The role of European big game (*Capreolus capreolus* and *Sus scrofa*) as hosts for ticks and in the epidemiological life cycle of tick-borne diseases

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Zusammenfassung

Rehe und Wildschweine gehören in Europa zu den häufigsten Kulturfolgern mit flächendeckender Verbreitung. Sie gelten als wichtige Wirte für Zecken und scheinen eine bedeutende Rolle im Lebenszyklus von zeckenübertragenen Krankheiten zu spielen. Von August 2011 bis Februar 2014 wurden im Bienwald (Rheinland-Pfalz) 247 Rehe und 344 Wildschweine auf ihren Zeckenbefall sowie auf bevorzugt befallende Körperstellen und Infektionen untersucht. Zecken und Organproben wurden durch PCR auf Rickettsien- und Borrelienspezies analysiert. Von 83 sequenzierten Zecken wurde in 9 *Rickettsia helvetica* gefunden. Eine statistische Auswertung, einschließlich Faktorenanalyse, Entscheidungsbäume, generalisierte lineare Modelle und logistische Regressionen, wurde verwendet, um die Schlüsselfaktoren bezüglich Zeckenbefall, befallener Körperstellen und Infektionen zu identifizieren. Dabei wurde eine Vielzahl neuer Erkenntnisse gewonnen: Geschlecht, Alter und körperlicher Verfassung von Rehen beeinflusste den Befall mit Zecken deutlich. Der Befall auf Wildschweinen war erheblich niedriger als auf Rehen. Klimatische Faktoren korrelierten signifikant mit der Zeckenabundanz, wobei Zecken die Körperstelle zur Blutmahlzeit aktiv auswählten und ganzjährig aktiv waren. Weibliche adulte Zecken zeigten dabei die niedrigste Spezialisierung. Keine der Organproben zeigte eine Rickettsien- oder Borrelieninfektion. Zecken von Rehen waren zu 47% mit *Rickettsia* spp. und zu 3,4% mit *B. burgdorferi* s.l. infiziert, während von Wildschweinen 41,7% der Zecken mit Rickettsien und keine mit Borrelien infiziert waren. Infektionen mit Rickettsien wurden durch Jahreszeit, Saugstadium und Geschlecht der Zecken sowie durch Wirtszustand und -geschlecht signifikant beeinflusst. Die Borrelien abtötende Wirkung von Rehblut in gesaugten Zecken wurde bestätigt. Eine ähnliche Wirkung für Wildschweinblut wird vermutet. Die Studie zeigt, dass Rehe gegenüber Wildschweinen die bevorzugten Wirte für *Ixodes ricinus* sind und somit eine herausragende Rolle für zeckenübertragene Krankheiten spielen. Dies ist weltweit die erste und umfangreichste Langzeitstudie von Zecken auf Rehen und Wildschweinen in einem gemeinsamen Habitat mit dem größten Datensatz.

Abstract

European wild boar and roe deer belong to the most common, synanthropic and widespread species of big game in Europe. They are potential key hosts for ticks and are hypothesized to play an important role in the life cycle of tick-borne diseases. From August 2011 to February 2014, 247 European roe deer and 344 wild boar were investigated for tick prevalence, abundance, preferred attachment sites and pathogen infections in a forest in southern Germany (the Bienwald, Rhineland-Palatinate). Ticks and organ samples were analyzed by PCR for infections with *Rickettsia* spp. and *B. burgdorferi* s.l. species. 83 tick samples were sequenced finding *Rickettsia helvetica* in 9 ticks. An extensive statistical evaluation, including factor analysis, decision trees, generalized linear modelling and logistic regressions, was used to identify, rank and analyze the key factors related to tick burden, feeding site selection and infections. The results included several novel findings. Host sex, age and condition influenced the tick burden on roe deer significantly. The infestation intensity on wild boar was considerably lower than on roe deer. Climatic factors correlated significantly with tick intensity, while ticks were active all-year and chose their feeding site actively preferring the abdomen, sternum and ear. Thereby, female ticks had the lowest feeding site specialization, which was lowest during the warmer months. None of the organ samples showed a *Rickettsia* or *Borrelia* infection. Ticks from roe deer were infected to 47.0% with *Rickettsia* spp. and to 3.4% with *B. burgdorferi* s.l. From wild boar 41.7% of the ticks had *Rickettsia* spp. and no *Borrelia* spp. were found. *Rickettsia* infections were significantly related with engorgement, the host's physical condition, sampling period and the sex of the ticks and hosts. The borreliacidal effect of roe deer is confirmed and a similar effect is proposed for wild boar. The results indicate that roe deer play a more important role than wild boar in the distribution and ecology of *Ixodes ricinus*, as well as in the epidemiological lifecycle of tick-borne diseases. This is the first and most comprehensive long-term study of ticks from roe deer and wild boar in a common habitat with the largest dataset currently available worldwide.

Content

Zusammenfassung iii

Abstract v

Content vii

Listing of Figures xi

Listing of Tables xv

Abbreviations xix

Acknowledgements xxiii

1 Introduction 1

1.1 Overview 1

1.2 Objectives 6

1.3 Outline 7

2 Related work 9

2.1 European big game 9

2.1.1 European roe deer 9

2.1.2 Central European wild boar 21

2.2 Ticks (Ixodida) 33

2.2.1 Taxonomy 33

2.2.2 Distribution 34

2.2.3 Ecology 36

2.2.4 Morphology of the Ixodidae 37

2.2.5 Distribution and ecology 39

2.3 Tick-borne diseases 45

2.3.1 Lyme borreliosis 45

2.3.2 *Rickettsia* spp. 59

3 Material and methods 69

3.1 Sampling methods 69

3.1.1 Sampling area 69

3.1.2 Hosts samples 71

3.1.3 Tick collection 73

3.1.4 Climate 75

3.2 Microbiological methods 76

3.2.1 Nucleic acid extraction from organ samples 76

3.2.2 Nucleic acid extraction from tick samples 78

3.2.3 Polymerase chain reaction 79

3.2.4 Real-time PCR 81

3.2.5 Specific detection of *Borrelia* spp. 84

3.2.6 Specific detection of *Rickettsia* spp. 89

3.3 Data supplementation 94

3.3.1 Measure of aggregation and relative tick density 94

3.3.2 Niche index – Levin's index 96

3.3.3 Niche overlap – Piankas' index 97

3.3.4 Group dependent mass index (GDMI) 97

3.3.5 Animal conditions 99

3.4 Statistical methods 99

3.4.1 Statistical modeling 101

4 Tick burden 105

4.1 Results 105

4.1.1 Tick burden on roe deer 105

4.1.2 Tick burden on wild boar 110

4.1.3 Identification of factors influencing tick activity 112

4.1.4 Ranking of influences on tick burden 116
4.1.5 Modeling of tick abundances 122
4.1.6 Modeling the composition of the tick population 140
4.1.7 Attachment site analysis 143
4.1.8 Niche breadth analysis 147
4.1.9 Spatial niche overlap 149
4.2 Discussion 152
4.2.1 Ticks on roe deer 152
4.2.2 Ticks on wild boar 166
4.2.3 Comparison of wild boar and roe deer 172
4.2.4 Attachment sites 172
4.2.5 Co-feeding 176
5 Pathogen prevalence 179
5.1 Results 179
5.1.1 Pathogens in organ samples 179
5.1.2 Pathogens in ticks from roe deer 181
5.1.3 Pathogens in ticks from wild boar 184
5.1.4 *Rickettsia* spp. sequencing 185
5.1.5 Host-tick-pathogen relationships 186
5.2 Discussion 192
5.2.1 *B. burgdorferi* s.l. infections 192
5.2.2 *Rickettsia* infections 196
6 Conclusion and perspectives 205
6.1 Summary 205
6.2 Future Work 209
References 213

Appendices 245

A Form for recording organ samples 247

B Mean group weights 249

C Equipment and consumables 251

D Solutions and buffers 255

Listing of Figures

Figure 1.1: Female *Ixodes ricinus* and *Erythema migrans* 2

Figure 1.2: Syntrophic wild boar and roe deer 3

Figure 2.1: Geographical distribution of the roe deer throughout Europe 11

Figure 2.2: Geographical distribution of the roe deer throughout Asia.....12

Figure 2.3: Worldwide geographical distribution of wild boar 23

Figure 2.4: Tree visualizing the taxonomy of ticks.....33

Figure 2.5: Map of georeferenced tick findings.....35

Figure 2.6: Comparison of *Ixodes ricinus* life history stages.....38

Figure 2.7: Comparison of adult female and male *Dermacentor marginatus*.....38

Figure 2.8: Map of the spatial distribution of *Ixodes ricinus* in Europe.....40

Figure 2.9: The development cycle of *Ixodes ricinus* 41

Figure 2.10: Morphological structure of *Borrelia burgdorferi* s.l. 49

Figure 2.11: Map of the geographical distribution of *B. burgdorferi* s.l. 54

Figure 2.12: Schematic progression Lyme borreliosis.....57

Figure 2.13: Taxonomical overview of the *Rickettsia* spp. 60

Figure 2.14: Electron microcopy image of *R. prowazekii* 62

Figure 3.1: Map of the Bienwald.....70

Figure 3.2: Culled roe deer and wild boar in the Bienwald (2004 – 2014).....71

Figure 3.3: Sample acquisition during driven hunts 72

Figure 3.4: Tick collection from roe deer.....73

Figure 3.5: Schemata of roe deer and wild boar body regions.....74

Figure 3.6: Average air temperature and precipitation depth 76

Figure 3.7: Maxwell® 16 DNA Purification Cartridge and sterile workbench 77

Figure 3.8: Reaction process of hybridization probes 82
Figure 3.9: Reaction process of the TaqMan® probe 83
Figure 3.10: *OspA*-PCR primer and probe in the LightCycler® 85
Figure 4.1: Sampled roe deer, tick abundances and tick prevalences 107
Figure 4.2: Estimated blood loss due to *I. ricinus* on roe deer 109
Figure 4.3: Sampled wild boar, tick abundances and tick prevalences 111
Figure 4.4: Decision trees for male and female ticks from roe deer 116
Figure 4.5: Decision trees for nymphs and larvae on roe deer 117
Figure 4.6: Decision trees for all ticks from roe deer 118
Figure 4.7: Decision trees for adult ticks and roe deer host parameters 119
Figure 4.8: Decision trees for immature ticks and roe deer host parameters 120
Figure 4.9: Fits of the NBI and the PO distribution against the dataset 122
Figure 4.10: Mean tick burden modeled by GAMLSS 125
Figure 4.11: Partial effects of temperature on tick life history stages 126
Figure 4.12: Box plots for average number of ticks per roe deer 127
Figure 4.13: Worm plots for GAMLSS models of tick life history stages 129
Figure 4.14: Effects of automatically selected parameters on adult ticks 130
Figure 4.15: Effects of automatically selected parameters on immature ticks 133
Figure 4.16: Climate in relation to tick life history stages 135
Figure 4.17: Worm plots for the parameter optimized models 136
Figure 4.18: Partial effects of roe deer body mass on tick burden 138
Figure 4.19: Partial effects of GAMLSS models for ticks from wild boar 139
Figure 4.20: Probabilities of all ticks life history stages 142
Figure 4.21: Relation between preferred feeding site and tick life stages 146

Figure 5.1: Detection of *Rickettsia* spp. in organ samples of wild boar.....180

Figure 5.2: Wild boar fetus.....180

Figure 5.3: Decision tree for *Rickettsia* spp. prevalence of ticks on roe deer.....190

Figure 5.4: Gregarious feeding of *I. ricinus* on roe deer.....199

Listing of Tables

Table 3.1: Primers and probes of the *OspA*-PCR on the LightCycler® 86

Table 3.2: Pipetting scheme of the *OspA*-PCR 86

Table 3.3: Temperature profile of the *OspA*-PCR 86

Table 3.4: Primers of the 5S23S rDNA-PCR 87

Table 3.5: Pipetting schemes of the 5S23S rDNA-PCR 88

Table 3.6: Temperature profiles of the 5S23S rDNA-PCR 89

Table 3.7: Primers and probe of the glta-PCR 90

Table 3.8: Pipetting scheme of the glta-PCR 91

Table 3.9: Temperature profile of the gltA-PCR 91

Table 3.10: Primers of the rOmpA-, rOmpB- and gltA-PCR 91

Table 3.11: Pipetting schemes for rOmpA-, rOmpB- and gltA-PCR 92

Table 3.12: Temperature profiles of the rOmpA-, rOmpB-, and gltA-PCR 92

Table 3.13: Pipetting scheme for the *Rickettsia* DNA-sequencing 93

Table 3.14: Mean surface area proportions of roe deer body 95

Table 3.15: Mean surface area proportions of wild boar body 95

Table 3.16: List of all variables contained in the final dataset 100

Table 4.1: Numbers of sampled roe deer and wild boar 106

Table 4.2: Contingency table of sampled roe deer and ticks 108

Table 4.3: Comparison of roe deer ages with tick burden 109

Table 4.4: Contingency table of sampled wild boar and ticks 110

Table 4.5: Correlation coefficients for ticks on roe deer and wild boar 114

Table 4.6: Correlation coefficients for individual periods 115

Table 4.7: AIC_c-values and degrees of freedom of the GAMLSS models 123

Table 4.8: Mean and dispersion coefficients of manually selected models 124
Table 4.9: Mean and dispersion coefficients for GAIC-optimized models 131
Table 4.10: Residuals of GAIC-optimized models for ticks from roe deer 135
Table 4.11: Coefficients of GAIC-optimized models for ticks from wild boar 139
Table 4.12: Coefficients of multinomial logistic regression 140
Table 4.13: Distribution of tick life stages in relation to body regions 143
Table 4.14: Friedman test for ranking tick densities of roe deer body areas 144
Table 4.15: Friedman test for ranking tick densities of wild boar body areas 147
Table 4.16: Standardized Levin index for ticks on roe deer 148
Table 4.17: GLMs for niche breadth of ticks on roe deer 149
Table 4.18: Pianka's index of spatial niche overlap for ticks on roe deer 150
Table 4.19: GLMs for spatial niche overlap of ticks on roe deer 151
Table 5.1: Analyzed ticks and found infections 181
Table 5.2: Tick engorgement and pathogen prevalence 182
Table 5.3: Pathogen prevalence and sampling periods 183
Table 5.4: *Rickettsia* spp. DNA-sequencing results of tick samples 186
Table 5.5: Correlation coefficients for tick, host and pathogen parameters 187
Table 5.6: Rotated components from factor analysis of infections 188
Table 5.7: Logistic regression for *Rickettsia* in ticks from roe deer 191
Table 5.8: *Rickettsia* infections in tick, organ and blood samples 196
Table A.1: Sample acquisition from 247
Table B.1: Mean body mass and deviations for groups of animals 249
Table C.1: All used devices and their manufactures 251
Table C.2: All used reagents and their suppliers 252

Table C.3: All used kits and their suppliers.....253

Table C.4: All used consumables and their suppliers.....253

Abbreviations

ACA	*Acrodermatitis chronica atrophicans*
AG	*Ancestral group*
AIC	*Akaike information criterion*
CATPCA	*Categorical principal component analysis*
CF	*Complement fixation*
CHAID	*Chi-square automated interaction detector*
CI	*Condition index*
DEPC	*Diethylpyrocarbonate*
DNA	*Deoxyribonucleic acid*
dsDNA	*Double-stranded DNA*
ELISA	*Enzyme-linked immunosorbent assay*
EM	*Erythema migrans*
EthBr	*Ethidium bromide*
FRET	*Fluorescence resonance energy transfer*
GAIC	*Generalized AIC*
GAMLSS	*Generalized additive models for location, scale and shape*
GDMI	*Group dependent mass index*
GIT	*Guanidine isothiocyanate*
GLM	*Generalized linear model*
GR	*Grass/roughage eaters*
IFAT	*Immune fluorescent antibody test*
IM	*Intermediate type eaters*
IS2	*Intergenic spacer 2*
JRA	*Juvenile rheumatoid arthritis*
LC-Kit	*LightCycler® DNA Master HybProbe kit*
LC-PCR	*LightCycler® PCR*
MSF	*Mediterranean spotted fever*
NBI	*Negative binomial distribution of type I*
NC	*Negative control*
NDVI	Normalized Difference Vegetation Index

osp	*Outer surface protein*
P1	*Pool 1*
P2	*Pool 2*
PC	*Positive control*
PCA	*Principle component analysis*
PCR	*Polymerase chain reaction*
PMP	*Paramagnetic particles*
PO	*Poisson distribution*
RH	*Relative humidity*
RMSF	*Rocky Mountain spotted fever*
RT-PCR	*Real-time PCR*
SD	*Standard deviation*
SFG	*Spotted fever group*
SPSS	*Statistical Package for the Social Sciences*
ssDNA	*Single-stranded DNA*
TBRD	*Tick-borne rickettsial disease*
TG	*Typhus group*
UDG	*Uracil-DNA glycosylase*
UTM	*Universal transverse mercator*
UV	*Ultraviolet*
VlsE	*Variable major protein-like sequence, expressed*

For Jens and Elina.

♡

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Senta Verena Muders

Images of "Belinda" the female egg laying tick (first row) taken by Prof. Dr. Urs Wyss (Entofilm, Kiel) for the ZDF documentation "planet e.: Zecken - Gefahr aus dem Wald" (second row).

1

Introduction

1.1 Overview

Worldwide, there is no other group of arthropods that transmits a greater variety of pathogens, including viruses, bacteria and protozoa, affecting humans, livestock and companion animals than ticks (Jongejan and Uilenberg 2004). In Europe, the substantial majority of vector-borne human diseases are transmitted by ticks with highly increasing incidence (Randolph 2001). The most prevalent pathogens are commonly transmitted to humans by the generalist ixodid tick *Ixodes ricinus* sensu stricto (Humair and Gern 1998). The increasing emergence of human diseases transmitted by ticks of the *I. ricinus* (L.) complex (see Figure 1.1) appears to be occurring simultaneously with changes in their spatial distribution, abundance and their associated pathogens (Pfäffle et al. 2013). However, evidence suggests that the current range expansion of ticks and the emergence of tick-borne diseases are also strongly correlated with changes in climate, human behavior and habitat modifications (e.g. habitat fragmentation) (Halos et al. 2010). Previous studies substantiate the importance of the effects of potential tick and reservoir hosts on the epidemiological life cycle of tick-borne diseases and disease outbreaks (McCoy et al. 1999, Estrada-Peña et al. 2006, Keesing et al. 2006). In particular, tick and pathogen prevalence is known to be strongly correlated with the presence and density of suitable host species as well as with their ecology (Gray et al. 1999, Dautel et al. 2006, Durden 2006, Ogden et al. 2008, Gilbert 2010, Kiffner et al. 2011c, Petney et al. 2011). As ticks have

Figure 1.1: Female *Ixodes ricinus* in on a leaf in search for a host (left, Picture: Holger Krisp, Ulm), female tick attached to human skin (center, Picture: Gabor Pozsgai, www.photogabor.com) and Rash (*Erythema migrans*) after tick bite (right, Picture: James Gathany, CDC).

only a limited ability to move horizontally (McCoy et al. 1999, Miller et al. 2007, Petney et al. 2011), vertebrate species play not only an outstanding role for tick and pathogen persistence in a specific area, but their movement also disperses ticks to new areas with the potential for subsequent human disease emergence (Randolph 2004, Estrada-Peña et al. 2006, Gilbert 2010, Pfäffle et al. 2013).

Immature life history stages of ixodid ticks are predominantly associated with small mammal hosts (Krasnov et al. 2002, Hanincová et al. 2003), whereas big game species appear to be potential key hosts favored by adult female ticks as they can provide large blood meals that encourage egg production (Smith et al. 1990, Pichon et al. 1999, Dobson et al. 2006, Ruiz-Fons et al. 2006, Gern 2008, Pound et al. 2010). In North America white-tailed deer (*Odocoileus virginianus*) are known to be a suitable and important big host species for *Ixodes scapularis* (Smith et al. 1990, Fish and Childs 2009), whereas in Europe red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) are usually heavily infested with *I. ricinus*, *Dermacentor marginatus* and, in southern Europe, *Hyalomma marginatum* ticks (Nelson et al. 2000, Fuller and Gill 2001). Red deer are thought to play an important role in tick distribution over long distances as they show distinct migration behavior similar to white-tailed deer (Nelson et al. 2004), but they tend to avoid urbanized areas (see Figure 1.2) (Mysterud 1999, Vor et al. 2010).

Figure 1.2: Syntrophic wild boar and roe deer. Adult female boar with its offspring on a Berlin front door (left, Picture: Th. Wiehle/Berliner Forsten). Roe deer passing by an industrial estate (right, Picture: Timo Jann/Bergedorfer Zeitung).

Contrary to this, adult roe deer form groups during winter and tend to be territorial in spring and summer, which involves the dominant male chasing away subadult or subdominant individuals, providing the chance for ticks to be distributed. This species occurs increasingly in urban and suburban habitats (Mysterud 1999, Vor et al. 2010, Pfäffle et al. 2013). Furthermore, their area-wide distribution, synanthropic mode of life and social behavior make roe deer potentially a key species of major concern for tick research (Pichon et al. 1999, de la Fuente et al. 2004, Ruiz-Fons et al. 2006, Kiffner et al. 2010a), as the emergence of diseases largely depends on the contact between humans and pathogens (Hughes and Randolph 2001a, 2001b, Wilson et al. 2002, Skuballa et al. 2010).

There are several studies that investigate tick burden on roe deer as well as the role of deer on the transmission of tick-borne diseases (Randolph 2001, 2009, Carpi et al. 2008, Kiffner et al. 2010a, 2011a, Vor et al. 2010, Vázquez et al. 2011, Alonso et al. 2012). Roe deer are reservoir hosts for several tick-borne pathogens (e.g. *Anaplasma* spp.) (Petrovec et al. 2003) and to play an important role with respect to the tick infection rate and the ecology of many pathogens (Kurtenbach et al. 1998b, Gern 2008). Although they are known to be dilution hosts for *Borrelia* spp. (Richter et al. 2004, Bhide et al. 2005) and other tick-borne pathogens (e.g. tick-borne encephalitis virus), their frequently high infestations with ticks also potentially makes them a platform for pathogen transmission via co-feeding (Jaenson and Tälleklint 1992,

Randolph et al. 1996, Ostfeld and Keesing 2000, Schmidt and Ostfeld 2001, Randolph 2004, Bhide et al. 2005, Kiffner et al. 2010a).

For cervids in general, a strong positive relationship between host population density and disease emergence was observed (Pichon et al. 1999, Randolph 2004, Brownstein et al. 2005, Fish and Childs 2009). As cervids serve demonstrably as amplifier hosts for ticks (Randolph 2004, Fish and Childs 2009), it is necessary to investigate their direct and indirect role in the ecological cycle of zoonoses and disease emergence. Besides host density, the composition of the species is considered to play an important role in disease emergence and risk of infection (Van Buskirk and Ostfeld 1995, Ostfeld and Keesing 2000, Schmidt and Ostfeld 2001, Keesing et al. 2006).

Similar to roe deer, wild boar (*Sus scrofa*) belong to the most common and widespread big game species of Europe. Both species share the same habitats and can reach very high population densities locally (de la Fuente et al. 2004, Ruiz-Fons et al. 2006, Rizzoli et al. 2009, Kiffner et al. 2010a, Vor et al. 2010, Keuling et al. 2013). Furthermore, roe deer and wild boar also profit from human influence on the environment (e.g. the change in land use, nutritional habitats and human life style) (de la Fuente et al. 2004, Ruiz-Fons et al. 2006, Keuling 2010a, Pfäffle et al. 2013). Moreover, wild boar show not only increasing population growth, a high spatial aggregation and overabundance (Gortázar et al. 2006, Keuling 2010b), but also enlarged home ranges which overlap substantially with highly urbanized areas (see Figure 1.2) (Vor et al. 2010, Keuling 2013, Keuling et al. 2013, Léger et al. 2013).

Although a variety of pathogens, such as piroplasms (e.g. *Babesia* spp. and *Theileria* spp.), *Ehrlichia* spp., *Anaplasma* spp. (i.e. *A. phagocytophilum* and *A. marginale*) and *Rickettsia* spp. (i.e. *Rickettsia slovaca*, *Rickettsia raoultii* and *Rickettsia sp. DnS28*), have been detected in ticks removed from wild boar (de la Fuente et al. 2004, Selmi et al. 2009, Michalik et al. 2012, Zanet et al. 2014), only little is known about the role

of these hosts in the epidemiological life cycle of tick-borne pathogens. Studies conducted in Spain, the Czech Republic and Poland found that wild boar serve as hosts for *A. phagocytophilum* (Petrovec et al. 2003, de la Fuente et al. 2004, Smetanová et al. 2006, Pfäffle et al. 2013). Kurtenbach et al. (1998a) considered wild boar to be possibly reservoir competent, at a low level, for *Borrelia burgdorferi* sensu stricto (s.s.). A serological survey conducted in the Czech Republic detected antibodies to *Borrelia burgdorferi* sensu lato (s.l.) in wild boar sera and pointed out the possible importance of wild boar in Lyme borreliosis ecology (Juricová and Hubálek 2009). Thus, wild boar are also of substantial interest in the transmission of zoonotic diseases.

In summary, roe deer, and to a lesser extent wild boar, are known to be natural hosts for ixodid ticks (de la Fuente et al. 2004, Ruiz-Fons et al. 2006, Kiffner et al. 2010a, 2010b), however, their role in tick-borne pathogen life cycle is not yet fully understood. In Germany, the role of European wild boar for tick-borne pathogen dynamics is less well investigated than for cervids. As far as known, investigations regarding prevalences of ticks on wild boar and tick-borne pathogens have not been conducted in Germany.

In terms of ticks sampled from roe deer and wild boar, my study is worldwide the most comprehensive study ever conducted to enlighten the role of both hosts for ticks and in the life cycle of tick-borne diseases. Insights from other studies gained in the US are not transferable to the situation in Europe. In particular in Germany, and particularly for wild boar, such studies are up to now completely missing, but essential for the understanding of the epidemiological life cycles of tick-borne diseases. It is the only study that investigated ticks and both host species in a common habitat over a long term period with a continuous sample acquisition throughout the years with the aim to acquire generally valid data and to reveal seasonal dynamics. In contrast to other studies (Kiffner et al. 2010a, Vor et al. 2010, Overzier 2013), I was the first to consider additional biotic parameters, such as host sex, age and

condition, tick engorgement and infestation with other parasites, as well as additional abiotic factors, in particular precipitation.

Simultaneously to my work, additional long-term studies of ticks and tick-borne diseases were conducted within the scope of the Bienwald project situated on the left bank of the Rhine (Moser 2012, Neumaier 2012, Schweikert 2012, Zöller 2014) and within the scope of the BWPLUS project on the right bank of the Upper Rhine near Karlsruhe (Petney et al. 2014, Sebastian et al. 2014, Pfäffle et al. 2015a, 2015b). These studies included drag sampling ("flagging") of ticks from the vegetation, tick sampling from small mammals (e.g. the bank vole *Myodes glareolus*) and from sheep. Both projects were established to gain long-term information about ticks, hosts and pathogens from both banks of the River Rhine with the aim of comparison. The BWPLUS project did not include the monitoring of ticks from big game species (i.e. roe deer and wild boar). However, the main goal of the two projects is to gain a complete set of information with respect ticks, host and pathogens in this area and to understand the differences between the left and right bank of the Rhine.

1.2 Objectives

The main aim of my work was to study concurrently roe deer and wild boar in conjunction with their parasitizing ticks over 3 years within a common sampling area. In addition to their ticks, the organs of sympatric wild boar and roe deer were collected and investigated for the *B. burgdorferi* s.l. and *Rickettsia* infections using PCR. A comprehensive statistical analysis was carried out in multiple steps in order to identify, rank and describe the key factors that influence tick prevalence, tick abundance and preferred tick attachment sites on the host body, as well as the pathogen prevalence in the collected ticks. In this context, models were constructed to gain additional information on how the key factors affect tick burden and pathogen prevalence with respect to both host species. The physical condition of the host as well as climatic parameters were also considered during this evaluation.

In combination with the other studies of the Bienwald project and the BWPLUS project, my work has the aim to gather, analyze and document the biotic and abiotic factors that influence ticks and tick-borne diseases. The information on these factors will allow us to analyze and thus understand changes in tick abundance and spatial distribution, as well as the occurrence of tick-borne diseases in humans. Such data are also crucial for the development of appropriate prevention and control strategies. Thus, the goal of my work was to provide a detailed analysis of key factors for the environment-tick-host-pathogen interactions in the Bienwald. Since studies on roe deer and wild boar as hosts for ticks have not been conducted within the BWPLUS project, my results, and in particular my generated models, can serve as a reference for future studies on these two host species, allowing a comparison of studies on the left and right bank of the River Rhine.

1.3 Outline

This thesis is structured as follows. Chapter 2 will give an overview on the current state of research in relation to European big game, ticks and their transmitted pathogens as well as the corresponding tick-borne diseases. This chapter will in particular discuss the ecology of roe deer, wild boar and ticks of the family Ixodidae. In Chapter 3 the material and methods will be explained, including a description of the sampling approach, the microbiological methods and the statistical evaluation techniques. Chapter 4 will present the results with respect to tick burden, including prevalences, abundances and the preferred feeding sites of ticks on roe deer and wild boar, followed by a discussion of the observations made. Chapter 5 takes a detailed look on the pathogen distribution in ticks from roe deer and wild boar with respect to *Rickettsia* spp. and *Borrelia* spp., whereby a discussion of these results completes the chapter. Finally, in Chapter 6 conclusions will be drawn from the results and perspectives on future work will be given.

2

Related work

2.1 European big game

Game represents a generic term for all wild living animals, which are subject to hunting rights. Based on biology, game laws and the practical aspects of hunting, a common classification of wildlife differentiates between furred game and feathered game, as well as between small and big game. The class of furred game comprises all mammalian species that are subject to hunting rights, while feathered game includes all hunted bird species. The category of hoofed game contains hunted cervids, bovids and wild boar. All hoofed game belong to the class of big game animals, as for example roe deer and wild boar. All other game species are assigned to the category of small game.

2.1.1 European roe deer

European roe deer (*Capreolus capreolus*), also known as western roe deer, belongs to the furred big game category and to the class of hoofed game, the so-called ungulates. Taxonomically, European roe deer belong to the suborder of ruminants (Ruminantia) within the order of even-toed ungulates (Artidodactyla). Within the family of Cervidae, roe deer fall within the subfamily of Capreolinae (Andersen et al. 1998). In addition to the European roe deer (Sempéré et al. 1996) , another roe deer species can be found in Asia, Siberia, Mongolia and China, the Siberian roe deer, also called eastern roe deer (*Capreolus pygargus*) (Ohtaishi and Gao 1990, Danilkin 1995). *Capreolus capreolus* has a smaller body size, a smaller cranium and smaller

antlers than *C. pygargus*. Two other roe deer subspecies are known: (1) the Tian Shan roe deer (*Capreolus c. tienschanicus*) inhabiting Chinese and Russian parts of the Tian Shan Mountains in central, and (2) a species only found in Spain, *Capreolus c. garganta*. However, for both species only very limited data is available and it is still unclear how to define the exact boundaries between *C. pygargus* and *Capreolus c. tienschanicus* in Asia and between *C. capreolus* and *Capreolus c. garganta* in Europe (Stubbe 2008). Indeed, the taxonomic status of *Capreolus c. garganta* is even still under discussion, as to whether it can be considered as a separate subspecies or just as an ecotype of the European roe deer (Stubbe 2008).

2.1.1.1 Morphology

In Germany, *C. capreolus* is the smallest native deer species. Its body length can vary between 95 and 135 cm and its shoulder height lies between 65 and 75 cm (Macdonald and Barrett 2002). A pronounced sexual dimorphism in relation to its body size cannot be determined. Adult animals have a live weight of up to 49 kg with a relatively narrow, stocky body and high, slender legs (Andersen et al. 1998). The hoofs are short and narrow with well-developed lateral hoofs (Andersen et al. 1998). Typical for roe deer is its cream white rump patch with a very short tail that is barely visible (2 to 3 cm). They have a short and slender neck with a triangular-shaped head. Roe deer have a yellowish-red to reddish-brown coat with shorter and thinner hair during summer, and a grey brown (even black) coat in winter. The face always has a greyish color (Apelt 2007, Maahs 2010). Only adult males carry antlers, which are relatively erect and short in comparison to larger deer species (e.g. red deer). The younger males usually have unbranched, short antlers (5 to 12 cm), whereas older bucks can carry antlers up to 25 cm with two, three or rarely even four prongs depending on their physical condition (Andersen et al. 1998). When the antlers start to grow they are covered in a velvet-like layer of fur that disappears over time. Roe deer are usually classified into three groups: (1) fawns (up to one year), (2) yearling bucks and does (about one year) and (3) adult animals. Disemboweled, an adult animal has a body mass of about 14 to 21 kg, while a one-year-old animal weights about 12 to 16 kg. Roe deer fawns are born from May to June with a

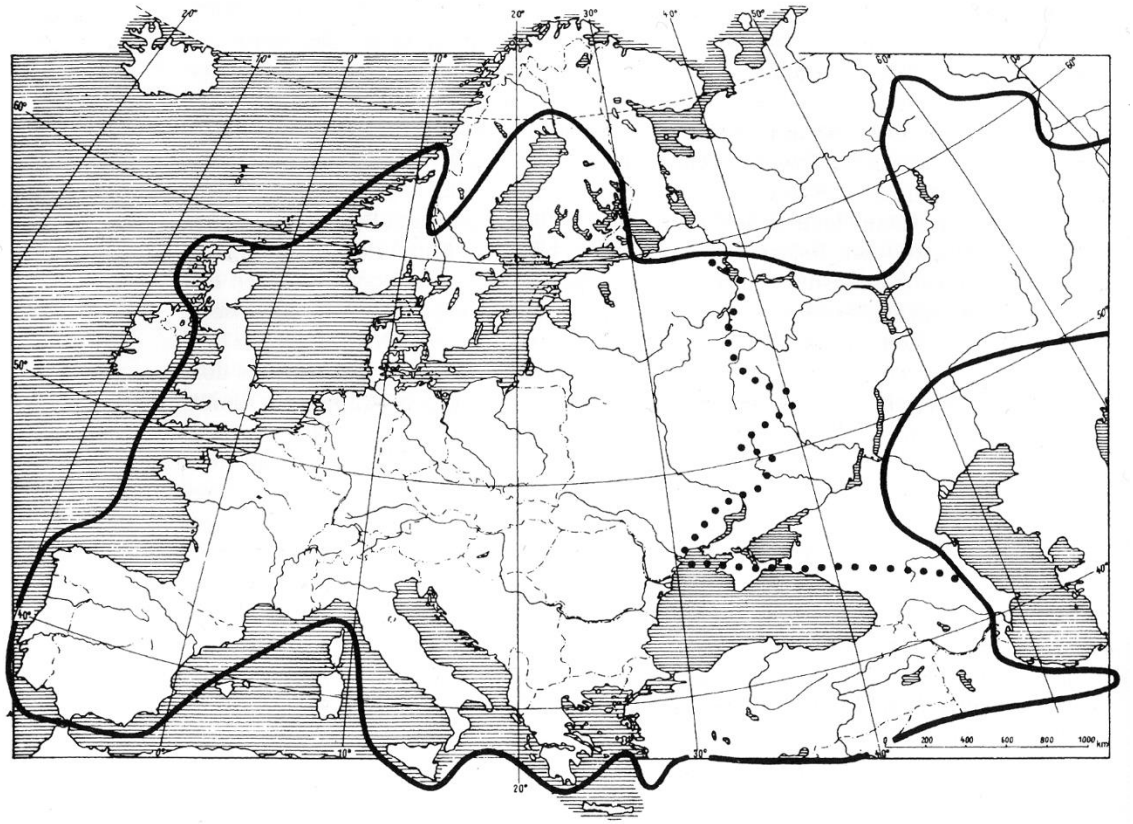


Figure 2.1: Geographical distribution of the roe deer throughout Europe. The boundary between European roe deer (*C. capreolus*) and Siberian roe deer (*C. pygargus*) is marked by the dotted line. The map is adapted from Heptner et al. (1966).

weight of 1 to 2 kg, but can reach a body mass of up to 13 kg. They are initially pale to dark brown with white spots on the top of their torso.

2.1.1.2 Distribution

Roe deer inhabit large parts of Europe and some areas of eastern Asia. They are not found in the northernmost regions of Scandinavia and have a scattered distribution in the Mediterranean region, where they are confined to mountainous areas due to climatic factors. Roe deer are wide spread in England and Scotland, whereas they do not inhabit Ireland (Figures 2.1 and 2.2). On most other European islands, such as Iceland and the Mediterranean islands, roe deer are also absent (Andersen et al. 1998, Stubbe 2008). Within Germany, *C. capreolus* is the most abundant deer species and is found from the coastal regions to the highlands, from floodplains to mountain forests, in agricultural steppe and parkland (Stubbe 2008).

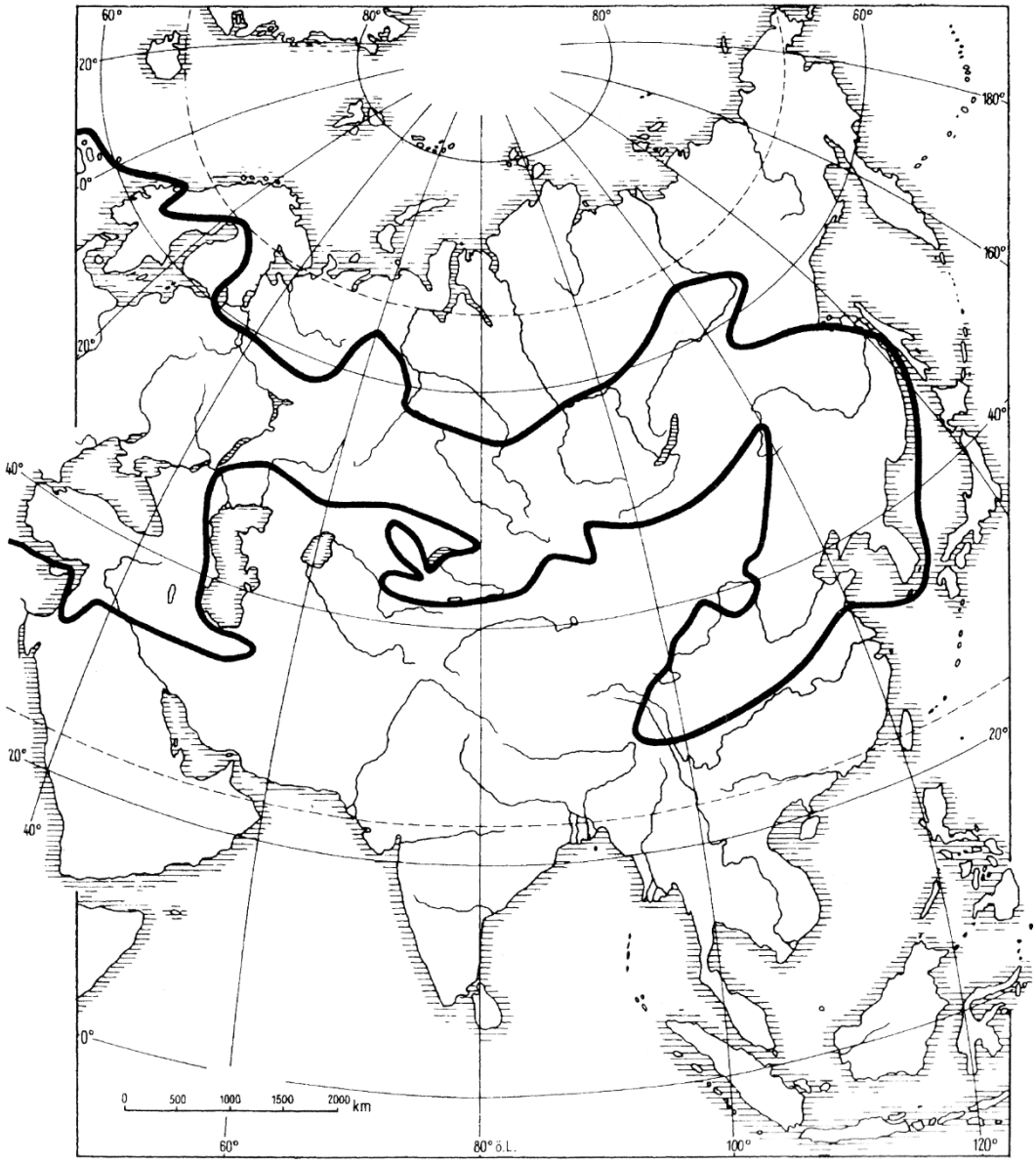


Figure 2.2: Geographical distribution of the Siberian roe deer throughout Asia. The map is adapted from Heptner et al. (1966).

Roe deer are considered to be very adaptable and their distribution is still expanding (Andersen et al. 1998). Through the widespread extinction of many predators, such as wolf and lynx, the European roe deer has almost no natural enemies (Linnell et al. 1995). However, the species has become more and more exposed to hazards of machines used during hay and grass harvesting, as well as to road traffic and wild

dogs (Deutscher Jagdverband 2014a). Nevertheless, there is a growing roe deer population in Germany, such that from 1993/1994 to 2012/2013 an increase in the number of culled roe deer from 1.032.821 to 1.192.583 was registered (Deutscher Jagdverband 2014b).

2.1.1.3 Habitat structure

Roe deer occupy a wide variety of habitats with optimal living conditions being found in the transition zone between open land and woodland that is rich in undergrowth. They prefer landscapes which are marked by the alternation of light forests, fields and meadows and thus can be considered as a forest edge dwellers (Hewison et al. 2001). Only high mountainous regions over the tree line, as well as wide open grasslands, are rarely inhabited by roe deer (Andersen et al. 1998). However, as a synanthropic species, roe deer have adapted to open farmlands, human settlements, as well as to park-like landscapes (Wölfel 2005, Stubbe 2008). As forest edges are good browsing habitats for roe deer, spatial heterogeneity has been determined to be a key factor that influences local population density (Saïd and Servanty 2005). In heterogeneous landscapes, habitat usage is constrained by potential sources of disturbance (i.e. human activities), such that roe deer tend to avoid buildings and roads (Kuehn et al. 2007, Coulon et al. 2008). In this context, Bonnot et al. (2014) have proposed the existence of a risk management syndrome which imposes constraints on how roe deer exploits high-risk habitats.

An additional important factor for roe deer habitat quality is the availability of shelter to escape from potential predators, including humans (Andersen et al. 1998, Bonnot et al. 2014). Due to their relatively small size, small forest remnants or hedges suffice as a shelter. Accordingly, roe deer also occupy the open agricultural plains. Consequently, a distinction is made between two ecotypes: forest roe deer and field roe deer (Jepsen and Topping 2004). While forest roe deer remain close to forest habitats, field roe deer resides in poorly sheltered open agricultural landscapes. Here they have switched to a diet mainly of crops. Field roe deer return to the forest and change their diet and behavior when the deer population density in

these areas decreases (Stubbe 2008). However, roe deer home ranges always included a minimum amount of woodland independent of the ecotype (Cargnelutti et al. 2002). Hewison et al. (2001) found that switch between forest and field behavior may involve woodland connectivity. Nevertheless, the behavioral plasticity of roe deer in response to landscape structure limits our ability to accurately predict the effects of landscape and landscape change (Jepsen and Topping 2004, Bonnot et al. 2014).

Furthermore, the species is quite resistant to climactic extremes, such that it can survive in the hot and dry regions of southern Europe (Aragón et al. 2006), as well as in the cold boreal forests of Scandinavia (Andersen et al. 1998). However, high and long-lasting snow is unfavorable for roe deer since they have difficulty moving and reaching food. Additionally, other deer species seem to influence the behavior of roe deer. In areas with high abundances of red deer and fallow deer, smaller roe deer population density has been observed (Stubbe 2008).

2.1.1.4 Feeding habits

With regard to their nutritional behavior, roe deer belong to the class of concentrate selectors and shows a remarkable range of digestive adaptations (Andersen et al. 1998). In comparison to the two other feeding types, the grass/roughage eaters (GR) and the intermediate (IM) types , the gastrointestinal tract of roe deer has a lower capacity, less subdivisions and larger openings (Van Soest 1994, Wölfel 2005). This leads to faster passage rates, such that a fiber-rich diet can only be poorly utilized. The low capacity requires roe deer to have several periods of feeding, which are distributed relatively evenly over 24 hours. Because of this, roe deer need nutritiously and easily digestible food (Stubbe 2008, Petrak 2013). Consequently, and in contrast to red deer which are IM feeders, roe deer is picky and its diet includes buds, herbs, flowers, young leaves and grasses. During fall roe deer also increasingly consumes berries, fruit, mushrooms, chestnuts, acorns, beechnuts as well as raspberries, raspberry leaves and lichens (Andersen et al. 1998, Stubbe 2008, Deutz et al. 2009).

In addition to a daily rhythm of food intake, roe deer have a yearly rhythm with regard to their energy demand. From October to December they have an increased need for food, which again reduces from mid-December to mid-February. Parallel to this development, a regression of the villi in the rumen can be registered (Deutz et al. 2009), and it also appears that roe deer are able to lower their basal metabolic rate during winter (Mauget et al. 1997, Andersen et al. 1998, Morellet et al. 2013). In the following months, until around June, the need for food is again increased, causing roe deer, particularly the bucks, to form body reserves of fat. The seasonal fluctuations in body mass are mainly caused through the change from summer to winter fur, differing food supply throughout the year, weather conditions and extraordinary burdens, such as the rut, pregnancy and lactation. Bucks reach their maximal body mass in June or July before the rutting season, while leading does show a reduction of their body mass during these months due to milk production (Deutz et al. 2009).

2.1.1.5 Home range and social behavior

Roe deer are considered to be extremely faithful to their immediate environment (Linnell and Andersen 1995). However, this assumption cannot be seen as strictly established, since the environment of roe deer includes a large variety of ecological parameters, such as population density, sex ratio, browsing range and shelter, disturbances (e.g. by humans) and climatic influences, the form of forestry and agriculture, as well as the presence or absence of predators (David 2012), the visibility and the food supply in the home range (Tufto et al. 1996). In principle, roe deer occupy differing summer and winter grazing areas within their annual cycle. These areas include day and night ranges, resting and feeding places, as well as breeding zones for does and territorial blocks of adult bucks (Stubbe 2008). Thereby, roe deer generally live as loners, but they gather together in fluid social communities during the winter months until spring (Andersen et al. 1998, Stubbe 2008). The size of the groups can vary considerably with population density and habitat structure, such that winter groups can contain more than 50 individuals in open agricultural plains and between 5 and 10 in woodlands (Andersen et al. 1995, 1998). During summer,

females live isolated from other individuals in order to raise their fawns, while adult males defend their mating territory (Andersen et al. 1998).

Recent findings show that territorial behavior seems to be dependent on multiple factors (David 2012). The size of the territory of a roe deer buck in wooded areas or structured field-forest districts is rarely more than 25 ha, in general they are much smaller (David 2012). However, the bucks do not show territorial behavior over the course of the entire year, but seem to abandon their territoriality after the rut due to hormonal changes (Stubbe 2008, David 2012). The synanthropic roe deer is very adaptable in its ranging behavior, such that the home range can become totally variable depending on the situation within the roe deer population (David 2012). Thus, older bucks often live non-territorially, but nonetheless remain in their original action space and even continue to participate in the rut. Despite this change in behavior, they are tolerated and not attacked by other roe deer bucks in the region. It is assumed that bucks know each other and that the younger ones respect the elders (David 2012). Moreover, field roe deer that permanently live in the open country, can give up their territorial behavior partially or entirely (David 2012). Food availability, local weather and components of seasonality (e.g. day length) are key factors that influence roe deer forage and ranging behavior (Morellet et al. 2013).

The behavior of female roe deer is quite different from that of the males (Tufto et al. 1996). Although they are faithful to their habitat, they seem to principally show no territorial behavior. David (2012) ascribes this behavior to the energy costs that territoriality requires, and that during breeding season female roe deer must pay attention to their energy budget being unable to waste unnecessary power. Moreover, there exist observations in which females reside almost immediately next to each other. At the same time, the females only roughly know the resting place of their offspring, since the fawns choose it for themselves and change their location independently from their mother by up to 100 m (Stubbe 2008). In forest areas with a high population density roe deer often live within very small areas and are organized in relational clans. Thereby, orphan fawns within such groups can be adopted

relatively easily by older sisters and aunts. All these behaviors indicate a non-territoriality of female roe deer (David 2012). Conversely, the setting and home ranges of female roe deer seem to overlap without the occurrence of serious conflicts (David 2012).

The reproductive cycle of roe deer has a unique status among ungulates. Females have only a single ovulation each year and the implantation of the embryo is delayed by five months (Andersen et al. 1998). The timing of this cycle is so precise that 98% of the females are fertilized during the rut, whereby 80% of the matings occur within two weeks. As a consequence, fawns are born aggregated closely in time during spring (Andersen et al. 1998, Wölfel 2005).

2.1.1.6 Parasites

Besides ticks (see Section 2.2), other ectoparasites have been recorded on roe deer (Duscher, 2006; Stubbe, 2008). For example, several findings of deer ked (*Lipoptena cervi*) have been reported (Välimäki et al. 2010, Handeland et al. 2013) and it has been proposed that deer support the reproduction of this parasite (Duscher 2006, Välimäki et al. 2010). In a Spanish study (Vázquez et al. 2011), roe deer were also infested by Hippoboscidae (*Hippobosca*, 3.3% and *Lipoptena*, 0.3%) and by Mallophaga (*Trichodectes meyer*, 3.1%). In the eastern Mediterranean findings of three hippoboscid flies (*Lipoptena capreoli*, *Hippobosca equina*, and *Hippobosca longipennis*) and one unidentified trombiculid mite species have been documented for roe deer reintroduced to Israel, while no exotic ectoparasites were collected (Wallach et al. 2008).

Furthermore, infestations by nasal and pharyngeal bot flies of the family Oestridae (e.g. *Cephenemyia stimulator*, *Cephenemyia ulrichii* and *Pharyngomyia picta*) have been described in several studies (Sugár 1974, Rivosecchi et al. 1978, Ruiz et al. 1993, Nilssen et al. 2008, Salaba et al. 2013). *C. stimulator* is the most common bot fly in roe deer with prevalence ranging from 11% up to 90% (Kusak et al. 2012). Roe

deer heavily infested with bot fly suffer severely having difficulty breathing, coughing and frequently sneezing. The infestation can be lethal, mostly in combination with other parasites (e.g. lungworms) (Duscher 2006, Stubbe 2008). In addition, warble flies (*Hypoderma*) occur regionally on roe deer. Severe infestations (100 to 200 larvae) with *Hypoderma diana* cause intense itching and scratching leading to skin abrasions, delayed hair change, dullness and weight loss (Yeruham et al. 1994, Duscher 2006). Deaths from *H. diana* alone are rare (Minar 1982), but in combination with stress and a poor physical condition high mortality rates (41%) have been observed in roe deer imported to Israel (Yeruham et al. 1994). Symptoms similar to those of *H. diana* are caused by the blood-feeding roe deer louse (*Solenopotes capreoli*) leading to a general state of restlessness in its hosts (Stubbe 2008). Moreover, several cases of sarcoptic mange caused by *Sarcoptes scabiei* mites have been reported for European roe deer (Duscher 2006, Menzano et al. 2008, Oleaga et al. 2008a, 2008b). This type of mite infestation causes skin inflammation, pruritus, and cutaneous hypersensitivity leading to physiological alterations in skin and organs, dehydration and occasionally to death (Oleaga et al. 2008b).

As endoparasites in roe deer, protozoa (unicellular) and metazoan (multicellular) have been reported. Protozoa, such as coccidia (e.g. *Eimeria capreoli*, *E. panda*, *E. ponderosa*, *E. rotunda* and *E. superba*) and sarcosporidia (e.g. *Sarcocystis gracilis* and *Sarcocystis capreolicanis*) infesting the intestinal tract, toxoplasmosis in the muscles as well as blood parasites (e.g. *Babesia capreoli*) and trichomonads, have been found in many studies in Germany (Rehbein et al. 2000). Trichomonad infections in female roe deer can cause fertility disorders (Rehbein et al. 2000). The group of endoparasitic metazoans includes helminths, pentastomids (i.e. tongue worms) and arthropods. For arthropods only the larval stages of *H. diana* and *C. stimulator* live endoparasitic in roe deer (Rehbein et al. 2000). Larvae of the pentastomid *Linguatula serrata* has been found in a liver of a roe deer in Bavaria (Rehbein et al. 2000). The helminth fauna of roe deer comprises liver flukes (e.g. *Fasciola hepatica, Fascioloides magna, Dicrocoelium dendriticum*), flat worms (e.g. *Paramphistomum cervi*), tape-

worms (e.g. *Moniezia expansa*, *Taenia cervi*) and lungworms (e.g. *Dictyocaulus capreoli* and *Varestrongylus capreoli*). In this context, Duscher (2006) and Stubbe (2008) provide a description of the symptoms and the consequences of an infestation for roe deer hosts.

The infestation rates of a study in Poland (Burlinski et al. 2011) showed gastrointestinal nematodes to be most frequent in roe deer (34.7%) followed by coccidia (13.1%). The most frequently observed roundworm in roe deer was *Chabertia* sp. (13.8%), while high counts of *Ostertagia* sp. eggs (11.6%) and *Trichostrongylus* sp. eggs (10.69%) were also reported in fecal samples. In other Polish studies (Drozdz et al. 1987, 1992, Drozdz and Dudzinski 1993) the rate of infestation with gastrointestinal nematodes were even higher (50 to 100%). In western Pomerania, the prevalence of gastrointestinal nematodes in roe deer was 84% (Cisek et al. 2004), while the overall observed parasite infection rate was 95.5% (Cisek et al. 2003). Czech studies described endoparasite infestation rates of 88% (Dyk and Chroust 1974) and 100% (Vetýška 1980). In the both studies the most frequent endoparasite species in roe deer was *Ostertagia leptospicularis* (88% and 83.9%, respectively). For four different Czech regions high infection rates in roe deer were also reported for *Capreocaulus capreoli* (78.4%), *Chabertia ovina* (77%), *Eimeria ponderosa* (60%), *Eimeria superba* (50%), *Haemonchus contortus* (100%), *Muflonagia podjapolskyi* (62.7%), *Ostertagia lasensis* (74%), *Ostertagia ostertagi* (57.4%), *Spiculopteragia böhmi* (55%), *Spiculopteragia spiculoptera* (57.4%) and *Trichocephalus ovis* (61.7%) (Vetýška 1980). Belarusian researchers (Shimalov and Shimalov 2003) also reported very high infestation rates (75%) of helminth species in roe deer, whereby *C. ovina* being the most frequent (50%) followed by *Trichuris ovis* (37.5%) and *Oesophagostomum venulosum* (31.3%). In Croatia, Kusak et al. (2012) also found that *C. ovina* (36%) was the most common parasite in roe deer followed by *Ostertagia sp.* (24%), *Trichostrongylus* sp. (20%) and *Haemonchus contortus* (16%). In Sweden, Aguirre et al. (1999) reported *V. capreoli* (30%), *Trichostrongylus axei* (25%) and *Dictyocaulus noerneri* (24%) as the most frequent endoparasites in roe deer. A study

from North Rhine-Westphalia (Rehbein et al. 2000) showed that 100% of the sampled roe deer were infested with gastrointestinal nematodes and 32.8% with lung worms (*D. eckerti*: 14.1% and *V. capreoli*: 29.7%), while liver flukes and flat worms were not found. The most frequently found nematode species were *Ostertagia leptospicularis* (95.3%), *Spiculopteragia böhmi* (87.5%), *Skrjabinagia kolchida* (85.9%), *Trichuris globulosa* (67.2%), *Trichostrongylus capricola* (60.9%) and *Oesophagostomum venulosum* (50%). Zaffaroni et al. (2000) determined the degree of specialization of endoparasites and found that particularly *H. contortus* and *T. axei* are most adaptable to different hosts and thus appear to be most important due to their potential pathogenic effects.

In addition, Aguirre et al. (1999) showed for roe deer collected from Sweden that parasitism was one of the most common (11%) causes of mortality. Rehbein et al. (2000) found out that stronger roe deer were significantly less intensely infested than weaker host individuals. Helminth abundance in roe deer was negatively correlated with physical host parameters, such as body length (Zaffaroni et al. 1997) and body mass (Segonds-Pichon et al. 1998, Body et al. 2011), and nutritional components, such as fat reserves (Rossi et al. 1997, Zaffaroni et al. 1997). In a Spanish study, the number of gastrointestinal worms was negatively correlated with faecal nitrogen and spleen mass, while landscape structure did not influence worm infestation intensity directly, but possibly indirectly, since open areas could provide a diet richer in nitrogen (Navarro-Gonzalez et al. 2010). Consequently, Navarro-Gonzalez et al. (2010) proposed that the risk of gastrointestinal nematode parasitism for roe deer might depend on access to high-quality food, enhancing immunocompetence.

Furthermore, for gastrointestinal nematodes in roe deer the infestation prevalence depends on the host density (Gortázar et al. 2006, Body et al. 2011) and human disturbance and restriction in roe deer home ranges has led to increased infestation intensity (Lutz and Kierdorf 1997). In an experimental roe deer population, Maublanc et al. (2009) have shown that very high host population densities can lead

to a rapid transfer of parasites between host animals causing significantly increased parasite loads and a high degree of stress that contributes to immunodepression. All of these factors can provoke demographic crashes, even before food becomes a limiting factor (Maublanc et al. 2009). In addition, Body et al. (2011) found strong age and sex-dependent patterns of parasitism, such that roe deer yearlings were less often infested and had lower fecal egg counts than fawns and adult individuals. Male roe were also more heavily infested than females. They also reported that *T. capricola* in roe deer was not affected by weather, whereas gastrointestinal strongylides were less frequent after dry summers (Body et al. 2011).

2.1.2 Central European wild boar

All wild boar (*Sus scrofa*) are members of the class of mammals (Mammalia) in the order of even-toed ungulates (Artiodactyla) and belong to the suborder of non-ruminants (Nonruminantia, also known as Suiformes). In addition to the infraorder of pig-like animals (Suina) there exists the family of Hippopotamidae to which hippos belong. Pig-like animals can be subdivided basically into two families: pigs native to the Old World (Suidae) and New World pigs (Tayassuidae). While New World pigs are divided into two genera (*Catagonus* and *Tayassus*), six genera are attributed to the family of Old World pigs: pig-deer (*Babyrousa*), giant forest hog (*Hylochoerus*), warthog (*Phacochoerus*), pygmy hog (*Porcula*), river pig (*Potamochoerus*) and pig (*Sus*). The pygmy hog, formerly named *Sus salvanius*, was recently placed into the monotypic genus *Porcula* (Funk et al. 2007). The genus *Sus* comprises 9 species, including for example the bearded pig (*Sus barbatus*), the Celebes warty pig (*Sus celebensis*), the Javan warty pig (*Sus verrucosus*) and wild boar (*Sus scrofa*). This species is further split up into more than 15 subspecies (exact number still controversial) including the domestic pig (*Sus s. domesticus*) and the Central European wild boar (*Sus s. scrofa*), which is the only wild representative of non-ruminant even-toed ungulates in Europe (Briedermann 2009, Kusza et al. 2014). Wild boar belongs to the class of hoofed game (Weindl 2014). Throughout this thesis the term “wild boar” and the species name *Sus scrofa* will be used to refer to the Central European wild boar (*Sus s. scrofa*).

2.1.2.1 Morphology

The Central European wild boar has a compact, massive and stocky body structure with short legs and marked withers. The animals have a strong, relatively long and wedge-shaped skull that almost seamlessly passes over into the barrel-shaped trunk. Adult males, also called tuskers, have a height at the withers of 80 to 100 cm and their disemboweled body mass lies between 100 and 200 kg. The snout-vent length of tuskers has a mean value between 150 and 160 cm, but can reach up to 180 cm. Adult females have a lower withers, a weigh disemboweled between 80 and 100 kg and their average snout-vent length is about 140 cm with a range up to 160 cm. The body mass of European wild boar varies greatly and depends heavily on the food supply, while its average value increases from east to west (Briedermann 2009). The subspecies has long bristles with a thick undercoat, whereby its overall appearance can vary from a brownish grey to grayish black color with a lighter underbelly. The bristles form a ridge along the back of the animals beginning between their relatively small ears. Their tail is unobtrusive, goes down as low as the ankles and ends in a bush of hair. Adult wild boar have striking and powerful canine teeth that are much more prominent in males and are used as a tool for breaking up the soil and as a weapon. The approximate age of an adult individual can be determined on the basis of its canines. From a hunter's point of view, wild boar are referred to differently depending on their age (Briedermann 2009): squeaker (0 to 12 months), juvenile (12 to 24 months), adult boar (2 to 5 years), old boar (6 year and above). The age-dependent differentiation of young wild boar can be very difficult, because size and nutritional status can vary substantially. However, for young wild boar (< 2 years) a classification can be made on the basis of the dental age (Stubbe 2001, Briedermann 2009).

2.1.2.2 Distribution

Wild boar are widespread over most of Europe and Asia, as well as the northern parts of Africa (Meynhardt 1989, Hennig 2007). It occurs in Great Britain, while in Sweden a new stock has emerged in recent years originating from wild boars that had escaped from game enclosures (Hennig 2007, Rosvold and Andersen 2008).

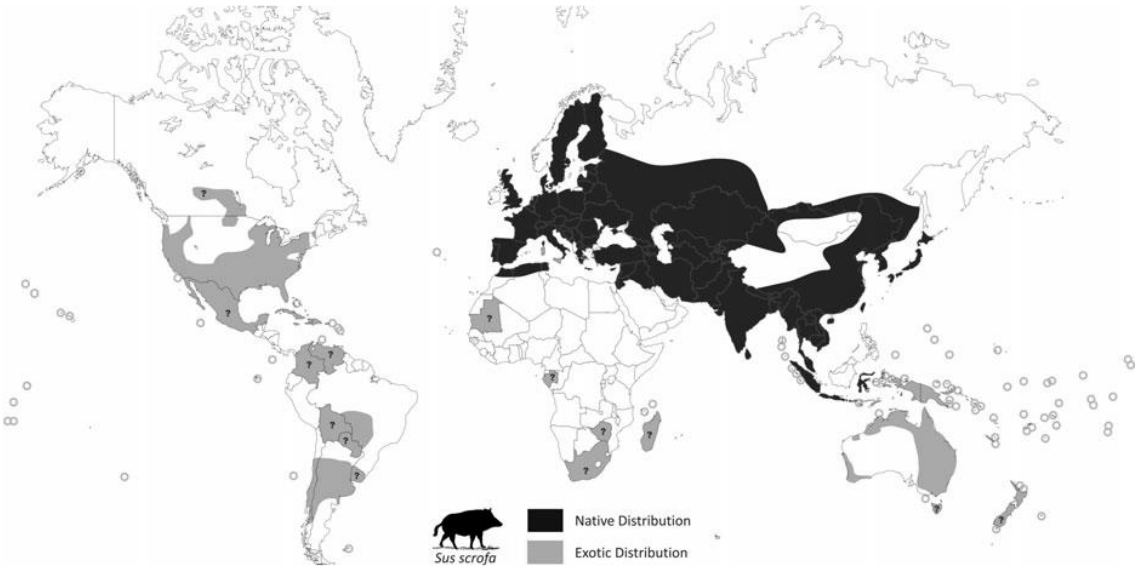


Figure 2.3: Worldwide geographical distribution of wild boar (*Sus scrofa*). Its native range is demarked in black, its introduced range in gray, islands where *S. scrofa* have been introduced are marked by gray circles and areas with an unknown distribution are demarked by a question mark. Map from Barrios-Garcia and Ballari (2012).

Wild boar is also found in South and Central America, Cuba, the Galapagos Islands, as well as in Australia and New Zealand, as well as in some regions of the United States, e.g. parts of California, Florida, South Carolina and Georgia, Hawaii and Puerto Rico (Hennig 2007). The species distribution also reaches the Far East, including Japan and Taiwan (see Figure 2.3) (Schley and Roper 2003).

Central European wild boar (*Sus s. scrofa*) inhabit West and Central Europe including France. In the south, its distribution is bounded by the Pyrenees and the Alps, while in Russia it can be found across to about the 25th meridian east. On the British Isles and in Scandinavia it was extinct, but a partial reintroduction has taken place (Hennig 2007, Briedermann 2009, Barrios-Garcia and Ballari 2012). The distribution of European wild boar in Germany extends over all federal states. In Mecklenburg-Western Pomerania, Brandenburg and Saxony-Anhalt, but also in certain areas of other federal states, such as the Eifel or the Hunsrueck, boars are particularly numerous (Gethöffer 2005, Anczikowski 2009, Keuling 2010a). Germany and other Central European countries show a massive increases in the total number of culled wild boars, illustrating the extreme population growth over the past several decades

(Schley et al. 1998, Schley and Roper 2003, Arnold 2008, Barrios-Garcia and Ballari 2012). Since the 1980s there has been an exponential growth in the wild boar harvest (Arnold 2008) with an increase from 339,232 culled wild boar during 1993/94 to 644,239 during 2012/13 (Deutscher Jagdverband 2014c). Von Rüden (2006) has shown that since the 1980s a six-fold increase has taken place in Germany, in particular in the federal state of Rhineland-Palatinate.

2.1.2.3 Habitat structure

The occurrence of wild boar depends on a multitude of factors, such as climate, food supply, and safety (Briedermann 2009). Wild boar are highly adaptable to different habitats and there are few conditions that are completely unsuitable for them (Keuling 2010a, 2013). However, mixed hardwood forests can be considered as their preferred habitat, since the fruit of the oak and the beech (i.e. the mast) is their main food (Meynhardt 1989, Hennig 2007, Briedermann 2009). Another factor, which is essential for the survival of wild boar, is the presence of swampy areas and pools, which mainly serve the body care. The sensory organs of wild boar are adapted to undergrowth and scrub land. This allows them to be active at night and to colonize habitats other than forests. For example, reeds have been a natural habitat of wild boar for a long time (Keuling 2013). Today, wild boar have spread increasingly across agricultural landscapes because many fields provide shelter throughout the year. As obligate omnivores, wild boar use grassland close to shelters in their search for food (especially earthworms and insect larvae) (Keuling 2013).

The distribution of wild boar stretches from areas with cold winters to warmer areas, whereby investigations in Norway (Rosvold and Andersen 2008) suggest that the establishment of *Sus scrofa* is mainly limited by food availability and not by climate, although the climate might by a limiting factor through its effect on the food supply. Briedermann (2009) also points out that the habitat choice of wild boar is strongly influenced by food supply. Moreover, shelter, water and rest areas are important habitat criteria. In Switzerland wild boar occur on high mountain pastures

with an altitude above 2000 m (Anczikowski 2009). In summary, wild boar can be found in forest, swamp and well-structured field landscapes, as well as areas with water and reeds, whereby their preferred habitats are deciduous and mixed forests with high proportions of oak and beech, meadows and marshy areas (Briedermann 2009).

2.1.2.4 Feeding habits

Wild boar are omnivorous consuming both plant and animal food, which is reflected in their teeth pattern and by their digestive organs (Briedermann 2009). The major part of their recorded food is plant material (93%), while only approximately 6% of their diet consists of animal material (Anczikowski 2009). The nutritional components of wild boar can basically be summarized into four groups (Meynhardt 1989). The first group comprises underground, plant-based food, such as roots, tubers and onions. To get this food, wild boar plough through the ground using their stable wedge-like head, their large canine teeth and their strong neck muscles (Hennig 2007). Social groups of wild boar, referred to as "sounders" (see Section 2.1.2.6), are able to plow up to several acres of forest or agricultural land within a very short time, causing great damage, particularly to cultivated areas. Green parts of plants such as different clover, grass and herbaceous species belong to the second nutritional group, which forms a large part of the overall food intake and is required to fulfill their essential needs of vitamin A (Hennig 2007, Briedermann 2009). Fruits and berries belong to the third group. This group includes two main components, acorns and beechnuts, which have a high nutritional value. Many field crops, including corn, peas, beans and potatoes, are also very popular, causing considerable losses to farmers. In addition, windfall and wild fruit belong to the third nutritional group. Corn has a special significance, since it is used as bait for hide hunting as well as to distract boars away from cultivated fields. Animal food forms the fourth group, which serves a source of the high vitamin B12 demands of wild boar. Insects (particularly pupae and larvae), earthworms, reptiles, small rodents, young game animals, but also clutches of ground breeding birds and carrion are preferred animal

food components. Due to the diversity of the ingested food, wild boar are very flexible having the ability to almost completely change their diet to adapt to the food supply available, thus optimizing their energy resources. The extraordinary adaptability of wild boar is one of the main reasons for its wide geographical distribution (Briedermann 2009).

2.1.2.5 Home range

Wild boar have limited territorial behavior. Sounders as well as individual tuskers have a more or less defined home range (Hennig 2007), such that their general space usage has been described as recurring and faithful to a habitat (Keuling 2013). Important places within those ranges are several sleeping places, fixed wallows, as well as permanent or temporary fixed feeding places that are connected by regularly followed paths (Keuling et al. 2009, Keuling 2010a). Furthermore, dunging areas are important within the home range (Meynhardt 1989), as the marking of territories is olfactory as well as visual in the form of marked trees. Adult males put markings on trees using their canines and their saliva foam has an additional marking effect during the mating season (Briedermann 2009). However, the precise home range borders are not particularly marked (Briedermann 2009). The places used within the territories must meet certain requirements. For example, the sleeping-place has to be safe and be adapted to the respective climatic influences, whereby the duration of its use also depends on the food supply and the size of the home range (Briedermann 2009). The excretory areas are located close to resting and sleeping areas, and if possible also close to a wet area which must contain enough mud and clay for exhaustive wallowing. This behavior serves for body care, assists with thermoregulation on warm days and provides protection from insects and parasites (Barrios-Garcia and Ballari 2012).

According to the descriptions of Meynhardt (1989), Stubbe et al. (1989) and Briedermann (2009) significant correlations between the size of the home ranges and factors such as habitat quality and structure, food supply and shelter or resting areas exist. Consequently, the action space of wild boar is highly variable and can

differ from region to region. Other aspects influencing the home range of sounders are the prevailing population structure in terms of its age composition and the experience of the leading adult female, who plays an essential role in territorial behavior (Sodeikat and Pohlmeyer 2003). Investigations using marked wild boar have shown that sounders under favorable conditions inhabit a home range of 500 to 1000 ha (Stubbe et al. 1989, Keuling and Stier 2009a). More recent telemetry studies came to a similar conclusion, but partially showed home ranges even larger than 1000 ha (Sodeikat and Pohlmeyer 2003, Keuling et al. 2008a, 2009, Keuling and Stier 2009a). In areas dominated by woodland, the home range is usually larger, with an approximate average of 800 ha, than in agricultural landscapes where an average size of about 500 ha can be assumed (Keuling and Stier 2009a, 2009b, Keuling et al. 2009, Keuling 2010a, 2013). Fears have been expressed that sounders are driven apart during hunts and that this could possibly lead to large wild boar migrations supporting the spread of wildlife diseases (von Rüden 2006). However, investigations showed that sounders under the influence of hunts do not exceeded the limits of their home ranges and that shortly after the hunts they can be found around the center of their main home range (Sodeikat and Pohlmeyer 2003). However, an important prerequisite for small escape distances and permanent home ranges of hunted sounders is that the social structure with a leading female wild boar remains intact during the hunt (Sodeikat and Pohlmeyer 2003).

Within their home range, sounders show territorial behavior which is lost once the leading adult female is killed. In this case, the lead of the sounder can be taken over by sub-adult or adult individuals, which possess less experience causing an increased and partially uncontrolled group activity that sometimes leads to the loss of the territory (Sodeikat and Pohlmeyer 2003). Moreover, a significant difference in the home range sizes of male and female wild boar has been described by several studies (Genov and Ferrari 1998, Keuling and Stier 2009a). Females are more faithful to their habitat than males, which could be explained through the reclusive lifestyle of tuskers (Anczikowski 2009). When the population density increases excessively juvenile male boars often travel vast distances until they find an appropriate

home range, because they are expelled by older males during the mating season (Briedermann 2009). Older adult wild boar often use the same sleeping-place over several months, which speaks for a relatively small home range (Hennig 2007). However, there have been observations that individual animals sometimes travel very long distances that can reach up to 250 km (Meynhardt 1989). The territories of sounders can overlap at certain places, such as at fields or mast-providing forests, as well as at wallows or game passes. In general, these common places will be shared peacefully, although literature considers a minimum distance between sounders that lies below 50 m as a cause for potential conflicts (Briedermann 2009).

2.1.2.6 Social behavior

Normally, wild boar are predominantly diurnal animals, but increasing disturbances, for example, human sport and recreational activities, as well as the increasing pressure through hunting, have forced them to become active at twilight and during the night (Weindl 2014). Thereby, wild boar are very social living animals. Only older males live alone most of the time, while all other individuals live in a family association, which consists of adult females and their offspring. Only closely related individuals form a sounder, while strangers are not, or only on rare occasions, accepted (Meynhardt 1989, Hennig 2007, Briedermann 2009). Both olfactory and acoustic signals are used for communication among the members of the sounder (Hennig 2007). There is also a strict hierarchy, which is usually maternally headed by a single adult female (Briedermann 2009). This order synchronizes all daily activities within the sounder, such as, for example, the search for food and resting places, as well as the time of ovulation of female members (Happ 2012). Only newborns are initially excluded from the hierarchy, with an integration beginning when they are about 3 to 4 months old (Meynhardt 1989). At an age from seven to thirteen months the juvenile pigs must compete in hierarchic encounters to fix their ranking. After this process, male juveniles basically hold the lowest ranks and are cast out of the family at the age of about 18 months (Meynhardt 1989). Often, they form their own sounders for a short time, until they finally split and live as solitary animals

(Weindl 2014). However, they return to the sounder for a limited time for the purpose of mating. The loner life of male wild boar protects the genetic diversity and inhibits inbreeding within the sounder (Anczikowski 2009).

The size of a sounder depends on season, food supply, population density and growth (Briedermann 2009). Consequently, there are small sounders from 2 to 4 wild boar, moderate ones with up to 15 individuals and large sounders with a maximum size of 30 to 40 animals. However, sounders split up once they reach a size of more than about 30 animals (Meynhardt 1989). Additionally, changes in the social association or in the hierarchy can lead to a division of the sounder. Such changes occur, for example, at the death of the leading female through hunting or disease. Under good environmental conditions and through good development, young boar can become sexually mature at an age of six months. Thus, they can already participate in their first year of life in reproduction and have their own offspring at an age of 13 to 14 months (Gethöffer 2005). This phenomenon can increase the number of wild boar dramatically leading to so-called rejuvenation of the population (Anczikowski 2009). Moreover, the leading female wild boar is unable suppress the heat of subordinate females or to synchronize the heat of the whole wild boar population. Therefore, the birth of squeakers at unusual times is caused by early-maturing juvenile females that come into heat at unusual times (Keuling 2013).

The mating season of wild boar occurs primarily in the months of November, December and January (Meynhardt 1989). Although (Briedermann 2009) observed longer mating periods, from October to May, the fertilization rate was highest during the winter months. The gestation period varies between 112 and 120 days and is equivalent to that of the domestic pig (*S. scrofa domesticus*) (Stubbe and Stubbe 1977, Heck and Raschke 1980, Meynhardt 1989). Similar to the variation in the mating season, the cubbing season shows a wide timeframe from November to August, with a core timespan between March and April (Briedermann 2009). Investigations have revealed that on average 80 to 100% of the juvenile and adult females take part in reproduction, while only 35% of the females younger 1 than year participate

(Stubbe and Stubbe 1977, Briedermann 2009). However, recent studies indicate the important role of younger females (< 1 year) for population growth (Bieber and Ruf 2002, Gethöffer 2005). The number of squeakers born per female varies between 1 and 10, whereby the litters of young females are always smaller than those of older ones (Meynhardt 1989). The ecological lifespan of wild boar lies between 8 and 10 years. Nevertheless, the average life expectancy is 18 to 25 months, which is due to the very high mortality during the first 2 years of life (Briedermann 2009). Because of their high adaptability and intelligence, the lack of predators and abundant food supply, wild boar have the highest rate of reproduction of our domestic game animals, with a prenatal growth rate of 260% minus a postnatal mortality rate of 15% leading to an average population increase of about 220% each year (Keuling 2013). The impact that these large numbers have on the animal community structure and on ecosystem function have been recently reviewed by Barrios-Garcia and Ballari (2012).

2.1.2.7 Parasites

In addition to tick infestations (see Section 2.2.3), a large variety of other ectoparasites have been reported on wild boar (Briedermann 2009). *Haematopinus apri* lice, which occur specifically on wild hogs, do not infest domestic pigs. They are distributed worldwide. However, the average prevalence varies regionally from 90% in Asia to under 10% in Central Europe (Kadulski 1974, Briedermann 2009, Fois et al. 2012). It is assumed that the lice predominantly infest sick boar, as well as young individuals that have a thinner skin in comparison to older individuals (Brütt 1955, Briedermann 2009). The clinical symptoms caused by *H. apri* are restlessness and skin injuries. The louse *Haematopinus suis* was found on wild boar in Corsica with the highest prevalence in spring (50%) followed by summer (18%) and autumn (14%).

A further ectoparasite found on wild boar, and on pigs in general, is the strongly host-specific mite *Sarcoptes suis* (Briedermann 2009) which causes sarcoptic

mange. This species also has a worldwide distribution and has been reported to occur in several other wild animal species (e.g. red deer, roe deer) in Central Europe (Pence and Ueckermann 2002). Reports of sarcoptic mange in wild boar mainly come from game enclosures (Duscher 2006, Briedermann 2009). Usually sarcoptic mange starts with a strong skin irritation and itching, then produces lesions with exudates that dry to crusts. It can lead to emaciation in serious cases (Briedermann 2009).

Endoparasitic infestations of wild boar are common and include protozoa (e.g. *Toxoplasma gondii, Sarcocystis suicanis, S. suihominis, Eimeria debliecki*, *E. sabra* and *E. polita*), trematodes (e.g. *Fasciola hepatica*, *Dicrocoelium dentriticum*, *Agamodistomum suis*), tapeworms (e.g. larval *Taenia hydatigena*, *Taenia solium L.*) and nematodes (e.g. *Ascaris suum*, *Ascarops strongylina*, *Physocephalus sexalatus*, *Globocephalus* spp., *Metastrongylus* spp., *Trichuris suis*, *Trichinella* spp.). Duscher (2006) and Briedermann (2009) have described the life cycles and clinical symptoms caused by these endoparasites. The first finding of the tapeworm *Echinococcus granulosus* in wild boar was made only quite recently in Romania with 45.5% of the worms identified as G1 and 39.4% as G7 genotypes (Onac et al. 2013). In a single boar from Switzerland the larvae of *Echinococcus multilocularis* was identified (Stephan et al. 2001). The infestation of wild boar with *Trichinella* spp. is monitored across Germany, since these parasites can cause a dangerous trichinellosis in humans who consume raw meat (Nöckler et al. 2006). Between 1991 and 2004 more than 3.7 million wild boar were analyzed for *Trichinella* spp. in Germany showing a mean infestation rate of 0.005% (Remde 2008). Although this prevalence seems to be relatively low, Nöckler et al. (2006) detected more than 900 *Trichinella* larvae per gram in the diaphragm of a single boar confirming that wild boar are a possible source of infection for humans.

The intestinal worm *Globocephalus urosubulatus* was found in wild boar in Corsica (Foata et al. 2006) with a prevalence between 30% and 70%, as well as in the Iran with a mean infestation rate of 74% (Eslami and Farsad-Hamdi 1992). The study

from Corsica also showed that adult *G. urosubulatus* were predominantly found during spring, while *Ascaris suum* was more frequent in winter (Foata et al. 2006). *G. urosubulatus* was significantly more frequent in wild boar older than one year (43% vs. 15%), whereas *Metastrongylus* sp. showed significantly higher infection rates in younger hosts (94% vs. 6%) (Foata et al. 2006). In Iran, in addition to *G. urosubulatus*, wild boar was also infested with *A. suum*, but at a low prevalence (5%), while gastrointestinal nematodes (e.g. *Ascarops strongylina*, 56% and *Physocephalus sexalatus*, 56%), lungworms (*Metastrongylus* spp., 14-16%) and the liver fluke *Dicrocoelium dentriticum* (21%) showed higher infection rates (Eslami and Farsad-Hamdi 1992). A study from Estonia reported that lung nematodes were the predominant helminths discovered in wild boar with a prevalence of 82%, whereby a significant negative correlation between wild boar body mass and the number of lungworms was determined (Järvis et al. 2007). *Ascarops strongylina* (87%) and *Metastrongylus* spp. (85%) were the most common helminths in Spain, whereby for the latter species the infection rate was greatest in wild boar younger than one year (de-la-Muela et al. 2001). In southwestern Spain, lung nematodes had a prevalence of 41.1%, with *Metastrongylus apri* (71.4%) also being the most frequent (García-González et al. 2013). Thereby, the infestation intensity and the prevalence were also higher in young wild boar, as well as in areas of higher altitude and with higher precipitation. A sex-biased lungworm parasitism was not detected (García-González et al. 2013). Similarly, studies in Germany showed that *Metastrongylus* spp. were most frequent (100%), followed by gastrointestinal nematodes (e.g. *G. urosubulatus*, 95.6% and *Physocephalus sexalatus*, 73.3%) (Walburga 1989, Barutzki and Richter 1990, Epe and Spellmeyer 1997). In contrast, in France, the prevalence of stomach nematodes (97%) was slightly higher than that of lung nematodes (92%), although a significantly higher nematode intensity was reported for young wild boar, similar to the Spanish studies (Humbert and Henry 1989). The development of parasites in the lungs and in gastrointestinal tract can be one of the main causes for increased mortality rates of young wild boar (Jezierski 1977).

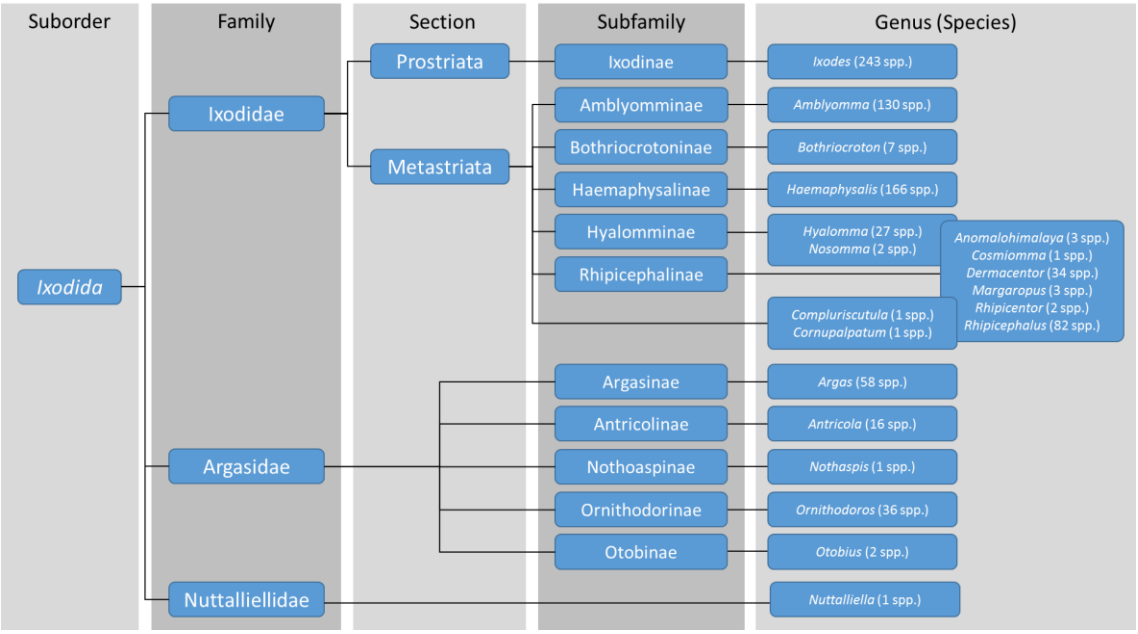


Figure 2.4: Tree visualizing the taxonomy of ticks (Ixodida) including the 3 families, all genera and the corresponding number of species in accordance with Guglielmone et al. (2010).

2.2 Ticks (Ixodida)

2.2.1 Taxonomy

Ticks belong to the class of Arachnida and form together with mites, the subclass of Acari. Ticks are ectoparasites living on the surface of the host. They belong to the order of Anactinotrichidea (Parasitiformes) and can be subdivided into 3 families (Oliver 1989, de la Fuente and Kocan 2006) which constitute the suborder Ixodida (Metastigmata): the Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae (see Figure 2.4). The family of Nuttalliellidae comprises only a single species, *Nuttalliella namaqua,* which so far has only been found in southern Africa in the areas from Tanzania to Namibia and in South Africa (Horak 2009, Sonenshine and Roe 2013a). This tick shares many features with the Ixodidae (e.g. anteriorly extending mouthparts and a dorsal shield) and the Argasidae (e.g. overall body structure). A recent study (Mans et al. 2011) has described *N. namaqua* as the closest living relative to the ancestral tick lineage. They indicate that it forms the evolutionary missing link between the other two tick families. The family of soft ticks includes approximately 5 genera (this is an area of some confusion) and approximately 190 species.

In contrast to soft ticks, the family of hard ticks contains 14 genera with approximately 700 species/subspecies and thus forms the largest of the 3 families (Guglielmone et al. 2013). Hard ticks are distinguished from soft ticks by the presence of a scutum, i.e. a hard shield. They comprise two sections: the Prostriata and Metastriata. The section Prostriata includes only the genus *Ixodes*, the members of which possess an anal groove that extends anterior to the anus, in contrast to other ixodid ticks when the groove lies posteriorly (Petney et al. 2012). The section Metastriata comprises all other ixodid tick genera (Nava et al. 2009). The tree in Figure 2.4 summarizes the taxonomy and shows the number of tick species belonging to each genus (Guglielmone et al. 2010).

2.2.2 Distribution

Ticks have adapted to all kinds of ecosystems, including tropical rainforests, wet grassy landscapes, deserts and oceanic islands. They are found from both polar areas, through the temperate zones into the tropics (Dautel and Kahl 1999, Barker and Murrell 2004, Dautel 2010). The distribution of ticks depends on both biotic and abiotic factors (Dautel 2010). Ticks react very sensitively to climatic changes: low temperatures inhibit their development from egg to larva, while a certain amount of humidity is required for them to molt successfully to the next life history stage. Moreover, there exist both minimum and maximum temperatures that are lethal for ticks and which influence their distribution (Dautel 2010). Consequently, three fundamental requirements are essential for an ecosystem to support ticks: (1) a temperature not lethal to ticks, (2) a high enough relative humidity allowing ticks to stay hydrated and (3) a host population density that is high enough.

In Germany 17 tick species have been described (Liebisch and Rahman 1976, Petney et al. 2011), whereby most of the species belong to the family of Ixodidae occurring within 5 genera: *Ixodes, Dermacentor*, *Haemaphysalis*, *Hyalomma* and *Rhipicephalus* (Petney et al. 2011, Rubel et al. 2014). A recent map of georeferenced tick findings recorded by Rubel et al. (2014) in Germany is shown by Figure 2.5. Ticks are

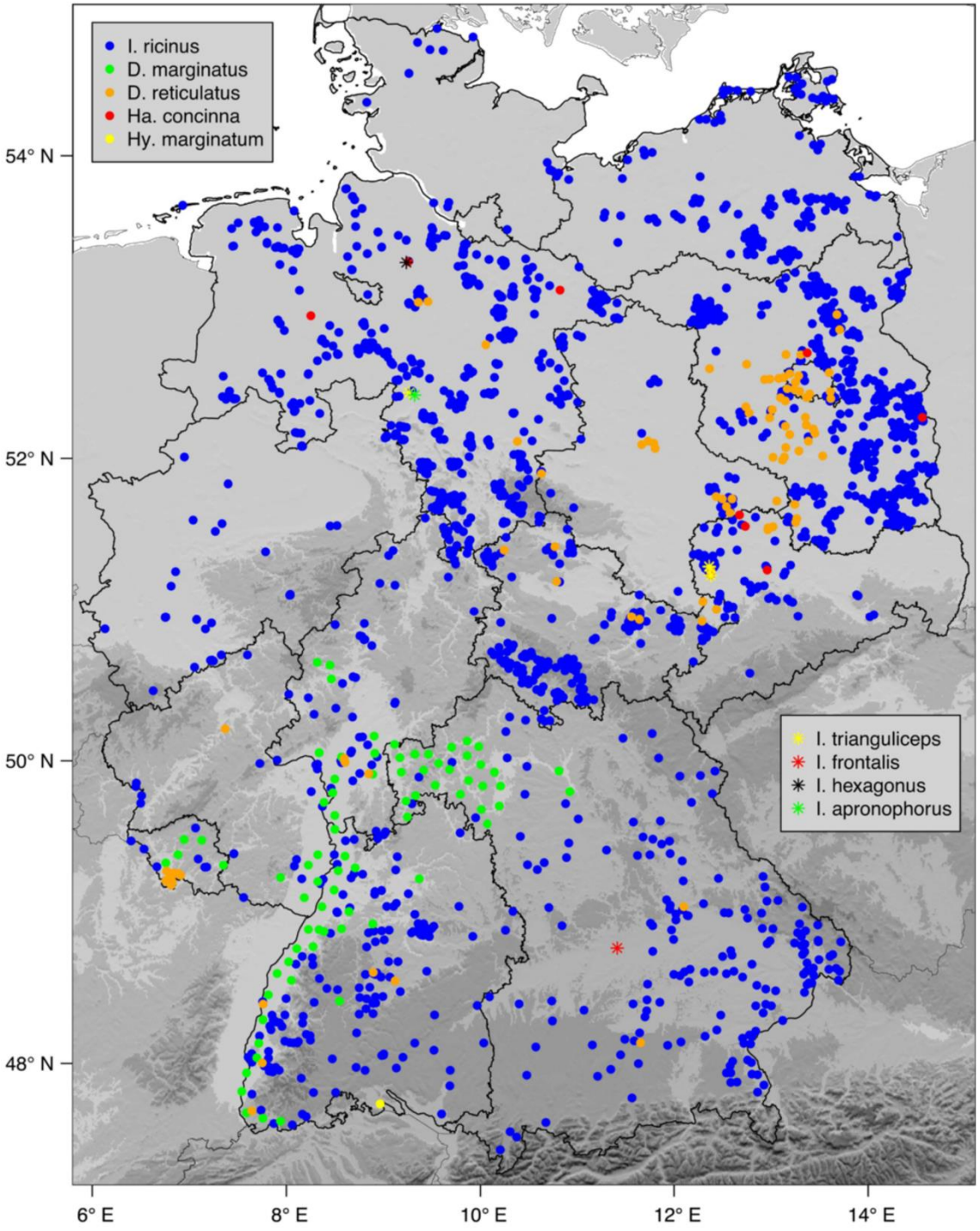


Figure 2.5: Map of georeferenced locations at which hard ticks were found in Germany (from Rubel et al. 2014).

occasionally imported from other countries, e.g. the brown dog tick, *Rhipicephalus sanguineus*, which occurs on dogs and comes from the Mediterranean into Germany (Rubel et al. 2014). However, as far as we know, these ticks are not able to survive

the cold temperate winters of Central Europe (Kimmig et al. 2010). In addition, ticks can be transported by migrating birds between different areas (Elfving et al. 2010, Plokarz 2010).

2.2.3 Ecology

Ticks feed on blood of vertebrates (Walter and Proctor 2013). While soft ticks take several relatively small blood meals over a short period of time (minutes to hours), hard ticks only feed once per life history stage (larva, nymph and adult adults: males of the genus *Ixodes* do not require a blood meal before mating) taking in a large amount of blood over several days. Most of the hard ticks are heteroxenous ticks and therefore need blood meals from different hosts to complete their developmental cycle (Wilson 1994). During each feeding ticks can deliver pathogens to and receive them from the hosts. Within the blood sucking arthropods, ticks transmit the largest variety of pathogens, including viruses, bacteria and protozoa (Aspöck 2008).

Crucial for the active host selection process of ticks is the ambient temperature. Like other arthropods, ticks have developed various mechanisms to adapt to daily and seasonal temperature fluctuations (Dautel 2010). This is in particular true with respect to phases of dormancy, which are used by ticks to survive unfavorable circumstances, i.e. extreme heat or coldness (Dautel 2010).

Some tick species are strictly host-specific and are only found on certain wild mammals and birds or in their dens and nests (Liebisch and Rahman 1976, Petney et al. 2011). For example, *Ixodes vespertilionis* feeds only on bats (Siuda et al. 2009), while other species like *Ixodes arboricola* are more specialized for birds (Schilling et al. 1981). In contrast to this, the most common European species, *Ixodes ricinus*, has no specific spectrum of hosts and infests more than 300 vertebrate species ranging through reptiles and birds to mammals (Baranton and De Martino 2009, Hubálek 2009). Because of its polyphagy, *I. ricinus* plays a central role in the propagation of *Borrelia* spp. and of other tick-borne pathogens (Faulde and Hoffmann 2001, Stanek 2005, Aspöck 2008, Reis et al. 2011).

2.2.4 Morphology of the Ixodidae

The dorsoventral flattened body of hard ticks is structured into 3 major regions: (1) the capitulum with its mandibles, (2) the body (the idiosoma), and (3) the legs (Sonenshine and Roe 2013b). The capitulum contains the basis capituli connecting the capitulum with the main body of the tick, as well as the mandibles that consist of the external pedipalps, a pair of chelicerae and the hypostome, which is immovable and covered with barbs anteriorly (Hillyard 1996, Sonenshine and Roe 2013b). The tick body is usually subdivided into an anterior, i.e. the podosoma, and a posterior region, i.e. the opisthosoma (the abdomen). The anterior region carries 4 pairs of walking legs and the genital pore, while the opisthosoma comprises the anal aperture and the spiracular plates. In contrast to adult hard ticks, larvae have only 3 pairs of legs (Petney et al. 2011). The tarsus of the two most anterior legs contain Haller's organ, which is an important sensory organ helping ticks to recognize heat, odors and other environmental factors (Petney et al. 2011, Sonenshine and Roe 2013b). All ixodid ticks have the characteristic hard, sclerotized plate, the scutum, which is important for species determination (Petney et al. 2011). For male hard ticks the scutum covers the whole idiosoma, whereas for females, nymphs and larvae the scutum is smaller and occupies only the anterior third of the dorsal surface.

The body of a male *Ixodes ricinus*, which is about 2.6 mm, appears in a uniform black brownish color. In comparison to females, males have seven sclerotized plates on the ventral side. The idiosoma of unfed *I. ricinus* females is usually reddish, but can vary from brown gray to beige after a blood meal. During feeding the body length of an *I. ricinus* female can grow from about 3.3 mm under unfed conditions up to 1.1 cm (Hillyard 1996). Males have a size of about 2.6 mm, and nymphs of approximately 1.4 mm. The larvae of *I. ricinus* are yellowish-translucent and have an approximate size of 0.5 mm in an unfed state. For adult *Ixodes* spp. the genital aperture lies in the anterior third, between the coxen of the fourth pair of legs (Hillyard 1996). Nymphs and larvae possess no genital aperture und are therefore not sexually differentiable. The life history stages of *I. ricinus* are displayed in Figure 2.6.

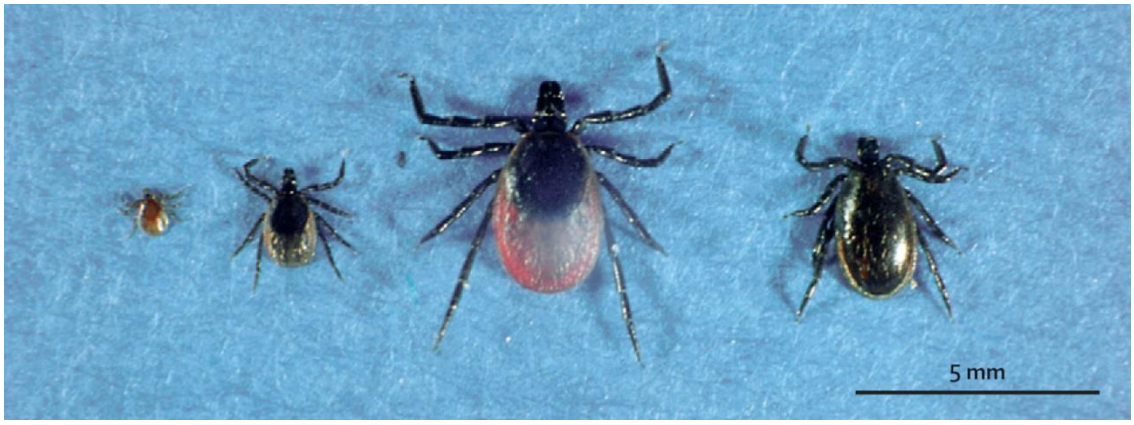


Figure 2.6: Comparison of *Ixodes ricinus* life history stages showing a larva (left), a nymph (second from the left), an adult female (second from the right) and an adult male tick (Stanek et al. 2012)

Figure 2.7: Comparison of adult female (left) and male (right) *Dermacentor marginatus*. The image has been acquired by Dragiša Savić (http://www.naturefg.com).

Adult *Dermacentor marginatus* (Figure 2.7) are about 3.5 to 5 mm long and the sexes show conspicuous differences in their appearances. The full-shielded males have a shimmering silver, dark grey and reddish coloring, while the partly shield-covered females appear in a brown to reddish color. Nymphs in an unfed state are approximately 1.6 mm long and 0.9 mm wide, while their shield covers about one-third of the body. Fully engorged females can reach a length of up to 1.5 cm. The larva of *D. marginatus* are about 0.75 mm long and about 0.5 mm wide, while their back shield covers approximately the half body (Mitrea et al. 2008).

2.2.5 Distribution and ecology

2.2.5.1 Ixodes ricinus

Ixodes ricinus is the most common tick species in Central Europe (Gern and Humair 2002, Stanek 2005, Aspöck 2008). It constitutes more than 90% of the overall tick fauna (Kimmig et al. 2010) and is the most commonly studied species (Petney et al. 2012). The geographic range of *I. ricinus* is related to temperate climate, which extends from the Atlas Mountains in North Africa to a latitude of about 65° north in Scandinavia and from Portugal to a longitude of 60° east in Central Asia (Hillyard 1996, Petney et al. 2012) , although there is some indication that the North African specimens may represent a sister species (Estrada-Peña et al. 2013). The eastern distribution area overlaps with the range of the near relative *Ixodes persulcatus*. Within this geographic range the local occurrence of *I. ricinus* over the whole year is restricted to regions with high humidity (Donnelly 1987). The range with respect to altitudes has expanded from 700 m at the end of the 1970s to over 1100 m after the turn of the millennium (Dautel 2010). Moreover, the distribution of *I. ricinus* seems to be expanding further northwards (Lindgren et al. 2000). In Germany, *I. ricinus* occurs in all states, where it can also be found within city areas (Petney et al. 2012). A recent map depicting the habitat suitability (see Estrada-Peña 1999) for *I. ricinus* in Europe and in the western parts of Asia is provided in Figure 2.8.

Like all other hard ticks, *I. ricinus* passes through multiple development stages, from egg over larva and nymph to adult tick (see Figure 2.9) and uses a passive strategy for finding hosts (Sonenshine and Roe 2013a). Thereby, host-seeking ticks lie in wait ("questing phase") on the vegetation, e.g. at the tip of grass stalks. During questing the ticks stretch out their front legs (Sonenshine and Roe 2013a). Situated on the front legs is Haller's organ, a small dent with sensory hairs, which ticks uses to detect mechanical, thermal and chemical stimuli. Movement and carbon dioxide emission of the hosts are recognized (Sonenshine and Roe 2013a). Once a host passes by, the tick releases the grass stalk, moves onto the host and searches its body for a suitable attachment site. Depending on the life history stage, the period that ticks stay attached to the host varies from 2 to 3 days for larvae, 4 to 5 days for nymphs and 7 to

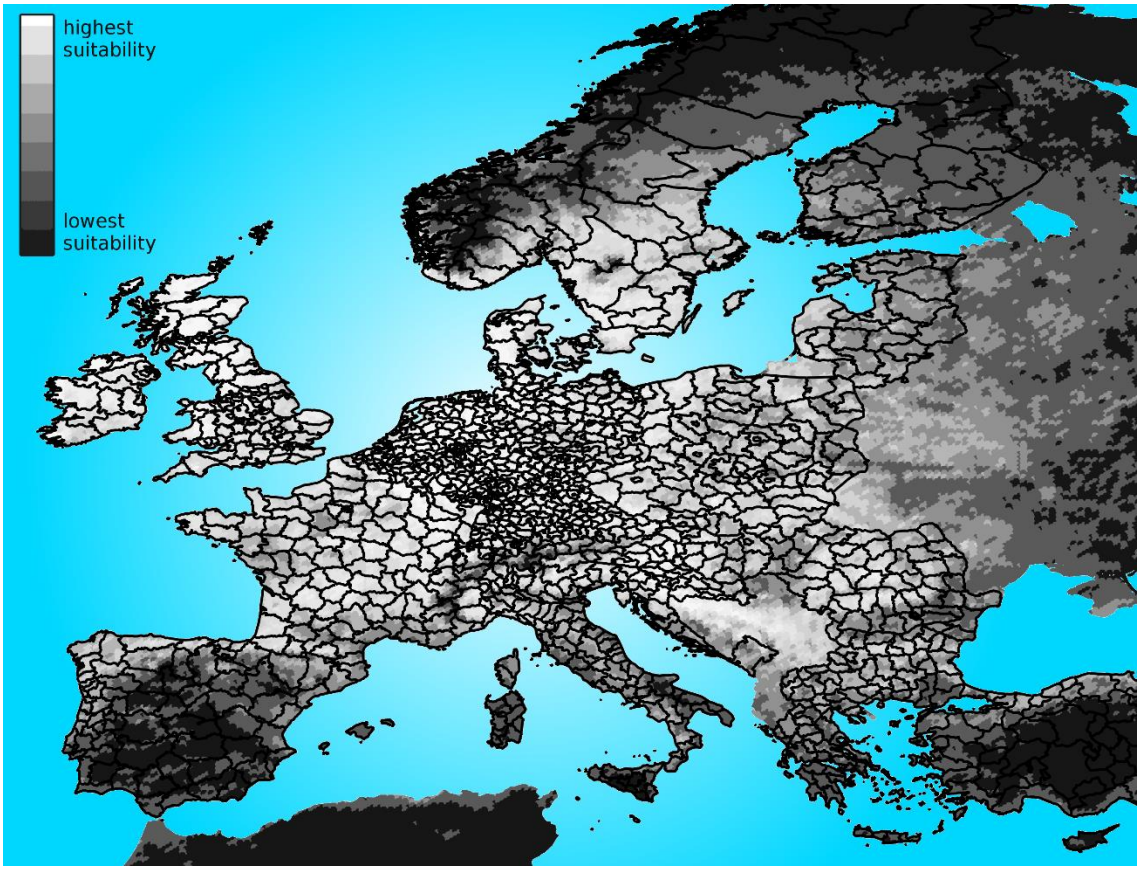


Figure 2.8: Map of the predicted spatial distribution of *Ixodes ricinus* in Europe. Habitat suitability is mapped as a range of grey values from dark (low suitability) to light (highest suitability). Black lines represent the NUTS administrative divisions. The map has been provided by Agustín Estrada-Peña and was generated from data of 2014.

9 days for adult females (Sonenshine and Roe 2013a). Mating ticks can be observed on the vegetation or on the host. After females are fully engorged, they detach from the host and can deposit between 2000 and 3000 eggs within the vegetation where they then die (Hillyard 1996). Larvae and nymphs detach from the host and molt on the ground within a few months to become nymphs and adult ticks, respectively. Each phase requires about 1 year to complete (Gray 1991). Therefore, the complete development cycle of *I. ricinus* usually takes over 3 years in natural surroundings. However, this period can vary between 2 and 6 years depending on the geographical location and on the associated climatic conditions (Gray 1991). Each life history stage can survive up to 2 years off-host (Brunnemann 2010).

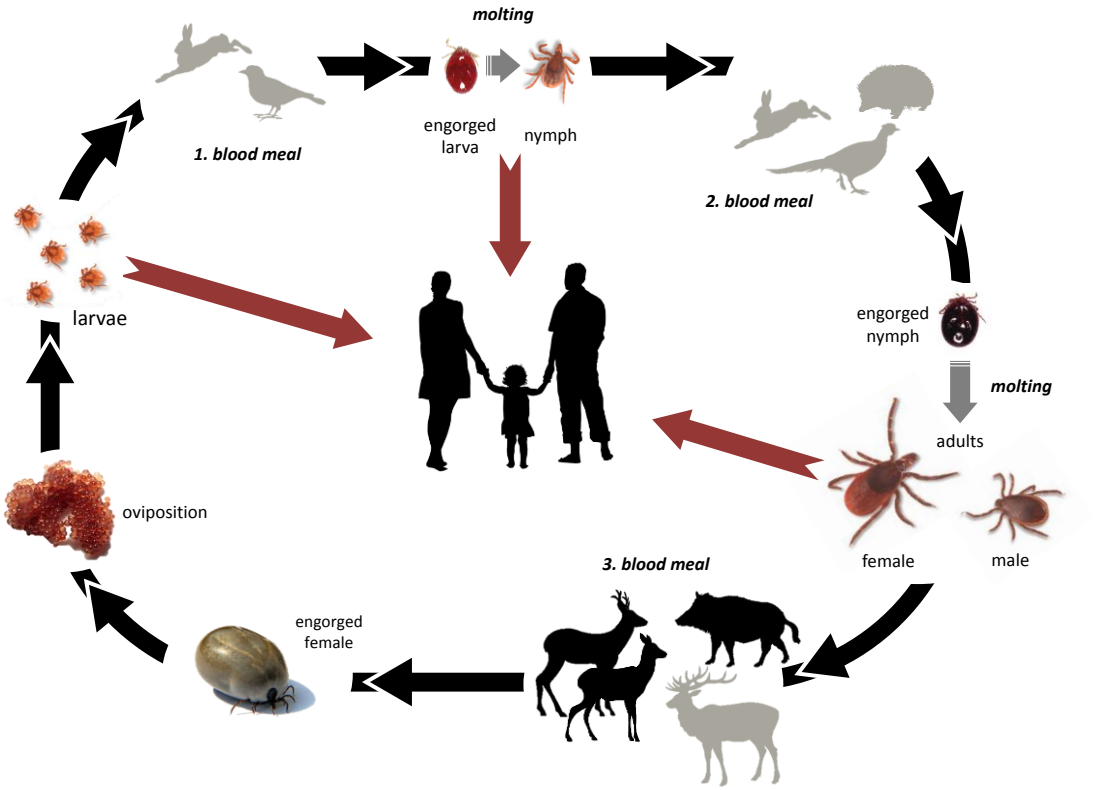


Figure 2.9: The development cycle of *Ixodes ricinus* in relation to the important host species serving for blood meals at each life history stage. Black host species (roe deer and wild boar) are studied within this thesis.

Although more than 300 vertebrate hosts are known for *I. ricinus* (Stanek 2005), the three life history stages prefer different hosts and seek their hosts at different heights within the vegetation (Mejlon and Jaenson 1997). Larvae are found near the ground, which is why they predominantly parasitize smaller mammals (Skuballa 2011). In a typical woodland habitat mice are the most important hosts for larvae. Nymphs wait on tall grasses and low ferns at an approximate height of 10 to 20 cm for their hosts and therefore can be found on a wider variety of vertebrate species, such as hedgehogs and squirrels, as well as on even larger mammals (Skuballa 2011). Under optimal conditions adult ticks can climb up to 100 cm (Mejlon and Jaenson 1997) and their horizontal movement is no more than 20 cm (Healy and Bourke 2008). Deer in general, and in particular roe deer in Europe, are important for adult females as they supply sufficient quantities of blood for egg production. Therefore, large deer hosts maintain and support the size of the tick population

(Alberdi et al. 2000, Stanek 2005, Vor et al. 2010, Handeland et al. 2013, Pacilly et al. 2014). A detailed review underlining the importance of roe deer as host for *I. ricinus* can be found in the thesis of Overzier et al. (2013). Conversely, the exact role of wild boar in the life cycle of *I. ricinus* is still unclear (Sprong et al. 2009, Pacilly et al. 2014).

I. ricinus prefers biotopes with humus-rich and slightly sour soils, which are characterized by an herbaceous vegetation with a cover of dead leaf and plant matter (Kurtenbach et al. 1995). Conifer, broadleaf and mixed forests with a large amount of undergrowth und near-ground vegetation provide an ideal habitat for their ticks. Additionally, these habitats constitute an outstanding biotope for *I. ricinus* hosts, such as roe deer, wild boar and small mammals (Gray 2002, Stanek 2005).

In general, the micro climate directly at ground level, in particular the high relative humidity, differs significantly from the areas predominant macro-climate. This micro-climate is essential for the activity and the survival of host-seeking ticks (Daniel and Dusbabek 1994, Perret et al. 2000). *I. ricinus* strongly depends on humidity and is only able to survive in biotopes in which the relative air humidity at the base of the vegetation is seldom below 85% (MacLeod 1935, Gray 2002). Within continental Europe, 2 characteristic seasonal abundance maxima (bimodal activity pattern) have been observed for adult and nymphal *I. ricinus*: the first seasonal peak lies between March and June, also called “spring peak”, and the second less intense peak occurs between August and October, also known as the “autumn peak” (Donnelly 1987, Kurtenbach et al. 2006). In some regions, for example the south of England and Ireland, the activity pattern can also be unimodal with only a single peak (Kurtenbach et al. 2006). Besides the relative humidity, the temperature also determines the seasonal activity and the abundance of host-seeking ticks. The lethal temperature for *I. ricinus* lies below -15 °C and above 30 °C (Dautel 2010), whereby a considerable increase of tick activity has been registered for daily air temperatures above 10 °C (Randolph et al. 2002). For eggs and larvae temperatures below -7 °C cause death after a few days (Lengauer 2004). The survival rate of *I. ricinus* depends

largely on the individuals' energy reserves. These are tightly coupled with water reserves which need constant renewal near to the ground to prevent the ticks from dehydrating (Perret et al. 2003).

2.2.5.2 Dermacentor marginatus

So far, *D. marginatus* has been reported in France, Germany, Hungary, Italy, Morocco, North Africa, Poland, Slovakia, Spain, Switzerland and former Yugoslavia as well as from East to South and Central Russia up to Western Siberia (Arthur 1960, Nosek et al. 1967, Darvishi et al. 2014). It was also found in northwestern provinces of Iran (Nabian et al. 2008). Evidence suggests that *D. marginatus* has been imported by the transportation of dogs from Mediterranean countries or Portugal to Central European countries in the mid-90s (Glaser and Gothe 1998). In Germany, findings of *D. marginatus* have been reported from Baden-Württemberg, Bavaria, Hesse, Rhineland-Palatinate and the Saarland (Petney et al. 2012). Recent georeferenced findings (Rubel et al. 2014) of *D. marginatus* in Germany are displayed in Figure 2.5. Thereby, *D. marginatus* is found up to heights of 3,500 m above sea level with a preferred range between 800 and 1,000 m (Estrada-Peña et al. 2004a, Selmi et al. 2009).

D. marginatus it a three-host tick and an entire life cycle takes one year under natural and 92 to 163 days under laboratory conditions (Nosek et al. 1967, Estrada-Peña et al. 2004a, Darvishi et al. 2014). Development of larvae takes about three weeks until they hatch during spring, as soon as suitable environmental conditions occur (Arthur 1960). The hatched larva remains some days in the litter layer and then starts to search for a host. Once a larva has found a suitable host, it feeds between 2 and 10 days depending on its age (Arthur 1960). Then it detaches from the host and molts on the ground. Once the nymphs have attached to a host, they feed 4 to 11 days, detach and molt (Arthur 1960, Nosek et al. 1967). Adult *D. marginatus* can be found on the vegetation, waiting for suitable hosts in the characteristic "questing position" at an altitude above the ground of ideally 20 to 50 cm (Arthur 1960). Females take far more blood than male *D. marginatus*, but in contrast to *I. ricinus*, male *D. marginatus* need a blood meal to become able to mate (Liebisch and Rahman

1976). After feeding males can mate with several females. Mating occurs on the host while the female is still feeding. After mating and feeding, the female detaches and finds a suitable place on the ground for oviposition (Liebisch and Rahman 1976). Adult ticks spend the winter just over the frozen ground in a cold rigidity with reduced metabolism. With increasing temperatures they become active (Arthur 1960).

Hosts of adult *D. marginatus* comprise a wide spectrum of wild animals, including roe deer, wild boar, horses, cattle and other even-toed ungulates as well as domestic animals, but in particular sheep (Petney et al. 2012). While adults prefer large mammals, the larvae and nymphs are found on mice and other small mammals (Arthur 1960, Nosek et al. 1967). *D. marginatus* attaches to domestic dogs, but is rarely found on humans (Immler et al. 1970, Liebisch and Rahman 1976). In Germany, sheep are the predominant hosts (Liebisch and Rahman 1976). In general, *D. marginatus* has a patchy distribution (Petney et al. 2012). One reason might be the lack of suitable hosts for the adult life stages in some areas (Petney et al. 2012). In Germany, the maintenance of *D. marginatus* populations is thought to depend on the presence of sufficient sheep (Liebisch and Rahman 1976).

D. marginatus has a relatively high resistance to drought (Immler et al. 1970) and cold (Dörr and Gothe 2001), but is rather sensitive to humidity (Meyer-König et al. 2001). Therefore, the species prefers dry, warm and sparsely vegetated habitats, such as bush- and grasslands (Immler et al. 1970), but it is also found along the courses of rivers where it inhabits distant, isolated areas (Liebisch and Rahman 1976). This behavior also explains the patchy distribution of *D. marginatus* (Petney et al. 2012). A Spanish study showed that the distribution correlated with various climatic and vegetation parameters, such that a relatively high Normalized Difference Vegetation Index (NDVI), a mean temperature between 14 °C and 15.8 °C and a maximum temperature around 27 °C where significant indicators for *D. marginatus* (Estrada-Peña et al. 2004b). In Slovakia, active *D. marginatus* adults prefer temperatures from 4 °C to 16 °C (Selmi et al. 2009). Adult individuals are most active

from the end of February until the beginning of May with an activity peak during March. During spring Liebisch and Rahman (1976) observed a prevalence of 100% with up to 200 ticks per sheep in several German states. A second phase of activity occurs from September to October and consists primarily of individuals that did not feed during spring or that belong to a new generation of adults (Liebisch and Rahman 1976). In Russia and Central Europe, larvae are most active during June and July, while nymphs reach their maximum abundance between July and September (Arthur 1960).

2.3 Tick-borne diseases

2.3.1 Lyme borreliosis

Lyme borreliosis, also known as Lyme disease, is an infectious disease that is caused by spirochetes of the *Borrelia burgdorferi* sensu lato species complex (see Section 2.3.1.2). These are transmitted by ticks of the genus *Ixodes* (see Section 2.3.1.5). Lyme borreliosis is the most common arthropod-borne disease in Europe where it has an important impact on public health (Wilske 2003, Rizzoli et al. 2011). The expanding geographical distribution of Lyme borreliosis is likely to become an increasingly relevant risk factor for public health in the near future. Thus, the study of the complex interactions between socio-economic and environmental influences on the ecology and epidemiology of Lyme borreliosis will become more and more important (Rizzoli et al. 2011).

2.3.1.1 Historical background

Only very recently, spirochetes-like cells were found in a tick larva that was trapped in amber some 15 to 20 million years ago, whereby a large grouping of cells showed a close resemblance to *Borrelia* (Poinar 2014). This finding suggests that tick-borne bacteria have been transmitted for millennia, potentially also in humans, for example the glacial body (Ötzi) was found to be infected with *Borrelia burgdorferi* s.l. (Keller et al. 2012). The first reporting of clinical sings of Lyme borreliosis go back

to the 19th century. In Europe, Buchwald (1883) observed an inflammatory skin lesion, acrodermatitis chronica atrophicans (ACA), also known as Herxheimer disease, which is usually related to the last stage of Lyme borreliosis. Afzelius, a Swedish dermatologist, identified the typical red rash on the skin, also known as erythema migrans (EM) in 1909 (Afzelius 1910). Further investigations (Garin and Bujadoux 1922) described radicular pains and neurological disorders that appeared after EM with many cases of chronic lymphocytic meningitis being reported (Bannwarth 1941). In the meantime, Hellerstrom (1930) had recognized a causal relation between ticks bites, characteristic dermatoses and neurological symptoms. Moreover, in 1953 Bäfverstedt (1953, 1960) reported lymphadenosis cutis benigna that is expressed by cutaneous disorders, such as swellings and stains on the skin (Lipsker and Jaulhac 2009, Brunnemann 2010), which were related to Lyme disease. The first successful treatments of EM (Hollström 1951) and ACA (Thyresson 1949) employed antibiotics (e.g. penicillin), although the infective nature of EM and ACA was not proven until 1955 (Binder et al. 1955, Götz 1955). Although the bite of *I. ricinus* was known to cause EM in Europe (Hellerstrom 1951), the real aetiology stayed unclear until arthritis and EM were found and investigated in the northeastern United States.

More than six decades after the first report by Afzelius, in the mid-1970s, an unusually clustered appearance of juvenile rheumatoid arthritis (JRA) in children, teenagers and few adults was investigated (Mast and Burrows 1976, Steere et al. 1977) in the coastal community of Old Lyme, Connecticut in the United States. Soon after the observations made in Old Lyme, a connection between skin rashes in Europe and arthritis was noted (Barbour and Fish 1993). On epidemiological grounds, a further study (Steere et al. 1978) that searched for the etiologic agent revealed that EM and Lyme arthritis are tick-transmitted diseases, with *Ixodes scapularis* as the vector in the United States, with many patients reporting tick bites preceding the disease. The isolation of spirochetes from the gut of *I. scapularis* ticks by Burgdorfer et al. (1982) first indicated the etiological agent causing Lyme borreliosis, since these bacteria showed a reaction with the immune sera from patients in the United States who had

had EM and Lyme disease. Thus, the pathogen and the vector of Lyme disease were identified at the same time. Only few months later, similar spirochetes were also isolated in Europe from *I. ricinus*, which is closely related to *I. scapularis* (Burgdorfer et al. 1983). Additional studies conducted during the same and in the following years verified the findings of Burgdorfer (Barbour et al. 1983, Steere et al. 1983, Pfister et al. 1988). In 1984, the spirochetes were named after their discoverer as *Borrelia burgdorferi* (Johnson et al. 1984). Moreover, the disease was named after the place of discovery as Lyme borreliosis (Gern and Falco 2000).

2.3.1.2 Systematics

In the context of taxonomy, the species of the *B. burgdorferi* s.l. complex are Eubacteria belonging to the order of Spirochaetales and the family of Spirochaetaceae. The order of Spirochaetales comprises three additional families: Brachyspiraceae, Brevinemataceae and Leptospiraceae (Krieg et al. 2011). However, with respect to human pathogenicity the genera *Borrelia*, *Treponema*, the agent of syphilis (*Treponema pallidum*) as well as *Leptospira*, the agent of leptospirosis (*Leptospira interrogans*) are of most importance (Porcella and Schwan 2001). The genus *Borrelia* is usually divided into 2 main groups: (1) the *B. burgdorferi* s.l. complex, which is responsible for Lyme disease, and (2) the agents of the relapsing fever (e.g. *B. duttoni*, Wang et al. 1999a, Olsen et al. 2000).

Borrelia spirochetes follow a strictly parasitic mode of life. Their lifecycle consists of phases that involve arthropods, particularly ticks, as vectors and vertebrates as host (Baranton and De Martino 2009). The *B. burgdorferi* s.l. spirochetes are vectored by ticks of the genus *Ixodes*, whereas most of the relapsing-fever *Borrelia* spirochetes are transmitted through argasid ticks of the genus *Ornithodorus*. Relapsing fever caused by *Borrelia recurrentis* is transmitted by the human body louse (*Pediculus humanus*) (Ras et al. 1996, Raoult and Roux 1999). Conversely, the relapsing-fever-related species *Borrelia miyamotoi* can also occur in ixodid ticks (Fraenkel et al.

2002, Barbour et al. 2009). In this thesis only species of the *B. burgdorferi* s.l. complex will be investigated. For this reason, and for the sake of simplicity, the used terms *Borrelia* and borreliosis refer to this complex.

When *B. burgdorferi* was discovered, it was assumed that it was a unique species (Burgdorfer et al. 1982). However, the molecular typing of a multitude of isolates from ticks, hosts and patients led to the insight that Lyme borreliosis is caused by multiple *Borrelia* species (Stanek and Reiter 2011). To date, at least 18 different genospecies have been included in the *B. burgdorferi* s.l. complex worldwide. In the northern part of the United States, *B. burgdorferi* sensu stricto is the only species that causes Lyme borreliosis in humans. Five species are clearly pathogenic for humans in Europe: *B. afzelii*, *B. burgdorferi* s.s., *B. garinii*, *B. spielmanii* and *B. bavariensis* (Baranton and De Martino 2009, Stanek et al. 2012). In comparison to the United States, the larger number of species involved could cause a wider variety of clinically detected symptoms in Europe (Stanek et al. 2012). The species *B. bissettii*, *B. lusitaniae*, and *B. valaisiana* seem to be less important pathogens, since they have been found only occasionally in patients (Stanek et al. 2012).

The term genospecies has been generally accepted over the term species in *Borrelia* research, because the different genospecies are not distinguishable by morphology and thus their descriptions are mostly based on genetic features. *B. burgdorferi* s.l. is a genetically very heterogeneous group of species. Between and within genospecies there can exist multiple strains, which can have considerably different genetic configurations. For this reason, taxonomic reorganization can happen after the initial naming of genospecies. For example, based on multilocus sequence analysis (MLST/MLSA) *B. garinii OspA*-serotyp 4 has been renamed to *B. bavariensis* (Margos et al. 2009) and a *B. bissettii* strain was renamed to *B. kurtenbachii* (Margos et al. 2010). The complex taxonomy of Lyme borreliosis spirochetes reflects their correspondence to ecotypes and thus their ecology (Margos et al. 2009, 2011).

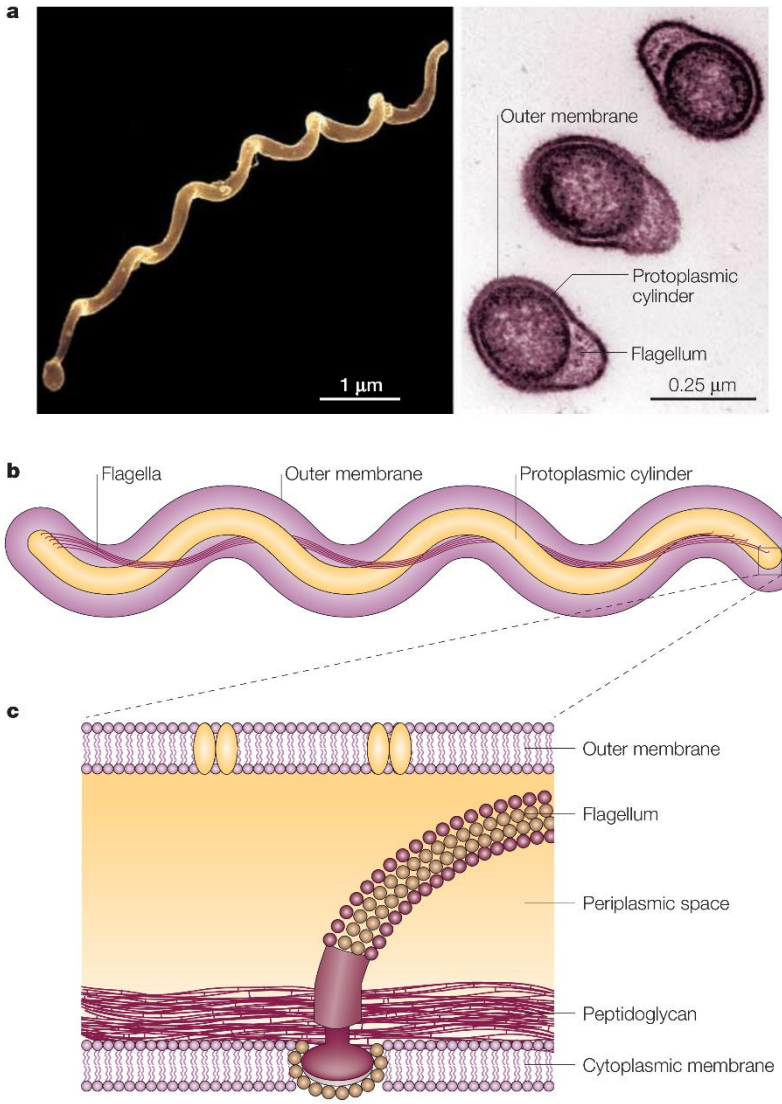


Figure 2.10: Morphological structure of *Borrelia burgdorferi* s.l. Top row (a): scanning (left) und transmission (right) electron micrographs. Second (b) and third (c) row: schematic view of the internal spirochete structure (adapted from Rosa et al. 2005)

2.3.1.3 Morphology

All species of the *B. burgdorferi* s.l. complex are gram-negative, helical-shaped bacteria (see Figure 2.10). Their length ranges from 10 µm to 20 µm, while their width varies between 0.2 and 0.5 µm (Barbour and Hayes 1986). Like other spirochetes, *Borrelia* possess an inner and outer membrane. The outer membrane encloses a protoplasmatic cylinder, which consist of the inner membrane and the cytoplasm. A further structural element is a 2 to 10 nm thin mucoid layer, which wraps around the

outer membrane and is quite unstable and easy to remove (cf. Brunnemann 2010). Between the outer and inner membranes of the cell lie 7-11 periplasmic flagella, which are used for locomotion (Barbour and Fish 1993, Wang et al. 1999b, Rosa et al. 2005). The flagella rotate in a counterclockwise direction as viewed from the back of the cell, causing waves to move from the anterior to the posterior ends of the cell (Li et al. 2000). However, different strains of *B. burgdorferi* s.l. can show morphological differences, such as a different number of flagella, differently shaped cell endings or the formation of vesicle (Hovind-Hougen 1984, Schulze et al. 1995).

2.3.1.4 Genetic features

The *B. burgdorferi* s.s. strain B31 was the first spirochete species for which the complete genome was sequenced (Fraser et al. 1997). It consists of a main chromosome of 910,725 base pairs and 11 plasmids, which have a size between 15 and 60 kb. The chromosome contains 853 genes, which encode proteins for DNA replication, transcription and translation, for solute transport and energy metabolism, for motility and chemotaxis, as well as for regulation of gene expression, repair and recombination of genes. However, genes for cellular biosynthetic reactions encoding enzymes for the synthesis of amino acids, fatty acids and nucleotides are lacking. Genes encoding proteins of the oxidative phosphorylation or tricarboxylic acid cycle were also not identified (Fraser et al. 1997). Therefore, the nutritional requirements of the spirochetes need to be satisfied by their environment (Stanek et al. 2012).

Complete sequences have also been obtained for other isolates, including *B. garinii* strain PBi and *B. afzelii* strain PKo (Casjens et al. 2011a, 2011b, Schutzer et al. 2011, 2012). All these analyses showed that all *Borrelia* have a main linear chromosome, which has a length of about 950 kb. In this way, *Borrelia* differs from the closely related genera *Leptospira* and *Treponema*, which have a circular chromosome. In addition to the main chromosome, *B. burgdorferi* s.l. genomes include multiple linear and circular plasmids (e.g. *OspA* plasmids). Among the different *Borrelia* strains the number of plasmids and their sizes (5 to 220 kbp) vary substantially (Fraser et al. 1997, Casjens 2000, Terekhova et al. 2006). On the plasmids lie genes for surface

proteins, for example, the so-called outer surface proteins (osp) or the lipoprotein variable major protein-like sequence, expressed (VlsE). The surface proteins and the VlsE are essential for pathogen-host interactions and for the survival of the complex life cycle of the spirochetes (Stewart et al. 2005, Marques 2010), respectively. In contrast to other well-studied bacterial pathogens, none of the plasmid genes of *B. burgdorferi* s.l. show similarity to known bacterial virulence genes suggesting that the plasmids encode functions that are specific to the spirochete infectious cycle (Rosa et al. 2005, Baranton and De Martino 2009).

An analysis of the metabolic pathways suggests that *Borrelia* gain their energy mainly by using the substrate phosphorylation during glycolysis. Due to its metabolic abilities, the existence of *Borrelia* is bound to a host which provides its nutrients (Fraser et al. 1997). Thereby, *Borrelia* have developed many different strategies to circumvent the immune system of their host (Embers et al. 2004, 2007, Singh and Girschick 2004, Coutte et al. 2009). These strategies include the suppression of innate and adaptive immune responses, retreat to niches not accessible to the immune system and the possibility to change their surface structure (antigen variation) (Ohnishi et al. 2001, Embers et al. 2007, Coutte et al. 2009). The change in the antigen structure is based on recombination events. This mechanism is common, especially in micro-organisms that cause a long-lasting or repeated infections (Coutte et al. 2009). Pathogens that are able to vary their antigen have an advantage over their hosts, which first have to adapt their immune system to the new surface coat. A protein that is subject to a very strong recombination is VlsE that is expressed by *Borrelia* only in vertebrate hosts. The constant changes in *Borrelia* surface complicates the production of a vaccine (Coutte et al. 2009). A vaccine for humans was successfully marketed in the U.S., but withdrawn from the market for economic reasons (Rizzoli et al. 2011). In Europe the development and the application of a vaccine is difficult due to the heterogeneity and diversity of the *Borrelia* genospecies (see Section 2.3.1.2).

2.3.1.5 Transmission

To maintain the cycles of *B. burgdorferi* s.l. in a certain habitat appropriate hosts must be present, in addition to competent vectoring ticks and suitable climatic conditions (Kurtenbach et al. 1998a, 1998b). Reservoir hosts play a key role in the epidemiological cycles, as only ticks feeding on them can become infected. In wooded areas, rodents, like the yellow-necked mouse (*Apodemus flavicollis*), the wood mouse (*Apodemus sylvaticus*) and the bank vole (*Myodes* formerly *Clethrionomys glareolus*), play an important role in the sense that they act as reservoirs for *B. burgdorferi* s.l. (Kurtenbach et al. 1998a, Piesman and Gern 2004). However, *Apodemus* and *Myodes* showed different transmission patterns, such that their reservoir competence appeared to be modulated by their immune response towards the pathogen and ticks (Piesman and Gern 2004). Moreover, another vole, *Microtus agrestis*, black rats (*Rattus rattus*) and Norway rats (*R. norvegicus*) may infect feeding *I. ricinus* ticks in urbanized environments (Piesman and Gern 2004). In France and Germany, edible dormice (*Glis glis*), the European hedgehog (*Erinaceus europaeus*) and garden dormice (*Eliomys quercinus*) have been confirmed as reservoir hosts for *Borrelia* (Piesman and Gern 2004). Many other vertebrate animals, particularly small- to medium-sized mammals, are classified as reservoir-competent (Gern et al. 1998). Larger mammals, such as deer and cattle, also play an important role in the cycle of *B. burgdorferi* s.l. because they provide the large blood meals necessary for adult female ticks, supporting oviposition, and thus helping to maintain the tick population size (Bhide et al. 2005, Pacilly et al. 2014). However, these larger hosts are reservoir incompetent (Telford et al. 1988, Gern et al. 1998, Richter and Matuschka 2010).

The risk for humans to become infected by Lyme disease spirochetes depends on the tick distribution and on the infection rate of *Ixodes* ticks. The main vector in Europe is *I. ricinus*, the main vector in Asia is *I. persulcatus*, the main vector in northeastern and Upper Midwestern USA is *I. scapularis* and the main vector in western USA is *I. pacificus* (Stanek et al. 2012). Usually, the prevalence of *Borrelia* in adult

ticks is highest because the blood feeding during larval and nymphal stages increases the likelihood of an infection by a factor of two (Hubálek and Halouzka 1997). Nymphs play a key role as vectors of *Borrelia* during spring and summer for humans and other vertebrates, because they have the opportunity to become infected as larvae on a reservoir host. Additionally, human out-door leisure behavior in spring and summer increases the risk of infection to humans because it commonly takes place in tick habitats (Stanek 2005, Hubálek 2009). A second period with increased infection risk arises in autumn by the activity of adult ticks. However, adult ticks are more easily recognized on the human body than immature life history stages and if removed in time (see below) do not transmit the infection. *Borrelia* spirochetes are injected through the tick saliva during a blood meal. At least 36 hours of feeding are necessary for a successful transmission of *B. burgdorferi* by *I. scapularis* or *I. pacificus* ticks to occur (Sood et al. 1997), while the transmission of *B. afzelii* by *I. ricinus* can happen within a shorter period (17 h) (Kahl et al. 1998). Most Lyme borreliosis cases are caused by bites from infected nymphs (Marques 2010). Predictors for *Borrelia* prevalence are the activity of nymphs and the intensity of human recreational activity, such that most infections occur between May and August (Marques 2010).

Several studies show an association between the different genospecies and preferred reservoir host (Lindgren and Jaenson 2006). For example, *B. afzelii* and *B. bavariensis* have predominantly been isolated from rodents (Humair et al. 1999, Huegli et al. 2002, Richter et al. 2004, Kurtenbach et al. 2006). Conversely, *B. garinii* and *B. valaisiana* are mainly specialized on various bird species (Hanincová et al. 2003, Kurtenbach et al. 2006, Gern et al. 2008). There are 2 principal transmission cycles for *B. burgdorferi* s.l.: (1) the rodent-tick cycle and (2) the bird-tick cycle. *B. burgdorferi* s.s. makes use of both cycles and seems least specialized to a certain host (Kurtenbach et al. 2006, Gern 2008).

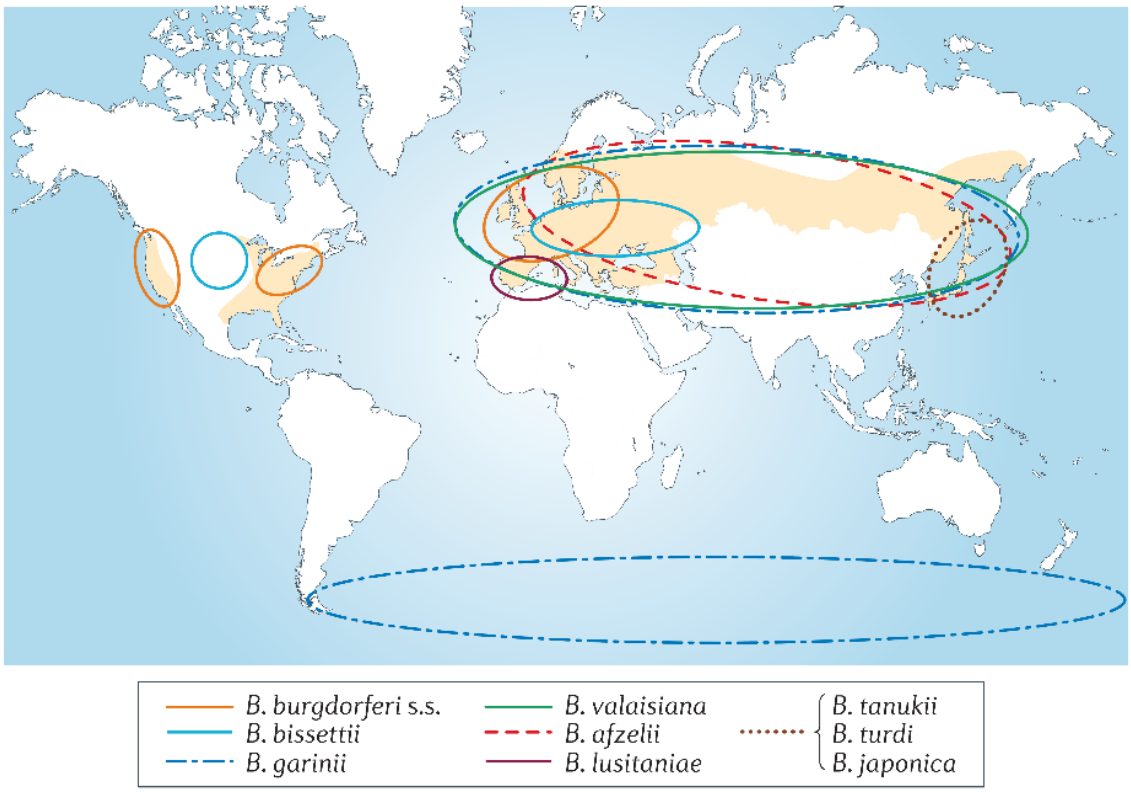


Figure 2.11: Map of the geographical distribution of some *B. burgdorferi* s.l. species (colored ellipses) and clinically recorded Lyme borreliosis cases (beige background) worldwide. (adapted from Kurtenbach et al. 2006). Species identified after 2006 are not included in the map.

2.3.1.6 Epidemiology

Species of the *B. burgdorferi* s.l. complex are distributed worldwide (see Figure 2.11). Seven *Borrelia* genospecies can be found in North America: *B. burgdorferi* s.s., *B. andersonii* (group 21038), *B. bissettii* (group DN127), *B. californiensis*, *B. carolinensis*, *B. americana* and *B. kurtenbachii.* The most common genospecies on the European mainland are *B. afzelii* and *B. garinii* including *B. garinii OspA* type 4 (recently renamed to *B. bavariensis*), which are widely spread across the continent (Rauter and Hartung 2005, Kurtenbach et al. 2006). The third-most common genospecies is *B. burgdorferi* s.s., which is found mainly in Eastern Europe (Rauter and Hartung 2005, Sonenshine and Roe 2013a) but rarely in the western areas. The limited spread of *B. lusitaniae* over South-West Europe might be explained with its close relationship with lizards (Younsi et al. 2005). On the British Isles, the distribution of genospecies differs fundamentally from the distribution patterns on the European

mainland: there *B. garinii* and *B. valaisana* are the dominant genospecies (Kurtenbach et al. 2006). To date, *B. spielmanii* has been found in Europe occasionally and *B. japonica* is mainly distributed in Asia (i.e. in Japan). Other genospecies present in Asia are *B. tanukii*, *B. turdi* and *B. sinica* (Stanek and Reiter 2011). In 2008, five *B. valaisiana*-related strains isolated from rodents and ticks in southwestern China were identified as a new genospecies and were named *B. yangtze* (Chu et al. 2008). In Germany 5 to 35% of *I. ricinus* are infected with *B. burgdorferi* s.l. dependent on year and locality (Oehme et al. 2002, Rauter et al. 2002, Kampen and Rotzel 2004). In highly endemic areas up to 44% is possible (Maiwald et al. 1995).

There is considerable variation in the annual incidence of human Lyme disease cases in Europe. One reason for this is that only few European countries have made Lyme borreliosis mandatorily notifiable (Rizzoli et al. 2011). Estimations assume an incident rate between 65,500 (Wilske 2003, Rizzoli et al. 2011) and 85,000 (Lindgren and Jaenson 2006) new borreliosis cases per year in Europe. Annual incidence rates (Hubálek 2009) are between 0.01 and 137 per 100,000 inhabitants in Turkey and Slovenia, respectively. In addition to other European countries such as Austria, Germany is a highly endemic area. Because there is no common federal reporting obligation for *Borrelia* infections, the exact number of new cases per year in Germany is not known. However, Lyme borreliosis is a notifiable disease in the new federal states of Germany and since June/July 2011 in Rhineland-Palatinate and Saarland. Krause and Fingerle (2009) assume an annual incidence rate of 25 to over 100 per 100,000 inhabitants/year, so that an average of 20,000 to over 80,000 new cases can be expected in Germany every year. According to recent estimates made by the National Reference Center for *Borrelia* (Nationales Referenzzentrum für Borrelien) of the Robert-Koch-Institute, about 60,000 to 100,000 people are infected by Lyme disease each year (Robert Koch-Institut 2011).

2.3.1.7 Pathogenesis and clinical symptoms

In Europe, Lyme disease in humans is mainly caused by *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. (Baranton and De Martino 2009). However, some DNA from *B. valaisiana*, *B. lusitaniae*, *B. spielmanii* and *B. bissettii* has occasionally been found in patient samples (Picken et al. 1997, Wang et al. 1999a, da Franca et al. 2005, Baranton and De Martino 2009). While the human pathogenicity of *B. burgdorferi* s.s., *B. afzelii, B. garinii*, *B. bavariensis* and *B. spielmanii* is beyond doubt, it is still not entirely clear for the other genospecies (Baranton and De Martino 2009). Lyme disease is characterized by a range of different clinical manifestations and disease outcomes, which depend on the state of the immune system of the infected organisms, as well as on the *Borrelia* genospecies involved (Stanek et al. 2012). The infection proceeds in more than 25% of clinical cases without any clinical signs (Krause and Fingerle 2009). An early sign of infection is an erythema migrans (EM) at the site of the tick bite characterized by a local, usually circular skin reddening. This occurs in 60 to 90% of the cases within 1 to 3 weeks after the tick bite (Krause and Fingerle 2009).

The genospecies pathogenic to humans seem preferentially to infect certain organ systems. For example, *B. afzelii* is associated with skin symptoms, particularly with EM, but also with the chronic skin lesions of ACA (Stanek et al. 2012). Similarly, *B. spielmanii* has been isolated mainly from skin biopsies of EM patients (Wang et al. 1999a, Földvári et al. 2005, Maraspin et al. 2006). Neurological dysfunction after infection with *B. garinii* strains or *B. bavariensis* occurs frequently, whereas *B. burgdorferi* s.s. is often mentioned in conjunction with severe Lyme arthritis and systemic signs of infection (Van Dam et al. 1993, Balmelli and Piffaretti 1995, Demaerschalck et al. 1995, Picken et al. 1998, Ornstein et al. 2001, Floris et al. 2007, Stanek et al. 2012). Lyme borreliosis is a multi-system or multi-organ disease in which the different organ systems can be infected individually or in combination. In particular, skin, joints and the central nervous system, but also heart muscles, eyes and vessels are affected (Kauffmann and Wormser 1990, Karma et al. 1995, de Carvalho et al. 2008, Palecek et al. 2010). However, apart from the EM, which may precede a late manifestation, most patients show only symptoms in a single organ

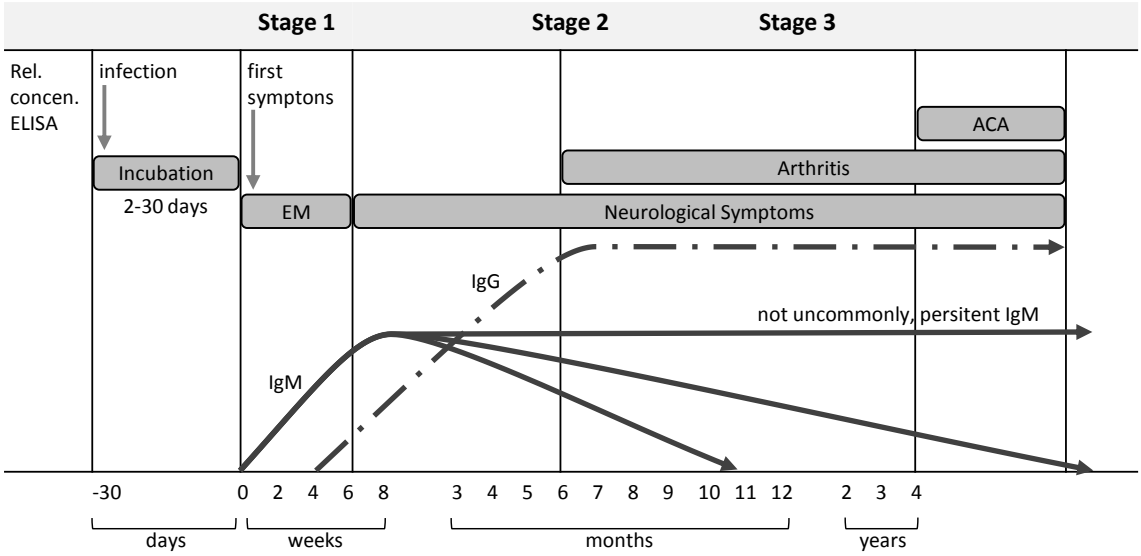


Figure 2.12: Schematic progression Lyme borreliosis and the corresponding antibody titers. Translated and adapted from Dörrschuck et al. (2014).

system (Stanek et al. 2012). The differing manifestations in organs are related to the heterogeneity of the *B. burgdorferi* s.l. complex (Baranton and De Martino 2009).

Lyme disease in humans is often divided into 3 stages (see Figure 2.12) that often merge fluently, whereby the first and second stage can also be seen as the early-stage and the third as the late-stage of Lyme borreliosis (Stanek et al. 2012). However, the disease can miss a certain stage. For example, local infections or even the spread of pathogens in the body can remain without tangible clinical symptoms, until finally organ manifestation of the third stage occurs. A detailed description of the 3 stages of Lyme disease with its clinical symptoms is given by Gern and Falco (2000), Skuballa (2011) and Steere et al. (2004).

Domestic animals can become ill with Lyme disease. The disease can be similar to that found in humans (Gall and Pfister 2006, Krupka and Straubinger 2010). It is assumed that in animals, especially in wild animals, the natural tick- and pathogen exposure has established a balanced host-parasite relationship in the course of evolution, and that infections usually run subclinically (Skotarczak 2002). Serological studies indicate frequent contact of wild living animals with *Borrelia* (Isogai et al. 1991, Gill et al. 1993, Juricová and Hubálek 2009). However, the course of infections,

their clinical manifestations and the persistence of the pathogen are largely unknown (Skotarczak 2002).

2.3.1.8 Diagnosis

In human medicine, the detection of specific antibodies against *B. burgdorferi* s.l. in the serum and cerebrospinal fluid is the main diagnostic method (Wilske et al. 2007). A verifiable infection normally begins with an increase of IgM antibodies after about 3 weeks and of IgG antibodies after about 6 weeks (Wang et al. 2007). A 2-stage diagnosis is recommended for the serological detection of Lyme disease. Enzyme-linked immunosorbent assay (ELISA) or the immune fluorescent antibody test (IFAT) is suitable for screening. If one of these tests is positive, an immunoblot is widely used as a confirmatory test (Wilske et al. 2007). However, the proof of a Lyme disease infection cannot be based solely on serological findings because a negative serology does not rule out an acute infection. On the other hand, high IgG antibody titers can persist after an earlier subclinical infection over many years (Wilske et al. 2007).

A direct proof of *B. burgdorferi* s.l. infection is made by growing the bacteria on a special culture media or by PCR on specific gene sections. The cultivation of *Borrelia* is very time consuming and expensive. Therefore, PCR has become the standard method of detection for the diagnosis of an unspecific *Borrelia* because it reliably allows the detection of the bacteria in epidemiological and clinical studies. In recent years, different PCR protocols have been developed, enabling the differentiation of different genospecies (Baranton and De Martino 2009). The targets are, for example, the *ospA* gene (Rauter et al. 2002), the *ospC* gene (Wang et al. 1999b) or the spacer region between 5S and 23S of the rRNA gene (Rijpkema et al. 1995). The method of Rauter et al. (2002) can be employed to determine the clinically most relevant genospecies (i.e. *B. burgdorferi* s.s., *B. garinii*, and *B. afzelii*) within a single PCR run.

2.3.2 *Rickettsia* spp.

Rickettsial diseases, also known as rickettsioses, are caused by bacteria of the genus *Rickettsia* within the family Rickettsiaceae. These intracellular bacteria are transmitted by arthropods (Raoult and Roux 1997, Schex 2011).

2.3.2.1 Historical background

Some of the oldest known infectious agents belong to the genus *Rickettsia*, such as *Rickettsia prowazekii*, the pathogen causing epidemic typhus. This disease is suspected of being responsible for the Athens plaque during the fifth century BC (Raoult and Roux 1997). In 1899, the first description of the Rocky Mountain spotted fever (RMSF) was given (Maxey 1899), while its causative agent, *Rickettsia rickettsii*, was reported some years later in wood ticks (*Dermacentor occidentalis*) by Ricketts (1906, 1909). Definitive evidence that *R. rickettsii* is maintained by ticks was provided experimentally by Wolbach (1919). During the 20th century, *R. rickettsii* was considered to be the only *Rickettsia* species in the Western hemisphere that was pathogenic to humans (Parola et al. 2005). Although many other *Rickettsia* were detected, these were considered as non-pathogenic (Raoult and Roux 1997, Raoult 2004). In Europe and Africa, *R. conorii* transmitted by the brown dog tick (*Rhipicephalus sanguineus*) was considered to be the only agent causing tick-borne rickettsioses (Parola et al. 2005). A similar situation is assumed for *R. sibirica* in the USSR and China, as well as for *R. australis* in Australia (Raoult and Roux 1997).

In comparison to classical serology that might have hindered the corrected identification of novel spotted fever group (SFG) rickettsioses, the advances in culture systems and molecular methods have greatly improved the identification process on the basis of rickettsial DNA (Raoult and Roux 1997). Since 1984 many new emerging tick-borne rickettsial diseases (TBRD) have been identified throughout the world (Parola and Raoult 2001, Parola et al. 2005). Moreover, more recent findings in Europe and Asia on *R. helvetica*, which has been considered nonpathogenic since its discovery in 1974 in Switzerland (Parola et al. 2005), show that this species is also pathogenic to humans (Raoult 2004).

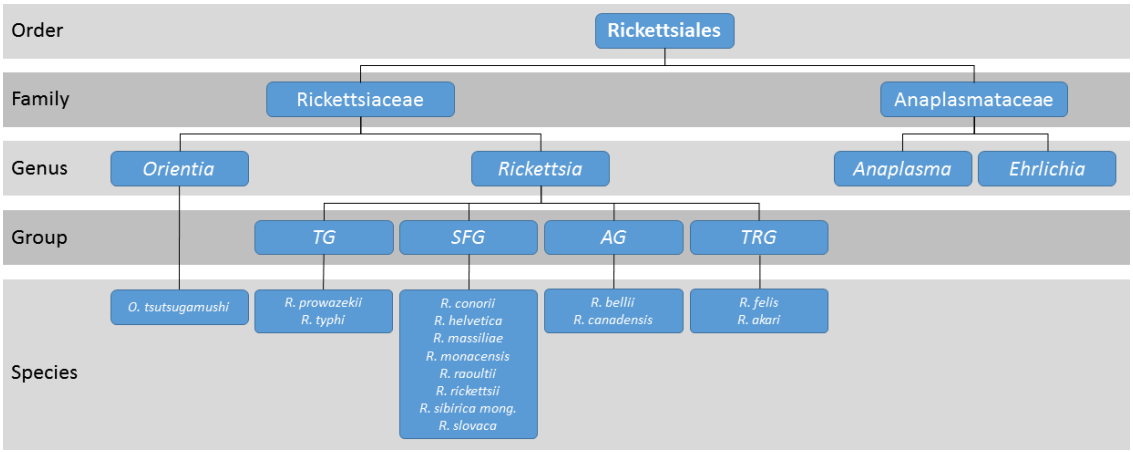


Figure 2.13: Taxonomical overview of the *Rickettsia* spp. of veterinary or medical interest with respect to Germany (cf. Sonenshine 2006, Benson et al. 2009, Schex 2011, Skuballa 2011, http://www.ncbi.nlm.nih.gov/taxonomy). The 4 *Rickettsia* groups are the typhus group (TG), the transitional group (TRG), the spotted fever group (SFG) and the ancestral group (AG).

In summary, highly significant developments have taken place in the field of rickettsiology over the last 20 years, with many previously unrecognized or incompletely described species of unknown pathogenicity being detected in or isolated from ticks (Parola et al. 2005). So far, for most representatives only few epidemiological data and only incomplete studies on their life cycle, possible vectors and their reservoir hosts exist (Sprong et al. 2009). However, recent developments in rickettsial genetics have facilitated our understanding of *Rickettsia* taxonomy (see Section 2.3.2.2) and the functional characterization of potential virulence determinants(see Section 2.3.2.7) (Sonenshine and Roe 2013a).

2.3.2.2 Systematics

Taxonomically *Rickettsia* are classified into the α-subdivision of the Proteobacteria, belonging to the order Rickettsiales (Figure 2.13). This subdivision comprises numerous pathogens of humans and animals, and includes the genera *Anaplasma*, *Ehrlichia* and *Orientia*, as well as several bacterial endosymbionts of invertebrates (Sonenshine and Roe 2013a). The latter genus contains the scrub typhus rickettsiae group, previously known as the tsutsugamushi group (Roux and Raoult 2000). In

comparison to the other genera, the species within this group lack lipopolysaccharide, peptidoglycan and a slime layer (Walker 1996).

The assignment of *Rickettsia* species to different groups has constantly changed with increasing knowledge (Roux and Raoult 2000, Lipsker and Jaulhac 2009). Quite recently, the complete sequencing of several rickettsial genomes has provided the basis for a phenotypic and genotypic classification of *Rickettsia* species (Raoult and Roux 1997). This classification has replaced the classical one, which consisted of 3 groups (typhus, spotted fever and scrub typhus) based on phenotype and clinical signs (Skuballa 2011). Currently, 4 groups are recognized (Sonenshine and Roe 2013a): (1) the large and strongly heterogeneous SFG containing the agent of RMSF; (2) the smaller typhus group (TG), which includes the agent of flea-borne murine typhus and louse-borne epidemic typhus; (3) a transitional group, which is based on shared characteristics of the TG and the SFG; and (4) an ancestral group (AG) including *R. bellii* and *R. canadenesis* differing from all other groups (Stothard et al. 1994, Pacheco et al. 2011). To date, almost 300 species exist or have been proposed within the SFG (Benson et al. 2009).

2.3.2.3 Morphology and genetic features

Rickettsiae are gram-negative bacteria that form spores. Most of them are very small and have a round to oval shape with a diameter of 0.3 to 0.5 µm and a length of 0.7 to 2.0 µm (Hackstadt 1996). As shown in Figure 2.14, they consist of a translucent zone, called the slime layer, which surrounds a trilaminar cell wall (Silverman et al. 1978). Only few variants of *Rickettsia* have been described as filamentous and long-formed (Sonenshine and Roe 2013a). Rickettsiae live obligate intracellularly and infest endothelial cells in small blood vessels. Because of their strict intracellular growth, rickettsiae cannot be cultivated on agar plates or in broth, but only in viable eukaryotic host cells (e.g., in cell culture, embryonated eggs, or susceptible animals) (Walker 1996).

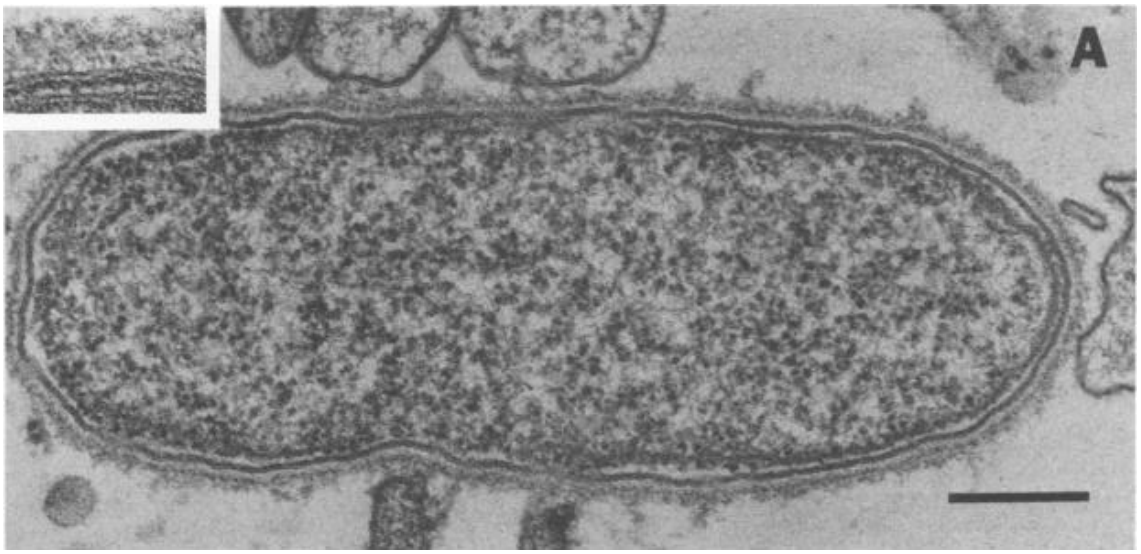


Figure 2.14: Electron microcopy image of *R. prowazekii*, the etiologic agent of epidemic typhus, also showing at detailed view of its outer envelope (from Silverman and Wisseman 1978).

The clinical picture of the two main groups of *Rickettsia*, the TG and the SFG, is clinically not clearly distinguishable. However, the average genome sizes of the 4 groups is different: 1.2 to 1.3 Mb in the SFG, 1.1 Mb in the TG, 1.2 to 1.5 Mb in the AG and 1.3 to 1.5 in the TRG (Sonenshine and Roe 2013a). Additionally, the groups SFG and TG can be differentiated on the basis of the surface protein OmpA which is absent in representatives of the TG, and by their vectors (Pérez-Osorio et al. 2008). For *R. rickettsii* the rOmpA protein appears to play a key role in the initial adhesion to the host cells (Li and Walker 1998). On the other hand, it has been demonstrated that the rOmpB protein is the immunodominant species of surface protein antigen for most of the rickettsiae, particularly of the SFG, underling its importance in rickettsial pathogenesis (Roux and Raoult 2000). In this context, it has been shown that rOmpB mediates the invasion of mammalian cells (Chan et al. 2009).

2.3.2.4 Transmission

The transmission of rickettsial diseases to humans is usually caused by chiggers, fleas, lice, mites or ticks (Telford and Parola 2007). For this reason, the geographic distributions of rickettsioses strongly depends on the infection rate, distribution and biting preferences of the corresponding arthropods. For *Rickettsia* found in

blood-feeding arthropods, e.g. in ixodid ticks, several transmission strategies, such as vertical (i.e. transovarial and transstadial), horizontal and mixed transmission, have been observed (Sonenshine and Roe 2013a). Transovarial transmission, for example, takes place for *Rickettsia* species that are pathogenic to vertebrates (Azad and Beard 1998), so that the pathogen is transmitted from female ticks to the larval life history stage. Once an arthropod has been infected with *Rickettsia*, the bacteria travel from the midgut to the salivary glands (Santos et al. 2002), from where the infection can be transmitted horizontally to a vertebrate host and thus to other blood-feeding arthropods (Sonenshine and Roe 2013a).

Initial studies have reported that SFG rickettsiae can be transmitted through at least 12 tick generations via transovarial transmission alone (Burgdorfer and Brinton 1975). In the case of rickettsiae that are mainly transmitted transovarially, ticks can act as reservoir hosts and as vectors of the rickettsial infection (Vitale et al. 1989). By this means, vertical pathogen transmission through all life history stages seems to maintain the *Rickettsia* populations when the number of available vertebrates is low (Munderloh and Kurtti 1995). Zanettii et al. (2008) observed 100% transovarial transmission to all laid eggs, suggesting a coupled growth between SFG *Rickettsia* and tick populations. However, some pairings of SFG *Rickettsia* and tick species do not produces infected offspring (Baldridge et al. 2007). In this context, it is still unclear which mechanisms regulate successful transmission (Sonenshine and Roe 2013a).

In *R. prowazekii*, the epidemic typhus agent, a rather unusual form of transmission has been observed (Azad and Beard 1998). *R. prowazekii* is pathogenic to its louse hosts generally killing them within few weeks, such that no vertical transmission is possible. Conversely, the pathogen seems to be better adapted to vertebrate hosts and thus relies on horizontal transmission (Azad and Beard 1998). Unlike the SFG, rickettsiae within the TG multiply in their arthropod vectors. They grow inside the epithelial cells of the intestinal tract and are excreted in the feces (Perlman et al.

2006). Thereafter, infections of humans with *R. prowazekii* occur via the dermis after scratching (Raoult and Roux 1997).

2.3.2.5 Epidemiology

Rickettsia species are widespread throughout the world. Their occurrence essentially depends the distribution of the vectors. As obligate intracellular bacteria, *Rickettsia* are transmitted from arthropods to vertebrates through saliva, feces, blood or aerosol (Sonenshine and Roe 2013a).

In Germany at least 7 *Rickettsia* species occur indigenously: *Rickettsia helvetica*, *R. felis*, *R. monacensis*, *R. massiliae*, *R. raoultii, R. aeschlimannii* and *R. slovaca* (Dobler and Wölfel 2009, Parola et al. 2013). *R. felis* is transmitted primarily by fleas, while *R. slovaca* and *R. raoultii* are primarily disseminated by *Dermacentor* ticks (Pluta et al. 2009, 2010). *R. aeschlimannii* was detected in *Hyalomma* ticks collected from several bird species (Parola et al. 2013). *R. helvetica*, *R. monacensis* and *R. massiliae* are predominantly found in *I. ricinus* (Simser et al. 2002, Parola et al. 2005), reaching prevalences of almost 50% (Milhano et al. 2010). In comparison to arthropods, the role that vertebrates play in the epidemiology of *Rickettsia* and how they contribute to their maintenance is little known. Potential reservoir hosts include rodents, lagomorphs, dogs and deer (Levin et al. 2011). Serological studies and direct detection of *Rickettsia* in tissue samples have shown that wild animals often come into contact with *Rickettsia* spp. (Smetanová et al. 2006, Stefanidesova et al. 2007, Selmi et al. 2009, Schex et al. 2011, Skuballa 2011, Overzier et al. 2013).

2.3.2.6 Pathogenesis and clinical symptoms

Rickettsiae cause human disease around the world (Walker 1996, Sonenshine and Roe 2013a). In Europe, particularly in the southern regions, *Rickettsia* are viewed as a growing health problem to humans (Ciceroni et al. 2006). During the years 1998 to 2002, 4604 clinical cases of MSF were described in Italy alone, of which 33 were lethal. The detected agent was mainly *R. conorii*, the trigger of MSF (Ciceroni et al.

2006). Other species such as *R. helvetica* seem to cause a milder disease in humans (Ciceroni et al. 2006, Lipsker and Jaulhac 2009).

For more than 20 species of *Rickettsia* a human pathogenic potential has been demonstrated (Parola et al. 2005). Depending on the *Rickettsia* species, the features and the course of the disease can range from mild, asymptomatic to life-threatening (Parola et al. 2005, Lipsker and Jaulhac 2009). Examples of diseases associated with SFG *Rickettsia* are: Rocky Mountain spotted fever (RMSF) caused by *R. rickettsii*, Mediterranean spotted fever (MSF) caused by *R. conorii*, Siberian tick typhus (*R. sibirica*), Queensland tick fever (*R. australis*), Japanese spotted fever (*R. japonica*), Flinders island spotted fever (*R. honei*), African tick-bite fever (*R. africae*) and tick-borne lymphadenopathy caused by *R. slovaca* and *R. raoultii*.

The bacteria enter the human body via the skin, then traverse the blood vessel walls and spread though the bloodstream. This spreading causes infection of the endothelium and more seldom the vascular smooth muscle cells. *Rickettsia* enter the cells of their host and reproduce by binary fission in the cytosol, thus damaging heavily parasitized cells directly (Walker 1996). Consequently, this behavior causes hyperplasia of the endothelial cells and thrombus formation leading to obstruction of blood flow and the escape of red blood cells into the surrounding tissue. This leads to a distinctive punctate bleeding from the fine capillaries in the skin or the mucous membranes (Kimmig et al. 2010). When inflammatory cells follow into the tissue papules can develop. The typical clinical sign of rickettsial infections, the eschar, is caused by necrosis in the center of the papule.

2.3.2.7 Diagnosis

The clinical diagnosis of rickettsioses can be difficult, because of the rather unspecific symptoms (e.g. fever, headache, nausea, vomiting, muscle aches, rash). Although infections can be diagnosed by serological assays, IgM and IgG antibodies that are reactive with *Rickettsia* could be hardly detectable during the first week of illness (Paddock et al. 1999). Immunofluorescence assays are not suited to distinguish

between the *Rickettsia* species because of the existence of a strong antigenic cross-reaction between the SFG and the TG (Ormsbee et al. 1978). Additionally, other serodiagnostic tools are available, such as the Weil-Felix test, complement fixation (CF) test, microagglutination, latex agglutination, ELISA and Western immunoblots (La Scola and Raoult 1997).

A more sensitive and specific diagnosis of rickettsial infections can be achieved through molecular analysis techniques. The analyzed material can be a tissue sample from an eschar or from any other possibly infected organ. For detection of rickettsial DNA there are several commonly used genes, such as the citrate synthase gene (gltA), the 16S rRNA gene, the genus specific 17-kDa antigen gene and the partial outer membrane proteins A (ompB) and B (ompA) (Reif and Macaluso 2009).

2.3.2.8 Rickettsia helvetica

In 1979 this pathogen was discovered in the Switzerland (Burgdorfer et al. 1979) and its original name "swiss agent" was proposed. This name was converted by Beati et al. (1993) into *R. helvetica*. *I. ricinus* seems particularly involved in the epidemiology of *R. helvetica* over the entire European continent (Hartelt et al. 2008) including France, Germany, Italy, Portugal, Slovenia, Spain and Sweden (Sanogo et al. 2003, Fernández-Soto et al. 2004, Goodman et al. 2005, Oteo et al. 2006, Wölfel et al. 2006, Dobler and Wölfel 2009), and in the north-west of Russia (Movila et al. 2011). Additionally, it has been shown that the distribution of *R. helvetica* could extend even further, since *Rickettsia* isolates from *I. ovatus*, *I. persulcatus*, and *Ixodes monospinosus* ticks collected in Japan had a close resemblance with *R. helvetica* (Fournier et al. 2002).

So far, insufficient information is available about the participation of other types of ticks in the epidemiology of *R. helvetica*. Hornok et al. (2010) noted that not *I. ricinus*, but *Haemaphysalis inermis* has the biggest vector potential for *R. helvetica* in their study area in Hungary. In Croatia, *R. helvetica* was detected in 10% of *Dermacentor reticulatus* (Dobec et al. 2009).

Our current state of knowledge about possible reservoir hosts is still very incomplete. Epidemiological studies have shown that migratory birds represent reservoirs for *R. helvetica* and that they can spread infected ticks over long distances, even onto islands (Elfving et al. 2010, Franke et al. 2010). Other vertebrate animals, including lizards, also possess reservoir competence (Tijsse-Klasen et al. 2011). Sprong et al. (2009) have found *R. helvetica* in the blood of mice, roe deer and wild boar.

R. helvetica was considered non-pathogenic to humans for about 20 year after its discovery. However, in Sweden *R. helvetica* was suspected of being involved in a case of fatal perimyocarditis in a young patient (Nilsson et al. 1999), a case of sarcoidosis (Nilsson et al. 2002), as well as a cases of febrile illness in France (Fournier et al. 2000, 2004). The latter infections were present during summer with fever, headache, arthralgia and myalgia but without any signs of a cutaneous rash (Fournier et al. 2004). Only quite recently, the human pathogenic potential, which has long been suspected, was confirmed by direct isolation of the pathogen from a patient with clinical signs of meningitis (Nilsson et al. 2010). However, there are only individual descriptions of human infections with different symptoms, so that there is currently no uniform picture of a disease (Fournier et al. 2000, Nilsson 2009). The extent to which the disease appears in animals is currently also unknown (Boretti et al. 2009).

3

Material and methods

3.1 Sampling methods

The collection of samples was carried out from August 2011 to February 2014 during periods of perennial hunting activity. Roe deer and wild boar were culled from January to October by hunting from a hide and from November to January by driven hunts. For each sample, the acquisition date was recorded and the sample was assigned to one of the following six sampling periods: January/February (Jan/Feb), March/April (Mar/Apr), May/June (May/Jun), July/August (Jul/Aug), September/October (Sep/Oct) and November/December (Nov/Dec).

3.1.1 Sampling area

The study took place in the Bienwald, a 10,275 ha state forest which is located in the southwest of the federal state of Rhineland-Palatinate in Germany (see Figure 3.1). The area is an irregular triangle lying between 48°59'0"N, 8°0'0"E and 49°7'0"N, 8°16'0"E (UTM) bordering France to the southwest along the river Lauter. The altitude ranges from 105 m above sea level in the north to 130 m in the west. The Bienwald is the largest coherent forest area of the Upper-Rhenish Lowlands where it lies on an alluvial fan landscape with numerous streams. Due to the geology of the Bienwald, there are also low hills ranging from 135 m a.s.l. at the border in the north, up to 152 m in the east. These consist of alluvially deposited dune sands (Rheinland-Pfalz Landesforsten 2015). Especially during winter, the soils in the west are rela-

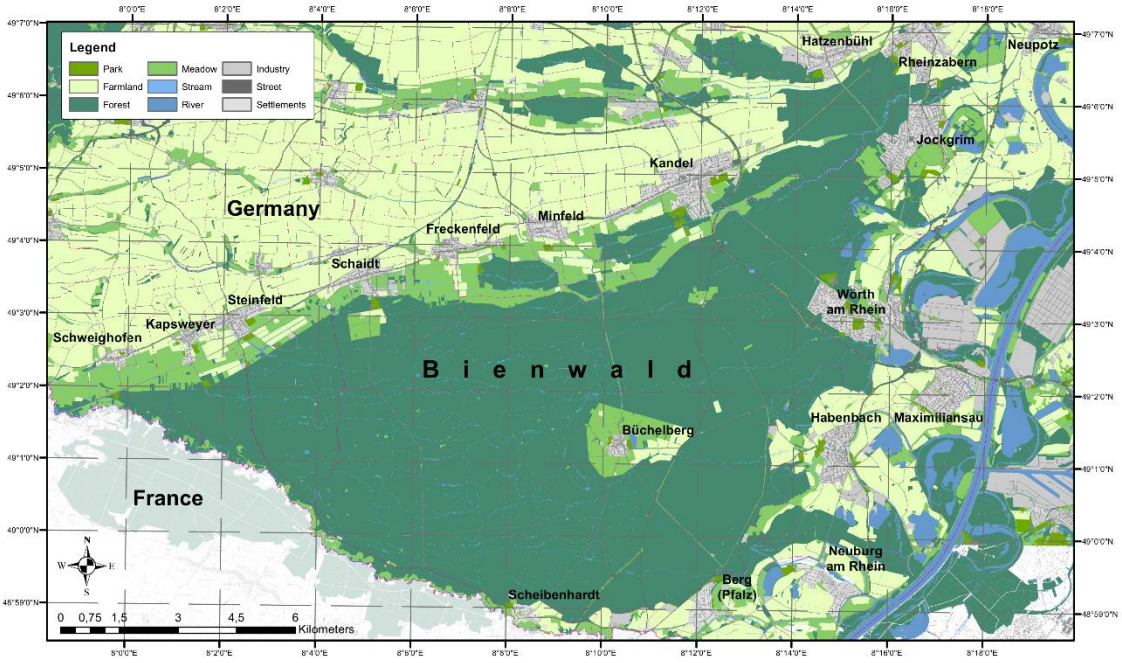


Figure 3.1: Map of the sampling area, the Bienwald, a 10275 ha state forest in Rhineland-Palatinate, Germany. The image was generated with ArcMap 10.2.1 (by ESRI) from data provided by the Office for Surveying and Geographic Information (Landesamt für Vermessung und Geobasisinformation), Rhineland-Palatinate, Germany.

tively wet, which is why the forest area is streaked by numerous channels for drainage into western tributary rivers of the Upper Rhine. During summer, many of these channels dry out but some also carry water throughout the year. In particular, during summer 2013 exceptionally high rainfall caused many parts of the western Bienwald to become waterlogged throughout the year.

Because of the small-scale habitat variation, sites of wet and dry, poor and rich soils, the area provides a wide variety of biotope patches. The vegetation of the Bienwald forest is composed of meadow forests (*Alnus glutinosa*), European ash (*Fraxinus excelsior*), oaks (*Quercus robur*, *Quercus petraea* and *Quercus rubra*), common beeches (*Fagus sylvatica*), hornbeams (*Carpinus betulus*) and pines (*Pinus sylvestris*). Pines dominate (56%) on the poor sand soils of the talus river fans and occupy 48% of the whole forest area, whereas deciduous trees amount to 44%, especially oaks with 25%. Although the forest is mainly used for forestry, the area also contains undisturbed biotopes.

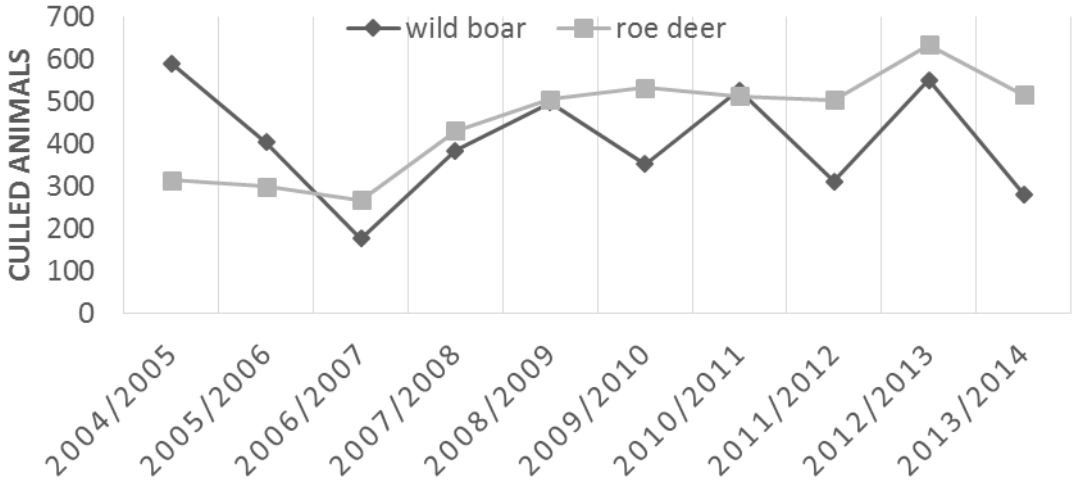


Figure 3.2: Numbers of roe deer and wild boar culled in the Bienwald between 2004 and 2014 during all hunting activities. Data from Forstamt Bienwald, 2014, pers. comm.

The Bienwald is inhabited by roe deer and wild boar, but red deer cannot be considered as a permanent resident of the local fauna since only very few occasional sightings have been reported. Recent estimations of the roe deer population density show an average of 6.0 animals per ha (Ehrhart 2012) with a distinctly increasing trend over the last decade (see Figure 3.2). To date, density estimations for the wild boar population in the Bienwald have not been conducted and the high variance in the hunting bags does not allow the identification of a clear population trend (see Figure 3.2). However, the numbers of culled wild boar in Rhineland-Palatinate have shown a vast increase over the last 30 years (see Section 2.1.2.2).

3.1.2 Hosts samples

For the studies presented in this thesis, organ samples were collected from roe deer and wild boar (see Figure 3.3). Collection occurred during the disembowelment of the animals no later than 60 min after the animal died. Tissue samples from the skin of the ear tip, heart, lung, diaphragm, liver, spleen, kidney, and urinary bladder, having a size of roughly 5 mm × 5 mm × 5 mm were collected. All organ samples were stored in Eppendorf tubes and cryopreserved at -70 °C. Each of the tubes was marked with the ear tag, which assigned a unique number to each animal, and an

Figure 3.3: Sample acquisition during driven hunts. Information on ticks, hosts and pathogens for hunters (left) as well as organ and tick sampling from wild boar (center) and roe deer (right).

abbreviation for the organ it contained. Moreover, from each animal two blood samples were collected, one in a serum tube and the second in an ethylenediaminetetraacetic acid (EDTA) tube, both labeled with the according ear tag. The blood samples have also been cryopreserved at -70 °C and are available for future investigation.

For each culled animal, the date of its sampling, its ear tag, species, sex, body mass and age were determined and recorded. This affords *inter alia* the possibility of drawing conclusions about the condition of the game animals. Body mass resulted from the measurement of the animal's carcass including the head without blood and with the intestines removed. It was measured no later than 1 hour after culling. Age was ascertained by analysis of the skulls and the individual dental abrasion, i.e. tooth wear (cf. Mysterud and Østbye 2006a). The animals were grouped into three classes: (1) game younger than 1 year, (2) yearlings and (3) individuals older than 2 years (adults). Additional data referring to high infestation with other ecto- and/or endoparasites, e.g. deer ked (*Lipoptena cervi*) and lungworm (*Metastrongylus apri*), obvious injuries, pregnancy and status of lactation for female individuals were recorded.

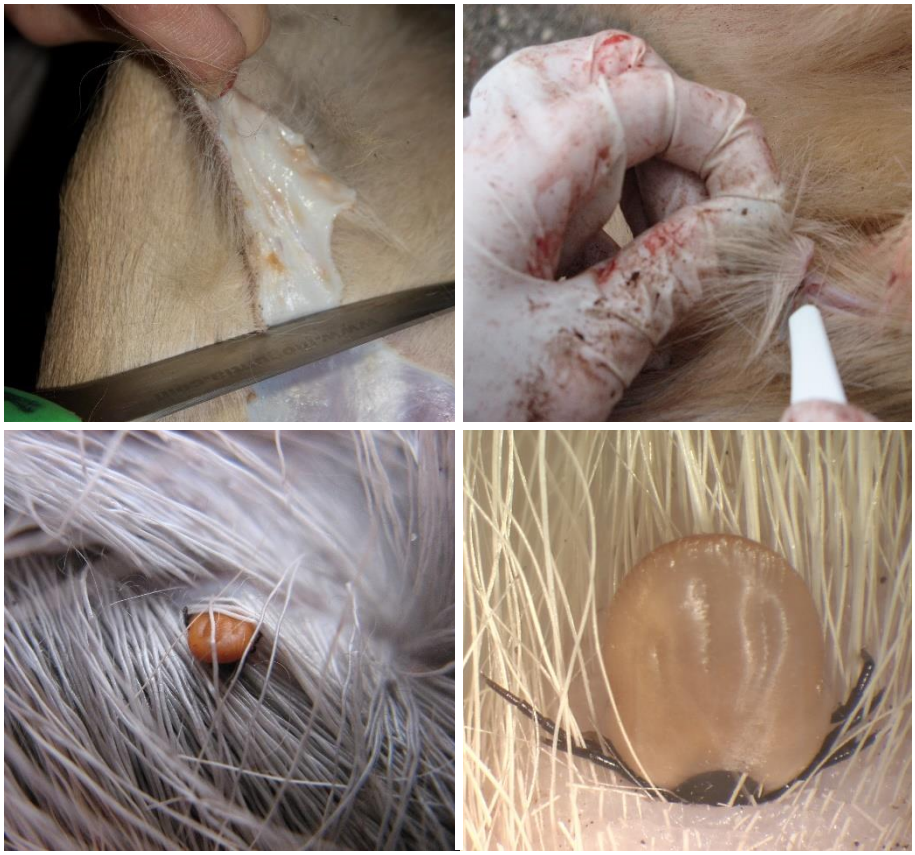
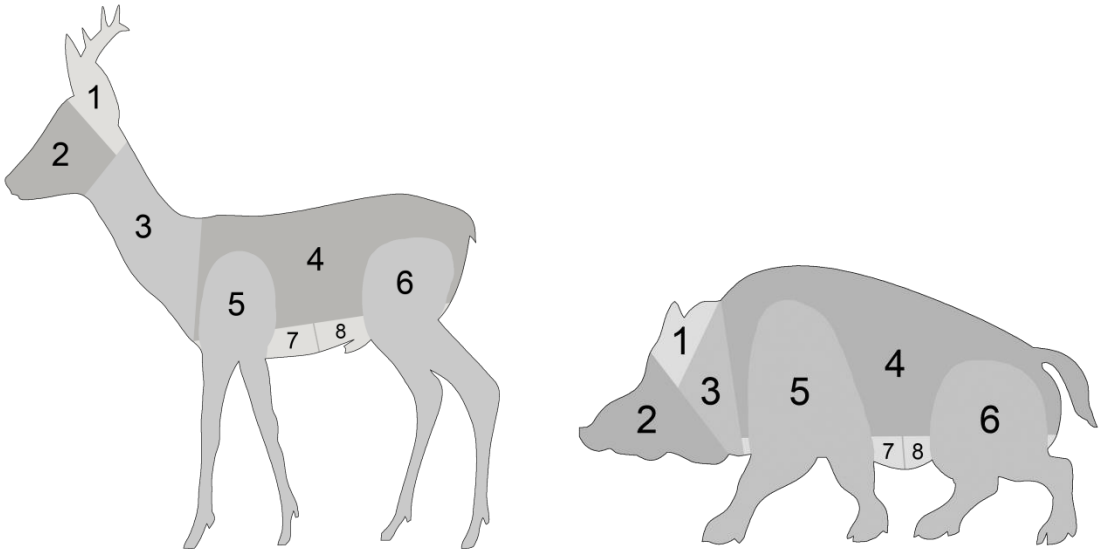


Figure 3.4: Tick collection from roe deer. Heavily infested skin areas were removed entirely with knife or scalpel (top, Pictures: Senta Verena Muders). Engorged ticks (bottom) were predominantly found on roe deer (see Section 4.1.1) (bottom left, Picture: Senta Verena Muders; bottom right, Material: Senta Verena Muders, Picture: Prof. Dr. Urs Wyss, Kiel)

All data was directly recorded on a form at the time of the sample acquisition. The form used for this purpose is provided in Appendix A.

3.1.3 Tick collection

Roe deer and wild boar were examined by palpation for ticks using latex gloves and fine forceps (Figure 3.4). Ticks were preferably removed directly after culling, but at the latest 60 min after the death to avoid effects of tick migration from the dead hosts. Hence, refrigerated bodies were excluded from the examination. Removed and loose ticks were stored directly in 70% ethanol in Eppendorf tubes. However, when tick infestation was very high, loose ticks were collected and preserved in ethanol before removing the organs and/or organ parts (e.g. skin and ears) entirely (see Figure 3.4). The highly tick infested skin parts and organs were stored in polythene



(a) (b)

Figure 3.5: Schemata of (a) roe deer and (b) wild boar to record tick location and to detect body regions preferred by ticks and different tick life history stages. Parts of the whole game corpus are numbered from 1 to 8. The regions 7 and 8 include armpit and groin, respectively.

bags and frozen at -20 °C. After removing all ticks from the cryoconservated skin and organs, the ectoparasites were also stored in 70% ethanol. In addition to this, at least two people swept the whole body of each animal for ticks, such that the probability of overlooking ticks was reduced as far as possible.

The body of the game animals was divided into regions numbered from 1 to 8 to facilitate the determination of preferred attachment sites of ticks. As displayed in Figure 3.5a and Figure 3.5b for roe deer and wild boar, respectively, the 8 regions have been defined as follows: (1) ears, (2) head, (3) neck, (4) main body, i.e. torso, (5) both front legs, (6) hind legs, (7) sternum including armpit and (8) abdomen with groin. All ticks were sorted in accordance with the body region on which they were found. The ticks from different body parts were preserved in separate tubes, which were labeled with the corresponding number of the attachments site and the ear tag of the host animal. Moreover, for each host the body regions that were infested by ticks were directly recorded on the sampling form (see Table A.1).

Afterwards, ticks were microscopically examined in the laboratory and determined to species, stage of development, sex, status of mating and engorgement. Species and age of the collected ticks was determined using the identification key of Hillyard (1996) The amount of engorgement of the ticks was classified into five levels (0 = loose and unengorged, 1 = attached and unengorged, 2 = attached and little engorged, 3 = attached and medium engorged, 4 = attached and fully engorged).

3.1.4 Climate

The mean annual rainfall in the Bienwald is between 680 and 700 mm (during the growing season from beginning of May to end of October: between 330 and 380 mm), and the average annual temperature is 10 °C (during the growing season 16.5 °C). Climatic data were recorded during the sampling period for every hunting day. This was provided by the German Meteorological Service (Deutscher Wetterdienst), and included cloud coverage (ranging from 1 to 8, whereby 1 = slight coverage and 8 = high coverage), relative humidity [%], air pressure [hPa], wind speed [m/sec], precipitation depth [mm], sunshine duration [h], snow height [cm], minimum air temperature 5 cm above the ground [°C], as well as minimum, maximum and mean daily air temperature 2m above the ground [°C]. All of this information was acquired at weather station number 4177 in Rheinstetten, Germany, which is located close to the Bienwald. From the daily mean air temperature (T) and the daily mean relative humidity (RH) the saturation deficit was calculated in accordance with Randolph and Storey (1999) and Perret et al. (2000) by the following empirical formula:

$$SAD = (1 - RH/100) \cdot 4.9463 \cdot e^{0.0621 \cdot T},$$

where SAD reflects the saturation deficit in millimeters of mercury, while T and RH are given in degree Celsius and percent, respectively. The saturation deficit is a measure of the drying power of the atmosphere (Randolph and Storey 1999, Perret et al. 2003). The precipitation depths and the average air temperatures for each sampling period are depicted in Figure 3.6.

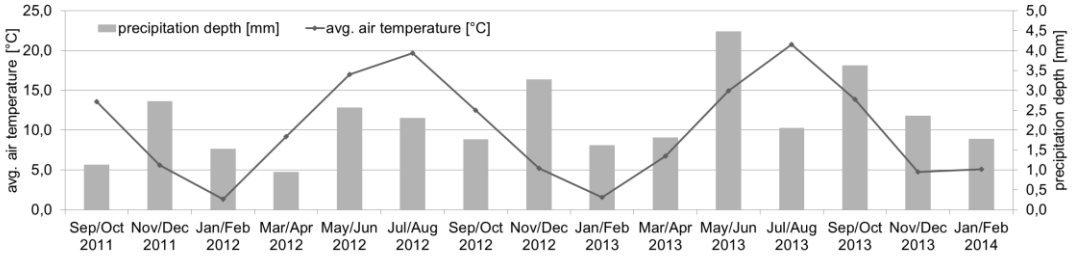


Figure 3.6: Average air temperature (line plot) and precipitation depth (bar plot) for each sampling period of the study.

3.2 Microbiological methods

The Sections 3.2.1 to 3.2.4 provide a general introduction to microbiological methods (i.e. nucleic acid extraction, polymerase chain reaction, agarose gel electrophoresis, and sequencing). Sections 3.2.5 and 3.2.6 describe the specific analyses that were used for *Borrelia* and *Rickettsia* detection. A detailed overview of the devices used and consumables is given in Appendix C, while the solutions and buffers used are listed in Appendix D.

3.2.1 Nucleic acid extraction from organ samples

The DNA isolation from organ samples was carried out using Maxwell® 16 Tissue DNA Purification Kits (Promega, Madison, WI, USA) with an automated DNA extraction device, the Maxwell® 16 system (Promega, Madison, WI, USA). This system consists of the pre-programmed device and prefilled reagent cartridges, which contain all required elements for nucleic acid extraction (i.e. prefilled buffers) as well as MagneSil® paramagnetic particles (PMPs). The silica particles are negatively charged quartz crystals, i.e. silicon dioxide (SiO_2) (Herrmann 2012a). The principle is based on the release of nucleic acids from the sample using a lysis buffer with high salt concentration, i.e. guanidine isothiocyanate. The high salt concentration and the low pH-value causes a reversible binding of DNA/RNA with the negatively charged, magnetic silica particles. Existing polysaccharides and proteins are unable to bind to the silica particles and are removed by repeat cleaning (i.e. washing) steps using a washing buffer with high salt concentration. At the end of the extraction, DNA/RNA

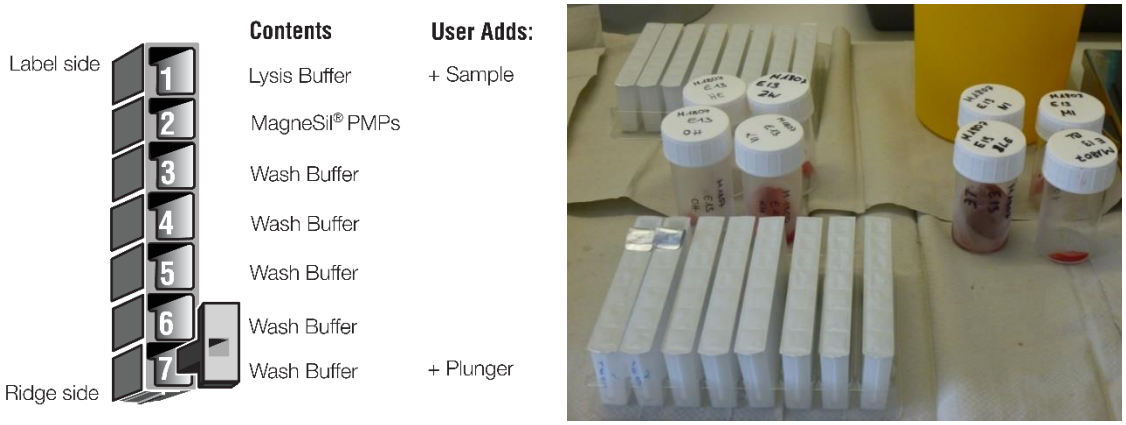


Figure 3.7: Maxwell® 16 DNA Purification Cartridge (left): well 1 contains the lysis buffer and the sample, well 2 contains the MagneSil® particles and wells 3 to 7 contain washing buffers. Sterile workbench (right) used to cut and transfer organs samples (2×2×2 mm) into the cartridges.

is dissolved from the PMPs by the use of an elution buffer with a low salt concentration and is present in solution (Herrmann, 2012a, unpublished; Skuballa, 2011).

A reagent cartridge of the Maxwell 16 system has 7 wells (see Figure 3.7). In a single run 16 samples can be processed simultaneously. The first well contains the lysis buffer and the sample material, which is crushed and dissolved by a magnetic plunger. Thereafter, PMPs (MagneSil®) inside the second well bind to the plunger and are transferred to the first well, such that the DNA can bind to the particles. After the PMPs have bound the source material, the plunger transfers the samples into the other wells of the cartridge (wells 3 to 7) for lysis and cleaning to remove bound contaminants (Herrmann 2012a). The purified and isolated DNA is transferred into an elution vial, which contains the elution buffer, a buffer with low salt concentration. Before the start of the purification, 250 µl of the elution buffer was prepared in the elution vial. Due to heating and the low salinity of this buffer, the DNA is again released from the silica particles. Then, the isolated DNA can be transferred into prepared 0.5 ml Eppendorf tubes. Traces of the black silica particles in the purified samples do not affect the following reactions (Herrmann 2012a). However, the eluates were centrifuged at 5 °C for 3 min with 16,000 g to keep these residues as low

as possible, before they were analyzed by PCR or cryopreserved at -70 °C. The entire purification process took about 45 min.

For the extraction of *Borrelia* spp. and *Rickettsia* spp. DNA two pools of tissue samples per animal were investigated. One pool contained a part (2×2×2 mm) of the skin (i.e. the ear), while the other consisted of tissue pieces (2×2×2 mm) from the heart, lung, diaphragm, kidney, liver, urinary bladder and spleen. Cutting of the tissue samples was performed under sterile conditions (see Figure 3.7). The first pool was denoted as P1 and the second pool as P2. A single cartridge was used per pool. Since the maximum inserted tissue material should not exceed 50 mg according to the manufacturer's instructions, the 2×2×2 mm tissue pieces of the pooled organ samples were finely crushed using a plastic pestle before they were inserted into the cartridges.

3.2.2 Nucleic acid extraction from tick samples

Ticks were investigated individually and homogenized in phosphate buffered saline using the NucliSENS® easyMag® system (bioMérieux Inc., Durham, NC, USA) to extract bacteria DNA. By the automatic addition of a chaotropic buffer, the lysis buffer, nucleic acids are released and are able to precipitate onto magnetic silica particles. Then, several cleaning steps take place to remove polysaccharides and proteins, as well as bonded impurities. For this step extraction buffers are used. Finally, the silica particles and the DNA are separated by an elution buffer of low salinity (Herrmann, 2012b, unpublished).

During the DNA purification by silica particles using the easyMag® system up to 24 samples can be processed simultaneously in three specimen strips with each having eight individual sample vessels. The device detects how many samples need to be processed using a barcode (Herrmann, 2012b). From the supernatant of the homogenized tick samples 100 µl was pipetted into the vessels and introduced into the easyMag®. After the barcodes were scanned, a new run was created specifying the sample type, the amount of inserted sample material (100 µl), and the desired

amount of eluate (50 µl), as well as a caption for each sample. Then, the lysis buffer was automatically added to the samples. Meanwhile, the silica particles were prepared and diluted with nuclease-free H_2O with a ratio of 1 to 2, such that for 8 samples 550 µl silica and 550 µl H_2O are needed. After lysis, 8 times 125 µl of the silica solution was prepared in a microtiter plate using the electronic Biohit eLINE® pipette (Biohit Oyj, Helsinki, Finland) programmed for the easyMag®. Then, 100 µl were recorded with each of the 8 tips of the pipette, transferred into the sample-lysis mixture and mixed (Herrmann, 2012b). Once the silica particles were added to the sample the purification could start and run in accordance with the principle described above. Finally, the eluates were transferred into prepared 0.5 ml Eppendorf tubes and centrifuged at 5 °C for 3 min with 16,000 g before they were analyzed by PCR.

3.2.3 Polymerase chain reaction

The polymerase chain reaction (PCR) was applied to detect infections of the *B. burgdorferi* s.l. complex and of *Rickettsia* spp. in the organ and tick samples. PCR replicates in several steps specific nucleic acid sequences that exist only in small quantities within the sample. For the PCR a sequence-specific, complementary oligonucleotide pair (forward and reverse primer), a thermostable DNA polymerase and a mix of the 4 nucleotides, adenine (A), thymine (T), guanine (G) and cytosine (C) are required. The primers have a length of 16 to 24 base pairs and are selected such that the target sequence is located between them. The PCR is performed in a thermocycler, which is able to change the temperature of the reaction block very quickly. Different temperatures are necessary to denature the DNA (formation of single-stranded DNA at 95 °C), then to allow the accumulation of the primers (annealing at 50-65 °C), and finally to ensure the elongation of the primers along the DNA matrix to a new daughter strain (temperature optimum of DNA polymerase at 72 °C). During annealing the forward primer binds to the 5‘ end of the single-stranded DNA (ssDNA, while the reverse primer attaches to the 3’ end of the ssDNA. The 3-step procedure (denaturation, annealing and elongation) represents a so-called PCR cycle and is repeated 30 to 40 times. During each cycle, the DNA section

between the primers doubles ideally, such that the obtained products are exact copies of the originally targeted DNA section. The cyclic repetition results in an exponential increase of DNA fragments (2^n, where n is the number cycles), whereby the overall reaction process takes about 1 to 2 hours. By means of this method, even the slightest traces of DNA can be detected and made accessible for diagnostic purposes.

In addition, the sensitivity and specificity of a PCR can be increased through a so-called nested PCR, which consists of 2 nested PCR responses. In the first run of the PCR a large product is formed, which is used as a template for the second run. The second PCR then reproduces a DNA sequence, which lies within the amplification product of the first PCR. The primers of the second PCR are chosen so that they lie completely within the first amplification product. One speaks of a semi-nested or half nested PCR, when only one primer of the second reaction lies within the amplified region and a primer of the first reaction is reused during the second run.

However, the high sensitivity of the PCR induces a large risk of contamination. To avoid contamination, all PCRs were carried out in compliance with the following safety aspects:

- The DNA preparation, the creation of the PCR master mix, the addition of purified DNA to the individual PCR vessels and the implementation of the PCR were carried out spatially separated.
- All pipetting steps were carried out on a sterile workbench with sterile pipettes.
- Overnight, the work areas were irradiated by UV light to destroy any residual DNA.
- To minimize false-positive results all tests included negative controls (NCs) consisting of sterile H_2O_{bidest} that was treated in the same way as the samples.
- Each test run included at least one positive control (PC).

3.2.4 Real-time PCR

Another reproduction method for nucleic acids is the real-time PCR (RT-PCR) or real-time quantitative PCR. These methods are based on the principle of conventional PCR, but allow the detection of positive samples during the PCR run through fluorescent dyes. This eliminates the detection of PCR products using gel electrophoresis and reduces the risk of contamination. Another advantage of real-time PCR in comparison to PCR on the thermocycler is the shorter detection times and thus the time saving. There exist different fluorescence detection systems. One way for the detection of PCR products is the use of DNA intercalating dyes (e.g. SYBR Green or ethidium bromide). In addition, sequence-specific probes marked with fluorescent dyes can be used. These exploit the energy transfer between 2 fluorophores, known as fluorescence resonance energy transfer or Förster resonance energy transfer (FRET). The following sections will explain the use of various fluorescent probes using the LightCycler® (Roche Diagnostics, Mannheim, Germany).

3.2.4.1 Real-time PCR using the LightCycler®

In contrast to conventional PCR, on the LightCycler® the PCR reaction takes place in glass capillaries (length: 45 mm; diameter: 1.55 mm), which are transported by a step motor, are translucent, allow a uniform temperature distribution, as well as higher heating and cooling rates, and thus shorten the overall reaction time. The template DNA is prepared with the PCR master mix in the capillaries, which reside in a ring, the so-called carousel. In the LightCycler® 32 samples can be examined simultaneously. In addition to the primers, sequence-specific fluorescence-labeled probes are used, which hybridize with the PCR products. The detection of the PCR products is not carried out by visualization of PCR fragments on an agarose gel, but by means of fluorescence, which is measured after each cycle and is dependent on the amount of product formed. The fluorescence measurement uses a system of filters and mirrors with a photometric diode and is carried out at a wavelength of 530 nm. The total procedure is a one-phase PCR, whereby the risk of contamination is significantly reduced by eliminating the gel electrophoresis. Another advantage of

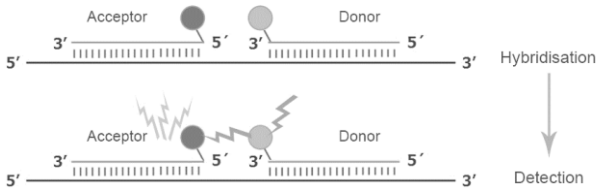


Figure 3.8: Reaction process of hybridization probes. Donor and acceptor hybridize to adjacent regions on the target DNA in close proximity (top). During FRET, the donor is excited by an external light source, its energy is transferred to the acceptor and the excited acceptor emits light, which can be detected and measured (bottom). Image from www.eurofinsgenomics.eu, 2015.

the LightCycler® is that the formation of the PCR products can be directly traced on the connected computer display (Skuballa 2011) and thus allows relatively quick sample diagnosis. The analysis of the PCR is based on threshold cycle (C_T). This value specifies the first reaction cycle, after which the fluorescence significantly raises above the background value (Applied Biosystems 2010).

The sequence-specific fluorescence detection method used for the visualization of the amplification employs probes that are marked with fluorescence dyes. Fluorescent probes are sequence-specific and complementary to the target sequence. Therefore, non-specific amplificates are not detected. There exist different types of FRET probes, such as hybridization probes and TaqMan® probes.

3.2.4.2 Hybridization probes

The two fluorescent dyes of hybridization probes are separated onto two different oligonucleotides: (1) the donor probe, also known as the anchor probe and (2) the acceptor probe, also called the sensor probe. They bind during the annealing phase to the complementary strand of the PCR product formed between the primers. Samples containing the target sequence, bind anchor and sensor probe in close proximity (distance $\leq$ 5 base pairs). Upon excitation from a light source, the sensor probe emits a light signal of 530 nm that stimulates the dye of the anchor probe and leads to the emission of a second signal (640 or 705 nm), which is measured by the detectors of the LightCycler®. The FRET can only take place when the binding of the probes occurs in close proximity (see Figure 3.8). This prevents the stimulation of

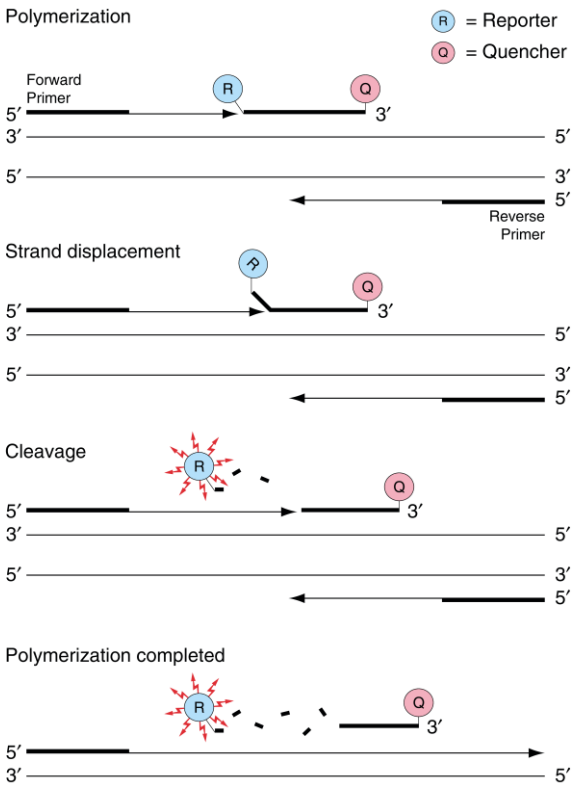


Figure 3.9: Reaction process of the TaqMan® probe. The probe is bound to the target DNA and is separated by the exonuclease activity of the *Taq* polymerase (Polymerization/Strand displacement), quencher and reporter are separated (Cleavage) and the fluorescence of the reporter can be measured by the LightCycler® (Polymerization completed). Image from Applied Biosystems (2010).

free probes and thus an incorrect determination. Since more amplificates are produced with increasing number of cycles, more probes can bind and the fluorescence intensity increases proportionally to the amount of the resulting PCR product. Fluorescence measuring is performed at the end of the annealing step of each PCR cycle. Hybridization probes can also be used for genotyping of samples that are marked with different fluorescent dyes.

3.2.4.3 TaqMan® probes

TaqMan® probes (see Figure 3.9), also known as hydrolysis probes, provide a way to detect only a specific DNS product during the PCR. They are short pieces of DNA that hybridize with a middle region of the template DNA. TaqMan® probes have a reporter fluorescent dye (R) (equivalent to donor fluorochromes) on one end and a

quencher (Q) on the other end. Quencher are molecules that intercept the fluorescence of dyes in their vicinity. In addition to its polymerase activity, the *Taq* polymerase of the PCR premix has a 5'-3' exonuclease activity making the breaking down of the TaqMan® probes possible. Once the polymerase cleaves the probe bound to the target sequence during the synthesis of the complementary strand on the 5' end, the quencher and reporter move away from each other, so that the reporter is able to fluoresce freely. The fluorescence, which depends on the amount of amplification product, is measured by the detectors of the LightCycler®. The fluorescence measurement takes place at the end of the elongation phase (Holzapfel and Wickert 2007). By this means, the fluorescence of the reporter is only measurable when the polymerase has actually copied the desired DNA strand. Each released molecule of reporter dye corresponds to a produced DNA strand and therefore can be employed to measure the amount of copied DNA. Consequently, TaqMan® probes allow the detection of amplificates as well as their quantification.

3.2.5 Specific detection of *Borrelia* spp.

For specific detection of *B. burgdorferi* s.l. in tissue 2 different PCR systems were used. For the first analysis a LightCycler® PCR (LC-PCR) was carried out targeting the *ospA* gene (*OspA*-PCR) (Rauter et al. 2002). A more time-consuming and more sensitive semi-nested PCR based on the method of Rijpkema et al. (1995) targeting the spacer region between 5S and 23S rRNA (5S23S rDNA-PCR) was carried out on the on the GeneAmp® thermocycler to verify positive LC-PCR results. The amplification products were investigated by gel electrophoresis in an agarose gel. For the negative control (NC) nuclease-free water and for the positive control (PC) a dilution of *B. burgdorferi* s.l. was used. Ticks samples were investigated for *Borrelia* spp. using only the LightCycler® method. Due to financial limitations, the *Borrelia* genospecies was not determined for any of the samples.

3.2.5.1 OspA-PCR on the LightCycler®

The *OspA*-PCR uses hybridization probes to detect *B. burgdorferi* s.l. (Rauter et al. 2002) and was applied to all samples in the present work. After denaturation of the

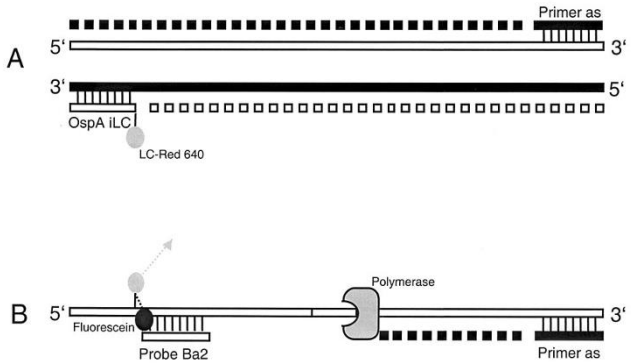


Figure 3.10: OspA-PCR primer and probe in the LightCycler®. The forward primer (OspA iLC) labeled with LC Red 640 and the fluorescein-labeled probe (Probe Ba2) bind to the first DNA strand. Their proximity induces LC Red 640 fluorescence by FRET. Adapted from Rauter et al. (2002).

double-stranded DNA to single strands, the forward primer (*OspA* ilC), which is marked with a LC red 640 probe (anchor probe), and the reverse of primer (*OspA* as) accumulate on the complementary sequences during the annealing phase (see Figure 3.10A). The polymerase will then synthesize the complementary strands. The sensor probe (Probe Ba2) is labelled with fluorescein and has its target sequence in the immediate vicinity of the anchor probe. During the following cycles of the PCR, the presence of *Borrelia* DNA leads to a spatial convergence of the sensor probe to the DNA strands that have been marked with LC red 640. The LC red 640 dye emits light at a wavelength of 640 nm, which is detected by the LightCycler® (see Figure 3.10B). The fluorescence signal is measured at the end of each annealing phase and increases with the PCR product formation in an exponential way (cf. Section 3.2.4.2).

Primers and probes of the *OspA*-PCR are given in Table 3.1. The corresponding pipetting scheme and the temperature profile are shown in Tables 3.2 and 3.3, respectively. $MgCl_2$, H_2O, and the enzyme mix were taken as components of the LightCycler® DNA Master HybProbe kit (LC-Kit). The prefabricated enzyme mix includes a DNA polymerase (Taq), a reaction buffer, 10 mM $MgCl_2$ as well as a dNTP mix with dUTP instead of dTTP.

Table 3.1: Primers and probes of the OspA-PCR on the LightCycler® for *Borrelia* spp.

Primer / Probe		Sequence (5' to 3')
forward primer & probe	OspA iLC	5`-AgCCTTAATAgCATgYAAgCAAAA®X`Tg-3`
reverse primer	OspA as	5`-CTAgTgTTTTgCCATCTTCTTTgAAAA-3`
probe	OspA Sensor Ba2	5`-gCgCTgTTTTTTTCATCAAggCTgCTAAC☼X-3`

®X'=LC Red 640-labeled base. ☼X= fluorescein-labeled base

Table 3.2: Pipetting scheme of the OspA-PCR for *Borrelia* spp.

Reagent	Amount
H_2O	8.8 µl
OspA as (5 pmol/µl)	2 µl
OspA ilc (10 pmol/µl)	1 µl
OspA Ba2 (10 pmol/µl)	1 µl
MgCl2 (25 mM)	3.2 µl
Enzyme mix (from LC-Kit)	2 µl
Sample DNA	2 µl
Total	**20 µl**

Table 3.3: Temperature profile of the OspA-PCR for the detection of *Borrelia* spp.

	Temp. (°C)	Time (sec)	Temp. change (°C/sec)	No. cycles
Denaturation				
	95	30	20	1
Amplification				
Denaturation	95	1	20	60
Annealing	57	10	20	60
Elongation	72	13	20	60
Cooling				
	40	30	20	1

This PCR protocol also allows the detection of several species of the *B. burgdorferi* s.l. complex by the creation of a melting curve following the amplification of the PCR product (Rauter et al. 2002, Skuballa 2011). However, *Borrelia* infections were not determined up to the genospecies in this thesis.

3.2.5.2 Semi-nested 5S23S rDNA-PCR

Extracted DNA from organ pools that positively during the LC-PCR were tested by 5S23S rDNA-PCR on the thermocycler in accordance with (Rijpkema et al. 1995) to avoid false positives. The target sequence of this semi-nested PCR is the intergenic spacer 2 (IS2), which is located between the 5S rRNA gene and the 23S rRNA gene. The first PCR run uses the forward primer 23SN1 and the reverse primer 23SC1 to

Table 3.4: Primers of the semi-nested 5S23S rDNA-PCR for the detection of *Borrelia* spp.

Primer / Probe		Sequence (5' to 3')
forward primer 1st PCR run	23SN1	5'-ACCATAGACTCTTATTACTTTGAC-3'
reverse primer 1st PCR run	23SC1	5'-TAAGCTGACTAATACTAATTACCC-3'
forward primer 2st PCR run	23SN2	5'-ACCATAGACTCTTATTACTTTGACCA-3'
reverse primer 2st PCR run	5SCB	5'-GAGAGTAGGTTATTGCCAGGG-3'

amplify a relatively long DNA piece of 380 bp, which is used as a source for the second run. The second PCR used the forward primer 23SN2, which is indented by 128 bp, and the reverse primer 5SCB (Table 3.4). The resulting PCR product has a length of 226 bp (Skuballa 2011). To each PCR run prepared DNA of the *Borrelia* strain B31 was added as positive control. This method has been applied by Oehme et al. (2002).

To prevent contamination with the amplificates of the nested PCR, the dUTPs were partially replaced by dNTPs. In the first cycle of the nested PCR a digestion step with uracil-DNA glycosylase (UDG) was integrated upstream. This enzyme causes interfaces on the DNA with the built-in uracil. As a result, the DNA which has built-in dTTPs instead of dUTPs can no longer by amplified. The pipetting schemes and temperature profiles are presented in Tables 3.5 and 3.6, respectively.

3.2.5.3 Detection of PCR products in Agarose gel

An agarose gel electrophoresis was used to detect the PCR amplification products obtained on the thermocycler. The amplificates are separated in an electric field according to their base size. Therefore, the amplified DNA is applied to a gel situated in an ionic buffer solution. The separation of the DNA occurs in an electrophoresis chamber, where the negatively charged, double-stranded DNA (dsDNA) fragments move in the electric field to the positive pole. The separation was carried out at a voltage of about 90 V for approximately 30 min. The differing movement of the DNA fragments through the agarose gel, which acts like a sieve, depends on the size of the molecules. The smaller the nucleobases are, the faster they move through the pores

Table 3.5: Pipetting schemes for the 1st and 2nd run of the semi-nested 5S23S rDNA-PCR for the detection of *Borrelia* spp.

1st PCR run		**2st PCR run**	
Reagent	**Amount**	**Reagent**	**Amount**
10 × Buffer	5 µl	10 × Buffer	5 µl
$MgCl_2$ (25mM)	5 µl	$MgCl_2$ (25mM)	5 µl
dNTP Mix (1 mM je dNTP)	10 µl	dNTP Mix (1 mM je dNTP)*	10 µl
Tris HCL (300 mM)	6.7 µl	Tris HCL (300 mM)	6.7 µl
23SN1 (6 pmol)	5 µl	23SN2 (6 pmol)	5 µl
23SC1 (6 pmol)	5 µl	5SCB (6 pmol)	5 µl
Nuclease-free H_2O	8 µl	Nuclease-free H_2O	11 µl
AmpliTaq® (5 U/µl)	0.3 µl	AmpliTaq® (5 U/µl)	0.3 µl
		Uracil-DNA glycosylase (1 U/µl)	0.5 µl
Sample DNA	5 µl	DNA of the 1st PCR run	1.5 µl
Total	**50 µl**	**Total**	**50 µl**

*dTTP : dUTP at a ratio of 4 to 1

of the gel. Thus, larger fragments remain closer to the top of the gel. The in-gel ethidium bromide (EthBr) intercalates with the double strands of DNA making possible their detection by excitation with ultraviolet (UV) light.

This allows the detection of single DNA fragments by fluoresces when the EthBr is exited with UV light. In addition to the PCR products, a defined marker (100 bp ladder) was applied to compare the DNA fragments with the length standard. The marker contains an 800 bp fragment with twice the intensity of the other bands. The bottom line is located at a size of 100 bp. In a 1.5% agarose gel 20 bands with a step-size of 100 bp emerge. Consequently, a precise determination of the size of DNA fragments between 100 and 2000 bp length is possible. Furthermore, a positive control, which must have a band in accordance with the amplified DNA fragment, was always used.

For the specific detection of amplification products of semi-nested *Borrelia* PCR (5S-23S rDNA fragments) a 1.5% agarose gel was prepared and loaded as follows:

Table 3.6: Temperature profiles of the semi-nested 5S23S rDNA-PCR for the detection of *Borrelia* spp.

	1st PCR run			2st PCR run		
	Temp. (°C)	**Time**	**No. cycles**	**Temp. (°C)**	**Time**	**No. cycles**
Denaturation / Decontamination						
	94	1.5 min.	1	37	4 min.	1
				96	2 min.	1
Amplification						
Denaturation	94	20 sec.	35	94	20 sec.	35
Annealing	52	30 sec.	35	55	30 sec.	35
Elongation	72	40 sec.	35	72	40 sec.	35
Final Elongation	72	5 min.	1	72	5 min.	1
Cooling						
	4	∞	1	4	∞	1

- Mix of 0.75 g agarose with 50 ml of TBE buffer (1×) in a glass flask,
- bring to a boil twice on a hotplate under constant stirring,
- cool down to approximately 50 °C,
- add 10 µl of a 0.1% ethidium bromide solution,
- pour liquid gel into a specific gel chamber equipped with combs to form slots,
- wait approximately 20 min until polymerized,
- put polymerized gel in an electrophoresis chamber filled with TAE buffer (1×),
- fill the slots with 8 µl PCR product and 2 µl loading buffer,
- and apply the marker (100 Base Pair ladder) and the positive control.

3.2.6 Specific detection of *Rickettsia* spp.

Infections with *Rickettsia* spp. in host organs and ticks were analyzed with the real-time TaqMan® real-time PCR protocol of Wölfel et al. (2008) using the LightCycler® targeting the *gltA* gene (Pluta et al. 2010, Pluta 2011). To avoid false-positive *Rickettsia* spp. results from the real-time PCR, two additional PCRs were performed on the thermocycler for those samples that showed positive on the LightCycler®. The

Table 3.7: Primers and probe of the gltA-PCR on the LightCycler® for the detection of *Rickettsia* spp.

Primer / Probe		Sequence (5' to 3')
forward primer	PanRick gltA 2 for	5'-ATAggACAACCgTTTATTT-3'
reverse primer	PanRick gltA 2 rev	5'-CAAACATCATATgCAgAAA-3'
TaqMan® probe	PanRick gltA taq	5´-6FAM-CCTgATAATTCgTTAgATTTTACCg-TMR-3´

first PCR targeted the rOmpA gene (*ompA*) following the method of (Roux and Raoult 2000). The second PCR targeted the citrate synthase gene (*gltA*) using primers from Nilsson et al. (1999). The temperature profiles and pipetting schemes were in accordance with Hartelt et al. (2004). The detection of the PCR amplification production from the thermocycler was carried out on an agarose gel electrophoresis (see Section 3.2.5.3).

3.2.6.1 gltA-PCR on the LightCycler®

The gltA-PCR on the LightCycler® used a TaqMan® probe (see Section 3.2.4.3) and 2 primers, which amplified a 70 bp region of the citrate synthase gene (gltA). PanRick gltA 2 for was used as forward primer, PanRick gltA 2 rev was the reverse primer and PanRick gltA taq the employed probe (Table 3.7). Each PCR run used purified of *Rickettsia rickettsii* (Dr. Kathrin Hartelt, Landesgesundheitsamt Baden-Württemberg) as positive control and H_2O as a negative control. The pipetting scheme employed and the temperature profile are displayed in Tables 3.8 and 3.9, respectively. This PCR method has also been used in other studies for the investigation of ticks (Pluta et al. 2010, Pluta 2011).

3.2.6.2 rOmpA-PCR on the thermocycler

This PCR protocol targets the rOmpA gene, a sequence of 532 bp. This gene encodes the rickettsial outer membrane protein (Roux and Raoult 2000). The purified DNA

Table 3.8: Pipetting scheme of the gltA-PCR for *Rickettsia* spp.

Reagent	Amount
PanRick gltA 2 for (5 pmol/µl)	2 µl
PanRick gltA 2 rev (5 pmol/µl)	2 µl
PanRick gltA taq (4 pmol/µl)	1 µl
$MgCl_2$ (25 mM)	1.6 µl
H_2O	6.4 µl
Enzyme mix (from LC-Kit)	2 µl
Sample DNA	5 µl
Total	**20 µl**

Table 3.9: Temperature profile of the gltA-PCR for the detection of *Rickettsia* spp.

	Temp. (°C)	Time (sec)	Temp. change (°C/sec)	No. cycles
Denaturation				
	94	60	20	1
Amplification				
Denaturation	94	4	20	50
Annealing	55	45	20	50
Elongation	72	30	20	50
Cooling				
	40	30	20	1

Table 3.10: Primers of the rOmpA-PCR (top), the rOmpB-PCR (middle) and the gltA-PCR (bottom) on the thermocycler for the detection of *Rickettsia* spp.

Primer / Probe		Sequence (5' to 3')
forward primer rOmpA-PCR	Rr190.70p	5'-ATGGCGAATATTTCTCCAAAA-3'
reverse primer rOmpA-PCR	Rr190.602n	5'-AGTGCAGCATTCGCTCCCCCT-3'
forward primer rOmpB-PCR	120-2788	5'-AAACAATAATCAAGGTACTGT-3'
reverse primer rOmpB-PCR	120-3599	5'-TACTTCCGGTTACAGCAAAGT-3'
forward primer gltA-PCR	RH314	5'-AAACAGGTTGCTCATCATTC-3'
reverse primer gltA-PCR	RH654	5'-AGAGCATTTTTTATTATTGG-3'

of *Rickettsia monacensis* (Dr. Kathrin Hartelt, Landesgesundheitsamt Baden-Württemberg, Stuttgart) was used as a positive control. The primers employed are shown in Table 3.10 and the pipetting scheme and the temperature profile used are shown in Tables 3.11 and 3.12, respectively.

3.2.6.3 gltA-PCR on the thermocycler

This PCR method amplifies a target sequence of 341 bp using the primers from (Nilsson et al. 1999). The temperature profile and the pipetting scheme were

Table 3.11: Pipetting schemes for the rOmpA-PCR (left), rOmpB-PCR (center) and gltA-PCR (right) on the thermocycler for the detection of *Rickettsia* spp.

rOmpA-PCR		**rOmpB-PCR**		**gltA-PCR**	
Reagent	**Vol.**	**Reagent**	**Vol.**	**Reagent**	**Vol.**
10 × Buffer	5 µl	Nuclease-free H_2O	8 µl	10 × Buffer	5 µl
$MgCl_2$ (2 mM)	5 µl	10 × Buffer	5 µl	$MgCl_2$ (2.5 mM)	5 µl
dNTP Mix (200 µM)	10 µl	$MgCl_2$ (2.5 mM)	5 µl	dNTP Mix (200 µM)	10 µl
Tris HCL (40.2 mM, pH9)	6.7 µl	dNTP Mix(1 mM)	10 µl	Tris HCL (40.2 mM, pH 9)	6.7 µl
Rr190.70p (0.5 µM)	5 µl	Tris HCL (40.2 mM, pH 9)	6.7 µl	RH314 (0.5 µM)	5 µl
Rr190.602n (0.5 µM)	5 µl	120-2788 (5 µM)	5 µl	RH654 (0.5 µM)	5 µl
Nuclease-free H_2O	8 µl	120-3599 (5 µM)	5 µl	Nuclease-free H_2O	11 µl
AmpliTaq® (1.5 units)	0.3 µl	AmpliTaq® (1.5 units)	0.3 µl	AmpliTaq® (1.5 units)	0.3 µl
Sample DNA	5 µl	Sample DNA	5 µl	Sample DNA	1.5 µl
Total	**50 µl**	**Total**	**50 µl**	**Total**	**50 µl**

Table 3.12: Temperature profiles of the rOmpA-PCR (left), rOmpB-PCR (center) and gltA-PCR (right) on the thermocycler for the detection of *Rickettsia* spp.

	rOmpA-PCR			**rOmpB-PCR**			**gltA-PCR**		
	Temp. (°C)	**Time (sec.)**	**cycles**	**Temp. (°C)**	**Time (sec.)**	**cycles**	**Temp. (°C)**	**Time (sec.)**	**cycles**
Denaturation									
	95	180	1	94	180	1	95	180	1
Amplification									
Denaturation	95	20	35	95	30	40	95	30	35
Annealing	60	30	35	50	30	40	54	30	35
Elongation	72	60	35	68	90	40	72	45	35
Final Elongation	72	300	1	68	420	1	72	300	1
Cooling									
	4	∞	1	4	∞	1	4	∞	1

adopted from (Hartelt et al. 2004). As positive control purified DNA of *Rickettsia helvetica* and *R. monacensis* (Dr. Kathrin Hartelt, Landesgesundheitsamt Baden-Württemberg, Stuttgart) was used. The primers used are shown in Table 3.10, while Tables 3.11 and 3.12 show the pipetting scheme and the temperature profile of the gltA-PCR, respectively.

Table 3.13: Pipetting scheme for the *Rickettsia* DNA-sequencing.

Reagent	Amount
BigDye Seq. Puffer (5 ×)	2 µl
Primer (5 pmol/µl)	2 µl
H_2O	10 µl
BigDye (v 1.1) Mix	4 µl
DNA Template (purified)	2 µl
Total	**20 µl**

3.2.6.4 Sequencing of Rickettsia spp.

From the PCR tick samples that tested positive, 83 were randomly selected and investigated to determine the species of *Rickettsia* present. The analyzed tick samples were all from roe deer. Therefore, at first a PCR protocol on the thermocycler was used to amplify a 811-bp sequence of the *ompB* gene using the primers 120-2788 and 120-3599 in Table 3.10 (Roux and Raoult 2000, Wölfel et al. 2008). As positive control the purified DNA of *R. monacensis* (Dr. Kathrin Hartelt, Landesgesundheitsamt Baden-Württemberg, Stuttgart) was used. The pipetting scheme and the temperature profiles of the rOmpB-PCR are given by Tables 3.11 and 3.12, respectively.

The amplification products of the rOmpB-PCR were then purified using the QIAquick Spin PCR Purification Kit (Qiagen, Venlo, Netherlands). Sequencing reactions were carried out on the thermocycler using the BigDye Terminator v1.1 Cycle Sequencing Kit and the ABI PRISM 310 Genetic Analyzer® on the basis of the chain termination method (Skuballa 2011). The BigDye (v1.1) Mix includes fragments of AmpliTaq® DNA polymerase, BigDye terminators, dNTPs, rTth pyrophosphatase, $MgCl_2$ and buffer. Thereby the polymerase is responsible for the amplification of the DNA template and for incorporation of the terminators. The master mix used for the sequencing reaction is described in Table 3.13.

The capillary gel electrophoresis on the ABI PRISM® 310 Genetic Analyzer used the Gel Performance optimized Polymer 6 (Skuballa 2011). After the resulting bases were checked and corrected manually where necessary, the DNA sequences were compared with existing data records on GenBank (NCBI taxonomy database, http://www.ncbi.nlm.nih.gov).

3.3 Data supplementation

3.3.1 Measure of aggregation and relative tick density

Shaw et al. (1998) demonstrated that parasites on wildlife animals usually show patterns of aggregation following a negative binomial distribution of type I (NBI), with a few hosts harboring most parasites. The dispersion parameter k of the NBI is often used as an inverse measure of aggregation. For each tick life history stage, the dispersion parameter can be calculated by the corrected moment estimate $k = (\mu_i^2 - \sigma_i^2/n)/(\sigma_i^2 - \mu_i)$, with μ_i representing the mean number of the ith tick life history stage per host and σ_i^2 being the corresponding variance, while n is the total number of sampled host individuals (Wilson et al. 2002). As k approaches zero the parasite aggregation increases, while larger values of k represent a lower level of aggregation (Wilson et al. 2002).

In order to estimate the relative tick density on each part (1 to 8, in Figure 3.5a and in Figure 3.5b) of the game carcass, the proportional surface area was determined by polygon measurements on photographs of roe deer and wild boar. Because the proportional surface area can vary with age and sex of the game animals, reference images of 25 roe deer and 20 wild boars were photographed for each group in accordance with the headings of Tables 3.14 and 3.15, respectively, such that 5 measurements were preformed per group. Based on the photographs, the proportional surface area of each body of the carcass was determined using ImageJ 1.48r (by National Institutes of Health) by performing the following steps:

1. Draw a polygon around each body part.

Table 3.14: Averaged surface area proportions (mean ± SD) of the roe deer body.

Body Part	Adult Male	Adult Female	Yearling Male	Yearling Female	Fawn Male/Female	Total Study
1 – Ear	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
2 – Head	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.00
3 – Neck	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.00	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01
4 – Body	0.23 ± 0.02	0.23 ± 0.01	0.24 ± 0.01	0.23 ± 0.01	0.20 ± 0.02	0.23 ± 0.01
5 - Front legs	0.23 ± 0.02	0.25 ± 0.02	0.22 ± 0.01	0.24 ± 0.02	0.26 ± 0.02	0.24 ± 0.01
6 - Hind legs	0.30 ± 0.01	0.27 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.30 ± 0.03	0.29 ± 0.01
7 - Sternum	0.03 ± 0.01	0.04 ± 0.00	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.00
8 - Abdomen	0.04 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.00

Table 3.15: Averaged surface area proportions (mean ± SD) of the wild boar body.

Body Part	Adult Male	Adult Female	Young Male/Female	Fawn Male/Female	Total Study
1 – Ear	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
2 – Head	0.12 ± 0.02	0.13 ± 0.02	0.11 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
3 – Neck	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.03	0.04 ± 0.01
4 – Body	0.29 ± 0.03	0.27 ± 0.03	0.26 ± 0.02	0.26 ± 0.04	0.27 ± 0.03
5 - Front legs	0.22 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.22 ± 0.02	0.23 ± 0.02
6 - Hind legs	0.23 ± 0.02	0.22 ± 0.01	0.24 ± 0.01	0.26 ± 0.02	0.24 ± 0.01
7 - Sternum	0.03 ± 0.00	0.03 ± 0.01	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
8 - Abdomen	0.03 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.03 ± 0.01

2. Calculate the area, i.e. contained pixels, within each polygon.
3. Compute the area sum over all polygons.
4. Divide each individual polygon area by the sum from step 3 to gain the proportional surface area.
5. Repeat step 1 to 4 for each photograph.

Subsequently, Microsoft Excel 2013 was used to calculate for each group the means and standard deviations (SDs) shown in Tables 3.14 and 3.15. Thereafter, tick densities for each part of the body were calculated based on the absolute tick numbers in relation to the relative surface area of the body region on which they were found. For a specific body part, denoted by j, the relative tick density can be calculated as

$$D_j = \frac{t_j}{a_j} ,$$

where t_j is the total number of ticks found at attachment site j and a_j is the proportional surface area determined by the procedure described above.

Using the tick densities calculated for each part of the game carcasses, a non-parametric Friedman test was carried out to identify body regions preferred by ticks, taking into account the life history stages of the ectoparasites as well as the season when they were collected. Consequently, the seasonal differences in the use of attachment sites could be determined with respect to the ticks' development. By this means, the potential places for tick attachment, mating and co-feeding were ranked. During the ranking, tick life history stages and seasons having a limited sample size were excluded.

In addition to this, the ideal free distribution hypothesis (IFDH) (Fretwell and Calver 1969) was tested at the host level using correlation and regression analyses to determine the relationship between the preferred attachment sites and the density of the ticks on the host body. In the context of ticks, this hypothesis can be interpreted such that with a higher tick infestation on the host body, the ectoparasites can be found increasingly in less preferred body areas (Sutherland 1996).

3.3.2 Niche index – Levin's index

After ranking the preferred attachment sites, Levin's index B (Levins 1968) was calculated to provide information on the niche breadth of the ticks per life history stage, sex and status of engorgement using the following expression:

$$B = \frac{1}{\sum p_j^2} ,$$

where p_j specifies the proportion of ticks found on the body part with the number j. This proportion can be calculated as $p_j = t_j/T$, with t_j being the number of ticks

at body part j and T the total number of ticks found on the host. Because the data used here do not follow a normal distribution, Levin's index was standardized by:

$$B_s = \frac{B - 1}{N - 1},$$

whereby N equals the total number of body parts, so that that the resulting values lie between 0 (narrow niche) and 1 (wide niche) (Hurlbert 1978). Thereby, a niche breadth of 0 indicates that all ticks were allocated on the same body part.

3.3.3 Niche overlap – Piankas' index

In addition to the ranking of the relative tick densities and the calculation of niche breadths, the Pianka index (Pianka 1973) was calculated to obtain information on the niche overlap of the ticks life history stages. This index was then used to evaluate the importance of co-feeding dependent on the body part and tick life history stage. Resource overlap was calculated by:

$$O_{jk} = \frac{\sum_{i=1}^{n} p_{ij} p_{ik}}{\sqrt{\sum_{i=1}^{n} p_{ij}^2 \sum_{i=1}^{n} p_{ik}^2}},$$

where p_{ij} represents the proportion of ticks of a certain life history stage, denoted by i, found at the body part with number j. The value of O_{jk} reflects the resource overlap between two tick life history stages j and k, whereby the index is symmetrical in the sense that $O_{jk} = O_{kj}$.

3.3.4 Group dependent mass index (GDMI)

The body mass of game animals allows conclusions about their individual's health and immune status (Pettorelli et al. 2002, Stubbe 2008, Briedermann 2009). It is assumed that the average body mass of big game depends on the sex and the stage of life, depending on changes over time during the year with the animal's individual development, i.e. growth. Nevertheless, the definition of a condition index (CI) that

reflects the physical condition of animals has been controversially discussed in ecological literature and has not yet been verified on roe deer (Peig and Green 2009, 2010). For this reason, the following derivation uses a naïve approach based on the sample population to determine an indicator for the physical condition of the studied host animals. A standardized group dependent mass index ($GDMI_s$) was calculated for both species in relation to the age, sex and sampling period of both species. Roe deer and wild boar were separated into groups formed by all possible constellations of sex, age and time period. For each group, denoted by g, an average body mass $\bar{m}_g$ was calculated over all related animals. Given an animal from the group g, the difference between its individual mass m and the mean body mass of its group $\bar{m}_g$ was then calculated as $M = m - \bar{m}_g$. Based on the difference M the standardized mass index $GDMI_s$ was calculated for each animal using the following formula:

$$GDMI_s = 2 \cdot \left(\frac{M - M_{min}}{M_{max} - M_{min}} - 0.5 \right) ,$$

where M_{max} and M_{min} are the maximal and minimal values of M within the whole dataset including all groups. The mean body mass of each group $\bar{m}_g$ and the differences$M$, M_{max} and M_{min} are displayed in Table B.1 of the appendix.

The index $GDMI_s$ lies between -1 and 1, whereby animals with low body mass tend to a value of -1 and those with high individual mass have an index tending to 1. Consequently, animals with a value of $GDMI_s > 0$ have an individual body mass higher than the group mean $\bar{m}_g$ and animals with $GDMI_s < 0$ have a body mass lower than the group average. As a result, the index allows an easier interpretation of the animal's body mass in relation to the groups formed, whereby higher indices might suggest healthier conditions and lower ones a less than optimal physical state. Nevertheless, the index should not be taken as an absolute quantity to judge an animal's physical condition. Rather it should be used as an indicator to support interpretation of other animal parameters.

3.3.5 Animal conditions

As mentioned above, for each animal infestation with additional ectoparasites was recorded, as were whether the individual was in a poor condition and if it had any deformation, i.e. was somehow crippled. In addition to these individual facts, a new variable, denoted by "HasCondition" has been introduced into the dataset, which has been set to 1 if any of the above conditions was encountered. Otherwise, the "HasCondition" parameter has been assigned a value of 0.

In addition, the tick induced blood loss BL was calculated for every animal sampled. For this purpose, the blood loss was estimated using the formula proposed by Tälleklint and Jaenson (1997):

$$BL = \frac{\left(2.62\ \mu l \cdot N_l + 15.86\ \mu l \cdot N_n + 732.8\ \mu l \cdot N_f\right) \cdot 10^{-6}}{m^{0.99} \cdot 0.055} \cdot 100\ ,$$

whereby N_l, N_n and N_f are the number of larval, nymphal and female ticks on the host, and m represents the body mass of the individual in kilograms. The resulting value represents the blood loss in percent.

3.4 Statistical methods

All parameters on ticks, game animals and pathogens were recorded in Microsoft Excel 2013. Indices, prevalences and intensities were also calculated in Excel. The resulting dataset was supplemented by the calculations described in Section 3.3. An overview of all variables contained in the resulting dataset is given by Table 3.16. Thereafter, the dataset was analyzed using IBM SPSS Statistics 22. Prior to statistical analysis, the data were tested for normal distribution using the Shapiro-Wilk test. For data not following the normal distribution, non-parametric tests (i.e. Mann-Whitney U-test and Kruskal-Wallis test) were used, whereas for normally distributed data a parametric tests (i.e. *t*-test) were employed.

Table 3.16: List of all variables contained in the final dataset about sampled host animals and ticks. Note that only relevant variables are shown in the results of the statistical analysis, although the complete dataset is available for future studies.

Category	**Variables**
Time data:	culling date, month group
Game information:	ear ID, species, age, sex, body mass, GDMI, hunting hide
Game samples*:	EDTA, serum, lung, liver, splenic, bladder, kidney, heart, muscle, ear
Game condition:	pregnant, nursing, poor condition, crippled, lungworms, deer fly, other ectoparasites, has condition
Organ infections*:	*Rickettsia* spp. in pool 1 and pool 2, *Borrelia* spp. in pool 1 and pool 2
Climate:	cloud coverage, relative humidity, air temperature, air pressure, wind speed, precipitation depth, sunshine duration, snow height
Number of collected ticks:	total ticks, males, females, nymphs, larvae, dead, mating
Proportional surface areas:	for ear, head, neck, body, front leg, hind leg, sternum, abdomen
Tick density:	one variable for all ticks, for each life history stage and for mating ticks at each body part (8 × 6 = 48 variables)
Niche breadth:	Levin index and standardized Levin index for all ticks and for each life history stage
Niche overlap:	Pianka's index for all combinations of tick life history stages
Notes:	type of hunt
Tick information:	tick ID, mate, host ID, species, age, engorgement, dead
Tick infections*:	*Rickettsia* spp., *Borrelia* spp., concurrent infection

* binary variables (yes = 1/no = 0)

To compare tick prevalences between groups of host animals contingency tables were generated, including a chi-squared test to identify significant differences, whereby for the comparison of 2 groups Fisher´s exact test has been used. Besides Pearson correlation analysis, linear regressions and generalized linear models (GLMs) as well as CHAID (chi-square automated interaction detector) classification trees were used to determine the significant and ranked impact of all analyzed factors comprising tick data, host data and climatic measurements (Sonquist and Morgan 1964). With CHAID the results were classified into groups (nodes) and chained in a ranked order describing the most important factors that influence tick infestation, while the GLMs were used to inspect seasonal niche behavior of the ticks.

In general, for all statistical tests P-values smaller than 0.05 were assumed to be significant, while values smaller than 0.001 were classified as highly significant.

Note that in the following P-values will be provided where they indicate significance or where they are relevant, otherwise they will be omitted for readability reasons.

3.4.1 Statistical modeling

A statistical modeling approach was used to estimate models for the infestation intensity of tick life history stages in relation to host and climate parameters. For reasons of flexibility and in accordance with Kiffner et al. (2011a, 2011c), generalized additive models for location, scale and shape (GAMLSS) were used to create regression type models. In comparison to other regression-based modelling techniques (e.g. linear regression or GLM), the GAMLSS approach (Rigby et al. 2005) can be used to model the mean as well as the dispersion of the dependent response variable with respect to a given parametric distribution. In relation to ticks on roe deer, Kiffner et al. (2011c) have shown that a variable dispersion term clearly improved the model fit in comparison to a conventional negative binomial additive model fitting a constant scale parameter. Additionally, the expression of the distribution parameters as functions of the exploratory variables can incorporate non-linear functions, such as smoothing terms.

In a first step, the dependent variables were tested as to whether they were better represented by a Poisson (PO) or a negative binomial (type I) distribution. This test was carried out by first fitting either distribution to the data using the maximum-likelihood estimation followed by the determination of the quality of the fit on the basis of the Akaike information criterion (AIC). After the selection of the distribution, the GAMLSS approach was used to calculate several models with respect to each developmental stage of the ticks. The first 4 models for each tick life history stage were estimated to determine the relationships between tick burden, air temperature, host sex and host body mass. Thereby, host body mass of each host was weighted by a value of 0.75 to simplify its relationship with parasite biomass (see Kiffner et al. 2011c). Then, the modeling formulas in accordance to the notation introduced by Chambers and Hastie (1992) are as follows:

1. Males ~ pb(Temperature)
2. Males ~ Sex + pb(Temperature)
3. Males ~ BodyMass + pb(Temperature)
4. Males ~ Sex + BodyMass + pb(Temperature)

A smoothing penalized B-spline (pb) was used to model the relationship between tick burden and temperature. The temperature itself was calculated as the average of four successive days, including the three days before the culling of the host animal and the day of its death. This averaging process ensured that the temperature values used corresponded to the attachment duration of the modeled ticks (compare with Kiffner et al. 2011c). Moreover, in each of the 4 models a heterogenic dispersion was assumed, which was related to the smoothing term "pb(Temperature)" during the model estimation procedure to achieve better fits (cf. Kiffner et al. 2011c). After successful generation of the models, the best fitting one was selected based on the sample size corrected AIC, denoted by AIC_c (Burnham and Anderson 2002).

In a subsequent step, a more complex model was estimated for each tick life history stage using the GAMLSS approach in combination with additional parameters, such that the inclusion of host sex, host age, host body mass, air temperature and precipitation depth was considered by an automatic parameter selection scheme. Based on the generalized AIC (GAIC) an optimization driven procedure selected the model which included those parameters that procured the best fit, whereby each parameter included was either represented linearly or by a non-linear term using a penalized B-spline. Finally, these automatically optimized models were compared to the corresponding aforementioned models selected by the AIC_c-values. The quality of the fits was additionally judged using worm plots (van Buuren and Fredriks 2001).

In a final step, the GAMLSS approach was used to estimate models for each tick life history stage with respect to the host body mass separately for each age group of the hosts to gain detailed information about their interdependencies. All of the above calculations were carried out in R (R Development Core Team 2013) using

the *gamlss* package (Stasinopoulos and Rigby 2007). In this context, the estimation of all models was performed using the RS algorithm (Stasinopoulos and Rigby 2007), whereby the automatic parameter selection made use of the *stepGAIC* function inside the *gamlss* package.

To investigate the composition of the tick population a multinomial logistic regression was used (Venables and Ripley 2002). For roe deer the proportions of collected male, female, nymphal and larval ticks were modelled depending on host related parameters. To find the best model from an information theory point of view, reflected by the AIC, all possible combinations of modelling formulas for the six parameters host species, host age, host sex, body mass and attachment site as well as the time of sample acquisition were used to perform 63 different multinomial logistic regressions. The sampling periods were represented by month, whereby months with a limited sample size were excluded from the modeling process. Additionally, body regions were aggregated to facilitate interpretation of results, such that four groups (Ears, Head & Neck, Front legs & Sternum, Hind legs & Abdomen) included all sampled ticks. Note that no ticks were found on the torso of any animal. The model with the lowest AIC was:

$$TickStage \sim Month + BodyMass + BodyPart + HostSex + HostSpecies.$$

This model was used for further interpretation of the effects on the composition of the tick population. Hereby it has to be considered that the reference group, i.e. the baseline, of the dependent variable was chosen such that female ticks were compared against all other life history stages. Moreover, January was used as reference period, while the ears, male hosts and roe deer were selected as the baselines in relation attachment site, host sex and host species, respectively. Predictions of the resulting model were then used to visualize the probability distribution of the individual tick life stages/sexes in relation to each of the independent model variables. The multinomial logistic regression was carried out in R (R Development Core Team

2013) using a maximum-likelihood estimation realized within the *vgam* package (Yee 2010).

With respect to infections of ticks with *Rickettsia* spp. and *Borrelia* spp., analyses included descriptive statistics, the identification of differences in the infection prevalences between groups of ticks using contingency tables in combination with chi-squared or Fisher's exact test, the study of linear relationships between parameters using Pearson's correlation coefficients, the detection of interdependencies between multiple variables by a factor analysis and, finally, the quantification of the parameters of importance using a decision tree and a logistic regression. The extraction of 5 orthogonal components was carried out by first applying an optimal scaling using categorical principal component analysis (CATPCA) to optimally quantify binary, ordinal and numerical variables (Meulman et al. 2004). This was followed by a factor analysis based on principle component analysis (PCA) in combination with a Varimax rotation and Kaiser Normalization. The decision tree was computed using the chi-square automated interaction detector (CHAID) classification algorithm to gain significant ranked impacts of the all analyzed parameters on the prevalence of the infections. The logistic regression was carried out using stepwise forward regression to inhibit the inclusion of non-significant parameters into the modelling process.

4

Tick burden

This chapter presents and discusses the results with respect to the tick burden on roe deer and wild boar.

4.1 Results

4.1.1 Tick burden on roe deer

Data were collected from September 2011 to February 2014 the examination including a total number of 247 roe deer composed of 83 fawns, 34 yearlings (age between 1 and 2 years) and 130 adult individuals (older than 2 years) (Table 4.1, Figure 4.1). Note that the maxima appear as a result of hunting and convalescence periods in Germany. Slight culling numbers during the warmer months (April to September, 2012 and 2013) were due to extremely wet weather conditions that made large parts of the sampling area extremely boggy and hunting difficult.

The total number of samples roe deer together with the number of collected ticks as well as the tick prevalence and intensities are listed by Table 4.2. Except for one loose and unengorged *D. marginatus* female (removed in October 2013), only *I. ricinus* was found on roe deer. A total of 1,584 ticks were recovered, whereby all *I. ricinus* life history stages were encountered: 154 larvae (9.7%), 492 nymphs (27.1%), 212 males (13.4%) and 789 females (49.8%).

Table 4.1: Numbers of sampled roe deer and wild boar by age with respect to year and month.

		Roe deer				Wild boar				Both species
		Adult	**Yearling**	**Fawn**	**Total**	**Adult**	**Young**	**Piglet**	**Total**	
2011	**Sep**	1	-	-	1	-	1	-	1	2
	Nov	24	2	21	47	22	23	19	64	111
	Dec	6	-	2	8	3	2	8	13	21
2012	**Jan**	13	-	6	19	7	1	14	22	41
	Apr	2	-	-	2	-	-	1	1	3
	Mai	2	9	-	11	-	1	-	1	12
	Jun	2	1	-	3	-	-	-	-	3
	Aug	1	-	-	1	-	-	-	-	1
	Sep	2	2	5	9	2	1	-	3	12
	Nov	20	3	15	38	8	17	55	80	118
	Dec	10	-	14	24	5	4	21	30	54
2013	**Jan**	3	-	6	9	3	4	32	39	48
	Mai	-	3	-	3	-	-	1	1	4
	Jun	2	2	-	4	-	1	-	1	5
	Sep	-	1	-	1	-	-	1	1	2
	Nov	12	6	3	21	13	10	7	30	51
	Dec	18	3	8	29	8	7	4	19	48
2014	**Jan**	12	2	3	17	12	11	15	38	55
Total		**130**	**34**	**83**	**247**	**83**	**83**	**178**	**344**	**591**

The highest average tick infestation per deer was recorded during May 2013 (intensity: 171.7 ± 63.2), followed by April 2012 with 35.5 ± 5.5 ticks per animal. The highest tick prevalences were recorded from April to September 2012 and from May to September 2013, although most of the animals were culled between November and January. For the whole period of the study the lowest tick prevalences were determined during the winter months, from November to January, with a minimal and maximal values of 12.5% (intensity: 0.1 ± 0.3) and 60.5% (intensity: 1.7 ± 1.7), respectively, during winter 2012 (see Figure 4.1a).

The tick burden per roe deer over the whole study period ranged from 0 to 261 ticks and averaged to 6.4 (± 21.8) ticks per deer. Most of the tick burden was caused by adult ticks with an intensity ranging from 0 to 62 ticks (intensity: 4.1 ± 9.0). Only females range from 0 to 53 (intensity: 3.2 ± 7.4). Fewer males were recovered, with

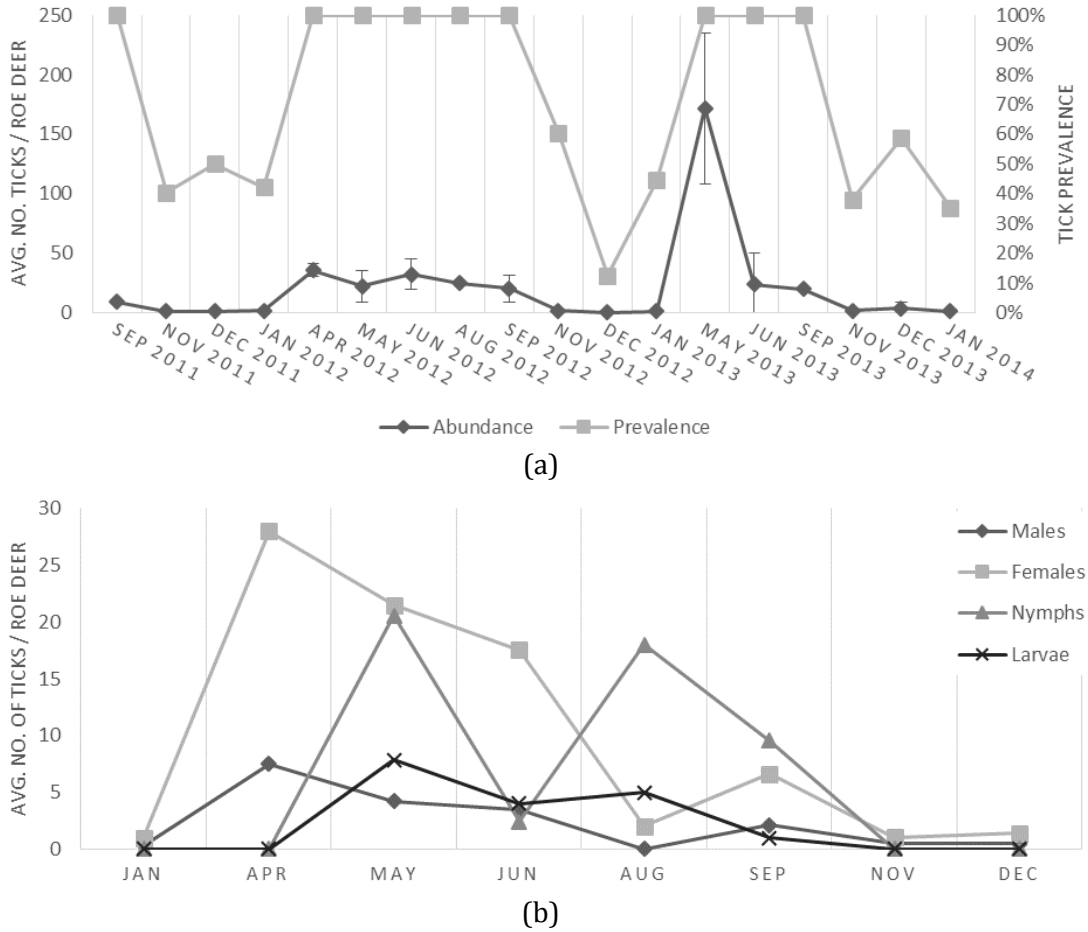


Figure 4.1: For the total period of sampling the bar plots in (a) show the average number of ticks per roe deer. The line plots in visualize the tick prevalences (dark gray) and the number of sampled animals (light gray) for roe deer. Dashed lines indicate time periods with zero animals between months with samples. The plots in (b) show the average number of tick life stage/sex per roe deer for each month. The standard deviations of the tick intensities are represented by the whiskers.

a range from 0 to 14 ticks per deer (intensity: 0.9 ± 1.9). The burden of mating adults per roe deer ranged from 0 to 22 with a mean of 1.1 (± 2.6). The widest range was recorded for nymphs with 0 to 149 individuals per host (intensity: 1.7 ± 11.8). Larvae abundance ranged from 0 to 50 (intensity: 0.6 ± 3.6) individuals. The average number of tick life history stages with respect to each sampling period is displayed in Figure 4.1b.

The highest tick prevalence (67.7%) was determined for young roe deer aged between 1 and 2 years, followed by individuals younger than 1 year (53.0%) and adults

Table 4.2: Number of ticks from roe deer, sampled roe deer and infested roe deer grouped by age and sex of the hosts together with the tick prevalence and the average number of ticks per roe deer (intensity).

	Roe Deer	Infested Roe Deer	Ticks	Prevalence	Intensity
Fawn	83	44	213	53.0%	2.6 ± 5.5
male	35	19	68	54.3%	1.9 ± 3.3
female	48	25	145	52.1%	3.0 ± 6.6
Yearling	34	23	864	67.7%	25.4 ± 51.8
male	17	11	546	64.7%	32.1 ± 66.8
female	17	12	318	70.6%	18.7 ± 31.1
Adult	130	60	507	46.2%	3.9 ± 9.4
male	46	26	351	56.5%	7.6 ± 14.5
female	84	34	156	40.5%	1.9 ± 3.4
Total	**247**	**127**	**1584**	**51.4%**	**6.4 ± 21.8**

with 46.2% tick infestation. The highest prevalences of larvae (50.0%) and nymphs (14.7%) were also found on yearlings. Dead ticks were only found on adult roe deer (0.8%). To verify dependence between tick infestation and the age groups of the hosts, a Kruskal-Wallis test with a subsequent post-hoc analysis was carried out. The results show that yearlings were highly significantly ($P < 0.05$) more infested than fawns and adult deer (Table 4.3). This test was also carried out for each tick life history stage leading to equal results (males, females, nymphs and larvae: $P < 0.05$).

To test if there are differences in tick infestation between male and female hosts, a Mann-Whitney U-test was performed with a result approaching the level of significance ($P = 0.065$) indicating that male deer tend to have more ticks than female deer. By splitting up the dataset by host age and repeating the test for each tick life history stage, the infestation intensity for adult and larval ticks on adult roe deer was significantly male-biased (males: $P = 0.039$; females: $P = 0.044$; larvae: $P < 0.001$), while for fawns and yearlings no imbalances were detected. However, nursing adult female roe deer had significantly higher tick burdens (Mann-Whitney U-test: $P = 0.024$) than females without fawns.

Table 4.3: Post-hoc analysis of the Kruskal-Wallis test comparing age groups of roe deer by tick burden.

Group 1	Group 2	Test Statistic	Std. Error	Std. Test Stat.	Sig.	Adj. Sig
> 1 year	< 1 year	0.607	9.431	0.064	0.949	1.000
> 1 year	1 year	47.304	12.930	3.658	0.000	0.001
< 1 year	1 year	-46.697	13.668	-3.416	0.001	0.002

Each row tests the null hypothesis that Group 1 and Group 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is 0.05.

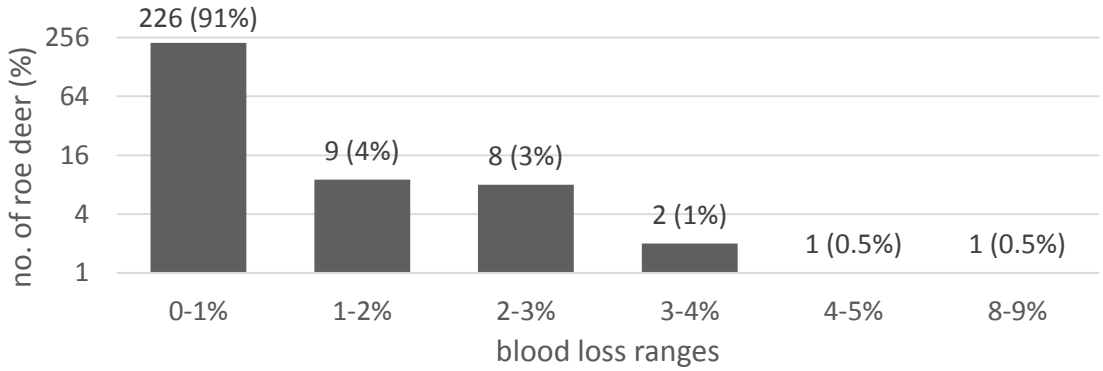


Figure 4.2: Estimated blood loss (not considering the production of new blood) due to infestation by *I. ricinus* of roe deer.

The levels of aggregation (see Section 3.3.1) on roe deer was highest for nymphs (k = 0.02), followed by larvae (k = 0.03), females (k = 0.19) and males (k = 0.26). Particularly on yearlings the level of aggregation was lower for all tick life history stages (adults: k = 0.59, nymphs: k = 0.07, larvae: k = 0.11) than on adult roe deer (adults: k = 0.19, nymphs: k = 0.02, larvae: k = 0.04) and fawns (adults: k = 0.45, nymphs: k = 0.02, larvae: k = 0.05).

The blood loss induced by the tick infestation (see Section 3.3.5) of roe deer is shown in Figure 4.2. More than 90% of the animals had an estimated blood loss lower than 1%, while about 8% of the deer lost between 1 and 4% of their blood. For only 1% of the individuals the blood loss was larger than 4%. As a result, the mean blood loss of all roe deer sampled was as low as 0.3%.

Table 4.4: Number of ticks from wild boar, total number of wild boar and number of tick infested wild boar together with the tick prevalence and the average number of ticks per host (intensity) grouped by host age and sex.

	Wild Boar	Infested Wild Boar	Ticks	Prevalence	Intensity
Piglet	178	10	29	5.6%	0.16 ± 0.97
male	88	8	22	9.1%	0.25 ± 1.22
female	90	2	7	2.2%	0.08 ± 0.64
Yearling	83	5	9	6.0%	0.11 ± 0.52
male	40	1	1	2.5%	0.03 ± 0.16
female	43	4	8	9.3%	0.19 ± 0.70
Adult	83	6	8	7.2%	0.10 ± 0.37
male	25	3	4	12.0%	0.16 ± 0.47
female	58	3	4	5.2%	0.07 ± 0.32
Total	**344**	**21**	**46**	**6.1%**	**0.13 ± 0.76**

4.1.2 Tick burden on wild boar

A total of 344 wild boar, with 178 piglets, 83 young boars (aged from 1 to 2 years) and 83 adult boars which were older than 2 years, were examined. The dataset includes 78 boars sampled in 2011, whereby 77 individuals were collected during November and December. In 2012, a total of 137 wild boar were recorded with the highest incidence (110 wild boars) from November to December. Similar to 2012, the 91 samples from 2013 were mainly collected during the winter months, i.e. January, November and December (88 wild boars). During January 2014 a number of 38 additional wild boars have been sampled (see Table 4.1).

Table 4.4 gives an overview over the total number of wild boar sampled and the number of ticks collected in relation to the tick prevalence and the average number of ticks per animal. From European wild boar only 46 *I. ricinus*, including 5 nymphs (10.9%), 4 males (8.7%) and 37 females (80.4%), were removed. Larvae as well as mating ticks were not found on boars. Most ticks showed little engorgement, while ticks with a higher level of engorgement were dead (11 ticks; 23.9%). Of the dead ticks, 4 were found on male and 7 on female boar. On adult wild boar 4 of 8 (50.0%) and on yearlings 5 of 9 (55.6%) ticks were dead, while on piglets only 2 of 29 (6.9%) were dead. The highest intensities of infestation was observed in May 2013 on a

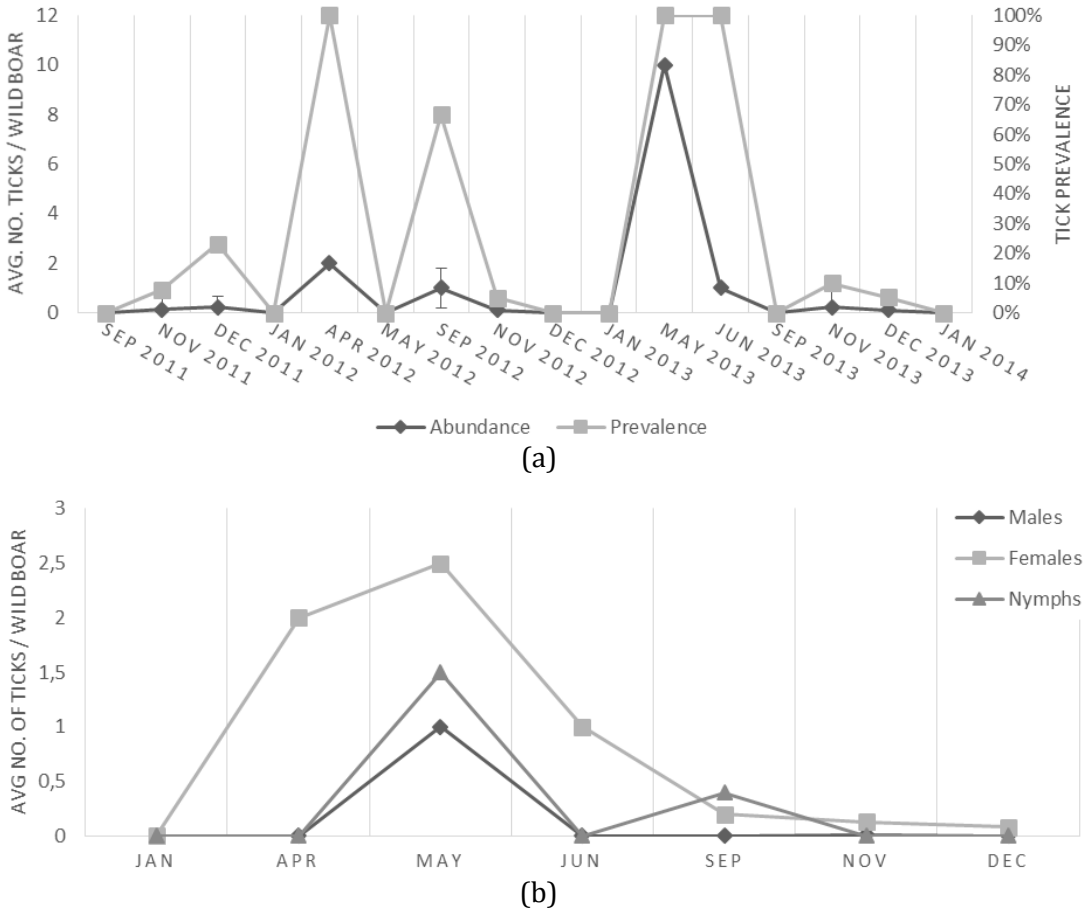


Figure 4.3: For the total period of sampling the bar plots in (a) show the average number of ticks, line plots visualize the tick prevalences (dark gray) and the number of sampled wild boar (light gray). Dashed lines indicate time periods with zero samples. The plots in (b) show the average number of tick life stage/sex per wild boar for each sampling period. The standard deviations of the tick abundances are represented by the whiskers.

single boar with 10 ticks, followed by April 2012 with a single animal carrying 2 ticks. During the whole study period tick prevalence was low (6.1%), whereby no ticks were found on wild boar in September 2011, in January, May and December 2012, in January and September 2013 as well as in January 2014 (Figure 4.3a).

The overall tick burden per wild boar ranged from 0 to 10 ticks (intensity: 0.13 ± 0.76), while female ticks showed the highest intensities, ranging from 0 to 5 (intensity: 0.11 ± 0.55), followed by nymphs ranging from 0 to 3 (intensity: 0.01 ± 0.19) and male ticks with a range of from 0 to 2 (intensity: 0.01 ± 0.15) individuals per boar. Females were also the most prevalent ticks on the boar (5.8%), followed by

males (0.58%) and nymphs (0.58%). The tick intensities grouped by sex and life history stage are shown in Figure 4.3b.Wild boars older than 1 year had the highest tick prevalence (7.2%), followed by animals with an age of between 1 and 2 years (6.0%) and piglets with a tick prevalence of 5.6%. However, the highest prevalence of dead ticks was registered on wild boar between 1 and 2 years old (4.8%), while older (3.6%) and younger (1.1%) individuals had fewer dead ticks. Most prevalent were female ticks on wild boar older than 1 year (6.0%), male ticks on individuals younger than 1 year (1.1%) and nymphs on boars older than 2 years (1.2%).

Piglets had the highest average number of ticks (0.16 ± 0.97), followed yearlings (0.11 ± 0.52 tick per boar) and adults (0.10 ± 0.37). Male ticks were only found on boars younger than 1 year (0.23 ± 0.21), while females were distributed over all of the age groups (piglets: 0.12 ± 0.64; yearlings: 0.11 ± 0.52; adults: 0.07 ± 0.30). Nymphs were only collected from piglets (0.02 ± 0.22) and adult boar (0.02 ± 0.21).

Similar to roe deer, a Kruskal-Wallis test and Mann-Whitney U-tests were carried out to test if tick infestation depended on the age or the sex of the hosts. The intensity of ticks, in particular that of female ticks, was significantly higher ($P < 0.05$) on male piglets (0.08 ± 0.06) than females (0.07 ± 0.04). However, none of the other tests showed significant differences between the individual groups, neither for age ($P = 0.890$), nor for sex ($P = 0.234$). The highest level of aggregation on wild boar was reached by nymphs ($k = 0.004$), followed by males ($k = 0.006$) and females ($k = 0.055$).

The largest amount of blood loss induced by ticks from wild boar was 0.4% and the average blood loss was 0.005%.

4.1.3 Identification of factors influencing tick activity

Pearson's correlation coefficients show for temperature related factors (i.e. air temperature, maximum air temperature, minimum air temperature, minimum air tem-

perature at the ground and sunshine duration) a highly positive significant relationship to tick burden on roe deer (Table 4.5). Could coverage was negatively correlated with tick burden on roe deer at a highly significant level. The correlation coefficients for relative humidity in relation to males, females and larvae were all negative and at a highly significant level, while for nymphs humidity no significance was determined. For air pressure, precipitation depth and wind speed no significant correlation was detected.

Significant ($P < 0.05$) negative correlations were found between tick burden and host health status (i.e. host body mass and $GDMI_s$), indicating that healthier animals have fewer ticks. In particular, a poor condition or crippled roe deer appear to be strongly associated with the presence of ticks and their status of mating ($P < 0.01$), such that more ticks were found on weak individuals. Moreover, highly significant correlations were found for nymphs on roe deer with a lower body mass and in bad physical condition ($P < 0.01$).

A closer look at the correlation coefficients with respect to individual sampling periods (Table 4.6) reveals the during spring (May/Jun) increasing temperature led to a significant decrease in larvae and nymphs on roe deer, while adult ticks were not significantly correlated. During the same period, relative humidity affected the nymphs significantly negatively. As a result of both effects, the saturation deficit has a significantly negative relationship with nymphal tick burden. A quite contrary observation can be made for autumn (Sep/Oct) correlation coefficients, when temperature was significantly positively related to nymphs and larvae, while relative humidity was not significantly associated with all life history stages and a higher saturation deficit increased the number of larvae found.

Table 4.5: Pearson's correlation coefficients for ticks on roe deer and wild boar in relation to climate and host condition with respect to the whole sampling period.

		Ticks	Adults	Females	Males	Nymphs	Larvae	Dead Ticks	Mating Ticks
Roe Deer	GDMI	-0.146*	-0.095	-0.089	-0.104	-0.167**	-0.100	-0.007	-0.114
	Body Mass	-0.142*	-0.082	-0.079	-0.079	-0.165**	-0.112	0.038	-0.113
	Poor or Crippled Condition	0.353**	0.179**	0.189**	0.111	0.388**	0.419**	-0.008	0.308**
	Relative Humidity	-0.273**	-0.417**	-0.426**	-0.316**	-0.121	-0.219**	-0.190**	-0.286**
	Air Temperature	0.319**	0.464**	0.478**	0.333**	0.170**	0.220**	0.073	0.278**
	Min. Air Temperature near Ground	0.247**	0.360**	0.377**	0.233**	0.132*	0.166**	-0.008	0.195**
	Min. Air Temperature	0.265**	0.400**	0.417**	0.266**	0.133*	0.176**	0.001	0.213**
	Max. Air Temperature	0.332**	0.460**	0.472**	0.337**	0.190**	0.241**	0.102	0.297**
	Sunshine Duration	0.237**	0.367**	0.373**	0.279**	0.097	0.204**	0.204**	0.270**
	Cloud Coverage	-0.180**	-0.259**	-0.262**	-0.204**	-0.087	-0.158*	-0.192**	-0.212**
	Precipitation Depth	-0.084	-0.100	-0.097	-0.096	-0.059	-0.067	-0.030	-0.086
	Air Pressure	-0.089	-0.099	-0.109	-0.043	-0.064	-0.081	0.000	-0.047
	Wind Speed	0.006	0.061	0.066	0.032	-0.028	-0.027	-0.053	-0.010
Wild Boar	GDMI	-0.027	-0.030	-0.049	0.048	-0.006	×	-0.080	×
	Body Mass	-0.046	-0.055	-0.055	-0.038	0.006	×	0.044	×
	Poor or Crippled Condition	0.061	0.073	0.088	-0.004	-0.004	×	-0.008	×
	Relative Humidity	-0.117*	-0.082	-0.064	-0.117*	-0.186**	×	-0.095	×
	Air Temperature	0.135*	0.080	0.072	0.086	0.261**	×	0.132*	×
	Min. Air Temperature near Ground	0.031	0.008	0.017	-0.025	0.092	×	0.094	×
	Min. Air Temperature	0.042	0.017	0.024	-0.016	0.111*	×	0.112*	×
	Max. Air Temperature	0.169**	0.103	0.085	0.137*	0.316**	×	0.098	×
	Sunshine Duration	0.222**	0.160**	0.123*	0.243**	0.333**	×	0.077	×
	Cloud Coverage	-0.146**	-0.119*	-0.105	-0.134*	-0.173**	×	-0.082	×
	Precipitation Depth	0.159**	0.145**	0.142**	0.112*	0.139**	×	0.078	×
	Air Pressure	-0.100	-0.102	-0.097	-0.088	-0.050	×	-0.030	×
	Wind Speed	-0.032	-0.030	-0.022	-0.048	-0.027	×	0.036	×

Significances: * $P < 0.05$;** $P < 0.01$; × live history stage not found

Table 4.6: Pearson's correlation coefficients for tick life history stages on roe deer and wild boar in relation to saturation related parameters with respect to individual periods.

	May/Jun	**Females**	**Males**	**Nymphs**	**Larvae**
Roe Deer	Relative Humidity	0.372	0.275	0.440*	0.345
	Saturation Deficit	-0.282	-0.309	-0.448*	-0.400
	Air Temperature	-0.218	-0.306	-0.538*	-0.511*
	Sep/Oct	**Females**	**Males**	**Nymphs**	**Larvae**
	Relative Humidity	-0.017	0.234	-0.134	-0.331
	Saturation Deficit	-0.375	-0.554	0.533	0.716*
	Air Temperature	-0.534	-0.602	0.612*	0.650*
Wild Boar	**May/Jun**	**Females**	**Males**	**Nymphs**	**Larvae**
	Relative Humidity	0.182	0.365	0.365	×
	Saturation Deficit	-0.991	-0.998*	-0.998*	×
	Air Temperature	-0.477	-0.302	-0.302	×

Significances: * $P < 0.05$; ** $P < 0.01$; × live history stage not found

On wild boar there were significant correlations ($P < 0.05$) between total tick burden and climate related parameters, such that higher air temperatures and a lower relative humidity are indicators for higher tick burden. Highly significant positive correlations ($P < 0.01$) can be observed for cloudy, warm days with longer sunshine duration and higher precipitation depths, which resulted in an increased tick burden, whereby the number of dead ticks found also correlated positively with higher temperatures ($P < 0.05$). In particular, the findings of nymphs on wild boars are related in a highly significant manner ($P < 0.01$) with these climatic factors. Significant relationships between sex, age, health status or any other host related parameter with the tick burden were not found. For the period of May/Jun with higher saturation deficits there were significantly reduced numbers of male and nymphal ticks on wild boar (Table 4.6), although air temperature and relative humidity were not significantly correlated with respect to all life history stages.

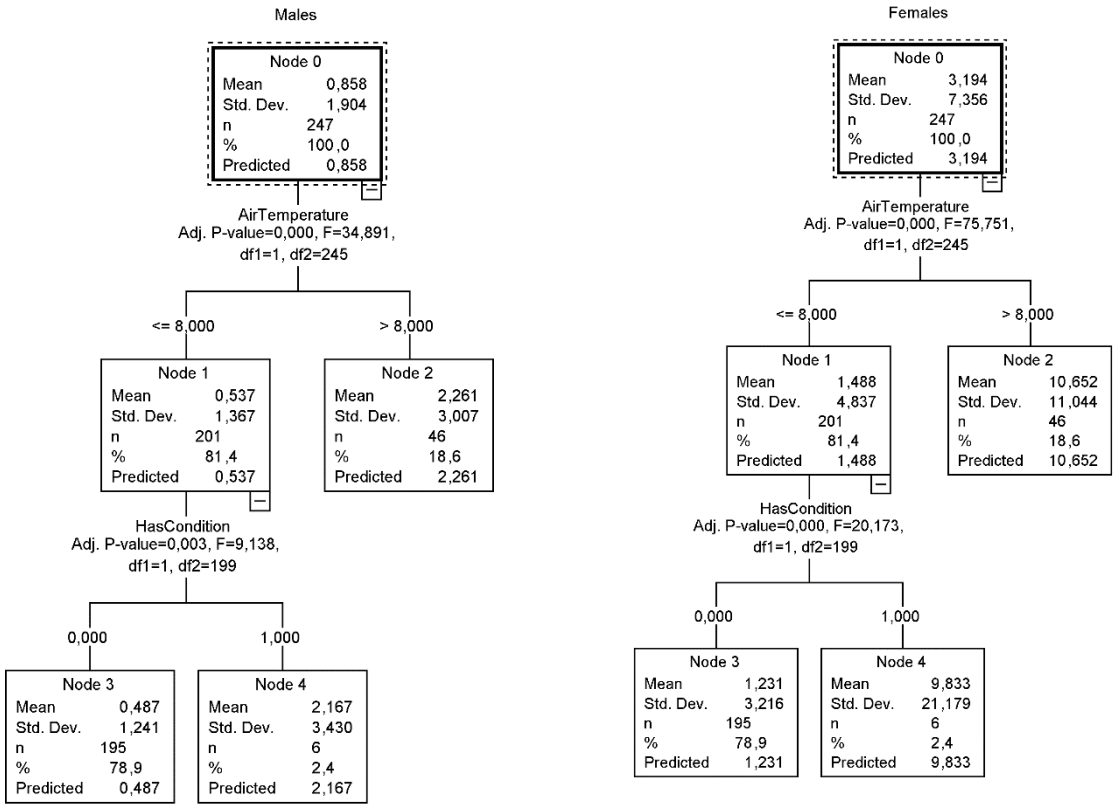


Figure 4.4: Decision trees for roe deer with respect to male (a) and female ticks (b) generated using the CHAID algorithm considering biotic (i.e. host age, sex and conditions) and abiotic factors (i.e. climate).

4.1.4 Ranking of influences on tick burden

The CHAID algorithm was first applied to the whole roe deer dataset, including host and weather data (Figures 4.4 to 4.8). It shows that climatic factors are highly important in their influence on tick burden, with daily air temperature being the strongest parameter for males and females, followed by the "HasCondition" variable, which is defined as association with the presence of any host infestations with other parasites (e.g. lungworms) in conjunction with poor or crippled host condition (Figure 4.4). The trees for non-adult ticks ranked the "HasCondition" parameter even higher than climatic factors (Figure 4.5), such that temperature and relative humidity were on the second level for nymphs and larvae. For both life history stages the CHAID algorithm isolated 6 roe deer having a condition (e.g. other ectoparasites or

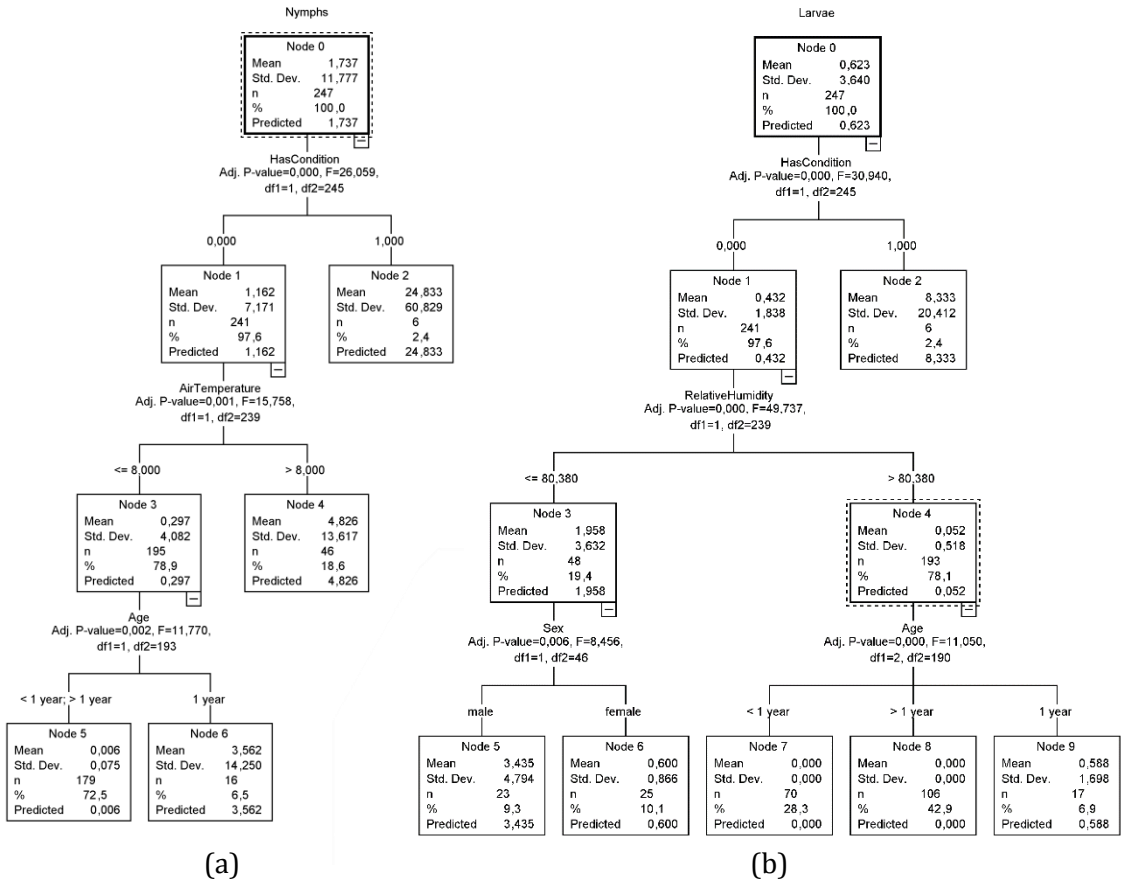


Figure 4.5: Decision trees generated using CHAID for nymphs (a) and larvae (b) on roe deer considering biotic and abiotic parameters.

were crippled) that were significantly more highly infested (Node 2) than the rest of the population (Node 1). Temperature was always positively associated with the intensity of infestation with the tick life history stages and the trees show a threshold of 8 °C at which the tick burden was significantly increased. For a low relative humidity (≤ 80.4%) more larvae were observed on roe deer. These observations stand in direct relationship to the correlation coefficients studied in the previous section, where significant and highly significant linear relationships between tick life history stage intensities, air temperature related parameters and relative humidity were observed. The lowest levels of the decision trees for nymphs and larvae suggest that roe deer related parameters could play a secondary role for ticks.

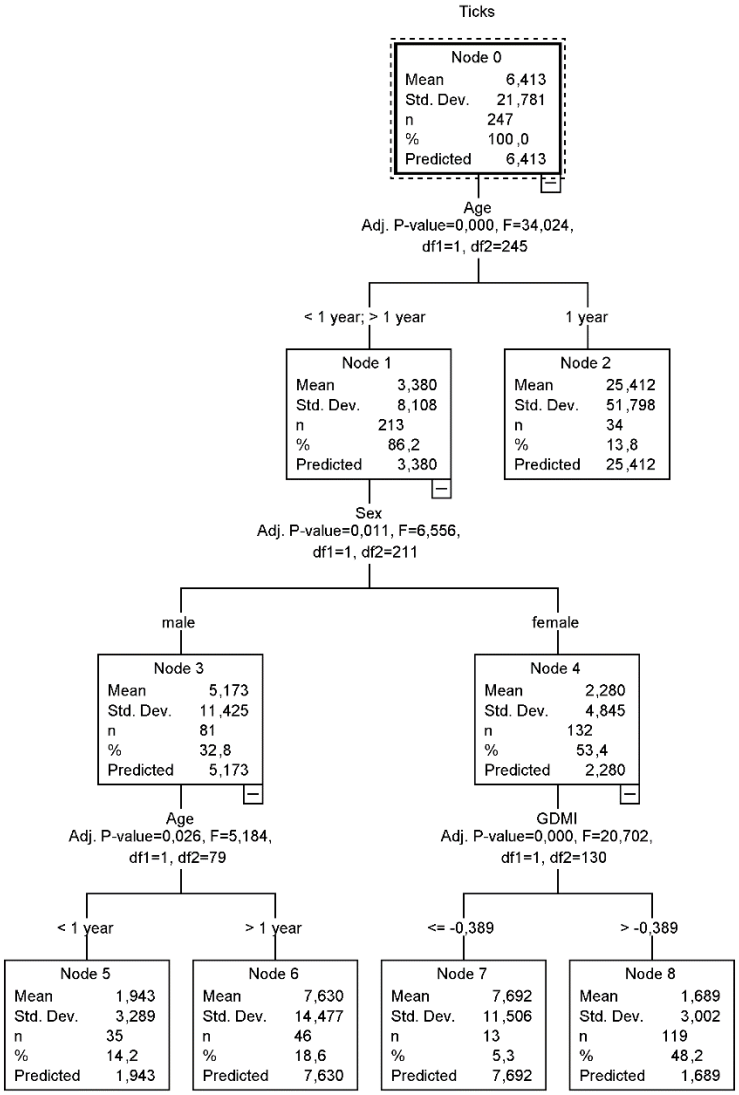


Figure 4.6: Decision trees for all ticks from roe deer generated using the CHAID algorithm considering host parameters only.

Therefore, host parameters were analyzed separately with CHAID using a first run that included all sampled ticks and additional runs for each tick life stage/sex. The results of the collective analysis shown in Figure 4.6 reveals that the most significant host parameter related to *Ixodes ricinus* burden is the age of roe deer, with significantly more ticks on yearlings (25.41 ± 51.80) than on fawns and adult individuals (3.38 ± 8.11). The second most significant factor with respect to fawns and adult roe deer is the sex of the hosts, whereby males showed higher tick infestations (5.17 ±

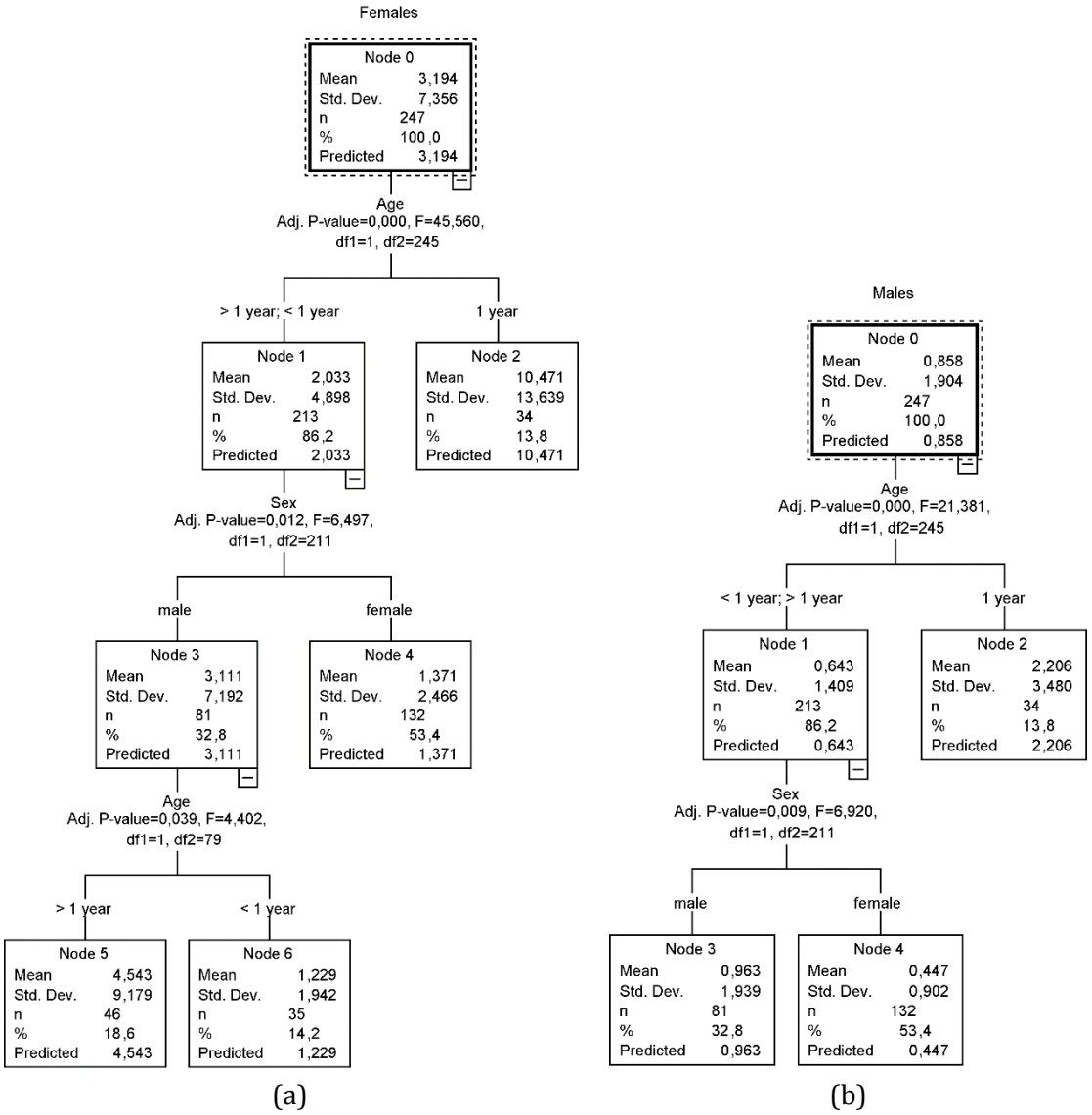


Figure 4.7: Decision trees for (a) male ticks and (b) female ticks with respect to roe deer host parameters generated with SPSS using the CHAID algorithm.

11.42) than females (2.28 ± 4.84). Furthermore, ticks were more abundant on adult male deer (7.63 ± 14.48) than on younger animals (1.94 ± 3.29). For female fawns and adults, their physical condition, represented by the $GDMI_s$, was relevant, as those animals in bad condition ($GDMI_s$ ≤ -0.389) had substantially more ticks than those in good condition.

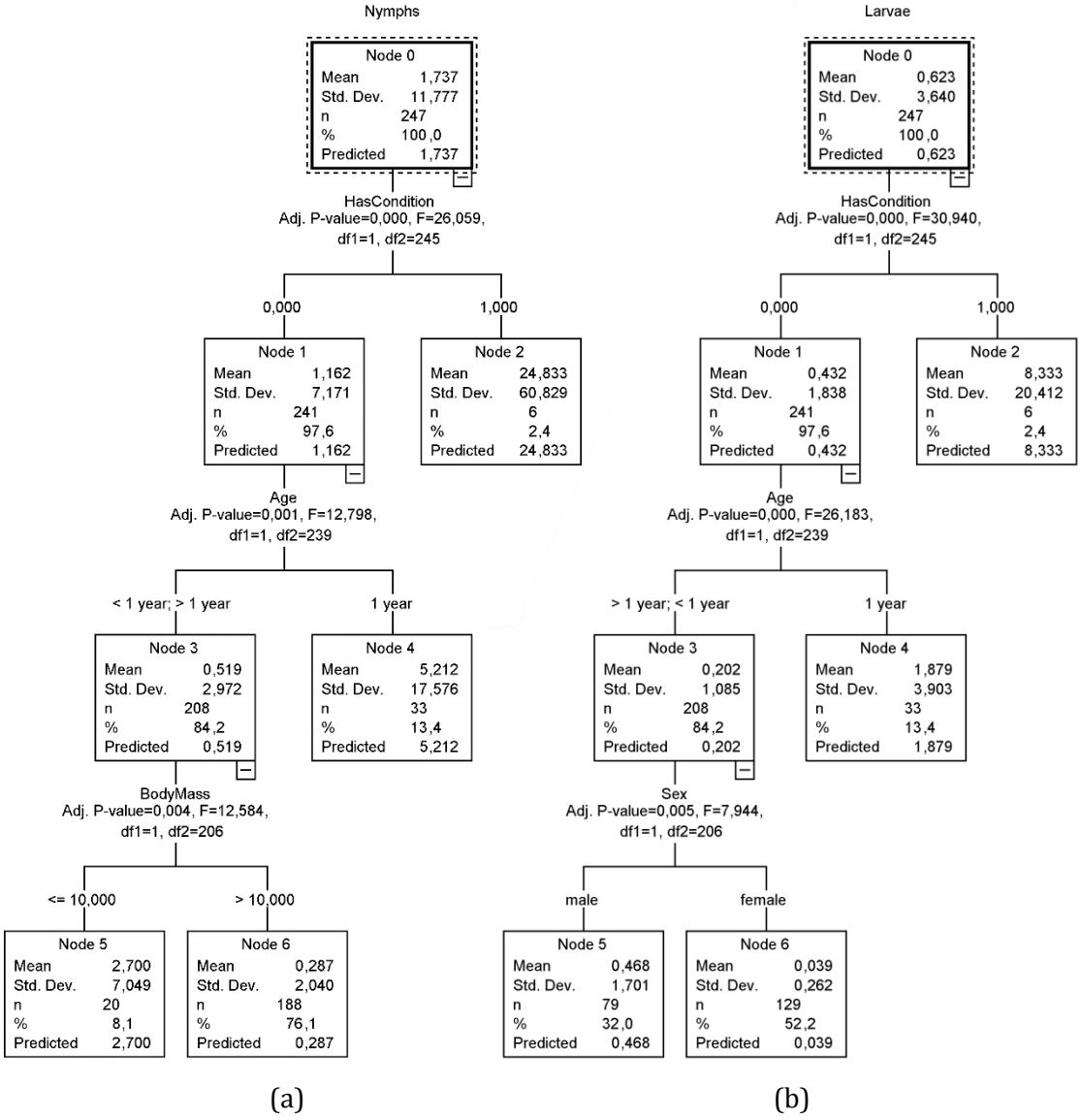


Figure 4.8: Decision trees for roe deer host parameters in relation to (a) nymphs and (b) larvae generated using the CHAID algorithm.

Analyzing parasitism by female ticks (see Figure 4.7a), the most important parameter is also given by the age of roe deer, with yearlings clearly favored by adult female ticks (10.71 ± 13.64) versus fawns and adults (2.03 ± 4.90). Male hosts (3.11 ± 7.19) are preferred by female ticks, whereas males older than one year showed a higher mean abundance (4.54 ± 9.18) than those younger than one year (1.23 ± 1.94). Analysis of the preferences of *Ixodes ricinus* males (Figure 4.7b) are comparable to the

findings for female ticks. The first division of the dataset was given by the host age, with yearlings showing a higher infestation (2.21 ± 3.48) than fawns and adult hosts (0.64 ± 1.41). Then, data was divided by sex, with male deer showing slightly higher tick infestations (0.96 ± 1.94) than female deer (0.45 ± 0.90). In contrast to adult ticks, the most outstanding parameter in nymphal abundance, denoted by the "HasCondition" variable, is defined as association with the presence of any host infestations with other parasites (e.g. lungworms) in conjunction with poor or crippled host condition.

The CHAID decision tree for nymphs shows that a high average number of nymphs can be expected on hosts having any of the aforementioned conditions (24.83 ± 60.83) versus lower tick infestations on deer in good condition carrying no other parasites (1.16 ± 7.17) (Figure 4.8a). The latter group is further divided by the age of the hosts, whereby yearlings had more nymphs (5.21 ± 17.58) than adults and fawns (0.52 ± 2.97). This non-yearling group is again subdivided by host body mass. Roe deer with a lower body mass carried slightly more nymphs (2.70 ± 7.05) than individuals who weighted more than 10 kg (0.29 ± 2.04). Similarly to nymphs, the decision tree for parameters effecting larval infestation (see Figure 4.8b) is also first divided by the host infestation with other parasites, whereby highly parasitized and poor conditioned individuals carried significantly more ticks (8.33 ± 20.41) than those in better condition (0.43 ± 1.84). Similar to nymphs, the group of ill conditioned individuals was subdivided by host age, with yearlings being more highly infested (1.88 ± 3.90) than fawns and adults (0.20 ± 1.08). The last important parameter pointed out by CHAID is the sex of the hosts, such that larval ticks slightly favored male roe deer (0.47 ± 1.70) versus females (0.04 ± 0.26).

Decision trees were also used to examine the host parameters of wild boar in relation to tick infestation. However, the CHAID algorithm did not identify any significant splitting parameter for the wild boar dataset. This is in accordance with the correlation analysis, where no linear relation between any of the host parameters and tick burden was found.

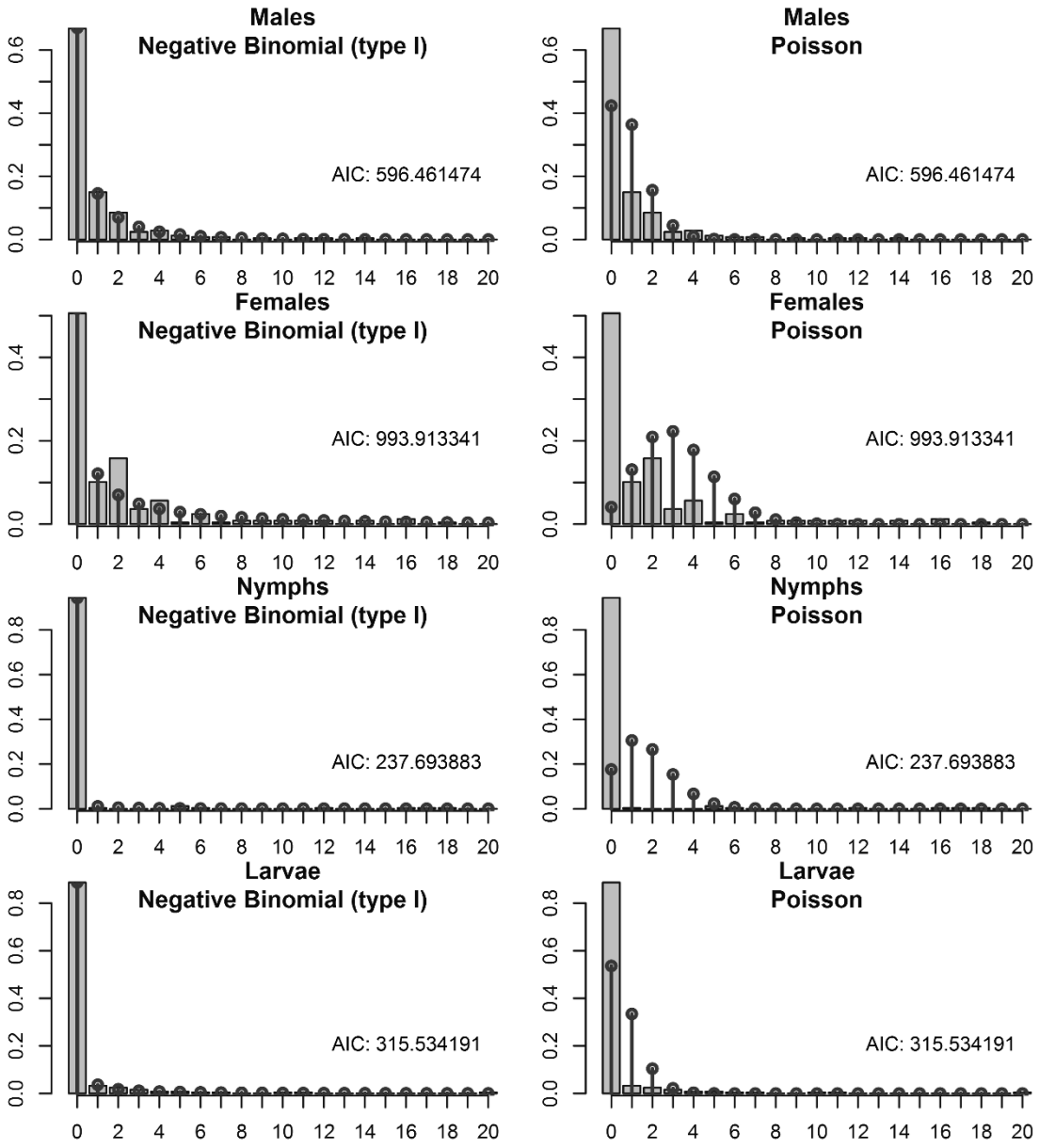


Figure 4.9: Fits (black lines with circles) of the negative binomial (type I) distribution and the Poisson distribution against the dataset (gray bars) of each tick life stage/sex. The AIC-value in the lower right of each bar plot provides information about the goodness of the fit (lower values are better), whereby the depicted values corresponds to the sample value corrected AIC.

4.1.5 Modeling of tick abundances

Figure 4.9 depicts the fitting of the negative binomial (type I) distribution and the Poisson (PO) distribution against the datasets of male ticks, female ticks, nymphs and larvae. For each case, by visual judgment and from an information theory point

Table 4.7: AIC_c-values and degrees of freedom (df) of the GAMLSS models describing the relationships between host sex, body mass, climate and tick burden. The two best fitted models for each tick life history stage are marked in bold.

	Males		**Females**		**Nymphs**		**Larvae**	
Model	**df**	**AIC_c**	**df**	**AIC_c**	**df**	**AIC_c**	**df**	**AIC_c**
Automatic parameter selection	**13.6**	**553.0**	**16.3**	**891.9**	**12.0**	**187.8**	**16.0**	**162.4**
pb(Temp.)	6.3	560.1	**8.0**	**904.2**	16.7	216.5	5.0	210.2
Body mass + pb(Temp.)	7.2	562.2	8.9	906.5	8.5	249.1	11.0	185.0
Sex + pb(Temp.)	**5.0**	**559.7**	8.5	905.2	10.1	246.9	**11.0**	**170.6**
Sex + Body mass + pb(Temp.)	6.0	560.7	9.3	907.6	**12.3**	**200.3**	9.3	907.6

of view with respect to the AIC-values, the NBI distribution represents the datasets better than the PO distribution. The low AIC suggests that the best fit of the NBI distribution was achieved for the nymphal data, while the largest AIC was reached for female ticks. However, the goodness of these fits has to be kept in mind during the estimation of the GAMLSS models in such a way that for tick life stages/sexes for which the NBI distribution fits more accurately the estimated models might be more reliable. In addition to the observations above, an over-dispersion of all four datasets is reflected by their variance-to-mean ratios (VMRs). For male and female ticks on roe deer the VMR has a value of 4.2 and 16.9, respectively, while the dispersion for nymphs and larvae was even higher with VMRs of 79.9 and 21.3, respectively.

For the 4 "simple" models estimated from the predefined formulas (see Section 3.4.1) the degrees of freedom (df) and the AIC_c-values are displayed in Table 4.7 with respect to each tick life history stage. From these models the ones with the lowest AIC_c and thus the ones which represented the information within the datasets most accurately have been selected (bold in Table 4.7). The corresponding model coefficients of the 4 chosen models are listed in Table 4.8, while the partial effects for each parameter included in these models are shown in Figure 4.10 with respect to the mean of the tick abundance on roe deer.

Table 4.8: Mean and dispersion coefficients with significance levels of the manually selected models for each tick life stage/sex from roe deer.

	mean coefficients				dispersion coefficients	
	(Intercept)	**Sex**	**Body mass**	**pb(Temp.)**	**(Intercept)**	**pb(Temp.)**
Males	-0.737 ± 0.268**	-0.637 ± 0.246**	not included	0.122 ± 0.021***	1.567 ± 0.359***	-0.098 ± 0.038**
Females	-0.454 ± 0.179*	not included	not included	0.179 ± 0.017***	1.333 ± 0.247***	-0.086 ± 0.026***
Nymphs	-5.427 ± 2.595*	0.967 ± 0.757	-0.335 ± 0.299	0.542 ± 0.093***	5.980 ± 1.409***	-0.271 ± 0.095**
Larvae	-10.441 ± 0.976***	-1.486 ± 0.333***	not included	0.783 ± 0.061***	6.539 ± 2.105**	-0.464 ± 0.154**

Significances: 'P < 0.1; * P < 0.05;** P < 0.01; *** P < 0.001

From an information-theoretic point of view the model for male ticks that included the smoothened temperature term *pb(Temperature)* in combination with roe deer host sex had the lowest AIC_c of all 4 predefined models and thus provided the best fit to the dataset, while models including roe deer body mass resulted in a lower AIC_c. The coefficients in the first row of Table 4.8 suggest that the female roe deer carry significantly fewer male ticks than male roe deer. This observation is underlined by the plot of the effect size in the first row and first column of Figure 4.10. Moreover, the number of male ticks increases almost linearly with temperature in a highly significant manner, although a B-spline smoothing has been used as basis for the model estimation procedure (see first column, second row of Figure 4.10). Furthermore, the dispersion in the male tick data decreases significantly with increasing temperature, which is modeled by the penalized B-spline as demonstrated in the upper left plot of Figure 4.11 (also see last column of Table 4.8).

Female ticks were best represented by the B-spline smoothened temperature model with a minimal value of the AIC_c of 904.2, while models for female ticks including additional parameters had consistently higher AIC_c-values. The GAMLSS model suggests that female tick burden on roe deer is monotonically rising with temperature, whereby the highest increase of tick abundance can be registered within the range of 5 to 10 °C. Similar to the model for male ticks, the dispersion in the data with respect to females decreased linearly on a highly significant level with increasing temperatures. This behavior is visualized in the upper right plot of Figure 4.11.

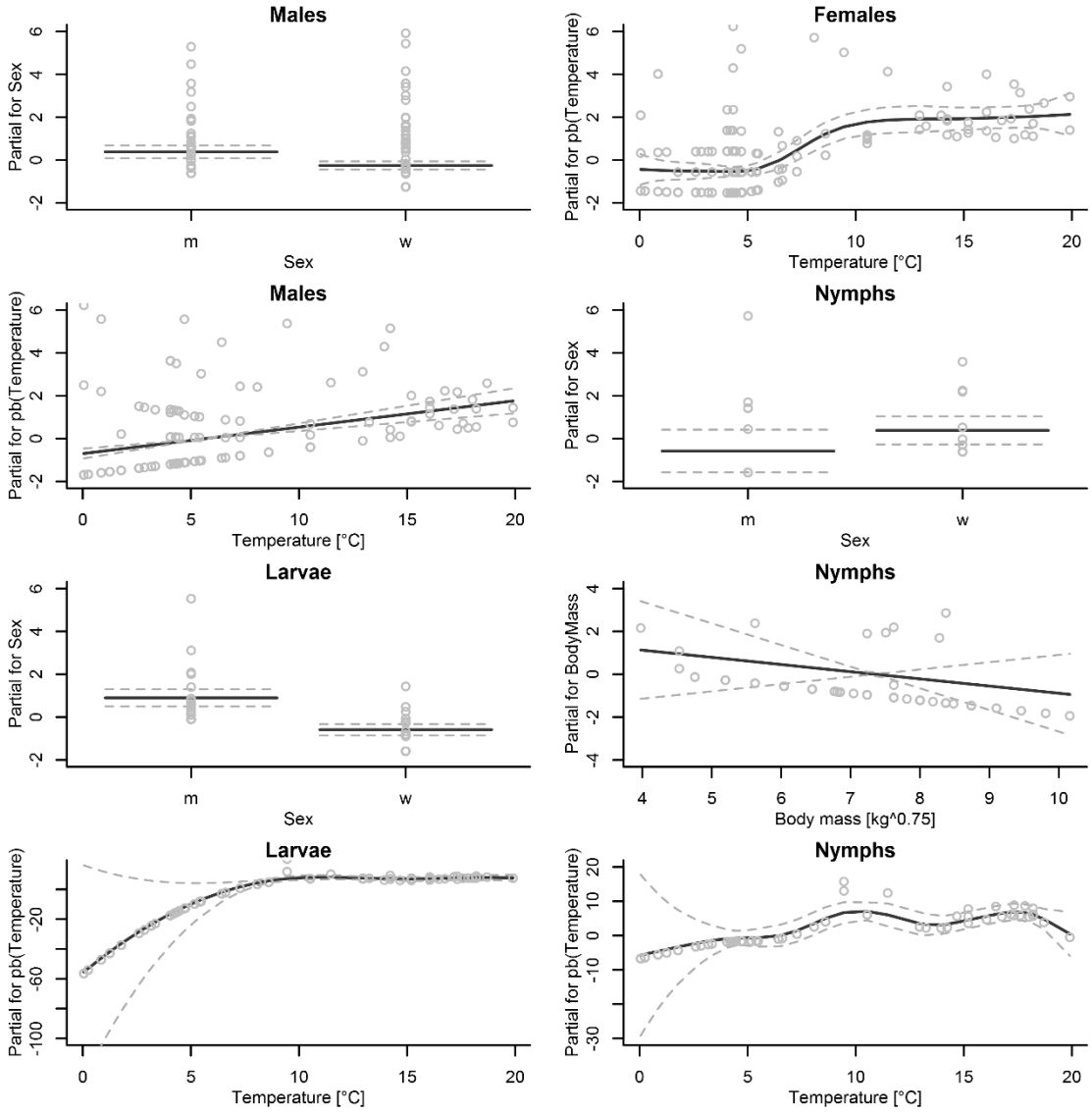


Figure 4.10: Mean male (upper left), female (upper right), nymphal (lower right) and larval tick burden modeled by the GAMLSS approach including relations to host sex, roe deer body mass and ambient temperature expressed by smoothing penalized B-spline function (black line). Dashed lines visualize the point-wise standard errors, while the gray circles represent the partial residuals of the fit.

In contrast to the models for mature tick life history stages, the nymphal tick burden is best expressed by the 3 parameters, i.e. host sex, host body mass and temperature, whereby only the *pb(Temperature)* term has been marked as highly significant by the GAMLSS approach. Additionally, in the third column of Table 4.7 the AIC_c-values of the nymphal models decrease with the number of included parameters. With the selected model more nymphs are predicted to be found on female roe deer and on

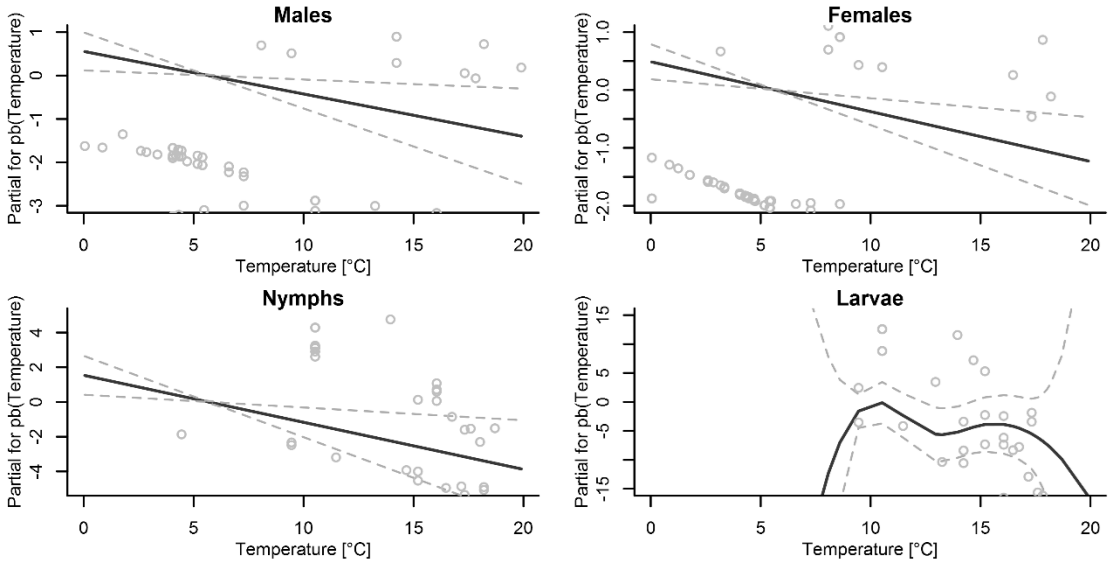


Figure 4.11: Partial effects of the B-spline smoothened temperature term on the dispersion of male, female, nymphal and larval tick burden (black lines) on roe deer. Standard errors are represented by the dashed lines and the partial residual are given by the gray dots.

roe deer with a lower body mass. However, as the standard errors of the mean coefficients are relatively high for host sex and body mass and as both parameters are not marked as significant, the statements about their relationship to nymphal tick burden can only be assumed and the predictions of the model should be handled cautiously. On the other hand, temperature has a highly significant non-linear effect on nymphs on roe deer. Within this context, the GAMLSS model shows two peaks of nymphal abundance for temperatures around 10 °C and 17 °C, whereby the standard errors increased at low temperatures (see last row, last column of Figure 4.10). Moreover and similar to the male and female model, the dispersion in the nymph dataset decreased with rising temperatures (see lower left plot of Figure 4.11).

Equivalently to the model for male ticks, the best model for larvae included host sex and the smoothened temperature term *pb(Temperature)* resulting in an AIC_c-value of 170.6, whereby all model coefficients are at a highly significant level. With this model, more larvae are predicted for male roe deer than for females. Moreover, with increasing temperature the larval tick burden is increasing, while the dispersion in

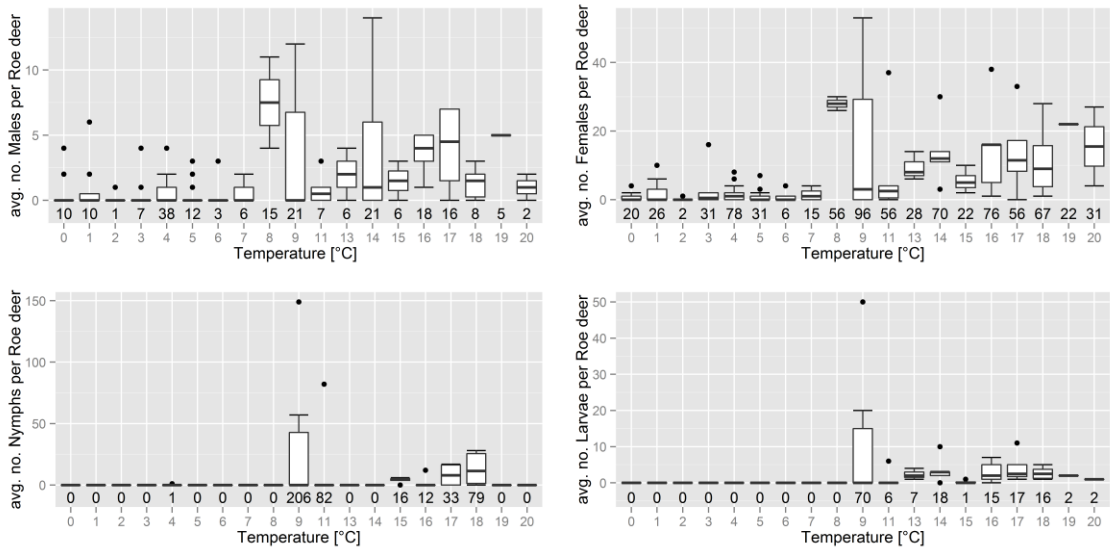


Figure 4.12: Box plots showing how the average number of ticks per roe deer changes with air temperature. Black numbers below each box provide the total number of ticks collected at the corresponding temperature. Whiskers correspond to the first and third quartiles and outliers beyond these error bars are marked by black dots.

the data decreases for temperatures larger than 10 °C as shown in the lower right plot of Figure 4.11. With respect to the partial effect of temperature and dispersion, no larvae were found for temperature below 9 °C (see Figure 4.12). However, the model does predict an increase of the number of larvae up to a temperature of 10 °C. Beyond this temperature threshold a level of saturation is predicted at which the intensity of the infestation stays almost constant.

However, for the partial effects of the temperature on the tick burden of nymphs and larvae predicted by each of the corresponding models the original tick samples have to be kept in mind. Therefore, Figure 4.12 shows box plots that visualize the changes in the tick burdens on roe deer for all tick life stages/sexes over the whole sampling period. For nymphs and larvae below a temperature of 9 °C almost no ticks were discovered. The exception was a single fully engorged female tick, which was collected from a female adult roe deer at 4.45 °C. However, since the models for nymphs and larvae represent the changes in the infestation intensity with temperature smoothly by penalized B-splines (see last row of Figure 4.10), a sudden occurrence

of ticks might not be able to be predicted reliably and the results have to be interpreted cautiously.

Throughout the models selected all intercepts and all temperature terms (mean and dispersion) were at least significant at a level of $P < 0.05$. Moreover, the models provide an adequate fit for the data of the different tick life history stages. The residuals of the male tick model showed a mean near zero (0.005) and a variance approaching a value of one (0.989), while their coefficients of kurtosis and skewness have values of 2.902 and 0.033, respectively. Similar values with respect to the residuals were achieved by the female tick model (mean = -0.044, variance = 1.112, kurtosis = 2.892 and skewness = -0.145). This is also true for the residuals of the nymphal (mean = -0.017, variance = 0.996, kurtosis = 2.890 and skewness = 0.053) and larval (mean = 0.003, variance = 1.050, kurtosis = 3.190 and skewness = 0.036) models. Moreover, the worm plots in Figure 4.13 underline the above observations that the 4 selected models fit adequately to the different tick life history stages, since all deviations lie inside the confidence regions defined by the elliptical curves. As a result, the residuals of the selected models approximate a normal distribution with a mean of 0 and a variance of 1. Note that the kurtosis and the skewness of the normal distribution are 3 and 0, respectively.

Based on van Buuren and Fredriks (2001), Figure 4.13 can be used to judge the quality of the estimated models more specifically. For all models the corresponding worms pass through the origin and show neither a clearly negative nor a clearly positive slope. Consequently, the fitted mean and variance are neither too large nor too small. Moreover, the skewness of the fitted distribution can be assumed correct, since none of the worms had a U-shape. However, all worms were S-shaped, such that the tails for the fitted distribution for the male and the female models are too heavy (S-shape on the left sloping upwards) and the tails of the distributions for the nymph and larva cases are too light (S-shape on the left sloping downwards). Overall, these facts underline the goodness of the fits, whereby a detailed analysis of the

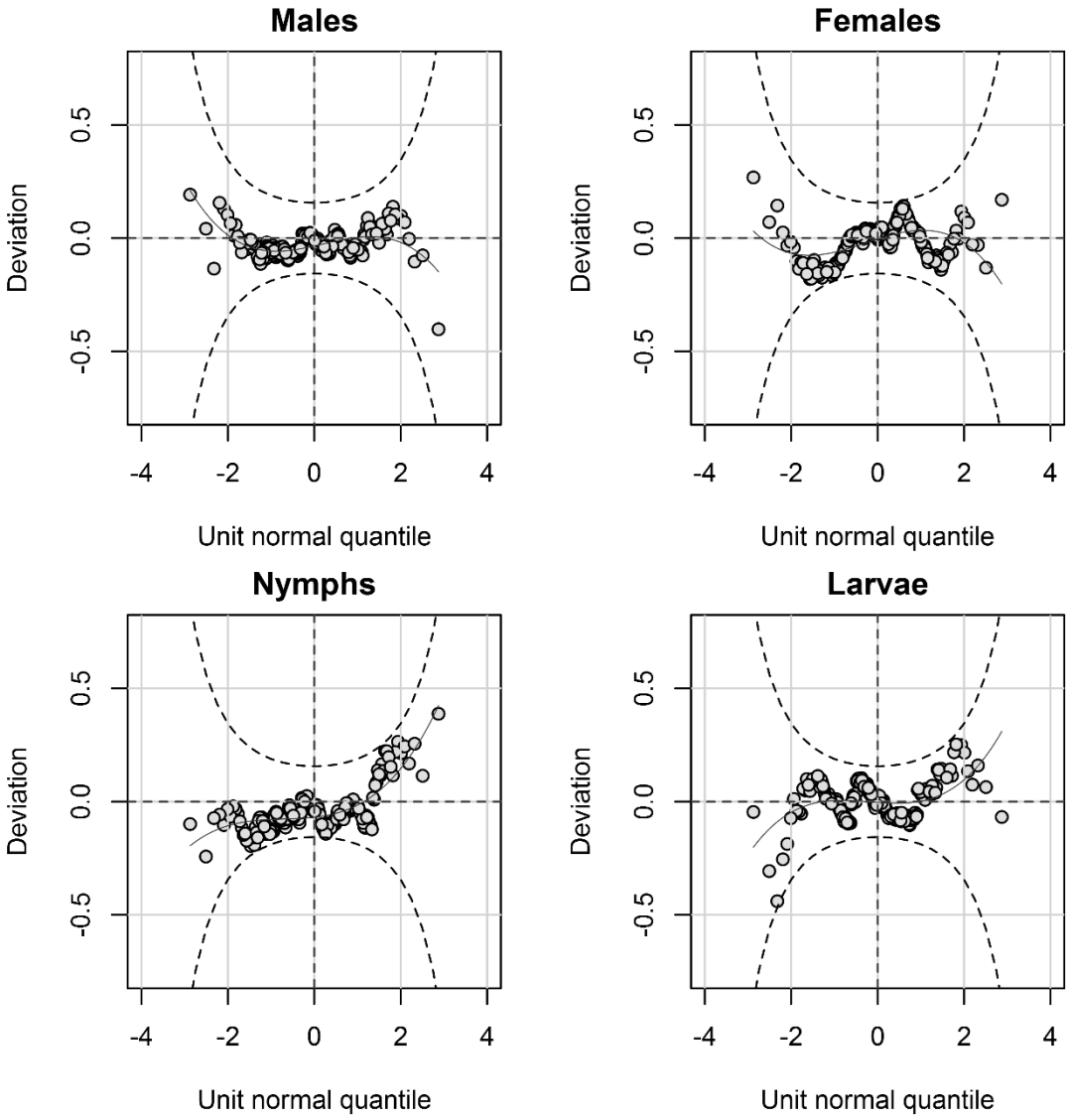


Figure 4.13: Worm plots with respect to female, male, nymphal and larval ticks providing a de-trended Q-Q plot for the GAMLSS models, for which the parameters have been selected manually, visualizing the deviations of the model residuals from the normal distribution. A polynomial fit through the deviations is given by the gray solid line, while the 95% confidence intervals of the unit normal quantiles are provided by the black dashed lines.

imprecisely fitted kurtosis with respect to the prediction of tick burden on roe deer is beyond the scope of this thesis.

For male ticks the stepwise parameter selection choose the age, sex and body mass of roe deer as well as temperature and precipitation depth to represent the tick distribution with a minimal AIC_c of 552.976 (see Table 4.7). Thereby, the precipitation

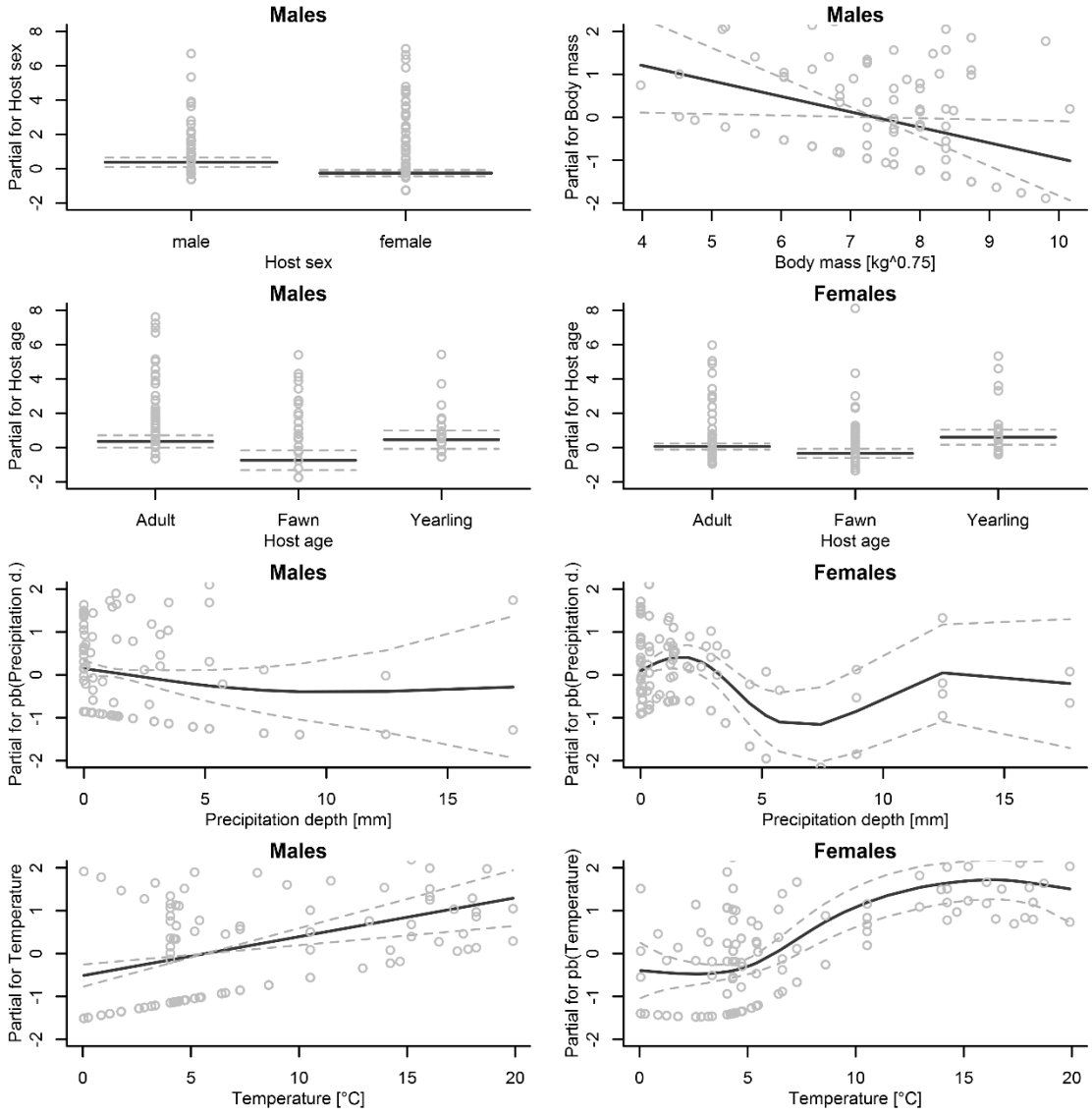


Figure 4.14: Partial effects with respect to the mean for the automatically selected parameters on mature tick burden (black lines) on roe deer. Dash lines represent the point-wise standard errors and gray circles correspond to the residuals of the fit.

depth was modeled with respect to the mean by a penalized B-spline term, which showed a lower abundance of male ticks on roe with increasing rainfall (see Figure 4.14). However, this observation was not marked as significant by the GAMLSS approach and higher standard errors can be observed with precipitation depths larger than 3 mm, such that the model becomes less reliable beyond this threshold. In contrast to this, the linear temperature and host body mass terms of the male tick model

Table 4.9: Mean and dispersion coefficients of the models generated by automatic parameter selection based on the GAIC for each tick life stage/sex.

		Males	Females	Nymphs	Larvae
mean	**(Intercept)**	2,471 ± 1,482'	-0,237 ± 0,232	-1,659 ± 3,520	-2,817 ± 2,354
	Fawns	-1,097 ± 0,469*	-0,401 ± 0,215'	-1,879 ± 1,443	-0,351 ± 1,197
	Yearlings	0.100 ± 0.364	0.542 ± 0.281'	2.554 ± 1.163*	-0.379 ± 0.387
	Female roe deer	-0.631 ± 0.231**	×	×	-1.026 ± 0.412'
	Temperature	0.091 ± 0.025***	×	0.511 ± 0.067***	×
	Body mass	-0.361 ± 0.170*	×	-0.675 ± 0.390'	-0.026 ± 0.310
	pb(Precip.)	-0.052 ± 0.041	-0.088 ± 0.032**	-0.535 ± 0.369	-0.022 ± 0.042
	pb(Temp.)	×	0.165 ± 0.020***	×	0.269 ± 0.068***
dispersion	**(Intercept)**	9.080 ± 3.600*	5.830 ± 2.140**	1.833 ± 0.655**	14.209 ± 3.377***
	Fawns	-3.038 ± 1.081**	-2.378 ± 0.726**	-37.963 ± 0.655***	×
	Yearlings	-1.422 ± 0.773'	-0.859 ± 0.491'	1.208 ± 0.852	×
	Temperature	-0.114 ± 0.052*	-0.104 ± 0.029***	×	-0.847 ± 0.207***
	Body mass	×	-0.519 ± 0.265'	×	×
	Precipitation	×	×	×	-2.023 ± 0.697**
	pb(Body mass)	-0.904 ± 0.470'	×	×	×

Significances: ' P < 0.1; * P < 0.05;** P < 0.01; *** P < 0.001; × = not included in model

were significant, whereby temperature correlated positively and body mass negatively with the mean of the tick burden. However, a comparison of the coefficients of both effects shows that body mass has a stronger influence on male ticks than temperature (Table 4.9). Moreover, the model predicts a decrease in dispersion with temperature and body mass, such that host body mass modeled by a smoothing spline has a larger impact on the dispersion than temperature. With respect to host sex, male roe deer are highly significantly more infested by male ticks than female roe deer. Furthermore, the models predicts significantly less male ticks on fawns than on adult roe deer, while yearlings showed not significantly deviations from adult individuals with respect to male tick burden. Additionally, the predictions of the model show a highly significantly lower dispersion for fawns and a marginally significantly lower dispersion for yearlings in comparison to adult roe deer.

For female ticks the GAMLSS approach selected the parameters host age, precipitation depth and temperature with respect to the minimization of the GAIC (Table 4.9 and Figure 4.14). The resulting AIC_c-value of the female tick model was 891.9 and thus lower than any of the manually composed models. Thereby, the precipitation and temperature terms were both modeled by a penalized B-spline and marked to

be highly significant. Within this context, a decrease of the female tick burden on roe deer was registered above a precipitation depths of about 2 mm, whereby the uncertainty of the model increased for higher precipitation depths. A peak for female infestation intensity was detected temperatures around 15 °C. A comparison of the magnitude of the influences of the two climatic factors on the female tick abundance shows that the impact of temperature is stronger by a factor of almost 2 than precipitation. In addition to this, the model predicts on a marginally significant level more females on yearlings and less on fawns in comparison to the group of adult roe deer. For the dispersion of the female tick data the model showed a highly significant decrease for fawns and an almost significant one for yearlings, while with rising temperatures the dispersion reduced in a highly significant manner. Moreover, host body mass has a marginally significant negative influence on the dispersion.

For the modeling of the nymphal tick burden on roe deer the host age and body mass, temperature and precipitation were selected to reach a minimal AIC_c-value of 187.8 (Table 4.9 and Figure 4.15). Temperature was modeled as a linear term, such that it correlated positively with nymphs at a highly significant level. In contrast to this, the precipitation was represented by a spline predicting a minimal intensity of nymphs at about 2 mm, while the standard errors beyond precipitation depths of 4 mm increased distinctly, suggesting a cautious handling of predictions within this range. Furthermore, a linear relationship of host body mass on the occurrence of nymphs approached a level of significance, whereby heavier roe deer carried less nymphs. In addition to this, the model predicts significantly more nymphs on yearlings than on adult roe deer, while fawns in comparisons to adult individuals, showed no significant differences. However, with respect to the dispersion of the nymphal data fawns had a highly significant negative influence, while yearling did not alter the dispersion significantly.

For larvae, host age, sex and body mass were included in the model as well as penalized B-spline terms of precipitation and temperature to represent the mean of the

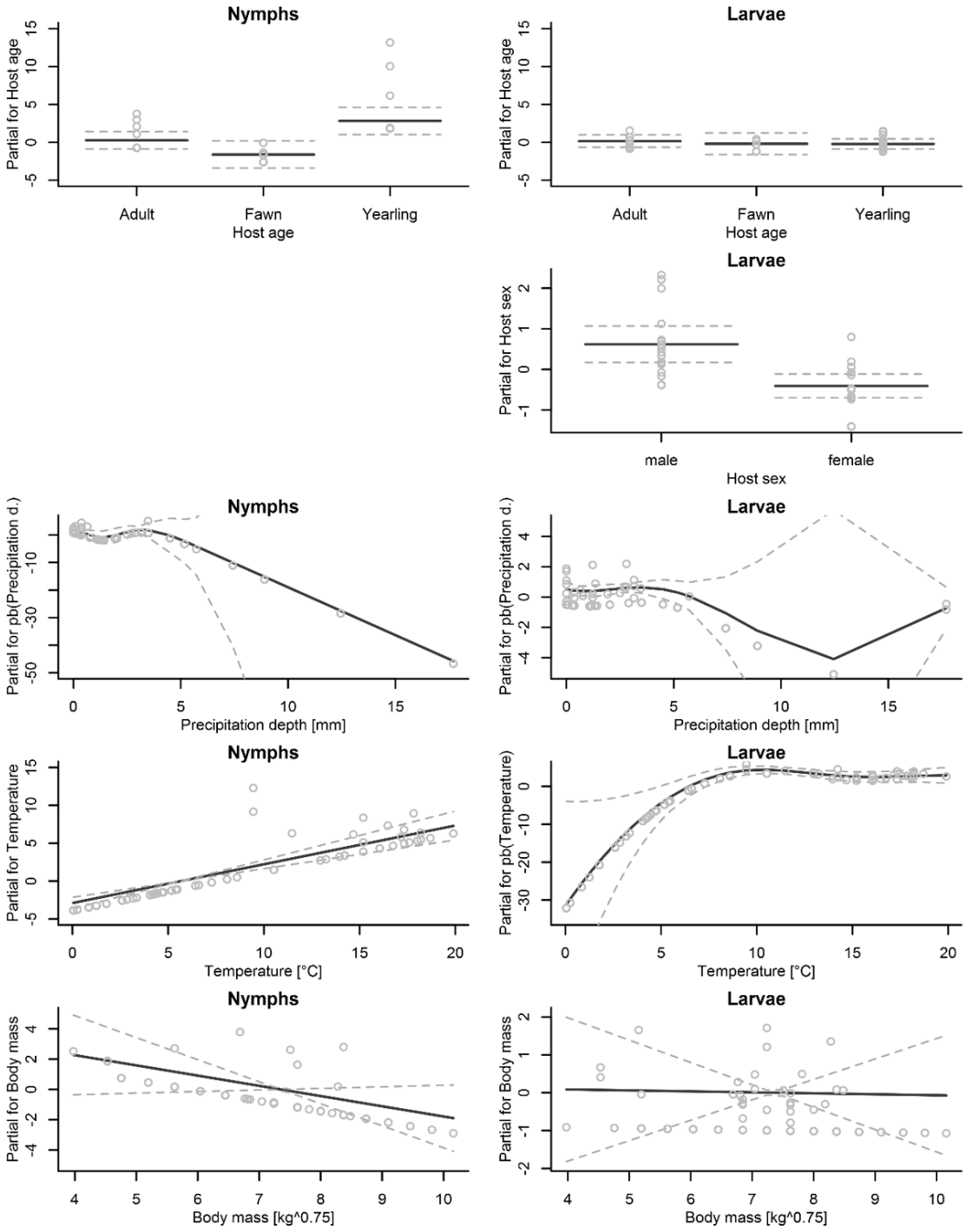


Figure 4.15: Mean partial effects of the models for nymphal and larval tick burden (black lines) on roe deer generated by the automatic parameters selection approach. Dashed lines correspond to the standard errors and gray circle are the partial residuals of the fit. Note that the sex of roe deer was not included into the nymph model.

dataset (Table 4.9 and Figure 4.15). Thereby, temperature had a highly significant impact on larval infestations of roe deer, which was constantly high for tempera-

tures above 9 °C. In contrast to this, the precipitation depth and host body mass were not marked by the GAMLSS approach as significant, although both parameters helped to reduce the AIC_c of the optimized model to a value of 162.4. The non-significance of both parameters is also underline by their corresponding plots in Figure 4.15, where they showed either partials effects close to 0 or large standard errors. Similar to this, the model predicts no significant changes of larval infestation with host age. Nevertheless, the predictions show, at a level approaching significance, that female roe deer have less larva than male roe deer. The modelling of the dispersion in the larval data reveals the highly significant influences of temperature and precipitation depth, which correlate negatively, while the effect precipitation is stronger than that of the temperature.

In summary, all automatically GAIC-optimized models had a lower AIC_c than the manually created ones (cf. Table 4.7) and thus represented the datasets more precisely from an information theoretical point of view. Additionally, all models contained temperature as a linear or as a spline-based smoothing term with a significant influence on the mean tick burden. The age of roe deer was also included in all 4 models, whereby fawns were at least significantly less infested by mature ticks than adult roe deer. The models also demonstrated that males and larvae preferred male roe deer over females, which could be an indication for sex-biased tick parasitism. Furthermore, precipitation depth was selected by the optimization scheme for all models, whereby a significant effect was only determined for female ticks. The effect of rainfall on tick burden on roe deer is illustrated by Figure 4.16, which shows that abundance of all three life history stages was close to zero when the mean monthly precipitation was approaching 2 mm.

Similar to the manually selected models, the fits estimated by the automatic parameter selection possess residuals that follow approximately a normal distribution. As shown in Table 4.10 the mean and the variance of each model approach 0 and 1, respectively, while their skewness is nearly 0 and their kurtosis is almost equal to 3. These, observations are underlined by the worm plot in Figure 4.17, which shows

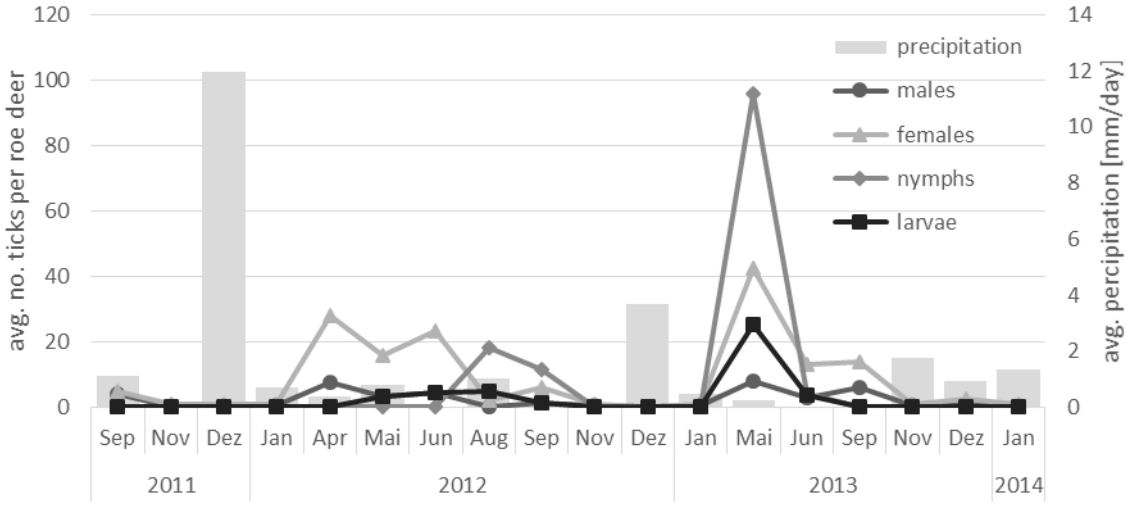


Figure 4.16: Average precipitation depth (bar plots) for each sampling period in relation to all tick life history stages (line plots).

Table 4.10: Statistical moments (i.e. mean, variance, kurtosis and skewness) for the residuals of the models generated by automatic GAIC-based parameter selection with respect to roe deer and each tick life history stage.

	Males	**Females**	**Nymphs**	**Larvae**
Mean	0.068	0.061	0.009	-0.055
Variance	0.892	0.896	0.873	0.995
Kurtosis	3.290	2.934	2.976	2.639
Skewness	-0.224	-0.030	-0.172	0.049

for all 4 models that salmost all observations are within the 95% confidence regions bounded by the elliptical curves.

Again, the interpretation patterns from (van Buuren and Fredriks 2001) can be used to judge the goodness of the fits. For the male model the plot in Figure 4.17 shows that the worm passes slightly above the origin, has an approximately negative slop and an inverted U-shape. Correspondingly, it can be assumed that the fitted mean is slightly too small, the variance marginally too large and fitted distribution is too skewed to the right. The observations with respect to the mean and the variance of the male models also hold true for the female model, whereas the skewness of the female tick model fitted more accurately to the dataset, since no U-shaped worm

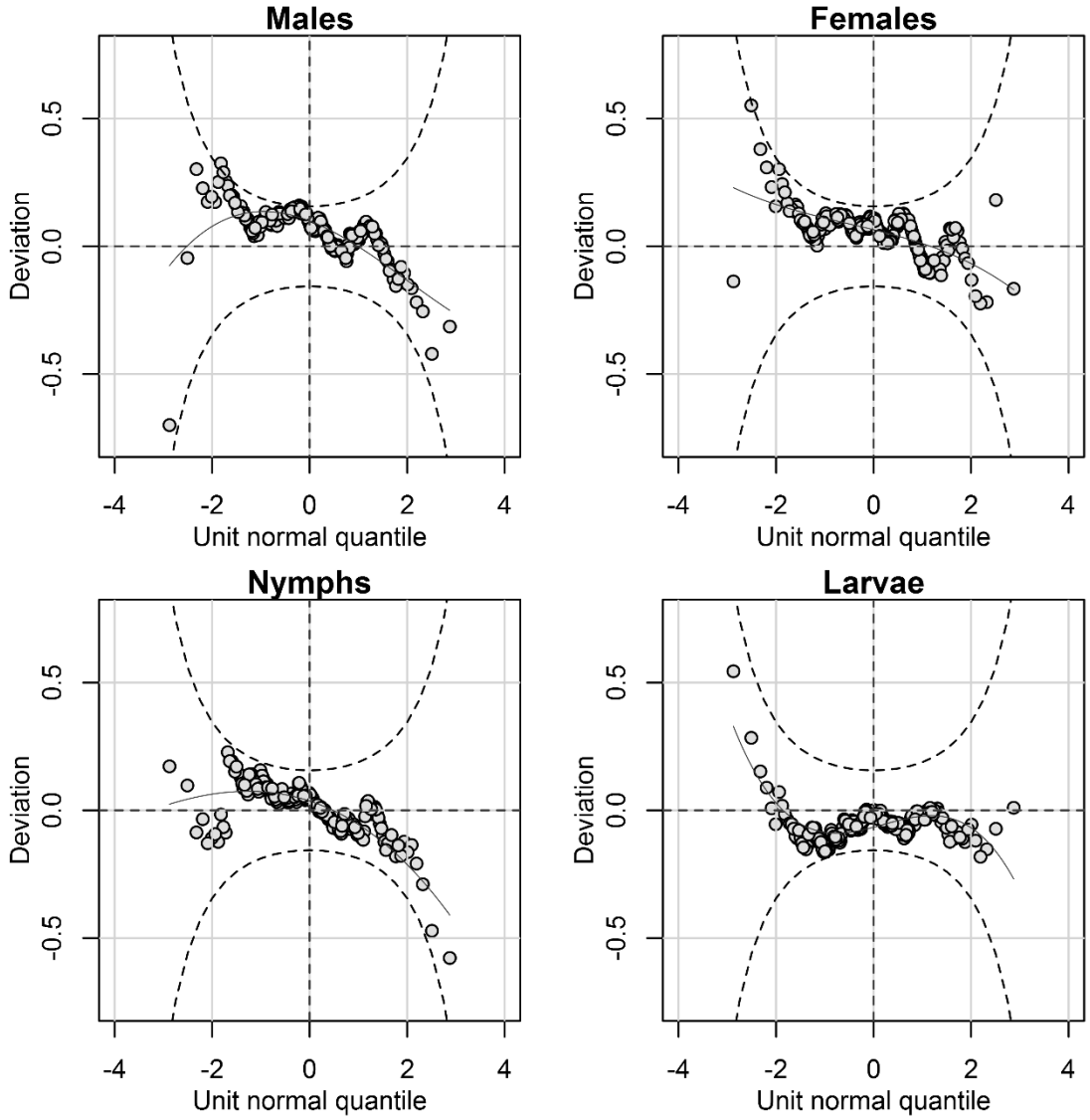


Figure 4.17: Worm plots, i.e. de-trended Q-Q plots, for the parameter optimized models with respect to each tick life history stage on roe deer. The solid gray line provides a polynomial fit through the residuals to facilitate the visual interpretation of the plots. The 95% confidence intervals of the unit normal quantiles are given by the black dashed elliptical lines.

was observed. The worm plot of the nymphal tick model revealed a similar behavior with respect the residuals to those of the male model (variance slightly too large and marginally too skewed to the right), whereby the worm passed closer to the origin and it can be assumed that the estimated mean fits more accurately to the distribution. In contrast to this, the worm plot of the larva model has an S-shape with its left sloping upwards, such that the tails of the fitted distribution are too heavy.

Although the aforementioned interpretations emphasize the minor imprecisions of the generated models, it has to be kept in mind that in none of the worm plots did the observations exceeded the confidence intervals, while all statistical moments of the residuals approached those of the normal distribution and the AIC_c-values of the automatically optimized models were lower than those of the ones generated by manual parameter selection. Consequently, the latter 4 models can be seen as the best possible fit to the tick datasets in terms of the GAMLSS approach.

In addition to the models above, a simple model of tick life history stage intensity in relation host body mass modeled by a penalized B-spline were computed using the GAMLSS algorithm. Figure 4.18 shows the partial effects for the host body mass on the tick burden with respect to each host age and each tick life stage/sex. In principle, on adult roe deer the number of ticks increases with rising host body mass, whereby the predictions of the simple models for nymphs and larvae show large uncertainties due to missing data for low and high masses. For yearlings the fits reveal peaks of the tick burden at a body mass of about $(5\ kg^{0.75})^{1/0.75} = 8.54$ kg and of about $(7\ kg^{0.75})^{1/0.75} = 13.4$ kg throughout all tick life history stages. However, when looking for an overall trend with respect to yearlings a slight marginally significantly negative correlation (Pearson's ρ = -0.313, P = 0.071) of host body mass with tick burden can be noticed. With respect to fawns, increasing host body mass reduced the intensity of tick infestations, whereby the models for nymphs and larvae show high standard errors for higher body masses, which are again caused by missing data. Nevertheless, the overall number of ticks on fawns correlated negatively with body mass at a highly significant level (Pearson's ρ = -0.495, P = 0.001).

Due to the limited number of ticks (n = 46) collected from wild boar, only a single model has been computed including all tick life history stages using GAMLSS with automatic parameter selection considering the same variables as for roe deer. The resulting model includes a smoothened precipitation depth term and a linear temperature term to represent the mean of the tick burden on wild boar as well as the temperature to model the dispersion of the distribution. The model coefficients in

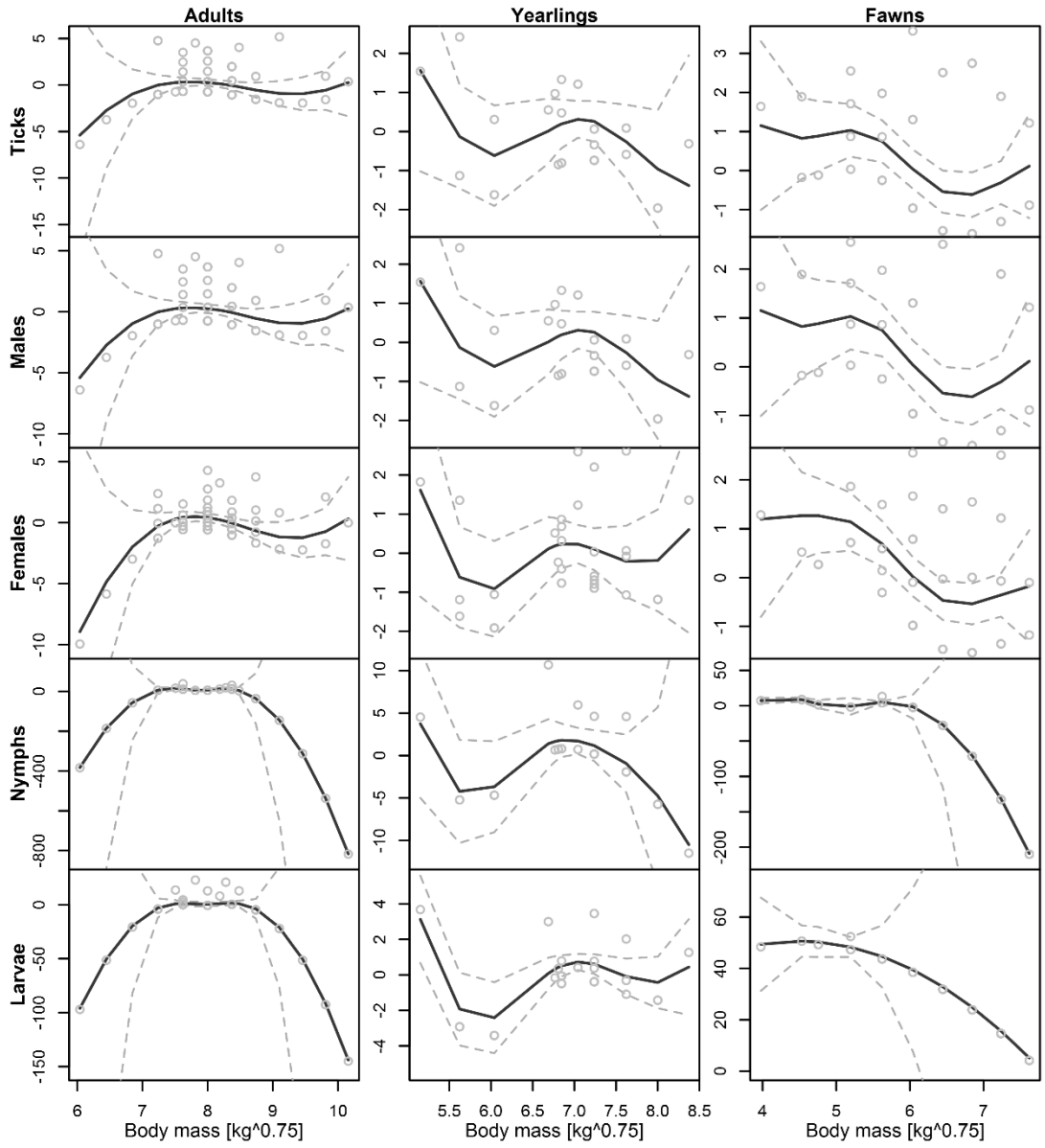


Figure 4.18: Mean partial effects for roe deer body mass on the tick burden with respect to each host age and each tick life stage/sex estimated by the simple model: Ticks ~ pb(Body-Mass).

Table 4.11 show that the intercepts and the temperature had a significant influence on the tick intensity, whereas precipitation depth was not marked as significant. However, a look at the plots on the left of Figure 4.19 suggests that the tick intensity on wild boar was highest for precipitation depths between 7 and 8 mm, while more ticks were generally present with rising temperature. Furthermore, the dispersion of the dataset decreased when the temperature were higher.

Table 4.11: Mean and dispersion coefficients with significance levels for the model generated using automatic parameter selection based on the GAIC for ticks on wild boar.

		Coefficients
mean	**(Intercept)**	-3.709 ± 0.572***
	pb(Precipitation depth)	0.048 ± 0.060
	Temperature	0.191 ± 0.057***
dispersion	**(Intercept)**	4.247 ± 0.854***
	Temperature	-0.268 ± 0.120*

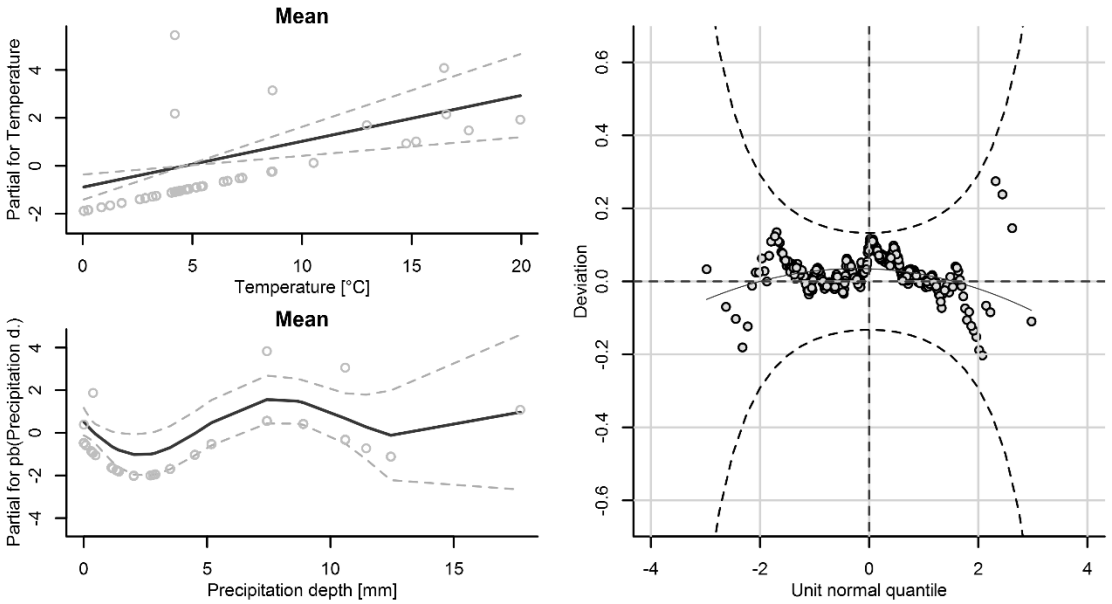


Figure 4.19: Mean partial effect plots (left) for the model generated by the GAMLSS approach using automatic parameter selection with respect to overall tick burden on wild boar. Standard errors and residuals in the effects plots are represented by the dashed lines and by gray circles, respectively. The corresponding worm plot is shown on the right.

With respect to the quality of the fit, the residuals of the resulting model approach a mean of 0 (0.037) and a variance of 1 (0.931). The kurtosis of the residuals was approximately 3 (3.060) and the skewness approached a value of 0 (-0.040). Consequently, the distribution of residuals follows approximately a normal distribution and, therefore, the model represents an accurate fit to the wild boar dataset. The worm plot on the right of Figure 4.19 underlines the quality of the model as all ob-

Table 4.12: Coefficients and odds ratios from the multinomial logistic regression with respect to the composition of the tick population. Reference categories, i.e. baselines, are displayed for ease of interpretation.

Effects		Males		Nymphs		Larvae	
		coeff.	**odds ratio**	**coeff.**	**odds ratio**	**coeff.**	**odds ratio**
(Intercept)		-1.393***	0.248	2.163***	8.696	-0.067	0.935
Host species	Roe deer	(Baseline)		(Baseline)		(Baseline)	
	Wild boar	-1.142	0.319	-1.747*	0.174	-20.186	0.000
Host sex	Male	(Baseline)		(Baseline)		(Baseline)	
	Female	-0.390*	0.677	-0.334	0.716	-1.597***	0.202
Body part	Ears	(Baseline)		(Baseline)		(Baseline)	
	Head & Neck	-18.237	0.000	-21.506	0.000	-1.353**	0.258
	F. legs & Stern.	-0.441	0.644	-5.394***	0.005	-3.274***	0.038
	H. legs & Abdom.	0.224	1.251	-4.128***	0.016	-6.234***	0.002
Month	Jan	0.478	1.613	-17.485	0.000	-14.654	0.000
	Apr	0.257	1.294	-17.363	0.000	-17.744	0.000
	May	(Baseline)		(Baseline)		(Baseline)	
	Jun	-0.167	0.846	-2.150***	0.116	-1.416***	0.243
	Aug	-15.989	0.000	1.233	3.431	0.233	1.262
	Sep	0.408	1.505	2.376***	10.758	2.585***	13.258
	Nov	0.685**	1.985	-4.023***	0.018	-17.606	0.000
	Dec	0.475'	1.608	-17.484	0.000	-14.836	0.000
Body mass		-0.005	0.995	-0.023	0.977	0.102*	1.107

Significances: ' P < 0.1, * P < 0.05, ** P < 0.01, *** P < 0.001

servations lie within the confidence interval and the worm only has a slightly inverted U-shape, which could suggest that the fitted distribution is marginally too skewed to the right.

4.1.6 Modeling the composition of the tick population

The results of the multinomial logistic regression are shown in Table 4.12. In principle, the regression approach results can be interpreted as three individual models, whereby each model compares female ticks, the reference group, to one of the other life history stages. The estimated coefficients and odds ratios of the intercepts show for the reference group (i.e. TickStage = Female, HostSpecies = roe deer, Host sex =

male, Body part = Ears and Month = May) that the chance of finding male ticks is significantly lower in comparison to female ticks. In contrast to this, the likelihood of finding nymphs on the ears of male roe deer during May is significantly higher than that of finding female ticks. For larvae no significant effect in relation to the reference group was detected.

The effect of host species on the tick population was only significant for nymphs. Here, the proportions of nymphs relative to females were significantly lower on wild boar than on roe deer. A similarly directed, but not significant effect can be seen registered for males and larvae. On the other hand, the sex of the hosts, played a significant role for the proportions of female and male ticks as well as for females and larvae, such that the occurrence of males and larvae was less likely than that of *I. ricinus* females on female hosts. Accordingly, female ticks appear to have a higher probability of occurrence on male hosts. The body part on which ticks were found, influenced the composition of the tick population with respect to the immature life history stages on a highly significant level. The ratio of nymphs to females and of larvae to females was considerably lower on legs, sternum and abdomen than on the ears. For larvae this negative effect was also observed with respect to head and neck.

Relating to the sampling periods, significant changes were determined for all three life history stages in comparison to female ticks, but for different months. During November and December male in comparison to female ticks were more likely to be found than in May. For nymphs a highly significant increase of the occurrences in relation to females were determined during June and November with respect the reference period. Moreover, nymphs were significantly more likely than females during September than during May. Similar seasonal proportions were observed for larvae, where the positive and negative effects were at a highly significant level during June and September, respectively. The body mass of both host species had a significantly positive effect on the proportions between larvae and females, such that larvae become more likely than female ticks with higher body mass.

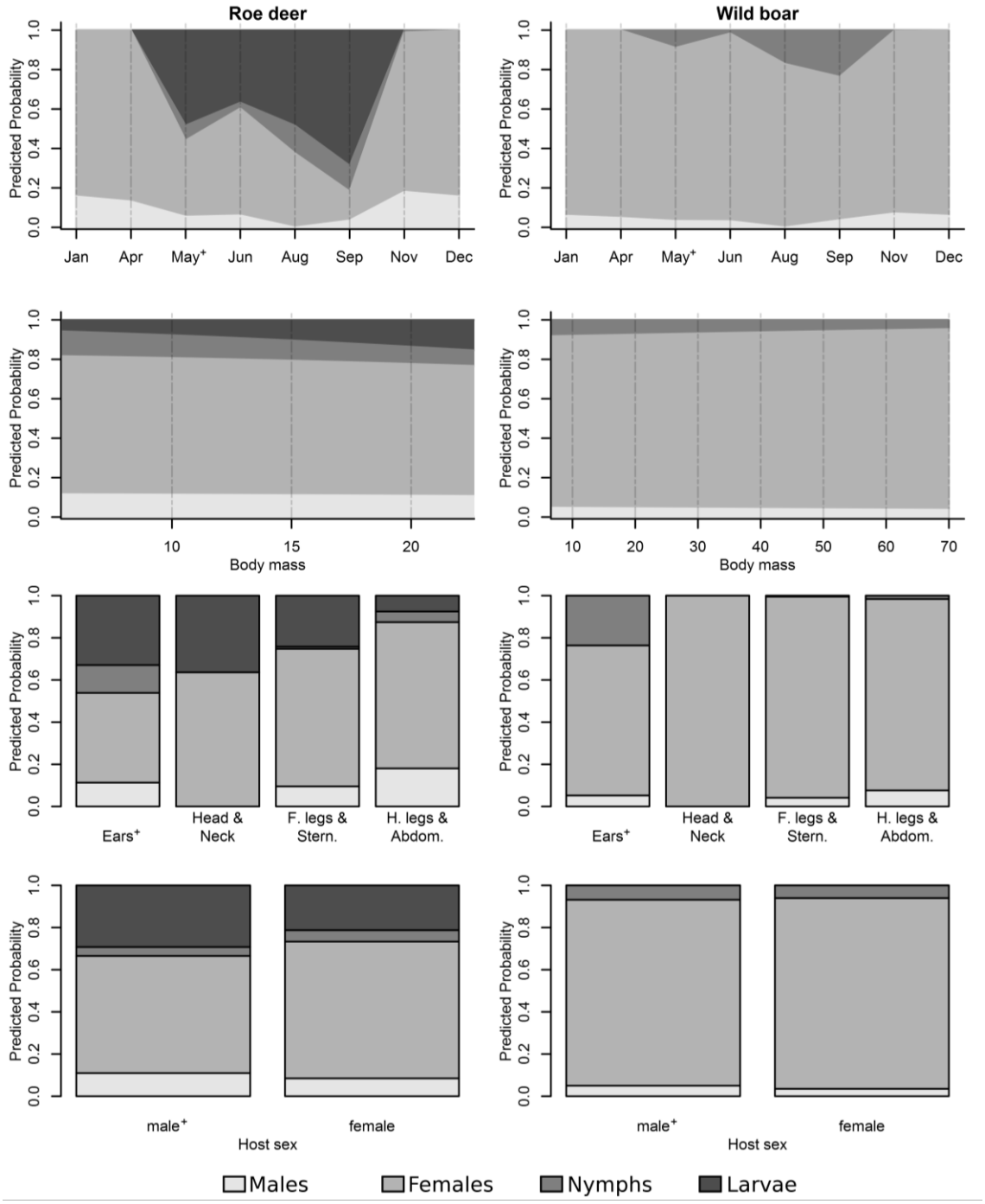


Figure 4.20: Predicated probabilities of all ticks life history stages models by multinomial logistic regression in relation to host species, host sex, body region, body mass and sampling period. Reference groups, i.e. baselines, are marked with †.

The predicted probabilities for the independent variables included in the model are presented Figure 4.20. The plots for roe deer (left) and wild boar (right) underline

Table 4.13: Distribution of collected tick life history stages in relation to roe deer and wild boar body regions.

	Ears		Head		Neck		Forelegs		Hind legs		Sternum		Abdomen		Total	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Roe deer	**577**	**35.4**	**8**	**0.5**	**13**	**0.8**	**7**	**0.4**	**39**	**2.4**	**217**	**13.3**	**723**	**44.4**	**1584**	**97.2**
Females	77	4.7	1	0.1	13	0.8	2	0.1	30	1.8	179	11.0	487	29.9	789	48.4
Males	16	1.0					1	0.1	9	0.6	27	1.7	159	9.8	212	13.0
Nymphs	352	21.6									3	0.2	74	4.5	429	26.3
Larvae	132	8.1	7	0.4			4	0.3			8	0.5	3	0.2	154	9.5
Mating	18	1.1					2	0.1	2	0.1	34	2.1	212	13.0	268	16.4
Wild boar	**14**	**0.9**									**5**	**0.3**	**27**	**1.7**	**46**	**2.8**
Females	9	0.6									5	0.3	23	1.4	37	2.3
Males	2	0.1											2	0.1	4	0.3
Nymphs	3	0.2											2	0.1	5	0.3
Total	**591**	**36.3**	**8**	**0.5**	**13**	**0.8**	**7**	**0.4**	**39**	**2.4**	**222**	**13.6**	**750**	**46.0**	**1630**	**100.0**

the effect of host species, as the composition of the tick population between both species is predicted to be considerably different. Moreover, the significant influence of the sampling periods on the proportions of males, nymphs and larvae in relation to females is supported by the visibility of the spring and autumn peaks for the immature tick life history stages (first roe of Figure 4.20). The positive effect of body mass on the proportions of larvae can only be observed for roe deer since no larvae were found on wild boar. Furthermore, the significant influences of attachment sites and host sex on the composition of the population is underlined by the corresponding bar plots.

4.1.7 Attachment site analysis

An overview of how the tick life history stages were distributed over the body parts of roe deer and wild boar is given in Table 4.13.

On roe deer, female ticks were predominantly found on the abdomen, followed by sternum and ears. Males were found with similar relative proportions, but with a lower intensity. Mating ticks were collected most frequently from the abdomen. In contrast to this, the immature life history stages were mostly collected from the ears. No males were found on the head and neck, nymphs were not collected from head, neck and legs, while no larvae were removed from the neck and hind legs.

Table 4.14: Friedman test for tick densities showing the mean rank for roe deer body parts with respect to each season and for the total study period.

	Jan/Feb		Mar/Apr		May/Jun		Sep/Oct		Nov/Dec		Study	
Ticks	5.90	Abdomen	8.00	Sternum	7.38	Ear	7.64	Abdomen	5.59	Abdomen	5.86	Abdomen
	4.37	Sternum	7.00	Abdomen	6.81	Abdomen	6.55	Ear	4.47	Hind Legs	4.76	Ear
	4.29	Ear	3.50	Ear	5.74	Sternum	4.09	Sternum	4.44	Sternum	4.54	Sternum
	4.29	Head	3.50	Head	3.55	Head	3.55	Head	4.43	Ear	4.26	Hind Legs
	4.29	Neck	3.50	Neck	3.29	Neck	3.55	Neck	4.28	Head	4.17	Head
	4.29	Front Legs	3.50	Front Legs	3.24	Front Legs	3.55	Front Legs	4.28	Front Legs	4.15	Neck
	4.29	Hind Legs	3.50	Hind Legs	3.00	Hind Legs	3.55	Hind Legs	4.26	Neck	4.15	Front Legs
Adults	5.90	Abdomen	8.00	Sternum	7.26	Abdomen	7.45	Abdomen	5.59	Abdomen	5.89	Abdomen
	4.37	Sternum	7.00	Abdomen	6.07	Sternum	4.73	Ear	4.48	Hind Legs	4.60	Sternum
	4.29	Ear	3.50	Ear	5.40	Ear	4.50	Sternum	4.44	Sternum	4.47	Ear
	4.29	Head	3.50	Head	3.64	Neck	3.86	Head	4.41	Ear	4.31	Hind Legs
	4.29	Neck	3.50	Neck	3.40	Head	3.86	Neck	4.28	Head	4.20	Neck
	4.29	Front Legs	3.50	Front Legs	3.40	Front Legs	3.86	Front Legs	4.28	Front Legs	4.18	Head
	4.29	Hind Legs	3.50	Hind Legs	3.40	Hind Legs	3.86	Hind Legs	4.26	Neck	4.18	Front Legs
Males	5.20	Abdomen	8.00	Abdomen	6.17	Abdomen	7.36	Abdomen	5.15	Abdomen	5.37	Abdomen
	4.47	Sternum	5.50	Sternum	5.29	Sternum	4.09	Ear	4.55	Hind Legs	4.50	Sternum
	4.39	Ear	3.75	Ear	5.26	Ear	4.09	Sternum	4.43	Ear	4.47	Ear
	4.39	Front Legs	3.75	Front Legs	3.86	Front Legs	4.09	Front Legs	4.42	Sternum	4.43	Hind Legs
	4.39	Hind Legs	3.75	Hind Legs	3.86	Hind Legs	4.09	Hind Legs	4.38	Front Legs	4.32	Front Legs
Females	5.90	Abdomen	8.00	Sternum	7.31	Abdomen	7.45	Abdomen	5.53	Abdomen	5.86	Abdomen
	4.37	Sternum	7.00	Abdomen	6.02	Sternum	4.73	Ear	4.49	Hind Legs	4.59	Sternum
	4.29	Ear	3.50	Ear	5.40	Ear	4.50	Sternum	4.45	Sternum	4.48	Ear
	4.29	Neck	3.50	Neck	3.64	Neck	3.86	Neck	4.42	Ear	4.32	Hind Legs
	4.29	Head	3.50	Head	3.40	Head	3.86	Head	4.29	Head	4.21	Neck
	4.29	Front Legs	3.50	Front Legs	3.40	Front Legs	3.86	Front Legs	4.29	Front Legs	4.19	Head
	4.29	Hind Legs	3.50	Hind Legs	3.40	Hind Legs	3.86	Hind Legs	4.27	Neck	4.19	Front Legs
Nymphs	--	--	--	--	5.17	Ear	5.86	Ear	4.52	Ear	4.65	Ear
	--	--	--	--	4.64	Abdomen	5.86	Abdomen	4.50	Abdomen	4.57	Abdomen
	--	--	--	--	4.52	Sternum	4.05	Sternum	4.50	Sternum	4.48	Sternum
Larvae	--	--	--	--	8.00	Ear	6.41	Ear	--	--	4.90	Ear
	--	--	--	--	4.50	Sternum	4.23	Sternum	--	--	4.49	Sternum
	--	--	--	--	4.36	Head	4.23	Head	--	--	4.47	Head
	--	--	--	--	4.05	Abdomen	4.23	Abdomen	--	--	4.45	Abdomen
	--	--	--	--	4.02	Front Legs	4.23	Front Legs	--	--	4.45	Front Legs
Matings	5.12	Abdomen	7.50	Abdomen	6.14	Abdomen	7.05	Abdomen	5.00	Abdomen	5.23	Abdomen
	4.48	Sternum	6.00	Sternum	5.14	Sternum	4.14	Sternum	4.47	Sternum	4.53	Sternum
	4.40	Ear	3.75	Ear	4.83	Ear	4.14	Ear	4.47	Ear	4.47	Ear
	4.40	Front Legs	3.75	Front Legs	3.98	Front Legs	4.14	Front Legs	4.43	Front Legs	4.36	Front Legs
	4.40	Hind Legs	3.75	Hind Legs	3.98	Hind Legs	4.14	Hind Legs	4.43	Hind Legs	4.36	Hind Legs

On wild boar, ticks were only found at the ears, sternum and abdomen, whereby no immature ticks were registered from the sternum. The abdomen was the preferred body part of males and females, while nymphs were slightly more likely on the ears.

The results for the seasonal analysis using the Friedman test on the tick densities of the roe deer body parts are given in Table 4.14. The ranking shows that larvae were only found on roe deer in the sampling seasons May/June and September/October

with significant differences ($P < 0.05$) in their preferred feeding sites. In May/June most larvae were found on the ears followed by sternum, head and abdomen. Similarly, in September/October the ears were clearly preferred.

Nymphs were found on roe deer in May/June, September/October and November/December, whereby significant feeding site preferences ($P < 0.05$) were observed during May/June and September/October. During these periods, nymphs preferred the ears followed by the abdomen. In contrast to this, female ticks were found on roe deer throughout the year, primarily selecting the abdomen ($P < 0.05$), except for March/June when females showed a slight preference for the sternum over the abdomen. The sternum was ranked second for females during January/February and May/June, while for September/October it was replaced by the ears and for November/December by the hind legs. The feeding site distribution of male *Ixodes* on roe deer was comparable to that of the female ticks: males preferred the abdomen, followed by sternum, except for September/October when the ears were ranked second and during November/December with the hind legs were ranked after the abdomen. Thus adult ticks selected the abdomen and the sternum as their favorite attachment and feeding sites, while the ears were more frequented in September/October and the hind legs during November/December.

Overall, the ticks on roe deer significantly preferred the abdomen during winter (November to February), but tended to sternum and ear during warmer months (March to June). Mating occurred predominantly at the abdomen and sternum, followed by the ear, with no seasonal variation present. For the total study period, the abdomen, ear and sternum were the top ranked attachment sites for all ticks. Males and females primarily preferred the abdomen, followed by the sternum, while nymphs and larvae choose the ear as their primary feeding site.

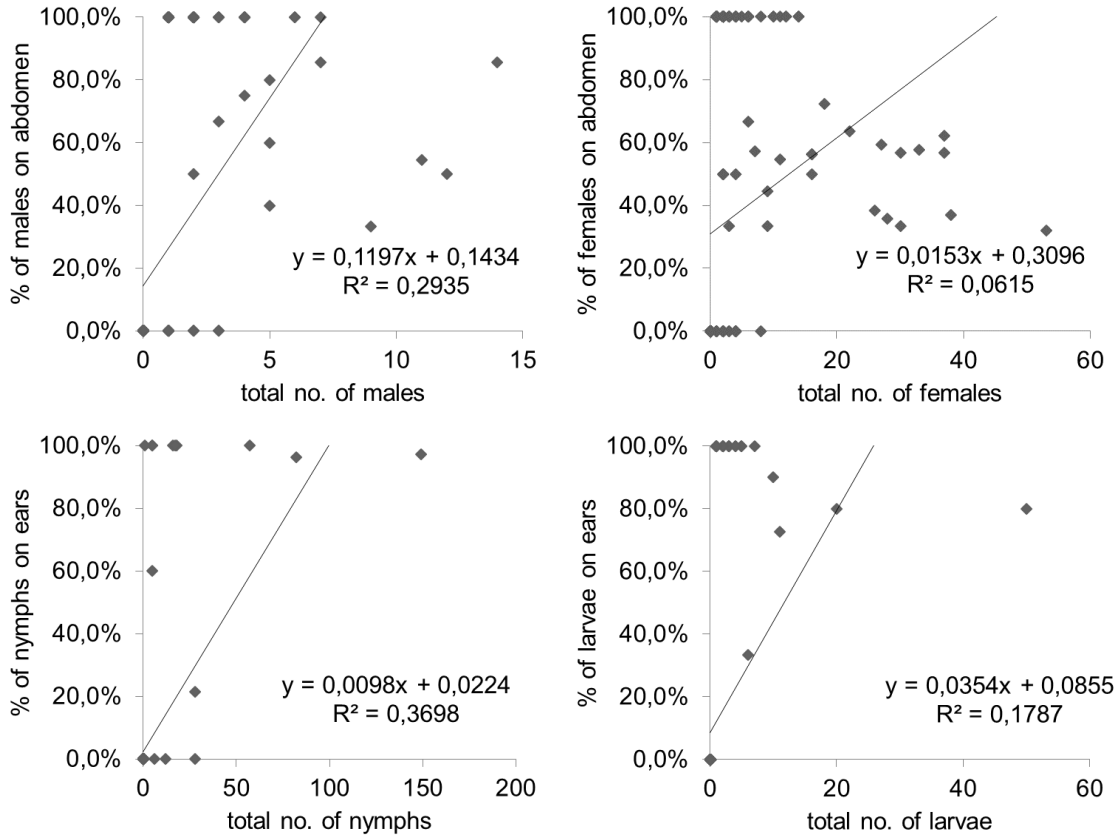


Figure 4.21: Regression analysis for roe deer showing the relation between the percentage of tick life stage/sex using the preferred attachment site and the total number of the same life stage/sex on the entire host body.

For the test of the IFDH on ticks from roe deer, Pearson's correlation coefficients (males: $\rho = 0.542$, $P < 0.001$; females: $\rho = 0.248$, $P < 0.001$, nymphs: $\rho = 0.608$, $P < 0.001$, larvae: $\rho = 0.423$, $P < 0.001$) and the regression analyses in Figure 4.21 show that in relation to each tick life stage/sex the percentage of ticks attached to the top ranked body part increased with the total number of the same life stage/sex found on the entire host body in a highly significant manner.

Ticks on wild boar were predominantly found on the abdomen (see Table 4.15) throughout the total period of sampling, although male ticks and nymphs were mainly found on the ears, which ranked second for female ticks. Ticks from wild boar were not found at the head, neck, front or hind legs or on the main body. Significant differences in feeding site selection were detected between May/June and Novem-

Table 4.15. Friedman test for tick densities showing the mean rank for wild boar body parts with respect to each season and for the total study period.

	May/Jun	Sep/Oct	Nov/Dec	Study
Ticks	6.83 Ear	5.10 Sternum	4.64 Abdomen	4.61 Abdomen
	4.17 Sternum	5.10 Abdomen	4.53 Sternum	4.53 Ear
	4.17 Abdomen	4.30 Ear	4.51 Ear	4.52 Sternum
Adults	6.83 Ear	5.20 Sternum	4.64 Abdomen	4.60 Abdomen
	4.17 Sternum	4.40 Ear	4.53 Sternum	4.53 Ear
	4.17 Abdomen	4.40 Abdomen	4.51 Ear	4.53 Sternum
Males	5.67 Ear	-- --	4.51 Abdomen	4.51 Ear
	4.33 Abdomen	-- --	4.50 Ear	4.51 Abdomen
Fe-males	6.83 Ear	5.20 Sternum	4.64 Abdomen	4.60 Abdomen
	4.17 Abdomen	4.40 Abdomen	4.53 Sternum	4.53 Sternum
	4.17 Sternum	4.40 Ear	4.51 Ear	4.53 Ear
Nymphs	5.67 Ear	5.20 Abdomen	-- --	4.51 Ear
	4.33 Abdomen	4.40 Ear	-- --	4.51 Abdomen

ber/December, with the ears ranking first in the warm months. In winter (November/October) the abdomen was the primary feeding site. Females slightly preferred the sternum second to the abdomen during November/December.

The correlation and regression analyses for the test of the IFDH on ticks sampled from wild boar did not deliver acceptable results due to the limited number of ectoparasites found.

4.1.8 Niche breadth analysis

During the total study period, adult female ticks showed the lowest specialization in their selection of the feeding site on roe deer, followed by larvae and adult male ticks (see Table 4.16). Nymphs were most specialized in their choice of feeding site. However, a paired *t*-test showed that niche indices differed significantly only between males and females (t = -4.322, df = 78, P < 0.001), females and nymphs (t = 2.568, df = 12, P = 0.025) and females in comparison with larvae (t = 3.881, df = 26, P = 0.001). In contrast to this, there were no significant differences between the Levin indices

Table 4.16. Monthly averaged standardized Levin index of niche breadth for roe deer with respect to each study period and for the total study.

	Sep/Oct 2011	Nov/Dec 2011	Jan/Feb 2012	Mar/Apr 2012	May/Jun 2012	Jul/Aug 2012	Sep/Oct 2012
Ticks	0 ± 0	0.005 ± 0.023	0.016 ± 0.044	0.136 ± 0.006	0.158 ± 0.072	0.025 ± 0.000	0.076 ± 0.050
Adults	0 ± 0	0.005 ± 0.023	0.016 ± 0.044	0.136 ± 0.006	0.103 ± 0.083	0.143 ± 0.000	0.042 ± 0.083
Males	0 ± 0	0 ± 0	0.038 ± 0.054	0.070 ± 0.070	0.048 ± 0.058	×	0 ± 0
Females	0 ± 0	0.006 ± 0.029	0.017 ± 0.045	0.121 ± 0.007	0.105 ± 0.084	0.143 ± 0.000	0.044 ± 0.087
Nymphs	×	×	×	×	×	0 ± 0	0.026 ± 0.047
Larvae	×	×	×	×	0.002 ± 0.008	0 ± 0	0 ± 0
	Nov/Dec 2012	Jan/Feb 2013	May/Jun 2013	Sep/Oct 2013	Nov/Dec 2013	Jan/Feb 2014	Study
Ticks	0.018 ± 0.044	0 ± 0	0.109 ± 0.084	0 ± 0	0.008 ± 0.029	0 ± 0	0.038 ± 0.068
Adults	0.019 ± 0.044	0 ± 0	0.157 ± 0.137	0 ± 0	0.008 ± 0.029	0 ± 0	0.033 ± 0.070
Males	0.007 ± 0.031	0 ± 0	0.100 ± 0.076	0 ± 0	0.007 ± 0.023	0 ± 0	0.018 ± 0.046
Females	0.024 ± 0.052	0 ± 0	0.156 ± 0.138	0 ± 0	0.011 ± 0.035	0 ± 0	0.035 ± 0.073
Nymphs	0 ± 0	×	0.005 ± 0.005	×	×	×	0.016 ± 0.037
Larvae	×	×	0.090 ± 0.122	×	×	×	0.024 ± 0.072

× = tick live history stage not found

of males and nymphs, males and larvae or between nymphs and larvae. Taking seasonal differences of attachment sites into consideration, the widest niche breadth overall was detected in May/June 2013 as well as in May/June 2012, followed by March/April 2012 and in July/August 2012 (see Table 4.16).

Female ticks showed the lowest specialization followed by males. Consequently, adult ticks showed less specialization in their feeding site selection than nymphs and larvae. An exception was the period between May and June 2013 when larvae showed a wide niche breadth, whereas nymphs were very specialized in their attachment site choice. As shown by the results of the seasonal analysis using the GLM in Table 4.17, niche breadth of female ticks was significantly higher from March to August compared to the reference season, i.e. the months November and December. In particular, in May and June these differences were highly significant ($P < 0.001$). For males, the niche breath from March to June was also significantly higher than during the colder months (September to February). In contrast to this, nymphs and

Table 4.17: Results of the estimated GLMs describing the variances of niche breadth (Levin index) for each tick life history stage and sex on roe deer.

	Ticks	Adults	Males	Females	Nymphs	Larvae
Intercept	0.011*	0.011	0.005	0.013	0.000	0.000
Jan/Feb	0.003	0.003	0.008	-0.006	×	×
Mar/Apr	0.125***	0.125**	0.065*	0.108*	×	×
May/Jun	0.131***	0.108***	0.059***	0.107***	0.005	0.032
Jul/Aug	0.015	0.132*	×	0.129*	-0.000	0.000
Sep/Oct	0.051***	0.024	-0.005	0.023	0.025	0.000
Nov/Dec [a]	0.000	0.000	0.000	0.000	0.000	×

[a] Reference period; × no tick life stage/sex found; Significances: * P < 0.05; ** P < 0.01; *** P < 0.001

larvae showed no significant differences with respect to the niche breadth. Nevertheless, for the entirety of the collected ticks the niche breadths of the months March to June, September and October were significantly different to the reference season.

Due to the low total number of ticks found on wild boar the computation of the Levin index and the subsequent estimation of the GLM did not deliver reasonable results. Therefore, an analysis of the niche breath and the evaluation of the spatial feeding site overlap, presented in the following section, was not conducted for the ticks from wild boars.

4.1.9 Spatial niche overlap

Niche overlap using Pianka's index was determined for the different tick life history stages and sexes on roe deer (Table 4.18). The highest niche overlap for the total sampling period was found between male and female ticks (0.72), followed by nymphs-larvae, females-larvae, males-larvae, females-nymphs and males-nymphs. For the individual sampling seasons, the highest overlap between males and females was observed during May/June 2012 and 2013. Similar peaks during May and June in existed for all other tick life stage/sex constellations. The high significances were verified by the GLM (Table 4.19).

Table 4.18: Pianka's index of spatial niche overlap for ticks on roe deer with respect to each study period and for the total study.

	Sep/Oct 2011	**Nov/Dec 2011**	**Jan/Feb 2012**	**Mar/Apr 2012**	**May/Jun 2012**	**Jul/Aug 2012**	**Sep/Oct 2012**
Adults-Nymphs	×	×	×	×	×	0 ± 0	0.257 ± 0.487
Adults-Larvae	×	×	×	×	0.301 ± 0.371	0 ± 0	0.210 ± 0.395
Males-Females	0 ± 0	0.195 ± 0.391	0.116 ± 0.491	0.716 ± 0.716	1.115 ± 1.965	×	0 ± 0
Males-Nymphs	×	×	×	×	×	×	0 ± 0
Males-Larvae	×	×	×	×	0.321 ± 0.437	×	0 ± 0
Females-Nymphs	×	×	×	×	×	0 ± 0	0.209 ± 0.392
Females-Larvae	×	×	×	×	0.281 ± 0.359	0 ± 0	0.210 ± 0.395
Nymphs-Larvae	×	×	×	×	0 ± 0	1.000 ± 0.000	0.251 ± 0.408
	Nov/Dec 2012	**Jan/Feb 2013**	**May/Jun 2013**	**Sep/Oct 2013**	**Nov/Dec 2013**	**Jan/Feb 2014**	**Study**
Adults-Nymphs	0 ± 0	×	0.251 ± 0.255	×	×	×	0.016 ± 0.120
Adults-Larvae	×	×	0.362 ± 0.323	×	×	×	0.035 ± 0.160
Males-Females	0.049 ± 0.226	0 ± 0	1.195 ± 1.891	0 ± 0	0.060 ± 0.417	0 ± 0	0.179 ± 0.720
Males-Nymphs	0 ± 0	×	0.195 ± 0.324	×	×	×	0.006 ± 0.063
Males-Larvae	×	×	0.223 ± 0.312	×	×	×	0.024 ± 0.142
Females-Nymphs	0 ± 0	×	0.246 ± 0.253	×	×	×	0.015 ± 0.103
Females-Larvae	×	×	0.372 ± 0.333	×	×	×	0.034 ± 0.158
Nymphs-Larvae	×	×	0.518 ± 0.459	×	×	×	0.028 ± 0.159

× = at least one live history stage not found

In addition, females, nymphs and larvae showed a significantly increased overlap in September/October compared to the reference months November and December. However, the strongest niche overlap on roe deer appeared during July and August between nymphs and larvae ($P < 0.001$). For other months and life stage/sex constellations no significant changes in the spatial feeding site overlap were detected. Thus niche overlap seems be positively associated with the warm summer months (May to August).

Table 4.19: Results of the parameter estimation using GLMs analyzing the variance of the spatial niche overlap (Pianka's index) between different constellations of tick life history stages and sexes on roe deer.

	Males-Females	Males-Nymphs	Males-Larvae	Females-Nymphs	Females-Larvae	Nymphs-Larvae
Intercept	0.100*	0.000	-0.000	-0.000	0.000	-0.000
Jan/Feb	-0.051	0.000	0.000	0.000	0.000	0.000
Mar/Apr	0.616	-0.000	0.000	0.000	-0.000	0.000
May/Jun	1.042***	0.065***	0.288***	0.082***	0.312***	0.173***
Jul/Aug	-0.100	-0.000	0.000	0.000	- 0.000	1.000***
Sep/Oct	-0.100	0.000	-0.000	0.171***	0.172***	0.206***
Nov/Dec[a]	0.000	0.000	0.000	0.000	0.000	0.000

[a] Reference period; Significances: * P < 0.05; ** P < 0.01; *** P < 0.001

4.2 Discussion

The necessity for long-term studies in parasite and vector ecology using a sufficiently large sample size to allow a comprehensive statistical evaluation has been confirmed by my study. Only such studies can provide generally valid, raw data able to evaluate the seasonal and annual dynamics of the epidemiology of tick-borne diseases. For example, the high larval abundance on roe deer in May 2013 was significantly different from that of the other year of the study.

4.2.1 Ticks on roe deer

4.2.1.1 Tick species and life history stages

I. ricinus was the dominant species on roe deer in several other European studies (Tälleklint and Jaenson 1997, Carpi et al. 2008, Skotarczak and Adamska 2008, Kiffner et al. 2010a, Vor et al. 2010, Vázquez et al. 2011, Handeland et al. 2013). However, single findings of *D. marginatus*, *D. reticulatus* and *I. hexagonus* on roe deer have been reported (Tälleklint and Jaenson 1997, Dautel et al. 2006, Vor et al. 2010, Vázquez et al. 2011). Recent studies using drag sampling and sampling from sheep (Moser 2012, Neumaier 2012) showed that *D. marginatus* occurs in my sampling areas in the Bienwald. Although the sampling period of my study included the main activity phase of *D. marginatus* (from March to April), the finding of only a single unattached female *D. marginatus*, suggests that roe deer do not play an important role for this species. This finding confirms that *I. ricinus* is the dominant species on roe deer.

My results show that ticks were active on roe deer throughout the sampling period. All tick life history stages were found, with adult females being the most frequent making up almost 50% of the total tick infestation. Other authors found that nymphs (Carpi et al. 2008, Kiffner et al. 2010a, Vor et al. 2010, Handeland et al. 2013) or males (Vázquez et al. 2011) were more common on roe deer than females. In comparison to my study, their approaches used significantly shorter sampling periods, seasonal sampling periods and/or collected ticks from individual body parts (e.g.

ears, head or legs) only. Consequently, it is possible that their results were biased towards a specific life history stage. By contrast, my finding relies on coherent long-term sample acquisition and on the collection of ticks from the entire roe deer body. The dominance of female *I. ricinus* supports the hypothesis that roe deer provide sufficient quantities of blood for egg production for large numbers of female ticks (see Section 2.2.5), although this host species also is an important host for all other life history stages.

The results of the multinomial logistic regression model show significant effects of host species, sex and body part and sampling period on the composition of the tick population (see Section 4.1.6). However, the model confirmed, that independently of host sex, body mass and attachment site, female ticks made up the largest part of the tick population on roe deer. Only seasonal influences led the proportions of immature stages to increase during the warmer months (May to September). A similar analysis with respect to host species, age and month was conducted by Handeland et al. (2013) for ticks collected from only the ears of free-ranging moose (*Alces alces*), red deer and roe deer in Norway, albeit for a considerably shorter sampling period and without the differentiation between female and male ticks. Similar to my model, they reported significant interactions between host species and sampling period with respect to the composition of the tick population. For roe deer, they described the relative maxima of larvae and adults occurring during August and September. Although my study had a longer total sampling period, the maximum for larvae also occurred consistently during these two months.

Moreover, Handeland et al. (2013) found a significant increase in the proportion of adult ticks with increasing host size, represented by species and age. The reverse was true for the nymphs. In contrast to this, the age of roe deer did not significantly influence the composition of the tick population in my model directly. However, I was able to determine a significant increase of larval proportions with body mass. A higher body mass could be related to healthier adult animals with increased home ranges, which therefore have a higher probability of contacting larvae, which have a

very limited movement range (see Section 4.2.1.3). Handeland et al. (2013) also reported larger proportions of nymphs on the ears than predicted by my models. Nevertheless, in comparison to the rest of the body the ears of roe deer carried proportionally the highest number of immature stages. This suggests that attachment sites have a major influence on the relative proportions and on the absolute intensity of ticks.

4.2.1.2 Tick infestation intensity

The mean tick intensity in my study was 6.4 ± 21.8 ticks reaching its maximum in May 2013 with 171.7 ± 63.2. Kiffner et al. (2010a) reported that the mean tick burden on roe deer was 64.5 ± 10.6, Vázquez et al. (2011) found a mean intensity of 43.2 ± 49.8 tick per roe deer, Handeland et al. (2013) had an average tick abundance on the ears of 25.0 and Carpi et al. (2008) reported that the mean number of nymphs from roe deer forelegs was 36.0 ± 5.2. For my data, the mean intensities are thus distinctly lower than those reported by others. However, the reported peak intensities for individual months were equally high or even higher in my study. There are multiple reasons that could lead to deviations in the average tick abundances between different studies, such as the sampling approach, seasonal dynamics caused mainly by climatic changes, prevailing vegetation within the sampling areas, as well as the size and the composition of host populations. For example, driven hunts were carried out during the cold periods leading to a higher sample size between November and January and thus to a reduction of average tick infestation intensity. Expectably, low abundances of ticks on roe deer in winter were also described by Kiffner et al. (2011c), while they tried to achieve a consistent sample size over the year that lay between 8 and 20 roe deer per month. On the other hand, Vázquez et al. (2011), Handeland et al. (2013) and Carpi et al. (2008) sampled over significantly shorter periods including the warmer months with higher tick activity (Randolph et al. 2000). Moreover, the latter two studies collected only samples from body parts (ears and forelegs) that showed high tick infestations in other studies (Kiffner et al. 2011a, Pacilly et al. 2014), such that tick activity on the other body parts can only be estimated with a bias towards higher abundances.

All of these facts suggest that a more detailed investigation of tick abundances is required in order to reveal the complex interactions between factors affecting tick burden and to allow a differentiated comparison of the tick infestation intensities over multiple studies.

4.2.1.3 Aggregation

The nymphs and larvae from roe deer appeared highly aggregated (k = 0.02 and k = 0.03, respectively). This result is clearly confirmed by my decision tree analyses (see Section 4.1.4) and underlines similar observations for immature ticks on cervids (Kiffner et al. 2010b, Pacilly et al. 2014) and smaller mammals (Tälleklint and Jaenson 1997, Kiffner et al. 2011c). Tälleklint and Jaenson (1997) observed an aggregated distribution for all life history stages of *I. ricinus* on roe deer. One of the main reasons for the aggregation of ticks on a few host seems to be the spatial clumping of questing ticks (Shaw et al. 1998, Wilson et al. 2002). In particular, for questing nymphs and larvae the degree of aggregation could be an indicator for spatial variation, as well as for limited seasonal activity (Randolph 2004). *I. ricinus* larvae commonly appear in "nests" (sites where females lay their eggs) within the leaf litter (Petney et al. 2011) and have a very limited range of movement (Mejlon and Jaenson 1997). The spatial locality of larvae could have the effect that only few potential hosts pass their questing positions resulting in an increased aggregation.

On the other hand, by means of the decision tree analysis, I was able to determine that for yearlings the lowest levels of aggregation were reached for all tick life history stages, in particular for immatures. The reason for this might lie in roe deer dispersal patterns that show increased mobility and home-ranges for yearlings in comparison to older roe deer (Pettorelli et al. 2003). This behavior could positively influence the probability of yearlings passing clumps of questing immature ticks. The results of the CHAID analysis also suggests that a worse physical condition of hosts supports the aggregation of nymphs and larvae. To a slightly lower extend this

seems also to be true for male and female ticks. This observation could be interpreted such that a poor host condition leads to a reduced movement range and thus to higher aggregation of the parasites on fewer deer (see Section 4.2.1.6).

4.2.1.4 Seasonal and annual dynamics

The intensities of infestation, ranging from 0 to 261 tick per roe deer, also corroborate the data presented by Randolph (2004), where ticks showed a high interannual variability (cf. Vor et al. 2010). With respect to the seasonal dynamics, I observed high tick activity of all life history stages from April to September (100%) with an intensity peak during spring, while during winter tick prevalences reached a minimum of 12.5%. Adult ticks peaked earlier in April, while the highest average intensity for nymphs was recorded in May, followed by a second peak in August. A second peak for adult life history stages was observed in September. The highest peak for larvae was during May with a second smaller one in August, while no larvae and nymphs were found from November to April and from December to April, respectively (see Section 4.1.1). The intensity peaks correspond to the findings for roe deer made by other authors (Walker et al. 2001, Carpi et al. 2008, Scharlemann et al. 2008, Tagliapietra et al. 2011, Vázquez et al. 2011, Handeland et al. 2013). However, in the study of Vor et al. (2010) larvae peaked later in July with prevalences between 57.4% in autumn and 100% in spring. Nevertheless, my study is the only one that compares results from 3 subsequent years demonstrating that tick abundance on roe deer not only follows seasonal changes, but also can have a high variability for the same season but between years within a single habitat.

The seasonal and annual variations in the tick burden of the different life history stages on roe deer can be explained by climatic changes (see Section 4.2.1.5) as well as by variations of host parameters (see Section 4.2.1.6). Additionally, the climatic dependence of host migration behavior (Morellet et al. 2013), body mass and population density (Mysterud and Østbye 2006b) might support the effects of climate and thus amplify the changes of tick burden on roe deer.

4.2.1.5 Climatic factors

The seasonal patterns of tick activity on roe deer are explained by Vázquez et al. (2011) using the corresponding climatic variables. Estrada-Peña has confirmed that abiotic factors, like ambient temperature and relative air moisture, are most important and vital factors for tick development also in Spain (Estrada-Peña 2001, Estrada-Peña and Venzal 2007). These factors have also been identified by others (Harvell et al. 2002, Gray et al. 2009, Gilbert 2010) in association with the effects of climate change on tick burden. In laboratory experiments, Randolph et al. (2002) showed that all stages of *I. ricinus* developed more rapidly with increasing temperatures, while Gern et al. (2008) proposed that for spring fed ticks high summer temperatures could lead to a second peak in autumn. This second intensity peak in September for immature tick life history stages on roe deer has been confirmed (Section 4.1.1).

Temperature: In addition, my correlation analyses (see Section 4.1.3) corroborate a highly significant positive linear relationship between tick burden and air temperature. Other temperature related parameters (positive correlation: min. and max. air temperature, sunshine duration; negative correlation: cloud coverage) were mostly significant for all life history stages. With the decision trees for males, females and nymphs, I was able to identify a rough temperature threshold of between 8 and 9 °C, above which the infestation intensity of roe deer increased significantly (Section 4.1.4). The boxplots in Section 4.1.5 for all life history stages confirm that the critical temperature for males and females was 8 °C, while for nymphs and larvae a higher temperature threshold of 9 °C was determined. The studies of Kiffner et al. (2010b) and Tagliapietra et al. (2011) found different temperature thresholds on deer, which are higher for larvae (10 °C) than for nymphs (7-8 °C). Perret et al. (2007) suggested that a temperature threshold of 7 °C could be employed to predict the emergence of ticks in different areas.

During my sample acquisition single male and female ticks were found at temperatures as low as -1.8 °C and very few nymphs and larvae were active at temperatures

lower than 5.1 °C and 7.2 °C, respectively. Critical temperatures for the questing activity of *I. ricinus* adults have been reported between -0.6 °C and 5 °C (Sixl and Josek 1971, Duffy and Campbell 1994, Duffy et al. 1994, Schulze et al. 2001). For nymphal activity the critical temperature has been reported to be 2 °C (Sixl and Josek 1971, Hubálek et al. 2003). My finding of adult ticks during the winter months is the first reporting of *I. ricinus* activity on roe deer at such low temperatures. Moreover, this observation shows that ticks in the Bienwald are active all-year.

The GAMLSS models developed here increase our understanding of the relations found above. All models included a temperature term showing the strong relation between ticks and climate, such that a clear trend with few ticks at low temperatures and an increase of intensity with rising temperature was present. In all models, male tick burden always increased in a significant linear fashion with temperature, while female and larval tick burden did not increase further beyond 10 °C. Nymphs, on the other hand, showed a bimodal behavior (peaks at 10 °C and 17 °C) with temperature (Section 4.1.5). This observation underlines the bimodal seasonal pattern observed for nymphs on roe deer. For the other three life history stages, the models did not identify a bimodal pattern of mean tick intensity in relation to temperature. One explanation for this behavior could be that the peaks during spring and/or autumn were more pronounced for nymphs than for females, males and larvae (see Section 4.1.1). However, for larvae a slight bimodality at 10 °C and 16 °C was observed for the modeling of the dispersion term, which could be an indication for bimodal seasonal behavior of larvae. In contrast to the models of Kiffner et al. (2011b) in which two intensity peaks appeared for adult ticks in relation temperature and not for nymphs, my models support to the bimodal seasonal pattern of nymphal behavior (Randolph 2004). This difference could be related to other environmental features or host community related parameters intrinsic to the different sampling areas.

Relative humidity: In addition to temperature, negative correlations have been reported for host seeking *I. ricinus* on vegetation in relation to relative humidity (Jensen 2000, Perret et al. 2000, 2004, Randolph et al. 2002, Hubálek et al. 2003,

Schwarz et al. 2009). For small mammals a significant decline of *Ixodes* spp. larvae infestations with increasing relative humidity was found (Boyard et al. 2008, Kiffner et al. 2011c). In the present study the daily average relative humidity was negatively correlated with tick abundance on roe deer at a highly significant level not only for larvae, but also for male and female ticks (see Section 4.1.3).For the first time, my study employed decision tree analysis on a sample population large enough to determine an activity threshold in relation to relative humidity for *I. ricinus* larvae. The results of the CHAID tree suggest that relative humidity plays an outstanding role for larvae on roe deer, such that a relative humidity above 80.4% reduces the infestation intensity significantly (0.052 ± 0.518 vs. 1.958 ± 3.632).

These observations stand in contrast to the results of the feeding experiments conducted by Randolph and Storey (1999), in which larvae did not quest high in the vegetation and contacted fewer rodents at low levels under drier conditions, while a higher relative humidity increased larval mobility and questing height allowing the ticks to feed on larger hosts. Nevertheless, it has also been suggested that larvae in particular are less mobile under wet conditions (Perret et al. 2003), that sufficiently wet conditions might lead to increased tick mortality (Randolph 2004) and that ticks might be positively affected by humidity only until an optimal value is reached (Tagliapietra et al. 2011). The combination and the interdependencies of the three effects, reduced mobility, increased mortality and optimal hydration, could be an explanation for the humidity threshold determined here by the CHAID analysis and the negative correlation between larval burden and relative humidity.

Saturation deficit: Various recent studies suggest that saturation deficit is a very important factor for the behavior and mortality rate of ticks (Randolph and Storey 1999, Perret et al. 2000, 2003, 2004, Randolph 2004, Gern et al. 2008, Gray et al. 2009, Tagliapietra et al. 2011). In quasi-natural arenas, Randolph and Storey (1999), and in the field Perret et al. (2000), recognized that a higher saturation deficit reduced the number of questing *I. ricinus* ticks along with a higher energy loss of

the ticks shortening their life span. Perret et al. (2003) observed that questing duration is inversely related to saturation deficit and that the effect of desiccation is more serve for nymphs than for the adult life stages (Perret et al. 2000). A high saturation deficit forces ticks to move from their questing position into quiescence on the ground to rehydrate (Gern et al. 2008). For ticks collected from deer, Tagliapietra et al. (2011) found out that saturation deficit was a key factor affecting nymphal abundance and predicting most of its variation. The correlation analyses conducted here (see Section 4.1.3) for spring underline the behavior described above. In May and June, nymphs were significantly negatively correlated with saturation deficit. For males and females, no significant correlations with respect to saturation deficit were detected. However, during autumn (Sep/Oct) larval infestation increased significantly with rising saturation deficits. This finding is in contrast to the studies of Randolph and Storey (1999), in which larvae avoided desiccation by moving into the litter layer and becoming quiescent. On the other hand, the results of Perret et al. (2003) indicate that higher saturation deficits lead to a larger distance traveled by ticks after quiescence. This might provide an explanation for the increased larval burden in the present study.

Precipitation: In addition to this, all models estimated by the GAIC-based parameters selection strategy included precipitation depth for all tick life history stages. High rainfalls above 2 mm and 4 mm have a negative influence on adult and immature tick burden, respectively, although a significant effect was only detected for female ticks (see Section 4.1.5). However, for females and larvae an increase in tick infestation was detected with precipitation above 7 mm and 12 mm, respectively. High precipitation depths during the study period were observed in winter and spring, particularly during May 2013 (see Section 4.1.5). Consequently, the non-linear relationship between ticks and rainfall revealed by the models can be interpreted twofold: (1) high rainfall can be seen as an indicator for the cold season and therefore reduced tick life history stage abundances, and (2) rain during spring supports tick hydration and leads to an increased tick occurrence. This non-linearity of

the seasonal patterns might be the reason why only the GAMLSS models include precipitation depths in comparison to the other statistical analyses. However, previous model-driven tick research on roe deer (Carpi et al. 2008, Kiffner et al. 2010a, 2011b, Vor et al. 2010, Tagliapietra et al. 2011, Handeland et al. 2013) did not consider rainfall in their analysis, making my study unique. By the use of the GAMLSS approach, I was able to show that precipitation plays an important role for ticks on roe deer and that such complex, non-linear effects can be revealed and better understood through an adequate modeling technique.

4.2.1.6 Host parameters

Host sex: With respect to biotic factors, there exists the hypothesis that tick parasitism might be sex-biased (Poulin 1996, Zuk and McKean 1996, McCurdy et al. 1998, Ferrari et al. 2003). In particular for white-tailed deer (*Odocoileus virginianus*) in the USA, many studies report a male-biased abundance of *Ixodes* spp. (Schulze et al. 1984, Kitron et al. 1992, Schalk and Forbes 1997, Schmidtmann et al. 1998). For roe deer in Spain, Vázquez et al. (2011) determined host sex as the most important factor influencing tick prevalence, while in German studies this factor was only marginally significant suggesting only a weak influence on the overall tick burden (Kiffner et al. 2011b), or that both roe deer sexes were almost equally infested (Vor et al. 2010). The GAMLSS models of Kiffner et al. (2011b) considered host sex for the immature tick life history stages, but not for male and female ticks. However, they suggested that host sex might play only a minor role in tick abundances on roe deer.

In contrast to this, my results indicate that the sex of roe deer has an influence on tick abundance, such that male deer had significantly more ticks than female animals. This observation is also underlined by the decision trees for males, females and larvae, which also included host sex. In particular, for adult ticks host sex seems to be a key factor since it appears at the second level of the decision trees. The GAMLSS models provide and even deeper insight into the interactions between host sex and tick life history stage abundance. The models show that host sex plays an im-

portant role for males, nymphs and larvae. A significantly male deer-biased parasitism was observed for males and larvae. With adult and larval *I. ricinus* showing a male-biased behavior in the CHAID analysis and in the GAMLSS models a sex-dependent parasitism of *I. ricinus* on roe deer seems to be very likely, although this is the first report of such a behavior in a German study.

There are basically two non-mutually exclusive theories for sex-biased tick behavior (cf. Zuk and McKean 1996, Pfäffle 2010): (1) the "exposure" hypothesis, which states that sex-specific features and behavior could influence the exposure to parasites (Poulin 1996), and (2) the "immunosuppressive" hypothesis suggests that adrenal hormones, like testosterone, might affect the intensity of tick infestation (Hamilton and Zuk 1982, Folstad and Karter 1992, Schalk and Forbes 1997, Christe et al. 2007). On small mammals, the experiments of Hughes and Randolph (2001a, 2001b) demonstrated that increased testosterone levels lead to male-biased tick parasitism. In accordance to the first hypothesis, Moore and Wilson (2002) found that a sex-bias could be a result of size dimorphism, such that parasites prefer the sex having an increased body size and mass. The idea behind this is that larger hosts provide more resources for the parasites (Perkins et al. 2003). For white-tailed deer, the size dimorphism between males and females could be the reason for male-based tick parasitism (cf. Kiffner et al. 2011b). However, in comparison to white-tailed deer, the sexual size dimorphism of roe deer is less strongly pronounced. For my dataset the mean body mass of each sex with respect to each age group (*t*-test for fawns: $P = 0.445$; for yearlings: $P = 0.069$; for adults: $P = 0.659$) showed no significant differences. This means that the body mass of coeval male and female roe deer was almost equal. Thus, sexual size characteristics alone are unlikely to lead to the sex-biased tick behavior on roe deer.

However, other behavioral patterns might cause the increased tick burden on male roe deer. The studies of Sempéré and Lacroix (1982), Blottner et al. (1996) and Mysterud (1999) suggest an increased migration behavior of male roe deer during spring (May/Jun), occurring simultaneously with a rise in testosterone levels, which

was stronger in adult males than in yearlings. This in turn coincides with the results of Schalk and Forbes (1997), who found a significant male-bias detected for adult hosts, but not for juveniles. My results underline this pattern by showing that adult and larval ticks had a higher abundance on adult male roe deer than on younger males. Thus, the combined effect of behavioral and hormonal changes suggests that a decoupling of the two hypotheses does not seem to make sense for roe deer.

Host age: Vázquez et al. (2011) observed an age-dependent distribution of tick abundance with yearlings having higher prevalences than adults and fawns, whereby their results did not reach a level of significance. Vor et al. (2010) found significant positive correlations between adult tick burden, host age and body mass. On the other hand, they found a decrease in the number of *Ixodes* spp. larvae with rising roe deer age indicted by a significantly negative correlation. They explained this by longer resting phases and thinner skin of younger animals. Handeland et al. (2013) complemented these results by showing that adult ticks were preferentially found on larger cervids, while immature ticks preferred smaller hosts.

My study confirms the above findings. For yearlings, a significantly higher tick intensity was found than for fawns and adults in relation to all tick life history stages (see Section 4.1.1). The CHAID analysis of the host parameters ranked host age as the most important factor for tick abundance, whereby yearlings where always separated from other individuals with significantly more ticks (Section 4.1.4). Moreover, the automatic parameter selection strategy included host age into the GAMLSS models of all tick life history stages. Tick intensity was estimated to be the lowest on fawns, while yearlings showed higher infestation for male, female and nymphal *I. ricinus* ticks. As host age was only significant for males and nymphs, its effect seems to be less important for females and larvae.

An explanation for my findings could be that adult ticks quest higher up in the vegetation and thus have an increased probability of attaching to larger hosts (Randolph 2004). In accordance with the “exposure” hypothesis, this observation

could be the reason why yearlings had higher tick abundances than fawns. Another explanation could be the immunosuppressive effect of testosterone leading to a tick preference towards yearlings. An equivalent argument works for the comparison of yearlings and adult roe deer. The lower mobility of adult roe deer (Pettorelli et al. 2003) might reduce their exposure to ticks in comparison to yearlings. In summary, and similarly to sex-biased behavior, it seems that both effects, i.e. exposure and immunosuppression, interact when considering roe deer hosts.

Body mass: Instead of host age, the models obtained by Kiffner et al. (2011b) included roe deer body mass suggesting a positive significant effect of host size on tick burden. In contrast to this, my simple model for nymphs and the GAIC-based models for male and larval *I. ricinus* showed a negative influence of body mass on the tick intensity of roe deer. This behavior stands in contrast to the assumption that larger animals provide more resources and could thus host more ticks (Smith et al. 1990, Pichon et al. 1999, Dobson et al. 2006, Ruiz-Fons et al. 2006, Gern 2008, Pound et al. 2010, Kiffner et al. 2011b). A more detailed view of this behavior has been provided by modeling tick life history stages in relation to body mass of roe deer age groups. These results showed the considerable influence of host body mass and reveal that the relationships between parasite intensity, tick life stage/sex, host body mass and host age are complex and highly non-linear (see Section 4.1.5), such that individual studies are needed to find out more about the interactions of these factors on roe deer.

Host condition: The $GDMI_s$ (see Section 3.3.4) correlated negatively with the overall tick burden and, in particular, with *I. ricinus* nymph numbers. Moreover, the CHAID algorithm determined the $GDMI_s$ as important for tick intensity on female fawns and adult deer. This might indicate that animals with a body mass that is increased relative to mean host age and sex have a lower tick abundance, such that healthier animals might carry less ticks. In conjunction with this, the correlation analyses revealed that a poorer condition or crippled host increases tick infestation for females, nymphs and larvae, only males were non-significantly correlated. These

patterns are in accordance with the decision trees which reveal the "HasCondition" variable as the most important factor for an increased number of nymphs and larvae (Section 4.1.4). Two questions arise from these results: (1) are ticks causing health problems to roe deer, and (2) do certain roe deer health issues increase tick burden?

An overview of the negative influence of parasites on the physical condition of hosts was given in Pfäffle (2010). Several deer species, including roe deer, are considered to be reservoir-incompetent for *Borrelia* spp. (Nelson et al. 2000, Bhide et al. 2005, Gern 2008, Pound et al. 2010, Alonso et al. 2012), but the effect of other tick transmitted diseases is largely unclear for roe deer. Vor et al. (2010) were not able to find any signs of deer health problems caused by high tick infestation. For small mammals, e.g. European hedgehogs (*Erinaceus europaeus*) (Pfäffle et al. 2009), a significant blood loss was noticed at high tick densities. This caused regenerative anaemia. For roe deer, Tälleklint and Jaenson (1997) estimated a median amount of tick induced potential blood loss at 2.0% of the host's total blood volume. For only 14% of their sampled roe deer was there a blood loss larger than 5%, while for the rest of the animals a lower reduction of blood volume was calculated. My estimate of the blood loss of roe deer was an order of magnitude smaller (see Section 4.1.1), with an average of 0.3%. This relatively low percentage of blood loss is an indicator that only very few roe deer suffer from tick induced blood loss.

With respect to the second question above, my results show that pregnant or nursing female deer were significantly more heavily infested with *I. ricinus* than other individuals (Section 4.1.1). Pregnancy and lactation increase the energy consumption of female roe deer considerably (Mauget et al. 1997). For fawns the correlation coefficients for all tick life history stages and body mass were significantly ($P < 0.01$) negative (females: $\rho = -0.393$; males: $\rho = -0.283$; nymphs: $\rho = -0.385$; larvae: $\rho = -0.376$), suggesting that fawns in better condition have less ticks. Moreover, the "HasCondition" variable including the infestation of the hosts with other parasites as well as deformities correlated negatively with host body condition suggesting a higher tick burden on hosts in poorer condition.

Concurrent infestations of roe deer with other parasites (i.e. *Damalinia* (syn. *Cervicola*) *meyeri* lice and the deer ked fly, *Lipoptena cervi*) were also observed in Norway (Handeland et al. 2013). Hosts in poor condition have fewer resources available than healthier ones and have to invest more energy in defensive responses to parasites and diseases at the same time (Wilson et al. 2002). This situation causes the infestation intensities of parasites to co-vary within a host population proportionally to host body condition (Wilson 1994, Holmstad and Skorping 1998, Stear and Wakelin 1998). Moreover, a reduced constitution and decreased mobility might increase attachment success and tick survival on these hosts (Wikel and Bergman 1997, Pfäffle et al. 2013). Consequently, my observations on roe deer show for the first time that roe deer in a poor physical condition have an increased probability of high tick infestation.

4.2.2 Ticks on wild boar

4.2.2.1 Species and life history stages

Only *I. ricinus* ticks were collected from wild boar. In contrast to roe deer, only little research on the tick infestation of wild boar has been conducted. Nevertheless, other Central European studies have also reported finding only *I. ricinus* on wild boar (Petrovec et al. 2003, Skotarczak and Adamska 2008, Michalik et al. 2012, Pacilly et al. 2014). In contrast, tick sampling in Spain described 8 different ixodid tick species parasitizing wild boar, mainly *Dermacentor* spp., *Hyalomma marginatum* and *Rhipicephalus bursa*, but only few *I. ricinus* (de la Fuente et al. 2004, Ruiz-Fons et al. 2006). Similarly, Selmi et al. (2009) report *D. marginatus* as the most common species on wild boar in Italy. Altogether, these studies indicate that *I. ricinus* is predominately found on wild boar in temperate areas, while the studies from southern Spain and Italy suggest that this species is replaced by *D. marginatus* in the Mediterranean region.

In relation to the tick life history stages, most of the *I. ricinus* collected were females, followed by nymphs and males, while no larvae and no mating ticks were found on wild boar. This distribution is quite similar to findings in Poland (Skotarczak and

Adamska 2008). However, quite the contrary was reported by Pacilly et al. (2014), where the infestation of wild boar in a Dutch national park during fall 2010 was dominated by larvae, followed by nymphs and adults. They did not find male ticks on wild boar. Michalik et al. (2012) also reported immatures to be more common on wild boar in Poland. One reason for the dominance of immatures over adults in those two studies could be seasonal influences due to a shorter sampling period. My results show that the abundance of nymphs on wild boar was also higher than that of adults in September, while the reverse was true for all other months (see Section 4.1.2). Consequently, it is important to consider the period of sampling and the total time of sample acquisition when comparing tick populations of different studies.

The multinomial logistic regression modelled the composition of a tick population from wild boar successfully for the first time (see Section 4.1.6). The results showed that the predicted probability of finding immature ticks was significantly lower on wild boar than on roe deer. Moreover, an increasing body mass of wild boar reduced the chance on finding nymphs even further. Thereby, my models confirm the findings of Skotarczak and Adamska (2008) in which adults ticks were also most abundant on wild boar. Pacilly et al. (2014) found higher proportions of the nymphs on the anterior axilla, whereas in my study nymphs where most common on the ears. In addition to this, I found that wild boar sex generally did not influence the composition of the tick population significantly, but male piglets hosted significantly more female ticks than female piglets.

4.2.2.2 Engorgement

Michalik et al. (2012) describe their *I. ricinus* as being partially engorged, in contrast to Skotarczak and Adamska (2008), who found fully engorged ticks. I found living *I. ricinus* individuals only little engorged, while the fully engorged ones were dead (23.9%). One explanation for the large proportion of dead engorged ticks might be the wallowing behavior of wild boar (Keuling and Stier 2009a, Morelle et al. 2014), which could cause ticks to detach from the host or die through friction. Additionally,

the wallowing of wild boar might also be one reason for the significantly lower overall tick burden of wild boar in comparison to roe deer. Another explanation for the dead ticks on wild boar could by an immune response of wild boar to ticks (see Section 4.2.2.7).

4.2.2.3 Tick infestation intensity

On average, the infestation intensity on wild boar was low, with 0.13 ± 0.76 ticks per animal and an average prevalence of 6.1%, whereby the highest values were reached during April and May. Michalik et al. (2012) and Skotarczak and Adamska (2008) reported 7.8 and 2.3 ticks per infested animal, respectively. However, the prevalence of *I. ricinus* on wild boar in the latter study was almost equal (6%) to my study.

A study on a managed wildlife population in a Dutch national park and reported considerably higher intensities with a mean of 15 ± 9.4 ticks per wild boar (Pacilly et al. 2014). Although controlling game densities, in particular of deer, by wildlife management is considered as a method for risk reduction with respect to tick infestations (Piesman 2006), the densities of the overall deer populations in the national park (in 1998: 11.2 deer per ha) (Kuiters and Slim 2002) were higher than those observed for free-ranging big game in the Bienwald (in 2012: 6.0 roe deer per ha) (Ehrhart 2012). In general, higher host population densities increase the size of the tick population and, therefore, the number of infested hosts (Lindgren et al. 2000, Estrada-Peña 2001, LoGiudice et al. 2003, Brownstein et al. 2005, Ruiz-Fons et al. 2006, Gilbert 2010, Tagliapietra et al. 2011). This is probably one of the main reasons why the Dutch study showed a considerably higher tick burden on wild boar. In addition to this, the absence of roe deer within the national park (Pacilly et al. 2014) could have led to a substitution of roe deer by wild boar for some tick life history stages.

On the other hand, a mean prevalence of 31% and an average intensity of 13.6 ticks per wild boar reported by Ruiz-Fons et al. (2006) appear quite similar to the observations of Pacilly et al. (2014), but are also distinctly higher than those described here. However, in northern Spain the registered intensities were as low as 1.5 ticks per boar, although *I. ricinus* is widely distributed in this region. In comparison to the Bienwald, the higher infestation of wild boars in Spain could be a result of climate related factors, as observed for roe deer above.

4.2.2.4 Aggregation

The wild boar in the Dutch national park (Pacilly et al. 2014) also showed highly aggregated tick dispersion, with the highest levels being reached by larvae, followed by nymphs. However, in my study, nymphs had the highest levels of aggregation, probably because I found no larvae on wild boar. Similarly to roe deer in the Bienwald, all three tick life history stages appeared highly aggregated on wild boar. The values for k were even lower than those of roe deer. Since aggregation is directly related to mean tick intensity (see Section 3.3.1), one explanation of the increased aggregation of *I. ricinus* on wild boar is the lower mean tick abundance and prevalence in comparison to roe deer. Nevertheless, these observations underline the findings and conclusions discussed for roe deer, such that immature tick life history stages appear on both species similarly aggregated due to nymphal and larval behavioral patterns, in particular through their spatially clumped occurrence.

4.2.2.5 Seasonal and annual dynamics

All three life history stages showed an intensity peak in May, whereby nymphs showed a second less intense peak during September. These intensity peaks match those found for roe deer (Section 4.1.2). In contrast to this, the *I. ricinus* collected by de la Fuente et al. (2004) and by Ruiz-Fons et al. (2006) were all found during winter and mainly in autumn, respectively. The reason for this might by the high summer temperatures and low relative humidity in their study areas. This is underlined by the fact that no *I. ricinus* were collected from the southern parts of Spain (de la

Fuente et al. 2004, Ruiz-Fons et al. 2006) where the position of this species is taken by the newly described *Ixodes inopinatus* (Estrada-Peña et al. 2013).

4.2.2.6 Climatic factors

As already discussed for roe deer, one of the main reasons for the seasonal appearance of *I. ricinus* on game animals is climatically related (Estrada-Peña 2001, Estrada-Peña and Venzal 2007). In comparison to roe deer, only little is known about the influence of climate on the tick burden of wild boar. The correlation coefficients (Section 4.1.3) for tick intensity in relation to sunshine duration and precipitation were significantly positive for all tick life history stages on wild boar. In contrast to this, temperature influenced only nymphal burden positively at a highly significant level. Cloud coverage and relative humidity were significantly negatively correlated with males and nymphs, while females showed no significant correlation. During spring, a high saturation deficit was associated with a significant decrease in the number of males and nymphs.

The positive influence of temperature on the total tick abundance on wild boar was also confirmed by the GAMLSS model. Although the effect of precipitation did not reach a level of significance in the GAMLSS model, I was able to estimate a tick intensity peak for precipitations between 7 and 8 mm (Section 4.1.5). These results are in accordance with those from roe deer and could be explained in a similar fashion (cf. Section 4.2.1.5), while they also support the findings of other studies on tick questing behavior (Jensen 2000, Perret et al. 2000, 2004, Randolph et al. 2002, Hubálek et al. 2003, Schwarz et al. 2009). In summary, I was able to successfully apply the GAMLSS modeling approach for tick burden on wild boar for the first time to gain a better understanding of the climatic factors of tick infestation.

4.2.2.7 Host parameters

Studies on the influence of host parameters in relation to the tick burden on wild boar are also largely missing. My study is the first that comprehensively addresses

this point. Adult wild boar had the highest tick prevalence (7.2%), followed by yearlings (6.0%) and piglets (5.6%). However, *I. ricinus* females and males were most prevalent on yearlings and on piglets, respectively, while the prevalence of nymphs was highest on adult boar. Piglets were most intensely infested (0.16 ± 0.97), followed by yearlings (0.11 ± 0.52) and adults (0.10 ± 0.37). For all three life history stages, piglets showed the highest intensity of infestation.

The higher tick abundance of piglets could be explained by the lower proportion of dead ticks found on piglets in comparison to older animals. The higher mortality of ticks on yearlings and on adult wild boars could be a first indicator that these age groups show an efficient immune response, i.e. an acquired resistance, to ticks. As far as known, such a resistance not been reported for wild boar, but for other species, for example mice, guinea pigs and cattle (McTier et al. 1981, Jones and Nuttall 1990, Brossard and Wikel 2004). Another reason for the higher infestation of piglets could be that older wild boar have thicker skin which inhibits tick attachment.

A third reason for the higher infestation of piglets might be the differing behavioral patterns of wild boar in relation to age. Wild boar employ three different movement strategies: (1) staying and short distance travel, (2) long ranging and (3) any combination of the former two strategies (Morelle et al. 2014). Females with piglets usually pursue the first strategy. They remain within a limited area and move over short distances at various speeds. Keuling et al. (2010) also indicates that piglets have a smaller home range than older animals. As a result, the limited, but dynamic mobility together with the thinner skin of young boars (Briedermann 2009) could make them more attractive to ticks in accordance with the “exposure” hypothesis (see Section 4.2.1.6). However, none of the aforementioned differences between the groups reached a significant level. Additionally, no significant deviations between male and female boar were observed. In contrast to my findings on roe deer, tick burden on wild boar might not be sex-biased. The results for the host parameters above are in line with the correlation coefficients where body mass, which is usually related to

host age, and $GDMI_s$ were not significantly correlated with the tick intensities (Section 4.1.3).

4.2.3 Comparison of wild boar and roe deer

Wild boar are rapidly increasing in abundance in Central Europe (Keuling et al. 2008b, 2013, Léger et al. 2013), particularly in Germany (Schwarz et al. 2009, Deutscher Jagdverband 2014c), and they are considered to play an essential role in determining overall tick abundances, as well as in the transmission of tick-borne diseases (Léger et al. 2013, Pfäffle et al. 2013). However, the intensity and the prevalence of the tick burden on roe deer and wild boar were significantly different for all life history stages (all Mann-Whitney *U*-tests: $P < 0.001$). This observation matches that described by Skotarczak and Adamska (2008), where the differences in infestation levels between roe deer and wild boars were also significant. The study of Pacilly et al. (2014) also reported a lower tick infestation of wild boar in comparison to other large grazers.

In summary, my results clearly confirm that wild boar do not constitute an important food source for ticks in the Bienwald. Although, they could serve as propagating hosts for *I. ricinus* (Ostfeld et al. 2006, Ruiz-Fons et al. 2006), the low infestation intensities with many dead ticks suggest the contrary. Roe deer are clearly the dominant host for adult female ticks.

4.2.4 Attachment sites

For the order of niche breath on roe deer, I found female ticks showing the lowest attachment site specialization, differing significantly from those of the other 3 life history stages, whereas nymphs were most specialized in their feeding behavior. This observation confirms the findings made by Kiffner et al. (2011a). Moreover, both studies demonstrate that niche breadths vary considerable over month and year with wider niche breadth during the warmer months.

The results of my attachment site analysis for roe deer showed that adult ticks preferred the abdomen, while immatures were predominantly found at the ears (Section 4.1.7). These observations are in sharp contrast to Kiffner et al. (2011) who found that female and male ticks reached their highest densities on the neck and head (including ears), except during winter when they preferred the front legs. For immature ticks Kiffner et al. (2011a) reported the front legs and the head (including the ears) as clearly preferred attachment sites. In contrast to Kiffner's study, the legs never reached a top rank for any of the tick life history stages in my study. In addition, the abdomen and sternum seemed to play a less important role than in my study. However, similar to the findings of Kiffner et al. (2011a), the legs in the current study showed an increased tick density during the winter months (Nov/Dec).

Also in contrast to my ranking, with the highest proportions of nymphs and larvae on the ears, is a study from Italy which showed the forelegs of roe deer, screened in September, to be occupied by larvae which made up 90% of all ticks (Carpi et al. 2008). Nevertheless, Carpi et al. (2008) did not sample other parts of the roe deer body, so that a further comparison was not possible.

Handeland et al. (2013) reported high tick burdens from the ears of roe deer in Norway. Their results show that nymphs occurred most frequently on the ear, followed by larvae and adults. Other parts of the roe deer body were not screened by Handeland et al. (2013). Thus, it is unclear if the composition of the tick population on the ears is representative for the entire body, and the attachment site preferences on these animals is unknown. In this context, my model of the composition of the tick population on roe deer shows that a single sampling of the ears can lead to a biased estimate of the overall tick burden (see Section 4.1.6) (cf. Mysterud et al. 2014).

The dominance of nymphs and larvae on the ears of Norwegian roe deer (Handeland et al. 2013) in combination with my results indicates that the ears are a preferred attachment site for immature ticks. The observation that adults prefer the anterior

and posterior axillae, while immature ixodid ticks select the head and particularly the ears, is in line with the findings on red deer (Pacilly et al. 2014), white-tailed deer (Bloemer et al. 1988, Schmidtmann et al. 1998), angora goats (Fourie et al. 1991) and impala (*Aepyceros melampus*) (Matthee et al. 1997). Thereby, the impala study found that one third of the total ixodid tick burden was located on the ears. This finding is almost equal to proportions found here. Approximately the same distribution were detected by Mysterud et al. (2014) on roe deer in Norway, but with more larvae on the legs (40.9%), nymphs on the ears (83.7%) and adults in the groin (89.2%) and on the neck (94.9%).

One possible explanation for this attachment behavior can be found in the differences in mobility between the tick life history stages. On larger animals, questing ticks will usually move onto the host over the body parts that contact the vegetation. Consequently, ticks climbing onto the host are likely to be predominantly found on the head and ears during grazing on the vegetation, as well as on the legs. After encountering a host, the immature life stages of *I. ricinus* travel only a short distance and begin to feed mainly on the ears and the legs, whereas male and female ticks travel a greater distance on the host surface to reach the sternum and the abdomen (cf. Mysterud et al. 2014). In particular, for female ticks the arrival at a body region where they can take larger blood meals but is protected from abrasion is vitally important for egg production and survival (Sonenshine and Roe 2013b). In comparison to this, nymphs and larvae have less energy resources that they can spent on movements and require only smaller blood meals (Oliver 1989).

The height of the vegetation in relation to the size of the host is likely to play an important role for the successful attachment of questing ticks (Mejlon and Jaenson 1997). Additionally, as climatic factors have an influence on the questing height of *I. ricinus*, these might also have an effect on the location at which ticks contact the host (Randolph and Storey 1999). The interacting effects of vegetation and climate might provide additional information on the attachment site selection of ticks on roe deer, but this has so far not been studied. Although my study and that of Kiffner et al.

(2011a) were both conducted in forest areas, the aforementioned interactions in relation to the types of prevailing undergrowth could be used to explain the deviations in the results of both analyses. For this reason, future studies of tick attachment sites on roe deer would also benefit from an additional classification scheme for the roe deer body regions using the height above the ground to define different categories, similar to Ogden et al. (1998).

On wild boar, the life history stages found preferred the abdomen and the sternum over the ears, except during spring when ticks occurred aggregated on the ears (Section 4.1.7). No ticks were found at the head, neck, front legs and hind legs, or on the main body of these hosts. Furthermore, males and nymphs were not collected from the sternum. In comparison to roe deer, a clear trend indicating that nymphs selected the ears over the abdomen could not be determined for wild boar. Until recently, the only other study on this host (Pacilly et al. 2014) also described adults mostly attached to the sternum and abdomen, while nymphs were found predominantly at the ear, whereas larvae were not collected. Despite some seasonal deviations, the attachment sites registered by Pacilly et al. (2014) are similar to my study. Both studies also show that no nymphs or male ticks were attached to the host's body, but were loose and unengorged. A certain number of ticks collected by Pacilly et al. (2014) had already detached from the host and dropped into water filled trays below the carcasses at the time of sample acquisition, such that a recording of attachment sites was not possible for these ticks. However, the site preferences presented by Pacilly et al. (2014) for wild boar resemble the ones in my study. Therefore, I can confirm that partially delayed sampling and the tray-based collection of ticks did not influence their results with respect to attachment sites.

My results for roe deer and wild boar show seasonal covariation for male and female ticks, such that the two top ranked body parts were similarly attractive for both sexes. This behavior is also in line with the fact the mating ticks were predominantly recorded on the abdomen and sternum (Section 4.1.7). In spite of some seasonal variations in the ranking of the tick densities, these results suggest that ticks actively

choose the body part they feed on. This is supported by the observations made with respect to the IFDH. For each tick life history stage, the total number of individuals found on the entire host correlated positively at a highly significant level with the percentage of ticks attached to the top ranked body part. This behavior stands in clear contradiction to the IFDH, which states that a higher tick life history stage abundance would increase the proportion of ticks at less preferred attachment sites (Kiffner et al. 2011a).

In summary, the above findings support the hypothesis of pheromone-based on-host aggregation of *I. ricinus*, which leads to a mutual attraction of conspecifics on a host (Oliver 1989, Grenacher et al. 2001, Sonenshine 2006, Healy and Bourke 2008, Sonenshine and Roe 2013b). On wild boar, such observations were not possible due to the limited number of ticks found. However, for the two boars on which more than 5 ticks were found, the infestations were completely aggregated at a single spot (once on the ear and once on the abdomen). This could be a first indicator that pheromones could lead to on-host aggregation on wild boar. Nevertheless, for roe deer and wild boar a multitude of other factors can be considered to influence the spatial aggregation of ticks on the hosts. These factors encompass features of fur and skin as a barrier for unfed *I. ricinus* ticks in combination with the amount of blood circulation at different attachment sites (cf. Ogden et al. 1998, Kiffner et al. 2011a). The collection of additional information on these interactions should be considered in future studies.

4.2.5 Co-feeding

The study on roe deer by Kiffner et al. (2011a) suggests that gregarious feeding might be beneficial for *I. ricinus* ticks causing an increased blood feeding rate and faster repletion. This statement is supported by my findings on roe deer, which show that interstadial attraction (low niche and active feeding site selection) occurred for all feeding life history stages (i.e. females, nymphs and larvae). The high niche overlaps between females, nymphs and larvae underline the gregariousness of *I. ricinus* (Section 4.1.7 to 4.1.9). In addition to the densities and niche indices calculated,

highly clumped occurrences of nymphs and larvae on the ears and abdomen were found during the sample acquisition. Very closely aggregated (< 1 cm) ticks on roe deer have been reported by other authors (Carpi et al. 2008, Vor et al. 2010, Handeland et al. 2013). The on-host aggregation of the feeding ticks might support non-systemic pathogen transmission between the feeding life history stages through co-feeding (Randolph et al. 1996), which is considered as an important factor in the epidemiology of tick-borne diseases (Randolph 2009).

Intra- and interstadial aggregation also seems be coupled with seasonal tick activity, induced mainly by climatic variations, such that niche overlap correlated positively with warmer months as pointed out by the GLM (see Section 4.1.8). A seasonal synchronicity with respect to co-feeding was reported Kiffner et al. (2011a). Carpi et al. (2008) modeled the significant influences of geographic location and autumnal cooling rate on the frequency of co-feeding in *I. ricinus*. For ticks on forest rodents, the presence of co-feeding was positively associated with the spring warming rate (Kiffner et al. 2011c). Nevertheless, it is still not clear whether non-systemic pathogen transmission on roe deer via co-feeding is possible. Therefore, co-feeding transmission experiments should be conducted (cf. Kiffner et al. 2011a).

Another recent study (Mysterud et al. 2014) on the feeding sites of *I. ricinus* on roe deer proposed a partial separation of the tick life history stages, which was also observed for some sampling periods in the current study (Section 4.1.9). This might limit the amount of co-feeding and the possibility of pathogen transmission. On the other hand, the highest spatial niche overlap in the entire study period was observed between male and female ticks. This result can be explained by the mating behavior of male ticks, which has been mainly observed at the abdomen and sternum. As males do not feed, co-feeding is not possible between the sexes.

Due to the limited sample size, it was not possible to calculate niche indices for wild boar. However, the aforementioned on-host aggregation on two boars in combina-

tion with the fact that ticks were only found at 3 of 8 possible attachment sites suggest that clumped feeding on wild boar is possible and can lead to co-feeding. Although, wild boar are considered to be important hosts in the ecology and dynamics of tick-borne pathogens through vectoring ticks (Juricová and Hubálek 2009), the low prevalences and intensities of infection found here make this doubtful for Germany.

5

Pathogen prevalence

The following chapter will present the pathogens found within the organ samples and the ticks collected from roe deer and wild boar.

5.1 Results

5.1.1 Pathogens in organ samples

In total, organ parts from 247 roe deer and 344 wild boar were collected during the study period. With respect to *Rickettsia* and *Borrelia* infections all samples have been processed in the lab. Only a single pool (P1 of sample 90) showed a weak positive result for *Rickettsia* spp. during the gltA-PCR on the LightCycler® (see Section 3.2.6.1) in comparison to the positive control (PC). The positive pool contained skin, i.e. the ear, of a male wild boar piglet (Figure 5.1) culled in January 2013. The body mass of the animal was 16 kg and the $GDMI_S$ was -0.38. The latter value indicates that the boar's body mass was distinctly below that of average male piglets and suggests that the individual was in poor condition, whereby no ticks were found on this animal at the time of the sample acquisition. However, for the pool testing positively on the LightCycler® (P1 of Sample 90) there was no indication of *Rickettsia* spp. during the two subsequent PCRs on the thermocycler (see Section 3.2.6). This underlines that the LC-PCR can generate false positives and that additional PCRs are necessary to verify positive results.

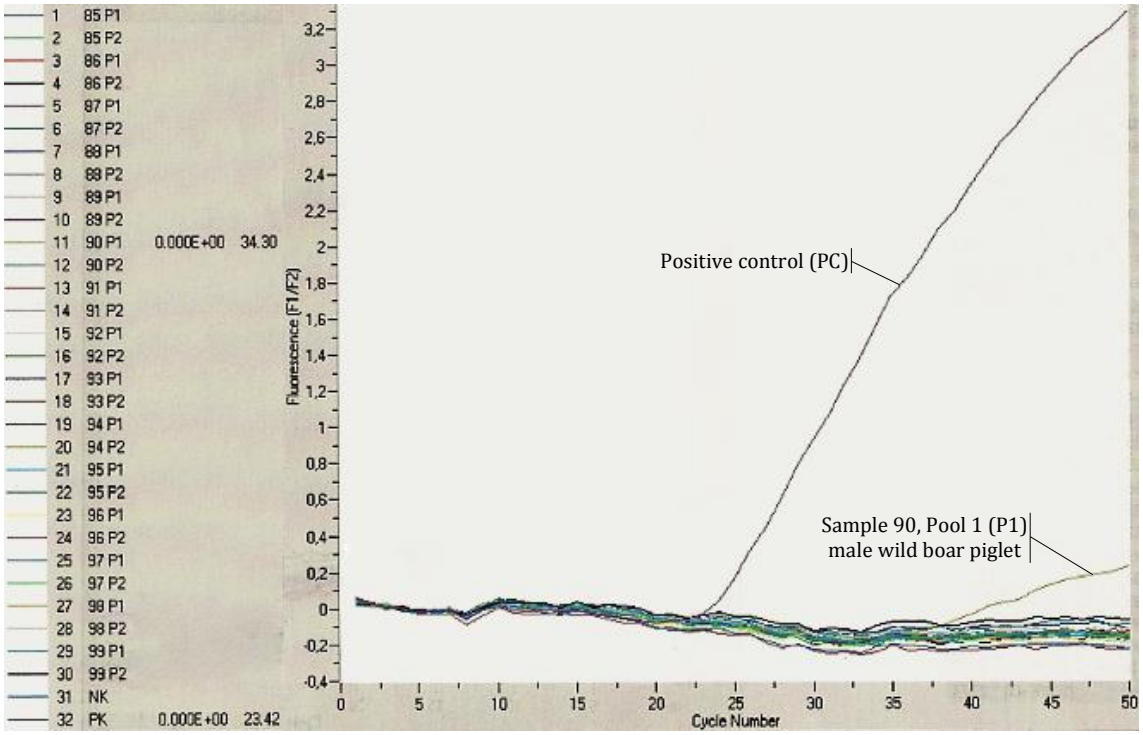


Figure 5.1: Detection of *Rickettsia* spp. in organs of wild boar by real-time gltA-PCR using the LightCycler® with 30 samples (left) with negative control (NC, i.e. NK) and positive control (PC, i.e. PK). The graph (right) plots the number of cycles against the fluorescence (F1/F2). Both positive samples are labeled accordingly: PK and a single positive pool of a wild boar.

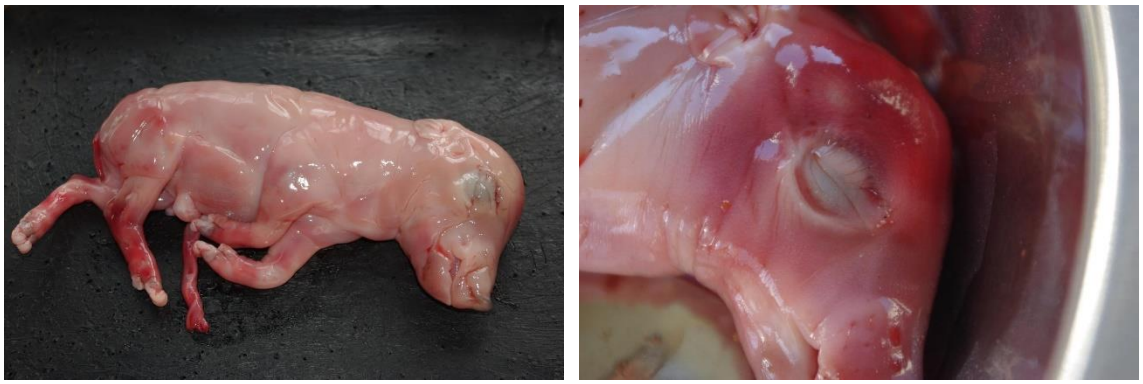


Figure 5.2: Wild boar fetus with the first hair over the eyes and still closed eyelids (right).

No other organ samples from wild boar, nor any of the roe deer organs were positive for *B. burgdorferi* s.l. species or *Rickettsia* spp. In addition to this, 15 wild boar embryos were collected (see Figure 5.2). The fetuses had a crown-rump length ranging from 9.5 to 18.3 cm and a body mass between 45 and 350 g. They were dissected, their organs were pooled equivalently to all other samples and investigated by gltA-

Table 5.1: Total number of ticks analyzed for *Rickettsia* and *Borrelia* infections along with the resulting counts of positives and the corresponding prevalences.

Host species / tick sex	Analyzed ticks	Unfed ticks		Positive ticks					
				Rickettsia spp.		*Borrelia* spp.[b]		Coinfection[b,c]	
Ticks from roe deer	**264**	**132**	**50.0%**	**124**	**47.0%**	**9**	**3.4%**	**4**	**1.5%**
male ticks	48	48	100%	26	54.2%	3	6.3%	2	4.2%
female ticks	216	84	38.8%	98	45.4%	6	2.8%	2	0.9%
Ticks from wild boar[a]	**12**	**6**	**50.0%**	**5**	**41.7%**	**0**	**0.0%**	**0**	**0.0%**

[a] all ticks were female, [b] all ticks were unfed, [c] concurrent infections with *Borrelia* and *Rickettsia* spp.

PCR for *Rickettsia* and *Borrelia* infections on the LightCycler®. None of the embryo organ pools tested positively.

5.1.2 Pathogens in ticks from roe deer

For the determination of *Rickettsia* and *Borrelia* infections, 256 ticks (16.7%, 47 males and 217 females) from 41 roe deer and 12 adult female ticks (26.1%) from 8 wild boar were analyzed.

5.1.2.1 Rickettsia species

The mean prevalence of *Rickettsia* from all analyzed ticks from roe deer was 47.0% (see Table 5.1), whereby male ticks showed slightly more infections (54.2%) than females (45.4%). However, none of the male ticks were attached on roe deer. In addition, *Rickettsia* were almost equally prevalent in ticks from fawns (47.4%), yearlings (46.2%) and adult deer (47.7%). For ticks from female deer, the *Rickettsia* prevalence (53.6%) was distinctly higher than for those from male deer (42.2%). However, Fisher's exact test revealed that the differences between the two groups approached the level of significance, but did not quite achieve it ($P = 0.080$). With respect to the ticks' state of engorgement the following prevalences of *Rickettsia* were observed: loose and unengorged (68.3%), attached and unengorged (46.0%), little engorged (36.7%), medium engorged (34.6%) and fully engorged (29.0%). The chi-squared analysis showed a high significance ($\chi^2_4 = 24.24$, $P < 0.001$) with respect

Table 5.2: Number of ticks per state of engorgement in relation to the ticks tested positively for *Rickettsia* spp. and *B. burgdorferi* s.l. species together with the corresponding prevalences.

	Ticks	*Rickettsia* spp.		*Borrelia* spp.		Coinfections[a]	
Ticks from roe deer	**264**	**124**	**47.0%**	**9**	**3.4%**	**4**	**1.5%**
loose, unengorged	82	56	68.3%	9	11.0%	4	4.9%
attached, unengorged	50	23	46.0%	0	0.0%	0	0.0%
attached, little engorged	49	18	36.7%	0	0.0%	0	0.0%
attached, medium engorged	52	18	34.6%	0	0.0%	0	0.0%
attached, fully engorged	31	9	29.0%	0	0.0%	0	0.0%
Ticks from wild boar	**12**	**5**	**41.7%**	**0**	**0.0%**	**0**	**0.0%**
attached, unengorged	6	4	66.7%	0	0.0%	0	0.0%
attached, medium engorged	6	1	16.7%	0	0.0%	0	0.0%

[a] concurrent infections with *Borrelia* and *Rickettsia* spp.

to the engorgement states, with a decrease of the *Rickettsia* prevalence with increasing engorgement (Table 5.2).

The most frequent *Rickettsia* infections were recorded for ticks from the hind legs (60.0%), followed by the sternum (49.3%) and abdomen (46.0%), while the lowest occurrence was detected for the ears (38.1%). When looking at the seasonal changes in *Rickettsia* prevalence, the highest values were registered in Sep/Oct when all analyzed ticks had *Rickettsia* (100.0%), whereas during all other period the prevalences were lower: Jan/Feb (29.4%), Mar/Apr (45.0%), May/Jun (41.7%) and Nov/Dec (60.7%). In Jul/Aug no ticks were analyzed. There was a highly significant difference between the seasons ($\chi^2_4 = 17.22$, $P = 0.002$) with a subsequent post-hoc test using pairwise comparisons with Bonferroni corrections of the P-values revealing that the periods Jan/Feb and May/Jun had a significantly lower ($P < 0.05$) *Rickettsia* prevalence than Sep/Oct. (Table 5.3).

Table 5.3: Number of ticks from roe deer and wild boar for each sampling period in relation to the number of infected individuals and their prevalences of *Rickettsia* spp. and *B. burgdorferi* s.l.

	Ticks	*Rickettsia* spp.		*Borrelia* spp.		Coinfections[a]	
Ticks from roe deer	**264**	**124**	**47.0%**	**9**	**3.4%**	**4**	**1.5%**
Jan/Feb	17	5	29.4%	0	0.0%	0	0.0%
Mar/Apr	20	9	45.0%	1	5.0%	1	5.0%
May/Jun	163	68	41.7%	6	3.6%	1	0.6%
Sep/Oct	8	8	100.0%	0	0.0%	0	0.0%
Nov/Dec	56	34	60.7%	2	3.6%	2	3.6%
Ticks from wild boar	**12**	**5**	**41.7%**	**0**	**0.0%**	**0**	**0.0%**
Nov/Dec	12	5	41.7%	0	0.0%	0	0.0%

[a] concurrent infections with *Borrelia* and *Rickettsia* spp.

5.1.2.2 B. burgdorferi s.l. species

In contrast to *Rickettsia*, the overall *B. burgdorferi* s.l. prevalence was distinctly lower in ticks from roe deer at 3.4%. *B. burgdorferi* s.l. were detected more frequently in male ticks (6.3%) than in females ones (2.8%) (Table 5.1). Furthermore, ticks from male deer had a lower prevalence of *Borrelia* (2.6%) than those from female deer (4.6%), whereby the pathogen was more prevalent in ticks from yearlings (5.1%) than in those from fawns (2.6%) and adult roe deer (1.8%).

A highly significant difference ($\chi^2_4 = 20.68$, $P < 0.001$) was determined between the engorgement states of the ticks: unengorged ticks had a *Borrelia* prevalence of 11.0%, whereas fed ticks were never infected (0.0%) independent of their level of engorgement. When considering only unfed ticks 17.6% of the females and 6.3% of the males were infected. Fisher's exact test showed that this difference was not significant ($P = 0.103$). In addition to this, no significant deviations between the body parts (ears: 4.8%, sternum: 4.2%, hind legs: 4.0%, abdomen: 2.4%) and the sampling seasons (Jan/Feb: 0.0%, Mar/Apr: 5.0%, May/Jun: 3.7%, Sep/Oct: 0.0%, Nov/Dec: 3.6%) were observed (Table 5.3).

5.1.2.3 Coinfections

The prevalence of concurrent infections of ticks from roe deer with *Rickettsia* and *Borrelia* was low (1.5%), whereby coinfections were only registered for loose unengorged ticks (4.9%). The deviation between attached and loose ticks approached significance with $\chi^2_4 = 9.0$, df = 4, P = 0.06. In addition to this, male ticks had more coinfections (4.2%) than female ticks (0.9%), although Fisher's exact test showed with that this difference was not significant (P = 0.15). Moreover, ticks from fawns were more frequently infected (2.6%) than those from adults (1.8%) and yearlings (0.9%). In accordance with the individual observations made for *Rickettsia* and *Borrelia*, male deer had less ticks with concurrent infections (0.7%) than female deer (2.7%), although significance has not been verified by Fisher's exact test (P = 0.31). With respect to the body parts, coinfections occurred in the following order: hind legs (4.0%), abdomen (1.6%) and sternum (1.4%). Moreover, for the sampling periods the following distribution of coinfections was found: Jan/Feb (0.0%), Mar/Apr (5.0%), May/Jun (0.6%), Sep/Oct (0.0%) and Nov/Dec (3.6%). In addition, there was no evidence found that *Borrelia* infections were significantly related to *Rickettsia* infections (Fisher's exact test: P = 1.0).

5.1.3 Pathogens in ticks from wild boar

5.1.3.1 Rickettsia species

The adult female ticks from wild boar had a *Rickettsia* prevalence of 41.7%, whereby no tick from female wild boar had *Rickettsia* (0.0%) and those from male boar showed a prevalence of 50.0%. With respect to the age of the wild boar, *Rickettsia* was most prevalent in ticks from piglets (44.4%), followed by those from adults and young wild boar (both 33.3%). Attached, unengorged ticks had a slightly higher prevalence (66.7%) than attached and medium engorged ones (16.7%). Although this difference was not significant by Fisher's exact test (P = 0.242), the decreasing *Rickettsia* prevalence with a higher state of engorgement could suggest a behavior similar to that of ticks from roe deer. Furthermore, *Rickettsia* were more frequent in ticks from the sternum (66.7%) than in those from ears and abdomen (both 33.3%).

The detection of prevalence changes with respect the sampling periods was not possible, since all analyzed ticks from wild boar were collected during November and December (compare with Table 5.3).

5.1.3.2 B. burgdorferi s.l. species

In contrast to *Rickettsia*, and contrary to the observations made for roe deer, neither fed nor unfed ticks from wild boar tested positive for *B. burgdorferi* s.l. For this reason, further descriptive and explorative statistical evaluation of *B. burgdorferi* s.l. in ticks from wild boar is omitted.

5.1.4 *Rickettsia* spp. sequencing

The *ompB*-PCR showed 24 positives out of 83 randomly selected samples (all from roe deer, see Section 3.2.6.4), whereby the sequencing determined *R. helvetica* in 9 ticks (Table 5.4). *R. helvetica* was the only species detected. From the ticks identified as having *R. helvetica*, 8 were adult females and 1 was a male, 8 were from roe deer yearlings and 1 was from an adult deer, 3 ticks were from male hosts and 6 were from a single female deer. All ticks with *R. helvetica* were found on the abdomen or the sternum. The hosts of the ticks having *R. helvetica* carried more than 20 ticks, expect for one male yearling with only 6 *I. ricinus*. Ticks infected with *R. helvetica* were attached and medium to fully engorged, except for a single female *I. ricinus* that was loose and unengorged. The eluates of 15 positively tested ticks could not be sequenced up to the species level.

Table 5.4: Tick samples positively tested for *Rickettsia* spp. with results of the DNA-sequencing in relation to *B. burgdorferi* s.l. infections (Borr.), host animal and tick parameters.

Host				Tick			Borr.	*Rickettsia*		
Species	**Age**	**Sex**	**No. Ticks**	**Life Stage**	**Attach. Site**	**State of Engorg.**	***ospA***	***gltA***	***ompB***	**Seq.**
roe deer	A	m	41	f	abd	4	-	+	(+)	NS
roe deer	A	m	24	f	abd	3	-	+	+	*R. helvetica*
roe deer	Y	m	22	f	abd	1	-	+	+	NE
roe deer	A	m	14	f	ste	1	-	+	+	NE
roe deer	Y	m	15	f	abd	2	-	+	(+)	NE
roe deer	Y	m	54	f	ste	3	-	+	(+)	NS
roe deer	Y	M	6	f	abd	4	-	+	+	*R. helvetica*
roe deer	Y	F	5	f	ste	0	-	+	+	NE
roe deer	Y	M	54	m	abd	0	-	+	+	*R. helvetica*
				m	abd	0	-	+	+	NE
roe deer	Y	F	27	f	ste	3	-	+	+	NE
				f	ear	3	-	+	(+)	NS
roe deer	Y	F	22	f	abd	3	-	+	(+)	NS
				f	abd	3	-	+	+	NE
				f	abd	0	-	+	(+)	NS
				f	abd	0	-	+	+	NE
roe deer	y	F	30	f	abd	3	-	+	+	*R. helvetica*
				f	abd	3	-	+	+	*R. helvetica*
				f	abd	3	-	+	(+)	NS
				f	abd	0	-	(+)	+	NS
				f	abd	3	-	+	+	*R. helvetica*
				f	abd	0	-	+	+	*R. helvetica*
				f	ste	4	-	+	+	*R. helvetica*
				f	ste	4	-	+	+	*R. helvetica*

a = adult; y = yearling; f = female; m = male; abd = abdomen; ste = sternum; ear = ears; + = positive; (+) = slightly positive; - = negative; NS = not sequenceable; NE = not evaluable

5.1.5 Host-tick-pathogen relationships

The following analyses will be restricted to exploring the interaction between ticks, roe deer and *Rickettsia* because of the limited tick sample size (n = 12) for wild boar and the few samples tested positively for *Borrelia* in relation to both host species.

Table 5.5: Pearson's correlation coefficients in relation to *Rickettsia* infections, level of engorgement, number of ticks found on the entire host body (roe deer), tick density at the preferred attachment site, host body mass and mass index.

	Rickettsia	**Tick count**	**Tick density**	**Body mass**	**$GDMI_s$**
Rickettsia	1.000	-0.093	-0.145*	0.091	0.083
Engorgement	-0.282**	-0.164**	-0.175**	0.067*	0.056*

Significances: * P < 0.05; ** P < 0.01

5.1.5.1 Correlation analysis

The correlation coefficients in Table 5.5 were calculated to investigate the occurrence of *Rickettsia* with respect to tick and roe deer parameters. Infections with *Rickettsia* were significantly negatively correlated with the tick density at the attachment site and had a highly significant negative linear relationship with tick engorgement. Consequently, a higher state of engorgement leads to a lower prevalence of *Rickettsia*. The state of engorgement depends significantly on the total number of ticks and on the relative tick density at the preferred attachment site in a negative way. In contrast to this, the effect of host body mass on the state of engorgement is significantly positive but an order of magnitude smaller than the influences of the tick burden.

5.1.5.2 Factor interdependency analysis

A subsequent factor analysis of the parameters revealed the interdependencies between roe deer, tick and pathogen parameters (Table 5.6). The first extracted factor represents 24.1% of the variance of the studied dataset and can be interpreted as the state of engorgement. Moreover, the positive loading of tick sex on the first factor underlines that only female ticks engorge (sex = 2), while male ticks (sex =1) were all loose and unengorged. The composition of the second component reveals that it has almost no effect on the *Rickettsia* prevalence and on the state of engorgement. This component reflects 19.9% of the dataset variance and demonstrates that a higher tick burden can be expected on male roe deer with increasing age and decreasing $GDMI_s$. Similar to the second component, the third and the fourth factors

Table 5.6: Rotated component matrix generated using optimal scaling followed by factor analysis with respect to *Rickettsia* spp. infections in relation to tick and roe deer host parameters.

Variables	Component				
(quantified by CATPCA)	1	2	3	4	5
Rickettsia	-0.118	-0.070	0.039	0.040	0.965
Engorgement	0.893	0.022	-0.024	0.073	-0.243
Tick sex[a]	0.939	0.022	0.022	-0.013	0.073
Tick count	0.007	0.723	0.416	0.062	-0.101
Tick density	0.069	0.806	0.022	-0.291	0.038
Host sex[a]	0.051	-0.662	0.061	-0.570	0.146
Host age[b]	-0.009	0.196	0.902	-0.102	-0.074
Host body mass	0.009	0.006	0.763	0.348	0.173
Host GDMIs	0.071	-0.136	0.146	0.886	0.074
% of Variance	24.1	19.9	16.5	12.1	9.5
Cumulative%	24.1	43.9	60.4	72.5	82.0

[a] sex encoding: male = 1, female = 2; [b] age encoding: fawn = 1, yearling = 2, adult = 3

show no large influence on the level of engorgement and on the number of infections. The third component represents older animals with higher body mass and $GDMI_S$ that have an increased numbers of ticks, while the fourth factor can be interpreted as "healthier" male roe deer. The latter factor is mainly identified by the high loading of the $GDMI_S$, which leads in combination with a higher body mass and decreasing age to a lower relative tick density at the preferred feeding sites.

Together with the first two components, the third and the fourth factor accumulate to reflect more than 70% of the variance in the studied data. Finally, the fifth extracted factor shows clearly that a lower states of engorgement leads to a higher *Rickettsia* prevalence, whereby this behavior stands in minor relation to the total number of ticks on the host, the host age and the host body mass. In this context, infections are more likely on male deer and are influenced negatively by the total number of ticks and positively by the body mass. In summary, the 5 factors represent 82.0% of dataset variance, while the analysis demonstrates that the ticks' state of engorgement, together with the sex of the ticks, are the most important parameters that influence the *Rickettsia* prevalence in ticks from roe deer. Furthermore, the

factor analysis has determined that tick burden (component 2), as well as host age and physical conditions (component 3 and 4), have only minor influences on the occurrence of *Rickettsia* infections.

5.1.5.3 Ranking of influences

The observations made above were confirmed by the decision tree analysis presented in Figure 5.3. Tick engorgement has been determined as the most important parameter splitting the initial dataset with respect to *Rickettsia* infections. The prevalence of *Rickettsia* in loose ticks is almost 30% higher than in attached ticks. On the next level of the tree, loose, unengorged ticks are divided by sex, revealing that females are significantly more likely to be infected than males. Furthermore, loose male ticks had considerably more *Rickettsia* infections from July to December than during the months January to June. For the first half of the year, the sex of roe deer is identified by the decision tree as splitting the group of loose, unengorged ticks even further, such that female deer carried not a single tick having *Rickettsia*, while for male roe deer the tick infection prevalence was at 52.4%. During the second half of the year the CHAID algorithm uses the $GDMI_S$ of the hosts to split loose male ticks into two groups. Males from roe deer with a $GDMI_S \leq 0.163$ had a *Rickettsia* prevalence of 100%, while those ticks from animals with a higher index had no *Rickettsia* at all. Similar to the correlation analysis and the factor extraction performed above, the decision tree determined the state of engorgement and physical condition of the hosts as important aspects for *Rickettsia* occurrence. Nevertheless, additional parameters, i.e. tick sex, sampling period and host sex, were incorporated to identify and rank significantly differing groups within the dataset.

5.1.5.4 Logistic regression model

The resulting model, estimated by the logistic regression, underlines the significance of the previously made observations, underlines their significance and reveals more details on how the parameters interact with the *Rickettsia* infection rate. The regression coefficients are displayed in Table 5.7 and have been validated on the

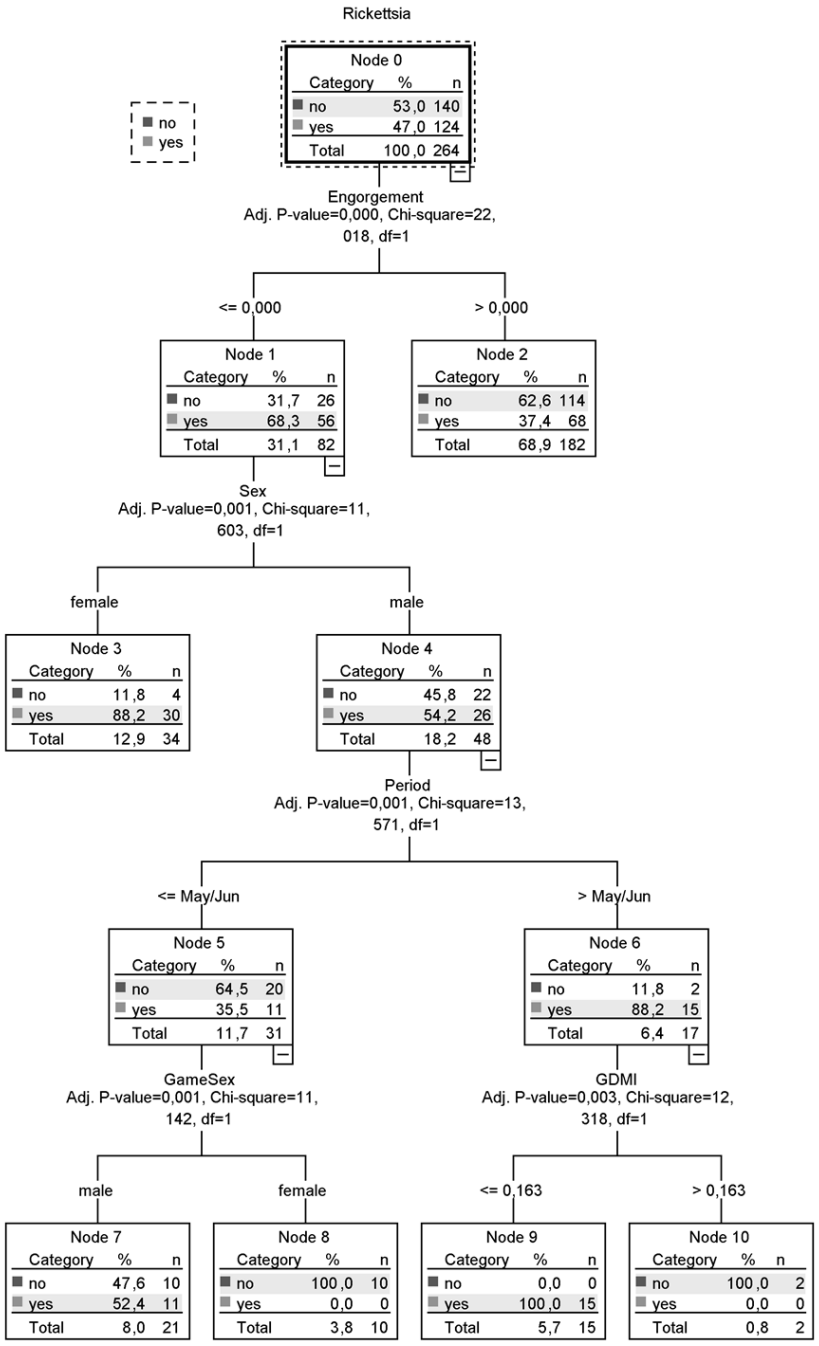


Figure 5.3: Decision tree identifying the most important parameters hat influence *Rickettsia* prevalence of ticks from roe deer.

original dataset, showing that the model was able to classify 66.3% of the cases correctly. For an increase in the host body mass the model predicts a significant rise in the number of the *Rickettsia* infections. There is a significant decrease in *Rickettsia*

Table 5.7: Model coefficients and significances resulting from the logistic regression with respect to *Rickettsia* infections in ticks from roe deer.

	Exp(B)	Sig.		Exp(B)	Sig.
(Intercept)	0.001	0.007	**Jan/Feb (Ref.)**		0.010
Body mass	1.563	0.004	**Mar/Apr**	2.265	0.294
Loose unengorged (Ref.)		0.000	**May/Jun**	0.705	0.607
Attached unengorged	0.467	0.053	**Sep/Oct**	$2.8 \cdot 10^{9}$	0.999
Attached little engorged	0.251	0.002	**Nov/Dec**	6.198	0.013
Attached medium engorged	0.231	0.000	**Fawn**	1.681	0.535
Attached fully engorged	0.223	0.002	**Yearling**	6.398	0.001
			Adult (Ref.)		0.003

infections with increasing levels of engorgement determined in relation to the reference state, i.e. loose and unengorged.

Moreover, the model demonstrates that the chance of a *Rickettsia* infection for ticks is significantly higher by a factor of 6.2 in Nov/Dec than during the reference period Jan/Feb. The extreme coefficient and the significance near 1 for the period Sep/Oct is caused by the fact the *Rickettsia* prevalence during these months was 100.0%. Finally, the logistic regression verified that ticks from yearlings have a significantly higher chance of being infected by *Rickettsia* than those from adult roe deer. Although the coefficient for fawns has not been determined as significant, its magnitude suggests that animals younger than 1 year could carry more infected ticks than adult hosts.

5.2 Discussion

5.2.1 *B. burgdorferi* s.l. infections

5.2.1.1 Roe deer

In the present study, no *B. burgdorferi* s.l. species were detected in any of the organ samples collected from roe deer, although *B. burgdorferi* s.l. was found in ticks collected from hedgehogs in the Bienwald (Skuballa 2011). A reservoir competence to *B. burgdorferi* has been shown for several small mammalian, bird and lizard species (Ostfeld et al. 2006, Gern 2008, Skuballa et al. 2012). Conversely, my findings confirm that roe deer have a reservoir incompetence for *B. burgdorferi* s.l. (Telford et al. 1988, Gill et al. 1993, Tälleklint and Jaenson 1997), such that none of the individuals was infected although they were infested by numerous *I. ricinus* ticks (Matuschka et al. 1993). Although Skotarczak and Adamska, (2008) report the presence of *B. garinii* in 2 out of 238 roe deer in western Poland, they assume that roe deer are not important in the transmission cycle of *B. burgdorferi* s.l.

Kurtenbach et al. (1998) demonstrated that the complement system of deer is involved in killing three human pathogenic strains of *B. burgdorferi* s.l. (i.e. *B. burgdorferi s.s.*, *B. garinii* and *B. afzelii*). An analysis of 12 *Borrelia* species showed that the borreliacidal activity of roe deer, red deer and fallow deer (*Dama dama*) sera was also observed regardless of the genospecies (Bhide et al. 2005). Complement-mediated killing of *B. burgdorferi* s.l. was also observed by Nelson et al . (2000) for sika deer (*Cervus nippon yesoensis*) in the presence or absence of antibodies (Isogai et al. 1991). However, for sika deer local infections of skin parts with co-feeding infected ticks have been found, although no generalized infection in the deer was present (Kimura et al. 1995). Consequently, the transmission of *B. burgdorferi* s.l. between ticks through co-feeding on locally infected skin might also be possible for uninfected roe deer, although they are known to be reservoir-incompetent.

The average *B. burgdorferi* s.l. infection rate of *I. ricinus* ticks (including engorged and unengorged ticks) from roe deer was 3.4% (Section 5.1.2.2). A distinctly lower

infection prevalence in ticks from roe deer was detected in a study from Norway (Kjelland et al. 2011) ranging from 0% for larval and adult ticks up to 2.9% for nymphs. In contrast, Rijpkema et al. (1996) reported considerably higher infection rates for *I. ricinus* from roe deer of up to 26% in three Dutch provinces. On red deer in the Netherlands the infection rate of unengorged male *I. ricinus* was 4.5% (Pacilly et al. 2014). In the United States (Maine), Lacombe et al. (1993) found that 13% of adult *Ixodes dammini* from white-tailed deer were infected with females having a higher prevalence of *B. burgdorferi* s.l. than males. This observation is in contrast to my findings with a higher prevalence of *B. burgdorferi* s.l. in male ticks (6.3%) than in female ticks (2.8%), although this difference was not significant (Section 5.1.2.2). A male tick-biased infection prevalence is also supported by the study of (Rijpkema et al. 1997). However, the *B. burgdorferi* s.l. prevalences found in the other studies differ from those I determined in the Bienwald. The high variation of the infection rates is an indicator to a multitude of factors influencing ticks, hosts and pathogen transmission (Kirstein et al. 1997, Halos et al. 2010).

In the present study, the state of engorgement was significantly related to the likelihood of a *Borrelia* infection, such that none of the fed females were infected by *B. burgdorferi* s.l. species and unfed ticks had an infection rate of 11.0% (unfed females: 17.7%). This observation underlines the findings of other studies that showed that the prevalence of *B. burgdorferi* s.l. in questing ticks from the vegetation or in unfed ones collected from wild cervids is significantly higher than in engorged individuals, such that only few ticks retain *Borrelia* spirochetes after feeding (Lacombe et al. 1993, Matuschka et al. 1993, Gray et al. 1999, Skotarczak and Adamska 2008, Rosef et al. 2009, Kjelland et al. 2011, Pacilly et al. 2014). The reason for this behavior is that the borreliacidal effect of deer sera is not only active in the host itself, but also in the ticks thorough the ingestion of host blood (Lacombe et al. 1993, Kurtenbach et al. 1998b). *B. burgdorferi* s.l. spirochetes that are sensitive to destruction by the alternative pathway of the complement system are lysed in the midgut of the feeding tick, although spirochetes in the salivary glands prior to the uptake of blood may be injected into the host and escape from killing (Kurtenbach et al. 2002, 2006). As a

consequence, increasing population densities of deer, which are the preferred hosts of adult female *I. ricinus*, led to a reduction in the number of infected ticks (Gray et al. 1999, Rosef et al. 2009, Brunnemann 2010). Thus, the apparent relationship between tick engorgement and the prevalence *B. burgdorferi* s.l. in the present study underlines the reservoir incompetence of roe deer (cf. Skotarczak and Adamska 2008, Kjelland et al. 2011).

This diluting effect can also decrease the infection rate in future tick generations, since it reduces the number of infected females and thus inhibits the transovarial transmission of *B. burgdorferi* s.l. spirochetes from adults to larvae of the *I. ricinus* complex (Magnarelli et al. 1987). The fact that a large proportion of ticks feeding on roe deer are adult females using deer as their main blood source for egg-production (Wilson et al. 1984, Tälleklint and Jaenson 1997, Pichon et al. 1999, Skotarczak and Adamska 2008, Kiffner et al. 2010a) might intensify the transovarial dilution even further, although only about 14% of infected females transfer the infection to their eggs (Bellet-Edimo et al. 2005). In addition, the borreliacidal effect of deer serum on feeding *I. ricinus* could reduce the number of infected small rodents and thus limit zoonotic transmission in the ecosystem, such that the timing of rodent-tick-deer interactions becomes a critical factor for the transmission of *B. burgdorferi* s.l. (cf. Pacilly et al. 2014). Current investigations in the Bienwald show that rodent population densities are at a low level (Schaeffer et al., unpublished data). This might also be an additional factor which keeps the *Borrelia* prevalence in this area at a relatively low level.

Other factors influencing the *Borrelia* infection rate could be seasonal changes in tick abundance, host age and attachment site. I registered a higher number of infections in April and May (both 5%). Other months had distinctly lower prevalences. This finding corresponds to the typical spring peak of tick activity (Gray 1991, Randolph et al. 2002), which was also found in my study (see Section 4.1.1). Therefore, tick abundance might be synchronized with *B. burgdorferi* s.l. prevalence, as has been observed for the tick-borne encephalitis (TBE) virus (Randolph et al. 2000). This in

turn could mean that an increased tick density on roe deer with higher chances of co-feeding leads to more *Borrelia* infections in the ticks. Although host age and attachment sites did not influence the infection rates of *I. ricinus* in general, the highest prevalences of *B. burgdorferi* s.l. spp. occurred in ticks from yearlings and in ticks attached to the ears (see Section 5.1.2.2). Over the whole study period, roe deer yearlings had the highest infestation intensity with ticks and the ears were one of the preferred feedings sites where ticks aggregated (Section 4.2.4). Such aggregations are a further indicator that co-feeding might increase the *B. burgdorferi* s.l. infection prevalences in ticks on roe deer (Randolph et al. 1996).

5.2.1.2 Wild boar

A study on wild boar blood and spleen tissue in Poland also showed no *B. burgdorferi* s.l. infected wild boar (Skotarczak and Adamska 2008). Domestic pigs, however, could support the circulation of *B. burgdorferi* s.s., (Kurtenbach et al. 1998), although they are completely borreliacidal for *B. afzelii*, *B. garinii* and *B. valaisiana*. A reported finding of an *I. ricinus* nymph infected with *B. afzelii* that had fed as a larva on a wild boar in North-Central Spain could indicate a reservoir competence for this genospecies (Estrada-Peña et al. 2005). Serological surveys of wild boar have shown seroprevalences to *B. burgdorferi* s.l. ranging from 19% up to 46.7% (Juricová and Hubálek 2009). Thereby, the evidence from IgG antibodies does not allow us to draw conclusions about current or past *Borrelia* infections, but only indicates contact of the animals with the agent of Lyme borreliosis.

Nevertheless, for wild boar my results suggest a reservoir incompetence in relation to *B. burgdorferi* s.l, since none of the organ samples and none of the ticks tested positively by PCR (see Section 5.1.3.2). The fact that during my study *B. burgdorferi* s.l. infections were determined in ticks from roe deer, while no infected ticks were found on wild boar, strengthens the assumption of a borreliacidal effect of wild boar serum and thus of a reservoir-incompetence of wild boar to *B. burgdorferi* s.l. regardless of the genospecies. Equivalently, no infection was found in adult female

Table 5.8: Literature review of prevalences of *R. helvetica* infections in feeding *I. ricinus* ticks, organ and blood samples in relation to roe deer and wild boar.

			Prevalence		
	Country	**Period**	**Ticks**	**Organs or Blood**	**Reference**
Roe deer	Germany	2010-2012	16.6%	0%	(Overzier et al. 2013)
	Poland	2005	12.5%	0%	(Stańczak et al. 2009)
	Denmark	2002-2003	n.a.	0%	(Skarphédinsson et al. 2005)
	Slovakia	2005-2006	n.a.	3.3 ± 6.5%	(Stefanidesova et al. 2007)
	Netherlands	2000-2002	n.a.	19%	(Sprong et al. 2009)
	Slovakia	2003-2004	n.a.	1 of 2	(Smetanová et al. 2006)
	Sweden	1996-1997	3 of 4	n.a.	(Nilsson et al. 1999)
Wild boar	Slovakia	2005-2006	n.a.	0%	(Stefanidesova et al. 2007)
	Netherlands	2000-2002	n.a.	6.9%	(Sprong et al. 2009)
	Spain	2004	0%	n.a.	(de la Fuente et al. 2004)

R. he. = *Rickettsia helvetica*; n.a. = not available

ticks collected from wild boar in the Netherlands (Pacilly et al. 2014). To gain a better understanding of the complement system of wild boar, studies using a procedure similar to that of Bhide et al. (2005) are recommended.

5.2.2 *Rickettsia* infections

5.2.2.1 Roe deer

Several PCR analyses of roe deer blood and organs did not find any positive *Rickettsia* samples (Skarphédinsson et al. 2005, Smetanová et al. 2006, Stańczak et al. 2009, Overzier et al. 2013) (see Table 5.8). These results suggest that wild cervids such as roe deer are not compatible hosts for *Rickettsia* spp. in the sense that they are reservoir incompetent. On the other hand, the study of Stefanidesova et al. (2007) detected a single roe deer in Slovakia the spleen of which tested positive for *R. helvetica*. In the Netherlands, the prevalence of *R. helvetica* in roe deer blood determined by PCR was 19% (Sprong et al. 2009), while red deer was not infected. In addition, *R. helvetica* was found in the peripheral blood of Sika deer in Japan, which leads to the hypothesis that deer may be potential reservoir hosts for this species, increasing

the geographical dispersion of the bacteria even further and thus playing a more significant role in its epidemiology (Sprong et al. 2009) than previously assumed.

In contrast to this, the organ samples from roe deer in this study all tested negative for *Rickettsia* spp. and thus roe deer in the Bienwald could not be confirmed as competent reservoirs of *Rickettsia* spp. In accordance with Skarphédinsson et al. (2005), one reason for this may be that *Rickettsia* infections, and particularly *R. helvetica* spirochetes, have a quite focal distribution, such that the bacteria might not occur in parts of the Bienwald. However, the high infection rates of the ticks collected within the present study and those found in another study (Speck et al. 2013) suggest the contrary. Another explanation for the observations made in the Bienwald may be that the roe deer analyzed were not rickettsiemic at the time of sampling, as the phase of an acute infection might be relatively short (Skarphédinsson et al. 2005, Stańczak et al. 2009). This result is in accordance with the absence of any clinical signs in roe deer in relation to *Rickettsia* spp. infections (cf. Sprong et al. 2009). My results suggest that roe deer are not a reservoir host for *Rickettsia* spp. Nevertheless, the definitive reason for the negativity of all roe deer organ samples with respect to *Rickettsia* spp. remains unclear.

The only *Rickettsia* species isolated from *I. ricinus* ticks in this study was *R. helvetica*, which has also been found in ticks in several European countries (Stefanidesova et al. 2007, Sprong et al. 2009). A Swedish study found 3 of 4 *I. ricinus* individuals collected from roe deer to be positive for *R. helvetica* (Nilsson et al. 1999). The *R. helvetica* infection rates of female ticks (15.7%) and male ticks (10.3%) from roe deer in Poland (Stańczak et al. 2009) were quite similar to those reported in a study from southern Germany (females: 18.4%, males: 13.6%) (Overzier et al. 2013). Both studies point out that the *Rickettsia* infection rate in female ticks was higher than in males, while the state of engorgement did not significantly influence rickettsial occurrence, similar to Dautel et al. (2006).

These findings are in contrast to my study. In the Bienwald, the mean prevalence of *Rickettsia* spp. with respect to all ticks collected from roe deer was considerably higher (47.0%) than found in previous studies. Thereby, male ticks (54.2%) were more likely to be infected than females (45.4%). All my analyses revealed that the prevalence of *Rickettsia* spp. was significantly lower in engorged *I. ricinus* than in unfed individuals. The decision tree showed that the state of engorgement was the most important factor influencing the *Rickettsia* infection rate, followed by tick sex. Unfed females showed a prevalence of over 80%, while the mean infection rate of feeding females reduced to under 40% (Section 5.1.5.3). Moreover, with the logistic regression (Section 5.1.5.4) I was able to show that with each higher level of engorgement the chance of an infection was reduced even further. The influence of feeding on female ticks seems to be the reason why in the present study the infection rate of females was significantly lower than in males. The negative correlation between feeding and infections might indicate that roe deer are able to kill *Rickettsia* spp. in the feeding ticks, similar to the situation found in *B. burgdorferi* s.l., although the effect does seem to be less intense. As far as is known, this is the first report of such a relationship between roe deer, *I. ricinus* feeding behavior and *Rickettsia* spp. Investigations similar to those of Bhide et al. (2005) and Kurtenbach et al. (1998) are highly recommended for *Rickettsia* spp. in ticks and roe deer.

One possible explanation for the high infection rates could be related to a rickettsiacidal effect of roe deer on feeding ticks, as discussed above. A higher roe deer population density would then lead to a reduction of the *Rickettsia* prevalence through the feeding ticks. Overzier et al. (2013), for example, estimated a roe deer population density at 10 animals per 100 ha in their sampling area. In contrast to this, the roe deer density in the Bienwald during the sampling period was lower with approximately 6 individuals per 100 ha (Ehrhart 2012). Consequently, the inverse relation between host density and infection rate might be the reason why more *Rickettsia* spp. infections in ticks were observed in the present study than in the investigation of Overzier et al. (2013).

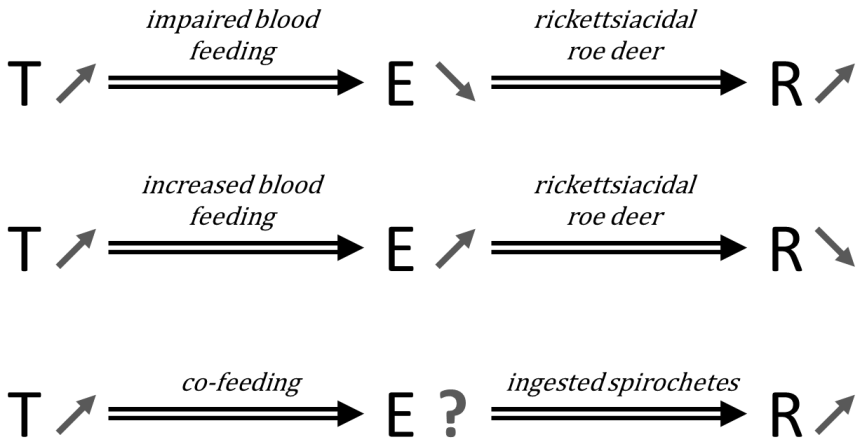


Figure 5.4: Gregarious feeding of *I. ricinus* on roe deer might cause three possible effects caused by tick abundance (T) onto the level of engorgement (E) and the prevalence of *Rickettsia* spp. (R) in the ticks. The direction of the effects are in accordance with the up- and down-pointing arrows.

My correlation analysis (Section 5.1.5.1) and in particular the factor analysis (Section 5.1.5.2) revealed that both the number of ticks on the entire roe deer body and the tick density at the preferred feeding site were associated with a reduced *Rickettsia* infection rate and with a lower level of engorgement. One explanation for the lower level of engorgement with increasing tick burden could be that only a few ticks feed rapidly, while a higher proportion feed more slowly. Such an observation was also made by Wang et al. (2001) showing that fast-feeding ticks seem to impair the blood-feeding success of slow-feeding females during gregarious feeding by causing host immune responses. Such a behavior is corroborated by my observation that at multiple attachment sites where more than 10 ticks were feeding gregariously only a very few ticks (1-3) were fully engorged (data not shown). However, Wang et al. (2001) also showed that gregarious feeding also leads to an increased blood feeding rate in female ticks. Thus, not all ticks will benefit from feeding aggregation, such that some will have to face increased costs (as discussed above).

In combination with the assumption of a rickettsiacidal effect of roe deer, the higher feeding rate could provide an explanation for the negative correlation between tick density at the preferred attachment site and *Rickettsia* spp. infections. As a result, a higher tick density leads to an increased blood feeding rate through aggregated

feeding, which in turn leads to a more rapid increases of the level of engorgement of some ticks (the faster feeding ones), such that the proposed rickettsiacidal effect of roe deer reduces the *Rickettsia* infection rate (second row of Figure 5.4). Moreover, the increased-blood-feeding hypothesis might be an explanation for why ticks from male roe deer have significantly less *Rickettsia* infections than ticks from female hosts (Section 5.1.5), because the tick intensity on male deer was significantly higher than on female deer (cf. Chapter 4). On the other hand, the impaired blood-feeding hypothesis (Wang et al. 2001) can be applied to explain the significantly higher number of *Rickettsia* infections in ticks from roe deer yearlings (Section 5.1.5.4). Since yearlings carry a higher number of ticks than adults (Section 4.1.1), the impaired blood-feeding success might be the reason for lower levels of engorgement and for the increased *Rickettsia* prevalence (first row of Figure 5.4). The higher infection rate might also be related to the transmission of *Rickettsia* spp. between attached ticks through co-feeding (last row of Figure 5.4). The discussion above underlines that the interdependencies of the three effects, which are summarized in Figure 5.4, have to be considered in the epidemiological cycle of rickettsial diseases with respect on roe deer and their ticks.

However, another explanation for the high prevalences found in this study cloud be that *R. helvetica* is efficiently vertically transmitted through the next generation by transovarial transmission and from one to the next life history stage by transstadial transmission. Under laboratory conditions these effects have been demonstrated for other *Rickettsia* spp. by Burgdorfer et al. (1979). In general, pathogens that benefit from both transmission modes are less dependent on vertebrate hosts. For this reason, *I. ricinus* can be considered as a reservoir host for *R. helvetica*. The high infection rates of *I. ricinus* in combination with the findings of only *R. helvetica* in these ticks underline the hypothesis that *I. ricinus* is an important reservoir host for *R. helvetica* (Sprong et al. 2009).

The interactions between the effects discussed above in combination with seasonal tick activity could have led to the significant changes in *Rickettsia* prevalences in

relation to the sampling periods shown by the logistic regression analysis (Section 5.1.5.4). Climatic parameters in relation to tick abundance could have affected the *Rickettsia* prevalence in ticks from roe deer. A correlation between tick burden and *Rickettsia* spp. prevalence has been suggested (Kantsø et al. 2010, Schorn et al. 2011). The study area of Overzier et al. (2013) was a forest area with an average temperature between 6.5 and 7.5 °C and rainfall between 950 and 1000 mm per year. A higher average temperature (10 °C) and lower precipitation depths (680 and 700 mm) were recorded in the Bienwald during the study period. The warmer and dryer climate in the Bienwald could constitute a more suitable habitat for *I. ricinus* and thus lead to higher tick prevalences and abundances (Perret et al. 2004, Gray et al. 2009, Gilbert 2010). This is underlined by a high tick intensity on small mammals in the Bienwald compared to that found in other studies (Skuballa 2011, Speck et al. 2013). Preliminarily sampling of ticks from the Bienwald vegetation supports these findings (Muders and Petney, unpublished data). As a result, the observed effect of low roe deer densities may be amplified further through higher tick abundance and might contribute to distinctly higher *Rickettsia* infection rates. Additionally, *Rickettsia* prevalences in ticks collected from European hedgehogs in the Bienwald did not exceed 17.3% (Speck et al. 2013). This fact underlines the importance of the role that that roe deer could play in the epidemiological cycle of *Rickettsia* spp.

Climatic and regional differences, together with differences in the vegetation, could lead to variation in *Rickettsia* prevalence (Halos et al. 2010). The Bienwald and the study area investigated by (Stańczak et al. 2009) have about the same proportion of woodland (≈78%), but have considerably different infection rates. In this context, roe deer prefer forest edges (Tufto et al. 1996), but these habitats in turn show higher tick mortality rates (Randolph 2004). The occurrence of *I. ricinus* seems to be higher in pure woodlands (Randolph 2004). Conversely, Halos et al. (2010) found that *Rickettsia* spp. prevalence in questing *I. ricinus* was maximal on pasture with medium forest fragmentation. Consequently, it is possible that the risk of infection with *Rickettsia* spp. depends on forest structure, as has been observed for the TBE

virus in Italy (Rizzoli et al. 2009). A study of the opposing effects of *I. ricinus* as vectors and roe deer as probably non-compatible hosts for *Rickettsia* spp. in relation to their habitat preferences could provide further insight on the pathogen distribution. Such investigations could also provide additional information on the possible reservoir incompetence of roe deer.

The screening of *D. reticulatus* from roe deer in Germany (Dautel et al. 2006) showed that 32.4% of ticks were infected with *Rickettsia*. Moreover, Dautel et al. (2006) reported an average prevalence of 23% for all *Rickettsia*-positive ticks from the investigated deer species (i.e. red deer, roe deer and fallow deer), while all rickettsial DNA was from the RpA4 strain. Comparing these findings to my results shows that the tick species, as well as the *Rickettsia* species, may lead to variation in the infection rates. One explanation of this behavior could be the differing modes of life of different tick species, including parameters such as host preferences and feeding behaviors (Sonenshine and Roe 2013b). However, the infection prevalences reported by Dautel et al. (2006) were averaged from all of Germany and matched those presented here most closely in comparison to the other studies, although *I. ricinus* was not considered. Therefore, it would be interesting to see the results of similar studies comparing the prevalence of *Rickettsia* spp. in *I. ricinus* considering multiple sampling sites within Germany and Europe.

5.2.2.2 Wild boar

Stefanidesova et al . (2007) found no evidence of *Rickettsia* spp. in spleen samples from wild boar. However, 7% of the whole blood from wild boar was positively tested by PCR for *R. helvetica* in the study of Sprong et al. (2009). In Spain, Ortuño et al. (2007) reported that 52.2% of wild boar were seropositive to *R. slovaca*. Skin biopsies from wild boar in Italy (Selmi et al. 2009) identified a single positive for *R. slovaca*, whereby a *D. marginatus* collected from the skin was also positive. Nevertheless, it is still unclear whether wild boar are able to develop a rickettsemia which could then infect feeding ticks. Equivalently to roe deer, wild boar in the Bienwald

appear not to harbor *Rickettsia* spp., since none of the organ samples showed positive during the PCR analyses (Section 5.1.3.1).

A study on *D. marginatus* collected from wild boar in France revealed an infection prevalence by *R. slovaca* of 15.7% (Sanogo et al. 2003). In Spain, de la Fuente et al. (2004) registered a higher infection rate of 18% in *D. marginatus* on wild boar, with mainly *R. slovaca* being detected. Ortuño et al. (2007) reported *R. slovaca* in 30.5% of the *D. marginatus* from wild boar in northern Spain, while their study showed no significant deviation between questing ticks and those feeding on wild boar. A similar spotted fever group (SFG) prevalence was found by Selmi et al. (2009) with 33.9% of ticks infected. However, none of these studies investigated the *Rickettsia* prevalence in *I. ricinus* on wild boar, nor did they find *R. helvetica* in any of the collected ticks.

In the present study the overall *Rickettsia* prevalence in *I. ricinus* from wild boar was distinctly higher (41.7%) than in the previous studies. In addition, I was able to show that *Rickettsia* spp. prevalence in ticks from wild boar decreased significantly with a higher level of engorgement (Section 5.1.3.1). This observation could be a first indicator for a rickettsiacidal influence of wild boar similar to roe deer. *I. ricinus* from female boar did not have any *Rickettsia* spp. and all ticks removed from piglets had a significantly higher infection rate than for those removed from older boar. This suggests that immunity had not been developed in piglets. To date, comparative studies in Europe, and particularly in Germany, are missing.

6

Conclusion and perspectives

6.1 Summary

This is the first and most comprehensive study of roe deer and wild boar in a common habitat, including the collection of ticks and host organ samples over 3 years. My study is not only comprehensive with respect to the number and continuity of the acquired samples, but also in the sense that it included potential biotic and abiotic factors that have not been considered any previous study. The data acquisition encompassed a multitude of parameters, such as host species, host age, host sex, host condition, pregnancy, lactation, body mass, infestation with other parasites, climate (e.g. temperature, rainfall, cloud coverage, relative humidity and sunshine duration), tick age/sex, attachment site, level of engorgement and mating status. In addition, I calculated the relative tick densities at the attachment sites, niche indices, the spatial niche overlap, the degree of tick aggregation, as well as $GDMI$ and tick induced blood loss. By this means, I gained the largest dataset concerning ticks, roe deer and wild boar currently available worldwide.

This dataset allowed the study of seasonal changes together with the analyses of annual variations of the tick burden on both hosts, as well as of the tick-host-pathogen interactions. In a first step, correlation and factor analyses were used to identify key factors influencing the dynamics of the ticks and the pathogens which they transmit. To rank the importance of these factors, I applied multiple decision tree analyses to different subsets of the data. To gain an even deeper understanding of

how the key factors acted on tick abundances, population composition and pathogen prevalence appropriate state-of-the-art modeling techniques were used. In Central Europe, and in particular in Germany, such a three-step statistical evaluation is unique. This approach shows how important a long sampling period is for the identification of the factors influencing inter-annual variation.

1) I was the first to find that tick aggregation was lowest on roe deer yearlings for all tick life history stages, while fawns and hosts in a poorer condition showed a higher aggregation of ticks. At the same time, tick abundance was highest on yearlings, followed by adults and fawns. I propose that these patterns might be related to the "exposure" hypothesis, and that thus roe deer movement patterns might significantly influence tick aggregation and abundance.
2) Wild boar piglets were most intensely infested by *I. ricinus*. The lower tick abundance on older boar could be explained by the higher proportion of dead ticks on these host animals. The higher tick mortality might be a first indicator that these age groups have a higher physical defense and/or an acquired resistance to ticks.
3) This study also showed that tick abundance on roe deer and wild boar not only follows seasonal changes, but can also have a high variability between years.
4) Climatic factors have a strong influence on roe deer and wild boar, explaining the bimodal seasonal peak densities of the ticks. Tick activity on roe deer started between 8 and 9 °C. I found the first evidence that ticks can be active on roe deer at temperatures as low as -1.8 °C. All of these observations indicate that ticks in the Bienwald are active all year.
5) For wild boar, this study was the first that examined tick burden in relation to climatic parameters. Although sample sizes were low, cloud coverage, relative humidity and saturation deficit were significantly negatively correlated with tick density on this species.
6) By using a decision tree analysis I determined an activity threshold in relation to relative humidity (80.4%) above which the tick infestation intensity of roe deer was reduced significantly.

7) Additionally, the GAMLSS models demonstrated for the first time that precipitation had a significant, but non-linear relationship with adult and immature tick burden on both roe deer and wild boar.
8) My results clearly confirmed a sex-biased tick behavior on roe deer, such that male deer were significantly more highly infested than females.
9) So far, this study is the only one that considers host condition as an important factor for tick infestation. I demonstrated that healthier roe deer burdened with fewer other parasites carried significantly fewer ticks than roe deer in a poorer or crippled condition. Pregnant or nursing female deer had a significantly higher infestation than other individuals.
10) I determined that only very few roe deer are likely to suffer from tick induced blood loss.
11) My attachment site analysis revealed that adult ticks preferred the abdomen and the sternum, while immatures were predominantly found on the ears. With respect to the IFDH, I confirmed that ticks actively choose their feeding site supporting the hypothesis of pheromone-based on-host aggregation of *I. ricinus* which plays a key role for co-feeding.
12) The overall tick abundance on wild boar, with many dead ticks, was significantly lower than on roe deer. Although both species share a common habitat, wild boar are a less important food source for ticks, while roe deer are the dominant host for adult female ticks.
13) In comparison to previous model-driven analyses that were based on significantly smaller datasets, I demonstrated that the GAMLSS approach applied to long-term, raw data can reveal details of the relationships between tick parasitism, biotic and abiotic factors, even if they are non-linear.
14) In particular, with respect to wild boar, I was able to successfully estimate GAMLSS models for the first time to gain a better understanding of the relationship between climatic factors and tick infestation.
15) The multinomial logistic regression that modelled the composition of tick population is also new in the sense that it included data from each season of the 3 years and from all body parts.

With respect to *Rickettsia* and *Borrelia* infections I was able to confirm past research result, but also made several novel findings:

1) *R. helvetica* was the only *Rickettsia* species found in the Bienwald with a mean prevalence in ticks from roe deer and wild boar reaching almost 50%. Such high infection rates in ticks have not been observed by any other previous study.
2) In my study, none of the organ samples showed positive for these pathogens, confirming that roe deer and wild boar are reservoir-incompetent hosts for *B. burgdorferi* s.l., as well as for *Rickettsia* spp.
3) One of my main novel findings was that the prevalence of *R. helvetica* was significantly lower in engorged *I. ricinus* than in unengorged individuals. This was also the reason why male ticks were more likely to be infected than females.
4) This observation was true independently of the host species.
5) In addition, with higher levels of engorgement the *Rickettsia* infection rate decreased significantly.
6) Therefore, I hypothesize that roe deer and wild boar blood is able to kill *Rickettsia* spp. in the feeding ticks. For both host species, this is the first report of a rickettsiacidal influence.
7) My study of this effect showed that interactions between multiple factors, such as impaired and increased blood feeding, co-feeding, climate, host population density and tick abundance, can be used to explain the lower infection prevalences in feeding *I. ricinus* ticks.
8) Thereby, I observed for the first time that ticks collected from wild boar piglets had a significantly higher *Rickettsia* infection rate than for those removed from older boar suggesting that immunity in piglets had not been developed.
9) The *B. burgdorferi* s.l. infection rate of *I. ricinus* in the Bienwald was relatively low (3.4%), with higher infection prevalences in male ticks than in females, while prevalences showed a seasonal pattern in synchrony with tick abundance.
10) Ticks from yearlings had the highest *Borrelia* prevalences.

11) Additionally, ticks found on the ears of roe deer and wild boar showed significantly increased infection rates.
12) My finding that engorged female ticks on roe deer were never infected, clearly confirmed that this host species is reservoir incompetent for *B. burgdorferi* s.l. This leads to the conclusion that increasing population densities of deer could reduce the infection rate significantly.
13) For the first time, my study proposes a borreliacidal effect for wild boar and thus of a reservoir-incompetence to *B. burgdorferi* s.l.

In summary, both host species can be considered as dilution hosts for *B. burgdorferi* s.l. species and *Rickettsia* spp., whereby roe deer play a key role as host for *I. ricinus* ticks providing a platform for co-feeding and tick propagation. The role of wild boar seems to be considerably less important in the epidemiological life cycle of tick-borne diseases, although this species could serve a propagation host for ticks and thus for pathogens as well.

Results from the northern (e.g. Norway) and southern parts (e.g. Spain and Italy) of Europe showed considerable differences to my study. This suggests that result from other regions are not transferable to Central Europe. My study showed that a continuous and coherent sample acquisition is essential to make general statements about tick behavior and pathogen life cycles. Therefore, additional long-term studies in Central Europe, and particularly in Germany are needed to gain results for comparative purposes. The presence of the agent of Lyme borreliosis and of SFG *Rickettsia* in the Bienwald pose a potential risk for humans and justifies further research.

6.2 Future Work

The population density of ungulate hosts, particularly roe deer, strongly affects tick abundance (Wilson et al. 1984, Hudson et al. 2001, Perkins et al. 2006, Rizzoli et al. 2009, Tagliapietra et al. 2011). Therefore, accurate estimates of population densities are unavoidable in order to make results from different studies comparable on an absolute scale. Concurrently to the collection of ticks and organ samples, the roe

deer density in the Bienwald has been estimated based on faecal density and camera-traps (cf. Ebert et al. 2012, Ehrhart 2012). Combining host density data with my dataset could provide more information on the relationships between ungulate host species and tick burden. For wild boar, very little research on the effect of population density on tick abundance has been conducted. Thus, a study that estimates host population density is strongly recommended.

In addition to this, tick samples from the vegetation by drag sampling (Neumaier 2012, Zöller 2014), from forest rodents (Schweikert 2012, Schaeffer and Petney, unpublished data) and from sheep (Moser 2012) were collected in the Bienwald during the period of my study. Within the BWPLUS project similar studies were carried out simultaneously (Fritschmann 2012, Petney et al. 2014, Sebastian et al. 2014, Pfäffle et al. 2015a, 2015b). Moreover, other environmental factors, such as vegetation and soil types, as well as the community structure of available hosts in the Bienwald could affect ticks and pathogens (Sonenshine and Roe 2013a, 2013b). A combination of those datasets was beyond the scope of this thesis, but could provide a more complete view of the tick and pathogen life cycles within the study area as well as on a larger scale (left vs. right bank of the Rhine). Additionally, future long-term studies on interactions of ticks, roe deer, wild boar and pathogens in the Bienwald and within the BWPLUS project are highly recommended using my results as a reference.

During the sample acquisition of this study blood samples were collected from all host animals. Their evaluation could provide more information on host condition, reservoir competence and pathogen transmission. For example, an analysis of host testosterone levels could provide additional confirmation of the sex-biased behavior of *I. ricinus*, and about the influence of ticks on host condition. The determination of *Borrelia* and *Rickettsia* genospecies was not possible for all samples due to financial constraints. However, the samples are still available for future analyses. Additionally, future investigations could determine whether other pathogens (e.g. *Babesia*

spp., *Bartonella* spp., *Ehrlichia* spp., etc.) are present in the ticks and/or in the organ samples collected in the Bienwald.

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Appendices

A

Form for recording organ samples

Table A.1: This from has been used during the sample acquisition to record all host data. For each animal the date of culling, its ear tag, species, age, sex and body mass has been noted down. To ensure that no blood or organ sample was forgotten during the acquisition, each sample has been ticked off. In the "Tick locations" column the numbers of the body regions with ticks have been recorded. The last column of the form was used to add notes about the physical condition of the animals and about additional parasites found on them.

Sample acquistion								
Species: Wild boar (WB), Reo Deer (RD) Sex: Female (F), Male (M) Age: Piglet (P), Fawn (F), Yearling (Y), Adult (A) Samples: EDTA (ED), Serum (SE), Lung (LU), Liver (LI), Splenic (SP), Bladder (BL), Kidney (KI), Heart (HE), Diaphragm (DI), Ear (EA)								
Date	**Ear tag**	**Species**	**Age**	**Sex**	**Body mass**	**Samples**	**Tick locations**	**Notes**
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		
						☐ ED ☐ SE ☐ LU ☐ LI ☐ SP ☐ BL ☐ KI ☐ HE ☐ DI ☐ EA		

B

Mean group weights

Table B.1: Mean body mass for groups of animals ($\bar{m}_g$) together with the total minimum (M_{min}) and maximum (M_{min}) deviation from the average masses (second last and last column) used to calculate the $GDMI_s$ of each individual as described in Section 3.3.4.

				Animals	$\bar{m}_g$	$min(m - \bar{m}_g)$	$min(m - \bar{m}_g)$
Roe Deer	**Adult**	**male**	**Jan/Feb**	5	15,40	-5,19	0,81
			Mar/Apr	2	15,00	-1,19	-1,19
			May/Jun	6	16,18	-1,19	1,11
			Jul/Aug	1	14,70	-1,49	-1,49
			Sep/Oct	2	15,50	-1,19	-0,19
			Nov/Dec	30	16,50	-4,19	5,81
		female	**Jan/Feb**	23	15,91	-2,32	1,68
			Sep/Oct	1	17,00	0,68	0,68
			Nov/Dec	60	16,47	-3,32	4,68
	Yearling	**male**	**May/Jun**	9	13,48	-3,76	4,34
			Nov/Dec	8	11,75	-2,66	1,34
		female	**Jan/Feb**	2	13,50	-0,82	0,18
			May/Jun	6	13,32	-1,22	1,18
			Sep/Oct	3	13,00	-3,82	1,18
			Nov/Dec	6	14,83	0,18	2,18
	Fawn	**male**	**Jan/Feb**	6	12,83	-0,01	1,99
			Sep/Oct	1	6,30	-5,71	-5,71
			Nov/Dec	28	12,04	-3,01	1,99
		female	**Jan/Feb**	9	11,44	-3,67	3,33
			Sep/Oct	4	8,50	-4,17	-1,67
			Nov/Dec	35	12,09	-3,67	3,33
Wild boar	**Adult**	**male**	**Jan/Feb**	7	59,86	-8,80	20,20
			Sep/Oct	1	67,00	5,20	5,20
			Nov/Dec	17	62,29	-13,80	12,20
		female	**Jan/Feb**	15	56,20	-11,34	28,66
			Sep/Oct	1	39,00	-17,34	-17,34
			Nov/Dec	42	56,81	-18,34	15,66
	Yearling	**male**	**Jan/Feb**	8	40,13	-15,13	14,87
			May/Jun	1	49,20	4,07	4,07
			Sep/Oct	2	27,00	-18,13	-18,13
			Nov/Dec	29	47,62	-16,13	23,87
		female	**Jan/Feb**	8	41,38	-20,53	13,47
			May/Jun	1	21,00	-23,53	-23,53
			Nov/Dec	34	45,97	-12,53	17,47
	Piglet	**male**	**Jan/Feb**	28	22,61	-16,28	16,72
			Mar/Apr	1	9,00	-14,28	-14,28
			May/Jun	1	18,70	-4,58	-4,58
			Sep/Oct	1	17,00	-6,28	-6,28
			Nov/Dec	57	24,05	-18,28	16,72
		female	**Jan/Feb**	33	23,42	-11,91	22,09
			Nov/Dec	57	22,61	-17,91	17,09
			Total	**608**	**26,72**	$\boldsymbol{M_{min}}$ **= -23,53**	$\boldsymbol{M_{max}}$ **= 28,66**

C

Equipment and consumables

Table C.1: All used devices and their manufactures.

Device	Manufacturer
Gel electrophoresis	
Agarose gel chambers	LTF Labortechnik, Wasserburg
Gel documentation system Bioprofil®	LTF Labortechnik, Wasserburg
Power Supply PowerPac™ 300	Bio-Rad Laboratories, München
Precision balance PM 2000	Mettler-Toledo, Giessen
Thermal Transfer Printer P90	Mitsubishi, Barcelona
General	
Centrifuge (Z233M-2)	Hermle, Wehingen
Minishaker MS 2	IKA, Staufen
Pipettes	Eppendorf, Hamburg
Refrigerated centrifuge (5417R, 5804R)	Eppendorf, Hamburg
Sterile workbench	BDK, Sonnenbühl
Tabletop centrifuge (5415D)	Eppendorf, Hamburg
Ultrapure water system	Millipore, Schwalbach
Vortex™ (Genie 2M)	Bender & Hobein, Schweiz
Nucleic acid extraction	
Biohit eLINE® electronic pipette	Sartorius Lab Instruments, Göttingen
Maxwell® 16	Promega, Mannheim
NucliSENS® easyMag®	bioMérieux, Nürtingen
PCR	
LightCycler® 1.5	Roche Diagnostics, Mannheim
Thermocycler: GeneAmp® PCR System 9700	Applied Biosystems, Weiterstadt
Sample storage	
Deep freezers (-20 °C; -70 °C)	Kendro, Hanau; Bosch, Gerlingen
Refrigerators	Liebherr, Biberach
Sequencing	
Sequencer: ABI Prism® 310 Genetic Analyzer	Applied Biosystems, Weiterstadt

Table C.2: All used reagents and their suppliers.

Reagent	**Supplier**
Gel electrophoresis	
1 × TBE Puffer (Tris, Boric acid, EDTA)	Merck-Millipore, Darmstadt
100 bp ladder	Amersham Pharmacia, Freiburg
Boric acid	Merck, Darmstadt
Bromophenol blue	Sigma-Aldrich, Taufkirchen
Ethidium bromide (1%)	Merck, Darmstadt
Ficoll	Sigma-Aldrich, Taufkirchen
peq Gold® Universal Agarose	Peqlab, Erlangen
General	
Diethylpyrocarbonate (DEPC)	Fluka/Sigma-Aldrich, Steinheim
Ethanol	Merck, Darmstadt
Hydrochloric acid (25%)	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt
Tris-HCl	Merck, Darmstadt
Nucleic acid extraction	
Guanidine isothiocyanate (GIT)	Carl Roth, Karlsruhe
RNasin®	Promega, Mannheim
Triton™ X-100	Sigma-Aldrich, Taufkirchen
PCR	
AmpliTaq® DNA-Polymerase, incl. 10 × buffer, $MgCl_2$ (25 mM)	Applied Biosystems, Weiterstadt
dNTP-Set (100 mM Solutions)	GE Healthcare, München
dUTP	Fermentas, St. Leon-Rot
Nuclease-free water	Promega, Mannheim
Primer	Tib Mol, Berlin
Tris HCl	Merck, Darmstadt
Uracil-DNA glycosylase	Fermentas, St. Leon-Rot
Sequencing	
HiDi™ formamid	Applied Biosystems, Weiterstadt
Performance optimized Polymer 6 (POP-6)	Applied Biosystems, Weiterstadt
Sodium dodecyl sulfate (SDS)	Serva, Heidelberg

Table C.3: All used kits and their suppliers.

Kit	Supplier
Nucleic acid extraction	
Reagent cartridges Maxwell® 16 Tissue DNA Purification Kit (AS1030)	Promega, Mannheim
PCR	
LightCycler® DNA Master HybProbe	Roche Diagnostics, Mannheim
Sequencing	
BigDye® Terminator v1.1Cycle Sequencing	Qiagen, Hilden
DyeEx™ 2.0 Spin Kit	Qiagen, Hilden
QIAquick® PCR Purification Kit	Qiagen, Hilden

Table C.4: All used kits and their suppliers.

Material	Manufacturer
General	
Disposable pipet tips, stuffed	Biozym, Hessisch Oldendorf
Disposable pipet tips, non-stuffed	Eppendorf, Hamburg
Safestock Eppendorf Cups (0.5 ml; 1.5 ml; 2 ml)	Eppendorf, Hamburg
Latex gloves	Asid Bonz, Herrenberg
Scalpels	Braun, Tuttlingen
PCR	
Glass capillaries: LightCycler® Capillaries (20 µl)	Roche Diagnostics, Mannheim
PCR vials (0.2 ml)	Biozym, Hessisch Oldendorf
Sample acquisition	
Multivette® for EDTA and Serum samples	Sarstedt, Nümbrecht
Sequencing	
Capillaries for Sequencer	Applied Biosystems, Weiterstadt

D

Solutions and buffers

For Nucleic Acid Extraction

All solutions and buffers were prepared with nuclease-free water or Diethylpyrocarbonate (DEPC) for the inactivation of RNAses. Vessels for the production of solutions and for their storage were hot air sterilized at 200 °C for 4 hours. Plastic materials were purchased nuclease-free.

DEPC Water

- make a 10% DEPC stock solution in absolute ethanol (100 ml DEPC + 900 ml ethanol) and store in a brown glass bottle at room temperature
- prepare a 0.1% utility solution by dilution of the stock solution at a ratio of 1 to 100 with H_2O_{bidest} (10 ml DEPC solution + 990 ml H_2O_{bidest}), let the utility solution incubate for 2 hours at 37 °C, and then store the DEPC utility solution at 4 °C in a glass jar.

The used DEPC solution was autoclaved. For the storage the DEPC solutions, the plastic screw caps were inserted for 2 hours at 37 °C in the DEPC utility solution and then autoclaved (30 min at 120 °C).

0.1 M Tris-HCl solution (pH 6.4)

- solubilize 12.11 grams of Tris in 800 ml of DEPC utility solution
- adjust the pH value with HCl (25%) to 6.4
- fill up to 1 l with DEP utility solution
- store in glass bottles with plastic screw caps.

Lysis Buffer for Tissue Pools

- solubilize 120 g guanidine isothiocyanate (GIT) in 100 ml 0.1 M Tris-HCl (pH 6.4) at 56 °C
- add 22 ml of 0.2 M EDTA (Na salt, pH 8.0) and 2.6 g Triton™ X-100 and store in a dark glass bottle at room temperature.

For Agarose Gel Electrophoresis

Tris/Borate/EDTA (TBE) buffer (5 ×)

- 53.9 g Tris
- 27.5 g boric acid
- 20 ml EDTA
- solubilize in H_2O and fill up to 1 l.

Ethidium Bromide Solution

- dilute ethidium bromide stock solution (1%) to a 0.1% utility solution with H_2O_{bidest} at a ratio of 1 to 10.

Loading Buffer

- 20% Ficoll
- 0.25% Bromophenol blue
- fill with H_2O up to 20 ml.

100 bp Ladder

- dilute stock solution with nuclease-free H_2O at a ratio of 1 to 8.