ENDOSYMBIONTS IN SPIDERS: DRIVING FORCE OR HITCHHIKERS?

ANTJE HUNDERTMARK MSc

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Table of Contents

Acknowledgments 5

Declaration 6

Abstract 7

List of Figures 9

List of Tables 12

Abbreviations 14

Chapter 1: Introduction 15

1.1 General background 15

1.2 Endosymbionts 17

1.2.1 Endosymbiont-induced phenotypes 17

1.2.2 Endosymbiont species 29

1.2.3 Endosymbionts and speciation 35

1.3 Why are spiders a good model system? 36

1.3.1 Diverse reproductive biology, behavioural strategies and ecology 36

1.3.2 Spider reproductive biology 40

1.3.3 Spiders as model systems 44

1.4 Endosymbiont infections in spiders 45

1.5 Hypothesis and aims 47

Chapter 2: *Wolbachia* infections in *Nephila senegalensis* 50

2.1 Introduction 50

2.2 Materials and methods 53

Nephila senegalensis 53

2.2.1 Spider husbandry 55

2.2.2 *Wolbachia* 56

2.2.3 DNA extraction and PCR 57

2.2.4 Statistics 59

2.3 Results 59

2.3.1 Endosymbiont analysis of stored individuals 59

2.3.2 Endosymbionts and sex ratio 59

2.3.3 Endosymbiont transmission 61

2.3.4 Endosymbionts and body weight 62

2.3.5 Endosymbionts and egg sacs 66

2.3.6 *Wolbachia* phylogeny 66

2.4 Discussion 69
Chapter 3: Life history and population genetics in *Linyphia hortensis* and *Linyphia triangularis*....76
3.1 Introduction 76
3.2 Materials and methods....80
Linyphia hortensis 80
Linyphia triangularis 82
3.2.1 Spider husbandry....86
3.2.2 Cephalothorax size 87
3.2.3 Ballooning 87
3.2.4 Mating experiments....89
3.2.5 DNA extraction and PCR 90
3.3 Results....91
3.3.1 Sex ratio 91
3.3.2 Cephalothorax size 93
3.3.3 Ballooning 101
3.3.4 Mating experiments....102
3.3.5 mtDNA analysis....110
3.4 Discussion 112
Chapter 4: The endosymbionts of *Linyphia hortensis* and *Linyphia triangularis* 119
4.1 Introduction 119
4.2 Materials and methods....124
4.3 Results....125
4.3.1 Endosymbiont infection frequencies....125
4.3.2 Endosymbionts and sex ratio....134
4.3.3 Endosymbionts and body size 139
4.3.4 Endosymbionts and egg sacs 149
4.3.5 Endosymbiont transmission 153
4.3.6 *Wolbachia* phylogeny 156
4.3.7 *Spiroplasma* phylogeny....158
4.3.8 *Rickettsia* phylogeny....161
4.3.9 Bacteriophage WO....164
4.4 Discussion 167

Chapter 5: Modelling the spread of bacterial endosymbionts through male mate choice and cytoplasmic incompatibility: Testing the model predictions with real-life data from spiders of the genus *Linyphia* ... 178
5.1 Introduction ... 178
5.1.1 Introducing the model ... 181
5.2 Materials and methods ... 185
5.2.1 The simulation ... 185
5.2.2 The real-life data ... 186
5.3 Results ... 189
Mermessus fradeorum ... 189
Linyphia triangularis ... 196
5.4 Discussion ... 203
5.5 Appendix ... 207
Methods ... 207
Results ... 211
Chapter 6: General discussion and future outlook ... 214
References ... 222
Appendix 1 ... 246
Permission for the use of Figures 1, 2 and 3. ... 246
Permission for the use of Figures 6 and 7. ... 247
Permission for the use of the photo in Figure 13, left. ... 248
Permission for the use of the photo in Figure 13, right. ... 249
Permission for the use of the photo in Figure 14, left. ... 250
Permission for the use of the photo in Figure 14, centre and right. ... 251

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Declaration

I declare that this thesis is the result of my own work which has been mainly undertaken during my period of registration for this degree at The University of Nottingham. I have clearly indicated any parts of this thesis in which collaborators were involved.

Abstract

Endosymbionts such as *Wolbachia* and *Rickettsia* and their effects on their hosts have been extensively studied. In recent years these endosymbionts have gained attention as potential drivers of speciation by inducing reproductive isolation in differentially infected host populations. Research in this area has so far provided support for, as well as against, this theory. Whilst a plethora of studies exist on endosymbiont infections in insects, the knowledge about the effect of endosymbionts on spiders is still limited. Only a few studies so far could attribute observed sex ratio distortions and incompatibilities to the presence of one or more endosymbiont species. The work presented in this thesis aimed to further our understanding of endosymbiont infections in spiders and the role of endosymbionts in the speciation process.

The first part of the thesis is dedicated to *Wolbachia* infections in *Nephila senegalensis*. A strong female bias has been found in a population from KwaZulu-Natal. However, so far it remains unknown if *Wolbachia* is involved in the sex ratio distortion. The finding that *Wolbachia* infects *N. senegalensis* was a serendipitous discovery which inspired the following studies on *Linyphia hortensis* and *Linyphia triangularis.* These two linyphiid species were found to carry the endosymbionts *Wolbachia*, *Rickettsia* and *Spiroplasma*. Furthermore, a reduced diversity in mtDNA haplotypes was detected which is consistent with endosymbiont-induced selective sweeps. All populations showed a female biased sex ratio, but since no matrilines could be successfully reared in the lab, it is not clear whether this sex ratio distortion is the result of the used sampling method, whether there is a skewed operational sex ratio or whether endosymbionts cause the observed bias. Potentially a combination of all three factors could be responsible.

The study could not confirm whether the endosymbiont infections cause incompatibilities between differently infected individuals in *L. hortensis* or *L.*

triangularis. No assumptions can be made at this point on whether endosymbiont infected males prefer to mate with infected compared to uninfected females. The spiders, which were collected from the United Kingdom, Germany, Denmark and Sweden, did not show any differences in mating behaviour in the few mating experiments that were conducted in the lab. Nevertheless, the sample size was not sufficient to be entirely sure that there are no differences and whether endosymbionts play a role in the mating system of these spiders.

All three spider species were infected with *Wolbachia* strains, which carry the temperate bacteriophage WO. This phage has been shown to influence the level of cytoplasmic incompatibility (CI) in insect populations. It is possible that the phage is not only able to disrupt the capability of *Wolbachia* to induce CI, but that it can also have an effect on the expression of one or more of the sex ratio distorting phenotypes. At this point the role of the phage in spider host systems remains a mystery.

The theoretical work presented in this thesis indicates that the endosymbiont infections found in the two *Linyphia* species do not show a high enough transmission fidelity to be able to successfully spread through the host populations. Choosy males, which discriminate against mating with uninfected females, would therefore not have a fitness advantage. Under these conditions a preference mutation would not be able to spread through a population either.

This thesis, though it cannot answer all the questions that were asked in the beginning, presents a good foundation for further research in the area of endosymbiont infections in spiders. The knowledge acquired about the three study species *N. senegalensis*, *L. hortensis* and *L. triangularis* will be useful in future studies, investigating the effects of endosymbionts on their spider hosts and their potential of driving speciation processes.

List of Figures

Figure 1. Lock-and-key model..21
Figure 2. Titration–restitution (= sink) model 222
Figure 3. Slow-motion model 233
Figure 4: The lytic and lysogenic cycles of the reproduction of bacterial viruses and bacteriophages 31
Figure 5: External appearance of a spider´s body 377
Figure 6: The life cycle of an orb-weaving spider as example for the stages in a spider’s life. 429
Figure 7: Genital structures (epigyne) of haplogyne and entelegyne female spiders and pedipalp structures of haplogyne and entelegyne male spiders 42
Figure 8: Mature *N. senegalensis* female (left) and male (right) 54
Figure 9: Female and male *N. senegalensis* in the female’s web..................54
Figure 10: a) Body weight of adult males in the 2012 KwaZulu-Natal population (P_{lab}), b) Body weight of adult females in the 2012 KwaZulu-Natal population (P_{lab})..64
Figure 11: a) Body weight of adult males in the 2015 KwaZulu-Natal population (P_{field}), b) Body weight of adult females in the 2015 KwaZulu-Natal population (P_{field})..65
Figure 12: Phylogeny of *Wolbachia* based on the *wsp* gene.......................688
Figure 13: left: female *L. hortensis*, right: male *L. hortensis* 80
Figure 14: left: Female *L. triangularis*, centre and right: male *L. triangularis* 82
Figure 15: left: MagentaTM GA7 vessels for housing spiders, right: Wham box for mating trials 86
Figure 16: left: large desktop fan, used at lowest setting, right: hand-held fan, only one setting 88
Figure 17: *L. hortensis* on a toothpick stuck unto a Styrofoam block 899
Figure 18: Cephalothorax width in mm for populations from each country of origin in both years..94
Figure 19: Cephalothorax width in mm for females and males 95

Figure 20: Male and female cephalothorax width in mm compared between 2016 and 2017.. 96
Figure 21: Cephalothorax width in mm in female and male *L. triangularis*...97
Figure 22: Cephalothorax width of *L. triangularis* from the three countries of origin in both years....99
Figure 23: Comparison of cephalothorax width of *L. triangularis* in 2016 and 2017 100
Figure 24: Comparison of male and female *L. triangularis* cephalothorax width in 2016 and 2017 for all locations 101
Figure 25: Number of eggs/offspring in populations from all four locations in both years....106
Figure 26: Egg sac hatching rates in populations from all four locations in both years....107
Figure 27: Correlation between *L. hortensis* females' cephalothorax width and their number of eggs / offspring 107
Figure 28: Correlation between *L. triangularis* females' cephalothorax width and their number of eggs / offspring 109
Figure 29: Phylogenetic tree of linyphiids based on the *cytochrome oxidase I* of the mtDNA....11111
Figure 30: Result of a *Wolbachia* PCR run on the UK *L. hortensis*....12626
Figure 31: Proportion of infected *L. hortensis* individuals 12929
Figure 32: Proportion of infected *L. triangularis* individuals....13030
Figure 33: Phylogenetic tree of *Wolbachia* 15858
Figure 34: Phylogenetic tree of *Spiroplasma*....15959
Figure 35: Nucleotide sequences (position 185 – 291) of SPIR1, SPIR2 and the reference sequences of other spiders and arthropods....**Error! Bookmark not defined.**60
Figure 36: Phylogenetic tree of *Rickettsia*.....16363
Figure 37: Phylogenetic tree of WO phage 16565
Figure 38: Relationship between the found WO phage strains and the *L. hortensis Wolbachia* they were isolated from 167

Figure 39: Relationship between the found WO phage strains and the *L. triangularis Wolbachia* they were isolated from 16767
Figure 40: Progress of endosymbiont frequencies..................................18383
Figure 41: Progress of endosymbiont frequencies...................................1844
Figure 42: Polymorphism of the endosymbiont infection and the choice mutation gene 18585
Figure 43: Spread of the *Wolbachia* infection through the *M. fradeorum* population in 100 generations 18989
Figure 44: a) Spread of a choice mutation (unconditional recessive model), b) Spread of a choice mutation (unconditional dominant model); *Wolbachia* 191
Figure 45: a) Spread of a choice mutation (conditional recessive model), b) Spread of a choice mutation (conditional dominant model); *Wolbachia*....193
Figure 46: a) Spread of a choice mutation (unconditional recessive model), b) Spread of a choice mutation (unconditional dominant model); *Wolbachia/ Rickettsia*...1955
Figure 47: a) Spread of a choice mutation (conditional recessive model), b) Spread of a choice mutation (conditional dominant model); *Wolbachia/ Rickettsia*...19696
Figure 48: Spread of the *Wolbachia/Rickettsia* infection through the *L. triangularis* population 19797
Figure 49: Spread of a choice mutation (unconditional recessive model); *Wolbachia/Rickettsia*...19999
Figure 50: Spread of a choice mutation (unconditional dominant model); *Wolbachia/Rickettsia*...200
Figure 51: Spread of a choice mutation (conditional recessive model); *Wolbachia/Rickettsia*...201
Figure 52: Spread of a choice mutation (conditional dominant model); *Wolbachia/Rickettsia*...202

List of Tables

Table 1: Overview of the locations where *N. senegalensis* were collected ...55

Table 2: *N. senegalensis* from Namibia and the Eastern Cape.....................599

Table 3: Parental matrilines of *N. senegalensis* from KwaZulu-Natal 60

Table 4: Transmission frequency of the *Wolbachia* infection........................61

Table 5: Number of collected individuals of both species 85

Table 6: Sex ratio in *L. hortensis* populations 92

Table 7: Sex ratio in *L. triangularis* populations 92

Table 8: Primers used for the screening for *Wolbachia*, *Spiroplasma*, *Rickettsia* and WO phage..1244

Table 9: Number of *L. hortensis* infected with *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Wolbachia*/*Rickettsia* and *Wolbachia*/*Spiroplasma* 12727

Table 10: Comparing the *Wolbachia* infection frequencies of *L. hortensis* 12828

Table 11: Infection frequencies of the endosymbiont species *Wolbachia (Wol)*, *Spiroplasma (Spir)*, *Rickettsia (Rics)* and multiple infections in *L. triangularis*..13030

Table 12: Comparing the endosymbiont infection frequencies of *L. triangularis* collected at three different locations in 2016 and 2017. 13131

Table 13: Inter-population comparisons of endosymbiont proportions. 13333

Table 14: Male sex ratio and endosymbiont infection pattern 13434

Table 15: Comparing the proportions of the different endosymbionts in both sexes of *L. hortensis* 1366

Table 16: Male sex ratio and endosymbiont infection pattern...............13737

Table 17: Comparing the proportions of the different endosymbionts in both sexes of *L. triangularis*.. 13939

Table 18: Comparison of cephalothorax width in *L. triangularis* individuals carrying the different endosymbionts 14444

Table 19: Intra-population comparison of cephalothorax width in *L. triangularis* carrying the different endosymbionts 14646

Table 20: Mean cephalothorax width and intra-sex comparison of spider size in male and female *L. triangularis* carrying different endosymbionts.....14848
Table 21: Transmission rates of the different endosymbiont for each individual *L. hortensis* female which produced an egg sac 15454
Table 22: Values for the simulation parameters taken from Curry *et al.* *(2015)*..187
Table 23: Values for simulation parameters taken from the study of the endosymbionts in *L. triangularis* 18888

Abbreviations

CI = cytoplasmic incompatibility

COI = cytochrome oxidase I

DK = Denmark

f = female

Fig. = figure

GER = Germany

GLM = Generalised Linear Model

gyrB = gyrase B

KZN = KwaZulu-Natal

m = male

m.ceph.w. = mean cephalothorax width

mtDNA = mitochondrial DNA

N = sample size

NC = negative control

NS_KZN = *Nephila senegalensis*_KwaZulu-Natal

PC = positive control

PCR = polymerase chain reaction

Rics = *Rickettsia*

Spir = *Spiroplasma*

SSD = sexual size dimorphism

SWE = Sweden

UK = United Kingdom

Wol = *Wolbachia*

Wsp = *Wolbachia* surface protein

Chapter 1: Introduction

1.1 General background

Endosymbionts are bacterial microorganisms that are found within cells of host organisms. They have been shown to infect a wide range of arthropod and nematode species. Since the discovery of one particular type of endosymbiont, *Rickettsia,* in insect cells in the 1920s (Arkwright *et al.*, 1921; Cowdry, 1923; Cowdry, 1925; Hertig and Wolbach, 1924) the research body on these microorganisms has steadily grown. It has now been established that endosymbionts are likely to infect at least 40% of all terrestrial arthropods (Zug and Hammerstein, 2012). In the field of bacterial endosymbionts, the terms 'infection' and 'being infected' are used to indicate that an individual/population/species carries an endosymbiont. They do not mean that the endosymbiont is contagious. Instead, the passing-on of the endosymbionts is usually referred to as 'transmission'.

The endosymbionts of arthropods can be divided in obligate and facultative endosymbionts. The obligate endosymbionts are necessary for the host, for example for survival or reproduction (Hoerauf *et al.*, 1999; Russell and Stouthamer, 2011). A well-studied example is the relationship of the pea aphid *Acyrthosiphon pisum* with its primary endosymbiont *Buchnera aphidicola*. *B. aphidicola* provides the aphid with essential amino acids and nutrients. Removing the *Buchnera* from its host e.g. by antibiotic treatment will lead to the host's sterility or death (Douglas, 1992). In tsetse flies *Wigglesworthia glossinidia* is essential for the reproduction and for digestive processes, connected to vitamin supplementation. Flies cured of their *W. glossinidia* are sterile and are not provided with essential vitamins. Since tsetse flies are vectors of trypanosome parasites, which cause the fatal sleeping sickness disease in humans, this knowledge could prove very helpful for the development of biological control mechanisms (Pais *et al.*, 2008).

Facultative endosymbionts are not needed for the host's survival or reproduction. Nevertheless, a mutualistic or parasitic relationship has to be present for the endosymbiont to prevail in the host population (Oliver *et al.*, 2010). It has been found that a facultative endosymbiont belonging to the genus *Serratia* can partly take over the function of *Buchnera* should the host *A. pisum* be deprived of this obligate microorganism (Koga *et al.*, 2003). This finding is particularly interesting, because it helps researchers to understand how a microorganism changes from the facultative to the obligate state. Endosymbionts can also provide protection to the host. Two of the facultative endosymbionts found in *A. pisum* were found to provide protection against parasitism by the parasitic wasp *Aphidius ervi* (Oliver *et al.*, 2003). North American *Drosophila neotestacea* are rescued from nematode-induced sterility by *Spiroplasma*. Females infected with *Spiroplasma* show an elevated tolerance to the nematode *Howardula aoronymphium*, which otherwise would cause complete sterility (Jaenike *et al.*, 2010).

Obligate endosymbionts are strictly vertically transmitted from one generation to the next. Horizontal transmission does not occur. Facultative endosymbionts are mainly transmitted vertically, but horizontal transmission does happen and, from an evolutionary perspective, could explain the incongruence of host and endosymbiont phylogenies, which has been found for example in whiteflies (Thao and Baumann, 2004). The endosymbionts *Rickettsia*, *Wolbachia*, *Cardinium* and *Spiroplasma* are mainly spread through populations of their host via vertical transmission from females to their offspring. Males are not thought to be able to transfer the endosymbionts, which makes them an evolutionary dead end for the endosymbionts (Hurst, 1993), with the occasional reports of paternal transmission appearing to be rather circumstantial. To promote their spread through the populations, endosymbionts have evolved sophisticated mechanisms to promote their own transmission that include skewing the sex ratio of populations towards females. Three so called phenotypes caused by the endosymbionts are known to lead to distorted sex ratios. These mechanisms are male-killing,

feminisation and parthenogenesis induction (Hurst, 1993) and will be explained later on in this thesis (see Chapter 1.2.1).

Sex ratio distortion is, however, not the only mechanism by which endosymbionts ensure their own transmission. They are also known in a wide range of cases to cause incompatibilities between differently infected males and females of the same species. This phenotype is called cytoplasmic incompatibility (CI) (Hurst, 1993; see section 1.2.1). The discovery of CI led to the hypothesis that endosymbionts could play a role in reproductive isolation and the speciation process (Laven, 1967; O'Neill and Karr, 1990; see Chapter 1.2.3). CI has already been utilised in the effort to fight the spread of serious diseases such as dengue fever, spread by mosquitoes (Blagrove *et al.*, 2013; Hoffmann *et al.*, 2011; Joubert *et al.*, 2016).

Whilst there is a plethora of studies which investigated the role of endosymbionts in insect hosts, our knowledge of the effect these bacterial microorganisms have on spiders is still quite scarce. In the group of the arachnids, endosymbionts in ticks are the only group to have received considerable attention because they are vectors of human pathogens (i.e. certain strains of *Rickettsia*), which cause serious health issues in humans such as Rocky Mountain spotted fever (Demma *et al.*, 2005).

1.2 Endosymbionts

In the following section the phenotypes induced by the endosymbionts and the four most common endosymbionts will be introduced in detail.

1.2.1 Endosymbiont-induced phenotypes

Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) leads to an incompatibility between males and females of the same species (Ghelelovitch, 1952). In diploid species CI leads to zygotic cell death and therefore no offspring, whereas in haploid

species it usually leads to the production of males (Werren, 1997). The male's sperm is modified by the endosymbiont and can only successfully fertilise eggs which were modified by the same (or a compatible) endosymbiont. This modification of the sperm happens during the early stages of spermatogenesis since the bacteria are shed from maturing sperm and are eliminated in cytoplasmic "waste bags" (Bressac and Rousset, 1993).

The incompatibility can be uni- or bidirectional. Unidirectional incompatibility occurs when an infected male copulates with an uninfected female. The reciprocal cross is compatible. Bidirectional CI occurs when a male and female infected with different, incompatible strains of the same endosymbiont mate (Breeuwer and Werren, 1990; O'Neill and Karr, 1990). By "punishing" uninfected females through brood failure, infected females have an advantage and the spread of the endosymbiont is promoted (Turelli, 1994). An infected male mating with an uninfected female will, even though his direct investment is lost, or the outcome is drastically reduced, provide an advantage to his infected sisters. It has been observed that endosymbionts inducing strong CI are often present in populations at levels near fixation. Nevertheless, endosymbionts inducing only weak CI have been found to exist at medium prevalence i.e. in *Drosophila melanogaster* (Duron *et al.*, 2008).

CI is set up by two components: the modification of sperm by the bacterium in the male's testes (mod function) and a bacterial rescue in the fertilised egg (resc function). If the bacterium is not existent or an incompatible strain is present in the egg the sperm cannot be rescued and the incompatibility occurs (Werren, 1997). In their publication from 2003, Poinsot *et al*. (2003) reviewed the different mechanisms of *Wolbachia*-induced CI . The following models were discussed and compared with the CI patterns discovered so far: (1) Lock-and-key model, (2) Titration-restitution model and (3) Slow-motion model.

The lock-and-key model like mechanism was first proposed in different studies in the 1990s (Hurst, 1991a; Werren, 1997). This model assumes that

the mod function is due to the bacteria producing a lock that binds to the paternal nucleus. The paternal material is locked in and embryonal mortality occurs in crosses where the maternal material stems from an uninfected individual, since mitosis cannot be performed properly. If the bacteria are present in the egg then it acts as a key to remove the lock, thereby acting as the resc function, and maternal and paternal material remain compatible (Fig. 1). Two features of the lock-and-key model are (1) the mod and resc function are not the result of the same molecular mechanism and are determined by different bacterial genes and (2) the mod function enters the egg with the paternal chromosomes thereby facilitating a physical interaction between the mod and resc products (Poinsot *et al.*, 2003).

The titration-restitution or sink model (Fig. 2) assumes that the mod function is provided by the endosymbiont removing (titrating-out) some proteins which are normally associated with the host chromosomes (Kose and Karr, 1995; Kose *et al.*, 1995). This happens in the spermatocyte before maturation. The endosymbiont and the proteins are shed from the spermatocyte in the "waste bag" during maturation. This renders the spermatocyte non-functional. Whilst the endosymbiont will titrate-out the proteins in an infected oocyte as well, the endosymbiont and the proteins stay in the cytoplasm. When the titrated-out sperm cell enters an infected oocyte, the endosymbiont gives back the titrated-out proteins, hereby providing the resc function, and mitosis can proceed normally. In this model the mod and resc function could either be determined by the same or by different gene(s) (Poinsot *et al.*, 2003).

In the slow-motion model (also called the mistiming model) the mod function delays but does not entirely block the entry of the paternal chromosomes into mitosis (Callaini *et al.*, 1997). The nuclear envelope breakdown, which marks the entry into mitosis, is delayed for the paternal material (Tram and Sullivan, 2002). Hence the modification is due to the endosymbiont producing a factor which binds to the paternal chromosomes and slows down their movements

(Reed and Werren, 1995). This leads to unsynchronised sets of paternal and maternal chromosomes during first mitosis (Fig. 3). The resc function in this model is provided by a similar modification of the maternal material in the egg of an infected female. The slowing down of the maternal material will restore the synchronous cycle between the paternal and maternal complements and mitosis can commence. The mod and the resc function therefore result from the same molecular mechanism and are determined by the same gene(s) (Poinsot *et al.*, 2003).

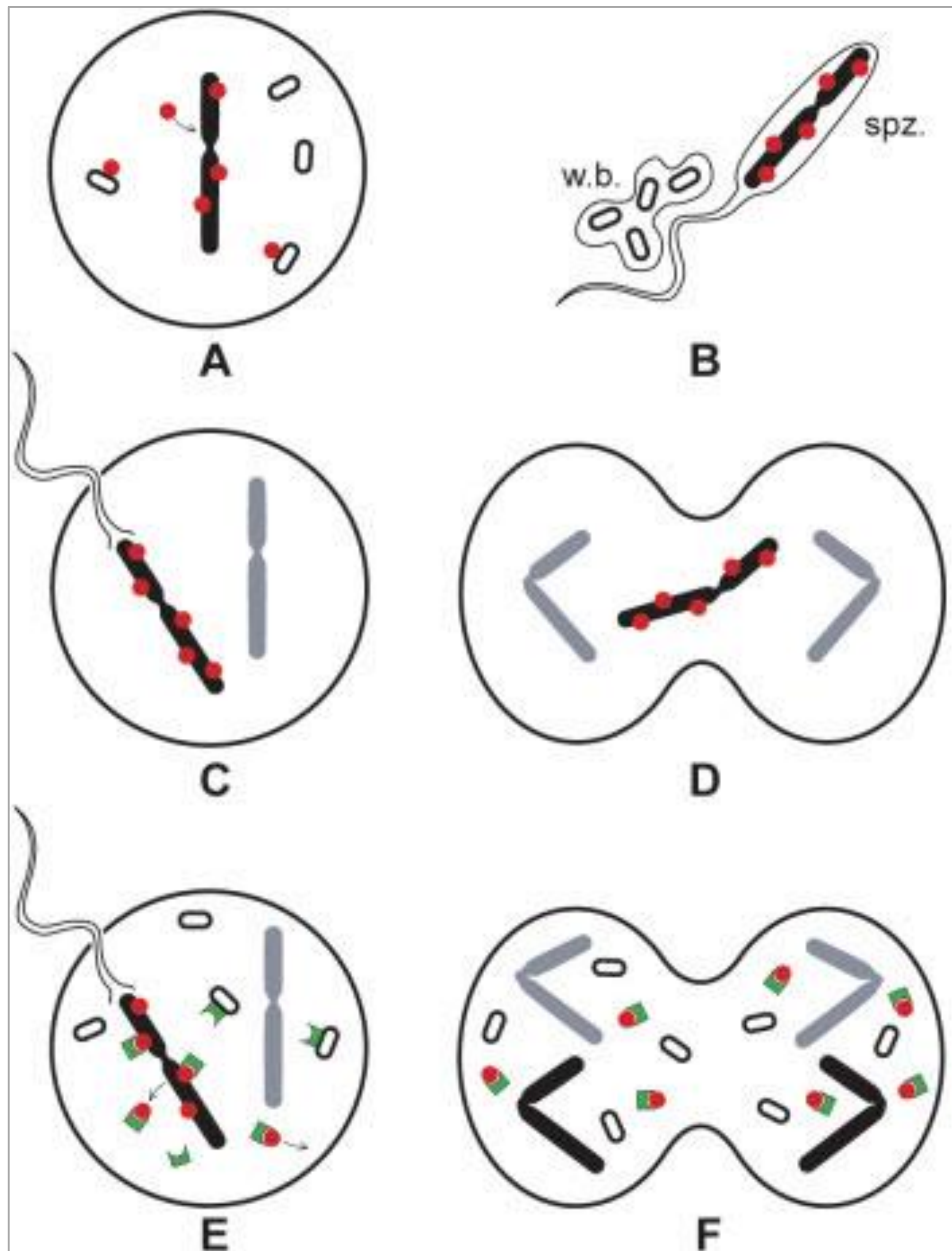

Figure 1. Lock-and-key model. A,B: Spermatogenesis in an infected male. A: *Wolbachia* (white symbols) produce a lock, (red circle) binding for example to paternal chromosomes (large black bar). B: The bacteria are then shed in a waste-bag structure (w.b.) with most of the cytoplasm and are therefore absent from the mature spermatozoon (spz). C, D: Incompatible cross between an infected male and an uninfected female. C: The sperm cell transporting ''locked'' paternal chromosomes enters an uninfected egg and meets unmodified maternal chromosomes (grey bar). D: In the absence of a key to remove the lock, paternal chromosomes are not functional and only maternal chromosomes take part normally in mitosis, which results in CI (death of the embryo in diplo-diploid species, production of a haploid male offspring in some haplo-diploid species). E,F: Compatible cross between two infected individuals. E: In an infected oocyte, *Wolbachia* produce a key (green symbol). F: The lock is thus removed from paternal chromosomes and mitosis takes place normally, rescuing the embryo. Taken with permission from Poinsot *et al*., 2003.

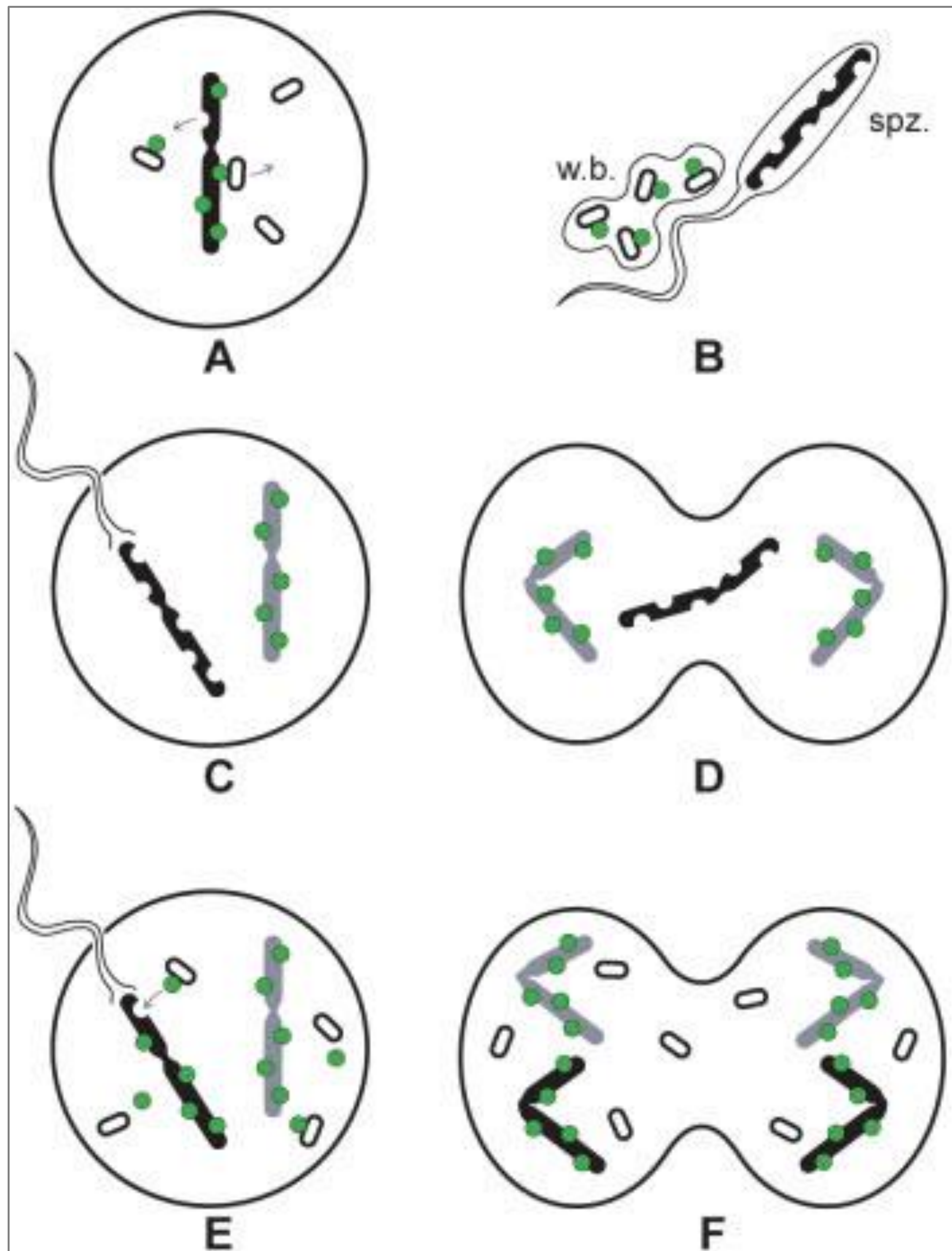

Figure 2. Titration–restitution (= sink) model. A,B: Spermatogenesis in an infected male. A: *Wolbachia* (white symbols) titrate-out a host protein (green circles) which is normally associated with chromosomes. B: The titrated protein is then expelled from the cell when *Wolbachia* are shed from the maturing spermatocyte, with most of the cytoplasm, in a waste bag structure (w.b.). Paternal chromosomes (black bar) in the mature spermatozoon (spz) are therefore missing the protein and are not functional. (NB: In an infected oocyte, the same phenomenon applies but the titrated-out protein is not lost and remains temporarily associated with *Wolbachia* in the cytoplasm of the oocyte). C,D: Incompatible cross between an infected male and an uninfected female. C: The ''titrated-out'' sperm cell enters an uninfected egg: due to lack of the host protein, paternal chromosomes are not functional and only maternal chromosomes (grey bar) take part normally in mitosis (D), which results in CI. E,F: Compatible cross between two infected individuals. E: In an infected oocyte, the *Wolbachia* give back to maternal and paternal chromosomes the host protein previously titrated-out. F: mitosis can now proceed normally, which rescues the embryo. Taken with permission from Poinsot *et al.*, 2003.

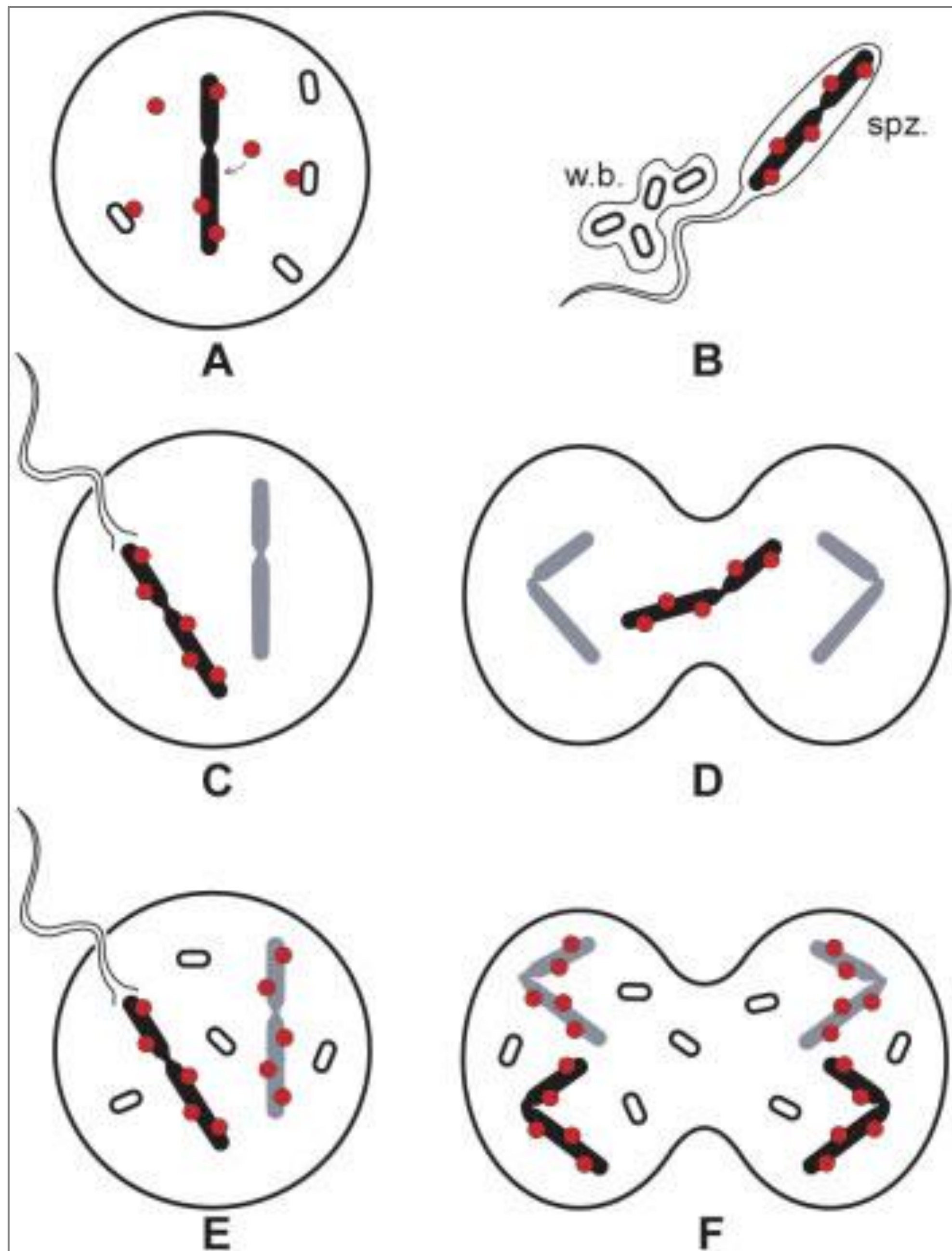

Figure 3. Slow-motion model. A,B: Spermatogenesis in an infected male. A: *Wolbachia* (white symbols) produce a slowing down factor (red circles) binding for example to paternal chromosomes (black bar) in such a way that they will go through the initial stages of mitosis at an abnormally slow pace. B: The bacteria are shed from the maturing spermatocyte, with most of the cytoplasm, in a waste-bag structure (w.b.). Paternal chromosomes in the mature spermatozoon (spz) remain loaded with the slowing down factor. (NB: in an infected oocyte, *Wolbachia* modify maternal chromosomes in the same way). C,D: Incompatible cross between an infected male and an uninfected female. C: a sperm cell bearing ''slowed-down'' paternal chromosomes enters an uninfected egg. D: by lagging behind maternal chromosomes (grey bar) during mitosis, paternal chromosomes are partially or totally lost, which results in CI. E,F: Compatible cross between two infected individuals. E: The sperm cell bearing ''slowed-down'' paternal chromosomes enters an oocyte infected by *Wolbachia*. F: Since maternal chromosomes are slowed down in the same fashion, both chromosome sets are synchronous, and the first mitosis proceeds normally (albeit at a slower pace than usual). Taken with permission from Poinsot *et al*., 2003.

Poinsot *et al*. (2003) concluded that the lock-and-key model, when confronted with the facts from different molecular studies on CI, "remains, in theory, the most parsimonious CI model currently available". Still, the molecular evidence for the lock-and-key model is lacking (Poinsot *et al.*, 2003).

Based on this conclusion and the fact that molecular evidence for the slow-motion model exists (Callaini *et al.*, 1997; Tram and Sullivan, 2002), Bossan *et al.* (2011) proposed another possible model for CI, namely the goalkeeper model. This model adds a quantitative dimension to the slow-motion model. It does not assume, in contrast to the lock-and-key model, that mod and resc are different functions determined by different genes. This makes it very suitable to explain known CI patterns. Also, two factors allow a countless number of endosymbiont strains that are bidirectionally incompatible to exist (Bossan *et al.*, 2011). Still, so far none of the proposed models has been confirmed as the true CI mechanism.

Another possible factor influencing the occurrence of CI is the density of bacteria. The bacterial density model proposes that CI is induced when bacterial density in the sperm and in the egg is not equal. In *Nasonia vitripennis* it was found that if males with a high bacterial load mate with females with a lower bacterial load, strong CI is induced. If the bacterial loads in the sperm and the egg are equal or the egg contains more bacteria then CI is weaker (Breeuwer and Werren, 1993).

CI has been shown in different species such as flour beetles (Wade and Stevens, 1985), mosquitoes (Yen and Barr, 1973), mites (Gotoh *et al.*, 2007) and parasitic wasps (Hunter *et al.*, 2003). So far only two of the common endosymbionts, *Wolbachia* and *Cardinium*, are known to cause this phenotype in their arthropod hosts (reviewed in Goodacre and Martin, 2012). The parasitic wasp *Encarsia inaron* is doubly infected with *Wolbachia* and *Cardinium*. Interestingly the study by White *et al.* (2009) showed that CI is only induced by the *Wolbachia* infection. *Cardinium*-induced CI could not be found.

The effect of the *Cardinium* infection could not be revealed in this study. *Cardinium* infected females are not able to rescue sperm from *Wolbachia* infected males. Neither does *Cardinium* lessen the occurrence of *Wolbachia*-induced CI. No sex ratio distorting effect of either of the endosymbionts was detected. It is therefore possible that *Cardinium* is neutral in *E. inaron* and is only kept in the population via perfect vertical transmission (White *et al.*, 2009). A recently conducted laboratory study showed that *Wolbachia* infections can also induce CI in spiders (Curry *et al.*, 2015).

Feminisation

Feminisation disrupts the sex ratio in a different way to CI, but the underlying mechanisms are also equally complex. Due to the infection of the female with an endosymbiont, male embryos develop into functional females. Feminisation was first described in *Armadillidium vulgar*e, a terrestrial isopod (Juchault *et al.*, 1992). This woodlouse species possesses heterochromosomes which are responsible for sex-determination (females: WZ, males: ZZ). Due to an infection with a feminising *Wolbachia* strain (F) this sex-determination system is overridden (Juchault *et al.*, 1992; Rousset *et al.*, 1992). The Z heterochromosome carries male genes which are responsible for the growth of the androgenic gland. Only this gland will secrete the hormones necessary for the development of the male gonads. *Wolbachia* inhibits the expression of these genes and thereby prevents the growth of the androgenic gland. Therefore, genetic males develop into functional females. Sometimes intersexes can be found. They can be either of female physiology with some male external characters or individuals with male physiology that are sterile.

A second parasitic sex factor (PSF) is at work in *A. vulgare*. This PSF (f) is part of the *Wolbachia* gene which was integrated into the host genome. It may be that this bacterial gene sequence is a transposable element. PSF f also induces the feminisation of genetic males but without the occurrence of intersexes (Juchault *et al.*, 1992). Feminisation has since been shown in a range of different species. In the butterfly *Eurema hecabe* an infection with *Wolbachia*

was found to be the cause of the feminisation of genetic males (Hiroki *et al.*, 2002). Further studies of *E. hecabe* and its relationship with *Wolbachia* have shown that also a CI inducing strain of *Wolbachia* is found in this butterfly species. Females that are doubly infected with the feminising and the CI inducing strain are capable of rescuing the sperm of CI infected males. Females carrying only the feminising strain are not capable of this rescue (Hiroki *et al.*, 2004). The doubly infected females produce all-female broods only, whereas females infected with the CI inducing strain alone produce broods with a 1:1 sex ratio (Narita *et al.*, 2011).

Feminisation of genetic males due to an infection with endosymbionts has further been observed in e.g. parasitic wasps and leafhoppers. The asexual reproduction of the parasitic wasp *Encarsia hispida* depends on the feminisation of genetic males induced by an infection with *Cardinium*. The asexuality is fixed but male production can be induced by feeding infected adult females with antibiotics. The antibiotic-treated females produce only uninfected male offspring. Like the females, these males are diploid. *Cardinium* feminises the unfertilised diploid eggs, which will develop into functional females. The antibiotic treatment of the infected females has a negative effect on offspring production. The most possible explanations are either that the diploid males suffer from a higher mortality rate during development or that *Cardinium* is necessary for a normal level fertility in this species. *Cardinium* may play a very early or no role at all in the diploidy restoration of unfertilised eggs. *Cardinium* is responsible for the feminisation of diploid male embryos and therefore must in some way interact with the host sex determination system (Giorgini *et al.*, 2009).

The leafhopper *Zyginidia pullula* is infected with a strain of *Wolbachia* which causes the feminisation of genetic males into females. The feminised males have a male genotype and a female phenotype but also show some outer male characteristics. Infected females that do not show the outer male characteristics are of the female genotype (normal females). If normal

females are treated with antibiotics, they produce broods with sons and daughters. The feminised males are vital and reproduce. Copulation is observed often but progeny are only seldom produced (Negri *et al.*, 2006).

Male-killing

Spiroplasma, Gamma-proteobacteria, *Rickettsia*, Flavobacteria and *Wolbachia* are known to induce the death of male offspring in different arthropod species, e.g. ladybirds (Hurst *et al.*, 1997; Hurst *et al.*, 1999b; Majerus and Majerus, 2010), fruit flies (Malogolowkin, 1958; Williamson *et al.*, 1999), butterflies (Hurst *et al.*, 1999a; Jiggins *et al.*, 1998) and pseudoscorpions (Zeh *et al.*, 2005). This leads to the loss of nearly 50% of an infected female's offspring in the case of particular species of butterfly (Jiggins *et al.*, 1998). This was also found in the ladybird *Adalia bipunctata* (Hurst *et al.*, 1992). The death of males is found to be induced at an early or a later stage in embryonic development (Hurst, 1993).

What appears to be a significant disadvantage to infected females and the males that mate with them does however provide benefits to their surviving, female biased, offspring. Three main ways in which male-killing benefits infected sisters are found in the literature: (1) Resources will be reallocated from killed males to surviving females, (2) inbreeding between siblings will be prevented and (3) cannibalism of females will be reduced (Hurst *et al.*, 1992; Hurst, 1991b).

Behavioural changes as a consequence of infection with a male-killing endosymbiont have also been observed, for example in the butterfly *Acraea encedon* and the closely related *Acraea encedana*. Due to an infection with *Wolbachia*, populations show a female-biased sex ratio. Jiggins *et al.* (2000b) found a direct connection between the infection with *Wolbachia* and the butterfly behaviour. Populations showing a normal sex ratio could be found at sites providing the larval food plants and mating pairs could be observed. In strongly sex ratio biased populations, females did form swarms at sites

lacking larval food and adult nectaring plants and tried to attract males for mating. This so-called lekking is known from other insect species but usually the males form leks and the females choose (Jiggins *et al.*, 2000b).

Parthenogenesis

From an endosymbiont's perspective, the ultimate manipulation of the reproductive system of its host to its own advantage would be to drive female development while males are made superfluous (Cordaux *et al.*, 2011). Parthenogenesis is caused by a disruption of the cell cycle during early embryonic development. This disruption causes the diploid development of unfertilised eggs (Werren *et al.*, 2008). At least three endosymbionts, namely *Wolbachia*, *Rickettsia* and *Cardinium*, are capable of this manipulation of their host's reproductive system. They induce parthenogenesis in haplodiploid insect and tick species (Cordaux *et al.*, 2011).

In haplodiploid species, males develop from haploid, unfertilised eggs while females develop from diploid, fertilised eggs. The endosymbionts are able to transform not-transmitting males to transmitting females. This is achieved by enabling unfertilised eggs to develop as females. Unfertilised eggs are rendered diploid through the doubling of chromosomes which leads to female development (Cordaux *et al.*, 2011). At least three mechanisms are known that lead to the chromosome doubling in unfertilised eggs: (1) In *Trichogramma*, a genus of parasitic wasps, the two haploid sets of chromosomes do not separate resulting in one diploid nucleus containing two sets of similar chromosomes (Stouthamer and Kazmer, 1994). (2) After a normal first mitotic division in *Muscidifurax uniraptor* which results in two cells with haploid nuclei, the two cell nuclei fuse to form one diploid nucleus (Gottlieb *et al.*, 2002). 3) In *Bryobia* mites, meiotic modification in infected eggs leads to diploid gametes (Weeks and Breeuwer, 2001).

In *Trichogramma,* it is possible to restore the production of males by the means of antibiotics or heat treatment (Stouthamer *et al.*, 1990). In *M.*

uniraptor the parthenogenic mode of reproduction is irreversible. A study by Gottlieb and Zchori-Fein (2001) showed that female *M. uniraptor* are not sexually receptive at all, regardless of their infection status. In addition to the never/rarely occurring matings, males are also not capable of producing mature sperm. Thus, even if matings occur, fertilisation of eggs is impossible. A third factor that leads to irreversible parthenogenesis is that a major muscle that impairs efficient sperm transport and storage is absent from the females' spermathecae. The receptivity of females and the function of the spermathecae are not reactivated by the elimination of *Wolbachia* (Gottlieb and Zchori-Fein, 2001).

The main difference between feminisation and parthenogenesis consists in the first changing genetic males into phenotypic females while the latter changes genetic males into genetic females (Cordaux *et al.*, 2011). Also, parthenogenesis does not require the female to mate with a male to reproduce whilst fertilisation is necessary for feminisation to occur.

1.2.2 Endosymbiont species

Wolbachia

One of the best studied obligate intracellular microorganisms is *Wolbachia pipientis*. Discovered by Hertig and Wolbach in the gonads of *Culex pipiens* in 1924 and named by Hertig in 1936 (Hertig, 1936; Hertig and Wolbach, 1924). *Wolbachia* are gram-negative, rod-shaped, alpha-proteobacteria belonging to the order Rickettsiales.

A big scale screening showed that at least 66% of all insect species are infected with *Wolbachia* (Hilgenboecker *et al.*, 2008). Transmission of *Wolbachia* happens mostly vertically from infected females to their offspring. Although, at least on an evolutionary timescale, some events of horizontal transmission must have occurred (Werren, 1997). Since its discovery many different strains of *Wolbachia* have been found. *Wolbachia* is currently defined as one species with further classification into 13 supergroups (A - N) (Ellegaard *et al.*, 2013).

The examples outlined earlier in this report show how drastic the effects of an infection with *Wolbachia* can be for the host. So far *Wolbachia* is the only endosymbiont known to induce all four phenotypes (CI, feminisation, parthenogenesis and male-killing) (reviewed in Goodacre and Martin, 2012).

Bacteriophage infecting *Wolbachia*

A bacteriophage has been identified that infects the *Wolbachia* found in arthropods, bacteriophage WO or WO phage. It was proposed that 89% of parasitic supergroup A and B *Wolbachia* are infected with the phage (Bordenstein and Wernegreen, 2004). The WO phage was first described by Wright in 1978 who, through morphological examination, detected it in *Wolbachia*. The *Wolbachia* seemed to by lysed by the phage which is consistent with the lytic cycle (Fig. 4) of bacteriophages and viruses (Wright *et al.*, 1978).

The reproduction of temperate phages is described using the *Escherichia coli* infecting phage lambda (or λ) as an example. A temperate phage has two possible ways to reproduce. One way is called the lytic the other the lysogenic cycle (Fig. 4). During the lytic cycle the phage replicates in the host cell and eventually lyses it, killing the host in the process. The now emerged phages go on to infect new cells. In the lysogenic phase the phage is integrated into the host genome as a prophage and the lytic gene expression is switched off. The phage DNA then replicates as part of the host genome during the following cell divisions. A phage can switch from the lysogenic phase to the lytic state, this is induced i.e. by DNA-damaging agent such as UV-light (Oppenheim *et al.*, 2005).

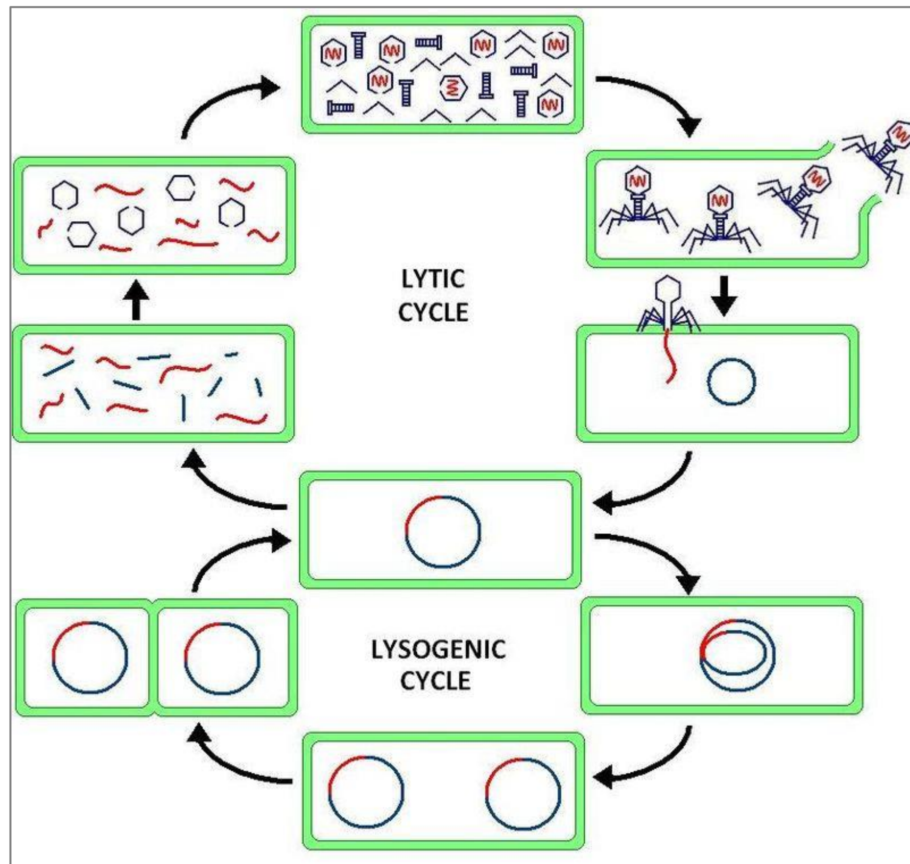

Figure 4: The lytic and lysogenic cycles of the reproduction of bacterial viruses and bacteriophages. Bacteriophages which use only the lytic cycle are called virulent phages whilst those using both cycles are referred to as temperate phages. Copyright: Suly12 [GFDL (http://www.gnu.org/copyleft/fdl.html)].

It was not until 2000 that the presence of WO phage could be molecularly confirmed (Masui *et al.*, 2000). It was established that most *Wolbachia* carry the phage and the association between the two seems likely to be evolutionarily ancient on the basis of the phylogenetic placement of phage and bacterial sequences. The phage is passed on vertically from mother to offspring, along with the bacterium, and as such the two might be expected to be in complete linkage disequilibrium with one another. At this stage the phage is dormant in the lysogenic phase.

Analysis of a phage gene indicated however that there is also horizontal transmission of phage amongst *Wolbachia* strains (Masui *et al.*, 2001). The WO phage is the first genetic element to be known to traverse the boundary between *Wolbachia* supergroups A and B. In addition to the apparent horizontal transmission of the WO phage, it has also been proposed that the

phage can be transmitted paternally into eggs that harbour a phage-free *Wolbachia* strain, during fertilisation (Kent and Bordenstein, 2010).

It is possible that the WO phage plays a role in the expression of the sexual parasitism of *Wolbachia*. A causal relationship with the strength of CI is suggested because it has been found that endosymbiont densities influence the strength of CI. CI is stronger when more *Wolbachia* are present in the sperm than in the egg (Breeuwer and Werren, 1993). Due to its lysing capability WO phage densities are inversely associated with *Wolbachia* densities. The higher the phage density the lower the *Wolbachia* density. Bordenstein *et al.* (2006) established the phage density model by showing that in *Nasonia vitripennis* CI was significantly reduced when phage density was high and *Wolbachia* density was low. This also provides endorsement for the bacterial density model of CI.

A study on the spider mite *Tetranychus urticae* suggests that the changing of WO phage from the lysogenic to the lytic cycle could be due to the accumulation of phage abundance with age of the host. Temperature also seems to have an influence. The abundance of WO phage was higher at temperatures extreme for the mites whilst *Wolbachia* frequency was lower. This negative correlation between WO phage and *Wolbachia* was positive at normal temperatures (Lu *et al.*, 2012). The change from lysogenic to lytic cycle will not happen in all *Wolbachia,* infecting a host, at the same time. The phage, which are still in the lysogenic cycle, can be transmitted to the next host generation as part of the *Wolbachia* genome. The phage will therefore not eradicate the *Wolbachia* from a population, but it will potentially impair the endosymbionts ability to express their phenotype.

So far only one study exists investigating the occurrence of WO phage in *Wolbachia* infecting spiders (Yan *et al.*, 2015). The study found WO phage in seven species from China: *Araneus alternidens*, *Nephila clavata*, *Hylyphantes graminicola*, *Pholcus crypticolens, Prosoponoides sinensis*, *Coleosoma*

octomaculatum and *Nurscia albofasciata*. Frequency of the phage ranged from 60 to 100%. Laboratory experiments with *H. graminicola* showed that WO phage is transmitted vertically from one generation to the next (Yan *et al.*, 2015). Unfortunately, the authors do not state where exactly in China the spiders were collected. The spiders which were found to harbour phage-infected *Wolbachia* belong to five different spider families: Araneidae, Linyphiidae, Pholcidae, Theridiidae and Titanoecidae. This could indicate that the prevalence of the WO phage in *Wolbachia* infecting spiders is high and widespread across family boundaries.

Rickettsia

Other well-characterised endosymbionts in the order Rickettsiales are the *Rickettsia* which give the order its name. *Rickettsia,* like *Wolbachia,* are gram-negative alpha-proteobacteria that live exclusively inside eukaryotic cells. Their shape can be coccoid, rod-like or thread-like.

In contrast to *Wolbachia*, *Rickettsia* can be harmful pathogens of humans. Ticks, for example, transfer *Rickettsia rickettsii,* which causes the dangerous Rocky Mountain spotted fever in humans (Demma *et al.*, 2005). In arthropods *Rickettsia* have been found to induce parthenogenesis and male-killing (reviewed in Goodacre and Martin, 2012). *Rickettsia*-induced male-killing is well-established e.g. in the ladybird species *Adalia bipunctata* (Hurst *et al.*, 1999b) and *Propylea japonica* (Majerus and Majerus, 2010).

Cardinium

Candidatus Cardinium belong to the Bacteroidetes phylum and are rod-shaped, gram-negative intracellular symbionts of eukaryotes that are transmitted transovarially (Zchori-Fein *et al.*, 2004). *Cardinium* has unique striate-like structures attached to its inner membranes. These structures have been found in bacteria associated with other invertebrate species, which suggests that *Cardinium* may indeed be widely distributed (Zchori-Fein and Perlman, 2004).

Cardinium is found in mites (Weeks *et al.*, 2001), ticks (Kurtti *et al.*, 1996), parasitic wasps (Zchori-Fein *et al.*, 2001; Zchori-Fein *et al.*, 2004), biting midges (Nakamura *et al.*, 2009), planthoppers (Marzorati *et al.*, 2006; Weeks *et al.*, 2003; Zchori-Fein and Perlman, 2004) and plant-parasitic nematodes (Noel and Atibalentja, 2006). Also, *Cardinium* seems to be common in spiders with approximately 20% of all species carrying the infection (Duron *et al.*, 2008; Martin and Goodacre, 2009; Perlman *et al.*, 2010).

The following phenotypes manipulating host reproduction are known to be caused by *Cardinium*: feminisation in mites (Weeks *et al.*, 2001), thelytokous parthenogenesis in parasitic wasps (Zchori-Fein *et al.*, 2001; Zchori-Fein *et al.*, 2004) and scale insects (Provencher *et al.*, 2005) as well as CI in parasitic wasps (Hunter *et al.*, 2003) and mites (Gotoh *et al.*, 2007; Ros and Breeuwer, 2009).

Spiroplasma

Spiroplasma are helical, motile mycoplasmas belonging to the Mollicutes. The absence of structures like a cell wall or axial filaments distinguished the *Spiroplasma* from spirochetes and other true bacteria (Tully *et al.*, 1977). *Spiroplasma* have been found across a wide range of different taxa e.g. plants (Clark, 1977), vertebrates (Tully *et al.*, 1977) and arthropods (Clark, 1977; Hurst *et al.*, 1999b; Jiggins *et al.*, 2000a; Williamson *et al.*, 1999).

Different studies have shown that *Spiroplasma* can induce male-killing in infected females. Male-killing has been found e.g. in fruit flies (Williamson *et al.*, 1999), ladybirds (Hurst *et al.*, 1999b) and butterflies (Jiggins *et al.*, 2000a). It is also a known pathogen that causes disease in a wide range of species. In the Chinese mitten crab *Eriocheir sinensis,* for example, infection with *Spiroplasma* causes tremor disease. Infected crabs show signs of weakness, anorexia, intense paroxysmal tremors and eventually die of the disease (Wang *et al.*, 2004).

1.2.3 Endosymbionts and speciation

In 1967, Laven discussed CI in the *Culex pipiens* complex and attributed the observed incompatibilities to factors that “are inherited through the cytoplasm” (Laven, 1967). Unknowingly he described endosymbiont induced CI. Yen and Barr were the first to propose that *Wolbachia pipientis* could be the CI inducing factor in *Culex* mosquitoes (Yen and Barr, 1971), which might have contributed to the speciation process. Since then the body of work on endosymbiont induced CI and its potential role in speciation has steadily been growing. Reproductive isolation of populations of the same species represents one step on the way to the evolution of new species (Dobzhansky, 1935). It has already been found that populations of the same species are differently infected with or harbour different strains of endosymbionts (Yun *et al.*, 2011). These unequal infections can lead to the occurrence of CI between these populations.

In the Northern Corn Rootworm (*Diabrotica barberi*) two populations in the US state of Illinois were found to carry different strains of *Wolbachia*. When the authors of the study investigated the mtDNA of both populations they found a genetic boundary between the two populations. Individuals of the two populations did not successfully reproduce with one another. This led to the assumption that the two *Wolbachia* strains are not compatible with one another and therefore lead to reproductive isolations of the two populations (Roehrdanz and Levine, 2007).

In another case, CI induced by *Wolbachia* represents one of two factors which, in combination, lead to the reproductive isolation of two species of *Drosophila*. *Drosophila subquinaria* males are not accepted by *D. recens* females because they do not display the correct mating/courtship behaviour. *D. subquinaria* females on the other hand do readily accept *D. recens* males for mating but due to the presence of a CI inducing *Wolbachia* these crosses lead to a very reduced number of hybrid progeny. Both factors together

reproductively isolate these two *Drosophila* from one another (Shoemaker *et al.*, 1999).

These examples show that not only evolutive changes in the host DNA but also in their endosymbiont composition and endosymbiont DNA can lead to reproductive isolation of different populations of the same species. The likelihood of populations being unequally infected is elevated if these populations are found at geographically distant locations from one another, especially if one acknowledges the potential of horizontal transfer of endosymbionts (Ahmed *et al.*, 2013; Schuler *et al.*, 2013).

1.3 Why are spiders a good model system?

1.3.1 Diverse reproductive biology, behavioural strategies and ecology

Spiders are a group of invertebrates belonging to the class Arachnida in the phylum Arthropoda. Like almost all arachnids, spiders have four pairs of walking legs, which makes them easily distinguishable from other arthropods i.e. insects or malacostraca. Their bodies consist of two parts, the prosoma or cephalothorax and the opisthosoma or abdomen. The chitinous exoskeleton of the cephalothorax is hardened and provides the attachment point for the walking leg pairs. An additional pair of leg-like appendages, the pedipalps, are situated at the front of the prosoma. In male spiders the pedipalps evolved into sophisticated copulatory organs. The much softer opisthosoma contains the vital organs such as heart, lungs and intestines as well as the silk glands (Fig. 5).

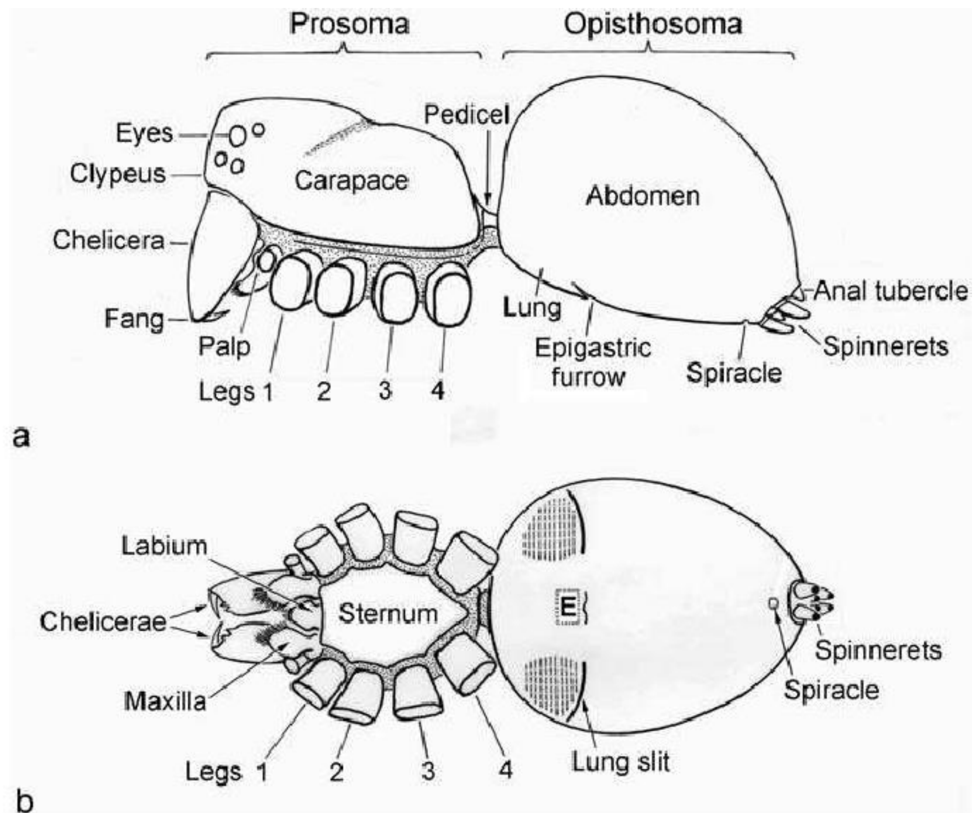

Figure 5: External appearance of a spider´s body: (a) side view, (b) ventral view. E = epigynum (in adult females), taken from: Biology of Spiders (Foelix, 2011)

Most spiders have eight eyes, with a few groups having only six eyes such as six-eyed spiders (Dysderidae) or spitting spiders (Scytodidae). Even fewer species show a reduction to four or two eyes and in some cave-dwelling spiders the eyes have been lost completely. The arrangement and size of the eyes can be used to determine the family an individual belongs to (Foelix, 2011).

In this thesis the following terms will be used to describe different developmental stages of spiders. **Spiderlings** will be used for juvenile spiders, which have just emerged from the egg sacs (yolk-feeding and prey-feeding) and have not dispersed yet. **Juveniles** are those spiderlings, that have dispersed from the hatching site and have built their own individual webs. These juveniles are exclusively feeding on prey, which they catch themselves. **Subadult** will be used for individuals, which will reach maturity with the next

moult. This is also called penultimate. Finally, **adult** spiders are males and females, which are sexually mature and can reproduce.

The life cycle of a spider, shown in Figure 6, can be roughly divided into seven stages. (1) The life of a spider starts as an egg in the egg sac. (2) The eggs hatch inside the egg sac and the spiderlings will spend the first 1 – 3 stages of their life in the egg sac, entirely depending on their yolk-sac for nourishment. (3) When the spiderlings emerge from their egg sac, they soon start to feed on prey. They often stay together in a shared web for a while. (4) The juveniles begin to disperse and to build their own webs. They catch prey on their own and continue to grow through moulting. (5) Subadult individuals, especially females, look very much like adult individuals apart from being smaller. (6) The spiders moult to maturity. Most spider species will stop moulting once they reached maturity. One exception are female tarantulas, which can continue to moult, and grow, until the end of their life (Foelix, 2011). At this stage, mature males will start to look for females. (7) Male and female copulate. In the case of orb-weavers, copulation happens in the web of the female. (1) Within a few weeks of copulation, the female will produce an egg sac. In orb-weavers the females often die shortly after egg sac production (depicted by the black cross and spider icon). However, in other species the females will look after the egg sac and the young. For example, in wolf spiders the female will carry the egg sac with her attached to her spinnerets. The mother will open the egg sac with her chelicera and will help the spiderlings to emerge. The spiderlings will then climb onto her back, where they will remain for about a week (Rovner *et al.*, 1973). In cases like this the female will die when the spiderlings are able to catch prey for themselves.

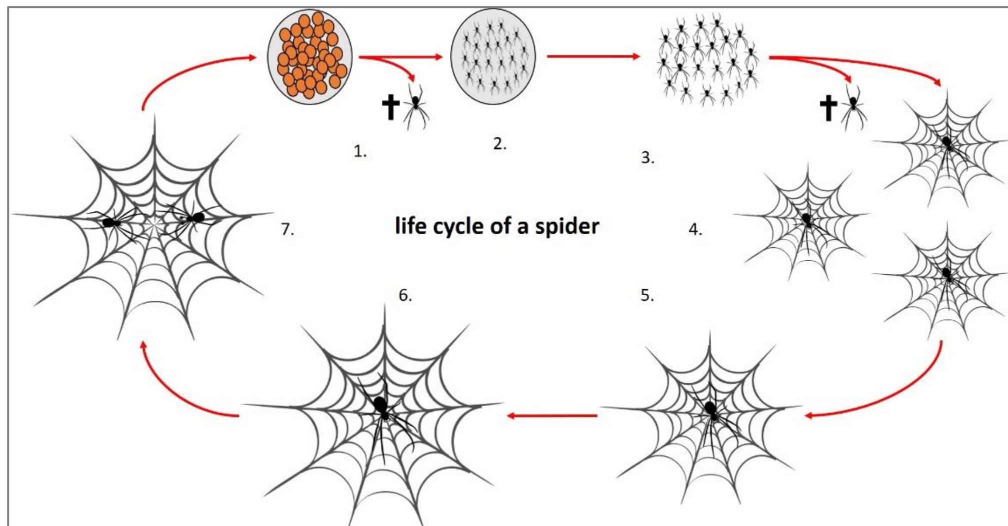

Figure 6: The life cycle of an orb-weaving spider as example for the stages in a spider's life. The black cross indicates the death of the mother. In most species the mother dies after production of her egg sac(s)(between 1. and 2.). In some species maternal care can be observed and the mother dies after the spiderlings have emerged from the egg sac (between 3. and 4.).

The World Spider Catalog currently lists 117 families of spiders containing 47,622 species in 4,093 genera (World Spider Catalog, 2018). Spiders have conquered every continent apart from Antarctica and are found in almost all habitats apart from the air and the sea.

Spiders are predators that prey mainly on insects and other spiders. Some species have been reported to occasionally prey on vertebrates (Dunbar *et al.*, 2018; Nyffeler *et al.*, 2017; Silva and Meirelles, 2016). They kill their prey by a venomous bite. At the same time digestion enzymes are injected which will liquify the prey from the inside. The spider can then suck up these liquid insides leaving only a desiccated husk of the prey. The only spider known off to be mostly herbivorous is *Bagheera kiplingi*, which feeds off Beltian food bodies found on ant-acacias in the dry forests of Central America (Meehan *et al.*, 2009). These Beltian food bodies are rich in proteins, sugars and lipids.

One very well characterised and diverse trait of spiders is the ability to produce webs made of silk to trap prey. The webs of the web-building spiders, or Araneae, can be divided into different types i.e. orb-webs, sheet-webs, frame or tangle-webs. These webs serve different functions. The orb-web and

tangle-webs contain sticky threads of silk and once prey get stuck in the web the spider can kill and consume them. Most parts of the sheet-webs of Linyphiids are not sticky themselves, but flying insects get trapped between so-called "knock-down" threads. The spider will shake these threads so that the prey falls onto the sheet (Foelix, 2011). However, not all spiders build webs, some species are sit-and-wait predators, such as members of the Lycosidae (wolf spiders) and others are active foragers, i.e. Salticidae (jumping spiders) (Nentwig, 1986).

Spiders can be grouped into three categories regarding their lifestyle: Cooperatively breeding social, colonial and solitary spiders. Social species such as *Anelosimus eximius* live in large communal webs in which they capture prey and feed together as well as cooperatively raise their young (Bilde and Lubin, 2011). Due to spiderling dispersal being lost in the social spiders, the geneflow between these social spider populations is restricted and the populations are often highly inbred (Bilde and Lubin, 2011). Colonial spiders, i.e. *Cyrtophora* do also inhabit large web structures, but every individual keeps its own web and defends its territories. No cooperation in prey capture or brood care takes place. The individual webs are interconnected and share common frame threads (Bilde and Lubin, 2011). Solitary spiders present the largest of the three groups. These spiders live a solitary lifestyle apart from during the mating season. The individuals hunt/catch their food by themselves (males usually stop foraging after reaching maturity) and other spiders in their territory will be seen as intruders. Cannibalism between solitary spiders does occur frequently with some, i.e. the salticid genus *Portia*, actively preying on other spiders (araneaophagic) (Jackson and Hallas, 1986).

1.3.2 Spider reproductive biology

Spiders have evolved very sophisticated copulatory organs and genital structures. During copulation the male inserts his pedipalps, which have evolved into copulatory organs, into the female's genital opening, the epigyne, to transfer sperm and fertilise the female's eggs. Spiders can be

divided in two groups in regard to their genital and copulatory structures, haplogyne and entelegyne.

Haplogyne females possess one genital opening from which insemination ducts lead to the spermathecae, where the male's sperm is stored. The sperm and the eggs "meet" closely behind the genital opening where fertilisation takes place (Fig. 7a). Entelegyne female spiders have two separate genital openings (gonopores) connected by insemination ducts to the spermathecae. The sperm are transported from the spermathecae via fertilisation ducts to "meet" the eggs released from the ovaries (Fig. 7b). Both, haplogyne and entelegyne spider males use their pedipalps to inseminate the females. At the end of their palps a structure called a bulb is used to transfer the sperm into the females. The structure of haplogyne bulbs is much simpler than that of the entelegyne species (Fig. 7c and 7d).

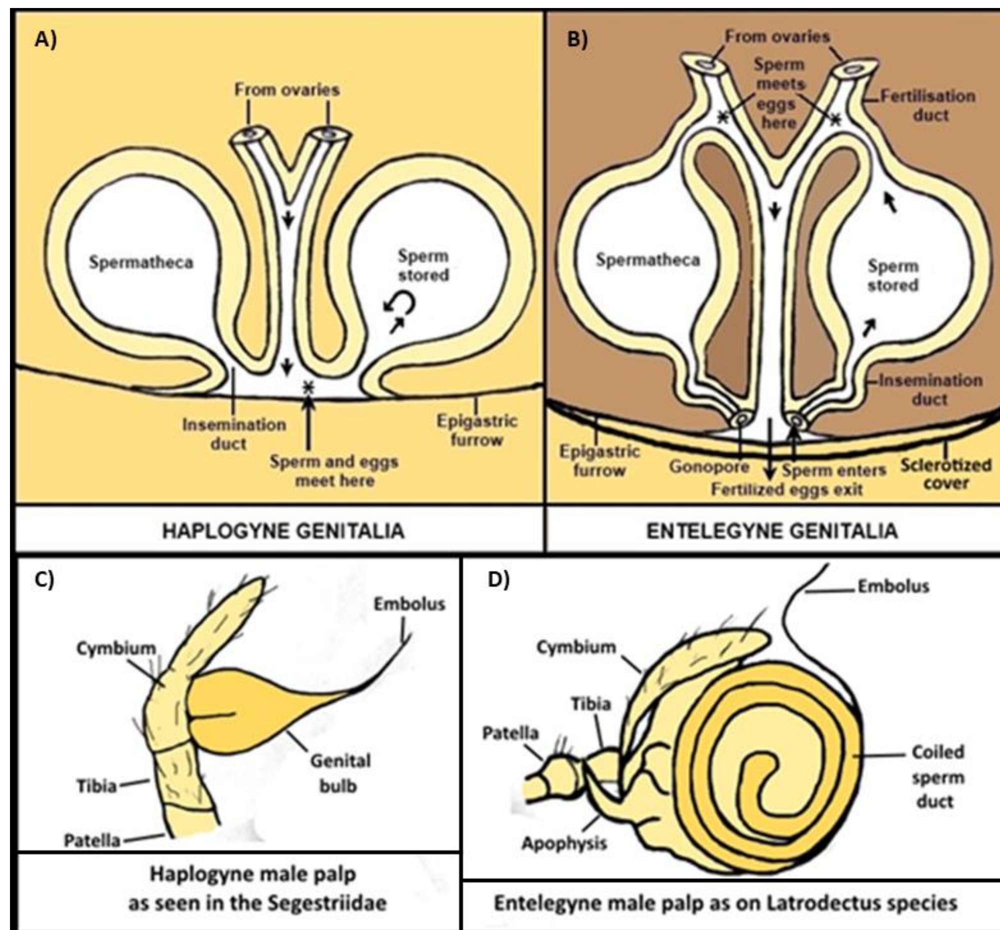

Figure 7: Genital structures (epigyne) of haplogyne (A) and entelegyne (B) female spiders. Pedipalp structures of haplogyne (C) and entelegyne (D) male spiders. Copyright: Ron Atkinson, www.findaspider.org.au/info/reproduction.htm.

Both male and female genital structures are used to unambiguously identify spiders since they are highly specialised in the different species (Foelix, 2011). Pedipalp and epigyne were previously viewed as a lock-and-key system but evidence suggests that often other mechanisms i.e. behaviour, habitat range or temporal factors prevent hybridisation of closely related species rather than physiological differences (Kunz *et al.*, 2015; Ware and Opell, 1989).

Spiders show a considerable variety of mating strategies and behaviours. Beginning with the courtship of the male, strategies include web reduction as in *Linyphia* (Watson, 1986), opportunistic mating e.g. in *Nephila* (Elgar and Fahey, 1996), nuptial gifts in *Pisaura mirabilis* (Stalhandske, 2001), substrate drumming in wolf spiders (Rovner, 1975) or the courtship dance of the Australian peacock spider *Maratus volans* (Girard *et al.*, 2011).

When accepted for mating by the female, there are a range of different behaviours that follow. In sheet-web spiders, so-called pseudo-copulation is often observed, where the male inserts his pedipalps into the female's epigyne without transferring sperm. He will leave the female after pseudo-copulation to build a sperm-web and fill his pedipalps with sperm. After that he returns to the female to copulate with her again, this time with sperm transfer (Gunnarsson *et al.*, 2004; van Helsdingen, 1965; Weldingh *et al.*, 2011). It remains unclear so far what purpose these pseudo-copulations fulfil, but it has been proposed that they function as copulatory courtship or mate guarding (Weldingh *et al.*, 2011).

In other species the males will break off the tip of their pedipalp after copulation to plug up the female's genital opening (reviewed in Uhl *et al.*, 2010). This genital mutilation prevents other males from successfully copulating with this female. It also limits the males to two possible copulations. This stands in contrast to those of polygynous/polyandrous species. In these species males and females can re-mate multiple times and the female can store the sperm of multiple males in her spermathecae over a long period of time (Snow and Andrade, 2005). This is a prerequisite for sperm competition to occur.

Sperm competition is the competition of the spermatozoa of two or more males for the fertilisation of the female's ova (Parker, 1998). Sperm competition has been observed in insects (Simmons and Siva-Jothy, 1998), birds (Birkhead, 1998), fish (Taborsky, 1998) and mammals (Stockley, 2004). The theory states that sperm of high quality should outcompete sperm of inferior quality. Many studies indicate that females are able to choose which male's sperm they use for the fertilisation of the eggs. This postcopulatory selective process is called cryptic female choice. Among the female processes utilised in the choice are: discarding of sperm, re-mating with other males, reducing the oviposition rate or the removal of spermatophores (Eberhard, 1997).

Multiple matings are not always easily archivable for males, since in some species females have developed the habit of cannibalising their mating partner after copulation. The most famous example for this behaviour is the black widow spider (*Latrodectus* spp.). The males of this species almost always get cannibalised by the females. In fact, it has been found that *Latrodectus hasselti* males do sacrifice themselves to prolong the copulation and thereby ensure a high paternity rate. The male will perform a somersault onto the female's ventral side with his abdomen and legs landing close to the females mouth parts. He will then copulate with her whilst she will start devouring him (Andrade, 1996; Andrade, 2003; Snow and Andrade, 2004).

1.3.3 Spiders as model systems

As important predators in the invertebrate world, spiders play a crucial role in the balance of the ecosystems they live in. The impact of endosymbionts on their spider host's ecology, their reproductive and dispersal behaviour has therefore the potential to affect the balance of said ecosystems.

Studying spider endosymbionts could shed light on evolutionary processes which led to the diversity visible in spiders today. Sexual selection is a strong driver of evolution and therefore the role of endosymbionts acting as reproductive parasites must not be ignored. It is possible that, due to the variability in spiders, different endosymbiont phenotypes will be observed in different spider groups. Also, the distribution of spiders in different climate zones could impact the effect of endosymbionts, e.g. spiders from hot climate zones might show signs of environmental curing.

Last but not least, many spider species have been shown to be good laboratory animals. Rearing of laboratory lines and experimentally curing of endosymbiont infections via antibiotic treatment has provided opportunity for controlled reproduction experiments (Goodacre, 2011).

1.4 Endosymbiont infections in spiders

Spiders do not escape manipulation by endosymbionts. In recent years, studies have emerged that demonstrated the influence of endosymbiont infections on spider hosts (Gunnarsson *et al.*, 2009; Oh *et al.*, 2000; Rowley *et al.*, 2004; Vanthournout *et al.*, 2011) . There is only a small body of evidence that shows the manipulation of reproductive behaviour by an endosymbiont.

In the sheet-web spider *Pityohyphantes phrygianus* it has been found that female postcopulatory abdominal position influences the sex ratio of the offspring (Gunnarsson *et al.*, 2009). In *Wolbachia* infected females a relevant relationship is present between body size and postcopulatory abdominal position. Larger infected females that mate with infected males produce fewer female-biased clutches of offspring compared to uninfected females. If these large infected females mate with uninfected males the sex ratio is more skewed towards females (Gunnarsson *et al.*, 2009). This leads to the assumption that female *P. phrygianus* are able to assess the infection status of their mating partners in respect to their own (Gunnarsson *et al.*, 2009). *Wolbachia* were also found in the linyphiid spider *Hylyphantes graminicola* from different locations in China. The study however did not report any reproductive manipulation by the *Wolbachia* infection (Yun *et al.*, 2011).

The sex ratio in populations of the dwarf spider *Oedothorax gibbosus* is female-biased but the strength of this bias varies considerably between populations (Vanthournout *et al.*, 2011). The daughters of females from sex ratio distorted matrilines also produce female-biased clutches of offspring. The sex ratio is maternally inherited. Nevertheless, there is no one-to-one relationship between the *Wolbachia* infection and the sex ratio distortion in *O. gibbosus*. Even though treatment of infected females with antibiotics will restore the normal sex ratio there are still considerable variations between females (Vanthournout *et al.*, 2011). In *Oedothorax retusus* a sister species of *O. gibbosus,* female biased offspring clutches have been found. The normal sex ratio can be restored via the administering of antibiotics. In an

endosymbiont screening *Wolbachia*, *Rickettsia* and *Cardinium* were found. All screened females carried the *Cardinium* infection. Only two females were found to be infected with *Wolbachia* and *Rickettsia*. *Wolbachia* and *Rickettsia* are probably the causative agents of the sex ratio bias. The most plausible mechanism of the sex ratio bias is male-killing which probably occurs during late embryonic development or during hatching. In *O. gibbosus Cardinium* and *Rickettsia* strains were found that are closely related to those found in *O. retusus* whereas the *Wolbachia* strains found in the two *Oedothorax* species are not closely related (Vanthournout *et al.*, 2014).

Although the distribution of *Cardinium* infections in spiders is known to be extensive, the consequences of such infection is still not well understood. Stefanini and Duron could not find any influence of *Cardinium* infection on the marbled cellar spider *Holocnemus pluchei* (Stefanini and Duron, 2012). A high prevalence of *Cardinium* was found in *Cybaeus* spiders. Nevertheless, no reproductive manipulation by the endosymbiont could be detected. The authors of the study were unable to determine whether the sex ratio of the screened *Cybaeus* population was distorted, since only one male was sampled. The only possible reproductive manipulation that could not be ruled out is cytoplasmic incompatibility, but this assumption could not be tested (Perlman *et al.*, 2010).

A recent study has shown that a sex ratio bias towards females in *Mermessus fradeorum*, a linyphiid spider, was found to be due to feminisation induced by a double infection with *Wolbachia* and *Rickettsia*. CI was found in individuals carrying a different single *Wolbachia* strain. In field populations all possible combinations of the two *Wolbachia* and the one *Rickettsia* strain could be found. Interestingly when males infected with the CI *Wolbachia* strain mated with doubly infected females (*Rickettsia-Wolbachia*) the resulting CI was much lower than when the males were mated with uninfected/cured females (Curry *et al.*, 2015).

A study on *Erigone atra* by Goodacre *et al*. (2009) presents the first report of an endosymbiont infection-induced non-reproductive phenotype in spiders. Female *E. atra* infected with *Rickettsia* showed a reduced long-distance dispersal tendency. The dispersal tendencies of males were not influenced by the infection. It remains unknown how such a reduced dispersal tendency in infected females benefits the endosymbiont (Goodacre *et al.*, 2009).

Vertical transmission between females and their offspring is widely accepted, while the frequency and impact of horizontal transmission between different insect species as well as between insects and spiders still needs to be established. Horizontal transmission describes the infection with an endosymbiont due to feeding on infected prey. It was proposed, that the incongruence of the phylogenies of *Wolbachia* and their arthropod hosts, is a result of horizontal transmission (O'Neill *et al.*, 1992). Rowley *et al.* (2004) tested 10 different spider families for *Wolbachia* and found that 7 were infected. Since some of these infections, according to the authors, seem to be of recent origin, they propose that these were acquired through horizontal transmission. Another study provides endorsement for this theory. Baldo *et al.* found that all of 9 tested *Agelenopsis* species were infected with different strains of *Wolbachia*. They propose that closely related species are more susceptible to horizontal transmission and that therefore the variety of strains found could be explained by numerous infective sweeps (Baldo *et al.*, 2008).

1.5 Hypothesis and aims

The coincidental finding of *Wolbachia* in a *N. senegalensis* population studying the heritability of sperm competition success in males, together with the further analysis of the nature of this infection, sparked those thought processes which eventually led to the questions and hypotheses that were studied in this PhD project. It has been mentioned before (section 1.3) that spiders are very diverse in terms of their reproductive anatomy and strategies. Therefore, *N. senegalensis* presents a different study system compared to the linyphiids used as the main study system in the project. Publication of the

work *on N. senegalensis*, in collaboration with Dr Gaetano Solazzo and Prof Jutta M. Schneider from the University of Hamburg, is in preparation.

The main study system chosen for this work were the closely related linyphiid species *Linyphia hortensis* and *Linyphia triangularis*. The aim of this study was to establish which endosymbionts are present in the two *Linyphia* species and to establish the potential effects of these infections. The focus was placed on the possibility of CI-inducing endosymbionts. As described earlier (section 1.2) endosymbiont induced CI is thought to play a part in the speciation process. Incompatibilities between populations of the same species due to different endosymbiont infections are more likely in populations that are divided by geographical distance or features (i.e. large water bodies, mountains). Also, CI could possibly have an effect on the mating behaviour of individuals, if they are able to assess their own and their mating partner's infection status. CI induced mate choice could promote reproductive isolation and the spread of the endosymbiont infection. This work therefore represents an attempt to provide new insights to the endosymbiont-mating behaviour-speciation-complex. The following hypotheses have been defined:

1. Individuals from spatially distant populations carry different strains of the same endosymbiont or are not equally infected with the same endosymbionts. This may lead to incompatibilities which could negatively impact the outcome of inter-population crosses.

2. If individuals are able to asses a potential mating partner's infection status in respect to their own, a difference in behaviour towards a compatible or incompatible mating partner should be observed. Incompatible mating partners might even be avoided completely.

Chapter 2 of this thesis will set the stage for the central study of the project. The study on *N. senegalensis* highlights the impact of endosymbiont infections on the study of arthropod systems. In particular research addressing

reproduction and mating behaviour can be affected by endosymbionts. Temporal and spatial fluctuations in endosymbiont presence emphasise that the collected data present only a snapshot in time. Chapters 3 and 4 represent a structured approach to investigate the presence and distribution of endosymbiont infection in two widespread endemic spider species. Considering the insights from the *Nephila* study, the *Linyphia* study first investigated the genetic background and distribution patterns of the two species (Chapter 3). In the second part (Chapter 4) the endosymbiont presence and distribution were analysed and put into context relevant to the information gathered in Chapter 3. Since many spider species are univoltine, in-depth investigation of endosymbiont frequencies and distributions in breeding experiments is often not feasible. Therefore, Chapter 5 presents a theoretical approach. The model presented and the respective simulation are tested with theoretical data and real-live data. Part of this real-live data was taken from the *Linyphia* study (Chapter 4).

Chapter 2: *Wolbachia* infections in *Nephila senegalensis*

2.1 Introduction

The study described in this chapter was conducted in cooperation with the spider lab at the University of Hamburg, Germany. Collaborators on this study were Dr Gaetano Solazzo, Prof. Jutta M. Schneider (both University of Hamburg), Dr Sara L. Goodacre and myself.

Following the discovery that spiders are not exempt from endosymbiont infections, there have been a rising number of studies on the effect of these endosymbionts on their hosts (Goodacre, 2011; Goodacre *et al.*, 2009; Goodacre *et al.*, 2006; Gunnarsson *et al.*, 2009; Martin and Goodacre, 2009; Rowley *et al.*, 2004; Vanthournout and Hendrickx, 2015; Vanthournout *et al.*, 2014). As yet there are only a few studies that show the direct impact, i.e. the phenotype induced by the endosymbiont or the effect on host fitness, of the infection on the spiders (Curry *et al.*, 2015; Gunnarsson *et al.*, 2009). In the case of this study the initial discovery of *Wolbachia* was more of a serendipitous finding. This had a significant effect on the original study, which was about sperm competition success and its inheritance, in such a way that the study as originally planned could not be completed.

The presence of an endosymbiont might have an influence on the sperm competition success of males. In *Drosophila simulans*, *Wolbachia*-infected non-virgin males had a reduced sperm competition success compared to uninfected males (De Crespigny and Wedell, 2006). This reduced success could be due to reduced sperm number and/or sperm quality (Price *et al.*, 2008). It has been shown that *Wolbachia* does reduce the sperm number of infected *D. simulans* males (Snook *et al.*, 2000). One possible strategy of these males to overcome the disadvantage of reduced sperm numbers has been proposed to be an increased mating rate (De Crespigny *et al.*, 2006). This is a good example to show that endosymbionts could have an impact on male mating strategy. Yet, no information is available on whether endosymbiont-

carrying spider males are affected in their sperm competition success by the infection.

Many other studies, which were done on spider reproductive ecology and mating behaviour, might have been, unbeknownst to the conductors of the studies, impacted by the presence of an endosymbiont acting as a reproductive parasite. So far, it remains unknown if an endosymbiont infection could influence male or female mate choice, including postcopulatory cryptic female choice, in spiders. Therefore, if an infection with endosymbionts is detected, it is worthwhile to study the effect further to be able to design future studies accordingly.

Differences in behavioural and reproductive ecology, but also in the environment (e.g. hot vs temperate climate) may have an impact on the extent of the infection and therefore the effect on the host. Reports of environmental curing of endosymbiont infections, i.e. via high temperatures (Johanowicz and Hoy, 1998; Stevens, 1989; Van Opijnen and Breeuwer, 1999), indicate that the habitat of a species could have an effect on e.g. the infection frequency or transmission rate of an endosymbiont. Also phenotype expression has been shown to differ between host species, even if the same endosymbiont strain is present (Jaenike, 2007; Jiggins *et al.*, 2002; Sasaki *et al.*, 2002; Sasaki *et al.*, 2005). In *Hypolimnas bolina* it was even found that two different phenotypes, male-killing and CI, could be induced by the same *Wolbachia* strain in the same host (Hornett *et al.*, 2008). Given that the *Linyphia* and *Nephila senegalensis* come from geographically distant parts of the world, they are expected to carry different endosymbiont strains. *N. senegalensis* is a large orb-weaver living in sub-Saharan Africa with a different reproductive ecology compared to the linyphiid species, the main study system of this thesis, which are widespread over the Northern hemisphere. It is to be expected that infections with endosymbionts will have different effects on these spider species because they do differ in their life history and the reproductive behaviour.

In the two *Linyphia* species, which were used as the study system for the other chapters in this thesis, multiple matings have been observed in the lab. However multiple mating does not seem to be the norm in this system (Stumpf, 1990; Weldingh *et al.*, 2011). Males and females can both re-mate with different partners. Re-mating frequencies are generally low but seem to be higher in *L. hortensis (Stumpf, 1996)*. In *L. triangularis* the female, once she becomes unreceptive a few hours after copulation, will not accept a male in her web and will chase him away (Stumpf, 1990; Weldingh *et al.*, 2011). It is unknown if, and for how long, spermatogenesis does continue in mature *Linyphia* males. Males have been observed, in the lab, to engage in copulation behaviour with up to four different females (pers. observation). Whether these males still transferred sperm could not be established.

The banded-legged golden orb-web spider *N. senegalensis* has an interesting reproductive system where males and females readily mate with more than one mating partner. Due to terminated spermatogenesis after maturation, male *N. senegalensis* have only a limited amount of sperm available in their pedipalps. Males have been found to split the load of each of their pedipalps into two loads which means that they can fertilise the eggs of a maximum of four females (Schneider and Michalik, 2011). Females accept almost every male that enters their web as mating partner (Schneider and Michalik, 2011). No visible pre-copulatory sexual selection takes place. Therefore, it is highly likely that post-copulatory sexual selection i.e. in form of cryptic female choice or sperm competition is present (Herberstein *et al.*, 2011). The presence of an endosymbiont that is already known to be a reproductive parasite could therefore have a significant impact on the reproductive strategies of this spider species. Sexual cannibalism, which has been observed in different spider taxa including other *Nephila* species (Schneider and Elgar, 2001), occurs very rarely and is not part of *N. senegalensis*' reproductive strategy.

Wolbachia infections have previously been reported from two nephilid species so far, *N. clavata* (Oh *et al.*, 2000) and *N. plumipes* (Rowley *et al.*,

2004). To date, no reports exist that explore the effect that these infections have on their hosts. In our study system, populations of laboratory kept *N. senegalensis* were shown to exhibit a strong sex ratio bias towards female offspring. An analysis of individuals from the matrilines indicated the presence of *Wolbachia* infection, which is the first report in this species.

The aim of this study was to characterise the *Wolbachia* infection found in *N. senegalensis.* One part comprised of establishing whether the observed sex ratio distortion discovered is directly linked to *Wolbachia* and, if possible, identifying the phenotype induced by the endosymbiont. Another part focussed on morphological parameters, such as body weight, egg sac weight and offspring number. Together, the acquired data from this study could be used to design future studies, researching the mating behaviour and mating system of *N. senegalensis*, incorporating the presence and effects of *Wolbachia*.

2.2 Materials and methods

Nephila senegalensis

The study was conducted with the species *Nephila senegalensis* of the family Nephilidae (Kuntner *et al.*, 2013; Kuntner *et al.*, 2008) which has a wide distribution across central and southern Africa. These spiders, also called golden orb-web spiders, build characteristic large orb-webs, often over one meter in diameter, made of golden coloured silk. Males and females of this species show a pronounced sexual size-dimorphism (Figs. 8 - 9) with giant females and dwarf males (Foelix, 2011).

Figure 8: Mature *N. senegalensis* female (left) and male (right)

Figure 9: Female and male *N. senegalensis* in the female's web for a mating experiment. Even though this male is quite big with long legs it is still dwarfed by the gigantic female.

Lab populations (see below for animal husbandry) were derived from collections of several mated females or egg sacs from sites in Namibia and South-Africa in the years 2007, 2008, 2009, 2011, 2012 and 2015 (Table 1). Permits for the collection of spiders and egg sacs were granted by the Ministry

of Environment and Tourism (Namibia), the Department of Economic Development and Environmental Affairs (Province of the Eastern Cape, South Africa) and the Department of Agriculture, Environmental Affairs and Rural Development (KwaZulu-Natal, South Africa).

Table 1: Overview of the locations where *N. senegalensis* were collected from 2007 to 2015. No GPS coordinates are available for the 2009 Namibia site.

Year	Location	Coordinates/Sites
2007	Namibia	22°34'42.2"S 17°07'53.6"E
2009	Namibia	
2010	Eastern Cape	32°20'24.0"S 25°45'00.0"E
2011	Khomas-Hochland, Namibia	22°26'24.0"S 16°58'12.0"E
2012	KwaZulu Natal, Mawana game reserve	28°00'19.6"S 31°12'20.9"E
2015	KwaZulu Natal	26°53'48.8"S 32°16'27.8"E

2.2.1 Spider husbandry

All spiders were raised in the laboratory at the Zoological Institute of the Universität Hamburg, Germany, following a standardized and establish protocol. In the wild a female will produce one to two egg sacs, whilst in the lab females have been observed to deposit up to seven (Schneider and Michalik, 2011). The reasons for this difference could be better nourishment in the lab and reduced mortality of the females compared to the field. The general procedure was that egg sacs collected in the field (2012) or first and second egg sacs produced by mated females in the laboratory (2015) were transferred to plastic containers. Each container was sprayed with water on 5 days a week. The containers are large enough for the hatchlings that are kept together until they moult for the first time. The spiderlings were sprayed with water and fed with small *Drosophila sp*. and pollen until their first moult. Cannibalism becomes more frequent after the first moult, so at this stage the spiderlings were transferred individually to labelled upturned plastic cups of 250ml.

Females grow much larger than males and were transferred to larger cups of 1000ml volume if they were too large for the small cups. The small and large cups have a hole in the top, which is closed with either mesh or cotton wool.

Spiders are given water from spray bottles every day to prevent dehydration and are fed *ad lib.* with *Drosophila sp.* and *Calliphora sp.* twice a week. The spiders were kept at 26 °C in a 12/12 h light cycle. Spiderlings from the first or second clutch of each matriline reached maturity. Adult females were transferred to Perspex frames (40 x 40 x 14 cm) in which they construct their typical webs that are important for mating. Before mating trials, males were gently introduced at the edges of the webs and observed until mating occurred. After mating, females were transferred back to 1000ml cups in which they produced egg sacs.

Spiderlings from all available matrilines were raised to adulthood while keeping track of family background and daily inspections recording moults and death. Due to the regular recording, dates of maturation are known for each male and female. Furthermore, all spiders are weighed on the day after maturation to an accuracy of 0.1mg on a Mettler Toledo Balance. In addition, all egg sacs were weighed within the first 2 days of production.

2.2.2 *Wolbachia*

In 2012 it was discovered that offspring raised from two (out of 8) field-collected egg sacs (further considered as matrilines) contained zero or very few males. Screening of between 15 and 125 females and males from each family of a total of 8 matrilines (named parental generation P_{lab} 2012) for endosymbionts revealed that *Wolbachia* were present in 6 of the 8 families, although only 3 showed a significant sex ratio distortion (Table 3). One of these 3 families contained males, but the statistical analysis revealed that the sex ratio significantly deviated from a 1:1 ratio. A F1 generation was raised originating from these 8 families. Males from different uninfected or infected matrilines were used as fathers. Out of 1069 individuals raised as F1 generation, 69 (between 3 - 14 per matriline) were tested for *Wolbachia*. Within each of the 6 matrilines that stemmed from an infected grandmother, uninfected individuals were found.

In 2015, 23 females and 10 males were collected from KZN, brought to the laboratory and screened for *Wolbachia* (named parental generation P_{field} 2015). With these samples, targeted research concerning *Wolbachia* was started. Ten females produced viable egg sacs of which 979 individuals were raised and 275 were randomly selected for *Wolbachia* screening (F1 generation) (Table 3). Among those 10 mothers, 6 were infected with *Wolbachia* and 4 were not. Please note that up to 1000 spiderlings hatch from each single *Nephila* egg sac so that it is impossible to raise complete clutches.

2.2.3 DNA extraction and PCR

The individuals that were screened for *Wolbachia* originated from different populations and different years. Important for the present study was the screening of the animals from KwaZulu-Natal (2012 and 2015), which comprised matrilines with a distorted sex ratio. In addition to that, spiders from the following populations and years were screened to test the distribution of *Wolbachia* through the populations that our research group had sampled during the years: Windhoek, Namibia (2007), Namibia (2009), Eastern Cape (2008) and Namibia (2011).

DNA for the KwaZulu-Natal (2012) individuals was extracted from spider legs using the Sigma GenElute™ Mammalian Genomic DNA Miniprep Kit. This extraction kit uses proteinase K to lyse the tissue and selectively binds DNA to a column for its purification. Proteinase K is an enzyme, that denatures proteins, including DNAses that might otherwise degrade the DNA. DNA from individuals from Namibia, Eastern Cape and KwaZulu-Natal (2015) was extracted in a different laboratory using the Chelex method, which also uses proteinase K to lyse tissue (Curry *et al.*, 2015; White *et al.*, 2009). In this case half femurs (females) and two to three legs (males) were pulverised in liquid nitrogen and incubated in 500 µl of Chelex 20%, with proteinase K, for 60 minutes at 37°C and then heat shocked for 10 min at 95°C to deactivate the enzyme (Curry *et al.*, 2015; White *et al.*, 2009).

Tests for *Wolbachia* were made using molecular tests for bacterial DNA, as reported previously for spiders (Goodacre *et al.*, 2006). Eluted DNA was used as a template in a PCR reaction, using primers that are designed to amplify a section of the *Wolbachia* cell surface protein gene wsp (WSP-F 5'-TGGTCCAATAAGTGATGAAGAAACTAGCTA-3' and WSP-R 5'-AAAAATTAAACG CTACTCCAGCTTCTGCAC-3', (Braig *et al.*, 1998). For the population from KwaZulu-Natal (2012) DNA was amplified in a total volume of 10µl containing 1µl DNA sample, 5µl BioMix™ Red PCR master mix, 0.5 µl of each primer (10 µmol) and 3µl of sterile distilled water to make up to the final volume. An initial denaturation at 94°C for 1 minute 30 seconds was followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50ºC for 30 seconds and extension at 72°C for 30 seconds. Final extension took 5 minutes at 72°C. The samples from 2015 were amplified using the same PCR protocol but with a larger final reaction volume (~25 µl). In each case bands were visualised by gel electrophoresis on a 1.5% agarose gel stained using ethidium bromide and viewed under ultraviolet light.

To study the transmission rate of the *Wolbachia* from one generation to the next, a number of offspring from positively tested females were screened for the infection. For the 2012 population a total of 62 spiderlings from six mothers were screened. Due to the more targeted approach, studying the *Wolbachia* infection in the 2015 population, a constant number of offspring was screened for every positive female. 197 spiderlings from six mothers were analysed. The DNA extraction technique and PCR protocols used were the same as for the adults of the 2012 and 2015 populations.

The identities of the bacterial strains present were confirmed through Sanger sequencing (Macrogen Inc.). Sequence analysis and phylogenetic construction were performed using the software packages ChromasLite (Technelysium Pty Ltd, 2012), BioEdit (Hall, 1999), NCBI BLAST search and MEGA6 (Tamura *et al.*, 2013).

2.2.4 Statistics

Data were analysed using Generalised Estimated Equations (GEE) in R using the package GEEpack (Høsgaard *et al.*, 2006; Yan, 2002; Yan, 2004). A Gaussian error structure and an exchangeable association structure were used. The ID of the mother was used as grouping variable to correct for dependency of data between offspring of the same family. Models with and without the factor *Wolbachia* infection were compared with Wald statistics. These analyses were conducted by the collaborators at the Universität Hamburg. All other statistical tests were computed using R 3.4.1 (R Core Team, 2017) and were done at the University of Nottingham. These included Chi² tests, exact binomial tests, Fisher's exact tests and Wilcoxon tests.

2.3 Results

2.3.1 Endosymbiont analysis of stored individuals

All populations from Namibia and Eastern Cape (Table 2) contained at least some individuals that were infected with *Wolbachia*, indicating that the endosymbiont has a wide distribution across the southern part of Africa. Small sample sizes precluded further interpretation of the data in terms of differences in infection prevalence between sites.

Table 2: *N. senegalensis* from Namibia (2007, 2009, 2011) and the Eastern Cape (2010). *Wolbachia* were detected in all screened populations.

Matriline	Year of collection	Males	Infected males	Females	Infected females	Total	Total infected %	Infection status
Namibia07	2007	-	-	10	8	10	80	yes
Namibia09	2009	2	2	8	7	10	90	yes
EasternCape	2010	10	2	10	7	20	45	yes
Namibia11	2011	-	-	10	4	10	40	yes

2.3.2 Endosymbionts and sex ratio

Table 3 lists the matrilines derived from KwaZulu-Natal (KZN) in 2012 and in 2015 and the numbers of males and females among their offspring that were raised and survived to maturity. The *Wolbachia* infection status is known for each mother. Sex ratio was estimated based on the random subsets of

spiderlings that were raised and survived to the stage when their sex could be determined. The unambiguous identification of males is possible once the bulbs on the pedipalps appear. Usually, it will take a one or two more moults for the male to mature.

A distorted sex ratio with a significant female bias was found in 10 out of 18 matrilines across both populations (Table 3). However, there was no significant association between the *Wolbachia* infection and the distorted sex ratio (N = 18, $Chi^2 = 0.112$, $p = 0.74$). In the 2012 population, only 3 of the 6 matrilines that were infected with *Wolbachia* showed a distorted sex ratio. The picture was different in the 2015 population where broods from 7 out of 10 matrilines showed a female biased sex ratio although only 4 of these were infected with *Wolbachia.* In 2015, we also found a female bias in 3 uninfected matrilines.

Table 3: Parental matrilines of *N. senegalensis* from KwaZulu-Natal (NS_KZN, 2012, 2015). Apart from six (two in the 2012 and four in the 2015 population from KwaZulu-Natal) all matrilines were infected with *Wolbachia*. Sex ratio was calculated as the proportion of males. The p-value shows, if the numbers of males and females differed from a 1:1 distribution (exact binomial test).

Matriline	Year of collection	Males	Females	Total	Male sex ratio	Infection status	Difference males and females (p-value)
NS_KZN_L1	2012	24	23	47	0.51	no	1
NS_KZN_L2	,,	47	43	90	0.52	yes	0.752
NS_KZN_L3	,,	39	54	93	0.42	yes	0.146
NS_KZN_L4	,,	0	80	80	0.00	yes	< 0.001
NS_KZN_L5	,,	60	45	105	0.57	no	0.172
NS_KZN_L6	,,	3	15	18	0.17	yes	0.008
NS_KZN_L7	,,	28	82	110	0.25	yes	< 0.001
NS_KZN_L8	,,	61	62	123	0.50	yes	1
NS_KZN_001	2015	16	24	40	0.40	yes	0.268
NS_KZN_200	,,	14	62	76	0.18	yes	< 0.001
NS_KZN_26b	,,	9	46	55	0.16	yes	< 0.001
NS_KZN_219	,,	17	47	64	0.27	yes	< 0.001
NS_KZN_26c	,,	27	48	75	0.36	no	0.020
NS_KZN_195	,,	41	58	99	0.41	yes	0.107
NS_KZN_26g	,,	29	62	91	0.32	no	< 0.001
NS_KZN_168	,,	12	84	96	0.13	yes	< 0.001
NS_KZN_150	,,	41	43	84	0.49	no	0.913
NS_KZN_13	,,	22	96	118	0.19	no	< 0.001

2.3.3 Endosymbiont transmission

Six out of 8 matrilines of the 2012 KwaZulu-Natal population (P_{lab}) must have had an infected mother. However, only a mean of 44.52% ± 5.17 of the tested offspring from these matrilines carried the infection (Table 4) suggesting imperfect transmission. Transmission frequency did not differ between the 3 distorted (43%) and the 3 non-distorted (48%) matrilines (Fisher's exact test, p = 0.7981, N = 6).

In the 2015 population, a lower proportion of the sampled females were infected (39%) but a significantly higher overall transmission rate from the P_{field} to the F1 generation was observed with 87%. Transmission frequencies significantly differed between the 2012 and 2015 populations (Wilcoxon test, Z = 2.80, p = 0.0051; Table 4). In contrast to the 2012 population, we found a significant difference in transmission frequencies between distorted (N = 4, 92%) and not distorted matrilines (N = 2, 81%) in the 2015 population. The transmission frequency was significantly higher in distorted matrilines (Fisher's exact test, p = 0.04209, N = 6).

Table 4: Transmission frequency of the *Wolbachia* infection from the P to the F1 generation for the 2012 and 2015 *N. senegalensis* populations from KwaZulu-Natal.

Mother	Infected	Total	Distortion	Transmission (%)
NS_KZN_L2	5	8	no	63
NS_KZN_L3	3	8	no	38
NS_KZN_L4	7	15	yes	47
NS_KZN_L6	6	12	yes	50
NS_KZN_L7	2	8	yes	25
NS_KZN_L8	5	11	no	46
NS_KZN_001	43	49	no	88
NS_KZN_200	30	31	yes	97
NS_KZN_26b	25	26	yes	96
NS_KZN_219	39	42	yes	93
NS_KZN_195	18	26	no	69
NS_KZN_168	18	23	yes	78

2.3.4 Endosymbionts and body weight

Families from the 2012 population differed markedly in body weights of females and males at maturation (Fig. 10). A GEE with "mother_ID" as grouping variable revealed that P_{lab}-females that hatched from egg sacs produced by infected mothers were significantly heavier than daughters from presumably uninfected mothers (N = 432, Wald statistics: Df = 1, $X^2 = 7.05$, p = 0.0079) and the same pattern emerged for sons (N = 260, Wald statistics: Df = 1, $X^2 = 5.68$, p = 0.017). The association of maturation weight and *Wolbachia* disappeared in the F1 generation (Wald statistics, female maturation weight: Df = 1, $X^2 = 3.33$, p = 0.068, N = 344, male maturation weight: Df = 1, $X^2 = 0.04$, p = 0.84, N = 176). Please note that the infection status of many F1 mothers was unknown (12 mothers with known infection status plus 12 with inferred infection status out of 61 mothers were included in the test). The infection status of some females had to be inferred since they were not tested for *Wolbachia* initially, but *Wolbachia*-positive offspring of these females implied that mothers carried the infection too.

The data from 2015 provide a more extensive set of data for statistical analyses because the mothers (P_{field}) and their offspring were tested for *Wolbachia.* F1 individuals were derived from 6 infected and 4 uninfected mothers. 69 daughters and 62 males from infected and 52 daughters and 59 sons from uninfected mothers were weighed at maturity, revealing large differences between families (Fig. 11). GEEs with mother ID as grouping variable detected no significant association between the infection status of the mother and the maturation weights of daughters and sons (Wald statistics, female maturation weight: Df = 1, $X^2 = 1.76$, p = 0.18, N = 121; male maturation weight: Df = 1, $X^2 = 0.105$, p = 0.75, N = 121).

Given the imperfect *Wolbachia* transmission, a GEE for a direct association of *Wolbachia* infection and body weight at maturation was also calculated correcting for mother_ID, but again failed to detect significant associations for

females (Wald statistics, Df = 1, X^2 = 0.885, p = 0.35, N = 121) or males (Wald statistics, Df = 1, X^2 = 0.434, p = 0.51, N = 121).

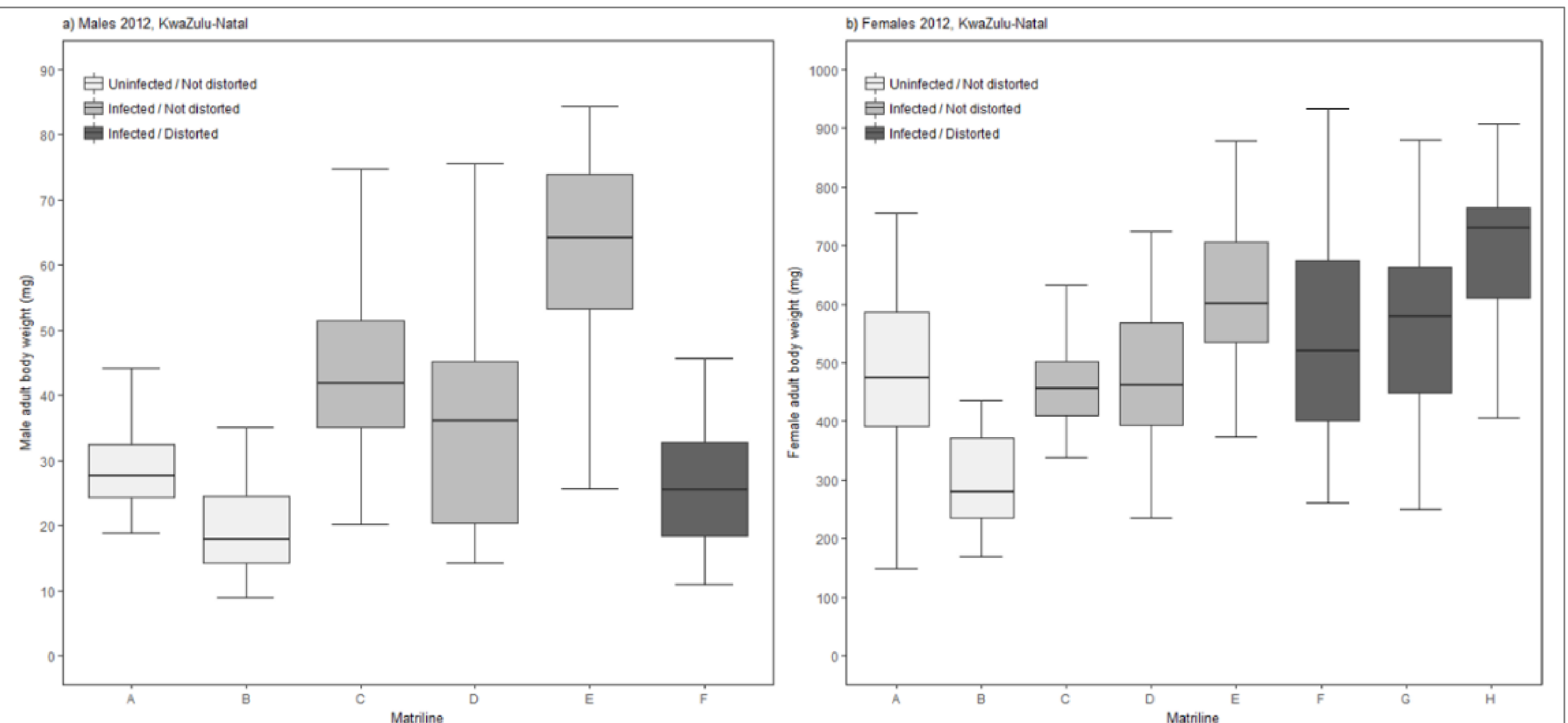

Figure 10: a) Body weight of adult males in the 2012 KwaZulu-Natal population (P_{lab}), b) Body weight of adult females in the 2012 KwaZulu-Natal population (P_{lab}). In both sexes body weight differed between the matrilines. Light grey = uninfected/not distorted, medium grey = infected/not distorted and dark grey = infected/distorted. The error bars represent the standard error.

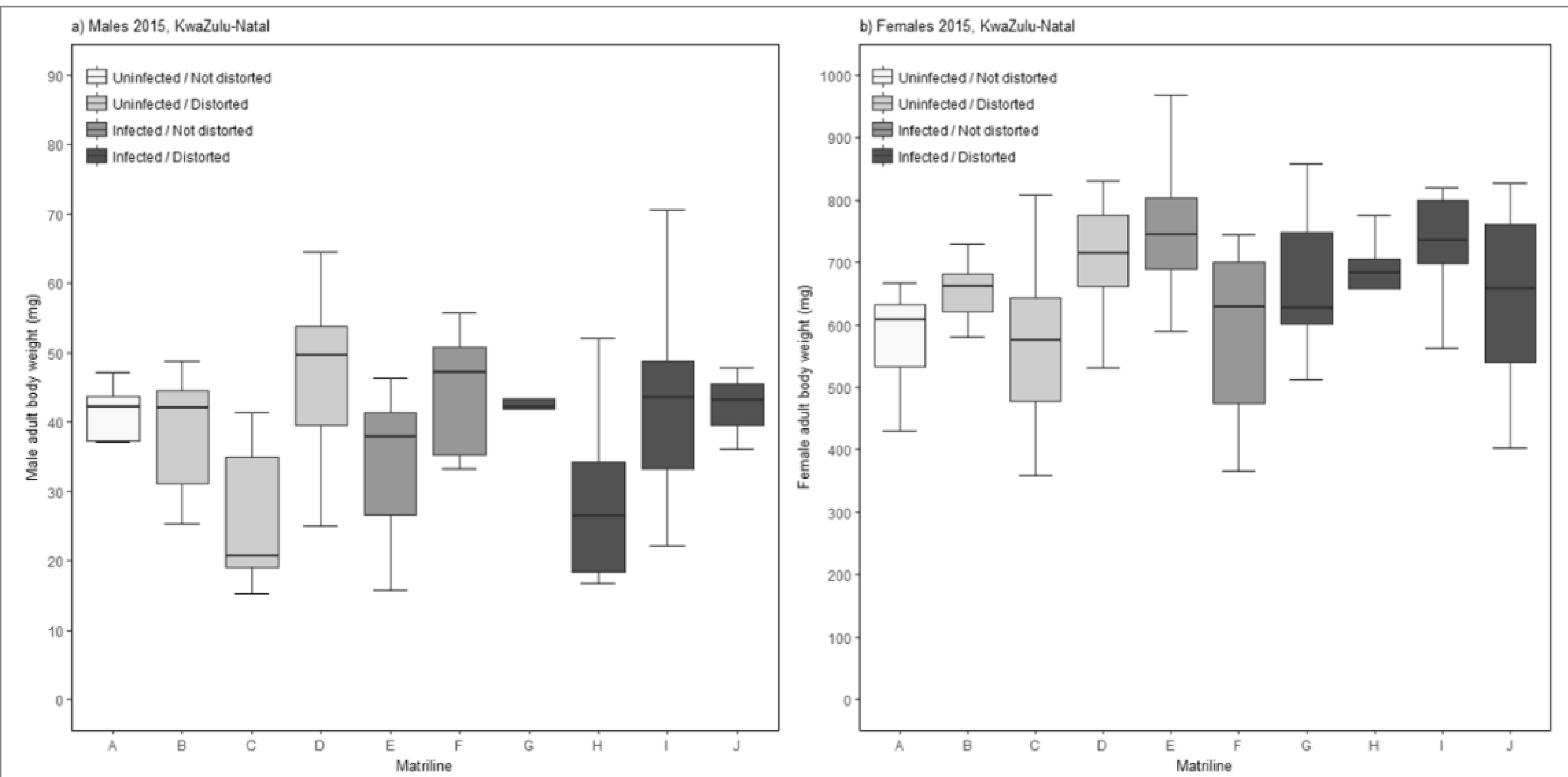

Figure 11: a) Body weight of adult males in the 2015 KwaZulu-Natal population (P_{field}), b) Body weight of adult females in the 2015 KwaZulu-Natal population (P_{field}). All individuals of this generation were screened for *Wolbachia*. In both sexes body weight differed between the matrilines. Light grey = uninfected/not distorted, medium grey = infected/not distorted and dark grey = infected/distorted. The error bars represent the standard error.

2.3.5 Endosymbionts and egg sacs

Egg sacs produced by *Wolbachia* infected P_{lab}-females were significantly heavier than egg sacs from presumably non-infected mothers (Wald statistics: Df = 1, X^2 = 11.4, p = 0.00074, N = 77). While this might be a direct effect of the larger body weights of infected females in this cohort (see above), we found the same effect in the 2015 population in which F1 female weights at maturation did not differ between infected and non-infected matrilines: GEEs revealed that egg sacs from infected F1 females of the 2015 population were significantly heavier than egg sacs from uninfected females (Wald statistics: Df = 1, X^2 = 8.85, p = 0.0029, N = 109). This association is even stronger if the infection status of the grandmother is entered in the model instead of the infection status of the mother while still being grouped for mother ID (GEE, Wald statistics: Df = 1, X^2 = 11.9, p = 0.00056, N = 109) suggesting a transgenerational effect even in the absence of *Wolbachia*.

2.3.6 *Wolbachia* phylogeny

Wsp sequence data obtained from 24 spiders of the 2012 population (8 males, 16 females) showed that at least two different, albeit closely related, strains of *Wolbachia* were present in this population of *N. senegalensis*. Five of the six infected matrilines harboured the same strain of *Wolbachia*. Only one matriline was infected with the other strain. No double infected individuals were found. DNA from 6 individuals of the 2015 population (one of each infected matriline) was also sequenced. All individuals were infected with what seemed to be the more common strain in the 2012 population. Again, no double infections were detected.

The two strains found are more closely related to each other than to any other published sequences, but they are not phylogenetically close to strains in other *Nephila* species such as *N. clavata* (GenBank: EF612772) and *N. plumipes* (GenBank: AY486100). Instead, the strains found in *N. senegalensis* cluster most closely with the *Wolbachia* strains of the northern corn

rootworm *Diabrotica barberi* (GenBank: EU188678) and the spider *Cybaeus penedentatus* (GenBank: GQ480746).

Figure 12 shows a maximum likelihood tree placing the newly found strains NSen1 and NSen2 in the B supergroup of the *Wolbachia* phylogeny. The tree shows examples of supergroup A *Wolbachia* which are also found in arthropods. Sequences from supergroup D which are found in nematodes were used as outgroup.

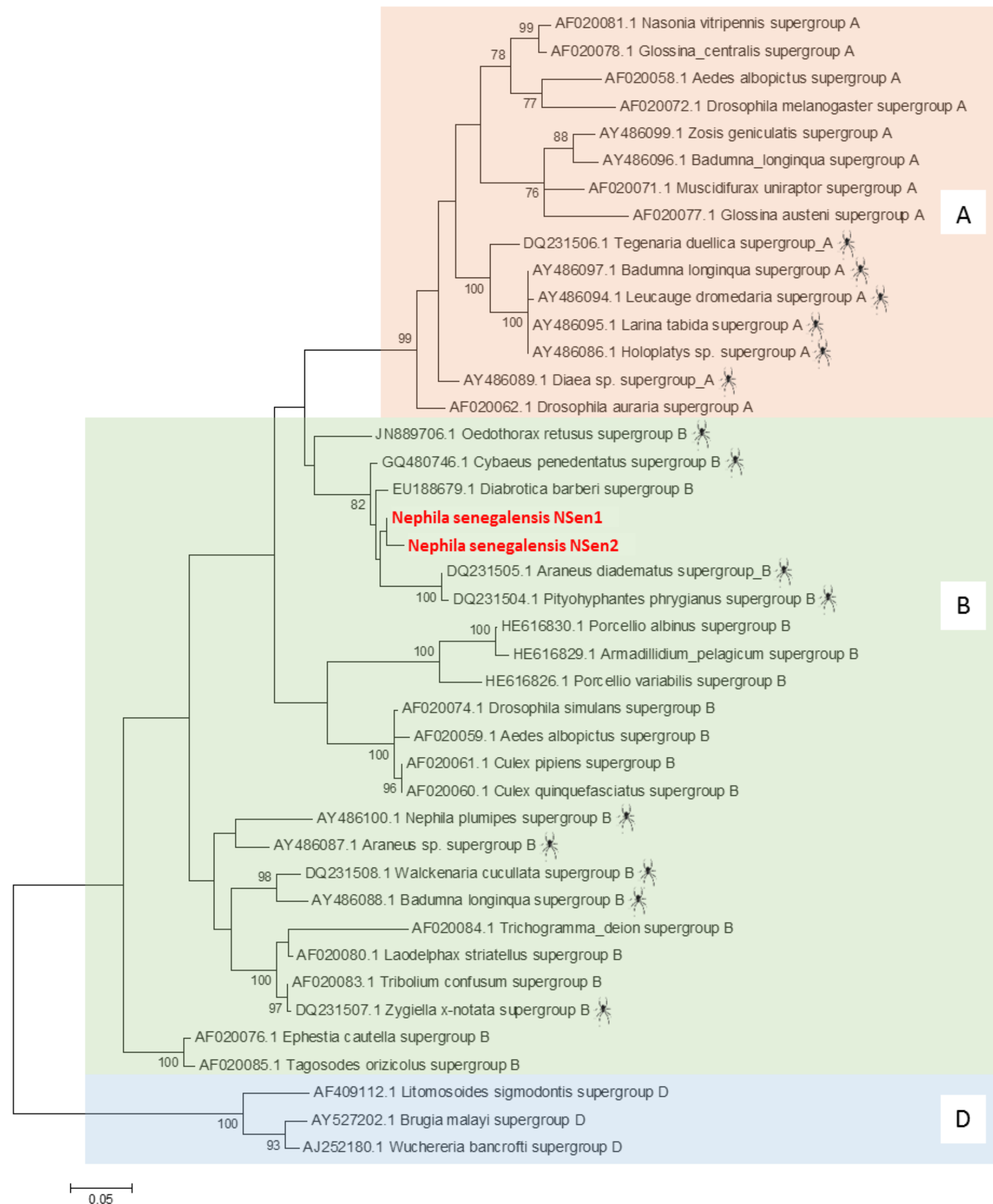

Figure 12: Phylogeny of *Wolbachia* based on the *wsp* gene (model: Tamura-Nei (Tamura and Nei, 1993), Maximum Likelihood Tree, log likelihood = -5671.4663, bootstrap: 100). Sequences were 622bp long and all positions were used. The newly found *Wolbachia* strains belong to supergroup B of the *Wolbachia* phylogeny. *Wolbachia* endosymbiont of *Nephila senegalensis* NSen1 = strain found in matrilines 2, 3, 4, 6 and 7, *Wolbachia* endosymbiont of *Nephila senegalensis* NSen2 = strain found in matriline 8. The spider icon indicates *Wolbachia* strains isolated from spider hosts.

A few individuals which carried the *Wolbachia* infection where also screened for the bacteriophage WO (data not shown) and it was found that the phage is present in the *Wolbachia* infecting *N. senegalensis.* Interestingly, the phage was not present in all tested individuals, but exclusively in those matrilines, that did not show a distorted sex ratio. At this point no further investigation of the distribution or the effect of the phage have been undertaken.

2.4 Discussion

Nephila spiders are well-established model systems for behavioural ecologists interested in studying the evolution and maintenance of different reproductive strategies. The finding, for the first time, that the maternally acquired endosymbiont *Wolbachia,* which is known to manipulate the reproduction of other arthropod hosts, is widespread throughout wild populations of *Nephila senegalensis* suggests that host/bacteria interactions may also have influenced the reproductive strategies employed by this species. The finding that all populations sampled to date contain a proportion of infected individuals and that the species itself contains two, very closely-related strains of *Wolbachia* that have not yet been reported from other species, supports the idea that these two strains diverged *in situ,* i.e. that the association between *Wolbachia* and their hosts may be long-established rather than having been more recently acquired through horizontal transmission. The potential longevity of the infection is interesting because it indicates that there will have been time for host/parasite co-evolution to have shaped the interaction.

The consequences of such interactions between host and endosymbiont are known to include rapid alterations in reproductive behavioural strategies, for example in the butterfly *Acraea encedon*. *A. encedon* is infected with a male-killing *Wolbachia* strain. The reduced number of males leads to a sex-role reversal. Female butterflies form leks to attract mating partners, a behaviour usually displayed by the males of this species (Jiggins *et al.*, 2000b). There are also direct genetic consequences for the host of the interaction with the microbe, for example in the rapid spread of resistance genes against male-killing *Wolbachia* as is observed in *Hypolimnas* butterflies (Charlat *et al.*, 2005; Hornett *et al.*, 2008).

In *N. senegalensis,* the two strains identified were found to cluster phylogenetically with strains belonging to supergroup B of the *Wolbachia* phylogeny. Within this they clustered most closely with a strain found in a

Cybaeus spider. These spiders live on the forest floor in a rather humid environment i.e. under logs or mossy areas. The genus *Cybaeus* was first described by Koch (Koch, 1868) and today includes 157 species (2018). Its representatives can be found in Japan and Western North America as well as Europe, China and Korea (Isaia and Chiarle, 2015). Whether any of the four phenotypes commonly found in these types of infection (CI, male-killing, feminisation and parthenogenesis) are induced by *Wolbachia* in the Cybeus case remains unknown (Perlman *et al.*, 2010). The next closest strains from spider hosts (*Araneus diadematus*, *Pityohyphantes phrygianus* and *Oedothorax retusus*) also belong to supergroup B. From *P. phrygianus* it is known that the infection with *Wolbachia* influences females' postcopulatory position, which in turn is associated with determining the sex ratio of their offspring (Gunnarsson *et al.*, 2009). In *O. retusus* triple infections with *Cardinium*, *Wolbachia* and *Rickettsia* were found. Reduced offspring numbers in female-biased clutches hint towards male-killing but it remains unclear so far which of the three endosymbionts is responsible for this (Vanthournout *et al.*, 2014).

The finding that more than one strain of *Wolbachia* infection may be present in a *Nephila* species agrees with studies of *N. clavata*, which also harbours two *Wolbachia* strains, one belonging to the A supergroup, the other to the B supergroup (Oh *et al.*, 2000; Wang *et al.*, 2010, sequences not included into the phylogeny in Figure 12). Whilst it was not possible to determine the mode of transmission in any of these individual cases, it is nevertheless interesting to note that there is some geographical congruence in the bacterial strains found in *Nephila* species. For example, the Eurasian *N. clavata* strains and that of a third infected *Nephila* species, the Australasian *N. plumipes,* (which is also infected with a single B-type *Wolbachia*), each group with bacteria found in species from the same respective geographic regions. The African *N. senegalensis* bacterial strains do not show this congruence. The closest related strains were isolated from species found in North America. It is possible that South African arthropod species with even closer related strains

exist but that they have not yet been identified. The different *Nephila* subclades are thought to have diverged around 21 – 26 million years ago (Kuntner *et al.*, 2013) thus our data are consistent with *Wolbachia* having invaded each clade independently, i.e. post-speciation. It would be exciting to screen other African *Nephila* species and to compare the strains with the ones we found in *N. senegalensis*.

It is also interesting that transmission efficiency appears to vary even under similar laboratory conditions, as evidenced by comparing populations with the same *Wolbachia* strain from KwaZulu-Natal. Lower transmission rates could be explained if some populations have a higher suppressing capability of the *Wolbachia* phenotype. This has been suggested to explain a similar phenomenon observed in the spider *Oedothorax gibbosus*. In this species, different degrees of *Wolbachia* suppression is proposed to be due to geographic heterogeneity in the distribution of 'modifier' genes in the host, although the biochemical basis of such bacterial suppression is not yet understood. The authors propose that the differences in Wolbachia suppression could be due to different strains of Wolbachia infecting different populations (Vanthournout and Hendrickx, 2016).

In a species, such as *N. senegalensis,* individual populations may represent unique opportunities for *Wolbachia*-host interactions either through the presence of spatially structured host modifier genes, or through spatial heterogeneity in the distribution of different bacterial strains. Previous studies on various taxa have already shown that *Wolbachia* infections differ between populations (Arthofer *et al.*, 2009; Cooper *et al.*, 2015; Sakamoto *et al.*, 2015; Sourassou *et al.*, 2014). *Nephila* spiders are predatory and cannibalistic species, both of which might facilitate horizontal transmission from the prey towards the predator (Le Clec'h *et al.*, 2013) although our results indicate that this may be less important because we would then expect the two strains discovered in *Nephila* to reflect the wide diversity of bacterial strains distributed amongst prey species. Any local differences in host-

bacterial interactions are thus more likely to be driven by differences in the host.

One unexpected finding from our study is that *Wolbachia* infection appears to be associated positively with increased fecundity. Egg sacs of infected females were found to be heavier than those of uninfected females, even accounting for female size, which is already a known factor influencing fecundity (heavier females usually produce heavier egg sacs (Harrington, 1978; Higgins, 1992; Skow and Jakob, 2003; Turnbull, 1962)). In our study, *Wolbachia* infected females appeared to produce egg clutches that were heavier than would be regular for their respective body weight. Our results fall in line with studies on different insect species that showed an increased fecundity of *Wolbachia* infected females (Mazzetto *et al.*, 2015; Unckless and Jaenike, 2012; Vavre *et al.*, 1999; Weeks *et al.*, 2007) but this is the first such finding in a spider. What remains to be seen however is whether this elevated fecundity is achieved by a greater number of eggs in a clutch or if the eggs themselves are heavier and larger. Both possibilities could lead to increased reproductive success either directly through increased offspring number, or through increased offspring survival if larger, heavier eggs contain higher quantities of yolk lipids, which are known to have a positive effect on the survival rate (Blamires, 2011; Geister *et al.*, 2009).

Significantly, our study confirms that although *Wolbachia* might benefit from local sex ratio skew in *Nephila* it is unlikely to be the sole cause of the observed sex ratio distortion in this species because not all infected matrilines produced an excess of female offspring and, conversely, some apparently uninfected individuals did produce such an excess. However, sex ratio estimates from raising a small subsample from a clutch to adulthood may not be very accurate. We will need better methods to sex spiders for an improvement of our estimates of the primary sex ratio. Unfortunately, so far, the sex chromosome system for N. senegalensis remains unknown. Therefore, sex determination at a chromosomal level is not feasible.

Our findings indicate that male-killing during the early embryonal stages is not the cause of the observed skew in the sex ratio since clutches of infected and uninfected females show no differences in size and in hatching success (data not shown). Male-killing could occur during or shortly after the hatching period for example through cannibalism of males by their sisters (Engelstadter and Hurst, 2009; Hurst and Majerus, 1993). Sibling cannibalism regularly occurs during the early gregarious phase after spiderlings leave the egg sac and would be an ideal fitness compensation mechanism. Feminisation cannot be ruled out as the reason for the distorted sex ratio at this point. The presence of infected males may be a result of host modifier or suppressor genes preventing the bacterial phenotype from being expressed. Similarly, interactions between *Wolbachia* and a yet unknown different endosymbiont could also explain the occurrence of infected males. Recently a linyphiid spider species has been found to be infected with multiple endosymbionts. The observed sex ratio distortion could not be attributed to either of the endosymbionts alone. Instead the female bias is likely the result of co-infecting endosymbionts (Curry *et al.*, 2015). Further study is needed to determine the possibility of feminisation. Parthenogenesis appears unlikely because unfertilised egg sacs were never observed to hatch (pers. obs.) as would be expected if parthenogenesis were common.

The presence of other undetected endosymbionts such as *Rickettsia*, *Cardinium* and *Spiroplasma* (Goodacre and Martin, 2012) or the action of some meiotic driver (Mercot *et al.*, 1995) might be the explanatory factor here - and it might also be the case that *Wolbachia* have additional as yet unidentified beneficial effects in *N. senegalensis* as has been found for other species (Braquart-Varnier *et al.*, 2015; Teixeira *et al.*, 2008). It is also possible that *Wolbachia* is maintained in *N. senegalensis* populations at least partially because of a beneficial effect on the host rather than simply as a reproductive parasite. Perhaps the symbiotic effect allows it to persist and diversify within its host in the face of reproductive manipulation by an alternative microbial

(or other) means, that would otherwise lead to its elimination in the longer term.

The finding that the *Wolbachia* identified in *N. senegalensis* carry the bacteriophage WO makes this system even more intriguing. Even though at this point it is not clear if the phage is active, it could be involved in the expression of the *Wolbachia* phenotype which in itself is still unknown. The phage WO has been found to be involved in the expression of CI in *Nasonia vitripennis.* The level of CI was significantly reduced when phage density was high and *Wolbachia* density was low (Bordenstein *et al.*, 2006). It is possible that the phage could influence the expression of the sex ratio distorting phenotypes in a similar way.

This study showed that the association between a host and its endosymbiont is not always straightforward and easy to disentangle. The data acquired are not sufficient to determine the phenotype which leads to the observed sex ratio distortion. It is unlikely that early male-killing or feminisation are the cause of the distortion since infected males were found, unless these male somehow escaped the manipulation by the endosymbiont. Potentially the phage could be involved in this. No unfertilised egg sacs have ever been observed to hatch in the laboratory, therefore it seems improbable that *Wolbachia* induces parthenogenesis in this spider species. No information is available at this stage as to whether CI is induced by the discovered *Wolbachia* strains. Further mating trials crossing differently infected individuals are needed to investigate the possibility of CI. Creating differently infected matrilines in the lab using antibiotics could be a way forward. On the whole *N. senegalensis* has proven to be a suitable system to study endosymbiont infections in spiders, yet many questions remain unanswered. The study presented here provides a good basis for further investigations.

Acknowledgements

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Chapter 3: Life history and population genetics in *Linyphia hortensis* and *Linyphia triangularis*

3.1 Introduction

Only a small number of studies exist on *L. triangularis* and even fewer on *L. hortensis*. Whilst the life history of both species has been studied through observation in the field and laboratory experiments (Rovner, 1968; Toft, 1987; Toft, 1989; Turnbull, 1960; Turnbull, 1962), no studies exist to date that investigate the population genetic diversity of either of these linyphiid species. *L. triangularis* has been studied more than *L. hortensis*, partially because of *L. triangularis*' highly ritualised mating behaviour. In this context male fighting behaviour and sperm precedence have been studied to some extent as well (Funke and Huber, 2005; Nielsen and Toft, 1990; Weldingh *et al.*, 2011). The studies that exist on *L. hortensis* are mainly comparative studies investigating traits such as the web-building behaviour of *L. hortensis* and *L. triangularis* or the mating behaviour of both species (Benjamin and Zschokke, 2004; Stumpf, 1990; Stumpf, 1996).

These closely related species were chosen for the present study because even though they are quite similar in some respects, e. g. habitat preference or prey capture, they also show differences in others. These differences make both species good comparative systems for different aspects of the study. Male *L. triangularis* show highly ritualised, easily observable courtship and mating behaviours that are divided into different phases (see below) (Stumpf, 1990). Therefore, studying these behaviours in depth could provide details about differences between individuals and make it possible to relate them to different traits such as body size, mating status (virgin vs mated), country of origin or of other factors such as the presence of endosymbiotic bacteria. However, not only the differences between the males may play a role here. Male mate choice and male investment in copulations have gained more attention over the years. In *Drosophila melanogaster* it was found that males transfer more sperm to mated compared to virgin females, probably due to

the elevated sperm competition risk. Males also transferred more sperm to young or large females, both of which are more fecund compared to old or small females. Lastly, the study found that males copulated significantly longer with larger females (Lupold *et al.*, 2011).

Both *Linyphia* species are widespread over the Northern hemisphere and are easily found and identified. This makes it easy to collect samples from populations at different locations. Comparing the mating behaviour in inter- and intra-population crosses and their respective reproductive outcome could provide information on potential divergence of populations. Studies exists on such differences between individuals from different populations, for example in vertebrates, insects and spiders. In the cricket frog *Acris crepitans* it was found that the two subspecies, *A. c. crepitans* and *A. c. blanchardi*, show differences in advertisement call structure. These variations are very likely the result of differences in the habitats of the two subspecies combined with sexual and social selection (Ryan *et al.*, 1990). A study on different strains of *Drosophila virilis* showed that variations in the mate recognition system of the strains exist. It was found that males and females did neither favour individuals from their own strain nor from a different strain for copulations. However, males of one strain were particularly successful in achieving copulations. The authors suggest that this might be the case because the males were the most persistent in terms of courting and started singing earlier (Saarikettu *et al.*, 2005). Males from different populations of *Schizocosa* wolf spiders in the South-eastern U.S. show significant inter-population variation in four courtship displays. When the conductors of the study experimentally paired males and females from different populations, breeding between two populations did not seem to be impacted, despite differences in the frequency of male courtship behaviours. A third population was nearly completely reproductively isolated from these two populations (Miller *et al.*, 1998).

Gene flow between populations of the same species can be restricted due to different factors. One of these is geographic isolation, which means that the

populations are separated from one another by geographical features, i.e. mountains, water bodies or sheer distance (Dobzhansky and Mayr, 1944). Populations from such different locations might diverge from each other over time, due to the effects of random genetic drift alone, and eventually reproductive isolation could occur. Such differentiation leading to reproductive isolation could in the long term lead to the evolution of new species (McPeek, 1996). In addition, if local environmental factors (e.g. habitat, climate, food/prey availability and diversity) differed amongst the isolated populations, it is possible that the process of divergence could be speeded up by natural selection for different local adaptations to their respective environments (e.g. different colour morphs, body size, food/prey choice) (Ayres and Scriber, 1994; McPeek, 1996; Taylor, 1991). Even though the two *Linyphia* species are closely related and are found in similar habitats, it is unlikely that population differentiation and local adaptations are the same for both of them. For example, the fact that *L. hortensis* is distributed more patchily throughout their preferred habitat range (Roberts, 1996) than *L. triangularis* could hint towards differences both in levels of inter-population divergence, as well as in levels of overall adaptability between the two species.

Studying the genetic diversity of the spiders can also provide information about the dispersal behaviour of these linyphiid species. Linyphiids are conspicuous for their ability for long distance dispersal via ballooning (Duffey, 1956; Weyman *et al.*, 2002). Juveniles of other species in most spider taxa are known to engage in this kind of behaviour as well, but linyphiids are rather unique in maintaining this ability throughout their life cycle. Ballooning is the term describing aerial dispersal of spiders using a strand of silk which is used to gain uplift. The spider will climb to an elevated point (plant stalks or the tops of shrubs), adopt a so-called tiptoeing posture where the spider will point its abdomen into the air. It will then release a strand of silk which will, given suitable wind speed and air pressure, provide enough uplift to take off (reviewed in (Weyman, 1993)). Spiders can cover large distances by this

method with some individuals travelling as high as the jet streams (reviewed in (Weyman, 1993)). It is currently unknown if the two *Linyphia* species studied here do disperse by this behaviour and how intermixed populations from the UK and mainland Europe are. The literature does not provide records of extensive ballooning behaviour by either *L. hortensis* or *L. triangularis*.

Less tractable aspects of the *Linyphia* system include that, for *L. triangularis,* the reproductive season only starts at the end of July and the egg sacs do not hatch until the following spring (Weldingh *et al.*, 2011). This makes *L. triangularis* a less good system when it comes to the assessment of hatching success and offspring survival. Here *L. hortensis* proves to be the more tractable species. The mating season starts in April and the offspring hatch in late spring (Stumpf, 1990). Therefore, *L. hortensis* is better suited to study factors that could influence compatibility between males and females, egg sac hatching or offspring survival.

This chapter focusses on the life-history and population genetic diversity within and among *L. hortensis* and *L. triangularis*. The aim of this part of the study was to establish lab populations, for both species, from wild caught individuals from the UK, Germany, Denmark (*L. hortensis* only) and Sweden. Intra-population and inter-population matings were attempted in order to breed a F1 generation and to collect mating behaviour data for the males. Rearing a F1 generation also aimed to provide information on the compatibility of individuals from the different populations. Testing the spiders' ability to disperse via ballooning in combination with the mtDNA analysis provided information on the level of geneflow between the populations. Additionally, this chapter also provides the basis for Chapter 4 which focuses on the endosymbiont composition and the impact of said endosymbionts on these spider species.

3.2 Materials and methods

Linyphia hortensis

The base colour of *L. hortensis* (Sundevall, 1830) is dark brown to black. The cephalothorax is plain-coloured in both sexes without any markings. The female's abdomen shows a pronounced white/creamy coloured leafy pattern (Fig. 13). A white band that completely circles the abdomen makes the female of this species easily identifiable. The female's legs are of a beige sometimes reddish colour. Whilst the juveniles of both sexes are patterned, the mature male's abdomen is mostly darkly coloured and only two white spots on the front end of the abdomen remain of the pattern. The male's legs will turn red after maturation (Fig. 13).

Figure 13: left: female *L. hortensis* (© Trevor and Dilys Pendleton, 2012, www.eakringbirds.com), right: male *L. hortensis* (© www.naturespot.org.uk, 2012)

The adult body size of this species ranges between 3.5 – 5.8mm for females and 3.1 – 4.7mm for males (Helsdingen, 1969). Females seem to have a larger size range. No information is available from the literature about sexual size dimorphism (SSD) in this species.

Individuals of both sexes mature from early April on. Males will reach maturity before the females and can often be found in close vicinity of penultimate (subadult, maturing with the next moult) females' webs to mate with them as soon as they have matured. All females mature in the course of six to seven weeks (Stumpf, 1996). Egg sacs will be deposited from May onwards and will

hatch after approximately two weeks (pers. obs.). The average number of eggs in an *L. hortensis* egg sac is not known. No studies exist that mention the operational sex ratio of this spider species. In *Oedothorax gibbosus* and *O. retusus* the operational sex ratio seems to be 1:1 unless the population carries an endosymbiont (Vanthournout *et al.*, 2011; Vanthournout *et al.*, 2014). Toft reported to have observed a 1:1 sex ratio among subadult *L. triangularis,* stating that the operational sex ratio was very likely to be skewed towards males (Toft, 1989). The reason for this is the asynchronous maturing of males and females. Males will reach maturity before the females start maturing. Therefore, more mature males will be present in a population than mature females. The operational sex ratio will be more strongly skewed towards the males the less synchronised the females are maturing (Toft, 1989). The juveniles will grow until approximately the fourth instar before hibernating over the autumn/winter to mature in the next spring. Sometimes males do mature before hibernation which gives them an advantage in the competition for females in the following spring.

The mating in this species shows the following pattern (Stumpf, 1990): The male enters the female's web and carefully moves towards her. He shakes the web with his legs, also known as courting. If she shows no aggression, he will move close to her, maybe touching her leg with his. He then climbs on her ventral side to perform pseudo-copulation. That means, he is inserting the tip of his pedipalps into the female's genital opening one at a time switching sides each time, without transferring any sperm. After pseudo-copulation, which can take up to 24 minutes (14 – 34 min, (Stumpf, 1990)), the male will leave the female to build a sperm-web. He will incorporate his own silk into the sheet-web of the female and place a droplet of sperm from his gonopore on this sperm web. He dips the tips of his pedipalps, alternating, into the droplet to 'suck up' the sperm. After this the male returns to the female to perform the actual copulation. Again, the male will insert the tip of his pedipalp into the female's epigyne opening, one at a time in an alternating pattern. This time he will transfer the sperm. The 'real' copulation can be distinguished

from the pseudo-copulation in that the duration of the male's palp insertions increases (3 - 15s pseudo-copula vs 85s in copula). During this copulation the whole droplet of sperm is transferred. After the copulation the male will leave the female's web.

Linyphia triangularis

The common hammock weaver *L. triangularis* (Clerck, 1757) is slightly larger than *L. hortensis* with body sizes between 4.6 – 6.5mm (females) and 4.0 – 7.2mm (males) (Helsdingen, 1969). Even though the size difference between the sexes is not very big and the males have a larger size range, a sexual size dimorphism is present with males being larger than females (Lang, 2001). The base colour of the female's cephalothorax is light brown with black markings in the shape of a tuning fork which makes this species easily identifiable. The abdomen is mostly creamy white with a leafy pattern in the middle and on the side (Fig. 14). The mature male's cephalothorax is darker in colour, a reddish brown, with the tuning fork pattern still visible. The abdomen is much slimmer than that of the female with the same colouration and pattern. Mature males have another distinctive feature, their enlarged chelicerae, which adds to the easy identification of this species (Fig. 14).

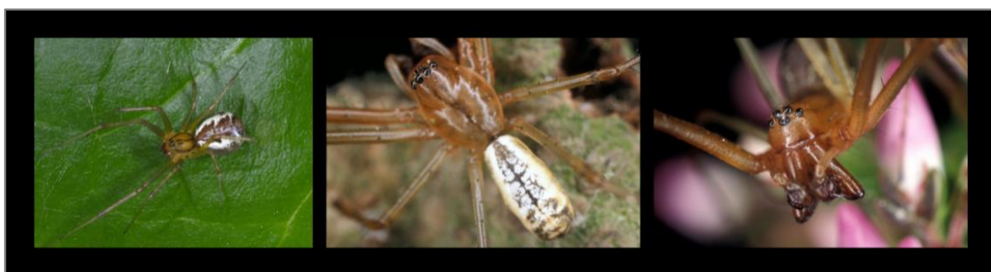

Figure 14: left: Female *L. triangularis* (© Saxifraga - Ab H. Baas, www.freenatureimages.eu), centre and right: male *L. triangularis* (© www.danmarks-edderkopper.dk)

Mature individuals can be found from July until October. As in *L. hortensis* males mature a few days before the females. All females mature over the course of three to four weeks (Stumpf, 1996). Males wait in the webs of penultimate females and mate with them immediately after they have moulted to maturity. Egg sacs are laid until late October and will not hatch

before late March the following year (Lang, 2001). The egg sacs will overwinter in the leaf litter during the cold month and developments commences once temperatures start to rise again in spring. The spiderlings hatch some time from mid to late spring. An egg sac contains around 30 eggs (Turnbull, 1960). Not much information is available about the operational sex ratio in this species. Toft observed a 1:1 sex ratio among subadults, concluding that the operational sex ratio should be skewed towards males (Toft, 1989). *L. triangularis* shows a more ritualised mating behaviour than *L. hortensis* even though some phases are similar i.e. pseudo-copulation, producing of a sperm-web and sperm induction directly before copulation takes place.

The mating pattern of *L. triangularis* is as follows (Stumpf, 1990): After entering the web of the female, the male will start to court her by shaking the web. This also helps him to assess if she is aggressive towards him. If she shows no aggression, the male will start to reduce the web around the female by destroying it (Rovner, 1968). Thereby he reduces the area of the web the female could retreat to. After web-reduction, he will move towards the female and gently make contact by touching one of her front legs with his own. If she accepts his touch, he will climb onto her ventral side and will start what is often a relatively long period of pseudo-copulation (48 – 86 min, (Stumpf, 1990)).The male will insert his pedipalp in an alternating pattern into the female's genital opening without transferring any sperm. After the pseudo-copulation the male will leave the female to produce a sperm web. He will place one droplet of sperm from his gonopore onto the sperm-web to then load both of his pedipalps from this droplet. After this sperm-induction the male returns to the female to begin the first copulation, again inserting one of his pedipalps at a time. Again, the elevated duration of the male's insertions is a good indicator of the "real" copulation (3 - 15s pseudo-copula vs 180s copula, (Stumpf, 1990)). The first copulation usually takes only a few minutes (Stumpf, 1990; Weldingh *et al.*, 2011) after which the males leaves the female again to build a second sperm-web and perform a second sperm induction. After this the male returns to the female again to copulate with her

for a second time. This second copulation takes much longer, often over one hour (54 – 76 min, (Stumpf, 1990; Weldingh *et al.*, 2011)). Once the male has finished the second copulation, he will leave the female's web. Even though the majority of the female's eggs can be fertilised in the first short copulation, the males (Stumpf, 1990) are always observed to attempt to mate twice with the same female.

Since males spend quite a long time in one female's web, it can happen that the pair is disturbed by the arrival of another male. In this case fierce and sometimes deadly fights over the female will occur. Usually the larger male will win these fights and will either chase the loser away or occasionally even kill him (Rovner, 1968). Both sexes in the species can re-mate, therefore sperm-competition and sperm precedence play an important part in *L. triangularis*' reproductive system. A study from 2011 showed that a first male precedence is present as long as the first male is allowed to transfer his first sperm droplet completely. In that case, even if the female re-mates, the first male will sire the majority of the female's offspring. Should the first male be interrupted before the complete transfer of the first sperm droplet and a second male mates with the females first male precedence changes to second male precedence and the second male sires more of the female's offspring (Weldingh *et al.*, 2011). The researchers used sterile males, which were sterilised by irradiation, in copulation trials, where females were mated with one fertile and one sterile male in succession. This made it possible to calculate the paternity percentage of the second male. All possible combinations between fertile and sterile males were used. (Weldingh *et al.*, 2011).

Spider collections were undertaken in spring and summer 2016 and 2017 in the UK, Germany, Denmark and Sweden (Table 5). Collections were made over two years for different reasons: (1) A larger overall sample size could be achieved. (2) Investigating possible fluctuations in spider density and habitat

usages. (3) Regarding Chapter 4: Looking at the fluctuation of the frequency of endosymbionts that are potentially present.

Table 5: Number of collected individuals of both species at all locations in 2016 and 2017

Species	Year	Country	Total	Males	Females
Linyphia hortensis	2016	Nottingham, UK	58	5	53
		Hamburg, Germany	22	2	20
		Lillebaelt, Denmark	-	-	-
		Muggetorp, Sweden	-	-	-
Linyphia hortensis	2017	Nottingham, UK	55	6	49
		Hamburg, Germany	15	0	15
		Lillebaelt, Denmark	12	0	12
		Muggetorp, Sweden	25	0	25
Linyphia triangularis	2016	Nottingham, UK	47	15	32
		Hamburg, Germany	45	11	34
		Muggetorp, Sweden	40	11	29
Linyphia triangularis	2017	Nottingham, UK	40	8	32
		Hamburg, Germany	42	4	38
		Muggetorp, Sweden	28	3	25

Whilst collecting *L. triangularis* was usually straightforward, in that once the appropriate type of habitat was spotted the spiders were usually there, this was not true for *L. hortensis*. *L. hortensis*' more ephemeral and patchy distribution, even when examining areas that had previously been found to be occupied, made it more often necessary to move from known locations to new ones. The best example was the location in Wollaton Park, Nottingham known as Arbour Hill. In 2016, *L. hortensis* were found in quite high abundance on leaves of bluebells close to the ground. When attempting to collect more spiders from that precise location in 2017, very few individuals were found. One potential reason for this might have been the difference in the mean temperature between the years, which potentially could have delayed the emergence of spiders in 2017 because temperatures stayed lower for longer than in 2016. Generally, *L. hortensis* were not always found at locations which seemed to be their ideal habitat, which again suggested that the distribution of this species was indeed patchy. *L. triangularis* were however reliably and repeatedly found in consecutive years at the same locations. This led to similar

number of individuals being collected at the different locations, in contrast to the wider range of sample sizes obtained for *L. hortensis* (Table 5).

3.2.1 Spider husbandry

Spiders were kept in MagentaTM GA7 vessels (77mm x 77mm x 97mm, referred to as “spider pots” or “pots2 from here on) for housing. In preliminary trials, the bottom of the pots was covered with cotton wool or by blue paper cloth, which was regularly moistened, to help keep the humidity in the pots at a sufficiently high level. Both of these were however prone to mould, especially when spider droppings and left-overs from prey items accumulated. The eventual solution was to place a moistened cotton wool ball in one corner of the pot and to replace it if it became mouldy. To provide anchor points for the spiders’ sheet webs, plastic straws were placed in the pots (Fig. 15). Females that took part in mating experiments were transferred to bigger plastic boxes (275 mm x 205 mm x 183 mm, (Wham - What more UK Ltd.), Fig. 15). At first, as for the housing pots, cotton wool was used to cover the bottom of the box but soon this was replaced by blue paper cloth alone, which was less prone to mould growth. Again, straws were used to provide attachment points for the sheet-webs.

Figure 15: left: MagentaTM GA7 vessels for housing spiders, right: Wham box for mating trials equipped with straws to provide anchor points for the sheet-web. The cotton wool shown in these pictures was replaced with blue paper cloth due to the quickly developing mould on the cotton wool.

To avoid web building on the underside of the lids of the MagentaTM vessels and of the mating boxes a thin layer of Vaseline was applied to the lids and upper part of the vessels/boxes. This procedure was dropped after the first experimental season because it appeared possible that the high spider mortality in the lab that year was connected to the use of Vaseline.

The spiders were fed fruit flies *ad libitum* once to twice a week. They were sprayed with water from plastic spray bottles five days a week to avoid dehydration. The dark/light cycle in the laboratory was equivalent to the natural photoperiod in spring/summer.

3.2.2 Cephalothorax size

The cephalothorax width of each spider was measured using a stereo dissection microscope with a built-in graticule. Measurements were taken at the widest point of the cephalothorax. Since the cephalothorax width was the only body size measurement the terms cephalothorax width, cephalothorax size and size will be used interchangeably during this chapter.

3.2.3 Ballooning

Ballooning experiments were conducted in June 2018, with mature individuals and spiderlings of *L. hortensis* and juvenile individuals of *L. triangularis*. To create airflow that would motivate and allow for the spiders to take off, a standard desktop fan and a less powerful hand-held fan (Fig. 16) were used.

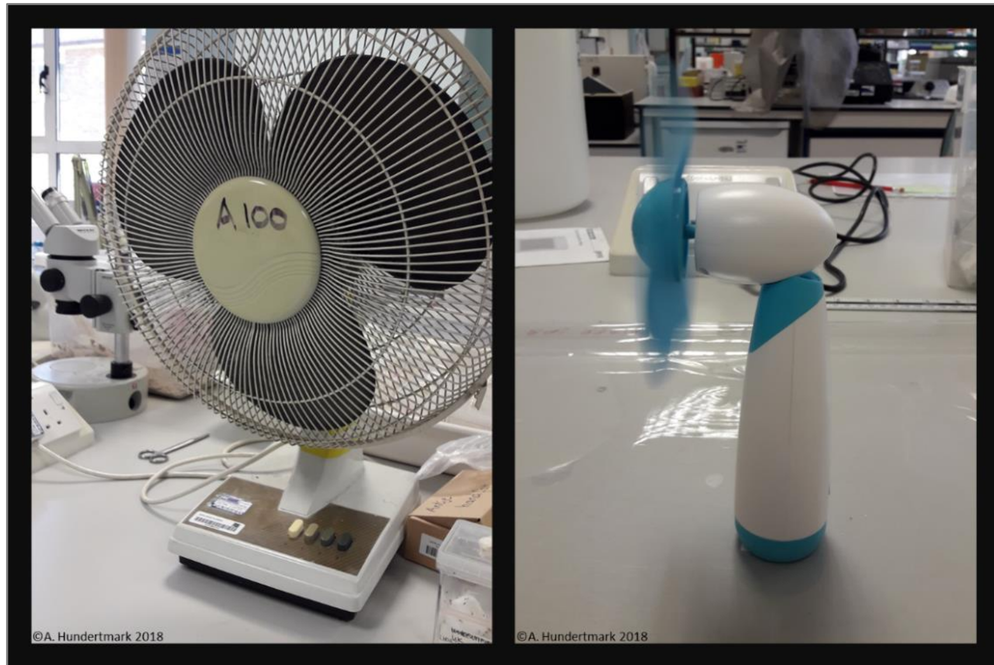

Figure 16: left: large desktop fan, used at lowest setting, right: hand-held fan, only one setting.

The desktop fan was placed 1.30m away from a toothpick which was stuck onto a small Styrofoam block (Fig. 17). The fan was switched on at the lowest possible speed and the spider was carefully placed onto the toothpick. It was then observed for 90s at this wind speed. After that the desktop fan was switched off and exchanged for the smaller, less powerful hand fan. This was placed 30cm away from the spider on the toothpick and switched on (Fig. 17). The spider was again watched for 90s.

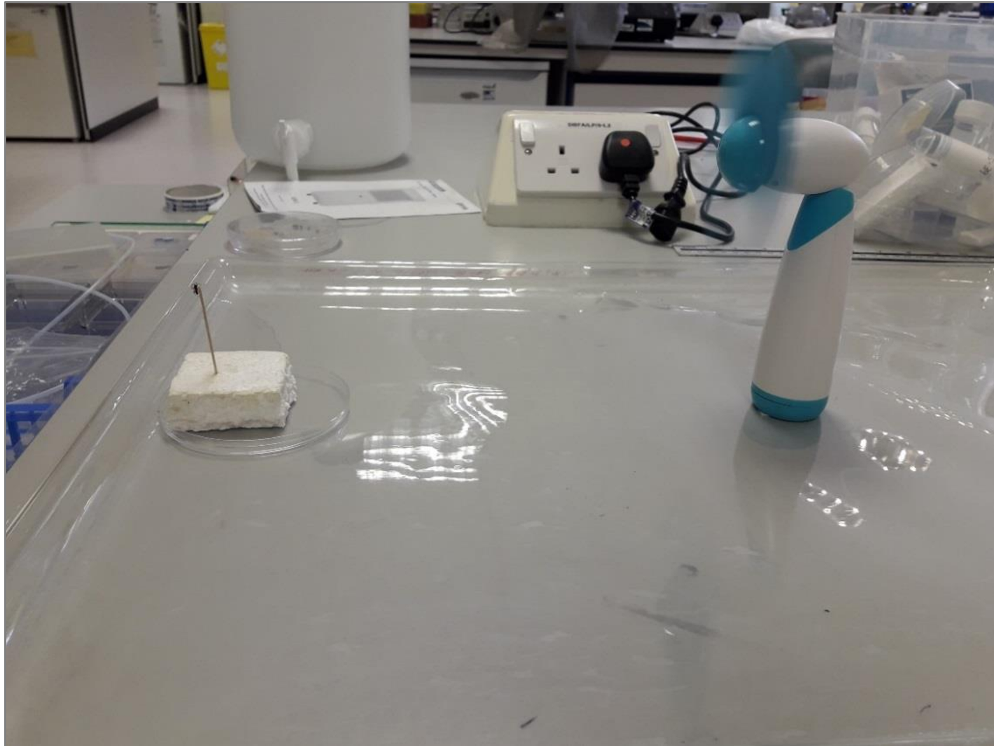

Figure 17: *L. hortensis* on a toothpick stuck into a Styrofoam block. A hand-held fan was placed 30cm away.

3.2.4 Mating experiments

For the mating experiments females were transferred from the spider pots into the larger mating boxes at least one day prior to the mating trial to allow them to build a sufficiently large enough sheet web. Males were introduced with a paint brush in a corner of the web far away from the females to avoid antagonistic behaviour between them. The time of the introduction was noted. If copulation did not occur in a time frame of two hours the trial was terminated by the experimenter. If the male showed interest, engaged in courtship and copulation seemed imminent, close to the two-hour cut-off point, the trial duration was extended until copulation had taken place.

For *L. hortensis* the following data were noted:

1. Time and date
2. Female and male ID
3. Female and males mating status (unmated/once/twice/unknown)
4. Time of male introduction
5. Courtship (yes/no)

6. 'Tasting' – moving legs through chelicerae, possibly to detect female silk-borne pheromones or gather other information about the female
7. Start and end of pseudo-copulation, to determine latency until pseudo-copulation and duration
8. Building of sperm web (yes/no)
9. Sperm induction (yes/no)
10. Start and end of copulation, to determine latency until copulation and duration
11. Termination of copulation (male/female)
12. Time of taking male out.

For *L. triangularis* data collection was similar to *L. hortensis* in points 1 – 7 and 12/16 respectively. From 8 onwards it was slightly different due to the two copulations occurring in *L. triangularis* matings. The following data were noted exclusively for *L. triangularis*:

8. Building of 1st sperm web (yes/no)
9. 1st sperm induction (yes/no)
10. Start and end of 1st copulation, to determine latency until 1st copulation and duration
11. Termination of 1st copulation (male/female)
12. Building of 2nd sperm web (yes/no)
13. 2nd sperm induction (yes/no)
14. Start and end of 2nd copulation, to determine latency until 2nd copulation and duration
15. Termination of 2nd copulation (male/female)
16. Time of taking male out.

3.2.5 DNA extraction and PCR

DNA was extracted from spider leg tissue with the salt DNA extraction method (Aljanabi and Martinez, 1997; Sunnucks and Hales, 1996). Proteinase K was used to digest the tissue, the DNA was re-suspended in sterile distilled water buffer. An approximately 900bp long fragment of a highly conserved region of

the mtDNA was amplified using specific primer pairs LCO1490 - GGT CAA CAA ATC ATA AAG ATA TTG G (Folmer *et al.*, 1994)/HCOoutout - GTA AAT ATA TGR TGD GCT C (Wheeler laboratory fide (Schulmeister *et al.*, 2002)). For reasons yet unknown the LCO1490/HCOoutout primer pair, which yielded good results amplifying *L. hortensis* mtDNA, did not work on the *L. triangularis* samples. A different primer pair ExtA - GAA GTT TAT ATT TTA ATT TTA CCT GG/ExtB – CCT ATT GAW ARA ACA TAR TGA AAA TG (Wheeler laboratory fide (Schulmeister *et al.*, 2002)) was used in testing these samples.

The following PCR protocol was applied to amplify the mtDNA: A total volume of 10µl contained 1µl DNA sample, 5µl 1x BioMix™ Red PCR master mix, 0.5 µl of each primer (10 µmol) and 3µl of sterile distilled water to make up to the final volume. PCRs were run under the following conditions: Initial denaturing at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30s, annealing at 50 °C (or optimal annealing temperature (Tm °C) for 45s and extension at 72 °C for 45 s followed by a final extension step at 72 °C for 5 min. The PCR products were visualised via gel-electrophoresis on 1.5% agarose gels stained with Ethidium Bromide under UV light.

For sequencing purposes, the reaction volume was increased to 30µl with still 1µl of the DNA sample (15µl BioMix™ Red PCR master mix, 1.5µl of each primer (10 µmol) and 11µl of sterile distilled water). Sanger sequencing was carried out at Macrogen Inc. and retrieved sequences were analysed using MEGA6 (Tamura *et al.*, 2013), BioEdit (Hall, 1999), NCBI BLAST search and ChromasLite (Technelysium Pty Ltd, 2012).

3.3 Results

3.3.1 Sex ratio

L. hortensis

The sex ratio of *L. hortensis* was distorted in all populations from every country in both years (Table 6).

Table 6: Sex ratio in *L. hortensis* populations from the UK, Germany, Denmark and Sweden in 2016 and 2017. Sex ratio was calculated as the proportion of males.

Year	Country	Males	Females	Male SR	Exact binomial test (p value)
2016	UK	5	53	0.09	3.496E-11
2016	GER	2	20	0.09	1.211E-04
2017	UK	6	49	0.11	1.823E-09
2017	GER	0	15	0	6.104E-05
2017	DK	0	12	0	4.883E-04
2017	SWE	0	25	0	5.960E-08

In 2016 the sex ratio did not differ significantly (Fisher's Exact test, $p = 1$) between the British and the German populations, it was equally skewed towards females. The same was true for the sex ratio of the populations from 2017 (Fisher's Exact test, $p = 0.1817$). Comparing the sex ratio of the populations from all locations and both years did not detect a difference in sex ratio (Fisher's exact test, $p = 0.4435$). A test for equality of proportions did not identify a significant difference in the sex ratio distortion between the years (2-sample test for equality of proportions, $Chi^2 = 0.29746$, $df = 1$, p-value = 0.5855).

L. triangularis

The sex ratio of *L. triangularis* populations from the UK, Germany and Sweden was distorted in both years (Table 7).

Table 7: Sex ratio in *L. triangularis* populations from the UK, Germany and Sweden in 2016 and 2017. Sex ratio was calculated as the proportion of males.

Year	Country	Males	Females	Male SR	Exact binomial test (p-value)
2016	UK	15	32	0.32	0.01862
2016	GER	11	34	0.24	0.0008241
2016	SWE	11	29	0.28	0.00126
2017	UK	8	32	0.20	0.0001822
2017	GER	4	38	0.10	5.653E-08
2017	SWE	3	25	0.11	1.40E-06

Applying a test for equality of proportions revealed that there was a significant difference in the proportion of males between 2016 and 2017 (2-sample test for equality of proportions, $Chi^2 = 6.1717$, $df = 1$, p-value =

0.01298). The proportion of males was significantly lower in 2017 (2016: 0.28%, 2017: 0.14%). Comparing the proportions of males between the three locations in 2016 and 2017 showed no significant difference in either of the years (3-sample test for equality of proportions, 2016: $Chi^2 = 0.64397$, df = 2, p-value = 0.7247, 2017: $Chi^2 = 2.1816$, df = 2, p-value = 0.3359). Despite the significant difference in male proportion between the years, a Mann-Whitney-U test could not confirm a significant difference in sex ratio between 2016 and 2017 (Mann-Whitney-U test, W = 47.5, p-value = 0.2313). Analysing the difference in sex ratio between the populations from Germany and the UK showed no significant difference, neither in 2016 (Mann-Whitney-U test, W = 9, p-value = 0.9009) nor in 2017 (Mann-Whitney-U test, W = 3, p-value = 0.6428), between the two locations.

3.3.2 Cephalothorax size

L. hortensis

Cephalothorax width was measured from a total of 172 spiders (UK: 105, GER: 33, DK: 12, SWE: 22) collected in 2016 and 2017. A GLM was used to analyse the complete dataset and to identify factors that potentially could affect cephalothorax width. The factors used in the GLM were year of collection, country of origin and sex. A Gamma error structure with log identity was applied. Of the aforementioned factors, only the year of collection had a significant effect on cephalothorax size. Spiders in 2016 were significantly smaller than 2017 (2016: N = 81, mean cephalothorax width (m.ceph.w.) = 1.46 mm ± 0.02, 2017: N = 107, m.ceph.w. =1.52 mm ± 0.01; GLM, year: $F_{1, 184} = 8.7426$, p = 0.003525). Additionally, there was a significant interaction between year of collection and country of origin (GLM, year:country: $F_{4, 180} = 3.0426$, p = 0.018571).

To investigate these significant factors further, the dataset was split into subsets, one for each year. There was no significant cephalothorax size difference between individuals from the UK 2016 and 2017 (Mann-Whitney-U test, W = 1321, p-value = 0.1415). German spiders were significantly smaller

in 2016 than in 2017 (Mann-Whitney-U test, W = 73, p-value = 0.003042). The populations in 2017 did not differ significantly in cephalothorax size (UK: N = 55, m.ceph.w. = 1.53mm ± 0.02, GER: N = 15, m.ceph.w. = 1.55mm ± 0.04, DK: N = 12, m.ceph.w. = 1.52mm ± 0.03, SWE: N = 22, m.ceph.w. = 1.48mm ± 0.02; Kruskal-Wallis test, Kruskal-Wallis Chi^2 = 5.24, df = 3, p = 0.155, Fig. 18).

Two separate GLMs were run, one on each of the subsets. In 2016 differences in cephalothorax size could be explained by sex and country of origin (GLM: sex: $F_{1,77}$ = 4.1249, p = 0.045709; country: $F_{1,78}$ = 7.0045, p = 0.009855). The difference in size between the German (N = 22, m.ceph.w. = 1.38mm ± 0.04) and the British (N = 59, m.ceph.w. = 1.48mm ± 0.02) population was significant (Mann-Whitney-U test, W = 400.5, p-value = 0.0085, Fig. 18). Females were also significantly smaller than males in that year (females: N = 73, m.ceph.w. = 1.45 ± 0.02, males: N = 7, m.ceph.w. = 1.57 ± 0.04; Mann-Whitney-U test, W = 371.5, p = 0.04271, Fig. 19). The interaction between the two factors was not significant (GLM: $F_{1,76}$ = 0.3868, p = 0.53587).

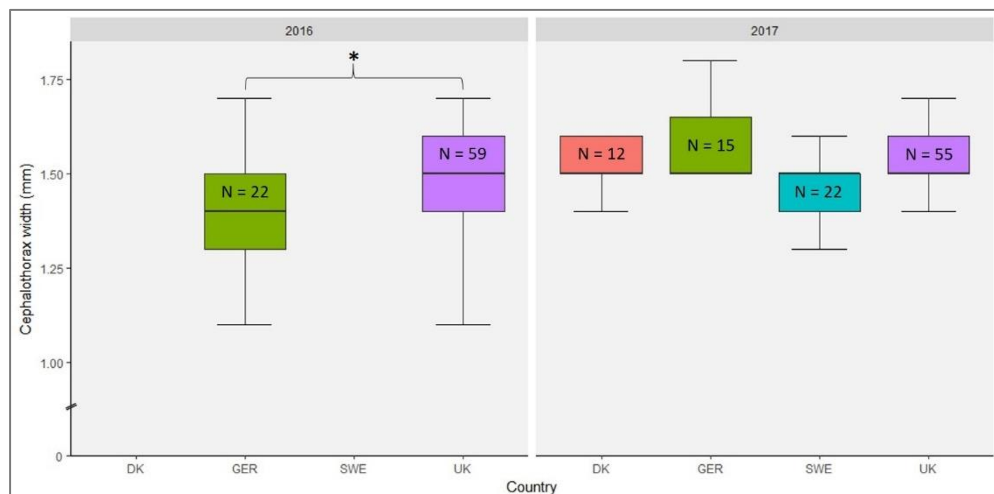

Figure 18: Cephalothorax width in mm for populations from each country of origin in both years. German and British spiders differed significantly in size in 2016. Green = Germany, purple = UK, red = Denmark, blue = Sweden. The * denotes a significant difference. The error bars represent the standard error.

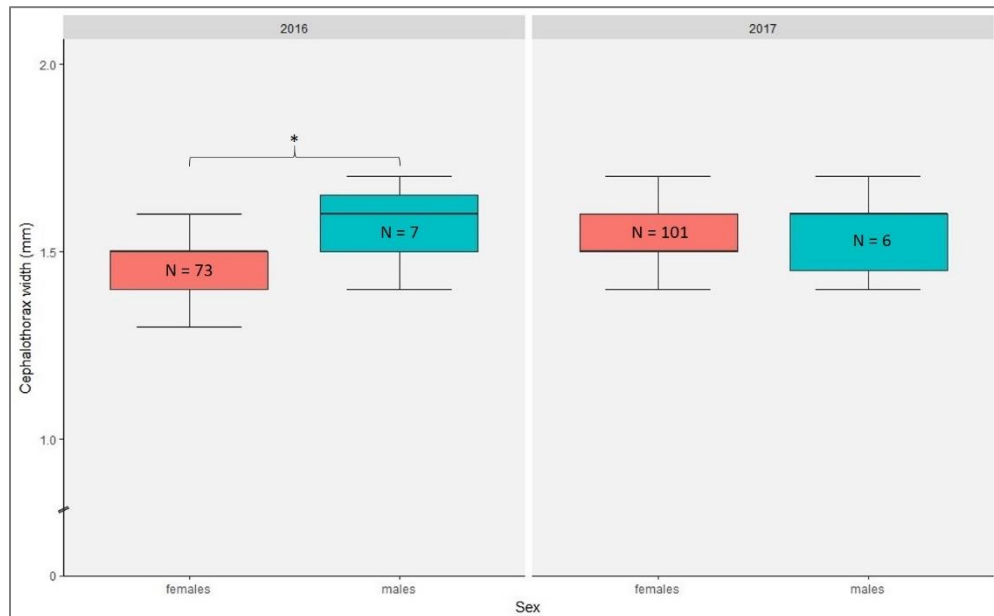

Figure 19: Cephalothorax width in mm for females and males in both years. In 2016 females were significantly smaller than males. There was no significant size difference in 2017. Red = females, blue = males. The * denotes a significant difference. The error bars represent the standard error.

In 2017 neither country of origin nor sex had a significant influence on cephalothorax width (GLM: sex: $F_{1,101} = 0.0725$, $p = 0.7882$; country: $F_{3,102} = 1.3866$, $p = 0.2512$). There was no significant size difference in males and females from 2017 (males: N = 6, m.ceph.w. = 1.52 ± 0.07, females: N = 101, m.ceph.w. = 1.52 ± 0.01; Mann-Whitney-U, W = 333, p-value = 0.6436). No significant size difference was found in males from 2016 and 2017 (2016: N = 7, m.ceph.w. = 1.57 ± 0.04, 2017: N = 6, m.ceph.w. = 1.52 ± 0.07; Mann-Whitney-U test, W = 23.5, p-value = 0.7668) but there was a significant size difference between females from 2016 and 2017 (2016: N = 73, m.ceph.w. = 1.45 ± 0.02, 2017: N = 101, m.ceph.w. = 1.52 ± 0.01; Kruskal-Wallis test, $Chi^2 = 8.2115$, df = 1, p-value = 0.004163); females from 2016 were smaller (Fig. 20).

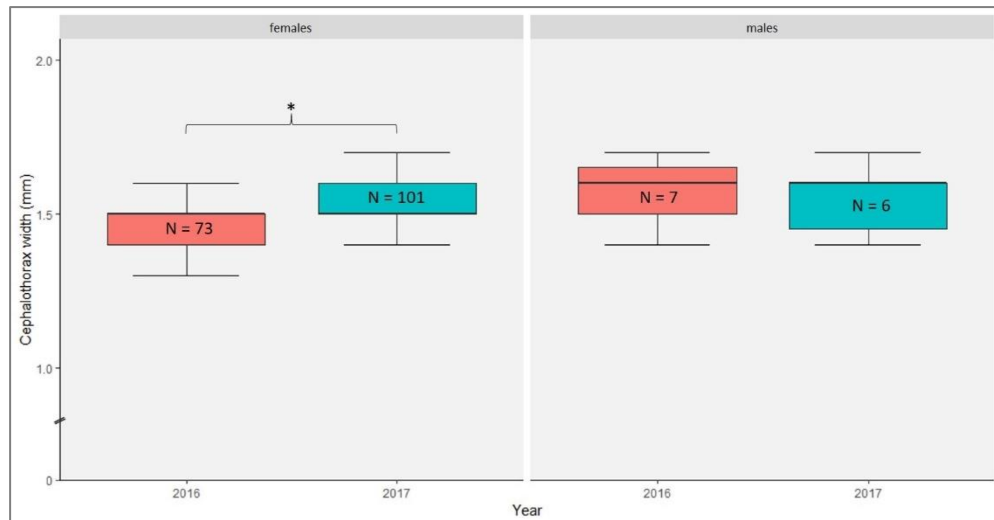

Figure 20: Male and female cephalothorax width in mm compared between 2016 and 2017. Females were significantly smaller in 2016 whilst males did not differ in size. Red = collection year 2016, blue = collection year 2017. The * denotes a significant difference. The error bars represent the standard error.

Comparing the size of British females from 2016 and 2017 showed no difference in cephalothorax width (Kruskal-Wallis Chi^2 = 2.8887, df = 1, p = 0.0892; 2016: N = 53, m.ceph.w. = 1.48mm ± 0.02, 2017: N = 49, m.ceph.w. = 1.53mm ± 0.02) whilst German females differed significantly in size between the years (Kruskal-Wallis Chi^2 = 10.141, df = 1, p = 0.00145; 2016: N = 20, m.ceph.w. = 1.37mm ± 0.04, 2017: N = 15, m.ceph.w. = 1.55mm ± 0.04). Females were significantly larger in 2017. Due to the low number of males in both years, no statistical comparison was attempted.

L. triangularis

A total number of 242 *L. triangularis* were collected in the year 2016 and 2017 (UK: N = 87, GER: N = 87, SWE: N = 68) and their cephalothorax width was measured. To analyse the data a GLM on the full dataset was run with cephalothorax width being the response variable and country of origin, year of collection and sex as explanatory factors. A gamma error structure with log identity was chosen.

As in *L. hortensis* a significant difference in cephalothorax width between 2016 (N = 132, m.ceph.w. = 1.66mm ± 0.02) and 2017 (N = 110, m.ceph.w. =

1.81 ± 0.02) was found; spiders in 2017 were significantly larger (GLM: year: $F_{1,217}$ = 25.567, p = 9.229e-07). Country of origin had no significant effect on the cephalothorax width of the spiders (GLM: country: $F_{2,215}$ = 1.7392, p = 0.1782) whilst the interaction between year of collection and country of origin was significant (GLM: year:country: $F_{4,212}$ = 10.681, p = 6.631e-08). Spiders collected at the three locations did not differ in size (mean cephalothorax width: UK: 1.75 mm ± 0.03, GER: 1.77mm ± 0.03, SWE: 1.68 mm ± 0.02). Nevertheless, an intra-population comparison revealed that Swedish spiders were significantly smaller than German spiders (Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 6.359, df = 1, p = 0.01168). whilst there were no size differences between German and British and British and Swedish spiders (UK vs GER: Mann-Whitney-U test, W = 2816.5, p-value = 0.5156; UK vs SWE: Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 2.749, df = 1, p-value = 0.09731). Looking at the difference between males and females an expected significant difference was found. Males were significantly larger than females (males: N = 52, m.ceph.w. = 1.87 mm ± 0.04, females: N = 190, m.ceph.w. = 1.7mm ± 0.02; GLM: sex: $F_{1,216}$ = 31.967, p = 5.029e-08; Fig. 21). Since the spiders differed in cephalothorax width between the years the dataset was split into two datasets.

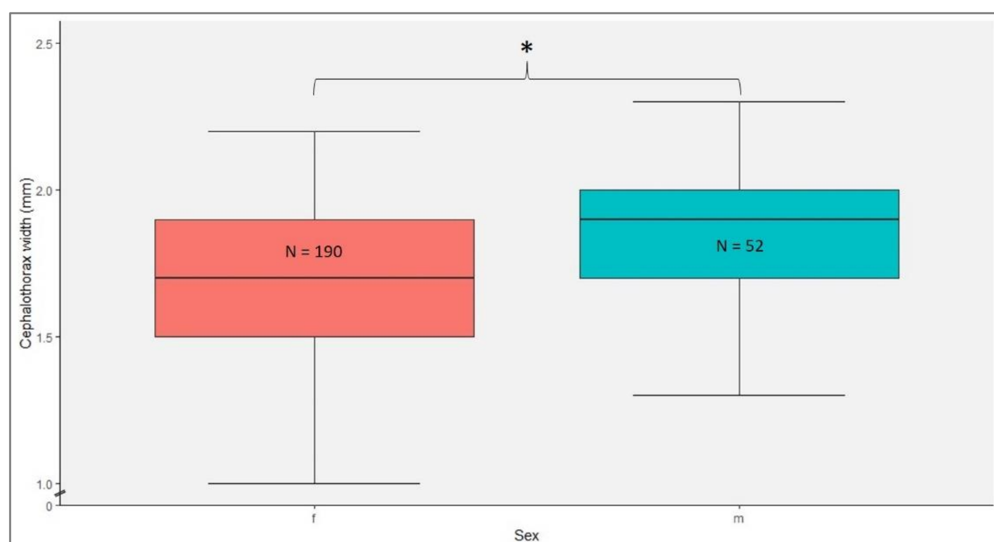

Figure 21: Cephalothorax width in mm in female and male *L. triangularis*. Males were significantly larger than females. Red = females, blue = males. The * denotes a significant difference. The error bars represent the standard error.

A GLM with a gamma error structure and log identity was used to investigate the potential effect of country of origin and sex on cephalothorax width in 2016. Country of origin had a significant effect on spider size (UK: N = 47, m.ceph.w. 1.58mm ± 0.03, GER: N = 45, m.ceph.w. 1.69mm ± 0.04, SWE: N = 40, m.ceph.w. 1.74mm ± 0.03; GLM: country: $F_{2,107} = 5.1772$, $p = 0.007142$; Fig. 22). British spiders were significantly smaller than Swedish spiders (Mann-Whitney-U test, W = 478, p-value = 0.004645) whereas there was no size difference neither between the British and the German (Mann-Whitney-U test, W = 520.5, p-value = 0.08767) nor the German and the Swedish spiders (Mann-Whitney-U test, W = 563.5, p-value = 0.58). Males and females differed in size with males being significantly larger (males: N = 37, m.ceph.w. = 1.77mm ± 0.04, females: N = 95, m.ceph.w. = 1.62mm ± 0.03; GLM: sex: $F_{1,109} = 8.1969$, $p = 0.005050$). The interaction between sex and country of origin was not significant (GLM: sex:country: $F_{2,105} = 1.1895$, $p = 0.308429$).

For the 2017 dataset, again a GLM with gamma error structure and log identity was used to analyse the potential effect of country of origin and sex on cephalothorax width. As in 2016, country of origin had a significant effect on cephalothorax width (SWE: N = 28, m.ceph.w. = 1.60mm ± 0.03, UK: N = 40, m.ceph.w. = 1.93mm ± 0.04, GER: N = 42, m.ceph.w. = 1.84mm ± 0.03; GLM: country: $F_{2,104} = 20.016$, $p = 4.424e-08$; Fig. 22). Swedish spiders were significantly smaller in this year than British (Kruskal-Wallis rank sum test, Kruskal-Wallis $Chi^2 = 28.581$, df = 1, p = 8.988e-08) and German spiders (Kruskal-Wallis rank sum test, Kruskal-Wallis $Chi^2 = 20.579$, df = 1, p = 5.723e-06) whilst there was no significant difference between the spiders from the UK and Germany (Mann-Whitney-U test, W = 990, p-value = 0.1048). Sex affected size with males being significantly larger than females (males: N = 15, m.ceph.w. = 2.11mm ± 0.07, females: N = 95, m.ceph.w. = 1.77mm ± 0.02; GLM: sex: $F_{1,106} = 38.225$, $p = 1.253e-08$). The interaction between country and sex was not significant (GLM: sex:country: $F_{2,102} = 1.2832$, $p = 0.2816$).

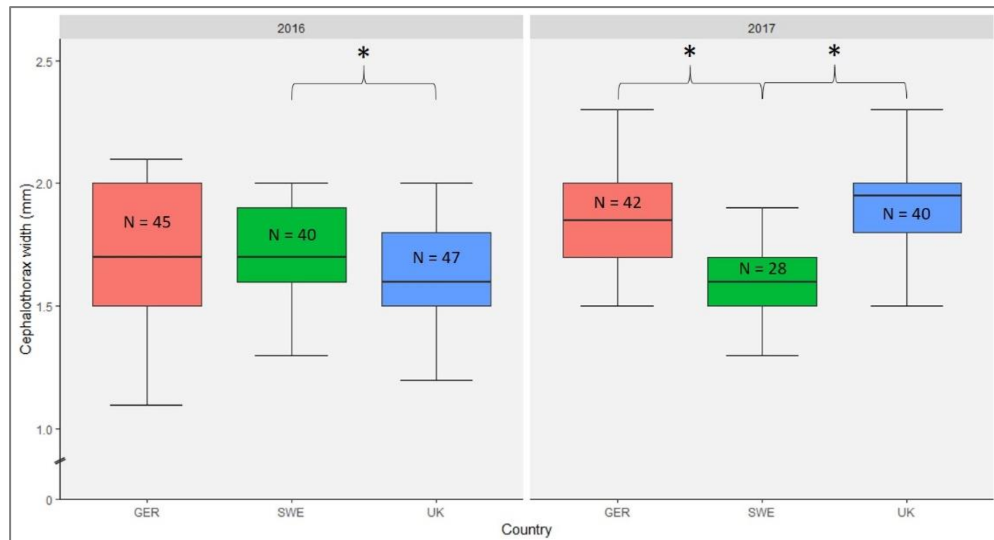

Figure 22: Cephalothorax width of *L. triangularis* from the three countries of origin in both years. In 2016 spiders from the UK were significantly smaller than Swedish spiders. In 2017 Swedish spiders were significant smaller than those from Germany and the UK. Red = Germany, green = Sweden, blue = UK. The * denotes a significant difference. The error bars represent the standard error.

To analyse the differences in cephalothorax width between the 2016 and 2017 further non-parametric tests were used to compare the different populations in both years (Fig. 23). Spiders collected in the UK differed in their size between 2016 and 2017 (2016: N = 47, m.ceph.w. = 1.58mm ± 0.03, 2017: N = 40, m.ceph.w. = 1.92mm ± 0.04), spiders were significantly smaller in 2016 (Mann-Whitney-U test, W = 238.5, p = 3.131e-08). The same pattern was found in the German spiders where individuals collected in 2016 were significantly smaller than those from 2017 (2016: N = 45, m.ceph.w. = 1.69mm ± 0.04, 2017: N = 42, m.ceph.w. = 1.84mm ± 0.04; Mann-Whitney-U test, W = 483.5, p = 0.03455). In the Swedish spiders the pattern was reversed where spiders were significantly larger in 2016 than in 2017 (2016: N = 40, m.ceph.w. = 1.74mm ± 0.03, 2017: N = 28, m.ceph.w. = 1.60mm ± 0.03; Kruskal-Wallis rank sum test, Kruskal-Wallis Chi² = 6.658, df = 1, p = 0.009871).

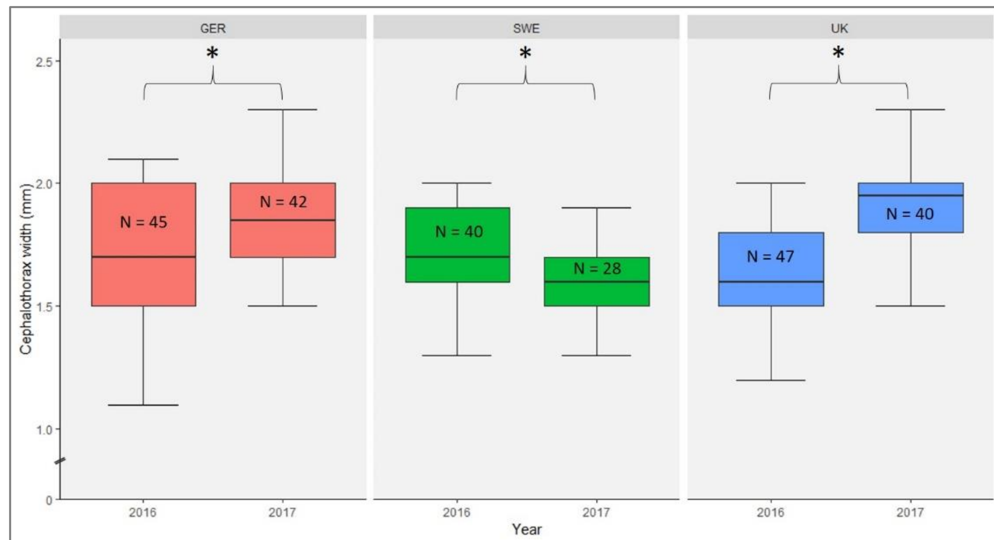

Figure 23: Comparison of cephalothorax width of *L. triangularis* in 2016 and 2017. German and British spiders were significantly smaller in 2016 than in 2017 whilst Swedish spiders were significantly larger in 2016. Red = Germany, green = Sweden, blue = UK. The * denotes a significant difference. The error bars represent the standard error.

When comparing the mean cephalothorax width of males in 2016 and 2017 it was found that males collected in 2016 were significantly smaller than those from 2017 (2016: N = 37, m.ceph.w. = 1.77mm ± 0.04, 2017: N = 15, m.ceph.w. = 2.11mm ± 0.07; Mann-Whitney-U test, W = 80.5, p = 0.0005746). Similarly, females were significantly smaller in 2016 than in 2017 (2016: N = 95, m.ceph.w. = 1.62mm ± 0.03, 2017: N = 95, m.ceph.w. = 1.77mm ± 0.02; Mann-Whitney-U test, W = 2449.5, p-value = 0.0001014).

Finally, intra-population comparisons of female and male cephalothorax width in both years were conducted (Fig. 24). The results for the males must be considered with care since only a small number of males was available from 2017. Comparing the size of German females in 2016 (N = 34, m.ceph.w. = 1.67mm ± 0.05) and 2017 (N = 38, m.ceph.w. = 1.8mm ± 0.03) showed that there was no significant difference (Mann-Whitney-U test, W = 317, p = 0.09674). In the populations from the UK, female size differed between the year, females were significantly smaller in 2016 (2016: N = 32, m.ceph.w. = 1.53mm ± 0.04, 2017: N = 32, m.ceph.w. = 1.86mm ± 0.04; Mann-Whitney-U test, W = 120, p-value = 9.461e-07). The Swedish females did not differ in cephalothorax width between the years (2016: N = 29, m.ceph.w. = 1.67mm

± 0.04, 2017: N = 25, m.ceph.w. = 1.6mm ± 0.04; Mann-Whitney-U test, W = 425, p-value = 0.1755). German male cephalothorax width differed between the years with males from 2017 being significantly larger than those from 2016 (2016: N = 11, m.ceph.w. = 1.72mm ± 0.08, 2017: N = 4, m.ceph.w. = 2.23mm ± 0.15). There was a significant difference in UK male size between 2016 and 2017 (2016: N = 15, m.ceph.w. = 1.68mm ± 0.05, 2017: N = 8, m.ceph.w. = 2.16mm ± 0.08), males in 2016 were smaller (Mann-Whitney-U test, W = 7.5, p = 0.001334). Males collected in Sweden in 2016 and 2017 did differ significantly in size (2016: N = 11, m.ceph.w. = 1.93mm ± 0.02, 2017: N = 3, m.ceph.w. = 1.7mm ± 0; Mann-Whitney-U test, W = 18, p = 0.03516). Nevertheless, since the sample size for 2017 was very low, it cannot be assumed that the difference identified is real.

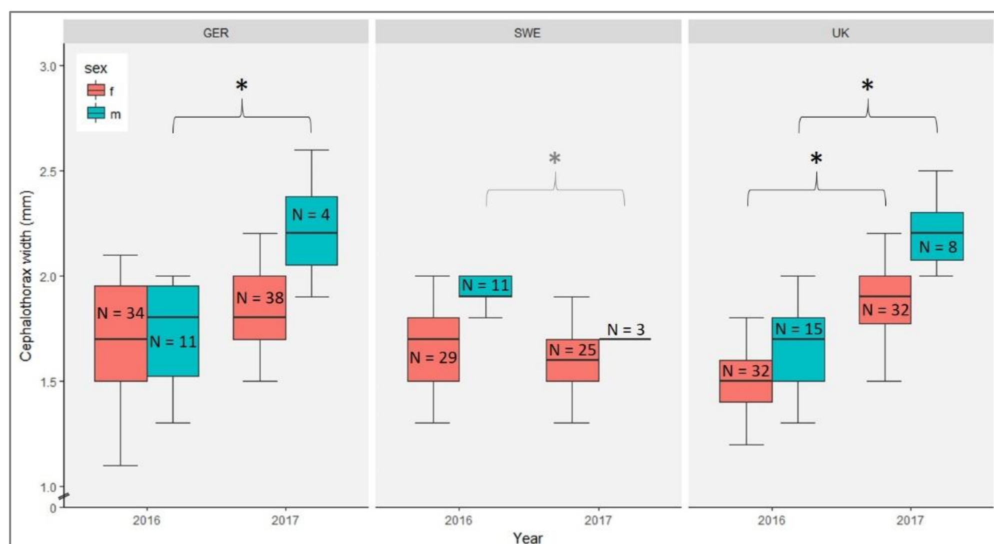

Figure 24: Comparison of male and female *L. triangularis* cephalothorax width in 2016 and 2017 for all locations. German males were significantly larger in 2017. There was a significant difference in Swedish males but since the sample from 2017 was small, this result must be interpreted with caution. British males and females were significantly larger in 2017. Red = females, blue = males. The * denotes a significant difference. The error bars represent the standard error.

3.3.3 Ballooning

A total of 31 *L. hortensis* (11 adult, 20 spiderlings) and six juvenile *L. triangularis* were tested in the ballooning trials. None of the spiders did attempt to take off and no pre-ballooning behaviour such as tiptoeing was observed. Spiders placed on the toothpick either immediately started running

down when the fan was switched on or they clung to the leeward side of the toothpick. This was true for the large as well as for the hand-held fan.

Additionally, a few trials with juveniles and adults placed on water to see if *Linyphia* is able to sail were attempted. Hayashi *et al*. (2015) state that if a spider can sail, landing on water due to ballooning is not an issue. Otherwise if the spider is not capable of sailing it should not balloon at all (Hayashi *et al.*, 2015). Even though all of those tested were capable of standing on the water, none of them used their legs and silk to attempt to sail. This was in line with trials conducted on *Linyphia*, which found that the spiders did not attempt to sail at all (Goodacre, pers. comm.).

3.3.4 Mating experiments

A total of 22 *L. hortensis* (males: 10, females: 12) and 47 *L. triangularis* (males: 19, females: 28) were used in mating trials in 2016 and 2017. It has to be mentioned at this point that the duration of the trials was often more than two hours if a mating did occur. In contrast, trials where no copulation was observed were often terminated earlier by the experimenter because the female behaved antagonistically towards the male, i.e. chasing him away, or the male showed absolutely no interest in the female and on some occasions even tried to exit the mating box. A few mating trials were recorded with a Samsung smartphone video camera since no other device, suitable for recording such small spiders, was available. Additionally, since it became clear that the behavioural analysis would not be feasible, recording of mating trials was ceased. The available footage has not been analysed so far since the focus of the thesis was shifted towards molecular investigations.

L. hortensis

In 2016 a few mating trials were conducted as a preliminary study to establish the experimental protocol. In total, 13 mating trials were set up but only in two cases were successful copulations observed. In 2017, four mating trials

were conducted to try and breed the spiders. These datasets were both too small for meaningful statistical analyses.

L. triangularis

Due to the high mortality of individuals from all three populations in 2016, only a limited number of trials was possible. A total number of 12 mating trials was set up. In four cases, successful mating was observed. One of these four trials must be classed a pseudo-replication, since the same male was used twice. Due to the issues in 2016, it was decided not to continue the mating behavioural trials in 2017. Nevertheless, a total number of 19 mating trials were set up to gain egg sacs which then could be used to study transmission rates of potentially present endosymbionts (Chapter 4). Due to the reduced number of males, many of the mating trials must be classed as pseudo-replications with some males being used as often as three times. No statistical analyses were attempted.

Egg sacs

L. hortensis

A total number of 138 egg sacs were laid by female *L. hortensis* brought to the lab in 2016 (N = 51) and 2017 (N = 87). For eight egg sacs from 2017 hatching success was unknown. Therefore, these egg sacs were removed from the analysis. Egg sacs were allowed to hatch and the offspring were kept and cared for until death. Some of the egg sacs laid in 2017 were split in half, with one half being put into EtOH directly after hatching. The other half of the hatched spiderlings were again kept and cared for until death. The aim was to rear these spiderlings until maturation and to set up a laboratory population. Spiderlings were fed with *Drosophila* sp. *ad libitum* once to twice a week and the cotton wool on top of the spider pots were moistened with water five days a week. Unfortunately, the husbandry measures applied were not suitable for the spiderlings since none of them survived until maturation. Additionally,

some of the 2017 egg sacs were put in EtOH directly after they had been discovered.

Egg/offspring numbers were very variable between females, ranging from 1 to 66 eggs/hatchlings over both years in total. The mean number of eggs/offspring differed significantly between the years (2016: N = 51, mean number eggs/offspring = 12.34 ± 1.99, 2017: N = 87, mean number eggs/offspring = 22.35 ± 1.43; Mann-Whitney-U test, W = 814.5, p = 4.182e-05). This difference must be considered with care since the number of eggs/hatchlings in 2016 was not assessed directly after hatching. Therefore, sibling cannibalism and spiderling mortality induced by other factors could have artificially lowered the number of offspring. Investigating the influence of country of origin on the egg/offspring number revealed no significant difference between the populations (Kruskal-Wallis Chi^2 = 3.9441, df = 3, p = 0.2676).

There was a significant correlation between the cephalothorax width of the females and the number of eggs/offspring she produced, larger females had significant more eggs/offspring (Kendall's rank correlation tau, tau = 0.1882919, z = 2.7274, p = 0.006384). The overall egg hatching rate was 78% with significant differences between 2016 and 2017 (2016: 92%, 2017: 72%; 2-sample test for equality of proportions, Chi^2 = 6.5764, df = 1, p = 0.01033). Country of origin also had a significant effect on the hatching success of egg sacs (Fisher's Exact Test, p-value = 0.02348). Due to the low number of egg sacs from German spiders (N = 4) in 2016 and the differences in egg/offspring number and hatching rate between 2016 and 2017, the dataset was partitioned into separate datasets for 2016 and 2017.

Analysing the number of eggs/offspring in 2016, a difference between the German and the British populations was found which was approaching significance (UK: N = 47, mean number egg/offspring = 13.26 ± 2.12, GER: N = 4, mean number egg/offspring = 2.33 ± 1.15; Kruskal-Wallis Chi^2 = 3.6115, df

= 1, $p = 0.05738$; Fig. 25). Nevertheless, because the number of egg sacs from the German population was low, it is not possible to establish at this point whether there truly is a difference between the locations. The correlation between the egg/offspring number and female cephalothorax width was highly significant (Kendall's rank correlation tau, tau = 0.3827472, $z = 3.0159$, $p = 0.002562$, Fig. 27), larger females had larger clutches. There was no significant difference in hatching success between egg sacs from German and British spiders (GER: 100%, UK: 91%; Fisher's Exact test, $p = 1$, Fig. 26). Again, because of the low German sample size it is unclear whether there was truly no difference between the populations.

In 2017, a significant difference in egg/offspring number between the four countries of origin was found (Kruskal-Wallis $Chi^2 = 13.384$, $df = 3$, $p = 0.003875$, UK: $N = 48$, mean number egg/offspring = 26.32 ± 1.76, GER: $N = 12$, mean number egg/offspring = 21.64 ± 4.64, DK: $N = 9$, mean number egg/offspring = 19.22 ± 4.67, SWE: $N = 18$, mean number egg/offspring = 12.94 ± 2.16). The differences were due to Swedish females producing significantly smaller offspring clutches than UK females (Mann-Whitney-U test, $W = 610$, $p = 0.0002253$; Fig. 25). There were no significant differences between the other populations (UK vs. GER: Mann-Whitney-U test, $W = 288.5$, $p = 0.5583$; UK vs. DK: Mann-Whitney-U test, $W = 288$, $p = 0.08982$; GER vs. DK: Mann-Whitney-U test, $W = 56.5$, $p = 0.6206$; GER vs. SWE: Kruskal-Wallis $Chi^2 = 1.3476$, $df = 1$, $p = 0.2457$; DK vs. SWE: Mann-Whitney-U test, $W = 94$, $p = 0.2225$).

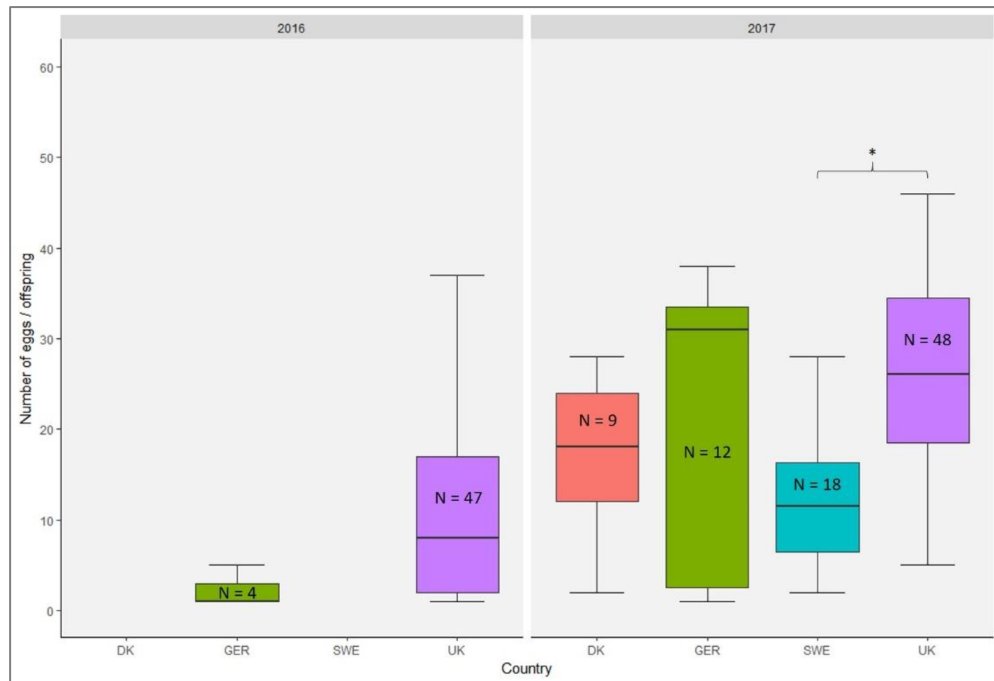

Figure 25: Number of eggs/offspring in populations from all four locations in both years. In 2017 British females had significantly larger clutches than Swedish females. The * denotes a significant difference. The error bars represent the standard error.

The hatching rates differed significantly between the locations (Fisher's Exact test, $p = 0.001857$). Hatching rate was significantly higher in egg sacs from Swedish females than egg sacs from the UK and Germany (SWE vs. UK: Pearson's Chi^2 test, $Chi^2 = 6.5162$, $df = 1$, $p = 0.01069$; SWE vs. GER: Pearson's Chi^2 test, $Chi^2 = 4$, $df = 1$, p-value = 0.0455, Fig. 26). No other significant inter-population differences were found (Pearson's Chi^2 test, DK vs. GER: $Chi^2 = 1.4$, $df = 1$, p-value = 0.2367, DK vs. SWE: $Chi^2 = 0.27$, $df = 1$, p-value = 0.6033, DK vs. UK: $Chi^2 = 2.375$, $df = 1$, p-value = 0.1233, GER vs. UK: $Chi^2 = 0.07177$, $df = 1$, p-value = 0.7888).

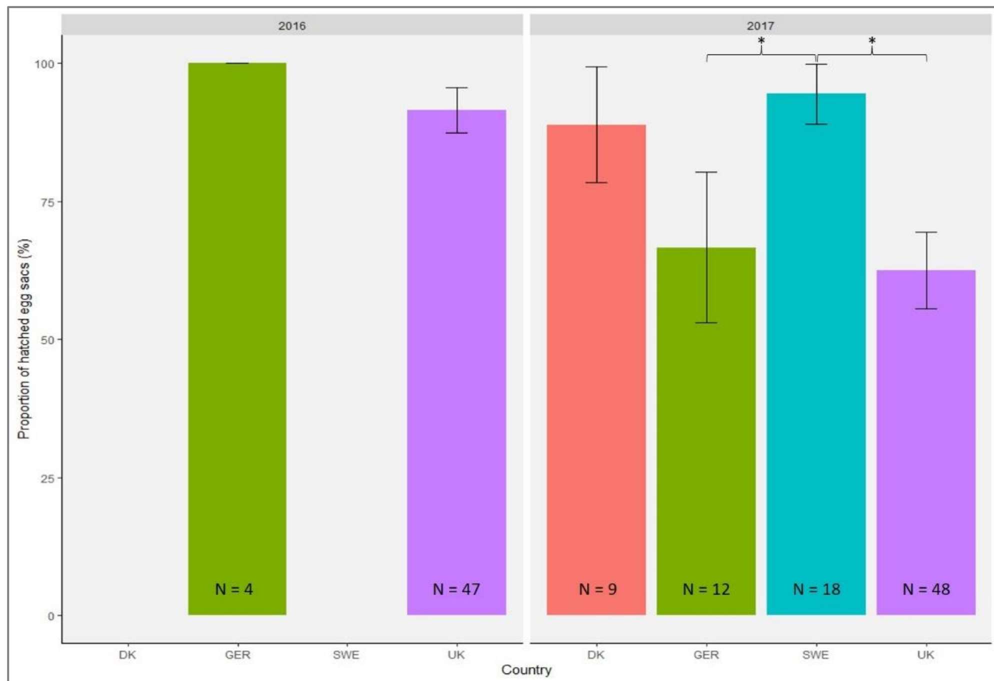

Figure 26: Egg sac hatching rates in populations from all four locations in both years. In 2017 egg sacs of Swedish females had a significantly higher hatching rate than egg sacs from British and German females. The * denotes a significant difference. The error bars represent the standard error.

In contrast to the 2016 population, in 2017 the correlation between female cephalothorax width and egg/offspring number was not significant (Kendall's rank correlation tau, tau = 0.09461473, z = 1.1309, p-value = 0.2581, Fig. 27).

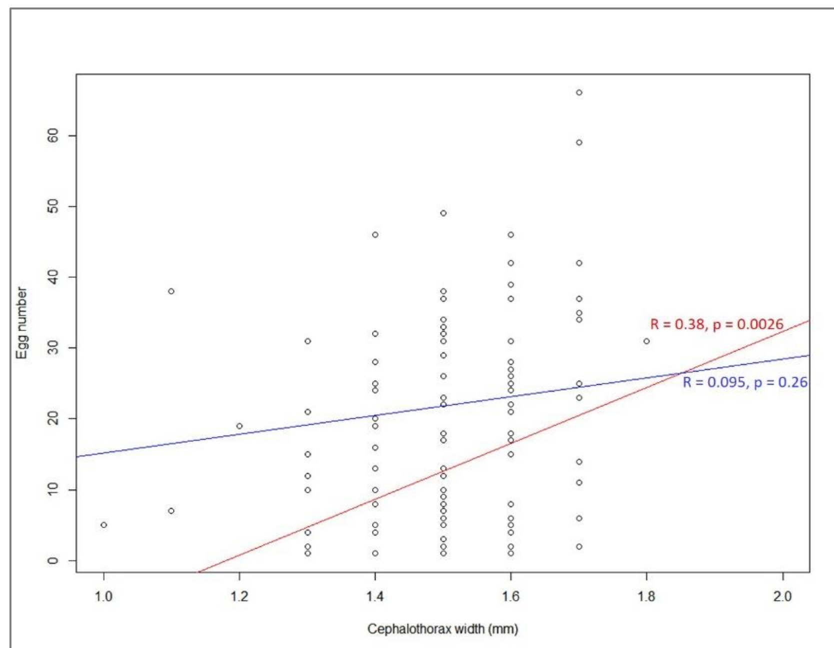

Figure 27: Correlation between *L. hortensis* females' cephalothorax width and their number of eggs / offspring. The correlation was significant in 2016 but not in 2017. Red line = 2016, blue line = 2017. R = correlation coefficient.

L. triangularis

In 2016, none of the mated females lived long enough to deposit an egg sac. Due to the high mortality in that year no egg sacs were derived from the initial collection. A few more females were collected in the UK and Germany in late September. These females produced 12 egg sacs in total (UK: N = 9, GER: N = 3). Unfortunately, it was not possible to get these egg sacs to hatch. They were kept in the fridge at approx. 4° C for six weeks and then brought back to room temperature to induce the continuation of development. After being brought back to room temperature egg sacs soon showed signs of mould and none of them hatched. When this became clear they were transferred to EtOH. In 2017, a total number of 18 egg sacs was produced (UK: N = 6, GER: N = 6, SWE: N = 6). Due to the experience from the 2016 batch of egg sacs, it was not attempted to hatch the 2017 egg sacs. All egg sacs were transferred to EtOH immediately after discovery.

The clutch size of females did vary from 18 to 105 eggs over both years (N = 24, mean egg number = 43.33 ± 1.58). There was no significant correlation between female cephalothorax width and clutch size (Kendall's rank correlation tau, tau = 0.2411705, z = 1.4741, p = 0.1404). No significant difference in egg number between the countries of origin was found (Kruskal-Wallis test, Kruskal Wallis Chi^2 = 3.567, df = 2, p-value = 0.168; UK: N = 14, mean egg number = 44.64 ± 1.83, GER: N = 9, mean egg number = 44.11 ± 3.66, SWE: N = 1, mean egg number: 18). Comparing the mean egg number of both years a significant difference between 2016 and 2017 was discovered (Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 8.0278, df = 1, p-value = 0.004607, 2016: N = 12, mean egg number: 58.42 ± 2.32, 2017: N = 12, mean egg number: 28.25 ± 0.66).

As in *L. hortensis*, due to the difference in mean egg number between the years, the dataset was subdivided into 2016 and 2017. Two Kendall’s rank correlation tests revealed that there was a correlation between clutch size and female cephalothorax width in 2016 but not in 2017 (2016: Kendall's rank

correlation tau, tau = 0.5656854, z = 2.197, p = 0.02802; 2017: Kendall's rank correlation tau, tau = 0.2911198, z = 1.2194, p = 0.2227; Fig. 28). This could explain why there was no significant overall correlation between female body size and clutch size.

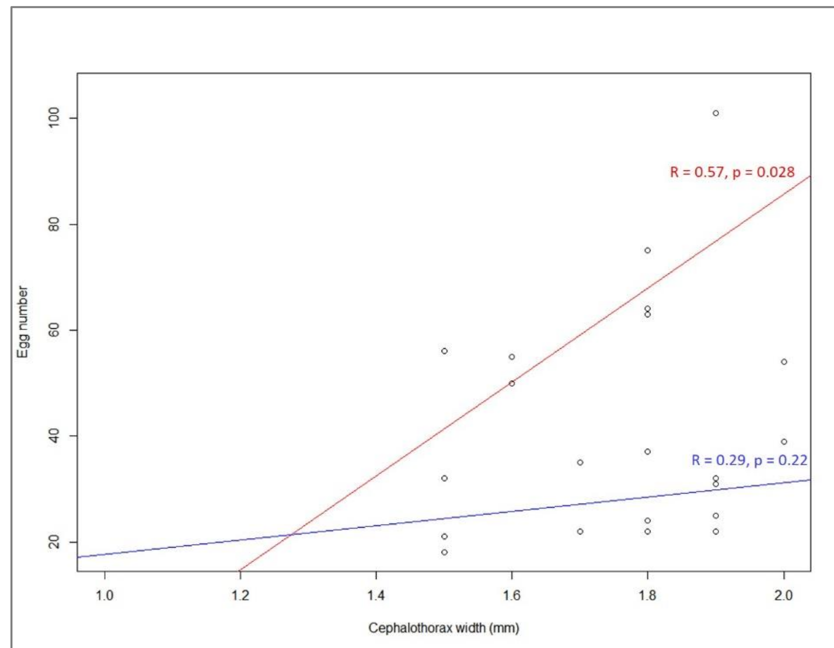

Figure 28: Correlation between *L. triangularis* females' cephalothorax width and their number of eggs / offspring. The correlation was significant in 2016 but not in 2017. Red line = 2016, blue line = 2017. R = correlation coefficient.

The clutch size of German and British females did not differ significantly in 2016 (Kruskal-Wallis Chi² = 0.69231, df = 1, p-value = 0.4054; UK: N = 9, mean egg number: 53 ± 2.28; GER: N = 3, mean egg number: 74.67 ± 7.32). Since no Swedish egg sacs were available for that year, a comparison with the UK and Germany was not possible. In 2017, there was again no difference between the egg number of females from the UK and Germany (Mann-Whitney-U test, W = 18, p = 0.6443; UK: N = 5, mean egg number: 29.6 ± 0.76; GER: N = 6, mean egg number: 28.83 ± 1.22). Only one egg sac was deposited by a Swedish female in 2017 so no statistical comparisons were possible. Lastly, inter-population comparisons of clutch sizes between 2016 and 2017 were conducted. British females had significantly smaller clutches in 2017 than in 2016 (Kruskal-Wallis Chi² = 5.4564, df = 1, p = 0.0195) whilst there was no difference in egg number of German females (Kruskal-Wallis Chi² = 0.62069,

df = 1, p-value = 0.4308). Due to the low egg sac number in both years these results must be interpreted with caution.

3.3.5 mtDNA analysis

Generally, the genetic diversity in both species was very low. *Linyphia hortensis* mtDNA sequences revealed two different haplotypes, one haplotype ('LhH1') seems to be mainly found in the UK but one Danish individual was LhH1 as well. The other haplotype ('LhH2') is spread over Germany, Denmark and Sweden. The difference is based on one polymorphic nucleotide site, but it does not cause a change in the amino acid sequence (*i.e.* they were all silent substitutions). A similar pattern was found in *L. triangularis* where again two different mtDNA haplotypes were present ('LtH1' and 'LtH2'). LtH1 was only found in individuals from the UK whilst LtH2 was found in Swedish and German but not in British spiders. The differences are due to six polymorphic nucleotide sites. Again, the amino acid sequence remains unchanged in each case.

The similar patterns in the two species could point towards the same factor reducing genetic diversity in *Linyphia* species in Europe. Figure 29 shows both species incorporated into a phylogenetic tree based on the cytochrome oxidase I gene of the mtDNA. Two different primer pairs were used in order to amplify DNA in both species. The same primer pair could not be used due to divergence between the species. It is possible, that a mutation at the primer binding site of the cytochrome oxidase I gene in *L. triangularis* prevents the amplification with the primer pair, which worked well for *L. hortensis*. Due to the use of two different primer pairs, only a short part of the sequences for both species overlap (64bp). Nevertheless, since this is a highly conserved region of the mtDNA and many reference sequences from other linyphiids are available from GenBank, the information derived from these sequences is suitable to build a phylogeny. The chosen linyphiid reference sequences were chosen on the basis on Wheeler *et al.*'s 'The spider tree of life' (Wheeler *et*

al., 2017). All sequences were trimmed, the final alignment consisted of 1240bp.

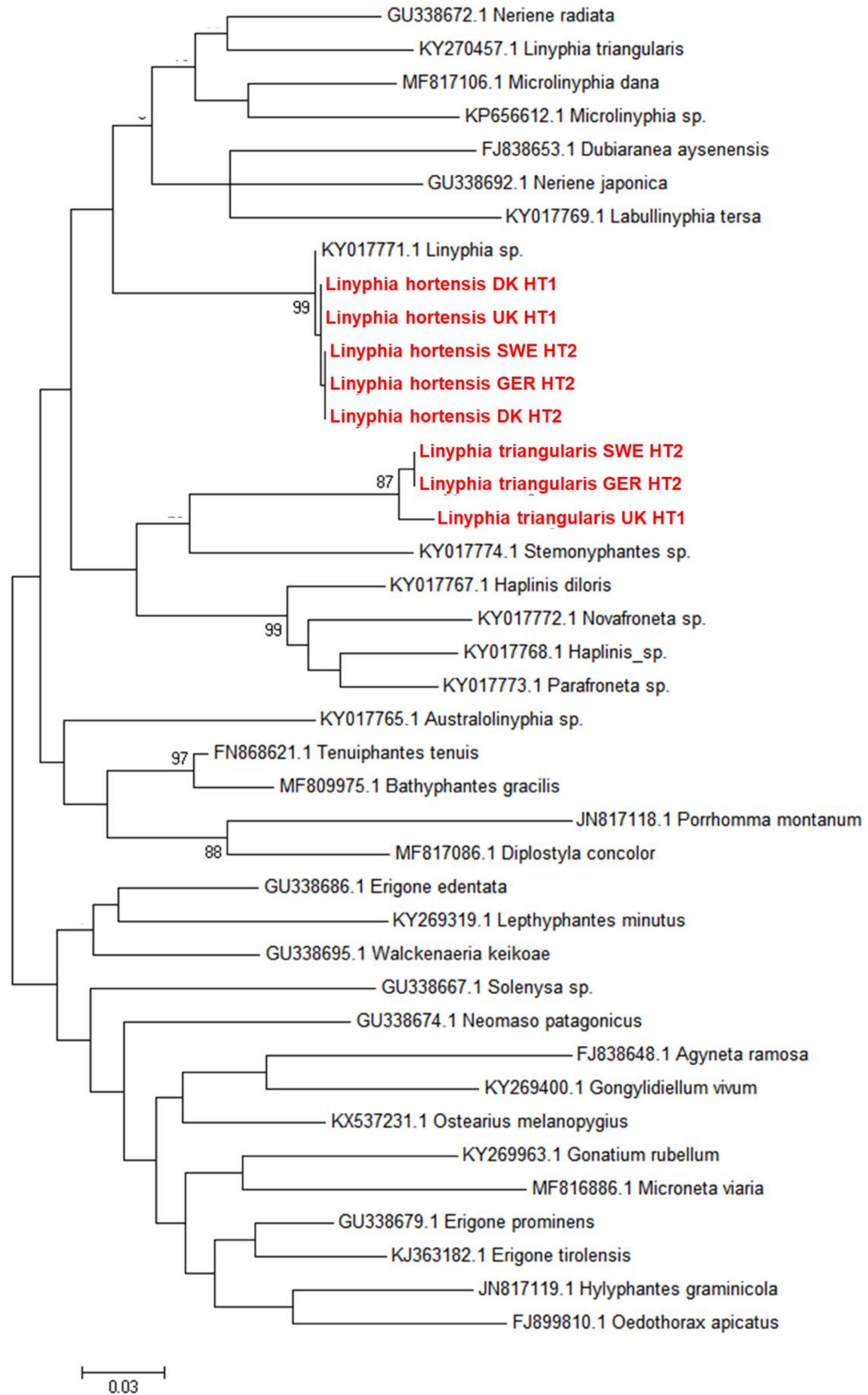

Figure 29: Phylogenetic tree of linyphiids based on the *cytochrome oxidase I* gene sequence of the mtDNA (bootstrap = 100). The tree was generated using the Maximum Likelihood method (log likelihood: -11292.0819, bootstrap = 100) based on the Tamura-Nei model (Tamura and Nei, 1993). The alignment consisted of 1240bp and all sites were used. Sequencing alignment and tree generation was conducted in MEGA6 (Tamura *et al.*, 2013).

3.4 Discussion

This study of the life-history and the population genetics of two closely related linyphiid species has added new pieces to the body of information already available for these species. The two species, *L. hortensis* and *L. triangularis*, proved to be good model organisms, each of them particularly suitable for a different part of the study. These closely related linyphiids showed spatial and temporal heterogeneity in different aspects of their morphology, ecology and genetics.

Generally, both species were found in the same kind of habitats in both collection years and especially *L. triangularis* was reliably found at more or less the same locations in both years. Only anthropogenic factors, *i.e.* taking down of hedge rows in a managed, park led to the spiders not being found at the same spot again. *L. hortensis* is known to show a patchier distribution and this was found to be true for both years. Weather conditions in spring 2017 led to a later emergence of subadult and adult individuals at locations where they had been found in 2016.

Differences in adult body size were found between the years of collection in both species. *L. triangularis* showed the expected sexual size dimorphism with males having a larger average cephalothorax width than females. The significant size difference between the year 2016 and 2017 with individuals being larger in 2017 could be the result of different mechanisms. Size fluctuations could be the effect of size limiting factors being at work in 2015, e.g. reduced prey availability (Reed and Nicholas, 2008) due to weather conditions that year. Generally, conditions in 2015 could have favoured smaller individuals which lead to them having a higher reproductive success. These conditions could have changed in 2016 to favour larger individuals.

Male – male competition is proposed to be a cause of male biased SSD in *L. triangularis* because males fight over mating opportunities and guard the females against other suitors. In these competitions usually the larger male

has an advantage (Rovner, 1968; Toft, 1989). So, it would make sense that, after a year with lower mean body size, larger males have a higher fitness and therefore their offspring could inherit their larger size.

In *L. hortensis* males were not significantly larger than females but low male sample size means this finding might not be representative of the population as a whole. The scarce literature available for *L. hortensis* does not state SSD to be present in this species. Again, individuals showed a higher mean cephalothorax width in 2017 which could be due to more general factors such as different ecological conditions influencing body size.

The sex ratio observed in the field whilst collecting the spiders was extremely female skewed in both species. Whilst female biased sex ratios are known from other linyphiids, e.g. *Pityohyphantes phrygianus* (Gunnarsson *et al.*, 2009), it is possible that the sampling method added an extra bias to the potentially naturally occurring skew. Males move through the undergrowth to find females. Therefore, they are much less conspicuous than the females in their webs. An earlier study on *L. triangularis* reported a sex ratio of 1:1 in subadult spiders. This could indicate that the operational sex ratio is male biased (Toft, 1989). No literature has been identified to date which states the operational sex ratio of *L. hortensis*. The sex ratio skew did not differ between the years in either species. This could mean that sex ratio distorting factors, even if a collector bias is acknowledged, are constantly present in both species.

If it is assumed that a female biased sex ratio, potentially with a weaker skew than observed, exists in *L. hortensis* and *L. triangularis*, different factors could be responsible for the distortion. One reason could be that mature males lead a riskier life than females. Mature males leave their webs on the hunt for premature female and will be constantly on the move in the herb and shrub layer (Rovner, 1968). This behaviour is widespread in different spider taxa (Foelix, 2011) and poses a higher risk of predation on the males (Kasumovic *et*

al., 2007). Additionally, mature males stop foraging themselves and the only nourishment they will gain is when they visit females in their webs and 'steal' their food (Kasumovic *et al.*, 2007). So, running out of energy resources might be an additional risk that could lead to a higher mortality in males (Andrade, 2003).

Another factor that might distort the sex ratio could be the presence of sex ratio distorting endosymbionts in these two linyphiid species. Bacterial endosymbionts e.g. *Wolbachia*, *Rickettsia* and *Spiroplasma* have been found to distort the sex ratio of host population towards females to promote their own transmission (see Chapter 1). This possibility and the potential impact of such an endosymbiont infection will be further discussed in Chapter 4. To reliably assess the degree of sex ratio distortion in *L. hortensis* and *L. triangularis,* breeding experiments to establish lab populations need to be conducted.

Differences in body size could be the result of an endosymbiont being present in the populations. This could also explain why we do not see the same pattern in all countries, populations could be unequally infected. Not many studies report a direct connection between body size and endosymbiont infection. In the whitefly *Bemisia tabaci,* 4th instar larvae were significantly larger if their parents carried *Wolbachia,* in comparison to offspring of individuals freed of the infection using antibiotics. This could indicate that *Wolbachia* infection and body size are positively correlated (Xue *et al.*, 2012).

It became clear during the experiments carried out in 2016, that high spider mortality under laboratory conditions was a significant constraint when carrying out mating trials. Nevertheless, the general set up of the mating trials and the methodology appeared to be sufficient if sample sizes of, preferably laboratory reared, virgin spiders, could be made available.

In order to set up such a laboratory population it would be necessary to be able to rear *L. hortensis* and *L. triangularis* juveniles from egg sacs in the lab. For *L. hortensis* this should be more easily achievable than for *L. triangularis*. It is not clear whether *L. triangularis* egg sacs need a phase of reduced temperature to successfully develop or if hatching in the lab could be accelerated by simply leaving them at room temperature and keeping humidity suitably high. Weldingh *et al*. tried to imitate the cold phase that *L. triangularis* egg sacs are subjected to in the wild by storing them in the fridge for a couple of months and then bringing them back to room temperature. They did experience problems with mould however, so that they had to cut the egg sacs open (Weldingh *et al.*, 2011). It is therefore unclear whether these egg sacs would have hatched of their own accord.

The analysis of the hatching rates and offspring numbers in *L. hortensis* were also impaired by the small German sample in 2016. Hence, the almost significant difference in hatching rates between British and German egg sacs in 2016 have to be considered with caution. The positive correlation between female size and clutch size is in line with studies on other spider species and on insects (Eberhard, 1979; Head, 1995; Honek, 1993; Skow and Jakob, 2003; Spence *et al.*, 1996). When the dataset was split by year of collection, this correlation was only significant in 2016 but not in 2017. The question is, whether there are factors that could lead to females not investing all available resources into their offspring clutch. In *Dolomedes triton* it has been shown that female would not reach their maximum reproductive capability if food was scarce at the time of egg production (Spence *et al.*, 1996). Maybe the prey availability was low in the field before collection or the feeding regime in the lab was not optimal and therefore not all females showed maximum fecundity. It also has to be considered that offspring were only counted once they emerged from the egg sac. It is possible that sibling cannibalism or other spiderling mortality could have occurred prior to this and altered offspring number.

It is also possible that a bacterial endosymbiont like *Wolbachia* is responsible for the loss of the positive correlation between female body size and clutch size. If male-killing was induced by an endosymbiont, leading to male embryos being killed in the early developmental stage, only half of a female's clutch will emerge from the egg sac (Hurst *et al.*, 1992). The endosymbionts of *L. hortensis* and *L. triangularis* are characterised and discussed in Chapter 4 of this thesis.

Interestingly, in 2017, the hatching rate of Swedish egg sacs was higher than that of British egg sacs, whilst having a smaller average clutch size. The smaller clutch size was possibly the result of the smaller size of Swedish spiders even though the difference in cephalothorax width between British and Swedish spider was not significant.

In *L. triangularis* there was no overall correlation between female cephalothorax width and clutch size. Splitting the dataset by year revealed a positive correlation in 2016 but not in 2017. Since this was found in *L. hortensis* collected from the same locations as well, it could be indicative that ecological reasons are responsible for the loss of the correlation. As mentioned before if food was scarce for females during egg production, they may not have been able to use their full reproductive potential (Spence *et al.*, 1996). The egg clutches were smaller in 2017, whilst individuals seemed to be larger. Since the egg sac weight was not recorded for most of the egg sacs it could not be determined, if the fewer eggs were potentially larger than those from larger clutches. Investing in fewer but resource-rich (i.e. higher yolk content) eggs could be a form of maternal investment which provided better offspring survival in less favourable conditions (Anderson, 1978; Turnbull, 1962).

The egg sacs of *L. triangularis* are produced in late autumn and overwinter in the leaf litter until the next spring when development commences (Weldingh *et al.*, 2011). In 2016, egg sacs were stored in the fridge for a couple of months

to imitate this cold phase. After this time, they were brought back to room temperature but none of them showed any sign of hatching. Instead, like Weldingh *et al.* reported previously, problems with mould occurred (Weldingh *et al.*, 2011). Therefore, it was not attempted to hatch *L. triangularis* egg sacs from 2017 and no information about hatching rates were available.

The genetic diversity in both species was low. Only two mitochondrial haplotypes were found in each species. This could be explained by selective sweeps which may have been induced by endosymbionts (Hurst and Jiggins, 2005; Jiggins, 2003; Johnstone and Hurst, 1996; Marcade *et al.*, 1999). The geographic pattern of the haplotype distribution indicates that only a low level of mixing between the populations is happening. This is not surprising given the fact that neither *L. hortensis* nor *L. triangularis* seem to be good ballooners. In fact, there are no studies which suggest that either of the species engages in ballooning at all. The more anecdotal data from the few sailing trials that were conducted in combination with observations by Goodacre (pers. comm) indicate that *L. hortensis* and *L. triangularis* will not drown immediately, if they land on water, but they do not seem to be able to sail either. It has been indicated that spiders should only engage in ballooning if they are able to sail since landing on water is one of the risks associated with this form of dispersal (Hayashi *et al.*, 2015). Another reason for the low genetic diversity could be that the environmental and climate conditions are too similar at the chosen location. Potential future work could include individuals from other regions of Europe.

If there is reduced geneflow between the populations due to the geographic distance and the restricted dispersal behaviour in both species, over time local adaptations could become visible. Nevertheless, the environmental conditions in all three countries do not seem to be vastly different from one another which therefore might lead to only very small, gradual changes. Investigations into local factors such as temperature difference throughout

the life cycle, humidity or diversity of prey and predator species, could help understanding of the local selective pressure that populations are subjected to. The few mating trials conducted did not indicate that males or females discriminated between mates from their own region and mates from 'abroad'. However, further studies are needed to establish whether males or females prefer local or foreign mating partners. Since no egg sacs were retrieved from mating trials conducted in the lab, it remains unclear whether any signs of reproductive isolation would be visible i.e. in hatching rates or viable offspring numbers/mortality.

If bacterial endosymbionts are present in one or both species, these could play an important role in the further evolution of individuals at different locations. The observed sex ratio distortion, the partial absence of correlation between female body weight and offspring number and the low genetic diversity in both species could all be connected to the presence of one or more endosymbionts. This possibility will be investigated in Chapter 4. The data presented in this chapter do not allow exclusion of any of the sex ratio distorting mechanisms potentially induced by an endosymbiont.

Whilst this study does not paint a complete picture for either of the two species investigated, it provides a solid basis for further research into the life-history and population genetics of *L. hortensis* and *L. triangularis*.

Chapter 4: The endosymbionts of *Linyphia hortensis* and *Linyphia triangularis*

4.1 Introduction

After investigating the life history and population genetics of *L. hortensis* and *L. triangularis* in Chapter 3, this chapter will study the consequences of endosymbiont infections for the biology of these two linyphiid spider species. The number of studies investigating endosymbionts in spiders is steadily growing and some of these, being part of the foundation of this research area, have been done on linyphiids (see Chapter 1.4.).

A plethora of studies exist, which describe the sex ratio-distorting effect of bacterial endosymbionts on their insect hosts. One famous example is the feminisation of male Armadillidium vulgare (terrestrial isopods). Due to a feminising Wolbachia strain genetic males are changed into fully functional females. Sometimes feminisation is not complete and intersexes appear. These intersexes can either have a female physiology with small male external characters or they possess developed male external characters and are sterile (Juchault *et al*., 1992; Rousset *et al*., 1992). Another example is the male-killing *Rickettsia* of the ladybird beetle *Adalia bipunctata* (Werren *et al.*, 1994). Male embryos are killed off at an early stage, leading to a strongly female-biased sex ratio. The killing of males could increase the fitness of bacteria inside the female siblings, therefore could be considered as kin selection. Another benefit of male-killing is the reduction of competition for resources and the reallocation the same to female siblings and thereby providing them with an advantage at the start of their life (Hurst *et al.*, 1992). Endosymbionts have been found to cause reversible (by antibiotic treatment) parthenogenesis in *Trichogramma* parasitic wasps (Stouthamer *et al.*, 1990; Stouthamer and Werren, 1993). Stouthamer *et al.* (1993) identified *Wolbachia* as parthenogenesis-inducing endosymbiont in *Trichogramma* wasps.

The research into cytoplasmic incompatibility (CI) has attracted equally as much attention as the sex ratio distorting phenotypes. CI had first been described in the 1950s (Ghelelovitch, 1952). Approximately 20 years later *Wolbachia* was identified as the endosymbiont inducing CI in the mosquito *Culex pipiens* (Yen and Barr, 1971; Yen and Barr, 1973). *Wolbachia* causes unidirectional incompatibility, where uninfected females and infected males are not compatible (Yen and Barr, 1973). CI has been utilised in the battle against dengue fever. *Wolbachia* from *Drosophila melanogaster* have been introduced into two Australian Ae*des aegypti* mosquito populations. *Wolbachia* successfully invaded these populations and reached near fixation in a few weeks after release of infected *A. aegypti*. In addition to inducing CI, Wolbachia disrupts the transmission of dengue via the mosquitoes' saliva (Hoffmann *et al.*, 2011).

As of yet the definite effects of endosymbionts on their spider hosts have only been identified in a small number of studies. The infection frequencies of the endosymbionts found in spiders differs across the studies available today. Haupt (2002) reported *Rickettsia* infection rates of 1.9 – 4.5% in four species of the Liphistiidae from Japan. He found that, even though infection rates were low, *Ryuthela nishihirai nishihirai* populations from two closely situated locations differed significantly, with almost 6% (n = 52) infected individuals in Suyeyoshi (Naha, Okinawa) but an almost negligible prevalence (sample size not stated) in Ryutan (Naha, Okinawa)(Haupt, 2002). A study across 21 species of Australian spiders found that 52.6% of all sampled spider genera carried *Wolbachia* (Rowley *et al.*, 2004).

Goodacre *et al.* (2006) conducted a large endosymbiont screening in 122 spider species belonging to 54 genera for *Wolbachia*, *Rickettsia* and *Spiroplasma*. The spiders were mainly collected in the southeast of England apart from specimens of two species, which were collected in Sweden. They found that 37 species in total carried *Wolbachia*. Among the screened spiders were 226 individuals belonging to 71 linyphiid species and all three

endosymbionts were present. In one individual of the linyphiid *Trichonchus scabriculus* all three endosymbionts were found and double infections, with *e.g*. *Rickettsia* and *Spiroplasma*, were present in other species. Thirty-two species were infected with *Wolbachia*, 21 species with *Rickettsia* and eight species with *Spiroplasma* (Goodacre *et al.*, 2006).

In *Pityohyphantes phrygianus*, wild-caught from three populations in southeast and southwest Sweden, it was found that this linyphiid spider species carries *Wolbachia* and *Rickettsia* endosymbionts. There was no difference in *Wolbachia* infection frequency between the sexes. In the experimental part of the study, spiders were either assigned to a water or an antibiotic treatment. *Wolbachia* infection rate in the water treatment group was 64.7% whilst the rate decreased to 10.7% in the antibiotic treatment group. The authors hypothesised that the antibiotic treatment may have been less effective in males. Since the used antibiotic, tetracycline, does only inhibit but not eliminate bacterial activity and the used tests did not discriminate between active and inactive bacteria, estimates of treatment efficiency were regarded as conservative (Gunnarsson *et al.*, 2009).

Wang *et al.* (2010) found that seven out of 31 tested spider species from Wuhan (Hubei Province, China) did carry *Wolbachia* with infection rates ranging from 13.3% to 73.3% (average 22.6%). None of the tested linyphiid species (*Hylyphantes graminicola*, *Neriene radiate*, *Erigone prominens*, *Neriene japonica*, *Neriene limbatinella*) were found to be infected with *Wolbachia* (Wang *et al.*, 2010). *Oedothorax gibbosus* from two different populations in Belgium showed no difference in *Wolbachia* infection frequency (Damvallei: females = 44% (N = 39), males = 57% (N = 7); Walenbos: females = 42% (N = 53), males = 64% (N = 11)) and males and females were equally infected (Fisher's exact test: $P > 0.2$) (Vanthournout *et al.*, 2011).

Yun *et al.* (2011) found that the sheet-web spider *Hylyphantes graminicola,* from different populations in China, harbours two different strains of

Wolbachia which belong to the supergroups A and B. The average infection frequency was 21.61% with distinct population infection rates ranging from 7.69% to 45.56%. No sex-biased prevalence of *Cardinium* in the marbled cellar spider *Holocnemus pluchei* (from Montpellier, France) was found. Males and females were equally infected with the endosymbiont (males = 83.9%, females = 74.6%). The study screened a total of 26 populations: 17 from France, four from California and one each from Israel, Spain, Corsica, Crete and Jordan. *Cardinium* was found only in populations from France and Israel. Infection rates in populations ranged from 7% to 86% with an average prevalence of 41%. Transmission was perfect from mother to offspring, all screened neonates of infected mothers were screened positive (Stefanini and Duron, 2012).

Regional differences in endosymbiont infection rates have been found between spider species from the UK and Germany in a study by Duron *et al*. (2008) 22% of the sampled species tested positive for *Cardinium* whilst *Wolbachia* was found in 37% of the screened species. The *Wolbachia* infection could only be detected in individuals from the UK but not in those collected in Germany. *Cardinium* was common in populations from both the UK and Germany and the frequency of infection did not differ between populations (Duron *et al.*, 2008). This study is one of the few that did screen *L. triangularis* for *Cardinium* and *Wolbachia* with infections of both kind being found. Interestingly, the *Wolbachia* strain that was found could not be assigned to any of the known *Wolbachia* supergroups at the time (phylogeny based on 16S sequences). The *Wolbachia* of *L. triangularis* clustered with the strain found in *Drosophila takahashii* (Duron *et al.*, 2008). No other 16S *Wolbachia* sequences are available on GenBank today, which are closer than the one found in *L. triangularis*.

A recent study on *Mermessus fradeorum* is the first to present evidence of endosymbiont feminisation in a spider. A co-infection of *Rickettsia* and *Wolbachia* was identified as the reason for a strong female-biased sex ratio.

Curing of this double infection restored the normal 1:1 sex ratio. The authors conducted crossing experiments, showing that males infected with a single *Wolbachia* strain induced CI when mated to females cured of the infection. If these males were crossed with females carrying the *Rickettsia/Wolbachia* double infection CI was significantly weaker showing that the double infection was capable of partially rescuing the sperm of singly infected males (Curry *et al.*, 2015).

The aim of this study was first to establish the presence or absence of three of the most common endosymbionts – *Wolbachia*, *Rickettsia* and *Spiroplasma* in *L. hortensis* and *L. triangularis*. Until this study limited information existed on endosymbiont infections in *L. triangularis* only. A systematic endosymbiont screening of *L. triangularis* and *L. hortensis* exclusively has not been done before. Relating the observed sex ratio and offspring numbers to any infection patterns identified, should shed light on the possible effects of these infections on the spiders' reproductive biology and ecology. The infection rates of the populations from Germany, the UK and Sweden will be analysed to establish if geographic infection variation is present. If regional differences are present the occurrence of CI would be much more likely than if all populations were equally infected/uninfected. Gaining information on the transmission frequency of the endosymbionts from one generation to the next will indicate how successful the endosymbionts are in invading the populations. Obtaining endosymbiont sequences through Sanger sequencing will make it possible to place the strains found in phylogenetic trees and hence identify how these endosymbiont strains relate to those found in other spiders and arthropods. Additionally, all *Wolbachia* positive spiders were screened for the WO phage to investigate whether sex ratio patterns could be explained by the presence of this bacteriophage. The endosymbiont data from this study will be considered in relation to the mtDNA haplotypes identified in the different populations. This could provide information on whether the presence of endosymbionts could have an influence on the genetic diversity in the two *Linyphia* species.

4.2 Materials and methods

A total number of 188 *L. hortensis* and 190 *L. triangularis* were screened for the endosymbionts *Wolbachia*, *Spiroplasma* and *Rickettsia*. These individuals were the same spiders which were collected in 2016 and 2017 from Germany, Denmark, Sweden and the UK. The DNA had already been extracted for population genetic studies of both species (see Chapter 3.2.5).

To screen the spiders for the different endosymbionts, PCRs with endosymbiont specific primers were executed (Table 8). *Wolbachia* was identified using primers targeting the *Wolbachia* surface protein (WSP-F/WSP-R) (Braig *et al.*, 1998). The primers used to screen for *Rickettsia* (Rics741F/Rcit1197R) amplifies a part of the citrate synthase gene (Davis *et al.*, 1998). To screen for *Spiroplasma* infections the 16S-23S ribosomal RNA intergenic spacer (ITS, Spits-J04/Spits-N55) was targeted using the primers described in Majerus *et al.* (1999). After the initial endosymbiont screen all *Wolbachia* positive individuals were also screened for the presence of the WO phage. The primers used (orf7f/orf7r) amplified a region of the minor capsid gene orf7 (Fujii *et al.*, 2004).

Table 8: Primers used for the screening for *Wolbachia*, *Spiroplasma*, *Rickettsia* and WO phage

Endosymbiont	Primer	Sequence
Wolbachia	WSP-F	TGGTCCAATAAGTGATGAAGAAACTAGCTA
	WSP-R	AAAAATTAAACGCTACTCCAGCTTCTGCAC
Rickettsia	Rics741F	CATCCGGAGCTAATCCTTTTGC
	Rcit1197R	CATTTCTTTCCATTGTGCCATC
Spiroplasma	Spits-J04	GCCAGAAGTCAGTGTCCTAACCG
	Spits-N55	ATTCCAAGGCATCCACCATACG
WO phage	orf7f	ATAAATTCTCCTATTTTTTCTGG
	orf7r	GAAATGCTTGTTCAGCTAATAGC

The following PCR protocol was applied to amplify the endosymbiont DNA: A total volume of 10µl contained 1µl DNA sample, 10µl 1x BioMix™ Red PCR master mix, 0.5 µl of each primer (10 µmol) and 3µl of sterile distilled water to make up to the final volume. PCRs to amplify the endosymbiont DNA were

run under the following conditions: Initial denaturing at 94°C for 1m 30s followed by 40 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s and extension at 72°C for 30s, completed by a final extension step at 72°C for 5m. WO phage PCRs were run using the following protocol: Initial denaturation at 94°C for 3m followed by 35 cycles of denaturation at 92°C for 30s, annealing at 57°C for 40s and extension at 72°C for 1m, completed by a final extension step at 72°C for 5m. The PCR products were made visible through gel-electrophoresis on 1.5% agarose gels stained with Ethidium Bromide under UV light.

For sequencing purposes, the reaction volume was increased to 30µl still using 1µl of the DNA sample (15µl BioMix™ Red, 1.5µl of each primer (10 µmol) and 11µl of sterile distilled water). Sanger sequencing was done at Macrogen Inc. and retrieved sequences were analysed using MEGA6 (Tamura *et al.*, 2013), BioEdit (Hall, 1999), NCBI BLAST search and ChromasLite (Technelysium Pty Ltd, 2012).

4.3 Results

4.3.1 Endosymbiont infection frequencies

L. hortensis

The screening methods for the three endosymbionts *Wolbachia*, *Rickettsia* and *Spiroplasma* yielded reliable results in all populations from the four locations. Figure 30 shows an example of a gel for *Wolbachia*.

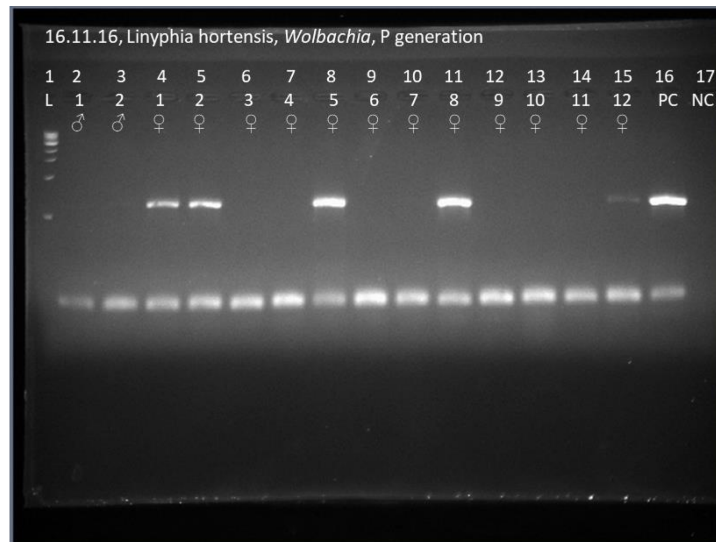

Figure 30: Result of a *Wolbachia* PCR run on the UK *L. hortensis* population. Four positives are visible on the gel under UV light.

A total of 188 spiders, collected at four different locations (UK, GER, DK*, SWE*; *only 2017) in 2016 and 2017, were screened for *Wolbachia*, *Rickettsia* and *Spiroplasma*. The most common endosymbiont was *Wolbachia* with a total proportion of 28% infected individuals. Infection rates with *Rickettsia* were significantly lower (5%, *Wolbachia* vs *Rickettsia*: 2-sample test for equality of proportions with continuity correction, $Chi^2 = 32.466$, $df = 1$, $p = 1.213e-08$) and only one individual was found to carry *Spiroplasma*. The rate of multiple infections was low with three individuals being doubly infected with *Wolbachia* and *Rickettsia* and two spiders carrying a *Wolbachia*/*Spiroplasma* double infection. 64% of the collected specimens were negative for all three endosymbionts. The raw numbers of infected individuals are presented in Table 9.

Table 9: Number of *L. hortensis* infected with *Wolbachia*, *Spiroplasma*, *Rickettsia*, *Wolbachia/Rickettsia* (*Wol/Rics*) and *Wolbachia/Spiroplasma* (*Wol/Spir*) in populations from the UK, Germany, Denmark and Sweden. Collections were made in 2016 and 2017. N = total number of collected individuals.

2016	***Wolbachia***	***Spiroplasma***	***Rickettsia***	***Wol/Rics***	***Wol/Spir***	**uninfected**	**N**
UK	22	1	1	2	1	32	59
Germany	8	0	2	1	0	11	22
2017							
UK	17	0	3	0	1	34	55
Germany	3	0	1	0	0	11	15
Denmark	1	0	0	0	0	11	12
Sweden	1	0	3	0	0	21	25

A Kruskal-Wallis test revealed that year of collection had a significant effect on infection status (Kruskal-Wallis $Chi^2 = 8.6656$, $df = 1$, $p = 0.003243$). Therefore, the dataset was subdivided into two datasets, 2016 and 2017, respectively.

The proportion of uninfected individuals increased significantly from 2016 to 2017 (2016: 53%, 2017: 72%, 2-sample test for equality of proportions, $Chi^2 = 6.3207$, $df = 1$, $p = 0.01193$) which means that the proportion of endosymbiont infected spiders decreased. Analysing the infection frequencies of the individual endosymbionts it was found that only the proportion of *Wolbachia* infections declined from 2016 to 2017 (2016: 37%, 2017, 21%, 2-sample test for equality of proportions, $Chi^2 = 5.4583$, $df = 1$, $p = 0.01948$). The infection frequencies for *Rickettsia* did not change significantly (2016: 4%, 2017: 7 %, 2-sample test for equality of proportions, $Chi^2 = 0.28155$, $df = 1$, $p = 0.5957$). Due to the low sample sizes for *Spiroplasma* and the double infections no statistical analysis was attempted.

Only the populations from the UK and Germany had enough *Wolbachia* infected individuals to make them suitable for statistical analysis. There was no significant difference in *Wolbachia* infection frequency between the two locations (UK: 34%, GER: 30%, 2-sample test for equality of proportions, $Chi^2 = 0.091321$, $df = 1$, $p = 0.7625$). Due to the difference in overall infection frequency between the years, the proportions of *Wolbachia* infected

individuals from the UK and Germany in 2016 and 2017 were compared. In both years *Wolbachia* was the most abundant endosymbiont found in the populations from the UK and Germany. In 2016, 37% and 36% of all collected individuals respectively carried the infection. Populations from both countries were equally infected (2-sample test for equality of proportions, Chi^2 = 7.3248e-31, df = 1, p = 1). The infection frequency declined in both populations from 2016 to 2017, with 31% of the British and 20% of the German spiders testing positive for *Wolbachia* (Fig 31). Again, both populations were statistically equally infected with *Wolbachia* in 2017 (2-sample test for equality of proportions, Chi^2 = 0.25667, df = 1, p = 0.6124) and the difference between the years was also not significant in either of the locations (2-sample test for equality of proportions, UK: Chi^2 = 0.27024, df = 1, p = 0.6032, GER: Chi^2 = 0.49406, df = 1, p = 0.4821).

For 2017, additional infection frequencies for populations from Denmark and Sweden were available. Inter-population comparisons between the four locations showed that there was a significantly higher frequency of *Wolbachia* in the UK than in Sweden. There were no other significant differences in *Wolbachia* infection rates between the countries of origin (Table 10).

Table 10: Comparing the *Wolbachia* infection frequencies of *L. hortensis* collected at four different locations in 2017. Prop. 1 = proportion of *Wolbachia* infected individuals in the first location, Prop.2 = proportion of *Wolbachia* infected individuals in the second location.

Comparison	Prop. 1	Prop. 2	2-sample test for equality of proportions
UK vs GER	31%	20%	Chi^2 = 0.25667, df = 1, p = 0.6124
UK vs DK	31%	8%	Chi^2 = 1.5354, df = 1, p = 0.2153
UK vs SWE	**31%**	**4%**	**Chi^2 = 5.6774, df = 1, p = 0.01718**
GER vs DK	20%	8%	Chi^2 = 0.091712, df = 1, p = 0.762
GER vs SWE	20%	4%	Chi^2 = 1.1852, df = 1, p = 0.2763
DK vs SWE	8%	4%	Chi^2 = 2.5713e-32, df = 1, p-value = 1

Rickettsia were also found in both populations but at very low frequencies (Fig. 31). In both years, infection rates at either location were below 10% (2016: UK = 1.69%, GER = 9%; 2017: UK = 5.45%, GER = 6.67%). As mentioned

above, only one individual was found to be infected with *Spiroplasma*. This individual was collected in the UK in 2016 (Fig. 31)

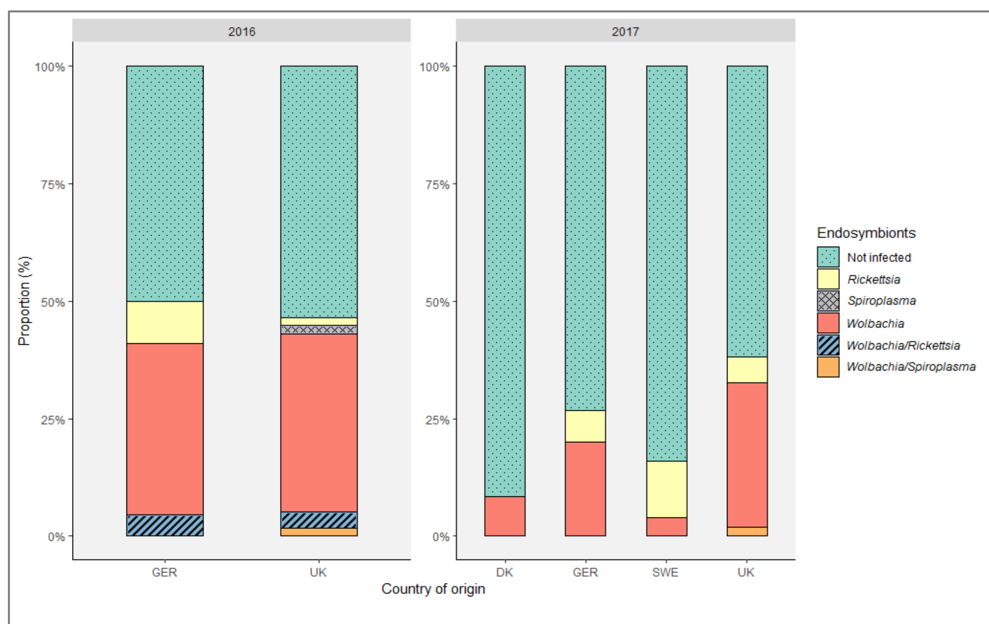

Figure 31: Proportion of infected *L. hortensis* individuals for the three different endosymbiont species in 2016 and 2017. Light blue/dotted = Not infected, light yellow = *Rickettsia* infected, grey/meshed = *Spiroplasma* infected, red = *Wolbachia* infected, blue/hatched = *Wolbachia/ Rickettsia* double infected, orange = *Wolbachia/Spiroplasma* double infected.

L. triangularis

In contrast to *L. hortensis*, *Wolbachia* was found at only low frequencies in both years and all populations from the UK, Germany and Sweden. The overall proportion of *Wolbachia* was 3%. A proportion of 5% of all collected spiders did carry *Spiroplasma*. The most abundant endosymbiont in *L. triangularis* was *Rickettsia,* with an overall infection rate of 22%, meaning significantly more *Rickettsia* than *Wolbachia* and *Spiroplasma* infections were found (2-sample test for equality of proportions, Wol vs Rics: Chi^2 = 39.715, df = 1, p-value = 2.939e-10, Rics vs Spir: Chi^2 = 29.511, df = 1, p-value = 5.561e-08). The proportions of *Wolbachia* and *Spiroplasma* were not significantly different (2-sample test for equality of proportions with continuity correction, Chi^2 = 0.87666, df = 1, p-value = 0.3491). A small number of multiple infections were found with 3% of all individuals carrying all three endosymbionts. Still, a significantly larger number of individuals was not infected with any of the three endosymbionts at all (59%, 2-sample test for equality of proportions,

Chi² = 16.066, df = 1, p-value = 6.116e-05). The proportion of the endosymbionts are presented in Figure 32 and the raw numbers of infected individuals are noted in Table 11.

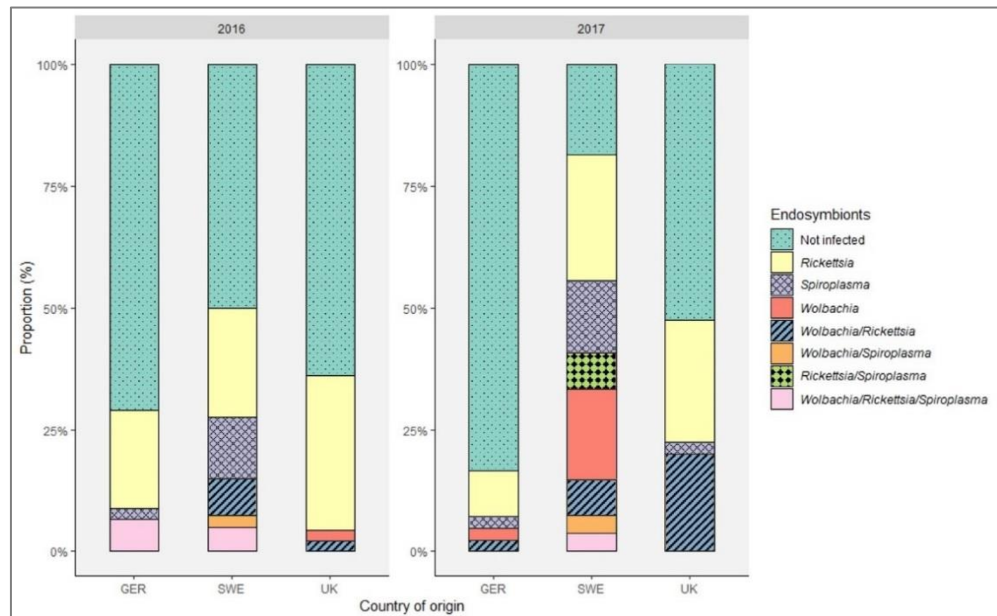

Figure 32: Proportion of infected *L. triangularis* individuals for the three different endosymbiont species in 2016 and 2017. The colours code for the following *L. triangularis* populations: Blue/dotted = Not infected, yellow = *Rickettsia* infected, grey/meshed = *Spiroplasma* infected, red = *Wolbachia* infected, blue/hatched = *Wolbachia/Rickettsia* double infected, orange = *Wolbachia/Spiroplasma* double infected, green/chequered = *Rickettsia/Spiroplasma* double infected and light pink = *Wolbachia/Rickettsia/Spiroplasma* triple infected.

Table 11: Infection frequencies of the endosymbiont species *Wolbachia (Wol)*, *Spiroplasma (Spir)*, *Rickettsia (Rics)* and multiple infections in *L. triangularis* populations from the UK, Germany (GER) and Sweden (SWE). Collections were made in 2016 and 2017. N = total number of collected individuals. Uninf. = uninfected individuals.

2016	***Wol***	***Spir***	***Rics***	***Wol/Rics***	***Wol/Spir***	***Rics/Spir***	***Wol/Rics/Spir***	**Uninf.**	**N**
UK	1	0	15	1	0	0	0	30	47
GER	0	1	9	0	0	0	3	32	45
SWE	0	5	9	3	1	0	2	20	40
2017									
UK	0	1	10	8	0	0	0	21	40
GER	1	1	4	1	0	0	0	35	42
SWE	5	4	7	2	1	2	1	5	27

There was no significant difference in the overall frequency of uninfected individuals between 2016 and 2017 (62% vs 56%, 2-sample test for equality of proportions, Chi²= 0.7004, df = 1, p-value = 0.4026). Comparing the

frequencies of the three endosymbionts and multiple infections found in 2016 and 2017 showed no significant differences between the years except for the proportions of *Wolbachia*/*Rickettsia* double infections (Table 12). There were significantly more individuals found carrying the double infection in 2017 than in 2016 (3% vs 10%, 2-sample test for equality of proportions, $Chi^2 = 3.9623$, df = 1, p-value = 0.04653).

Table 12: Comparing the endosymbiont infection frequencies of *L. triangularis* collected at three different locations in 2016 and 2017.

Endosymbiont	Prop. 2016	Prop. 2017	2-sample test for equality of proportions
Wolbachia	1%	6%	$Chi^2 = 3.2355$, df = 1, p-value = 0.07206
Rickettsia	25%	19%	$Chi^2 = 0.82327$, df = 1, p-value = 0.3642
Spiroplasma	5%	6%	$Chi^2 = 0.0018667$, df = 1, p-value = 0.9655
Wolbachia/Rickettsia	3%	10%	**$Chi^2 = 3.9623$, df = 1, p-value = 0.04653**
Wolbachia/Spiroplasma	1%	1%	$Chi^2 = 2.6568e-31$, df = 1, p-value = 1
Rickettsia/Spiroplasma	0%	2%	$Chi^2 = 0.72159$, df = 1, p-value = 0.3956
Wol./Rick./Spir.	4%	1%	$Chi^2 = 1.0164$, df = 1, p-value = 0.3134

Due to the low overall frequency of *Wolbachia* infections in all populations no statistical analysis of inter-population comparisons was attempted. Comparing the proportions of *Rickettsia* infections in the three countries of origin showed that spiders from the UK were significantly more likely to be infected with *Rickettsia* than spiders from the German populations (29% vs 15%, 2-sample test for equality of proportions, $Chi^2 = 4.0739$, df = 1, p-value = 0.04355). The *Rickettsia* frequencies did not differ significantly neither between the British and the Swedish nor between the German and the Swedish populations (UK vs SWE: 29% vs 24%, 2-sample test for equality of proportions, $Chi^2 = 0.24199$, df = 1, p-value = 0.6228; GER vs SWE: 15% vs 24%, 2-sample test for equality of proportions, $Chi^2 = 1.4368$, df = 1, p-value = 0.2307).

Spiroplasma were present in significantly larger proportion in the Swedish population than in the UK and in Germany (SWE vs. UK: 13% vs 1%, 2-sample test for equality of proportions, $Chi^2 = 7.4915$, df = 1, p-value = 0.006199; SWE

vs GER: 13% vs 2%, 2-sample test for equality of proportions, $Chi^2 = 5.4953$, df = 1, p-value = 0.01907). Infection frequency did not differ between the British and the German populations (1% vs 2%, 2-sample test for equality of proportions, $Chi^2 = 0$, df = 1, p-value = 1).

The British population was found to harbour significantly more *Wolbachia*/*Rickettsia* double infections than the German population (10% vs 1%, 2-sample test for equality of proportions, $Chi^2 = 5.1988$, df = 1, p-value = 0.0226). The differences between the British and the Swedish, or between the German and the Swedish populations were not significant (UK vs SWE: 10% vs 8%, 2-sample test for equality of proportions, $Chi^2 = 0.11162$, df = 1, p-value = 0.7383; GER vs SWE: 1% vs 8%, 2-sample test for equality of proportions, $Chi^2 = 2.5194$, df = 1, p-value = 0.1125). Since the remaining multiple infections were only detected in low numbers, no comparisons were conducted.

To investigate possible differences between the years 2016 and 2017 in the populations, intra-population comparisons of endosymbiont infection frequencies were conducted. There was no significant difference in infection frequency with any of the three endosymbionts between the UK populations from 2016 and 2017 (2-sample test for equality of proportions, *Wolbachia*: 2% vs 0%, $Chi^2 = 5.1933e-33$, df = 1, p-value = 1; *Rickettsia*: 32% vs 25%, $Chi^2 = 0.22339$, df = 1, p-value = 0.6365; *Spiroplasma*: 0% vs 3%, $Chi^2 = 0.0065917$, df = 1, p-value = 0.9353). There were significantly more *Wolbachia*/*Rickettsia* double infections found in 2017 than in 2016 (20% vs 2%, 2-sample test for equality of proportions, $Chi^2 = 5.64$, df = 1, p-value = 0.01756). Infection frequencies did not differ between the years for any of the endosymbionts in the German populations (2-sample test for equality of proportions, *Wolbachia*: 0% vs 2%, $Chi^2 = 0.0012043$, df = 1, p-value = 0.9723; *Rickettsia*: 20% vs 10%, $Chi^2 = 1.1422$, df = 1, p-value = 0.2852; *Spiroplasma*: 2% vs 2%, $Chi^2 = 5.6829e-32$, df = 1, p-value = 1; *Wolbachia*/*Spiroplasma*: 0% vs 2%, $Chi^2 = 0.0012043$, df = 1, p-value = 0.9723).

In the Swedish populations from 2016 and 2017 there was a significant difference in *Wolbachia* infection rates. In 2017 more individuals did carry a *Wolbachia* infection than in 2016 (*Wolbachia*: 18.52% vs 0%, 2-sample test for equality of proportions, $Chi^2 = 5.5478$, df = 1, p-value = 0.0185). No further significant differences between the two years in *Rickettsia*, *Spiroplasma* and *Wolbachia/Rickettsia* double infection frequencies were detected (2-sample test for equality of proportions, *Rickettsia*: 22.50% vs 25.92%, Chi^2 = 0.00093132, df = 1, p-value = 0.9757; *Spiroplasma*: 12.50% vs 14.81%, Chi^2 = 9.0361e-31, df = 1, p-value = 1; *Wolbachia/Rickettsia*: 7.50% vs 7.41%, Chi^2 = 4.3142e-31, df = 1, p-value = 1). Country-wise inter-population comparisons between 2016 and 2017 were conducted with most results being insignificant (Table 13). The British and Swedish populations differed in *Spiroplasma* infection rate in 2016 as well as in *Wolbachia* frequency in 2017 (Table 13). Significantly more *Wolbachia/Rickettsia* double infections were found in the UK than in Germany in 2017 (Table 13).

Table 13: Inter-population comparisons of endosymbiont proportions. P1, P2 = proportion one and proportion two.

Comparison		UK vs GER		UK vs SWE		GER vs SWE	
Endosymbionts	**Year**	**P1 vs P2**	**2-sample test for equality of proportions**	**P1 vs P2**	**2-sample test for equality of proportions**	**P1 vs P2**	**2-sample test for equality of proportions**
Wolbachia	2016	2% vs 0%	Chi^2 = 2.2429e-32 df = 1 p = 1	2% vs 0%	Chi^2= 5.1933e-33 df = 1 p = 1	0% vs 0%	NA
Rickettsia	2016	32% vs 20%	Chi^2 = 1.1311 df = 1 p = 0.2875	32% vs 23%	Chi^2 = 0.54547, df = 1 p = 0.4602	20% vs 23%	Chi^2 = 0.00024473 df = 1 p = 0.9875
Spiroplasma	2016	0% vs 2%	Chi^2 = 0.00047801 df = 1 p = 0.9826	0% vs 13%	**Chi^2 = 4.1392 df = 1 p = 0.0419**	2% vs 13%	Chi^2 = 2.023 df = 1 p = 0.1549
Wolbachia /Rickettsia	2016	2% vs 0%	Chi^2 = 2.2429e-32 df = 1 p = 1	2% vs 8%	Chi^2 = 0.46085 df = 1 p = 0.4972	0% vs 8%	Chi^2 = 1.6425 df = 1 p = 0.2
Wolbachia	2017	0% vs 2%	Chi^2 = 4.775e-31 df = 1 p = 1	0% vs 19%	**Chi^2 = 5.5478 df = 1 p = 0.0185**	2% vs 19%	Chi^2 = 3.5497 df = 1 p = 0.05955
Rickettsia	2017	25% vs 10%	Chi^2 = 2.459 df = 1 p = 0.1169	25% vs 26%	Chi^2 = 4.298e-31 df = 1 p = 1	10% vs 26%	Chi^2 = 2.189 df = 1 p = 0.139
Spiroplasma	2017	3% vs 2%	Chi^2 = 4.5019e-32 df = 1 p = 1	3% vs 15%	Chi^2 = 1.9812, df = 1 p = 0.1593	2% vs 15%	Chi^2 = 2.1567, df = 1 p- = 0.142
Wolbachia /Rickettsia	2017	20% vs 2%	**Chi^2 = 4.8308, df = 1 p = 0.02796**	20% vs 7%	Chi^2 = 1.1435, df = 1 p = 0.2849	2% vs 7%	Chi^2 = 0.15557, df = 1 p = 0.6933

4.3.2 Endosymbionts and sex ratio

L. hortensis

All *L. hortensis* populations were female biased and infected with *Wolbachia*. It is possible that there is a direct link between the observed sex ratio skew and the endosymbiont infection. To analyse the influence of *Wolbachia*, *Rickettsia* and *Spiroplasma* on the sex ratio distortion further, categories describing the population infection status were introduced (WNN = *Wolbachia*, WNR = *Wolbachia* & *Rickettsia* and WSR = *Wolbachia*, *Spiroplasma* and *Rickettsia*). The number of males and females as well as the sex ratio for each population are shown in Table 14.

Table 14: Male sex ratio and endosymbiont infection pattern of each population collected in the UK, Germany, Denmark and Sweden in 2016 and 2017. ArHi = Arbour Hill, Wollaton Park, Nottingham; BeLo = Beeston Lodge, Wollaton Park, Nottingham; PuBA = "Planten un Blomen", Hamburg; OHL/OHL17 = Ohlsdorf Cemetery, Hamburg; WOLL17 = Wollaton Park, Nottingham; UP17 = University Park, Nottingham; BERG17 = Bergedorf, Hamburg; LILLE17 = Lillebælt car park; MUSW17 = Muggetorp, Vissefjärda. Infection pattern: WSR = *Wolbachia/Spiroplasma/Rickettsia*, WNN = *Wolbachia*, WNR = *Wolbachia/Rickettsia*.

Year	Country of origin	Population	Males	Females	Male SR	Exact binomial test (p - value)	Population infection pattern
2016	UK	ArHi	5	37	0.12	4.43E-07	WSR
2016	UK	BeLo	0	16	0.00	3.05E-05	WNN
2016	GER	PuBA	1	9	0.10	0.02148	WNR
2016	GER	OHL	1	11	0.08	0.006348	WNR
2017	UK	WOLL17	1	30	0.03	2.98E-08	WNR
2017	UK	UP17	5	18	0.22	0.01062	WSR
2017	GER	OHL17	0	6	0.00	0.03125	WNR
2017	GER	BERG17	0	7	0.00	0.01563	WNN
2017	DK	LILLE17	0	12	0.00	0.0004883	WNN
2017	SWE	MUSW17	0	23	0.00	2.38E-07	WNR

The sex ratio seemed to be significantly affected by the population infection pattern (Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 6.3062, df = 2, p-value = 0.04272). To investigate this further, comparisons between the different infection patterns were conducted. No significant differences between populations with the infection patterns WNN and WNR were found (WNN vs WNR: Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 2.3625, df

= 1, p-value = 0.1243; WNR vs WSR: Mann-Whitney-U test, W = 0, p-value = 0.07864).

The difference in sex ratio of populations carrying only *Wolbachia* infections and populations in which all three endosymbionts were present is not significant (WNN vs WSR: Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 3.75, df = 1, p-value = 0.05281). Populations infected with only *Wolbachia* had a more skewed sex ratio. A larger sample size is needed to establish if the infection pattern truly has an effect on population sex ratio. Additionally, since *Wolbachia* was detected in all populations and all populations showed a skewed sex ratio, it is possible that *Wolbachia* is the culprit here. Since no uninfected or not distorted populations were found a statistical analysis of *Wolbachia* impact was not possible. Therefore, only the impact of *Spiroplasma* and *Rickettsia* infections on the sex ratio was analysed.

Spiroplasma infections seemed to have an impact on population sex ratio (Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 4.9655, df = 1, p-value = 0.02586) with the population carrying the endosymbiont being significantly less female biased (Mann-Whitney-U test, W = 16, p-value = 0.0367). It has to be noted though that *Spiroplasma* was only found in populations which also harboured *Wolbachia* and *Rickettsia*. No significant effect of *Rickettsia* infections on population sex ratio was found (Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 3.3251, df = 1, p-value = 0.06823).

Investigating further, to assess if there were sex-related infection patterns present in the study populations, infection proportions of the different endosymbionts in the two sexes were compared. Males and females were equally infected with *Wolbachia* (males vs females: 29% vs 27%), no significant difference was found (2-sample test for equality of proportions, Chi^2 = 4.8996e-31, df = 1, p-value = 1). The proportions of *Rickettsia* infections in males and females did not differ significantly (males vs females: 14% vs 5%,

2-sample test for equality of proportions, $Chi^2 = 0.88746$, df = 1, p-value = 0.3462).

Infection rates with only *Spiroplasma* were low in both sexes with only one male carrying the infection whilst no female infected with only *Spiroplasma* was found (males vs females: 7% vs 0%, 2-sample test for equality of proportions, $Chi^2 = 2.6591$, df = 1, p-value = 0.103). Two females from the 'ArHi 2016' population were doubly infected with *Wolbachia* and *Spiroplasma*. Therefore, the population was labelled as 'WSR', but the females were not used in analyses of singly infected individuals. Comparing the proportions of the different endosymbionts in the sexes showed that in females *Wolbachia* was more abundant than *Rickettsia* and *Spiroplasma* with the *Rickettsia* proportion being significantly higher than *Spiroplasma* (Table 15).

Table 15: Comparing the proportions of the different endosymbionts in both sexes of *L. hortensis*. Prop.1 = proportion of the first endosymbiont in the comparison, Prop.2 = proportion of the second endosymbiont in the comparison.

Comparison	Sex	Prop. 1	Prop. 2	2-sample test for equality of proportions
Wol vs *Rics*	m	29%	14%	$Chi^2 = 0.21212$, df = 1, p = 0.6451
	f	27%	5%	**$Chi^2 = 32.334$, df = 1, p = 1.298e-08**
Wol vs *Spir*	m	29%	7%	$Chi^2 = 0.97391$, df = 1, p = 0.3237
	f	27%	0%	**$Chi^2 = 53.335$, df = 1, p = 2.812e-13**
Rics vs *Spir*	m	14%	7%	$Chi^2 = 0$, df = 1, p = 1
	f	5%	0%	**$Chi^2 = 6.2683$, df = 1, p = 0.01229**

L. triangularis

In Chapter 3, the sex ratio data for *L. triangularis* was pooled as populations for country of origin. All populations turned out to be significantly skewed towards females. To analyse the influence of endosymbionts on sex ratio, the data was pooled for the individual populations. Of these populations five populations showed a not significantly female biased sex ratio. The same categories of population patterns were used as for *L. hortensis* (Table 16).

Table 16: Male sex ratio and endosymbiont infection pattern of each population collected in the UK, Germany and Sweden in 2016 and 2017. Nottingham, UK: UP16, UP16_2, LT_UP17 = University Park; Woll16, LT_WOLL17 = Wollaton Park; Hamburg, Germany: BGHH16, LT_BGHH17 = Botanical Garden; AWHH16 = "Am Weiher" public park; EPHH16 = "Eppendorfer Park" public park; EE_HH16 = "Entenwerder Elbdeich" public park; LT_OHLHH17 = Ohlsdorf Cemetery; Vissefjärda, Sweden: LT_MUSW17 = Muggetorp. Infection pattern: NNN = not infected, NNR = *Rickettsia* infected, WNN = *Wolbachia*, NSR = *Spiroplasma/Rickettsia* infected, WNR = *Wolbachia/ Rickettsia*, WSR = *Wolbachia/Spiroplasma/Rickettsia*.

Year	Country of origin	Population	Males	Females	Male SR	Exact binomial test (p-value)	Population infection pattern
2016	UK	HiFi16	6	4	0.60	0.7539	NNN
2016	UK	UP16	5	7	0.42	0.7744	NNR
2016	**UK**	**UP16_2**	**0**	**10**	**0.00**	**0.001953**	**WNR**
2016	UK	Woll16	4	10	0.29	0.1796	NNR
2016	**GER**	**BGHH16**	**5**	**15**	**0.25**	**0.04139**	**NSR**
2016	GER	AWHH16	5	5	0.50	1	NNR
2016	**GER**	**EPHH16**	**1**	**8**	**0.11**	**0.03906**	**NNR**
2016	**GER**	**EE_HH16**	**0**	**6**	**0.00**	**0.03125**	**NNR**
2016	**SWE**	**MUSW16**	**11**	**29**	**0.28**	**0.006427**	**NSR**
2017	UK	LT_UP17	5	12	0.29	0.1435	NSR
2017	**UK**	**LT_WOLL17**	**3**	**18**	**0.14**	**0.00149**	**NNR**
2017	**GER**	**LT_BGHH17**	**0**	**21**	**0.00**	**9.54E-07**	**WNN**
2017	**GER**	**LT_OHLHH17**	**4**	**16**	**0.20**	**0.01182**	**NSR**
2017	**SWE**	**LT_MUSW17**	**3**	**25**	**0.11**	**2.74E-05**	**WSR**

A GLM with sex ratio as the response and country of origin, endosymbiont infection status and year of collection as explanatory variables, with a quasipoisson error structure was run on the dataset. None of the factors had a significant effect on population sex ratio (GLM, year: $F_{1,5} = 0.6239$, p = 0.4654; country: $F_{2,11} = 0.5419$, p = 0.5964; country:year: $F_{1,4} = 0.0593$, p = 0.8195), most importantly neither infection status (GLM, $F_{5,6} = 1.9248$, p = 0.2242) nor the interaction between country of origin and infection status ($F_{1,4} = 0.0070$, p = 0.9387) seemed to have an impact.

Comparing the sex ratios of populations showing the two most common infection patterns NNR and NSR showed no significant difference (Kruskal-Wallis rank sum test, Kruskal-Wallis $Chi^2 = 0.045455$, df = 1, p-value = 0.8312). When analysing the effects of the individual endosymbionts it seemed that *Wolbachia* had a significant influence on sex ratio distortion (Kruskal-Wallis

rank sum test, Kruskal-Wallis $Chi^2 = 5.1422$, df = 1, p-value = 0.02335). Populations not carrying *Wolbachia* had a significantly less biased sex ratio (Mann-Whitney-U test, W = 2, p-value = 0.02856). This result has to be considered with care since two of the populations carrying *Wolbachia* did also harbour *Spiroplasma* and/or *Rickettsia*. An interaction between the endosymbionts cannot be ruled out. *Spiroplasma* and *Rickettsia* infections did not seem to impact sex ratio significantly (Kruskal-Wallis rank sum test, *Spiroplasma*: Kruskal-Wallis $Chi^2 = 0.040355$, df = 1, p-value = 0.8408; *Rickettsia*: Kruskal-Wallis $Chi^2 = 0.033629$, df = 1, p-value = 0.8545).

Analyses of infection rates between males and females were conducted to establish if there were any other infection patterns present in the two sexes. No significant difference in the number of uninfected males and females was found (2-sample test for equality of proportions, $Chi^2 = 0.51655$, df = 1, p-value = 0.4723 males and females: 64.71% vs 57.89%). *Wolbachia* infection rates were low in both sexes with only 4% of all females carrying the endosymbiont. It is noteworthy that no *Wolbachia*-infected males were found. Still, statistically there was no difference in *Wolbachia* infection rates between males and females (2-sample test for equality of proportions, $Chi^2 = 0.84926$, df = 1, p-value = 0.3568).

There was no significant difference in the frequency of *Rickettsia* infections in males and females (2-sample test for equality of proportions, $Chi^2 = 0.00075428$, df = 1, p-value = 0.9781, males vs females: 23.53% vs 22.11%). Both sexes were equally infected with *Spiroplasma* with males showing a slightly higher but not significantly different infection frequency (2-sample test for equality of proportions, $Chi^2 = 0.48504$, df = 1, p-value = 0.4861, males vs females: 7.84% vs 4.21%).

The number of *Wolbachia*/*Rickettsia* double infections did not differ significantly between the two sexes (2-sample test for equality of proportions, $Chi^2 = 0.19373$, df = 1, p-value = 0.6598; males vs females: 3.92% vs 6.84%).

The *Wolbachia/Spiroplasma* and *Rickettsia/Spiroplasma* double infections as well as the *Wolbachia/Spiroplasma/Rickettsia* triple infections were only found in females and therefore, no comparisons between the sexes were possible for these infection patterns. In both sexes *Rickettsia* was significantly more abundant than *Wolbachia* and *Spiroplasma* whilst there was no difference in *Wolbachia* and *Spiroplasma* frequencies in either sex (Table 17).

Table 17: Comparing the proportions of the different endosymbionts in both sexes of *L. triangularis*. Prop.1 = proportion of the first endosymbiont in the comparison, Prop.2 = proportion of the second endosymbiont in the comparison.

Comparison	Sex	Prop. 1	Prop. 2	2-sample test for equality of proportions
Wol vs *Rics*	m	0%	24%	**Chi² = 11.428, df = 1, p = 0.0007235**
	f	4%	22%	**Chi² = 27.084, df = 1, p = 1.948e-07**
Wol vs *Spir*	m	0%	8%	Chi² = 2.3418, df = 1, p = 0.1259
	f	4%	4%	Chi² = 0, df = 1, p = 1
Rics vs *Spir*	m	24%	8%	**Chi² = 3.6323, df = 1, p = 0.05667**
	f	22%	4%	**Chi² = 25.08, df = 1, p = 5.5e-07**
Wol vs *Wol/Rics*	m	0%	4%	Chi² = 0.51, df = 1, p = 0.4751
	f	4%	7%	Chi² = 1.3194, df = 1, p = 0.2507
Rics vs *Wol/Rics*	m	24%	4%	**Chi² = 6.7062, df = 1, p = 0.009608**
	f	22%	7%	**Chi² = 16.667, df = 1, p = 4.455e-05**
Spir vs *Wol/Rics*	m	8%	4%	Chi² = 0.17708, df = 1, p = 0.6739
	f	4%	7%	Chi² = 0.80647, df = 1, p = 0.3692

4.3.3 Endosymbionts and body size

L. hortensis

A GLM with cephalothorax width as the response and infection status, year of collection and country of origin as explanatory variables with a Gamma error structure and log identity was run on the pooled data. Sex is not included in this model as a factor since it had already been established that males and females are equally infected, and males are significantly larger than females. Cephalothorax width is therefore linked with sex and because of the equal infection rates in males and females the interaction between these two factors would not provide new insights.

Year of collection and the interaction between year of collection and country of origin was significant again as was already reported in Chapter 3.3.2 (GLM; year: $F_{3,174} = 6.4709$, $p = 0.011867$; year:country: $F_{1,168} = 5.6983$, $p = 0.018092$). Infection status did have a significant effect on cephalothorax width (GLM; $F_{2,178} = 6.6758$, $p = 0.001622$) as did the interaction of infection status and country of origin (GLM; $F_{5,169} = 3.5659$, $p = 0.004324$).

Further analyses were conducted to establish how the significant factors and interactions influenced spider cephalothorax width. Due to low infection rates with *Spiroplasma* and just a few multiple infections only the potential impact of *Wolbachia* and *Rickettsia* on cephalothorax width was investigated. Data from both years and all populations were pooled and the analysis showed that *Wolbachia* had no influence on body size. *Wolbachia* infected individuals did not differ significantly in size from uninfected individuals (Kruskal-Wallis rank sum test, Kruskal-Wallis $Chi^2 = 0.78384$, df = 1, p-value = 0.376; *Wolbachia*: N = 52, m.ceph.w. = 1.49 mm ± 0.02; uninfected: N = 120, m.ceph.w. = 1.51 mm ± 0.01). Interestingly the variance of cephalothorax width seemed to be significantly larger in *Wolbachia* infected than in uninfected individuals (F test to compare two variances, F = 0.54291, num df = 119, denom df = 51, p-value = 0.007064).

Rickettsia infected individuals are significantly smaller than uninfected individuals (*Rickettsia*: N = 10, m.ceph.w. = 1.37 mm ± 0.06; Kruskal-Wallis rank sum test, Kruskal-Wallis $Chi^2 = 4.8083$, df = 1, p-value = 0.02832). Since only two of the individuals infected with *Rickettsia* were males, an analysis pooled by sex was not possible. Again, the variance in size was significantly larger in the *Rickettsia* infected compared to the uninfected spiders (F test to compare two variances, F = 0.36179, num df = 119, denom df = 9, p-value = 0.01136). There was no sig. size difference between *Wolbachia* and *Rickettsia* infected spiders (Mann-Whitney-U test, W = 344.5, p-value = 0.1011).

To investigate the connection between endosymbiont infections and cephalothorax width further, intra-populations analyses were conducted. The data, pooled for both years, was split by country of origin. As in the pooled analysis, no significant difference between British *Wolbachia* infected and uninfected individuals could be found (uninfected: N = 66, m.ceph.w. = 1.53mm ± 0.01; *Wolbachia*: N = 39, m.ceph.w. = 1.51mm ± 0.02; Mann-Whitney-U test, W = 1184, p-value = 0.481). There was an almost significant difference between *Rickettsia* infected and uninfected individuals (*Rickettsia*: N = 4, m.ceph.w. = 1.35mm ± 0.10; Mann-Whitney-U test, W = 57.5, p-value = 0.05157) and no significant difference between *Wolbachia* and *Rickettsia* infected individuals (Mann-Whitney-U test, W = 37.5, p-value = 0.08745).

In the German population the variance in size was significantly different between *Wolbachia* infected and uninfected individuals (F test to compare two variances, F = 2.8413, num df = 10, denom df = 21, p-value = 0.04201) with *Wolbachia* infected spiders showing a more variable cephalothorax width. Nevertheless, no difference in cephalothorax width was found (uninfected: N = 22, m.ceph.w. = 1.50mm ± 0.03; *Wolbachia*: N = 11, m.ceph.w. = 1.4mm ± 0.07; Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 1.7118, df = 1, p-value = 0.1907). Again, *Rickettsia* infected spiders were significantly smaller than uninfected individuals (*Rickettsia*: N = 3, m.ceph.w. = 1.2mm ± 0.06; Mann-Whitney-U test, W = 2, p-value = 0.007364). The cephalothorax width did not differ significantly between *Wolbachia* and *Rickettsia* infected spiders (Mann-Whitney-U test, W = 8, p-value = 0.2017).

Due to generally low infection rates in the Swedish populations, only the cephalothorax width of *Rickettsia* infected individuals could be compared to that of uninfected individuals. There was no significant difference in size between *Rickettsia* infected and uninfected individuals (uninfected: N = 21, m.ceph.w. = 1.47mm ± 0.02; *Rickettsia*: N = 3, m.ceph.w. = 1.57mm ± 0.03; Mann-Whitney-U test, W = 49.5, p-value = 0.1095). It seems that the significant interaction of country of origin and infection status is due to

Rickettsia infected individuals in Germany and the UK being smaller than uninfected spiders from these locations.

A few inter-population analyses were attempted even though the sample sizes were quite small. Therefore, the results must be considered with care and can only be interpreted as a suggestion of potential differences. There were no significant size differences between *Rickettsia* infected individuals from Germany and the UK (Mann-Whitney-U test, W = 3, p-value = 0.359; GER: N = 3, m.ceph.w. = 1.2mm ± 0.06; UK: N = 4, m.ceph.w. = 1.35mm ± 0.10). Swedish *Rickettsia* infected individuals did neither differed significantly in cephalothorax from German (Mann-Whitney-U test, W = 0, p-value = 0.07652; SWE: N = 3, m.ceph.w. = 1.57mm ± 0.03) nor British individuals (Mann-Whitney-U test, W = 2, p-value = 0.1947).

The size comparison of *Wolbachia* infected spiders from Germany and the UK showed that the variance in cephalothorax width was significantly larger in the German than in the British population (F test to compare two variances, F = 2.8111, num df = 10, denom df = 38, p-value = 0.02075). However, the mean cephalothorax width did not differ between the two *Wolbachia* infected populations (Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 2.0733, df = 1, p-value = 0.1499).

The different variance in cephalothorax width found in uninfected and *Wolbachia* infected populations was also found in the two sexes. The size variance in *Wolbachia* infected males was significantly larger than in uninfected males (F test to compare two variances, F = 0.11525, num df = 5, denom df = 3, p-value = 0.03994). Males infected with *Wolbachia* seem to be slightly smaller than uninfected males, even though this difference was not significant (uninfected: N = 7, m.ceph.w. = 1.61mm ± 0.03, *Wolbachia*: N = 4, m.ceph.w. = 1.48mm ± 0.11; Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 1.0058, df = 1, p-value = 0.3159). A larger sample size is required to confirm whether *Wolbachia* has an influence on male body size.

The same difference in body size variance was found when comparing uninfected with *Wolbachia* infected females (F test to compare two variances, F = 0.549, num df = 112, denom df = 47, p-value = 0.01074). Females infected with *Wolbachia* were of equal size as uninfected individuals (uninfected: N = 114, m.ceph.w. = 1.51mm ± 0.01, *Wolbachia*: N = 48, m.ceph.w. = 1.49 ± 0.02; Kruskal-Wallis rank sum test, Kruskal-Wallis $Chi^2 = 0.49033$, df = 1, p-value = 0.4838). Comparing *Wolbachia* infected males and females showed no difference in cephalothorax width (Mann-Whitney-U test, W = 95.5, p-value = 1).

L. triangularis

A GLM was run on the pooled dataset. The response variable was cephalothorax width with infection status, year of collection and country of origin as explanatory variables and a Gamma error distribution with the log link function. As in the model for *L. hortensis,* sex was not included in the model since it was a predictor for body size and again no differences in endosymbiont infection rates between males and females were present. Year of collection and the interaction of year and country of origin had significant effects on cephalothorax width (GLM; year: $F_{1,201} = 23.8467$, p = 2.090e-06; year:country: $F_{2,206} = 17.9554$, p = 6.484e-08) but since this was already discussed in Chapter 3.3.2, no further analyses were conducted in this regard. Neither infection status nor any of the interactions with infection status were predictors for cephalothorax width in *L. triangularis* (GLM; infection status: $F_{7,211} = 1.0848$, p = 0.3742). Even though, according to the GLM, infection status cannot explain the differences in cephalothorax width, analyses of possible differences between the three endosymbionts and the different countries of origin were conducted. Additionally, the interaction of sex and endosymbiont infection on spider body size was analysed.

In Table 18 the mean cephalothorax width of uninfected individuals and spiders infected with the different endosymbionts are presented. These groups were compared with one another. The *Wolbachia*/*Spiroplasma* and

Rickettsia/*Spiroplasma* double infected individuals were not included in the analysis since the sample size was too low in these groups (Table 18).

Table 18: Comparison of cephalothorax width in *L. triangularis* individuals carrying the different endosymbionts. Uninf. = uninfected, Wol. = *Wolbachia*, Rics. = *Rickettsia*, Spir. = *Spiroplasma*, Wol/Rics = *Wolbachia*/*Rickettsia*. Mann-Whitney-U test.

Endosymbiont	N	M.ceph.w. ± SD	Uninf.	Wol.	Rics.	Spir.	Wol/Rics
Uninfected	143	1.78mm ± 0.02					
Wolbachia	7	1.70mm ± 0.08	W = 443, p = 0.5744				
Rickettsia	54	1.74mm ± 0.04	W = 3127, p = 0.8106	W = 156.5, p = 0.6707			
Spiroplasma	12	1.73mm ± 0.06	W = 838, p = 0.6702	W = 33.5, p = 0.8479	W = 293, p = 0.8416		
Wol/Rics	15	1.76mm ± 0.07	W = 973, p = 0.9922	W = 41, p = 0.7822	W = 351, p = 0.9868	W = 86, p = 0.8628	
Wol/Spir/Rics	6	1.52mm ± 0.06	**W = 505.5, p = 0.03435**	W = 22.5, p = 0.1887	W = 180, p = 0.05208	W = 47, p = 0.07581	W = 56, p = 0.1123
Wol/Spir	2	1.70mm ± 0.2	-	-	-	-	-
Rics/Spir	2	1.50mm ± 0.2	-	-	-	-	-

First, the cephalothorax width of uninfected spiders was compared to the size of spiders carrying one or multiple endosymbionts. The only significant size difference was found between uninfected and *Wolbachia*/*Spiroplasma*/*Rickettsia* triple infected spiders, with uninfected individuals being significantly larger. No significant differences were found between uninfected and *Wolbachia*, *Rickettsia*, *Spiroplasma* or *Wolbachia*/*Rickettsia* double infected spiders (Table 18). Comparing the cephalothorax width of individuals with the different endosymbionts revealed no significant differences either (Table 18). The size difference between *Rickettsia* infected and *Wolbachia*/*Spiroplasma*/*Rickettsia* triple infected spiders was almost significant; the triple infected individuals were smaller than the *Rickettsia*-carrying ones.

To investigate a possible interaction of country of origin and infection status which could influence cephalothorax width, intra- and inter-population comparisons were conducted. The statistics for the intra-population analysis are presented in Table 19. Comparisons were only attempted between groups with a sample size of at least five individuals. For the German populations the

only comparison possible was between the uninfected and the *Rickettsia* infected individuals. No size differences were found.

In British spiders, uninfected, *Rickettsia* infected and *Wolbachia*/*Rickettsia* double infected spiders showed no significant difference in cephalothorax width. Swedish spiders carrying a *Wolbachia*/*Rickettsia* double infection were significantly smaller than uninfected and *Rickettsia* infected individuals. The size difference between these double infected individuals and *Spiroplasma*-carrying spiders was almost significant, with double infected individuals being smaller. These results must be considered with care since only five double infected spiders were found. None of the other comparisons in the Swedish populations showed any significant differences (Table 18).

Table 19: Intra-population comparison of cephalothorax width in *L. triangularis* carrying the different endosymbionts. Uninf. = uninfected, Wol. = *Wolbachia*, Rics. = *Rickettsia*, Spir. = *Spiroplasma*. Mann-Whitney-U test.

Endosymbiont	N	M.ceph.w. ± SD	Uninf.	Wol.	Rics.	Spir.
UK						
Uninfected	51	1.73mm ± 0.04				
Wol	1	NA	-			
Rics	25	1.75mm ± 0.07	W = 554, p = 0.8557	-		
Spir	1	1.50mm	-	-	-	
Wol/Rics	9	1.89mm ± 0.09	W = 143, p = 0.09528	-	W = 73, p = 0.2641	-
GER						
Uninfected	67	1.76mm ± 0.04				
Wol	1	2.00mm	-			
Rics	13	1.82mm ± 0.06	W = 301, p = 0.5228	-		
Spir	2	1.90mm ± 0.1	-	-	-	
Wol/Rics	1	1.80mm	-	-	-	-
Wol/Spir/Rics	3	1.60mm ± 0.08	-	-	-	-
SWE						
Uninfected	25	1.75mm ± 0.04				
Wol	5	1.64mm ± 0.07	W = 72.5, p = 0.2808			
Rics	16	1.69mm ± 0.05	W = 144.5, p = 0.3522	W = 47.5, p = 0.5561		
Spir	9	1.71mm ± 0.06	W = 87, p = 0.6099	W = 27, p = 0.5854	W = 68.5, p = 0.8625	
Wol/Rics	5	1.52mm ± 0.05	**W = 19, p = 0.02359**	W = 6, p = 0.1797	**W = 64, p = 0.0483**	W = 8, p = 0.05456
Wol/Spir	2	1.70mm ± 0.2	-	-	-	-
Rics/Spir	2	1.50mm ± 0.2	-	-	-	-
Wol/Spir/Rics	3	1.47mm ± 0.09	-	-	-	-

Due to the very small sample sizes for some of the infection statuses, only a limited number of inter-population comparisons could be conducted. German uninfected individuals did not differ in size from British (Mann-Whitney-U test, W = 1564, p-value = 0.4629) or Swedish uninfected spiders (Mann-Whitney-U test, W = 684, p-value = 0.7112). No significant difference in cephalothorax width between British and Swedish spiders could be found either (Mann-Whitney-U test, W = 559, p-value = 0.806).

Rickettsia infected spiders did not differ in size between the three countries of origin (Mann-Whitney-U test, GER vs UK: W = 114.5, p-value = 0.51, GER vs SWE: W = 97, p-value = 0.1604; Kruskal-Wallis rank sum test, UK vs SWE: Kruskal-Wallis Chi^2 = 0.28833, df = 1, p-value = 0.5913). Interestingly, the variance in size differed significantly between the Swedish and the British *Rickettsia*-carrying individuals (F test to compare two variances, F = 0.30121, num df = 15, denom df = 21, p-value = 0.02088). Overall, the interaction between country and infection status did not seem to have an influence on spider cephalothorax width.

As pointed out in the previous chapter, males and females differed significantly in cephalothorax width with males on average being larger (see Chapter 3). To investigate the influence of the interaction of endosymbiont infection and sex on cephalothorax width, comparisons between uninfected and differently infected males and females were conducted. The data were pooled and not split by year or country of origin for the same reason. The mean cephalothorax width for both sexes with different infection status is shown in Table 20, as well as the results of the intra-sex comparisons. Infection status did not have a significant effect on male cephalothorax width.

Furthermore, there were no differences in mean cephalothorax width between uninfected and *Rickettsia* infected, uninfected and *Spiroplasma* infected or *Rickettsia* and *Spiroplasma* infected males. As in the males, infection status had no significant influence on female cephalothorax width (Kruskal-Wallis rank sum test, Kruskal-Wallis Chi^2 = 6.6368, df = 7, p-value = 0.4677). In the females, the only significant difference was found between uninfected and triple infected females. Triple infected females were significantly smaller than uninfected females (Table 20).

Table 20: Mean cephalothorax width and intra-sex comparison of spider size in male and female *L. triangularis* carrying different endosymbionts. U = uninfected, W = *Wolbachia*, R = *Rickettsia*, S = *Spiroplasma*. Mann-Whitney-U test.

Endosymbionts	N	M.ceph.w. ± SD	U	W	R	S	W/R
				Males			
Uninfected	33	1.83mm ± 0.06					
Rickettsia	12	1.93mm ± 0.07	W = 124.5, p = 0.2915				
Spiroplasma	4	1.85mm ± 0.09	W = 54, p = 0.8457	-	W = 26, p = 0.6395		
Wol/Rics	2	2.2mm ± 0	-	-	-	-	
				Females			
Uninfected	110	1.72mm ± 0.02					
Wolbachia	7	1.70mm ± 0.08	W = 333, p = 0.6864				
Rickettsia	42	1.69mm ± 0.04	W = 2019.5, p = 0.3208	W = 106, p = 0.9565			
Spiroplasma	8	1.66mm ± 0.07	W = 486.5, p = 0.3355	W = 21.5, p = 0.7881	W = 156, p = 0.7229		
Wol/Rics	13	1.69mm ± 0.06	W = 727.5, p = 0.5256	W = 37, p = 0.8933	W = 237.5, p = 0.9453	W = 50, p = 0.9117	
Wol/Spir/Rics	6	1.52mm ± 0.06	**W = 386.5, p = 0.04438**	W = 7.5, p = 0.1887	W = 128.5, p = 0.1255	W = 28, p = 0.2553	W = 28, p = 0.2553
Wol/Spir	2	1.70mm ± 0.2	-	-	-	-	-
Rics/Spir	2	1.50mm ± 0.2	-	-	-	-	-

When comparing male and female cephalothorax width with regards to their infection status, it was found that uninfected individuals from both sexes did not differ significantly in size (Mann-Whitney-U test, W = 1719, p-value = 0.1517). This is interesting given the fact that *L. triangularis* is known to show a sexual size dimorphism with males being the larger sex. In *Rickettsia* infected individuals the expected size difference between males and females was present again, males were significantly larger than females (Mann-Whitney-U test, W = 298.5, p-value = 0.01125). *Spiroplasma* infected males and females again showed no significant size difference (Mann-Whitney-U test, W = 25, p-value = 0.141).

4.3.4 Endosymbionts and egg sacs

4.3.4.1 Hatching rates

L. hortensis

A total number of 138 first egg sacs were produced by *L. hortensis* females from all four sample locations (UK: 93, GER: 12, DK: 8, SWE: 17) in two years. For eight egg sacs from 2017, hatching success was unknown. Therefore, these egg sacs were not included in the analyses. The influence of infection status on egg sac hatching was first analysed pooling the data of both years and all populations.

Infection status did not seem to have a significant effect on egg sac hatching rates (Kruskal-Wallis rank sum test, Kruskal-Wallis $Chi^2 = 2.6162$, df = 4, p-value = 0.624). The hatching rates of egg sacs from uninfected females did not differ from that of *Wolbachia*-carrying females (2-sample test for equality of proportions, $Chi^2 = 9.0971e-31$, df = 1, p-value = 1; uninfected: N = 88, hatching rate = 84%, *Wolbachia*: N = 40, hatching rate = 85%). There was also no significant difference in hatching rates between egg sacs from uninfected and *Rickettsia* infected females (2-sample test for equality of proportions, $Chi^2 = 0.096661$, df = 1, p-value = 0.7559; *Rickettsia*: N = 6, hatching rate = 100%).

Due to the difference identified in hatching rates between 2016 and 2017, the hatching rates of eggs from uninfected and *Wolbachia* infected females were compared between the years. No difference was found between the hatching rates of uninfected females' egg sacs from 2016 and 2017 (2-sample test for equality of proportions, $Chi^2 = 4.6419e-30$, df = 1, p-value = 1; 2016: 92%, 2017: 95%). Hatching rates did also not differ between the years in *Wolbachia* infected females' egg sacs (2-sample test for equality of proportions, $Chi^2 = 1.3582e-30$, df = 1, p-value = 1; 2016: 95%, 2017: 94%).

Egg sacs from *Wolbachia*/*Rickettsia* and *Wolbachia*/*Spiroplasma* double infected females were not included in any of the analyses since the sample sizes were too small with only two egg sacs from each group. Since only some

egg sacs in the UK populations did not hatch, further analyses of the influence of endosymbiont infection status on hatching rates were only conducted on egg sacs laid by UK females. No differences were found between the egg sac hatching rates of uninfected compared to *Wolbachia* infected UK females (2-sample test for equality of proportions, $Chi^2 = 0.24216$, df = 1, p-value = 0.6227; uninfected: N = 53, hatching rate = 82%; *Wolbachia*: N = 34, hatching rate = 75%). Due to the low number of egg sacs from *Rickettsia*, *Wolbachia*/*Rickettsia* and *Wolbachia*/*Spiroplasma* infected females, no further comparisons were conducted.

L. triangularis

Since none of the egg sacs produced by *L. triangularis* females in 2016 hatched and egg sacs from 2017 were directly transferred into EtOH after discovery, no analysis was possible (see also Chapter 3).

4.3.4.2 Egg / Offspring number

L. hortensis

Egg/offspring number was analysed in connection with endosymbiont infections for 120 egg sacs. Comparisons were made between egg sacs from uninfected, *Wolbachia* infected and *Rickettsia* infected females. According to a Kruskal-Wallis test, endosymbiont infection status did not have a significant influence on egg/offspring number (Kruskal-Wallis rank sum test, Kruskal-Wallis $Chi^2 = 3.6729$, df = 4, p-value = 0.4521).

There was no significant difference in egg/offspring number between egg sacs from uninfected and *Wolbachia* infected females (Mann-Whitney-U test, W = 1462.5, p-value = 0.1069; uninfected: N = 70, mean egg/offspring number = 20.26 ± 1.67; *Wolbachia*: N = 35, mean egg/offspring number = 15.23 ± 1.94). Egg sacs from uninfected females did not differ significantly in egg/offspring number from those produced by *Rickettsia* infected females (Mann-Whitney-U test, W = 194, p-value = 0.694; *Rickettsia*: N = 5, mean egg/offspring number

= 16.8 ± 5.59). *Wolbachia* and *Rickettsia* infected females did not differ significantly in the number of eggs/offspring they produced (Mann-Whitney-U test, W = 80, p-value = 0.7743).

Two egg sacs each were produced by females carrying the *Wolbachia*/ *Rickettsia* or the *Wolbachia*/*Spiroplasma* double infection. Due to the low sample size, they were excluded from the statistical analyses. The comparisons presented confirmed that infection status had no effect on egg/offspring number.

Further analyses were conducted to investigate the potential interaction between egg sac hatching, egg/offspring number and infection status. The number of offspring from hatched egg sacs was significantly lower than that of unhatched egg sacs (Mann-Whitney-U test, W = 1162.5, p-value = 0.0002991; hatched: N = 98, mean number of offspring = 16.51 ± 1.28; unhatched: N = 20, mean number of eggs= 31.4 ± 3.11). As mentioned in Section 1.3.2, the spiderlings hatch from their eggs inside the egg sac. They will moult at least once before emerging from the egg sac. Therefore, sibling cannibalism and other spiderling mortality could have occurred in the egg sac before the juveniles emerged. This could explain why the number of offspring from hatched egg sacs was lower. Also, the juveniles were not counted directly after emerging, therefore this difference has to be considered with care.

The data were then split into groups of egg sacs from uninfected and *Wolbachia* infected females. The significant difference in egg/offspring number between unhatched and hatched egg sacs was found in both groups with unhatched egg sacs showing a higher egg number (Mann-Whitney-U test, uninfected: W = 434.5, p-value = 0.02441; *Wolbachia*: W = 142, p-value = 0.001685). Comparing the mean number of eggs of unhatched egg sacs from *Wolbachia* infected and uninfected females revealed no significant difference

(Mann-Whitney-U test, W = 30, p-value = 0.5809; uninfected: N = 10, mean egg number = 30.3 ± 4.47; *Wolbachia*: N = 5, mean egg number = 33.6 ± 3.67).

The picture was different when comparing the number of offspring from hatched egg sacs laid by uninfected and *Wolbachia* infected females. First the variance in offspring number was significantly different between the two groups (F test to compare two variances, F = 0.45728, num df = 29, denom df = 59, p-value = 0.02363) and second uninfected females had significantly more offspring than *Wolbachia* infected females (Kruskal-Wallis rank sum test, Kruskal-Wallis Chi² = 4.141, df = 1, p-value = 0.04186; uninfected: N = 60, mean offspring number = 18.58 ± 1.73; *Wolbachia*: N = 30, mean offspring number = 12.17 ± 1.68). *Wolbachia* infected females had about one third fewer offspring than uninfected females.

L. triangularis

A total of 27 females produced egg sacs in 2016 and 2017. As in *L. hortensis*, infection status did not seem to have an impact on egg number (Kruskal-Wallis rank sum test; Kruskal-Wallis Chi² = 3.3713, df = 5, p-value = 0.6429). Comparing mean egg numbers from uninfected and *Rickettsia* infected females showed no significant differences (Mann-Whitney-u test, W = 38, p-value = 0.4629; uninfected: N = 16, mean egg number = 39.17 ± 5.21; *Rickettsia*: N = 6, mean egg number = 56.13 ± 11.79).

In Chapter 3 it was reported that, using the pooled data from 2016 and 2017, female size and egg number did not correlate. This was an unexpected finding and therefore the effect of infection status on the correlation of female cephalothorax width and egg number was investigated. There was no correlation between uninfected females' cephalothorax width and the number of eggs they produced. This was true for the pooled data (Kendall's rank correlation tau, z = -0.65137, p-value = 0.5148, tau = -0.1543033) as well as for the data split in subsets for the years 2016 (Kendall's rank correlation

tau, z = 1.0835, p-value = 0.2786, tau = 0.5477226) and 2017 (Kendall's rank correlation tau, z = 0.79789, p-value = 0.4249, tau = 0.2502173).

In *Rickettsia* infected females there was again no correlation between size and mean egg number (Kendall's rank correlation tau, z = 1.5191, p-value = 0.1287, tau = 0.48795). The data was split into subsets for 2016 and 2017 but since only one observation from a *Rickettsia* infected female was present in 2017, the correlation was only researched for 2016. As before no correlation was found (Kendall's rank correlation tau, T = 12, p-value = 0.1361, tau = 0.6).

4.3.5 Endosymbiont transmission

An integral part of studying the effects of endosymbionts on their host was establishing the rate with which the endosymbionts are transmitted from one generation to the other, *i.e.* the vertical transmission rate.

L. hortensis

A total of 107 offspring from 54 females in both years was screened for the endosymbionts found in their mothers. Unfortunately, for the majority of females, fewer than three offspring were available for screening. Therefore, the transmission rates presented in Table 21 cannot be regarded as representative of the sample set. The table only shows transmission rates for offspring from infected females and no further statistical analysis was attempted. It was obvious that, as soon as more than one juvenile was available for screening, transmission rates dropped below 100%, making it clear that transmission was imperfect for any of the endosymbionts found.

Table 21: Transmission rates of the different endosymbiont for each individual *L. hortensis* female which produced an egg sac. Year = year of collection, Pop. = population, C = country of origin, M. I. = mother infection status, N = number of offspring screened, W = *Wolbachia*, S = *Spiroplasma*, R = *Rickettsia*, U = uninfected.

Year	Pop.	C	M. I.	N	W	S	R	U	W%	S%	R%	U%
2016	PuBA	GER	W	1	0	0	0	1	0	0	0	100
2016	OHL	GER	W	1	0	0	0	1	0	0	0	100
2016	OHL	GER	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	W	1	0	0	0	1	0	0	0	100
2016	ArHi	UK	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	W	2	2	0	0	0	100	0	0	0
2016	ArHi	UK	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	W	1	1	0	0	0	100	0	0	0
2016	BeLo	UK	W	1	1	0	0	0	100	0	0	0
2016	BeLo	UK	W	14	2	0	0	12	14	0	0	86
2016	BeLo	UK	W	1	1	0	0	0	100	0	0	0
2016	BeLo	UK	W	1	1	0	0	0	100	0	0	0
2016	BeLo	UK	W	1	1	0	0	0	100	0	0	0
2016	BeLo	UK	W	1	1	0	0	0	100	0	0	0
2016	BeLo	UK	W	1	1	0	0	0	100	0	0	0
2016	ArHi	UK	WR	1	1	0	0	0	100	0	0	0
2017	WOLL17	UK	R	5	0	0	4	1	0	0	80	20
2017	OHL17	GER	R	9	0	0	5	4	0	0	56	44
2017	MUSW17	SWE	R	4	0	0	0	4	0	0	0	100
2017	MUSW17	SWE	R	4	0	0	0	4	0	0	0	100
2017	MUSW17	SWE	R	2	0	0	0	2	0	0	0	100
2017	UP17	UK	W	4	2	0	0	2	50	0	0	50
2017	WOLL17	UK	W	4	3	0	0	1	75	0	0	25
2017	WOLL17	UK	W	1	0	0	0	1	0	0	0	100
2017	WOLL17	UK	W	1	1	0	0	0	100	0	0	0
2017	WOLL17	UK	W	6	5	0	0	1	83	0	0	17
2017	WOLL17	UK	W	2	2	0	0	0	100	0	0	0
2017	WOLL17	UK	W	1	0	0	0	1	0	0	0	100
2017	WOLL17	UK	W	3	2	0	0	1	67	0	0	33
2017	WOLL17	UK	W	2	2	0	0	0	100	0	0	0
2017	WOLL17	UK	W	1	1	0	0	0	100	0	0	0
2017	OHL17	GER	W	3	0	0	0	3	0	0	0	100
2017	BERG17	GER	W	1	0	0	0	1	0	0	0	100
2017	MUSW17	SWE	W	1	0	0	0	1	0	0	0	100
2017	UP17	UK	WS	3	1	2	0	0	33	67	0	0

L. triangularis

The quality of the *L. triangularis* egg sacs produced by females in 2016 was poor due to desiccation. When the egg sacs were brought back to room temperature after being stored in the refrigerator to imitate a cold phase, they started to shown signs of mould. To prevent more moulding the egg sacs were not sprayed with water as often as previously. It turned out that this led to desiccation of the egg sacs. Consequently, eggs appeared shrivelled and were hard to squash for DNA extraction. It had to be considered that the DNA extraction procedure did not work as well as if the eggs had been 'fresh'. Hence, these egg sacs, even though they were screened for endosymbionts, if their mothers were infected, were not included in the assessment of the transmission rates. Three *Rickettsia*-infected females from 2016 produced egg sacs and at least some of their eggs did test positive for the infection as well. None of the eggs of one *Wolbachia*-infected female seemed to be carrying the infection. Due to aforementioned problems with DNA quality it is possible that the absence of *Wolbachia* transmission is an artefact.

Only two females infected with endosymbionts produced egg sacs in 2017. This meant that the sample size was too low to conduct statistical analyses. Both females were carrying multiple infections. The female from the UK (LT_WOLL17/2062) was infected by a *Wolbachia*/*Rickettsia* double infection and the Swedish female (LT_MUSW17/2092) carried all three endosymbionts. In both egg sacs all eggs were found to be infected by at least one endosymbiont.

Of the 32 eggs from LT_WOLL17/2062's egg sac 26 eggs (81%) tested positive for the *Wolbachia*/*Rickettsia* double infection. Four eggs (13%) were single infected with *Rickettsia* and two eggs (6%) carried only *Wolbachia*. The egg sac from LT_MUSW17/2092 contained only 18 eggs. None of these eggs was triple infected like the mother. Fourteen eggs (78%) tested positive for *Rickettsia*, three eggs (17%) carried a *Wolbachia*/*Rickettsia* double infection and one (6%) egg was infected with *Wolbachia* only.

The double infection of the British female was transmitted with high efficiency whilst only a few eggs carry only one of the endosymbiont species. The triple infection found in the Swedish female is not transmitted to the next generation. Instead *Rickettsia* is transmitted at a high rate whilst *Wolbachia*/*Rickettsia* double infections and *Wolbachia* were only found in a small number of eggs.

When looking at the infection rates of the pooled UK populations, *Wolbachia*/*Rickettsia* double infections were only at a frequency of 10%. This could mean that transmission of double infections is not always as effective as in LT_MUSW17/2092 or individuals carrying this double infection are rarer for other reasons.

The triple infection of LT_MUSW17/2092 was not transmitted to any of her eggs. Frequency of triple infections was low with only 4% of Swedish spiders showing this infection pattern. Occasionally females must transmit these triple infections to their offspring, otherwise triple infections would not persist in the population. It is possible, however, that these are the result of de novo infection via horizontal transmission.

4.3.6 *Wolbachia* phylogeny

In both *Linyphia* species, different strains of the *Wolbachia* endosymbiont, namely LH1, LH2 and LH3 in *L. hortensis* and LT1 and LT2 in *L. triangularis*, were found. LT1, found in individuals from Germany, the UK and Sweden, was the most different from the other strains. Comparing LT1 and LT2, 76 nucleotide differences were found which resulted in 30 differences in the amino acid sequence.

The different *Wolbachia* types found in *L. hortensis* also showed differences in the nucleotide and amino acid sequences. LH1 and LH2 were polymorphic for 23 nucleotide positions which translated into differences in seven amino acids. The same number of nucleotide and amino acid differences found

between LH1 and LH3 suggested that LH2 and LH3 were likely to be similar. Inspecting the nucleotide and amino acid sequences of LH2 and LH3 only two nucleotide polymorphisms were found which resulted in one difference in the amino acid sequence.

LH1, which was found in *L. hortensis* from Germany, Denmark and the UK, clustered with LT2 found in Swedish *L. triangularis*. These two strains seem to be closely related to the *Wolbachia* found in linyphiids, *Oedothorax retusus*, collected in Belgium (Fig. 33). The very different LT1 strain groups with *Wolbachia* found in Australian *Badumna longinqua*, a spider belonging to the Desidae, although, unlike other species of this group, it does not live in intertidal areas but in urban areas and agroecosystems.

LH2 and LH3 clustered with *Wolbachia* strains found *Araneaus diadematus* from the UK and *Pityohyphantes phrygianus* from Sweden. Given that LH2 was only present in *L. hortensis* from the UK and LH3 was only found in *L. hortensis* from Sweden the positioning of these strains in the phylogeny seemed adequate. The presented phylogeny shows that all *Wolbachia* strains found in *L. hortensis* and *L. triangularis* did cluster with strains belonging to the *Wolbachia* supergroup B (Fig. 33). Many *Wolbachia* found to infect spiders are known to fall into this group. The fact that the *Wolbachia* identified do not seem to co-locate with the host could indicate that the endosymbiont invaded these linyphiid species multiple times via horizontal transmission.

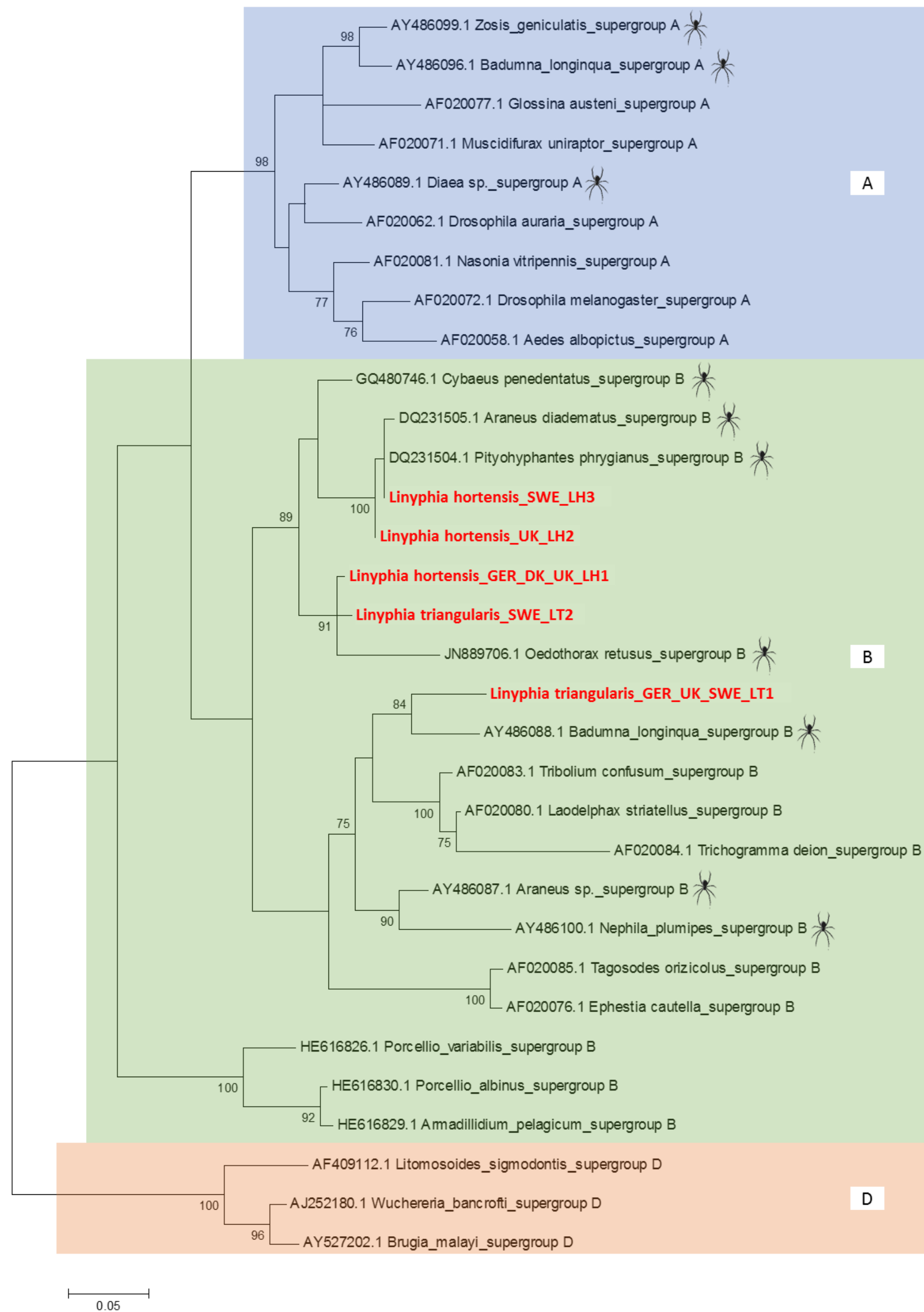

Figure 33: Phylogenetic tree of *Wolbachia* based on the outer *Wolbachia* surface protein (WSP). The alignment consisted of 559 base pairs with all sites being used. The tree was generated using the Maximum Likelihood method (log likelihood: -4724.3076, bootstrap = 100) based on the Tamura-Nei model (Tamura and Nei, 1993). Sequencing alignment and tree generation was conducted in MEGA6 (Tamura *et al.*, 2013). The spider icon indicates *Wolbachia* strains isolated from spider hosts.

4.3.7 *Spiroplasma* phylogeny

Due to the low occurrence of *Spiroplasma* infections in *L. hortensis*, no sequences suitable for phylogeny construction were available. Therefore, the

phylogeny presented here only includes *Spiroplasma* sequences retrieved from *L. triangularis*. Two different strains of *Spiroplasma* were identified in *L. triangularis*, namely SPIR1 and SPIR2. Both strains were found in German and Swedish spiders.

The constructed phylogeny (Fig. 34) showed that SPIR2 is very similar to the *Spiroplasma* found in *Gonatium rubellum*, a linyphiid spider, collected in the UK. SPIR1 was only different in three nucleotide positions. Interestingly, both strains showed heterozygote nucleotide positions, four in SPIR1 and three in SPIR2. They were found at positions 112 (A/G), 173 (T/C), 237 (T/C) and 280 (A/T). Position 280 was only heterozygote in SPIR1, in SPIR2 A and T were found in serial order at positions 280 and 281 (Fig. 35). It is not possible to establish which nucleotide 'belongs' at the heterozygote position. Therefore, it is uncertain if really two strains of *Spiroplasma* exist in *L. triangularis*.

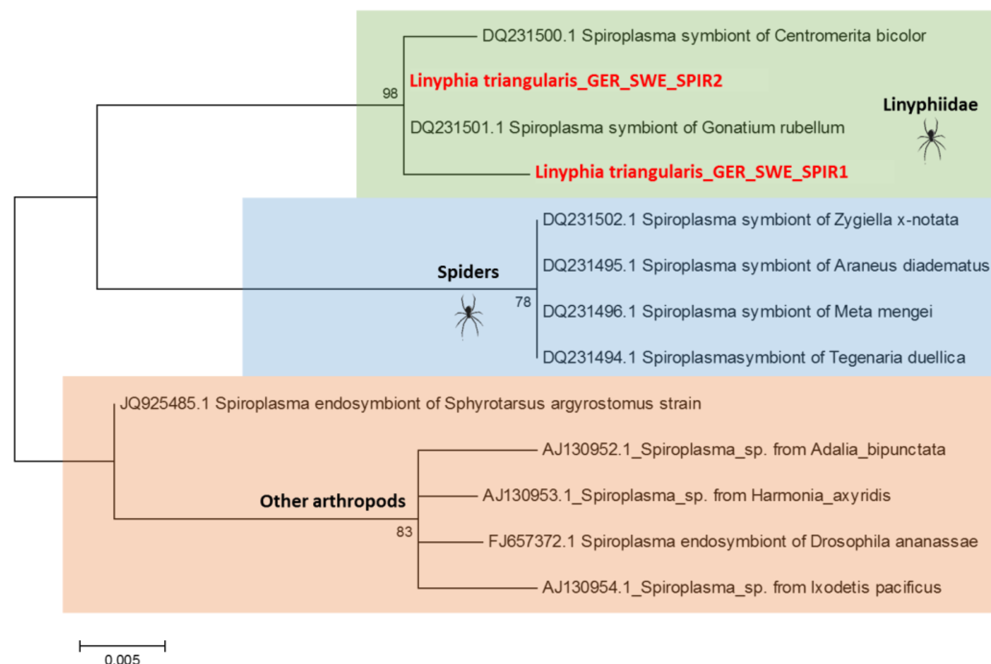

Figure 34: Phylogenetic tree of *Spiroplasma* based on the 16S-23S ribosomal RNA intergenic spacer. The alignment consisted of 291 base pairs with all sites being used. The tree was generated using the Maximum Likelihood method (log likelihood: -562.1337, bootstrap = 100) based on the Tamura-Nei model (Tamura and Nei, 1993). Sequencing alignment and tree generation was conducted in MEGA6 (Tamura *et al.*, 2013). The spider icon indicates *Spiroplasma* strains isolated from spider hosts.

Figure 35: Nucleotide sequences (position 185 – 291) of SPIR1, SPIR2 and the reference sequences of other spiders and arthropods. Heterozygote positions 237 and 280 are highlighted. In position 280 and 281 the differences between SPIR1 And SPIR2 are visible.

4.3.8 *Rickettsia* phylogeny

Partial sequences were obtained for the *Rickettsia* endosymbiont from *L. hortensis* and *L. triangularis*. 114bp could be successfully aligned to reference sequences from other arthropods, including spiders. In both *Linyphia* species two different strains of *Rickettsia*, namely R1 and R2, were present. Within the 114bp long region six nucleotide polymorphisms were found that defined these two strains, but none of the changes caused differences in amino acid sequence. Even though both strains seem to infect *L. hortensis* and *L. triangularis*, strain R1 could only be found in *L. hortensis* from the UK whilst it was detected in *L. triangularis* from all three locations. Strain R2 was only present in German and Swedish *L. hortensis* and German and British *L. triangularis* (Fig. 36).

Looking at the available larger fragments (for R1 and R2 in *L. triangularis* and R1 in *L. hortensis*) it was possible to find additional 39 polymorphisms between the two strains. These accounted for nine changes in the amino acid sequence. It is not clear, whether the shorter sequences would show the same polymorphisms but assuming this is the case it is possible that the two *Rickettsia* strains are significantly different from one another and could potentially have different effects on their hosts.

The *Rickettsia* strains clustering with R1 were isolated from linyphiids collected in England, Sweden and Belgium whilst the arthropods belonging to the Coleoptera and Diptera were collected in Germany, Switzerland, Belgium, France and Spain. R1 seems to be widely distributed in arthropods throughout Europe. The same Europe-wide distribution pattern could be observed for strain R2 which was found in linyphiids from England and other arthropods from Switzerland, Belgium, France and Austria.

Strain R1 clustered with *Rickettsia* found in other spiders, including linyphiids (Fig. 36). It also seemed to be closely related to *Rickettsia* found in fly species belonging to the Diptera and beetles from the order Coleoptera. *Rickettsia*

strain R2 clustered also with strains found in Linyphiidae but more species belonging to the Diptera fell into this group (Fig. 36). *Rickettsia* strain R1 appeared to be more common in linyphiids than *Rickettsia* strain R2.

Figure 36: Phylogenetic tree of *Rickettsia* based on the citrate synthase gene. The alignment consisted initially of 392 base pairs but due to sequence quality all missing sites were deleted completely, leaving a 102bp long alignment. The tree was generated using the Maximum Likelihood method (loglikelihood: -207.7676, bootstrap = 100) based on the Tamura-Nei model (Tamura and Nei, 1993). Sequencing alignment and tree generation was conducted in MEGA6 (Tamura *et al.*, 2013). Green = Araneae, orange = Diptera, blue = Coleoptera. The spider icon indicates *Rickettsia* strains isolated from spider hosts.

4.3.9 Bacteriophage WO

All *L. hortensis* and *L. triangularis* individuals which tested positive for *Wolbachia* were also screened for the presence of the WO phage. Out of 86 tested spiders, 63 carried the phage (*L. hortensis*: WO^+ = 46 = 82%, WO^- = 10 = 18%; *L. triangularis*: WO^+ = 17 = 57%, WO^- = 13 = 43%). The frequency of WO phage differed significantly between the two species (2-sample test for equality of proportions, Chi^2 = 5.2365, df = 1, p-value = 0.02212).

In neither species did males nor females differ in WO phage frequency (*L. hortensis*: males vs females = 100% vs 81%, 2-sample test for equality of proportions, Chi^2 = 0.084281, df = 1, p-value = 0.7716; *L. triangularis*: males vs females = 50% vs 57%, 2-sample test for equality of proportions, Chi^2 = 2.6779e-32, df = 1, p-value = 1).

There was no difference in the proportion of individuals carrying the WO phage between the countries of origin in *L. hortensis* (4-sample test for equality of proportions, Chi^2 = 1.2879, df = 3, p-value = 0.732; UK: N = , WO^+ = 34 = 79%; GER: N = , WO^+ = 10 = 91%; DK: N = 1, WO^+ = 1 = 100%; SWE: N = 1, WO^+ = 1 = 100%). The same was true when comparing the WO phage frequencies of the different countries of origin in *L. triangularis* (3-sample test for equality of proportions, Chi^2 = 0.27149, df = 2, p-value = 0.8731; UK: N = 10, WO^+ = 5 = 50%; GER: N = 5, WO^+ = 3 = 60%; SWE: N = 15, WO^+ = 9 = 60%). In both species all populations from all sample locations showed at least some individuals which did harbour *Wolbachia* infected with the WO phage. Disentangling a potential connection between the WO phage and sex ratio distortion was therefore not possible at this point.

A 101bp alignment was created for WO phage sequences retrieved from *L. hortensis* and *L. triangularis* which would translate into amino acids. Two WO phage strains were found in *L. hortensis* and three in *L. triangularis* (*L. hortensis*: WOLh1, WOLh2; *L. triangularis*: WOLt1, WOLt2, WOLt3). Due to the short length of the sequences, the phylogenetic analysis was limited.

Nevertheless, suitable reference sequences were retrieved from GenBank and a phylogeny was created (Fig. 37).

The phylogeny presented shows that all identified WO phage strains clustered with WO phage isolated from *Wolbachia* in other linyphiids. The phage found in *N. senegalensis* was included into this tree as well and seemed to be quite different from those of other spider and arthropod hosts.

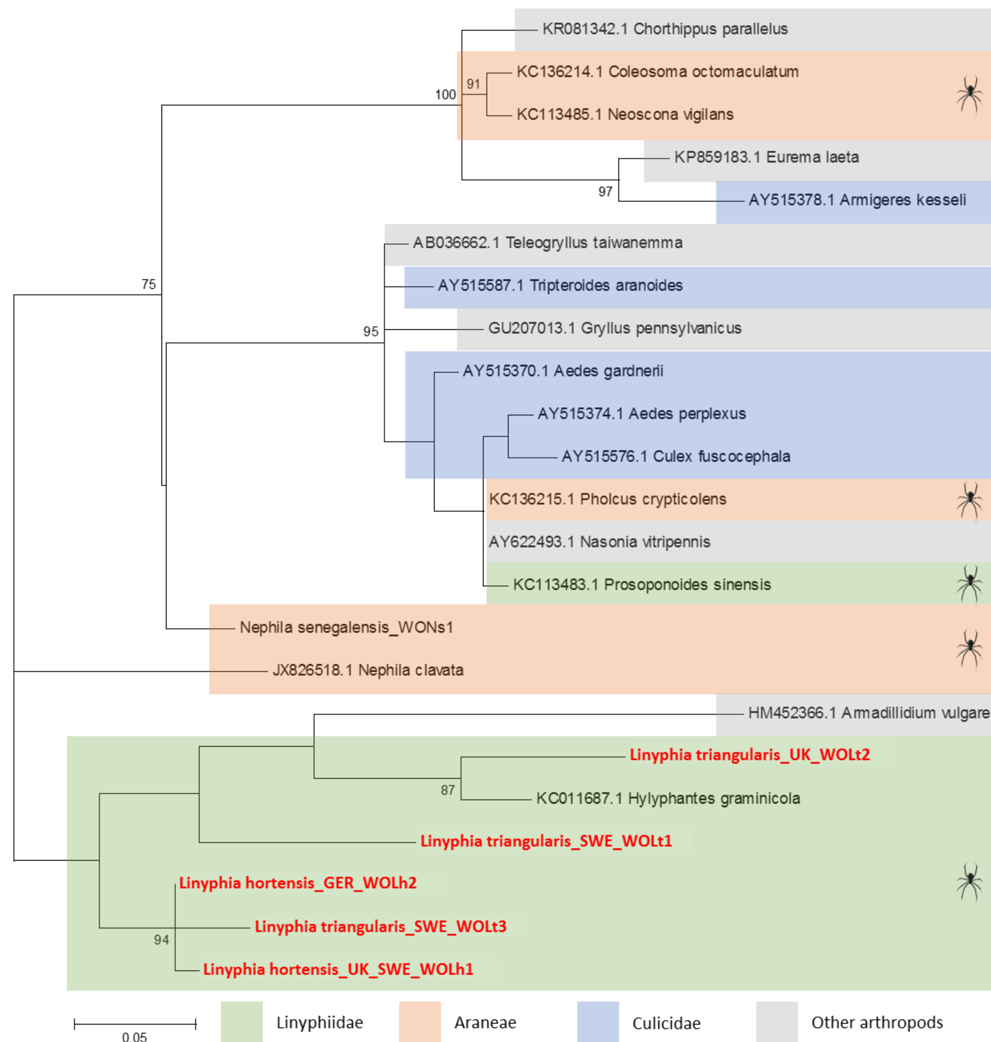

Figure 37: Phylogenetic tree of WO phage based on the minor capsid gene orf7. Due to mediocre sequence quality, all positions with less than 98% site coverage were eliminated. That is, fewer than 2% alignment gaps, missing data, and ambiguous bases were allowed at any position. The final alignment included 101bp. The tree was generated using the Maximum Likelihood method (loglikelihood: -755.1915, bootstrap = 100) based on the Tamura-Nei model (Tamura and Nei, 1993). Sequencing alignment and tree generation was conducted in MEGA6 (Tamura *et al.*, 2013). The spider icon indicates WO phage strains isolated from spider hosts.

The relationship between the *Wolbachia* and the WO phage found in *L. hortensis* was difficult to decipher. WOLh1 was found in Swedish and British spiders. The British spiders were very likely to carry *Wolbachia* strain LH1 whilst the Swedish spiders definitely were infected with LH3 since none of the Swedish *L. hortensis* showed an infection with LH1 or LH2. Therefore, it follows that LH1 and LH3 share the same WO phage, namely WOLh1. WO phage strain WOLh2 was only found in German spiders. All of them carried *Wolbachia* strain LH1, hence it seemed as if LH1 *Wolbachia* could be infected by both WO phage strains. The potential *Wolbachia*/WO phage pattern is presented in Figure 38.

Unfortunately, the WO phage sequencing of *L. triangularis* failed to produce usable sequences in 50% of the cases. Hence, only WO phage sequences from Swedish and British spiders were available for analysis. Three different WO phage strains were found, namely WOLt1, WOLt2 and WOLt3. *Wolbachia* strain LT1 which was found in German, British and Swedish spiders was carrying the WO phage WOLt1. This phage could be identified in Swedish *Wolbachia* infected individuals only. WOLt2 was isolated from British *Wolbachia* infected spiders. Since these spiders only carried *Wolbachia* strain LT1, it can be assumed that LT1 is infected by WO phage WOLt1 and WOLt2. Phage WOLt3 was only found in Swedish spiders and these spiders were the only ones which carried *Wolbachia* strain LT2. As mentioned before, it is not clear at this point which WO phage strains were present in German *L. triangularis* (Fig. 39).

The quality of the data for the analyses of the WO phage and its relationship with the different *Wolbachia* strains identified was not sufficient to draw definite conclusions. Not enough sequences of acceptable quality were retrieved for either the *Wolbachia* or the phage. Therefore, the WO phage phylogeny presented here, as well as the proposed relationship between the different strains of *Wolbachia* and WO phage, have to be considered with care.

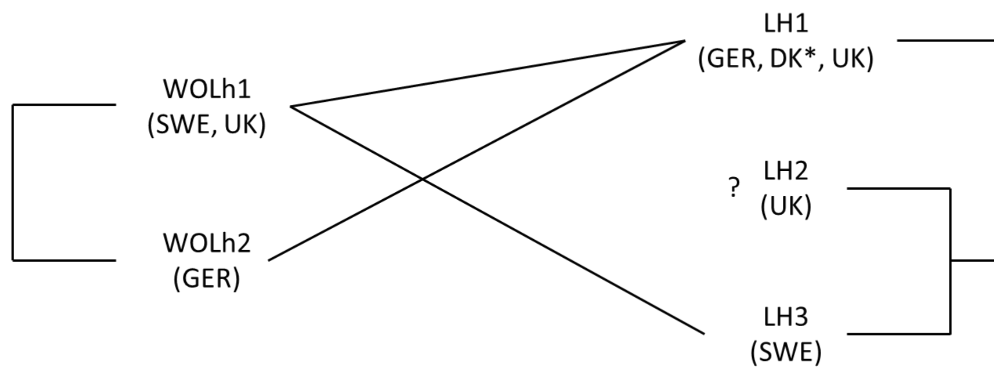

Figure 38: Relationship between the found WO phage strains and the *L. hortensis Wolbachia* they were isolated from. The ? means that the relation between the WO phage and the *Wolbachia* strain is not clear. An * indicates that no WO phage was found in *Wolbachia* infected individuals in this population.

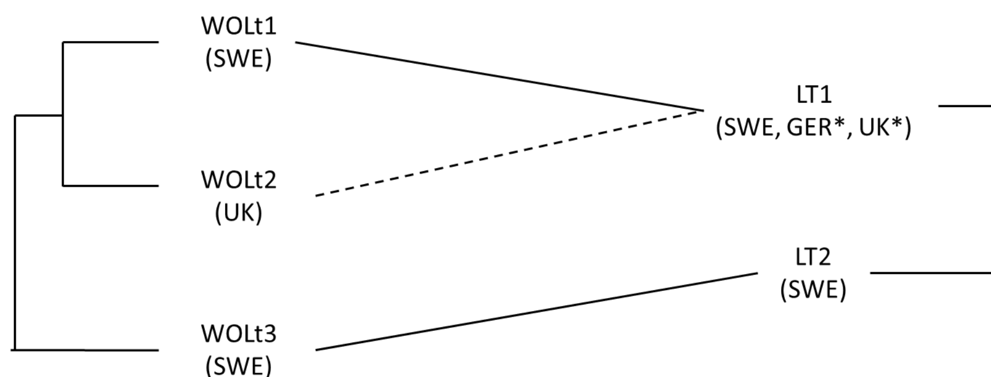

Figure 39: Relationship between the found WO phage strains and the *L. triangularis Wolbachia* they were isolated from. The dashed line shows an uncertain relation. An * indicates that no WO phage was found in *Wolbachia* infected individuals in this population.

4.4 Discussion

All three endosymbionts were found in both *Linyphia* species. Still, the infection patterns seemed to differ between these closely related species. *Wolbachia* was the most abundant endosymbiont in *L. hortensis* whilst in *L. triangularis* predominantly *Rickettsia* was found. In both species the other two endosymbionts were only present at low frequencies. It appears as if the endosymbionts are in some form of competition with each other, with one type becoming the dominant bacterial species. This would be in line with competitive exclusion principle, which states that two species competing for the same limiting resource cannot coexist. The species with even the slightest advantage will eventually replace the other (Hardin, 1960). Multiple infections

were very rare in *L. hortensis*. More of these, especially *Wolbachia*/*Rickettsia* double infections, were found in *L. triangularis*. A few *L. triangularis* individuals were found to be infected with all three endosymbionts at once.

In *L. hortensis* no inter-population differences in *Wolbachia* infection rates were found. This contrasts with *L. triangularis* where populations from the different countries of origin seemed to differ in endosymbiont infection rates. Nevertheless, no obvious infection pattern in relation to country of origin was present. Infection frequencies did differ between the years, but this was not always true for the whole dataset. In *L. hortensis Wolbachia* frequency declined significantly from 2016 to 2017. In *L. triangularis* the double infection *Wolbachia*/*Rickettsia* was more abundant in 2017 than in 2016. Again, no overall pattern in infection frequencies regarding the year of collection was apparent.

Wolbachia infections were significantly more abundant in female *L. hortensis* than *Rickettsia* or *Spiroplasma*. The only single *Spiroplasma* infection was found in a male. Due to the low infection rate of *Spiroplasma* it is unclear whether females do carry the infections or if indeed only males are infected by this endosymbiont. Males and females were equally infected with *Wolbachia* and *Rickettsia* which makes it unlikely that male-killing induced by either of them is the cause for the observed sex ratio skew. However, male-killing cannot be excluded completely at this stage since it is possible that infected males are the result of host suppression (Hornett *et al.*, 2006). Maybe infected males are those males which were superior in some shape or form to their brothers and were therefore able to evade being killed off. Feminisation of genetic males can also not be ruled out entirely, but infected males did not show any particular female traits. One *L. hortensis* individual could not be unambiguously identified as male or female. This immature individual showed male (bulbs on the pedipalps) and female (size and shape of abdomen) characteristics. However, it could not be confirmed if this individual was an

intersex. The individual was screened for *Wolbachia*, *Rickettsia* and *Spiroplasma*, but tested negative for all of them.
In all *L. hortensis* populations at least some individuals infected with *Wolbachia* were found. Few of the populations did carry only *Wolbachia*, most also had individuals infected with *Rickettsia*, *Spiroplasma* or even multiple infections. This made it difficult to establish if the sex ratio distortion could be induced by any of the endosymbionts alone. The fact that the sex ratio of populations carrying only *Wolbachia* was almost significantly more skewed than the sex ratio of populations carrying all three endosymbionts could be a hint that the effect of *Wolbachia* is counteracted by the other two endosymbionts. *Spiroplasma* was only found in the populations carrying all three endosymbionts. *Rickettsia* in combination with only *Wolbachia* did not seem to influence the sex ratio.

No apparent connection between the endosymbionts and the observed sex ratio distortion was found in *L. triangularis* either. *Rickettsia* were found at equal frequencies in males and females and therefore are probably not responsible for the sex ratio distortion. *Wolbachia* alone were only found in females but the frequency was too low to and the sample size too small be sure that males are never infected. Also, the observed sex ratio bias is too strong given the low *Wolbachia* frequencies. The *Wolbachia*/*Rickettsia* double infection was also found in males and females whilst *Wolbachia*/*Spiroplasma* and *Rickettsia*/*Spiroplasma* double infections as well as the triple infections were only present in females. As in *L. hortensis* male-killing and feminisation as cause for the sex ratio distortion seem unlikely. Again, host suppression could explain why occasionally infected males are found.

To reliably establish whether one of the endosymbionts acts as a sex ratio distorter in one or both *Linyphia* species, experimental curing using antibiotics would be useful. It has already been shown that *Wolbachia*, *Rickettsia* and *Spiroplasma* are all sensitive to tetracycline (Goodacre *et al.*, 2009). A sufficient number of populations have to be sampled to find single infected

populations. Females from these populations can then be cured from their infection and rearing their offspring in the lab will show if a 1:1 sex ratio can be restored. If curing the females of their endosymbiont infection does not restore the 1:1 sex ratio, it is unlikely that the endosymbiont acts as a sex ratio distorter. In *Mermessus fradeorum* the curing of the *Rickettsia*/*Wolbachia* infection restored the 1:1 sex ratio, thereby confirming that this double infection lead to the observed sex ratio distortion (Curry *et al.*, 2015).

British and German *Rickettsia*-infected *L. hortensis* were significantly smaller than uninfected individuals. Due to the low number of males, it was impossible to establish whether this was true for both sexes. Interestingly, it seemed as if the variance in body size was higher in endosymbiont-infected individuals. This was found for *Wolbachia*- as well for *Rickettsia*-infected spiders. *Wolbachia*-infected males and females did not differ significantly from their uninfected counterparts but in both sexes the size variance was significantly higher in infected individuals.

The benefit for the endosymbiont to "create" a greater variance in spider body size remains unknown. It is possible that males of different size classes follow different mating strategies. It has been found in *L. triangularis* that, in aggressive male – male encounters, usually the larger male is victorious (Rovner, 1968). Nevertheless, small males have been found, after being chased away, to interfere in the mating of the larger male with the female (Toft, 1989). If a smaller male manages to interrupt the first male's copulation with the female before the first sperm droplet is completely transferred, he might mate with her and sire the majority of her offspring by inducing second-male precedence (Weldingh *et al.*, 2011). Potentially, smaller males adopt a sneaker male strategy. Such a male mating strategy has been found in side-blotched lizards *Uta stansburiana*, where the males of the yellow morph are not territorial and less aggressive than the other two morphs but are able to sneak matings with females from the other males (Sinervo and Lively, 1996).

In *L. triangularis* the only significant size difference was found between uninfected and triple-infected spiders, with triple-infected individuals (all female) being significantly smaller. This could indicate that carrying three endosymbionts does have an influence on overall body condition. In the pea aphid *Acyrthosiphon pisum,* females carrying the endosymbionts *Serratia symbiotica* and *Hamiltonella defensa* showed a significantly reduced fecundity and a smaller adult weight compared to uninfected individuals (Oliver *et al.*, 2006). For females a reduced body size could mean a lower fecundity than uninfected females (Skow and Jakob, 2003) whilst smaller males might have a disadvantage in male – male encounters (Rovner, 1968; Toft, 1989).

Looking at regional differences, it was found that Swedish spiders double-infected with *Wolbachia* and *Rickettsia* were significantly smaller than uninfected or *Rickettsia*-infected individuals. Cephalothorax width did not vary significantly between the differently infected individuals, apart from a significant difference which was found between Swedish and British *Rickettsia*-carrying individuals.

Infection status had no influence on male size. No differences in cephalothorax width between uninfected or endosymbiont-carrying males has been found. Uninfected individuals from both sexes did not differ significantly in size which is interesting given the fact that *L. triangularis* is known to show a sexual size dimorphism (SSD) with males being the larger sex (Lang, 2001). In *Rickettsia* infected individuals the SSD was present whilst it was not found in *Spiroplasma* infected spiders. Another study (Funke and Huber, 2005) did not find an SSD and described males and females as being of the same size. The authors did note though that a potential sampling bias had been induced, since they only collected co-habiting pairs and the females might not have been virgin. Larger males could have been missed since they would be on the search for the more valuable virgin females (Funke and Huber, 2005).

It seems, even though the intra-sex comparisons between the differently infected males and females showed no significant difference, that *Rickettsia*-infected males were slightly bigger than the average whilst *Rickettsia*-carrying females were of average size. These larger males would have an advantage in the competition for females (Rovner, 1968; Toft, 1989). It is unclear how *Rickettsia* would benefit from being in a superior male, since the males cannot pass the infection on to the next generation. However, if *Rickettsia*-infected males win more competitions, therefore mate with more females, they could potentially induce CI in uninfected females which would benefit their infected sisters.

So far *Wolbachia* and *Cardinium* have been shown to cause CI (Goodacre and Martin, 2012), it is unknown whether *Rickettsia* strains could also induce this phenotype. In *Erigone atra* it has been found that *Rickettsia* infected individuals showed a reduced dispersal tendency than uninfected individuals (Goodacre *et al.*, 2009). *L. triangularis* do not seem to engage in ballooning, therefore dispersal is already limited. If *Rickettsia* lowers this dispersal tendency even further it is possible that infected males would stay closer to infected sisters and could therefore successfully reproduce with them, thereby avoiding possible CI and promote the spread of the endosymbiont in the population.

Egg sacs of *L. hortensis* and *L. triangularis* were retrieved from both years, but only the *L. hortensis* egg sacs hatched and therefore provided information about hatching success and offspring numbers. Endosymbiont infection did not have an influence on the hatching success of egg sacs in *L. hortensis* in 2016 or 2017. It was found that the number of offspring emerging from hatched egg sacs was significantly lower than egg number in unhatched egg sacs. This is not surprising since hatching failure of individual eggs as well as sibling cannibalism in the egg could reduce the number of emerging offspring compared to the number of eggs produced. The most striking result was that,

comparing the offspring number of hatched uninfected egg sacs with that of *Wolbachia* infected egg sacs, there was a significant difference. *Wolbachia* infected females had one third fewer offspring emerging from their egg sacs. It seems this is the cost for being infected. It is possible that these reduced offspring numbers indicate a weak form of CI or even male-killing. If male-killing is at play, an endosymbiont infected female can have up to 50% less offspring than an uninfected female (Jiggins *et al.*, 1998). It is nevertheless possible that a small number of male somehow avoid being killed off, potentially through host suppression (Martin and Gage, 2007). This would explain why a sex ratio distortion is observed in *L. hortensis* but still a few infected males were found.

Multiple strains were found for all endosymbionts in the two *Linyphia* species. *Spiroplasma* sequences could only be retrieved for *L. triangularis,* since infection rates with this endosymbiont were very low in *L. hortensis*. Two strains (SPIR 1, SPIR2) were found which clustered closely with *Spiroplasma* found in other linyphiids. No amino acid differences were found between the strains. Three heterozygote nucleotide positions were shared by both strains whilst one position was only showing heterozygosity in SPIR1.

Five different *Wolbachia* strains were identified, three of those where isolated from *L. hortensis* (LH1, LH2 and LH3) and the other two from *L. triangularis* (LT1 and LT2). All five strains belong to the *Wolbachia* supergroup B in which, together with supergroup A, most strains isolated from spiders are found (Rowley *et al.*, 2004). Strains LH2 and LH3 were very similar to one another and clustered with other strains found in spiders, being especially close to other linyphiid *Wolbachia*. LH1 was different to the other two strains found in *L. hortensis* with differences in the amino acid sequence which could be a sign that these strains could potentially persist differently in their host (Jiggins *et al.*, 2002; Jiggins *et al.*, 2001). The strains LT1 and LT2, isolated from *L. triangularis*, did also show differences in the amino acid sequence. Overall LT1

was quite different from the other four strains, clustering with strains found in insect and other non-linyphiid spiders.

Strains LH1 and LT2 were closely related to a *Wolbachia* found in the linyphiid *Oedothorax retusus*. This *Wolbachia* in combination with a *Rickettsia* infection is thought to be the cause for a distorted sex ratio in this spider (Vanthournout *et al.*, 2014). Unfortunately, it is not known if *Wolbachia/Rickettsia* infected *L. hortensis* or *L. triangularis* do produce skewed offspring sex ratio clutches. The transmission rate of this double infection seems to be quite high but since this assumption is only based on one observation it is unclear whether this is a reliable result. The strains LH2 and LH3 cluster with a *Wolbachia* found in *Pityohyphantes phrygianus* (Goodacre *et al.*, 2006). In a separate study it was indicated that *Wolbachia* has an influence on the post-copulatory abdominal position of female *P. phrygianus* which influences the sex ratio of their offspring (Gunnarsson *et al.*, 2009). However, the study did not present any information on the *Wolbachia* strain these females carried. It is therefore not clear if it is the same strain which clusters with LH2 and LH3.

Two *Rickettsia* strains (R1 and R2) were identified which were shared by *L. hortensis* and *L. triangularis*. To create a phylogeny, only a short fragment was chosen and there were no amino acid differences found between the strains. When looking at a couple of larger fragments it was found that the two strains differed in their amino acid sequence. Even though it is not clear whether the shorter fragments would show the same differences, it is very likely that the two strains are considerably different from one another. As for the *Wolbachia* strains this could mean that the different strains have different effects on their hosts. R1 was found to cluster with more *Rickettsia* strains found in spiders than R2.

Strain R1 fell into the same group of *Rickettsia* found in *O. gibbosus* and *O. retusus*. It is known that a double infection of *Rickettsia* and *Wolbachia* causes a sex ratio distortion in *O. retusus*. In *O. gibbosus* the *Rickettsia* infection is

fixed, and it is therefore unlikely that this endosymbiont is responsible for the sex ratio skew observed in this spider. Instead, it seems to be an infection with *Wolbachia* distorting the sex ratio. This is a good example how different strains of the same endosymbiont can have different effects on closely related hosts. *Rickettsia* strain R2 clustered with strains, some of which were isolated from spiders, but most of which were found in fly species belonging to the Diptera. No information exists about the effect these *Rickettsia* have on their host. Most of these strains were identified in one large scale study investigating endosymbiont infections in the true fly superfamily Empidoidae (Martin *et al.*, 2013).

There were different geographical patterns of infection in *L. hortensis* and *L. triangularis*. In *L. hortensis* strain R1 was only found in British spiders whilst it was isolated from *L. triangularis* from all three locations. R2 was restricted to Germany and Sweden in *L. hortensis* and to Germany and the UK in *L. triangularis*. It is possible that the higher frequency of *Rickettsia* in *L. triangularis* is responsible for the observed pattern. The fact that multiple strains of all three endosymbionts were identified in *L. hortensis* and *L. triangularis* opens up the possibility of a multitude of interactions in these spider species. Different strains might not only have different effects on their hosts but also their interactions might vary. At this stage it is unknown whether any of the found strains do induce one of the known endosymbiont phenotypes.

Since no egg sacs were retrieved from the few mating trials conducted, no data are available regarding the possibility that the endosymbionts identified could contribute to a reproductive isolation between individuals from the different populations. To investigate this possibility further, mating trials between individuals infected with different strains of the same endosymbiont have to be conducted. Given the fact that neither males nor females seemed to reject mating with partners from other populations, reproductive isolation

at a behavioural level seems unlikely. However, the data are not sufficient to draw a definite conclusion.

Three different WO phage strains were isolated from *L. triangularis* and two from *L. hortensis*. All five strains cluster with another one found in a linyphiid spider. The retrieved sequences did align and translated into amino acids. Nevertheless, the alignment was quite short hence it would be good to sequence longer WO phage from both species to get a better idea of the differences between the strains. It seems that one *Wolbachia* strain can carry more than one WO phage strain and one WO phage strain can infect different *Wolbachia* strains. Since the *Wolbachia* strains identified in the two *Linyphia* species are still quite close to one another, this is not too surprising.

At this stage it is not clear whether the WO phage is active in *Wolbachia* infected *Linyphia*. No pattern could be identified so far that could explain the observed sex ratio distortion. The way forward would be to investigate further whether *Wolbachia* acts as a sex ratio distorter in either or both species. Since the WO phage has been associated with CI (Bordenstein *et al.*, 2006), it would be equally important to establish whether *Wolbachia* does induce CI in this system.

In conclusion, it can be said that, even though both *Linyphia* species do harbour different endosymbiont strains, no obvious effect of these infections is visible. No evidence was produced to confirm any of the four known phenotypes induced by endosymbionts. The presence of infected males makes it unlikely that male-killing or feminisation are acting in either of the species. Nevertheless, host suppression of the endosymbiont could lead to infected males emerging, due to escaping being killed or feminised.

On the other hand, infected males could be an indicator that CI is induced by one of the three endosymbiont identified. For CI to be successful, infected males are needed because they penalise uninfected females and provide an

advantage to their infected sisters. This will lead to the spread of the endosymbiont (Turelli, 1994). What contradicts the expression of CI is that endosymbiont strains inducing this phenotype are typically found at high frequencies near fixation (Jiggins *et al.*, 2001). Additionally, the presence of high levels of CI is unlikely due to the fact that egg sacs of uninfected and endosymbiont infected *L. hortensis* did not differ in hatching success. If strong CI was at play, the mean hatching rate should be higher for egg sacs of infected than of uninfected females (Stefanini and Duron, 2012). Potentially, the discovery of the WO phage in *Wolbachia* infected individuals of both *Linyphia* species could provide an explanation as to why no high levels of CI were detected. WO phage has been found to lower the level of CI in *Nasonia vitripennis*. The more WO phage were found the lower was the *Wolbachia* titre and CI was significantly weakened (Bordenstein *et al.*, 2006).

The results retrieved from this study are not adequate to draw a definite conclusion on the effects of *Wolbachia*, the WO phage, *Rickettsia* and *Spiroplasma* on the two *Linyphia* species. However, they present a first intriguing insight into this study system which can be used as a stepping stone for future research on the role of the endosymbionts in *L. hortensis* and *L. triangularis*.

Chapter 5: Modelling the spread of bacterial endosymbionts through male mate choice and cytoplasmic incompatibility: Testing the model predictions with real-life data from spiders of the genus *Linyphia*

5.1 Introduction

Mate choice has been studied intensively in different study systems ranging from arthropods to birds, fish and mammals. Often these studies focus on female mate choice since usually females are the choosier sex. However, male mate choice has received more attention in recent years and has been the subject of numerous studies. Males from various taxa have been found to choose females *e.g.* based on body size as an indicator for high female fecundity (*Poecilia reticulata* Herdman *et al.*, 2004; *Drosophila melanogaster* Lupold *et al.*, 2011), low probability of sperm competition (*Nephila senegalensis* Schneider *et al.*, 2011; *Spermophilus tridecemlineatus* Schwagmeyer and Parker, 1990), female mating status (*Acanthoplus discoidalis* Bateman and Ferguson, 2004; *Poecilia reticulata* Guevara-Fiore *et al.*, 2009) or female age (*Colaphellus bowringi* Liu *et al.*, 2014; *Drosophila ananassae* Prathibha and Krishna, 2010).

A number of studies exist that have investigated male mate choice and the factors influencing it in spider mating systems. In *Argiope keyserlingi* it was found that males preferred females with narrower abdomens because these females are more likely to be virgin. The males thereby try to avoid potentially fatal cannibalism by the female and to obtain a higher fertilisation success (Herberstein *et al.*, 2002). In the myrmecomorphic spider *Micaria sociabilis* there is reversed sexual cannibalism, where males attack and kill females instead of copulating with them. In this spider two generations, one from the spring and one from the summer, overlap. Young males from the summer generations were found to discriminate against old females from the spring generations. These old females were significantly more often attacked and

cannibalised by the young males. The young females from the summer generation represented high-quality mates and were therefore chosen over older females, which, due to reproductive senescence, were classed as low-quality mates (Sentenska and Pekar, 2013).

Age is also an important factor influencing male mate choice in *Argiope bruennich*i. It was found that, in contrast to *M. sociabilis,* old and heavy females, which were close to oviposition, attracted more males than young and lighter females (Cory and Schneider, 2016). In *Latrodectus hesperus*, males showed a near-universal preference for virgin, well-fed females. Wild males showed the same preferences as laboratory-reared males. Virgin, well-fed females represent a lower sperm competition risk combined with a high fecundity (MacLeod and Andrade, 2014).

The issue of whether the presence of endosymbionts in a population could influence mating decisions of males or females has previously been discussed (*e.g*. Arbuthnott *et al.*, 2016; De Crespigny and Wedell, 2007; Markov *et al.*, 2009; Ming *et al.*, 2015; Vala *et al.*, 2004). In their study of the spider mite *Tetranychus urticae*, Vala *et al.* (2004) report that uninfected females prefer to mate with uninfected males compared to males carrying CI-inducing *Wolbachia*. Thereby the females avoid the incompatibility caused by the endosymbiont. The study further showed that *Wolbachia*-infected females aggregated their offspring and thereby promoted mating between siblings. This will ensure that individuals are compatible and *Wolbachia* is spread further. Females also preferred to place their offspring clutches on leaves which held clutches with the same infection status. Females seemed to be able to detect the infection status of the clutches already present on the leaves, or of the female placing them and adapt their oviposition behaviour accordingly (Vala *et al.*, 2004).

Randerson *et al.* (2000) developed a theoretical model to present a new solution to the ‘paradox of the lek’. In lekking species males form leks around

females and the females choose. Intense directional selection for certain male traits, imposed by female choice, would quickly reduce the genetic variance in this trait. This raises the question why females should continue to choose, if the genetic benefits of choice are small. This was termed the paradox of the lek (Kirkpatrick and Ryan, 1991).

In *Acraea encedon* infection with a male-killing *Wolbachia* lead to a reversal of the behaviour of males and females. Due to a shortage of males in an infected population, female *A. encedon* start lekking in large swarms and the males choose (Jiggins *et al.*, 2000b). The model by Randerson *et al.* (2000) describes how a population harbouring the male-killer could be invaded by a choice gene, which would cause males to discriminate against infected females and thereby prevent killing of their male-offspring. Three conditions were identified, which need to be fulfilled, in order for the male-killer and the choice gene to persist at a high frequency equilibrium in a population. (1) Male mistakes are common, *i.e.* males choose infected or reject uninfected females. (2) The cost of mate searching is low. (3) Male-killing imposes no costs or is beneficial to females. The model is presented as a potential solution to 'the paradox of the lek', because, due to the occasional choice errors of males, the male-killer is never driven out of the population. The benefit of choosing is not lost and the paradox does not arise (Randerson *et al.*, 2000). However, an empirical study on *A. encedon* could not provide any evidence for male mate choice. Males did not prefer uninfected over infected females (Jiggins *et al.*, 2002). It is possible, that males do not choose because they are unable detect the *Wolbachia* infection in the females.

A study of the linyphiid *Pityohyphantes phrygianus* indicates that female spiders may be able to assess a potential mating partner's infection status with respect to their own, and as a consequence adopt a post-copulatory abdominal position that will lead to weaker or stronger sex ratio distortion in the offspring clutch (Gunnarsson *et al.*, 2009). In the face of possible CI, being able to detect a mating partner's infection status would be highly beneficial

because mating with an incompatible partner could lead to complete reproductive failure. A theoretical model by De Crespigny *et al.* investigated the situation from the female's perspective (De Crespigny *et al.*, 2005). The key finding was that mate preference genes have the potential to spread through populations infected with CI-inducing *Wolbachia*. The authors further concluded that such a preference gene was most likely to evolve if the following prerequisites were fulfilled: (1) The allele creating the preference is dominant. (2) *Wolbachia* incurs fitness costs in the host in addition to CI *e.g*. reduced female fecundity. (3) *Wolbachia* is not transmitted with perfect fidelity (De Crespigny *et al.*, 2005).

The aim of this chapter was to apply real-world data to the theoretical model to investigate whether any predictions of the model are transferrable to populations from the wild. The data collected from wild populations can only provide a snapshot in time. The two *Linyphia* species and *M. fradeorum* only produce one generation per year. Following the development of endosymbiont infections *in situ* is therefore only possible to a certain extent. Running simulations with real-world data could provide information on how the population might develop.

5.1.1 Introducing the model

The model described in this chapter looks at the situation from the male's perspective. As much as for the females, it is detrimental to the male's fitness if he chooses an incompatible partner. The ability to detect and avoid differently infected and therefore potentially incompatible females would thus be highly beneficial for the male. Under such circumstances a 'choosiness' gene would be able to spread through a population. The gene would stop infected males from mating with uninfected females, which is the cross that leads to CI. The disadvantage of being a choosy male however is that, depending on the frequency of the endosymbiont in the population, more males would compete for copulation with a smaller number of females. Whether being choosy is a successful strategy is also expected to depend on

the level of CI. If incompatible crosses always lead to complete failure, the male should always avoid potentially incompatible females and should continue searching for compatible mates. As soon as CI is incomplete however, the advantage of finding a new mate is reduced by the cost of searching for this mate. In the current model, no costs, related to wasting energy, are imposed on the males, but we note that this and other factors might also be important.

It is unknown so far if males of *L. hortensis* or *L. triangularis* are choosy when it comes to mating. No studies have investigated the acceptance rate of females by males. In both *Linyphia* species females mature over a short period of time. In *L. triangularis* this timeframe is especially short with all females moulting to maturity within two weeks. A choosy male might risk remaining unmated if competition for uninfected females is high and the number of those is low.

A theoretical model has been developed by Prof John Brookfield taking these facts into consideration. The details about the designing of the model and the respective equations can all be found in his paper draft in the appendix of this thesis. The model investigates two different scenarios. In the first scenario, males will always choose the infected females over uninfected females regardless of their own infection status. This is called the unconditional model. The second scenario assumes that males choose females in respect to their own infection status. Infected males should therefore prefer the infected females whilst uninfected males should not discriminate between infected and uninfected females. This is the conditional model. Based on his model J. Brookfield designed a simulation to test the theoretical assumptions of the model. These simulations were based on the conditional model.

The simulations run on theoretical data revealed two situations in which equilibrium points were identified. For information about the choice of values refer to the appendix of this chapter (5.5 Appendix). These simulations

described the situation when the preference allele was absent from the population. In both situations, endosymbiont transmission c was high but the fertility of crosses f between infected males and uninfected females differed. When the endosymbiont transmission was c = 0.9 and the fertility of crosses f = 0.5 three equilibrium points were found for initial endosymbiont frequency W. These were at W = 0 (stable), at W = 0.307 (unstable) and at W = 0.804 (stable, Fig. 40). When the transmission frequency was lower at c = 0.8 and the fertility of crosses f = 0, complete reproductive failure, also three equilibrium points were found. Again, one at W = 0 (stable), at W = 0.3455 (unstable) and at W = 0.9045 (stable, Fig. 41). In both cases the endosymbiont frequencies move away from the unstable middle equilibrium points. For W = 0.307 the endosymbiont frequency decreased whilst for W = 0.3455 it increased. These changes in endosymbiont frequency illustrate the instability of the equilibria.

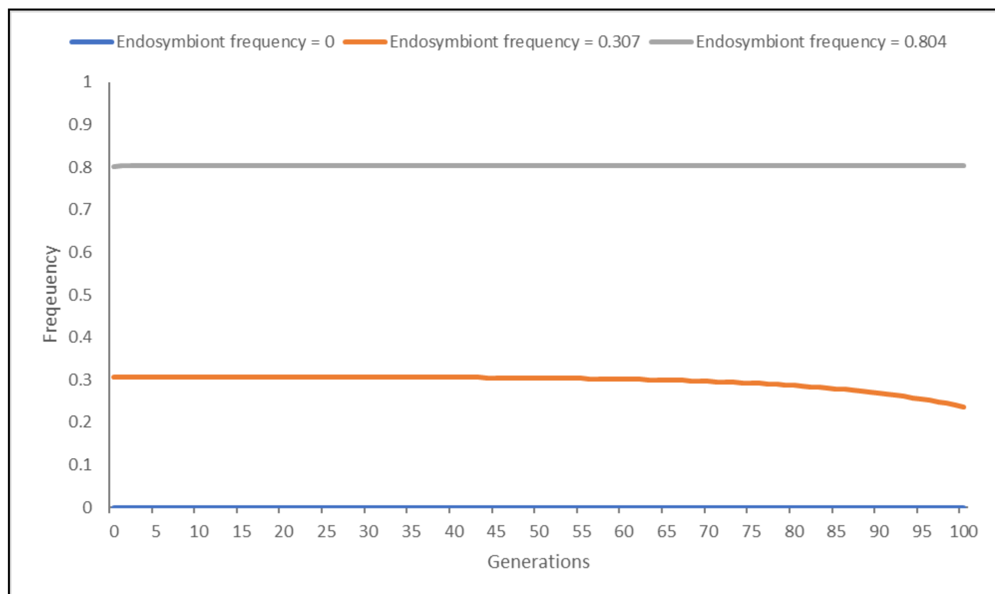

Figure 40: Progress of endosymbiont frequencies starting at the three equilibrium points for a very high transmission rate and medium fertility. Transmission rate c = 0.9 and a fertility of crosses f = 0.5 at three initial endosymbiont frequencies, W = 0 (blue, stable), 0.307 (orange, unstable) and 0.804 (grey, stable). The spread of the endosymbiont is shown for 100 generations.

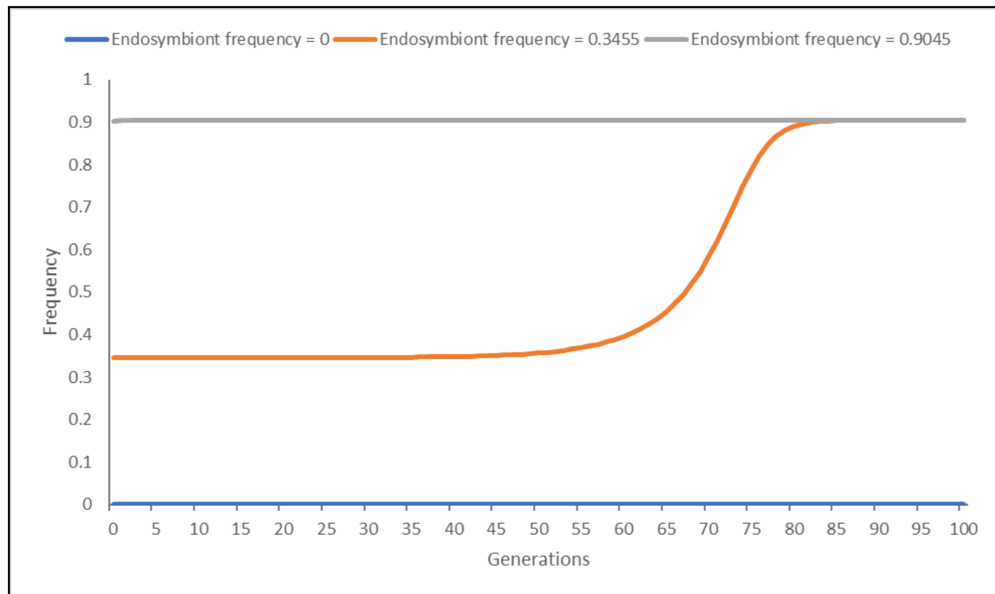

Figure 41: Progress of endosymbiont frequencies starting at the three equilibrium points for a high transmission rate and low fertility. Transmission rate c = 0.8 and a fertility of crosses f = 0 at three initial endosymbiont frequencies, W = 0 (blue, stable), 0.3455 (orange, unstable) and 0.9045 (grey, stable). The spread of the endosymbiont is shown for 100 generations.

When the choice mutation was introduced into the model a threshold for the maintenance of the mutation was identified. When c = 0.981, f = 0.5 and W = 0.6287 an initial frequency of the choice mutation M >= 0.2561 will lead to maintenance of the mutation (Fig. 42). Depending on whether the mutation is dominant or recessive the frequency reaches a frequency of 40% and 77% respectively after 500 generations. The spread of the endosymbiont was not impaired by the introduction of the choice mutation in either case. It reaches a frequency of 97% after 13 generations.

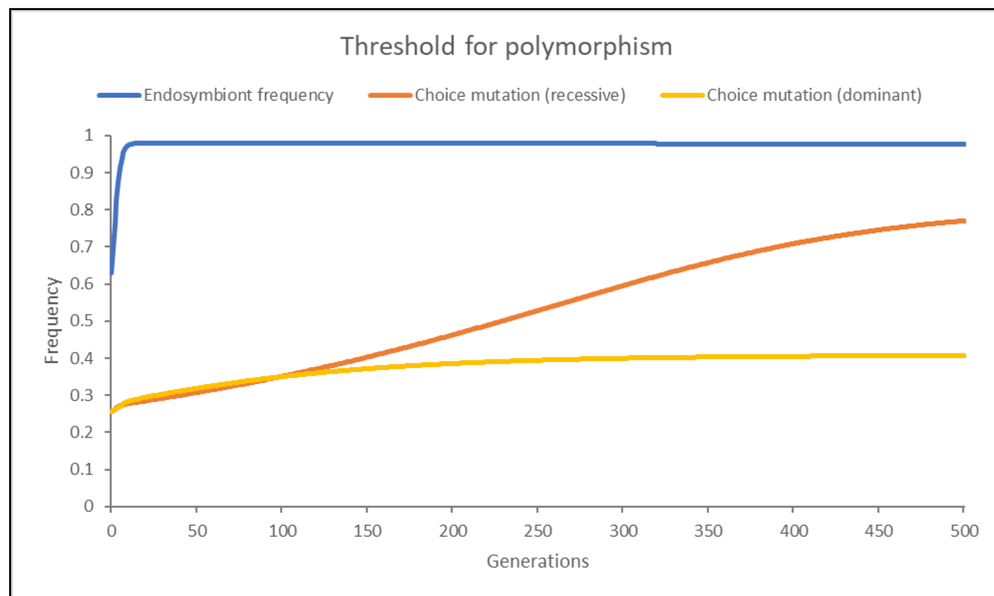

Figure 42: Polymorphism of the endosymbiont infection and the choice mutation gene. The initial values were transmission rate c = 0.981, fertility of crosses f = 0.5, endosymbiont frequency W = 0.6287 and choice mutation gene frequency M = 0.2561. The spread of the endosymbiont (blue), the recessive choice mutation (orange) and the dominant choice mutation (yellow) is shown for 500 generations.

With his permission Brookfield's simulation was used with the data from *L. hortensis* and *L. triangularis (*see Chapter 4*)*. Since it is not known whether CI or a choice mutation are present in either of the species' populations these values had to be chosen based on other studies. Data from Curry *et al*.'s study on *Mermessus fradeorum* (Curry *et al.*, 2015) was used in the simulation since it provides values for all parameters except the choice mutation. Since reliable transmission rates were only available for *L. triangularis* no extensive simulations have been run on *L. hortensis*.

5.2 Materials and methods

5.2.1 The simulation

The simulation requires the following parameters: strength of CI/offspring fidelity 0 – 1 (0 – 100%), endosymbiont starting frequency 0 - 1 (0 – 100%), endosymbiont transmission frequency 0 – 1 (0 – 100%), starting frequency of preference gene 0 -1 (0 – 100%), dominance of the preference gene (mm, Mm or MM = 0, 0.5 or 1) and strength of the preference mutation 0 - 1 (default = 1). The dominance of the preference gene describes to which extent the Mm

genotype resembles the MM genotype. If dominance is 0, Mm is identical to the mm phenotype whilst if dominance is 1, Mm is phenotypically identical to MM. The allele creating the preference is M. Males with the Mm genotype and dominance of the preference gene = 1, will show the same preference as males with the MM genotype. If the dominance of the preference gene is = 0, males with the Mm genotype will show the same preferences as males with the mm genotype.

5.2.2 The real-life data

M. fradeorum

In the study on *Wolbachia* and *Rickettsia* infections on *M. fradeorum* by Curry *et al.* (2015) two laboratory lineages were established of which one carried a *Wolbachia* (W1) and the other a *Rickettsia*/*Wolbachia* (R1W1) double infection. The values for the parameters taken from this study are listed in Table 22. Since no information was available as to whether a choice mutation is present, three different initial frequencies were chosen: 0.05, 0.1 and 0.2561 (theoretical polymorphism threshold for c = 0.981, f = 0.5, and W = 0.6287).

Table 22: Values for the simulation parameters taken from Curry *et al.*., 2015. CI = cytoplasmic incompatibility/the proportion of failed egg sacs, c = endosymbiont trans-mission rate, f = fertility/the proportion of successful egg sacs, W = endosymbiont frequency, M = choice mutation frequency, Meff = effect of choice mutation (default = 1), Mdom = dominance of choice mutation (mm, mM, MM; 0, 0.5, 1).

Endosymbiont	c	f	W	M	M_{eff}	M_{dom}
Wolbachia	0.98	0.27	0.55	0	0	0
	0.98	0.27	0.55	0.05	1	0
	0.98	0.27	0.55	0.05	1	1
	0.98	0.27	0.55	0.1	1	0
	0.98	0.27	0.55	0.1	1	1
	0.98	0.27	0.55	0.2561	1	0
	0.98	0.27	0.55	0.2561	1	1
Wolbachia/Rickettsia	1	0.65	0.39	0	0	0
	1	0.65	0.39	0.05	1	0
	1	0.65	0.39	0.05	1	1
	1	0.65	0.39	0.1	1	0
	1	0.65	0.39	0.1	1	1
	1	0.65	0.39	0.2561	1	0
	1	0.65	0.39	0.2561	1	1

L. triangularis

As already mentioned in Chapter 4, only two *L. triangularis* females, carrying endosymbionts, produced egg sacs. The UK female's egg sac contained 31 eggs of which 81% were infected with the *Wolbachia/Rickettsia* double infection and 13% tested positive for a single *Rickettsia* infection. The egg sac from the Swedish female contained 18 eggs which were mainly infected with *Rickettsia* (78%). The *Wolbachia/Rickettsia* double infection was present in 17% of the eggs. In both egg sacs *Wolbachia* alone was found in 6% of the eggs.

For the simulation the transmission rate and the infection frequency of the *Wolbachia/Rickettsia* double infection from the UK female was used. Since no information was available on CI levels or the presence of a choice mutation, values had to be chosen for these parameters. For the level of CI, the same values as in the Curry *et al.* study were used (f = 0.27 and f = 0.65). Additionally, very weak (f = 0.99) and very strong (f = 0.01) CI was simulated

(Table 23). For the choice mutation initial frequencies of 0.05, 0.1 and 0.2561 (theoretical polymorphism threshold) were chosen.

Table 23: Values for simulation parameters taken from the study of the endosymbionts in *L. triangularis* (see Chapter 4). CI = cytoplasmic incompatibility/the proportion of failed egg sacs, c = endosymbiont transmission rate, f = fertility/the proportion of successful egg sacs, W = endosymbiont frequency, M = choice mutation frequency, Meff = effect of choice mutation (default = 1), Mdom = dominance of choice mutation (mm, mM, MM; 0, 0.5, 1). a) Values taken from Curry *et al.*, 2015. b) Values chosen to represent very weak and very strong CI.

Endosymbiont	c	f	W	M	M_{eff}	M_{dom}
	0.81	0.99[b)]	0.2	0	0	0
	0.81	0.99[b)]	0.2	0.05	1	0
	0.81	0.99[b)]	0.2	0.05	1	1
	0.81	0.99[b)]	0.2	0.1	1	0
	0.81	0.99[b)]	0.2	0.1	1	1
	0.81	0.99[b)]	0.2	0.2561	1	0
	0.81	0.99[b)]	0.2	0.2561	1	1
	0.81	0.65[a)]	0.2	0.05	1	0
	0.81	0.65[a)]	0.2	0.05	1	1
	0.81	0.65[a)]	0.2	0.1	1	0
	0.81	0.65[a)]	0.2	0.1	1	1
	0.81	0.65[a)]	0.2	0.2561	1	0
Wolbachia/Rickettsia	0.81	0.65[a)]	0.2	0.2561	1	1
	0.81	0.27[a)]	0.2	0.05	1	0
	0.81	0.27[a)]	0.2	0.05	1	1
	0.81	0.27[a)]	0.2	0.1	1	0
	0.81	0.27[a)]	0.2	0.1	1	1
	0.81	0.27[a)]	0.2	0.2561	1	0
	0.81	0.27[a)]	0.2	0.2561	1	1
	0.81	0.01[b)]	0.2	0.05	1	0
	0.81	0.01[b)]	0.2	0.05	1	1
	0.81	0.01[b)]	0.2	0.1	1	0
	0.81	0.01[b)]	0.2	0.1	1	1
	0.81	0.01[b)]	0.2	0.2561	1	0
	0.81	0.01[b)]	0.2	0.2561	1	1

For both species the spread of the endosymbionts was simulated with no CI and without a choice mutation present. These simulations were run for 100 generations since *M. fradeorum* as well as both *Linyphia* species are univoltine and their life cycle lasts a year. Therefore, 100 generations in the simulation equals 100 years. For the simulations which were run at different CI levels and

with different choice mutation frequencies, the number of generations was set to 500 generations to observe long-term dynamics. Since generations would translate to years it must be considered that other factors *e.g.* host suppression and other mutations in the host and/or the endosymbiont will have occurred after all this time which has the potential to make them significantly different from today's organisms.

5.3 Results

Mermessus fradeorum

All simulations run on the single *Wolbachia* infection showed that *Wolbachia* will reach an equilibrium close to fixation (99%) in a few generations. With an almost perfect transmission rate of 98%, a fertility of 27% and a *Wolbachia* frequency of 55% the spread of the endosymbiont through the populations seems inevitable. The infection reaches equilibrium after eight generations (Fig. 43).

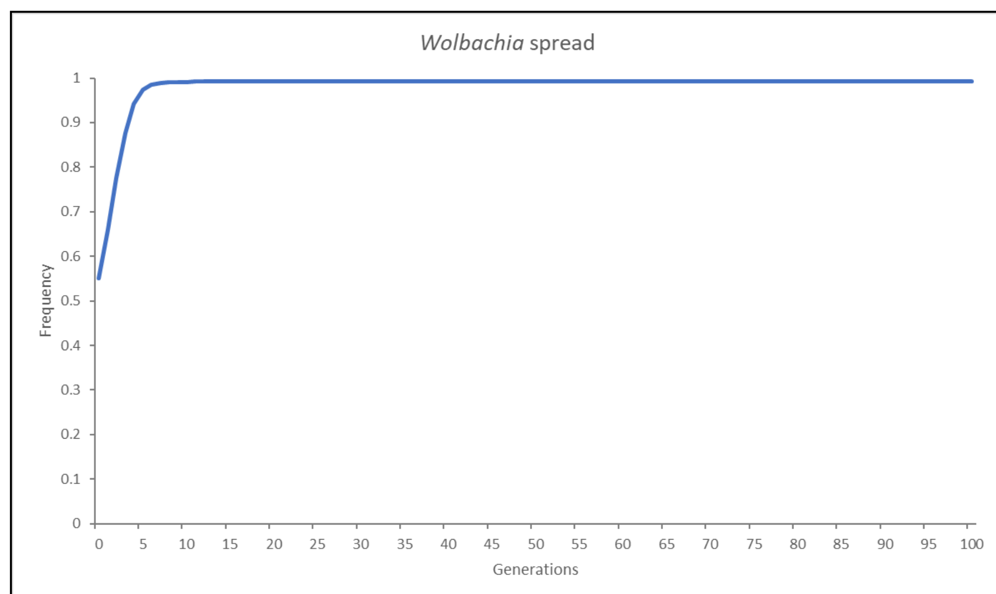

Figure 43: Spread of the *Wolbachia* infection through the *M. fradeorum* population in 100 generations. The infection reaches equilibrium in the populations after eight generations. Transmission frequency c = 0.98, CI fertility f = 0.27, initial *Wolbachia* frequency W = 0.55.

A recessive choice mutation was introduced into the unconditional model at three different initial frequencies: 0.05, 0.1 and 0.2561. In the unconditional model, males prefer infected females over uninfected females irrespective of

their own infection status. 0.2561 represents the threshold for the maintenance of the mutation found by Brookfield. The simulations were run for 500 generations. In each case the choice mutation was still present after 500 generation but only for the threshold frequency was a considerable spread of the mutation observed. It reached a frequency of 0.84. Only a slight increase in frequency was detected for the other two initial frequencies. The slow spread of the mutation was due to the fact that only males which are homozygous for the preference allele show a preference phenotype. Selection for the preference was also weak since the preference is only visible in the avoidance of uninfected females and the frequency of uninfected females is low (< 1%). *Wolbachia* spread to fixation in eight generations (Fig. 44a).

The situation was different when a dominant choice mutation was introduced, at the same three initial frequencies as before, into the unconditional model. For all three initial frequencies the choice mutation spread through the population in the course of 500 generations to reach frequencies of 0.54, 0.56 and 0.57 (Fig. 44b). As in the recessive, unconditional model the *Wolbachia* infection spread to fixation in eight generations.

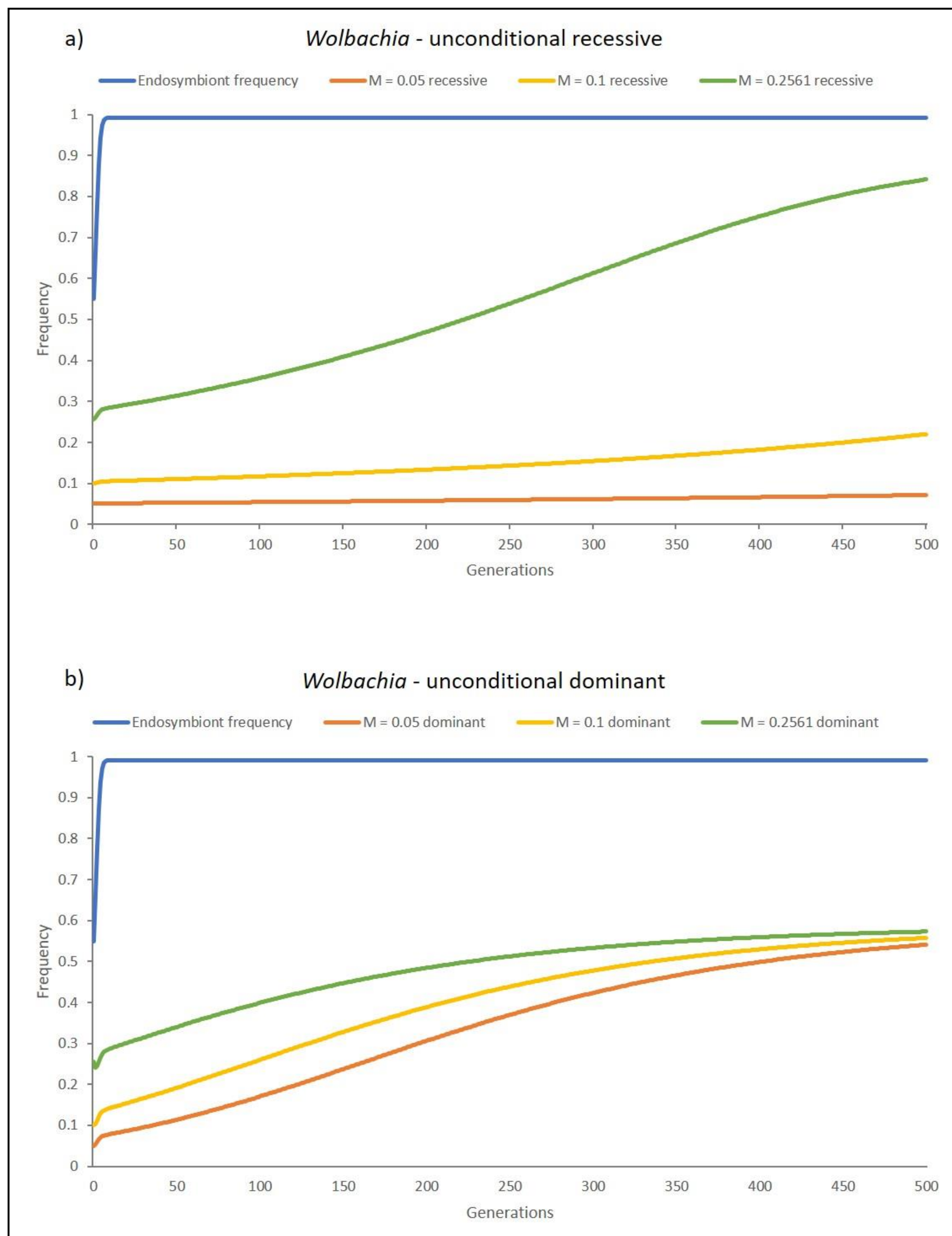

Figure 44: a) Spread of a choice mutation in the unconditional recessive model. The spread is shown for three different initial mutation frequencies. Orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561. Transmission frequency c = 0.98, CI fertility f = 0.27, initial *Wolbachia* frequency W = 0.55. Blue = *Wolbachia* frequency. b) Spread of a choice mutation in the unconditional dominant model. The spread is shown for three different initial mutation frequencies. Orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561. Transmission frequency c = 0.98, CI fertility f = 0.27, initial *Wolbachia* frequency W = 0.55. Blue = *Wolbachia* frequency.

In the conditional model males choose females dependent upon their own infection status, consequently infected males will prefer infected females whilst uninfected males should not discriminate between infected and uninfected females. The choice mutation was introduced into the recessive

conditional model at the same initial frequencies as in the unconditional model: 0.05, 0.1 and 0.2561. The spread of the choice mutation in this model was equal to the spread in the unconditional recessive model. Only for the threshold initial frequency did the choice mutation spread through the population to reach a frequency of 0.85 after 500 generations. The *Wolbachia* infection invaded the population as fast as in the unconditional model and again reached fixation after eight generations (Fig. 45a). When the choice mutation was introduced as dominant at the three initial frequencies, the spread was equal to that observed in the unconditional model. In all three cases the choice mutation did spread to frequencies between 0.55 and 0.58 over 500 generations (Fig. 45b). *Wolbachia* became fixed in the population after eight generations.

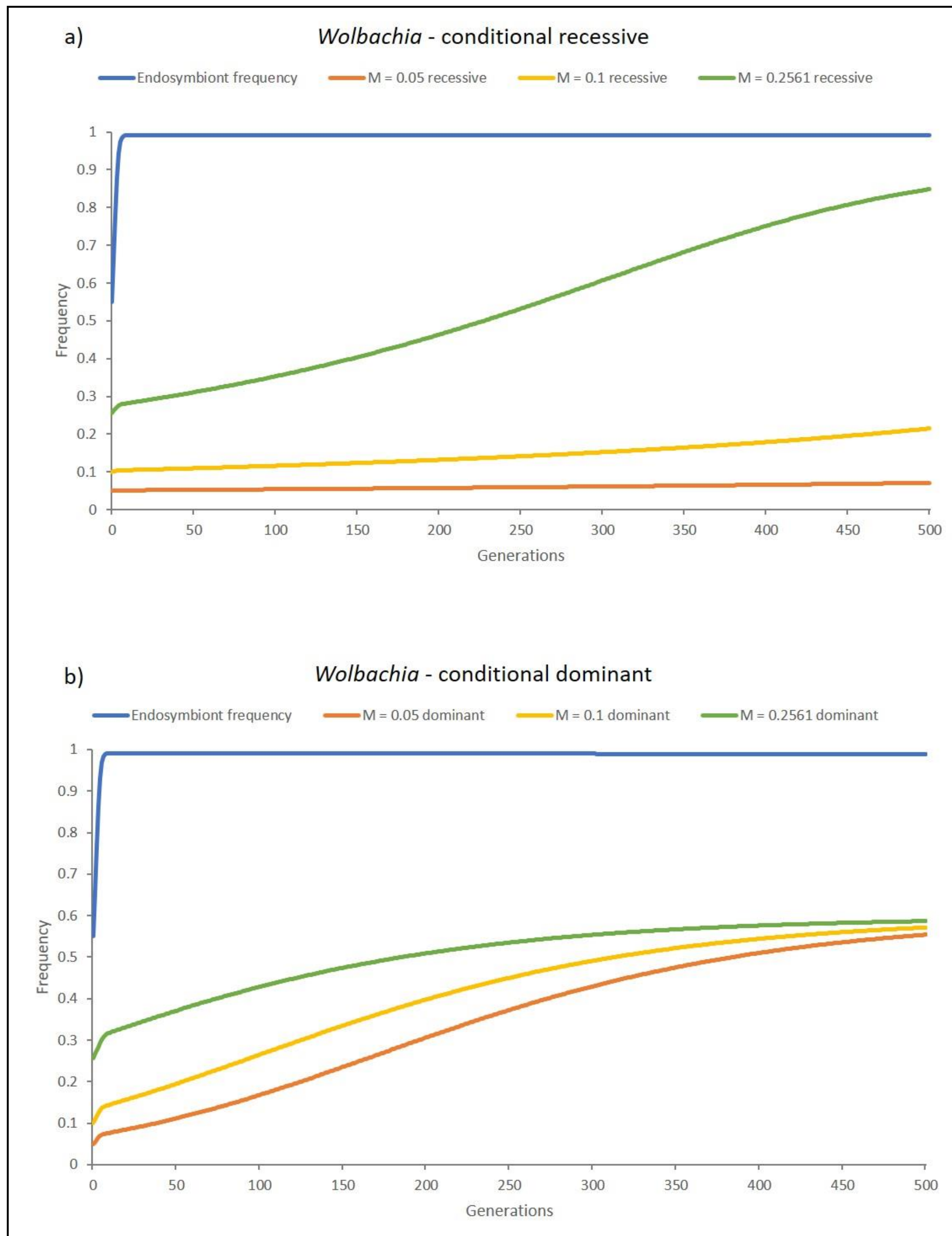

Figure 45: a) Spread of a choice mutation in the conditional recessive model. The spread is shown for three different initial mutation frequencies. Orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561. Transmission frequency c = 0.98, CI fertility f = 0.27, initial *Wolbachia* frequency W = 0.55. Blue = *Wolbachia* frequency. b) Spread of a choice mutation in the conditional dominant model. The spread is shown for three different initial mutation frequencies. Orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561. Transmission frequency c = 0.98, CI fertility f = 0.27, initial *Wolbachia* frequency W = 0.55. Blue = *Wolbachia* frequency.

Apart from the single *Wolbachia* infection, the study of Curry *et al*. (2015) also states frequency, transmission frequency and CI level for a *Wolbachia*/ *Rickettsia* double infection. These results were also used as values for the simulation parameters (Table 22). Transmission of the double infection was

perfect. In the simulation the infection became fixed in all four situations (unconditional/recessive, unconditional/dominant, conditional/recessive, conditional/dominant, Fig. 46a - b, Fig. 47a - b).

The choice mutation, introduced at the three aforementioned frequencies, does persist in the population. In the unconditional/recessive and the conditional/recessive simulations, a slight increase in frequency was observed before the mutation reached equilibrium (Fig. 46a, Fig. 47a). In the unconditional/dominant situation a small decrease in choice mutation frequency was seen for the first few generations. After this 'dip' the frequency increases again and reaches equilibrium. In the case of M = 0.05 and M = 0.1 the equilibrium was reached above the initial frequencies. However, for M = 0.2561, the equilibrium was observed below the initial frequency (Fig. 46b). The same situation was found for M = 0.2561 in the conditional/dominant simulation. For M = 0.05 and M = 0.1 it was different. The 'dip' that was seen for M = 0.2561 was not present, instead in both cases the choice mutation frequency increased and reached equilibrium at a higher level than the initial frequency (Fig. 47b).

Whereas in the unconditional model the choice mutation frequencies did rise to the same equilibrium, in the conditional simulations with c = 1 *Wolbachia* becomes fixed and no uninfected females are left to avoid. The preference mutation is no longer subject to selection since it no longer affects the phenotype. The mutation might eventually be lost due to genetic drift.

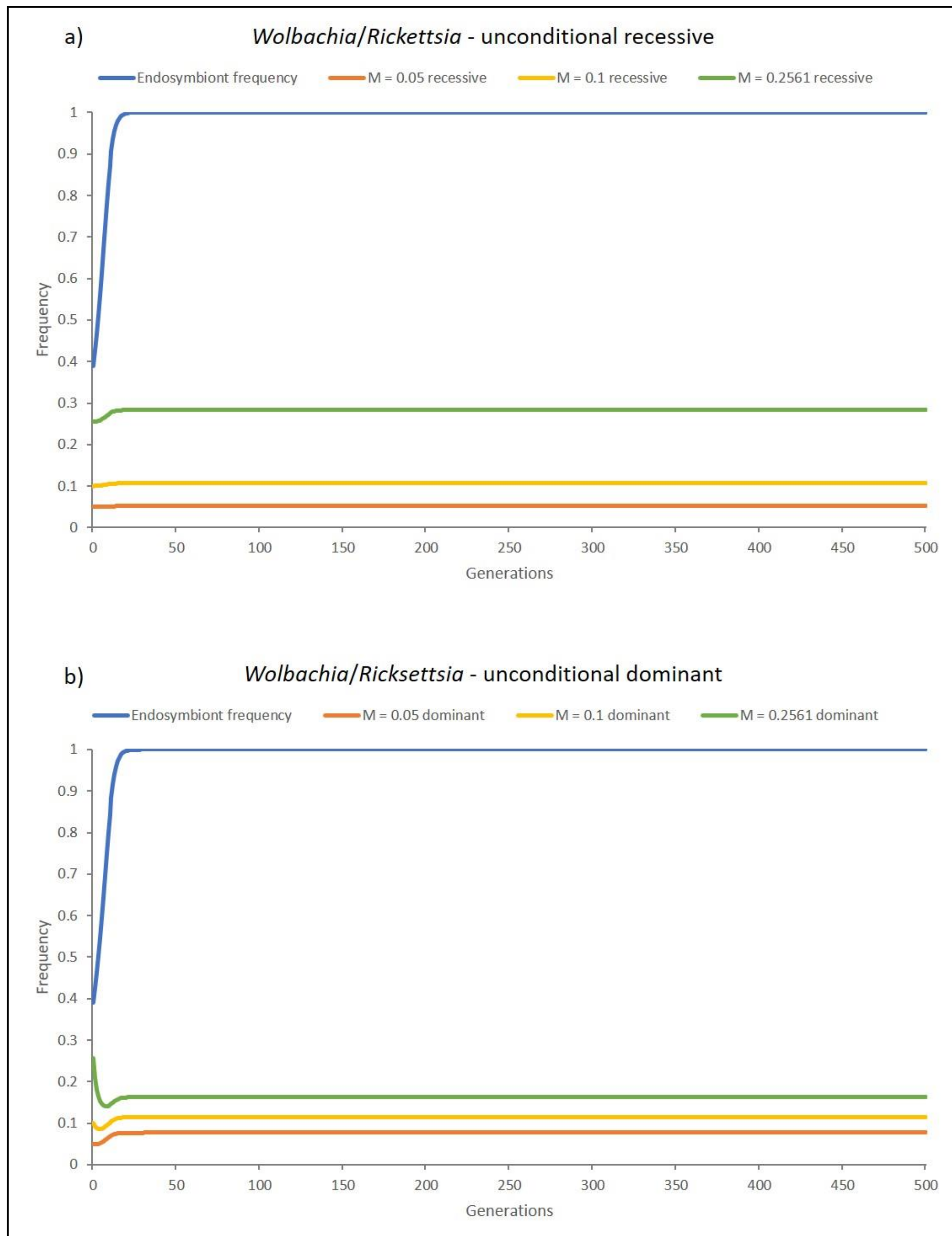

Figure 46: a) Spread of a choice mutation in the unconditional recessive model. The spread is shown for three different initial mutation frequencies. Orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561. Transmission frequency c = 1, CI fertility f = 0.65, initial *Wolbachia*/*Rickettsia* frequency W = 0.39. Blue = *Wolbachia*/*Rickettsia* frequency. b) Spread of a choice mutation in the unconditional dominant model. The spread is shown for three different initial mutation frequencies. Orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561. Transmission frequency c = 1, CI fertility f = 0.65, initial *Wolbachia*/*Rickettsia* frequency W = 0.39. Blue = *Wolbachia*/*Rickettsia* frequency.

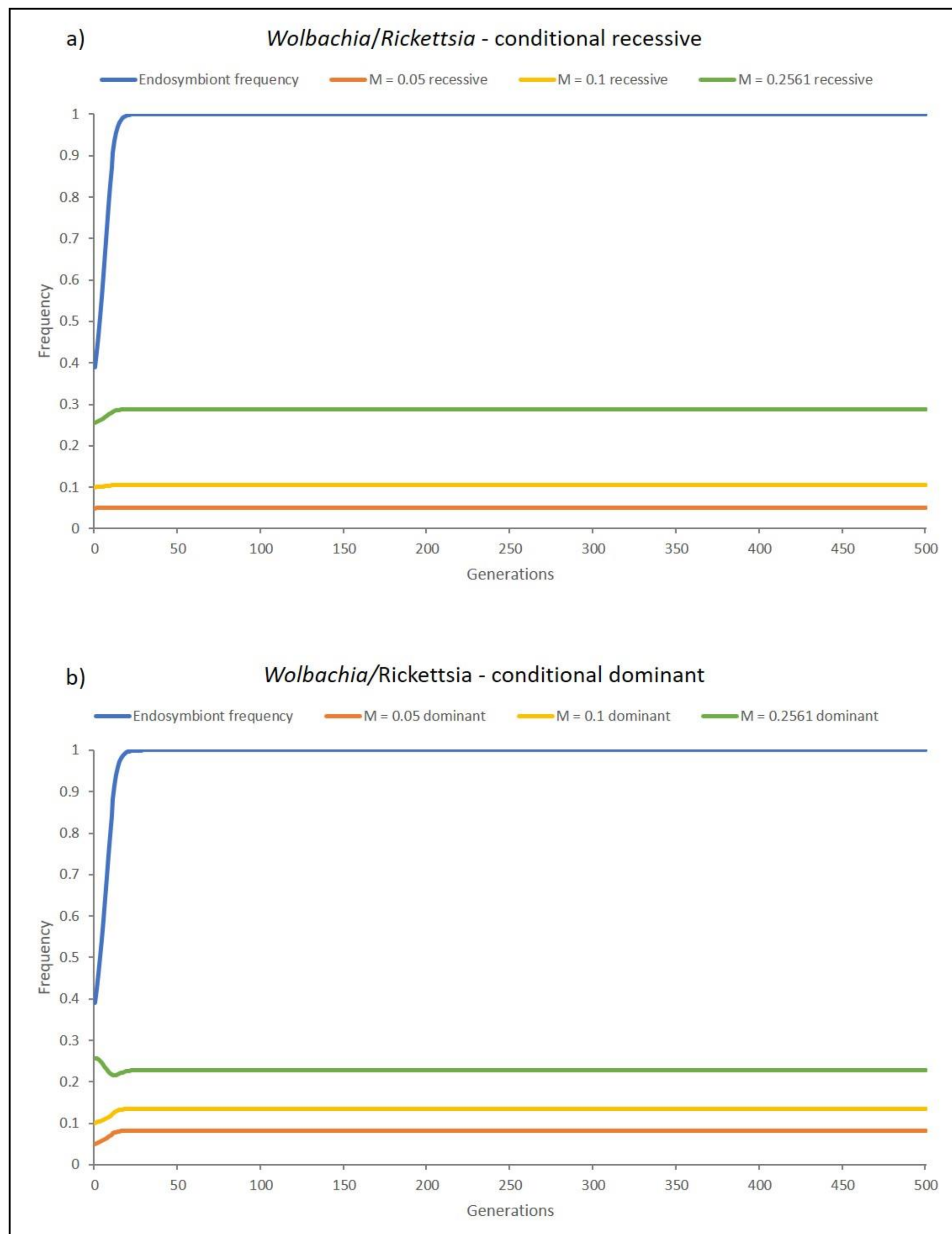

Figure 47: a) Spread of a choice mutation in the conditional recessive model. The spread is shown for three different initial mutation frequencies. Orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561. Transmission frequency c = 1, CI fertility f = 0.65, initial *Wolbachia/Rickettsia* frequency W = 0.39. Blue = *Wolbachia* frequency. b) Spread of a choice mutation in the conditional dominant model. The spread is shown for three different initial mutation frequencies. Orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561. Transmission frequency c = 1, CI fertility f = 0.65, initial *Wolbachia/Rickettsia* frequency W = 0.39. Blue = *Wolbachia/Rickettsia* frequency.

Linyphia triangularis

The simulation run on the data acquired from the *L. triangularis* study showed that the *Wolbachia/Rickettsia* double infection is unlikely to spread through

the population. The initial frequency of the infection was low (W = 0.2) whilst transmission frequency was quite high (c = 0.81). This combination did not seem to enable the infection to spread. After about 20 generations the infection was lost from the population (Fig. 48).

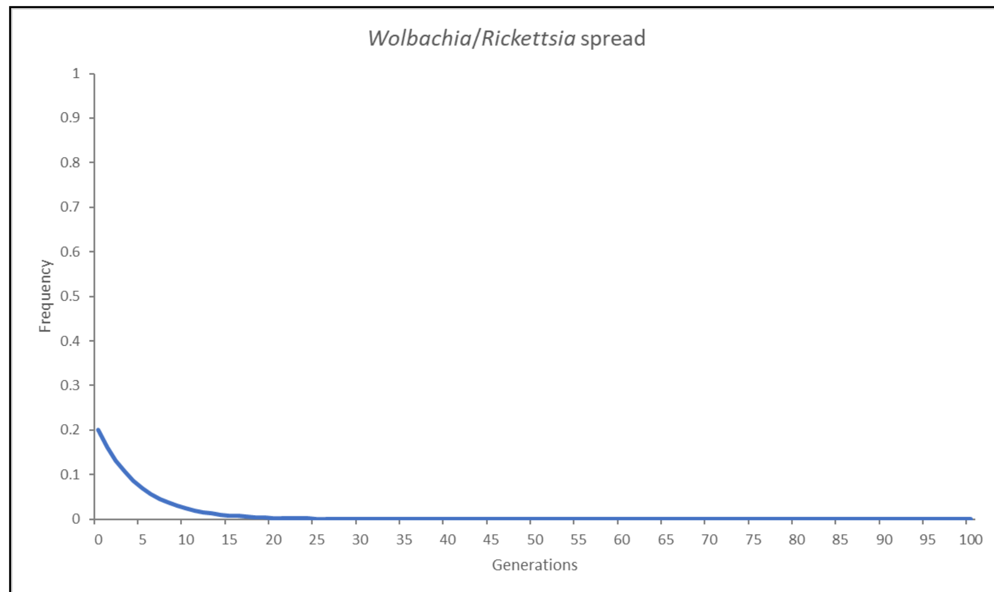

Figure 48: Spread of the *Wolbachia/Rickettsia* infection through the *L. triangularis* population in 100 generations. The infection declines steadily in 15 generations and disappears from the population. Transmission frequency c = 0.81, CI fertility f = 0.99, initial *Wolbachia* frequency W = 0.2.

The weak CI combined with rapid loss in transmission will lead to the *Wolbachia/Rickettsia* infection being lost from any starting frequency.

To investigate the potential spread of a choice mutation three different scenarios were simulated. The initial frequencies of the mutation chosen were M = 0.05, 0.1 and 0.2561. Since no information was available on whether CI is present in the *L. triangularis* system, four different CI levels were introduced into the model as well. Two of these were taken from Curry *et al.* (f = 0.27 and 0.65) whilst the two others were chosen to represent very strong (f = 0.01) and very weak (f = 0.99) CI.

In the unconditional recessive model, the different levels of CI and the different initial choice mutation frequency made no difference to the

outcome of the simulation. In all four situations the *Wolbachia*/*Rickettsia* infection and subsequently the choice mutation was lost (Fig. 49 a - d). The loss of the endosymbiont infection and the choice mutation happened even quicker in the unconditional dominant model. After fewer than 20 generations both are lost from the population (Fig. 50 a - d). The choice mutation cannot persist if the *Wolbachia*/*Rickettsia* are lost because all males carrying the mutation avoid mating with the uninfected females. As *Wolbachia*/*Rickettsia* frequency diminishes the females in the populations are all uninfected and males with the choice mutation never mate. The *Wolbachia*/*Rickettsia* infection was lost from the population in the conditional recessive model after about 25 generations in all four CI scenarios. The choice mutation persisted at equilibrium which did not change from the initial frequency. This was true for all three different initial frequencies (Fig. 51 a – d). The situation was similar in the conditional dominant model. The endosymbiont infection vanished from the population whilst the choice mutation persisted (Fig. 52 a - d). The choice mutation does persist after the *Wolbachia*/*Rickettsia* vanished from the population since it only shows its effect in infected males. Since no infected males are left the mutation does not have a phenotype.

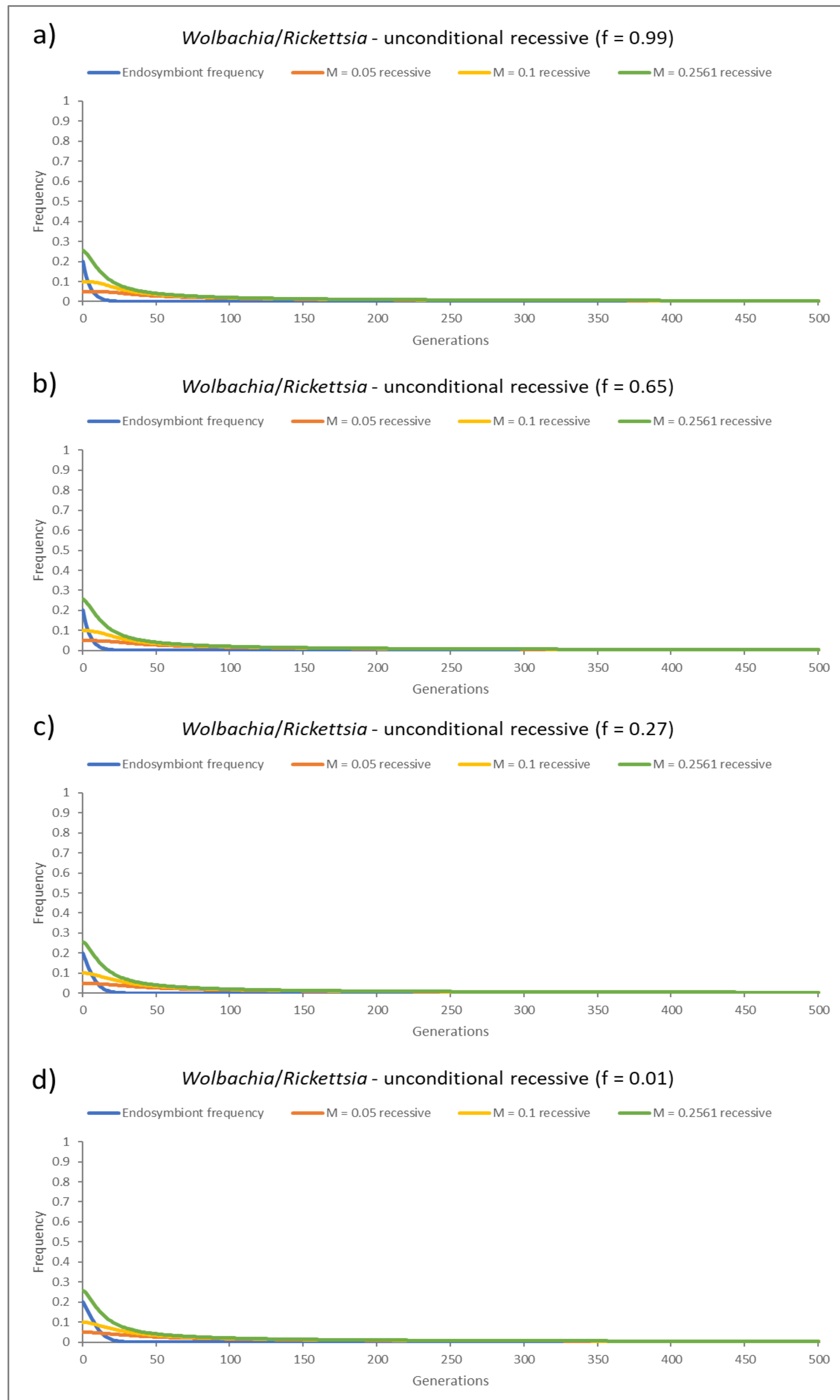

Figure 49: Spread of a choice mutation in the unconditional recessive model. The spread is shown for three different initial mutation frequencies (orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561) and four values for CI fertility (a) f = 0.99, b) f = 0.65, c) f = 0.27, d) f = 0.01). *Wolbachia/Rickettsia* transmission frequency c = 0.98, initial *Wolbachia/Rickettsia* frequency W = 0.2. Blue = *Wolbachia/ Rickettsia* frequency.

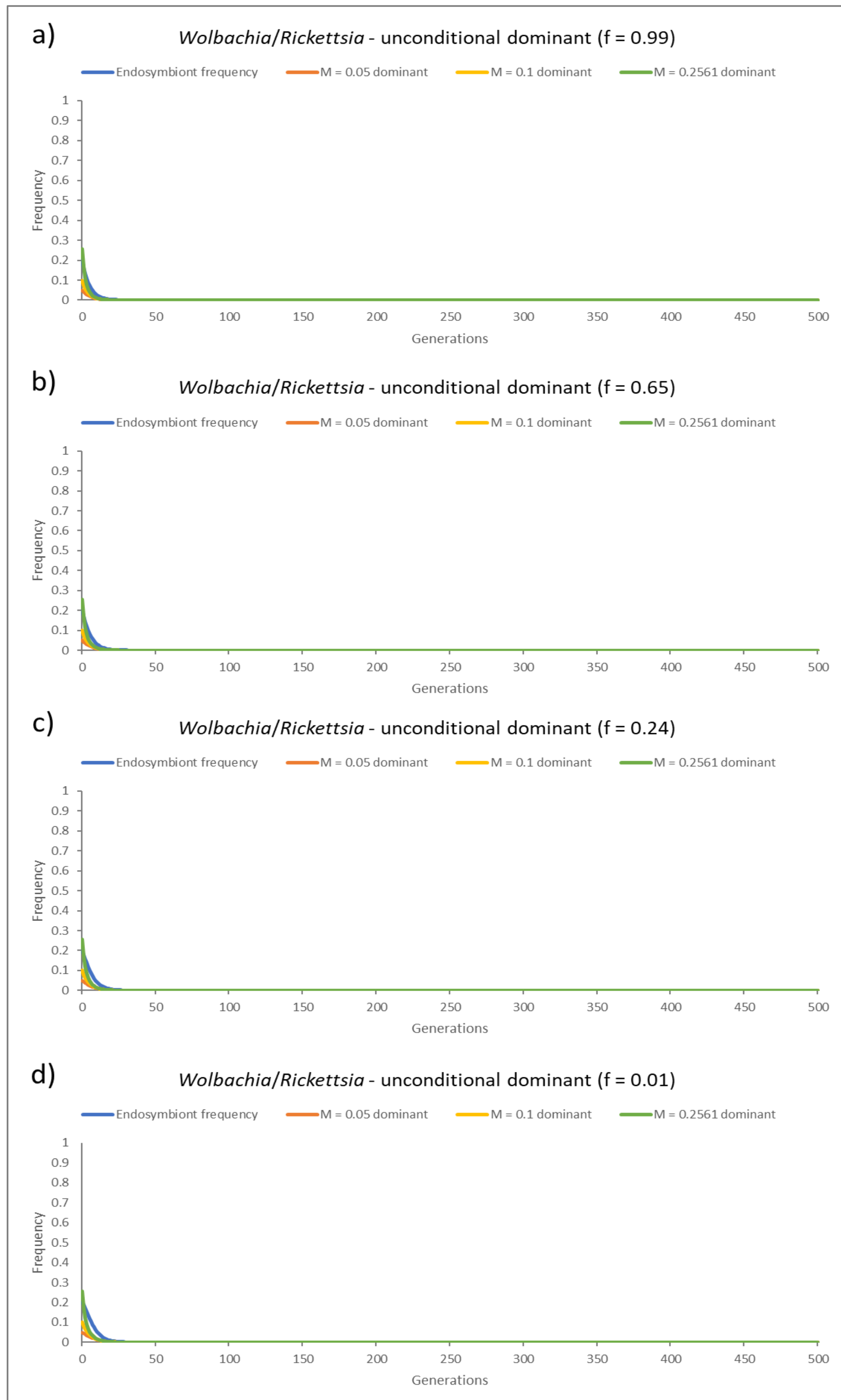

Figure 50: Spread of a choice mutation in the unconditional dominant model. The spread is shown for three different initial mutation frequencies (orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561) and four values for CI fertility (a) f = 0.99, b) f = 0.65, c) f = 0.27, d) f = 0.01). *Wolbachia/Rickettsia* transmission frequency c = 0.98, initial *Wolbachia/Rickettsia* frequency W = 0.2. Blue = *Wolbachia/ Rickettsia* frequency.

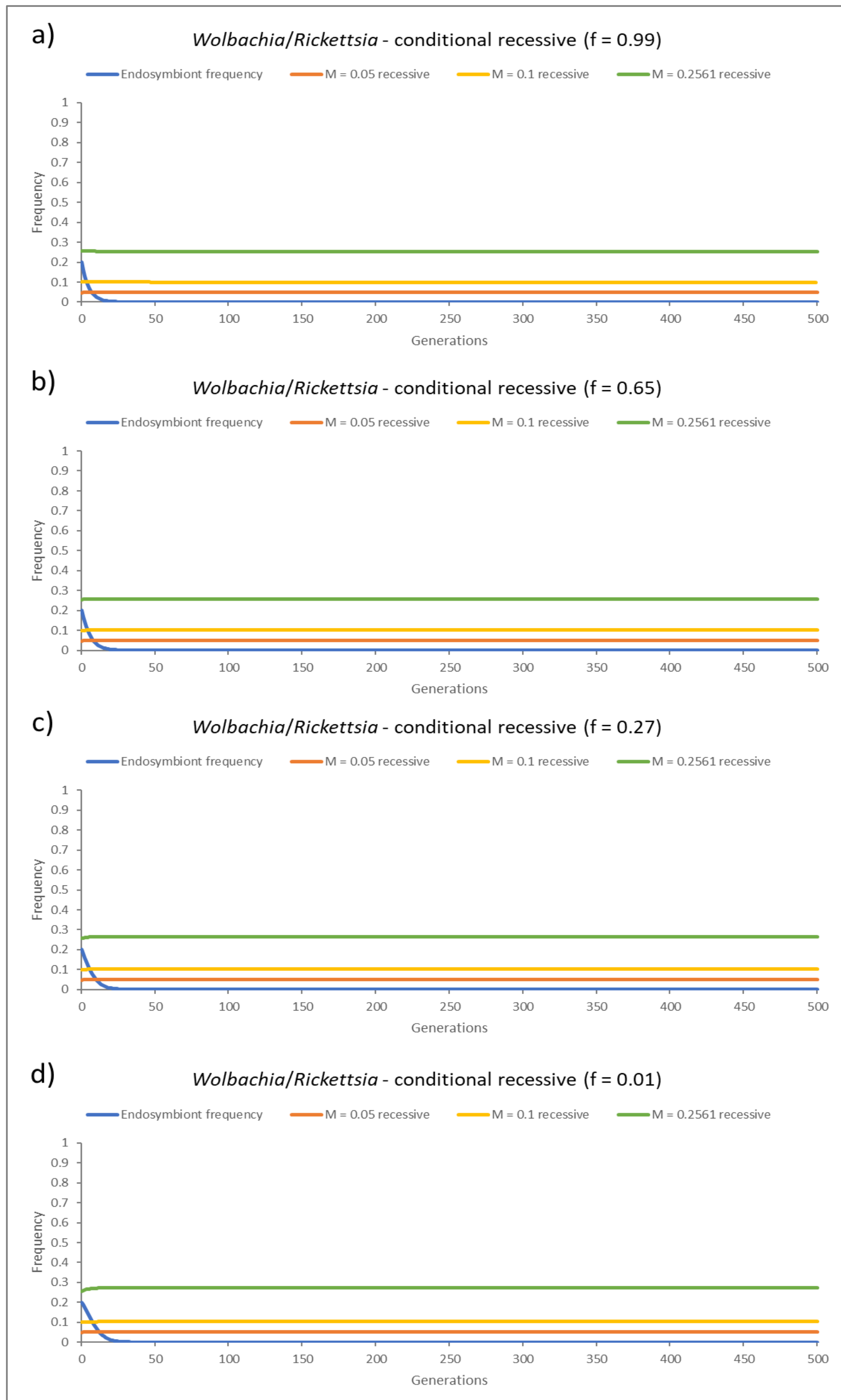

Figure 51: Spread of a choice mutation in the conditional recessive model. The spread is shown for three different initial mutation frequencies (orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561) and four values for CI fertility (a) f = 0.99, b) f = 0.65, c) f = 0.27, d) f = 0.01). *Wolbachia/Rickettsia* transmission frequency c = 0.98, initial *Wolbachia/Rickettsia* frequency W = 0.2. Blue = *Wolbachia/ Rickettsia* frequency.

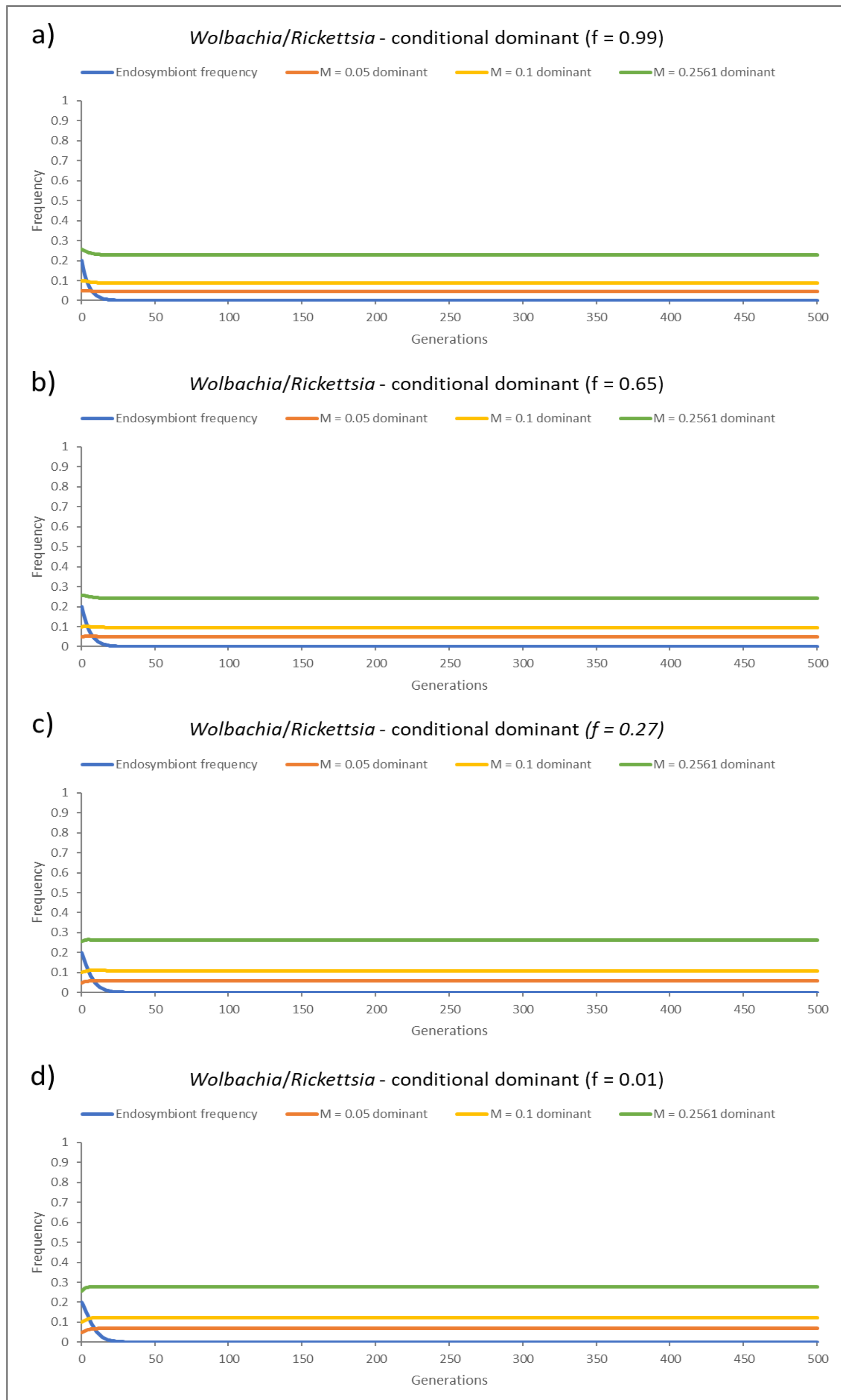

Figure 52: Spread of a choice mutation in the conditional dominant model. The spread is shown for three different initial mutation frequencies (orange: mutation frequency M = 0.05, yellow: mutation frequency M = 0.1, green: mutation frequency M = 0.2561) and four values for CI fertility (a) f = 0.99, b) f = 0.65, c) f = 0.27, d) f = 0.01). *Wolbachia/Rickettsia* transmission frequency c = 0.98, initial *Wolbachia/Rickettsia* frequency W = 0.2. Blue = *Wolbachia/ Rickettsia* frequency.

5.4 Discussion

In all four situations (unconditional recessive, unconditional dominant, conditional recessive and conditional dominant) *Wolbachia* spread to a high equilibrium frequency in the *M. fradeorum* population. After eight generations, choosy males that could discriminate against uninfected females, no matter whether unconditionally or dependent on their own infection status, would no longer need to be able to make this distinction, because the vast majority of all available females would be infected. The choice mutation not only persisted in all four scenarios, but in the case of the unconditional and conditional recessive models, starting from the theoretical threshold frequency (M = 0.2561 with c = 0.981, f = 0.5 and W = 0.6287), the mutation spread to high levels in the populations.

The *Wolbachia*/*Rickettsia* double infection spread to fixation in all simulations. As in the case of the single *Wolbachia* infection, at some point all individuals will carry the double infection and males carrying the choice mutation would not need to choose based on female infection status anymore. The choice mutation would stay in the population since males with the mutation and males without would have the same fitness at a neutral equilibrium frequency, assuming that the mutation would have no other costs. Male carrying the preference mutation would not have an advantage anymore.

The difference between the single and double infections was the level of CI. The single *Wolbachia* infection induces stronger CI than the *Wolbachia*/*Rickettsia* double infection (Curry *et al.*, 2015). Additionally, the two infections differed in their transmission fidelity. The *Wolbachia*/*Rickettsia* infection showed a perfect transmission whilst the single *Wolbachia* infection showed a high, yet imperfect, transmission rate. In the simulations for the single infection, the choice mutation will spread through the populations due to the strong CI level and the imperfect transmission. Males carrying the

mutation will have a higher chance of avoiding incompatible crosses and subsequently have a higher fitness than males without it.

The *Wolbachia*/*Rickettsia* double infection found in *L. triangularis* did not seem to have the ability to successfully invade the population. The initial infection frequency and the transmission rate were not sufficient for the endosymbionts to spread, and the infection vanished in all run simulations. In the unconditional model simulations, the choice mutation vanished as well. This was due to the fact that in the unconditional model, males, which carry the choice mutation, will avoid mating with uninfected females irrespective of their own infection status. After the extinction of the endosymbiont infection, all females would be uninfected and therefore discriminated against by males carrying the mutation. The fitness of these males would be below that of males without the mutation and hence it would not be able to persist, let alone spread through the population.

In the conditional models the situation was different. Even though the endosymbiont infection did not spread and also disappeared in all simulations, the choice mutation persisted. The absence of the *Wolbachia*/*Rickettsia* infection after approximately 20 generations did render the choice mutation redundant. In the conditional model the males with the choice mutation would choose mating partners conditional on their own infection status. After the disappearance of the endosymbiont infection, no infected males or females would be left in the populations. Uninfected males carrying the mutation would not discriminate against uninfected females. These males, assuming no other costs due to the choice mutation, would have the same fitness as uninfected males without the choice mutation.

The different levels of CI introduced into the models did not make a difference since the *Wolbachia*/*Rickettsia* double infection disappeared too quickly from the population and, without the endosymbiont, CI would simply not occur.

The two systems, *M. fradeorum* and *L. triangularis*, showed vast differences in their endosymbiont infection frequency and transmission rates. In *M. fradeorum* the transmission of both the single *Wolbachia* infection, as well as the *Wolbachia*/*Rickettsia* double infection, showed an (almost) perfect transmission. According to the models, both infections will spread to fixation in the next 10 generations. The transmission rate of *L. triangularis*, even though not particularly low, combined with a lower infection frequency was not sufficient to allow the endosymbiont infection to spread.

The model and simulation designed by Brookfield showed that the success of an endosymbiont and the spread of a potential choosiness gene are dependent on a fine balance between the parameters. Endosymbionts need to be very efficient in their transmission to be able to spread through a population. The lower the initial endosymbiont frequency the higher transmission rate has to be. The spread and persistence of a potential choice mutation through a population is dependent on the frequency of the endosymbiont. De Crespigny *et al.* (2005) found that a choice mutation cannot overcome the advantage of a high endosymbiont transmission rate, but that a rapidly spreading mutation can slow down the spread of the endosymbiont. In the simulations presented here, the endosymbionts either reached fixation or vanished very quickly from the populations.

The choice mutation did not have a significant effect on the endosymbiont spread. However, there are parameter values where an otherwise stably persisting endosymbiont infection would be driven to extinction by the spread of a preference mutation. When $c = 0.981$, $f = 0.5$, $W = 0.6287$ and the initial frequency of the choice mutation was $M >= 0.2561$ the endosymbiont infection and the preference mutation both persist in the population (see 5.1.1). A reduced transmission rate $c = 0.915$, a CI level of $f = 0.5$ and an initial endosymbiont frequency of $W = 0.6287$ would still allow the endosymbiont to spread through the population to reach a high equilibrium value. Introducing the choice mutation into the model at $M = 0.2561$ would alter this. The

endosymbiont would be driven to extinction whilst the choice mutation would decrease but, instead of vanishing as well, would reach a low equilibrium (data not shown).

Even though the data for the model presented in this chapter were acquired from two linyphiid spider species, the results were quite different. Whilst in *M. fradeorum* the endosymbionts seem to be very successful, to a point that they could (hypothetically) become fixed in the population, the endosymbionts of *L. triangularis* will not be able to spread and eventually will be lost. These differences emphasise that systems can vary immensely. Different strains of the same endosymbiont can differ in their transmission fidelity and consequently in their ability to spread. In the butterfly *Eurema hecabe*, two co-infecting *Wolbachia* strains were discovered (Nomura *et al.*, 2007). One strain causes perfect CI whilst the other strain causes feminisation of genetic males. The feminising strain only occurs as a double infection with the CI-inducing strain and is able to rescue male sperm carrying the CI-inducing strain. The authors of the study state that the two strains show significantly different transmission rates. The CI-inducing strain is transmitted perfectly to the next generation whilst the transmission fidelity of the feminising strain is only 80% (Nomura *et al.*, 2007).

The strength of CI represents a crucial factor in the spread of the endosymbiont and of a choice mutation. If CI favours endosymbiont infected individuals, the infection will spread and individuals carrying a choice mutation, which enables them to avoid incompatible crosses, will have a higher fitness than individuals without the mutation. Thereby facilitating the spread of the choice mutation.

If the predictions of the model were correct for the *L. triangularis* population, the expectation is that the *Wolbachia*/*Rickettsia* double infection will not persist. For the simulations, CI was assumed to be the endosymbiont induced phenotype. However, it is possible that not CI but one of the sex ratio

distorting phenotypes is at work in this spider species. The data from the population genetics and endosymbiont analyses presented in Chapter 3 and Chapter 4 can neither confirm nor reject the possibility that one of the endosymbionts found in this system acts as a sex ratio distorter. The data make it seem unlikely that male-killing or feminisation are acting in *L. triangularis* and parthenogenesis can be ruled out because unfertilised egg sacs never hatched. In *M. fradeorum* the infection should spread to fixation in less than 10 years according to the simulations. CI has been established to act in this system and the *Wolbachia*/*Rickettsia* double infection was found to induce weaker CI than *Wolbachia* alone.

To assess whether the model can be applied to data from the field, further study of field populations is needed. Nevertheless, populations in the field might not be as easy to track since they might not stay at one location for a prolonged period of time. A potential way forward would be to establish lab populations which would provide reliable data on transmission rates, sex ratio and thereby endosymbiont phenotype and infection rates. The combination of a theoretical model with data from live populations would provide a valuable insight into the host – endosymbiont relationship.

5.5 Appendix

From manuscript draft: *Wolbachia*-induced Cytoplasmic Incompatibility can create a stable equilibrium for preference alleles operating in males

John F.Y. Brookfield, Antje Hundertmark, Sara L. Goodacre

Methods

Modelling

As has been appreciated, the expected dynamics of CI-inducing *Wolbachia* are complex. We can produce a simple analytical model predicting the three equilibria for the infection frequency in the absence of mating preference but

with less than 100% maternal transmission fidelity. The modelling assumes that the population consists of six genotypes and is a single population that is panmictic except for any mating preferences shown by males.

The genotypes are defined by *U* and *I* to imply uninfected and infected, and *MM*, *Mm* and *mm* to imply the genotypes at the preference locus. The frequencies of the six possible genotypes are the same in males and females, and are p_{UMM}, p_{UMm}, p_{Umm}, p_{IMM}, p_{IMm}, and p_{Imm}. And p_U and p_I are the proportions of animals that are uninifected and infected, respectively. x represents the strength of avoidance of uninfected females, and f is the fertility of crosses between infected males and uninfected females.

For a Conditional Dominant Preference

For an uninfected female genotype i, of frequency p_{iU}, the relative probabilities of mating with different males are

Infected *MM* probability is $\frac{(1-x)p_{IMM}}{1-x(p_{IMM}+p_{IMm})}$

Infected *Mm* probability is $\frac{(1-x)p_{IMm}}{1-x(p_{IMM}+p_{IMm})}$

Infected *mm* probability is $\frac{p_{Imm}}{1-x(p_{IMM}+p_{IMm})}$

Uninfected *MM* probability is $\frac{p_{UMM}}{1-x(p_{IMM}+p_{IMm})}$

Uninfected *Mm* probability is $\frac{p_{UMm}}{1-x(p_{IMM}+p_{IMm})}$

Uninfected *mm* probability is $\frac{p_{Umm}}{1-x(p_{IMM}+p_{IMm})}$

The avoidance, of strength x, by infected MM and Mm males, of uninfected females, will release *MM* and *Mm* males to compete for the infected females.

The proportion of the males that are competing for the infected females that are infected *MM* is

$$\frac{p_{IMM}(1 + x\,p_U/p_I)}{1 + (p_{IMM} + p_{IMm})x\,p_U/p_I}$$

The proportion that are infected *Mm* is

$$\frac{p_{IMm}(1 + x\,p_U/p_I)}{1 + (p_{IMM} + p_{IMm})x\,p_U/p_I}$$

The proportion that are infected *mm* is

$$\frac{p_{Imm}}{1 + (p_{IMM} + p_{IMm})x\,p_U/p_I}$$

The proportion that are uninfected *MM* is

$$\frac{p_{UMM}}{1 + (p_{IMM} + p_{IMm})x\,p_U/p_I}$$

The proportion that are uninfected *Mm* is

$$\frac{p_{UMm}}{1 + (p_{IMM} + p_{IMm})x\,p_U/p_I}$$

And the proportion that are uninfected *mm* is

$$\frac{p_{Umm}}{1 + (p_{IMM} + p_{IMm})x\,p_U/p_I}$$

The crosses have fertility f in the CI crosses (I father and U mother). A proportion c of the offspring of infected mothers are infected and a proportion (1-c) are not infected. The proportions of *MM*, *Mm* and *mm* in the offspring are calculated from Mendelian segregation of alleles in their parents.

For a Conditional Recessive Preference

The situation is the same except that, only for IMM males is the preference against U females shown. Thus the denominator is

$$1 - xp_{IMM}$$

For crosses with uninfected females, and

$$1 + p_{IMM}x\,p_U/p_I$$

For crosses with infected females.

For an Unconditional Dominant Preference

For an uninfected female genotype *i*, of frequency p_{iU}, the relative probabilities of mating with different males are

Infected *MM* probability is $\frac{(1-x)p_{IMM}}{1-x(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})}$

Infected *Mm* probability is $\frac{(1-x)p_{IMm}}{1-x(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})}$

Infected *mm* probability is $\frac{p_{Imm}}{1-x(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})}$

Uninfected *MM* probability is $\frac{(1-x)p_{UMM}}{1-x(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})}$

Uninfected *Mm* probability is $\frac{(1-x)p_{UMm}}{1-x(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})}$

Uninfected *mm* probability is $\frac{p_{Umm}}{1-x(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})}$

The avoidance, of strength *x*, by all *MM* and *Mm* males, of uninfected females, will release *MM* and *Mm* males to compete for the infected females. The proportion of the males that are competing for the infected females that are infected *MM* is

$$\frac{p_{IMM}(1+x\,p_U/p_I)}{1+(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})x\,p_U/p_I}$$

The proportion that are infected *Mm* is

$$\frac{p_{IMm}(1+x\,p_U/p_I)}{1+(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})x\,p_U/p_I}$$

The proportion that are infected *mm* is

$$\frac{p_{Imm}}{1+(p_{IMM}+p_{IMm}+p_{UMM}+p_{UMm})x\,p_U/p_I}$$

The proportion that are uninfected *MM* is

$$\frac{p_{UMM}(1 + x\,p_U/p_I)}{1 + (p_{IMM} + p_{IMm} + p_{UMM} + p_{UMm})x\,p_U/p_I}$$

The proportion that are uninfected *Mm* is

$$\frac{p_{UMm}(1 + x\,p_U/p_I)}{1 + (p_{IMM} + p_{IMm} + p_{UMM} + p_{UMm})x\,p_U/p_I}$$

And the proportion that are uninfected *mm* is

$$\frac{p_{Umm}}{1 + (p_{IMM} + p_{IMm} + p_{UMM} + p_{UMm})x\,p_U/p_I}$$

For an Unconditional Recessive Preference

The situation is the same except that, only for IMM males is the preference against U females shown. Thus, the denominator is

$$1 - x(p_{IMM} + p_{UMM})$$

for crosses with uninfected females, and

$$1 + (p_{IMM} + p_{UMM})x\,p_U/p_I$$

for crosses with infected females.

Results

Cytoplasmic Incompatibility

Our simplified model includes random mating, and the absence of any cost from the bacterium other than from CI. *p* is the proportion of surviving offspring that come from mothers with *Wolbachia* (mothers called I as opposed to U). These offspring are half male and half female. But the transmission of *Wolbachia* is not 100%. Rather, only a proportion c of the offspring received the *Wolbachia*. This means that the proportion of I animals, p’, is

$$p' = pc$$

Then there is the random mating, and, if an I male mates with a U female, then the fertility of the cross is f, where f<1.

Thus, there is a loss of fitness of the population, which affects only these offspring. As a proportion p' of males are I and a proportion (1-p') of females are U, and a proportion of 1-f of their offspring die, the population fitness is 1- p'(1-p')(1-f).

Thus, the frequency of I in the surviving offspring, and the next generation, p'', is given by

$$p'' = \frac{p'}{1 - p'(1 - p')(1 - f)}$$

But $p'=pc$,

So

$$p'' = \frac{pc}{1 - pc(1 - pc)(1 - f)}$$

If we are interested in an equilibrium value of p, $p'' = p$, so

$$p\big(1 - pc(1 - pc)(1 - f)\big) = pc$$

or

$$p\big(1 - c + p^2c^2(1 - f) - pc(1 - f)\big) = 0$$

So, either p=0, or

$$p^2c^2(1 - f) - pc(1 - f) + 1 - c = 0$$

which allows two solutions of a quadratic equation

$$p = \frac{c(1 - f) \mp \sqrt{c^2(1 - f)^2 - 4c^2(1 - f)(1 - c)}}{2c^2(1 - f)}$$

which simplifies to

$$p = \frac{(1-f) \mp \sqrt{(1-f)^2 - 4(1-f)(1-c)}}{2c(1-f)}$$

given that the square root term is positive. This requires $f<4c-3$.

If $f<4c-3$ there is a stable equilibrium point at $p=0$, an unstable equilibrium at

$$p = \frac{(1-f) - \sqrt{(1-f)^2 - 4(1-f)(1-c)}}{2c(1-f)}$$

and a stable equilibrium point at

$$p = \frac{(1-f) + \sqrt{(1-f)^2 - 4(1-f)(1-c)}}{2c(1-f)}$$

We show two situations. When c=0.9, and f=0.5, there are equilibrium points at 0 (stable), 0.307 (unstable) and 0.804 (stable). When c=0.8 and f=0, there are equilibrium points at 0 (stable), 0.3455 (unstable), and 0.9045 (stable). Thus, for a given set of parameters of the *Wolbachia* infection, initial conditions will determine whether the population moves to the high stable equilibrium point, or whether the bacterium will be lost.

Chapter 6: General discussion and future outlook

The work presented in this thesis aimed to further the knowledge of endosymbiont infection in spiders. The study on *N. senegalensis* presented in Chapter 2 initiated the thought processes which shaped the study on the two linyphiid species *L. hortensis* and *L. triangularis*.

N. senegalensis is quite well studied in different aspects of reproductive behaviour (Neumann and Schneider, 2015; Schneider *et al.*, 2011; Schneider and Michalik, 2011). This polygamous species is a good system to study mechanisms such as sperm competition. During a study aimed at investigating the heritability of sperm competition success in *N. senegalensis* males, a sex ratio distortion in some of the matrilines and subsequently an infection with *Wolbachia* was detected. Following this discovery, Chapter 2 presents the first attempts at characterising the *Wolbachia* infection. It was found that *N. senegalensis* carries two different *Wolbachia* strains, but the phenotype induced by the endosymbiont remains unknown. The two different *Wolbachia* strains identified are very closely related which indicates that they diverged in their host. Therefore, it is possible that the association between *N. senegalensis* and *Wolbachia* is not recent but actually quite old.

It is possible that *Wolbachia* is the cause of the sex ratio distortion discovered, limited data around the infection and the distortion patterns make it currently impossible to identify the mechanism that is acting in this system. The presence of the bacteriophage WO could be involved in the expression of the phenotype, thereby making it less obvious what the primary sex ratio distortion mechanism is. There is also still the possibility that the sex ratio distortion was present in the host before *Wolbachia* invaded it and thereby the endosymbiont would rather be a hitchhiker than a driver of the distortion. Another intriguing finding of this study was that infected *N. senegalensis* females had heavier egg sacs than uninfected females. It is unclear whether

this is due to more eggs per clutch or larger eggs, the latter of which might provide a larger amount of resources for the offspring.

Although *N. senegalensis* is an established system and easy to breed in the lab they have a disadvantage. Generation time is quite long and only one generation per year can be bred. Studying endosymbiont transmission, sex-ratio distorting effects, and other factors like offspring survival or longevity will therefore take a long time. One specific limitation of the study presented here was that the discovery of *Wolbachia* was coincidental. Therefore, only the second part of the study aimed at characterising the *Wolbachia* infection. The data acquired from the first part of the study must be interpreted with care. Additionally, the study only focussed on *Wolbachia*, but it is possible that other endosymbionts, e.g. *Rickettsia*, *Spiroplasma* or *Cardinium*, are also present in *N. senegalensis*. It is possible therefore that the observed sex ratio distortion is caused by another endosymbiont or even by multiple infections.

In contrast to *N. senegalensis* the two *Linyphia* species have not gained much attention to date. Research on *L. hortensis* is especially scarce. The first part of the study was therefore to gain more knowledge about the life history and population genetics of *L. hortensis* and *L. triangularis* (Chapter 3). The second part of the study was aimed at identifying possible endosymbiont infections in both species and also characterising the potential effects of such infections (Chapter 4). The bigger question behind this part of the study was to investigate the possible involvement of endosymbionts in the evolution of species. The role of endosymbionts as drivers of speciation is a controversial topic, with some believing that they indeed are a driving force (Roehrdanz and Levine, 2007; Yen and Barr, 1971) whilst others suggest they are just hitchhiking on speciation processes (Amit *et al.*, 2017; Chen *et al.*, 2016). By analysing the genetic background of spiders from different geographical locations in combination with their endosymbionts this work tried to add new information to the existing knowledge about the endosymbionts' role in speciation.

For the first part of the study, wild-caught spiders were brought to the lab from different locations in Europe: Germany, Denmark, Sweden and the UK. Mating experiments were planned to look for potential differences in male mating behaviour in inter-population and intra-population crosses. The mating behaviour of both species is characterised by different phases but *L. triangularis* shows a more ritualised behaviour than *L. hortensis* (Stumpf, 1990; Stumpf, 1996).

As mentioned in Chapter 3, the mating experiments on *L. triangularis* were not completed as originally planned due to a high mortality of the spiders in 2016 and a repetition was deemed not feasible in 2017. Therefore, it is unclear whether differences in mating behaviour between individuals from different locations exist. The few successful mating trials did not show any signs of rejection of mating partners from a different geographical regions. Since no egg sacs were retrieved from either inter- or intra-population matings it is not clear whether these would be equal in clutch size, hatchability or offspring viability. Further investigation is needed to find out if the geographic distance between the populations has a quantifiable impact on the mating behaviour or whether inter-population crosses are equally as fertile as intra-population crosses.

The endosymbiont screening revealed that both species carry *Wolbachia*, *Rickettsia* and *Spiroplasma*. The infection frequencies differ between the two *Linyphia* species, and also between different geographic locations and the years of collection. This fluctuation could be due to different factors such as low transmission rates, natural curing or host resistance (Hornett *et al.*, 2006; Martin and Gage, 2007; Negri *et al.*, 2006). Likewise, sampling errors cannot be excluded, and false positives or negatives could have biased the screening results. Also, when working on univoltine species, which have an almost year-long life cycle, only a snapshot of the endosymbiont situation can be captured in the course of three years. It is possible that the observed lowered infection rates in 2017 were due to the fact that the endosymbionts are in a declining

phase. Assuming that the endosymbionts are not at equilibrium in the populations, it is unlikely that the same infection frequencies are recorded at different locations and in different years. It is also possible that individuals occasionally migrating into a population, introduce another strain or a different endosymbiont. Different strains of each of the endosymbionts were identified in both *Linyphia* species. Like in *N. senegalensis* the *Wolbachia* found in *Linyphia* are infected with the bacteriophage WO. In both species different strains of the phage were found and it seems that one strain of *Wolbachia* can harbour more than one phage whilst one phage can infect more than one *Wolbachia* strain. Whether the phage does impact the *Wolbachia's* ability to express their sex ratio distorting phenotypes remains unknown for now.

Even though sex ratio distortion was noted for both species, no conclusive statement about the endosymbiont-induced phenotype can be made at this stage. In both species and both years, males turned out to be the rarer sex, but it is unclear whether one of the endosymbionts found is responsible for the observed skew. It is possible that, inadvertently, a collection bias was introduced when collecting spiders, since males are far less conspicuous than females. Once mature they leave their webs and move through the herb and shrub layer, in search of females (Rovner, 1968). This puts them at a higher risk of being a victim of predation, or depleting their energy resources since they stop feeding once they leave their web (Andrade, 2003; Kasumovic *et al.*, 2007). Therefore, it is possible that there is a skewed sex ratio in *L. hortensis* and *L. triangularis* but that it is not induced by an endosymbiont. The fact that endosymbiont infected males were found reduces the probability that early male-killing or feminisation are at work here. However, they cannot be excluded completely since factors like host suppression or the presence of the bacteriophage WO could impact phenotype expression.

Since no egg sacs from inter-population matings were available for analysis, it is not known whether any level of CI is present in *L. hortensis* or *L. triangularis*.

One of the hypotheses of this piece of work was that individuals potentially avoid mating partners which would be incompatible due to endosymbiont infection status. Chapter 5 investigated a theoretical system where infected males, which carry a preference gene, would discriminate against uninfected females and would only mate with infected ones. The model and simulation were first used on theoretical data which identified threshold points at which a choice mutation and an endosymbiont infection would both remain in a population (Hundertmark *et al.*, in prep).

Data from a study on *M. fradeorum* (Curry *et al.*, 2015), another linyphiid spider, and data from the *Linyphia* study, presented in this work, were used in the simulation to study the potential spread of the found endosymbiont infections. Whilst the endosymbiont infection in *M. fradeorum* spread to fixation, due to highly efficient transmission, the endosymbiont infections in *L. triangularis* vanished from the population in every simulated scenario. The observed transmission rates are not sufficient to keep the infection in the population. In *M. fradeorum* a polymorphism of endosymbiont infection and preference gene would be possible under the conditions assessed whilst this would not be the case for *L. triangularis*. Whereas data for all parameters, except for the preference gene, were available from *M. fradeorum*, more data from the field are needed for *L. triangularis*, to establish more reliable results about infection frequency, transmission rates and levels of CI.

The analyses of the host mtDNA revealed a low genetic diversity in both species which is in line with the finding that both species carried endosymbionts. The low genetic diversity could be the result of a selective sweep induced by the endosymbionts (Hurst and Jiggins, 2005; Jiggins, 2003; Johnstone and Hurst, 1996; Marcade *et al.*, 1999). The geographic patterns of haplotype distribution indicate that mixing between the populations is limited in both *Linyphia* species. This is not surprising given that neither *L. triangularis* nor *L. hortensis* show any sign of ballooning behaviour (pers. obs.; Goodacre pers. comm.). No distinctive geographic pattern in the endosymbiont

infections was detected. This could indicate that the endosymbiont infections are not old but rather recent. If dispersal is restricted by the inability to balloon and the endosymbiont infections were old, geographic patterns in endosymbiont distribution would be expected. Due to the elevated mutation rate of *cytochrome oxidase I* gene (Hebert *et al.*, 2003), differences in the genome caused by endosymbiont selective sweeps, could be detected quite early on.

Choosing *L. hortensis* and *L. triangularis* as study system to investigate the role of endosymbionts in speciation had its advantages and its limitations. A definite advantage was that the spiders were abundant and easy to identify in the wild. Their widespread distribution over the northern hemisphere made it easy to retrieve spiders from different geographic locations. Establishing a lab population on the other hand proved to be quite difficult. The high mortality rates, egg sac hatching failure (*L. triangularis*) and inability to rear offspring to maturity (*L. hortensis*) prevented the successful set up of a lab population. Investigating offspring clutch sex ratios and endosymbiont transmission were therefore very restricted. The spider mortality also directly impacted the planned behavioural observations. Not enough mating trials could be set up to obtain a sufficiently large sample size.

The aim was to collect at least 40 spiders per location to be able to conduct enough intra- and inter-population crosses. Due to the aforementioned issues, this number turned out to be too low. A more sensible number would have been 90 - 120 individuals per population. However, keeping such a large number of spiders in the lab would have been very time consuming and probably not feasible in the scope of this study. Screening a larger sample of spiders per population for endosymbionts would also have benefitted the analysis of infection frequencies in the wild. The number and quality of endosymbiont and mtDNA sequences was not always sufficient to thoroughly analyse the phylogenetic relationships. Therefore, collecting larger quantity

of spiders, even if they are not kept alive in the lab, would definitely be beneficial for the molecular investigations.

In conclusion it must be said that, even though this project could not establish what effect the endosymbionts have on their spider hosts *N. senegalensis*, *L. hortensis* and *L. triangularis*, the data gathered in the past three years provide a solid background for further research. This work was the first to attempt a characterisation of endosymbiont infections in all three species studied. *Linyphia* mtDNA analyses did show signs of selective sweeps which could be linked to the endosymbionts present in these species. The limited behavioural data are inconclusive at this point and more studies are needed to establish whether behavioural differences exist that could eventually lead to reproductive isolation of populations. Connecting any potential behavioural differences to existing endosymbiont species, in combination with more data from mtDNA analyses could provide an idea of whether endosymbiont-driven speciation processes are at work here.

Another route to follow would be to look at local adaptation in more depth. The *Linyphia* were collected from regions, which do not differ vastly from one another. Maybe they were too similar to one another to see local adaption in terms of *e.g.* prey choice, habitat choice, pigmentation of abdomen (*e.g.* Northern individuals could be darker). Retrieving individuals not only from Northern Europe but also from Central and Southern Europe could provide more information about potential morphological differences as well as mtDNA and endosymbiont variation.

To investigate the actual phenotype of the endosymbiont infection in *N. senegalensis* and the two *Linyphia* species, mating trials between differently infected individuals need to be conducted. *N. senegalensis* can be bred very reliably in the lab and, with the use of antibiotics, matrilines of different infection status can be established. To be able to do the same with *Linyphia*, firstly the difficulties encountered in keeping and breeding the spiders in the

lab need to be overcome. *L. hortensis* might be more promising in this regard than *L. triangularis*. These laboratory bred populations would also provide the opportunity to investigate the role of the bacteriophage WO further.

This work presents the first record of endosymbionts in *N. senegalensis* and *L. hortensis*. Whilst *L. triangularis* had been part of an endosymbiont study before (Duron *et al.*, 2008), the information available is still very limited. Even though this thesis may not provide a definite answer to the question of whether endosymbionts play a role in speciation processes, or whether individuals have the ability to avoid potentially incompatible mates, it still adds new information to the body of knowledge available on endosymbionts in spider hosts. It also emphasises again that the relationships between endosymbionts and their hosts are rarely straightforward. The advantages and limitations of *N*. *senegalsensis* and the two *Linyphia* species as study systems could be used to design future studies accordingly.

Endosymbionts in spiders: Driving force or hitchhikers? The questions raised in the title of this thesis can hardly be answered with the knowledge gained in the different parts of the study. Interpreting the compiled data did not exclude or endorse either of the possibilities. Future work in this area willbring more clarity, but maybe there is no definite answer and endosymbionts can actually be either, driver or hitchhiker, depending on the host system they are in.

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From: [redacted]
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Good luck with your PhD work.

Ron Atkinson

On Fri, Mar 22, 2019 at 6:07 AM Antje Hundertmark <Antje.Hundertmark@nottingham.ac.uk> wrote:

> Hi Ron,
>
> I'm a final year PhD student at the University of Nottingham and I'm currently writing up my thesis on endosymbiont infections in spider.
>
> When I was on the hunt for some good pictures of spider genitalia I came across the drawings (female and male) on your website. I assume that you created those and I would like to kindly ask if I could use them in my thesis. The thesis will eventually be uploaded to the uni's ethesis system and will be freely accessible. I obviously would include your name as the copyright holder and also include the address of the website to the thesis. I do not intend to make any financial profit from this. I would be very grateful, if you could let me know if you are happy for me to use these drawings.
>
> Thank you very much in advance and have a good evening.
>
> Kind regards,
>
> Antje
>
> **Antje Hundertmark**
>
> PhD Student
>
> The University of Nottingham

1

Permission for the use of the photo in Figure 13, left.

Antje Hundertmark

From: [redacted]
Sent: 22 March 2019 19:16
To: Antje Hundertmark
Subject: Re: Use of a photo for PhD thesis

Hi Antje and thanks for getting in touch.

Yes we would be delighted for you to use our image for your thesis. Thank you for taking the time to ask permission. It is very much appreciated. If you need a copy of the original photograph, just let us know.

Best wishes for the future.
Trevor and Dilys

On Thu, 21 Mar 2019, 20:33 Antje Hundertmark, <Antje.Hundertmark@nottingham.ac.uk> wrote:

Dear Trevor and Dilys.

I'm a final year PhD student at the University of Nottingham and I'm currently writing up my thesis on endosymbiont infections in spider.

When I was on the hunt for some good pictures of the sheet-web spider Linyphia hortensis. I found a nice photo of a female (http://www.eakringbirds.com/eakringbirds3/linyphiahortensis03.jpg) and wondered if you would approve of me using this picture in my thesis.

The thesis will eventually be uploaded to the uni's ethesis system and will be freely accessible. I obviously would include your names as the copyright holders and also include the address of the eakringbirds website into the thesis.

I do not intend to make any financial profit from this. The photo will only be used for the thesis and it will not be used in any potential future publications that may come out of the thesis.

Please let me know if you would be happy for me to use your photo and how much you would charge for this kind of use.

Thank you very much in advance and have a good evening.

Antje Hundertmark

1

Permission for the use of the photo in Figure 13, right.

Antje Hundertmark

From:	
Sent:	22 March 2019 06:56
To:	Antje Hundertmark
Cc:	Dave Nicholls
Subject:	Use of a photo for PhD thesis
Attachments:	Spider (Linyphia hotensis male) Field near Granitethorpe Quarry Sapcote SP49349383 (taken 12.5.2012).JPG

Hello Antje,

David Nicholls of NatureSpot has forwarded your request to use my image of Linyphia hortensis for your thesis on endosymbiont infections in spiders, and I am happy for you to do so. The image is attached.

If you credit the image, would you please credit it to www.naturespot.org.uk ?

Regards

Permission for the use of the photo in Figure 14, left.

Antje Hundertmark

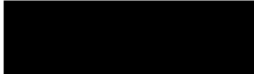
From:
Sent: 21 March 2019 20:55
To: Antje Hundertmark
Subject: Re: Saxifraga: Use of a photo in PhD thesis

Dear Antje Hundertmark,

This is Ok, you can use this picture free of charge in your thesis.

With kind regards,

Dr Jan van der Straaten
Saxifraga Foundation



Saxifraga schreef op 2019-03-21 21:46:
> Dit is een e-mailbericht via http://www.saxifraga.nl/ van:
> Antje Hundertmark <Antje.Hundertmark@nottingham.ac.uk>
>
> Dear Saxifraga team,
> I'm a final year PhD student at the University of Nottingham and I'm
> currently writing up my thesis on endosymbiont infections in spiders.
>
> When I was on the hunt for some good pictures of the sheet-web spider
> Linyphia triangularis I found a nice photo of female (Linyphia
> triangularis 2, Saxifraga-Ab H Baas.jpg, Linyphia triangularis
> #03992).
> I downloaded the low-resolution photo to include it in my thesis. I
> hope that is in line with your terms and conditions.
>
> The thesis will eventually be uploaded to the uni's ethesis system and
> will be freely accessible. I obviously would include the
> photographer's name as the copyright holder, as stated in your terms
> and condition, and also include the address of the Saxifrage website
> to the thesis.
>
> I do not intend to make any financial profit from this. Please let me
> know if you are not happy with me using this photo.
>
> Thank you very much in advance and have a good evening.
> Antje Hundertmark

1

Permission for the use of the photo in Figure 14, centre and right.

Antje Hundertmark

From:	
Sent:	21 March 2019 21:48
To:	Antje Hundertmark
Cc:	
Subject:	Re: Use of photos in PhD thesis

Dear Antje

Please go ahead and use any images of the species you like. You are welcome to cite our website. You are also welcome to send a link to your thesis once uploaded. Seems like an interesting topic.

Good luck with your thesis!

Jørgen Lissner

Hjemmeside: www.jorgenlissner.dk
ResearchGate: https://www.researchgate.net/profile/Jorgen_Lissner2
Danmarks Edderkopper: http://www.danmarks-edderkopper.dk

Fra: "Antje Hundertmark" <Antje.Hundertmark@nottingham.ac.uk>
Til: "Jørgen Lissner"
Sendt: torsdag, 21. marts 2019 21:57:52
Emne: Use of photos in PhD thesis

Dear Jørgen and Nikolaj.

I'm a final year PhD student at the University of Nottingham and I'm currently writing up my thesis on endosymbiont infections in spiders.

When I was on the hunt for some good pictures of the sheet-web spider Linyphia triangularis. I found two nice photos of a male (https://www.danmarks-edderkopper.dk/lissner/Linyphia_triangularis_han_1888.jpg, https://www.danmarks-edderkopper.dk/lissner/Linyphia_triangularis_han_1043.jpg) and wondered if you would approve of me using these pictures in my thesis.

The thesis will eventually be uploaded to the uni's ethesis system and will be freely accessible. I obviously would include your names as the copyright holders and also include the address of the Danmarks Edderkopper website into the thesis.

I do not intend to make any financial profit from this. The photos will only be used for the thesis and it will not be used in any potential future publications that may come out of the thesis.
Please let me know if you would be happy for me to use your photos.

Thank you very much in advance and have a good evening.

Antje Hundertmark
PhD Student
The University of Nottingham