

The Microbiome of Social Spiders



PhD dissertation by
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SECTION 1

Introduction

Beneath our superficial differences we are all of us walking communities of bacteria.
The world shimmers, a pointillist landscape made of tiny living beings.

- Lynn Margulis, *Microcosmos*

A short history of ancient interactions

In 1683 Antony van Leeuwenhoek discovered that we are not alone. Using an early microscope, he made the first ever observation of symbiotic bacteria when he saw “little animals” in plaque from his own teeth (reviewed in Bardell 1983). Although Leeuwenhoek’s skill with the microscope was recognized and respected in his own time, it would be many years before the importance of his discoveries was widely understood, and his “little animals” taken seriously.

In the mid 19th century, Louis Pasteur disproved the theory of spontaneous generation and founded the field of microbiology as we know it (Bordenave 2003 on Pasteur). The knowledge of bacteria quickly grew, but for a while they were mostly studied either in relation to disease and decay, or for their role in the global geochemical cycle (Dworkin 2012 on Winogradsky).

In 1878 Heinrich Anton de Bary coined the term symbiosis (“living together”) to describe, among other things, how fungi and cyanobacteria come together to form lichen (translated in Oulhen, Schulz, and Carrier 2016). At the turn of the century, several independent researchers were looking at the cells of plants and animals, and suggesting that the chloroplasts and mitochondria found there, were actually bacteria (reviewed in Sapp 1994). If true, this would mean that all eukaryotic life had originated from ancient symbioses, a theory called symbiogenesis (Mereshkowsky 1910).

Symbiogenesis was not generally accepted until the 1960’s when technology was finally beginning to catch up to theory, and Lynn Margulis published a seminal paper collecting molecular evidence for the bacterial origins of chloroplasts and mitochondria (Margulis 1967). A big point in this paper was that the organelles contained their own DNA, and had therefore once been independently reproducing lifeforms. Many researchers were still skeptical, but with the advent of DNA sequencing and Carl Woese’s rRNA-based tree of life (Woese and Fox 1977), the DNA found inside of the organelles, could finally be compared to that of free-living bacteria. Although the organelle genomes were greatly reduced in size and content (Anderson et al. 1981; Shinozaki et al. 1986), we now know that chloroplasts are indeed closely related to current-day Cyanobacteria (Bonen and Doolittle 1976), and that mitochondria are related to bacteria from the genus *Rickettsia* (Andersson et al. 1998), providing strong evidence that the symbiogenesis theory is correct.

The evidence of ancient symbiosis lies in each of our cells, but the effect of bacterial symbiosis on animal evolution did not stop there. Several other great leaps in evolution, such as the beginning of multicellular life, are believed to have been directly affected by interactions with bacteria (McFall-Ngai et al. 2013), and these symbiotic interactions – big and small – carry on today.

Classifying symbioses

Animal-bacterium symbioses come in all varieties, and are generally classified on two main axes, One describing the effect of the association (mutualist – parasitic) and one describing the interdependence of the symbiotic partners (obligate – facultative) (Fisher et al. 2017).

Some symbioses are mutually beneficial with fully co-dependent (obligate) symbiotic partners such as the pea aphid (*Acyrtosiphon pisum*) and its bacterial symbiont *Buchnera aphidicola*. The aphid subsists on plant sap which is high in carbs but low in certain essential amino

acids, and it owes its success to the obligate symbiont which lives inside specialized cells and produces these amino acids (Akman Gündüz and Douglas 2012). In return, *Buchnera* gets to live in a protected environment where it is supplied with nutrients. Questions have been raised about how beneficial this truly is for the symbiont (Garcia and Gerardo 2014), but at this point, the genome of *Buchnera aphidicola* is so reduced that it cannot live outside its host (Gil et al. 2002).

Other animal-bacterium symbioses are commensal, meaning beneficial for one partner (usually the bacterium) and irrelevant for the other. The typical example being many of the gut bacteria of humans where symbiosis is facultative and both partners can get by without the other. In some instances, gut bacteria are so transient that it is discussed whether the relationship even counts as a symbiosis (Hammer et al. 2019). This is the case with several moth and butterfly species, in which the caterpillar gut microbiomes contain no specific members and seem to be made up entirely by bacteria from the caterpillars' diet (Staudacher et al. 2016). Other caterpillars show no response to antibiotics treatment (Phalnikar et al. 2019), meaning their gut bacteria must be truly commensal, if they are symbionts at all.

The de Bary definition of symbiosis (Oulhen et al. 2016), also includes relationships between animals and parasitic bacteria. Although they can make their host sick, parasitic bacteria are often dependent on their animal host for growth. Chlamydia, for example are obligate intracellular parasites, and cannot replicate outside of a host cell (Jones et al. 2015), so from these bacteria's perspective, the symbiosis is obligate (Fields et al. 2011).

Symbioses are not always easily classifiable. Even between the same two partners, the costs and benefits experienced are highly dependent on circumstance. Many commensal or even mutualistic gut bacteria become parasitic if they colonize other places than the gut, as exemplified by the human symbiont *E. coli* which is harmless in the gut, but causes disease if it gets into the urinary tract or bladder (Mazumdar et al. 2006). In other cases, the net fitness effect of a certain symbiont on a certain host may change with the diet of the host (Whon et al. 2017), with the predator pressure on the host (Polin et al. 2015), or even with temperature (Montllor et al. 2002), making it very important to consider the context when attempting to classify a symbiosis.

Other factors to take into account when studying animal-bacterial symbiosis, are transmission and the taxonomic specificity of the symbiotic partners. These are often correlated to each other and to the degree of interdependence (Fisher et al. 2017). Intracellular animal-bacterium symbioses that are obligate for both partners, such as the aphid-*Buchnera* symbiosis, generally rely on direct symbiont transmission from parent to offspring, via the egg or in the embryo (Wilkinson et al. 2003). This is called vertical transmission, and it ensures that symbiotic partners can stay together across generational gaps. Because the symbiotic relationship is stable in the long term, vertically transmitted symbionts can co-evolve with their host, which sometimes leads to co-speciation (Baumann 2005), resulting in a very high level of host-symbiont taxonomic specificity.

Symbionts do not have to be intracellular to be vertically transmitted. Some animals have evolved behaviors that aid in transmitting symbionts to the next generation, such as the subsocial stinkbug, *Parastrachia japonensis*. In this species, mothers guard their eggs for many days after laying them, and then, with very precise timing, excrete a special mucus on the eggs just before they

hatch. This mucus contains the obligate gut symbiont *Candidatus Benitsuchiphilus tojoi*, and when the stinkbug nymphs hatch and eat the mucus, the symbiont is transmitted with very high fidelity (Hosokawa et al. 2012). However, this kind of indirect vertical transmission opens up the possibility for colonization by bacteria from the environment or from other animals (horizontal transmission), and makes co-speciation less likely (De Vienne et al. 2013).

Symbioses that depend on horizontal transmission can still be taxonomically specific. The immune system of the host can be highly selective (Ryu et al. 2010), allowing only certain species of bacteria to colonize. The specificity can also be driven by the symbiont's adaptation for the specific host, giving them a competitive advantage over other bacteria (Mandel 2010). On the other hand, not all symbioses are taxonomically specific. Some bacteria (e.g. *E. coli*) are generalists, able to colonize different host species. On the other side of the interaction, many animals rely on their microbiome providing certain functions, but do not require a specific bacterium to provide them. For example the human gut microbiome is very functionally redundant (Moya and Ferrer 2016), meaning that different symbionts can perform the same function.

Viewing animal-microbe symbiosis as relationships between pairs of organisms is a vast simplification. In reality, most animals are hosts to a whole ecosystem of different types of bacteria. The collection of microorganisms inhabiting a host is called its "microbiota" or its "microbiome". These words are often used interchangeably, but many newer publications (and this thesis) favor "microbiome" although some argue it has a broader definition (Marchesi and Ravel 2015). The microbiome includes archaea, fungi, protozoa and viruses as well as bacteria, but here the emphasis will be on the latter, since it has been the sole focus of all the papers presented in this thesis. Even when considering only the bacterial microbiome, the complexity of symbiotic interactions taking place within the body of any animal is breathtaking. Not only do all the different bacterial strains interact with the host and the host immune system in some way, they also interact with each other and with whatever environmental effects influence the host. With the introduction of next generation sequencing, research has taken a great leap in revealing the identity and diversity of microbiota in a wide range of animals (Kostic et al. 2013), but we still only have a rudimentary understanding of the full complexity of microbiomes and their functions.

The importance of microbiome research

During the last 15 years, research into the human microbiome has increased dramatically (e.g. Turnbaugh et al. 2007). This research has led directly to several advances in medicine: Infections of *Clostridium difficile* are now treated effectively with fecal transplants from healthy donors (Gough et al. 2011), and infants born by C-section are seeded with microbiome from the mother (Dominguez-Bello et al. 2016). It has also greatly improved our understanding of the normal healthy human gut microbiome (Shanahan 2012), and shows promising advances in understanding and treatment of conditions such as obesity and autoimmune disorders (Castaner et al. 2018; De Luca and Shoenfeld 2019). However, to really achieve a deeper understanding of the functional basics of animal-bacteria interactions, we need study systems that are both simpler and easier to work with (Kostic et al. 2013). Invertebrates typically have less complex microbiomes than mammals, they are

easier to handle in the lab, have much faster generation times, and can be used for more invasive studies.

Invertebrate microbiome research has also proven relevant in the (increasingly urgent) search for novel antibiotics. Several insects have been shown to harbor protective symbionts (often belonging to the bacterial genus *Streptomyces*), which produce antimicrobial compounds to combat parasitic fungi and bacteria (Chevrette et al. 2019).

Lastly, I believe there is inherent value in understanding the natural world around us, and microbiomes are a big part of it. All animals live in a world suffused by microbes, and many aspects of animal ecology, physiology, and evolution will not be fully understood without taking the microbiome and bacterial symbioses into account.

Social spiders

The social spiders *Stegodyphus dumicola* (family Eresidae) are peculiar animals. The specifics of the spiders and their ecology are summarized elsewhere in this thesis (paper II and III) and described in detail by experts (Lubin and Bilde 2007). Here I will present only a short summary emphasizing the traits that are most important in relation to microbiome research.

First and foremost, these spiders have an extremely low genetic diversity. With a species-wide nucleotide diversity (π) of 0.00048% (Settepani et al. 2017), *S. dumicola* are some of the least genetically diverse animals ever studied (arthropods have mean $\pi = 1.25\%$ (Leffler et al. 2012), and even the famously inbreeding naked mole-rat have population-level $\pi = 0.0007\%$ (Kim et al. 2011)). This alone makes *S. dumicola* interesting for microbiome research, as it might aid in distinguishing host phenotype effects caused by differences in microbiome, from those caused by host genetics. Such a scenario can also be set up in a lab using inbred lines of e.g. *Drosophila*, but laboratory rearing affects the microbiome (Staubach et al. 2013), which may confound the results. *S. dumicola* is a naturally occurring low-diversity system in which the undisturbed wild microbiome can be investigated.

The low genetic diversity of *S. dumicola* raises some questions about the ecological success of the spiders. *S. dumicola* has a wide geographical distribution (Majer et al. 2013), covering several climatic zones in Southern and Central Africa (Peel et al. 2007), but how are they able to adapt to such different environments, if not through genetic variation? One possibility is adaption through epigenetics, and *S. dumicola* has been shown to regulate gene expression by methylation (Liu et al. 2019). A different possibility is adaptation through microbiome. The combined microbiome usually contains more genes than its host (Qin et al. 2010), and can contribute important functions to the host phenotype, e.g. temperature tolerance and behavioral changes (Morse et al. 2012; Rohrscheib et al. 2015). These functions are more plastic than the inherent abilities of the host, since the composition of species in the microbiome is usually not fixed and the microorganisms themselves evolve at higher rates than their multicellular hosts.

Another theoretical consequence of low genetic diversity, is vulnerability to pathogens (Read 1994). In *S. dumicola* this is compounded by the close living quarters and frequent exchange of bodily fluids through communal extra-oral digestion. In other invertebrates, bacterial symbionts have been shown to protect their hosts from pathogens. The beewolf (a type of solitary wasp) has a symbiotic *Streptomyces* bacterium which produces a veritable cocktail of antimicrobial compounds that protect the wasp's egg cocoons against both fungus and other bacteria (Kroiss et al. 2010). Defensive symbioses can also work by symbionts priming their hosts immune systems so they can better withstand pathogens (Moreira et al. 2009) or just by mutualistic symbionts taking up the space and resources and outcompeting virulent pathogens (Khan et al. 2019). However, even without any intervention from beneficial bacteria, parasitic symbionts of *S. dumicola* may be under a significant selection pressure for decreased virulence (Hughes et al. 2008). The sameness of the spiders and the likely constant transmission of symbionts between spiders within a nest would mean that a highly virulent and harmful symbiont could very quickly kill the whole nest. An obligate symbiont (as most pathogens are), would then be out of hosts, and would likely also die out, unless it had some route of transmission between nests.

The transmission of symbionts between nests is probably minimal. Most of the spiders will spend their whole life in the same nest, and – because they rely on their capture webs for prey – they never have to leave. Because of their inbreeding mating system, *S. dumicola* from different nests do not blend or interact in any systematic way. The spiders show no signs of kin recognition, and will accept social spiders from other nests (and even other social species!) into their community, sharing food and everything (Seibt and Wickler 1988). However, this sort of mixing probably happens rarely in nature, and based on genetic evidence, most nests contain spiders from only a single matri-lineage (Johannesen et al. 2002). The spider's prey animals don't leave the nest either (except as dry chitin husks going no further than the ground below the nest). Other spider species have been observed in *S. dumicola* nests (Tharina Bird, personal communication), and these might act as vectors for bacterial symbionts, but this has yet to be studied.

Transmission within the nest is a different story. Not only do *S. dumicola* in the same nest share both a tight living space and digestive juices, they also practice a very high level of brood care. The brood care is collaborative, meaning that all females participate in raising the next generation, whether they are the biological mothers or not (Salomon and Lubin 2007; Junghanns et al. 2017). The care includes a special form of regurgitation feeding, in which adults feed the spiderlings a mixture of digested prey and dissolved intestinal lining, and it all ends with matrphagy when the adults give themselves up to be eaten by the new generation (Salomon and Lubin 2007; Junghanns et al. 2017). These behavioral traits likely provide ample opportunity for symbiont transmission, both within and between generations of spiders living in the same nest. In section 3 of this thesis I show preliminary findings that indicate a lack of direct vertical symbiont transmission via the eggs in *S. dumicola*. However, the intense brood care of *S. dumicola* probably leads to a type of behavior-mediated vertical transmission, similar to what happens in the stinkbug mentioned earlier. In theory this transmission of symbionts between the generations, could allow for co-evolution and result in symbiotic partners that are well adapted to each other.

The microbiome of (other) spiders

In order to provide a comparative background to my studies of the social *Stegodyphus* microbiome, I present here a small review/meta study on published spider microbiome studies. Not much work has been published on the full microbiome of spiders; I have found 22 studies using molecular methods to identify bacterial spider symbionts (not including paper II & III from this thesis), but 15 of these only screen for specific bacteria, and just 7 use amplicon sequencing with general bacterial 16S primers to identify the full spider microbiome (Table 1.1).

SCREENING FOR A SMALL GROUP OF SYMBIOTNS

Most studies on spider-bacterium symbiosis have focused on a small group of so-called endosymbionts¹: *Wolbachia*, *Rickettsia*, *Cardinium*² and *Spiroplasma*. The first three are obligately intracellular symbionts (Zchori-Fein et al. 2004; da Rocha-Lima 2015; Scola et al. 2015). They are found in a diverse selection of arthropods, and have been shown to affect the reproductive biology of their hosts in various ways (Charlat et al. 2003; Gotoh et al. 2007; Giorgini et al. 2010). While *Cardinium* belong to the bacterial phylum Bacteroidetes, *Wolbachia* and *Rickettsia* are both Proteobacteria and belong to the same order (Rickettsiales) as the ancient symbiont/organelle mitochondria. *Spiroplasma* is a more functionally diverse genus, belonging to the Mollicutes and containing both plant- and arthropod extracellular symbionts (Williamson et al. 2015). *Spiroplasma* can also affect the reproductive biology of its host, e.g. by creating a female-biased sex-ratio via male killing (Harumoto and Lemaitre 2018).

Based on a total of 24 studies (table 1.1), I have compiled an overview of the distribution of these symbionts in spiders. The data is available on GitHub (link in Fig 1.1 figure text). All four symbionts have been found across many different spider families (Fig. 1.1). *Wolbachia* in 98 out of 259 species screened, *Rickettsia* in 40 out of 147 species screened, *Cardinium* in 48 out of 119 species screened, and *Spiroplasma* in 35 out of 164 species screened (Species level plot also available in same GitHub repository). For many of the species, only one individual has been screened, so there could easily be some “false” negatives, as species with more individuals sequenced rarely show 100% prevalence. The symbionts co-occur in the same species (and in the same individual) in several instances, indicating that there is no fierce competition between them. There is not much of a phylogenetic pattern in the prevalence of the symbionts. The only thing that really jumps out from my analysis, is the high incidence of *Cardinium* in the Salticidae (the jumping spiders) (Fig. 1.1).

¹ People sometimes use the term “endosymbiont” to describe intracellular symbionts, but others use it to describe any symbiont living inside the body of a host. This ambiguity has rendered the word practically meaningless, so I will mostly refrain from using it.

² No bacteria of the *Cardinium* genus have yet been isolated, so it is technically “*Candidatus Cardinium*”. Here, I will use *Cardinium*, as others have done in contemporary publications (Li et al. 2020). Several full *Cardinium* genomes have been published, and I think that the isolation criterium is too rigid when it comes to symbionts.

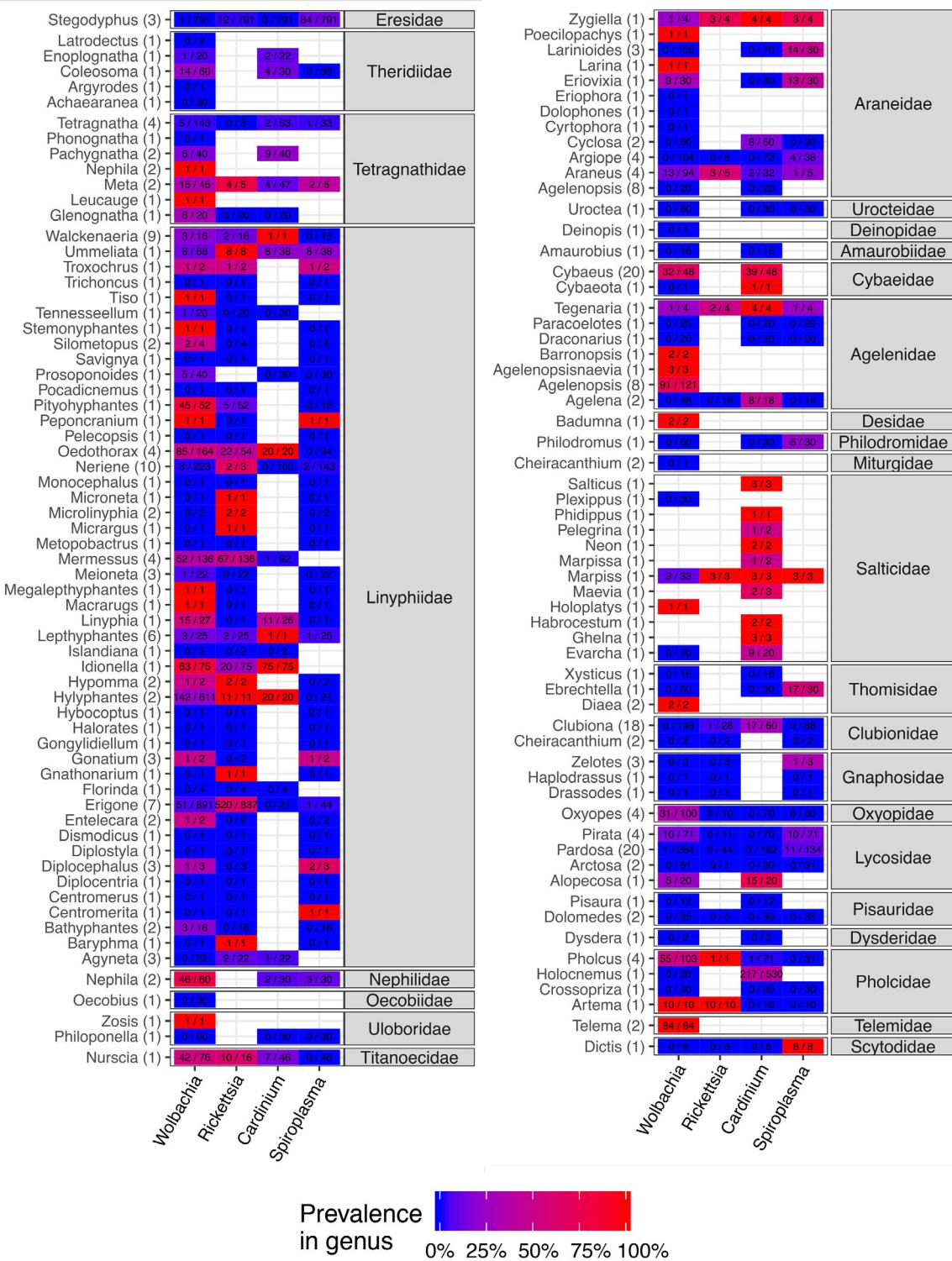


Figure 1.1 – Prevalence of Wolbachia, Rickettsia, Cardinium and Spiroplasma in spiders. Each row represents a spider genus, number in parentheses shows the number of spider species screened from each spider genus (but not all species were screened for all symbionts). The genera are grouped by family (grey boxes). Cells are colored by prevalence, and indicate number of symbiont-positive individuals/number of individuals screened. Data compiled from the 24 studies summarized in table 1.1. The data (on species level) is available here:

https://github.com/Mettetron/SpiderMicrobiome/blob/master/spider_symbionts.csv

Table 1.1 – Sequencing studies of bacterial spider symbionts.

Study	Method	n	Spider species investigated	Symbionts Investigated*
(Rowley et al. 2004)	Directed PCR	24	20 different	W
(Goodacre et al. 2006)	Directed PCR	312	119 different	W, R, S
(Duron et al. 2008)	Directed PCR	516	26 different	W, C
(Baldo et al. 2008)	Directed PCR	126	10 different	W
(Gunnarsson et al. 2009)	Directed PCR	36	<i>Pityohyphantes phrygianus</i>	W, R
(Goodacre et al. 2009)	Directed PCR	1568	<i>Erigone atra</i>	W, R
(Martin and Goodacre 2009)	Directed PCR	44	16 different	C
(Perlman et al. 2010)	Directed PCR	49	21 different	W, C
(Vanthournout et al. 2011)	Directed PCR	110	<i>Oedothorax gibbosus</i>	W
(Yun et al. 2011)	Directed PCR	560	<i>Hylyphantes Graminicola</i>	W
(Stefanini and Duron 2012)	Directed PCR	510	<i>Holocnemus pluchei</i>	C
(Jin et al. 2013)	Directed PCR	1000	37 different	W, C, S
(Curry et al. 2015)	Directed PCR	44	<i>Mermessus fradeorum</i>	W, R
(Yan et al. 2015)	Directed PCR	970	33 different	W
(Vanthournout and Hendrickx 2015)	Full16s	20	<i>Oedothorax gibbosus</i>	all
(Wang et al. 2016)	Directed PCR	84	<i>Telema cordata, Telema cucurbitina</i>	W
(Zhang et al. 2017)	Full 16s	3	<i>Marpiss magister</i>	all
(Zhang et al. 2018)	Full 16s	74	8 different	all
(Vanthournout et al. 2018)	Full 16s	6	<i>Stegodyphus dumicola, Stegodyphus mimosarum</i>	all
(White et al. 2019)	Directed PCR & Full 16s	267 (110)	14 different	W, R, C (all)
(Hu et al. 2019)	Full 16s	18	<i>Nurscia albofasciata, Pardosa astrigera, Pardosa laura</i>	all
(Sheffer et al. 2020)	Full 16s	6	<i>Argiope bruennichi</i>	all
Paper II	Full 16s	216	<i>Stegodyphus dumicola, Stegodyphus mimosarum, Stegodyphus sarasinorum</i>	all
Paper III	Full 16s	569	<i>Stegodyphus dumicola</i>	all

*W: *Wolbachia*, R: *Rickettsia*, C: *Cardinium*, S: *Spiroplasma*, all: any bacterium whose 16S rRNA gene matches the commonly used bacterial 16S PCR primers.

Some studies have looked further into the different species and strains of the symbionts, revealing a lack of phylogenetic grouping of spider-associated *Wolbachia* and *Spiroplasma* (Goodacre et al. 2006), which indicates that these symbionts are less host-specific and can be transmitted horizontally between distantly related hosts, e.g. a spider and its insect prey. On the other hand, spider-associated *Rickettsia* and *Cardinium* each form nearly monophyletic groups (Goodacre et al. 2006; Duron et al. 2008) so they might rely more heavily on vertical transmission.

Few studies have investigated the effect of these symbionts on spider phenotype, but three studies have found connections between *Wolbachia* infection and female-biased sex-ratio (Gunnarsson et al. 2009; Vanthournout et al. 2011; Curry et al. 2015), and one study tested for, but found no effect of *Cardinium* on the sex-ratio of their spider host (Stefanini and Duron 2012). In spite of their very female-biased sex ratio, social *Stegodyphus* spiders have low prevalence of *Wolbachia* and *Rickettsia*, and have no *Cardinium* at all (Fig. 1.1). A significant amount of *Spiroplasma* were present in one *S. dumicola* population (paper III), but in paper I we showed that the skewed sex ratio of *S. dumicola* and *S. mimosarum* is most likely caused by a bias in sperm production, rather than by symbionts (Vanthournout et al. 2018).

MICROBIOME COMPOSITION

Seven studies have been published on full body or gut microbiome of spiders (table 1.1). Adding results from the two unpublished studies presented in this thesis (paper II & III), 28 spider species belonging to 12 families have had their microbiome sequenced.

The first striking result from the compiled microbiome studies is that the four “usual suspects”, *Wolbachia*, *Rickettsia*, *Cardinium* and *Spiroplasma*, are not only very prevalent, but also dominate the microbiome of many species (Zhang et al. 2018), often making up 50-95% of individual relative abundance (White et al. 2019). These four are not the only symbiont genera capable of dominating spider microbiomes. Several other symbionts are found in high relative abundance across spider species and families: The same *Diplorickettsia/Rickettsiella*³ appear in both *Stegodyphus* (paper II & III) and *Glenognatha foxi* (White et al. 2019). Bacteria belonging to the Chlamydiales are found dominating individuals in two different spider species from the Linyphiidae family (Vanthournout and Hendrickx 2015; White et al. 2019), as well as in *S. sarasinorum* (paper II). Lastly, different symbionts belonging to the phylum Tenericutes and very distantly related to *Mycoplasma* and to each other, are very abundant and very prevalent in the European wasp spider (*Argiope bruennichi*) (Sheffer et al. 2020), and in two of the *Stegodyphus* species (paper II & III).

Phylogenetic analysis of some of the dominant spider symbionts group them with other Arachnid symbionts. This is the case for the *Rickettsiella* (/*Diplorickettsia*) in *Glenognatha foxi* (White et al. 2019), and in *Stegodyphus* (paper II), which is closely related to *Diplorickettsia* isolated from a tick (Mediannikov et al. 2010), but not for the Chlamydiales in the same studies or in (Vanthournout and Hendrickx 2015), which finds its closest relatives in amoeba-associates.

³ In (White et al. 2019) it is classified as *Rickettsiella*, but shares 100% sequence identity with the *Diplorickettsia* found in *S. dumicola* and *S. mimosarum* (paper II & III),

The studies generally show that individuals belonging to the same species have similar microbiome compositions (Zhang *et al*, 2018; Hu *et al*, 2019; White *et al*, 2019; paper II), but many of the studies only sample one population of each species, making it difficult to tell the difference between species similarities and population similarities. In the few cases where several populations were sampled, it is clear that microbiome composition can vary between both populations and individuals of the same spider species (Vanthournout and Hendrickx 2015; Sheffer *et al*, 2020; paper II & III), and although some studies show all sampled individuals of a spider species carrying a certain symbiont, there is no proof of any bacterial symbiont being obligate for any spider species.

Microbiome alpha diversity is generally lower in insects (mean Shannon div. < 2) than it is in mammals (mean Shannon div. > 6) (Reese and Dunn 2018), and this seems to be true for spiders as well, with mean Shannon diversities below two in most spider species (Zhang *et al*. 2017, 2018; White *et al*. 2019, paper II & III) even though some of these studies pooled several spiders into each sample. The low diversities are even more striking when considering that many of the insect and spider studies sample the whole body of individuals, while most mammal studies only sample the gut.

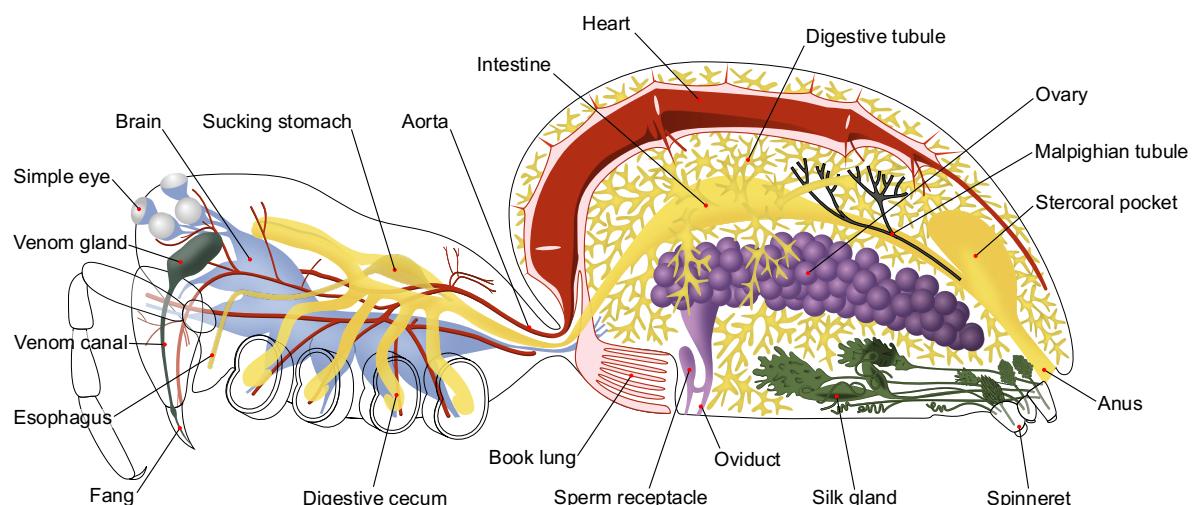


Figure 1.2 – Spider anatomy. Showing the extreme branching of the midgut.

Image from: https://commons.wikimedia.org/wiki/File:Spider_internal_anatomy-en.svg, based on original diagram by John Henry Comstock, vectorized by Ryan Wilson.

The most recently published study on spider microbiomes sequenced different body parts of their spider separately, and showed that there was no significant difference in microbiome composition (Sheffer *et al*. 2020). Similarly, many of the “endosymbiont” screening studies summarized above, only sample the legs of spiders because it has been shown that DNA extractions from legs produce the same results as DNA extractions from the abdomen (Goodacre *et al*. 2006). Symbionts being present in the legs of spiders could be an indication of an intracellular or hemolymph localization, which is consistent with such infections being much more common in arthropods than in mammals (Douglas 2011). However, the peculiar anatomy of most spiders, means that parts of their midgut often branch out into the legs (Fig. 1.2), so these results do not

rule out a gut-localization of some of the symbionts, which is indeed what we found in *S. dumicola* (paper II, but see end of Section 3).

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SECTION 2

Thesis papers

Stories are like spiders, with all they long legs, and stories are like spiderwebs, which man gets himself all tangled up in but which look pretty when you see them under a leaf in the morning dew, and in the elegant way that they connect to one another, each to each.

- Neil Gaiman, Anansi Boys

Paper I:

Male spiders control offspring sex ratio through greater production of female-determining sperm

Bram Vanthournout, Mette Marie Busck, Jesper Bechsgaard, Frederik Hendrickx, Andreas Schramm & Trine Bilde

Published in *Proceedings of the Royal Society B: Biological Sciences* (2018)

With this study we intended to identify the mechanism or cause of the female-biased sex ratio seen in social *Stegodyphus*. Certain bacterial symbionts are known to affect the sex ratio of their hosts, so we screened two 16S gene amplicon datasets for these bacterial genera. The known sex ratio distorting symbionts were neither prevalent nor abundant enough in *S. dumicola* and *S. mimosarum* to explain the female-bias in these species. Instead, using flow-cytometry, we showed a bias in sperm production of the social species, with an average of 70% of sperm cells produced by social *Stegodyphus* being female-determining.

My role in this study was to analyze the 16S rRNA gene amplicon data, make figures and tables with this data, write the microbiome sections of the methods and results and review the paper in general. The “large dataset” used in this paper is part of the Paper II dataset, for which I also did a large part of the lab work.

Paper II:

The microbiome of social spiders (Genus *Stegodyphus*): host and population-specific patterns

Mette M. Busck, Virginia Settepani, Jesper S. Bechsgaard, Marie B. Lund, Trine Bilde & Andreas Schramm.

Intended for publication in *The ISME Journal*

With this study we characterized the microbiome of social *Stegodyphus*, comparing composition in hosts at four levels: Individuals, nests, populations and species (*S. dumicola*, *S. mimosarum* and *S. sarasinorum*). Our main findings were that individual spiders tended to have low diversity microbiomes dominated by one or a few symbionts. These dominant symbionts belonged to a small but diverse group of bacteria, several of which were only distantly related to any bacteria described in the literature. Some dominant symbionts were shared between spider species and some not, the South African species (*S. dumicola* and *S. mimosarum*) being more similar, and the Indian species (*S. sarasinorum*) standing out. Spiders shared microbiomes within nests, but two nests from the same population could be very different, making it unlikely that the dominant symbionts play a big role in local adaptation.

The initial idea and the sampling for this study was already in place when I began my PhD, but I did a large part of the DNA extraction and amplicon library preparation lab work. I also designed and tested a new *Borrelia*-specific FISH (Fluorescence in situ hybridization) probe, and I did the FISH and the imaging. I analyzed the data, producing all figures, phylogenies and figures. I wrote the manuscript in collaboration with my co-authors.

Paper III:

Temporal and spatial dynamics of the social spider *Stegodyphus dumicola* microbiome

Mette M. Busck, Marie B. Lund, Tharina Bird, Jesper S. Bechsgaard, Trine Bilde & Andreas Schramm

Intended for publication in *FEMS Microbiology Ecology*

The variation in microbiome composition between spiders from closely related nests found in study II, raised questions about the stability of social spider microbiomes. With this paper, we investigated the temporal stability of both microbiomes and the spider nests themselves, focusing on *S. dumicola* which is the most extreme of the 3 social *Stegodyphus* species, both in terms of low genetic diversity and low diversity microbiome. We monitored six *S. dumicola* populations for two and a half years*, sampling every 3 months. We also had temperature and humidity loggers in many of the nests, so we could follow the local climate, but analysis of this data showed no correlation of climate and microbiome composition. The microbiome composition within nests turned out to be surprisingly stable, not even changing much with generational shifts. This supported the theory that the dominant bacterial symbionts are not virulent parasites, but live in stable relationships with their spider hosts. Something was killing our nests, however, as we kept having to include new nests in our study to keep a representative number of replicates. This led to an analysis of the spider nest turnover, showing ~50% yearly survival rate. We also used qPCR to estimate the bacterial load experienced by the spiders, and found that this was mostly stable, except for some cases where the spiders would experience an extreme increase in bacterial load within the months before nest death. There was a clear correlation between nest death and bacterial load, but no correlation with either of these and microbiome composition, indicating that the increase in bacterial load might be an effect of a weakened nest, rather than the initial cause of it.

I played a big role in the idea and planning phases of this study, basing the methodology on our findings in study II. I did not participate in field work for this specific study, but I did go to Namibia and visited several of the same *S. dumicola* populations to collect samples for other studies. I kept track of 1000+ sampled spiders, prioritizing which were most important for the different analyses. I analyzed all the data, continuously collaborating with co-workers to choose the best figures and statistics. I wrote the draft manuscript with input from the co-authors.

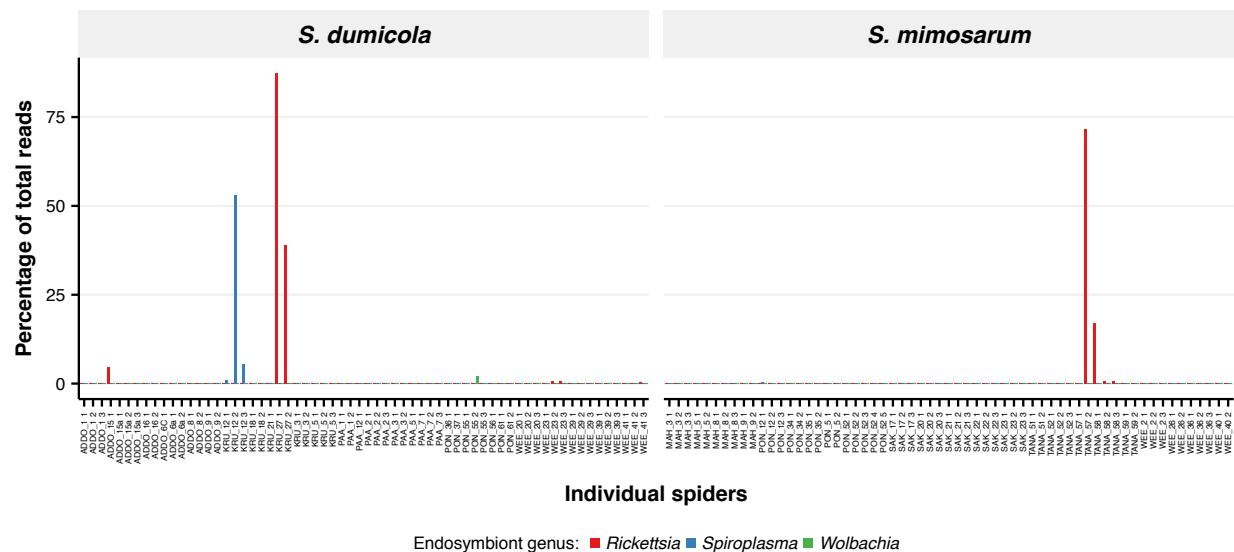
*NOTE: The version of paper III presented in this thesis lacks microbiome data from the last three sampling trips. At the time of writing, this is currently being sequenced and will be incorporated before publishing (and maybe presented at the defense).

Paper I

Male spiders control offspring sex ratio through greater production of female-determining sperm

Bram Vanthournout, Mette Marie Busck, Jesper Bechsgaard, Frederik Hendrickx,
Andreas Schramm & Trine Bilde

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Research



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Male spiders control offspring sex ratio through greater production of female-determining sperm

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Sex allocation theory predicts that when sons and daughters have different reproductive values, parents should adjust offspring sex ratio towards the sex with the higher fitness return. Haplodiploid species directly control offspring sex ratio, but species with chromosomal sex determination (CSD) were presumed to be constrained by Mendelian segregation. There is now increasing evidence that CSD species can adjust sex ratio strategically, but the underlying mechanism is not well understood. One hypothesis states that adaptive control is more likely to evolve in the heterogametic sex through a bias in gamete production. We investigated this hypothesis in males as the heterogametic sex in two social spider species that consistently show adaptive female-biased sex ratio and in one subsocial species that is characterized by equal sex ratio. We quantified the production of male (0) and female (X) determining sperm cells using flow cytometry, and show that males of social species produce significantly more X-carrying sperm than 0-sperm, on average 70%. This is consistent with the production of more daughters. Males of the subsocial species produced a significantly lower bias of 54% X-carrying sperm. We also investigated whether inter-genomic conflict between hosts and their endosymbionts may explain female bias. Next generation sequencing showed that five common genera of bacterial endosymbionts known to affect sex ratio are largely absent, ruling out that endosymbiont bacteria bias sex ratio in social spiders. Our study provides evidence for paternal control over sex allocation through biased gamete production as a mechanism by which the heterogametic sex in CSD species adaptively adjust offspring sex ratio.

1. Introduction

Equal production of male and female offspring is generally considered an evolutionarily stable strategy [1,2], however, when sons and daughters have different reproductive values, sex allocation theory predicts parents to adjust offspring sex ratio towards the sex with the higher fitness return [3–6]. While adaptive sex ratio bias is well understood in haplodiploid systems, species with chromosomal sex determination (CSD) have been presumed to be constrained by Mendelian segregation in their ability to strategically adjust sex ratio [3]. There is accumulating evidence against this idea [7,8], but we still have limited understanding of possible sex biasing mechanisms that are mainly limited to theoretical hypotheses (such as steroid and glucose levels [9–14]), and almost no empirical evidence for mechanisms through which CSD species with adaptive sex ratio bias exert control over offspring sex. One hypothesis proposes that control over offspring sex is more likely to evolve in the heterogametic sex through a bias in the production of gametes [15,16].

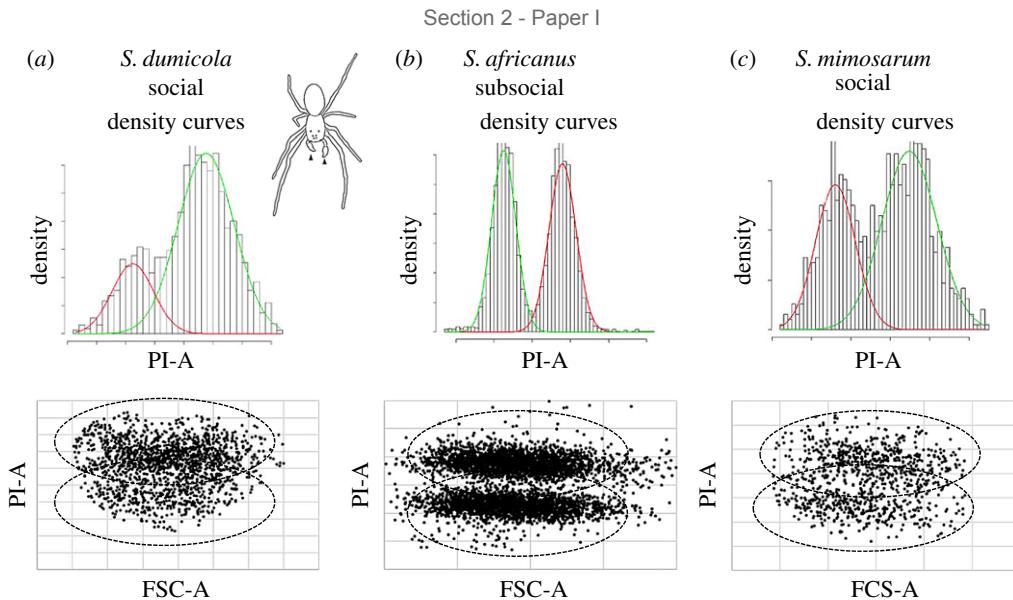


Figure 1. Dot plot of propidium iodide-stained sperm nuclei (PI-A, corresponding to DNA content) and forward scatter of the nucleus (FSC-A, corresponding to particle size) + PI-A histogram, isolated from one pedipalp of one social *S. dumicola* (a), subsocial *S. africanus* (b) and social *S. mimosarum* (c) male. For *S. dumicola*, a graphical representation of a male spider, with pedipalps indicated by arrowheads, is added. Circles indicate populations of X-sperm (top circle) and 0-sperm (bottom circle). Circles are used for illustrative purposes only and are not used in estimating density curves. Estimated proportion of X-sperm for the three individual males is 0.77, 0.51 and 0.63, respectively. Sperm sample sizes consist of 1620 (*S. dumicola*), 5049 (*S. africanus*) and 959 (*S. mimosarum*) sperm nuclei. (Online version in colour.)

A striking example of CSD species with highly female-biased sex ratio is found in social spiders, which are cooperative breeders that live in permanent groups with an obligatory inbreeding mating system [17–20]. Social spider groups are usually composed of around 85% females [17], and female bias is considered adaptive by reducing competition for fertilization between brothers (local mate competition [5]) and/or increasing the number of females as the helping sex (local resource enhancement [2,4,6,21,22]). However, the mechanism of sex ratio bias is unknown. By contrast, closely related subsocial species that cooperate in the juvenile stage but disperse prior to reproduction show equal sex ratios [17]. In most spiders, sex is determined by an X0 sex chromosome system where males are heterogametic with one copy of the X chromosome (X0) and females are homogametic (XX) [23]. Males produce two types of sperm cells, female-determining with X chromosomes (X-sperm) and male-determining without X chromosomes (0-sperm). These sperm types are expected to be produced in equal numbers after Mendelian segregation during meiosis. However, a bias towards X-sperm in social spiders would produce more female offspring and thereby function as a mechanism for adjusting offspring sex ratio. To test this hypothesis, we used flow cytometry to quantify the proportion of X- and 0-sperm cells from two social spider species of the genus *Stegodyphus*; *Stegodyphus dumicola* and *Stegodyphus mimosarum* with female-biased sex ratio [17]. We also included one subsocial species *Stegodyphus africanus* with equal sex ratio [24] as control.

Female-biased sex ratio in arthropods, including spiders, can also occur through inter-genomic conflict caused by infection with certain endosymbiotic bacteria ([25–32], but see [33]). The effect of a cytoplasmic distorter on offspring sex ratio can vary with environmental (i.e. temperature) and genetic (i.e. host suppression) effects that influence the bacteria's abundance [34]. To control for potential confounding effects caused by endosymbiont bacteria, we screened the microbiome of the two social species (*S. dumicola*, *S. mimosarum*) for the five endosymbiont genera known to be able to cause female-biased sex

ratio, i.e. *Wolbachia*, *Rickettsia*, *Candidatus Cardinium*, *Spiroplasma* and *Arsenophonus*.

2. Methods

(a) Biased X-sperm proportion

(i) Flow cytometry

We quantified the proportion of male- (0-sperm) and female-determining sperm (X-sperm) using flow cytometry. Using a DNA stain, flow cytometry allows the automated determination of the DNA content of thousands of sperm nuclei, based on fluorescence intensity [35,36]. Males from two social species (*S. mimosarum* and *S. dumicola*) and one subsocial species (*S. africanus*, sister species to *S. mimosarum* [37]) were used. Spiders load sperm into reproductive organs called pedipalps, which are used for external transfer of sperm to the female sperm storage organ [38] (pedipalps are indicated by arrowheads in figure 1). Sperm present in the pedipalp, therefore, represents the male ejaculate. Both live males and males that were stored at -80°C were included (see the electronic supplementary material, table S1 for sample details). Previous analysis revealed that, next to haploid sperm cells, substantial amounts of diploid cells are also present in the pedipalp [39]. In order to correctly identify sperm cells we, therefore, included a leg sample where diploid somatic cells are present but haploid sperm cells are absent (electronic supplementary material, figure S1). DNA of the isolated nuclei was stained with propidium iodide (PI) using the protocol described in Vanthournout *et al.* [39] (adapted from [40] and [41]). Preparations were stored at 4°C for up to 2 h and protected from light using tin foil. DNA-content analysis of prepared nuclei was performed on a BD Biosciences FACStarflow cytometer and Fortessa (Argon laser emitting at 488 nm).

(ii) Data analysis

We visualized the PI intensities (representing DNA amount) as a function of the forward scatter (FSC) (representing cell size) using FCS Express 6 (DeNovoSoftware). We manually selected populations representing sperm nuclei (see the electronic supplementary material), and exported the data into a text file. We

observed a positive correlation between FSC and PI intensity, and corrected the PI intensity values according to the strength of the correlation (for sperm scatter plots, see data accessibility section). We estimated the proportion of X- and 0-sperm using the normalmixEM function (with the following specifications: $k = 2$, mean.constr. = NULL, sd.constr = NULL) in R. This function allows us to fit two normal distributions (representing 0-sperm and X-sperm) to the PI intensity [42] and hence provides estimates of sperm proportions. This ensures an objective approach by taking into account potential overlap in X- and 0-sperm populations. We bootstrapped the PI intensities from each sample 100 times, and ran the normalmixEM on the bootstrapped datasets to obtain confidence intervals (CIs). Because this approach produces point estimates of sperm proportions, these are not expected to follow a binomial distribution. Indeed, normality tests (PROC UNIVARIATE, SAS v. 9.4, SAS Institute Inc. (2002–2012)) indicated that the distribution of the point estimates did not deviate significantly from a normal distribution (see the electronic supplementary material). For this reason, we performed a one-sample *t*-test (assuming normal distribution, PROC TTEST, SAS v. 9.4, SAS Institute Inc. (2002–2012)) on the point estimates to test whether sperm ratios within a species are significantly different from 0.5. A generalized linear model (assuming normal distribution, PROC GLM, SAS v. 9.4, SAS Institute Inc. (2002–2012)) was used to analyse the differences in X-sperm production between species. If two pedipalps were analysed of the same male, the average proportion was used.

(b) Microbiome

(i) Small dataset: Ion Torrent

16S rRNA amplicon libraries were prepared for three adult females from each of the social spider species *S. mimosarum* and *S. dumicola*, along with one individual of the solitary dwarf spider *Oedothorax retusus* originating from a laboratory-reared line that is confirmed to be infected with *Wolbachia*, *Rickettsia* and *Ca. Cardinium* [26]. DNA was extracted from whole spiders using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. 16S rRNA gene amplicon libraries of variable region V4 were prepared according to Life Technologies protocol (Ion Plus Fragment Library Kit), using primers Univ519F and Univ802R [43,44], and sequenced on an Ion Torrent PGM (Life Technologies) (average read length = 285 bp). Raw sequence reads were quality-screened using fastQC software v. 0.96 (Babraham Bioinformatics), trimmed to 260 bp and quality filtered and clustered with usearch v. 8.1.1861 and the UPARSE pipeline [45]. Operational taxonomic units (OTUs) were generated based on a high-quality subset of the data (maxee = 1.0), and the remaining reads were mapped onto the obtained OTUs with a 97% similarity cut-off. OTUs were classified to genus level using mothur v. 1.36.1 [46] with the Silva SSU Ref NR release 123 database as reference [47].

(ii) Large dataset: MiSeq

To increase the small sample size of microbiome data, a larger-scale sampling of the social spiders was also screened for sex ratio distorting endosymbionts. Adult female *S. dumicola* ($n = 64$) and *S. mimosarum* ($n = 61$) were collected from six populations across South Africa and Madagascar in April–June of 2012. Several nests were sampled from each population, and several individuals were sampled from each nest (see the electronic supplementary material for sample details). The spiders were placed in animal tissue lysis buffer (Qiagen) and frozen in the field. Whole spider DNA extraction was performed as described above. 16S rRNA gene amplicon libraries of variable regions V3 and V4 were prepared according to Illumina's 16S Metagenomic Sequencing Library Preparation guide, using Bac 341F and Bac 805R primers

[48] and sequenced on a MiSeq desktop sequencer (Illumina). Sequence analysis, OTU clustering and taxonomic classification was done using mothur v. 1.39.0 [46], with Silva SSU NR release 128 as a reference [47]. The analysis protocol is available at https://github.com/ianpgm/AU_microbio_16S_protocol.

Further data analysis of both datasets was done in R v. 3.3.1 [49] using in-house scripts. The data were filtered to exclude non-bacteria and OTUs classified as common laboratory contaminants [50]. The OTU lists were screened for five endosymbiont sex ratio distorters: *Wolbachia*, *Rickettsia*, *Ca. Cardinium*, *Spiroplasma* and *Arsenophonus*. Results of the screenings were plotted using the R package ggplot2 [51].

3. Results and discussion

(a) Biased X-sperm proportion

We screened the sperm of males of two social species *S. dumicola* ($n = 8$) and *S. mimosarum* ($n = 9$, with female bias), and one subsocial species *S. africanus* ($n = 7$). In the dot plot of PI-stained sperm nuclei (corresponding to DNA content) and FSC of the nucleus (corresponding to particle size), originating from a single pedipalp, two populations of sperm nuclei are visible based on DNA content (figure 1). The lower population consists of 0-sperm nuclei (lower amount of DNA), while the upper population consists of X-sperm nuclei (higher amount of DNA). This corresponds to the left (0-sperm) and right (X-sperm) peaks of the PI-histograms in figure 1. Males of the two social species both produced significantly higher numbers of X-sperm nuclei compared to 0-sperm: *S. dumicola*: 0.70 ± 0.07 ; ($t_7 = 7.85$, $p < 0.0001$) and *S. mimosarum*: 0.68 ± 0.12 ; ($t_8 = 4.47$, $p = 0.001$). For males of the subsocial *S. africanus* a lower, yet significant, bias was found: 0.54 ± 0.03 ($t_6 = 4.01$, $p = 0.004$; figure 2). *Stegodyphus africanus* is an outcrossing species with pre-mating dispersal, and we, therefore, expected unbiased sperm cell production. This bias was significantly lower compared to the social species (generalized linear model: $F_2 = 7.57$, $p = 0.003$). Our results indicate substantial variation in sperm ratio between males, even originating from the same colony (figure 2).

Our result of an average bias in X-sperm of *S. dumicola* males of 70% corresponds with the primary sex ratio bias found by karyotyping eggs from a single nest, where female embryos ranged from 68 to 92%, with a median of 83% [52]. While our data provide evidence for direct paternal influence over sex ratio, we cannot rule out the possibility of additional maternal influence on offspring sex ratio, as spider sperm is transferred in an inactive state, stored and subsequently activated in the female tract. This leaves ample opportunity for female control [53] and makes it unlikely that other factors such as difference in motility between X- and 0-sperm play an important role.

In mammals, several studies found bias in the proportion of X- and Y-sperm produced by a male ([54,55] and 19 studies reviewed in [16]), while to our knowledge, only three studies exist that link a bias in X/Y sperm proportion (either directly through fluorescence *in situ* hybridization of sperm cells [56] or indirectly using sperm nucleus area as a proxy [57]) or differences in male fertility [58] to offspring sex ratio. However, the potential adaptive function of sex ratio bias is not well understood. By contrast, female-biased sex ratio in social spiders applies across populations and species and is considered adaptive [17,18]. Social spiders have an obligatory

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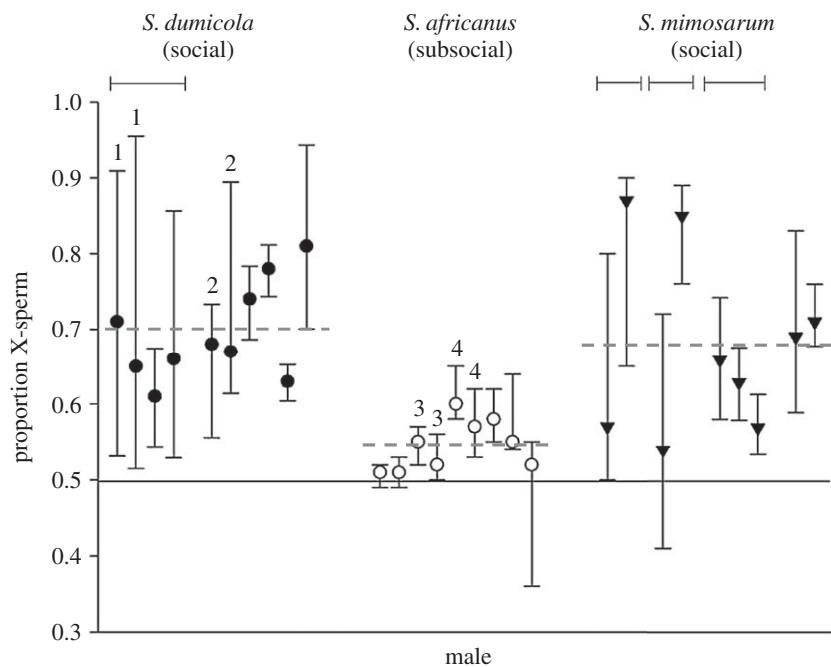


Figure 2. Proportion of X-sperm in the pedipalps of social *S. dumicola* and *S. mimosarum* males and subsocial *S. africanus* (average \pm 95% CI). Striped lines depict the average proportion for each species and the solid line depicts an equal sperm ratio. Data points indicated with the same number originate from two pedipalps (left and right) of the same male. Bars indicate males originating from the same nest.

inbreeding mating system and as a consequence, extraordinary high homozygosity and almost no genetic variation [20]. This social spider system can, therefore, be viewed as an extended version of the local mate competition model that predicts increased allocation to daughters, to reduce competition between highly related males within the nest over access to females over multiple generations (haystack model) [2,19,59]. Similarly, the local resource enhancement model [6] predicts allocation to daughters as the helping sex in social spiders, where females cooperate in tasks such as web building, prey capture and brood care [21,22]. Both of these explanations imply that females and males have aligned interests over the production of daughters. It is, therefore, unlikely that sexual conflict over offspring sex ratio exists in this system, as is frequently the case in other species [60].

Biased sperm production in arthropods is often associated with sex chromosome meiotic drive, in which males produce a biased proportion of sperm types because the driving sex chromosome is over-represented [61–63] (several *Drosophila* species, stalk eyed flies, mosquitos, etc.). Driving loci are considered as examples of selfish genetic elements rather than a mechanism of adaptive sex ratio adjustment. Our results are highly consistent with the actions of X chromosome meiotic drive. Although there is no sex chromosome towards which the driving X chromosome can be directed (X0 males in spiders), meiotic drive has been recorded in a nematode with a similar X0 sex chromosome system and was suggested as a way to control offspring sex ratio [64]. Furthermore, meiotic drive is hypothesized to play a role in the facultative adjustment of sex ratio in birds, where females are the heterogametic sex [65] and in the adult sex ratio in tetrapods [66]. The evolution of meiotic drive could lead to species extinction and should be strongly selected against [63]. However, it is possible that an incomplete suppressor of the meiotic drive action has evolved in social spiders. Further investigations into the cytological characteristics of spermatogenesis, identification of genomic regions involved in meiotic drive and the construction of a

suitable genetic model are needed to establish the presence of a meiotic drive system. If confirmed, this would be a striking example of how a selfish genetic element can be recruited to function as a mechanism to adjust sex ratio adaptively [64].

(b) Microbiome

(i) Small dataset: Ion Torrent

The screening of six social spiders and one positive control revealed 509 bacterial OTUs. The number of reads classified as known sex ratio distorting endosymbionts are presented in table 1, along with total read numbers for each sample. No OTUs were classified as *Spiroplasma* or *Arsenophonus*, indicating that these bacteria were not present. *Rickettsia*, *Wolbachia* and *Ca. Cardinium* were each represented as a single OTU, and only showed high abundance (25–48% of total reads) in the positive control sample of *Oedothorax retusus*, a dwarf spider previously shown to be infected with these bacteria, causing a female bias [26]. In the social *Stegodyphus* species, these endosymbionts were present in a very low number of reads and never exceeded 0.02% of the total reads; this minimal detection might result from contamination by ingesting infected prey or by misassignments during de-multiplexing [67].

(ii) Large dataset: MiSeq

This larger screening confirmed the absence of OTU's classified as *Ca. Cardinium* or *Arsenophonus* in this dataset. More than half of all social spiders sampled were completely free of sex ratio distorting endosymbionts (*S. mimosarum* 33 out of 61, *S. dumicola*: 45 out of 64; figure 3). In infected individuals, endosymbionts are generally found at very low abundances, only comprising more than 1% of total reads in eight individuals (figure 3). Of those eight, three *S. dumicola* and two *S. mimosarum* stand out, having clear infections of either *Rickettsia* or *Spiroplasma*. Detailed data are available in the electronic supplementary material, table S2.

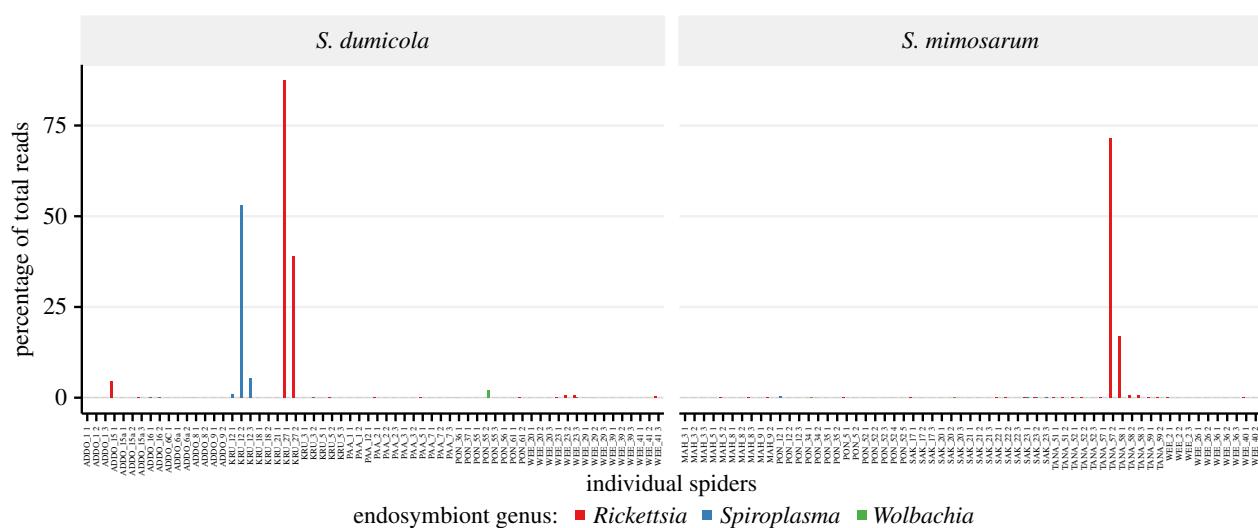


Figure 3. Percentage of total 16S rRNA gene amplicon reads assigned to *Rickettsia*, *Spiroplasma* and *Wolbachia* endosymbionts in individuals of social *S. dumicola* and *S. mimosarum*. Localities are indicated with MAH (Madagascar, Mahavanana), SAK (Madagascar, Isalo National Park), TANA (Madagascar, Antananarivo), PON (South Africa, Pongola Game Reserve), WEE (South Africa, Weenen Game Farm), KRU (South Africa, Kruger National Park), ADDO (South Africa, Addo), PAA (South Africa, Paarl). First number indicates colony number while second number is the number of the individual spider, belonging to the respective colony.

Table 1. Counts of OTUs classified as known sex ratio distorters in positive control *Oedothorax retusus* and social species *Stegodyphus dumicola* and *Stegodyphus mimosarum*.

	control		social				
	<i>O. retusus</i>	<i>S. dumicola</i>	<i>S. dumicola</i>	<i>S. dumicola</i>	<i>S. mimosarum</i>	<i>S. mimosarum</i>	<i>S. mimosarum</i>
<i>Rickettsia</i>	3641	0	3	0	6	0	1
<i>Wolbachia</i>	6985	3	4	0	3	0	1
<i>Candidatus Cardinium</i>	3721	0	1	0	2	0	0
<i>Spiroplasma</i>	0	0	0	0	0	0	0
<i>Arsenophonus</i>	0	0	0	0	0	0	0
total reads	14 495	17 788	15 154	51 631	27 799	11 983	7969

Because of differences in library preparation methods and sequencing platforms, the data from the two sequencing experiments are not directly comparable. However, both datasets point to the same conclusion: sex ratio distorting endosymbionts are not responsible for sex ratio bias in social *Stegodyphus*.

4. Conclusion

Our results show the production of more female- than male-determining sperm cells as the likely mechanism underlying female-biased sex ratio in two social *Stegodyphus* spider species. This supports the hypothesis of sex ratio adjustment through a bias in gamete production in the heterogametic sex. We also show that five common genera of bacterial endosymbionts known to affect sex ratio are largely absent, ruling out that endosymbiont bacteria influence sex ratio bias in social spiders. In CSD species, offspring sex ratio adjustment is often expected to occur in the female reproductive tract at fertilization, suggesting female control [11]. Our study instead lends support for the heterogametic sex—here males—to

evolve the ability to strategically bias sex ratio in a low cost manner by skewing gamete production [15,16].

Ethics. Research on the species in this study does not require ethical licences.

Data accessibility. Flow cytometry data have been uploaded onto FlowRepository (<http://flowrepository.org/id/RvFr4wSgP6tERF9mx-pOW30F941OaQDY73ZVArWWoQzG3eCocpmvKWPmnytoSiUZT8>).

EXCEL files containing the FSC-A and PI-A data for the gated sperm nuclei populations per individual spider can be found in the Dryad repository: <http://dx.doi.org/10.5061/dryad.r3206> [68]. Source data for figure 2 can be found in the electronic supplementary material, table S1. Microbiome sequence data are available at NCBI, Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) accession numbers SRP130747, SRP130740 and SRP130742.

Authors' contributions. All authors contributed to the design of the study and to writing of the manuscript. B.V., J.B., F.H. and T.B. performed the flow cytometry experiment and analysed the data, microbiome screening and data analysis was performed by M.M.B. and A.S.

Competing interests. We declare we have no competing interests.

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Section 2 - Paper I

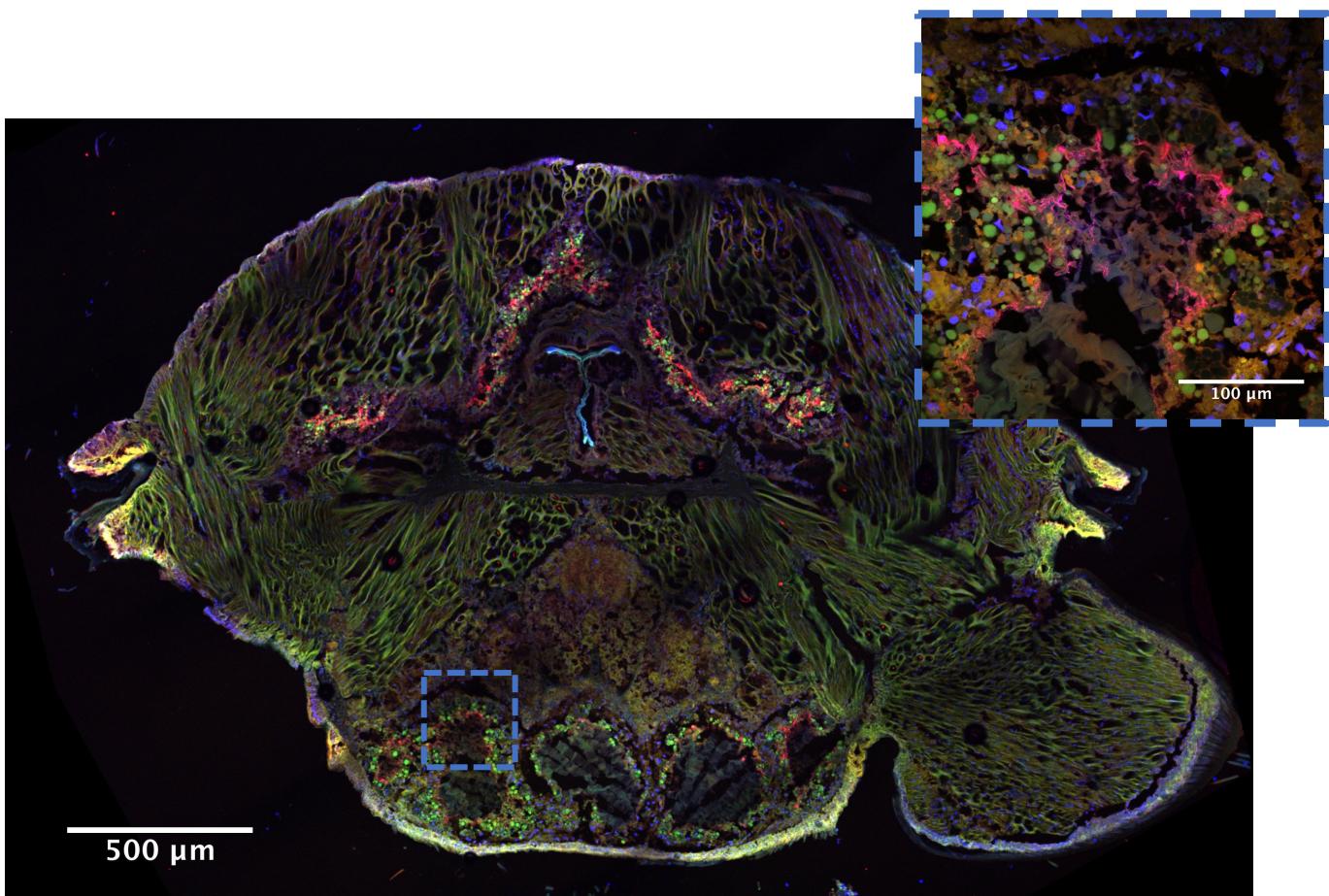
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Paper II

The microbiome of social spiders (Genus *Stegodyphus*): host and population-specific patterns

Mette M. Busck, Virginia Settepani, Jesper S. Bechsgaard, Marie B. Lund, Trine Bilde & Andreas Schramm.

Intended for publication in *The ISME Journal*



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2 **The microbiome of social spiders (Genus *Stegodyphus*): host and**
3 **population-specific patterns**

4

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14 ABSTRACT

15 Social spider species of the genus *Stegodyphus* have remarkably low species-wide
16 genetic diversities, providing a suitable system for investigating whether their
17 microbiome potentially facilitates host adaptive responses. Here we establish the host
18 specific microbiome and its patterns of variation within and between populations. We
19 characterized the bacterial community in populations of three species of social
20 *Stegodyphus* by 16S rRNA gene sequencing. The microbiomes differed between host
21 species, showed low diversity (Shannon index 0.5-1.7), strong dominance of one out
22 of 15 amplicon sequence variants (ASVs) in single spiders (McNaughton's
23 dominance index, DMN=0.68-0.93), and a core microbiome consisting of five (*S.*
24 *dumicola* and *S. mimosarum*) or seven (*S. sarasinorum*) distinct ASVs. The most
25 abundant and prevalent ASVs, classified as Chlamydiales, *Borrelia*, and *Mycoplasma*,
26 represent novel, presumably *Stegodyphus*-specific lineages; *Borrelia* and *Mycoplasma*
27 were shown by fluorescence in situ hybridization (FISH) to colonize the spider
28 midgut. Individual spider microbiomes were similar within a nest, differed between
29 nests of the same population, and differed slightly more between populations. A
30 population-specific microbiome pattern was found in the Indian species *S.*
31 *sarasinorum*, and microbiome dissimilarity between nests correlated with their
32 geographical distance. The two South African species showed hardly any population
33 pattern. The lack of strong population-specific patterns in the microbiome may arise
34 from random forces such as drift-like processes, which could hamper processes of
35 microbiome-facilitated responses to the local environment.

36 INTRODUCTION

37 Microbiomes are ubiquitous in the animal world and can provide their hosts with
38 beneficial and sometimes essential functions like energy, nutrition, or protection

39 against pathogens (McFall-Ngai *et al.* 2013). If the microbiome composition varies
40 among host individuals and provides habitat-specific beneficial functions,
41 microbiomes may contribute to local host adaptation (Henry *et al.* 2019). The
42 composition and distribution of microbiomes among host individuals and populations
43 may therefore provide insights into the potential of the microbiome to facilitate
44 adaptive responses to the local environment. Depending on the transmission fidelity
45 of the microbiome and the origin of microbiome variation, such symbiont-mediated
46 mechanisms of host adaptation can be classified as either evolutionary (inherited),
47 plastic (environmentally induced) or transgenerational plastic (environmentally
48 induced and transmitted across a number of generations) (Kaltenpoth and Steiger
49 2014; Zolnik *et al.* 2016; Näpflin and Schmid-Hempel 2018; Rock *et al.* 2018;
50 Scanlan 2019; Vujanovic, Islam and Daida 2019). A significant role in host
51 adaptation predicts population-specific patterns with host individuals from the same
52 population carrying more similar microbiomes compared to host individuals from
53 different populations. However, random forces such as low transmission fidelity and
54 drift-like processes caused by host population dynamics may cause a less structured
55 pattern of microbiome distribution within and across populations. The biotic and
56 abiotic factors that shape the diversity distribution of the microbiome within
57 individuals and populations remain poorly understood (Adair and Douglas 2017;
58 Reese and Dunn 2018), although they have important implications for our
59 understanding of the functional significance of the microbiome in host adaptation.

60 We propose that symbiont-mediated adaptations may be particularly important
61 in facilitating adaptive host responses in species with low genetic diversity (Settepani
62 *et al.* 2017), since the possibility of evolutionary responses based on standing genetic
63 variation of such hosts is reduced (Ørsted *et al.* 2019). The spider genus *Stegodyphus*

64 (family Eresidae) represents a unique opportunity for assessing the assumptions
65 underlying this hypothesis by establishing microbiome composition and distribution
66 within and between host populations. The Genus contains three species (*S.*
67 *sarasinorum*, *S. mimosarum*, *S. dumicola*) that evolved social behavior independently
68 (Figure 1A) (Johannesen *et al.* 2007; Settepani, Bechsgaard and Bilde 2016), harbor
69 extraordinarily low genetic diversity (Settepani *et al.* 2017), and exist across large
70 geographic and climatic distances (Figure 1B), suggesting that adaptative responses
71 could occur by other means than evolutionary responses based on host genetic
72 variation.

73 These social spiders live in nests of up to 1000 individuals and cooperate on
74 web building, prey capture and consumption, and brood care (Lubin and Bilde 2007).
75 They have a highly female-biased sex ratio (Vanthournout *et al.* 2018) and a
76 reproductive skew with less than half of the females reproducing (Salomon, Mayntz
77 and Lubin 2008). Mothers and non-reproducing female helpers (allomothers) practice
78 a special form of regurgitation feeding, in which they feed the spiderlings a mixture of
79 digested prey and dissolved intestinal lining (Junghanns *et al.* 2019). When the
80 spiderlings are old enough to begin capturing prey, they consume the adult females
81 (matriphagy), and subsequently mate and reproduce with their siblings within the nest
82 (Lubin and Bilde 2007). Dispersal is rare and happens either by nest fission or by
83 single, mated females using silk strands to “balloon” over longer distances (Schneider
84 *et al.* 2001).

85 The combination of female-biased sex ratio, reproductive skew, and
86 inbreeding ('social syndrome') leads to extraordinary low levels of genetic diversity
87 within spider nests and populations but also to enhanced drift due to the very low
88 effective population size (Settepani *et al.* 2017). This should theoretically result in

89 drift-induced differentiation between populations. However, genetic diversity is also
90 extremely low between populations, which has been explained by the homogenizing
91 effect of recurrent extinction-recolonization events, i.e., a population dynamics with
92 rapid nest turnover and long-distance dispersal by the ballooning females (Settepani *et*
93 *al.* 2017).

94 In order to investigate the potential for variation in microbiome composition to
95 assist in host adaptation, it is first necessary to characterize the microbiome of the
96 three social *Stegodyphus* species, to identify and localize putative specific symbionts,
97 and to establish microbiome composition within and across individuals, nests, and
98 populations. The objective of our study was thus to assess whether there are species-
99 specific core microbiomes, and to establish patterns of variation in the microbiome
100 within and between populations. To this end, we carried out 16S rRNA gene amplicon
101 analysis (approx. 400 bp, V3-V4 region) on whole spider DNA extractions of the
102 three social *Stegodyphus* species. For each species, multiple nests were sampled from
103 five to eight geographically distinct populations in South Africa, Madagascar, Sri
104 Lanka, and India (Figure 1B). In addition, full-length 16S rRNA gene sequences for
105 proper phylogenetic placement of the most dominant symbionts were obtained via
106 clone libraries, and two of the most prevalent symbionts were localized within the
107 host by fluorescence in situ hybridization.

108 **MATERIALS AND METHODS**109 **Sample collection**

110 Spiders were collected from populations spanning large climatic gradients in South
111 Africa, Madagascar, Sri Lanka, and India (Figure 1B). For each of the three species,
112 up to five individuals from four to eight nests were collected from more than five
113 populations each. *S. sarasinorum* spiders were collected in October through
114 December 2010 and transported at ambient temperature inside intact nests to the
115 laboratory in Denmark, where they were frozen at -20°C. *S. dumicola* and *S.*
116 *mimosarum* were collected in April through June 2012, cut in half and submerged in
117 ATL buffer (from DNeasy Blood and Tissue kit, Qiagen) in the field, and transported
118 to the laboratory at ambient temperature where they were stored at -20°C. These
119 samples were previously used for investigating population genetics of these species
120 (Settepani *et al.* 2017). For a summary of all samples see Table S1.

121 Spiders used for imaging were adult female *S. dumicola* collected in Namibia
122 in June 2017 and transported to Denmark in intact nests.

123 **DNA extraction and 16S rRNA gene amplicon sequencing**

124 DNA was extracted from whole spiders using DNeasy Blood and Tissue Kit (Qiagen)
125 according to the manufacturer's protocol. 16S rRNA gene amplicon libraries were
126 prepared according to Illumina's 16S Metagenomic Sequencing Library Preparation
127 guide, with slight modifications, using Bac341F and Bac805R primers to amplify
128 variable regions V3 and V4 (Herlemann *et al.* 2011). Sequencing was done in five
129 runs on a MiSeq desktop sequencer (Illumina). Some samples did not yield a PCR
130 product after the first or second PCR steps and we therefore had to optimize PCR
131 conditions for these samples individually by e.g., increasing PCR cycle number, using

132 a different DNA polymerase, or including gel extraction (for details see
133 supplementary methods).

134 **PCR, clone libraries, and sequencing of full-length 16S rRNA genes**

135 Six *S. dumicola* samples (M897, M538, X435, M898, M754, and M562) were
136 selected to obtain full-length 16S rRNA gene sequences of the dominant symbionts,
137 especially of those with low identity to published sequences, for proper phylogenetic
138 identification. The near full-length 16S rRNA gene was amplified using the general
139 bacterial primers Eub26 F (Hicks, Amann and Stahl 1992) and 1492R (Loy *et al.*
140 2002). Clone libraries were prepared using the pGEM®-T Vector System (Promega)
141 according to the manufacturer's protocol. Sanger sequencing of clones was performed
142 by GATC Biotech (Konstanz, Germany). Sequences were trimmed, assembled, and
143 checked for chimeras using Geneious v11.0.5 (Biomatters Ltd.).

144 **16S rRNA gene amplicon analysis**

145 All analyses were run on local servers using R v. 3.4.4 (R Core Team 2018) and
146 custom shell scripts. Raw sequences were trimmed to remove barcodes and PCR
147 primers using cutadapt v1.18 (Martin 2011). Filtering, denoising, paired-end merging,
148 and classification was done using the R package 'dada2' v. 1.6.0 (Callahan *et al.*
149 2016). Sequences from each of five sequencing runs were filtered and denoised
150 separately, so the Divisive Amplicon Denoising Algorithm (DADA) could make
151 independent error models for each run. After denoising, the five data sets were
152 merged for chimera finding, and ASVs were classified using the Silva SSU reference
153 database nr. 132 (Quast *et al.* 2013). The R package 'phyloseq' v. 1.22.3 (McMurdie
154 and Holmes 2013) was used to extract data as separate ASV, taxonomy, and sample
155 tables, ASVs were filtered to exclude non-bacteria and ASVs < 400 bp. After

156 excluding samples with < 3000 reads after filtering, 58 *S. dumicola* samples, 60 *S.*
157 *mimosarum* samples, and 98 *S. sarasinorum* samples remained for further community
158 analysis.

159 Normalization of amplicon data was done in one of two ways: For any
160 analysis involving diversity measures, amplicon reads were subsampled to a common
161 depth of 3000 reads. For everything else, amplicon reads are reported as fractions of
162 all reads per sample. If subsampling was used, it is stated in the figure caption.

163 All remaining analyses and visualizations of community data were done with
164 custom R scripts using several R packages such as 'ggplot2' v. 3.0.0 (Wickham 2009),
165 'vegan' v. 2.5.2 (Oksanen *et al.* 2017), and 'Biostrings' v. 2.46.0 (Pag'es *et al.* 2016).
166 Custom scripts and their use are listed in Table S2; they are available from the GitHub
167 repository

168 https://github.com/Mettetron/16S_amplicon_communityAnalysis_R.

169 Read statistics, detailed sample information, raw and normalized ASV data,
170 and microbiome taxonomic information including ASV sequences are available in the
171 supporting information (Supplementary tables S1, S3, S4, S5, S6).

172 Phylogenetic analysis

173 Phylogenetic trees based on full length 16S rRNA gene sequences were constructed
174 using Bayesian inference. Sequences were aligned using SINA, Silvas's online
175 aligner (Pruesse, Peplies and Glöckner 2012), and alignments were manually curated
176 in ARB (Ludwig *et al.* 2004). Trees were constructed with MrBayes (Ronquist *et al.*
177 2012) using a generalized time reversible substitution model with a gamma
178 distributed among-site rate variation and a proportion of invariable sites (GTR+I+Γ),
179 the analysis was run with 1,000,000 generations and a sample frequency of 100. The
180 shorter ASV sequences were added to the resulting consensus trees by maximum

181 parsimony using the ‘Quick add’ function in ARB without changing the overall tree
182 topology.

183 **Design and optimization of probes for FISH**

184 A new 16S rRNA-targeted oligonucleotide probe for FISH-detection of *Borrelia* spp.
185 (Bor744: 5'-ACTCAGCGTCAGTCTTGA-3') was developed using the probe design
186 function in ARB (Ludwig *et al.* 2004). The probe matches all spider-associated
187 *Borrelia*-like ASVs and full-length sequences found in this study, as well as 123 out
188 of 129 published *Borrelia* and *Borrelia* 16S rRNA sequences in the Silva SSU Ref
189 NR 99 release 128 database (Quast *et al.* 2013), with no exact matches outside of this
190 group. The probe binds to *E. coli* position 744-761, an area of low accessibility
191 (Fuchs *et al.* 1998); thus four helper probes (H726SPB: 5'-
192 CCTAGAAGTTGCCTTCG-3'; H649SPB: 5'-TCCCCTATCAGACTCTAGCTT-
193 3'; H576SPB: 5'-AAACCGCCTACTCACC-3'; H762BOR: 5'-
194 CTCCCTACGCTTCGTG-3') were designed specifically for the target
195 *Borrelia*. Hybridization efficiency and optimal formamide concentration were
196 modeled using MathFISH (Yilmaz, Parnerkar and Noguera 2011) and experimental
197 optimization with *Borrelia sinica* DSM23262 showed that probe Bor744 required
198 25% formamide in standard FISH buffer (Pernthaler *et al.* 2001) for specific
199 hybridization. In addition, probe LGC0355b was used with 35% formamide to
200 specifically detect *Mycoplasma* spp. (Neulinger *et al.* 2009), and probes EUB I-III
201 (Daims *et al.* 1999) and NON (Manz *et al.* 1992) were used as positive and negative
202 controls, respectively. All probes were synthesized by biomers.net.

203 **Sample preparation and FISH**

204 Spiders were sedated with CO₂ gas and dipped in 70% ethanol to reduce surface
205 hydrophobicity. The prosoma and opisthosoma were cut apart, and the tissue was
206 fixed in 4% paraformaldehyde for 24 hours at 4°C. Embedding, cryosectioning,
207 mounting, FISH and microscopy were performed as previously described in detail
208 (Kroer et al., 2016), with the following modification: embedded and frozen samples
209 were kept at -80°C for long term storage, moved to -20°C at least 24 h before
210 sectioning, and spiders were cut into 20 µm thick transverse sections at -15°C; cover
211 slips of slides after FISH were sealed with nail polish, and slides were stored at -20°C
212 until use. Confocal imaging was done on a Zeiss LSM700 confocal laser scanning
213 microscope (CLSM) with 405, 488, and 639 nm lasers and ZEN software. All images
214 were processed using FIJI (Schindelin *et al.* 2012).

215 **Data availability**

216 Full length 16S rRNA gene sequences were submitted to NCBI with the accession
217 numbers MH627291-MH627379. The V3-V4 16S rRNA gene amplicon sequences
218 were submitted to NCBI Sequence Read Archive (SRA) with the accession numbers
219 SRP130746, SRP130740 and SRP130742.

220

221 **RESULTS**

222 **Social *Stegodyphus* carry low diversity microbiomes that in each individual
223 spider are dominated by a shifting, single ASV.**

224 Amplicon sequencing of 216 individual spiders from 85 nests revealed a total of 772,
225 1,470, and 1,567 bacterial 16S rRNA gene ASVs in *S. dumicola*, *S. mimosarum*, and
226 *S. sarasinorum*, respectively (Table S1). ASV richness (expressed as the number of

227 observed ASVs per individual spider) was significantly lower in *S. dumicola* than in
228 *S. mimosarum* and *S. sarasinorum* (Figure 2A) but with 24-47 ASVs overall fairly
229 low. Likewise, ASV diversity (Shannon index) was significantly lower in individuals
230 of *S. dumicola* than in the other two species (Figure 2B, Table S1). The majority of all
231 ASVs (83%) occurred in only 1-2 samples and at relative abundances <0.1% and thus
232 can be categorized as transient microbiome. Most individual spiders were dominated
233 by a single ASV, accounting for more than 50% of the microbiome (Figure S1), but
234 not all spiders were dominated by the same ASV. The McNaughton's dominance
235 index (DMN, the sum of the two most abundant ASVs, Figure 2C) shows that
236 dominant ASVs are more common in *S. dumicola* than in *S. mimosarum* and *S.*
237 *sarasinorum*, which matches the much lower alpha diversity in *S. dumicola*. Only a
238 small set of 15 ASVs consistently showed such a dominant role (Figure 3); this
239 includes the most abundant and most prevalent ASVs (Figure 4), which were
240 classified as Chlamydiales (ASV_1), *Diplorickettsia* (ASV_2), *Borrelia*, (ASV_3),
241 *Mycoplasma* (ASV_4), and Rickettsiaceae (ASV_6). In addition, a few taxa with
242 overall lower abundance and prevalence (*Rickettsia*, *Entomoplasma*, *Brevibacterium*,
243 *Acaricomes*, *Staphylococcus*, Weeksellaceae) were occasionally dominant in *S.*
244 *dumicola* or *S. sarasinorum* (Figure 3).

245 **The three social *Stegodyphus* species carry distinct overall and core microbiomes**
246 **but no ubiquitous endosymbionts.**

247 Analysis of similarities (ANOSIM) based on Bray-Curtis dissimilarities revealed that
248 the overall microbiome composition differed significantly ($p < 0.01$) between the
249 three *Stegodyphus* species, but that it was more similar between the two African
250 species, *S. dumicola* and *S. mimosarum* ($R=0.094$), than between any of them and the

251 Indian species, *S. sarasinorum* (R=0.554 with *S. dumicola* and R=0.499 with *S.*
252 *mimosarum*).

253 The interspecies similarities and differences of the microbiomes were even
254 more evident when looking at each host species' core microbiome (here defined as
255 ASVs present in >50% of the individuals with >0.01% relative abundance): The two
256 African species, *S. dumicola* and *S. mimosarum*, each contained five core ASVs, of
257 which four were shared: *Diplorickettsia* (ASV_2), *Borrelia* (ASV_3), *Mycoplasma*
258 (ASV_4), and *Rickettsiaceae* (ASV_6). The fifth core ASV in *S. dumicola* was
259 *Chlamydiales* (ASV_1), in *S. mimosarum* it was *Delftia* (ASV_16) (Figure 3A, B).
260 The core microbiome of the Indian species *S. sarasinorum* contained seven ASVs, of
261 which only *Chlamydiales* (ASV_1) was shared with the *S. dumicola* core (Figure 3C).
262 No single ASV could be detected in all spider individuals, and the only occurrence of
263 an ASV being found in all nests in a species, was *Diplorickettsia* (ASV_2) in *S.*
264 *mimosarum*. However, all core ASVs were detected in all populations of their
265 respective host species (Figure 4). Although not part of any of the three core
266 microbiomes, *Acaricomes* (ASV_15) was also detected in all populations of *S.*
267 *dumicola* and in most populations of *S. mimosarum* (3 out of 5) and *S. sarasinorum* (7
268 out of 8). ASV_3 (*Borrelia*) was overall most prevalent even though it was not part of
269 the *S. sarasinorum* core microbiome (Figure 3, 4).

270 **Phylogenetic analysis of core and dominant ASVs indicates the presence of**
271 ***Stegodyphus*- and arachnid-specific lineages.**

272 Of the core microbiome members, the ASVs classified as *Chlamydiales* (ASV_1),
273 *Borrelia* (ASV_3), and *Mycoplasma* (ASV_4) were with 89%, 95%, and 86% only
274 distantly related to their closest match in the database. Phylogenetic analysis based on
275 partial (ASV_1) or full-length (ASV_3, ASV_4) 16S rRNA gene sequences identified

276 each of these three putative symbionts as a novel lineage, hitherto only found in
277 *Stegodyphus* (Figure 5A-C). ASV_1 branched deeply within the Chlamydiales
278 (Figure 5A); the *Borrelia*- and *Mycoplasma*-lineages each formed a distinct
279 monophyletic group with sequences obtained from scorpions, indicating that these
280 lineages were arachnid-specific (Figure 5B,C). The ASVs classified as *Diplorickettsia*
281 (ASV_2) and Rickettsiaceae (ASV_6) were both >99% identical to sequences
282 obtained from ticks, and clustered separate from non-arachnid hosts (Figure S2A,B).
283 The remaining ASVs of the core microbiome (*Delftia* in *S. mimosarum*;
284 *Staphylococcus*, *Brevibacterium*, *Pseudomonas*, *Enterobacter*, and *Leucobacter* in *S.*
285 *sarasinorum*; Figure 2) were all 100% identical to published sequences of diverse
286 (non-arachnid) host-associated or environmental bacteria.

287 Besides members of the core microbiome, three other ASVs could dominate in
288 individual spiders: ASV_15 grouped within the genus *Acaricomes* and showed 98%
289 identity to *A. phytoseiuli*, isolated from a mite (Figure S2C); ASV_12, classified as
290 Weeksellaceae, was 97% identical to a shrimp gut-derived sequence and formed a
291 distinct cluster with two more ASVs within the *Bergeyella-Chryseobacterium* group
292 (Figure S2D); and ASV_7, which grouped with three other ASVs within the genus
293 *Entomoplasma* and was 98% identical to *E. freundtii*, isolated from a beetle (Figure
294 S2E).

295 **Specific symbionts reside in a complex intestinal tract**

296 Fluorescence *in situ* hybridization (FISH) revealed the social spiders' digestive
297 system as the primary site for their microbiome. Spiders have a branching midgut,
298 which fills most of the abdomen and even has lobes extending into the legs and head
299 region of the cephalothorax (Foelix 2011) The gut terminates in a rectal sac (the
300 cloaca), where waste products are stored prior to excretion through the anus. In *S.*

301 *dumicola*, the entire midgut, including all branchings, was densely colonized with
302 bacteria (as detected by the general bacterial probe EUB), which mostly lined the
303 epithelial tissues and completely filled the rectal sac (Figure 6A-D). FISH with probes
304 specific for *Borrelia* and *Mycoplasma* (including the ASV sequences of this study)
305 proved that these putative symbionts and members of the core microbiomes of *S.*
306 *dumicola* and *S. mimosarum* were located extracellularly, lining the epithelium of the
307 branching midgut, both of them interspersed with other bacteria (Figure 6E,F).

308 **Beta diversity estimates show higher microbiome similarities within nests than
309 between nests in all three host species but different degrees of population
310 patterns.**

311 For each host species, we calculated microbiome beta diversity at three levels; within
312 individual nests, within populations, and between populations (Figure 7). The
313 microbiomes of spider individuals from the same nest were moderately to highly
314 similar, with mean Bray-Curtis dissimilarities (BC) <0.4, and were with few
315 exceptions dominated by the same ASV, although differences in relative abundance
316 of the ASVs shared between individuals did sometimes occur (Figure S1).

317 Microbiomes within populations were more dissimilar (BC 0.71-0.78) but still
318 significantly ($p<0.01$) more similar than between populations (BC 0.83-0.92). This
319 pattern was also evident when calculating the Sørensen dissimilarity index (Figure
320 S3), except that it was not significant for *S. dumicola*. Microbiome dissimilarity
321 (expressed as BC dissimilarity) did not correlate with geographical distance for nests
322 of *S. dumicola* and *S. mimosarum* (Figure S4A,B); for *S. sarasinorum*, there was a
323 weak but significant positive correlation between nest distance and microbiome
324 dissimilarity (Figure S4C).

325 To test for host population-specific microbiome patterns, we conducted non-
326 metric multidimensional scaling (NMDS) and ANOSIM analyses based on BC
327 dissimilarities (Figure 8). Microbiomes grouped by host species as stated above but
328 not consistently by host population: for *S. dumicola*, microbiomes were not
329 significantly different between populations; in fact, the differences within a
330 population were often larger than between populations (indicated by negative R
331 values; Figure 8). For *S. mimosarum*, only the Madagascan populations MAH and
332 SAK had microbiomes significantly different ($p<0.01$) from each other and from the
333 remaining three populations ($R=0.217-0.57$). Finally, all populations of *S.*
334 *sarasinorum* had distinct ($p<0.01$) microbiomes ($R=0.24-0.99$) except for populations
335 E and RAO, whose microbiomes were indistinguishable (Figure 8).

336 **DISCUSSION**

337 **Social spider microbiomes resemble those of other arachnids and social insects in
338 structure but contain unique, *Stegodyphus*-specific endosymbionts.**

339 The simple microbiome structure observed in the individuals of all three social
340 *Stegodyphus* species, with low alpha diversity, strong dominance of 1-2 bacterial
341 species (or ASVs), and a core genome of <10 species (Figures 2, 3, S1), appears
342 typical for the gut microbiomes of social arthropods (Figure S5; (Corby-Harris, Maes
343 and Anderson 2014; Sapountzis *et al.* 2015), with the notable exception of wood-
344 feeding termites that rely on a complex microbiome for lignocellulose degradation
345 (Benamino and Graf 2016). Solitary spider microbiomes are generally low diversity
346 (Zhang *et al.* 2018), and tend to be even more dominated by single symbionts than the
347 social arthropods (White *et al.* 2019; Sheffer *et al.* 2020).

348 Three of the most abundant and most dominant members of the social
349 *Stegodyphus* core microbiomes define novel lineages indicative of specific
350 endosymbionts: according to the 94.5% 16S rRNA similarity threshold proposed for
351 bacterial genus delineation (Yarza *et al.* 2014), ASV_1 represents a novel genus
352 within the obligate intracellular Chlamydiales (Jones, Rake and Stearns 2015). Its
353 phylogenetic affiliation with amoeba endosymbionts (Figure 5A) suggests that it may
354 not reside within spider cells but within spider-associated amoeba. ASV_3 is only
355 distantly (92%) related to the obligate host-associated genus *Borrelia* (Barbour 2018)
356 and forms a novel genus together with a putative scorpion symbiont. Based on this
357 phylogenetic information, a discriminant phenotype (spirochete morphology and
358 extracellular inhabitant of the spider midgut including the diverticular tissue; Figure
359 6E), and its high prevalence in all three social *Stegodyphus* species (Figure 3, 4), we
360 propose ASV_3 to represent a novel species within a novel genus, with the tentative

361 name *Candidatus Arachnospira stegodyphii*. Finally, ASV_4 groups with ASV_26
362 and a >99% identical sequence from the African sub-social spider *Stegodyphus*
363 *tentoriicola* as a novel, presumably *Stegodyphus*-specific sister genus to the recently
364 described Scorpion Mycoplasma Clade (Bolaños *et al.* 2019). These two clusters thus
365 delineate an arachnid-specific evolutionary lineage of *Mycoplasma*, a typically host-
366 associated bacterial group without cell wall and often intracellular infections (Brown
367 *et al.* 2018); in the spider gut however, they were extracellular (Figure 6F).

368 Of the remaining core or dominant ASVs with specificity or preference for
369 arachnid hosts, ASV_6 (*Rickettsiaceae*) and ASV_2 (*Diplorickettsia*) represent likely
370 intracellular endosymbionts given their >99% identity to obligate intracellular
371 symbionts of ticks, spiders, and insects (da Rocha-Lima 2015) and ticks
372 (Mediannikov *et al.* 2010), respectively. In contrast, ASV_15 (*Acaricomes*) may
373 represent an extracellular gut parasite similar to its close relative, *A. phytoseiuli*,
374 which upon gut colonization in mites causes degradation of the epithelium (Schütte *et*
375 *al.* 2008).

376 Most research into bacterial symbionts of spiders, has focused on a set of
377 bacteria which are sometimes involved in sex manipulation of insects and arachnids
378 (Duron *et al.* 2008; Goodacre 2011; Vanthournout, Vandomme and Hendrickx 2014).
379 These bacteria, *Wolbachia*, *Rickettsia*, *Cardinium* and *Spiroplasma*, were largely
380 absent in social *Stegodyphus* (except for two *S. dumicola* individuals from nest
381 KRU_27 that had 37% and 87% relative abundance of *Rickettsia*; Table S5), and the
382 skewed sex ratio in social *Stegodyphus* has been explained by a biased production of
383 X chromosome-carrying sperm cells (that give rise to females) in *S. dumicola* and *S.*
384 *mimosarum* (Vanthournout *et al.* 2018).

385 **Patchy colonization and shifting dominance of core microbes in social spiders**
386 **may indicate low transmission fidelity and dispersal/mixing events in their**
387 **microbiomes.**

388 One of the proposed benefits of group living is access to and sharing of beneficial
389 endosymbionts and gut microbes (Lombardo 2008). In fact, social insects like ants,
390 termites, bees, and bumblebees transmit and homogenize their gut microbiomes
391 between colony members by fecal-oral transmission, trophallaxis, oral exchanges, and
392 via the shared nest environment (Powell *et al.* 2014; Brune and Dietrich 2015; Billiet
393 *et al.* 2017; Zhukova *et al.* 2017). The high microbiome similarity between
394 individuals from the same nest observed in the social spiders (Figure S1, Figure 7)
395 may likewise be explained by continuous transmission and homogenization within the
396 nest: both (allo)maternal care and matriphagy (Junghanns *et al.* 2017, 2019) are
397 potential routes of vertical transmission, while communal feeding (Schneider and
398 Bilde 2008) and the shared nest environment may lead to horizontal transfer and
399 homogenization of the microbiome.

400 In contrast, the social spider microbiomes are more dissimilar on population- and
401 species-level (Fig. 7), the dominating ASVs differ from nest to nest (Fig. S1), and
402 only a single ASV was detected in all nests of its *Stegodyphus* host species (ASV_2
403 *Diplorickettsia* in *S. mimosarum*, Fig. 3, 4). This pattern of patchy colonization and
404 shifting dominance indicates that no single symbiont is obligate for survival, and that
405 stochasticity together with a founder effect (as hypothesized for the human
406 microbiome; (Litvak and Bäumler 2019)) rules microbiome assembly of social
407 *Stegodyphus*: when new nests establish by fission or long distance ballooning of
408 single, mated females (Schneider *et al.* 2001), only a subset of the core microbes may

409 be transferred from the nest of origin, and the first symbiont to colonize the offspring
410 in the new nest may end up dominating.

411 Despite this apparent low transmission fidelity during the establishment of
412 new nests and populations, identical ASVs are found across the entire geographic
413 range, from South Africa to Northern India, and across all three social spider species
414 (Figures 1, 3, 4). How can these core microbes be maintained in the host species? We
415 see two possibilities, which may apply to distinct subsets of the core microbes:
416 selection, and uptake from environmental vectors.

417 The first scenario (most likely for the intracellular and most prevalent
418 symbionts, like *Diplorickettsia* or *Ca. Arachnospira*) implies that a given core ASV is
419 actually present in (almost) every nest but sometimes below our detection limit, and
420 that the symbiont provides an essential function at least once during a nest's lifespan.
421 Only nests that maintain that symbiont will then proliferate; a fluctuating relative
422 abundance of the symbiont (from nearly undetectable to dominant) may be driven by
423 host life stage, prey type, or environmental conditions, and may even have an
424 adaptive function. Time series studies are needed to resolve whether such predicted
425 fluctuations occur in the field.

426 The second scenario suggests that core microbes can be acquired from distant
427 nests or environmental reservoirs via migrating vectors. This appears plausible for not
428 host-restricted taxa like *Delftia*, *Pseudomonas*, or *Brevibacterium*, which may be
429 selected by the host for a beneficial function. The *Stegodyphus*- or arachnid-specific
430 symbionts, however, would need a more specific vector. Migration of social spiders
431 between nests is rare (Johannesen *et al.* 2002) but social and more migratory sub-
432 social *Stegodyphus* species co-occur in both Africa and India (Settepani *et al.* 2017),
433 and different social and sub-social species can be observed in the same nest (Trine

434 Bilde, personal observation). This level of mixing may suffice to spread symbionts
435 and maintain them in the host populations; it probably also explains that the co-
436 occurring African *S. dumicola* and *S. mimosarum* share almost all core ASVs and
437 have more similar microbiomes than *S. sarasinorum* (Fig. 3, 8), although *S. dumicola*
438 is more closely related to the Indian *S. sarasinorum* (Settepani, Bechsgaard and Bilde
439 2016).

440 **Host population-specific microbiomes were detected in *S. sarasinorum* but not in**
441 **the African social spiders.**

442 The microbiomes of all three spider species were more similar within a host
443 population than between populations, based on significant differences in BC
444 dissimilarity (Figure 7). These patterns could be explained by random processes of
445 drift, or possibly some degree of local adaptation to the host population or to the
446 environment. However, only for the Indian species *S. sarasinorum* and two of the
447 Madagascan populations of *S. mimosarum*, the microbiomes clustered according to
448 host populations (Fig. 8), and only for *S. sarasinorum* there was a correlation between
449 microbiome dissimilarity and distance between nests (Fig. S4). The population
450 structure of social *Stegodyphus* is driven by extinction-re-colonization dynamics
451 (Settepani *et al.* 2017), which over time will homogenize both genetic variation
452 among host populations (Settepani *et al.* 2017), and possibly also their associated
453 microbiome. In addition, mixing of symbiont populations via immigration, vectors,
454 and environmental acquisition is likely to occur (see above). The extent of mixing and
455 dispersal should then play a key role for the degree of homogenization (Settepani,
456 Bechsgaard and Bilde 2014). Given the shorter geographic distances between
457 populations and the greater number of co-existing social and sub-social *Stegodyphus*
458 species (Settepani *et al.* 2017), it is conceivable that the African populations (except

459 for the *S. mimosarum* “island” populations on Madagascar) experience a higher
460 degree of microbiome homogenization than *S. sarasinorum* in India, and therefore do
461 not show detectable microbiome population patterns.

462 Another factor may be the diversity of the microbiome or the number of core
463 or dominating ASVs, which is lowest in *S. dumicola* and overall highest in *S.*
464 *sarasinorum* (Fig. 2, 3, 4): this may facilitate the detection of population-specific
465 patterns at the 16S rRNA gene amplicon level in *S. sarasinorum*, while we cannot
466 exclude that patterns may first occur at higher (genomic) resolution in the African
467 species.

468 **Conclusions and Perspective**

469 Our study shows that social spiders harbour low-diversity (gut) microbiomes with a
470 species-specific pattern and core microbiomes that include novel, *Stegodyphus*- or
471 arachnid-specific endosymbiont lineages with unknown function. Individual spider
472 microbiomes were dominated by one out of 4-15 single ASVs, which varied between
473 nests. The microbiome of the Indian *S. sarasinorum* but not that of the two African
474 species showed a host population-specific pattern, consistent with either drift-like
475 processes, or a potential role of microbial symbionts in host adaptation. Whether the
476 microbiome indeed contributes to the fitness or local adaptation of the spider host
477 now remains to be tested.

478

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485 **COMPETING INTERESTS**

486 The Authors declare no competing financial interests in relation to the work
 487 described.

488

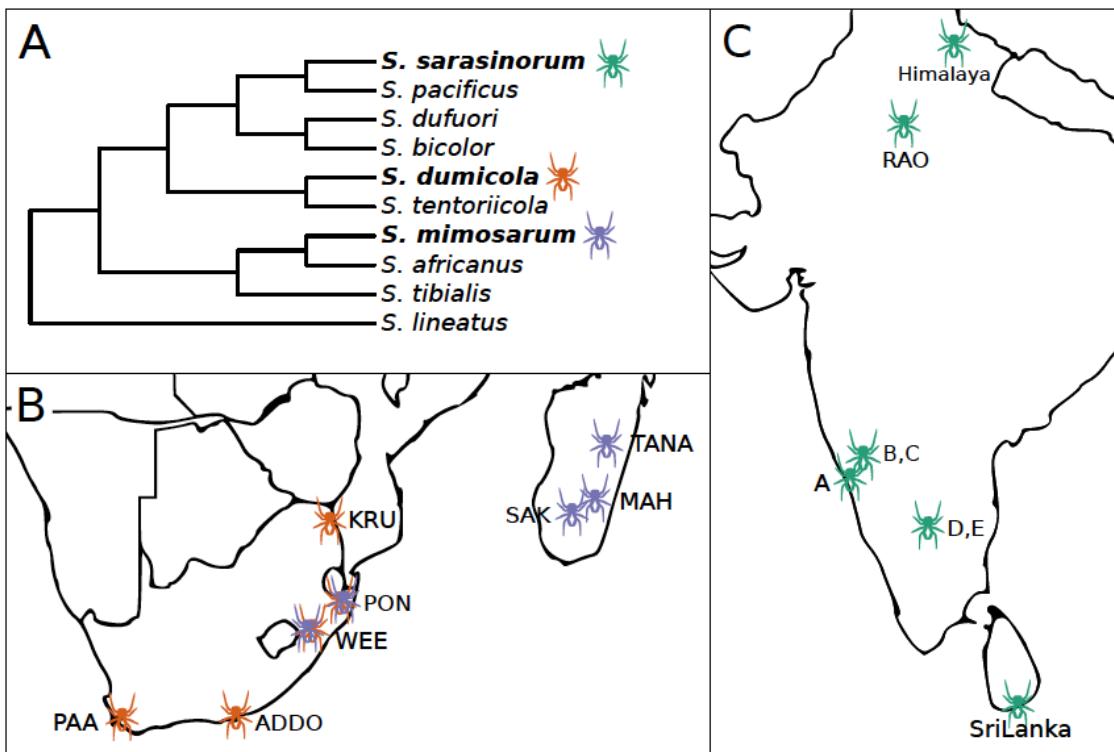
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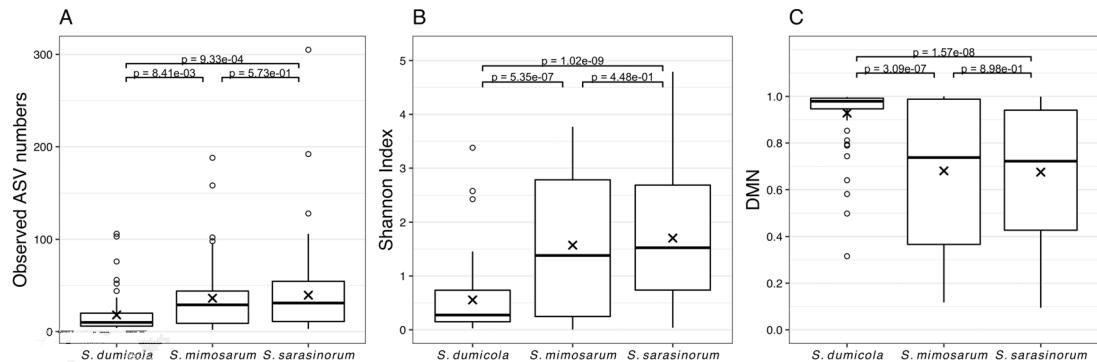
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- 661
- 662

663 **FIGURES**

664

665 **Figure 1**

666 (A) Phylogeny (cladogram) of the three social *Stegodyphus* (in bold) and their sub-
 667 social sister species (redrawn from (Settepani, Bechsgaard and Bilde 2016)). (B) Map
 668 of the sampling locations for the three social *Stegodyphus* species across South
 669 Africa, Madagascar, Sri Lanka, and India. Orange, *S. dumicola*; blue, *S. mimosarum*;
 670 green, *S. sarasinorum*.
 671

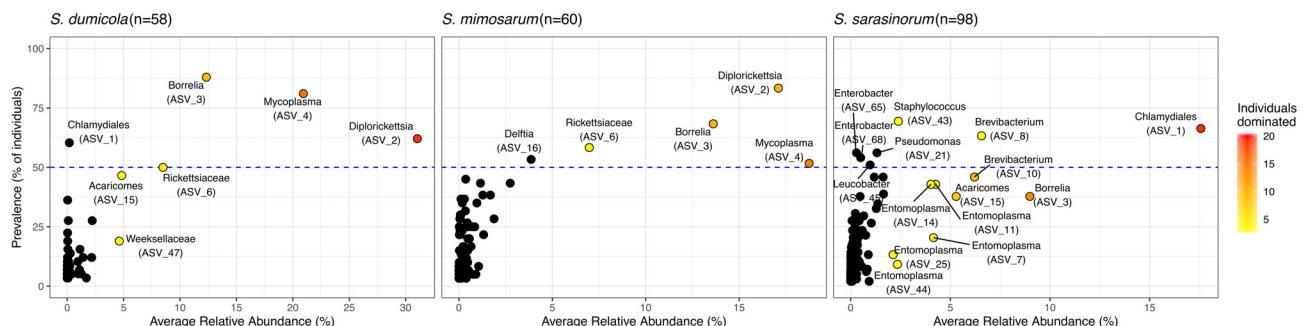


672

Figure 2

Microbiome richness, diversity and dominance metrics for each of the three social *Stegodyphus* species based on subsampled data. Each data point is an individual spider. (A) Microbiome richness, represented by the number of observed ASVs. (B) Symbiont diversity represented by the Shannon index. (C) Symbiont dominance represented by McNaughton's dominance (DMN) = sum of the relative abundances of the two most abundant ASVs in each sample. X indicates the means, p-values are based on pair-wise t-tests with Benjamini-Hochberg adjustment for multiple comparisons. Boxes span the interquartile range (IQR) with the median as the middle horizontal line, the whiskers extend 1.5*IQR from each end of the box, and anything outside of this min-max span is depicted as outliers with a circle point. All based on data subsampled to 3000 reads/sample.

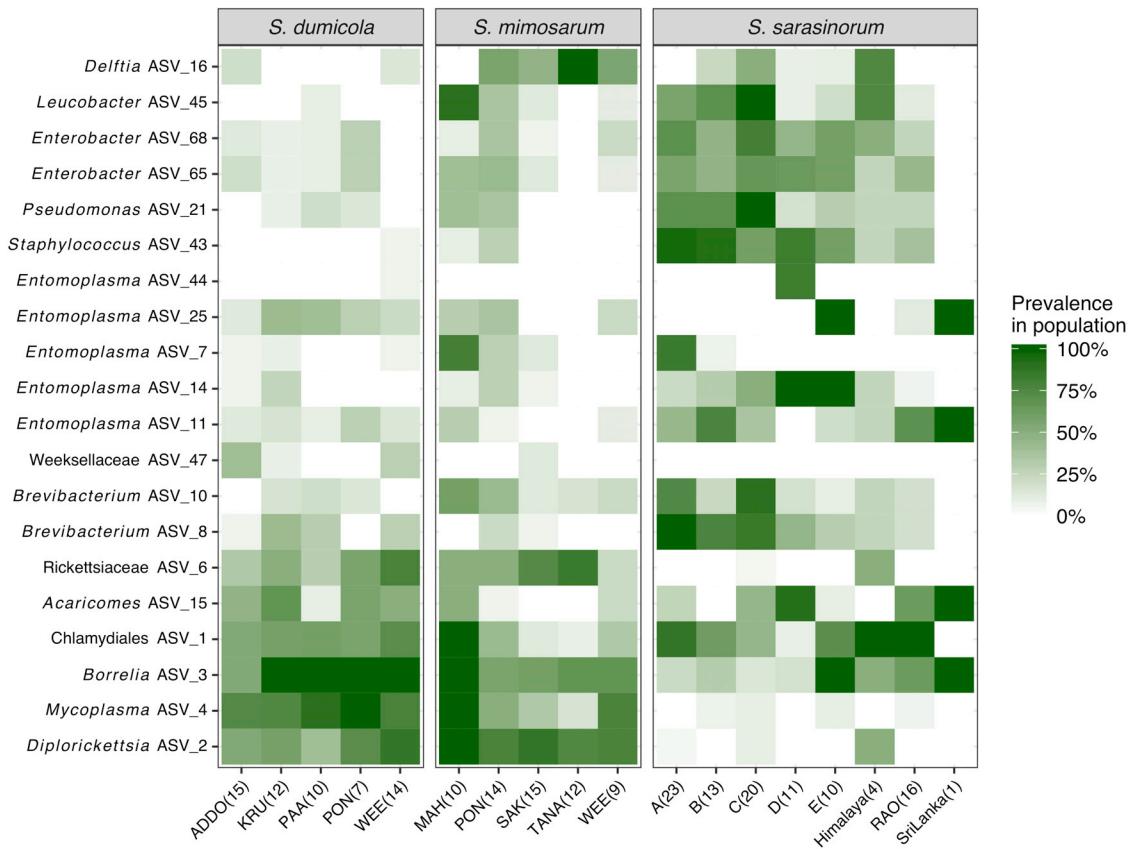
685



686

Figure 3

Prevalence (presence threshold: Relative abundance $\geq 0.01\%$), average relative abundance, and cases of dominance of ASVs in the three social *Stegodyphus* species. Colors indicate in how many individual spiders the ASV reached $>30\%$ relative abundance. Stippled line indicates 50% prevalence; ASVs above the line are included in the core microbiome of the respective spider species.



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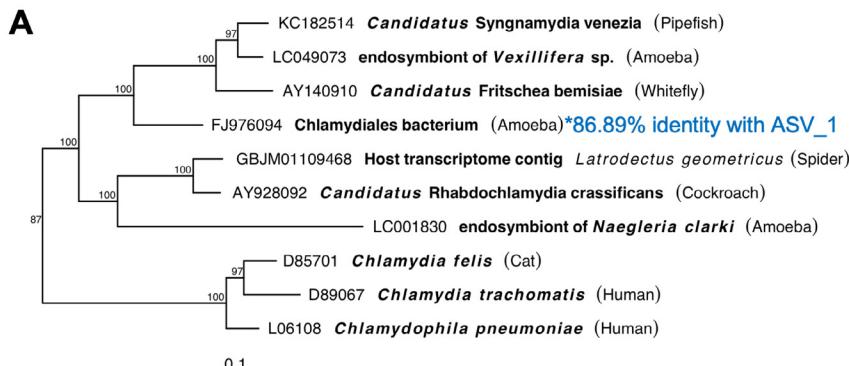
694

Figure 4

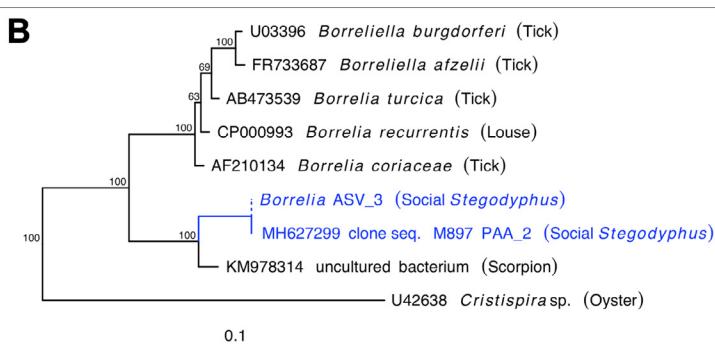
695 Heatmap of the core and dominant ASVs across the three social *Stegodyphus* species.
 696 (presence threshold: Relative abundance $\geq 0.01\%$). Each column represents a
 697 population. The prevalence in a population is calculated as percent of individual
 698 spiders in that population with that ASV. The number of spider individuals in each
 699 population is shown in brackets.

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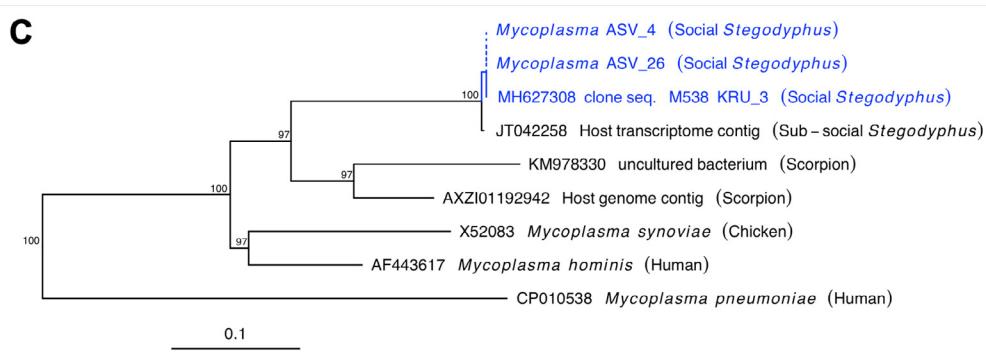
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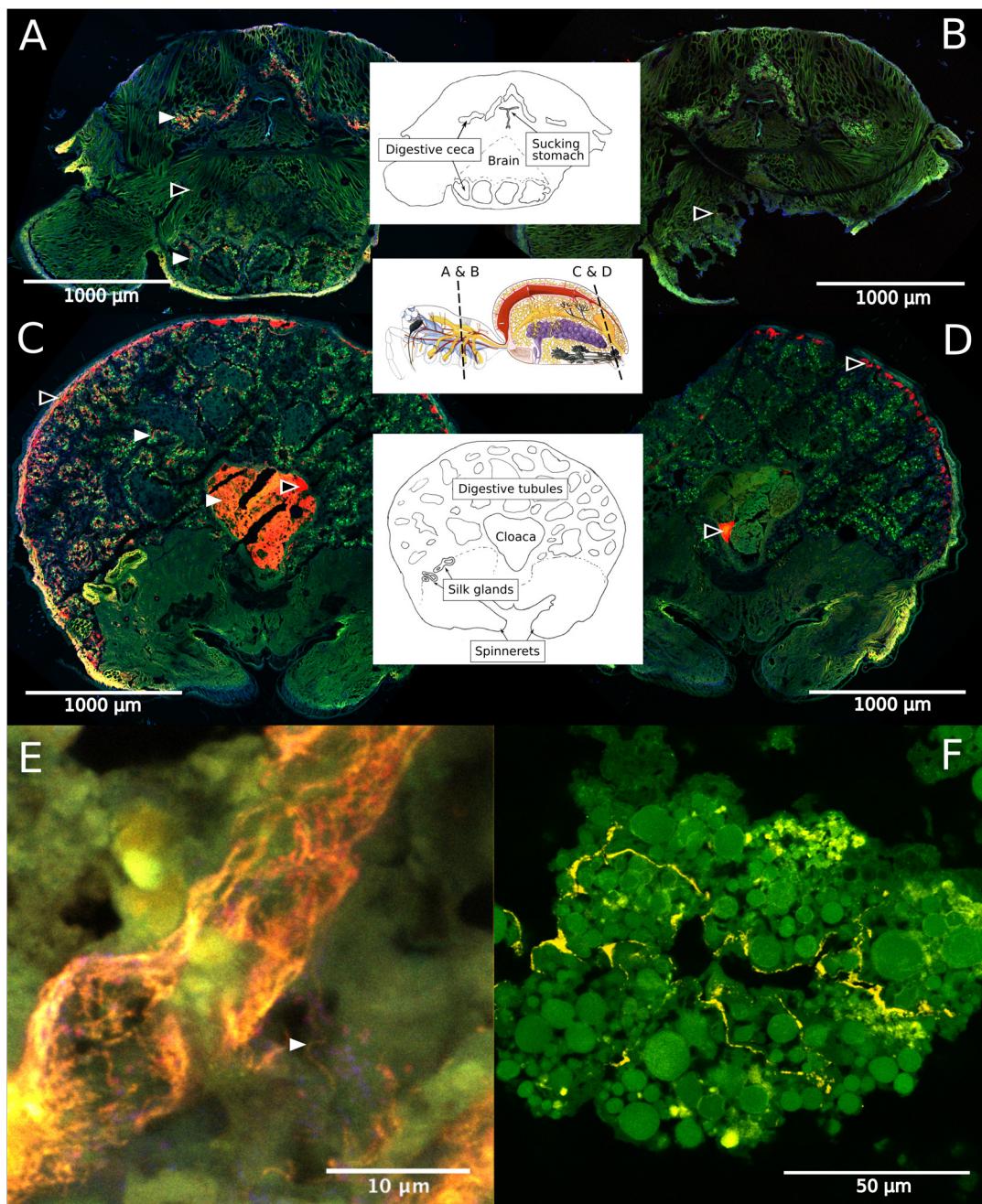


703

Figure 5

704 Phylogenetic position of three abundant, prevalent, and putatively specific
705 *Stegodyphus* symbionts classified as (A) Chlamydiales, (B) *Borrelia*, and (C)
706 *Mycoplasma*. Sequences obtained from *Stegodyphus* spiders are shown in blue, host
707 organisms are given in parenthesis. Trees are based on near full-length 16S rRNA
708 gene sequences (identified by GenBank accession numbers) and calculated by
709 Bayesian Inference. Short sequences (indicated by stippled lines, e.g. of ASVs) were
710 added without changing the tree topology. Numbers on nodes are posterior
711 probabilities. Scalebar: 0.1 estimated substitutions per site.





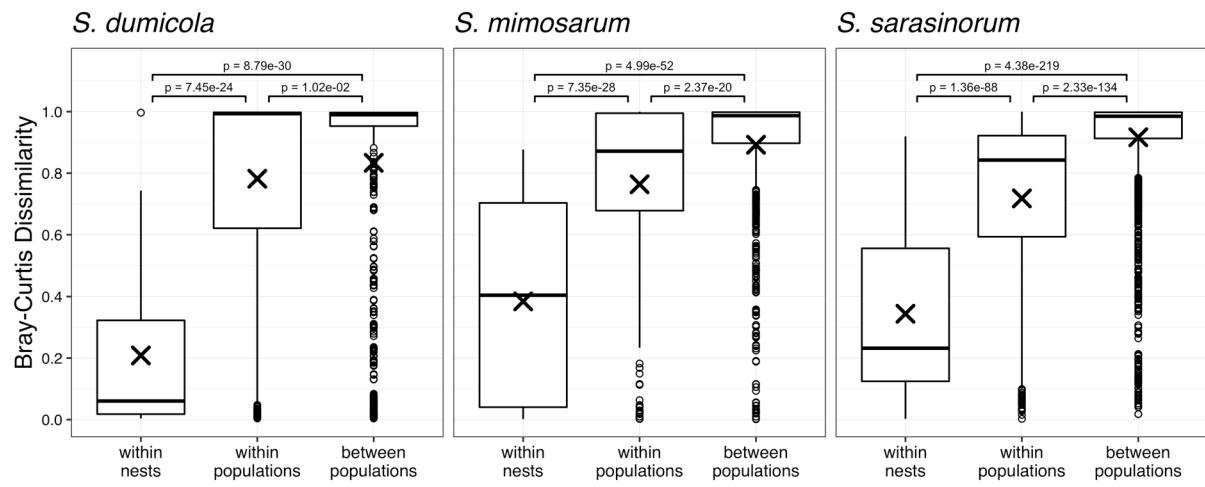
713

714

Figure 6

715 Fluorescence in situ hybridization in cryosections of adult female *S. dumicola*. Middle
 716 panels show anatomy and location of sections. All panels: Green is autofluorescence,
 717 blue is DNA (dapi). (A & C) Red is Eub I-III probe, white arrows point to examples
 718 of bacteria. (B & D) Red is NON probe. (A – D) Black center arrows point to
 719 unspecific binding. (E) Orange is Bor477 probe binding to *Borrelia*, white arrow
 720 pointing to clear spirochaetal cell. Red is Eub I-III probe. (F) Yellow is LGC0355b
 721 probe binding to *Mycoplasma*.

722

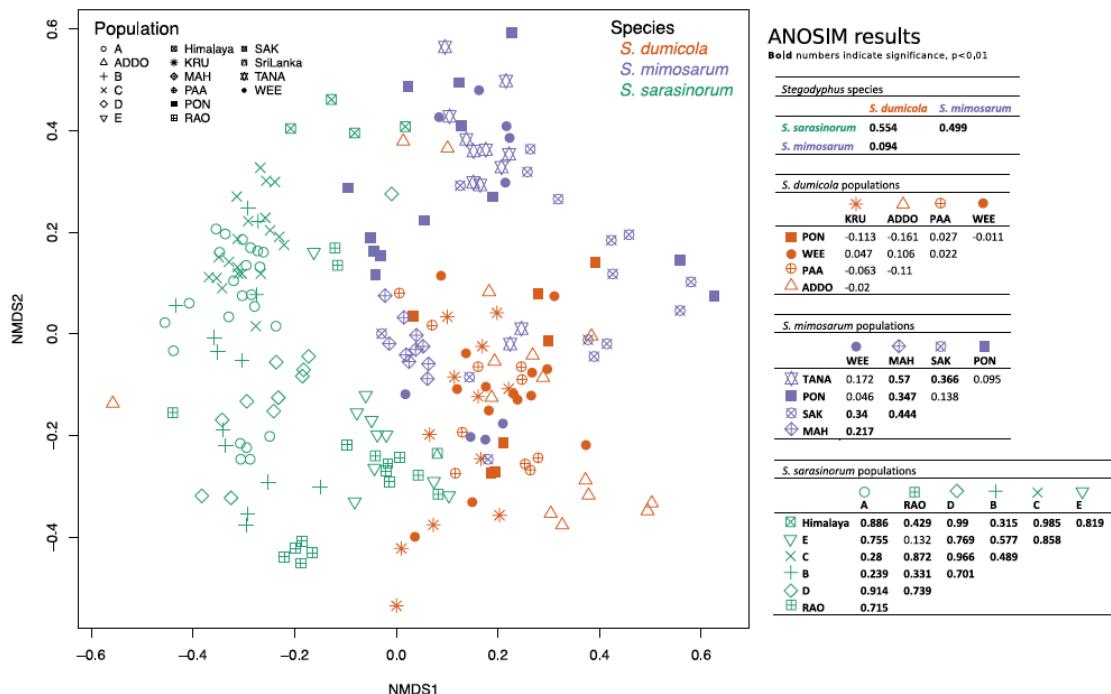


723

Figure 7

Beta diversity at three different host organization levels (within nest, within population, and between populations) in the three species of social *Stegodyphus*. Each data point compares the microbiomes of two individual spiders. For estimating the Bray-Curtis dissimilarities the data were subsampled to 3000 ASVs per spider. Group means are indicated with “x” and compared using one-way ANOVA and pairwise t-test with Benjamini-Hochberg adjusted p-values. Boxes span the interquartile range (IQR) with the median as the middle horizontal line, the whiskers extend 1.5*IQR from each end of the box, and anything outside of this min-max span is depicted as outliers with a circle point. All based on data subsampled to 3000 reads/sample.

733



734

735 **Figure 8**

736 NMDS ordination of spider microbiomes based on Bray-Curtis dissimilarities. Each
 737 point is an individual spider. Orange: *S. dumicola*, blue; *S. mimosarum*, and green *S.*
 738 *sarasinorum*. Symbols denote the different populations according to the legend and
 739 Fig. 1B. For estimating the Bray-Curtis dissimilarities the data was subsampled to
 740 3000 ASVs per spider. Stress = 0.23. The ANOSIM R metric indicates degree of
 741 difference between groups; R=0 indicates no difference in microbiome composition
 742 between groups, R=1 indicates a high degree of difference in microbiome
 743 composition between groups, and negative R values indicate more difference within a
 744 group than between groups. Bold numbers indicate significance ($p < 0.01$). The *S.*
 745 *sarasinorum* Sri Lanka population was removed from the ANOSIM analysis because
 746 it contains only one sample.

SUPPORTING INFORMATION**The microbiome of social spiders (Genus *Stegodyphus*): host and population-specific patterns**

Mette M. Busck, Virginia Settepani, Jesper S. Bechsgaard, Marie B. Lund, Trine Bilde, Andreas Schramm

Supporting methods – PCR protocols

PCR protocol for amplicon run 0, 1, 2, and 3:

Three PCR amplifications were carried out for each sample, the first using Bac 341F and Bac 805R primers to amplify variable regions V3 and V4 (Herlemann *et al.* 2011), the second adding overhang adapters with modified primers and finally an index PCR with Nextera XT index primers (Illumina) for sample identification. Each PCR had a total reaction volume of 25 µl containing 2.5 µl DNA sample, 0.5 µl of each primer (primer stock concentration = 10pmol/µl), 12.5 µl 2x KAPA HiFi HotStart Ready Mix and 9 µl sterile H₂O. The PCRs were performed on a Veriti® 96-Well Thermal Cycler (Applied Biosystems®) with the following program: Denaturation at 95°C for 3 min. Cycles of 95°C, 55°C and 72°C for 30 secs each, and finally a 5 min extension step at 72°C. PCR 1 with 20 cycles, PCR 2 with 10 cycles and PCR 3 with 8 cycles. After each PCR, samples were purified using AMPure XP beads (Illumina).

PCR protocol for amplicon run 4:

Run 4 was a trouble shooting run, redoing PCRs and sequencing for samples which had failed to provide adequate read numbers in previous runs. Based on several trials and optimization iterations, the PCR protocol for samples in amplicon run 4 was carried out differently for different groups of samples. Table S7 summarizes these differences while the text below explains the details.

For several samples PCR 1 and PCR 2 were carried out normally as described for amplicon run 0-3, But some samples went through the following optimized protocol: PCR 1 was run with a diluted template and added BSA giving a total reaction volume of 25 µl containing 2.5 µl DNA sample (diluted 1:50), 0.5 µl of each primer (primer stock concentration = 10pmol/µl), 0.5 µm BSA (10mg/ml), 12.5 µl 2x KAPA HiFi HotStart Ready Mix and 8.5 µl sterile H₂O. This was run for 30 cycles rather than the 20 cycles normally run for PCR1. PCR 2 was run in triplicate for each sample with 3 µl DNA sample, 0.5 µl of each primer (primer stock concentration = 10pmol/µl), 12.5 µl 2x KAPA HiFi HotStart Ready Mix and 8.5 µl sterile H₂O, and for 12 cycles instead of the normal 10. For all samples in amplicon run 4, PCR 2 products were size separated by agarose gel electrophoresis and the desired band was extracted from the gels using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek). Several samples (see Table S7) in run 4 had such low DNA concentration after gel extraction that AMPure XP beads (Illumina) were used to bind and resuspend in a smaller volume. PCR 3 adding index primers was run as described for amplicon run 0-3, but with differing amounts of template depending on DNA concentration (see Table S7).

Table S1 Summary statistics of samples, sequencing, and community data

	<i>S. dumicola</i>	<i>S. mimosarum</i>	<i>S. sarasinorum</i>
Samples	58	60	98
Nests	28	23	34
Populations	5	5	8
Raw read number^{a,c}	43079 (5278-675594)	51551 (14601-96639)	44425 (3959-280637)
Filtered read number^{a,d}	30270 (3131-438346)	30368 (4059-68412)	31146 (3004-195228)
ASV number^d	772	1470	1567
ASV number per sample^{a,d}	24.52 (4-152)	44.73 (3-211)	46.97 (3-343)
Shannon diversity^{b,d}	0.56 ± 0.67	1.58 ± 1.30	1.71 ± 1.11
Simpson diversity^{b,d}	0.77 ± 0.25	0.48 ± 0.38	0.40 ± 0.29
DMN^{b,d}	0.93 ± 0.13	0.68 ± 0.32	0.68 ± 0.27

Note: Based on samples with minimum 3000 reads after filtering

a: Mean (minimum-maximum)

b: Mean ± standard deviation

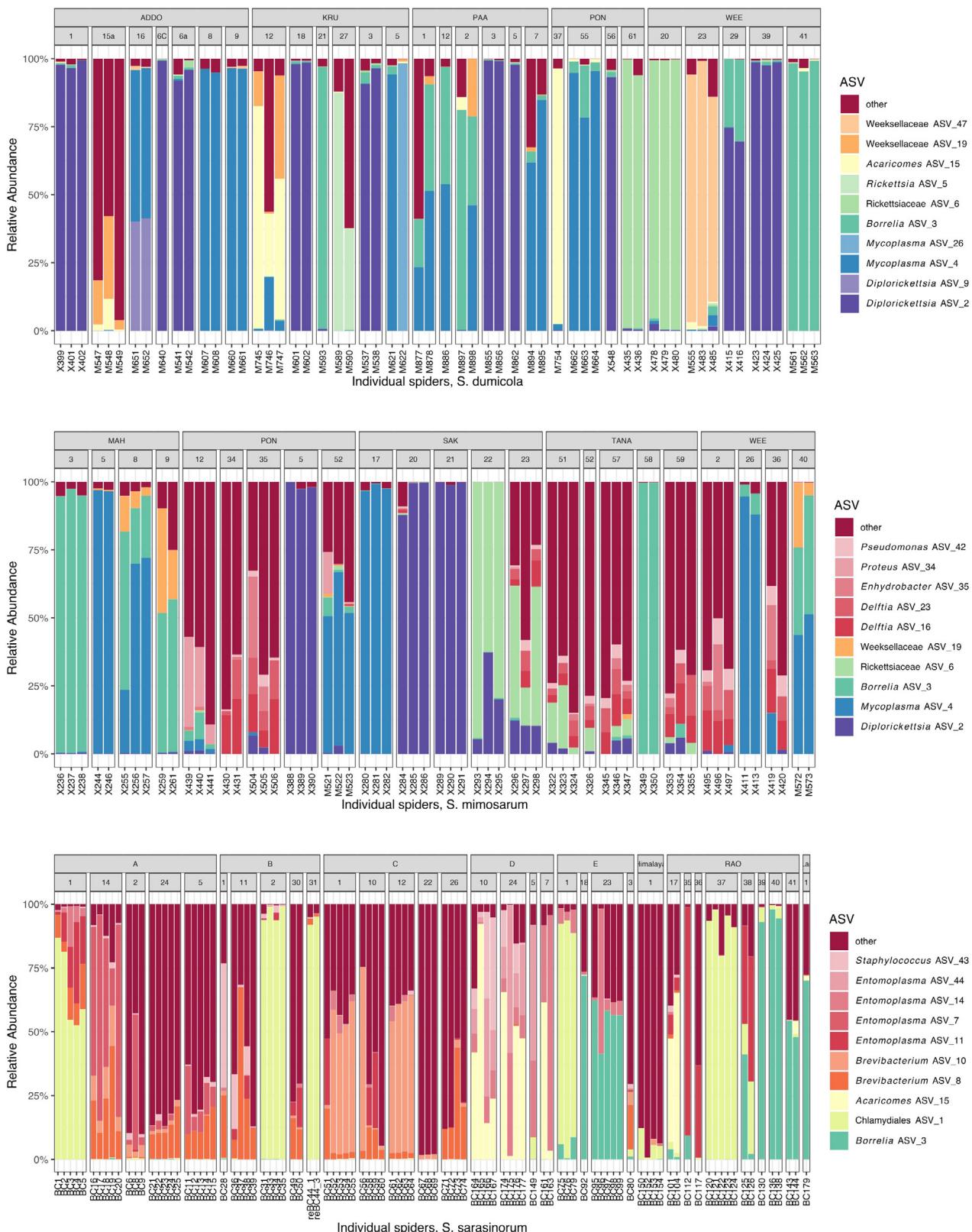
c: Unfiltered forward read numbers from Miseq

d: After quality filtering, denoising, paired-end merging, chimera finding, length filtering (minimum 400bp) and taxonomic filtering

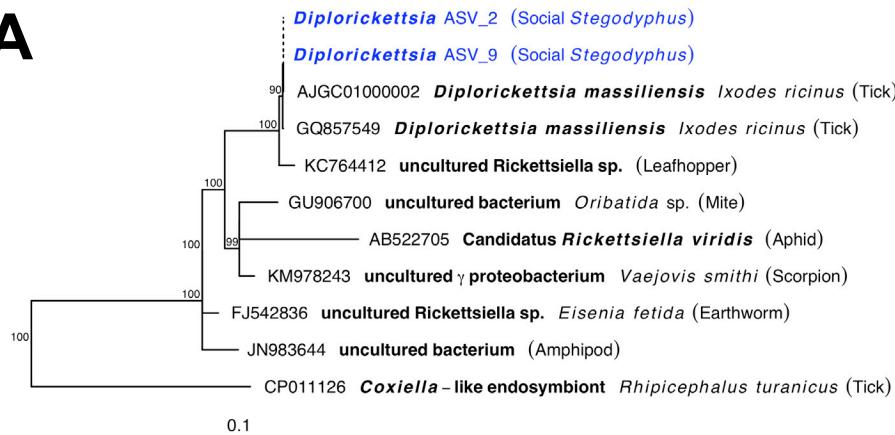
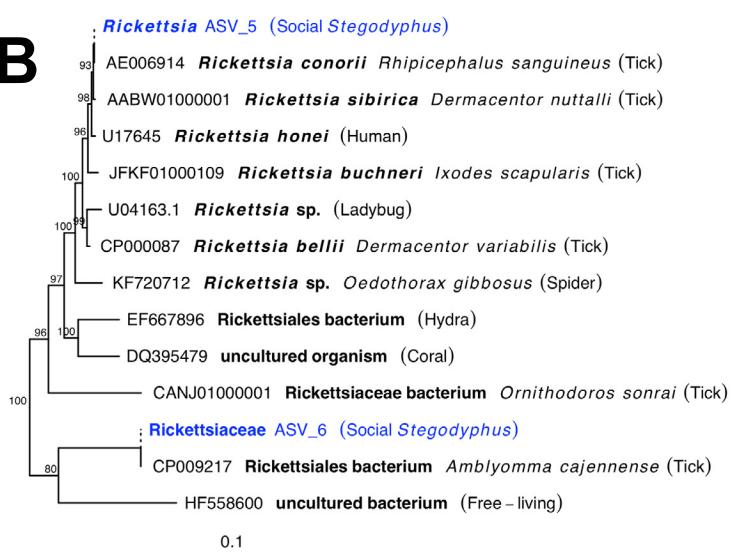
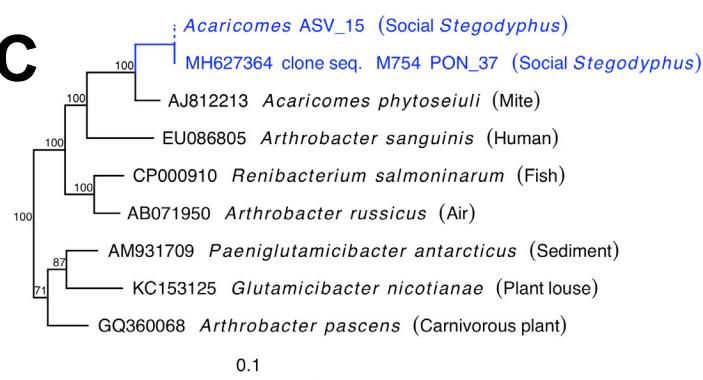
Table S2 R scripts used for analysis and data visualization

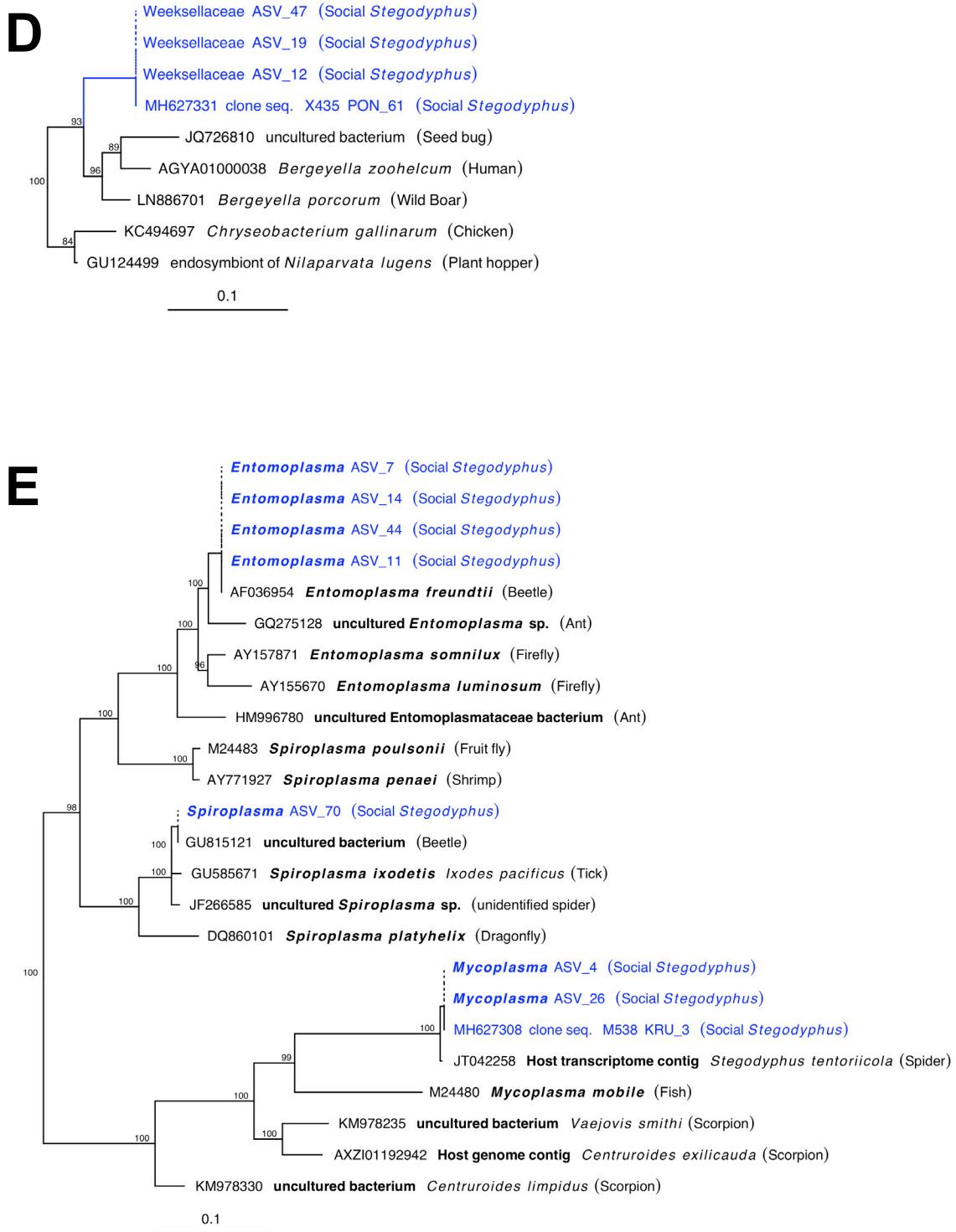
Use	Figure	GitHub link
Basic DADA2 analysis	-	https://github.com/Mettetron/3Species/blob/master/DADA2_basicAnalysis.R
Merging of ASV data from several sequencing runs	-	https://github.com/Mettetron/3Species/blob/master/DADA2_combRuns_tax.R
Data formatting	-	https://github.com/Mettetron/3Species/blob/master/DADA2_to_Phylseq.R
Decontamination and normalization	-	https://github.com/Mettetron/3Species/blob/master/DADA2_filterAndNorm.R
Richness, alpha diversity and dominance boxplots	2	https://github.com/Mettetron/3Species/blob/master/ASV_divDom_box.R
Prevalence vs. abundance scatter plots	3	https://github.com/Mettetron/3Species/blob/master/ASV_coreDom_scatter.R
Prevalence heatmap	4	https://github.com/Mettetron/3Species/blob/master/ASV_prevalenceHeatmap.R
Diversity boxplots	7	https://github.com/Mettetron/3Species/blob/master/ASV_Bdiv_boxplots.R
Ordination	8	https://github.com/Mettetron/3Species/blob/master/ASV_NMDS_ordination.R
Stacked barchart for individual hosts	S1	https://github.com/Mettetron/3Species/blob/master/ASV_barchartsIndividuals_dumicola.R
Diversity vs. Geographical distance	S4	https://github.com/Mettetron/3Species/blob/master/ASV_betaDist_vs_geoDist.R

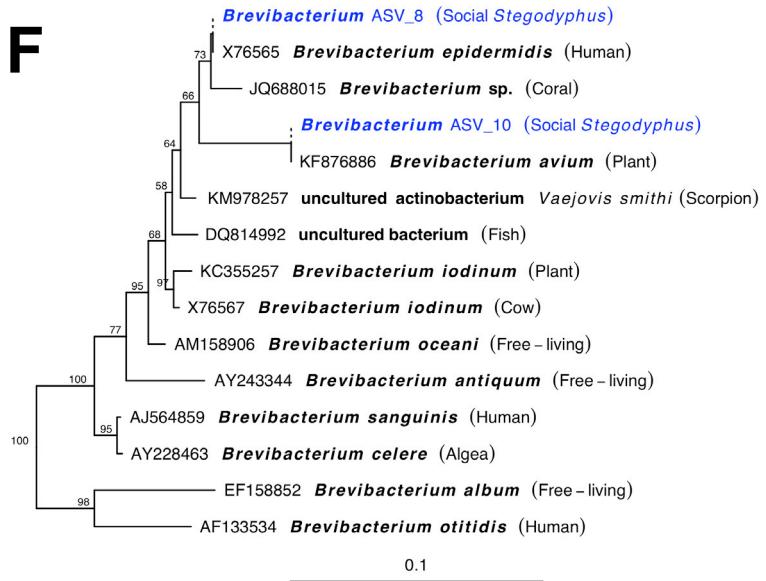
Note: Example data for running scripts also available on GitHub <https://github.com/Mettetron/3Species>

**Figure S1**

Stacked bar charts showing relative abundances of the most abundant bacterial symbiont ASVs in individuals belonging to 3 species of social *Stegodyphus*. Each chart shows the 10 most abundant ASVs found in each spider species. The columns are grouped by spider population (top bar) and nest (bottom bar, numbers)

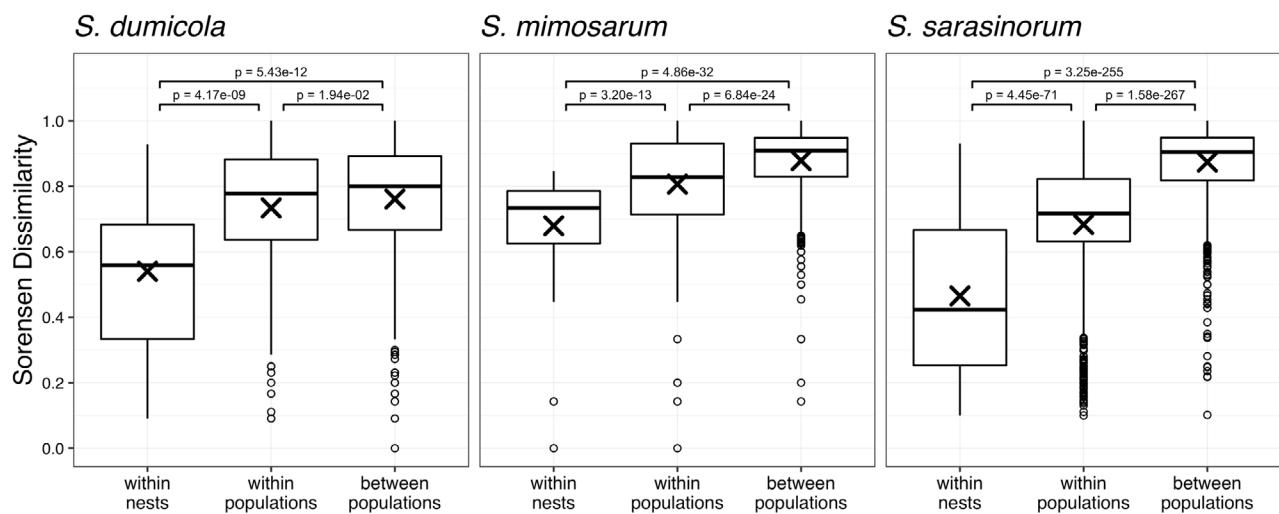
A**B****C**



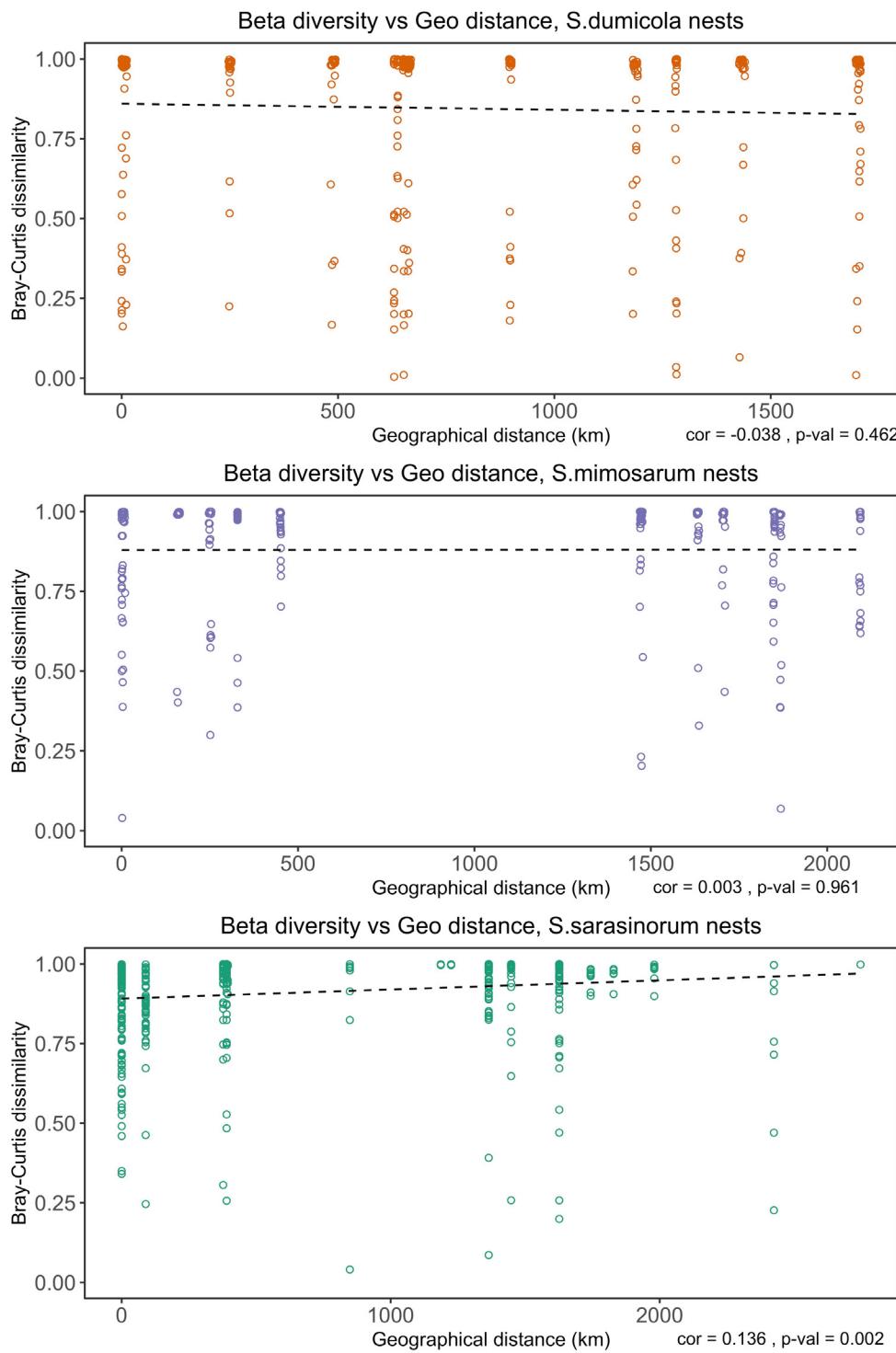
**Figure S2**

Phylogenetic trees with dominant *Stegodyphus* ASVs.

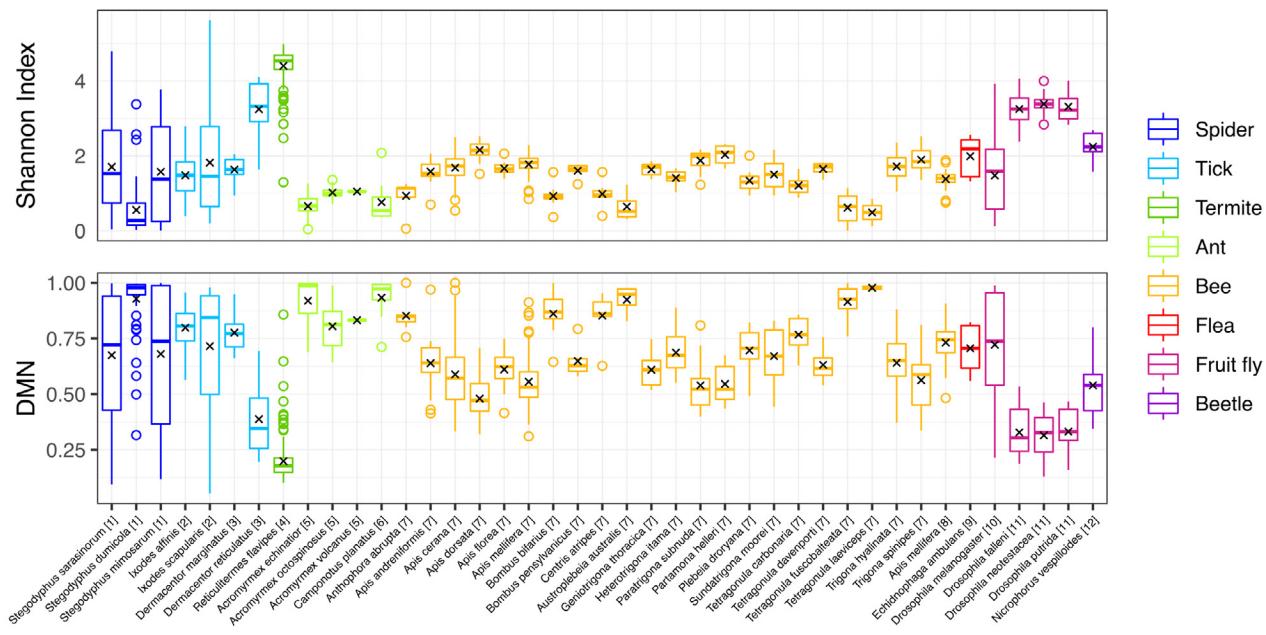
A) *Diplorickettsia*, B) *Rickettsia/Rickettsiaceae*, C) *Acaricomes*, D) *Weeksellaceae*, E) *Entomoplasma/Spiroplasma/Mycoplasma*, F) *Brevibacterium*. Bayesian inference consensus trees based on full length 16S rRNA sequences from bacteria closely related to social *Stegodyphus* symbionts found in the silva database (v128). Numbers on nodes show posterior probabilities. ASVs inserted into trees with ARB. Tip labels: Accession number, **full name**, and (host) of bacteria.

**Figure S3**

Beta diversity at three different host organization levels (within nest, within population, and between populations) in the three species of social *Stegodyphus*. Each data point compares the microbiomes of two individual spiders. For estimating the Sørensen dissimilarities the data were subsampled to 3000 ASVs per spider. Group means are indicated with “x” and compared using one-way ANOVA and pairwise t-test with Benjamini-Hochberg adjusted p-values. Boxes span the interquartile range (IQR) with the median as the middle horizontal line, the whiskers extend 1.5*IQR from each end of the box, and anything outside of this min-max span is depicted as outliers with a circle point.

**Figure S4**

Correlation of beta diversity and geographical distance between social *Stegodyphus* nests. Each data point represents comparison between average microbiomes of two nests. Beta diversity measures based on subsampled ASV data. Pearson's product-moment correlation

**Figure S5**

Comparison of symbiont diversity and dominance metrics for 40 species of arthropod using data from 12 different studies (represented by the numbers on the x-axis – see table S8). Each data point represents a sample. Depending on the study, some samples are whole host animals, some are guts, some samples are tissues pooled from several animals, while some only contain tissue from one individual (see table S7). DMN = McNaughton's dominance, the sum of the relative abundances of the two most abundant ASVs in each sample. X marks the mean.

Table S7 Meta Data for Diversity and Dominance Comparison

Number	Study	Sample Pooling	Body Part	16S rRNA region	Sequencing Platform	ASV/OTU (clustering threshold)
1	This study	No	Whole	V3 + V4	Illumina MiSeq	ASVs
2	(van Treuren <i>et al.</i> 2015)	No	Whole	V4	Illumina MiSeq	OTUs (97%)
3	(Zhang <i>et al.</i> 2019)	4	Whole	V3 + V4	Illumina MiSeq	OTUs (97%)
4	(Benjamino <i>et al.</i> 2018)	No	Hindgut	V4	Illumina MiSeq	OTUs (97%)
5	(Sapountzis <i>et al.</i> 2015)	5	Gut	V3 + V4	Illumina MiSeq	OTUs (97%)
6	(Ramalho, Bueno and Moreau 2017)	No	Whole	V4	Illumina MiSeq	OTUs (97%)
7	(Kwong <i>et al.</i> 2017)	No	Gut	V4	Illumina MiSeq	OTUs (97%)
8	(Corby-Harris, Maes and Anderson 2014)	No	Gut	V1 + V2	Roche 454	OTUs (97%)
9	(Lawrence <i>et al.</i> 2015)	No	Whole	V4	Illumina MiSeq	OTUs (97%)
10	(Adair <i>et al.</i> 2018)	No	Whole	V3 + V4	Illumina MiSeq	OTUs (97%)
11	(Bost <i>et al.</i> 2018)	20-30	Gut	V3 + V4	Illumina MiSeq	OTUs (97%)
12	(Vogel <i>et al.</i> 2017)	4	Gut	V1 - V3	Roche 454	OTUs (97%)

SI: The microbiome of social spiders (Genus *Stegodyphus*): host and population-specific patterns

Table S8 Comparison of Symbiont Diversity and Dominance in Different Arthropod Hosts

Number	Study	Host species	n	ASVs/OTUs total	ASVs/ OTUs per sample	Shannon	DMN
1	This study	<i>Stegodyphus sarasinorum</i>	98	1310	39.5 ± 41.76	1.7 ± 1.11	0.68 ± 0.27
		<i>Stegodyphus dumicola</i>	58	540	18.07 ± 21.9	0.56 ± 0.66	0.93 ± 0.13
		<i>Stegodyphus mimosarum</i>	60	1164	36.23 ± 36.02	1.57 ± 1.3	0.68 ± 0.32
2	(van Treuren <i>et al.</i> 2015)	<i>Ixodes affinis</i>	11	1081	159.91 ± 63.29	1.48 ± 0.72	0.8 ± 0.11
		<i>Ixodes scapularis</i>	81	5246	143.42 ± 106.76	1.82 ± 1.37	0.72 ± 0.27
3	(Zhang <i>et al.</i> 2019)	<i>Dermacentor marginatus</i>	6	280	115.67 ± 36.54	1.63 ± 0.41	0.78 ± 0.11
		<i>Dermacentor reticulatus</i>	6	385	175.33 ± 55.58	3.25 ± 0.97	0.39 ± 0.2
4	(Benjamino <i>et al.</i> 2018)	<i>Reticulitermes flavipes</i>	169	812	318.85 ± 62.76	4.4 ± 0.5	0.2 ± 0.09
5	(Sapountzis <i>et al.</i> 2015)	<i>Acromyrmex echinatior</i>	9	38	7.33 ± 4.58	0.66 ± 0.4	0.92 ± 0.12
		<i>Acromyrmex octospinosus</i>	9	32	7 ± 4.03	1.02 ± 0.21	0.81 ± 0.11
		<i>Acromyrmex volcanus</i>	1	8	8 ± NA	1.05 ± NA	0.83 ± NA
6	(Ramalho, Bueno and Moreau 2017)	<i>Camponotus planatus</i>	9	304	46.67 ± 45.2	0.76 ± 0.57	0.93 ± 0.1
7	(Kwong <i>et al.</i> 2017)	<i>Anthophora abrupta</i>	6	15	6.33 ± 2.5	0.93 ± 0.45	0.85 ± 0.08
		<i>Apis andreniformis</i>	25	23	8.8 ± 2	1.58 ± 0.28	0.64 ± 0.12
		<i>Apis cerana</i>	102	63	9.17 ± 3.01	1.68 ± 0.34	0.59 ± 0.14
		<i>Apis dorsata</i>	27	35	15.85 ± 2.68	2.16 ± 0.24	0.48 ± 0.09
		<i>Apis florea</i>	14	14	8.93 ± 1.07	1.67 ± 0.16	0.61 ± 0.09
		<i>Apis mellifera</i>	84	36	9.63 ± 2.04	1.78 ± 0.25	0.56 ± 0.11
		<i>Bombus bifarius</i>	7	10	4.14 ± 1.57	0.93 ± 0.36	0.86 ± 0.12
		<i>Bombus pensylvanicus</i>	5	15	9.4 ± 0.55	1.61 ± 0.21	0.65 ± 0.09
		<i>Centris atripes</i>	6	18	6.17 ± 0.75	0.98 ± 0.38	0.85 ± 0.12
		<i>Austroplebeia australis</i>	3	11	5 ± 3.46	0.65 ± 0.51	0.92 ± 0.08
		<i>Geniotrigona thoracica</i>	10	15	8.5 ± 1.51	1.64 ± 0.18	0.61 ± 0.08
		<i>Heterotrigona itama</i>	21	21	7.05 ± 1.66	1.41 ± 0.19	0.69 ± 0.1
		<i>Paratrigona subnuda</i>	11	26	10.82 ± 1.6	1.88 ± 0.31	0.54 ± 0.13
		<i>Partamona helleri</i>	5	21	15.4 ± 2.07	2.03 ± 0.31	0.55 ± 0.11
		<i>Plebeia droryana</i>	8	15	5.88 ± 2.8	1.34 ± 0.33	0.7 ± 0.11
		<i>Sundatrigona moorei</i>	8	21	9.38 ± 2.77	1.5 ± 0.43	0.67 ± 0.14
		<i>Tetragonula carbonaria</i>	6	16	6 ± 1.41	1.21 ± 0.29	0.77 ± 0.1
		<i>Tetragonula davenporti</i>	3	12	9.33 ± 1.53	1.65 ± 0.25	0.63 ± 0.11
		<i>Tetragonula fuscobalteata</i>	15	21	4.33 ± 2.38	0.62 ± 0.42	0.92 ± 0.08
		<i>Tetragonula laeviceps</i>	2	7	4 ± 1.41	0.49 ± 0.52	0.98 ± 0.02
		<i>Trigona hyalinata</i>	19	33	12.21 ± 3.1	1.72 ± 0.37	0.64 ± 0.15
		<i>Trigona spinipes</i>	14	42	13.57 ± 3.06	1.9 ± 0.34	0.56 ± 0.14
8	(Corby-Harris, Maes and Anderson 2014)	<i>Apis mellifera</i>	26	74	17 ± 2.58	1.38 ± 0.26	0.73 ± 0.09
9	(Lawrence <i>et al.</i> 2015)	<i>Echidnophaga ambulans</i>	16	1700	220.62 ± 37.95	1.99 ± 0.49	0.71 ± 0.1
10	(Adair <i>et al.</i> 2018)	<i>Drosophila melanogaster</i>	101	1193	58.23 ± 39.21	1.48 ± 0.92	0.72 ± 0.22
11	(Bost <i>et al.</i> 2018)	<i>Drosophila falleni</i>	8	416	134.5 ± 41.93	3.25 ± 0.59	0.33 ± 0.13
		<i>Drosophila neotestacea</i>	8	413	154.62 ± 30.63	3.39 ± 0.38	0.31 ± 0.12
		<i>Drosophila putrida</i>	8	424	148.12 ± 30.39	3.31 ± 0.45	0.33 ± 0.12
12	(Vogel <i>et al.</i> 2017)	<i>Nicrophorus vespilloides</i>	4	86	51 ± 9.76	2.24 ± 0.51	0.54 ± 0.2

Note: all numbers based on OTU and ASV tables subsampled to 3000 symbionts/sample. Numbers shown as counts or means ± standard deviations.

SI: The microbiome of social spiders (Genus *Stegodyphus*): host and population-specific patterns

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Paper III

Temporal and spatial dynamics of the social spider *Stegodyphus dumicola* microbiome

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Intended for publication in *FEMS Microbiology Ecology*



Photo: Tharina Bird

Temporal and spatial dynamics of the social spider *Stegodyphus dumicola* microbiome

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ABSTRACT

All organisms harbor a microbiome that may form an obligatory core microbiome with specific functions, or facultative associations that are context-dependent and vary with temporal or spatial conditions. To assess the temporal and spatial dynamics of associations between a host and its microbiome requires long-term data from multiple natural populations. We characterized the temporal and spatial variation in the microbiome within and between six populations of the social spider *Stegodyphus dumicola* using 16S rRNA gene amplicon sequencing. We show that the microbiome composition of *S. dumicola* varies between nests within populations, and that individuals within a nest share a highly similar microbiome, which remains stable over several generations and is not predictably affected by seasonal variation in temperature or humidity. qPCR analyses showed that the bacterial load is relatively low and stable in healthy nests, but sometimes shows dramatic increases in dying nests. These increases are not driven by any specific bacterium, and are likely caused by a general loss of host immune control under deteriorating conditions. *Stegodyphus dumicola* nests show an annual survival rate of approximately 50%, but nest death is not correlated to microbiome composition, and the bacteria found in *S. dumicola* are not considered to be high virulence pathogens.

INTRODUCTION

All animals live in close association with microbes (Feldhaar 2011; Ferrari and Vavre 2011), which may form an obligatory core microbiome with specific functions (Douglas 2009; Feldhaar 2011; Morse *et al.* 2012; Engel and Moran 2013; Rohrscheib *et al.* 2015), or facultative associations that are context-dependent and vary with temporal or spatial conditions (Sela *et al.* 2008; Muegge *et al.* 2011). Due to short bacterial generation times, host-bacterial symbioses have the potential to facilitate rapid host responses to local ecological or environmental conditions (Kjeldsen *et al.* 2012), possibly providing a direct link between ecological conditions and host function. However, our ability to assess host-microbiome relationships is limited by a general lack of data on temporal and spatial variation of the microbiome in natural populations.

The social spider *Stegodyphus dumicola* (Eresidae) occupies a wide geographical range across Central and Southern Africa (Majer, Svenning and Bilde 2013) and its population dynamics are characterized by frequent extinction and colonization events. Colonization happens by long-distance dispersal of single mated females, whose offspring continue the family lineage (Lubin and Bilde 2007). Families of spiders live in communal nests containing tens to hundreds of individuals (Avilés 1997), who collaborate on all tasks from brood care to prey capture (Lubin and Bilde 2007). Nests are geographically aggregated in populations, and because new nests can arise from the fission of existing nests, these populations are likely to descend from a single colonization. Nests experience high rates of turnover (Bilde *et al.* 2007), as also documented in another social *Stegodyphus* species (Crouch and Lubin 2001). Combined with a biology that includes a strictly inbreeding mating system and female bias (Lubin and Bilde 2007; Vanthournout *et al.* 2018 - Paper I; Bechsgaard *et al.* 2019), this has severe consequences for genetic diversity, which is extremely low both within and between populations across its range (Settepani *et al.* 2017). This population genetic structure is not mirrored in the spiders' microbiome. *Stegodyphus dumicola* have low

diversity microbiomes, which tend to be dominated by a one or two bacterial symbionts (Busck *et al.* in prep. – Paper II). Individual spiders belonging to the same nest have very similar microbiome compositions, but this similarity does not extend to the population level (Busck *et al.* in prep. – Paper II). There is high variability in microbiome composition between closely related nests, and along with the dominance of single symbionts in individual spiders, this is indicative of unstable microbiomes. The current knowledge of the *S. dumicola* microbiome is limited to relative abundances of symbionts, measured in spiders sampled at single time points. Therefore, we are currently unable to distinguish between long-term stable symbiotic relationships, and a series of transient infections.

To assess the temporal dynamics of the microbiome composition within and across generations, we conducted an *in situ* time-series analyses of the *S. dumicola* microbiome by sampling six natural populations for a period of two and a half years. An earlier study showed that spiders sharing the same nest have extremely similar microbiome composition (Busck *et al.* in prep. – Paper II), which provides a unique opportunity to assess temporal variation in the microbiome by sampling new closely related individuals from the same nest within and across generations. Every three months, we determined the microbiome composition of three individuals sampled from each nest by whole-body 16S rRNA gene amplicon sequencing, and subsequently we performed qPCR to estimate bacterial load. By analyzing microbiome composition and bacterial load of *S. dumicola*, sampled across a 2.5-year period we address the following questions: (i) How is the variation in microbiome composition distributed across nests and populations, and is the distribution similar across the species distribution range? (ii) How does the microbiome composition and bacterial load of *S. dumicola* vary within and across generations of a nest? (iii) What is the survival rate of *S. dumicola* nests, and is nest mortality correlated to microbiome?

MATERIALS AND METHODS

Sample collection

Stegodyphus dumicola spiders were collected approximately every 3 months from 5 populations in Namibia (around Otavi, Windhoek, Stampriet, Betta and Karasburg) and 1 population in Botswana (around Palapye) in the period from April 2017-November 2019. In each population, the same 5-10 nests were sampled at each time point, while a replacement nest was included if a nest died or disappeared during the study period. Three spiders were sampled from each nest at each time point (see Table S1 and Fig. S1). Sampled spiders were lured out by vibrating the capture web. The total body length (in mm from front of prosoma until tip of abdomen) was measured before individuals were placed in ATL buffer (Qiagen) in Eppendorf tubes.

Environmental data collection

iButton™ temperature/humidity loggers (Maxim integrated) were placed in several nests in each population, logging nest temperature and relative humidity every hour. During each sampling trip, the data was extracted from the iButtons using a dual Blue Dot™ Receptor and OneWireViewer software (Maxim integrated).

DNA extraction and 16S rRNA gene amplicon sequencing

DNA was extracted from whole spiders using DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol (specifically "Purification of total DNA from animal tissue – spin-column protocol"). The spiders extracted in Denmark (see Table TS_SI_PCRtable.xlsx) were homogenized using liquid nitrogen and a sterile pestle, spiders extracted in Namibia/Botswana were homogenized without the use of liquid nitrogen. Fully grown female spiders (~1cm) were extracted using double reagent volumes and two spin-columns per spider.

16S ribosomal RNA gene amplicon libraries were prepared according to Illumina's 16S Metagenomic Sequencing Library Preparation guide, with slight modifications (see SI Table and SI methods – Not included in thesis) using Bac 341F and Bac 805R primers to amplify variable regions V3 and V4 (Herlemann *et al.* 2011). For each run, a pooled library containing amplicons from all samples was sequenced on a MiSeq desktop sequencer (Illumina) according to the provided preparation guide.

16S rRNA gene amplicon analysis

All analysis was done in R v 3.6.1 (R Core Team 2019). Sequences were trimmed to remove barcodes and primers using cutadapt (Martin 2011). Each sequencing run was processed separately for quality filtering, denoising, and paired-end merging using the R package ‘dada2’ v. 1.12.1 (Callahan *et al.* 2016) with filter settings maxEE=(2, 2), truncLen=230, and truncQ=2 to identify ASVs (Amplicon Sequence Variants). Data from the separate sequencing runs were then merged for chimera finding and classification using dada2 and Silva SSU reference database nr. 132 (Quast *et al.* 2013). ASVs were filtered to a minimum length of 400 and to exclude chloroplasts, mitochondria and non-bacteria. Samples with fewer than 7000 remaining reads were excluded from further analysis.

For any analysis involving diversity measures, amplicon reads were subsampled to a common depth of 7000 reads (with seed=42). For everything else, amplicon reads are reported as fractions of all reads per sample. If subsampling was used, it will be clear from the Fig. caption.

All remaining analysis and visualization of community data was done with custom R scripts, using several useful packages such as 'ggplot2' v. 3.2.1 (Wickham 2009), 'vegan' v. 2.5.6 (Oksanen *et al.* 2019).

qPCR

Quantitative real-time PCR (qPCR) was used to determine the total bacterial load (16S copies/spider gene copies) in whole spiders. qPCR was only run on a subset of the samples used for 16S amplicon sequencing (working from the same DNA extraction). Initially we ran qPCR on samples from 6 relatively long-lived nests belonging to two populations. When this showed interesting results in the last samples before nest death, we added samples taken from the last two sampling trips of 15 nests which died in the course of our study.

The ratio between bacterial DNA copies and spider DNA copies was measured by amplifying a 167 bp fragment of bacterial 16S rRNA gene using the universal primer pair Bac908F/Bac1075R (Mateos-Rivera *et al.* 2018) and a 147 bp fragment of a conserved *Stegodyphus* gene using the primer pair 5F/5R (Settepani, Bechsgaard and Bilde 2016). To ensure comparable standard curves, a plasmid containing a hybrid of the two fragments was constructed and synthesized (Eurofins) to be used as a qPCR standard. A 10x dilution series of the plasmid was run with every plate to create accurate standard curves. All PCRs were run in triplicate in a 20 µl reaction volume containing 10 µl LightCycler 480 SYBR Green I Master (Roche), 2 µl Bovine Serum Albumin (10 µg/ml), 1 µl of forward primer (10 pmol/µl), 1 µl of reverse primer (10 pmol/µl), 4 µl dH₂O and 2 µl template DNA. qPCR assays were run on the Stratagene Mx3005P qPCR system with the following conditions: 5 min. initial denaturation at 95 °C, 45 cycles of [30 sec. denaturation at 95 °C, 30 sec. annealing at 59 °C, 20 sec. elongation 72 °C, 15 sec. acquisition at 80 °C for the spider fragment and 78 °C for the bacterial fragment] followed by a final melting curve with 95 °C for 1 min. and ramping temperatures 59-95 °C, 30 sec. at each degree.

qPCR data was filtered based on Ct values of both bacterial and spider fragments. Samples were only included in analysis if standard deviation of the technical replicates was below 0.167. In some instances, one technical replicate was excluded to satisfy this condition.

Survival analysis

Kaplan-Meier analysis was run on a dataset of 73 nests. Not all nests included in the survival analysis were used for 16S amplicon sequencing, and several nests used for 16S amplicon sequencing were excluded from the survival analysis because sampling of them stopped without a sure observation of natural death (e.g. nests gone because of road-side maintenance) (Fig. S1). The analysis was run in R using the package ‘survival’ v. 2.44.1.1 (Therneau 2015) and plotted using ‘survminer’ v. 0.4.6 (Kassambara, Kosinski and Biecek 2019). Nests still alive at the end of the study were right censored. Nest sampling times were corrected to days since sampling start, meaning the first sampling of each nest was set to T_0 , even though sampling start were staggered in real time.

Data availability

Sequences in SRA. qPCR, ASV tables, taxonomy tables and sample data in SI

RESULTS AND DISCUSSION

***Stegodyphus dumicola* have low diversity microbiomes with clear nest structure**

We identified 12914 bacterial amplicon sequence variants (ASVs) in 569 *S. dumicola* samples.

Only 789 ASVs appeared in 5 or more samples, and no ASV appeared in all spiders sampled, indicating that no obligate symbiosis with any specific bacterium exists. Detailed sample information and read statistics, as well as ASV sequences and classifications are available in the supporting information (Table S1, meta- tax- and asv-tables).

The individual spider microbiomes had an average Shannon diversity index of 0.65 ± 0.68 (mean \pm sd), which is low compared to other spiders (Zhang *et al.* 2018) and to eusocial arthropods (Raymann, Shaffer and Moran 2017; Benjamo *et al.* 2018; Chua *et al.* 2018). Note that two factors in our study have likely increased the estimated alpha diversity: First, the denoising algorithm we used to group reads into ASVs allows for a higher taxonomic resolution than the clustering algorithms used in many comparable studies (Callahan, McMurdie and Holmes 2017). This is exemplified in the two main *Mycoplasma* ASVs in this study (ASV_1 and ASV_3), which differ by only 1/426 base pairs, and would have been grouped together in the same operational taxonomic unit by a clustering algorithm. Secondly, many arthropod microbiome studies are based on surface sterilized animals (Zhang *et al.* 2018), or even dissected guts (Raymann, Shaffer and Moran 2017), while we have sampled whole, unwashed spiders.

As previously shown (Busck *et al.* in prep. – Paper II), spiders within a nest typically had the same dominant symbiont, and shared overall highly similar microbiome composition (Fig. S2), suggesting a shared “nest-mate microbiome”. 10 dominant ASVs belonging to 4 taxonomic groups (*Mycoplasma*, *Diplorickettsia*, Spirochaetaceae and Weeksellaceae) made up $> 80\%$ of all 16S gene amplicons sequenced in this study. These dominant symbionts were not evenly distributed between individual spiders, instead different ASVs dominated in different hosts (i.e. making up $> 50\%$ of

relative abundance), and did so in 491 out of 569 individual spider microbiomes. Some of the dominant taxonomic groups were represented by 2-5 ASVs, but all shared > 99% sequence identity within the group. There were other ASVs (classified as *Acaricomes*, *Rickettsiella*, *Spiroplasma*, and *Rickettsiaceae*) which dominated individual microbiomes, but they were not as prevalent as the four dominating ASVs mentioned above (Fig. 2). The four dominant ASVs also occurred at low abundances in spiders from other nests with different dominating ASVs, and there seemed to be no strict inhibition between the taxonomic groups as they all could co-occur in different configurations in the same host (Fig. 2, Fig. S2). The 4 main dominant taxonomic groups matched the dominant symbionts found in a study of South African *S. dumicola* populations (Busck *et al.* in prep. – Paper II), several of them being identical at the ~400 bp ASV level. These dominant symbionts are not common to all spiders (Vanthournout and Hendrickx 2015; Zhang *et al.* 2018), but do seem to share phylogenetic histories with other arachnid symbionts (Busck *et al.* in prep. – Paper II). Except for Weeksellaceae, all the dominant symbionts are most closely related to obligately host-associated bacteria (Mediannikov *et al.* 2010; Brown *et al.* 2015; Barbour 2018), which means they most likely cannot survive outside the host, and are dependent on direct host-to-host transmission. These facts suggest that the dominant symbionts are not random bacteria acquired from the environment, but are specific symbionts that have evolved to depend on their association with *S. dumicola*.

Nest microbiome composition changes only slightly across sampling trips and generations

We found that the composition of *S. dumicola* nest-mate microbiomes was surprisingly stable over time (Fig 2, Fig. 3). The dominant ASV rarely changed, and there were no clear cyclical changes indicating any seasonal effects (Fig. 2). Using Bray-Curtis dissimilarity (BC) to compare individual spider microbiomes (Fig. 3), we found a very low dissimilarity between nest-mates sampled at the same timepoint (median BC = 0.081). This dissimilarity was slightly increased when comparing

nest-mates sampled at different timepoints (median BC = 0.113), and additionally increased if a generational shift had happened between sampling trips (median BC = 0.145). All these increases in dissimilarity were significant (Fig. 3), indicating that small changes in spider nest-mate microbiomes occurred over time, and that the rate of change was increased in generational shifts. However, within-nest Bray-Curtis dissimilarities, were not correlated to the time between sampling (Fig. S3), indicating that the microbiome composition was undergoing random fluctuations rather than directed change. Meanwhile, spiders from different nests (but in the same population) sampled at the same time point had a much higher median Bray-Curtis dissimilarity (median BC = 0.950), which was almost as high as the dissimilarity between populations (median BC = 0.994), showing that nest identity was a better predictor of microbiome composition than either sampling time, generation or population (Fig. 3).

Little is known about the transmission routes of *S. dumicola* symbionts, but there is no evidence of strict vertical transmission e.g. via eggs. The spiders have an annual life cycle with several months overlap between generations where extensive brood care including regurgitation feeding of juveniles (Salomon and Lubin 2007) likely facilitates high-fidelity horizontal transmission, which can be viewed as a form of vertical transmission (Schmid-Hempel 1998). Communal feeding (Schneider and Bilde 2008) and collaborative brood care (Salomon and Lubin 2007; Junghanns *et al.* 2017) means that symbionts can not only be passed from mother to offspring, but are likely continuously passed between nest-mates both within and between generations. This – together with the fact that the spiders depend on their capture web for prey, and never leave the nest – likely explains how the microbiome can remain relatively stable within a nest for years, even without any of the symbionts being obligate for their spider hosts.

The stability of microbiome composition within nests stands in stark contrast to the microbiome dissimilarity between nests from the same population (many of which are likely to be

closely related). We believe that the founding of new nests, offers opportunities for larger shifts in microbiome composition than what is seen within a nest over time. When an individual dispersing female establishes a new nest (Lubin and Bilde 2007), she is in an isolated condition without the constant symbiont-exchange with a larger group of nest-mates, and random fluctuations in microbiome composition are more likely to become fixed. The female will subsequently propagate her changed microbiome to her offspring, giving rise to a new, changed but stable nest-mate microbiome. It is also possible that a dispersing female joins or visits an existing nest, although genetic data suggests this is rare (Johannesen *et al.* 2002). This could lead to mixing of different microbiomes, and similarly provide an explanation for the observed variation in microbiome composition between nests (Busck *et al.* in prep. – Paper II).

Bacterial load is stable in healthy nests but increases before nest death

Initial qPCR of samples from 6 selected nests ($n= 84$) showed a generally low and stable bacterial load (median = 1.37 16S copies/spider gene copies). It did not fluctuate predictably with the seasons (Fig. 4) as seen in bees (Kešnerová *et al.* 2019), nor was it correlated to body size (Fig. 5 B). There were, however, some extreme outliers in the data (max = 6486.67 16S copies/spider gene copies) (Fig. 4, SI table qPCR results). Interestingly, these high bacterial load outliers were all found in nests that were either clearly dying (the spiders were in bad condition and their numbers in the nest declining, T Bird pers obs), or would be found dead by the next sampling trip (Fig. 4). To investigate this connection further, we used qPCR to estimate bacterial load in spiders belonging to several other nests which had died in the course of our study, focusing on the last sampling (~3 months before the nest was found dead) and the second-to-last sampling (~6 months before the nest was found dead). Here, we will refer to these nests as “dying”, although most seemed healthy at the time of sampling. In 6 out of 18 dying nests, spiders had high bacterial loads at the last sampling,

and in 3 of these nests, the increased bacterial load was already apparent - though less pronounced - in the second-to-last sampling (Fig. 5 A, Fig. S4).

It is tempting to view these increased bacterial loads as a sign that pathogenic bacteria are causing nest death, but the bacterial load increases were not tied to any specific ASV (Fig. 5 C, Fig. S4), and happened in nests with very different microbiome compositions. Furthermore, only a few of the affected spiders had atypical microbiome compositions (compared to healthy nest mates from earlier samplings), while many seemed “normal” when looking only at relative abundances of symbionts (Fig. S4, Fig. S2).

In the absence of specific bacterial pathogens, the dramatic increase in bacterial load might be caused by a general loss of host immune control (Schneider and Ayres 2008). In such cases, bacteria which are normally beneficial or commensal can become opportunistic pathogens, possibly by invading parts of their hosts which were previously protected by the immune system. We cannot be certain what could cause such impairment of immune function in *S. dumicola*, but we suspect that fungal infections in the nest or in the spiders themselves may play a role (Henschel 1998). Fungi are known pathogens of arthropods (Lovett and St Leger 2017), and their ability to spread between nests as spores, would allow them to be much more virulent than the non-spore forming obligately host-associated bacterial symbionts we have identified (Hughes, Pierce and Boomsma 2008). We have attempted to identify fungi present in or on the spiders by ITS amplicon sequencing, but have not been successful.

Nest survival rate is low, and is not correlated to microbiome composition

Of the 40 nests chosen at the start of the study, only 6 (15%) were left alive at the end of the 2.5-year study period (Fig. S1). In order to keep all populations represented, new nests were added to the study when existing nests died. This high turnover of social *Stegodyphus* nests has been reported elsewhere (Crouch and Lubin 2001; Bilde *et al.* 2007). Great care was taken not to disturb the nests during sampling, but we cannot rule out that sampling might have decreased the nests' chance of survival. Furthermore, we cannot estimate the full lifespan of nests, since we do not know the age of the nests at the start of sampling. Kaplan-Meier survival analysis showed a median nest survival time of 312 days from sampling start, and an average survival rate of 48% in the first year sampled (Fig. 6 A). Once again, there were no clear differences in microbiome composition between healthy spiders, and spiders sampled ~3 months before their nests was found dead. However, as discussed above, nest death may not be driven by bacterial pathogens.

Evaluating each population separately, they fell into two groups: Karasburg, Betta and Windhoek had higher survival rates, and Otavi, Stampriet and Botswana had lower survival rates (Fig. 6 B). Otavi and Botswana stood out from the other populations by experiencing relatively higher humidity (data from loggers, Fig. 1 B). It may be coincidental that these two populations also had some of the lowest survival rates (especially because Stampriet had both low survival and low humidity), but a correlation of nest humidity and mortality could support the fungal pathogen hypothesis, as fungus load increases with humidity.

CONCLUSIONS

The microbiome composition of *S. dumicola* varies between nests within populations, however spiders within a nest share a highly similar “nest-mate microbiome” which is stable over time. The microbiome composition is not predictably affected by seasonal changes in temperature or

humidity, it remains stable through the lifecycle of the spider, suggesting it is passed on to the next generation. The bacterial load experienced by *S. dumicola* is low and stable in healthy nests, and does not fluctuate predictably with either seasons or body size, but sometimes shows massive increases in dying nests. These increases are not driven by any specific bacterium, and are likely caused by a general loss of host immune control under deteriorating conditions. *Stegodyphus dumicola* nests show an annual survival rate of approximately 50%, but nest death is not correlated to microbiome composition, and the bacteria found in *S. dumicola* are not considered to be high virulence pathogens.

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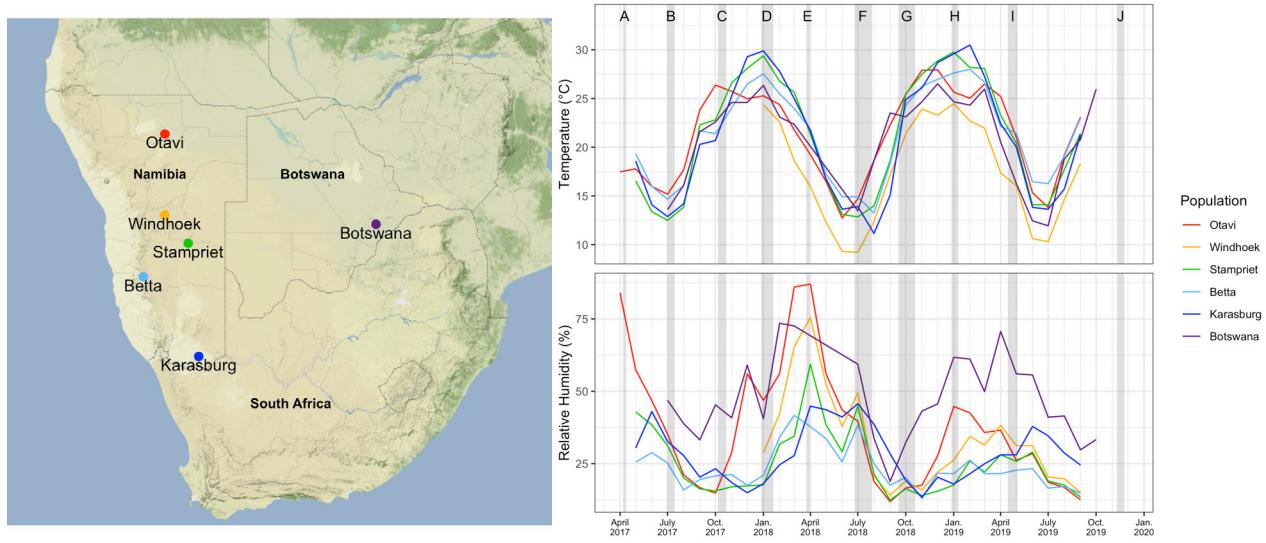
FIGURES

Figure 1 – A) Geographical location of *S. dumicola* populations sampled for this study. **B)** Monthly averages of temperature and relative humidity for each population. Sampling trips (A-J) indicated by letters on top panel and light grey shading. Data collected by several iButtons in each population placed both inside and outside of spider nests.

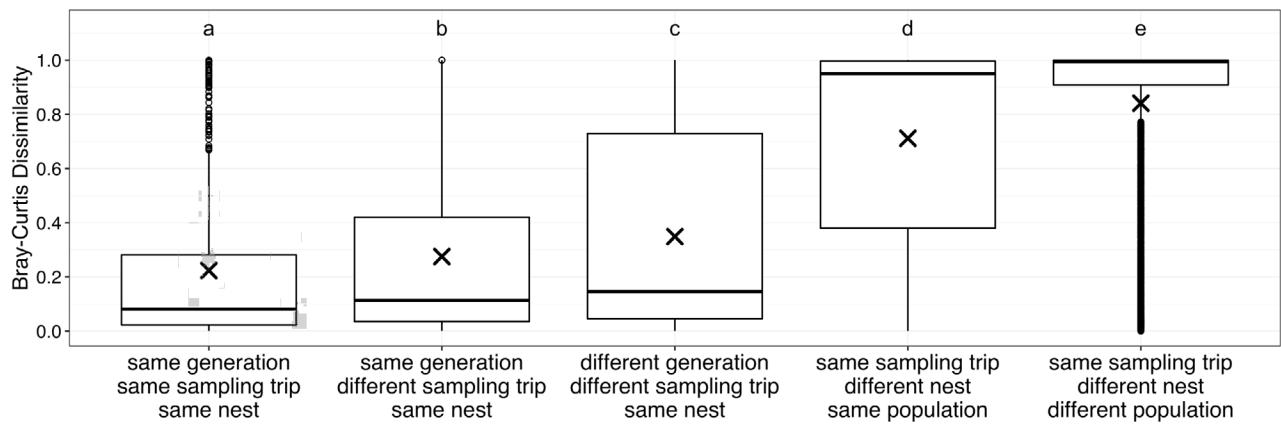


Figure 3 - Bray-Curtis dissimilarity between microbiomes of individual spiders. Grouped at different levels of comparison. Beta diversity measure based on data subsampled to 7000 ASVs per sample, including only ASVs above 0.1% relative abundance which were present in 5 or more samples across the whole data set. Significant differences between groups at $p < 0.01$ were determined using pairwise Wilcoxon rank sum test (BH adjusted), and are indicated by different letters (a, b, c, d, e) above boxes. X marks the mean.

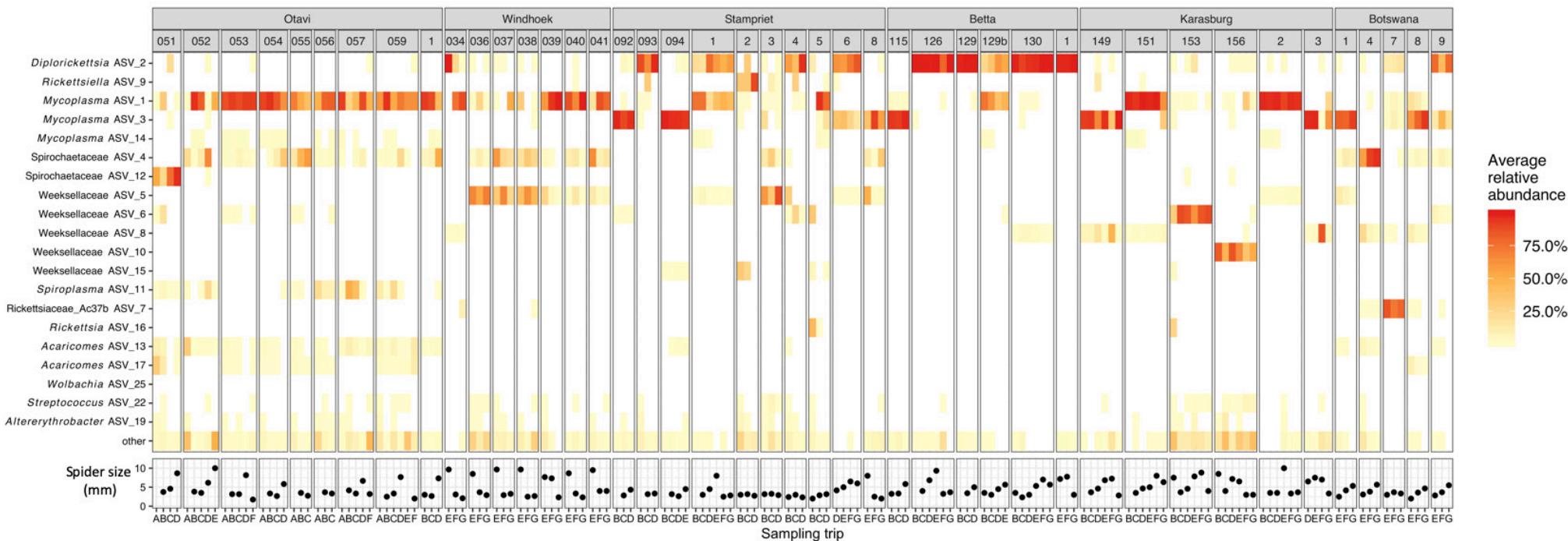


Figure 2 – ASV heatmap. Relative abundance and distribution of the 20 most abundant ASVs in populations and nests of *S. dumicola* across several sampling trips. Only nests sampled 3 or more times are included. Each column represents the nest average at a single sampling event (1-3 individual spiders). Bottom panel plots average size of sampled spiders. White cells signify relative abundances < 0.1%.

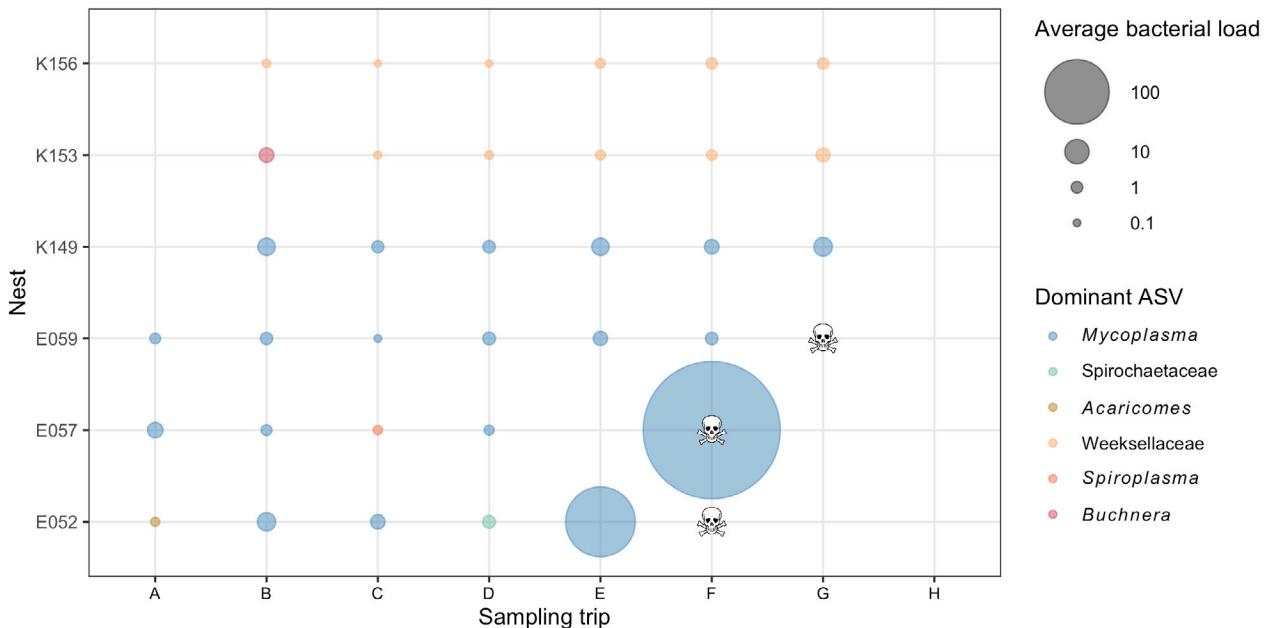


Figure 4 – Bacterial load in selected nests across sampling trips. Total n = 84. Each circle represents the average bacterial load (16S copy no./Spider gene copy no.) in the 1-3 spiders sampled from a given nest at a given sampling trip. Circle area is scaled to indicate bacterial load and circle color indicates taxonomic group of dominant ASV. Skull and crossbones symbols indicate the nests being found dead (or actively dying in the case of E057). White spaces indicate no sampling. E052, E057, E059, belong to the Otavi population, K149, K153 and K156 belong to the Karasburg population.

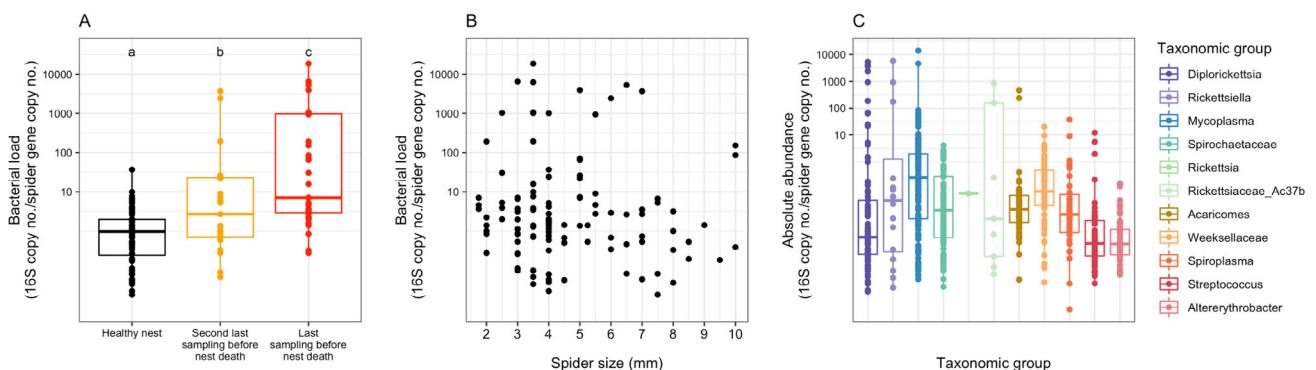


Figure 5 – Bacterial load correlations. All figures based on qPCR data from a total of X individual spiders. Note the log transformed y-axes **A)** Bacterial load in individual spiders sampled from nests at different timepoints before natural nest death. Healthy nest is defined by being found alive at the following two sampling trips. Significant differences between groups at p < 0.05 were determined using pairwise Wilcoxon rank sum test (BH adjusted), and are indicated by different letters (a, b, c) above boxes. **B)** Bacterial load versus spider body size. **C)** Absolute abundance of most abundant ASVs. ASVs are grouped and color-coded by lowest identified taxonomic level.

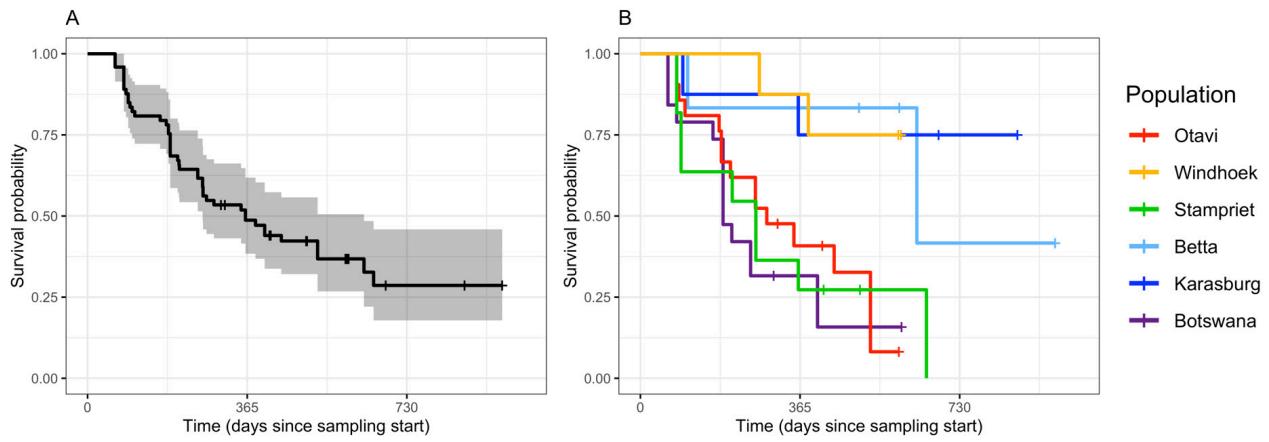


Figure 6 – Kaplan-Meier survival analysis of nests. t_0 = sampling start. Nests that were still alive at the end of the study period are right censored (indicated as vertical tick marks on the graphs). **A)** all nests in study (n=73) **B)** nests separated into populations.

SUPPORTING INFORMATION

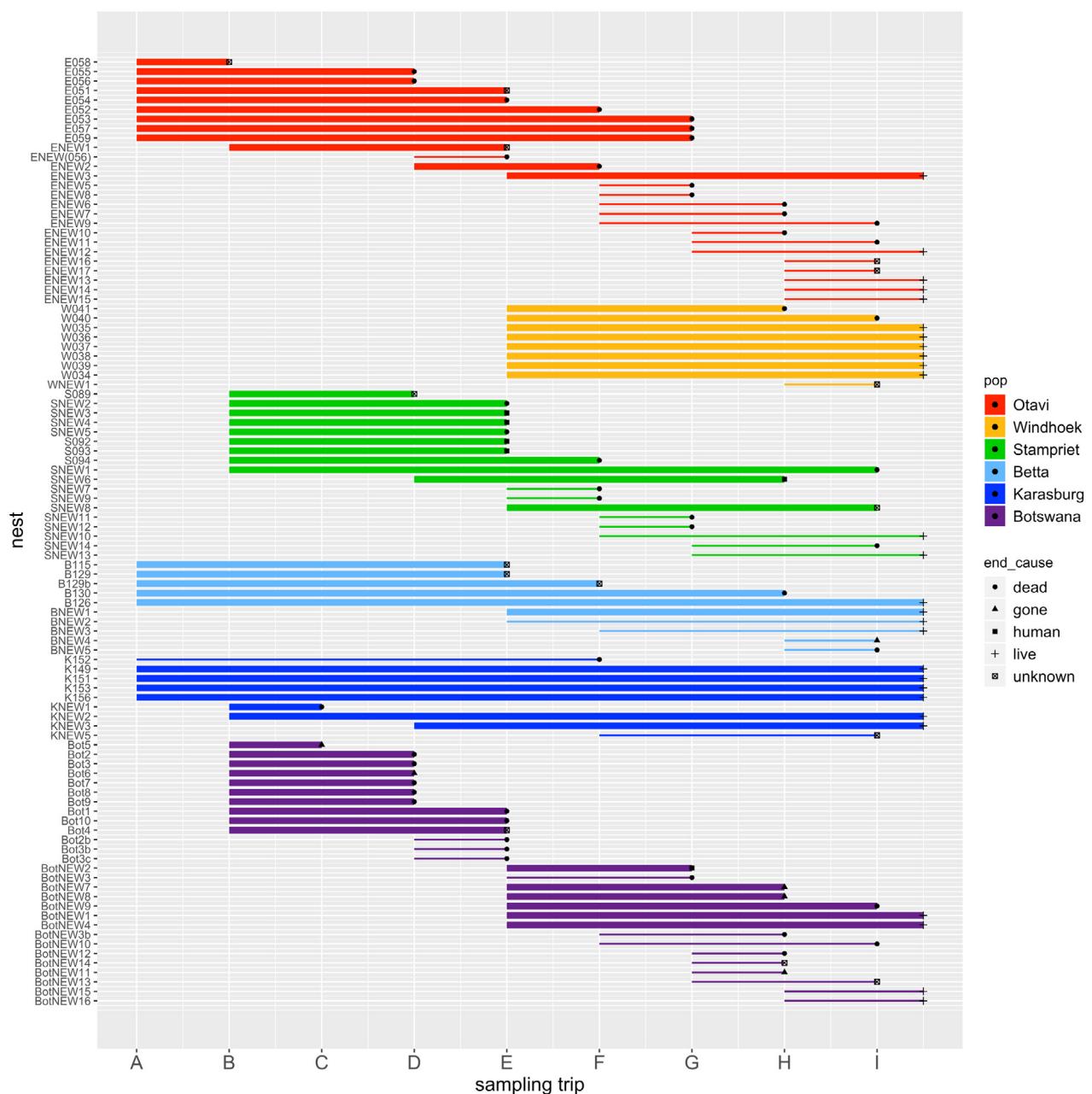


Figure S1 – Nest lifespan plot. Indicating start- and stop times of all nests used in the study. Line ends at the trip where nest was excluded from the study (see end_cause). Hence there are no samples from the last point on a nest's time line. Thick lines indicate nests that were used in the 16S amplicon sequencing part of this study.

Table S1 – Summary statistics of samples, sequencing, and community data

	All	Otavi	Windhoek	Stampriet	Betta	Karasburg	Botswana
Samples	569	123	68	105	73	97	103
Nests	61	13	8	11	6	7	16
Sampling start	20170413	20170413	20180325	20170701	20170704	20170702	20170715
Sampling end	20181019	20180921	20180917	20180918	20180920	20180919	20181019
Raw read number ^{a,c}	52262 (7682-500429)	51114 (7682-190371)	36320 (11076-85542)	53339 (12223-185197)	61936 (14635-477814)	54055 (7896-500429)	54512 (9877-133165)
Filtered read number ^{a,d}	39794 (7224-426198)	36990 (7372-122739)	29168 (7224-66210)	38758 (8543-158028)	47930 (12747-363826)	42756 (7354-426198)	42657 (9039-113305)
ASV number	12914	4443	3611	1669	566	4229	1851
ASV number per sample ^{a,d}	49.37 (1-858)	70.15 (2-858)	82.21 (4-452)	28.86 (3-237)	12.89 (1-198)	67.41 (2-466)	32.65 (4-307)
Shannon diversity ^{b,d}	0.648 +- 0.68	0.818 +- 0.79	0.925 +- 0.81	0.597 +- 0.54	0.243 +- 0.32	0.601 +- 0.74	0.645 +- 0.54
Simpson diversity ^{b,d}	0.257 +- 0.22	0.309 +- 0.25	0.335 +- 0.25	0.274 +- 0.2	0.124 +- 0.18	0.19 +- 0.2	0.285 +- 0.2
Dominance DMN ^{b,d}	0.931 +- 0.11	0.896 +- 0.14	0.9 +- 0.12	0.952 +- 0.09	0.989 +- 0.03	0.931 +- 0.12	0.931 +- 0.09

Note: Based on samples with minimum 7000 reads after filtering

a: Mean (minimum-maximum)

b: Mean ± standard deviation

c: Unfiltered forward read numbers from Miseq

d: After quality filtering, denoising, paired-end merging, chimera finding, length filtering (minimum 400bp) and taxonomic filtering

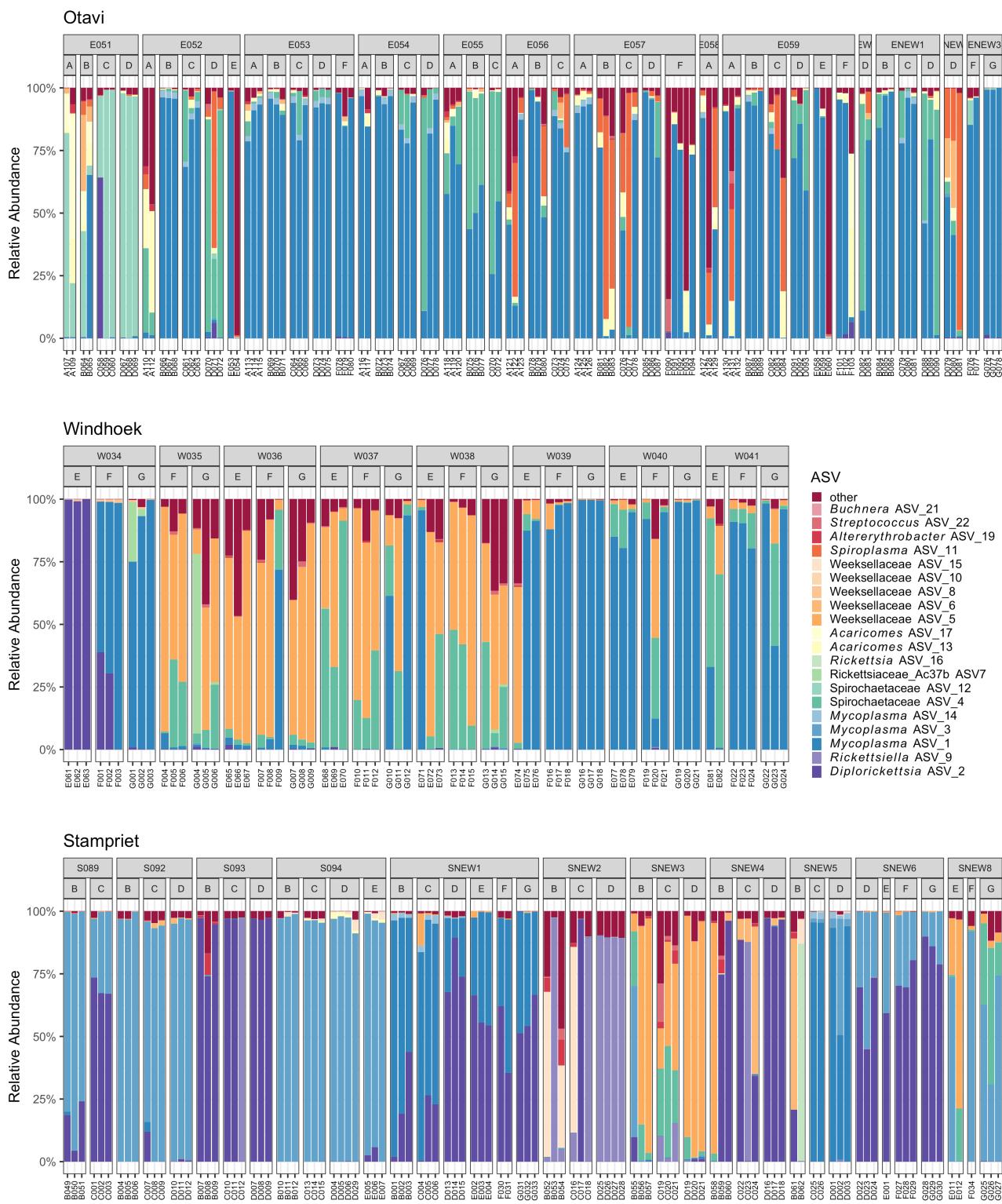
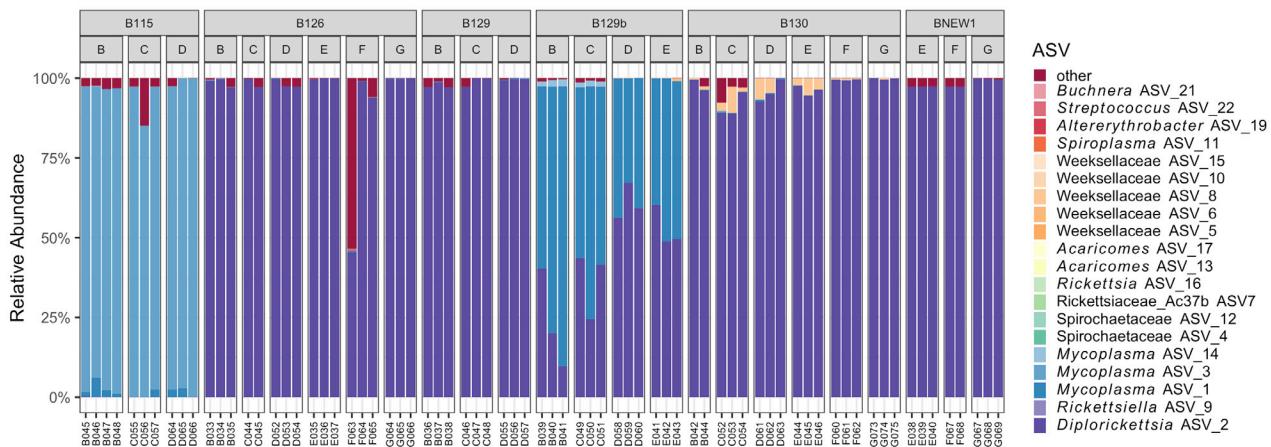
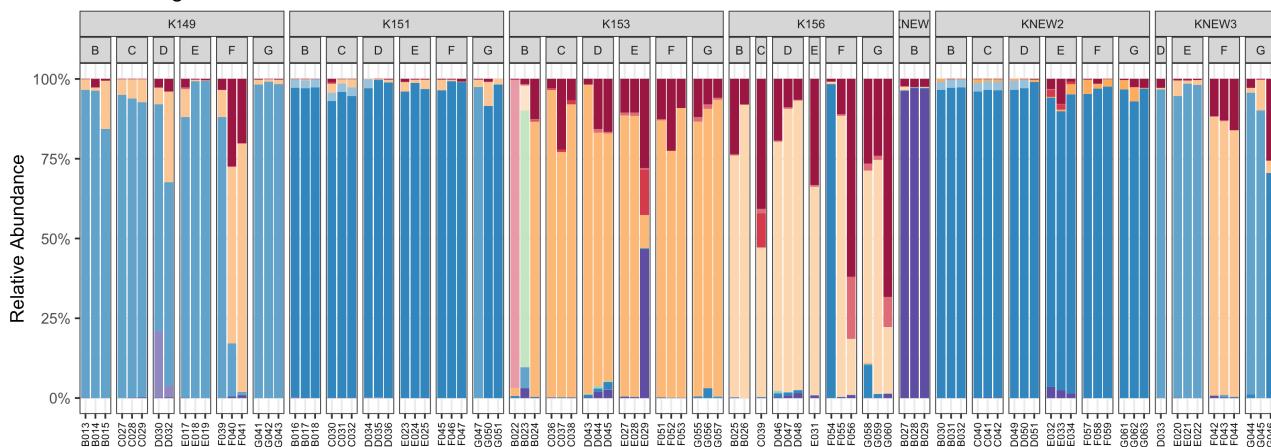


Figure S2 – Relative ASV abundance in all samples used in the 16S amplicon analysis, separated by population. Color is same for all populations (see legend by Windhoek and Betta plots). Facets indicate nest (top line) and sampling trip (bottom line).

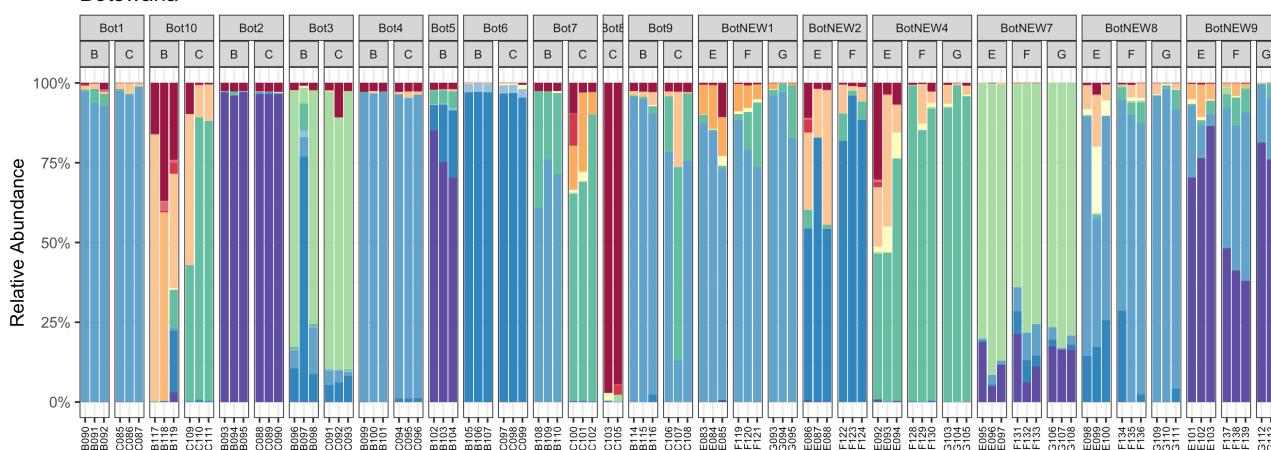
Beta



Karasburg



Botswana



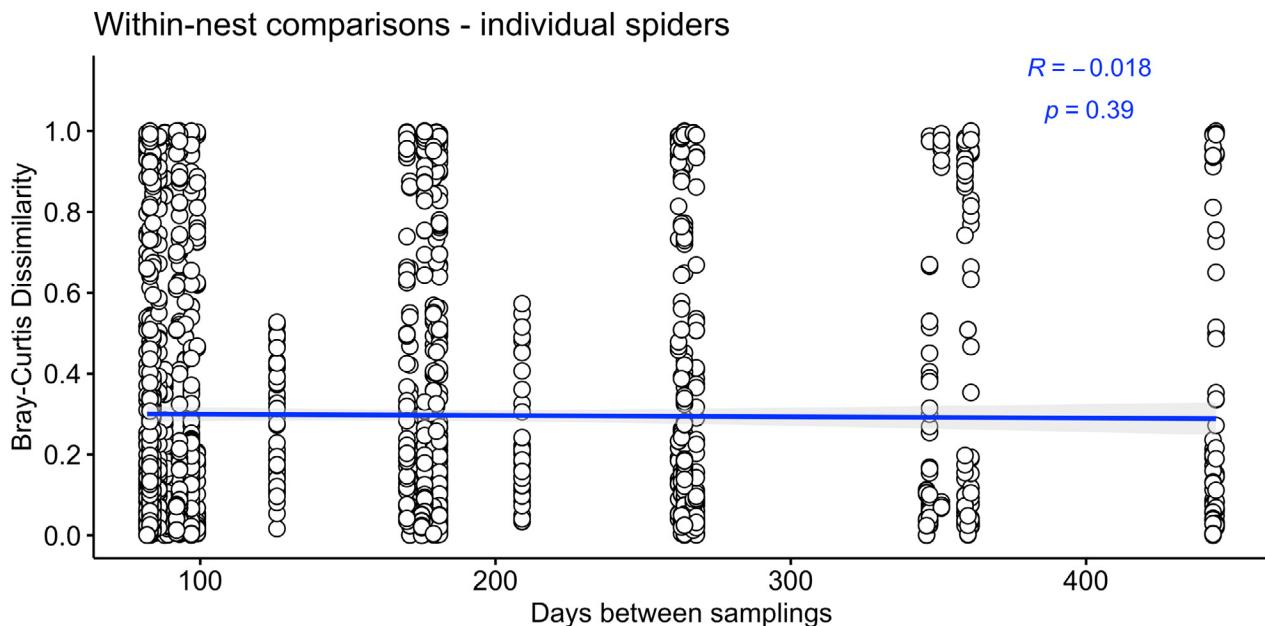
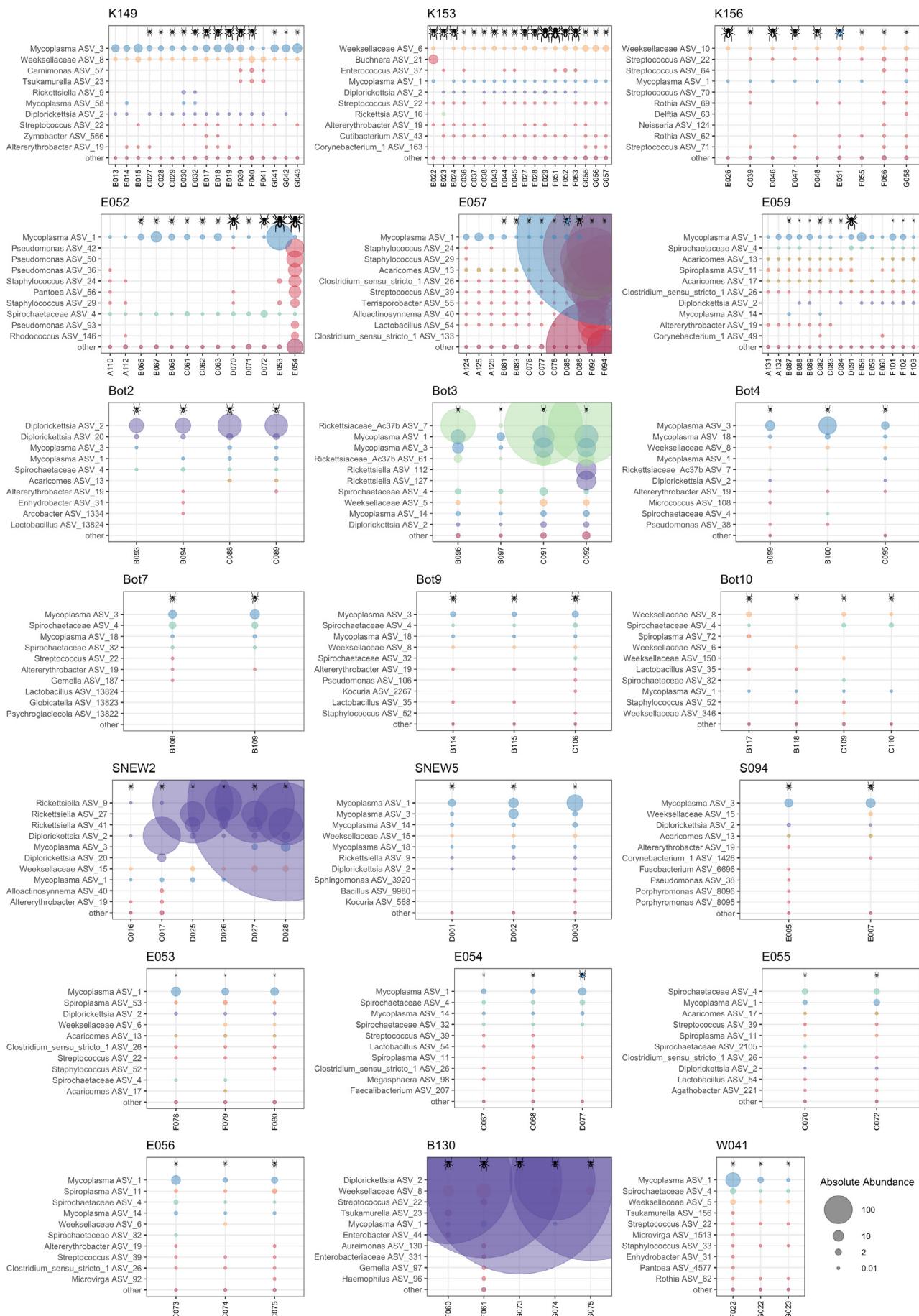


Figure S3 – Within nest Bray-Curtis dissimilarities vs. days between samplings. Each dot is a comparison of microbiome composition between two individual spiders from the same nest. Does not include same-trip comparisons. Regression based on Spearman Rank Correlation.

Figure S4 – (NEXT PAGE) Bubble plots showing absolute abundance (in 16S copies/spider gene copies) of the 10 most abundant ASVs in each of the selected nests. Each column is a single spider, used for qPCR and 16S, the starting letter of the sample name indicates sampling trip. Bubble size legend in the lower right corner. The last samplings before death are shown for all nests except for the top row (K149, K153 and K156), which lived beyond this sampling point.

Section 2 - Paper III



SECTION 3

Side projects and preliminary findings

Presented here is a group of smaller studies that I have been involved in during my PhD. Most of the hard work has been done by the students, lab techs, and supervisors mentioned throughout the text, and I have mostly just taken part in the planning and/or data analysis. I collect these findings here for future reference, but most of them are described in more detail in the bachelor-, master's- and project reports of the respective students.

The very last section contains pictures of varying scientific importance.

Transmission studies

To understand the functional relationship between host and bacterial symbiont, it is important to understand the transmission route. In *S. dumicola*, this is still under active investigation, but based on the preliminary results presented in this section we believe that transmission is indirectly vertical. Meaning that bacteria are transmitted between generations through interactions between adult females and young nymphs/ spiderlings (Fig 3.1), rather than by direct transmission in egg cells.

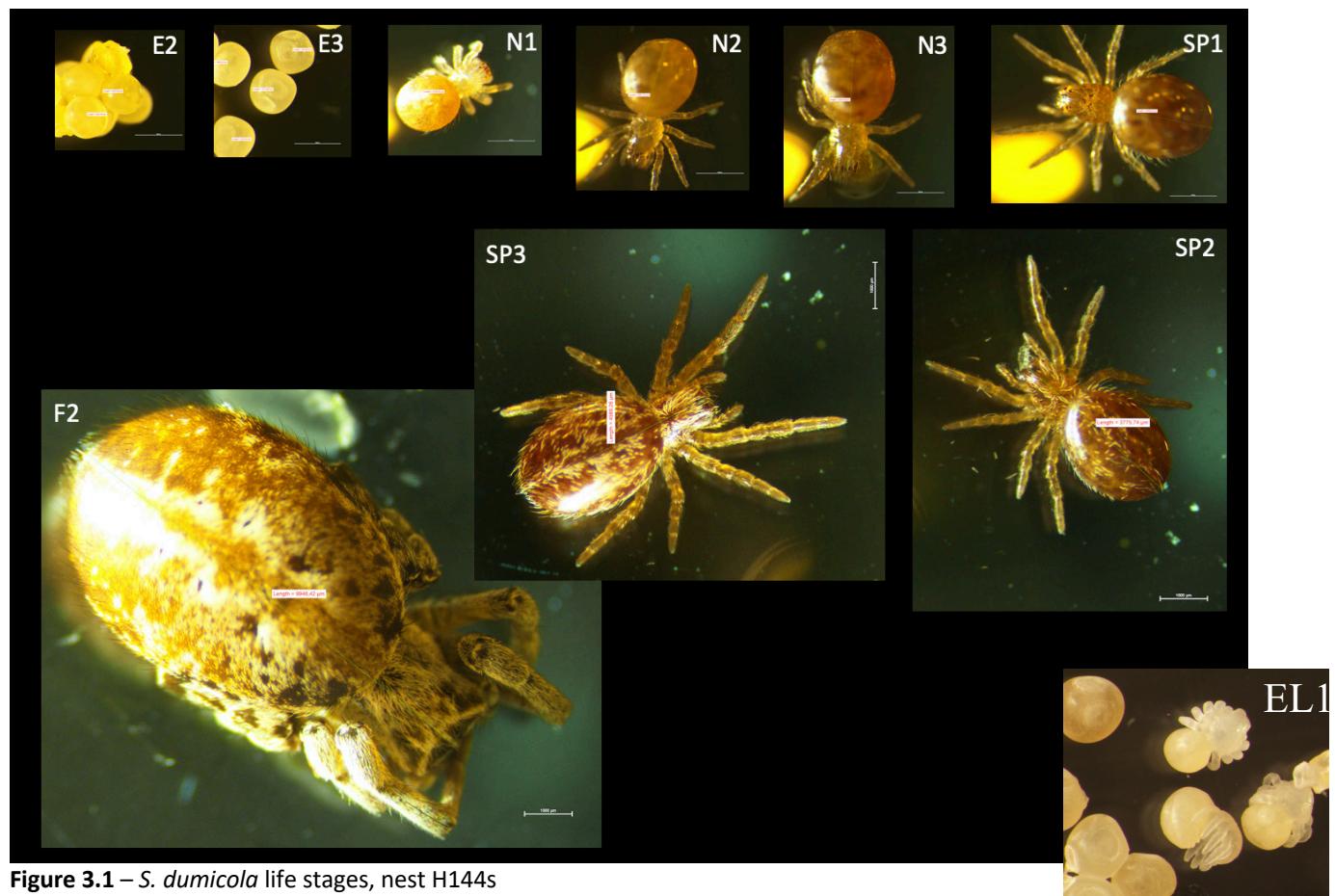


Figure 3.1 – *S. dumicola* life stages, nest H144s

Scale bar on all images = 1mm. E: Egg, N: Nymph, SP: Spiderling, F: adult Female.

Insert: "Eggs with legs" from a different nest. This stage falls between Egg and Nymph.

Photos by: Marie Rosenstand Hansen

LIFECYCLE 16S

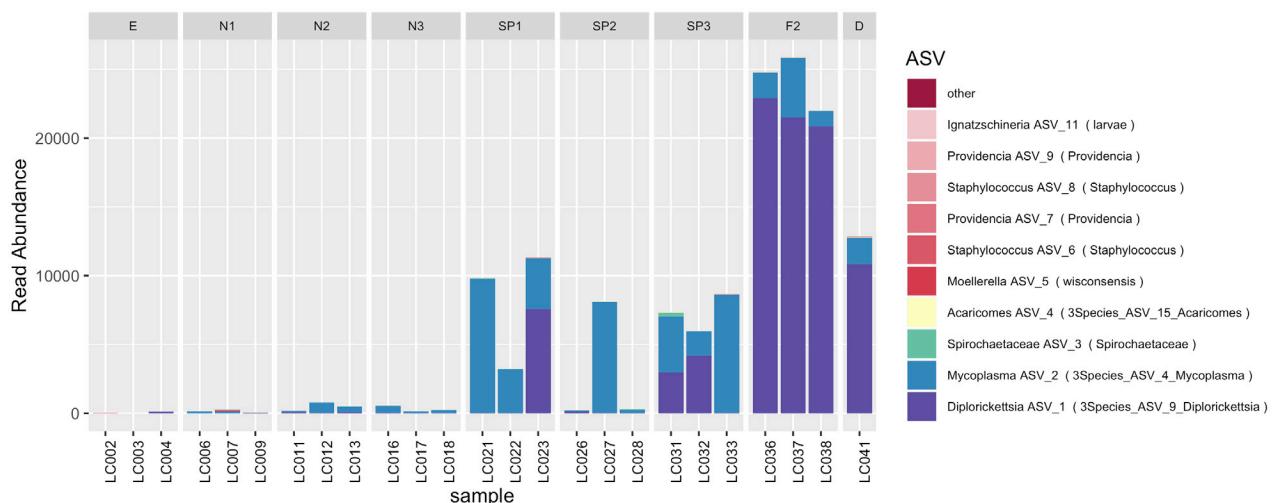


Figure 3.2 – 16S rRNA gene amplicon results, *S. dumicola* different life stages, nest H144s. Each column is a single spider or egg. E: Egg, N: Nymph, SP: Spiderling, F: adult Female. D: Dead female. F2 and D are from the parent generation relative to the other samples.

Amplicon sequencing of the 16S rRNA gene region V3-V4 was done as described in paper III, on spiders at different life stages coming from the same nest (Fig. 3.2). Although all samples were treated the same, very few reads were recovered from eggs and nymphs, indicating that these samples may not contain many bacteria at all. Nymphs mainly rely on regurgitation feeding, while spiderlings participate in prey capture and communal feeding. The maternal generation is eaten some time at the end of the nymph stage and the start of the spiderling stage (personal communication – Marie Rosenstand Hansen, spider caretaker extraordinaire). The fact that bacterial colonization of spiders only really takes hold during the spiderling stage, could mean that these bacteria derive from prey, but I believe that the difference between nymph and spiderling bacterial load (Fig. 3.2) is caused more by a lag phase in bacterial growth and maybe also by the effect of matrphagy. I would argue that the very low read numbers in the eggs could easily be caused by contamination or tag-switching. However, amplicon sequencing is not a quantitative method, so these results need to be backed up by qPCR before we can truly conclude anything.

EGG FISH

Meanwhile, a preliminary fluorescence in situ hybridization (FISH) study done by bachelor student Emma Skou Rasmussen (using methods and probes developed for, and described in paper II), shows no trace of bacteria in the eggs (Fig. 3.3). Supporting our theory that no direct vertical transmission is taking place.

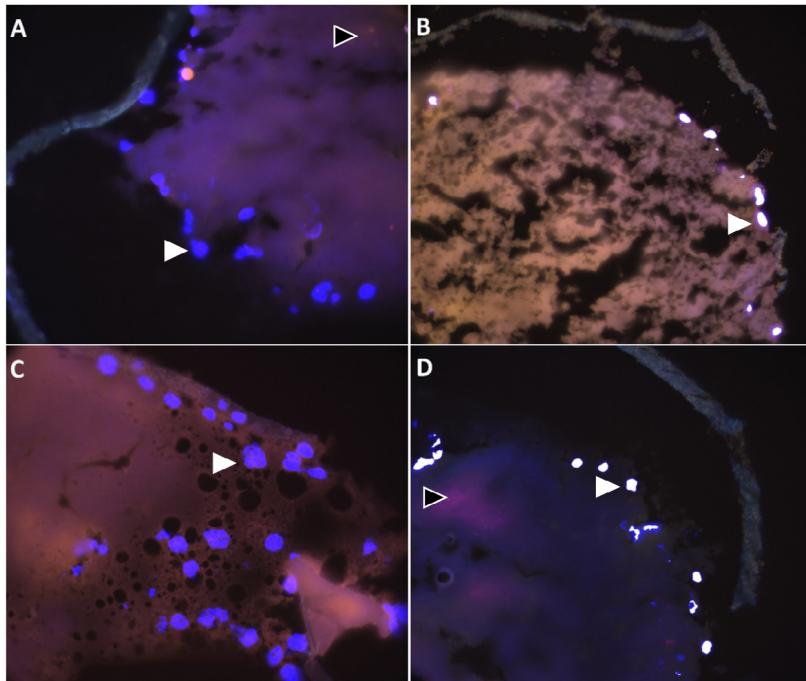


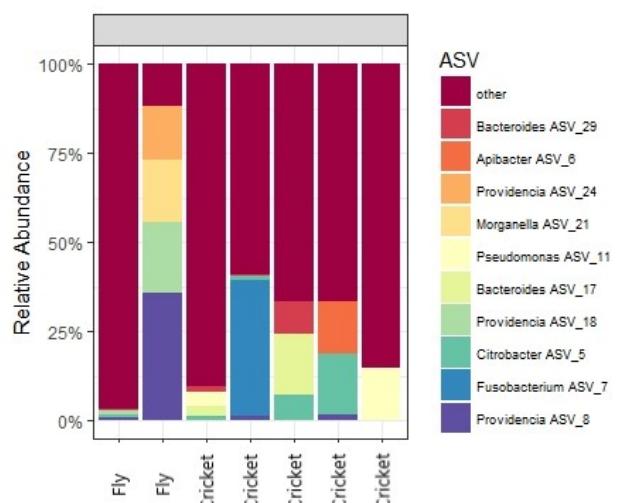
Figure 3.3 – FISH on *S. dumicola* eggs, cryosections. Blue: DAPI (DNA), Orange: EUB 338 I-III 16S probe (all bacteria). Red: LGC0355b 16 probe (*Mycoplasma*) in panel A, Bor477 16 probe (*Borrelia*) in panel B & C, NON probe in panel D. Filled white arrows point to examples of host nuclei. Black arrows point to unspecific binding or autofluorescence (looks identical with NON and LGC0355b probe). Images by: Emma Skou Rasmussen

PREY 16S

To investigate the likelihood of bacterial transmission from prey, Emma Skou Rasmussen also ran a 16S rRNA gene amplicon study on the prey animals we use for feeding our *S. dumicola* in the lab. They turned out to have completely different bacterial symbionts than what we find in the spiders we keep in the lab (compare Fig. 3.2 and Fig. 3.4, and please note that the colors do not match between the two plots), so transmission from prey does not seem to be very efficient, if it occurs at all.

Figure 3.4 – 16S rRNA gene amplicon results, laboratory prey animals. Relative abundance of the 10 most abundant ASVs are shown, the rest are grouped in “Other”. Each column is one prey animal.

Figure by: Emma Skou Rasmussen



EGG SWAP EXPERIMENT

To further investigate the transmission of bacterial symbionts between *S. dumicola* generations, Master's student Andrea Mjelva Søgård conducted an experiment in which she swapped egg-sacs between nests in the lab. Social *Stegodyphus* spiders do not seem to have much kin recognition, and freely accept other social *Stegodyphus* in to their nest (Seibt and Wickler 1988). And, as this study showed, they will also accept egg-sacs from other nests, and rear the spiderlings as their own.

Andrea formed experimental rearing nests composed of 15 adult female spiders (originating from the same nest) and a single egg-sac originating from either a different nest than the adults (SWAPPED) or from the same nest as the adults (CONTROL). The egg-sac was marked, and any new egg-sacs were removed, so only spiderlings from the intentionally placed egg-sac were reared. Spiderlings and mothers were sampled when spiderlings reached stage SP3 (see fig 3.1), and 16S rRNA gene amplicon sequencing was run on one mother and one spiderling from each rearing nest (Fig 3.5 A).

Results from the egg swap experiment showed very clearly that spiderling microbiome is not transmitted by strict/direct vertical transmission in the eggs, but by interactions with adults in the rearing nest. Spiderlings had similar microbiomes to their rearing mothers no matter if they had been swapped or not (Fig. 3.6A, Fig. 3.5A). Furthermore, the microbiome of spiderlings was much more dependent on the nest their rearing mothers came from, than on the nest their egg-sac had come from (Fig. 3.6B, Fig. 3.5B&C). Exceptions to the rule of near-perfect transmission from rearing mother to spiderling, were seen in experimental rearing nests 24_C and 24_1 (Fig. 3.5A). In these nests, the mothers' microbiomes were dominated by the intracellular symbiont *Diplorickettsia*, and the spiderlings only had a low abundance of this symbiont. However, This was true for both the non-swapped control spiderling (in 24_C), and the spiderling originating from a different nest (in 24_1), so may be due to a delayed transmission or delayed growth of these intracellular symbionts. A similar pattern was seen in the life-cycle 16S results (Fig. 3.2) where the adults were dominated by *Diplorickettsia*, but many of the younger spiders had mostly *Mycoplasma*.

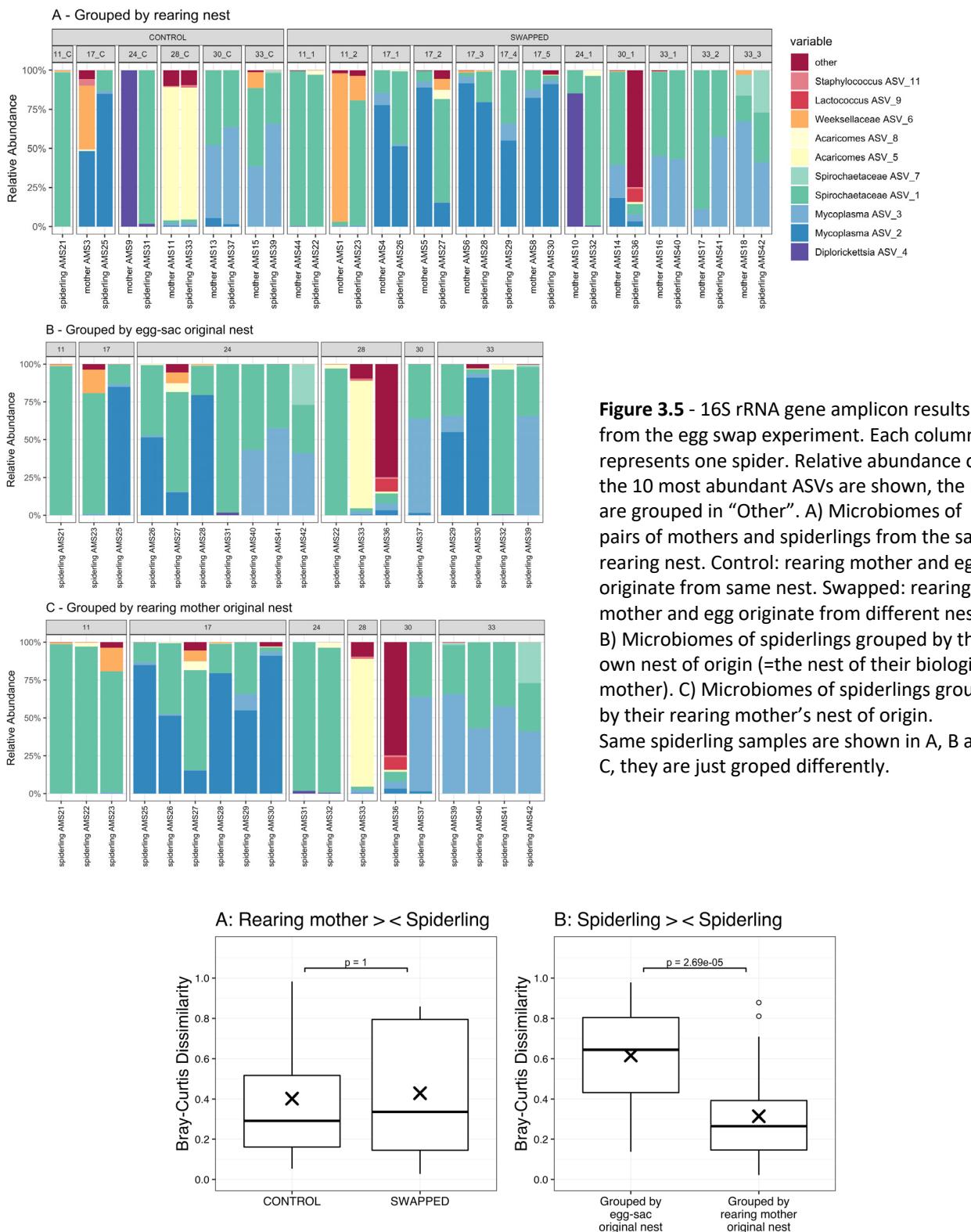


Figure 3.6 - 16S rRNA gene amplicon results from the egg swap experiment. Data was subsampled to 10 000 reads/sample. A) Each data point is the beta diversity between microbiomes of a rearing mother and a spiderling from the same experimental rearing nest. Control: rearing mother and spiderling from same original nest. Swapped: rearing mother and spiderling from different original nests. B) Each data point is the beta diversity between two spiderlings, either from the same original nest, or having rearing mothers that shared an original nest. A & B) Groups were compared using pairwise Wilcoxon rank sum test. p-values indicated in plots.

Symbiont knock-down studies

One of the best ways of getting an understanding of the effect of bacterial symbiosis on the host, is to knock down the symbionts using antibiotics. This has been done in e.g. honey bees (Raymann, Shaffer and Moran 2017), ticks (Zhong, Jasinskas and Barbour 2007), and also in spiders (Vanthournout, Swaegers and Hendrickx 2011).



Figure 3.7 – Experimental setup for antibiotics exposure.
Photo: Emma Hvidtfeldt Jensen.

Table 3.1 – Spider survival, Control vs Antibiotic treatment

Treatment	n start	n survived	Survival	z	p
Control	172	157	0.913		
Ampicillin	189	160	0.847	-1.898	0.058
Tetracycline	187	149	0.797	-3.015	0.003

Note: z- and p-values derive from binomial GLM.

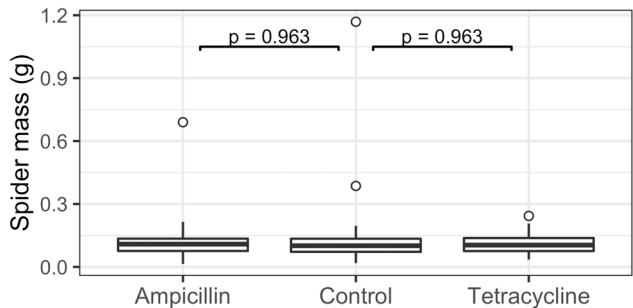


Figure 3.8 – Weight of spiders at the end of 24 days treatment. Benjamini-Hochberg adjusted p-values from pairwise t-test.

With help from Jesper Smærup Bechsgaard and Marie Braad Lund, Bachelor student Emma Hvidtfeldt Jensen ran a study to test the effects of antibiotics on *S. dumicola* and their bacterial symbionts. Using spiders from 8 natural nests collected in Namibia, and acclimated to our lab, she set up several experimental groups of 5-7 spiders in petri dishes (Fig. 3.7), and sprayed them with a 0.1 w/v water solution of antibiotics (or pure water for the control group) every day for 24 days. A similar method has worked in other spiders (Vanthournout, Swaegers and Hendrickx 2011). The antibiotics used were chosen based on a preliminary 16S study showing that these spiders were dominated by *Mycoplasma* and *Borrelia*. Tetracycline has been shown to be effective against both *Mycoplasma* (Taylor-robinson and Bébéar 1997) and *Borrelia* (Hunfeld *et al.* 2000), and should work against a broad spectrum of bacteria because of its mode of action, binding to the 30S rRNA subunit and inhibiting protein synthesis. Ampicillin on the other hand, works by inhibiting cell wall synthesis, and should therefore not affect *Mycoplasma*, but is likely to work against *Borrelia* just like penicillin and amoxicillin have been shown to do (Barbour, Todd and Stoenner 1982; Sicklinger, Wienecke and Neubert 2003).

After 24 days of antibiotics treatment, spiders treated with tetracycline showed a slight but significant decrease in survival rate compared to the control group (Table 3.1). However, the body mass of surviving spiders was not affected by treatment (Fig. 3.8).

Emma went on to test whether the antibiotic treatments had any effect on the microbiome composition (16S rRNA amplicon sequencing) or the bacterial load (qPCR) of the spiders, but the composition showed only a tentative decrease of relative *Borrelia* abundance (Fig. 3.9), and the qPCR results showed no reduction in bacterial load (Fig. 3.10).

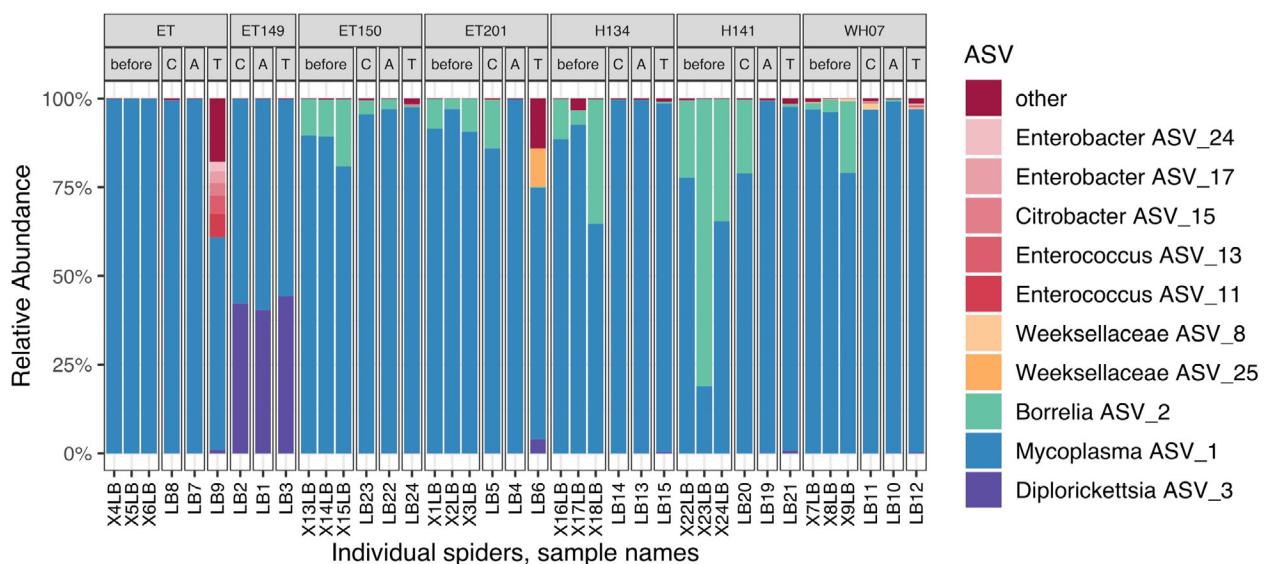


Figure 3.9 – 16S rRNA gene amplicon results, *S. dumicola* before and after antibiotics treatment. Each column is a single spider. The top bar indicates original nest. The bar below it indicates treatment. Before: spiders were sampled for sequencing before treatment. C: Control, spiders were treated with pure water. A: spiders were treated with Ampicillin. T: spiders were treated with Tetracycline.

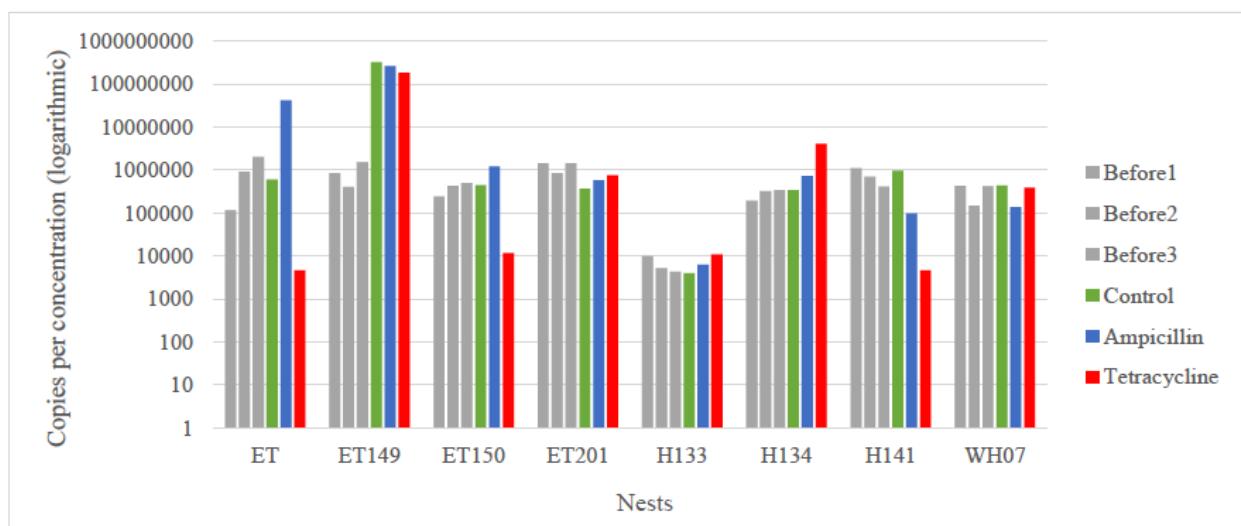


Figure 3.10 – qPCR results, *S. dumicola* before and after antibiotics treatment. Each column is a single spider. Color indicates treatment. Figure by: Emma Hvidtfeldt Jensen.

The lack of effect of antibiotics on the bacterial load found in Emma's study, was likely due to an ineffective qPCR protocol in which the spider DNA dominated so much that differences in bacterial DNA were difficult to spot. Marie Braad Lund solved this problem by developing the qPCR protocol described in paper III: We run parallel PCR reactions on the same sample, one amplifying a fragment of a spider gene and one amplifying a fragment of bacterial 16S rRNA gene. A plasmid containing a hybrid of both fragments is used as the qPCR standard, and the same dilution series is used for both reactions. This allows us to normalize the 16S copy number to the spider gene copy number, thus getting a measure of bacterial load that is comparable between samples.

Andrea Mjelva Søgård had a chance to repeat antibiotics treatments of *S. dumicola* as part of her master's study. The treatment scheme and dosing were similar to Emma's, but Andrea used only tetracycline and the spiders were kept at constant temperatures. Perhaps for that reason, Andrea only lost 3 out of 600 spiders, giving a much higher survival rate, and indicating that tetracycline is not really a problem for the health of the spiders. Andrea ran qPCR (using the new and improved protocol) on 60 of the spiders from her antibiotics study (one from each treatment box), and found a dramatic decrease in bacterial load in spiders treated with tetracycline compared to spiders treated with pure water (Fig. 3.11A)

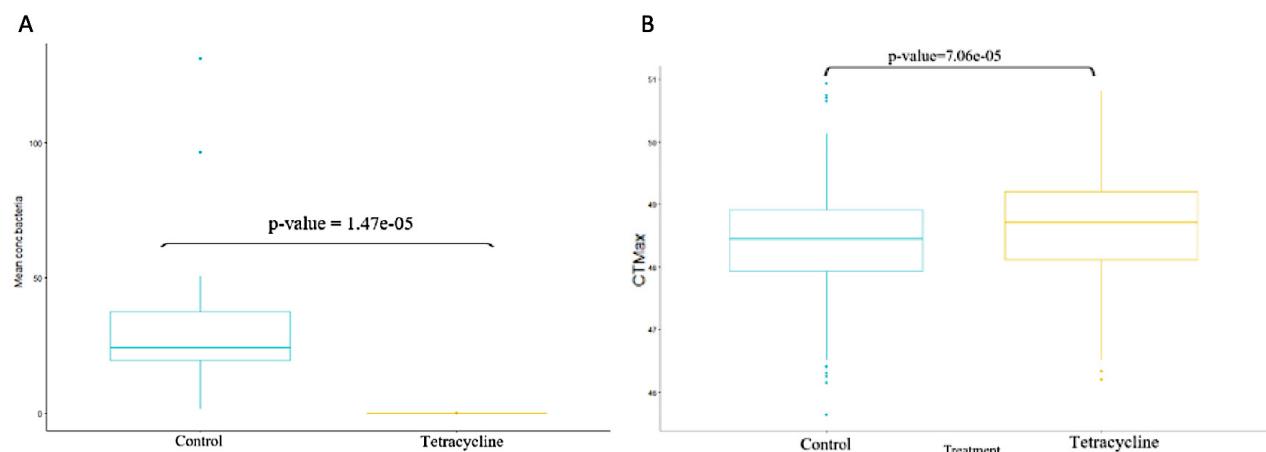


Figure 3.11 – Effects of tetracycline treatment on *S. dumicola*. A) qPCR results of 60 spiders (30 in each group), Concentration of bacterial DNA in water-treated vs. tetracycline-treated spiders. B) Temperature tolerance in water-treated vs. tetracycline-treated spiders, n=600 (300 in each group). P-values in both plots derive from pairwise t-tests. Figures by: Andrea Mjelva Søgård.

Andrea ran a temperature tolerance test on the tetracycline-treated spiders (and controls), measuring each spider's critical maximum temperature (CT_{max}) by subjecting it to steadily increasing temperatures ($0.1\text{ }^{\circ}\text{C}$ every 10 minutes) until movement ceased as the spider died. Surprisingly, CT_{max} was increased in the tetracycline-treated spiders compared to controls (Fig. 3.11B), meaning spiders with a knocked down microbiome could tolerate higher temperatures. This interesting result becomes even more interesting when you consider the fact that the same pattern was found by

Emma Hvidtfeldt Jensen when she tested her spiders' temperature tolerance: A significant increase in the CT_{max} of the tetracycline-treated spiders (Fig. 3.12).

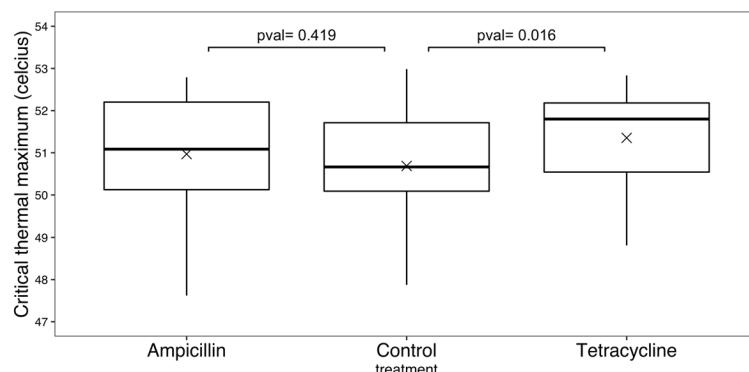


Figure 3.12 – Effect of antibiotics treatment on *S. dumicola* CT_{max} (Emma Hvidtfeldt Jensen's study).

The problem with the studies recounted here, is that we cannot be sure if the effect on CT_{max} is caused via the microbiome, or directly by the tetracycline itself acting on the spider in some way. In any good knock-down study, the cause of the achieved phenotype is tested by re-introducing whatever was knocked down, to see if the phenotype is reversed. In this case, that would mean re-introducing the microbiome.

We currently only have one dominant *S. dumicola* symbiont in culture (a *Spiroplasma* isolated by Sofie Vangkilde), so we do not have the possibility of re-introducing many of the natural microbiome members in a controlled manner. However, Marie Braad Lund is working to find out, if transmission between adults is as effective as transmission from adults to spiderlings. And if it is, we could let antibiotic-treated spiders get re-infected, simply by letting them share nests with non-treated spiders for a while.

Elusive symbiont genomes

As mentioned in the previous subsection, many of the dominant bacterial symbionts of *S. dumicola*, are still uncultured, and it is very likely that most of them will remain that way. Symbionts are notoriously difficult to culture, as they often have very specific requirements for growth.

Not having pure cultures, but still wanting the genomes of the spider symbionts, we attempted to assemble genomes from metagenomic sequencing. This work was carried out mostly by master's student Sofie Vangkilde, helped by Marie Braad Lund and Ian Marshall.

Sofie has described the methods she used for DNA extraction, sequencing and assembly in great detail in her master's thesis, so I will just summarize the salient points. A special kit (Molysis) was used to selectively degrade host DNA and then extract bacterial DNA from dissected spider guts. The DNA was sequenced using an Illumina MiSeq system, resulting in short paired-end reads. After trimming, quality filtering and merging, these (still very short) reads were assembled using Megahit v1.1.3, producing a number of variable-length scaffolds. The great hurdle when trying to get full bacterial genomes from metagenomic sequencing, is to separate the reads belonging to the different bacteria from each other. If the assembly is made from at least two samples with different relative abundances of the bacteria, differential coverage can be used for this "binning". Sofie's assembly was based on 3 samples, and differential coverage was used to "bin out" the reads belonging to a dominant symbiont classified as Weeksellaceae ("Bin1" Fig. 3.13A). These reads were then re-assembled which resulted in a 98.5% complete genome, fragmented in only 30 scaffolds. This genome was analyzed by project student Samuel Greenrod, who identified it as belonging to a novel genus related to *Chryseobacterium* and *Bergeyella*. Sam's proposed name for this *S. dumicola* symbiont is *Interanea botswani*.

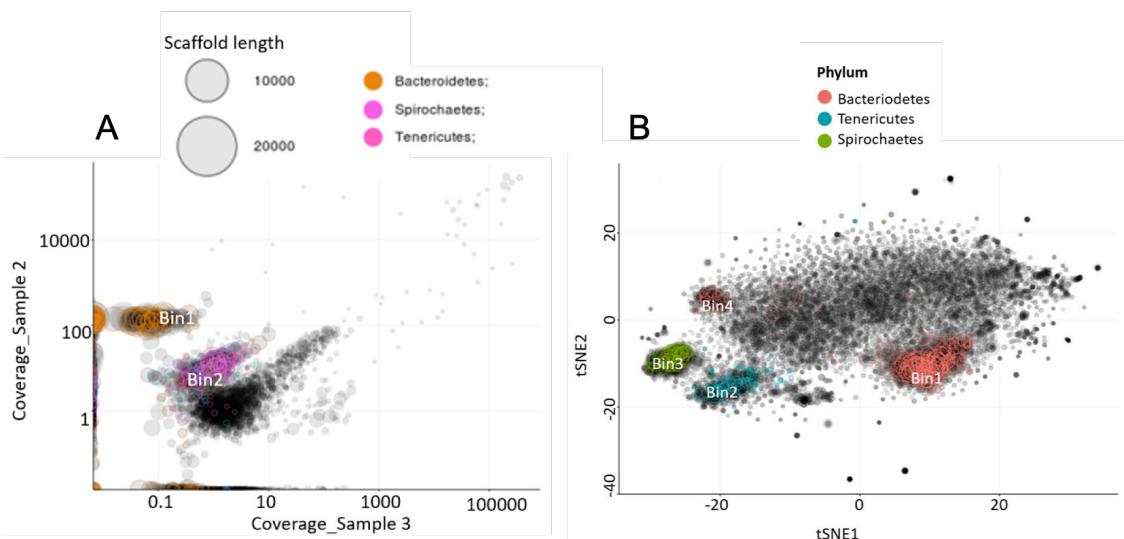


Figure 3.13 – Metagenome binning. A) Differential coverage binning B) Binning based on tetranucleotide frequency. Bacteroidetes contains Weeksellaceae, Tenericutes contains *Mycoplasma*, Spirochaetes contains *Borrelia*. Both figures by Sofie Vangkilde using R package mmgenome2.

The assembly contained many scaffolds belonging to Spirochaetes and Tenericutes (which matched *Borrelia*/Spirochaetaceae and *Mycoplasma* ASVs from paper II & III), but these could not be easily separated by differential coverage (Fig. 3.13A). Sofie instead used tetranucleotide frequency to separate the two dominant symbiont genomes (“Bin2” and “Bin3” Fig. 3.13B). All reads were mapped back onto the scaffolds belonging to each bin, and reassembly was run for each bin’s reads using SPAdes, resulting in one *Mycoplasma* draft genome and one *Borrelia* draft genome.

Note that I will call these *Borrelia* and *Mycoplasma* although they are in fact both so distantly related from other known members of those genera, that they probably should be in their own genera.

Both of the draft genomes were of very poor quality, *Mycoplasma* being only 57.33% complete and with a N50 of just 2072 bp (meaning that the genome is very fragmented), and *Borrelia* being even worse with a completeness of 31.16% and a N50 of a dismal 869 bp (Table 3.2). Genomes of such low quality are not worth a lot in functional or comparative analysis, so we have used them mainly for phylogenetic analysis, using GTDB-Tk and IQtree to construct multi-gene phylogenies (Nguyen *et al.* 2015; Chaumeil, Hugenholtz and Parks 2019), and Kostas lab’s online calculator ANI/AI distances (Rodriguez-R and Konstantinidis 2016).

Table 3.2 – Draft genome features

	<i>Mycoplasma</i> sp. Bin2	<i>Borrelia</i> sp. Bin3
Contigs	668	5978
Genome size(bp)	705814	305678
GC content (%)	28.12	31.16
N50 (bp)	2072	869
Completeness (%)	57.33	32.58
Contamination (%)	5.51	0.05

Mycoplasma values from Sofie’s assembly and analysis, *Borrelia* values from mine.

These poor results from metagenome sequencing were probably caused by a high level of strain variation within the symbiont genera, which makes assembly very difficult. For the *Borrelia* specifically, strain variation is probably not the only problem. *Borrelia* (and *Borrelia*) have some of the least metagenome-friendly genomes I have ever come across, generally being made up of a single linear chromosome and up to 21 circular or linear plasmids (Jabbari *et al.* 2018). Marie Braad Lund is currently working on getting better quality genomes via long-read sequencing (nanopore), so hopefully that will result in some deeper understanding of these symbionts.

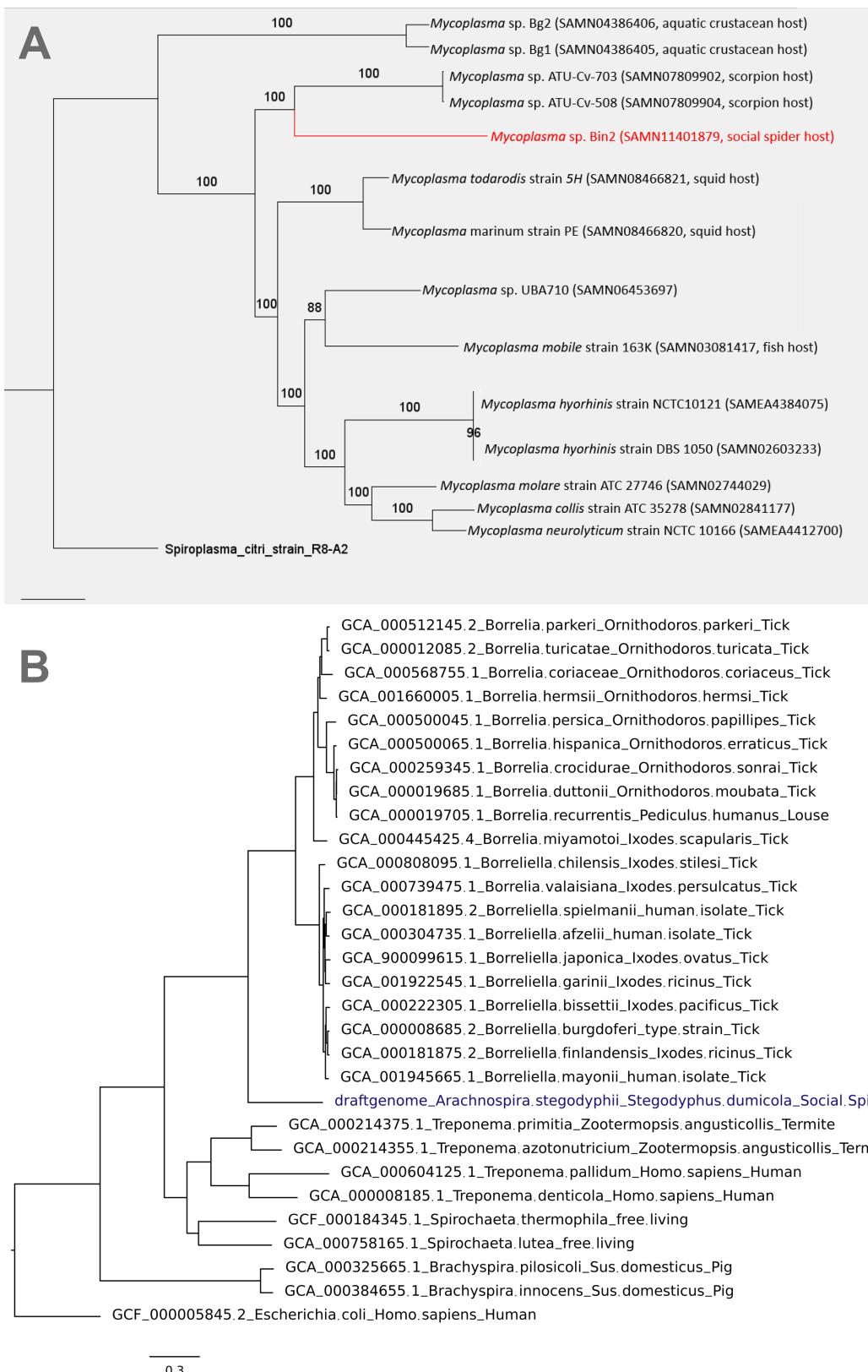


Figure 3.14 – Multi-gene phylogenies of *S. dumicola*-associated bacteria (colored) and the closest related published genomes. Both figures made with IQtree, based on concatenated multi-gene alignments obtained with GTDB-Tk. A) *Mycoplasma* tree by Sofie Vangkilde. B) *Borrelia*/Arachnospira tree by me, quick and dirty, hence the missing bootstrap values.

Candidatus Arachnospira stegodyphii (Or A. dumicola if you're silly)

The spirochete ASV that represents one of the most prevalent and dominant bacterial symbionts found in social *Stegodyphus*, is sometimes classified as a *Borrelia*, and sometimes as a member of the family Spirochaetaceae, I will argue it is neither, but instead forms its own genus within the (relatively) recently proposed family Borreliaceae (Gupta, Mahmood and Adeolu 2013). My proposed name for this genus is Arachnospira (roughly “arachnid spiral” in ancient Greek), and the name for this specific species could be stegodyphii (because it was found in the spider genus *Stegodyphus*).

Borreliaceae includes the genera *Borrelia* and *Borreliella* and also includes *Cristispira*. *Cristispira* is interesting in its own right; a huge (30-180 µm!) spirochete living on the crystal style of mollusks (Paster *et al.* 1996), which has been known about since 1910, but has never been isolated nor sequenced (other than 16S clone sequences). Our *Stegodyphus*-associated spirochete is placed right between *Cristispira* and the *Borrelia/Borreliella* group in the full-length 16S phylogeny from paper II (Fig. 3.15A), and commonly used 16S rRNA gene identity thresholds includes it in the Borreliaceae family, in its own genus with an unidentified scorpion symbiont (Fig. 3.15B).

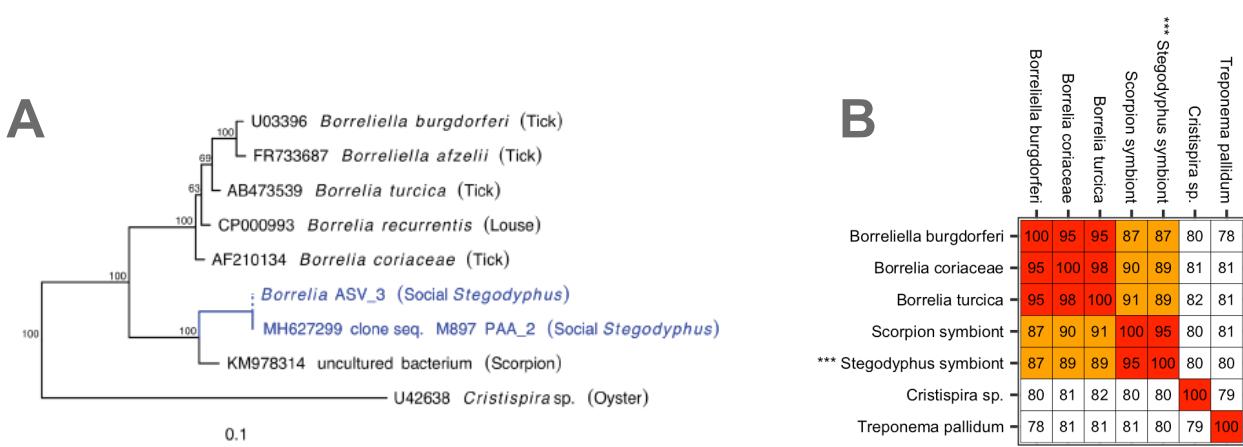


Figure 3.15 – (A) Full length 16S phylogeny of *Candidatus Arachnospira stegodyphii* (Borrelia ASV_3), and closely related bacteria. See paper II for details. **(B)** 16S identity matrix comparing (almost) full length 16S rRNA gene sequence of *Candidatus Arachnospira stegodyphii* draft genome (“*** Stegodyphus symbiont” in plot) to those of related bacteria. Identity values obtained using NCBI blast. Colors set by thresholds in (Yarza *et al.* 2014): Red: >94.5% indicating same genus, Orange: >86.5%, indicating same family.

An analysis of the AAI (average amino acid identity) between the *Candidatus Arachnospira stegodyphii* draft genome and selected representatives from different clades of spirochetes gave similar results (Fig. 3.16). A AAI threshold of >65% has been proposed for bacteria in same genus, and >45% for family (Konstantinidis, Rosselló-Móra and Amann 2017). This fits with our 16S-based taxonomic placement of *Candidatus Arachnospira stegodyphii*. Sadly, full genomes have not been published for neither *Cristispira* nor the scorpion-associated spirochete shown in the 16S phylogeny (Fig. 3.15), so these could not be included in the AAI analysis.

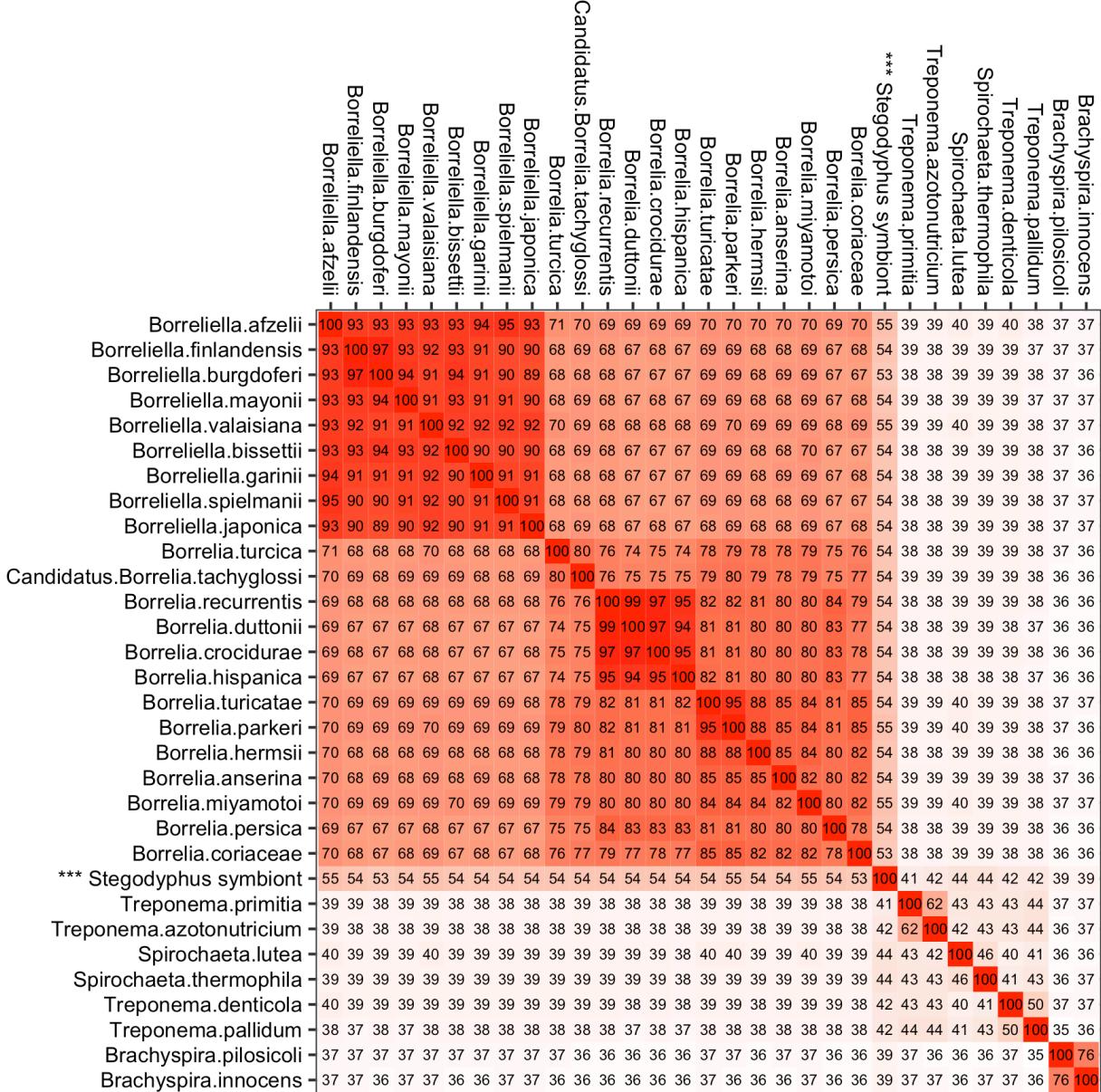


Figure 3. 16 – AAI matrix comparing amino-acid coding sequences of the *Candidatus Arachnospira stegodyphii* draft genome (“*** Stegodyphus symbiont” in plot) to those of related bacteria. Numbers indicate % identity of amino acids. AAI values calculated using Kostas lab’s online calculator (Rodriguez-R and Konstantinidis 2016).

Looking at figure 3.15B and 3.16 you might notice that all of the *Borrelia/Borrelia* fall into the same genus based on the thresholds. The legitimacy of the split of the old *Borrelia* genus into these two genera is still debated (Margos *et al.* 2018; Gupta 2019), but no matter what, I think that the family *Borreliaeae* makes sense, since other members of the *Spirochaetaceae* such as *Treponema* sp. have AAIs well below the family threshold when comparing to *Borrelia*.

In general, however, I believe that the characteristics of a bacterium are also important for taxonomic placement, and we need to both get a better genome of our spider-associated spirochete and find out more about its capabilities, before we can truly propose it as the founding member of the *Arachnospira* genus.

Picture show

ANOMALOUS FISH RESULTS

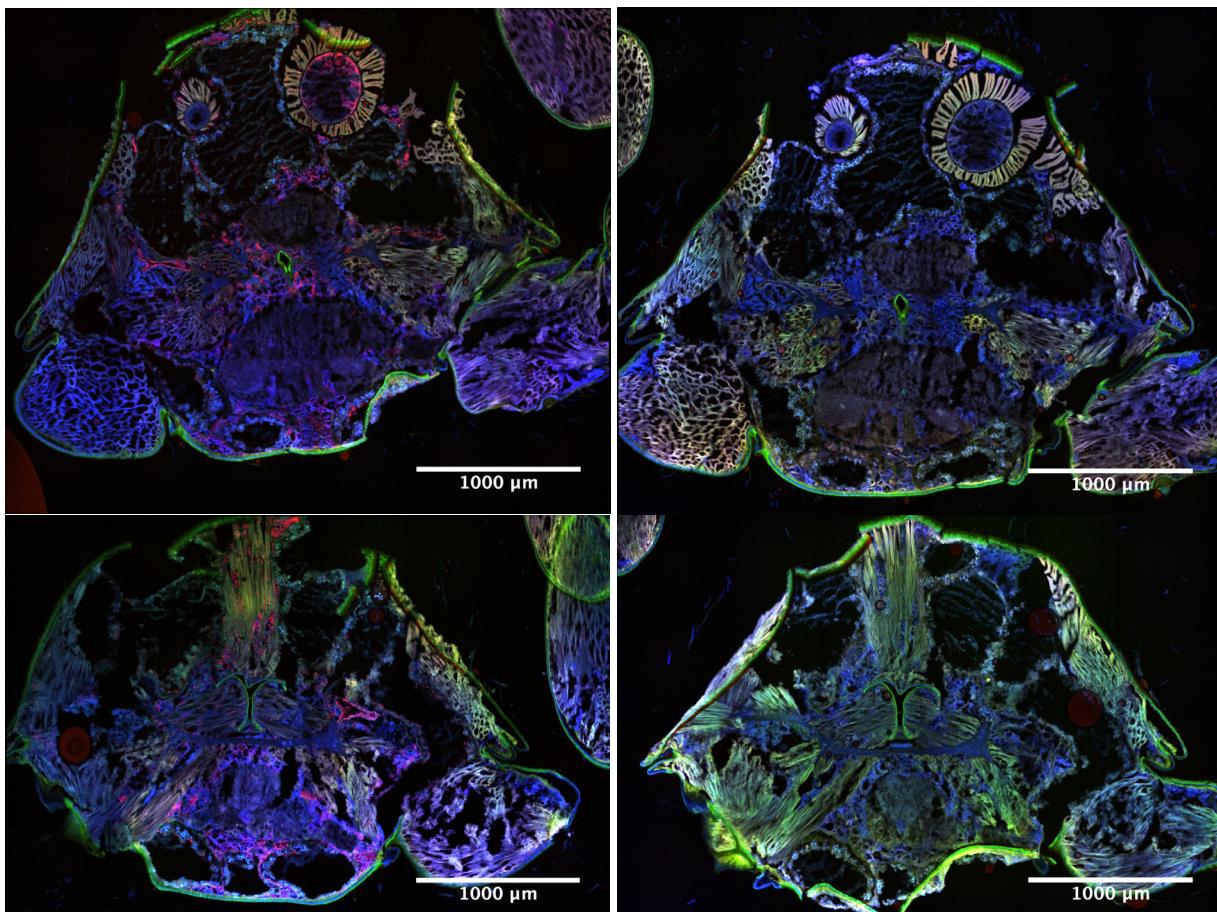


Figure 3. 17 – Fluorescence in situ hybridization on cryosections of *S. dumicola* cephalothorax. Method for FISH as described in paper II, Images on the left: **EUB probe**. Images on the right: **NON probe**.

For paper II, I ran FISH on several spiders, and generally only saw bacteria inside of the gut. The spider showed in figure 3.17 is the exception; seemingly having bacteria in its muscle tissue, brain (?) and venom glands (?). As this was never seen in any other spider, and could conceivably have been a product of poor tissue fixation, or dragging bacteria around with an unsharp microtome knife, we chose to focus on the general pattern, rather than this anomaly. Sadly, this spider (or other spiders from its nest) was not sequenced for 16S analysis, so we do not know what type of symbiont was dominating it. For future studies, it would be interesting to compare FISH and 16S results from the same nests, to try FISH on spiders known to carry symbionts belonging to obligately intracellular taxonomic groups such as *Diplorickettsia*, and to develop or find specific FISH probes for these symbionts.

TRANSMISSION ELECTRON MICROSCOPY

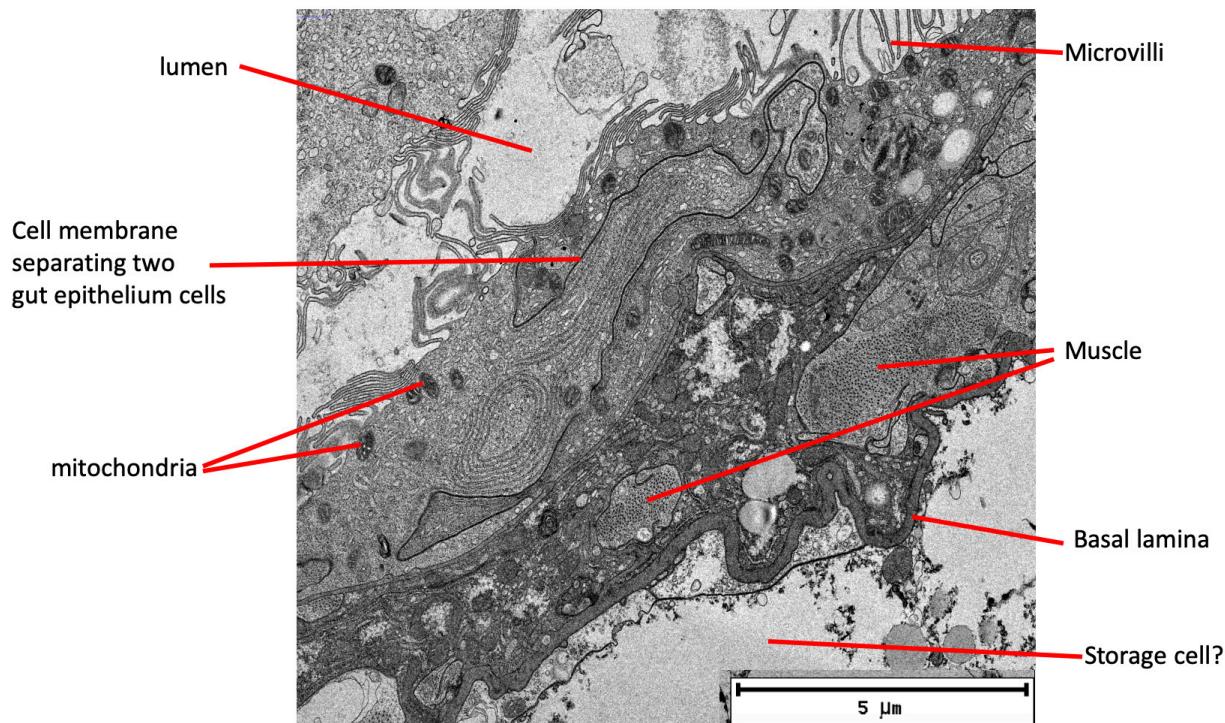


Figure 3. 18 – Transmission Electron Microscopy (TEM) of *S. dumicola* gut tissue.

The image in figure 3.18 was made by Pia Bomholt Jensen at iNANO, AU, and annotated with help from Peter Funch, AU. After showing that symbionts were lining the midgut epithelium of *S. dumicola* (Paper II) we wanted to get a better understanding of their interaction with the host. Pia got us these amazing pictures, but as far as we can tell, there is no bacteria there. This may just be a question of this particular place, in this particular spider being symbiont free, but we are also not completely sure that we would recognize the bacteria if they were there. This is a work in progress, and might require tagging the bacteria with particles, involving bacteria TEM experts, or a combination of both. My colleague Tobias Sandfeld Jensen is currently working on a study describing the physical and chemical conditions experienced by *S. dumicola* gut symbionts, and TEM imaging might become a part of that.

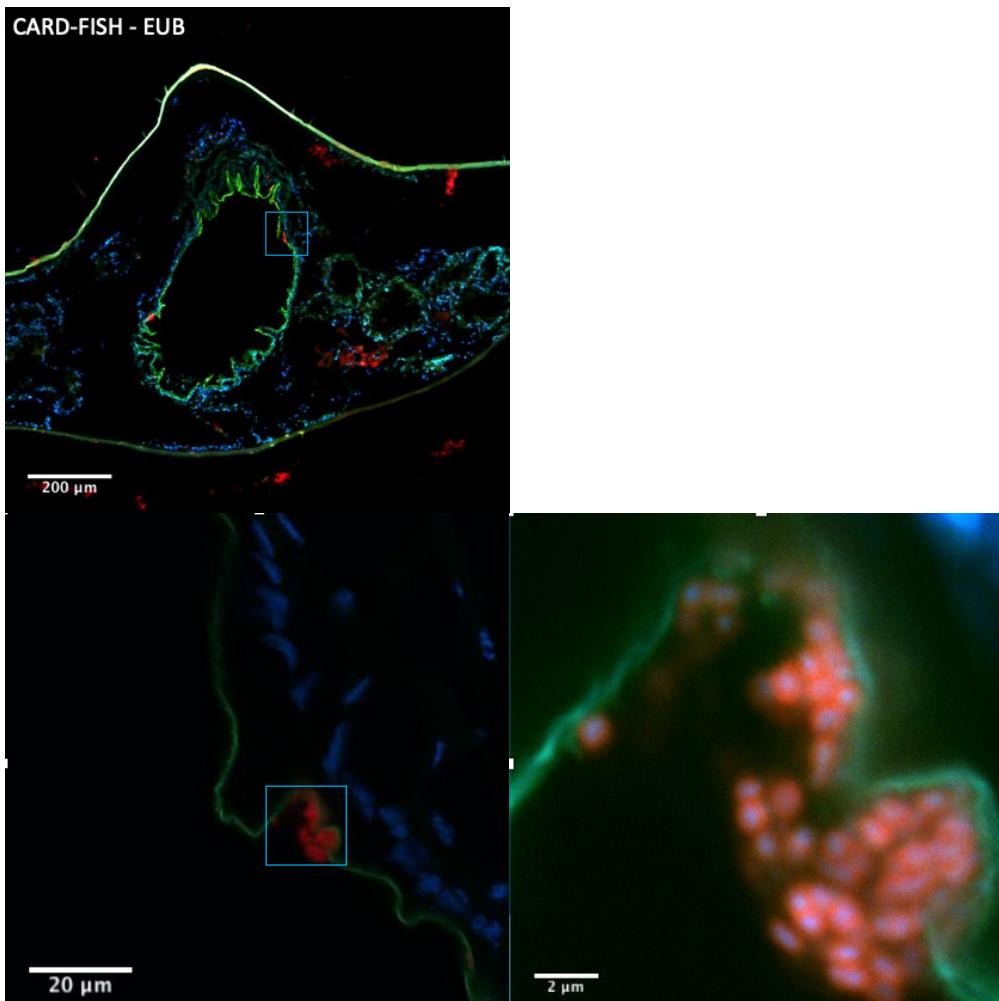
HORSESHOE CRAB FISH

Figure 3.19 – My best-ever FISH images, showing gut bacteria in the wrong host a horseshoe crab.

Each picture is a zoomed-in view of the one before it. Roughly illustrated by the blue boxes. Green=autofluorescence (mostly chitin), Blue=DNA(DAPI), Red=bacterial 16S (and some unspecific binding to amoebocytes in the first picture).

As a part of my PhD study, I was fortunate enough to get to attend the microbial diversity summer course at the MBL in Woods Hole, Massachusetts. Here, I investigated the microbiome of horseshoe crabs (*Limulus polyphemus*) for my individual project, attempting to isolate symbionts as well as visualize them in situ. This resulted in some of the best FISH images I have ever taken with close-ups very neatly showing a clump of bacteria colonizing the chitinous gut wall of a juvenile horseshoe crab (Fig. 3.19). I have never been able to get pictures of this quality from spiders, and maybe it was the CARD-FISH, or the top-of-the-line Zeiss equipment, but I suspect it might have been the science-magic of Woods Hole helping me to see these symbionts so clearly.

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Project summary in English

All animals live in close association with bacteria, but only with the recent advances in DNA sequencing techniques have we truly become aware of the scope and importance of these interactions. The complexity of the relationships and interactions between animal hosts and their bacterial microbiomes imposes the need for simple study systems to increase our understanding.

Because of an obligate inbreeding mating system, *Stegodyphus dumicola* has one of the lowest genetic diversities ever measured in a natural animal species. Not only does this simplicity make *S. dumicola* well suited for microbiome research in its own right, it also poses several questions about the relative ecological success of the spiders, which may be answered with more insight into their microbiome. With this PhD study, we have taken some of the first steps on the road to understanding the relationship between social spiders and their bacterial symbionts.

The first step was to identify the bacterial symbionts by DNA sequencing and to verify their existence within the host, using imaging. We found out that the spiders are hosts to many bacteria, which mostly live in their huge branching gut, and that a small group of bacteria seem especially well suited to life as social spider symbionts. These dominant symbionts are a diverse group with members from several bacterial phyla, but most of them belong to taxonomic groups which are known to be obligately host-associated. The closest relatives to many of the spider-associated bacteria were symbionts found in other arachnids such as scorpions and ticks, indicating that the bacteria share a long evolutionary history with their hosts. Each spider does not contain all of the dominant symbionts, but is instead dominated by one or two. We do not yet know what determines which symbiont a certain spider has, but we do know that spiders sharing a nest, also share the same dominant symbiont.

We still do not know the function of these symbiotic bacteria within the spiders, but after following several nests and their microbiome for more than two years, we know that the relationships are stable. The microbiome composition is not affected in any predictable way by seasons or climate but remains mostly unchanged with time, even crossing generational shifts in the nests. This stability is an indication that the bacteria are not virulent pathogens in the social spiders. Whether the bacteria perform any important functions for their spider hosts is still an open question. We are working both on identifying the bacteria's functional potential via genome analysis, and on the manipulation of the social spider microbiome in the lab.

Resumé af projektet på dansk

Alle dyr lever i tæt sammen med bakterier, men kun med de nylige fremskridt inden for DNA-sekventering er vi blevet opmærksomme på omfanget og betydningen af disse interaktioner. Forholdene og interaktionerne mellem dyr og deres mikrobiom er meget komplekse, og for at kunne få en dybere forståelse har vi brug for simple systemer at undersøge dem i.

På grund af indavl har *Stegodyphus dumicola* en ekstrem lav genetisk diversitet. Faktisk er det en af de mindst genetisk diverse naturlige dyrearter man har registreret. Ikke alene gør dette *S. dumicola* velegnet til mikrobiom forskning i sig selv, det stiller også adskillige spørgsmål til edderkopernes relative økologiske succes, som måske kan besvares med større indblik i deres mikrobiom. Med dette PhD studie har vi taget nogle af de første skridt på vejen til en større forståelse af forholdet mellem sociale edderkopper og deres bakterielle symbionter.

Det første skridt var at identificere de bakterielle symbionter med DNA-sekventering og at verificere deres eksistens i værten ved hjælp af mikroskopi. Vi fandt ud af, at edderkopperne er værter for mange bakterier, som for det meste lever i deres enorme forgrenede tarmsystem, og at en lille gruppe af bakterier synes særlig godt egnede til livet som sociale edderkoppe symbionter. Disse dominerende symbionter er en forskelligartet gruppe med medlemmer fra adskillige bakterie rækker, men de fleste af dem hører til taxonomiske grupper, kendt som obligatorisk værts-associerede. De nærmeste slægtninge til mange af de edderkop-associerede bakterier var symbionter fundet i andre arachnider, såsom skorpioner og flåter, hvilket er et tegn på, at bakterierne deler en lang evolutionær historie med deres værter. Hver edderkop indeholder ikke alle de dominerende symbionter, men domineres i stedet af en eller to. Vi ved endnu ikke, hvad der bestemmer, hvilken symbiont en bestemt edderkop har, men vi ved, at edderkopper der deler rede også deler den samme dominerende symbiont.

Vi ved stadig ikke hvilken effekt disse symbiotiske bakterier har på edderkopperne, men efter at have fulgt flere edderkoppereder og deres mikrobiom i over to år, ved vi at forholdet er stabilt. Sammensætningen af edderkopernes mikrobiom påvirkes ikke på nogen forudsigelig måde af årstider eller klima, men forbliver stort set uændret over tid, selv på tværs af generationsskift. Denne stabilitet tyder på at bakterierne ikke er sygdomsfremkaldende for de sociale edderkopper. Om bakterierne udfører vigtige funktioner for deres værter, er stadig et åbent spørgsmål. Vi arbejder både på at vurdere bakteriernes funktionelle potentiale via genom analyse, og på at kunne manipulere edderkopernes mikrobiom i laboratoriet.

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