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Introduction

Chlamydiae as a health issue

Chlamydiae, more specifically members of the *Chlamydiaceae*, are the cause of several diseases affecting millions of humans, predominantly living in developing countries. Currently, more than 1.9 million humans worldwide are visually impaired or blinded as a result of trachoma, a disease caused by infection of the eye with *Chlamydia trachomatis* [1]. The variety of *C. trachomatis* biovars includes, besides the already mentioned trachoma biovar, the genital tract biovar, acting as a sexually transmitted disease, leading to ectopic pregnancy and infertility, and the lymphogranuloma venereum biovar, which disseminates to the lymph nodes of patients [2,3]. More recently, the chlamydial pathogen *Chlamydia pneumoniae* was discovered and linked to community-acquired pneumonia and other diseases like Alzheimer and Diabetes, although, apart from pneumonia, the causal relationships could not be shown so far [4]. *C. pneumoniae* can also be found in several animals, thus presenting the potential for zoonosis, a fact that also applies to other members of the *Chlamydiaceae*, like *Chlamydia psittaci* [4, 5]. While chlamydial infections can be treated with antibiotics, no effective vaccine has been developed hitherto [2].

The general life cycle of chlamydiae

All described chlamydiae share a biphasic life cycle, consisting of an extracellular and an intracellular phase, with respect to their host (Figure 1) [6]. The extracellular form of chlamydiae, the elementary body (EB), is released in the environment by infected host cells, either by host cell lysis or extrusion, and sustains there, until it is taken up by a new, native host cell [2]. While EB's were long considered to be cyst-like cells and therefore metabolically inactive, recent research suggests that EB's are actively transcribing genes and producing proteins [3]. Once an EB is ingested into an inclusion by a host cell, it begins to transform to a reticulate body (RB), a process accompanied by a vast shift in gene expression and cell metabolism [7]. Inside the host cell, chlamydiae thrive upon the energy provided by the host, in the form of nucleotides, amino acids or lipids [8]. A third cell form, the aberrant body, is described as a chlamydial stress response and potentially cause persistence and chronic infections [2, 9].

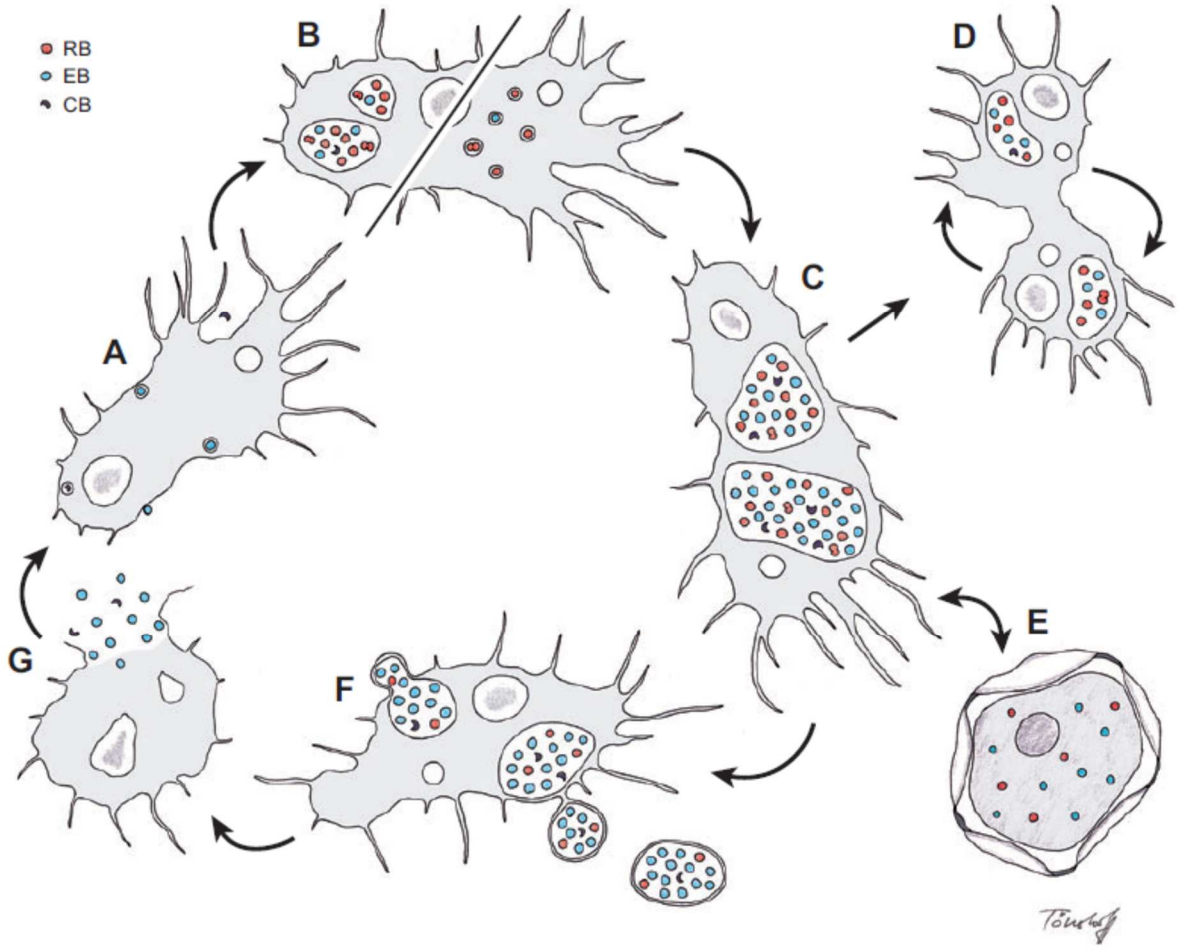

Figure 1: Schematic representation of the chlamydial, biphasic life cycle. The actively replicating reticulate bodies (RBs) are depicted in red, the infectious extracellular elementary bodies (EBs) are depicted in blue. Indicated in Step D is the long-term coexistence of chlamydiae and host cells, by coordinated cell replication cycles. Indicated in Step E is the ability of chlamydiae to survive inside cysts of certain amoebae. Figure taken from Horn, 2008 [10].

Infection by *Chlamydia spp.*

Although chlamydiae (especially *Chlamydia spp.*) have been excessively studied since their discovery, many of the mechanisms involved in infection persistence and release of chlamydiae are still far from understood. When the extracellular form of a chlamydia, the EB, encounters a potential host cell, it attaches by means of the major outer membrane protein (MOMP) which binds to glycosaminoglycans presented on the outer membrane of the eukaryotic cell, a process known from numerous other pathogens [11]. Various proteins facilitate this process by the same or similar mechanisms, e.g. OmcB, a highly conserved and the second most abundant membrane protein on the outer membrane of *C. trachomatis* [12].

Additionally, host proteins play a crucial role in the initial step of chlamydial infection: The ER chaperone Gp96, which is transported to the outer membrane by the eukaryotic cell, is not only utilized by *C. trachomatis* to attach to the cell, but is also differently expressed during the course of infection[13]. While Gp96 is upregulated in the earlier stages of the infection thereby increasing the probability of

EB attachment it is downregulated in the later phase of the infection, thereby decreasing the probability of EB attachment – this mechanism avoids the reinfection of the host cell by EBs and the detection of the infection by the immune system [13]. The host protein disulfide isomerase (PDI), which acts in processing of disulfide bonds and as a chaperone, is not only crucial for attachment, but also enables the entry of *Chlamydia spp.* into the host cell, probably by reducing either a host or bacterial component of the outer membrane [14].

The characterization of polymorphic membrane proteins (PMP) forming the chlamydial outer membrane complex (COMC) of EBs have been of great medical interest, since they play a crucial role in infection, acting as autotransporters and porins [15]. At least 17 different proteins were found to contribute to the COMC of *C. trachomatis* serovar L2 [16], many of them without homologues in environmental chlamydiae [17]. PMPs represent a substantial part of the chlamydial genome, accounting for more than 5% of the total coding capacity in *C. trachomatis* and are strikingly heterogeneous, although they all contain two characteristic, otherwise seldom, motifs [18]. The adhesion capacity of *C. trachomatis* and *C. pneumoniae* PMPs is species-specific and characteristic for the human cell type they interact with, thus the presence of PMPs of the same but not of a different species decreases infectivity [19]. Most PMPs in *C. psittaci* studied so far, are transcribed and produced in the late stages of chlamydial infection, which underpins their role during the EB stage, the beginning of a new infection and possibly before the egress of the bacteria [20].

Despite years of concentrated effort, the mechanisms by which chlamydiae enter the host cell are largely unresolved, e.g., whether Chlatrin-coated pits mediate endocytosis [7]. One of the virulence factors present in all chlamydiae species found so far, is the type three secretion system (T3SS), which is encoded in several coding regions dispersed over the genome [6]. Interestingly, the dispersion pattern is the same in various chlamydiae, suggesting that the T3SS is an ancient feature, present in the last common ancestor 700 million years ago [21]. The effector proteins ejected by the T3SS, include various chaperones, the actin remodeling TarP and IncA, which contains SNARE-like motifs[22]. Together those proteins facilitate the uptake of *Chlamydia spp.* and prevent or likewise facilitate the fusion of the inclusion with vesicles containing lysozymes or nutrients respectively [22]. Despite their importance, Inc proteins are highly variable depending on the species and Tarp are not present in other chlamydiae than the *Chlamydiaceae* [17].

To avoid the leakage of inclusion vesicle lumen into the cytosol, the inclusion membrane needs to be stabilized by F-actin and intermediate filaments [23]. Inside the host, the inclusion vesicle is transported to the nutrient-rich peri-Golgi region, a migration process requiring dynein (like many DNA viruses), but no dynactin [24]. Other organelles interacting with the inclusion vesicle include multivesicular bodies, lipid droplets, mitochondria and the lysosomes [23]. During the intracellular life stages, *C. trachomatis* is

to a great extent depending on carbohydrates and amino acids supplied by the host as substrates to build up the cell envelope and proteins, due to the limited set of enzymatic capabilities encoded in the genome [25].

To infect new host cells, chlamydiae exit the infected host cells by two fundamentally different mechanisms: lysis and thereby death of the host cell or extrusion of chlamydiae by pinching off a part of the inclusion into the environment, leaving the host cell intact [2]. Both pathways occur at a similar frequency in *Chlamydia spp.* [26].

Environmental chlamydiae are diverse and prevalent

Despite the efforts invested in the research on *C. trachomatis*, the huge diversity of chlamydial species was long overseen and only brought to light recently, especially by the advent of readily applicable sequencing techniques [6]. With respect to the origin of those novel chlamydiae, they were named environmental chlamydiae or chlamydiae-like organisms, in contrast to the already known *Chlamydiaceae* [27]. Analysis of 16S rRNA sequences taken from the integrated microbial next-generation sequencing database show an increase of known existing chlamydial families from 181 to 1157 in only 5 years [6]. Despite this potential diversity cultured representatives are available for only 6 families [6, 28]. Deep metagenomic sequencing and single-cell genomics promise to overcome the problem of accessing low-abundance chlamydiae genomes in environmental and clinical samples [29].

The first environmental chlamydia discovered was *Waddlia chondrophila* in 1990 via isolation from an aborted bovine fetus [30]. At the time, its morphological similarities to *Chlamydiae* were noticed and described, however, since it reacted weakly with antisera of *Cowdira ruminatium,* it was classified as a member of the *Ehrlicheae* tribe, thus a *Rickettsiales* [30]. A few years later, 16S rRNA gene sequencing revealed the affiliation of *W. chondrophila* to the chlamydiae, thus it was placed in the thereby founded family *Waddlia* [31].

This classification took place only after a second obligate intracellular bacteria was discovered and described as chlamydia-like organism [32]. The organism named “Z”, derived from a contaminated HeLa cell culture and raised attention due to its chlamydia-like cell cycle, showing the characteristic EB and RB stages, and its resistance to penicillin, a feature which was also described for *W. chondrophila* [33]. Shortly after the first description, “Z” was identified as a relative of the pathogenic *Chlamydiae* and a member of a new genus (and family), by 16S rRNA gene sequencing [34].

At that time, more and more evidence for the prevalence of chlamydia-like organisms arose, as a similar bacterium was detected in an *Acanthamoeba* strain, isolated from human nasal mucosa [35]. This novel bacteria could be maintained in *Acanthamoeba* strains and its life cycle resembled the life cycle of pathogenic chlamydiae [35]. Again, 16S rRNA gene sequencing revealed the affiliation of the

endosymbiont to the family *Chlamydiaceae* where it was classified as a member of the newly proposed genus *Parachlamydia* [36]. Inspired by this discovery, older isolates were revisited and reclassified by 16S sequencing. Thus, it was found, that "Hall's coccos", described in 1989 [37] as an endosymbiont of *Acanthamoebae,* was indeed a *Parachlamydia sp.* [38].

It took two more years to reclassify the phylum *Chlamydiae* and to introduce the new families of chlamydia-like organisms, besides the well described *Chlamydiaceae* (including *C. pneumoniae* and *C. trachomatis*). The new families were named *Simkaniaceae* (including the endosymbiont "Z"), *Parachlamydiaceae* and *Waddliaceae* [27, 31].

The utilization of free-living amoebae (FLA) as potential hosts of chlamydiae-like organism yielded the discovery and characterization of two more genera in the family *Parachlamydiaceae*, namely *Neochlamydia* and *Protochlamydia* [39, 40, 41].

The endosymbiont *Neochlamydia hartmannellae* was originally isolated from the FLA *Vermamoeba vermiformis* (formerly *Hartmannella vermiformis*) found in the water conduit system of a dental unit [39]. Instead of utilizing a vacuole, *N. hartmannella* executes its life cycle directly in the host cytosol and lyses the host to release the EBs into the environment [39]. It prevents the formation of *V. vermiformis* cysts, a behavior already described for *P. acanthamoeba* [35], and could not infect other amoebae species apart from *D. discoideum*, which however, did not carry the endosymbionts into the multicellular stages and the spore [39].

The species *Protochlamydia amoebophila* originated from *Acanthamoeba sp.,* isolated from a soil sample and showed a different distribution in the host, than the other *Parachlamydiaceae* members, being distributed in low numbers in vacuoles inside the host cell [40]. Most importantly, *Protochlamydia amoebophila* was also the first chlamydia-like organism whose genome was completely sequenced [21]. With a total genome size of roughly 2.4 megabases, it was twice as big as the hitherto known pathogenic chlamydiae genomes and had a substantially lower G+C content [40]. The analysis of the *P. amoebophila* genome showed, among other features, a high number of plant gene homologs, ATP/ADP translocase genes, all genes required for the TCA cycle, while at the same time the lack of many amino acid synthesis genes, no homolog for the major outer membrane protein, but all genes to build a type three secretion system [21, 40]. Overall the analysis implied, that the last common ancestor of all chlamydiae at least 700 million years ago, was already adapted to an intracellular lifestyle [21].

Today environmental chlamydiae are found in virtually every habitat [6] and environmental chlamydiae have been isolated from various other host species, often after reclassifying known symbionts [42].

Increasing diversity in the *Rhabdochlamydiaceae*

One example of how misleading the classification based on morphological features can be, is the case of *Rhabdochlamydia porcellionis*, found in the gland cells of the hepatopancreas of the isopod *Porcellio scaber*, where it replicates in vacuoles [43]. It was originally described as a chlamydia-like organism due to its life cycle [44], then proposed to belong to the order *Rickettsiales* [43] due to the rod shaped EBs, only to be reclassified as chlamydia-like organism by 16S rRNA gene sequencing [45]. Hence, it was the first member of the newly proposed family *Rhabdochlamydiaceae*, which was placed as a sister group of the *Simkaniaceae*. The most striking features of the novel family were the rod-shaped EBs, intermediate bodies with electron-dense areas and the white vesicles they form, large enough to be visible with the naked eye [45].

A similar fate of reclassification befell *Rhabdochlamydia crassifans*, an endosymbiont found mainly, but not only, in the fat body of the oriental cockroach *Blatta orientalis*, where it was found and described as *Rickettsiella crassifcans* [46]. Due to the different size of the symbionts, they were not thought to be *R. blattae*, described decades earlier [47], albeit the localization and the life cycle of the endosymbionts as well as the origin of the hosts was the same in both studies. The species was later reclassified by 16S rRNA gene sequencing as *Rhabdochlamydia crassifans* and is thereby a close relative to *R. porcellionis* [48].

More similar cases will likely appear in the years to come, as the example of *Porochlamydia buthi* shows, which was described as a pathogen of the scorpion *Buthus occitanus* [49]. Apart from the initial description of the chlamydia-like cell cycle and the cell morphology, most noteworthy EBs displaying a five-layered cell wall, there was no further investigation hence no molecular confirmation of the phylogeny [50].

Sequencing of *Rhabdochlamydia helvetica*, endosymbiont of the tick *Ixodes ricinus* shed light on the relation of the two organisms [42]. The presence of characteristic chlamydial genes such as the T3SS or ATP/ADP antiporters, as well as the absence of several genes encoding for the biosynthesis of amino acids, suggest a parasitic rather than a mutualistic lifestyle, underscored by the low prevalence of *R. helvetica* in tick populations and the vice versa high numbers of *R. helvetica* per tick [42]. Comparative genomics also revealed the occurrence of horizontal gene transfer (HGT) with other endosymbionts inhabiting the same niche [42].

A potential niche for HGT could be the dwarf spider *Oedothorax gibbosus,* in which four endosymbionts were found to be predominant: *Cardinium, Wolbachia, Rickettsia,* and *Rhabdochlamydia* species [51]. Interestingly, the abundance of *Rhabdochlamydia* 16S rRNA gene reads was substantially different between the two dwarf spider populations sampled at different locations [51]. Additionally, the

prevalence of *Rhabdochlamydia* was higher in female than in male specimens, implying a potential reproductive effect on the host [51].

Up to this day the diversity in the family *Rhabdochlamydiaceae* is only captured to a small extent [52]. Many genera are still to be discovered as the example of *Renichlamydia lutjani* shows. This parasite of the blue-striped snapper infects the kidneys of the fish and appears similar to epitheliocystis, which is affecting the gills [53]. The organism was phylogenetically placed between the arthropod-infecting *Rhabdochlamydia spp.* and the *Simkaniaceae*, thus at a very basal position within the family *Rhabdochlamydiaceae* [53].

A high prevalence of *Rhabdochlamydiaceae* and *Simkaniaceae* in arthropods was shown for ticks in Australia, which could serve as potential vectors and possess high zoonotic potential [54]. Of the two tick species collected mainly from koalas, around 20% were affiliated with chlamydiae, composed of 6 genotypes (3 *Simkaniaceae* and 3 *Rhabdochlamydiaceae*). No *Chlamydia spp.* were detected in this study, albeit *Chlamydia pecorum* is endemic in the sampled region [54]. The zoonotic potential of *Rhabdochlamydiaceae* was highlighted in a study of bats in Finland, where 16S rRNA of *Rhabdochlamydiaceae* was present in the bat feces as well as in the main prey *Chironomidae* which were considered the original host species [55]. *Rhabdochlamydiaceae* 16S rRNA gene sequences also occurred in sponges collected at Hawaii [56], fresh water samples taken from fountains and ponds in Italy and France [57], water and waste water treatment plants [57, 58] and respiratory samples taken from patients suffering from community-acquired pneumoniae [59].

The analysis of metagenomic and amplicon-based data suggested, that the *Rhabdochlamydiaceae* are in fact, the most diverse family of the phylum *Chlamydiae*, which is attributed to the diversity of their arthropod hosts [52]. Only very recently, *Rhabdochlamydiaceae* were also found in free-living amoebae [60], despite the central role these eukaryotes played in the discovery of the environmental chlamydiae.

Free living amoebae and their position in the tree of life

While the *Amoebozoa* form a monophyletic group in the tree of life, as a sister taxon of the *Ophistokonta* [61], the term “amoeba” refers to cells which are moving via pseudopods and feeding by phagocytosis, hence, it covers a broad range of organisms and is polyphyletic [62, 63]. Amoebae can further be specified as parasitic or free-living amoebae (FLA), albeit the lines are blurred in some cases of opportunistic pathogens classified as FLA, seldomly called amphizoic amoebae [63]. Free-living amoebae are important parts of a wide variety of ecosystems, mostly due to their bacteria grazing properties[64], making them key players in nutrient fluxes [65].

As described above, amoebae have been used repeatedly to isolate environmental chlamydiae and are a promising reservoir to reveal even more of the hidden chlamydial diversity [10, 62]. The two fundamental

approaches to isolate amoebae endosymbionts, are the co-incubation of naïve, unifected amoebae with environmental samples containing putative endosymbionts or alternatively, the isolation of natively infected amoebae [66]. Of course the latter approach is preferred, since co-cultivation means loss of the original host, which is valuable information on the relation between host and chlamydia [57]. Only few FLA (draft) genome sequences are available to date, e.g. *Acanthamoeba castellanii*, *Naegleria spp.* [62], *Polysphindilum pallidium* and *Dictyostelium spp.* [67].

The intensively studied model organism *Dictyostelium discoideum*

The cellular slime mold *Dictyostelium discoideum* first described in 1935 is one of the best characterized eukaryotic model organisms in existence [68]. The cellular mechanisms, researched with this organism are numerous and span from chemotaxis over phagocytosis to cytokinesis [69]. The feature that drew most attention to *D. discoideum* is without doubt, its complex life cycle consisting of several distinct phases and requiring sophisticated signaling mechanisms [70]. *D. discoideum* was not the first Dictyostelid in which a social life cycle was observed (*D. mucoroides*, *D. purpureum*, etc.), but at the time of its first characterization, it generated additional attention due to the migrating slug-like stage which was not known hitherto [68]. In 2005 researchers also succeeded in sequencing the genome of *D. discoideum,* which was thereby the first genome sequence of a free-living protist to be fully sequenced [71].

The life cycle of dictyostelids

The most extensively studied aspect of *D. discoideum* and the *Dictyostelids* in general, is their extraordinary life cycle, thus it has been described numerous times in reviews and papers alike. A short but detailed summary of the different stages of the *Dictyostelid* life cycle is provided by Romeralo et al. [70], the most precise descriptions can be found in the book "The *Dictyostelids*" by the luminary of *Dictyostelid* research K.B. Raper [72].

The vegetative cells of the *Dictyostelids* called myxamoebae (or trophozoites) divide by mitosis and are grazing the environment for bacteria to digest them by phagocytosis. If food is scarce, dictyostelids have several mechanisms to cope with this type of stress, namely the encystation cycle, the sexual cycle and the social (sorocarp) cycle (Figure *2*). While the first two cycles are relatively unexplored, the latter has been thoroughly investigated in the last decades [70].

The social cycle starts with an aggregation process, during which trophozoites stream towards a common center where they form a pseudoplasmodium, in which the cells aggregate but do not fuse in contrast to a plasmodium [73]. Aggregation is induced by the pulsed release of a chemoattractant (originally named acrasin) in the aggregation centers [74], which is cAMP in *D. discoideum* [75], but can be other substances like folic acid in other species [76]. Cell aggregation is the next complex step in the social

cycle, heavily dependent on at least three types of glycoproteins: DdCAD-1, CsaA and LagC [77]. The protein DdCAD-1 (formerly called gp24) is important for the initial aggregation. It is Ca^{2+}-dependent and can thus be inhibited by EDTA [78]. The transcription of CsaA (gp80) is upregulated during the early aggregation steps and the protein is not EDTA-sensitive [77, 79]. Only after the initial aggregation steps, LagC (gp150) is produced and mediates aggregation, sorting of pre-spore and pre-stalk cells and differentiation [77].

After trophozoites aggregate, members of the monophyletic clade of the *Dictyostelium spp.* referred to as group 4 (including *D. discoideum* and *D. giganteum*)[80], proceed to form a slug-like structure, which moves towards heat and light sources and away from ammonia [81]. This process is thought to lead to migration towards the soil surface in nature, where the progeny can disperse [82], e.g. by transportation in the gut of nematodes and isopods [83]. In the slug, cells differentiate to fulfill different tasks, including the defense against invading bacteria, executed by sentinel cells, which resemble the innate human immune system, in their response to potential pathogens (e.g., in utilizing a Toll/ interleukin-1 receptor (TIR) domain protein) [84, 85].

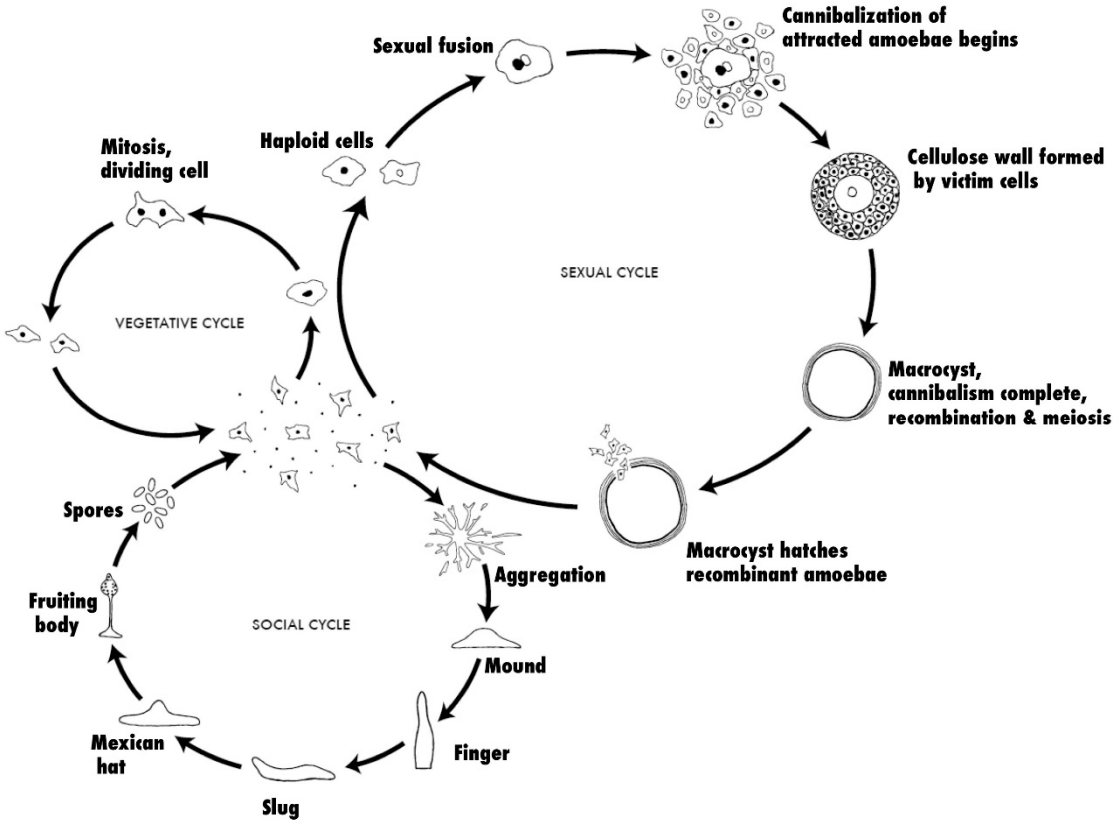

Figure 2: The life cycles of Dictyostelium discoideum by David Brown & Joan E. Strassmann, CC BY 3.0 (http://dictybase.org/Multimedia/DdLifeCycles/index.html).

After migrating to a favored spot, the slug transforms into a sorocarp, consisting of a basal disc, a stalk, which descends from the anterior 20% of the slug cells, and spores, which are formed from the posterior 80% of slug cells [86], during a concerted and tightly regulated process [87]. Stalk cells die in an autophagic process during stalk formation and remain filled with vacuoles and covered with cellulose [88]. This sacrifice arose the question for altruism in *Dictyostelids*, more precisely, how genes encoding for altruistic behavior sustain in a population [89]. To date, *D. discoideum* is one of the most important model systems to study this question [89].

Diversity of the dictyostelids

Apart from the thoroughly studied model organism *D. discoideum* over 100 dictyostelid species are known, divided in 8 phylogenetic groups[90]. Culture-independent approaches show, however, that there is still an enormous undiscovered diversity, which can to-date only be accessed with specific primers in culture-independent approaches [91]. Furthermore, the morphological diversity in the dictyostelids and the families included in this clade is high and while the phylogenetic groupings haven't changed substantially in recent years, the nomenclature was renewed [80, 92]. The speciose family *Dictyosteliaceae* contains the genus *Dictyostelium* including the two species *D. discoideum* and *D. giganteum*[92]. The latter species was first described by Singh in 1947, who collected it from dung and interestingly described the lack of a migrating pseudoplasmodium [93].

Compared to *D. discoideum* the studies focusing on *D. giganteum* are rare and almost exclusively cover the sexual cycle. It is known that macrocyst formation in *D. giganteum* is a single-locus-multiple-allele system similar to meiosis, thus, three of the four mating types involved, are lost in the process [94]. The variation in small subunit rRNA of *D. giganteum* sequences depending on geographical distribution is low and there is no significant influence of genetic or geographical distance on macrocyst formation [95]. While macrocyst formation is a heterothallic process, thus only different mating types fuse [94], the formation of fruiting bodies involves clustering of related cells, thus exclusion of genetically different cells [96]. The sexual cycle is dependent on hierarchies, with mating-types predominantly secreting, others predominantly responding to chemoattractants [97].

The microbiome of dictyostelids

Dictyostelids are known to live in soils all over the world to prey on the bacterial population in their environment [70]. Numerous studies revealed, however, that the interactions between predator and potential prey are much more complex than originally thought. Already at the first description of *D. giganteum*, the inability to grow on several bacterial strains could be shown [93]. Further, different dictyostelids have different preferences for food bacteria and can outcompete other dictyostelids depending on the prey species [98]. Several genes influence the ability to prey on either Gram(+) or Gram(-) bacteria, which is likely to derive from defense pathways against potential pathogens [99]. To

efficiently prey on bacteria *D. discoideum* excretes the glycoprotein DdCAD-1, which is also produced in the aggregation process, and induces the agglutination and thereby facilitated uptake of bacteria [100].

Specific strains of *D. discoideum*, called farmers, are also capable of associating with life bacteria and incorporate them in the otherwise sterile fruiting body, where they can sustain and be used as a food source once the spores disperse and hatch [101]. It is suggested that this farming-capability of a *D. discoideum* strain is depending on the presence of *Burkholderia* strains, which allow for the uptake of additional species into the fruiting body [102]. Further, it was shown that strains of *D. discoideum* carrying the farmer-associated bacteria *Burkholderia* were much more resilient to toxins, such as ethidium bromide, compared to *Burkholderia*-free strains, despite their decreased number of sentinel cells [103].

Those observations emphasized the importance of the microbiome for the survival and reproduction of Dictyostelids in the environment. Brock et al. could show that more than a third of wild *D. discoideum* harbored culturable, aerobe bacteria species of which almost 90% were edible [104]. The culture independent method of 16S rRNA gene sequencing, added the genera *Amoebophilus*, *Procabacter*, and *Chlamydia* to the list of *D. discoideum* microbiome associated bacteria [60]. The chlamydial species discovered in various dictyostelids, are phylogenetically diverse and especially prevalent in *D. giganteum*, although the most abundant chlamydiae are generalists, not limited to one dictyostelids host [60, 105].

Aims of this study

In this study, we analysed the symbiosis between a *D. giganteum* isolate and its chlamydial endosymbiont, member of the *Rhabdochlamydiaceae*. The symbiosis is maintained throughout the *D. giganteum* social life cycle, as we could confirm using light microscopy and FISH. By combining these techniques with ddPCR, we could further reveal ongoing infection in *D. giganteum* populations, despite the lack of infectious EBs. Based on these results, we hypothesize that the role of EBs is neglectable in the transmission of these chlamydial symbionts. As an adaption to the social life cycle of its host, the here described chlamydiae use the aggregated life stages of *D. giganteum* to infect neighboring cells. Thanks to the readily accessible data on the genetic, metabolic and ecological properties of *D. discoideum*, a close relative of *D. giganteum*, this system shows potential to be a model system for chlamydia-host interactions, particularly with respect to the emergence of multicellularity.

Materials & Methods

Instruments

Accu-jet pro pipetting aid	Brand GmbH & Co KG, Wertheim, Germany
Biorad T100	BioRad, Laboratories, Inc., Hercules, USA
Centrifuge Z 366 K	Hermle Labortechnik, Wehingen, Germany
Eppendorf Research® pipettes, 1-channel, 1000 µL/ 200 µL / 20 µL / 2 µL	Eppendorf AG, Hamburg, Germany
Holten Safe 2010 Class II Cabinet 1.8	Thermo Scientific, Vienna, Austria
Leica DM IL LED	Leica Microsystems GmbH, Wetzlar, Germany
Micro TS Compact Plate Sealer	Vitl Life Science Solutions, Ashland, USA
Milli-Q Biocel System Ultrapure Water	Merck Millipore, Darmstadt, Germany
MiniSpin centrifuge	Eppendorf AG, Hamburg, Germany
MIR-254 Incubator	Sanyo/Panasonic Marketing Europe GmbH, Wiesbaden, Germany
NanoDrop 1000 Spectrophotometer	Thermo Fisher Scientific, Wilmington, USA
pH-meter: inoLab pH Level 1	Wissenschaftlich-Technische Werkstätten, Weilheim, Germany
PIPETBOY acu 2	INTEGRA Biosciences GmbH, Biebertal, Germany
Qubit 4 Fluorometer	Thermo Fisher Scientific, Waltham, USA
QX200 Droplet Generator	Bio-Rad Laboratories, Inc., Hercules, USA
QX200 Droplet Reader	Bio-Rad Laboratories, Inc., Hercules, USA
Sartorius BL6100	Sartorius AG, Goettingen, Germany
Vortex-Genie 2	Scientific Industries, Inc., New York, USA
White Light Laser Confocal Microscope Leica TCS SP8 X	Leica Microsystems GmbH, Wetzlar, Germany

Software

BLASTn	National Center for Biotechnology Information, Bethesda, USA
MAFFT version 7 [106]	
EPA-ng [107]	
Python 3	Python Software Foundation, Beaverton, USA
QuantaSoft Version 1.7.4.0917	Bio-Rad Laboratories, Inc., Hercules, USA

Leica Application Suite X Version 3.5.1	Leica Microsystems CMS GmbH, Mannheim, Germany
NanoDrop 1000 Operating Software, version 3.8.1	Thermo Fisher Scientific, Wilmington, USA
Mendeley Desktop Version 1.19.8	Mendeley Ltd., London, UK
RStudio Version 1.2.5001	RStudio, Inc.
Microsoft® Word für Microsoft 365 MSO (16.0.14131.20278) 32-Bit	Microsoft Corporation, Redmond, WA

Consumables

Cell scraper 240 mm/300 mm	TPP Techno Plastic Products AG, Trasadingen, Switzerland
CitiFluor™ AF1	Science Services GmbH, Munich, Germany
Cover glasses 24 x 50 mm	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
DG8 Cartridges	Bio-Rad Laboratories, Inc., Hercules, USA
DG8 Gaskets	Bio-Rad Laboratories, Inc., Hercules, USA
Fast-Read 102® plastic counting chamber	Kisker Biotech GmbH & Co. KG, Steinfurt, Germany
Fisherbrand™ Polystyrene Petri Dishes (d=55mm)	Fisher Scientific (Austria) GmbH, Vienna, Austria
Glass beads 0,25 - 0,5 mm	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Greiner centrifuge tubes, 50 mL/15 mL	Greiner Bio-One GmbH, Frickenhausen, Germany
innuPREP DNA Mini Kit	Analytik Jena GmbH, Jena, Germany
Microscope slides with 10 reaction wells	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Minisart® Syringe Filters (5 µm, 1.2 µm)	Sartorius AG, Göttingen, Germany
PCR Softstrips (0.1 mL)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Pipette tips (various sizes)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Reaction tube, 2 mL / 1.5 mL, PP	Greiner Bio-One GmbH, Frickenhausen, Germany
Serological pipette, plugged, 50 ml/25 ml/10 mL, PS, sterile	SARSTEDT AG & Co. KG, Nümbrecht, Germany
Thermo Scientific™ Nunc™ EasYFlask™ Cell Culture Flasks 75 cm^2/175 cm^2	Fisher Scientific (Austria) GmbH, Vienna, Austria
Sterican® Safety G 25 x 1 1/2'' 0,5 x 40 mm	B. Braun SE, Melsungen, Germany

Chemicals

16% Formaldehyde (w/v)	Fisher Scientific (Austria) GmbH, Vienna, Austria
Agar	Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany
Calcium chloride (CaCl)	Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany
DAPI (4',6-Diamidino-2-phenylindole dihydrochloride)	Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany
dNTP Mix (10 mM)	Fisher Scientific (Austria) GmbH, Vienna, Austria
DreamTaq Green DNA Polymerase (5 U/µL)	Fisher Scientific (Austria) GmbH, Vienna, Austria
DreamTaq Green PCR Master Mix (2X)	Fisher Scientific (Austria) GmbH, Vienna, Austria
Droplet Reader Oil	Bio-Rad Laboratories, Inc., Hercules, USA
EDTA magnesium disodium salt dihydrate	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol, Absolute (200 Proof), Molecular Biology Grade	Fisher Scientific (Austria) GmbH, Vienna, Austria
Formamide, deionized	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Magnesium sulfate ($MgSO_4$)	Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany
Potassium phospate monobasic (KH_2PO_4)	Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany
QX200 ddPCR EvaGreen Supermix	Bio-Rad Laboratories, Inc., Hercules, USA
QX200 Droplet Generation Oil for EvaGreen	Bio-Rad Laboratories, Inc., Hercules, USA
Rifampicin	Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH und Co KG, Karlsruhe, Germany
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany
Tris	Carl Roth GmbH und Co KG, Karlsruhe, Germany
Trisodium citrate dihydrate	Sigma-Aldrich Chemie GmbH, Steinheim am Albuch, Germany
Tryptone	Fisher Scientific (Austria) GmbH, Vienna, Austria
Yeast Extract Powder	Fisher Scientific (Austria) GmbH, Vienna, Austria

Isolation and maintenance of *D. giganteum*

D. giganteum was isolated from soil samples derived from Harvard Forest, Massachusetts, USA. The original soil was put on non-nutrient agar (NNA) PAS plates (1 g/l sodium citrate, 0.4 mM $CaCl_2$, 4 mM $MgSO_4$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, 15 g/l agar) overlayed with a suspension of live *E. coli*. After incubation at 20°C and onset of amoebal growth, trophozoites were picked from the NNA-PAS agar plate and transferred to a fresh *E. coli* covered NNA PAS plate.

Isolated *D. giganteum* were kept in monoxenic liquid cultures in Page's amoeba saline buffer (PAS buffer: 1 g/l sodium citrate, 0.4 mM $CaCl_2$, 4 mM $MgSO_4$, 2.5 mM Na_2HPO_4, 2.5 mM KH_2PO_4, in MiliQ water), enriched with *E. coli* at 20°C. Cured *D. giganteum* were incubated under equal conditions.

Maintenance of *E. coli*

To obtain food bacteria for *D. giganteum*, *E. coli* were inoculated in 500 mL LB-Medium (10 g/L tryptone, 5 g/L yeast-extract, 10 g/L NaCl), distributed to five 300 mL Erlenmeyer flasks and incubated overnight at 37°C and 180 rpm. Subsequently, the cultures were centrifuged at 5,000 x g for 15min, resuspended in PAS buffer to a final concentration of $2.5x10^{10}$ cells per mL and stored at 4°C.

Identification of the chlamydial symbiont by PCR

DNA was extracted from infected *D. giganteum* isolates by using the innuPREP DNA mini Kit following the manufacturers protocol.

The primers used to amplify a fragment of the 16S rRNA gene sequence chlamydiae were SigF2 (5'-CRGCGTGGATGAGGCAT-3') and SigR2 (5'-TCAGTCCCARTGTTGGC-3') [108]. To conduct the PCR, 2.5 µL Dream Taq Green Buffer (10x), 0,25 µL 10 mM dNTP Mix, 0,625 µL SigF2 Primer, 0,625 µL SigR2 Primer, 1 µL extracted DNA, 0,125 µL Dream Taq DNA Polymerase and 19.875 µL ddH_2O were mixed in a reaction tube. Thermal cycling was carried out as follows: an initial denaturation step at 98°C for 2 min followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 60°C for 15 s, and elongation at 68°C for 90 s. Cycling was completed by a final elongation step at 68°C for 5 min.

Sanger sequencing was conducted by Microsynth Austria GmbH using the primers SigF2 and SigR2. The sequences obtained were initially analysed using the BLASTn tool on the NCBI homepage (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with default settings.

Curing *D. giganteum* from its symbiont

NNA PAS plates were overlayed with 200 µL rifampicin (c=2.5 mg/mL) and 200 µL E. coli ($2.5x10^{10}$ cells/mL). One fruiting body of *D. giganteum* was identified by eye, picked with a sterile pipette tip and placed in the middle of the freshly prepared plate, which was allowed to dry and incubated at 20°C.

Fruiting bodies picked from these plates were used to repeat the procedure. Loss of the symbiont was confirmed by FISH and PCR.

Fluorescence *in situ* hybridization and confocal laser scanning microscopy

D. giganteum cells used for fluorescence *in situ* hybridization (FISH) were detached from the culture flask by using a cell scraper. An aliquot of 40µL was put on a Teflon-coated microscope slide and incubated for 15 to 30 minutes at room temperature to allow attachment of the trophozoites. Subsequently, the droplet was replaced by 20µL of 4% (w/v) paraformaldehyde (PFA) in PAS buffer, which was left for incubation for 10 minutes. The fixation was terminated by taking off PFA/PAS and shortly washing each well with 50µL H_2O.

The fixed cells were covered with 10µL of hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, 0.01% sdS, 25 % formamide) mixed with one 1µL of the respective probes and hybridized at 46°C for 90 minutes in a hybridization chamber consisting of a paper towel soaked with hybridization buffer inside a 50 mL centrifugation tube. Subsequently, the slides were incubated in washing buffer (20 mM Tris-HCl, 0.25 mM EDTA and 0.149 M NaCl) at 46°C for 20 minutes and washed in ice cold water for 10 seconds. The probes used in this study were Chla-0232 (5'- TAG CTG ATA TCA CAT AGA -3') [109] 5'-labelled with the fluorescent dye Cy3 and Euk-516 (5'- GGA GGG CAA GTC TGG T -3') 5'-labelled with Cy5 [110].

To stain DNA, all wells were covered with 10µL DAPI in PAS buffer (c = 1 µg / mL) and subsequently washed 7 minutes in cold 96% EtOH. Citifluor AF1 was used to mount a cover glass.

The slides were analysed using a Leica TCS SP8 X confocal laser scanning microscope equipped with a 93x glycerol objective and the Leica application suite X software.

Infection of *D. giganteum* with extracted chlamydiae

A total of 1.3 x 10^7 *D. giganteum* trophozoites, entering the stationary phase of growth, was detached and centrifuged at 5000 x g for 5 min. The pellet was resuspended in 1 mL PAS buffer, transferred to a 2mL reaction tube filled with 0.5 mL of glass beads (d=0.25-0.5mm) and horizontally vortexed for 2 minutes at 2700 x g. The lysate was transferred to a new 2 mL reaction tube pooled with 1mL of PAS buffer, used to wash debris off the glass beads, and homogenized using a 25-gauge injection needle. Subsequently, the lysate was filtered through a 1.2µm filter, resulting in 900µL filtrate containing chlamydiae, and used for infection and ddPCR.

Cultures consisting of 10^6 cured *D. giganteum* trophozoites, 1.2 x 10^9 *E. coli* cells and 150µL of the chlamydiae filtrate, inoculated in 75cm² cell culture flasks containing 10 mL of PAS buffer were incubated at 20°C. Immediately after inoculation, 1mL of each culture was used for DNA extraction

with the innuPREP DNA mini Kit following the manufacturers protocol. To assess abundance of chlamydiae in the super natant and the trophozoites, 1 mL of supernatant was taken off and filtered through 1.2 µm filter, 5, 12 and 19 days after inoculation. Subsequently, trophozoites were detached, 100 µL were taken off and diluted in 1 mL PAS buffer, which was used for cell counting and FISH. The rest of the sample was used for DNA extraction with the innuPREP DNA mini Kit, following the manufacturers protocol, and subsequent quantification of chlamydial 16S rRNA sequences by ddPCR.

Evaluating amoeba growth

Infected and cured cultures of *D. giganteum* and a 1:5 (infected:total) mixture of these cultures, were inoculated in PAS buffer, enriched with *E. coli*, starting at an amoebal cell density of 10^4 trophozoites per mL, with the starting cultures being in the state of nascent aggregation. All cultures were incubated in triplicates at 20°C and growth was assessed after 6, 24, 30, 48 and 72 hours of incubation, by using a cell counter. After 72 hours of incubation DNA was extracted from an aliquot, using the innuPREP DNA mini Kit following the manufacturers protocol.

Testing influence of EDTA on infection rate in mixed cultures

Infected and cured cultures of *D. giganteum* were mixed in a 1:5 (infected:total) ratio in PAS buffer enriched with *E. coli*, starting at an amoebal cell density of 10^4 trophozoites per mL, with the starting cultures being in the state of nascent aggregation. Cultures were incubated in triplicates at 20°C. Half of the cultures were inoculated with EDTA at a concentration of 5mM, which was raised to 10mM after 24 hours of incubation. After 6, 24, 30, 48 and 72 hours of incubation, growth was assessed using a cell counter, FISH was performed and DNA was extracted using the innuPREP DNA mini Kit following the manufacturers protocol.

Digital droplet PCR

Digital droplet PCR (ddPCR) was performed using the QX200 Droplet Digital PCR System. The final PCR reaction mixture contained 1x QX200™ ddPCR™ EvaGreen Supermix, SigF2/SigR2 primers (0.18pM), 2µL of the respective DNA sample and ddH_2O in a total volume of 22µL. A DG8 cartridge was loaded with 20µL of this mixture and 70µL droplet generation oil for the generation of droplets in the QX200™ Droplet Generator. Forty microliters of these droplets were transferred to a 96-well plate and sealed with pierceable foil using the Micro TS Compact Plate Sealer. PCR was performed with the following steps: enzyme activation at 95°C for 5 minutes, 40 steps of denaturation at 95°C for 30 seconds and annealing at 59°C for 1 minute, signal stabilization at 4°C for 5 minutes and a final step at 90°C for 5 minutes. The signal of the droplets was analyzed with the QX200 droplet reader combined with the QuantaSoft software. To distinguish positive from negative droplets, a threshold in the fluorescence amplitude of 11500 was set manually for every sample.

Phylogenetic analysis

Near-full length 16S rRNA gene sequences (>1200nt) from chlamydiae and other PVC members, were downloaded from SILVA v132 and aligned using SINA [111]. A phylogenetic tree was constructed by IQtree [112] using ultrafast bootstrap "-bb 2000" [113] and single branch test "-alrt 2000". Best model as calculated by ModelFinder [114]: TVMe+R8. Short 16S rRNA gene sequences (<300nt) derived from wild isolates of *D. discoideum* by Haselkorn et al. [60], were aligned and added to the tree using MAFFT[106] and EPA-ng [107].

Statistical tests

All statistical tests were conducted using RStudio Version 1.2.5001 and the package rstatix and ggpubr. All data was analyzed for normal distribution using Shapiro-Wilk test and further analyzed by using two-tailed student's t-test or ANOVA. If the data was not normally, distributed Wilcoxon rank-sum test was applied. The null hypothesis was rejected when $p < 0.05$.

Results

A chlamydial symbiont is persistent and prevalent in a wild isolate of *D. giganteum*

Soil from Harvard Forest was used to isolate *D. giganteum*, growing readily on plates and in PAS buffer, with *E. coli* as a food source. After depletion of *E. coli*, aggregates formed, which were subsequently transforming to slugs and fruiting bodies (Figure 3). Using FISH and PCR with chlamydia-specific primers, infection of *D. giganteum* with chlamydiae was confirmed.

Although the symbiont occurred mainly solitarily in the amoeba host cells, we could still observe clusters of aggregated chlamydiae sporadically (Figure 4a). It was not possible to make a clear distinction between different chlamydia cell types, like EBs or RBs. The symbiont was spherical, independent of the state of its host. Chlamydiae were almost evenly distributed in the cytoplasm, with a slight incline to locate at the nucleus of the host. While FISH was the standard technique to identify chlamydiae in the following experiments, staining of the DNA with DAPI often generated characteristic signals under the microscope, which could be used to identify chlamydial infection. The size of the symbiont can hardly be determined by fluorescence microscopy but chlamydiae extracted from host cells fitted through a 1.2µm filter, thus the diameter of chlamydiae is likely below this threshold, which is consistent with reported cell sizes of other environmental chlamydiae. To assess how persistent the infection of *D. giganteum* with chlamydiae is, every phase of the social life cycle was analyzed by fluorescence *in situ* hybridization. Chlamydiae were found inside *D. giganteum* trophozoites, the slug stage and the spores and are thus stably transmitted through the spores into the next host generation by vertical transmission (see Figure 4).

The characteristic feature of this symbiosis is the high prevalence of chlamydiae in the host population, combined with a low symbiont load per host cell. The estimated prevalence is higher than 90% in a stably infected population, while 3 to 7, but no more than 10 chlamydial cells can be found in a single host cell, based on interpretation of FISH signals (Figure 4a).

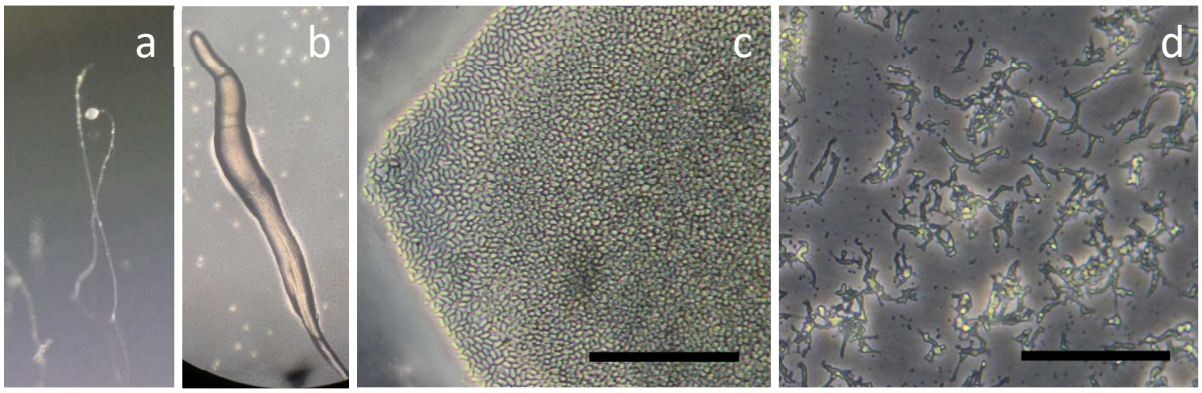

Figure 3: The different life stages of *D. giganteum*: (a) fruiting body; (b) aggregation of cells forming a slug; (c) spores in the fruiting body; (d) aggregating trophocytes. Bar, 100µm.

We successfully and repeatedly cured *D. giganteum* artificially from its chlamydial symbiont by rifampicin. The absence of chlamydiae in *D. giganteum* was investigated by PCR (data not shown) and FISH (see Figure 4d). The symbiosis is therefore not obligate for the host but for the symbiont, although the potential of the symbiont to infect other eukaryotes, apart from cured *D. giganteum* and *D. discoideum*, was not assessed. It was not possible to distinguish infected from aposymbiotic *D. giganteum* cultures by light microscopy, neither by the shape or behavior of the trophozoites, slugs and fruiting bodies, nor by the appearance of the supernatant. Even after longer incubation times, we observed no increase of extracellular content, like EBs, in the supernatant of *D. giganteum* cultures.

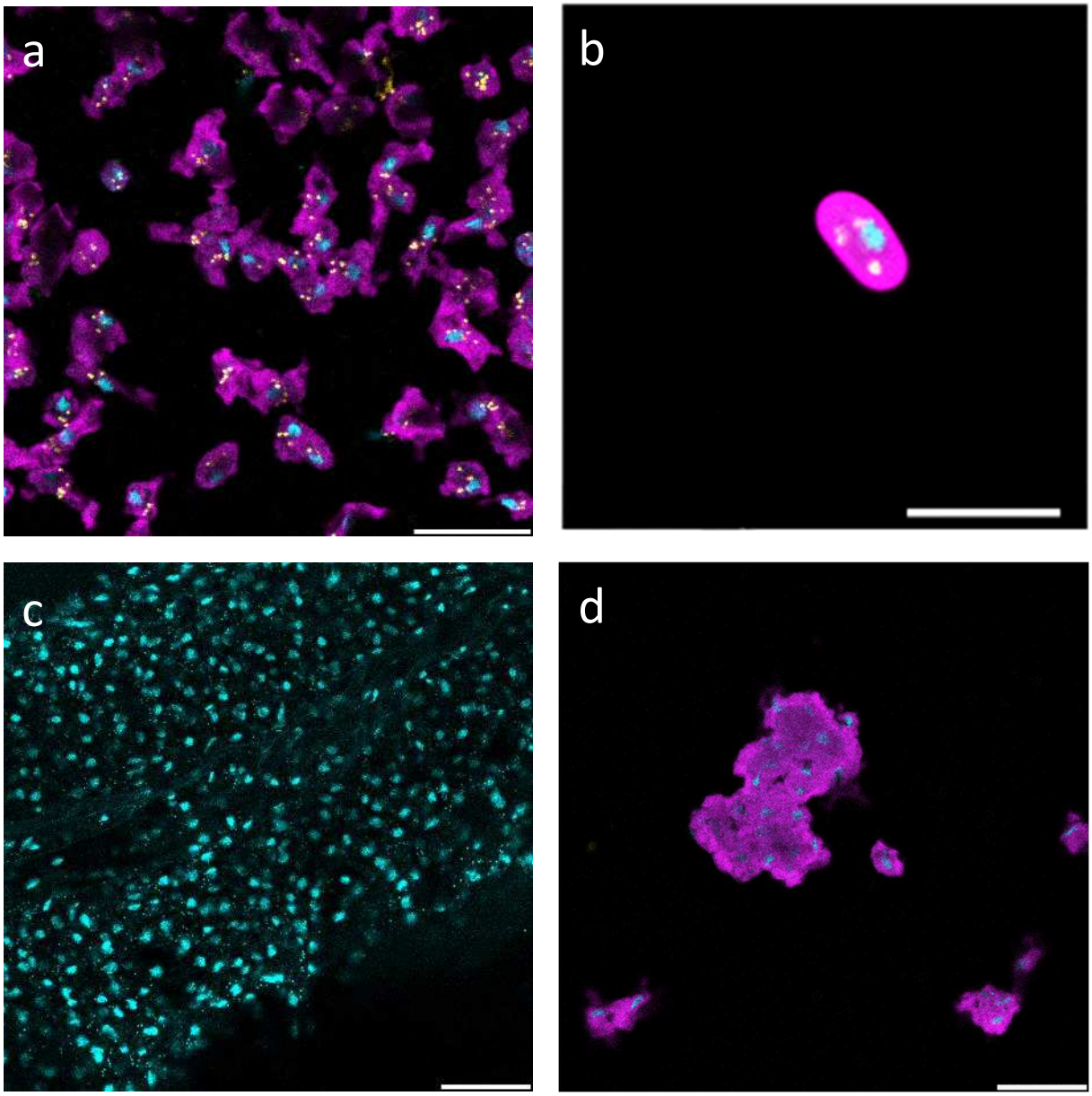

Figure 4: Identification of chlamydiae inside D. giganteum by FISH with the chlamydiae-targeting probe Chla-0282-Cy3 (yellow), eukaryote-targeting probe Euk-516-Cy5 (magenta) and DAPI for DNA staining (cyan). Chlamydiae signal can be seen in the infected trophozoites (a), spores (b) and slug (c), but not in the cured trophozoites (d). Due to autofluorescence at the Cy3-excitation wavelength in the slug stage, only DAPI staining is depicted to visualize the characteristic spots, deriving from intracellular chlamydiae. Bar, 20µm (b: 10µm).

Sequencing the chlamydial 16S rRNA gene reveals affiliation to *Rhabdochlamydiaceae*

To identify the symbiont, the 16S rRNA gene sequence extracted from infected *D.* giganteum was used to perform a Basic Local Alignment Search Tool (BLAST) search on the homepage of the National Center for Biotechnology Information (NCBI). The most similar sequence in the NCBI database, according to the BLAST search, was the entry "Chlamydiae bacterium isolate ChlamHap2 16S ribosomal RNA gene, partial sequence" with a percentage identity of 99.19% and a calculated E-value of 1e-121. This sequence derives from a chlamydial symbiont present in wild isolates of *D. discoideum* and reported very recently [60].The sequence was placed at a basal position of the *Rhabdochlamydiceae* clade in the tree constructed from chlamydial 16S rRNA gene sequences downloaded from the SILVA database (Figure 5).

Figure 5: Phylogenetic tree of the *Rhabdochlamydiaceae* based on 16S rRNA sequences derived from SILVA. Branches consisting of more than two OTUs without a mention in the introduction are collapsed. The chlamydia described in this study is written in bold.

Spread of infection, despite the absence of extracellular chlamydiae

Attempts to infect cured *D. giganteum* trophozoites by inoculation with supernatant of an infected *D. giganteum* culture, failed repeatedly (data not shown), although infectious EBs should naturally be released into the supernatant. To understand if and how chlamydiae can be horizontally transmitted in a *D. giganteum* population, the number of chlamydial 16S rRNA gene copies in 1:5 (infected:total) mixed cultures were measured by ddPCR and used to estimate the number of symbionts per host at the start and the end of a 72 hour incubation. After 72 hours of incubation, ddPCR showed that the ratio of 16S rRNA gene copies per trophozoite was 6.17 (sd=1.55) in mixed cultures and 7.60 (sd=1.79) in infected cultures (Table 1). Student's t-test showed no significant difference between these two treatments (p=0.358). In the supernatant, the amount of chlamydial 16S rRNA gene copies per trophozoite, after 72 hours, was 0.0033 (sd=0.0014) in mixed and 0.062 (sd=0.056) in infected cultures.

Table 1: Comparison of amoebal cell density (trophozoites per mL), and the abundance of 16S rRNA gene copies in mixed and infected cultures, after 72 hours of inoculation. Values in brackets show the standard deviation.

Culture	Trophozoites per mL	16S rRNA sequences per mL		16S rRNA sequences per host	
		Trophozoites	Supernatant	Trophozoites	Supernatant
Mix	1.12×10^6 (2.89×10^5)	6.64×10^6 (2.31×10^5)	3570 (1110)	6.17 (1.54)	0.0033 (0.0014)
Infected	1.50×10^6 (2.93×10^5)	1.11×10^7 (7.33×10^5)	1.01×10^5 (1.06×10^5)	7.60 (1.79)	0.062 (0.056)

A positive control was conducted with *A. castellanii* infected with *P. acanthamoeba* incubated for three days, resulting in 100% infected trophozoites. By using ddPCR at the end of the incubation, we measured 10.23 (sd=9.54) and 21.23 (sd=18.48) 16S rRNA gene copies per host cell when sampling the supernatant and the trophozoites, respectively.

Reinfection of cured trophozoites with isolated chlamydiae occurs at a low rate

Cured *D. giganteum* cultures could not be infected with the supernatant of infected *D.* giganteum cultures. Thus, to assess the infectivity of the intracellular chlamydiae, we extracted chlamydiae from infected *D. giganteum* cultures and inoculated them with cured *D. giganteum* trophozoites. With DNA extracted from both, detached trophozoites and the filtered supernatant at several incubation times, the ratio of chlamydiae to *D. giganteum* was measured using ddPCR (Figure 6). The starting ratio of 0.38 chlamydial 16S rRNA gene sequence copies per trophozoite declined substantially during the first five days to an average ratio of 0.025, when sampling the trophozoites, or 0.00, when sampling the supernatant. This ratio remained at 0.00 during the next 14 days in the supernatant but increased to 0.11 when trophozoites were sampled after 12 days and slightly decreased to 0.09 after 19 days.

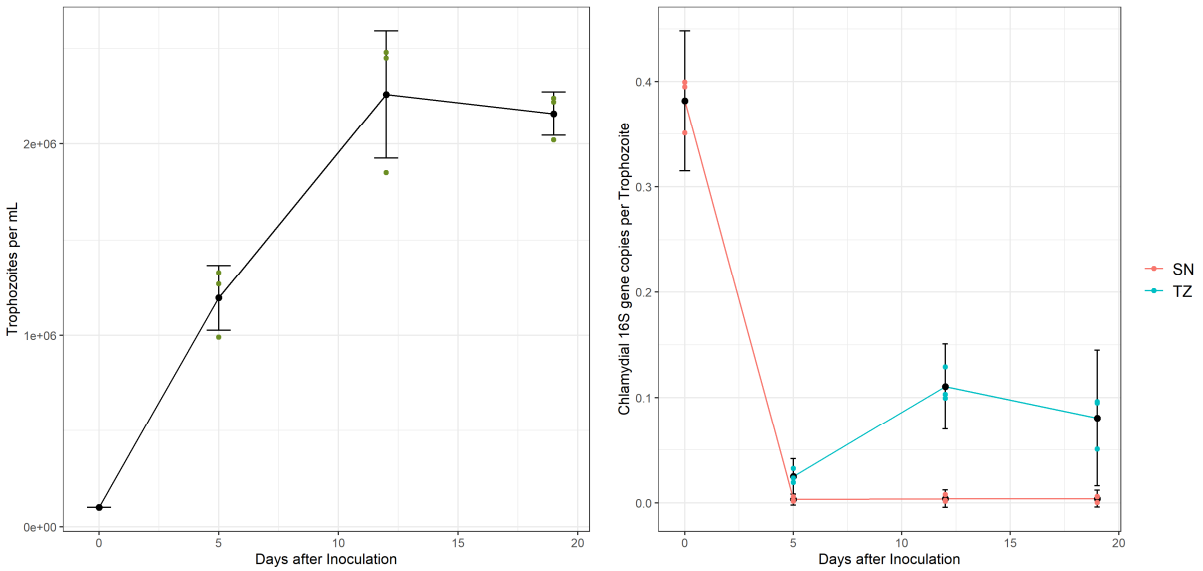

Figure 6: Left: Growth of *D. giganteum* over a time course of 19 days. Right: Ratio of 16S rRNA gene copies per trophozoite assessed with DNA extracted from the supernatant (SN) and the trophozoites (TZ). Colored and black dots depict the measured, individual and the calculated, mean amoebal cell density, respectively, for every point of time and culture. Bars depict the 95% confidence interval.

The growth curve of the cultures gives a decent picture of the trophozoites state of health. While the cultures were steadily growing during the first 12 days of incubation, amoebal cell density declined during the last seven days of sampling (Figure 6). The effect was also visible under the microscope, considering the small, spheric and detached cells floating in the buffer. At several incubation times, we could observe nascent aggregation of trophozoites, induced by the lack of *E. coli*.

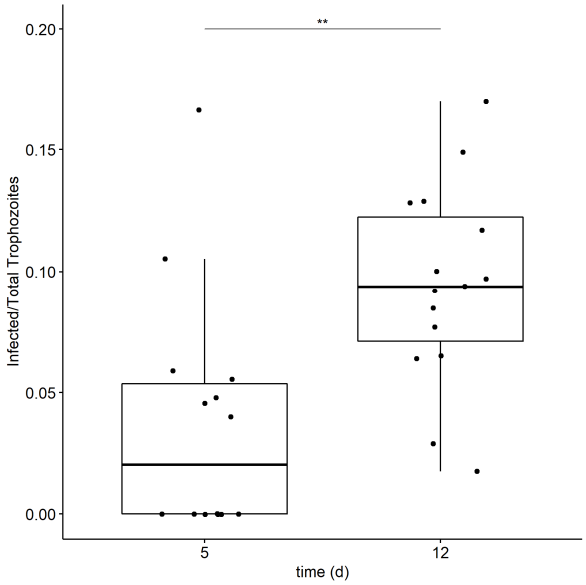

Figure 7: Proportion of infected trophozoites in a *D. giganteum* population after incubation with extracted chlamydiae for 5 and 12 days. p=0.00195, according to Wilcoxon rank-sum test

Analyzing the cultures by FISH, showed an increasing ratio of infected to total trophozoites in all replicates, in the course of time. In total, this ratio increased from 0.037 (sd=0.05), after 5 days, to 0.094 (sd=0.041), after 12 days. The proportion of infected cells in the total population was significantly increasing during these time points according to Wilcoxon rank-sum test (p=0.00195) (Figure 7).

Growth rates of *D. giganteum* are not depending on the infection state

To assess and compare the influence of chlamydiae on the growth rate of *D. giganteum*, liquid cultures were set up, consisting of infected, cured and 1:5 (infected:total) mixed trophozoites. The latter served the additional purpose of observing infection events during the time course, as described above. All cultures started at the same density of host cells, but with an excess of *E. coli* to ensure optimal conditions for growth. The amoebal cell density was measured 6, 24, 30, 48 and 72 hours after inoculation (Figure 8). The differences in amoebal cell density between the three conditions were not significant according to ANOVA, (6h: F=0.212, df=2, p=0.815; 24h: F=0.051, df=2, 0.951; 30h: F=4.553, df=2, p=0.063; 48h: F=1.644, df=2, p=0.270; 72h: F=2.818, df=2, p=0.137), although amoebal cell density in the mixed culture exceeded amoebal cell density in the infected and cured cultures.

The maximal growth rate and – vice versa – the lowest doubling time of the populations, was measured at the beginning of the incubation, i.e., between 0 and 6 and between 6 and 24 hours. Using the formula $x = (t_2-t_1)/\log_2(cd_{t2}/ cd_{t1})$, in which x represents the doubling time of the cell culture in question, t_2 and t_1 represent the incubation time and cd_2 and cd_1 represent the amoebal cell density (trophozoites per mL) at the respective time, it was possible to calculate the doubling time for each infection state during the different phases of incubation (Table 2).

Additionally, we wanted to specifically compare the fitness of infected and cured populations, thus applied student's t-test to test for significant differences in doubling time and amoebal cell density. No significant differences in the density of *D. giganteum* cells could be observed at any time point (6h: p=0.92; 24h: p=0.81; 30h: p=0.98; 48h: p=0.77; 72h: p=0.13). While exponential growth was observed in the beginning, growth rates decreased towards the end of the time course.

Table 2: Doubling times and standard deviation of cured, infected and 1:5 (infected:total) mixed *D. giganteum* cultures, from start to 6, and from 6 to 24 hours of incubation. The last two columns show the p-value for the t-test, comparing infected and cured (I-C), and the ANOVA, comparing infected, cured and mixed (I-C-M) cultures doubling time.

Time (h)	Doubling time (h)						p-value	
	Cured		Infected		Mixed		t-test	ANOVA
	Mean	sd	Mean	sd	Mean	sd	I-C	I-C-M
0-6	4.34	0.77	4.74	2.05	3.97	1.03	0.77	0.80
6-24	6.79	1.31	6.68	1.37	6.93	0.81	0.92	0.97

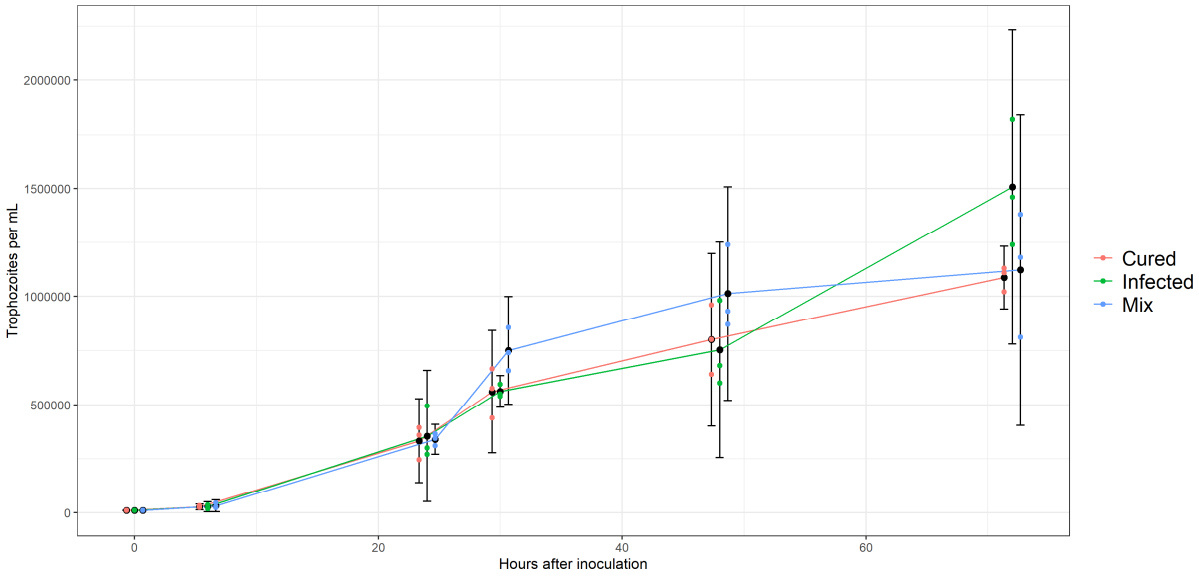

Figure 8: Influence of the infection state on the growth of *D. giganteum*. All cultures were inoculated at an amoebal cell density of 10^4 cells per mL and grew exponentially until a plateau was reached. No significant differences in the amoebal cell density could be observed at any point of time, using ANOVA. Colored and black dots depict the measured, individual and the calculated, mean amoebal cell density, respectively, for every point of time and culture. Bars depict the 95% confidence interval.

EDTA addition does not affect the reproduction but the distribution of chlamydiae

To understand the role of cell-to-cell contact in horizontal transmission of chlamydiae, EDTA was added to *D. giganteum* cultures during exponential growth, to prevent the aggregation of trophozoites. During the course of incubation, aggregation efficiency in cultures with added EDTA was evaluated using a light microscope and compared to an EDTA-free culture. While the EDTA-free cultures developed typical aggregation streams 48 hours after inoculation, trophozoites in the EDTA treated cultures remained predominantly solitary, i.e., hardly attached to each other (Figure 9). Amoebal cell densities did only vary significantly after 30 hours of incubation (6h: p=0.068; 24h: p= 0.092; 30h: p=0.019; 48h: p=0.13; 72h: p=0.11), the time point after the concentration of EDTA was augmented from 5mM to 10mM (Figure 10). Evaluating the relative growth of chlamydiae during the incubation using ddPCR, revealed a 2050-fold and a 2770-fold increase of chlamydial 16S rRNA gene copies during the 72 hours of incubation, in the EDTA-free cultures and EDTA-treated cultures, respectively.

Subsequently we assessed the ratio of 16S rRNA gene copies per trophozoite and revealed significant differences in the two set-ups, after 6 and 24 hours of incubation using student's t-test (6h: p=0.045; 24h: p=0.026; 30h: p= 0.73; 48h: p=0.080; 72h: p=1.00). The supernatant was almost completely devoid of any chlamydial 16S rRNA gene copies. In both set-ups the ratio of chlamydial 16S rRNA gene copies to trophozoites was rising in the first 24 hours declining at 30 hours and rising again until the end of the incubation (Figure 11).

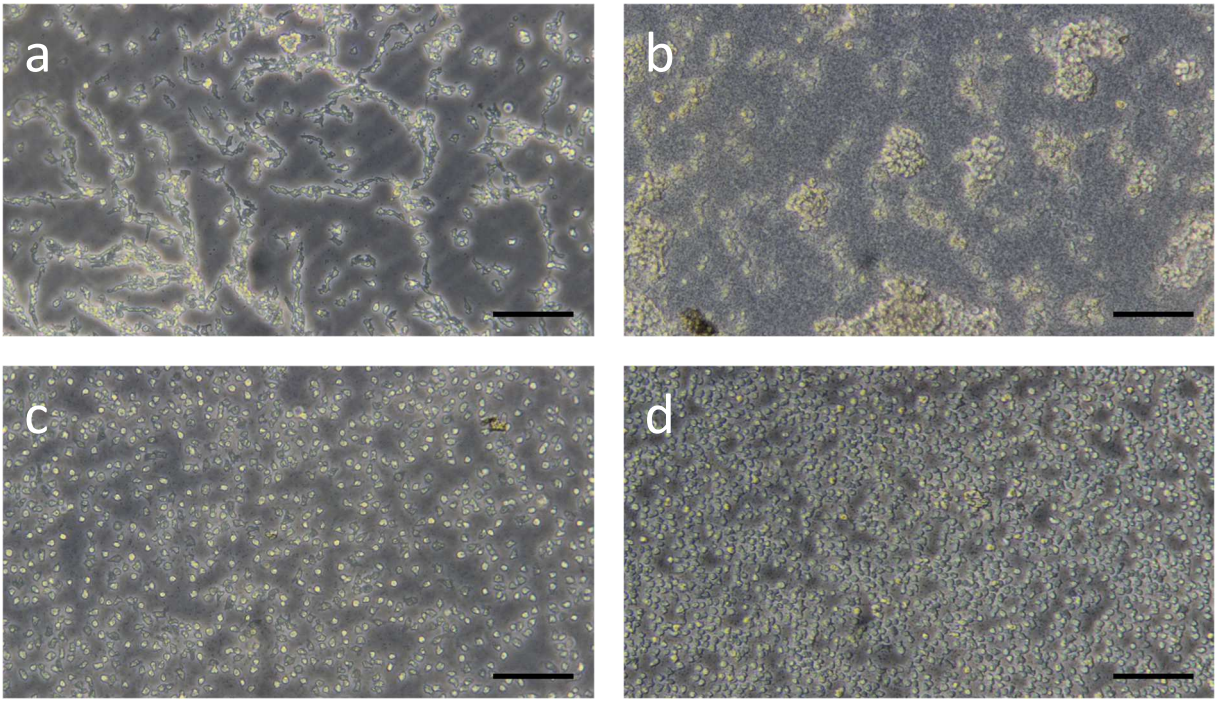

Figure 9: Aggregation state of D. giganteum populations in absence (a, b) and presence (c, d) of EDTA, after 48 (a, c) and 72 hours (b, d) of incubation. Bar, 100µm

To assess the distribution of chlamydiae in the *D. giganteum* population, i.e., the ratio of infected to aposymbiotic trophozoites and the number of chlamydial symbionts per host cell, FISH was conducted with the cell cultures fixed after 72 hours of incubation (Figure 12). Quantitative analysis of the resulting images showed, that 49% (n_{total} = 261) and 33% (n_{total} = 338) of the counted trophozoites were infected, in the EDTA-free cultures and the EDTA-treated cultures, respectively (Figure 13). Thus, although ddPCR showed no significant differences in the overall abundance of chlamydiae in both set-ups after 72 hours incubation, the distribution of the symbionts in the *D. giganteum* population varied significantly, according to student's t-test (p=0.0049).

Due to problems in the detection of FISH signals, only the last time point, 72 hours after inoculation, was analyzed using FISH. It was however possible, to evaluate the chlamydial distribution in EDTA-free cultures 48 hours after inoculation, by counting the characteristic dots, originating from chlamydiae after DAPI staining. According to the image analysis, 32% of the counted trophozoites (n_{total} = 185), were infected by the chlamydial symbiont (Figure 13).

Higher infection rates occurred at later time points, since the proportion of infected cells in the cultures increased 1.6-fold during the first 48 hours of incubation and 1.5-fold, during the subsequent 24 hours.

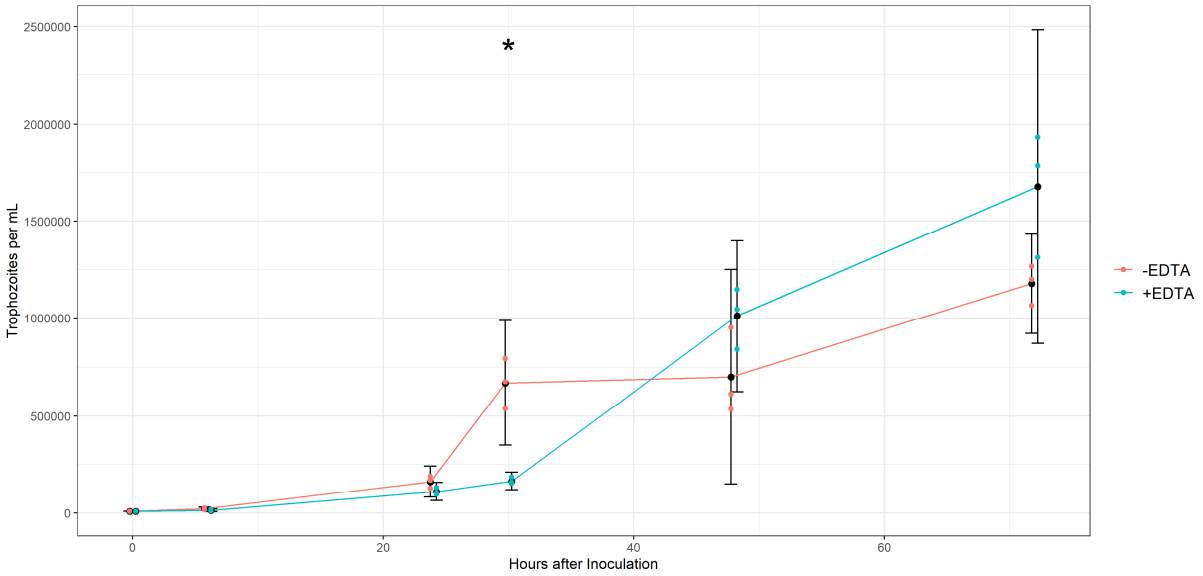

Figure 10: Growth of *D. giganteum* cultures mixed in a 1:5 (infected:total) ratio. Colors indicate the presence/absence of EDTA. Black dots depict the mean amoebal cell density at the respective point of time and culture, bars depict the 95% confidence interval. Significant difference in amoebal cell density ($p<0.05$), indicated by a star, was measured after 30 hours.

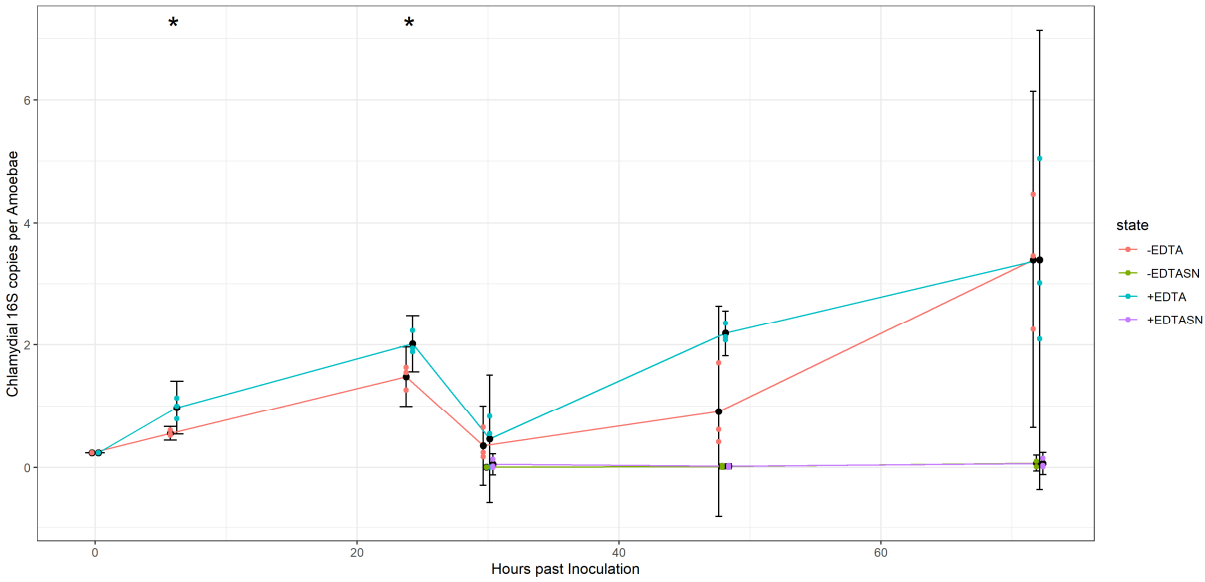

Figure 11: Ratio of 16S rRNA gene copies per trophozoite assessed with DNA extracted from the supernatant (SN) and the trophozoites (TZ) of cultures with and without added EDTA. Black dots depict the mean amoebal cell density at the respective point of time and culture, bars depict the 95% confidence interval. Significant difference in the ratio of the sampled trophozoites ($p<0.05$), was measured after 6 and 24 hours and is indicated by a star.

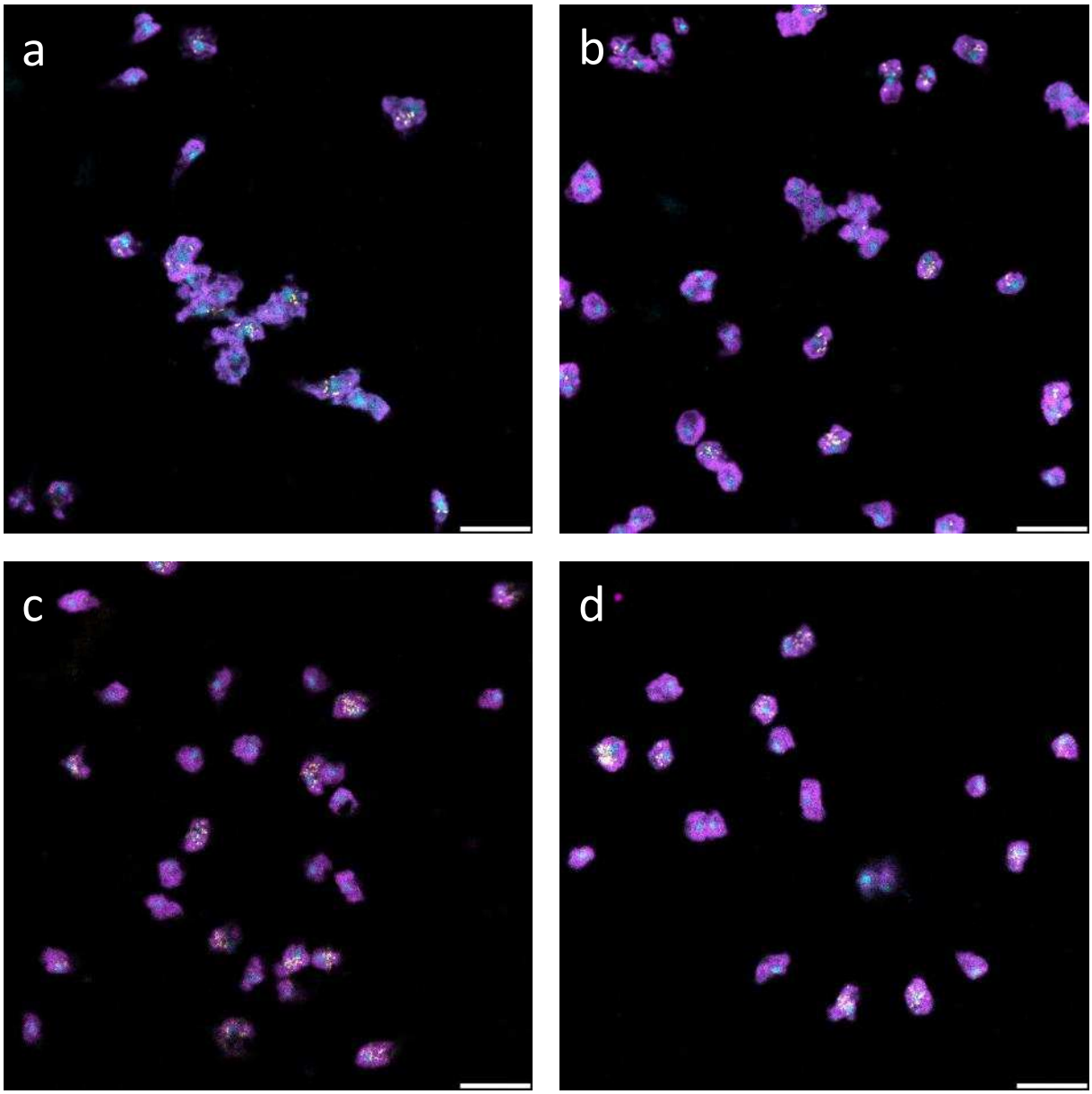

Figure 12: In situ identification of infected and aposymbiotic *D. giganteum* trophozoites in EDTA-free (a, b) and EDTA-treated (c, d) cultures, using the chlamydiae-targeting probe Chla-0282-Cy3 (yellow), eukaryote-targeting probe Euk-516-Cy5 (magenta) and DAPI for DNA staining (cyan).

Considering these observations, it was possible to calculate the absolute numbers of infected and aposymbiotic trophozoites in the cultures after 48 and 72 hours of incubation. After 48 hours, of roughly 7.00 x 10^5 cells per mL, 2.31 x 10^5 were infected, while 4.69 x 10^5 were aposymbiotic. During the subsequent 24 hours, these numbers increased to 1.18 x 10^5, 5.77 x 10^5 and 6.01 x 10^5 cells per mL, respectively. Thus, at the same time the total amount of trophozoites per mL multiplied by a factor of 1.68, the amount of infected trophozoites per mL increased by a factor of 2.50 while the amount of aposymbiotic trophozoites per mL increased only by a factor of 1.28.

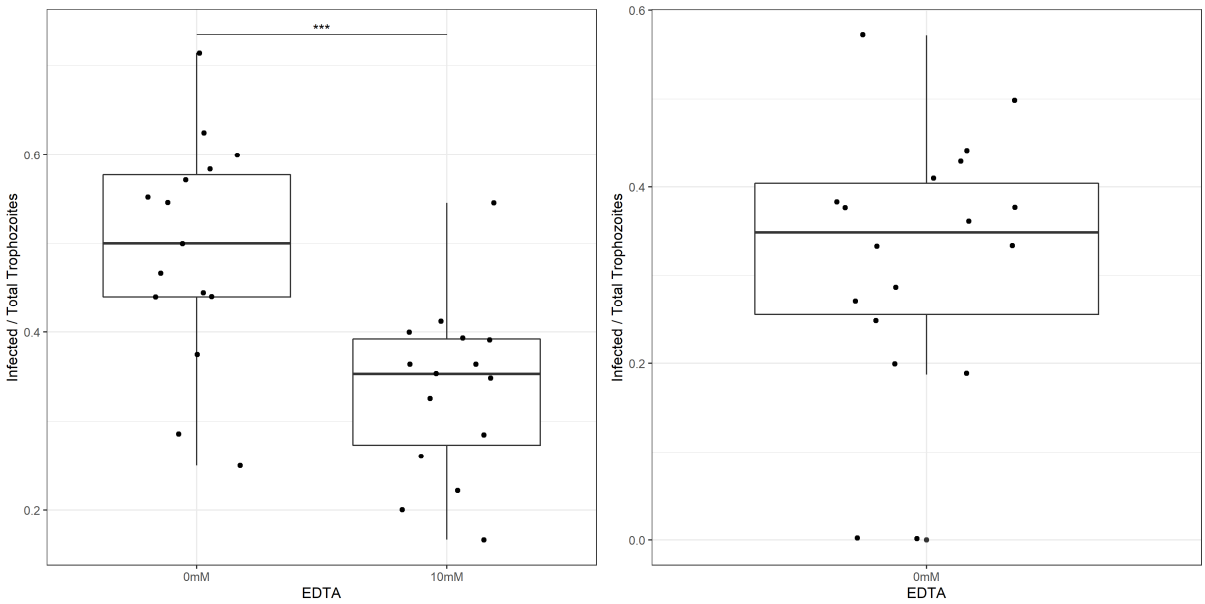

Figure 13: Left: Proportion of infected trophozoites in the cultures 72 hours after inoculation depending on the presence or absence of EDTA. Right: Proportion of infected trophozoites in EDTA-free cultures 48 hours after inoculation.

Discussion

Free-living amoebae are historically an important source for the study and characterization of environmental chlamydiae, since they are often naturally infected and can thus be used to study the relations between chlamydiae and their original host in controlled laboratory set-ups [10]. With the isolation of an infected *D. giganteum* strain, we have the possibility to study a member of the *Rhabdochlamydiaceae*, the phylum which is currently suggested to be the most diverse chlamydial family [52], in a host that is closely related to *D. discoideum*, one of the best-known model systems in existence [71,72].

No pathogenic effect of chlamydiae could be observed in *D. giganteum* isolates

A prominent feature of the symbiosis described in this study was its virtual low impact on the physical appearance of the infected *D. giganteum* populations. No major changes in the phenotype could be observed, neither with the naked eye, nor under the microscope, at any stage of the social life cycle.

Measuring the growth rate of amoebae is a very intuitive approach to compare fitness of two populations and can easily be measured by counting trophozoites in a counting chamber. No significant difference in growth rate could be observed when the two populations of *D. giganteum*, one infected, one cured, both provided with an excess of *E. coli*, were compared (Figure 8). Thus, the chlamydial symbiont is most likely not impairing nor accelerating the growth rate of *D. giganteum* under optimal growth conditions, contrary to the results of similar studies using other amoebae-chlamydiae systems [115]. This is to a certain degree surprising, since we could show in other parts of this study, that chlamydiae are actively replicating in the host cells. Parasitic chlamydiae like *C. trachomatis* but also *P. amoebophila* are exploiting nutrients, amino acids, nucleotides and lipids of the host cell to thrive [25,108], which thereby lowers the fitness and growth rate of the host and cause diseases in humans and animals [116]. Which mechanisms compensate for this parasitism in the symbiosis presented here, remains to be elucidated. However, positive effects on the host growth rate have also been reported in other studies [115]. The absence of negative or positive effects could also be a result of the low symbiont load, compared to other chlamydial symbionts, e.g., *P. acanthamoeba* [35]. Yet, the absence of clear negative effects on *D. giganteum* is an indicator for an established symbiosis in which vertical transmission is dominant and horizontal transmission is only playing a minor role [117]. Thus, according to our current knowledge, we must consider this chlamydial species a mutualistic or commensalistic, rather than a parasitic symbiont.

However, assessing growth at optimal laboratory conditions, only gives a very limited few on the fitness of an organism, especially an organism with a complex life cycle such as *D. giganteum.* Completely different parameters, such as the commitment to aggregate under unfavorable conditions,

the spore size or the spore viability, are most likely more important than the maximal growth rate of trophozoites to survive in harsh environments [118]. Even the food bacteria, which can alter the relative fitness between different dictyostelid species [98], could influence the competition between infected and cured *D. giganteum* populations.

Preliminary observations indicated, that cured trophozoites inoculated on a bacterial lawn on NNA PAS plates were faster to form fruiting bodies than infected trophozoites (data not shown). Despite the fact, that we did not further investigate this behavior, this observation exemplifies, that different approaches to quantify fitness can yield different impressions on how it is affected by an endosymbiont.

Horizontal transmission does not occur by secreted EBs

One of the key issues in symbiosis research is to understand the transmission mode of the symbiont, since it has a range of implications, including the history of (co-)evolution and the dependence of the partners of one another [119]. Chlamydiae, albeit considered obligate intracellular, need to leave their host cell to infect new host cells, a process mediated by a cell form called elementary bodies (EBs), specialized for extracellular survival, regarding their metabolism, morphology and proteome [3, 2, 120]. Size and especially shape of the EBs of different species can vary to a certain degree, ranging from circular to rod-shaped [121, 43]. Yet, EBs are an essential part of the chlamydial life cycle and hitherto have been described in every chlamydial isolate [6, 9].

The data presented in this study suggests that the symbiont described in this thesis lacked the transition from RB to EB, hence the EB life stage in general. The microscopic appearance of chlamydiae did not change during the time course of infection, we could not observe clustering, any form of specific localization or changes in shape or size (Figure 4). Yet, FISH was the sole microscope technique applied to visualize the symbiont, making clear predictions on the cell morphology almost impossible. The application of electron microscopy could render further, more explicit, insights on the presence or absence of specialized cell forms and their morphology.

The release of chlamydiae in the supernatant by infected amoebae cultures, can often be readily seen by light microscopy, as in the case of *P. acanthamoeba*, used as a positive control in this study. The daily observation of infected *D. giganteum* cultures and the comparison with cured *D. giganteum* cultures under the light microscope further strengthened the impression that chlamydiae, more generally speaking “extracellular particles”, are virtually absent in the supernatant of the infected *D. giganteum* cultures.

Artificial infection of native amoebae can be achieved with EBs collected from the supernatant of infected cultures in the case of *P. acanthamoebae* [117] and *P. amoebophila* [108]. Several, slightly varying

attempts to copy this technique failed to infect cured *D. giganteum* populations, either because EBs are not infective, which would contradict their main natural purpose, or because EBs were not or only sparsely present in the supernatant of infected cultures. The probable reasons for a low concentration of EBs in the supernatant may be an incubation time too short for the effective production and excretion of EBs by the host cells or the general absence of EBs in the supernatant, which would fall in line with our previous observations.

By inoculating cured *D. giganteum* populations with chlamydiae extracted by mechanical lysis from an infected culture, transmission via extracellular chlamydiae was finally achieved. The drop in 16S rRNA sequence copies measured between the inoculation of the cultures and the first sampling time point after 5 days, indicates that the majority of chlamydiae did not survive the extraction or the extracellular conditions present in the supernatant (Figure 6). Possible scenarios for this drastic decrease of chlamydial abundance in the supernatant include lysis of the chlamydiae immediately after inoculation, as the extracted chlamydiae rather resembled RBs than EBs, which would then not survive the harsh extraction conditions. Another possible scenario is the uptake of the extracted chlamydiae by *D. giganteum* trophozoites. Yet, if the latter was the case, EBs did only infect negligible low numbers of *D. giganteum* and were most probably digested. Even after 19 days of incubation, levels of 16S rRNA gene copies in the supernatant did not in the least, reach similar heights as *P. acanthamoebae* infecting *A. castellanii*, after an incubation time of 3 days, used as a positive control. It should be considered, however, that only approximately 10% of the *D. giganteum* population were infected after 12 days, compared to a 100% infection rate of the *A. castellanii* positive control.

In summary, no or only very limited release of EBs by infected *D. giganteum* could be observed in this thesis. These results fall in line with recent studies, in which the soil surrounding chlamydiae-infected *D. discoideum* cultures was almost devoid of any chlamydial 16SrRNA gene copies [105].

Yet, it is possible, that the development of EBs depends on environmental or host-derived cues, which were absent in the laboratory so far. Since all chlamydiae described so far, showed a biphasic life cycle, the potential to leave *D. giganteum* host cells was most likely preserved in the chlamydial genome.

Infection occurs, despite the absence of EBs

The characteristic feature of the chlamydial life cycle, found in all chlamydiae described hitherto [6], is the regular transition between two cell forms, RB and EB, the latter being the infectious form sustaining in the environment[7]. Thus, the complete absence of EBs in the supernatant of infected *D. giganteum* cultures implied the absence of horizontal transmission.

By applying ddPCR over a time course of 72 hours to *D. giganteum* cultures consisting of both, symbiotic and aposymbiotic host cells, we could prove that the ratio of chlamydial 16S rRNA gene

copies to host cells, thus the ratio of symbionts to host cells in the total population, increased over time, which implies ongoing infection. At the same time, chlamydial 16S rRNA gene copies were virtually absent in the supernatant, consistent with our earlier observations and excluding the classic chlamydial infection route via extracellular EBs (Table 1).

Of note, these results depict the ratio of chlamydial 16S rRNA gene copies to trophozoites as a mean over the whole population and do not provide information about the distribution of chlamydiae, with respect to *D. giganteum*. Chlamydiae possibly replicated in their original host cells, which were either non-dividing, or dividing and thereby passing on chlamydiae to daughter cells by cytokinesis. Alternatively, chlamydiae were replicating and leaving their host cells to infect cured trophozoites during the incubation. The first two scenarios would result in extremely high symbiont loads for the individual, infected host cells. Since the ratio of chlamydial 16S rRNA gene copies to trophozoites increased 15-fold (Figure 11) and the total amount of trophozoites increased 120-fold (Figure 10) during the incubation, each chlamydiae produced 1800 descendants, which do not fit into a single trophozoite. If no infection occurred during the time course but chlamydiae were passed on to daughter cells with each host cell division, while the proportion of 1:5 (infected:total) trophozoites was maintained, each host cell should contain 10 times the starting chlamydial load, which would result in 30 to 80 chlamydiae per host cell. While this is still impossible, another, theoretically probable scenario, is outcompetition of cured by infected trophozoites. This is, however, unlikely, due to the similar growth rates observed earlier, when comparing the two different infection states (Figure 8), yet, we do not know how the populations influence each other. However, the measured doubling time of the mixed culture was not smaller than that of infected and cured cultures (Table 2). Thus, if infected trophozoites by any means outcompete cured trophozoites, they would need to exceed the growth rate measured in pure cultures to reach the cell densities measured in mixed cultures.

The last alternative to ongoing infection in mixed cultures is apoptosis or necrosis of cured cells after the growth rate of the populations decreased and left the exponential phase, while infected cells grow and even nourish from the remains of the dying cells. In fact, cannibalistic dictyostelid species have been reported, actively feeding on each other [122], yet, this goes hand in hand with declining cell densities and was never reported for *D. giganteum*. Additionally, we are confident that cell death or cannibalism in the mixed *D. giganteum* populations would not have remained unnoticed, since we monitored the state of the trophozoites continuously during the time course, by microscopic observations.

Taken together, these considerations lead us to the conclusion, that infection processes are ongoing in the mixed cultures, but they are not dependent on EBs in the supernatant.

Infection of *D. giganteum* depends on the aggregation of trophozoites

Data derived from ddPCR only provides a global view of the processes occurring in the respective culture and is influenced by the availability of suitable DNA stretches for PCR and the efficiency of the used DNA extraction protocol. Since the number of 16S rRNA gene copies per genome can vary in different chlamydial species, e.g. 1 in *S. negevensis* [123], 2 in *W. chondrophila* [124], 3 in *P. acanthamoebae UV-7* [125] and *P. amoebophila UWE25* [126], 16S rRNA sequence copies in a culture can only provide information on the trend of abundance, but no absolute quantification of chlamydiae in the *D. giganteum* population, without knowing the whole chlamydial genome.

A more precise insight into the distribution of chlamydiae in an infected *D. giganteum* culture and the shifts in the abundance of endosymbionts per host cell, is provided by FISH, with the drawback that this technique is operator dependent and challenging with respect to quantification of intracellular microbes, and it has a relatively low throughput.

Still, by FISH, we could show that not only the abundance but also the prevalence of chlamydiae was increasing in mixed cultures of *D. giganteum* over the course of time (Figure 13). This adds to the results provided by the ddPCR measurements and underpins the hypothesis of a propagation of the infection in the observed cultures. It provides further evidence for a higher infection rate at later time points. While it took 48 hours for the chlamydial prevalence to increase by a factor of 1.6, it took only 24 hours more to increase by a factor of 1.5. We consider two main factors to contribute to this phenomenon, firstly the high rate at which *D. giganteum* grew during the first 48 hours of incubation, which can hardly be exceeded by chlamydiae, and secondly the reduced distance between neighboring cells by higher cell densities towards the end of the incubation, resulting in an increased possibility of infection by vicinity. The question how fast chlamydiae can replicate and how this affects the infection rate, remains to be elucidated.

Thus, we hypothesize that the infection is not dependent on the well-known extra-cellular chlamydial EB stage, demanding for transformation and excretion of the symbiont, but on an alternative route not yet observed or understood. By its reoccurring social life cycle, *D. giganteum* and in fact all other dictyostelids [70], provide optimal conditions for chlamydiae to infect adjacent, tightly-aggregated, potential host cells. In this melting pot, chlamydiae could be transmitted from one *D. giganteum* cell to the next, thereby avoiding the potential risks linked to the release into the environment, yet, facing challenges like the sentinel cells, which clear pathogens in the dictyostelids slug stage [84].

By adding EDTA to mixed *D. giganteum* cultures we could successfully diminish the aggregation of trophozoites even at high cell densities (Figure 9), enabling us to compare the effects of aggregation on the infection events. These results strongly suggest that aggregation is a prerequisite for chlamydial

transmission in *D. giganteum*, further underpinned by the high number of infections occurring only in later stages of the co-cultivation experiment, during which aggregation increased (Figure 13). However, the side effects of EDTA in the supernatant and on the cells, be it symbionts or hosts, may be diverse and are hardly known. In a similar way EDTA interferes with DdCAD-1, the glycoprotein responsible for the early aggregation of *D. giganteum*, it could interfere with any other glycoprotein dependent on Ca^{2+}. The glycoprotein gp96, expressed by HeLa cells, was shown to facilitate the attachment of *C. trachomatis* and EDTA was used to detach chlamydiae from the host cells [13]. Thus, we can not exclude the possibility of EDTA affecting similar mechanisms in the symbiosis described here, albeit the absence of EBs in the media renders transmission through the supernatant almost impossible. Yet, if the uptake of chlamydiae was hindered by EDTA, additional accumulation of EBs would likely occur in the supernatant of the cultures. We did not observe accumulation of 16S rRNA gene sequences in the supernatant using ddPCR (Figure 11).

Implications of the transmission mode on the chlamydial genome are yet to be studied

Symbiosis is often tightly linked to genome reduction of the symbiont, due to the reduced selection pressure on the already small population of symbionts [119]. Thus, it is not surprising that the obligate life cycle of chlamydiae has led to substantial genome reduction and genome sizes of 0.9 to 2.6 Mb according to metagenomic studies [127]. A general pattern is, that genomes of chlamydiae associated with multicellular eukaryotes are smaller than genomes of chlamydiae associated with protists [128, 127], thus *Chlamydiaeceae* possess highly reduced genomes (1 Mb), compared to *P. acanthamoebae* UV-7 (3 Mb) [17].

Over the course of this study, we repeatedly extracted genomic DNA from the chlamydial symbiont but could not successfully sequence its genome yet.

Whole genome sequencing will show which impacts the lack of a free-living phase has on the genome of this *D. giganteum* symbiont. Potential gene loss could affect genes involved in the infection mechanism such as the major outer membrane protein (MOMP), other components of the outer membrane complex (OMC) or the polymorphic membrane proteins (PMP), which are all highly versatile [17]. Additionally, genome sequencing of environmental chlamydiae uncovered a surprisingly high potential for diverse metabolic pathways as compared to *Chlamydiaceae*. All environmental chlamydiae encode a glucokinase and a complete TCA cycle, giving them the ability to utilize D-glucose and acetyl-CoA in their energy metabolism [3]. Possibly, considering the absence of a negative effect on the maximum doubling time of *D. giganteum,* the metabolism of the chlamydial symbiont is to a certain degree independent or even supporting the metabolism of its host, by a yet unknown mechanism.

Furthermore, genomic and transcriptomic data could reveal the impact of chlamydiae on the aggregation behavior of *D. giganteum*. Large proportions of *D. discoideum* research, has concentrated on the effects of kin on the aggregation behavior and the influence of cheating genotypes on the population [89]. However, symbionts introduce a new variable into the equation of relatedness and cheating. Depending on the negative or positive effects of chlamydiae on the hosts fitness, aposymbiotic dictyostelids would likely avoid or induce aggregation with infected neighbors. At the same time, chlamydiae could either induce aggregation to spread in the population or repress aggregation to avoid coinfection with other pathogens. We do not know yet, if endosymbionts effect the behavior of dictyostelids and how they underlying mechanisms would work, due to the lack of research in this field. However, it has been shown, that *Burkholderia sp.* has diverse effects on the *D. giganteum* fruiting body morphology and microbiome [102].

The here described symbiosis, also offers the possibility to elucidate HGT, not only between different chlamydial species, which could possibly infect dictyostelids, such as *D. giganteum* [60] but also bacteria like *Burkholderia spp.*, known to be actively sustained through the dictyostelids social life cycle [129], and various other bacteria which are apparently persistently carried through the social life cycle [104]. Amoebae are already considered as melting pot for pathogens, including giant viruses, in which considerable transfer of genetic material, thus huge leaps in evolution can occur [130]. This adds on and extends the hypothesis of amoebae as a training ground for pathogens [131]. Both phenomena are likely to occur in this symbiosis.

The *D. giganteum* life cycles offer additional routes for symbiont transmission

Finally, while the focus of this thesis lies on the transmission during the solitary phase of *D. giganteum* and the early phase of the social life cycle, another transmission route is presented by the sexual life cycle of dictyostelids, in which thousands of cells fuse to form a zygotic macrocyst, which divides in a meiosis-related manner, giving rise to recombinant progeny [132]. The fusion event presents an ideal opportunity for chlamydiae to spread in a dictyostelid population, because it reduces the need for the time and energy consuming transition to EBs. Instead, chlamydiae could maintain the RB or AB state inside the host, until host cells fuse. Thereby chlamydiae would also avoid the threats presented by the environment outside of the host cell. Additionally, macrocyst formation is depending on mating types, which discriminate against closely related kin, leading to the exchange of genetic material [132]. *D.* giganteum is even heterothallic, thus exclusively fusing with different mating types in macrocyst formation [94]. Thereby, the spread of chlamydiae to distant relatives of the current host, with a lower likelihood of being already infected, could be facilitated.

Conclusion and future directions

In this study, a chlamydial species, member of the *Rhabdochlamydiaceae*, was isolated in its host *D. giganteum* and identified using FISH and 16S rRNA gene sequencing. In contrast to *Chlamydia spp.* the symbiosis did not show pathogenic characteristics, since it was not decreasing the fitness of infected *D. giganteum* in the conducted experiments. The symbiont was highly prevalent in the infected cultures but did not exceed a certain threshold in maximum symbiont load per host cell. Furthermore, we could provide evidence for a vertical transmission mode, through the social life stages of *D. giganteum.* All these factors indicate a well-established relationship between the two partners. Similar patterns have recently been described in wild isolates of dictyostelids, which show high prevalence of chlamydial infections [105]. Thus, chlamydiae seem to be an essential symbiont of various dictyostelids in soil forests.

Typically, horizontal transmission is often complementing vertical transmission [119] and our results strongly suggest, that this is the case in the here described symbiosis. However, EBs, representing the infectious extracellular form of chlamydiae, were scarce, virtually absent from the supernatant of infected cultures. This falls in line with recent studies stating the absence of chlamydiae in soil surrounding infected dictyostelid populations [105]. By interrupting the aggregation behavior of *D. giganteum*, we were able to significantly decrease infection events, without disturbing the replication of chlamydiae. Thus, we consider aggregation of trophozoites, more explicitly, cell-to-cell contact, as a prerequisite for horizontal transmission, in which chlamydiae use a yet unknown mechanism to be passed on directly from an infected to an aposymbiotic host cell.

Using electron microscopy techniques, it will be possible to morphologically characterize the chlamydial symbiont of *D. giganteum*. Future studies could further clarify whether chlamydiae are passed on from infected to aposymbiotic hosts, by utilizing cell trackers to stain the populations, as it was done to distinguish the origin of *D. discoideum* cells in different stages of the social life cycle [96]. The diversity presented by chlamydiae in other dictyostelids, like *D. discoideum* and *D. giganteum* [60] also opens the door for comparative genomics, which can further elucidate the evolutionary history of chlamydiae and chlamydiae associated virulence genes, like the type three secretion system [17]. The long history of *D. discoideum* as a model system in biology and the existence of genetic tools to manipulate its organism, present new possibilities for environmental chlamydiae research. The work presented here, is another piece adding to a yet incomplete picture of chlamydiae, their impact on free-living amoebae in the environment and, in the long run, could expand our understanding of chlamydial infections in humans and animals.

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133. The life cycles of Dictyostelium discoideum by David Brown & Joan E. Strassmann, CC BY 3.0 (http://dictybase.org/Multimedia/DdLifeCycles/index.html).

Ich habe mich bemüht, sämtliche Inhaber*innen der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

Abstract

The phylum Chlamydiae is best-known for the species *Chlamydia trachomatis*, the cause for trachoma and several other diseases, affecting millions of people worldwide. Overshadowed by this important human pathogen, the real diversity of chlamydiae, infecting various animals and protists, has long been overlooked. Today, these so-called environmental chlamydiae, represent the majority of all known chlamydial species and have greatly expanded our perception of the ecological potential of chlamydiae. Especially free-living amoebae are frequently infected with environmental chlamydiae and have been used as a tool to isolate novel chlamydial species. All environmental chlamydiae, despite being obligate intracellular symbionts, undergo a biphasic life cycle, in which they temporarily leave the host cell to infect new potential hosts. Certain free-living amoebae, the dictyostelids, show a similar potential to change their phenotype, by performing a social life cycle, consisting of several multicellular stages, as a reaction to environmental cues. In this study, we isolated the free-living amoeba *Dictyostelium giganteum*, infected with a chlamydial symbiont, member of the originally arthropod-associated *Rhabdochlamydiaceae*. Using fluorescence *in situ* hybridization and digital droplet PCR, we found that the chlamydial symbiont is not only vertically transmitted through the social life cycle of *D. giganteum* but lacks the extracellular phase found in all chlamydiae described so far. Yet, it is capable of horizontal transmission, but only during the aggregating stages of the *D. giganteum* life cycle. We thus hypothesize, that this chlamydial species has adapted to the social life of its host during long periods of co-evolution.

Zusammenfassung

Chlamydien haben vor allem durch einen Vertreter ihres Phylums große Bekanntheit erlangt: *Chlamydia trachomatis*, den Erreger der Augenkrankheit Trachoma und weiterer, Millionen von Menschen betreffender, Krankheiten. Im Schatten dieses bedeutenden Krankheitserregers, wurde lange die natürliche Diversität der Chlamydien übersehen, welche noch zahlreiche Tiere sowie Protisten infizierende Spezies enthält. Erst nach der Entdeckung der sogenannten Umweltchlamydien, die heute den größten Teil der bekannten Chlamydien darstellen, wurden die vielfältigen Auswirkungen der Chlamydien auf zahlreiche Ökosysteme erkannt. Zur Isolierung neuer Chlamydien wurden, damals wie heute, vor allem frei-lebende Amöben untersucht. Alle Umweltchlamydien besitzen einen zweiteiligen Lebenszyklus, der eine infektiöse, extrazelluläre Phase beinhaltet, in welcher potenzielle Wirte infiziert werden können. Einige freilebende Amöben, nämlich die Dictyosteliden, besitzen ebenfalls die Fähigkeit ihren Phänotyp drastisch zu ändern, um, als Reaktion auf widrige Umweltbedingungen, einen sozialen Lebenszyklus durchzuführen, der aus mehreren multizellulären Stadien besteht. In dieser Studie konnten wir Symbionten, die zur Familie der *Rhabdochlamydiaceae*, eine Familie innerhalb der Umweltchlamydien, in *Dictyostelium giganteum* identifizieren und charakterisieren. Durch Fluoreszenz *in-situ* Hybridisierung und digital droplet PCR konnten wir sowohl die vertikale Übertragung des Symbionten durch den Lebenszyklus des Wirtes als auch die untergeordnete Rolle der extrazellulären Phase nachweisen. Nichtsdestotrotz können die hier beschriebenen Chlamydien auch aposymbiotische *D. giganteum* Zellen infizieren, allerdings nur in den aggregierten Phasen des sozialen Lebenszyklus. Daraus schließen wir, dass sich die symbiontischen Umweltchlamydien in *D. giganteum* in einer langanhaltenden Phase der Co-Evolution an den sozialen Lebenszyklus ihres Wirtes angepasst haben.