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MASTERARBEIT

Titel der Masterarbeit

How Intracellular Bacteria Affect the Fate of their Amoeba Host: Endosymbiotic versus Parasitic Interactions

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"In every walk with nature one receives far more than he seeks"

John Muir

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CHAPTER 1

Introduction

1.1 Symbiosis

1.1.1 Definition of Symbiosis

The term "symbiosis" came up at the end of the 19th century in context with observations made on the "dual nature" of lichens (Sapp, 1994). In 1877, Albert Bernhard Frank defined "Symbiotismus" as a situation where two different species live on or in one another under a comprehensive concept (Frank, 1877). Nevertheless, the term symbiosis was coined one year later by Anton de Bary as "a phenomenon in which dissimilar organisms live together" (De Bary, 1878). Symbiosis is often equated with mutualism, which can be traced back to Beatrix Potter. She studied fungi and lichen in the late 19th century and first proposed that both the fungus and the alga benefit from the symbiosis, so the relationship is mutualistic. Nowadays, symbiosis can be understood as an association between two or more organisms of different species that is integrated at the behavioural, metabolic or genetic level (Moya, 2008). Symbiosis can be obligate, describing a relationship required for the survival of one or both partners, or non-obligate.

1.1.2 The Nature of Symbiosis

In accordance with A. de Bary´s rather broad definition of symbiosis, three major categories of interspecies interaction can be distinguished. Van Benden classified them in terms of "parasitism", "commensalism" and "mutualism" (Sapp, 1994). Parasitism can be defined as a relationship in which one species increases its own fitness on the cost of the other species fitness. In commensalism one of the partners benefits in term of is fitness without affecting the other species. Mutualism describes a beneficial interaction in which both partners increase their fitness (Moya et al., 2008). However, symbioses between organisms do often not fit perfectly into any single category, especially if a mix of positive, negative and neutral effects occurs. Moreover, it has to be considered that the predominant outcome of a symbiotic interaction, impacting fitness of the organisms involved at any one point in time, may be relative to the conditions that the organisms are experiencing at that time (White & Torres, 2009)

In symbiosis one can distinguish between ecto- and endosymbiosis depending on the localisation of the symbiont in regard to the host cell. The term endosymbiosis is mainly used for associations between organisms of an unequal size in which the entire body of the smaller partner (the endosymbiont) is located within the larger partner (the host) and which are overtly not

parasitic (Douglas & Smith, 1989). The establishment of an endosymbiotic relationship often seems to be driven through complementation of the host's limited metabolic capabilities by the biochemical versatility of the endosymbiont. This may enable the host to thrive in environments or on diets previously inaccessible. For the endosymbiont the symbiosis may provide a nutrient-rich, sheltered environment (Douglas & Smith, 1989). Hosts in these relationships are typically eukaryotes, owing to their larger cell size, phagocytosis and restricted metabolic capabilities, whereas the endosymbiont partners may be either pro- or eukaryotes (Nowak & Melkonian, 2010).

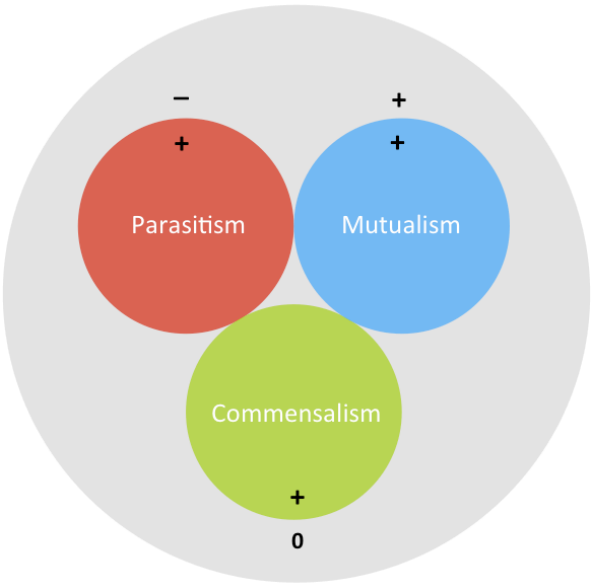

Figure 1. Parasitism, mutualism and commensalism in host-symbiont relationships.
In symbiosis there are advantages or disadvantages for either the host or the symbiont. – reduced fitness; + improved fitness; 0 unaltered fitness.

1.2 *Acanthamoeba* and its Bacterial Symbionts

1.2.1 Acanthamoeba

Acanthamoeba, as well as *Hartmanella*, *Balamuthia* and *Naegleria*, belong to the diverse protozoan group of free-living amoebae. These organisms are ubiquitous, they can be found in soil, in freshwater lakes, in marine sediments and even in the air (Khan, 2006). Acanthamoebae replicate by binary fission. There are basically two main developmental stages, the so-called trophozoite, representing a metabolically active, vegetative form, and the cyst, which is metabolically inactive,

but resistant to harsh environmental conditions like adverse pH, osmotic pressure, UV-radiation or heat (Khan, 2006). A switch back to the vegetative form takes place when surrounding conditions become favourable again. Acanthamoebae take up food by phagocytosis or pinocytosis. It mainly consists of bacteria, algae, yeast or other protists. By grazing on microorganisms, they contribute to soil mineralization and plant growth (Bonkowski, 2004). Acanthamoebae are also recognized as opportunistic human pathogens causing severe diseases, like *Acanthamoeba* keratitis and *Acanthamoeba* granulomatous encephalitis (Khan, 2003).

1.2.2 Bacteria Resistant to Amoebal Grazing

Free-living amoebae mainly feed upon bacteria (figure 2). Usually ingested microorganisms are rapidly killed and digested. However, microbial infections almost exclusively occur via ingestion. Some bacteria have evolved mechanisms allowing them to resist digestion and to escape the phagosome (reviewed by Molmeret et al., 2005; Casadevall, 2008). Those bacteria may either adapt to intracellular life or overgrow the host cell. Bacteria which can either transiently or stably thrive inside the amoebae are referred as endosymbionts. Bacteria which adversely affect and finally kill their host are considered as parasites. In many cases amoebae serve as vehicles transferring their symbionts to new hosts (Barker and Brown, 1994). It is thought that many of the so called bacterial pathogens first learned to survive and live inside protozoans and that mechanisms acquired from this relationship later granted them the potential to broaden their host range and to invade higher eukaryotes (reviewed by Molmeret et al., 2005). With the development of culture-independent methods to identify prokaryotes, namely the 16S rRNA full-cycle approach, bacteria capable of propagating in amoebae have been increasingly found. It is supposed that about 25 % of acanthamoebae contain bacterial symbionts (Fritsche et al., 1993). Phylogenetic analysis showed that they belong to different bacterial taxa, stable associations of bacteria and amoebae were reported for members of the *Alphaproteobacteria*, the *Betaproteobacteria*, the *Bacteroidetes* and the *Chlamydiae* (Horn & Wagner, 2004; Schmitz-Esser et al., 2008).

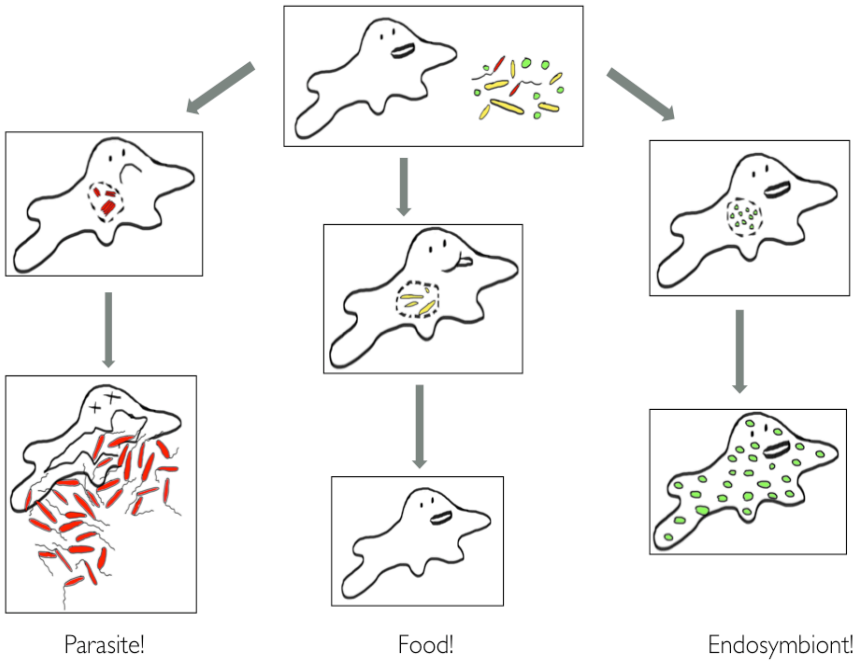

Figure 2. Schematic representation of the outcome of amoebal grazing on bacteria.

The outcome of grazing varies depending on the type of ingested bacteria. Usually bacteria taken up are digested and supply the amoebae with nutrients and energy. Bacteria resistant to phagocytosis could show either parasitic or endosymbiotic behaviour towards their amoebal host.

1.2.3 The phylum *Chlamydiae*

Chlamydiae are obligate intracellular bacteria with an unique biphasic developmental-cycle (Moulder, 1982). *Chlamydiaceae*, especially *Chlamydia trachomatis,* became known as the world´s major causes for preventable blindness and as causative agents for the most common bacterial sexually transmitted disease (Grayston & Wang, 1975). Until recently only chlamydiae capable of infecting higher eukaryotes were known and they were thought to form a distinct phylogenetic group whose members are closely related to each other. Within the last two decades more and more representatives of this phylum were identified, and an unexpected diversity within the *Chlamydiae* could be shown (Horn, 2008; Fig. 3). Several new families were described, e.g. the *Parachlamydiaceae*, the *Simkaniaceae* or the *Waddliaceae* (Horn, 2008). Many of the newly found taxa could not directly be related to disease in higher eukaryotes, but some of them enter human epithelial cells or macrophages and may even multiply (Greub et al. 2003).

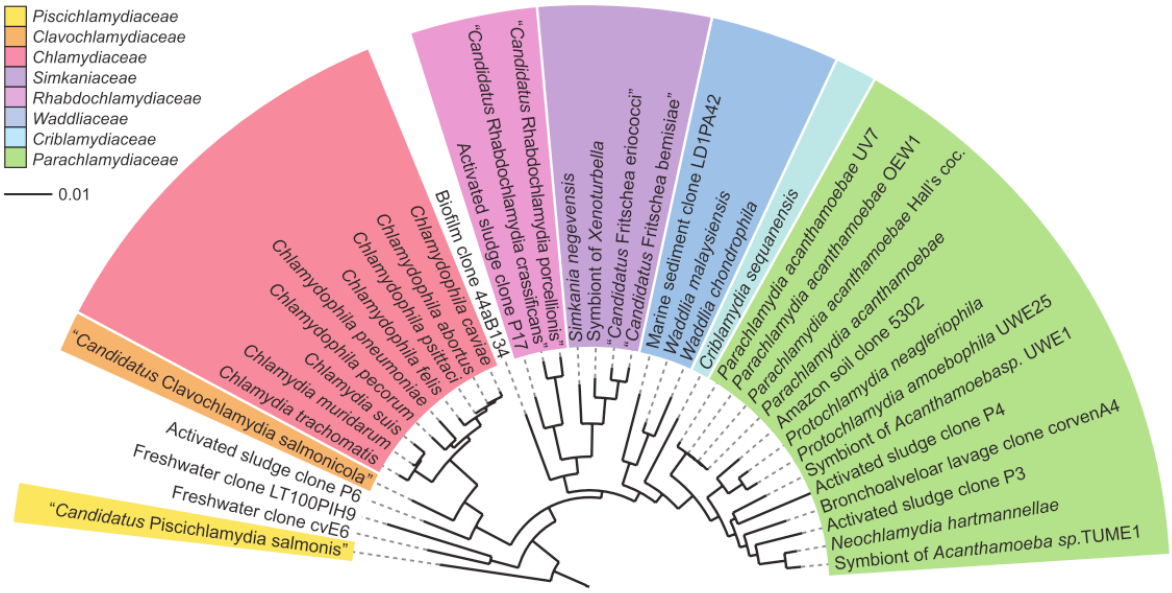

Figure 3. Diversity of the phylum *Chlamydiae*.

Phylogenetic 16S rRNA tree displaying relationships among members of the phylum *Chlamydiae*. Shown are representatives of all so far recognized families. Bar, 10% estimated evolutionary distance; adapted from Horn, 2008

1.2.3.1 *Protochlamydia amoebophila*

Protochlamydia amoebophila UWE25 was initially isolated from an *Acanthamoebae sp.* found in soil in the Washington State, USA. (Fritsche et al., 1993). As it had a cytopathic effect on its original amoebal host, *P. amoebophila* was transferred to *Acanthamoeba sp.* UWC1 (Fritsche et al., 1998) and later to *Acanthamoeba castellanii* Neff. Recently, there were also other groups isolating *P. amoebophila* from the environment, underlining how widespread this organism is (Schmitz-Esser et al., 2008; Matsuo et al., 2009, our lab, unpublished). *P. amoebophila* was described as obligate intracellular, coccoid, gram-negative bacteria belonging to the family *Parachlamydiaceae* (Fritsche et al., 2000). These bacteria are distributed throughout the amoebal cytoplasm and every cell seems to be surrounded by an inclusion membrane (Collingro et al., 2005). This clearly differs from the larger inclusions often found in other members of the *Chlamydiacea*. *P. amoebophila* could also be detected in acanthamoebae cysts (Fritsche et al., 2000).

Complete genome sequencing of *P. amoebophila* showed its genome to be twice the size of the *Chlamydiaceae* genomes (Horn et al., 2004). It has retained several key features of the last common chlamydial ancestor like a complete tricarboxylic-acid cycle and it also possesses major virulence

factors of pathogenic chlamydiae like a type three secretion system (Horn et al., 2004). Recent studies on the developmental cycle (Diplomathesis Lena König, 2009) as well as the availability of continuous and stable co-cultures in the laboratory suggest an overall non-detrimental relationship between *P. amoebophila* and *A. castellanii*. Thus, the endosymbiont *P. amoebophila* is considered non-parasitic.

1.2.3.2 *Parachlamydia* sp.

In co-cultivation experiments using *Acanthamoeba sp.* strain UWC1 to isolate potentially novel endosymbionts of amoebae from activated-sludge of an industrial wastewater treatment plant, an unknown *Parachlamydia* strain was found (Collingro, 2005). It showed a 16S rRNA similarity of 98.7 % to *Parachlamydia acanthamoebae* Bn9 and was designated as *Parachlamydia* sp. strain UV-7 (University of Vienna, isolate number 7). These coccoid bacteria displayed the typical chlamydial developmental forms and resided in large host derived vacuoles typical for *Chlamydiaceae* (Collingro et al., 2005). For *P. acanthamoeba* a temperature-dependent lytic or endosymbiotic relationship in *A. polyphaga* was shown: temperatures beyond 30 °C allowed a stable coexistence with the host, whereas temperatures above 32 °C lead to host-lysis (Greub et al., 2003). Latest insights into the developmental cycle of *Parachlamydia* sp. in *A. castellanii* do also suggested a detrimental effect on the host, even at 20 °C (König, 2009). Thus, in contrast to *P. amoebophila*, it is considered to be an amoebal parasite. Moreoveer, *Parachlamydia* sp. was shown to be capable to infect and thrive in human cells, what indicates a possible role as an emerging pathogen (Collingro et al., 2005)

1.2.4 *Legionella* – Common Parasites of *Acanthamoeba*

Members of the genus *Legionella* are gram-negative, mainly rod shaped bacteria that belong to the class of *Gammaproteobacteria*. In the environment they can be found worldwide in nutrient-rich biofilms, in freshwaters systems and anthropogenic systems like showers, cooling towers and swimming pools (Lau & Ashbold, 2009). Legionellae are thought to multiply mainly in protozoa in the environment, but can be cultivated without host cells in the laboratory and therefore considered facultative intracellular bacteria (Fields, 1996, Lau & Ashbold, 2009). They resist amoebal phagocytosis and exploit their amoebal host in terms of energy and replication. Legionellae are known as parasites as they finally lyse their hosts. However, they do not exclusively infect protozoans. Especially *Legionella pneumophila* is widely known as an opportunistic

human pathogen, as it is capable to thrive in human macrophages, where it causes pulmonal diseases like severe Legionnaires disease or the milder Pontiac-fever (Winn, 1988). *L. pneumophila* enters the human lungs through aerosol formation or by aspiration of contaminated water. The infection of humans seems to be disadvantageous for legionellae as person-to-person spread has not been observed so far, thereby reducing the chance of further propagation. (Steinert et al., 2002; Casadevall, 2008).

1.3 Developmental Cycles of Intracellular Bacteria

Obligate or facultative intracellular bacteria have evolved developmental cycles displaying efficient symbiont-host interactions. Key steps in the life cycles of those bacteria are the entry into the host, the establishment of the infection, the manipulation of the host, the replication and finally the transmission to new hosts.

1.3.1 *Chlamydiae*

Characteristic to members of Chlamydiae is a unique biphasic developmental cycle. The alternation between two morphologically and functionally distinct cell types can be observed, namely the infectious elementary body (EB) and the non-infectious but metabolically active reticulate body (RB) (Moulder, 1991; Abdelrahman & Belland, 2005). The putative host takes up chlamydial EBs by endocytosis. EBs resist amoebal phagocytosis, establish the infection and switch to RBs, which then start to replicate by binary fission within a vacuole termed inclusion. The energy necessary for this process is acquired from the amoebal host in the form of nucleic acids, amino acids and cofactors which are imported into the chlamydial cells by numerous protein transporter systems. An ATP/ADP translocase seems to play a major role in the endosymbiont-host metabolism and can so far be found in all *Chlamydiae* (Schmitz-Esser et al., 2004). Another import protein for the interaction with the host is a type three secretion system (T3SS), commonly found in members of *Chlamydiae* (Peters et al., 2007). It may facilitate the injection of effector proteins into the host cytoplasm, which then may alter different cellular processes (Hueck, 1998). After several rounds of replication a yet unknown signal triggers the fulfilment of the developmental cycle and a re-differentiation into EBs occurs. Chlamydial exit from the host cell is either mediated by host cell lysis or by extrusion of membrane-engulfed bacterial packages, leaving an intact host cell as well as a residual inclusion behind (Hybiske& Stephens, 2007).

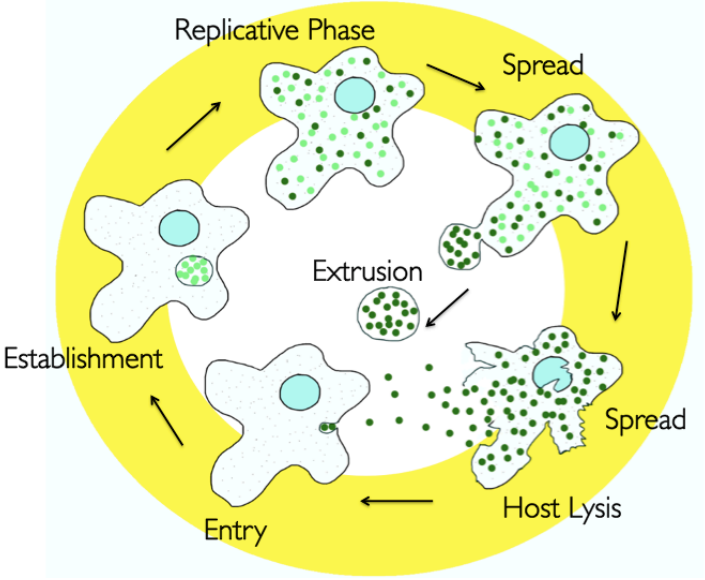

Figure 4. Schematic representation of the developmental cycle of *Chlamydiacea*.

Uptake of infectious EBs (dark green) by endocytosis, transformation to replicating RBs (light green) and establishment of infection in a vacuole termed inclusion.. Re-transformation to EBs and spread of infection by either extrusion of membrane-engulfed bacterial packages or by host lysis. Host cell nucleus is depicted in blue.

1.3.2 *Legionella pneumophila*

L. pneumophila is able to grow independent from a host in a nutrient-rich, well-buffered medium supplemented with iron and L-cystein (Ewann & Hoffmann, 2006; Johnsen et al., 1991). In vitro growth resembles intracellular growth in many ways: the exponential phase models the replicative phase in vivo, and the post-exponential stationary phase (SP) the transmissive phase (Molofski et al., 2004; Weissenmayer et al. 2011). Legionella in the SP are characterised as motile, infective, cytotoxic, stress resistant and sodium sensitive (Byrne and Swanson 1998). Additionally, in vivo a further differentiation into the so-called mature infective form (MIF) was observed (Garduno et al., 2002). MIF was described to occur exclusively in the intracellular milieu, to be metabolically dormant, to exhibit a unique cell wall structure, to express HSP60 and a unique protein profile. In addition, it could be shown to be 10-fold more infective compared to SP bacteria and it is suggested to represent the predominant infective form of *Legionella* found in the environment (Garduno et al., 2002).

The first step in the developmental cycle of *L. pneumophila* is its uptake by the host through receptor-mediated phagocytosis (Venkataraman et al., 1997; Harb et al, 1998). Phagosome-lysosome phusion is inhibited and therefore legionellae resist amoebal phagocytosis (Cazalet et

al., 2004). Moreover, for the successful establishment of an infection, the expression of the macrophage infectivity factor (mip) seems to be crucial (Cianciotto & Fields, 1992). The next step in the developmental cycle is the fusion of the legionellae containing phagosome with the membrane of the endoplasmatic reticulum of the host cell, leading to a remodelling of the endosome membrane by recruiting organelles (Swanson & Isberg, 1995). Throughout the developmental cycle, effector proteins are supposed to be injected into the host cell by a Icm/Dot type IV secretion system (Vogel et al., 1998). These proteins manipulate the host to the advantages of the pathogen, subverting the endocytic pathway of the host and bringing forward the replication of *L. pneumophila* (Cazalet at al., 2004). As long as favourable nutrient levels are present, *L. pneumophila* multiplies at a maximum rate. When amino acids become limiting, intracellular bacteria begin to produce factors to lyse the host cell, to survive osmotic stress, to disperse in the environment, and finally to re-establish an intracellular niche protected from lysosomal degradation (Byrne & Swanson, 1998; Sauer et al., 2005). The exit of Legionella is mainly triggered by a release of pore forming toxins, inducing wholes into the host´s cytoplasma membrane and finally host cell lysis occurs (Byrne and Swanson 1998). In addition, a non-lytic exit of Legionella was described in *Dictyostelium*, in which a membrane engulfed bacteria containing package is released from an intact amoeba (Chen et al., 2004).

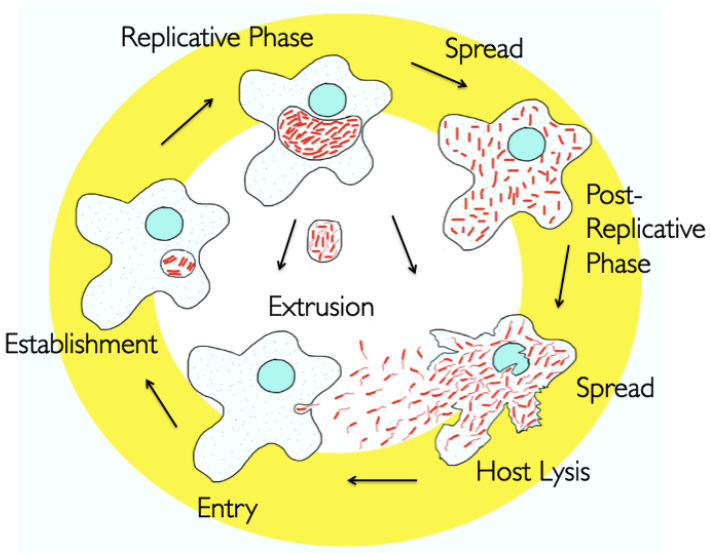

Figure 5. Schematic representation of the developmental cycle of *Legionella pneumophila*.

The uptake of *L. pneumophila* is mediated by endocytosis. After inhibition of phagosome-lysosome phusion, a recruitment of the endoplasmatic reticulum as a protective layer around replicating legionellae occurs. A switch from the replicative to the transmissive form happens after nutrients are used up (Bryne & Swanson, 1998). The transmission of infectious legionellae occurs mainly by host lysis and sometimes by extrusion of membrane engulfed bacterial packages. Host cell nucleus is depicted in blue.

1.4 Effort in research on symbiont-host systems

Within the last years, there has been a large increase in understanding the relationships between intracellular bacteria and their hosts. Nevertheless, many details on mechanisms, and especially the establishment of symbiosis and the following steps in the developmental cycles still remain elusive. However, next generation sequencing allows a quicker and easier access to whole genomes. The application of comparative genomics represents an important in the study of symbiont-host systems, as genetic manipulations of the symbiont are often not applicable. At the moment, several genomes of the environmental chlamydia are available like *Protochlamydia amoebophila* UWE25 (Horn et al., 2004), *Parachlamydia* sp. UV7 (our lab, not published), *Simkania negenvensis* (our lab, not published) and *Waddlia chondrophila* (Bertelli et al., 2010) as well as different genomes of *Legionella pneumophila* strains (Cazalet et al., 2004; Chien et al., 2004). However, even with this large amount of data, there is still a lack of knowledge in bridging predicted genes with what actually is happening in symbiont-host systems. To close these gaps, transcript and proteome studies as well as metabolic profiling are necessary, as recent studies have implicated (Albrecht et al., 2010; Weissenmayer et al., 2011; Haider et al., 2010; Sixt et al., 2011).

1.5 Project Aims

The first project represents preliminary work for a transcriptomic project, which final goal is to uncover differences in gene expression between an endosymbiont- and a parasite-host system. A special emphasis lies on key factors contributing to mutualistic or parasitic interactions. The aim of the study at hand was the search for key time points during interaction between intracellular bacteria and their host. Therefore, selected aspects of the developmental cycles of three model systems were studied in the course of this thesis. As an endosymbiont-host system *Acanthamoeba castellanii* Neff infected by *Protochlamydia amoebophila* UWE25 was chosen. Moreover, two parasite-host systems were compared: *A. castellanii* Neff infected by *Parachlamydia* sp. UV7 and *A. castellanii* Neff infected by *Legionella pneumophila* Lp02. Two different temperatures were used to determine the one at which endosymbiotic and parasitic effects were most pronounced, either 20 °C mimicking natural habitats or 30 °C addressing to anthropogenic environments.

The second project followed the question about a possible mutualistic nature of the endosymbiont-host system *A. castellanii*/*P. amoebophila*. So far, only benefits for *P. amoebophila* could be revealed, considering the transfer of nucleotides, amino acids and cofactors from the host to the symbiont. The question about reciprocity lay at hand when considering the fact that *Protochlamydia* stably thrives in *Acanthamoeba* already for several hundred of million years (Horn et al., 20004). One potential benefit for the host might be a symbiont-provided protection against infection by other bacteria. To test this hypothesis the model parasite *L. pneumophila* was used to infect *A. castellanii* either carrying *P. amoebophila* as an endosymbiont or not. Over a timescale of several weeks the course of infection was monitored by FISH. Furthermore, host viability was checked with propidium iodide death stain, amoebae were enumerated and *L. pneumophila* viability was determined with plate counts.

CHAPTER 2

Material and Methods

2.1 Material

2.1.1 Chemicals

All chemicals used in this study were purchased in p.a. quality, if not stated otherwise.

Table 1. Chemicals used.

Chemicals	Manufacturer
ACES ($C_4H_{10}N_2O_4S$)	NeoLab, Heidelberg, Germany
Activated charcoal	Fisher Scientific, UK
Agar	Fluka, Steinheim, Germany
4',6-Diamidino-2-phenylindole (DAPI)	Lactan Chemikalien und Laborgeräte GmbH, Graz, Austria
Calcium chloride dihydrate (CaCl2*2 H2O)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Citifluor AF1	Agar Scientific Ltd., Stansted, UK
di-Sodiumhydrogen phosphate dihydrate (Na2HPO4*2 H2O)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Ethanol absolute (EtOHabs.)	AustrAlco Österreichische Alkoholhandels GmbH, Spillern, Austria
Ferrous ammonium sulfate hexahydrate (Fe(NH4)2(SO4)2*6 H2O)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Formaldehyde 37% (w/w) Rotipuran®	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Formamide deionized	Carl Roth GmbH & Co KG, Karlsruhe, Germany
α-D(+)-Glucose monohydrate	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Hydrochloric acid 37% (w/w) (HCl)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Iron (III) nitrate	Sigma-Aldrich, Steinheim, Germany
L-Cysteine	MISSING
L-Glutamic acid	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
L-Thymidine	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Magnesium chloride hexahydrate (MgCl2)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Magnesium sulfate heptahydrate (MgSO4*7 H2O)	Merck GmbH, Vienna, Austria
Potassium dihydrogen phosphate (KH2PO4)	Mallinckrodt Baker B.V., Deventer, Holland
Propidium iodide (PI)	Invitrogen Molecular Probes Inc., Eugene, OR, USA
Proteose peptone	Oxoid Ltd., Hampshire, England
Sodium acetate (Na acetate)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sodium hydrogen carbonate (NaHCO3)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Sucrose	Merck GmbH, Vienna, Austria
Trypticase Soy Broth	Oxoid Ltd., Hampshire, England
BactoYeast Extract (for *Legionella* cultivation)	BD Bioscience, Sparks, USA
Yeast Extract (for amoebae cultivation)	Oxoid Ltd., Hampshire, England

2.1.2 Disposable Items

Table 2. Disposable items used.

Disposable items	Manufacturer
25 cm2 Tissue culture flasks	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
500 cm2 Tissue culture flasks	Nunc, Roskilde, Denmark
Cover glasses (24 x 50 mm, 24 x 60 mm)	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Coverslips (12 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Glass beads (0.75-1.0 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Greiner tubes (15 ml, 50 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Microscope slides (76 x 26 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Microscope slides, 10 wells	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Multiwell dishes, polystyrene (12 wells)	Nunc, Roskilde, Denmark
Needles Sterican® (Ø 0.45 x 25 mm, Ø 0.90 x 40 mm)	B. Braun Melsungen AG, Melsungen, Germany
Parafilm® M laboratory film	American National Can Company, Chicago, IL, USA
Plastic cuvettes	Greiner Bio-One GmbH, Frickenhausen, Germany
Plastic pipettes (2 ml, 10 ml)	Barloworld Scientific Ltd., Staffordshire, UK
Plastic inoculation loop	Nunc Roskilde, Denmark
Plastic tips (various sizes)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Reaction tube 1.5 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Reaction tube 2 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
SafeSeal-Tips® Premium (various sizes)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Syringe filter, cellulose acetate (0.2 µm)	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
Syringe Injekt®-F 1 ml	B. Braun Melsungen AG, Melsungen, Germany
Syringe Omnifix® 50 ml	B. Braun Melsungen AG, Melsungen, Germany

2.1.3 Technical Equipment

Table 3. Technical equipment used.

Instrument	Manufacturer
Accu-jet® pro pipette aid	Brand GmbH+Co KG, Wertheim, Germany
CCD camera AxioCam HRc	Carl Zeiss MicroImaging GmbH, Jena, Germany
Centrifuges	
OptimaTM L-100 XP ultracentrifuge	Beckman Coulter, Inc., Palo Alto, CA, USA
Centrifuge 5804 R	Eppendorf AG, Hamburg, Germany
Mikro 20 benchtop centrifuge	Andreas Hettich GmbH & Co KG, Tuttlingen, Germany
Incubators	
Microbiological incubator KB 115	Binder GmbH, Tuttlingen, Germany
Hybridization oven UE 500	Memmert GmbH & Co KG, Schwabach, Germany
Laminar flow hood, model 1.8	Holten, Jouan Nordic, Allerød, Denmark
Magnetic stirrer RCT basic	IKA® Werke GmbH & Co KG, Staufen, Germany
Microscopes	

Epifluorescence microscope	
Axioplan 2 imaging	Carl Zeiss MicroImaging GmbH, Jena, Germany
Inverse microscope Axiovert 25	Carl Zeiss MicroImaging GmbH, Jena, Germany
Confocal Laser Scanning Microscope	
LSM 510 Meta	Carl Zeiss MicroImaging GmbH, Jena, Germany
Neubauer counting chamber	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
pH meter inoLab pH Level 1	Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Rollerdrum TC-7	New Brunswick Scientific, Edison, USA
Scales	
OHAUS® Analytical Plus balance	Ohaus Corporation, Pine Brook, NJ, USA
Sartorius BL 3100	Sartorius AG, Göttingen, Germany
Spectral photometer SmartSpecTM 3000	Bio-Rad Laboratories GmbH, Munich, Germany
Vortex-Genie 2	Scientific Industries Inc., Bohemia,NY, USA
Water baths	
Haake DC10-P5/U Heating circulator bath	Thermo Fisher Scientific Inc., Waltham, MA, USA
Incubation bath GFL 1004	Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Water purification system	
MILLI-Q® biocel	Millipore GmbH, Vienna, Austria

2.1.4 Software

Table 4. Software used.

Software	Manufacturer
AxioVision 4.8	Carl Zeiss MicroImaging GmbH, Jena, Germany
GraphPad Prism 5.0 for Mac	GraphPad Software Inc., La Jolla, CA, USA
LSM Image Browser 4.2	Carl Zeiss MicroImaging GmbH, Jena, Germany
Microsoft Office 2011 for Mac	Microsoft Corporation, Redmond, WA, USA

2.1.5 Media, buffers and solutions

All buffers, media and solutions were produced utilizing double distilled and filtered water. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were used to adjust the pH if not stated otherwise. All buffers and general media were sterilized for 20 min at 121°C and 1.013 x 105 Pa pressure using a water-vapour high pressure autoclave, and were stored at RT prior to usage if not stated otherwise.

Table 5. Media, buffers and solutions.

AYE Medium	
ACES	10 g
BactoYE	10 g
$_{dd}H_20$	ad 1000 ml
	pH 6.9 adjust with KOH
CYE Agar	
AYE Medium	1000 ml
Agar	18 g
Active Charcoal	2 g
L -Cysteine 100x	100 µl
Ferric Nitrate 100x	100 µl
	Supplements (sterile) were added after autoclaving
CYET Medium	
AYE Medium	1000 ml
L -Cysteine 100x	100 µl
Ferric Nitrate 100x	100 µl
Thymidine 100x	100 µl
	Supplements (sterile) were added after autoclaving
L -Cysteine 100x	
L-Cysteine	400 mg
$_{dd}H_20$	10 ml
	sterile filtration prior to use
DAPI Solution	
DAPI stock solution 1 mg/ml	
$_{dd}H_20$	1:10000 dilution
Ferric Nirate 100x	
Ferric Nitrate	135 mg
$_{dd}H_20$	10 ml
	sterile filtration prior to use
HCS Solution 2 µg/ml	
Hybridisation Buffer for FISH	20%
NaCl 5M	180 µl
Tris/HCl 1M	20 µl
SDS 10 % [w/v]	1 µl
$_{dd}H_20$	599 µl
Formamide [FA]	200 µl
PYG	
Peptone	20 g
Glucose	18 g
Yeast-extract	2 g
Sodiumcitrate	1 g
$MgSO_4$ x 7 H_2O	0.98 g
Na_2HPO_4 x 2 H_2O	0.36 g
KH_2PO_4	0.34 g
$Fe[NH_4]_2[SO_4]_2$ x 6 H_2O	0.02 g
$_{dd}H_20$	ad 1000 ml
	adjust to pH 6.5
	Medium was autoclaved at 110 °C
PAS 10x	
NaCl	1.2 g

$MgSO_4$ x $7H_20$	0.04 g
$CaCl_2$ x H_20	0.04 g
NaH_2PO_4 x $2H_20$	1.78 g
KH_2PO_4	1.36 g
$_{dd}H_20$	ad 1000 ml
PFA 4%	
Paraformaldehyde 37 %	1 ml
$_{dd}H_20$	9.25 ml
Propidium Iodide (PI) Solution	
PI stock solution 1 mg/ml	
1x PAS	1:1000 dilution
Sucrose-Phosphate-Glutamate Buffer (SPG)	
Sucrose	75 g
KH_2PO_4	520 mg
Na_2HPO_4 x 2 H_2O	1.53 g
Glutamic acid	720 g
$_{dd}H_20$	ad 1000 ml
	adjust to pH 7.2
Thymidine 100x	
Thymidine	100 mg
$_{dd}H_20$	10 ml
	sterile filtration prior to use
Washing Buffer for FISH	20%
NaCl 5M	2.15 ml
Tris/HCl 1M	1 ml
EDTA 0.5M	0.5 ml
$_{dd}H_20$	ad 50 ml

2.1.6 Organisms

Table 6. Organisms used.

Host	Endosymbiont	Source	Reference
Acanthamoeba castellanii Neff	Endosymbiont-free	ATCC, USA	*A. castellanii*. 1957
	P. amoebophila	Our lab	In preparation
	Parachlamydia sp.	This study	
Acanthamoeba sp. UWC1	*Parachlamydia* sp.	Our laboratory	Collingro et al., 2005

2.1.7 FISH probes

Table 7. FISH probes used.

Probe	Specifity	5´- 3´ Sequence	Reference	FA [%]
EUK516	most *Eukarya*	ACCAGACTTGCCCTCC	Amann et al., 1990	0-50
E25-454	*P. amoebophila*	GGATGTTAGCCAGCTC		20
UV-763	*Parachlamydia* sp.	TGCTCCCCCTTGCTTTCG	Collingro et al., 2005	20
LEGPNE1	*L. pneumophila*	ATCTGACCGTCCCAGGTT	Grimm et al., 1998	20

2.2 Methods

2.2.1 Cultivation of Amoebae and Symbionts

A. castellanii as well as the continuous culture of *A. castellanii*/*P. amoebophila* were cultivated in 25 cm^2 polystyrene culture flasks containing 8 ml PYG medium. To obtain higher amoebal biomass, for example to isolate EBs of *Parachlamydia* sp., amoebae were grown in 500 cm^2 polystyrene culture flasks containing 150 ml TSY medium. Depending on the temperature needed for upcoming experiments amoebae were cultivated either at 20 °C or at 30 °C. To maintain the cultures medium was exchanged every 2-3 weeks. If amoebae were needed for experiments, fresh media was supplied 2-3 days prior to usage. Morphological characteristics of the amoebae were monitored regularly by light microscopy. A culture was considered as well-grown and ready to use when its surface was densely covered with attached amoebal cells, and only a low proportion of floating cells in the surrounding medium being present. To maintain such cultures, the flasks were gently beaten before replacing the media. Doing so should allow pouring out most of the weakly attached and free-floating amoebae together with the old growth medium. Medium then was exchanged and/or new culture flasks were prepared by adding fresh PYG, and inoculated with 1-2 ml cell suspension (2.2.2) from well-grown cultures.

2.2.2 Harvesting of Amoebae

Culture flasks containing well-grown amoebae were shaken vigorously to detach amoebae from the surface. The resulting cell suspension was poured into 50 ml Greiner tubes. Amoebae were obtained by a centrifugation step at 3900 rcf for 8 min at RT. The supernatant was decanted and the pellet washed once by re-suspending it in 10 ml 1xPAS, followed by another centrifugation step at 3900 rcf at RT for 8 min. The supernatant was poured off and the pellet finally resuspended in 5 ml 1xPAS.

2.2.3 Counting of Amoebae

Amoebal cell concentrations were determined using a Neubauer haemocytometer. Depending on the pellet size a 1:10 or 1:100 dilution of the cell suspension was prepared and 20 µl were applied at each side of the counting chamber. The amoebae residing in 8 big squares were counted and amoebal numbers in 1 µl of the cell suspension was determined as follows. The total number of

counted cells was divided by 8 and then multiplied with the dilution factor (10 or 100). The result was divided by the size of the counting area (8 mm^2) and multiplied by the depth of the chamber (0.1 mm).

2.2.4 Cultivation of *Legionella*

AYE plates were inoculated with *L. pneumophila* and incubated for 4 days in a wet chamber at 37 °C. Single well grown colonies were selected and used for the inoculation of test tubes containing CYE liquid medium with thymidine. This was followed by an overnight incubation step on a roller drum at a cycling speed of 850 rpm. The next day OD_{600} was determined and legionellae were diluted to an OD_{600} of 0.3 in CYE liquid medium with thymidine and again grown overnight at 37 °C on a roller drum at the same cycling speed. The following day the OD_{600} was checked and as a value of 3.8 to 4.5 was reached, motility of legionella was monitored by light microscopy. If the number of motile legionella exceeded 10 % those cultures were directly used for infection experiments.

2.2.5 Establishing *A. castellanii*/*Parachlamydia* sp. Continuous Cultures

To finally derive *Parachlamydia* sp. from *A. castellanii*/*Parachlamydia* sp., first those cultures had to be established. Therefore, C1/*Parachlamydia* sp. cultures were taken to purify *Parachlamydia* sp. EBs. One small culture flask *A. castellanii* was infected with 50 µl *Parachlamydia* sp. EB suspension. After 4 days the infected *A. castellanii* were transferred to a large culture flask containing well-grown *A. castellanii*. Due to the rapid rate of amoebal lysis every 3-4 days half of the growth medium was replaced by fresh PYG and uninfected *A. castellanii*, grown in a small culture flask, were added. The success of the infection was monitored by DAPI stain.

2.2.6 Purification of *Parachlamydia* sp. EBs

Two large culture flasks containing *A. castellanii* UWC1/*Parachlamydia* sp. or *A. castellanii* Neff/*Parachlamydia* sp. were harvested as described before (2.2) and cells were pooled. The volume was adjusted to 5 ml with 1xPAS. Initially, amoebae were broke up by applying two successive freeze/thaw steps (-20 °C/45 °C). To further lyse the host cells, 2.5 ml sterile glass beads were added, the suspension was vortexed for 3 min and amoebal cell debris was pelleted by centrifugation at 300 rcf for 10 minutes at 4 °C. In order to break up clusters of bacteria the

supernatant was passaged through a single-use 0.45 mm needle for 6 times. The resulting suspension was centrifuged at maximum speed for 30 min at 4 °C. Finally, the pellet was resuspended in 4 ml ice-cold SPG. To check if no intact amoebae were left, a 10 µl sample of the *Parachlamydia* sp. suspension was fixed on a FISH slide and DAPI staining was performed (2.2.13). Afterwards 1 ml aliquots were prepared. Before storage at -80 °C, 10 µl per aliquot were taken to test for contamination and to quantify the purified endosymbionts.

2.2.7 Contamination Tests

After purification of EBs (2.2.6) two wells in a 12-well dish, each containing 2 ml PYG, were inoculated with 10 µl of a single chlamydial EB aliquot. Incubation was performed at RT for 7 days. If there was no growth of any organisms detectable, the EB purification was considered to be contamination free.

Cultures of uninfected *A. castellanii* or *A. castellanii/P. amoebophila* were routinely checked for contaminations. It was assumed that silent contaminations could be present in continuous amoebal cultures. Those contaminants could theoretically be kept beyond a certain threshold due to amoebal grazing. But after a decrease in amoebal fitness they would start to overgrow amoebae. To test this, 2 ml amoebal suspensions were taken from each culture flask in use and lysed as described in (2.2.16). The resulting lysate was used for an inoculation of 2 ml PYG in a 12-well-dish. Incubation was performed at RT for 7 days. If there was no growth of any organisms detectable, the amoebal culture was considered to be contamination free.

2.2.8 Test Infections

Test infections were undertaken with freshly purified chlamydial EBs to determine the optimal MOI for upcoming infection experiments.

In a 6-well plate containing 3 ml PYG in each well 5x10^5 uninfected *A. castellanii* were placed in each well. Amoebae were given 2 hours at 20 °C to attach to the surface of the wells. In the meanwhile *Parachlamydia* sp. or *P. amoebophila* EBs were thawed at 37 °C and put immediately on ice afterwards. EB suspensions were diluted 1:10 in ice-cold SPG. For infection, diluted EBs were added to attached amoebae at a MOI of 1, 5, 10, 20 and 50. To support infection a centrifugation step was performed at 1000 rpm for 15 min at 20 °C. This was followed by

incubation for 2 h at 20 °C, to let the infection occur. Afterwards the PYG was gently removed by pipetting, and attached amoebae were washed once with 1x PAS. To each well 2 ml fresh PYG were added and amoebae were harvested by pipetting. The resulting cell suspension was spun down at 3900 rcf for 8min at RT, washed once with 1xPAS and the pellet finally resuspended in 100 µl 1x PAS. Per used MOI 20 µl and 40 µl of the cell suspension were put on a well on a 10-well microscope slide. After 20 min the droplet was removed and the samples were fixed with 10 µl 4 % PFA. Prior to determining the infection rates, a DAPI stain was performed. In addition, the number of chlamydiae within single amoebae was determined.

2.2.9 Infection Cycles

2.2.9.1 Preparations and Infection

Continuous cultures (20 °C, 30 °C) of uninfected *A. castellanii* were used for infection experiments. Amoebae were harvested (2.2.2) and counted (2.2.3). In each well of a 12-well dish 2 ml PYG were prepared and $3x10^5$ amoebae added. To let amoebae attach to the surface, the 12-well dishes were incubated over night at 20 °C or 30 °C, respectively. The experiments were undertaken in biological duplicates meaning a handling on separate 12-well dishes.

2.2.9.2 *L. pneumophila*

Legionellae used for infection were obtained from independently grown *L. pneumophila* overnight cultures. To calculate the theoretical MOI, it was assumed that an MOI of 1 corresponds to $1x10^9$ *L. pneumophila*/ml and hence an MOI of 4 to $4x10^9$ *L. pneumophila*. To determine the real MOI the 1:10 *L. pneumophila* dilution was further diluted to 1:100 000 and 15 µl were streaked on a CYE plate and incubated in a wet chamber for 4 days at 37 °C.

Motile *L. pneumophila* (2.2.4) were diluted 1:10 with sterile $_{dd}H_20$ and added at a theoretical MOI of 20 to *A. castellanii*. To stop the initial infection, extracellular legionella were removed after 2 hours by performing three subsequent washing steps with prewarmed (30 °C) PYG. Before every washing step the 12-well dishes were shaken carefully, the supernatant was removed by pipetting and 2 ml of prewarmed (30 °C) PYG added.

2.2.9.3 *P. amoebophila* and *Parachlamydia* sp.

Bacteria were thawed in a water bath at 37 °C and immediately placed on ice afterwards. A 1:10 dilution of chlamydial EBs was prepared with ice-cold SPG buffer. Bacteria with a theoretical MOI of 20 were added to *A. castellanii*. To ensure an effective uptake, *Parachlamydia* sp. was spun onto the amoebae at 1500 rpm for 15 minutes. Afterwards medium was removed and 2 ml fresh PYG added.

2.2.9.4 Monitoring the Course of Infection

The course of the infection was monitored over 168 hours and samples were taken every 24 hours. The first time point to harvest the amoebae was directly after the initial washing step. Amoebae were detached by re-suspending (50x) in the surrounding media with a 1 ml pipette tip. From the resulting suspension 50 µl were used for enumerating amoebae, 950 µl for FISH (2.2.12) and 950 µl for propidium iodide (PI) death stain (2.2.15).

2.2.10 Short Term Effect of *L. pneumophila* Infection on *A. castellanii* and *A. castellanii*/*P. amoebophila*

Continuous cultures (30 °C) of *A. castellanii*/*P. amoebophila* as well as uninfected *A. castellanii* were harvested (3.2) and amoebae counted (2.2.3). In each well of a 12-well dish 2 ml PYG were placed first and $3x10^5$ amoebae added. Prior to infection the 12-well dishes were incubated over night at 30 °C to let amoebae attach to the surface. Motile *L. pneumophila* (2.2.4) were diluted 1:10 with sterile $_{dd}H_20$ and added at a theoretical MOI between 10 and 30, depending on the number of motile *L. pneumophila*, to *A. castellanii*/*P. amoebophila* as well as to *A. castellanii*. As controls served in each case *L. pneumophila* -uninfected *A. castellanii* and *L. pneumophila* -uninfected *A. castellanii*/*P. amoebophila*. To determine the real MOI the 1:10 *L. pneumophila* dilution was further diluted to 1:100 000 and 15 µl were streaked on a CYE plate and incubated in a wet chamber for 4 days at 37 °C. To stop the initial infection, extracellular legionella were removed after 2 hours by performing three subsequent washing steps with prewarmed (30 °C) PYG. Prior to each washing step the 12-well dishes were shaken carefully, the supernatant was removed by pipetting and 2 ml of prewarmed PYG added. The course of the infection was monitored over 168 hours by taking samples every 24 hours. The initial time point for harvesting of the amoebae was immediately after the washing (2 hpi). Amoebae were detached by re-suspending (50x) in the

surrounding medium with a 1 ml pipette. From the resulting suspension, 100 µl were taken for legionella plate counts (2.2.17) and 50 µl to determine amoebal cell concentrations. For FISH (2.2.12) and for PI death stain (2.2.15) 900 µl of the suspension were used. The experiment was undertaken in biological duplicates, meaning a handling on separate 12-well dishes. If the experiments involved legionellae, than those used for infection were obtained from independently grown *L. pneumophila* overnight cultures.

2.2.11 Long Term Effect of *L. pneumophila* Infection in *A. castellanii* and *A. castellanii/P. amoebophila*

The experiment was undertaken in biological and technical duplicates. Continuous cultures (20 °C, 30 °C) of *A. castellanii/P. amoebophila* as well as uninfected *A. castellanii* were harvested (2.2.2) and amoebae counted (2.2.3). For each biological replicate two 25 cm^2 culture flasks were prepared containing 8 ml PYG and 5x10^5 amoebae. Motile *L. pneumophila* (3.4) were diluted 1:10 with sterile $_{dd}H_20$ and added at a theoretical MOI of 20 to *A. castellanii/P. amoebophila* as well as to uninfected *A. castellanii.* As controls served in each case 3 uninfected *A. castellanii* and *A. castellanii/P. amoebophila* 25 cm^2 culture flasks. To determine the real MOI the 1:10 *L. pneumophila* dilution was further diluted to 1:100,000 and 15 µl were streaked on a CYE plate and incubated in a wet chamber for 4 days at 37 °C. To stop the initial infection after 2 hours extracellular legionella were removed by exchanging the medium twice. The course of the infection was monitored weekly. Before harvesting the amoebae at each time point 5 representative phase contrast pictures were taken. The initial time point to harvest the amoebae was immediately after the washing (2 hpi). To detach amoebae the flasks were shaken vigorously 10 times. At every time point 3 ml amoebae suspension were removed from each flask. To ensure reattachment of amoebae, after 2 hours at RT the residing 5 ml medium were poured and replaced by 8 ml fresh PYG. The collected 3 ml of amoebae suspension were proceeded as follows: 900 µl were taken for legionella plate counts (2.2.17), 100 µl for the determination of amoebal cell concentrations, 1000 µl for FISH directly (2.2.12) and 1000 µl for PI death stain (2.2.15).

2.2.12 Fluorescence-In-Situ-Hybridisation

2.2.12.1 Sample Preparation

After harvesting samples were spun down using an Eppendorf 5804R microcentrifuge at 3900 rcf for 8 min at RT. The supernatant was decanted and the pellet washed with 1 ml 1x PAS. Again amoebae were centrifuged at the same speed, the supernatant removed and the resulting pellet resuspended in 50-200 µl 1x PAS, depending on its size.

2.2.12.2 Sample Fixation

On a Teflon-coated glass slide 20-40 µl sample volume were spotted per well, followed by an 20 min incubation step to let amoebae attach to the surface. Excess 1x PAS was removed by pipetting and 20 µl 4 % PFA were applied onto the sample for 10 min at RT. PFA was removed and amoebae were washed once with 20 µl $_{dd}H_20$. Excess liquid was taken off and the slide then was dried at RT. Slides prepared in this way were used directly for FISH or stored at -20 °C.

2.2.12.3 Hybridisation

Hybridisation was performed using 10 µl hybridisation buffer plus 1 µl of each probe on each spot. Formamide concentrations of the hybridisation buffer were chosen depending on the probes used and are indicated in tables 5 and 7. Slides were incubated at 46 °C in a wet chamber. Afterwards slides were washed in prewarmed (48 °C) washing buffer for 10 min. Finally a hyper-stringent washing step was undertaken for 2 s in ice-cold $_{dd}H_20$ and the slides were immediately dried by compressed air.

2.2.12.4 Image Analysis

Prior to analysis samples were mounted with Citifluor and visualised by epifluorescence microscopy (Zeiss Axioplan 2). Alternatively slides were analysed using a confocal laser scanning microscope (LSM 510, Carl Zeiss) equipped with two helium-neon lasers (633 nm, 543 nm), an argon-krypton laser (488 nm) and a UV laser (351–364 nm). Image analysis processing was performed with the standard software package delivered with the instrument (version 4.2).

2.2.13 DAPI Stain

To perform a DAPI stain 20 µl of the 1:10,000 DAPI working solution were applied per spot containing fixed cells on a 10-well microscope slide. After an incubation period of 4 min at RT it was removed by pipetting and washed once with 20 µl $_{dd}H_20$. Afterwards the slides were immediately dried by compressed air.

2.2.14 HCS Stain

For the HCS stain 20 µl 2 µg/ml HCS working solution was placed onto the spots containing the fixed samples on a 10-well microscope slide. After 30 min incubation at RT HCS was removed and the spot was washed twice with 20 µl $_{dd}H_20$. Afterwards the slides were immediately dried by compressed air.

2.2.15 PI Stain and Measurement

All necessary steps for PI staining of cells were undertaken in the dark. Samples were spun down using an Eppendorf 5804R microcentrifuge at 3900 rcf or 8 min at RT. The supernatant was decanted and the pellet washed once with 1 ml 1x PAS. After washing the sample it was resuspended in 500 µl 1.5 µM PI solution and incubated for 20 min at RT. After a centrifugation step of 3900 rcf at RT the supernatant was removed and the pellet washed twice first with 1000 µl, then with 500 µl 1xPAS. Again amoebae were centrifuged, the supernatant decanted and the resulting pellet resuspended in 150 µl 1x PAS and transferred to a black plate reader dish. The PI fluorescence intensity was measured with a fluorescence-reader (shaking for 5s; fluorescence top reading without lid; gain 100; 4 reads per well; A_{exc}= 535 nm, A_{em}= 617 nm).

2.2.16 Lysis of Amoebae

Amoebal suspensions were frozen on a cold-block for about 1 h at -20°C and afterwards rapidly thawed at 45 °C in a water bath. Cells were resuspended by 8 passages through a 26-gauge needle plugged on a 1 ml syringe.

2.2.17 Legionella Plate Counts

A dilution series was prepared from the resulting amoebae lysate. Depending on the expected legionella numbers three different dilutions were chosen and were then streaked on CYE plates and incubated in a wet chamber for 4 days at 37 °C.

2.2.18 Preparation of Chlamydial EB Lysate

P. amoebophila EBs were thawed at 37 °C and immediately placed on ice afterwards. To generate bacterial lysate, EBs were diluted 1:10 in ice-cold SPG and then placed into a 1 ml bead-beater cap and bead-beaten 4-times for 15 seconds. To prevent the sample to be exposed to high temperatures during the processing, it was placed on ice for 10 seconds between each bead-beating step.

2.2.19 Statistical Analysis

For statistical analysis Graphpad PRISM was used. Depending on the experiment, mean proportions and standard deviations (SD) were calculated for the proportion of infected amoebae, PI fluorescence values, amoebal numbers and [%] correlation of DAPI and FISH signal at each time point. To test for the presence of overall statistically significant differences between used temperatures and/or systems, a two-way analysis of variance (ANOVA) was performed. In addition, individual time point pairs within a fraction were post-tested for statistical significance of observed differences using the Bonferroni test.

CHAPTER 3

Results

3.1 Developmental Cycles

3.1.1 Performance of *A. castellanii*

As a control for the upcoming infection experiments, uninfected amoebae were grown in PYG at 20 °C or 30 °C, respectively. Amoebae were harvested every 24 hours, counted (figure 6 a) and PI fluorescence values determined (figure 6 b).

Initially 1.5 x 10^5 amoebae/ml were seeded and incubated at the related temperature for about 12-16 hours, than the monitoring of amoebal cell concentrations was started. The doubling from 1.5x 10^5 to 3 x 10^5 amoebae/ml took approximately 24 hours. The doubling time then slowed down to approximately 72 hours and after attaining cell densities of about 6 x 10^5 amoebae/ml, cell concentrations increased or decreased only marginally. No statistically significant differences in amoebal growth rates at the different incubation temperatures could be detected.

In uninfected *A. castellanii*, there was no increase in PI fluorescence intensities detectable within the first 144 hours. Only after reaching the plateau phase of amoebal growth, also the PI values slowly start to rise and obtain a maximum after 192 hours. This effect was even more distinct at 30 °C ($p<0.01$).

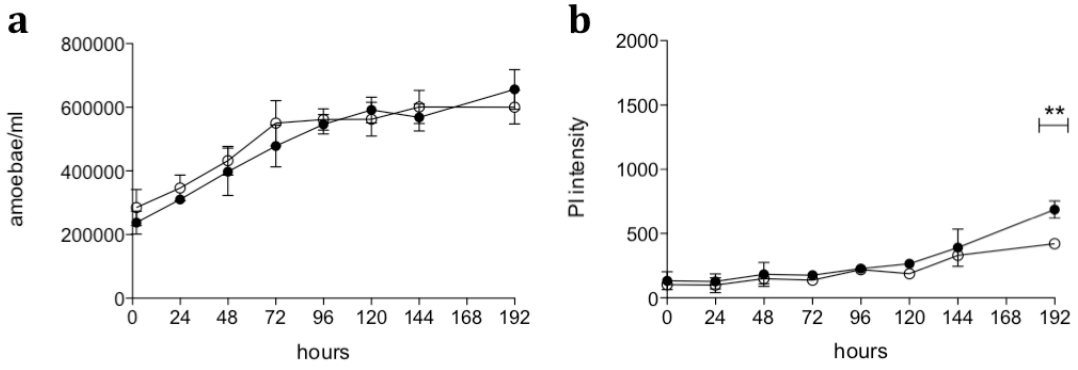

Figure 6. Amoebae fitness over a course of 192 hours.

Uninfected *A. castellanii* were grown in PYG at 20°C (open circles) or 30 °C (filled circles), respectively. Within the first 168 hours, amoebae were counted and PI fluorescence values determined every 24 hours. At both temperatures, an approximately linear amoebal growth was observed within the first 120 hours. Afterwards, plateau phase was reached with cell densities of about 600,000 amoebae/ml. Data represent the mean ± SD, and are representative of at least two independent experiments. For statistical analysis a 2-way ANOVA test together with a Bonferroni post-test were performed. *P < 0.05; **P < 0.01; ***P < 0.001.

3.1.2 Developmental Cycle of *P. amoebophila* in *A. castellanii*

3.1.2.1 Course of *P. amoebophila* Infection Monitored by FISH

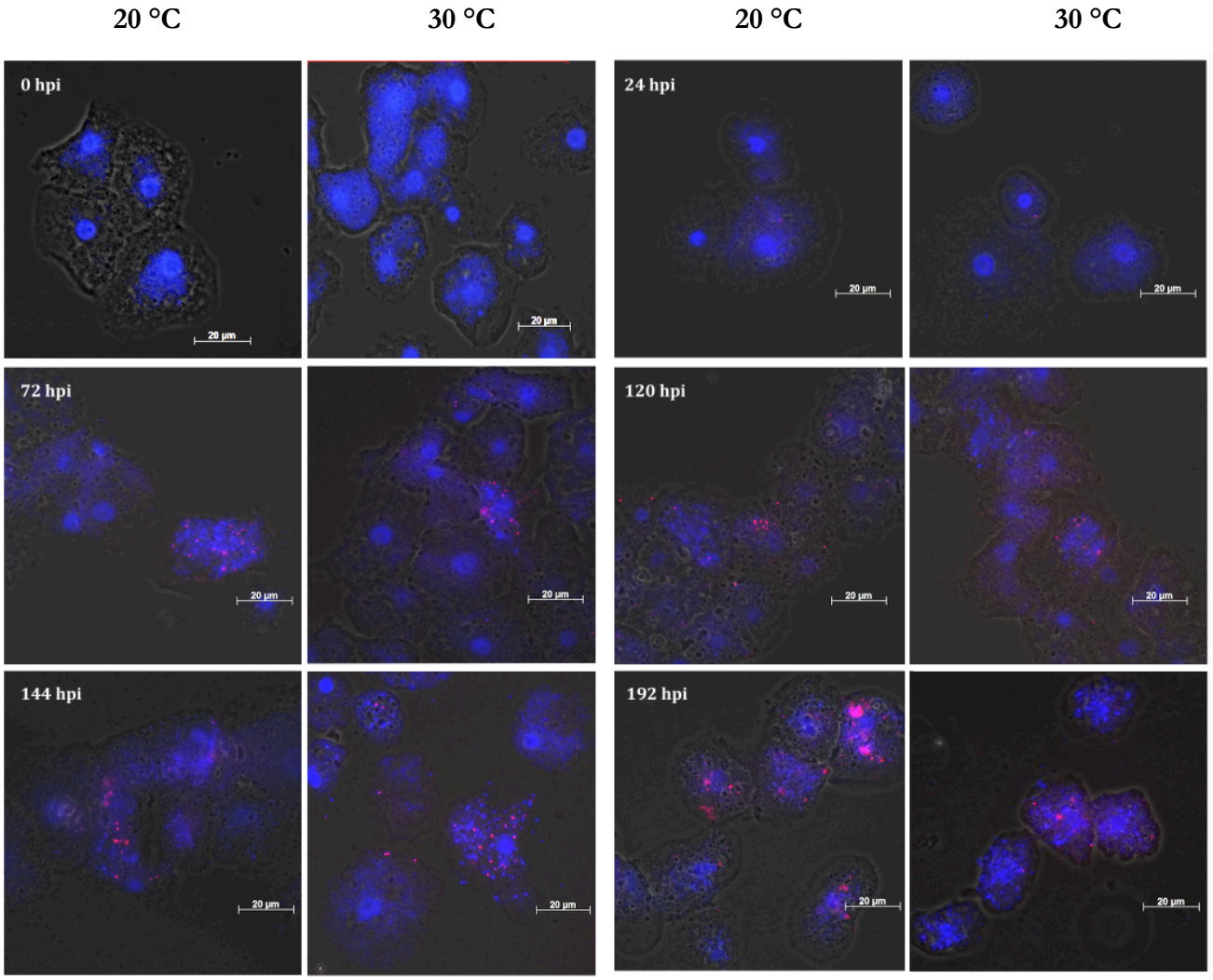

Figure 7. Developmental cycle of *P. amoebophila* within *A. castellanii* at 20 °C and 30 °C.

The cycle was being followed by DAPI staining and FISH at various time points within the first 192 hpi. *P. amoebophila* was stained with the Chlamydiales-specific probe E25-454 labelled with Cy3 (red); the nucleic acid stain DAPI is shown in blue. Cells stained with Cy3 and DAPI are purple. Fluorescence images overlay the corresponding phase contrast images. Images were taken with a Zeiss Axioplan 2 epifluorescence microscope. The scale bar corresponds to 20 µm.

DAPI staining, FISH and bright field analysis of amoebae gave insights into cell morphologies (figure 7), infection levels (figure 7, figure 8 a) and metabolic activity of *P. amoebophila* (figure 7, figure 8 c) over the course of the experiment.

Immediately after infection most bacterial particles gave no signal with the FISH probe, confirming that they were still present as EBs. Infected amoebae contained 1-2 bacteria in most cases.

From infection rates of about 50 % in the beginning a drop to about 20 % occurred until 24 hpi. At that time point there were between 1 and 5 *P. amoebophila* particles present in infected amoebae. Chlamydiae had presumably already switched from EBs to metabolically active RBs, as the FISH signal now correlated in most cases with the DAPI signal.
This trend even stronger manifested after 48 hours, where infected amoebae already contained more than 20 bacteria. Infection rates did not increase further between 24 and 48 hours. After 72 hours the switch from RBs to infectious EBs had already occurred partially, since most chlamydiae were still visible in their metabolic form, but a growing discrepancy between the FISH and the DAPI signal became obvious. The infection rates did also increase from about 20 % to approximately 30 %.

From this point on, the course of infection was asynchronous, as re-infections of already infected amoebae could not be excluded. The proportion of metabolically active bacteria did not change much after 96 hpi, with only a slightly increased amount of intracellular EBs. Remarkably, at the same time the number of infected amoebae had almost doubled, thereby clearly indicating an efficient spread of infection facilitated by the presence of extracellular EBs.

A notably high amount of intracellular EBs could be observed 120 hpi, whereas only in about 40 % of bacterial cells the FISH signal corresponded with the DAPI signal. Again, the number of infected amoebae was highly elevated. After 144 hours, most amoebae were infected by *P. amoebophila* and there was a reoccurrence of metabolic activity in intracellular *P. amoebophila*. This second metabolic peak was about 25 % lower compared to the first peak, which appeared 48 hpi.

The end of the second round of the UW25 infection was suspected to occur between 168 and 192 hpi, since the fraction of EBs in the cytoplasm was strongly increased during this period of time.
The spread of the *P. amoebophila* infection in *A. castellanii* was not statistically significantly different between 20 and 30 °C. The same was true for time points with high or low chlamydial metabolic activity.

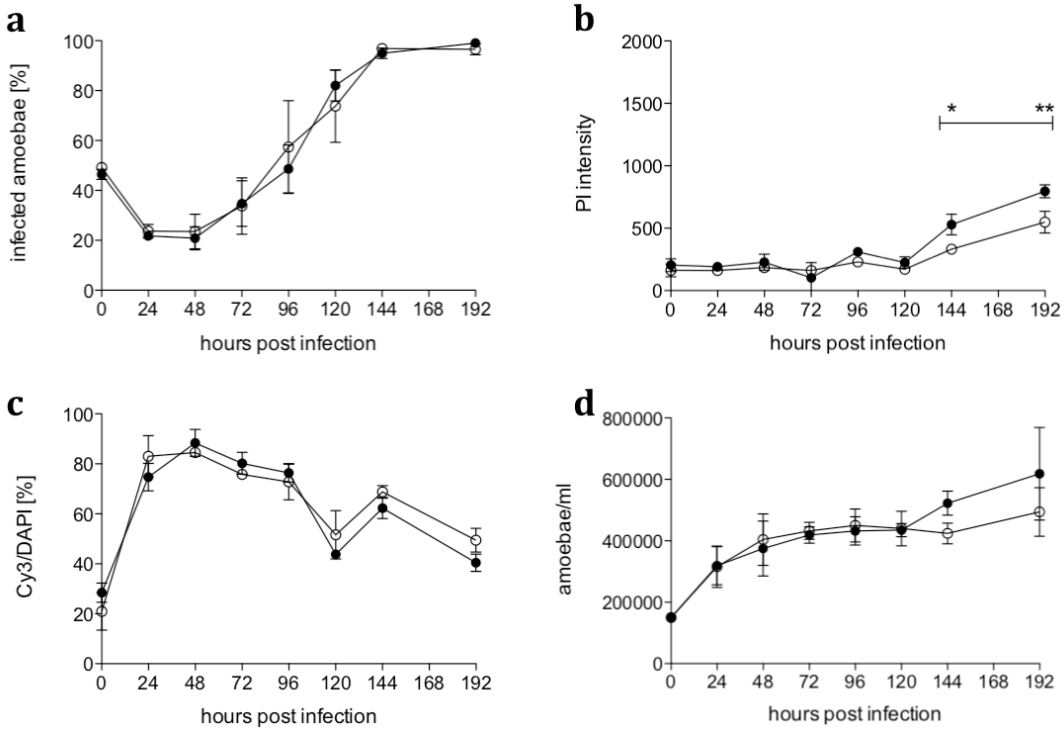

Figure 8. Effects of *P. amoebophila* infection in *A. castellanii*.

The course of infection was monitored over 192 hours at 20 °C (open circles) and 30 °C (filled circles), respectively. Shown is: (a) the percentage of *P. amoebophila*-infected amoebae; (b) the amoebal viability visualised by fluorescence intensity resulting from the propidium iodide death stain; (c) the different phases of metabolic activity represented by the amount of correlation between specific FISH signal and DAPI stain; (d) the development of amoebal cell concentrations during the cycle. Data represent the mean ± SD, and are representative of at least two independent experiments. For statistical analysis a 2-way ANOVA test and a Bonferroni post-test were performed. *P < 0.05; **P < 0.01; ***P < 0.001.

3.1.2.2 Influence of *P. amoebophila* Infection on Amoebal Fitness

PI staining followed by PI fluorescence measurement (figure 8 b) and enumeration of amoebae (figure 8 d) gave insights into amoebal fitness over the course of the experiment.

Overall, amoebal viability during *P. amoebophila* infection followed a curve quite similar to the uninfected control independent from the temperature. Elevated PI fluorescence intensities as a result of amoebal cell death were found 144 hpi and reached a maximum after 192 hours. At those late time points, the PI fluorescence signal was about 20 % stronger if amoebae were incubated at 30 °C, and therefore statistically significantly different (144 hpi: $p<0.5$; 192 hpi: $p<0.01$). This trend fit the rise in absolute amoebal numbers after 120 hours.

3.1.3 Developmental Cycle of *Parachlamydia* sp. in *A. castellanii*

3.1.3.1 Course of *Parachlamydia* sp. Infection Monitored by FISH

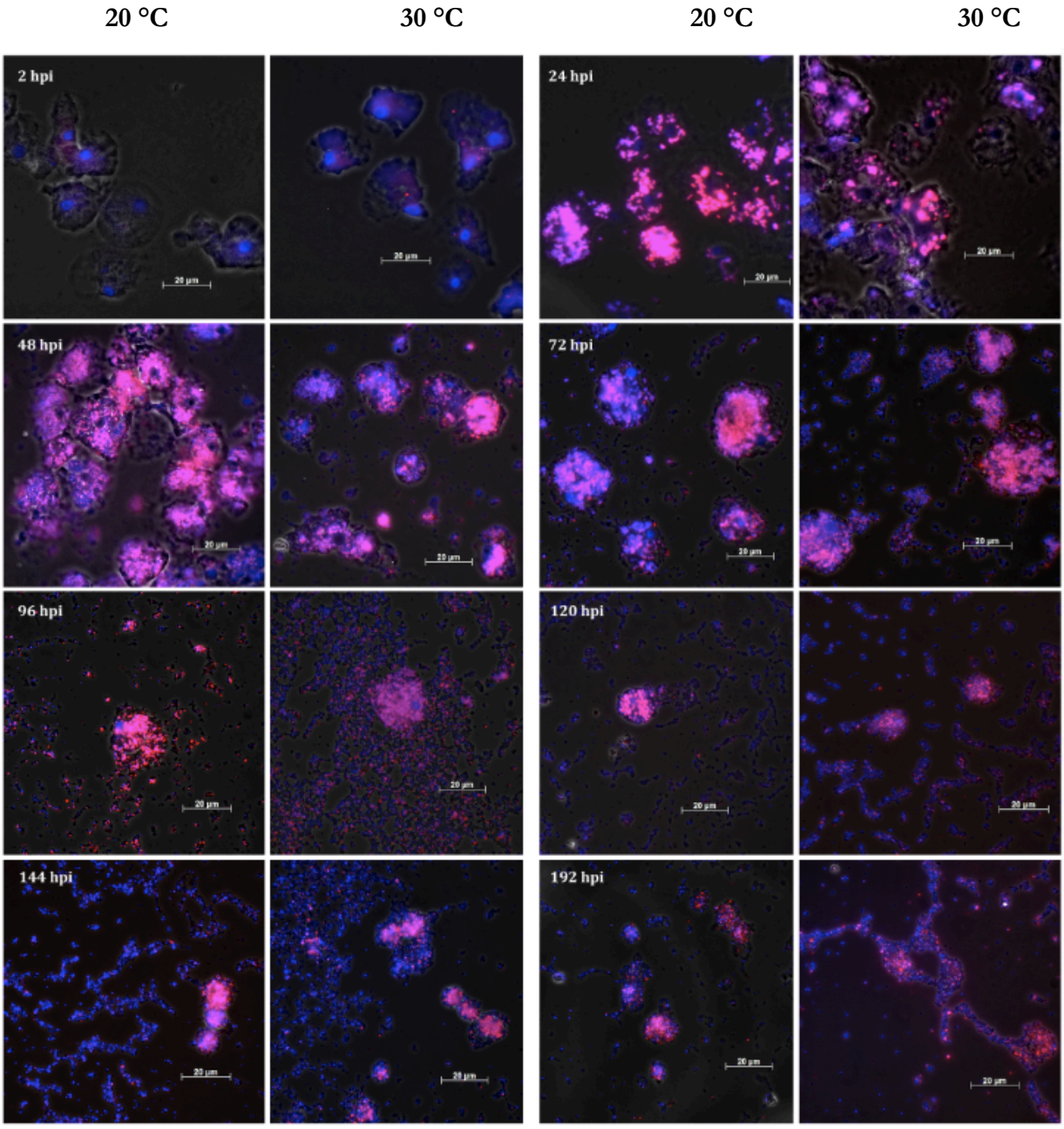

Figure 9. Developmental cycle of *Parachlamydia* sp. in *A. castellanii* at 20 °C and 30 °C.

The cycle was being followed by DAPI staining and FISH at various time points within the first 192 hpi. *Parachlamydia* sp. was stained with the *Parachlamydia* sp. -specific probe *UV*7-763 labelled with Cy3 (red); the nucleic acid stain DAPI is shown in blue. Cells stained with Cy3 and DAPI are purple. Fluorescence images overlay the corresponding phase contrast images. Images were taken with a Zeiss Axioplan 2 epifluorescence microscope. The scale bar corresponds to 20 µm.

DAPI staining, FISH and bright field analysis of amoebae gave insights into cell morphologies (figure 9), infection levels (figure 9, figure 10 a) and metabolic activity of *Parachlamydia* sp. (figure 9, figure 10 c) over the course of the experiment.

Directly after infection, 1-10 *Parachlamydia* sp. particles could be found in about 70 % of amoebae. Most *Parachlamydia* sp. were still present as EBs, as only fewer than 10 % of FISH signals corresponded to the DAPI signal. The infection rates increased dramatically between 0 and 24 hours, so that almost all amoebae were infected by *Parachlamydia* sp. by 24 hpi. At the same time the correlation of the FISH and the DAPI signal reached a maximum of 80-90 %, indicating an overall peak of *Parachlamydia* sp. metabolic activity. RBs were arranged in dense cauliflower-like clusters, distributed throughout the cytoplasm of infected amoebae.

After 48 hours those aggregates had increased in size and still gave a bright FISH signal. However, more and more exclusively DAPI stained and needle-shaped single bacteria became visible, and were assigned as *Parachlamydia* sp. EBs due to the lack of a FISH signal. At an incubation temperature of 30 °C, the correlation between the DAPI and the FISH signal was about 50 %, but not significantly different compared to the approximately 60 % at 20 °C. With the occurrence of an increasing amount of EBs, also a release of those particles was more and more likely, coming along with reinfection of already *Parachlamydia* sp.-infected amoebae.

At the following time points there was a high variability of the correlation of DAPI signal and FISH signal, indicating a strongly asynchronous course of infection. For both temperatures used, there were in total 3 metabolic peaks visible over a course of 192 hours, subsequently dropping in intensity. At both temperatures, the first peak appeared after 24 hours, and the second peak could be found 72 hpi at 30 °C, whereas at 20 °C it appeared after 96 hours. The third peak became visible after 144 hours at both temperatures. After 192 hours, only about 40 % of *Parachlamydia* sp. were detectable by FISH. It should also be noted that after 96 hours increasing numbers of extracellular *Parachlamydia* sp. were found. Occasionally, these extracellular cells still gave a FISH signal, but the correlation was at the following time points subsequently reduced and in the end most extracellular bacteria were exclusively DAPI stained.

In general, the infection rates as well as the metabolic activity of *Parachlamydia* sp. were not statistically significantly different between both temperatures.

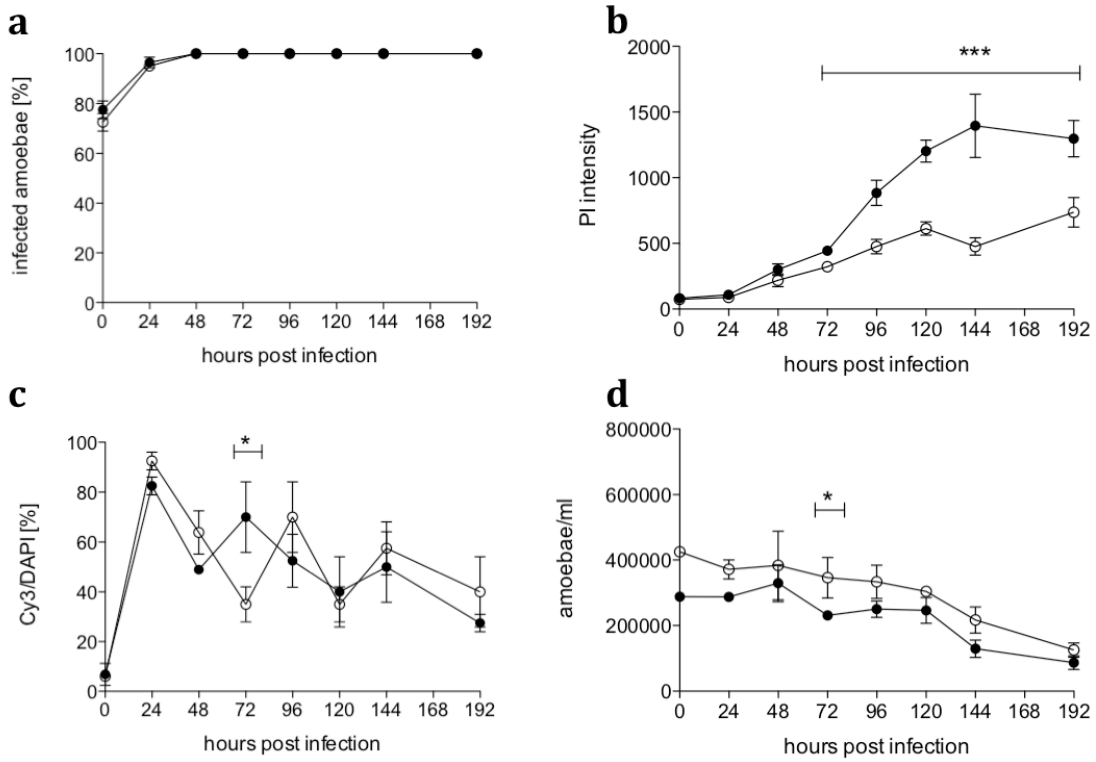

Figure 10. Effects of *Parachlamydia* sp. infection in *A. castellanii*.

The course of infection was monitored over 192 hours at 20 °C (open circles) and 30 °C (filled circles), respectively. Shown is: (a) the percentage of *Parachlamydia* sp.-infected amoebae; (b) the amoebal viability visualised by fluorescence intensity resulting from the propidium iodide death stain; (c) the different phases of metabolic activity represented by the amount of correlation between specific FISH signal and DAPI stain; (d) the development of amoebal cell concentrations during the cycle. Data represent the mean ± SD, and are representative of at least two independent experiments. For statistical analysis a 2-way ANOVA test and a Bonferroni post-test were performed. *P < 0.05; **P < 0.01; ***P < 0.001.

3.1.3.2 Influence of *Parachlamydia* sp. Infection on Amoebal Fitness

PI staining followed by PI fluorescence measurement (figure 10 b) and enumeration of amoebae (figure 10 d) gave insights into amoebal fitness over the course of the experiment.

Within the first 24 hours no rise in amoebal cell concentrations was observed. In addition, there was almost no amoebal lysis as indicated by a low amount of PI stained cells. Between 24 to 48 hpi, a slight increase in PI fluorescence intensities was recorded, which increased further after 72 hours. However, this effect was much more distinct at 30 °C, where PI fluorescence values were 2-3 fold higher compared to the situation at 20 °C ($p<0.001$). A maximum in amoebal lysis was detected after 144 hours and stayed at a similarly high level until 192 hpi. At 20 °C two time points characterized by a high rate of amoebal lysis became visible (120 and 192 hpi) and an

increase of about 20 % between both maxima became obvious. Overall, the effect of temperature on the PI fluorescence intensities was statistically significant ($p=0.0003$).

Amoebal cell concentrations decreased at both temperatures, starting 48 hpi. A large drop was observed between 48 and 72 hours at 30 °C, and between 120 and 144 hpi at 20 °C. Nevertheless, in the end amoebal cell concentrations were similarly low at both temperatures. Between the two temperatures used, cell concentrations were only significantly different 72 hpi ($p<0.05$).

3.1.4 Developmental Cycle of *L. pneumophila* in *A. castellanii*

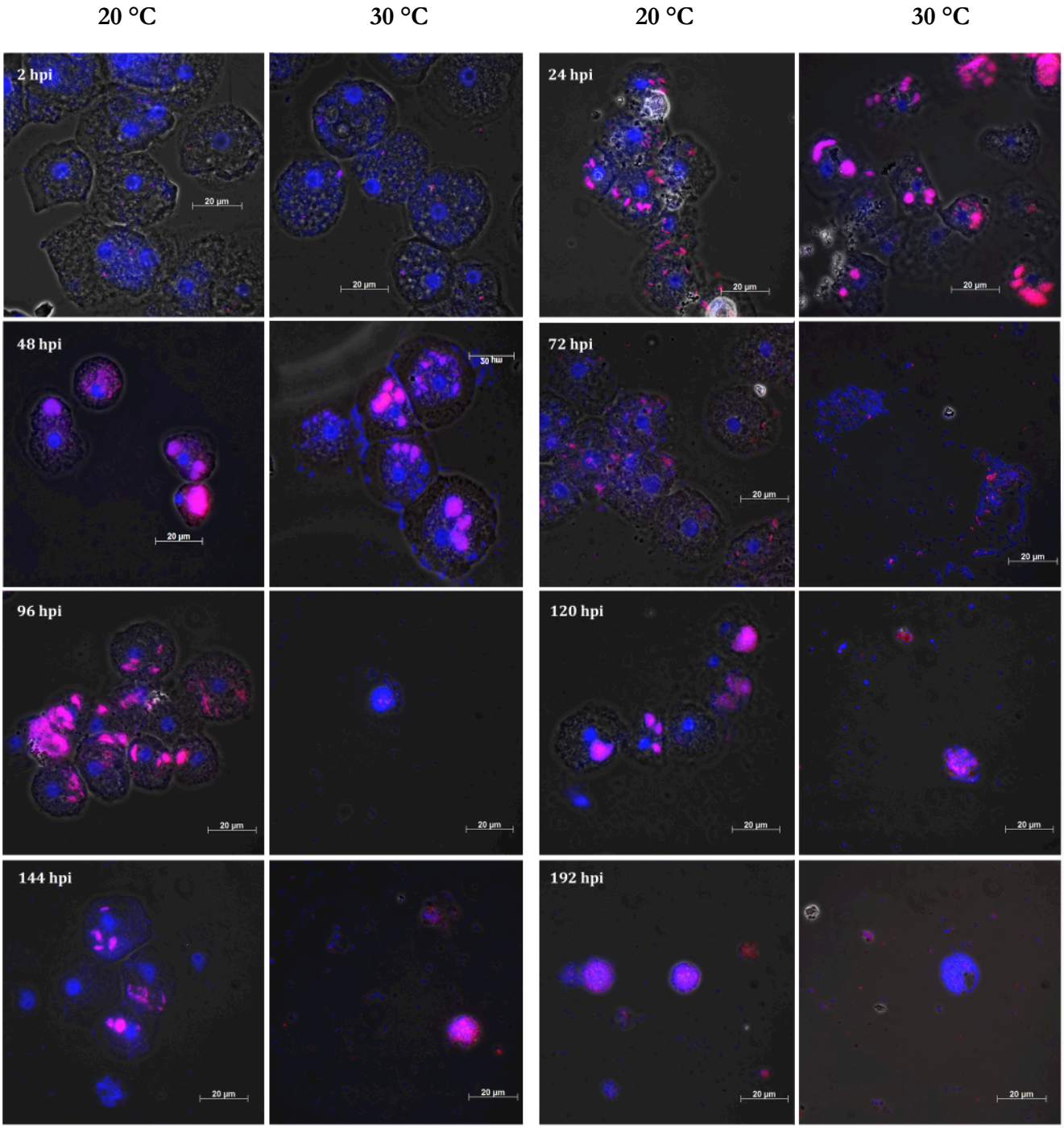

Figure 11. Developmental cycle of *L. pneumophila* in *A. castellanii* at 20 and 30 °C.

The cycle was being followed by DAPI staining and FISH at various time points within the first 192 hpi. *L. pneumophila* was stained with the *L. pneumophila*-specific probe LEGPNE1 labelled with Cy3 (red); the nucleic acid stain DAPI is shown in blue. Cells stained with Cy3 and DAPI are purple. Fluorescence images overlay the corresponding phase contrast images. Images were taken with a Zeiss Axioplan 2 epifluorescence microscope. The scale bar corresponds to 20 µm.

3.1.4.1 Course of *L. pneumophila* Infection Monitored by FISH

DAPI staining, FISH and bright field analysis of amoebae gave insights into cell morphologies (figure 11), infection levels (figure 11, figure 12 a) and metabolic activity of *L. pneumophila* (figure 11, figure 12 c) over the course of the experiment.

Two hours after infection 1-5 single *L. pneumophila* could be detected in approximately 70 % of amoebae. In addition, at that time point already about two-thirds to three-quarters of bacteria gave a signal with the FISH *L. pneumophila*-specific FISH probe. At both temperatures 24 hours after infection, the correlation between DAPI and FISH signals was higher than 95 %. After 48 hours it had slightly decreased to 90 %.

Infection rates increased at both temperatures, but 3-4 fold faster at 30 °C, where after 48 hours already all amoebae were infected ($p<0.001$). At 20°C, the spread of *L. pneumophila* infection among *A. castellanii* was relatively slow until 48 hpi, where a stronger increase in infection rate was observed. Between 72 and 96 hpi the majority of amoebae were infected, even at 20 °C. At both temperatures, the proportion of legionellae detectable by DAPI and FISH dropped steeply between 48 and 72 hpi. This decrease was milder at 20 °C, where after 72 hours a correlation between DAPI and FISH signal of about 60 % could be found, whereas at 30 °C it was only 30 % ($p<0.05$). Subsequently, at 20 °C the proportion of *L. pneumophila* with a FISH signal rose again, reaching a second peak and increasing the gap between both temperatures 96 hpi ($p<0.001$). After 120 hours there was still a statistically significant difference between both temperatures ($p>0.05$), but afterwards correlation values converge due to the second peak in metabolic activity at 30 °C after 144 hours.

In summary, there was a statistically significant influence of temperature on infection rates and metabolic activity of *L. pneumophila* in *A. castellanii*.

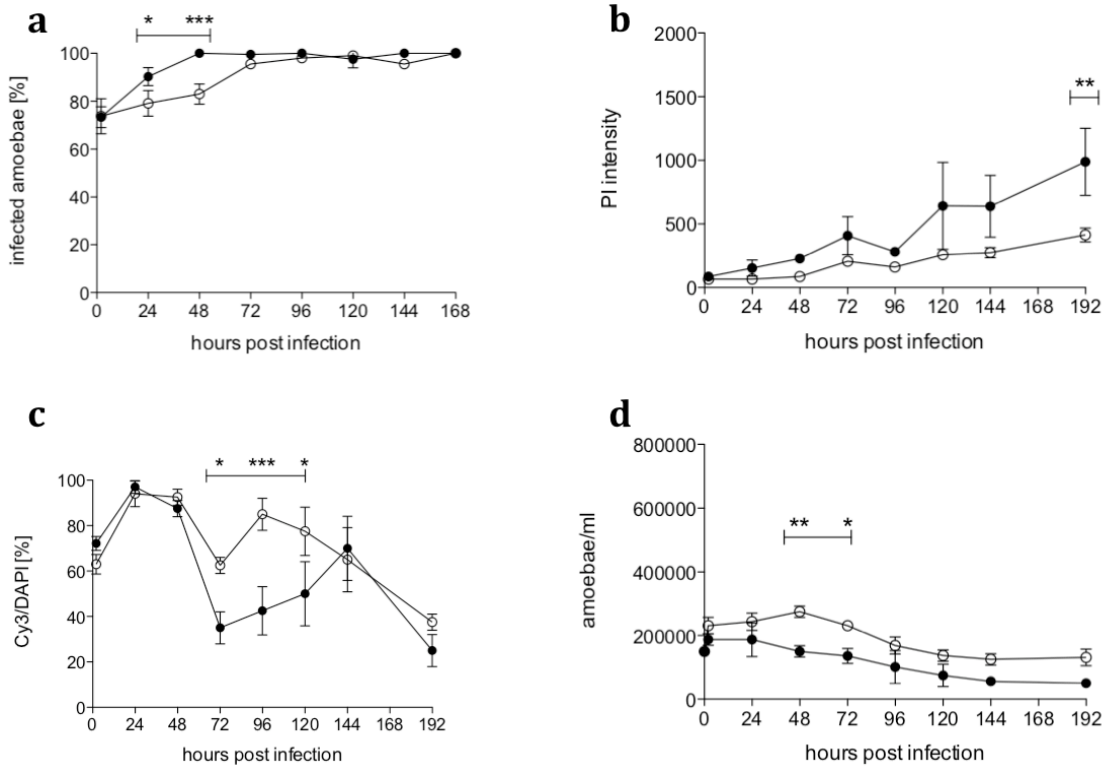

Figure 12. Effects of *L. pneumophila* infection in *A. castellanii*.

The course of infection monitored over 192 hours at 20 °C (open circles) and 30 °C (filled circles), respectively. Shown is: (a) the percentage of *L. pneumophila*-infected amoebae; (b) the amoebal viability visualised by fluorescence intensity resulting from the propidium iodide death stain; (c) the different phases of metabolic activity represented by the amount of correlation between specific FISH signal and DAPI stain; (d) the development of amoebal cell concentrations during the cycle. Data represent the mean ± SD, and are representative of at least two independent experiments. For statistical analysis a 2-way ANOVA test and a Bonferroni post-test were performed. *P < 0.05; **P < 0.01; ***P < 0.001.

3.1.4.2 Influence of *L. pneumophila* Infection on Amoebal Fitness

PI staining followed by PI fluorescence measurement (figure 12 b) and enumeration of amoebae (figure 12 d) gave insights into amoebal fitness over the course of the experiment.

At 20 °C, the PI fluorescence intensities in *L. pneumophila*-infected amoebae were quite similar to those in the uninfected control. This finding is opposing to the situation at 30 °C, where an initial slight rise in PI fluorescence occurred. At both temperatures a first peak in amoebal lysis was observed after 72 hours. Subsequently, stronger peaks followed between 120 and 192 hpi. Remarkably, at 30 °C PI fluorescence intensities did more than double between 72 and 192. At 20 °C, amoebal lysis was pronounced initially 72 hpi, and a second time after 192 hours.

The decrease in amoebal cell concentrations was reflected elevated PI fluorescence intensities, at least in the beginning. At 30 °C, amoebae depleted already after 24 hours. After 48 hours there was a statistically significant difference in cell concentrations between both temperatures ($p<0.01$). At 20 °C amoebal cell concentrations initially started to drop down 48 hpi. These trends were reflected by elevated PI fluorescence intensities at those time points. Later on, the correlation was much lower, as PI fluorescence intensities still continued to rise, whereas cell concentrations only slightly decreased or stayed the same. The most statistically significant difference between both temperatures regarding PI fluorescence values occurred 192 hpi ($p<0.01$).

Taken together, amoebal lysis was statistically significant different between both temperatures, reflected by either elevated PI fluorescence intensities ($p=0.0008$), or decrease in amoebal cell concentrations ($p=0.0058$) at 30 °C.

3.2 Co-Infection of *A. castellanii* with *P. amoebophila* and *L. pneumophila*

3.2.1 Short Term Effect of *L. pneumophila* Infection *A. castellanii*/*P. amoebophila*

For infection of continuous cultures of *A. castellanii*/*P. amoebophila* with *L. pneumophila*, a theoretical MOI of about 20 was used for the first replicate, where about 20 % of *L. pneumophila* were motile. This was different to the second biological replicate, in which approximately 40 % motile *L. pneumophila* were found. From previous experiments in our lab it was known, that initial infection rates positively correlate with the initial proportion of motile legionellae. To ensure a comparable course of infection a theoretical MOI of 10 was chosen for the second replicate. With the help of viable counts a real MOI of about 1 was determined in each of both biological replicates.

3.2.1.1 Co-infection of *A. castellanii* with *P. amoebophila* and *L. pneumophila* monitored by FISH

The course of infection in the two systems *A. castellanii*/*L. pneumophila* and *A. castellanii*/*P. amoebophila*/*L. pneumophila* was visualised by FISH (figure 14) and the percentage of infected amoebae for each time point (figure 15 a), as well as the infection level (figure 13 a, b) were determined. In both systems the situation was quite similar 2 hpi, about 20 % of amoebae were infected with 1-5 legionellae in their cytoplasm. After 24 hours the first differences became visible. Putative food vacuoles could be detected exclusively in *A. castellanii*/*P. amoebophila*/*L. pneumophila*, showing up as round to oval inclusions appearing yellow due to the overlay of Cy3-stained legionella and FLUOS-stained *P. amoebophila* (figure 14; 24 hpi). After 48 hours, two clearly distinguishable amoebal morphologies could be observed for the first time in *A. castellanii*/*P. amoebophila*/*L. pneumophila*. On the one hand, small and roundish amoebae containing densely accumulated *P. amoebophila* became apparent, on the other hand the regular *A. castellanii*/*P. amoebophila* trophocyte morphology was observed. Both forms were found being infected by *L. pneumophila*, often at a high level (>25 *L. pneumophila*/amoebae). In *A. castellanii*/*L. pneumophila* only trophozoites containing *L. pneumophila* were visible, but compared to *A. castellanii*/*P. amoebophila*/*L. pneumophila* the infection level was still in an early (0-5 *L. pneumophila*/amoebae) or an average stage (5-25 *L. pneumophila*/amoebae). After 72 hours in *A. castellanii*/*P. amoebophila*/*L. pneumophila* a higher number of roundish amoebae appeared and

their number increased continuously at the following time points. This trend was reciprocally correlated with the proportion of infected amoebae. Amoebae in an early stage of infection could rarely be found at later time points.

An increasing number of highly infected amoebae was detected in *A. castellanii*/*L. pneumophila* after 48 hours and continued to increase until 96 hpi. Then the amoebal number started to decline, as indicated by the presence of many lysed amoebae. Based on the large number of freshly infected amoebae between 48 and 144 hpi, it is not surprising that most of amoebae are in a high infection level after 168 hours, which may have already represented the peak of the second round of infection.

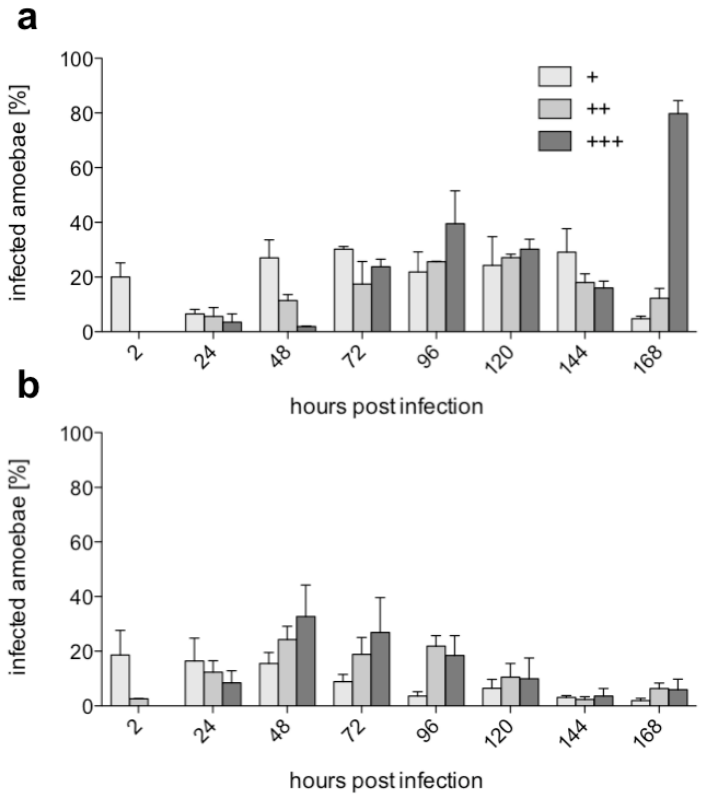

Figure 13. *L. pneumophila* infection levels in in *A. castellanii*/*P. amoebophila*

The course of *L. pneumophila* infection in *A. castellanii* (a) and *A. castellanii*/*P. amoebophila* (b) was monitored over 168 hours. The infection level was classified into three categories: + corresponds to an early infection stage with 0-5 *L. pneumophila*/amoebae; ++ as an average infection stage with 5-25 *L. pneumophila*/amoebae; +++ as a high level infection with > 25 *L. pneumophila*/amoebae. Error bars indicate SD, n =2.

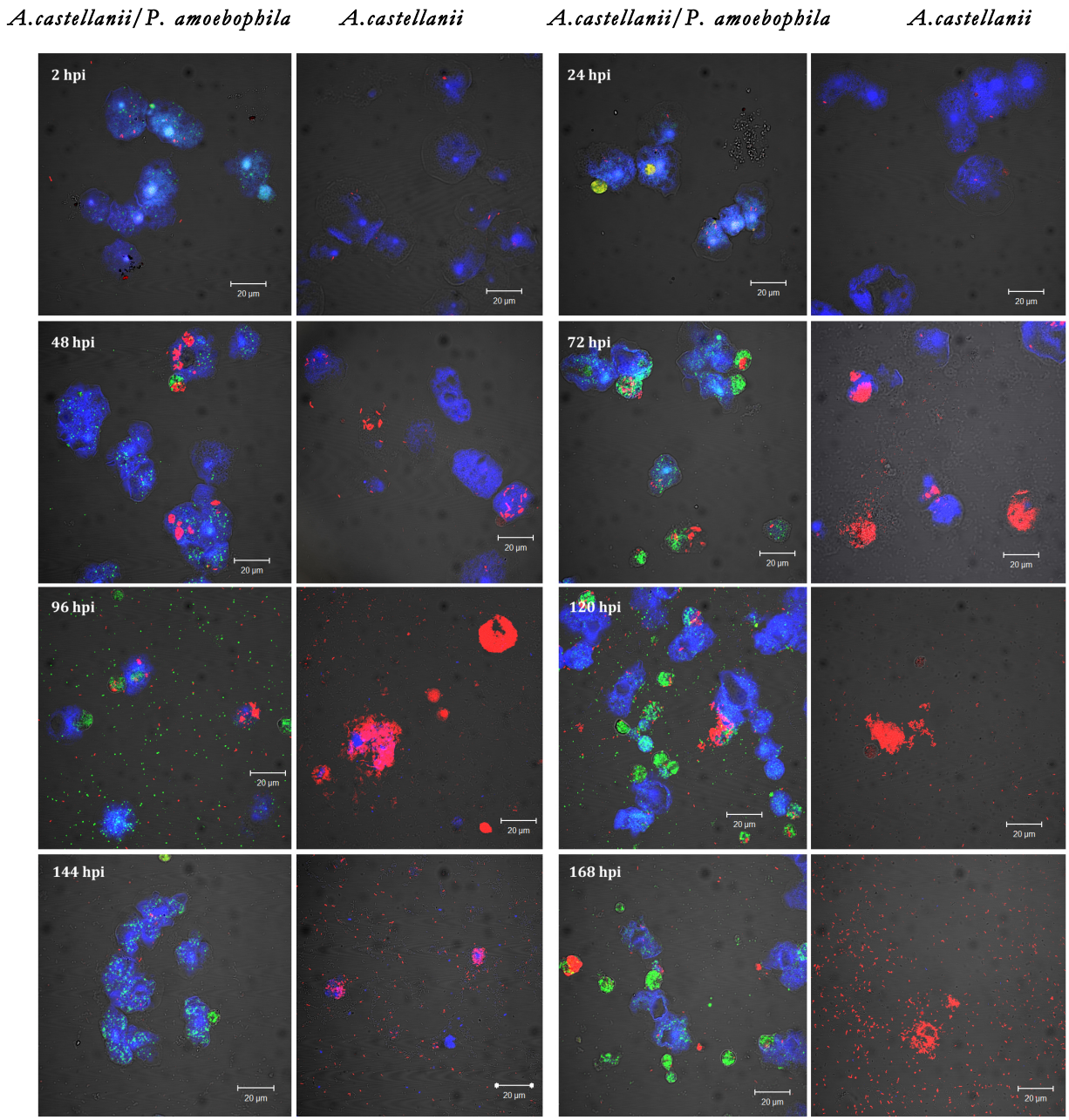

Figure 14. Short-term co-cultivation of *L. pneumophila* in *A. castellanii/P. amoebophila*.
The course of infection was monitored and visualized by FISH with LEGPN-Cy3 (red), Chls523-FLUOS (green) and HCS stain (blue) over 168 hours at 30 °C, The scale bar shows 20 µm.

When taking a closer look at the proportion of *L. pneumophila*-infected *A. castellanii/P. amoebophila* (figure 14 a), it becomes obvious that in the beginning the number of *L. pneumophila* infected amoebae raise almost exponentially. Directly after infection, approximately 20 % of amoebae showed *L. pneumophila* in their cytoplasm. This proportion started to increase and reached a maximum of 72 % *L. pneumophila* -infected amoebae after 48 hours. This was almost twice as high

compared to the control *A. castellanii*/*L. pneumophila* at that time point. Nevertheless, after this initial peak the number of infected amoebae steadily declined with a rate of about 15 % each 24 hours, and finally came down to 9 % infected amoebae 144 hpi.

These findings were contrary to what happened in *A. castellanii*/*L. pneumophila*. where initially a comparable rise of infected amoebae was observed, reaching a first maximum of more than 80 % infected amoebae after 96 hours. Similarly to *A. castellanii*/*P. amoebophila*/*L. pneumophila*, there was a delay as the proportion of *L. pneumophila* -infected amoebae decreased within the first 24 hours. A reason could have been an unsuccessful establishment of the infection in *A. castellanii*, as non-motile legionellae very likely were used as food source and successfully digested. Alternatively, the already low initial number of infected amoebae could have been further reduced by cell division of uninfected amoebae. Nevertheless, the number of infected amoebae doubled for the first time between 24 and 48 hours and a second time between 48 and 72 hpi.

After 96 hours the number of infected amoebae slightly dropped down, while a second round of infection began. This observation was statistically significantly different to *A. castellanii* with endosymbiont, where after 120 hours the proportion of *L. pneumophila*-infected amoebae continued to drop down and finally was lower than 10 % (120 hpi & 144 hpi, $p<0.05$; 168 hpi, $p<0.001$).

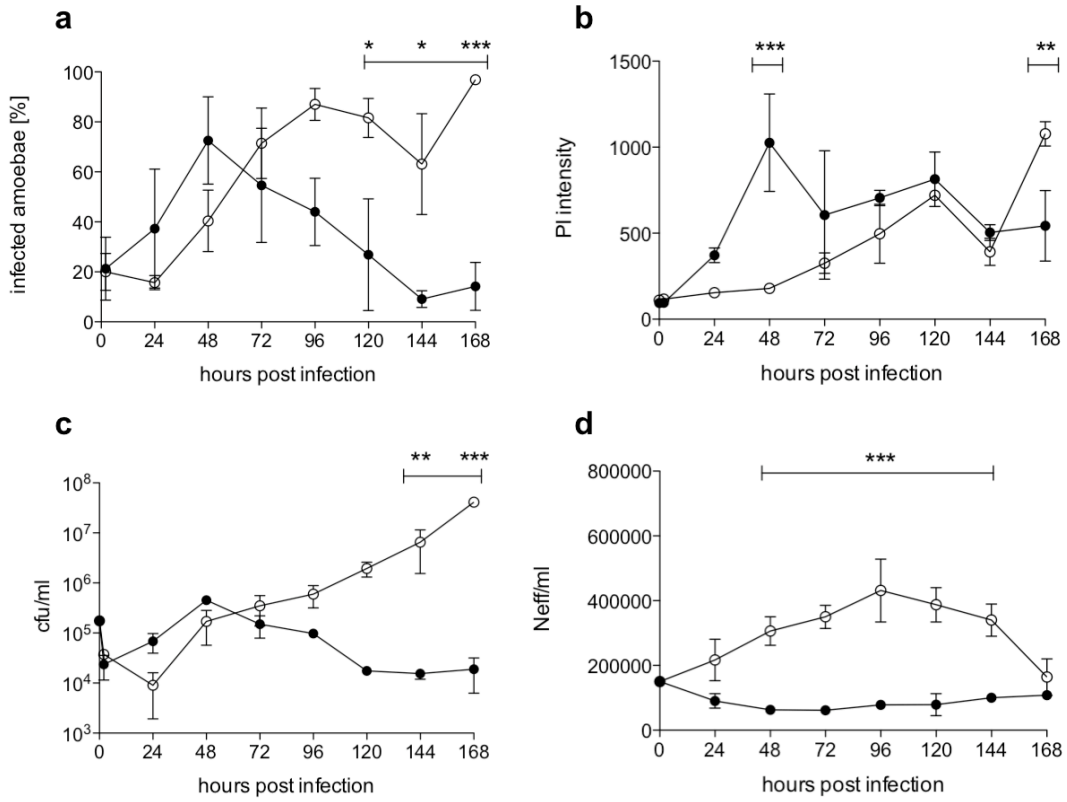

Figure 15. Effects of co-cultivation of *L. pneumophila* in *A. castellanii/P. amoebophila*.

The course of infection was monitored over 168 hours at 30 °C for the continuous culture *A. castellanii/P. amoebophila* infected with *L. pneumophila* (filled circles). The results are displayed in comparison to the control, represented by *A. castellanii* infected by *L. pneumophila* exclusively (open circles). Shown is: (a) the percentage of *L. pneumophila* - infected amoebae; (b) the amoebal viability visualised by the fluorescence intensity resulting from the propidium iodide death stain; (c) the number of viable *L. pneumophila*; (d) the development of amoebal cell concentrations during the cycle. Data represent the mean ± SD, and are representative of at least two independent experiments. For statistical analysis a 2-way ANOVA test and a Bonferroni post-test were performed. *P < 0.05; **P < 0.01; ***P < 0.001.

3.2.1.2 Impact of *L. pneumophila* Infection on Amoebal Fitness in the Presence or Absence of *P. amoebophila*.

Regarding *A. castellanii/P. amoebophila/L. pneumophila*. the PI fluorescence signal intensity strongly positively correlated with the number of infected amoebae until 72 hpi (figure 15 b). The first peak in PI signal intensity measured 48 hpi was statistically significantly different to the control ($p<0.001$). At that time already more than 70 % of amoebae were infected at a high infection level, which obviously resulted in a loss of cell viability reflected by high PI fluorescence intensities.

In the case of *A. castellanii*/*L. pneumophila* the first peak in PI fluorescence intensity was observed 120 hpi, representing a shift of about 24 hours compared to the high infection rate after 96 hours. This observation was in line with a drop of amoebal cell concentrations as wells as a decrease in percentage of infected amoebae. Simultaneous with the first PI fluorescence peak in *A. castellanii*/*L. pneumophila* 120 hpi, a second peak in the *A. castellanii*/*P. amoebophila*/*L. pneumophila* system was recorded.

In the presence of *P. amoebophila*, amoebal cell concentrations did only drop down directly after *L. pneumophila* infection (figure 15 d). After the first 48 hours they stayed at a similarly low level or did even rise slightly. This observation is statistically significant opposing to what was found in *A. castellanii*/*L. pneumophila*. There amoebal cell concentrations first increased until 96 hpi ($p=0.0313$), which was followed by a massive host lysis, reflected by strongly elevated PI values. The second round of infection of *A. castellanii* by *L. pneumophila* taking place after 120 hours seemed to be enhanced to some extend. After 144 hours, the biggest increase in the proportion of infected amoebae go along with an increased in PI fluorescence intensity and consequently also with a large decline in amoebal cell concentrations.

3.2.1.3 Performance of Viable *L. pneumophila*

To monitor changes in the number of viable *L. pneumophila* during the course of the infection, legionellae were enumerated by plate counts (figure 15 c). Between 2 and 48 hpi, a 19-fold increase in cfu was found in *A. castellanii*/*P. amoebophila*/*L. pneumophila*, which is more than twice as high compared to *A. castellanii*/*L. pneumophila*. In *A. castellanii*/*L. pneumophila* between 2 to 24 hpi there was an initial decrease in viable *L. pneumophila*. But after 24 hours this drop was followed by a steep increase in cell number, which continued to increase in contrast to *A. castellanii*/*P. amoebophila*/*L. pneumophila*. If *P. amoebophila* were present in the amoebae, at this time point numbers of viable *L. pneumophila* slowly went down until 120 hpi. Afterwards, the number of viable *L. pneumophila* stayed approximately the same. When comparing legionella counts in the two systems 168 hpi, a strongly significant ($p<0.001$) and approximately 2200-fold difference in CFU/ml could be observed.

3.2.2 Long term effect of *L. pneumophila* infection on *A. castellanii/P. amoebophila*

For the infection of the continuous *A. castellanii/P. amoebophila* culture with *L. pneumophila*, a theoretical MOI of 20 with about 30-40 % motile *L. pneumophila* was used for all replicates. By plating Legionella cultures right before infection, a real MOI of about 0.5 was determined for the first, and of about 0.35 for the second biological replicate.

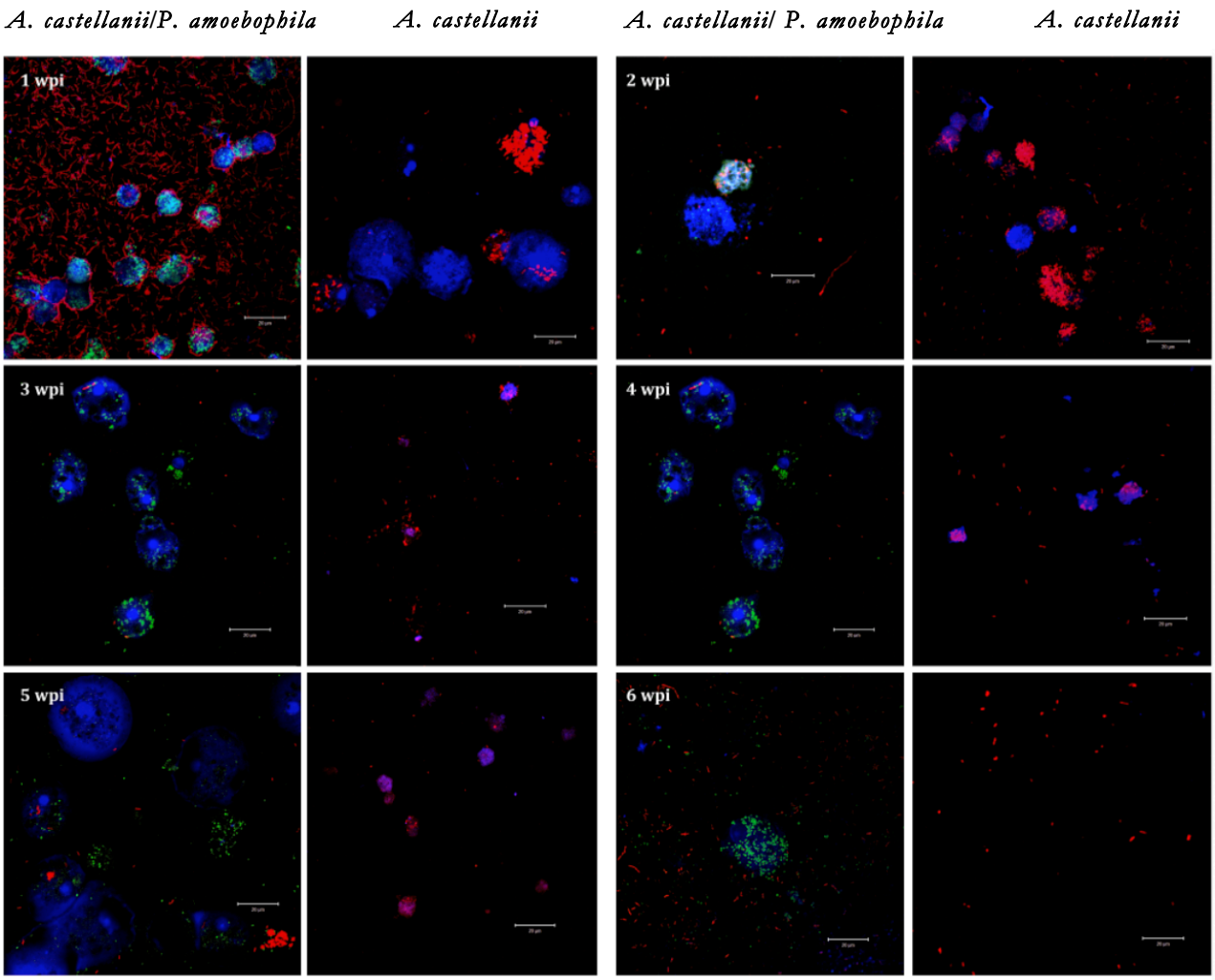

Figure 16. Long term co-cultivation of *L. pneumophila* in *A. castellanii/P. amoebophila*.
The course of infection over 5 weeks at 30 °C monitored and visualized by FISH with LEGPN-Cy3 (red), Chls523-FLUOS (green) and HCS stain (blue). The scale bar shows 20 μm.

3.2.2.1 Co-infection of *A. castellanii* with *P. amoebophila* and *L. pneumophila* monitored by FISH

The long-term effect of *L. pneumophila* infection either on *A. castellanii* or *A. castellanii/P. amoebophila*, was monitored by FISH (figure 16) and the proportion of infected amoebae was determined (figure 18).

After one week, infection rates in both systems were not statistically significantly different. Nevertheless, amoebae were in the presence of *P. amoebophila* small and roundish, compared to the larger and often trophozoite-like amoebae in the absence of an endosymbiont. Furthermore, in *L. pneumophila* in *A. castellanii/P. amoebophila* partially showed a filamentous morphology. Similarly to the observations made on short time scale, the proportion of *L. pneumophila* infected amoebae rapidly decreased in the *A. castellanii/P. amoebophila* system. Between weeks 2 and 6 more than 90 % of amoebae were uninfected. This was reflected by a high amount of amoebal trophozoites visible by FISH. In the absence of *P. amoebophila* the course of infection was dramatically different. More than 70 % of amoebae were already infected after the first week and this proportion did not change significantly until 5 weeks post infection. Amoebae detected in this system by FISH were usually very small, roundish and infected by *L. pneumophila* to a high level. The proportion of infected amoebae without endosymbiont after week 6 could not be quantified, due to the low number of amoebae on the FISH slide, but the infection rates were estimated to be similarly high.

As reflected by the differences between the courses of infection in both systems are considered as extremely significant ($p=0.0007$).

3.2.2.2 Impact of *L. pneumophila* Infection on Amoebal Fitness in the Presence or Absence of *P. amoebophila*

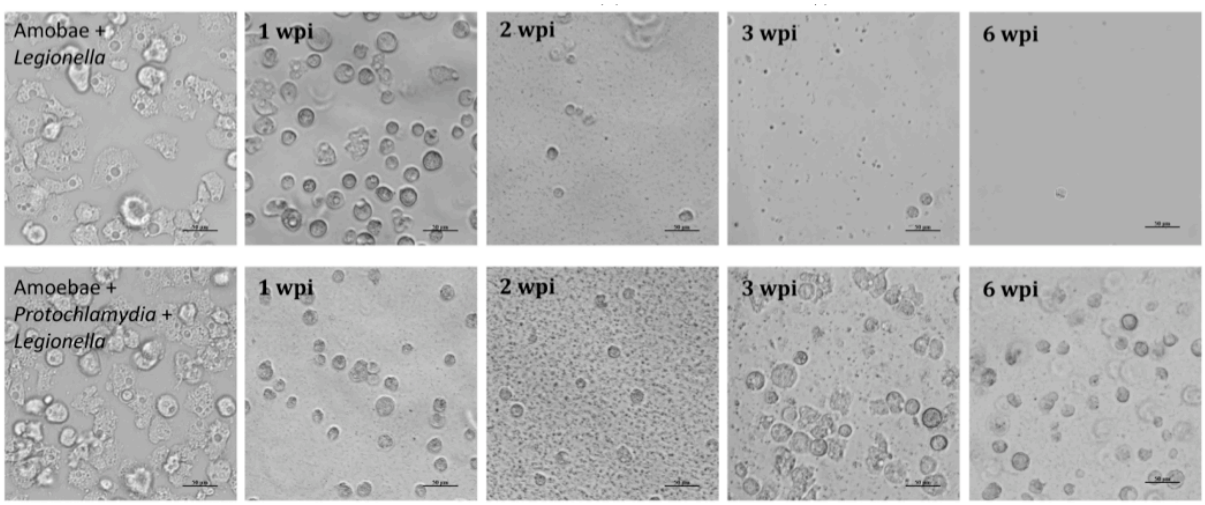

Figure 17. Impact of *L. pneumophila* infection on *A. castellanii* in the presence or absence of *P. amoebophila*.

The course of infection monitored over 6 weeks by phase contrast microscopy. Bars indicate 20 μm.

Every week amoebal cell concentrations were determined (figure 18 c,d) and amoebal cell densities and morphologies were monitored by phase contrast microscopy (figure 17).

Amoebal cell concentrations initially dropped down shortly after *L. pneumophila* infection, in the presence as well as in absence of *P. amoebophila*. Nevertheless, amoebal numbers were significantly lower in *A. castellanii*/*L. pneumophila* after 1 week ($p<0.05$). Amoebae continued to deplete, but in *A. castellanii*/*P. amoebophila*/*L. pneumophila* absolute numbers of amoebae began to rise again after 2 weeks. There, amoebae slowly recovered and almost reached amoebal densities found in the uninfected control *A. castellanii*/*P. amoebophila* after 6 weeks. This was in contrast to *A. castellanii* without endosymbiont where lysis constantly lowered amoebal cell numbers and only 0.3 % of initial amoebae were left after 5 weeks. Within weeks five and six this value approximately stayed the same and was equivalent to a more than 110-fold difference compared to amoebae numbers in the presence of *P. amoebophila* (18 d).

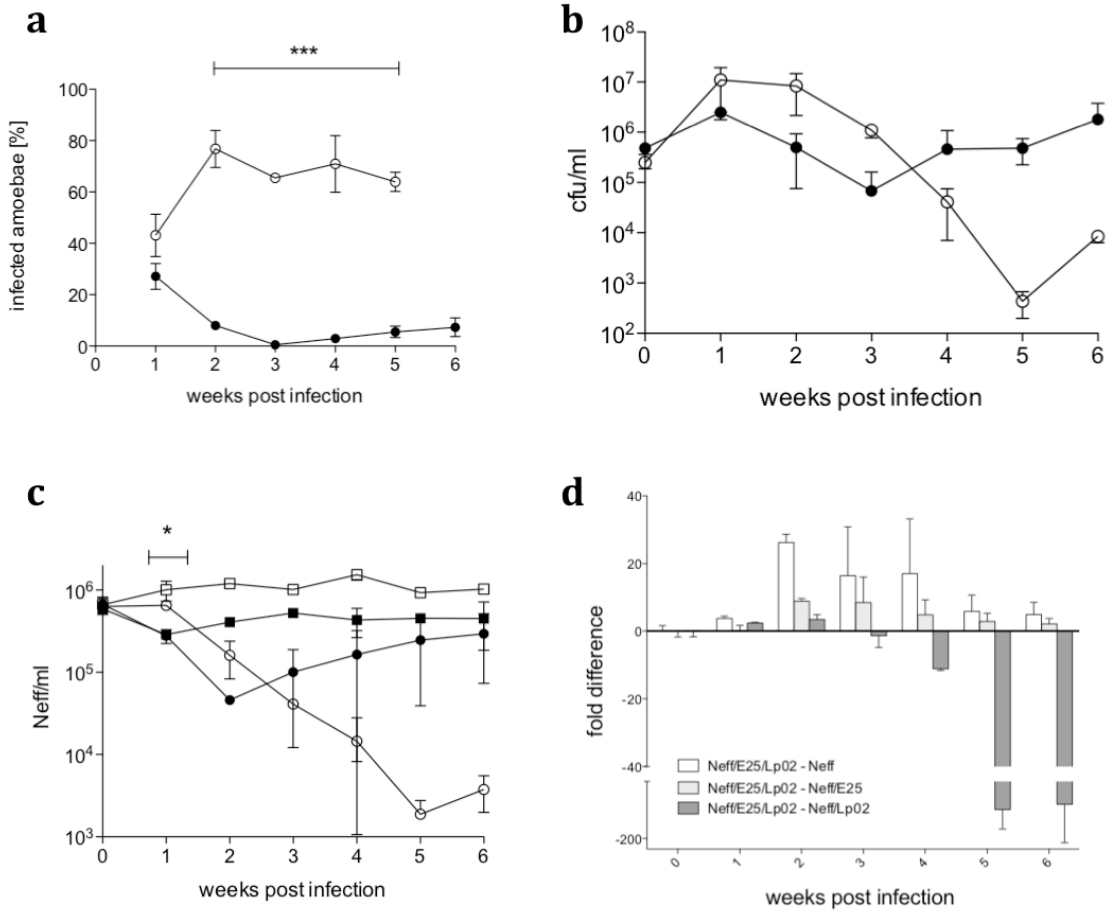

Figure 18. Effects of long term co-cultivation of *P. amoebophila*, *L. pneumophila* and *A. castellanii*.

The course of infection was monitored over 168 hours at 30 °C for the continuous culture *A. castellanii*/*P. amoebophila* infected with *L. pneumophila* (filled circles). The results are displayed in comparison to the control, represented by *A. castellanii* infected by *L. pneumophila* exclusively (open circles). Uninfected *A. castellanii* (open squares) and uninfected *A. castellanii*/*P. amoebophila* (filled squares) served as additional controls. Displayed are: (a) the percentage of *L. pneumophila* -infected amoebae; (b) the developing of amoebal numbers; (c) the number of viable *L. pneumophila* (d) the fold difference in amoebal concentrations between *A. castellanii*/*P. amoebophila*/*L. pneumophila* and the three controls. Bars showing a negative fold difference indicate an x-fold increase of amoebal cell concentrations compared to the controls. Data represent the mean ± SD, and are representative of at least two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001.

3.2.2.3 Performance of Viable *L. pneumophila*

Following the *L. pneumophila* numbers over 6-weeks it became clear, that in *A. castellanii*/*P. amoebophila*/*L. pneumophila* the numbers roughly remained in a range of 10^5 to10^6 per ml. This was contrary to the situation observed in *A. castellanii*/*L. pneumophila*, where within the first week a more than 44-fold increase in Legionella/ml occurred. These numbers stayed at a

similar level for about one week, whereas in the presence of *P. amoebophila* the proportion of culturable *L. pneumophila* already declined. Between weeks two and three, in the *A. castellanii*/*L. pneumophila* system *L. pneumophila* numbers were also reduced, this time at a rate similarly to the one found in *A. castellanii*/*P. amoebophila*/*L. pneumophila*. In contrast to this system in *A. castellanii*/*L. pneumophila* the dropdown of culturable *L. pneumophila* continued at an even higher rate, and after week 6 there were almost 30 times less legionellae compared to directly after infection.

As already mentioned above, it could be shown by FISH (figure 16), that over the course of the experiment *L. pneumophila* were not only present as rods or coccoid rods but also form filamentous structures. Short filaments appeared already one-week post infection and to a lesser extend between week 2 and week 5. Surprisingly this changed 6 weeks post infection and even stronger after 7 weeks, where filamentous *L. pneumophila* seemed to be the predominant form of legionellae. In contrast to the filaments found initially, those were enhanced in length to an even larger extend (figure 19). In addition to the prevalence of filaments, *L. pneumophila* overgrew most amoebae in the system 7 weeks post infection. These observations were made in all replicates. With the help of 16S rRNA sequencing, a successful cultivation on BCYE agar, and a lack of growth on nutrient rich LB-agar, the identity of those legionellae could be confirmed (data not shown).

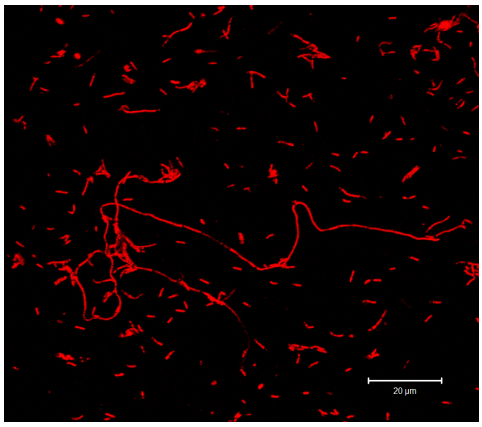

Figure 19. Filamentous morphology of *L. pneumophila* of a continuous *A. castellanii*/*P. amoebophila* culture infected with *L. pneumophila* 7 weeks post infection.

Visualisation by FISH with the LEGPN-Cy3 (red) probe. The scale bar shows 20 µm.

3.2.3 Inhibitory effects on *L. pneumophila* replication rates in *A. castellanii*

To elucidate a possible effect of *P. amoebophila* EBs on the uptake and the replication of *L. pneumophila*, *A. castellanii* was infected with *L. pneumophila* at theoretical MOI of 20. Prior to infection, different MOIs of either intact or lysed *P. amoebophila* EBs were added. Extracellular bacteria were not removed 2 hpi. Amoebae were harvested after 48 hpi, lysed and *L. pneumophila* viable counts were determined after 48 hours.

Dependent on the used MOI of chlamydial EBs, after 48 hours the *L. pneumophila* cfu/ml was lowered (figure 20) *L. pneumophila* numbers determined by viable counts were reduced by 45 % at a MOI of 10, and by 90 % at a MOI of 50. Using a MOI of 100 could only increase the observed effect slightly more, as *L. pneumophila* numbers were now reduced by 95 %. It did not matter if *P. amoebophila* EBs were lysed before addition or if they were intact. The effectiveness of EB lysis controlled by FISH revealed that only very few intact EBs were left (data not shown).

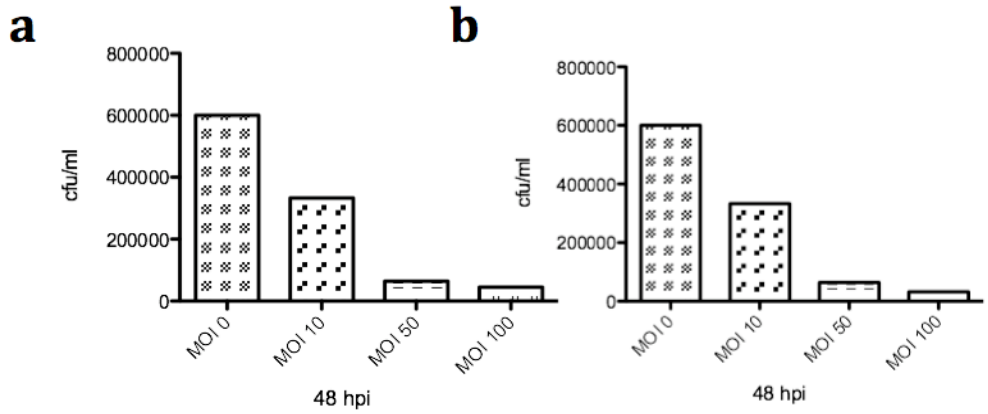

Fig. 20. Inhibitory effect of E25 EBs on *L. pneumophila* replication in *A. castellanii*.

A. castellanii was infected with *L. pneumophila* at a MOI of 20 in absence or presence of different MOIs of E25 EBs either (a) intact or (b) lysed prior to addition. The incubation period was 48 hours at 30 °C, The number of colony forming units determined by *L. pneumophila* viable counts is shown. Data shown were obtained from a single experiment.

CHAPTER 4

Discussion

4.1 Key Time Points in Developmental Cycle of Intracellular Bacteria

4.1.1 Considerations about the Experimental Setup

The initial aim of this project was to decipher key time points in parasite- or endosymbiont-host interactions of selected model-systems. The results showed clearly, that this could be achieved. However, there are some basic considerations to be taken into account regarding the methods used.

Within the last years, developmental cycles of intracellular bacteria have been studied in some detail, mainly by TEM and light microscopic analysis (Kahane et al., 2002; Wolf et al., 2000; Greub & Raoult, 2002; Tilney & Portnoy, 1989; Holden et al., 1984). In this project FISH was chosen to study the developmental cycles of *P. amoebophila*. *Parachlamydia* sp. and *L. pneumophila*. Fluorescence-labelled probes were used, which specifically target organisms of interest. In contrast to TEM, FISH allows for identification of organisms at a phylogenetic level. This is a big advantage over TEM, as with FISH morphologically similar or even identical organisms can be distinguished. Moreover, a differentiation between metabolically active bacteria, non-viable bacteria or bacteria in their infectious form may be undertaken. This is possible due to the binding of FISH probes to rRNA, in which signal strength tends to correlate positively with the ribosome content of a cell. Regarding bacteria, which are metabolically inactive or only weakly active, it is very likely that detection with FISH probes is not possible anymore. A combination of FISH with DAPI circumvents a total loss of detection, as DAPI stains nucleic acids independent from metabolic activity. Recent studies in our lab made use of this concept while studying the developmental cycle of *P. amoebophila* in *A. castellanii* (Diplomathesis König, 2009; PhD Thesis Haider, 2009). It could be shown, that the described method enables to roughly distinguish between the two main chlamydial developmental stages, as RBs always gave a FISH signal, whereas EBs usually did not (Poppert et al., 2002). Considering these facts, FISH seems to be a straightforward method for this project that allows answer questions about host infection rates and metabolic activity of bacteria simultaneously.

However, infection rates quantified by FISH should be considered with care, as a bias due to a possible influence of intracellular bacteria on the host´s replication rates might be possible. If that is the case, infected amoebae may suffer from increased generation times, and might more or less

be overgrown by uninfected amoebae. This "dilution effect" could play a significant role in the determination of the proportion of infected amoebae at a certain time point.

Another problem regarding the infection rates may be early cell death as a reaction on bacterial infection. In *Acanthamoeba* as well as in *Dictyostelium* the occurrence of apoptosis-like cell death was described (Feng, 2009; Cornillon, 1994). Even though it was previously shown in mammalian cells, that legionellae as well as chlamydiae apply strategies to inhibit host apoptosis, to successfully complete their developmental cycle (Santic et al., 2007; Paschen et al., 2008), nothing is known if and how this works in a protozoan host. If apoptosis-like processes happens to some extend, the outcome would be a reduced amount of infected amoebae. Nevertheless, in this study no further investigation on the impact of the "dilution effect" or apoptosis on infection rates were undertaken.

In addition to metabolic activity of intracellular bacteria, the impact of these bacteria on their host´s fitness, reflected by amoebal viability, was studied. Adversely influenced fitness may finally result in host cell death. In this study, cell death was monitored with propidium idodide (PI), which is a widely used dye to distinguish between live and dead cells. It does not pass through intact membranes, and can thus only enter a cell after its plasma membrane integrity got disrupted. If this is the case, PI intercalates with nucleic acids, which results in the emission of a strong fluorescence signal.
One disadvantage of PI is that if used alone, it is not possible to elucidate if cell death resulted from apoptosis or necrosis (Vermes et al., 1995). In the context of this study, this disadvantage can be disregarded, as only general cell death had to be considered. A possible bias of the measured PI fluorescence intensities could arise from accumulated free nucleic acids from previously died cells. This might lead to false positive results, especially at later time points. Nevertheless, its possible impact is assumed to be irrelevant for the overall outcome of this study, as the influence of two different temperatures on the system was compared and the bias may affect both to a similar level as well as the uninfected control.
Another problem of the PI stain may be a false positive result due to the presence of a high amount of non-viable bacteria, whose nucleic acids may also be targeted by PI and influence the measured fluorescence signal.

Besides host lysis, also the cell concentrations of amoebae were checked during the course of infection. This should give further insights into growth or lysis of amoebae at certain time points.

Harvesting cells and counting them with a haemocytometer is a standard method to estimate cell concentrations. However, it cannot be said for sure, if the harvesting strategy used in this project does completely de-attach all amoebae on a given surface. To keep error rates low and consistent, the harvesting procedure was standardised by re-suspending amoebae in the surrounding liquid medium always by pipetting 50 times. When trying to interpret similar cell numbers between two time-points, it remains elusive if it is the result of an inhibition of amoebal replication, or a steady-state between on-going replication and amoebal cell death. Therefore, amoebal numbers always have to be considered together with PI fluorescence intensities and image analysis by FISH.

In experiments involving *L. pneumophila*. colony forming units (cfu) were determined by plating, after lysing the amoebal cells. To obtain values reflecting the real situation in the system, the used lysis protocol plays an important role. There are several different methods described in literature and the performances of those methods are currently assessed in our lab (König, unpublished). In previous experiments, the lysis method used here, consisting of a freeze and thaw step followed by repeated passage through a needle, has proven to be fast, simple and still effective. Nevertheless, it cannot be excluded, that some amoebae did not lyse during this procedure, resulting in a lower amount of countable legionellae. However, the error would be present in different samples at a similar level, so the results are still comparable.

4.1.2 Developmental Cycle of *P. amoebophila* in *A. castellanii*

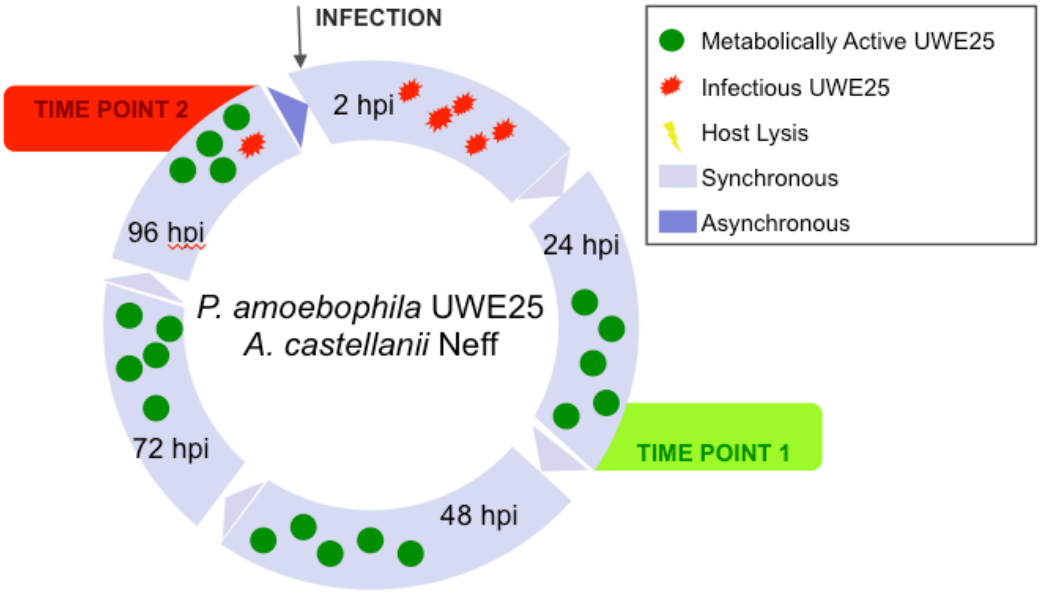

Figure 21. Key time points for transcriptomic analysis in the synchronous developmental cycle of *P. amoebophila* in *A. castellanii*.

The highest metabolic activity of the symbiont was determined to be 24 to 48 hpi. *P. amoebophila* is mainly present in its infectious form between 96 to 120 hpi. Incubation temperature was 30 °C.

The developmental cycle of *P. amoebophila* in *A. castellanii* was recently investigated in detail with similar methods used in this project (Diplomathesis König, 2009). Nevertheless, in this previous study the incubation temperature was 20 °C. Now the question had to be addressed, if the observed endosymbiotic effects are similar at 30 °C, or if there are any significant differences. This could be an option, regarding the fact that intracellular bacteria may switch from an endosymbiotic to a parasitic lifestyle, depending on the surrounding temperature. This was shown for *Parachlamydia acanthamoeba*, which was suggested to be endosymbiotic for *Acanthamoeba polyphaga* at 25−30°C and lytic at 32−37°C (Greub et al., 2003). Also, the interaction of *L. pneumophila* with *A. castellanii* does also seem to be temperature-dependent (Ohno et al., 2008).

Consequently, the developmental cycle of *P. amoebophila* was investigated at 20 and 30 °C. Considering the results obtained in this study, no significant differences were found at both temperatures used, neither regarding the course of infection, the metabolic activity of the

symbiont, nor the host´s fitness. The only temperature-related difference was an increase in PI fluorescence intensity at 30 °C after 144 hpi. However, the same effect was observed in the uninfected control. Assuming higher metabolic turnover rates in *A. castellanii* at 30 °C, a quicker depletion of nutrients in the surrounding medium would be the consequence, and might explain the observed higher cell death rates.

The course of infection of *P. amoebophila* in *A. castellanii* was quite similar to the outcome of previous studies in our lab (Diplomathesis König, 2009). After internalisation of chlamydial EBs, the transition from this infectious form to the metabolically active RBs took place within the first 24 hours. A bright signal from the *P. amoebophila* specific FISH probes signalled a phase of high metabolic activity between 24 and 72 hours, and the number of bacteria within infected amoebae increased subsequently. After 72 hours, the appearance of some exclusively DAPI-stained bacteria indicated the beginning transformation from RBs back to EBs. The proportion of EBs iteratively increased and reached a first maximum 120 hpi. This trend suggested the end of the developmental cycle to occur already between 72 to 96 hpi. After more and more EBs were released, new infections of previously uninfected, as well as already infected amoebae occured and the course of infection was be considered to be asynchronous.

The obtained results showed no detrimental effect of *P. amoebophila* on amoebal fitness, neither at 20 nor at 30 °C. Nevertheless, compared to the uninfected control, there was an initial delay in amoebal growth. This had already been reported previously for *P. amoebophila* in *A. castellanii*. regarding freshly infected amoebae (Collingro, 2004). However, after 120 hours at 30 °C and after 144 hours at 20 °C, amoebal numbers started to increase further and almost reached cell concentrations found in uninfected amoebae. This lets one assume, that already asynchronously infected amoebae propagate without handicap. The obtained data confirmed existing observations made recently in our lab (Diplomathesis König, 2009).

When following *P. amoebophila* infection rates, it becomes clear that immediately after infection, bacteria were more commonly found in the amoebal cytoplasm compared to the situation 24 hours later. Only half of amoebae were still infected then. This may be explained due to a failure in the establishment of infection and a digestion of those bacteria. As described above, another possibility may be a slow-down in the generation time of infected amoebae, which then get diluted by faster-dividing uninfected amoebae. However, the latter assumption can most likely be excluded, as in contrast to the first 24 hours, the infection rates did not vary between 24 and 48

hours, and exactly this would be assumed, if a "dilution effect" further plays a role. The proportion of infected amoebae began to rise between 48 to 72 hpi. This was contradicting to the finding, that after 48 hours most bacteria were still present as non-infectious RBs.. One could assume the transformation of RBs to EBs took place between 48 and 72 hours post infection. An alternative explanation for the observed spread of infection might be the transfer of bacteria to daughter cells during cell division of infected amoebae. After the occurrence of EBs 72 hpi, infection rates increased exponentially until 120 hpi. Considering the overall non-detrimental effect on amoebal fitness reflected by increasing amoebal numbers and non-elevated PI fluorescence intensities, bacteria-induced host-lysis most-likely played no role in spreading of infection. It is suggested that non-lytic extrusion of EBs made up the major part of the transmission of infective particles. This mode of exit from the host was reported to occur in addition to lysis for *Parachlamydia* sp. (Greub & Raoult, 2002) and for *C. trachomatis* (Hybiske & Stevens, 2007).

Taken together, it can be stated that *P. amoebophila* overall behave endosymbiotic in its amoebal host. Moreover, novel insights regarding different incubation temperatures allowed for the conclusion that the system is stable at 20 as well as at 30 °C.

Regarding the time points for the transcriptome analysis, the first should be chosen between 48 and 72 hpi, as there the highest metabolic activity of the symbiont was recorded. The second time point, shortly before release of bacteria already transformed to their infectious forms, should be selected between 96 and 120 hpi, where a massive reoccurrence of protochlamydial EBs was found..

4.1.3 Developmental Cycle of *Parachlamydia* sp. in *A. castellanii*

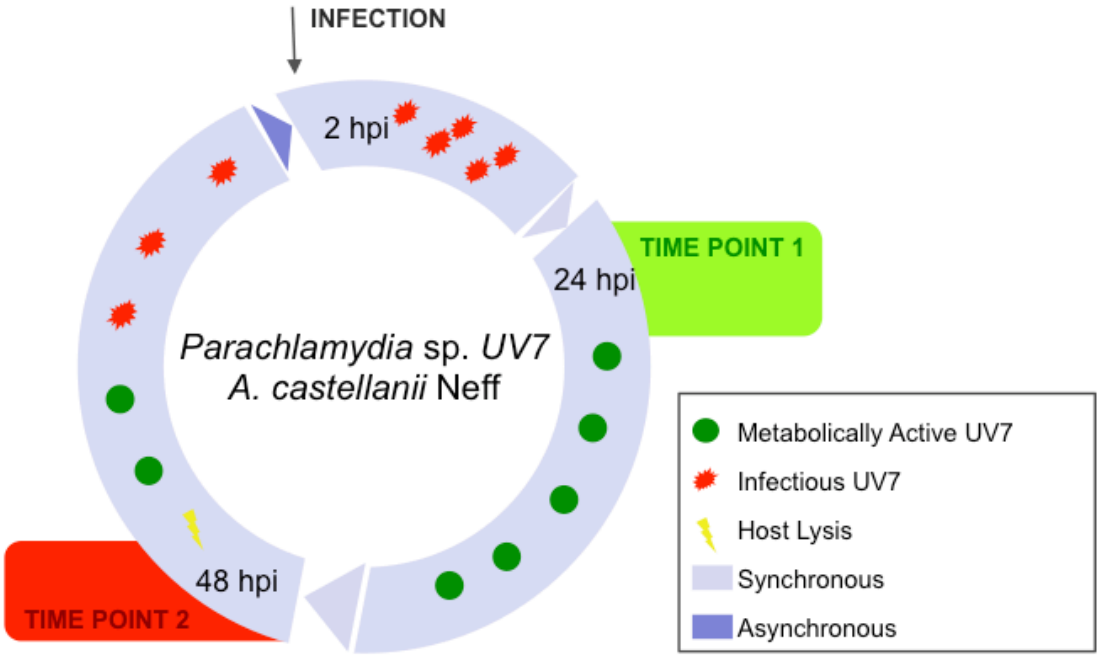

Figure 22. Key time points for transcriptomic analysis in the developmental cycle of *Parachlamyida* sp. in *A. castellanii*

The highest metabolic activity of the symbiont was determined around 24 hpi. *Parachlamydia* sp. is mainly present in its infectious form prior to host lysis around 48 hpi. Incubation temperature was 30 °C.

The first of the two parasite-host system studied, was *A. castellanii*/*Parachlamydia* sp.. *Parachlamydia* sp. is a close relative to *Parachlamydia acanthamoebae*, for which a lytic effect on its host was already shown at temperatures above 32 °C (Greub et al., 2003). In our lab, the developmental cycle of *Parachlamydia* sp. was recently studied in some detail in Vero cells (Collingro et al., 2005) and briefly also in *A. castellanii* (Diplomathesis König, 2009). Similar to experiments on the developmental cycle of *P. amoebophila*, the incubation temperature used here was 20 °C. But in contrast to *P. amoebophila*, *Parachlamydia* sp. showed parasitic traits at this temperature. This effect was supposed to be even more pronounced at a higher temperature like 30 °C. Taken together the results of the project at hand, this hypothesis could be confirmed. Beginning at 72 hours at 30 °C the PI fluorescence intensities increased strongly above already elevated levels at 20 °C, indicating massive amoebal lysis. In addition, this was also reflected by a decrease in amoebal cell densities 72 hpi.

In contrast to *P. amoebophila*, *Parachlamydia* sp. seemed to have a much higher replication rate. Within the first 24 hpi, initially single bacteria have multiplied to a high extend and were present in dense aggregates showing strong metabolic activity, as indicated by a bright FISH signal. As bacterial replication seemed to be accelerated, also the developmental cycle appeared to be. As soon as 48 hpi many bacteria were lacking a FISH signal. This indicated, that the transformation of RBs to EBs started already between 24 and 48 hours. In addition, some lysed amoebae were already found with light microscopy and FISH. These observations were confirmed by elevated PI fluorescence intensities and decreased amoebal cell numbers between 48 to 72 hpi, as described above. Moreover, FISH analysis revealed high amounts of extracellular *Parachlamydia* sp. EBs and already a large amount of lysed amoebae 72 hpi, which was not found to that extend after 48 hours. Thus, it can be concluded that the developmental cycle of *Parachlamydia* sp. was completed within 48 hours.

The key time points for the transcriptomic analysis were determined as follows. As there was only a narrow peak in metabolic activity and as it cannot be said when exactly the transition to EBs started, the key time point for metabolic activity of *Parachlamydia* sp. should be chosen as early as 24 hpi. Following the observations made, the time point where most bacteria were present in their infectious form and host lysis has not yet occurred to a high extend, was around 48 hpi. Compared to 20 °C, parasitic effects on the host were much more distinct at 30 °C. Therefore, this is the preferential temperature to be used in follow-up experiments.

4.1.4 Developmental Cycle of *L. pneumophila* in *A. castellanii*

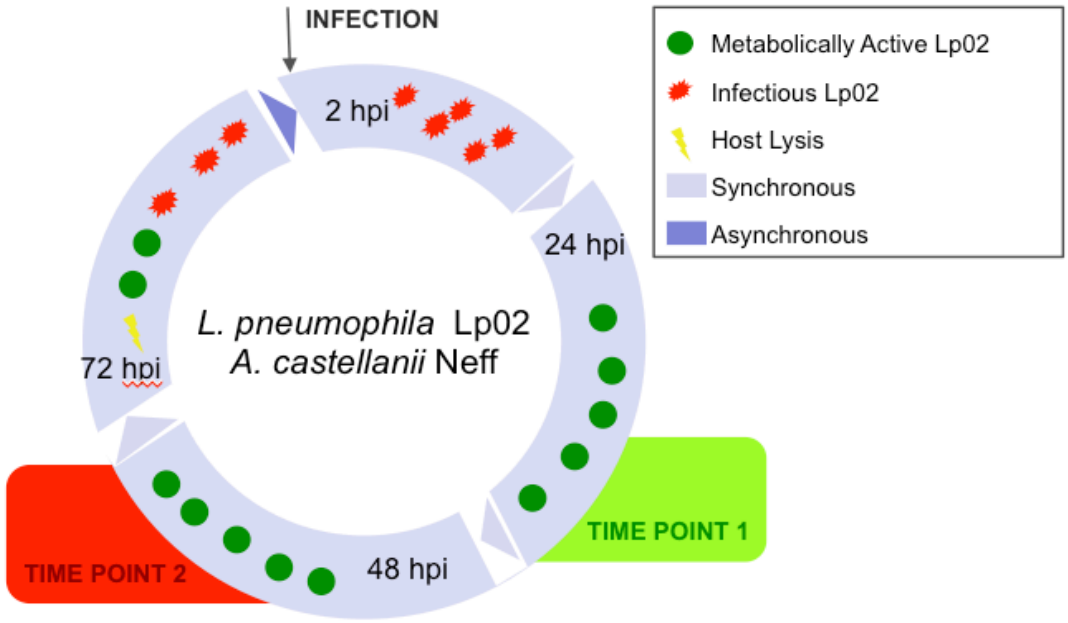

Figure. 23. Key time points for transcriptomic analysis in the developmental cycle of *L. pneumophila* in *A. castellanii*

The highest metabolic activity of the symbiont was determined 24 to 48 hpi. *L. pneumophila* is mainly present in its infectious form prior to host lysis between 48 to 72 hpi. Incubation temperature was 30 °C.

The second parasite-host system investigated in this study was *L. pneumophila* in *A. castellanii*. There are already different studies available about the developmental cycle of legionellae in amoebae (Gao et al., 1997). Nevertheless, so far no study has monitored a detailed *L. pneumophila* infection in amoebae by FISH. The in-vitro grown post-exponential and infective *L. pneumophila* are, in contrast to *P. amoebophila* or *Parachlamydia* sp. EBs, not reported to be metabolically inactive. Therefore, it could not be assumed, that *L. pneumophila* lack a FISH signal in this developmental stage. In this study in-vitro-grown *L. pneumophila* have not been tested if they give a FISH signal directly before they were used to infect *A. castellanii*.

At least 2 hpi, most legionellae could be detected by FISH. However, the correlation of FISH and DAPI signal showed that at later time points extracellular, as well as legionellae located in and around amoebae seemed to be actually lysed, often lacked a FISH signal. The appearance of legionellae not detectable by FISH could be a hint towards the presence of the mature infective form (MIF). This form was previously described as a highly infective form of *L. pneumophila* occurring exclusively in vivo after the exploitation of amoebal hosts, and was described to lack

metabolic activity (Garduno et al., 2002). At 30 °C some DAPI-only stained *L. pneumophila* appeared already after 48 hours, but to a much higher extend after 72 hours. An alternative option to the MIF, may be that legionellae lacking a FISH signal are present in metabolically inactive "viable but not culturable" form (Oliver, 2004). But the occurrence of the VBNC at those time points is very unlikely, as this stage usually is found in nutrient-depleted systems or as a reaction to the presence of certain deleterious substances, like disinfectants (Türetgen, 2008). Furthermore, co-cultivation of *L. pneumophila* and *Acanthamoeba* was even described as a way to recover legionellae from the VBNC (Steinert et al., 1998).

As mentioned above, directly after infection bacteria seemed to be metabolically active and started to replicate quickly. This led to the presence of legionellae in densely packed cytoplasmatic inclusions already 24 hpi. The FISH signal for those legionellae was very bright, indicating high metabolic activity. After 48 hours the situation was still similar. But then, after 72 hours there were more and more legionellae not showing a FISH signal and also the shape of the amoebae was strongly altered, indicating beginning host lysis.

When studying the developmental cycle of *L. pneumophila* in *A. castellanii*, the parasitic nature of those bacteria quickly becomes obvious and a higher incubation temperature seems to play a major role in the shape and strength of the observed effects. There was a quick spread in infection levels, indicating a continuous release of infective particles, throughout the developmental cycle. At 30 °C, the first increase in amoebal lysis occurred after 24 hours, as indicated by a depletion of absolute amoebal numbers. Nevertheless, PI fluorescence intensities were initially elevated only slightly, but then more remarkably after 72 hpi. *L. pneumophila* was expected to massively lyse and in turn re-infect other amoebae. Based on these observations, it can be stated, that the synchronous developmental cycle of *L. pneumophila* was completed after 48-72 hours.

Regarding the key time points, the peak of metabolic activity of *L. pneumophila* in *A. castellanii* was found between 24 and 48 hpi, and should be chosen as first time point for transcriptomic analysis. As described above, massive host lysis set in after 72 hours. Therefore, the second time point for transcriptomic analysis should be situated between 48 and 72 hpi. Similarly to *Parachlamydia* sp., parasitic traits were more pronounced in *L. pneumophila* at 30 °C, and follow up experiments should be undertaken at this temperature.

4.2 Co-Infection of *A. castellanii* with Protochlamydia and Legionella

4.2.1 Preface

The motivation for this study was to address the question, if *P. amoebophila* grant its host a better survival in the face of parasite infections. The reason to choose *L. pneumophila* for co-infecting acanthamoebae harbouring *P. amoebophila* lies at hand. Due to the worldwide occurrence of those three organisms, thriving in freshwater biofilms as a potentially shared habitat, it would be very likely that in the environment an established system like the endosymbiont-host system *A. castellanii*/*P. amoebophila* is the target of an infection by parasitic bacteria, like *Legionella*.

For different microbial systems a so-called "defensive mutualism" has been described (White & Torres, 2009). For example, *Paramecium* can harbour *Caedibacter caryophilus* as an intracellular bacterium, releasing toxins and therefore granting its host advantages in competition with other ciliates (Kusch et al., 2002). Furthermore, the ciliate *Euplotidium* has been described to live in a mutualistic relationship with certain *Verrucomicrobia*, which, in the form of ejectable epixenosomes, grant their host an effective defensive tool host against predation (Rosati et al., 1999; Petroni et al., 2000).

If a similar defensive mutualism could be shown for the model-system *A. castellanii*/*P. amoebophila*. this would contribute much to the understanding of the question why *Protochlamydia* is stably thriving in *Acanthamoeba* and the system is so successful, as indicated by its wide-distribution.

4.2.2 The course of *L. pneumophila* Infection in *A. castellanii* is altered in the Presence of *P. amoebophila*

Considering the results, it becomes clear, that the presence of *P. amoebophila* in *A. castellanii* strongly altered the course of *L. pneumophila* infection. Surprisingly, *A. castellanii*/*P. amoebophila* initially appeared to be more susceptible to infection by *L. pneumophila*. This was reflected by a steeper increase of infection rates in the beginning. In addition, higher replication rates of *L. pneumophila* were found, displayed by elevated infection levels within the first 48 hpi. Furthermore, *L. pneumophila*-infected amoebae with endosymbionts suffered in the beginning stronger, compared to amoebae without endosymbiont, as underlined by highly increased PI fluorescence intensities, as well as a quicker depletion of amoebae.

In contrast to *A. castellanii* without endosymbiont, in the presence of *P. amoebophila* amoebal cell concentrations dropped immediately after infection by *L. pneumophila*. Considering the experiments on the developmental cycle of *L. pneumophila* in *A. castellanii* it can most likely be excluded, that this was the effect of *L. pneumophila*-induced host lysis. Necrotic cell death was supposed to happen due to pore-forming activity of *L. pneumophila* after its developmental cycle is completed. But the detrimental effect on amoebae observed here already happened within the first 48 hours, where *L. pneumophila* was supposed to be actually still establishing the infection and replicating. As these observations were opposing to on-going amoebal growth found in the absence of an endosymbiont, the effect could be directly related to the presence of *P. amoebophila*. One possible explanation for the immediate decrease of amoebal cell numbers might be an induction of apoptosis-like cell death in the host (Feng et al., 2009). One could speculate if *L. pneumophila* modulates and inhibits apoptosis-like cell death in amoebae as it does with apoptosis in mammalian cells, and that this process is interfered by proteins expressed by *P. amoebophila*. Following this hypothesis, *L. pneumophila* infected amoebae would selectively be driven towards apoptosis-like cell death, but this is contradicting to the observed fast rise of *L. pneumophila* infection rates in *A. castellanii*/*P. amoebophila*. It remains elusive if early apoptosis-like processes in *A. castellanii*/*P. amoebophila*/*L. pneumophila* were linked to the depletion of amoebae in the beginning of the experiment. However, an improved protocol to specifically detect the impact of this mode of cell death might give an answer.

Another and more likely reason for early amoebal cell death might be host lysis as an outcome of an accelerated developmental cycle of *Legionella*. As already mentioned above, another remarkable

feature exclusively found in the presence of *P. amoebophila* is the fact that *L. pneumophila* was present in already highly elevated infection levels 24 and 48 hpi, indicating higher parasite replication rates. In addition, within the first 48 hours not only the infection levels were more advanced in the presence of *P. amoebophila,* also the number of viable *L. pneumophila* was 2-3 times larger. It looks like *L. pneumophila* propagation was supported in amoebae containing *P. amoebophila* as an endosymbiont. One possible explanation may be that amoebae partially digested their endosymbiont and obtained additional nutrients from it. As *L. pneumophila* replication is considered to be highly resource-demanding for the host, the presence of *P. amoebophila* as an additional food source may to some extend support intracellular growth of *L. pneumophila*. Contributing to this argument is the relatively high abundance of putative food vacuoles found in *A. castellanii*/*P. amoebophila* 24 hpi by FISH analysis. Interestingly, those vacuoles seemed to contain not only chlamydial RBs but often also legionellae. One could speculate that *P. amoebophila* was transferred into vacuoles containing replicating *L. pneumophila*. and subsequently digested due to the acidic environment within the Legionella-containing vacuole. Thereby, *L. pneumophila* could have been directly supplied with set-free nutrients. Follow-up TEM studies, targeting the composition of the observed vacuoles, have the potential to shed more light on this issue.

When the course of *L. pneumophila* infection in *A. castellanii*/*P. amoebophila* was further followed, after 48 hours sudden changes became obvious. The initial trend of a higher *L. pneumophila* susceptibility reverted and consequently, the proportion of infected amoebae was reduced. Nevertheless, PI fluorescence intensities stay high throughout the first week post infection and amoebal cell concentrations remained fairly low, indicating on-going host cell lysis. However, as judged by the increasing proportion of uninfected amoebae, one could guess, if amoebae either replicated staying uninfected, or if previously infected amoebae were cured. This finding was in contrast to what happened in the absence of *P. amoebophila*, as in the control system ultimately all amoebae got infected by *L. pneumophila* and eventually lysed. When considering the results further, it became obvious that the observed trend in *A. castellanii*/*P. amoebophila* continued and that following 2 weeks post infection amoebal numbers increased again and had almost fully recovered after 5 weeks.

Overall, the observed effect can already carefully be appraised to be a kind of acquired resistance against a parasitic infection granted by an endosymbiont. Several open questions regarding the causes and possible explanations are being discussed in the next chapter.

4.2.3 Possible Mechanisms Contributing to the Observed Effects

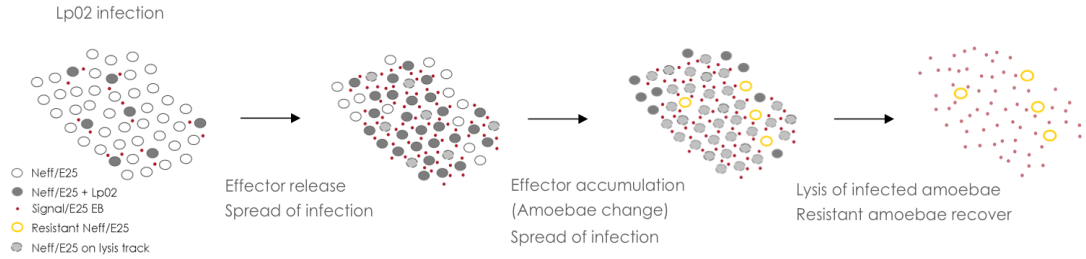

Figure 24. Model describing the influence of a putative *P. amoebophila* -dependent effector on the course of *Legionella* infection of a continuous *A. castellanii*/*P. amoebophila* co-culture.

The multiplication and dissemination of *L. pneumophila* in amoebae/*P. amoebophila* co-culture contribute to the spread and accumulation of a putative effector, which can either be a signal molecule or intact *P. amoebophila* EBs. After the effector has reached a certain concentration threshold, there is an effect on the system. The outcome could be a "warning" of surrounding Legionella-free amoebae, which then acquire resistance against further *L. pneumophila* infections. Finally, the subpopulation of resistant amoebae recovers.

One possible way how *P. amoebophila* may trigger the increased survival of its amoeba host in the face of parasitic infections could be the release of an effector into the amoebal cytoplasm as a response to *L. pneumophila* infection. After *L. pneumophila* -induced amoebal lysis, such an effector might then be set free into the surrounding medium and accumulates there. After a certain threshold is passed, an effect on surrounding amoebae might occur. Possible outcomes might either be a general unspecific inhibition of phagocytosis or a specific stop of *L. pneumophila* uptake. An alternative way to take up food would be pinocytosis, which might work especially in the given artificial system, containing the nutrient rich growth medium PYG. It is questionable however, if amoebae would obtain enough food in a natural environment, where phagocytosis is necessary for grazing.

Therefore, an alternative way such a putative effector might function would be by directly affecting *L. pneumophila* and diminish its virulence. In this case, taken up legionellae would simply be digested. Such an *P. amoebophila* -triggered effect on *L. pneumophila* could take effect either after the accumulation of the mentioned putative effector in the surrounding medium, or alternatively even directly in infected amoebae. Quorum-sensing would be a likely trigger, as it might ensure the survival of a population *A. castellanii*/*P. amoebophila* in an emergency-situation, without being of a too high disadvantage if only a few amoebae are infected. However, one should consider that up to now, no quorum sensing has been described for members of the *Chlamydiacea* and that also

the genome of *P. amoebophila* does not provide any homologues to quorum-sensing related molecules (Horn et al., 2004).

Besides the speculation about an effector molecule, the explanation of the observed effect could be much easier if *P. amoebophila* would act as an effector by itself. After amoebal lysis, *P. amoebophila* RBs and EBs are set free and may partially be degraded. Therefore again, an accumulation of an "effector" in the surrounding medium takes place, which could be either chlamydial EBs or chlamydial degradation products.

The question now is, how extracellular *P. amoebophila* or its degradation product might act and influence *L. pneumophila* infection in amoebae? A closer look at the mode of *L. pneumophila* uptake in *Acanthamoeba* might help. It is mediated by a lectin receptor (Harb et al., 1997) and furthermore, *L. pneumophila* uptake can be inhibited by adding certain sugars, like for example mannose (Declerck et al., 2007). If similar molecules, for example glycoproteins are present in the protochlamydial outer membrane, then the result would be a competition for the receptors responsible for *L. pneumophila* uptake. If now the competing protochlamydial substances have a higher affinity to the receptor, the outcome might be even a complete inhibition of *L. pneumophila* uptake into *A. castellanii*. This may explain observations, ranging from decreased infection rates to almost no *L. pneumophila* new-infections in *A. castellanii* after the first amoebae got lysed.

This competition hypothesis was followed up, by adding either intact or lysed protochlamydial EBs at different MOIs to *A. castellanii* without endosymbiont prior to *L. pneumophila* infection. During infection of *A. castellanii*, compared to *Legionella* numbers, there were either 0.5, 2.5 or 5 times as many protochlamydiae present. Surprisingly, the number of viable *L. pneumophila* was negatively correlated with the applied MOI of EBs or lysed EBs. Compared to numbers of viable legionellae in the control where no *P. amoebophila* EBs were added, an up to 19-fold decrease in *Legionella* yield after 48 hours of intracellular growth was found.

The observations made were strikingly different to the outcome of a similar study (Declerck, 2005). Although 1000 times more non-*Legionella* bacteria (*Escherichia coli*, *Aeromonas hydrophila*, *Flavobacterium breve*, and *Pseudomonas aeruginosa*) were added prior to the infection with legionellae. *L. pneumophila* uptake by *A. castellanii* was only reduced by less than a factor 3, and its replication rates were even increased.

Those findings confirm a causal relationship between the presence of either intact or degraded *P. amoebophila* EBs in the supernatant and the course of *L. pneumophila* infection in amoebae. However, due to the lack of image analysis by FISH, it remains elusive, if the observed lower number of viable *L. pneumophila* is the outcome of lower infection rates due to competition for receptors, apoptosis-like events in the host immediately upon infection as described above, generally decelerated *L. pneumophila* replication rates in the host, or a direct adverse influence on the viability and/or the culturability of *L. pneumophila*. Also, it should be noted that this experiment was undertaken without biological replication and that therefore the results have to be taken with care.

The next step would be to repeat the experiment and to refine it. The most important point would be to use FISH analysis for the quantification of infection rates and infection levels. EBs degraded by heat-inactivation could be included as an additional control. This treatment will alter and destroy protein compounds and might lead to a loss of the observed effect, if it is based upon those molecules. Furthermore, the experiment could be improved by using supernatants obtained from the co-infection experiment at certain key time-points. This would be followed by a filter-sterilization step to remove all viable particles. The supernatants could then be added to the growth medium prior to infection with *L. pneumophila*. If lowered infection rates in *A. castellanii* without *P. amoebophila* can be found, this would indicate that a soluble effector is present and related to the observed effects. Moreover, the supernatants should then be analysed regarding sugar, lipid and protein patterns.

4.2.4 Adaption of *Legionella* to its Environment

The observations made so far let one conclude, that *A. castellanii* harbouring *P. amoebophila* in the end overcomes *L. pneumophila* infection. Surprisingly, after 6 weeks there was a dramatic change in the co-infected system, as suddenly filamentous Legionella grew up in floc-like structures. Finally, after 7 weeks almost all amoebae were overgrown by *L. pneumophila*.

Filamentous legionellae were already detected one week post infection, followed by an only sporadically appearance until week 6. However, it has to be considered that the proportion of filamentous *L. pneumophila* detectable by FISH did not necessarily correspond to the real number legionellae showing this morphology at a certain time point. An explanation could be, that filamentous structures may get damaged easily during cell harvesting procedure, centrifugation steps and general handling.

In the literature the filamentous form in *L. pneumophila* has been reported several times in artificial systems (Berg et al., 1985; Loma et al., 1992; Pia et al., 2006), and it is thought to be the predominant *L. pneumophila* morphology in biofilms, especially at higher temperatures (Declerck, 2010). In the environment, the filamentous form was speculated to allow *Legionella* to anchor at locations were nutrients are present, to increase rapidly in biomass while accessing nutrients by eliminating the process of septum formation, and to proliferate and disperse in great numbers when conditions are appropriate (Pia et al., 2006).

It seems in the artificial system used in this experiment, *Legionella* was capable to build up a biofilm-like structure on the polystyrene surface of the culture flaks. This allowed these bacteria to persist in the system, as reflected by constantly high *Legionella* numbers, even after exchange of amoebal growth medium.

Assuming that *P. amoebophila* interferes with *L. pneumophila* virulence as discussed above, the outcome would be a much higher grazing pressure on *L. pneumophila*. If this hypothesis holds true, the formation of long filaments may represent a survival tactic. In other bacteria, the formation of a filamentous form has previously been described as such a strategy, allowing to resist phagocytosis by protozoans or to even evade cells of the human immune system (reviewed by Justice et al., 2008).

Regarding the low number of infected *A. castellanii* it can be speculated, that thriving in amoebae played a minor role, and *Legionella* were capable to grow extracellularly at low rates. Growth outside a host has recently been described as the so-called "necrotrophic growth", which allows *Legionella* to replicate at reduced rates on the debris of other organisms, like other bacteria or amoebae (Temmerman et al., 2006). In the study at hand, this phenomenon was only observed in the presence of *P. amoebophila*. Therefore, release and degradation of protochlamydiae, or metabolic byproducts of the endosymbiont-host system, could have provided proper nutrients. After amoebae recovered in the system, nutrients were used up faster due to a higher overall metabolic turnover, resulting in a depletion of oxygen in the system followed by a massive increase of amoebal cell death. In consequence, the high amount of nutrients set free at once, could have led to a sudden booth of *Legionella* growth.

Considering these facts, under the given conditions in the *A. castellanii*/*P. amoebophila* system, a switch of *L. pneumophila* to a grazing-resistant filamentous form would be a logical adaptation to a hostile environment for Legionella, in which there is increased grazing pressure and a lack of important nutrients, as propagation in amoebae is not possible anymore. It has to be kept in mind that the formation of filaments is only a compromise, as together with grazing resistance also the loss of infectivity is the outcome (Bornstein, 1984). This is a high price to pay, regarding the efficient replication of *L. pneumophila* in its amoebal host (reviewed by Declerck, 2010). But in the end the adaption is worth it, as legionellae now are grazing-resistant and have adapted to thrive extracellularly under suboptimal growth conditions.

4.2.5 Conclusion

The results obtained in this project do clearly show that the presence of *P. amoebophila* as an endosymbiont in *A. castellanii* alters the course infection of *L. pneumophila*. Within the first days *L. pneumophila* thrives more quickly in its host and that within 5 weeks post infection almost a full recovery of the co-infected system *A. castellanii*/*P. amoebophila* can be found. Therefore, *P. amoebophila* seems to grant its host an advantage regarding long-term survival in the face of parasite infections. It remains elusive if a putative effector released by *P. amoebophila* is responsible for the observed phenomenon or if *P. amoebophila* itself is on act. Nevertheless, *L. pneumophila* seems to adapt to this artificial environment by switching to its filamentous form and finally overgrows *A. castellanii*/*P. amoebophila*.

The findings obtained in this study may have wide-ecological consequences regarding the prevalence of *Acanthamoeba* harbouring bacterial endosymbionts. If one assumes that similar defensive mechanisms are present in other stable amoeba-endosymbiont associations, this may have contributed much to the evolutionary success of these systems, and may have limited the dissemination of parasites in natural and anthropogenic habitats. Furthermore, the occurrence of the filamentous form of *Legionella* in the artificial system used in this study, adapted to grow under nutrient-limited conditions, confirms and enhances recent findings how these bacteria persist in biofilms, and may finally broaden the understanding of the ecology of this widespread parasite and human pathogen.

LIST OF ABBREVATIONS

16S rDNA	small subunit ribosomal RNA-encoding gene of prokaryotes
%	percentage
°C	degree celcius
µ	micro (10^{-6})
16S rRNA	small subunit ribosomal RNA of prokaryotes
A.	*Acanthamoeba*
A260, A280	absorbance at 260, 280 nm
ANOVA	analysis of variance
C.	*Chlamydia* or *Chlamydophila*
cfu	colony forming units
Cy3	indocarbocyanine
DAPI	4',6-diamidino-2-phenylindole
$_{dd}H_2O$	double distilled and filtered water
DIC	differential interference contrast
DNA	deoxyribonucleic acid
EB(s)	elementary bodies
EDTA	ethylenediaminetetraacetic acid
$EtOH_{abs.}$	ethanol absolute
Fig.	figure
FISH	fluorescence *in situ* hybridization
FLUOS	fluorescein N-hydroxysuccinimidester
g	gram
hpi	hours post infection
Hsp60	heat-shock protein 60
kb	kilobases
l	liter
L.	*Legionella*
Lp02	*L. pneumophila* Lp02
m	milli (10^{-3}), meter
M	molar

m^2	square meter
Mb	megabases
Mg	magnesium
MIP	macrophage infectivity potentiator
MOI	multiplicity of infection
n	nano (10^{-9})
p	P value
P.	*Protochlamydia*
p.a.	pro analyticum (grade of purity)
PAS	Page's amoebic saline
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI	propidium iodide
PYG	peptone-yeast extract-glucose
RB(s)	reticulate bodies
rcf	relative centrifugal force
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	room temperature
s	seconds
SD	standard deviation
SEM	standard error of the mean
sp.	species
SPG	sucrose-phosphate-glutamate
TCA	tricarboxylic acid cycle
u	unit
UV7	*Parachlamydia* sp. UV7
v/v	volume to volume
vs.	versus
w/v	weight to volume
WB	washing buffer
x	times

ABSTRACT

Within the last years, there has been an effort in understanding the relationship between intracellular bacteria and their hosts. However, many details on mechanisms underlining endosymbiotic or parasitic interactions still remain elusive. The aims of the study were to (1) compare the developmental cycles of the amoebae endosymbiont *Protochlamydia amoebophila* and two amoebae parasites *Parachlamydia* sp. and *Legionella pneumophila*, and (2) address the hypothesis of increased amoebal survival in the face of parasitic infection when the endosymbiont was present. Developmental cycles in *Acanthamoeba castellanii* Neff at two different temperatures were roughly characterized by combining fluorescence-in-situ-hybridization (FISH) with the nucleic acid stain DAPI in order to estimate the symbiont's metabolic activity, and in addition, by assessing host viability using propidium iodide. The obtained quantitative data allowed for specification of crucial time points during our model stable and parasitic interactions, thereby facilitating a follow-up study on the transcriptomes of these systems. In order to test for a benefit of the host *A. castellanii* Neff in the presence of *P. amoebophila*, co-cultures and cultures of endosymbiont-free amoebae were infected with the amoebal parasite *L. pneumophila*. The course of infection was compared by FISH, and by quantification of amoebal as well as *L. pneumophila* cell numbers. Within the first days post infection *Legionella* replication was enhanced in amoebae with endosymbiont, resulting in higher rates of host cell death. After 2 weeks most amoebae with endosymbiont were uninfected and started to recover, reaching cell densities more than 100-fold increased relative to endosymbiont-free amoebae. However, in the presence of *P. amoebophila*, *Legionella* finally grew up as filaments in a biofilm-like structure, allowing it to persist in high numbers. The results obtained clearly show that the presence of *P. amoebophila* as an endosymbiont in *A. castellanii* alters the course infection of *L. pneumophila*. Considering filamentous *Legionella* as an outcome of the used artificial system, the host-recovery in the presence of *Protochlamyida* lets one conclude from a mutualistic relationship. Given the abundance of free-living amoebae in the environment as well as the frequent presence of endosymbionts in these protozoa, the observed phenomenon may hold widespread ecological and evolutionary implications.

ZUSAMMENFASSUNG

In den letzten Jahren gab es große Fortschritte im Verständnis der Beziehung zwischen intrazellulären Bakterien und ihren Wirten. Viele Details endosymbiotischer und parasitischer Interaktionen sind jedoch weiterhin unklar. Ziel dieser Arbeit war es Entwicklungszyklen des Amöbenendosymbionten *Protochlamydia amoebophila* und der beiden Amöbenparasiten *Parachlamyida* sp. und *Legionella pneumophila* zu vergleichen. Zum anderen wurde die Hypothese getestet, ob das Vorhandensein des Endosymbionten seinem Amöbenwirt eine erhöhte Überlebenswahrscheinlichkeit bei einer Parasiteninfektion verleiht. Die Entwicklungszyklen in *Acanthamoeba castellanii* Neff wurden bei zwei unterschiedlichen Temperaturen charakterisiert. Dabei wurde eine Kombination aus Fluoreszenz-in-situ-Hybridisierung (FISH) und DAPI angewendet , um Rückschlüsse auf die metabolische Aktivität der Symbionten ziehen zu können. Die Lebensfähigkeit des Wirtes wurde mit Propidiumiodid untersucht. Die erhaltenen quantitativen Daten ermöglichten die Bestimmung charakteristischer Zeitpunkte der endosymbiotischen bzw. parasitischen Interaktionen unserer Modellsysteme, welche die Basis für später darauf aufbauende Transkriptomik Studien bilden. Amöben-Kulturen mit und ohne Endosymbiont wurden mit dem Modell-Parasit *L. pneumophila* infiziert, um zu überprüfen, ob das Vorhandensein von *P. amoebophila* einen Vorteil für seinen Wirt *A. castellanii* bringt. Der Infektionsverlauf wurde mittels FISH und Quantifizierung von Amöben und Legionellen untersucht und verglichen. In endosymbiontentragenden Amöben kam es bereits innerhalb der ersten Tage nach Infektion zu einem beschleunigten Legionellen-Wachstum. Dies führte zu einer erhöhten Sterberate des Wirtes. Amöben mit Endosymbiont waren nach zwei Wochen uninfiziert und begannen sich zu erholen. Schließlich erreichten sie Zelldichten, die jene endosymbiontenloser Amöben um mehr als das Hundertfache überstiegen. Letztendlich kam es jedoch in der Gegenwart von *P. amoebophila* zu einem filamentösen Legionellen-Wachstum mit Ausbildung Biofilm-ähnlicher Strukturen, was das Überleben einer großen Anzahl von Legionellen zu ermöglichen schien. Die Ergebnisse zeigen deutlich, dass die Gegenwart von *P. amoebophila* in *A. castellanii* den Verlauf einer Legionellen-Infektion verändert. Versteht man das Auftreten filamentöser Legionellen als Folge des hier verwendeten künstlichen Systems, so kann man aufgrund der beobachteten Erholung des Wirts, auf eine mutualistische Beziehung zwischen *A. castellanii* und *P. amoebophila* schließen. Betrachtet man die weite Verbreitung freilebender Amöben in der Natur und die Häufigkeit ihrer bakteriellen Endosymbionten, so wird klar, dass die in dieser Arbeit beobachteten Vorgänge bedeutende Auswirkungen auf ökologische und evolutionäre Prozesse haben könnten.

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