Experimental approaches to understand the role of genetic and environmental influences on the microbial community associated with *Daphnia*

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Summary

Every multicellular organism on this planet is associated with a diverse community of microorganisms referred to as its microbiota. In recent years, myriads of effects resulting from this interaction have been revealed. The series of experiments condensed in this thesis aimed at investigating this relationship in more detail in planktonic crustaceans from the genus *Daphnia* and in particular the water flea *Daphnia magna*. The findings from these experiments provide important basic knowledge essential for future host-microbiota related research in this system.

Part one of the thesis comprises all experiments conducted by myself. In the first chapter I reciprocally transplanted the microbiota between the two species *Daphnia magna* and *Daphnia pulex* to examine the degree of specificity in these associations and determine the effects on host fitness as a consequence thereof. We found that being associated with a foreign microbial community did not negatively affect maturity, fecundity and size in both *Daphnia* species, letting us speculate that the two partners did not adapt to each other. This hypothesis was further supported by the insights gained when investigating the transmission of the whole microbial community, which was the purpose of the second chapter. Compositional comparisons between maternal and offspring microbiota revealed a stable fraction of microbes that gets transmitted, neither influenced by the origin nor the diversity of the microbial community.

The third chapter of the thesis aimed at assessing the stability of the *Daphnia*-microbiota association and to what degree established microbial communities were still modifiable. We paired and raised juveniles from three different locations (Belgium, Germany and Switzerland) in a full factorial design and compared their microbiota. We found that the microbiota is easily modifiable and that microbial communities greatly influence each other's composition. In addition, the results allowed the conclusion that the microbiota is not genetically controlled. We further investigated this in the fourth chapter dedicated to evaluate if the homozygosity level of the host influences microbial diversity by comparing the microbiota of selfed and outcrossed animals. From the results we conclude that the host genotype has little influence on the diversity of the microbiota.

Taken together, these results suggest that although microbial communities play a crucial role in *Daphnia*, the two partners did not adapt to one another leading us to propose a scenario of how transmission in the *Daphnia*-microbiota association might take place.

Part two of the thesis encompasses work resulting from collaborations. In chapter five, Alexandra Mushegian tested the role of bacteria for animal functioning, showing a positive effect on embryonic development under warm temperature conditions.

The goal of the last chapter, conducted by Karen Sullam, was to investigate the effect of temperature, host clone, and their interaction on host-associated microbiota. The experiment showed that the interaction of the factors affected microbial community structure while their diversity was more affected by host clonal background.

Part 1

Introduction

Starting the moment it is born, every eukaryote inevitably comes into contact with myriads of microorganisms, culminating in the formation of life-long mutualistic associations with a diverse community of microbial symbionts termed its microbiota. In recent years, these microbes have been shown to benefit the host in an incredible variety of ways. They help breaking down food components (Mackie, 2002; Poulsen et al., 2014), synthesize vitamins (LeBlanc et al., 2013; Snyder & Rio, 2015) and metabolize therapeutics into active compounds (Claus et al., 2011; Nicholson, Holmes, & Wilson, 2005). They provide colonization resistance to pathogens (Koch & Schmid-Hempel, 2011; Lawley & Walker, 2013), promote development (Bates et al., 2006) and train the immune system (Hill & Artis, 2010; Renz, Brandtzaeg, & Hornef, 2012). They even manipulate a host's behavior (Bravo et al., 2011; Neufeld, Kang, Bienenstock, & Foster, 2011), mood (Zheng et al., 2016) as well as food and mate choices (Leitão-Goncalves et al., 2017; Sharon et al., 2010). But the effects are not unidirectional and there are also host factors which in turn affect the microbial community. While some of these are unchangeable properties of the host such as age (Odamaki et al., 2016; Saraswati & Sitaraman, 2015), gender (Haro et al., 2016; Org et al., 2016), genotype (Benson et al., 2010; Estellé et al., 2014) and mode of birth (Dominguez-Bello et al., 2010; Rutayisire et al., 2016), others like diet (Martínez et al., 2013; Wu et al., 2011), medication (Becattini, Taur, & Pamer, 2016; Francino, 2016) and travel activities (David et al., 2014) can be deliberately influenced by the host. The long term persistence, evolution and consequences of these host-microbiota associations depend on two crucial aspects: (I) specificity of the host-microbiota interaction and (II) mechanisms by which microbes get acquired, maintained and transmitted.

Specificity can be categorized as either obligate or facultative. In the latter both partners are able to live on their own without negative effects. This is the case in several aphid species harboring symbionts not crucial for their survival despite affecting them positively, which are still able to return to their free-living form (Moya, Peretó, Gil, & Latorre, 2008; Oliver, Russell, Moran, & Hunter, 2003). In the former case at least one of the two or both would face negative fitness consequences. The most intensively studied case where both partners are not anymore able to survive on their own is the symbiosis of aphids and their endosymbiotic Gammaproteobacterium *Buchnera aphidicola*, living within specialized host cells (bacteriocytes) providing the host with essential nutrients lacking in the phloem diet (Baumann, 2005; Engel & Moran, 2013). The Hawaiian bobtail squid *Euprymna scolopes* and its bacterial symbiont *Vibrio fischeri* represent a classic example for a unidirectional

dependency. While the regulation of the circadian rhythm of the squid is highly dependent on the presence of *V. fischeri*, the bacterium can also be found free-living (Heath-Heckman et al., 2013; Ruby & Lee, 1998). Although some of these partnerships exist since millions of years (Douglas, 1998; Kwong et al., 2017; Munson et al., 1991), they need to be reestablished each generation anew. Thus, in the course of their long evolutionary history, elaborate mechanisms evolved to ensure acquisition of the desired microbes and transmission to the next generation.

There are two main transmission modes termed horizontal and vertical by which symbionts are transmitted, irrespective of whether they are beneficial or harmful. Vertical refers to the transmission of microbes from parents to their offspring, from one generation to the next. The afore-mentioned symbiont *Buchnera aphidicola* and the intracellular bacterial parasite *Wolbachia* (Taylor, Bandi, Hoerauf, & Lazdins, 2000; Werren, Windsor, & Guo, 1995) are prominent examples of this type. Vertical transmission often leads to obligate associations due to coevolution of host and symbiont (Moran, 2006) which can go thus far, that the symbiont begins to resemble an organelle (Dyall, Brown, & Johnson, 2004; Russell, Bouvaine, Newell, & Douglasa, 2013). Horizontal transmission, on the other hand, is characterized by the transmission between individuals not related in direct line either via host to host contact, a vector or uptake from the environment. The previously mentioned bioluminescent *Vibrio fischeri* as well as the influenza virus (Cowling et al., 2013; Killingley & Nguyen-Van-Tam, 2013) are well-known representatives using this route. But there are cases like the human immunodeficiency virus (HIV) that use both routes (Sirengo et al., 2014). This mode is termed mixed mode and probably the most common mode of transmission (Ebert, 2013).

In terms of acquisition, the same distinctions as described above for transmission apply. However, horizontal acquisition requires elaborate recognition mechanisms in one or both partners to ensure the selection of specific microbes from the environment for colonization (Bright & Bulgheresi, 2010). For instance, species-specific antimicrobial peptides have been shown to be responsible for the different microbial communities in four species of the cnidarian *Hydra* (Franzenburg et al., 2013). While vertical acquisition generally leads to microbial communities with rather reduced complexities, horizontal acquisition, in contrast, establishes taxonomically diverse communities. Once established, a stable state is maintained through cross-talk and cross-regulation between host and microbiota. In mice, symbiont-specific factors of *Bacteroides fragilis* were found to control the stability and specificity of the gut microbiota (Lee et al., 2013). Nevertheless, our understanding of the factors influencing

the establishment and maintenance of symbioses in non-model organisms is rather limited and rudimentary.

The ubiquity and stunning diversity of the effects required us to revise some of the basic assumptions about how living systems function through the lens of the mutualistic associations between microbes and multicellular organisms. With the constant development of new imaging technologies and the advances in molecular methods our knowledge continues to expand, altering our understanding of host-microbiota associations even more.

The study system

Daphnia are Cladocerans that that inhabit a variety of standing water bodies such as rock pools, ponds and lakes worldwide, often being the dominant member of the zooplankton community (Cottenie, Nuytten, Michels, & De Meester, 2001; Steiner, 2004). As a result of this, it has become one of the oldest model organisms in biological research with a well-studied natural history and ecology (Ebert, 2011; Lampert, 2011).

Daphnia reproduce by cyclic parthenogenesis, meaning that during the growth season they produce asexual offspring, switching to sexual reproduction when environmental conditions deteriorate (Ebert, 2005). The asexually produced eggs are deposited in the mother's brood chamber, an open system with water circulation located under the carapace, where they complete their development before being released. With the production of their first clutch of eggs after going through 4-6 juvenile instars the cycle starts over again. The outcomes of sexual reproduction are long-lasting diapausing embryos enclosed in chitinous shells composed of often melanized parts from the mother's carapace called ephippia, which can withstand harsh conditions. They are able to remain dormant for many years in the sediment before the embryos resume development as soon as the environmental conditions become favorable. *Daphnia* from this reservoir are the main contributors starting new populations at the beginning of the season (Hairston, 1996).

All these features make *Daphnia* an ideal study system for diverse questions. Besides experimental data based on life history traits such as growth, reproduction and survival that are straightforward to obtain, genotype effects can be relatively easy assessed too. Their mode of reproduction allows to maintain clonal lineages and to test multiple replicates of a genotype. Further, the ability of ephippia to be stored for long periods of time and embryos to survive harsh treatments (Sison-Mangus, Mushegian, & Ebert, 2014) are additional useful experimental features.

This work focused mainly on the species *Daphnia magna* although in some parts work with the species *Daphnia pulex*, *Daphnia longispina* and *Scapholeberis rammneri* was included. While the latter belonged to the genus *Scapholeberis* Schoedler 1858 and the other three species to the genus *Daphnia* Müller 1785, all belonged to the family Daphniidae Straus 1820.

Aim of this thesis

Daphnia-microbe related research has a long history, mainly focusing on the aspects of microbes as food (Brendelberger, 1991; Degans, Zollner, Van der Gucht, De Meester, & Jurgens, 2002; Gophen & Geller, 1984; Langenheder & Jürgens, 2001; Martin-Creuzburg, Beck, & Freese, 2011; Modenutti, C., Balseiro, & Reissig, 2003; Pace & Cole, 1994) or as causative agents of diseases (Ebert et al., 2016; Ebert, Rainey, Embley, & Scholz, 1996; Green, 1974; Vizoso & Ebert, 2004). For this reason, the ecologically important beneficial interactions between *Daphnia* and microbes remained unexplored.

In recent years, sequencing projects showed that different *Daphnia* species, although their ecological niches often strongly overlap, harbor distinct but similar microbial communities with relative low complexities that are different from the surrounding water and sediment (Eckert & Pernthaler, 2014; Freese & Schink, 2011; Qi, Nong, Preston, Ben-Ami, & Ebert, 2009). Further, these associations have been found to be stable for a prolonged period of time, as clonal lineages of *Daphnia magna* still harbor different microbial communities even after years of being kept under standard laboratory conditions (Pichon et al., unpublished). Experimental manipulation of the microbial community showed that the microbiota is crucial for *Daphnia* survival and development (Callens et al., 2016; Gorokhova et al., 2015; Mushegian et al., 2016; Sison-Mangus et al., 2014), emphasizing the complex interplay between *Daphnia* and microbiota affecting important aspects of an organisms life.

The first goal of this work was to investigate possible fitness consequences in two *Daphnia* species (*Daphnia magna* and *Daphnia pulex)* that might arise from being habituated to the coexistence with a specific community of microbes (**chapter 1**). The second objective was to determine how these microbial communities get transmitted to the next generation and if there are microbiota-specific transmission differences (**chapter 2**). Following up on this, we evaluated how already established microbial communities affect each other's composition when coming into close contact (**chapter 3**). In the last part of this work the aim was to get an idea of the influence the genotype of *Daphnia magna* exerts on the

diversity of its acquired microbiota by linking their homozygosity level to differences in microbial diversity (**chapter 4**). Taken together, we tried to move away from describing the composition of the microbial communities and move towards unraveling the principles regarding their establishment, stability and dynamics.

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Chapter 1

Reciprocal microbiota transplantation reveals specificity but no evidence for coevolved mutualism in sympatric zooplankton

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Author contributions: TMMS conceived the study. TMMS and DE designed the experiment. TMMS performed the experiment, all analyses and wrote the paper. DE revised the paper.

Abstract

Microbial communities form associations with their hosts that influence, among other things, the nutritional status and well-being of these hosts. As host-microbial relationships are generally considered to be specific, hosts may be expected to display poor health (dysbiosis) when they harbor microbiota transplanted from other host species. To experimentally test whether the origin of microbiota affects host fitness. We measured life history fitness traits (age at maturity, fecundity and body size) of two naturally co-occurring Cladocera species, *Daphnia magna* and *D. pulex*, in treatments with their native microbiota and with microbiota from three other, closely related Cladocera species. We found strong main effects in the microbiota from different host species, as well as host species by microbiota interactions. However, contrary to our hypothesis, we did not find adverse fitness effects for hosts with non-native microbiota. We conclude that zooplankton species from the same habitat benefit equally, on average, from the microbes they harbor, irrespective of their origin, and that, although specific interactions exist between hosts and their microbial consortia, they do not support the idea of coevolved mutualistic relationships.

Introduction

Throughout its lifetime, every organism inevitably comes into contact with diverse microorganisms. These interactions can range from parasitism, on one end of the spectrum, to mutualism, on the other end; however, it is often impossible to categorize these relationships clearly, as these categories can be fluid and depend on various factors. The importance of beneficial microbe-host associations (Moran, 2006) has been shown in many animal systems, and these associations can influence diverse aspects of an organism's life, such as nutrient uptake (Hehemann et al., 2010), protection against pathogens (Koch & Schmid-Hempel, 2011b; Silva et al., 2004) and mate choice (Sharon et al., 2010). Establishing and maintaining beneficial interactions between a host and its associated microbiota requires a certain level of specificity between the involved partners. While some associations are facultative, where both host and microbe could live on their own, others are obligate, where at least one of the two needs the other to survive. For example, the association between the Gammaproteobacterium *Buchnera aphidicola* and almost every aphid species is obligatory for both partners (Douglas, 1998; Munson et al., 1991). However, several aphid species harbor

additional symbionts that, although beneficial, are not essential for their survival (Moya et al., 2008; Oliver et al., 2003). This example elucidates the complex and diverse nature of interactions between hosts and their associated microbes.

For eukaryotes, it seems logical that the absences of bacteria would lead to negative fitness consequences (Brummel et al., 2004; Chung et al., 2012; Houthoofd et al., 2002), and that normal fitness could be restored by reestablishing the microbiota, if it is added early enough (Lenaerts et al., 2008; Rawls et al., 2004). Reciprocal transplant experiments have shown that even microbiota from another organism can be sufficient for such rescue efforts, though they do not restore health to the same degree as the native microbiota (Rawls et al., 2006; Salem et al., 2013). The reduced effectiveness of foreign microbiota has suggested that beneficial effects depend to some degree on the specificity of the host-microbiota interaction.

Specificity can be defined in a statistical sense as significant host-type times symbiont-type interaction. Thus, the expression of host traits depends not only on the potential main effects of the host and the symbiont, but also on the particular combination of the two. Specificity does not assume a particular form of interaction, but is indicated by the non-additivity of the host and symbiont effects. However, certain hypotheses posit explicit forms of specificity. For example, local adaptation testing requires that natural (coevolved) combinations have average trait expressions different from those of newly created combinations (Kawecki & Ebert, 2004).

Specificity in host-microbiota interactions can be investigated through reciprocal transplant experiments in which each host type is tested in combination with each microbiota type (Macke et al., 2017). Multiple studies have shown that resident microbiota support host nutrition (Chaston et al., 2016; Dobson et al., 2015; Hacquard et al., 2015; Huang et al., 2015) and, thus, directly affect host well-being. It is often assumed that these host-microbiota combinations have coevolved (Bäckhed et al., 2005; Frese et al., 2013; McFall-Ngai et al., 2012; Moeller et al., 2016), suggesting that a mismatch between microbiota composition and host species would reduce host fitness, a state known as dysbiosis (Martins dos Santos et al., 2010; Nicholson et al., 2012). We tested this hypothesis by conducting reciprocal microbiota transplant experiments with two *Daphnia* host species that co-occur in nature, replacing the host's native microbiota with microbiota from a different host to see if fitness was reduced.

The freshwater crustacean *Daphnia* is a promising model for microbiota research, given the large amounts of data available (Ebert, 2005, 2011; Stollewerk, 2010). With their strongly overlapping niches, the different species harbor distinct but similar microbial

communities (Qi et al., 2009) with relatively low complexity (Freese & Schink, 2011). Despite being crucial for host survival, the microbiota is not transmitted transovarially (Peerakietkhajorn et al., 2015; Sison-Mangus, Mushegian, et al., 2014) and is reestablished after hatching from resting eggs from the environment (Mushegian et al., 2017). Moreover, microbiota of *D. magna* have been shown to affect ecologically important functions (Macke et al., 2017). In our experimental transplant experiment, we found strong evidence for specificity in *Daphnia* – microbiota interactions, but did not find evidence to support the hypothesis that hosts with native microbiota perform better than those with foreign microbiota.

Material and Methods

<u>Organism and sampling site</u>

Four species of the filter-feeding Cladocera, all belonging to the family *Daphniidae* Straus 1820, were collected from the Ägelsee near Frauenfeld, Switzerland (site code = CH-H; coordinates = N 47.557769° E 8.862783°), a permanent pond that is covered with ice in winter. The species were *Scapholeberis rammneri*, *Daphnia magna*, *D. pulex,* and *D. longispina*. Field collected animals from each species were placed individually in jars filled with artificial *Daphnia*-medium (ADaM) (Klüttgen et al., 1994) to establish clonal isofemale lines. In addition, samples of surface sediment containing ephippia, chitinous shells that enclose the sexually produced resting eggs, were collected and stored in cold (4°C), dark conditions until further use.

Unless stated otherwise, all *Daphnia* cultures were kept under standard laboratory conditions for several generations before the experiment: 400-mL jars, ADaM, fed every other day with 50 Mio cells of the green alga *Scendesmus sp*., 16L:8D light:dark cycle and 20°C.

<u>Experimental set up</u>

Ephippia from *D. magna* and *D. pulex,* which are easily distinguishable and abundant, were collected from the pond sediment sample. Each egg is a unique sexually produced offspring. The resting eggs were separated from their protective shell, placed in falcon tubes filled with ADaM and kept in the fridge at 4°C overnight. Before assigning them to the different treatments, we surface-sterilized the eggs by removing excess ADaM, adding 2 ml of a 5% sodium hypochlorite solution (bleach), inverting the tube gently for 5 min, and then washing them three times with autoclaved ADaM. The sterile eggs were transferred in sets of three to

2-mL Eppendorf tubes containing 850 µL autoclaved ADaM and 150 µL of a microbiota inoculum. The microbiota inocula were obtained by homogenizing adult hosts, which involved filtering the suspension through a 7 µm mesh and diluting it with sterile ADaM to roughly the same final optical density (OD600 = 0.07 ± 0.01). Three independent clonal lineages of each of the four microbiota source species were selected (=origin), resulting in 12 unique microbiota inocula. Eggs of both host species (*D. magna* and *D. pulex*) were also treated in addition to the 12 microbiota inoculates. For each host-inocula combination, fifteen replicate tubes were prepared. Two types of controls were produced: Germ-free controls in which animals were bleach treated as before, but without adding microbiota, and natural microbiota controls, which were handled in the same way as treatment eggs, but received neither the bleach treatment nor the microbiota solution. Control animals contracted their native microbiota from the egg surface and the water. For each combination, additional replicates were prepared and frozen in TE buffer for later PCR screening to check for successful treatment application.

To induce hatching, all tubes with resting eggs were placed horizontally under a constant light source at 21°C. Hatchlings were left in the tubes for 24h before being transferred to 100-mL DURAN laboratory glass bottles containing autoclaved ADaM and axenic algae and sealed with membrane screw caps (SCHOTT AG, Mainz, Germany). Only one hatchling from each hatching tube was transferred to avoid pseudoreplication. In this way, 10 independent replicates per host – inocula combination (12 inocula x 2 host species x 10 replicates =240) and per control group (2 host species x 2 control types x 10 replicates = 40) were produced. The animals were fed every other day with axenic algae ad libitum and checked daily for eggs in their brood pouch, i.e. onset of reproduction (=maturity). When offspring were present, the adult animals were transferred to new bottles containing autoclaved ADaM and axenic algae, and the offspring were counted. Fecundity was monitored until day 21. At this time, body size was measured, and the animals were frozen individually in Eppendorf tubes containing 200 µl TE buffer. All procedures requiring sterile conditions were done in a laminar flow cabinet.

PCR screening of animals

To evaluate the successful removal and transfer of bacteria, PCR screening of a subset of three *Daphnia* per treatment was conducted. DNA was extracted using a slightly modified protocol from Edwards *et al.* 1991: TE buffer was removed from the frozen animals, and 200 µL

extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) was added. This mixture was homogenized with a pestle and centrifuged at 14'000 rpm for 5 min at 4 °C. We then transferred 150 µL of the supernatant to a new Eppendorf tube, added 150 µL of cold isopropanol (100 %), briefly vortexed it, and left at room temperature for 2 min before putting it at -20 °C for 15 min. Following centrifugation at 14'000 rpm for 5 min at room temperature, the supernatant was discarded, and the pellet was washed with 500 µL cold ethanol (70 %), vacuum dried and resuspended in 50 µL ddH_2O. Samples were stored at -20°C until further use. The bacterial 16s rDNA was amplified using the universal bacterial primer pair fD1 (AGAGTTTGATCCTGGCTCAG) and rP2 (ACGGCTACCTTGTTACGACTT). PCR conditions were as follows: 95°C for 5 min, 10 cycles of touch-down PCR of 94°C for 30 s, 55-45°C for 30 s, 72°C for 1 min, followed by 30 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 1 min and final extension at 72°C for 7 min. The DNA extracted from an untreated adult *Daphnia* and nuclease-free water served as positive and negative controls, respectively. To verify that DNA extraction was successful, samples were additionally screened with the primer pair mdh-F (TGCCTCGAAAAGAGGGTATG) and mdh-R (ATTGGCAGGATTACCCACAA) targeting the malate dehydrogenase (MDH) encoding region of *Daphnia*.

Axenic algae

Bacteria-free *Scenedesmus sp.* cultures were obtained using a procedure similar to the one described by Sison-Mangus et al. (2014). In short, algae cultures were treated with 1 mg ml^{-1} ampicillin, 50 µg ml^{-1} kanamycin and 50 µg ml^{-1} tetracycline simultaneously for three culture passages. We then conducted axenicity screening by PCR and phase contrast microscopy. DNA extraction, PCR conditions and bacterial primers were conducted as described above. The DNA extracted from an untreated algae culture and nuclease-free water were used as positive and negative controls, respectively. The success of DNA extraction was verified by screening the samples with the primer pair ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC), amplifying parts of the 5.8S rDNA encoding region and the internal transcribed spacer 2 of the algae.

Statistical analysis

Analyses were conducted separately for the controls and the reciprocal transplantation parts of the experiment using the statistics software JMP 11.0 (Cary, NC, USA). Life history data (age at maturity, fecundity and body size) were analyzed by fitting the following model: trait =

host + origin + inoculum (origin) + host x origin + host x inoculum (origin), followed by Dunnett's comparison tests where appropriate. Mortality was analyzed using Cox proportional-hazards regression, with the above-mentioned variables included in the model. Differences were considered significant when $P \leq 0.05$.

Results

PCR screening

Successful DNA extraction was verified for *Daphnia* and algae using the *Daphnia* and algae specific primers, respectively. No bacteria were detected in either the negative control treatment or in the axenic algae. Bacteria were, however, confirmed in the untreated controls and in the inoculated treatments.

Life history traits

Hatching rates of the resting eggs across the different treatments varied between 50-100%, which is within the normal range observed in other experiments (Allen, 2010; Haghparast et al., 2012; Vandekerkhove et al., 2004). In accordance with Sison-Mangus et al. (2014), germ-free *Daphnia magna* showed much higher mortality than individuals with natural microbiota. This finding also extended to *D. pulex* (Cox proportional-hazards regression, germ-free vs. natural microbiota: $X^2 = 49.04$, $p<0.0001$; host species: $X^2 = 2.12$, $p=0.15$; host x germ-free/natural: $X^2 = 0.77$, $p = 0.38$). In the reciprocal microbiota transplant treatment, individual mortality differed significantly among host species, with *D. pulex* having a higher mortality rate than *D. magna* (21.6 vs. 4.1 %; Cox proportional-hazards regression, host species: $X^2 = 19.75$, $p<0.0001$). Other factors did not affect mortality, and no harmful effects of the transplant treatments were detected (origin: $X^2 < 0.0001$, $p = 1.0$; inoculum (origin): $X^2 = 9.5$, $p = 0.3$; host x origin: $X^2 <0.0001$, $p = 1.0$; host x inoculum (origin): $X^2 = 9.91$, $p = 0.27$).

In both the transplant treatments and the controls, *D. magna* matured (i.e. produced first eggs) earlier than *D. pulex* (6.6 vs. 7.4 days) (Table 1A, Figures 1A and 2A). Time to maturity differed significantly among the microbiota origins as well as among the inocula within an origin, irrespective of the host species, suggesting that both the microbiota from the different source species as well as the inocula within different host individuals from the same source host species vary in their effects. Moreover, the two species responded differently to

the three inoclula within an origin (Figure 2A), resulting in a significant host by inoculum interaction (Table 1A).

Host fecundity differed significantly between the two control treatments, with germ-free animals from both host species producing many fewer offspring (Table 1B, Figure 2B). However, this difference is confounded by the fact that all germ-free animals died during the course of the experience, while the animals from the natural microbiota control treatment did not. In the microbiota transplant treatments, host fecundity differed significantly among the microbiota origins (Table 1B). Furthermore, the two host species interacted differently with the microbiota origin and the three inocula within an origin (Figure 1B, Figure 2B).

As expected, *D. magna* individuals in all treatments were substantially larger than *D. pulex* individuals (3.4 vs. 2.6 mm) (Table 1C, Figure 1C, Figure 2C). Otherwise, microbiota influenced body size differently depending on its origin, and the two host species responded differently to the inocula (Figure 2C). Body size of the germ-free animals could not be measured at day 21, as no germ-free animals survived that long.

Discussion

This reciprocal transplant experiment investigated the effect of native and foreign microbiota on fitness in two sympatric species of the freshwater Crustacean *Daphnia*. Although transplanting the microbiota from one individual to another is a standard procedure in microbiota research and has been done in diverse organisms (Ellekilde et al., 2014; Hosokawa et al., 2016; Ridaura et al., 2013; Thaiss et al., 2014), only few studies have transplanted microbiota reciprocally and monitored host fitness (Koch & Schmid-Hempel, 2012; Lau & Lennon, 2012; Salem et al., 2013; Sison-Mangus, Jiang, et al., 2014). Our study demonstrates that interspecies transfer of microbiota is possible in *Daphnia*, resulting in viable animals with normal phenotypes, and that the transfer of microbiota from related host species does influence host fitness, but is not generally harmful. While germ-free *Daphnia* suffer from substantial fitness loss, we found no adverse effects of foreign microbiota on host fitness, which counters our hypothesis that host species are associated with species-specific beneficial microbiota. These findings counter a study in two species from the insect family *Pyrrhocoridae,* where symbiont-deprived animals inoculated with their native microbiota had a significantly higher fitness than cross-inoculated animals (Salem et al., 2013). Our design, in which each host individual had a different genotype (hatchlings from sexually produced

resting eggs), did not allow us to test for a contribution of the host genotype, but instead emphasized the species level effect. The experiment uncovered host species specific microbiota interactions for all measured life-history traits (Table 1), indicating that host species contribute to the interaction and the outcome. These species-specific effects support findings from other organisms where host species specific microbiota associations have been described (Bolaños et al., 2016; Fraune & Zimmer, 2008; Koch & Schmid-Hempel, 2011a; Samad et al., 2017; Schultze & Kondorosi, 1998), with the bobtail squid - *Vibrio fischeri* system being the best studied example (Nyholm & McFall-Ngai, 2004; Visick & McFall-Ngai, 2000).

The apparent absence of significant benefits for *Daphnia* treated with native microbiota versus those treated with foreign microbiota, might stem from the *Daphnia*'s life cycle, which includes a resting phase during which bacteria are not vertically transmitted (Mushegian et al., 2017). Thus, the microbial community of *Daphnia* must be reestablished from the environment at the beginning of the growth season, which may involve a complex interplay between available microbes, the environment and host genetic effects. Together this creates a founder effect, producing a microbiota characteristic to the individual host. Some of these microbes may be selected by the host and perform specific functions, while others may be accidental opportunists that are able to colonize the host but have no specific function for it. Thus, a combination of deterministic and stochastic factors brings about the microbiota, producing a lasting community footprint. As *Daphnia* are able to reproduce asexually and care for their developing eggs in an open brood pouch, transmission of microbes from the mother to her offspring is possible, which maintains this footprint across asexual generations (unpublished results). When the *Daphnia* go into diapause, the individual clone-lineage effects disappear, while the species-specific effects are likely re-created every year after emergence from diapause. Similarly, although *Daphnia* brought into the laboratory maintain a characteristic microbiota, their microbial diversity decreases over time with captivity (Sullam et al., in prep.), as has also been observed in woodrats and Atlantic cod (Dhanasiri et al., 2011; Kohl et al., 2014) in captivity and in two species of the cnidarian *Hydra*, which maintain specific microbial communities under laboratory conditions for decades (Fraune & Bosch, 2007).

Our experiment revealed specific interactions between hosts and the microbiota they come in contact with, suggesting that hosts would benefit by selecting the best composition of microbes; however, this does not seem to happen, at least not on the species level. While we

cannot fully exclude that it happens on the host genotype level, the finding that our un-manipulated controls fared no better than the other *Daphnia*, makes this conclusion unlikely as well. Indeed, this host-centric view ignores the role of the microbes. A microbiota cannot be regarded as a fully functional and optimized community, but rather as an assemblage of diverse lineages with individual needs, some of which may be beneficial in a given context, others detrimental, all of them opportunistic. Furthermore, microbes interact not only with the host, but also with other microbes, creating a net outcome for the host that is difficult to predict. Finally, microbiota are also influenced by bacteriophages (Łusiak-Szelachowska et al., 2017; Ventura et al., 2011) that may have cascading effects on the host. In this light, it would be surprising not to find specificity in host-microbiota interactions. A host-centric view of microbiota function, thus, captures too little of the picture. But why do some studies find beneficial effects of native vs. foreign microbiota? The answer may be related to the yearly cycles of diapause in *Daphnia*, that possibly purge the microbiota encountered in the previous season completely. The formation of specific and, on average, beneficial microbiota may require more stability than is inherent in the *Daphnia* system. Furthermore, since *Daphnia* inhabits short-lived standing freshwater habitats, there may not be enough long term stability for highly specific associations to evolve with horizontally acquired symbionts, just as in the marine highly specific bobtail squid – Vibrio system (McFall-Ngai, 2014). Thus, while highly dependent on microbiota for normal functioning, *Daphnia* may have evolved to cope with diverse microbiota that provide, on average, the functions needed. In addition, environmental factors within specific seasons may also select for hosts that carry the most beneficial microbiota. It has been shown, for example, that certain microbiota in the *D. magna* system,protect hosts from the harmful effects of cyanobacteria (Macke et al., 2017). Whether host genotypes select for these microbiota to gain a beneficial function, or whether associations are largely a chance result of the post-diapause lottery is not clear, but would be an important point to explore further.

Because Cladocerans of the family Daphniidae are freshwater planktonic filter feeders, it is tempting to assume that every host is constantly exposed to all local microbes in the water. This assumption, however, is inconsistent with our observation that the inocula showed clear differences. Such differences in the light of constant homogenization seem as if they would be difficult to maintain, as they would require strong selection on the side of the host (McFall-Ngai et al., 2012; Nyholm & McFall-Ngai, 2004). An alternative possibility is that the differences are produced by founder events during hatching from diapause, which

helps to maintain this observed diversity. Such priority effects (advantage for the first colonizers) have been well observed in community ecology (Lockwood et al., 1997; Louette & De Meester, 2007; Weidlich et al., 2017) and may play a role in the *Daphnia* system as well.

Microbes can manipulate the host through a variety of ways (Hooper et al., 2001; Larsson et al., 2012; O'Shea et al., 2012). The most prominent, and probably the best studied, way is host nutrition (Hacquard et al., 2015; Wong et al., 2014). Metabolites produced and released by the microbes can, among other things, provide energy that influences the host's nutritional status (Tremaroli & Bäckhed, 2012). Thus, if the composition of the microbiota changes, the metabolic profile of the community and, subsequently, the quality and quantity of the provided nutrients may change too. In crustaceans, bacteria contribute to the breakdown and absorption of essential compounds (Bui & Lee, 2015), and these nutrients, in turn, affect host fitness (Martin-Creuzburg & Von Elert, 2004; Taipale et al., 2012). The functional significance of *Daphnia* life history traits are well documented as fitness indicators and are strongly affected by diet (Bradley et al., 1991; Cuhra et al., 2015; Ebert, 1994; Vanni & Lampert, 1992). Thus, we expected that mismatches between microbiota and hosts would impact these life history traits. However, our results showed the opposite: that host-microbiota mismatches did not inevitably weaken life history traits; these traits are influenced to different degrees even by the microbiota within the native host-microbiota combinations of the same species. These results were unexpected, since we expected hosts with foreign microbiota to do poorly compared to native combinations and we expected those native combinations to be fairly stable. At the same time, the results indicate that microbial communities probably differ in their ability to effectively harvest and provide additional energy.

Conclusion

Although *Daphnia* benefit from functions provided by microbes, it does not seem to matter whether a native or a foreign microbial community provides these functions. This finding suggests the absence of long-term coevolved mutualism between *Daphnia* and their microbiota. Nevertheless, there are strong *Daphnia* - microbiota interactions, with some combinations providing more benefits than others. Whether this pattern is caused by stochastic effects (e.g. founder events after breaking diapause) or host genetic effects is not clear, but would be important to explore further. It also remains to be determined if the

apparent absence of host-specific beneficial microbiota is an adaptation to the instability of the *Daphnia* system, which is characterized by strong seasonal patterns, yearly diapause and short-lived freshwater habitats, or if the instability prevents the evolution of stable beneficial microbiota.

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Table 1: Analysis of variance of A) maturity, B) fecundity and C) body size for the control and the reciprocal transplant treatments. The factors have the following categories: host (*D. magna*, *D. pulex*), condition (natural microbiota, germ-free), origin (*D. magna*, *D. pulex*, *D. longispina*, *S. rammneri*), inoculum (Magna [M1, M2, M3], Pulex [P1, P2, P3], Longispina [L1, L2, L3], Scapholeberis [S1, S2, S3]). Body size data for germ-free animals are missing, as all animals died before being measured. Significant p-values are shown in bold.

A

Maturity	Source	df	MS	F	P
Controls	Host	1	11.23	4.33	**0.045**
	Condition	1	4.49	1.73	0.19
	Host x condition	1	0.02	0.01	0.93
	Error	34	2.59		
Reciprocal transplant	Host	1	28.78	26.57	**<.0001**
	Origin	3	13.33	12.31	**<.0001**
	Inoculum (origin)	8	2.81	2.59	**0.0106**
	Host x origin	3	2.80	2.58	0.054
	Host x inoculum (origin)	8	3.45	3.19	**0.021**
	Error	180	1.08		

B

Fecundity	Source	df	MS	F	P
Controls	Host	1	11.58	0.14	0.72
	Condition	1	13684	159.72	**<.0001**
	Host x condition	1	798.95	9.33	**0.0044**
	Error	34	85.67		
Reciprocal transplant	Host	1	846.74	2.58	0.11
	Origin	3	1500.03	4.57	**0.0041**
	Inoculum (origin)	8	606.41	1.85	0.071
	Host x origin	3	1239.97	3.78	**0.0116**
	Host x inoculum (origin)	8	683.58	2.08	**0.0397**
	Error	180	328.32		

C

Body size	Source	df	MS	F	P
Controls	Host	1	3.94	190.50	**<.0001**
	Error	17	0.02		
Reciprocal transplant	Host	1	31.72	1258.87	**<.0001**
	Origin	3	0.09	3.57	**0.0152**
	Inoculum (origin)	8	0.02	0.94	0.48
	Host x origin	3	0.03	1.06	0.37
	Host x inoculum (origin)	8	0.05	2.04	**0.0442**
	Error	180	0.03		

Figure 1: Interaction between host species and microbiota origin: Bar plot shows the mean (± SEM) per origin of the life history traits A) maturity, B) fecundity and C) body size. N indicates native host species-microbiota origin combinations. Microbiota origins: M: *D. magna*, P: *D. pulex*, L: *D. longispina*, S: *Scapholeberis*. Statistical comparisons were performed using analysis of variance, followed by Dunnett's test. *** p<0.001, ** p<0.01, * p<0.05, + p<0.1.

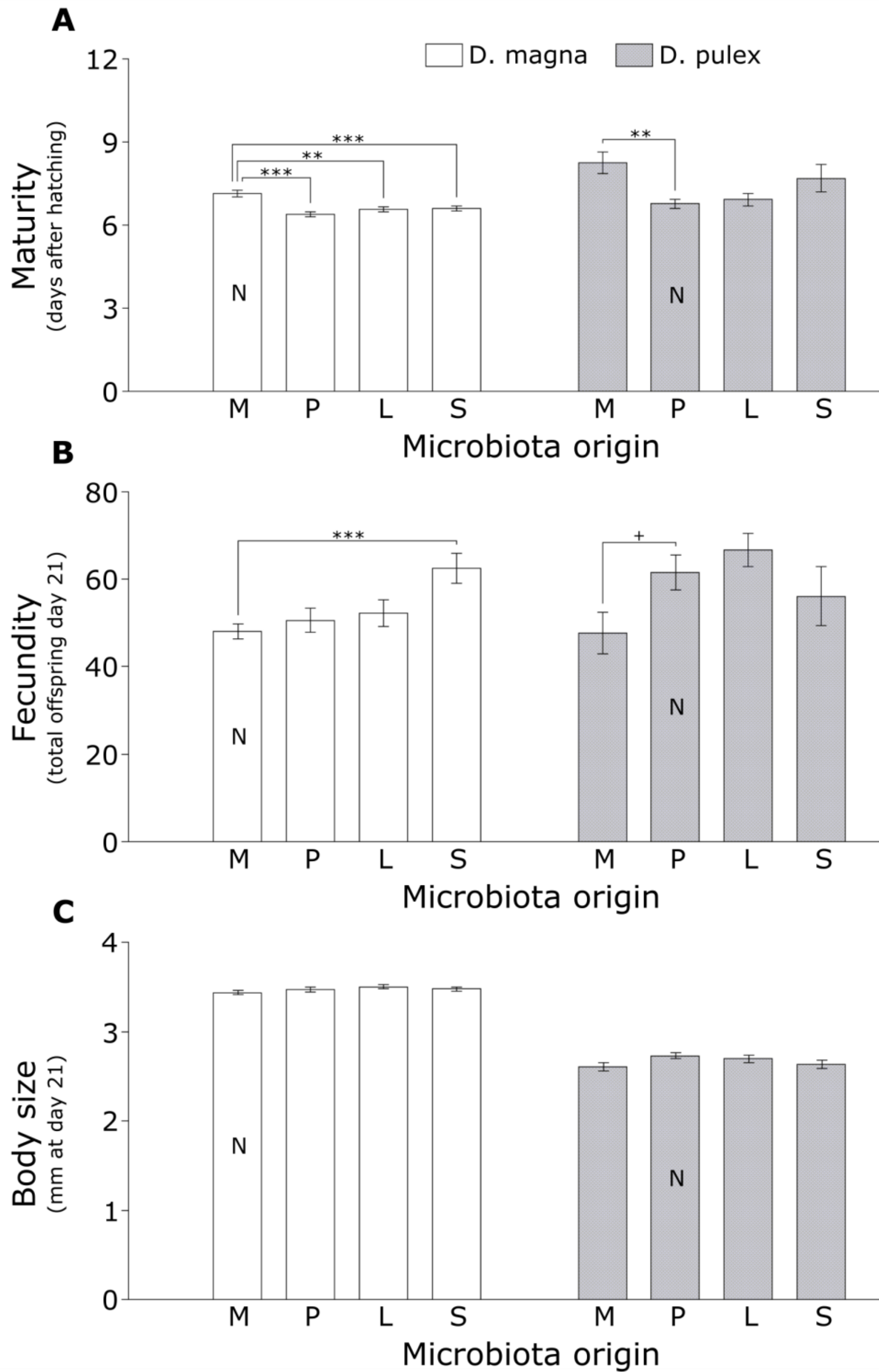
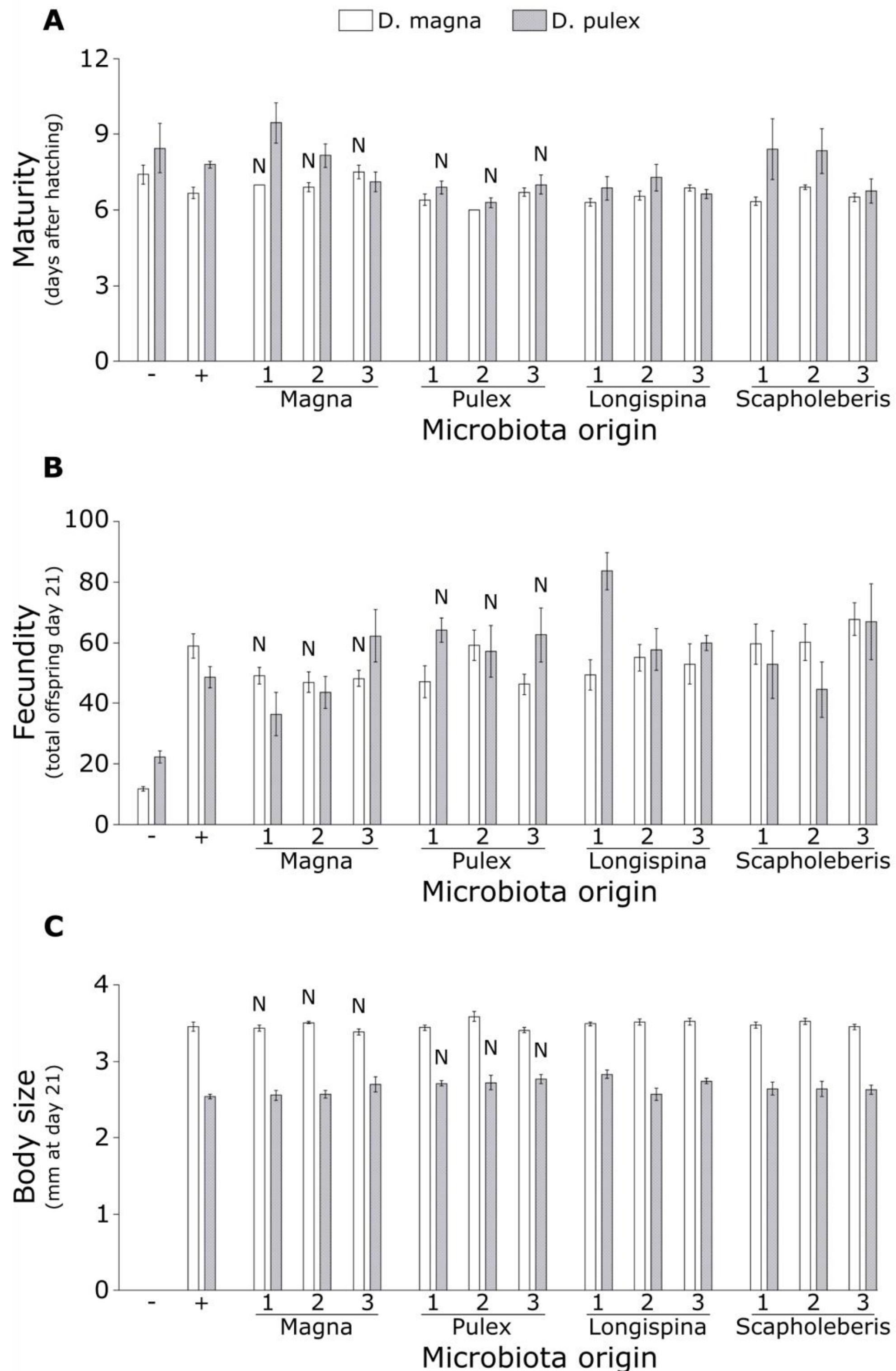


Figure 2: Interaction between host species and inoculum: Bar plot shows the mean (± SEM) per inoculum of the life history traits A) maturity, B) fecundity and C) body size. N indicates native host species-microbiota origin combinations; - stands for germ-free animals; + stands for natural microbiota (untreated control).



Chapter 2

Origin of microbiota does not influence transmission but composition of the microbial community in the water flea *Daphnia magna*

Tobias M.M. Schär, Jean-Claude Walser and Dieter Ebert

Author contributions: TMMS conceived the study. TMMS and DE designed the experiment. TMMS performed the experiment, sequencing library preparation and analyses. JCW performed sequence quality control, OTU selection and taxonomic assignment procedures. TMMS wrote the paper. DE revised the paper.

Abstract

Specific host-microbiota associations can be ancient, dating back millions of years. A key aspect for such associations is transmission to the next generation. With time the partners may coevolve, leading to mutualistic partnerships. To better understand transmission of microbiota, we study microbial communities of the freshwater Cladoceran *Daphnia magna*. Germ-free juveniles were exposed to microbiota coming from either conspecifics or three other species belonging to other members of the family of *Daphniidae*. Their microbial composition and that of their offspring was analyzed using 16S rRNA amplicon sequencing. Compositional microbiota comparisons between mothers and offspring revealed that the fractions of shared and unique operational taxonomic units (OTUs) were quite uniform across the different treatments and not affected by microbial diversity. If the partners coevolved and adapted to each other one would expect to find a larger fraction of shared OTUs in native host-microbiota combinations as compared to associations with a foreign microbiota. Since all here tested communities get equally well transmitted, there is no support for improved transmission due to coevolution of host and microbiota in this system. We propose an alternative scenario explaining transmission in the *Daphnia*-microbiota association.

Introduction

All eukaryotes form life-long mutualistic associations with diverse communities of microbial symbionts, resulting in diverse ecological functions (Engel et al., 2016; McFall-Ngai et al., 2013; Moran, McCutcheon, & Nakabachi, 2008). Some of these relationships are facultative as in several aphid species that harbor symbionts not crucial for their survival despite affecting them positively (Moya, Peretó, Gil, & Latorre, 2008; Oliver, Russell, Moran, & Hunter, 2003). These microbes are still able to return to their free-living form. Others became obligate symbionts and have been shown to coexist with their host for millions of years: For example, the five core bacterial lineages of modern social bees were acquired around 80 million years ago by their last common ancestor, still forming a major part of their gut microbiota (Kwong et al., 2017). Also the dependency between the Gammaproteobacterium *Buchnera aphidicola* and almost every aphid species began about 200 million years ago and is obligatory for both partners in order to survive (Douglas, 1998; Gil, Sabater-Muñoz, Latorre, Silva, & Moya, 2002; Munson et al., 1991). Although these partnerships are ancient, they need to be

reestablished each generation anew. In the course of their evolutionary history, elaborate mechanisms evolved to ensure a close association from generation to generation, with highly reliable transmission.

There are two main transmission modes termed horizontal and vertical by which symbionts are transmitted, independent of whether they are beneficial or harmful. Vertical transmission is the transfer from parents to their offspring, from one generation to the next. The aphid symbiont *Buchnera aphidicola* and the intracellular bacterial parasite *Wolbachia* (Taylor, Bandi, Hoerauf, & Lazdins, 2000; Werren, Windsor, & Guo, 1995) are prominent examples for this. Horizontal transmission, on the other hand, is characterized by the transmission between individuals not related in direct line either via host to host contact, a vector or uptake from the environment. Well-known examples for this route of transmission are the human influenza virus (Cowling et al., 2013; Killingley & Nguyen-Van-Tam, 2013) and the bioluminescent Gammaproteobacterium *Vibrio fischeri* of the Hawaiian bobtail squid *Euprymna scolopes* (Heath-Heckman et al., 2013; Ruby & Lee, 1998). However, the human immunodeficiency virus (HIV) for instance does not simply fall in one of these two categories. Although mainly transmitted sexually, it also frequently passes to infants of infected mothers (Sirengo et al., 2014). HIV is only one of many examples belonging to the third and probably most common form of transmission, combining vertical and horizontal transmission, the mixed mode transmission (Ebert, 2013).

Studies of microbiota suggest, that some microbes are transmitted strictly vertically (Cary & Giovannoni, 1993; Sacchi et al., 1988; Schmitt, Angermeier, Schiller, Lindquist, & Hentschel, 2008; Sharp, Eam, John Faulkner, & Haygood, 2007) and others horizontally (Di Meo et al., 2000; Kikuchi, Hosokawa, & Fukatsu, 2007). Nevertheless, considering the entire host associated microbial community, mixed mode transmission is likely the predominant mode of microbiota transmission. This is supported by the observation that the microbiota of mothers and offspring are similar, but not identical (Ley, Peterson, & Gordon, 2006). But what is the relative contribution of vertical and horizontal transmission to the composition of a newly establishing microbiota? What proportion of the maternal microbiota is transmitted to the offspring? Inoue & Ushide (2003) showed that the main component of the intestinal microbiota of rat pups were vertically transmitted bacteria, but that horizontal transmission influenced the diversity during growth. Regarding the proportion, Browne et al. (2016) report that in humans, bacterial genera capable of forming spores represented 30 % of the total intestinal microbiota, specialized for host-to-host transmission.

The limited knowledge of microbiota transmission prompted us to pursue this topic in more detail using the Cladoceran *Daphnia magna*. We inoculated animals with native and foreign microbial communities, respectively, monitoring their microbiota composition and that of their offspring using a sequencing-based approach. That way we were not only able to determine what proportion was incorporated initially and transmitted to the next generation subsequently, but also whether the proportion depended on the origin of the microbial community which would hint at a particular close interaction between the involved partners. We find that microbiota can be transmitted both vertically as well as horizontally, but that irrespective of the origin, the transmitted proportions of the microbiota were not influenced by past associations.

Material and Methods

Organism and Study Site

Four species of small filter-feeding Cladocera, all belonging to the family *Daphniidae* Straus 1820, were collected from the Ägelsee in Hohliberg, Switzerland. One species (*Scapholeberis rammneri*) belonged to the genus *Scapholeberis* Schoedler 1858, while the other three (*Daphnia magna*, *D. pulex* and *D. longispina*) belonged to the genus *Daphnia* Müller 1785. Clonal isofemale lines were established by placing collected adult females individually in jars filled with artificial *Daphnia* medium (ADaM) (Klüttgen, Dülmer, Engels, & Ratte, 1994). Besides, sediment samples containing resting eggs (ephippia) were collected and stored under cold (4 °C) and dark conditions until further use.

Unless stated otherwise, all *Daphnia* cultures were kept under standard laboratory conditions for several generations before the experiment: 400-mL jars, ADaM, fed every other day with 50 Mio cells of the green alga *Scendesmus sp.*, 16L:8D cycle and 20 °C.

Experimental set up

Ephippia from *D. magna* were collected from the sediment. The resting eggs were isolated from the protective shell, placed in falcon tubes filled with ADaM and kept in the fridge at 4 °C overnight. Before assigning them to different treatments (Table 1), the eggs were surface sterilized by removing excess ADaM and adding 2 ml of a 5% sodium hypochlorite solution, inverting the tube gently for 5 min, followed by washing three times with autoclaved ADaM. The sterile eggs were transferred in sets of three to 2-mL Eppendorf tubes containing 850 µL

autoclaved ADaM and 150 µL of a microbiota inoculum. For each inoculum, six replicates were set up. The inocula were prepared by homogenizing adult hosts in sterile ADaM and adjusting them to the same OD600 (0.7 ± 0.03). Three clonal lineages per host species were selected to serve as microbiota source. To induce hatching, the tubes were placed horizontally under a constant light source at 21 °C. Within 24 hours of hatching, hatchlings were transferred to 100-mL DURAN laboratory glass bottles sealed with membrane screw caps containing autoclaved ADaM and axenic algae. To avoid pseudoreplication only one hatchling from each tube was used. The animals were fed every other day with axenic algae ad libitum. Mothers were transferred to new bottles containing autoclaved ADaM and axenic algae when offspring was present. At the third clutch, the offspring was left in the jar together with the mother for 2 days before the mother was frozen individually in an Eppendorf tube and stored at -20 °C. The offspring were kept for additional 7 days and then also frozen individually and stored. These 9 days old *Daphnia* are just mature. All procedures requiring sterile conditions were done in a laminar flow hood.

Axenic algae

Bacteria-free *Scenedesmus sp.* cultures were obtained by following a similar procedure as described by Sison-Mangus et al. (2014). In short, algae cultures were treated with 1 mg ml^{-1} ampicillin, 50 µg ml^{-1} kanamycin and 50 µg ml^{-1} tetracycline simultaneously for three culture passages, followed by axenicity screening by PCR and phase contrast microscopy. DNA extraction, PCR conditions and bacterial primers were as described above. The DNA extracted from an untreated algae culture and nuclease-free water served as positive and negative control, respectively. Success of DNA extraction was verified by screening the samples additionally with the primer pair ITS3 (GCATCGATGAAGAACGCAGC) and ITS4 (TCCTCCGCTTATTGATATGC), amplifying parts of the 5.8S rDNA encoding region and the internal transcribed spacer 2 of the algae.

DNA extraction for sequencing analysis

One sample per inoculum (N=12) and randomly chosen subsets of four samples out of the six replicates per inoculum (N=96) were used for DNA extraction and subsequent microbial community analysis by sequencing. Total genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) protocol. A PVP K90 (20 %) and CTAB 2x (150 mM Tris-HCl pH 8, 4 % CTAB, 2.8 M NaCl) solution were placed in a water bath at 65 °C.

Excess ADaM was removed from the frozen animals, 310 µL Lysis Buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M Saccharose) was added, the animals were homogenized with a sterile pestle and 20 µL Lysozyme solution (10 mg/mL) was added. After 45 minutes of incubation at 37 °C at 850 rpm, 5 µL Proteinase K (20 mg/mL) was added and again incubated for 1 hour at 55 °C at 850 rpm. Following the treatment with 15 µL RNase A (20 mg/mL) for 10 minutes at room temperature, 300 µL CTAB 2x, 12 µL β-mercaptoethanol (0.2 %) and 60 µL PVP K90 (20 %) were added, gently mixed and incubated at 65 °C at 300 rpm for 1 hour. An equal volume of Chloroform:Isoamyl alcohol (24:1) was added and mixed with care by inversion. After centrifugation at 12'000 rpm for 8 minutes at 15 °C, the upper phase was transferred to a new Eppendorf tube and all steps starting with adding Chloroform:Isoamyl Alcohol were repeated once. 50 µL Sodium acetate (3 M, pH 5.2) and 900 µL cold Isopropanol were added to the separated upper phase and stored over night at -20 °C. Following centrifugation at 14'000 rpm for 30 min at 4 °C, the supernatant was discarded, the pellet washed with 1 mL cold ethanol (70%) and centrifuged again (14'000 rpm, 5 min, 4 °C). After discarding the supernatant, the pellet was washed once again with 500 µL, centrifuged, vacuum dried and resuspended in 30 µL TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). Samples were kept one night at 4 °C and then stored at -20 °C until further use.

All samples were processed over the course of 8 days and the different treatments were randomly distributed between the days to avoid processing batch effects. Furthermore, a negative control using Nuclease-Free Water (Ambion) instead of animal tissue was included every day.

Library preparation

DNA samples were processed for sequencing on an Illumina MiSeq platform following the adapted protocol of Lundberg et al. (2013) provided by the Genomic Diversity Centre at the ETH Zürich where the sequencing was carried out. Two PCR reactions were performed on the template DNA. First, the V3-V4 variable region of the bacterial 16S rRNA gene was amplified using the primer pair 341F and 785R with Illumina adapter sequences and 0-3 bp random frameshifts (Table 2). Each PCR reaction contained 12.5 µl 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 0.5 µl of each primer (10 µM/µl), 1.25 µl DMSO (Sigma-Aldrich), 7.25 µl ddH2O and 3 µl extracted DNA adding up to a final volume of 25 µl. PCR conditions consisted of initial denaturation at 95 °C for 5 min, followed by 29 cycles of 98 °C for 20 s, 57 °C for 15 s, 72 °C for 15 s and final extension at 72 °C for 5 min. All samples were set up in triplicates to

increase the yield and reduce the risk of failed amplification. The PCR products of the triplicates were pooled and purified using the magnetic beads Agencourt AMPure XP system (Beckman Coulter) at 1:1 a beads/PCR product volume ratio. The second PCR to index each sample was performed in 50 µl reaction volume containing 25 µl 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 5 µl of each primer from the Nextera XT Index Kit v2 (Illumina), 2.5 µl DMSO (New England Biolabs), 7.5 µl ddH2O and 5 µl purified PCR product. PCR amplification was carried out as follows: initial denaturation at 95 °C for 3 min, followed by 10 cycles of 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 5 min. After additional purification as described above, the concentration of the libraries was quantified with the Spark 10M Multimode Microplate Reader (Tecan) and qPCR. Samples were normalized and pooled in an equimolar fashion. To remove residual impurities, the library pool was bead purified once again and diluted to a concentration of 2 nM. Afterwards the pool was denatured (NaOH 0.2N), diluted to 10 pM and 15 % (v/v) PhiX was added. Finally, the mixture was loaded onto the Illumina MiSeq according to the manufacturer's instructions using the MiSeq Reagent Kit v3 (2x300 bp Paired-End Reads).

Negative controls from the DNA extraction step as well as negative controls using Nuclease-Free Water (Ambion) in place of the extracted DNA were included throughout the whole process of library preparation.

Quality assessment and quality control of the sequencing data

The raw MiSeq sequencing data were processed in a series of control steps. First, the reads were quality controlled with FastQC (Babraham Institute, UK). Then the paired reads were merged (FLASH v1.2.9), primer sequences got trimmed (Cutadapt v1.9.1) and they got size selected and quality filtered (PRINSEQ-lite v0.20.4). The clustering into Operational Taxonomc Units (OUT), including abundance sorting and chimera removal, was conducted at 97% sequence similarity using USEARCH v9.0.2132 implemented in the UPARSE pipeline (Edgar, 2013). Only OTUs passing the selection criterion of being represented by 5 or more reads were considered for further analysis. The taxonomical annotation of the OTUs was performed by UTAX using the GreenGenes v13/5 database.

Statistical data analysis

The software package R 3.3.1 (R Core Team) and the Bioconductor library phyloseq (McMurdie & Holmes, 2013) were used to perform data filtering, statistical analyses and prepare figures.

As a first step, the samples were decontaminated by excluding OTUs present in the negative controls if they represented more than 5 % of the corresponding OTUs total reads. Subsequently singletons and OTUs appearing in only one sample were removed and the data were rarefied to an even sampling depth of 10'313 reads per sample. Alpha diversity was calculated using the three indices Shannon, inverse Simpson and observed species richness. To determine statistical dissimilarities in the 16S profiles between animals of different treatments analysis of variance (ANOVA) were run. The relationship between the microbial diversity in the different samples was visualized using non-metric multidimensional scaling (NMDS).

The ten most abundant OTUs were examined in more detail, focusing on their transmission from mother to offspring and their relative abundance.

Results

A total of 6.5 million reads were obtained and after quality control we were left with 4.1 million reads for analysis of which 3.7 million remained after decontamination and filtration. Using the 97 % similarity cutoff, the reads were assigned to 363 OTUs.

We evaluated whether diversity differed among groups (Figure 1), focusing on three alpha diversity measures. In most indices, stage, origin and inoculum nested within origin had marginal to significant effects on microbial diversity whereas all but one of their interactions were not significant (Table 3). We concentrated on the Shannon index for further analyses as it accounts for both, species richness and evenness. Closer examination of mother, offspring and inocula stages separately showed that the origin had no effect on the microbial diversity of the inocula, but did so in both, mothers and offspring. The individual inocula nested within an origin only affected diversity significantly in the offspring (Table 4).

A non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities was performed to visualize the differences in the diversity of microbial community composition across treatments and stages (Figure 2). ADONIS analysis using Bray-Curtis distances showed that there were significant differences in microbiota

composition between the inocula and the hosts (R^2=0.1; p=0.001) as well as between the treatments (R^2=0.17; p=0.001), with *Scapholeberis* samples being clearly distinct.

Classification into shared and unique OTUs among samples and treatments revealed that the number of shared OTUs between any two or all three samples within a treatment, respectively, was uniform between treatments (Figure 3A). This pattern was not influenced by the total number of OTUs of a sample. The identical analysis carried out for the different inocula revealed that the majority of the OTUs belonged to the two categories not shared between inocula and shared among all inocula (Figure 3B).

Focusing on the ten most abundant OTUs (Table 5), comprising 66 % of the reads, revealed differences in their abundances in the inocula, mothers and offspring (Figure 4) illustrating the previous findings from the NMDS analysis. The abundance patterns of the inocula, representing the starting situation, varied between as well as within most origins. These differences between origins were carried over to the mothers. However, the microbial abundance patterns of mothers exposed to the same inocula were not identical. A similar pattern was seen when comparing mothers with their corresponding offspring. Though they resembled each other, marked dissimilarities between mothers and offspring could be observed in some places.

Transmission of these ten OTUs between mothers and offspring was investigated by individually looking at their presence-absence patterns (Figure 5). While some microbes always got transmitted from mothers to offspring irrespective of the origin (e.g. OUT 2), others seemed to only transmit well in certain origins (e.g. OUT 11, 12, 23) or not at all (e.g. OUT 15). Overall, OTUs originating from *Scapholeberis* were not as reliably transmitted as those from the three *Daphnia* origins.

Discussion

The aim of this study was to obtain a better understanding of the microbiota transmission dynamics of individual microbes and whole microbial communities. Using microbiota derived from different sources (sympatric hosts of the family Daphniidae) allowed us to address the question whether the microbial consortium and the host evolved more efficient vertical transmission.

Our results showed that only a fraction of the community derived from homogenized tissue of different hosts (inocula) was incorporated into the microbiota of the mothers of

which in turn also only a portion was shared between mothers and their offspring (Figure 3). While the shared OTUs got transmitted vertically, the remaining microbes were likely acquired horizontally from the environment, as for instance from the water or the food. Previous experiments showed that both, sexually and asexually produced eggs do not contain transovarially transmitted bacteria (Sison-Mangus, Mushegian, & Ebert, 2014). However, as a result of the close proximity of the developing offspring to their mothers in the open brood pouch, it is reasonable to assume that vertical transmission occurs, for example through feces and body surface contact. This form of vertical transmission is similar to what has been reported in many other systems (Bakula, 1969; Crowell-Davis & Caudle, 1989; Kovács et al., 2006; Osawa, Blanshard, & Ocallaghan, 1993). For example, Termites transmit their microbiota to newly hatched juveniles by feeding them feces (Brune, 2011; Brune & Dietrich, 2015) or female brown-winged green stinkbugs (*Plautia stali*) cover the egg surface with excretions containing their specific bacterial symbiont (Hosokawa et al., 2016). However, the process of transmission in *Daphnia*, in contrast to the mentioned examples where the mothers actively ensure that the symbionts are transmitted, seems of a passive nature and no active control by the mother is apparent. If passive vertical transmission is reliable, specific mechanisms may never evolve.

The number of shared OTUs in the inocula, mothers and offspring were similar among the native and foreign microbiota treatments (Figure 3A). Nevertheless, clear abundance differences within their microbial communities were found when looking at the ten most abundant OTUs at the family level (Figure 4) which were still detectable in the microbiota of the offspring, showing that these differences are transmittable (Figure 5) and potentially long lasting. While the latter result was expected since we knew that *Daphnia* clones kept in the lab for years under identical conditions still harbored different microbial consortia (Samuel Pichon, unpublished), the finding that native and foreign microbiota have similar ratios of shared OTUs was rather surprising. A prediction of the phylosymbiosis hypothesis is that the relatedness of host-associated microbial communities parallels the phylogeny of related host species (Brooks, Kohl, Brucker, van Opstal, & Bordenstein, 2016). This was found in many other systems such as ants (Sanders et al., 2014), apes (Ochman et al., 2010), bats (Phillips et al., 2012), sponges (Easson & Thacker, 2014) and wasps (Brucker & Bordenstein, 2011, 2012). We hypothesized that the number of shared OTUs should be higher in native combinations than in foreign ones. While some evidences for microbial community differences based on phylogeny were found in *Daphnia* (Figure 2), no differences in transmissibility for the

different origins were found (Figure 3A). Thus, inferring from these results, we conclude that *Daphnia* and their microbial communities did not coevolve and in addition to it, as each mother represented a different genotype, we speculate that host genetic background did no influence transmission.

Taken together, we showed that mothers transferred a certain proportion of their microbiota vertically to their offspring while the remaining microbes were likely of environmental origin, fitting the mixed mode transmission expectation when looking at the microbiota as an entity. Since transmission efficiency (= number of shared OTUs) was quite uniform, irrespective of the origin or composition of the microbial community, we suggest that these stable proportions resulted from the interplay of an undirected transmission process and the anatomical features of *Daphnia*.

Our results suggest a scenario of how transmission might take place in *Daphnia magna*: At the beginning of the growth season, the entire microbiota of hatchlings from resting eggs consists of horizontally acquired environmental microbes. From that point on, a rather stable proportion of the microbial community is vertically transmitted to the next generation through mother-offspring contact via feces and body surface and further microbes may be acquired from the environment. The microbes that are vertically transmitted may fulfill a certain function and by this contribute to the host's well-being. Different microbes may be able to fulfill the same function. Thus, microbial communities within a habitat may reflect more the needs for certain functions and be less specific to the hosts. As a consequence, hosts with similar needs will have similar microbiota if they live sympatric. The hosts used here are all freshwater planktonic Cladocera, collected in the same pond. Therefore, our model would not predict much differences among the species. However, it remains unclear if the vertically transmitted microbes are somehow determined by the mother or if it is according to the random principle.

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Table 1: List of microbiota inocula used in the experiment. Each inocula was transferred to six replicates.

Microbiota origin	Inoculum	Clone
D. magna	M1	CH-H-4
	M2	CH-H-149
	M3	CH-H-434
D. pulex	P1	CH-H-DP-1
	P2	CH-H-DP-2
	P3	CH-H-DP-3
D. longispina	L1	CH-H-DL-1
	L2	CH-H-DL-3
	L3	CH-H-DL-5
S. rammneri	S1	CH-H-S-1
	S2	CH-H-S-2
	S3	CH-H-S-3

Table 2: Primer pairs (5'-3') used to target the variable region V3-V4 of the 16S rRNA for sequencing

Primer pairs	Tail	Linker	Primer sequence
351F_GA_fs0	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	GA	CCTACGGGNGGCWGCAG
785R_CA_fs0	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	CA	GACTACHVGGGTATCTAATCC
351F_GA_fs1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NGA	CCTACGGGNGGCWGCAG
785R_CA_fs1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NCA	GACTACHVGGGTATCTAATCC
351F_GA_fs2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NNGA	CCTACGGGNGGCWGCAG
785R_CA_fs2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NNCA	GACTACHVGGGTATCTAATCC
351F_GA_fs3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NNNGA	CCTACGGGNGGCWGCAG
785R_CA_fs3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NNNCA	GACTACHVGGGTATCTAATCC

Table 3: Summary of analysis of variance of the effect of stage, origin and inoculum nested within origin on the three diversity indices Shannon, Inverse Simpson and Richness. The factors have the following levels: stage (mothers, offspring), origin (*D. magna*, *D. pulex*, *D. longispina*, *Scapholeberis*), inoculum (M1, M2, M3, P1, P2, P3, L1, L2, L3, S1, S2, S3). Significant p-values are shown in bold.

Shannon

Source	df	MS	F	P
Stage	1	0.716	3.256	0.075
Origin	3	2.012	9.150	**3.31e-05**
Inoculum (Origin)	3	0.611	2.781	**0.0097**
Stage x Origin	8	0.471	2.146	0.102
Stage x Inoculum (Origin)	8	0.265	1.208	0.307
Error	72	0.220		

Inv. Simpson

Source	df	MS	F	P
Stage	1	15.140	3.592	0.062
Origin	3	25.951	6.157	**0.0009**
Inoculum (Origin)	3	8.122	1.927	0.069
Stage x Origin	8	15.052	3.571	**0.018**
Stage x Inoculum (Origin)	8	5.690	1.350	0.233
Error	72	4.215		

Richness

Source	df	MS	F	P
Stage	1	3.01	0.03	0.86
Origin	3	247.71	2.43	0.07
Inoculum (Origin)	3	79.88	0.78	0.62
Stage x Origin	8	53.01	0.52	0.67
Stage x Inoculum (Origin)	8	52.48	0.51	0.84
Error	72	102.04		

Table 4: Summary of analysis of variance of the effect of origin and inoculum nested within origin on the Shannon diversity index of mothers, offspring and inocula. The factors have the following levels: origin (*D. magna*, *D. pulex*, *D. longispina*, *Scapholeberis*), inoculum (M1, M2, M3, P1, P2, P3, L1, L2, L3, S1, S2, S3). Significant p-values are shown in bold.

Mother stage

Source	df	MS	F	P
Origin	3	1.296	5.266	**0.004**
Inoculum (Origin)	8	0.171	0.695	0.694
Error	36	0.246		

Offspring stage

Source	df	MS	F	P
Origin	3	1.188	6.133	**0.002**
Inoculum (Origin)	8	0.706	3.645	**0.003**
Error	36	0.194		

Inocula stage

Source	df	MS	F	P
Origin	3	0.48	1.59	0.27
Error	8	0.30		

Table 5: Detailed information about the classification of the ten most abundant OTUs.

	Class	**Order**	**Family**	**Genus**
OTU_2	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Shinella
OTU_28	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobium
OTU_18	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bosea
OTU_12	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingobium
OTU_13	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas
OTU_11	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rubrivivax
OTU_23	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovorax
OTU_5	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas
OTU_15	Gammaproteobacteria	Alteromonadales	Chromatiaceae	Rheinheimera
OTU_7	Actinobacteria	Actinomycetales	Microbacteriaceae	Yonghaparkia

Figure 1: α Diversity quantified by Shannon index of the different stages (color coded) and inocula (symbols) across the four treatments (origin of microbiota). Error bars represent standard error of the mean.

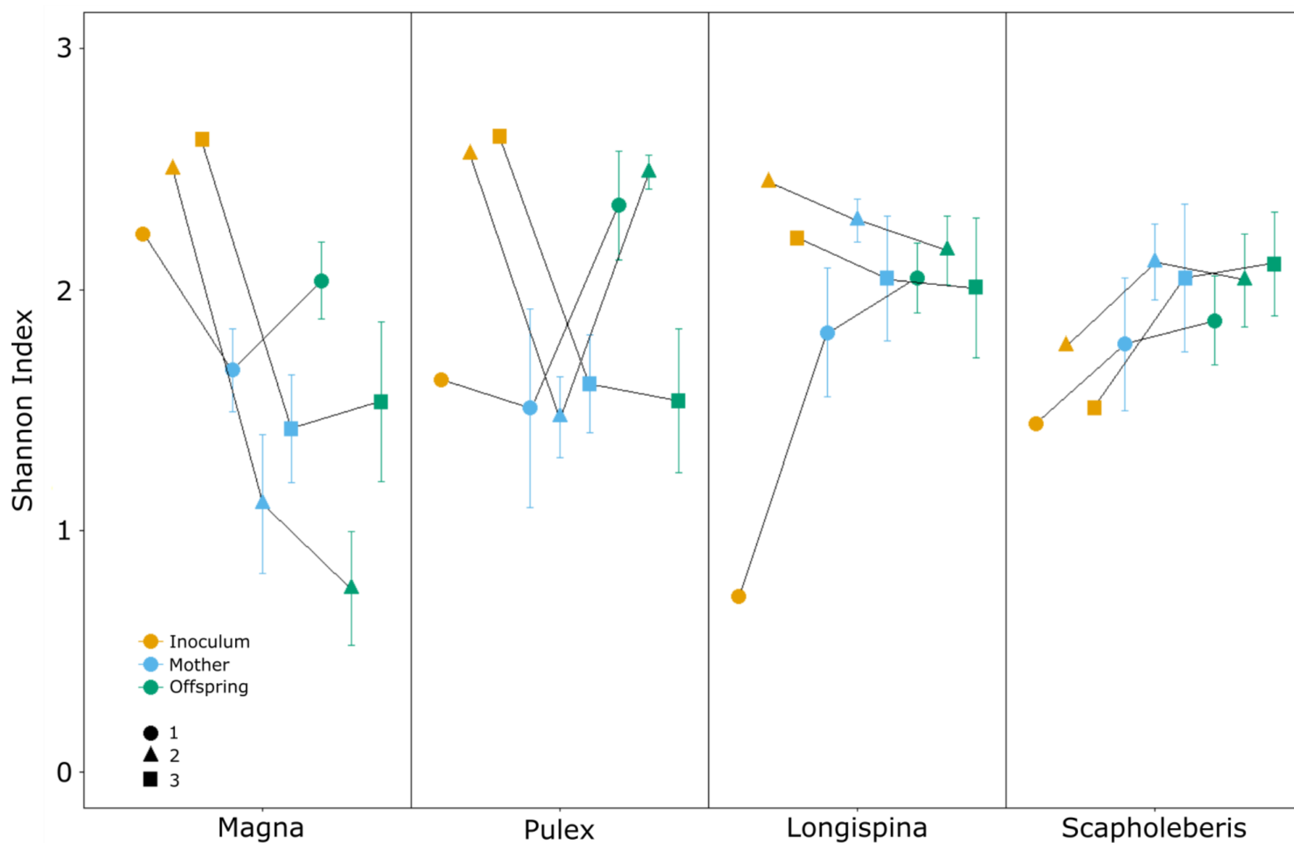


Figure 2: Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity between samples. The goodness of fit (or stress) associated with this ordination is 0.17. Each data point represents the microbial profile of one sample. The different stages are represented by different symbols color coded by treatment.

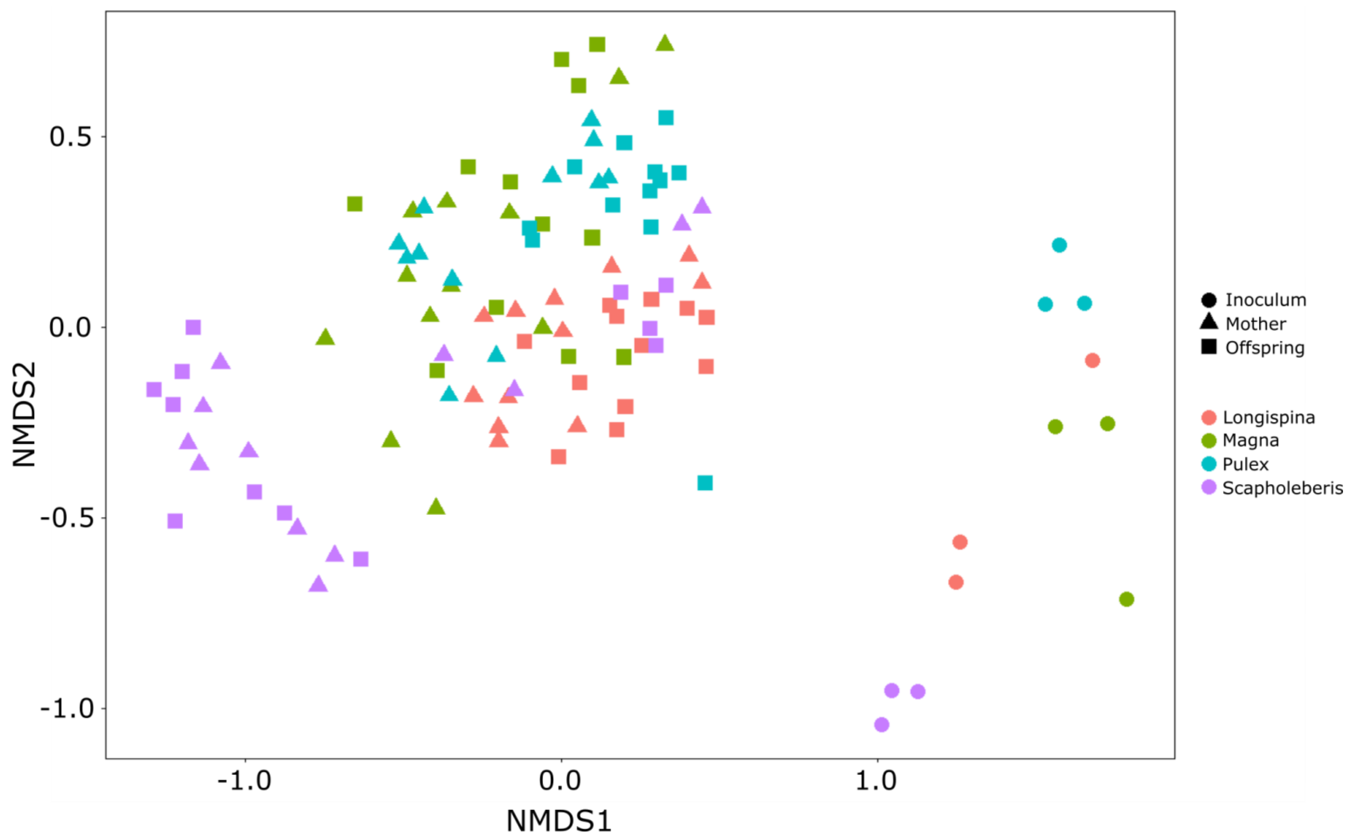
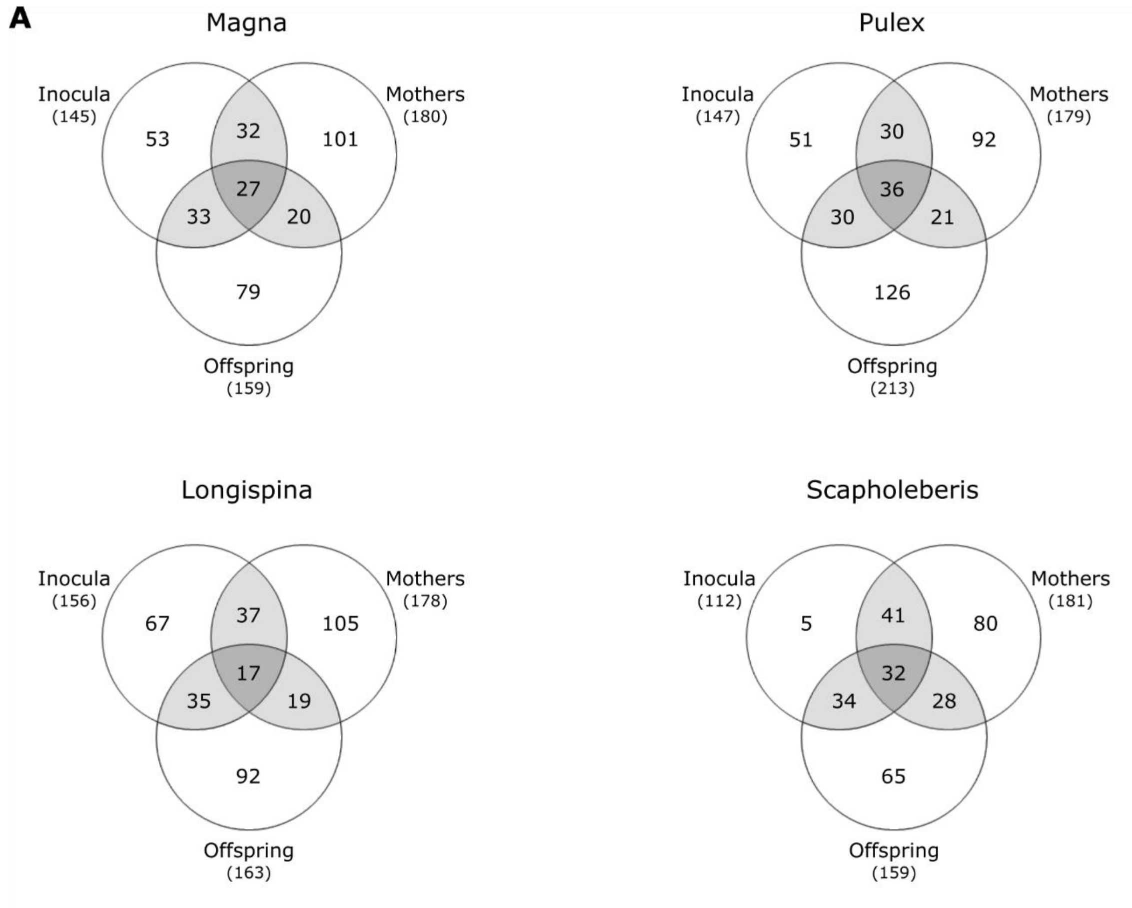


Figure 3: Venn diagrams showing the number of shared and unique OTUs between A) inocula, mothers and offspring for all treatments and B) the different inocula. Within a treatment, samples belonging to the same stage were combined: inocula (N=3), mothers (N=12), offspring (N=12).



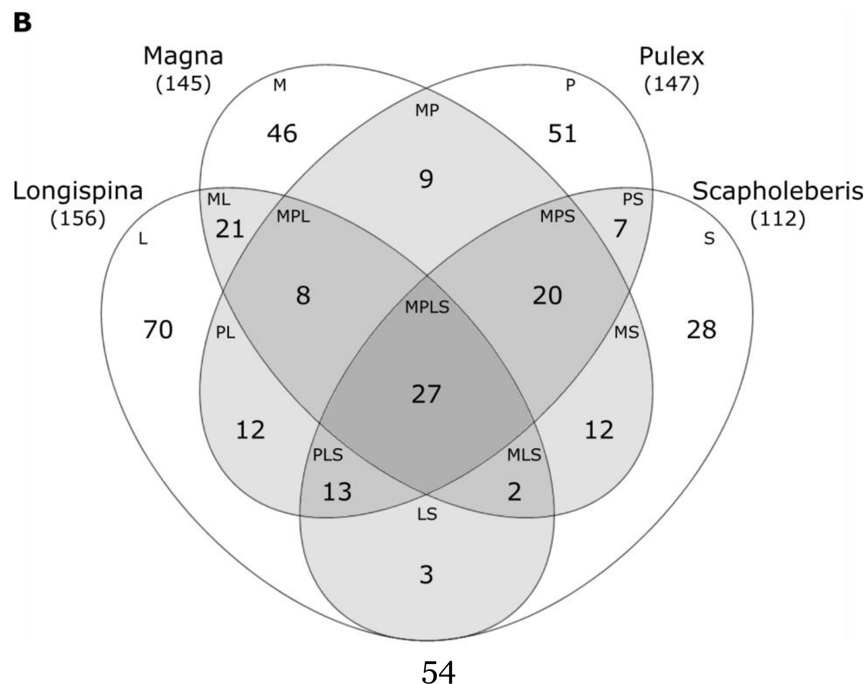


Figure 4: Stacked bar graph showing the relative abundance of the 10 most abundant OTUs at the family level, accounting for 66 % of all reads. Each bar represents one sample, starting with the inoculum (I) followed by four pairs of mother (M) and their corresponding offspring (O). Row wise the plots belong to the same origin (Magna = M, Pulex = P, Longispina = L, Scapholeberis = S) while column wise they are arranged by replicates within the treatment (Inoculum 1, Inoculum 2, Inoculum 3).

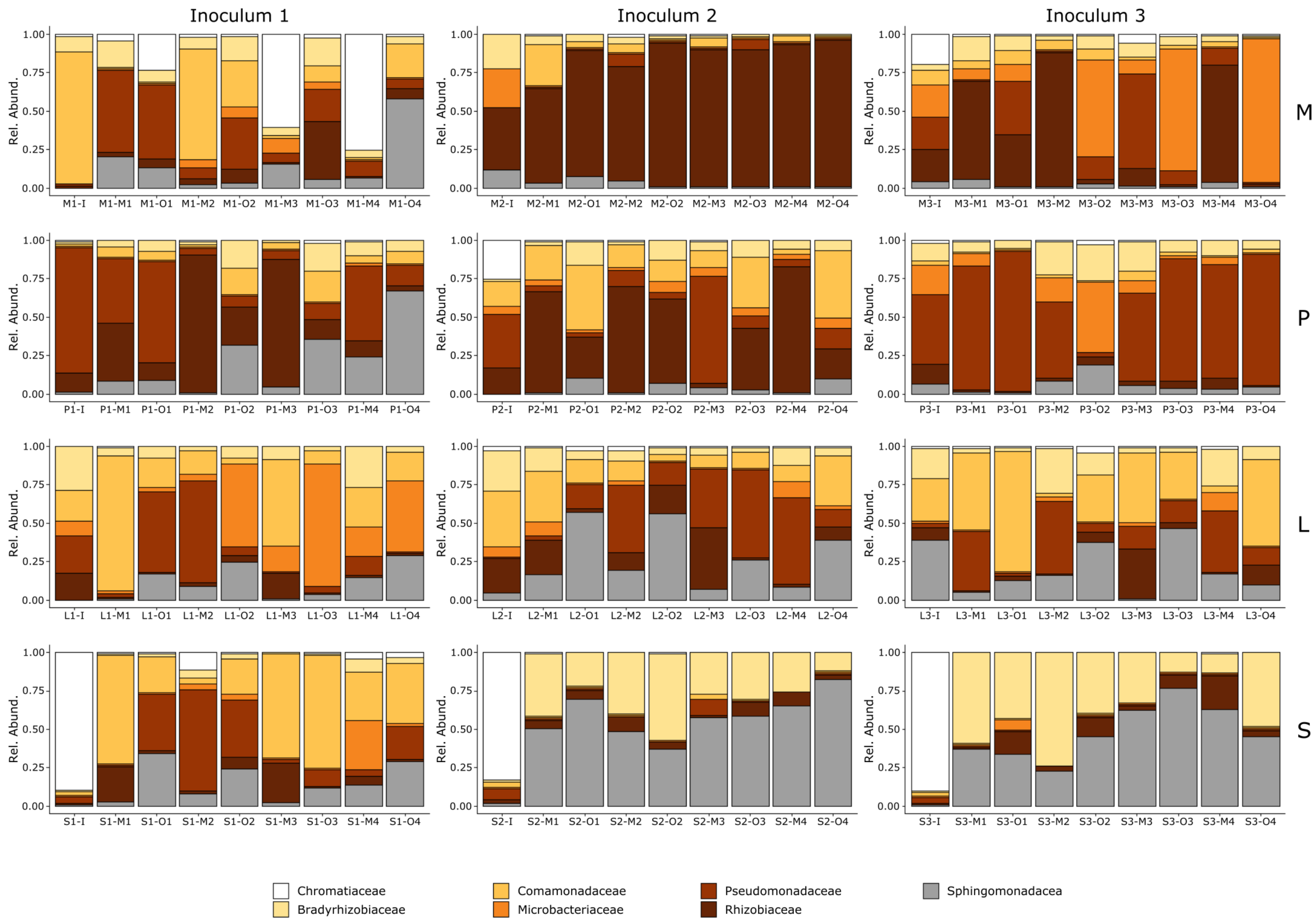
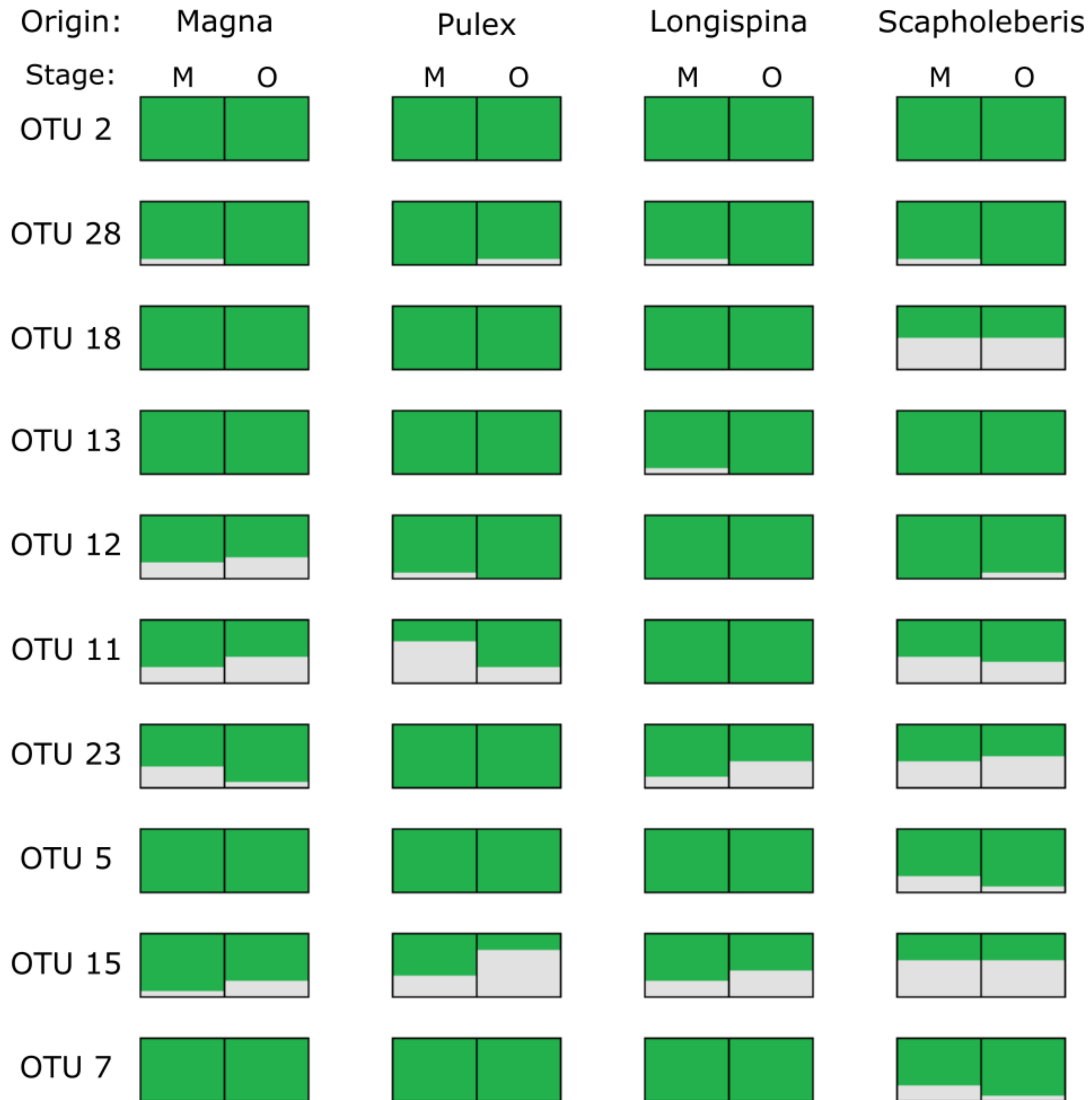

Inoculum 1
Inoculum 2
Inoculum 3
Rel. Abund.
M
P
L
S
M1-I
M1-M1
M1-O1
M1-M2
M1-O2
M1-M3
M1-O3
M1-M4
M1-O4
M2-I
M2-M1
M2-O1
M2-M2
M2-O2
M2-M3
M2-O3
M2-M4
M2-O4
M3-I
M3-M1
M3-O1
M3-M2
M3-O2
M3-M3
M3-O3
M3-M4
M3-O4
P1-I
P1-M1
P1-O1
P1-M2
P1-O2
P1-M3
P1-O3
P1-M4
P1-O4
P2-I
P2-M1
P2-O1
P2-M2
P2-O2
P2-M3
P2-O3
P2-M4
P2-O4
P3-I
P3-M1
P3-O1
P3-M2
P3-O2
P3-M3
P3-O3
P3-M4
P3-O4
L1-I
L1-M1
L1-O1
L1-M2
L1-O2
L1-M3
L1-O3
L1-M4
L1-O4
L2-I
L2-M1
L2-O1
L2-M2
L2-O2
L2-M3
L2-O3
L2-M4
L2-O4
L3-I
L3-M1
L3-O1
L3-M2
L3-O2
L3-M3
L3-O3
L3-M4
L3-O4
S1-I
S1-M1
S1-O1
S1-M2
S1-O2
S1-M3
S1-O3
S1-M4
S1-O4
S2-I
S2-M1
S2-O1
S2-M2
S2-O2
S2-M3
S2-O3
S2-M4
S2-O4
S3-I
S3-M1
S3-O1
S3-M2
S3-O2
S3-M3
S3-O3
S3-M4
S3-O4
0.00
0.25
0.50
0.75
1.00
Chromatiaceae
Bradyrhizobiaceae
Comamonadaceae
Microbacteriaceae
Pseudomonadaceae
Rhizobiaceae
Sphingomonadacea

Figure 5: Presence (green) and absence (grey) of the ten most abundant OTUs in mothers (M) and their offspring (O) across the different microbiota origins (Magna, Pulex, Longispina, Scapholeberis). Within a treatment, samples belonging to the same stage were combined: mothers (N=12), offspring (N=12). For detailed information of the OTUs see Table 5.



Chapter 3

Microbial community of *Daphnia magna* is an open system

Tobias M.M. Schär, Jean-Claude Walser and Dieter Ebert

Author contributions: TMMS conceived the study. TMMS and DE designed the experiment. TMMS performed the experiment, sequencing library preparation and analyses. JCW performed sequence quality control, OTU selection and taxonomic assignment procedures. TMMS wrote the paper. DE revised the paper.

Abstract

All eukaryotes are colonized by a diverse community of microorganisms leading to the formation of long-lasting associations that are often essential for the well-being of the host. However, microbial communities associated with hosts are open systems with the possibility that new microbes arrive and others being lost. Thus, their stability might be constantly challenged by microbes from the environment. The present study aims at evaluating how microbial communities established in young animals react to the arrival of other communities in the environment. For this purpose, *Daphnia magna* from three different locations (Belgium, Germany and Switzerland) were raised with their native microbiota for a few days. Then pairs of animals from the same or different location were housed together for three weeks. Subsequently, their microbiota were analyzed and compared using 16S rRNA amplicon sequencing. The microbial diversity and richness in animals from the mixed pairs was higher than in those from same host clone pairs, indicating that microbial communities intermixed rapidly. These findings were not influenced by the host genotype. We conclude that the microbiota of *Daphnia magna* is easily modifiable by the environment and not tightly controlled by host genetics. These findings challenge the assumption that microbiota are tightly coevolved with their host.

Introduction

From the moment an animal is born, it is in constant contact with environmental microbes and experiences rapid colonization, forming essential mutualistic associations. The establishment of the initial microbiota is a crucial step during development influencing the short and long term health status of the host by affecting traits like gut maturation (Bates et al., 2006; Smith et al., 2007) and immune system education (Lathrop et al., 2011; Weng & Walker, 2013). To ensure successful colonization with specific microorganisms specific mechanisms evolved, often involving complex and well-regulated molecular signaling (M. J. McFall-Ngai et al., 2005; Rader & Guillemin, 2013). For example, Franzenburg et al. (2013) showed that specific antimicrobial peptides of several closely related species of the Cnidarian *Hydra* accounted for different bacterial communities. Following the acquisition of the right microbes from the environment, the next challenge is to ensure the temporal stability of this

association, which might be achieved by cross-talk and cross-regulation between the microbes and the host (DeGruttola et al., 2016).

Host associated microbiota are open systems, constantly exposed to and challenged by external microorganisms potentially invading the microbiome. Invaders may originate from other hosts or may be free living in the environment. While some invaders may be transient, others may be compatible with the host and compete with the resident microbiota for access to the host habitat to establish new associations (Bäumler & Sperandio, 2016). This offers the host the potential to modify the composition of its microbiota if environmental conditions require it, as in the case of corals that respond to higher temperatures by hosting specifically adapted symbiotic algae (Rowan, 2004). At the same time, members of the host's microbiota spread to the environment and to other hosts. As a result, the altered composition of the microbial communities in the host's proximate environment may serve as a microbial source for other hosts (Castro-Sanguino & Sánchez, 2012; Sweet, 2014). In habitats populated by the Hawaiian Bobtail Squid, for instance, the concentration of its symbiont *Vibrio fischeri* was up to 30 times that of similar habitats without squids and decreased with increasing distance from inhabited sites (Lee & Ruby, 1994). In summary, hosts and environment reciprocally affect each other's microbial composition (Chandler et al., 2011; Mistry et al., 2017), making it harder for the host to maintain a temporally stable microbial community, but at the same time allowing a host to adapt its microbiota to foster its needs (Macke et al., 2017). Longitudinal studies of microbiota in individual hosts showed that the microbial composition slowly changes as the host gets older (Arboleya et al., 2016; O'Toole & Jeffery, 2015; Odamaki et al., 2016; Ottman et al., 2012), but little is known about the factors affecting this change and consequently the stability of the microbial community.

For a better understanding of microbiota stability, we used *Daphnia magna* and its associated microbes as a model. The aim of this study was to determine if two hosts with different microbiota alter the composition of their respective communities in response to the presence of other hosts in their vicinity. We hypothesized that the microbiota of individuals is rather stable and only minor shifts, if any, would occur upon challenge with new microbes in the environment. However, our results do not support this hypothesis but revealed that the microbial communities of the hosts changed quickly.

Material & Methods

Study Organism

Clonal lineages of *Daphnia magna* (Crustacea: Cladocera) originating from populations in Switzerland (CH-H-434), Belgium (BE-OM2) and Germany (DE-K3-11-34) served as source for the animals used in this study. They were started 2-5 years earlier by placing females individually in jars and ever since then kept the clonal populations under standard laboratory conditions in 400-mL jars filled with artificial *Daphnia* medium (ADaM) (Klüttgen et al., 1994), fed every other day with 50 Mio cells of the green alga *Scendesmus sp.*, 16:8 light:dark cycle and 20 °C.

Mitochondrial marker analysis

DNA was extracted using the "DNeasy Blood & Tissue Kit" (Qiagen) following a slightly modified version of the instructions for "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)". In short, adult *Daphnia* were individually placed in Eppendorf tubes and excess ADaM was removed. 200 µL buffer ATL was added, homogenized with a pestle, followed by the addition of 20 µL Proteinase K (20 mg/mL) and incubation at 750 rpm for 2 hours at 56 °C. After adding 200 µL Buffer AL and 200 µL ethanol (100 %) the sample was vortexed and transferred to a DNeasy Mini spin column placed in a 2 mL collection tube. Following centrifugation at 8'000 rpm for 1 min, the supernatant was discarded, the column placed in a new collection tube and 500 µL Buffer AW1 was added. After repeated centrifugation at 8'000 rpm for 1 min the column was placed again in a new collection tube, 500 µL Buffer AW2 was added and centrifuged at 14'000 rpm for 3 min. Afterwards the column was placed in an Eppendorf tube, 100 µL ddH_2O was added and incubated for 1 min before centrifuged at 8'000 rpm for 1 min. Samples were stored at -20 °C until further use.

In order to be able to assign animals to the different lineages the products of 4 mitochondrial markers (Table 1) were tested. One marker was universal for invertebrates (Folmer et al., 1994) whereas the remaining three were newly designed for *Daphnia magna* (Table s1). PCR reactions were performed in 50 µl reaction volume containing 1 µl of each forward and reverse primer (10 µM/µl), 1 µl dNTPs (10nM), 5 µl 10x PCR Buffer, 0.3 µl Taq (5 units/µl), 40.7 µl ddH_2O and 1 µl extracted DNA. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 48 °C for 50 s, 72 °C for 60 s, followed by final extension at 72 °C for 5 min. Subsequently PCR products were analyzed by Sanger sequencing to type clone specific SNPs. It turned out that marker ND5_2,

amplifying parts of the ND5 subunit of the NADH dehydrogenase, was sufficient to reliably distinguish the three host clones used here.

Experimental setup

Juveniles from mass cultures of each of the three clonal lineages were transferred individually to 80-mL jars filled with ADaM. Unless stated otherwise, all *Daphnia* cultures were kept under standard laboratory conditions. The jars were checked daily and in case offspring were present the mother was transferred to a new jar. The free swimming offspring of the third clutch was left in the jar together with the mother for 2 days to ensure transmission of maternal microbes. The mother was then frozen individually in an Eppendorf tube and stored at -20 °C. The offspring on the other hand were transferred to new jars assigned to monoclonal or mixed clone pair treatments in a full factorial design (Figure 1) and each combination was 10 times replicated. An additional jar was produced with one offspring of each location (triplets). Within a replicate, all individuals belonging to the same population originated from a single mother. The jars were randomly arranged in trays and monitored daily for the presence of hatchlings. If offspring were present they were removed and the adults were left in the jar. After 21 days all animals were frozen individually in Eppendorf tubes at -20 °C until further use. Only replicates where all animals survived until the end of the experiment were included in sequencing analysis, leaving us with four replicates (N = 72).

DNA extraction and library preparation for amplicon sequencing

Total genomic DNA was extracted using a cetyltrimethylammonium bromide (CTAB) protocol. A PVP K90 (20 %) and CTAB 2x (150 mM Tris-HCl pH 8, 4 % CTAB, 2.8 M NaCl) solution were placed in a water bath at 65 °C. Excess ADaM was removed from the frozen animals, 310 µL Lysis Buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M Saccharose) was added, the animals were homogenized with a sterile pestle and 20 µL Lysozyme solution (10 mg/mL) was added. After 45 minutes of incubation at 37 °C at 850 rpm, 5 µL Proteinase K (20 mg/mL) was added and again incubated for 1 hour at 55 °C at 850 rpm. Following the treatment with 15 µL RNase A (20 mg/mL) for 10 minutes at room temperature, 300 µL CTAB 2x, 12 µL β-mercaptoethanol (0.2 %) and 60 µL PVP K90 (20 %) were added, gently mixed and incubated at 65 °C at 300 rpm for 1 hour. An equal volume of Chloroform:Isoamyl alcohol (24:1) was added and mixed with care by inversion. After centrifugation at 12'000 rpm for 8 minutes at 15 °C, the upper phase was transferred to a new

Eppendorf tube and all steps starting with adding Chloroform:Isoamyl Alcohol were repeated once. 50 µL Sodium acetate (3 M, pH 5.2) and 900 µL cold Isopropanol were added to the separated upper phase and stored over night at -20 °C. Following centrifugation at 14'000 rpm for 30 min at 4 °C, the supernatant was discarded, the pellet washed with 1 mL cold ethanol (70%) and centrifuged again (14'000 rpm, 5 min, 4 °C). After discarding the supernatant, the pellet was washed once again with 500 µL, centrifuged, vacuum dried and resuspended in 30 µL TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). Samples were kept one night at 4 °C and then stored at -20 °C until further use.

All samples were processed over the course of 4 days and the different treatments were randomly distributed between the days to avoid processing batch effects. Furthermore, a negative control using Nuclease-Free Water (Ambion) instead of animal tissue was included every day.

DNA samples were processed for sequencing on an Illumina MiSeq platform following the adapted protocol of Lundberg et al. (2013) provided by the Genomic Diversity Centre at the ETH Zürich where the sequencing was carried out. Two PCR reactions were performed on the template DNA. First, the V3-V4 variable region of the bacterial 16S rRNA gene was amplified using the primer pair 341F and 785R with Illumina adapter sequences and 0-3 bp random frameshifts (Table 2). Each PCR reaction contained 12.5 µl 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 0.5 µl of each primer (10 µM/µl), 1.25 µl DMSO (Sigma-Aldrich), 7.25 µl ddH2O and 3 µl extracted DNA adding up to a final volume of 25 µl. PCR conditions consisted of initial denaturation at 95 °C for 5 min, followed by 29 cycles of 98 °C for 20 s, 57 °C for 15 s, 72 °C for 15 s and final extension at 72 °C for 5 min. All samples were set up in triplicates to increase the yield and reduce the risk of failed amplification. The PCR products of the triplicates were pooled and purified using the magnetic beads Agencourt AMPure XP system (Beckman Coulter) at 1:1 a beads/PCR product volume ratio. The second PCR to index each sample was performed in 50 µl reaction volume containing 25 µl 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 5 µl of each primer from the Nextera XT Index Kit v2 (Illumina), 2.5 µl DMSO (New England Biolabs), 7.5 µl ddH2O and 5 µl purified PCR product. PCR amplification was carried out as follows: initial denaturation at 95 °C for 3 min, followed by 10 cycles of 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 5 min. After additional purification as described above, the concentration of the libraries was quantified with the Spark 10M Multimode Microplate Reader (Tecan) and qPCR. Samples were normalized and pooled in an equimolar fashion. To remove residual impurities,

the library pool was bead purified once again and diluted to a concentration of 2 nM. Afterwards the pool was denatured (NaOH 0.2N), diluted to 10 pM and 15 % (v/v) PhiX was added. Finally, the mixture was loaded onto the Illumina MiSeq according to the manufacturer's instructions using the MiSeq Reagent Kit v3 (2x300 bp Paired-End Reads).

Negative controls from the DNA extraction step as well as negative controls using Nuclease-Free Water (Ambion) in place of the extracted DNA were included throughout the whole process of library preparation.

Quality assessment of sequencing data and statistical analysis

The raw MiSeq sequencing data were processed in a series of control steps. First, the reads were quality controlled with FastQC (Babraham Institute, UK). Then the paired reads were merged (FLASH v1.2.9), primer sequences were trimmed (Cutadapt v1.9.1), size selected and quality filtered (PRINSEQ-lite v0.20.4). The clustering into Operational Taxonomc Units (OUT), including abundance sorting and chimera removal, was conducted using USEARCH v9.0.2132 implemented in the UPARSE pipeline (Edgar, 2013). Only OTUs passing the selection criterion of being represented by 5 or more reads were considered for further analysis. As a last point, taxonomy was assigned using UTAX against the GreenGenes v13/5 database.

The software package R 3.3.1 (R Core Team) and the Bioconductor library phyloseq (McMurdie & Holmes, 2013) were used to perform data filtering, statistical analyses and to prepare figures. As a first step, the samples were decontaminated by excluding OTUs present in the negative controls if they represented more than 5 % of the corresponding OTUs total reads. Subsequently singletons and OTUs appearing in only one sample were removed and the data were rarefied to an even sampling depth of 17'370 reads per sample. Alpha diversity was calculated using the three indices Shannon, inverse Simpson and observed species richness. Samples from the triplet treatment were excluded for these analyses.

To test for the effect of microbiota mixing, we simulated datasets for the mixed pair treatments by combining the real data from their respective monoclonal treatments. This approach simulates a mixed community where members of both communities were pooled without bias in relative abundance. To determine statistical dissimilarities in the 16S profiles between animals of different clones and treatments analyses of variance (ANOVAs), Principal Coordinates Analysis (PCoA) and Welch Two Sample t-test were used.

Results

Microbial diversity of the mothers differed significantly between the three clonal lines from Belgium, Switzerland and Germany (F=16.09, P=0.0011), showing that the animals harbored distinct microbiota at the beginning of the experiment. In the offspring generation, we found that regardless of the alpha diversity index used, microbial diversity differed significantly between the monoclonal and the mixed pairings. The microbiota communities of host individuals exposed to microbiota of different hosts (mixed pairs) were consistently more diverse and species rich (Table 3, Figures 2, 3).

An unweighted UniFrac distance based Principal Coordinates Analysis (PCoA) was used to cluster the microbial community diversities by pairing (Figure 4). While the samples from the monoclonal pairs tended to be more at the periphery, the mixed clone pairs tended to be between their respective monoclonal origins with the triplets being in the center.

The microbial diversities of the simulated mixed pairs were found to be less diverse than the real data (Figure 5), suggesting that the process of microbiota mixing in our experiment was more complex than expected from an additive model.

Discussion

We investigated the stability of host-associated microbial communities, by exposing hosts to conspecifics with the same or a different microbiota. Contrary to our hypothesis, our experiment revealed that the microbiota of *Daphnia magna* was susceptible to invasion by microbes from conspecific hosts, demonstrating low stability. Furthermore, the newly formed communities were not simply a mix of the communities of the two hosts being kept together as they were more species-rich (OTU-rich) and diverse than a simulated dataset where we pooled *in-silico* the microbiota of hosts not exposed to other microbiota.

Low stability

We hypothesized that the microbiota of *Daphnia* magna is rather resistant to the invasion of foreign microbiota, but were unable to support this assumption. Host-associated microbial communities are generally considered to be rather stable (Reveillaud et al., 2014; Sommer et al., 2017). This assumption is based on diverse lines of circumstantial evidence. For example, longitudinal studies of microbiota across individual hosts reveal small changes over time

(Rajilić-Stojanović et al., 2013; Voreades et al., 2014). Microbiota communities show patterns of clade specificity, i.e. related host species have similar microbiomes (Ley et al., 2008; Ochman et al., 2010). And finally, if microbiota coevolve with their hosts, as is often assumed (M. McFall-Ngai et al., 2012, 2013; Shapira, 2016), the association with the host must be stable across many generations. Circumstantial evidence was also used to suggest that our here used model organism, *Daphnia*, has a stable bacterial community (Freese & Schink, 2011; Qi et al., 2009).

On the other hand, there is also evidence for low stability of the microbiota composition. Cohabiting humans, particularly couples, shared more of their microbiota than individuals from different households (Song et al., 2013). This finding mirrors our finding. A low stability of the microbial community is expected in applied aspects of host-microbiota interactions. For instance, microbiota transplants are becoming increasingly important in the medical field of microbiota-mediated health (Greenhalgh et al., 2016; Scott et al., 2015). If resident microbes would prevent invaders to settle, the transplantation of microbes would not be successful. Likewise, probiotics, the enrichment of food with beneficial microbes, would be pointless if resident microbes would prevent the uptake of bacteria from the food.

Our experiment revealed that the microbiota of a host cohabiting with a host carrying a different microbiota becomes more species-rich and more diverse. This effect is symmetric in the sense that both hosts involved give and receive microbiota, excluding the interpretation that one of the two hosts has a healthy and the other a sub-optimal microbiota. We cannot rule out that all our *Daphnia* lines had sub-optimal microbiota, and used the chance to improve it, by picking up microbes from the environment. However, transplantation experiments showed that microbiota are easily transferable among clones and *Daphnia* species without any recognizable effect for the health of the host (Schär & Ebert, submitted). Our experimental design also allowed us to exclude that the uptake of microbiota was necessary because the environment changed (Sullam, Pichon, Schaer, & Ebert, in press). All clonal lines had been kept for years under standard laboratory maintenance culture conditions, and the experiment was carried out under the same conditions, i.e. the same food, medium, temperature, day-night cycle, handling regimes and more.

In our experiment we allowed the transfer of microbes among clonal lines of the same host species. The three host lineages had a native microbiota that was suitable for their genotype, and presumably for the species. Thus, the ease with which microbes among lines were mixed, may be taken as evidence for a species-specific, rather than host-line specific

microbiota. Same-species hosts are known to have more similar microbiomes to one another than to hosts of different species (Ley et al., 2008). However, a comparison of the microbial diversities of the mothers, representing the initial state of the microbiota in our experiment, revealed strong differences. These mothers came from three different populations in Belgium, Switzerland and Germany, and apparently maintained unique microbiota despite being kept for years in the same culture room under the same maintenance conditions. Apparently, this difference among the microbiota of these clonal lines cannot be taken as evidence that an intimate tight association exists (Mushegian & Ebert, 2016), possibly reflecting simply a historical effect. The lines kept what they had when they were first cultivated, and were kept under conditions that prevent the exchange of microbes among culture vials (Sullam et al., in press).

Our experiment tested the hypothesis that the microbiome of a host is resistant to the invasion by microbes from conspecific hosts. This was clearly not the case. However, this does not rule out that the microbiome includes a stable core that is protected against invaders and that only a part of the microbiome is unstable. We cannot rule this out with our data. Nevertheless, our data suggest that a substantial part of the microbiota is unstable, as otherwise the mixed microbiota would not fall in between the controls in the UniFrac Principal Coordinates Analysis plot (Fig. 4).

Non-random uptake of microbiota

Our experiment included the assessment of the microbial communities in monoclonal pairs. This allowed us to simulate *in-silico* an expected microbial composition resulting from a simple one to one pooling of the communities of two hosts. To our surprise, the species-richness and diversity of the real data were significantly higher than those of these simulated data. Simulating data with unequal contributions would make this effect even stronger. Thus, the process of microbiota intermixing was not simply of additive nature. Invading microbes did not generally replace resident microbes, but were in many cases incorporated into the existing community, resulting in more even species distributions than expected by chance.

More complex ecological communities have been shown to be more stable and show high resilience after disturbance (Folke et al., 2004; Peterson et al., 1998). High microbiota diversity and species-richness have also been suggested to be signs of a healthy microbiome (Le Chatelier et al., 2013; Pflughoeft & Versalovic, 2012; The Human Microbiome Project Consortium, 2012). If so, one might speculate that it is in the interest of the host to add

further species to its microbiome. But this is a double edge sword, as it also opens up the door for harmful microbes to invade. On the other hand, microbes are selected to spread and to grow opportunistically. Thus, an increase in diversity and species-richness may be a side effect of general microbe strategy, rather than a benefit for the host. Experiments are needed which track not only the movements of microbes, but also the resulting fitness effects for the host.

Our here presented experiment sheds light on the understudied interplay between microbiota with regard to their stability and composition, which in turn is of particular importance for research in the field of microbiota-mediated health (Carding et al., 2015; de Vos & de Vos, 2012; DeGruttola et al., 2016; Greenhalgh et al., 2016; Scott et al., 2015). Experimental studies, more than circumstantial evidence, can help us to establish cause effect relationships and to obtain a basic understanding of the mechanisms and factors influencing microbiota stability. This will be vital to be able to treat diseases by manipulating the microbiota to the hosts benefit and maintaining it in a healthy state.

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Table 1: Mitochondrial primers (5'-3') used for the identification of host genotypes.

Gene	Primer pairs	Primer sequence
mtCOI	LCOI490	GGTCAACAAATCATAAAGATATTGG
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA
mtCOI	COI_DM_FL	CGAGCTGAGTTAGGGCAATC
	COI_DM_RL	CCGCAGGATCAAAGAATGAA
ND5	ND5_1_F	TTAGCGTCGGGGGTTACTGT
	ND5_1_R	CATTTTGATTGGAAGTCAAG
ND5	ND5_2_F	TTTCTATCTACTGATGGGGT
	ND5_2_R	AATGTAACCTTTACTTCAGA

Table 2: Primer pairs (5'-3') used to target the variable region V3-V4 of the 16S rRNA for sequencing.

Primer pairs	Tail	Linker	Primer sequence
351F_GA_fs0	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	GA	CCTACGGGNGGCWGCAG
785R_CA_fs0	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	CA	GACTACHVGGGTATCTAATCC
351F_GA_fs1	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NGA	CCTACGGGNGGCWGCAG
785R_CA_fs1	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NCA	GACTACHVGGGTATCTAATCC
351F_GA_fs2	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NNGA	CCTACGGGNGGCWGCAG
785R_CA_fs2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NNCA	GACTACHVGGGTATCTAATCC
351F_GA_fs3	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NNNGA	CCTACGGGNGGCWGCAG
785R_CA_fs3	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NNNCA	GACTACHVGGGTATCTAATCC

Table 3: Summary of analysis of variance of the effect of population and pairing on the three diversity indices Shannon, Inverse Simpson and Richness. The factors have the following levels: population (Belgium, Switzerland, Germany), pairing (monoclonal, mixed). Samples from the triplet treatment were excluded. Significant p-values are shown in bold.

Shannon

Source	df	MS	F	P
Population	2	0.19	0.77	0.4691
Pairing	1	3.17	12.92	**0.0008**
Population x Pairing	2	0.18	0.73	0.4870
Error	42	0.25		

Inv. Simpson

Source	df	MS	F	P
Population	2	2.52	0.59	0.5568
Pairing	1	49.84	11.75	**0.0014**
Population x Pairing	2	6.02	1.42	0.2536
Error	42	4.24		

Richness

Source	df	MS	F	P
Population	2	154.19	2.02	0.145
Pairing	1	456.33	5.98	**0.019**
Population x Pairing	2	44.77	0.59	0.560
Error	42	76.26		

Figure 1: Detailed setup of the experiment. Each combination was 10x replicated. Within a replicate, all five individuals belonging to the same population originated from a single mother. The colored boxes represent the clones of the three different origins, the small circles are the offspring, the ovals are the monoclonal (periphery) and mixed (center) pairs. The very center shows the triplet treatment.

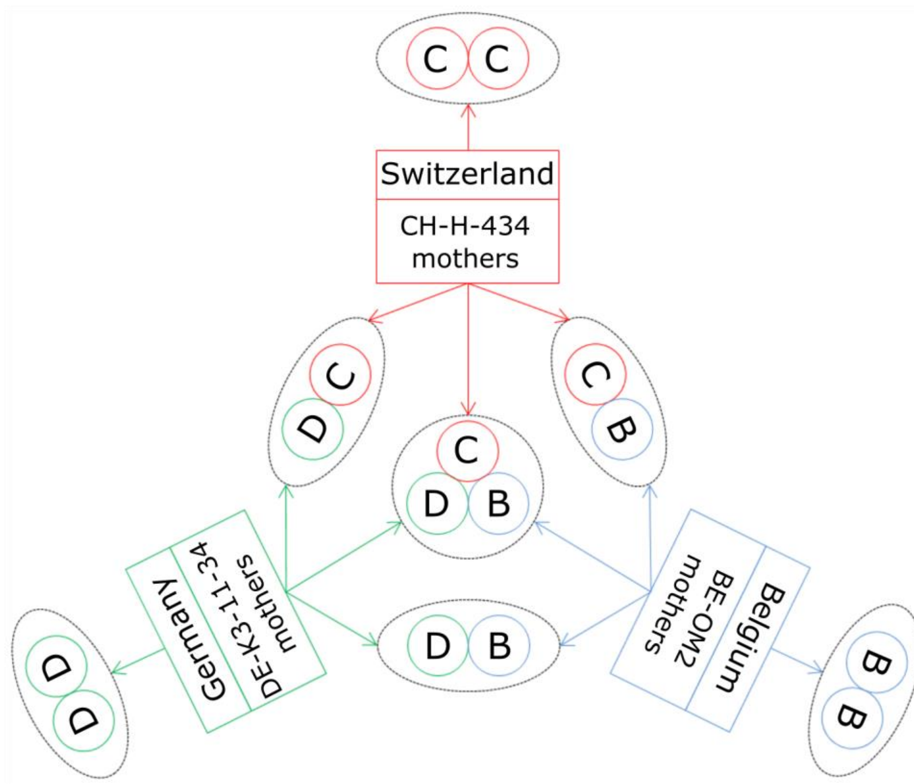


Figure 2: Boxplots illustrating α diversity measures (quantified by Shannon index) of all individuals belonging to the different origins split up by their treatment. Bold horizontal lines indicate medians while box limits show first and third quartiles. Whiskers extend to the most extreme values within 1.5x the inter-quartile range and dots show outlying data points.

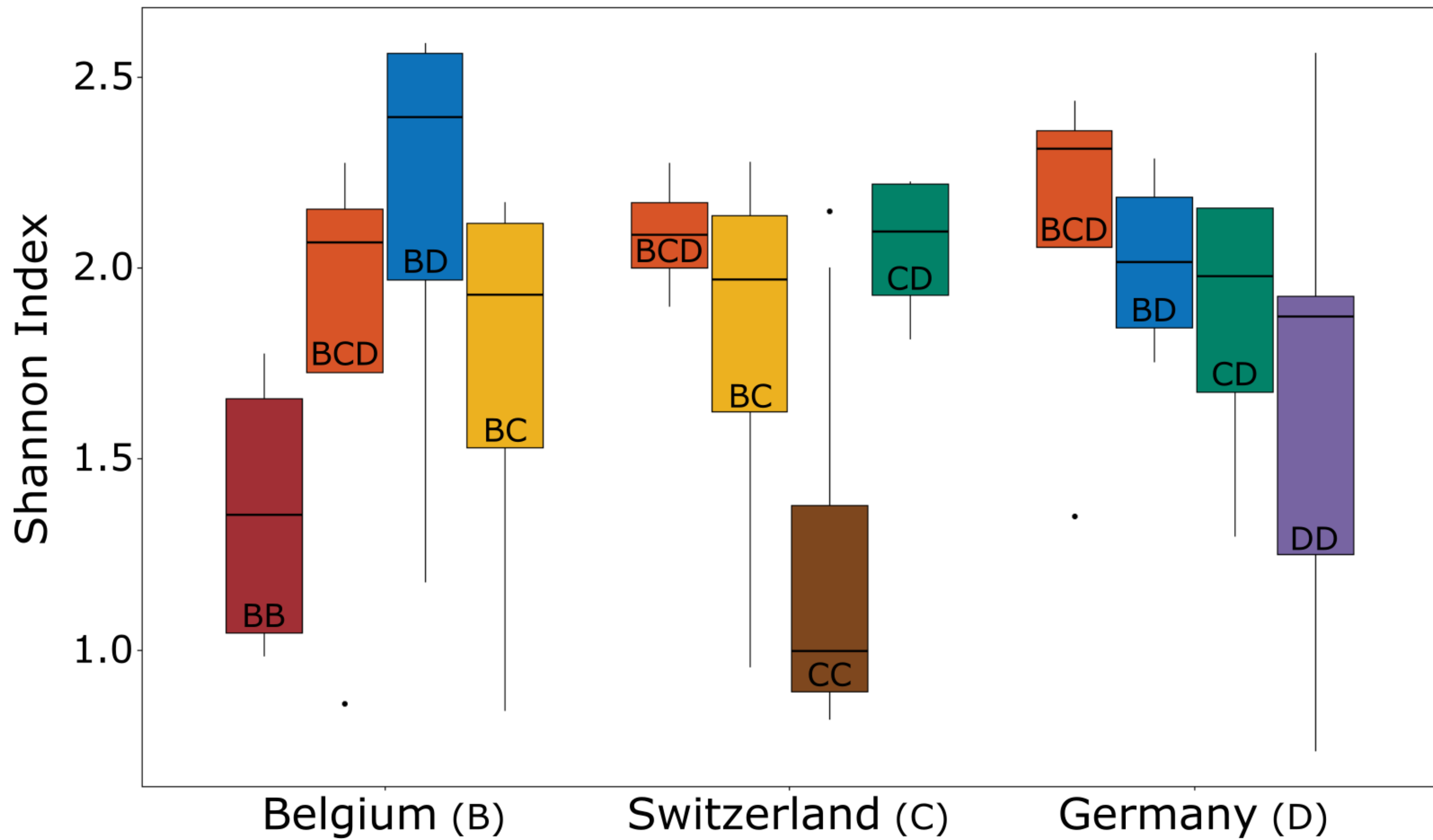


Figure 3: Boxplots showing the alpha diversity indices over all populations for the two treatments using the three diversity indices Shannon, Inverse Simpson and Richness. Bold horizontal lines indicate medians while box limits show first and third quartiles. Whiskers extend to the most extreme values within 1.5x the inter-quartile range and dots show outlying data points.

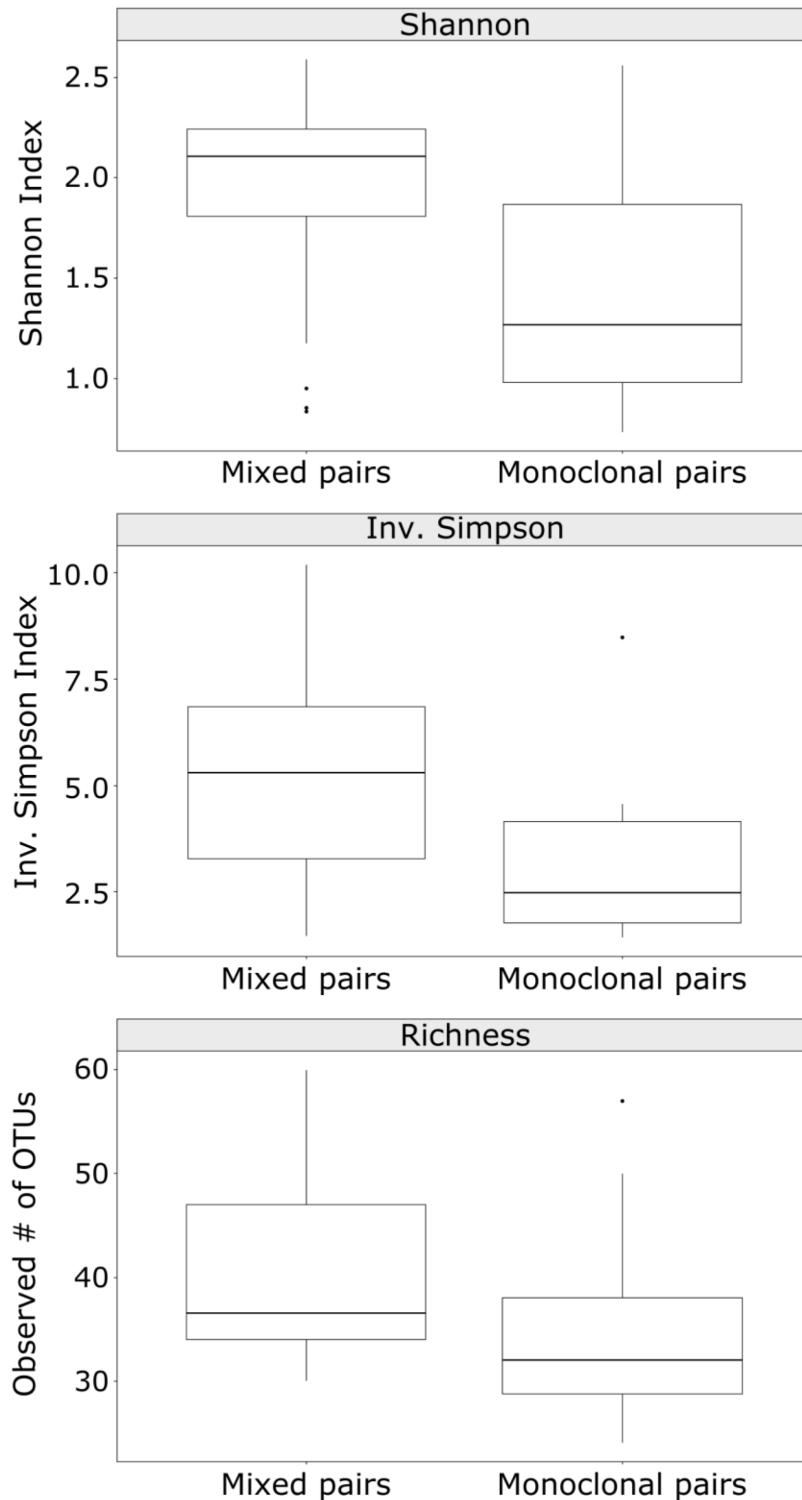


Figure 4: Unweighted UniFrac Principal Coordinates Analysis (PCoA) plot showing microbial community diversity of monoclonal and mixed paires. Each data point represents the microbiota profile of an individual. Samples belonging to the same pairing are color-coded and connected by lines. Labels indicate the different pairings between the used clones from Belgium (B), Switzerland (C) and Germany (D). The variance explained by the PCs is indicated in parentheses on the axes.

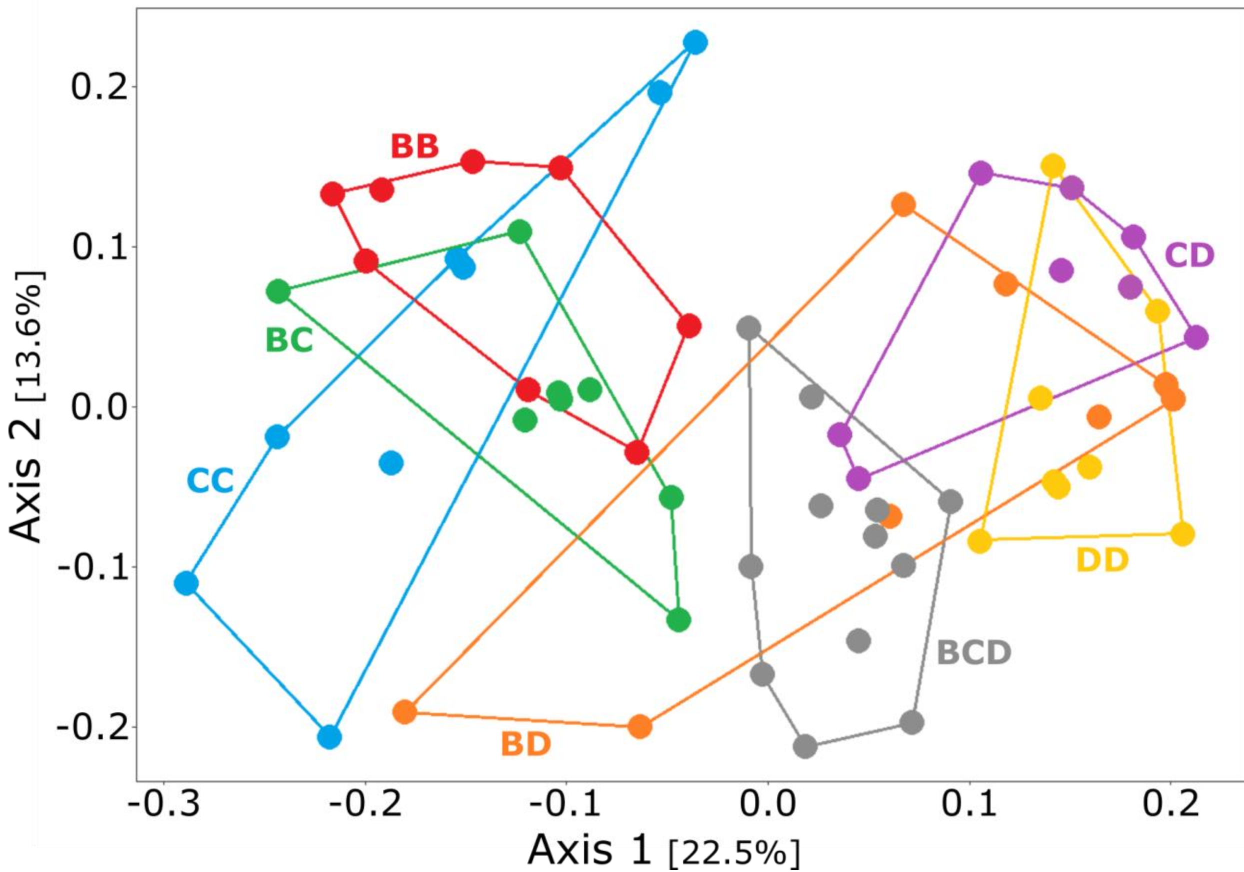
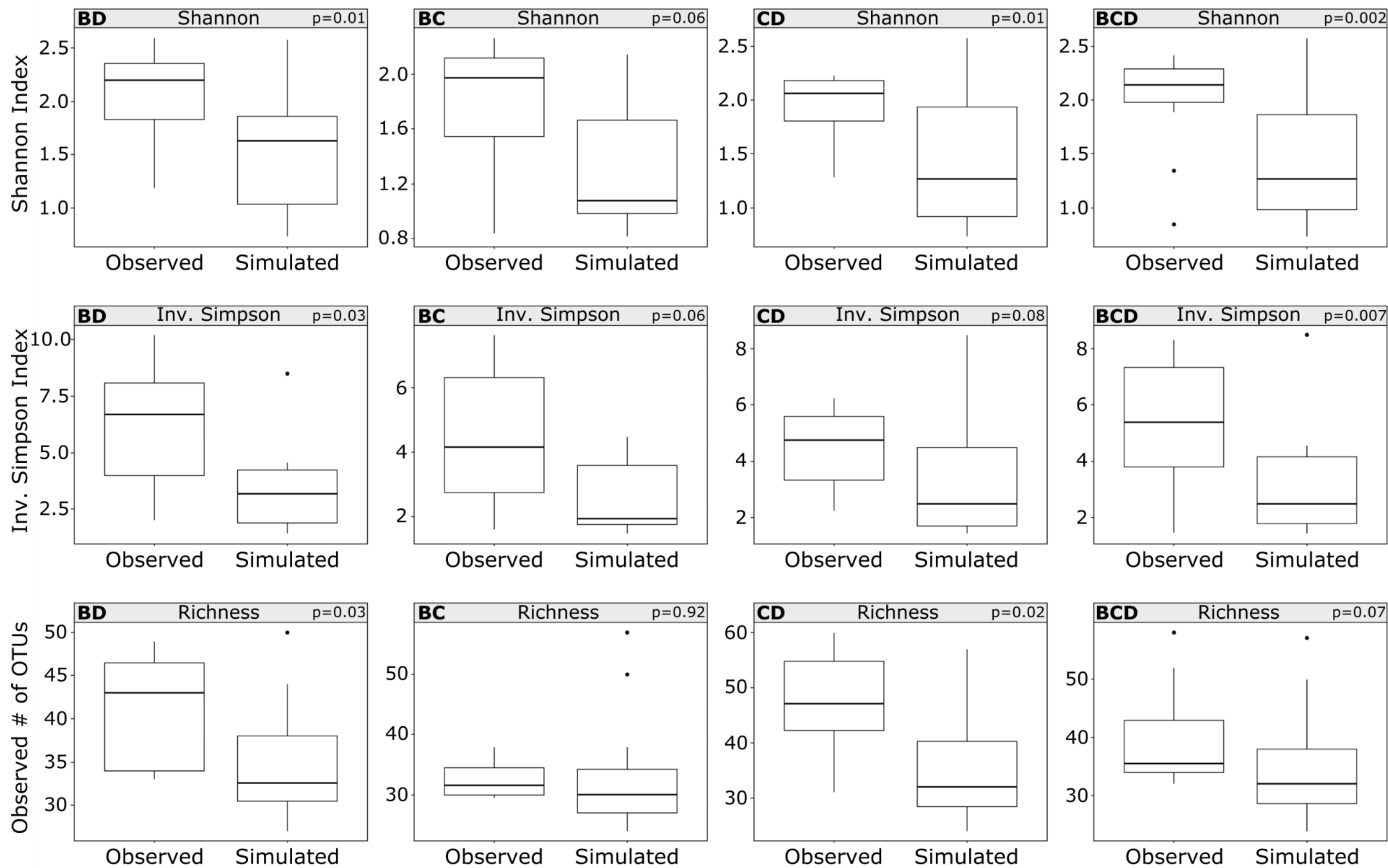


Figure 5: Boxplots showing the Shannon index comparisons between real and simulated number of observed OTUs for all mixed treatment combinations. A Welch two-sample t test was used to determine significance between the groups within a treatment. Holm correction was applied to p values account for multiple testing. Bold horizontal lines indicate medians while box limits show first and third quartiles. Whiskers extend to the most extreme values within 1.5x the inter-quartile range and dots show outlying data points.


BD Shannon p=0.01
BC Shannon p=0.06
CD Shannon p=0.01
BCD Shannon p=0.002
Shannon Index
BD Inv. Simpson p=0.03
BC Inv. Simpson p=0.06
CD Inv. Simpson p=0.08
BCD Inv. Simpson p=0.007
Inv. Simpson Index
BD Richness p=0.03
BC Richness p=0.92
CD Richness p=0.02
BCD Richness p=0.07
Observed # of OTUs
Observed
Simulated

Chapter 4

Consequences of host inbreeding on microbiota diversity in the planktonic crustacean *Daphnia*

Tobias M.M. Schär, Jean-Claude Walser and Dieter Ebert

Author contributions: TMMS conceived the study. TMMS and DE designed the experiment. TMMS performed the experiment, sequencing library preparation and analyses. JCW performed sequence quality control, OTU selection and taxonomic assignment procedures. TMMS wrote the paper. DE revised the paper.

Abstract

Inbreeding, the mating between related individuals, and the resulting negative fitness consequences termed inbreeding depression, have been studied in great detail for diverse traits. Although microbiota are known to contribute significantly to host fitness, up to now they have not been investigated in the context of inbreeding. The freshwater Cladoceran *Daphnia magna* offers the ideal system to investigate this connection since it can be selfed easily and its microbiota is essential for its well-being. Assuming negative consequences from inbreeding depression and knowing that the microbiota can be affected by host genotype, we hypothesized that microbial diversity would decrease in inbred hosts. This was experimentally tested by comparing the microbiota of selfed and outcrossed individuals reared in the same environment. The results showed that differences in the inbreeding status of the hosts did not affect their microbial consortia. We conclude that host homozygosity and microbial diversity are not correlated in *Daphnia magna* and - more generally – suggest that the host's genotype has little influence on the microbiota.

Introduction

Inbreeding, the mating between relatives, results in an increase of homozygous genotypes in the offspring. As a consequence thereof, recessive deleterious alleles may become unmasked leading to a decrease in fitness such as lowering fertility, survival and growth, causing developmental defects and genetic diseases (Jiménez, Hughes, Alaks, Grahamt, & Lacyt, 1994; Mccune et al., 2002; Radha Rama Devi, Appaji Rao, & Bittles, 1987). For example, Sletvold et al. (2013) report that a chlorophyll deficient mutant phenotype in *Arabidopsis lyrata* is responsible for 81 % of seedling mortality in inbred offspring which is absent in outcrossed ones. This phenomenon called inbreeding depression has been documented in many plant and animal species and regularly occurs in natural populations (Crnokrak & Roff, 1999; Keller & Waller, 2002). The genetic basis of this interdependency and the consequent fitness implications have been extensively studied for a wide range of traits (Charlesworth & Willis, 2009; Haag, Hottinger, Riek, & Ebert, 2002).

An understudied factor in the context of fitness and inbreeding is its effect on microbiota, the prokaryotic community living in close physical association with multicellular eukaryotes (Lederberg & McCray, 2001). Numerous studies elucidated the importance of

host-associated microbial communities for diverse aspects of an organism's life in various animal and plant systems (Hehemann et al., 2010; Koch & Schmid-Hempel, 2011; Moran, 2006; Müller, Vogel, Bai, & Vorholt, 2016; Sharon et al., 2010; Silva et al., 2004). Host genotype, among others, was found to be an influential factor for the host-microbiota interplay (Alexander et al., 2006; Olivares, Moisés Laparra, & Sanz, 2013; Spor, Koren, & Ley, 2011). In the simplest scenario, different host alleles would lead to differences in the microbiota. Indeed, as shown by Benson et al. (2010) in mice, variation at a specific locus affects the diversity and population structure of the gut microbiota by influencing the abundance of specific microbial taxa. While some studies are in agreement with these findings (Goodrich et al., 2014; Mcknite et al., 2012), others found no effect of host genetic background on the microbial profiles (Carmody et al., 2015; Friswell et al., 2010). Therefore, it is not yet clear to what degree host genotypes influence the microbiota. But if host genotypes influence the microbial community, the host's degree of homozygosity can be expected to do so as well. Here we tested this hypothesis.

In order to understand how host genotype and microbiota are interconnected, manipulation of the level of inbreeding can be a powerful tool. The aim of this study was to assess the link between host genetic factor and microbial diversity by comparing the microbiota of selfed and outcrossed *Daphnia magna* in a semi-natural environment. Ensuing from a positive correlation between genetic and microbial diversity, we expected selfed animals to harbor less diverse microbial communities compared to outcrossed animals as a consequence of their reduced genetic variation. Surprisingly, our results showed that the level of homozygosity has no systematic effect on microbial diversity.

Material and Methods

<u>Outline of the experiment</u>

We kept pairs of different *Daphnia magna* genotypes in mesocosms and collected the sexually produced resting eggs (ephippia) resulting from selfing and outbreeding. After hatching under natural conditions, the animals were kept to maturity and then genotyped by using microsatellite information to determine the homozygosity level and subsequently used for microbiota analysis by 16S rRNA sequencing.

Study Organism

Daphnia magna individuals were sampled from the Ägelsee in Hohliberg, Switzerland between 2010 and 2014 in regular intervals during the reproductive season from March to October. Clonal isofemale lines were started by placing females individually in jars filled with artificial *Daphnia* medium (ADaM) (Klüttgen, Dülmer, Engels, & Ratte, 1994) and keeping them under standard laboratory conditions: 400-mL jars, ADaM, fed every other day with 50 Mio cells of the green algae *Scendesmus sp.*, 16L:8D light:dark cycle and 20 °C. In this way, as part of an ongoing study, 938 clonal lineages were established.

DNA extraction for initial microsatellite analysis

A subset of 72 clones from the 938 clonal lineages was randomly chosen for analysis with 18 microsatellite markers to find clones with unique genotypes. DNA was extracted following a slightly modified protocol from Edwards *et al.* (1991). In short, animals were individually placed in Eppendorf tubes and excess ADaM was removed, 200 µL extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5 % SDS) was added, homogenized with a pestle and centrifuged at 14'000 rpm for 5 min at 4 °C. 150 µL of the supernatant was transferred to a new Eppendorf tube, 150 µL of cold isopropanol (100 %) was added, briefly vortexed and left at room temperature for 2 min before being put at -20 °C for 15 min. Following centrifugation at 14000 rpm for 5 min at room temperature, the supernatant was discarded, the pellet washed with 500 µL cold ethanol (70 %), vacuum dried and resuspended in 50 µL ddH_2O. Samples were stored at -20 °C until further use.

Microsatellite analysis

On the basis of product size, 18 microsatellite primers (Andras & Ebert, 2013; Colson, Du Pasquier, & Ebert, 2009) were assigned to four multiplex PCR reactions (Table 1). Additionally, the forward primers within a multiplex were uniquely labelled with fluorescent dyes. The varying concentrations of the primer pairs (Table 1) resulted from optimization processes to obtain approximately equal product ratios. PCR reactions were performed on a PTC-200 Peltier Thermal Cycler (MJ Research) in 5 µl reaction volume composed of 2.5 µl Multiplex PCR Master Mix (Qiagen), 1 µl extracted DNA and variable volumes of Q-Solution (Qiagen), H_2O and primers (see Table 1). PCR conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 90 s, 72 °C for 90 s, followed by 8 cycles of 94 °C for 30 s, 52 °C for 90 s, 72 °C for 90 s and final extension at 72 °C

for 10 min. PCR products were analyzed using an ABI 3130 Automated Capillary DNA Sequencer in combination with GeneScan™ 500 LIZ™ dye Size Standard (Applied Biosystems). Allele sizes were determined by eye using GENEMAPPER version 4.0 (Applied Biosystems). After this analysis 46 clones with a unique genotype remained.

Preparation of microbe-enriched culture medium

To be able to determine the influence of the homozygosity level on microbiota diversity, it was preferable to provide hatching *Daphnia* an environment with a diverse microbiota. We produced microbe-enriched ADaM by filling three new 100-L tanks (Semadeni AG, Ostermundigen, Switzerland), thoroughly rinsed to remove putative chemical residues from the production process, with 80 L ADaM, placing them side by side on the roof of the building and leaving them open for three weeks.

Before usage, equal amounts of water from all three tanks were mixed to even out differences in the microbial composition between the tanks. Furthermore, the water was filtered through a 250 µm nylon mesh to get rid of larger particles. To later analyze its microbial composition, 1 L microbe-enriched ADaM was sequentially filtered through a 3 µm Cellulose Nitrate Filter (Sartorius Stedim Biotech), a 1 µm Polycarbonate Membrane (Poretics Corporation) and a 0.2 µm Polycarbonate Track Etch Membrane (Sartorius Stedim Biotech) and the membranes were individually stored at -20 °C in 2-mL Eppendorf tubes containing 400 µL TE-Buffer.

Experimental setup

The 46 clones were paired based on their microsatellite profile in such a way that up to 4 homozygous markers differed among them, which was enough to distinguish selfed from outcrossed offspring (Figure 1). Three 400-mL jars filled with ADaM were set up for every pair, each containing an equal number of individuals from both *Daphnia* clones. To increase ephippia production they were kept under shorter day length and lower temperature: fed every other day with 50 Mio cells of the green alga *Scendesmus sp.*, 8L:16D light:dark cycle and 18 °C. Over the course of 9 months ephippia were collected at regular intervals, placed in 2-mL Eppendorf tubes and stored in the dark at 4 °C in the fridge for at least 3 months.

All resting eggs from a pair were collected by separating them from the protective shell and placed in a 2-mL Eppendorf tube filled with ADaM. Ten 80-mL jars filled with microbe-enriched ADaM were set up for every pair, each supplemented with 12 randomly picked eggs.

The jars were then randomly arranged in trays, placed outside in natural conditions in early August and checked every other day for the presence of hatchlings. As soon as the hatchlings had their first clutch, the animals were frozen individually in Eppendorf tubes at -20 °C until further use.

DNA extraction for sequencing analysis

Total genomic DNA was extracted using the CTAB method. A PVP K90 (20 %) and CTAB 2x (150 mM Tris-HCl pH 8, 4 % CTAB, 2.8 M NaCl) solution were placed in a water bath at 65 °C. Excess ADaM was removed from the frozen animals, 310 µL Lysis Buffer (50 mM Tris-HCl pH 8.3, 40 mM EDTA pH 8.0, 0.75 M Saccharose) was added, homogenized with a pestle and 20 µL Lysozyme solution (10 mg/mL) was added. After 45 minutes of incubation at 37 °C at 850 rpm, 5 µL Proteinase K (20 mg/mL) was added and again incubated for 1 hour at 55 °C at 850 rpm. Following the treatment with 15 µL RNase A (20 mg/mL) for 10 minutes at room temperature, 300 µL CTAB 2x, 12 µL β-mercaptoethanol (0.2 %) and 60 µL PVP K90 (20 %) were added, gently mixed and incubated at 65 °C at 300 rpm for 1 hour. An equal volume of Chloroform:Isoamyl Alcohol (24:1) was added and mixed with care by inversion. After centrifugation at 12'000 rpm for 8 minutes at 15 °C, the upper phase was transferred to a new Eppendorf tube and all steps starting with adding Chloroform:Isoamyl alcohol were repeated once. 50 µL Sodium acetate (3 M, pH 5.2) and 900 µL cold Isopropanol were added to the separated upper phase and stored over night at -20 °C. Following centrifugation at 14'000 rpm for 30 min at 4 °C, the supernatant was discarded, the pellet washed with 1 mL cold ethanol (70%) and centrifuged again (14'000 rpm, 5 min, 4 °C). After discarding the supernatant, the pellet was washed once again with 500 µL, centrifuged, vacuum dried and resuspended in 30 µL TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA pH 8). Samples were kept one night at 4 °C and then stored at -20 °C until further use.

DNA extraction took place over the course of 7 days. Samples from all the different pairings were haphazardly distributed across the extraction days to avoid batch effects. Additionally, every day an extraction negative control using Nuclease-Free Water (Ambion) instead of animal tissue was included.

Library preparation

DNA samples were processed for sequencing on an Illumina MiSeq platform following the adapted protocol of Lundberg et al. (2013) provided by the Genomic Diversity Centre at the

ETH Zürich where the sequencing was carried out. Two PCR reactions were performed on the template DNA. First, the V3-V4 variable region of the bacterial 16S rRNA gene was amplified using the primer pair 341F and 785R with Illumina adapter sequences and 0-3 bp random frameshifts (Table Y). Each PCR reaction contained 12.5 µl 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 0.5 µl of each primer (10 µM/µl), 1.25 µl DMSO (Sigma-Aldrich), 7.25 µl ddH2O and 3 µl extracted DNA adding up to a final volume of 25 µl. PCR conditions consisted of initial denaturation at 95 °C for 5 min, followed by 29 cycles of 98 °C for 20 s, 57 °C for 15 s, 72 °C for 15 s and final extension at 72 °C for 5 min. All samples were set up in triplicates to increase the yield and reduce the risk of failed amplification. The PCR products of the triplicates were pooled and purified using the magnetic beads Agencourt AMPure XP system (Beckman Coulter) at 1:1 a beads/PCR product volume ratio. A second PCR to index each sample was performed in 50 µl reaction volume containing 25 µl 2x KAPA HiFi HotStart ReadyMix (Kapa Biosystems), 5 µl of each primer from the Nextera XT Index Kit v2 (Illumina), 2.5 µl DMSO (New England Biolabs), 7.5 µl ddH2O and 5 µl purified PCR product. PCR amplification was carried out as follows: initial denaturation at 95 °C for 3 min, followed by 10 cycles of 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 5 min. After additional purification as described above, the concentration of the libraries was quantified with the Spark 10M Multimode Microplate Reader (Tecan) and qPCR. Samples were normalized and pooled in an equimolar fashion. To remove residual impurities, the library pool was bead purified once again and diluted to a concentration of 2 nM. Afterwards the pool was denatured (NaOH 0.2N), diluted to 10 pM and 15 % (v/v) PhiX was added. Finally, the mixture was loaded onto the Illumina MiSeq according to the manufacturer's instructions using the MiSeq Reagent Kit v3 (2x300 bp Paired-End Reads).

Negative controls from the DNA extraction step as well as negative controls using Nuclease-Free Water (Ambion) in place of the extracted DNA were included throughout the whole process of library preparation.

Quality assessment and quality control of the sequencing data

The raw MiSeq sequencing data were processed in a series of control steps. First, the reads were quality controlled with FastQC (Babraham Institute, UK). Then the paired reads were merged (FLASH v1.2.9), primer sequences got trimmed (Cutadapt v1.9.1) and they got size selected and quality filtered (PRINSEQ-lite v0.20.4). The clustering into Operational Taxonomc Units (OUT), including abundance sorting and chimera removal, was conducted

using USEARCH v9.0.2132 implemented in the UPARSE pipeline (Edgar, 2013). Only OTUs passing the selection criterion of being represented by 5 or more reads were considered for further analysis. As a last point, taxonomy was assigned using UTAX against the GreenGenes v13/5 database.

Statistical data analysis

The software package R 3.3.1 (R Core Team) and the Bioconductor library phyloseq (McMurdie & Holmes, 2013) were used to perform data filtering, statistical analyses and to prepare figures.

As a first step, samples with less than 5'000 reads were excluded. The remaining samples were decontaminated by excluding OTUs present in the negative controls if they represented more than 5 % of the corresponding OTUs total reads. Subsequently singletons and OTUs appearing in only one sample were removed and the data were rarefied to an even sampling depth of 5'029 reads per sample. Alpha diversity was calculated using three indices: Shannon, inverse Simpson and observed species richness. To determine statistical dissimilarities in the 16S profiles of the animals, analysis of variance (ANOVA) and non-metric multidimensional scaling (NMDS) were run.

Results

From the 23 host clone pairs 22 produced enough ephippia to be used in the experiment. For 11 out of these 22 pairs all three types (two selfed, one outcrossed) could be recovered within a replicate. These 11 families were then sequenced. After sequencing, one pair was excluded due to low number of reads. In the pairs not fulfilling the requirements only one selfed type was present in three cases, the outcrossed type was missing in four cases and one of the selfed types was absent in four pairs. These differences were most likely the result of one clone out-competing the other, biased hatching success (inbreeding depression) or a combination of both.

First, a nonmetric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarities allowed us to visualize the differences in the diversity of community composition across families (Figure 2). ADONIS analysis using Bray-Curtis distances showed that there were significant difference in community composition between families (R^2=0.69; p=0.001).

Second, using the three different measures of alpha diversity, we looked at how bacterial diversity differed between selfed and outcrossed offspring (Figure 3). Microbial diversity differed marginally to significantly between the families, but cross and its interaction with the families had no effect on diversity in all measured indices (Table 2). Thus, microbiota of selfed offspring did not systematically differ from those of outcrossed offspring.

Discussion

Inbreeding is well known for its negative effects on various traits in many species, including *Daphnia* (De Meester, 1993; Deng, 1997; Haag et al., 2002; Innes, 1989; Swillen, Vanoverbeke, & De Meester, 2015). However, our study found no influence on microbiota composition. Microbial diversity did not differ between selfed and outcrossed individuals. More generally we infer that there is no correlation between genetic and microbial diversity in *Daphnia* and conclude that the diversity of microbiota of *Daphnia magna* is not or hardly influenced by the genotype of the host.

Our result that the level of homozygosity has no effect on microbial diversity contrasts findings from Hufeldt et al. (2010), who showed that in mice individuals from an outbred colony had lower gut microbiota similarities (corresponding to a higher diversity) than those from an inbred colony. This discrepancy might result from differences in the experimental conditions. While they compared separately kept colonies, we compared selfed and outcrossed offspring cohoused under identical conditions. Thus, based on our experimental design, potential confounding factors were excluded and the only difference between the individuals within a family was their level of homozygosity.

Inbreeding depression and competition between clones are known to be strong in *D. magna* and may have contributed to the circumstance that we were not able to recover the three types of offspring (two selfed, one outcrossed) from all our pairs. Differences in the inbreeding levels of the parental clones and clonal variation in the production of males and sexual eggs (Roulin et al., 2013; Yampolsky, 1991) might have contributed to this. Further, selection against unfit selfed offspring might have alleviated the observed effect of inbreeding and might explain why we do not see differences in the microbiota between selfed and outcrossed animals. Inbreeding has been found to affect development (Chai & Degenhardt, 1962; Mccune et al., 2002; Michaud, Bultman, Stubbs, & Woychik, 1993; Réale & Roff, 2003). In the Hemiptera *Delphacodes pellucida* Fabricius, for example, Kisimoto & Watson (1965)

showed that in the first generation of inbreeding many eggs failed to hatch and embryos became abnormal at various stages of growth.

Looking at the individuals within families provides further evidence supporting our findings. Although each family encompassed three genotypes, they harbored microbial communities with similar diversities, while the opposite was found between families, showing distinct differences in their microbial diversities. This is in contrast to results from Org et al. (2015) demonstrating that in a controlled environment the microbiota of mice is defined by their genetic background. But since our experiment was carried out under semi-natural conditions, the environment, a well-known factor affecting the microbiota (Claus, Guillou, & Ellero-Simatos, 2016; Yun et al., 2014), was not well controlled. This raises the question of whether this effect can be attributed to host genetics or if the environment could be the driving force. A plausible scenario is that even though the conditions at the beginning were identical for all families, they changed over the course of the experiment due to stochastic events. As a result, unique microcosms established within the jars. Picking up these environmental differences in the absence of genetic regulations the animals closely resembled their surroundings. Therefore, environmental heterogeneity needs to be taken into account when examining effects of inbreeding on *Daphnia* microbiota.

Our general conclusion from the current study is that in *Daphnia magna* genetic and microbial diversity are not or hardly correlated. Our findings indicate that the genotype of the host has little to no influence on the composition of the microbiota. The environment may be the main source for microbial differences. The degree to which our finding is universally valid is not clear. Thus it is important to run experiments powerful enough to disentangle the effects of genetics from environmental influences.

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Figure 1: Crossing scheme of two parental clones (P) and the resulting selfed and outcrossed offspring (F1) from sexual reproduction. All individuals of a certain generation were always kept together, thus experiencing the same environmental conditions.

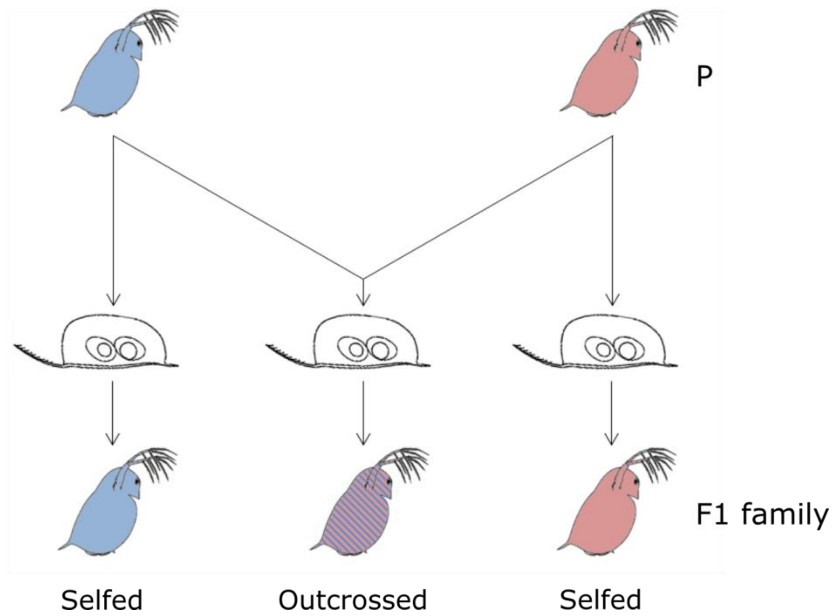


Figure 2: Non-metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarity between samples. The goodness of fit (or stress) associated with this ordination is 0.26. Each data point represents the microbiota profile of an individual (circle=selfed; asterisk=outcrossed). Samples belonging to the same family are color-coded and connected by lines.

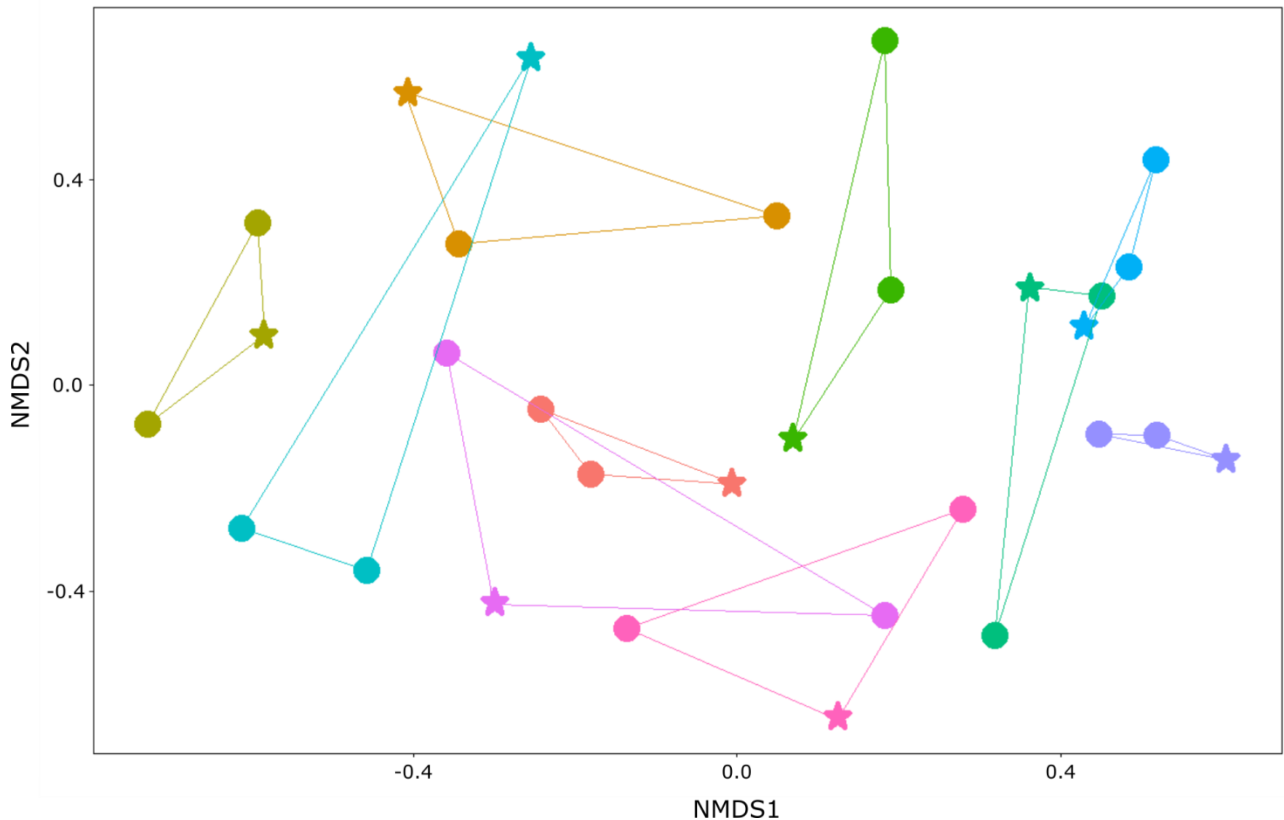
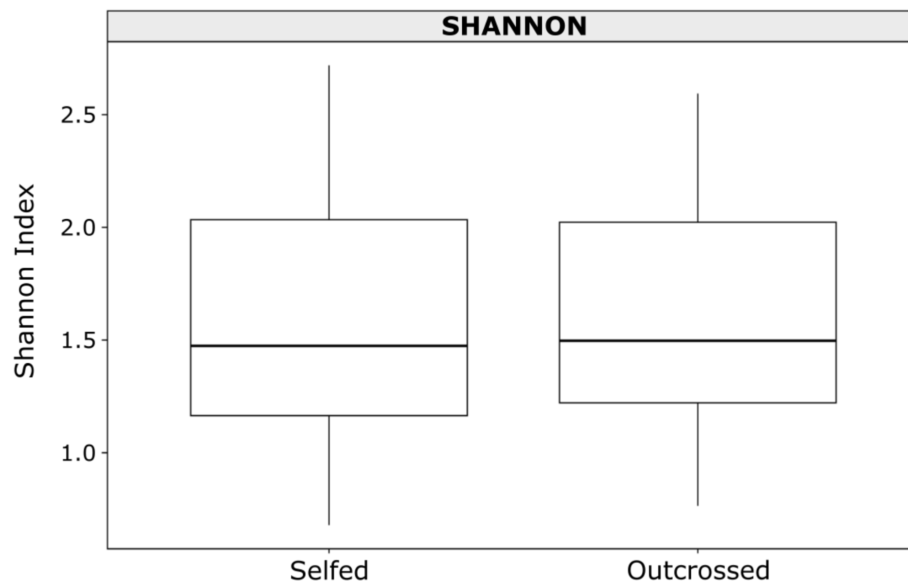


Figure 3: Boxplots showing the alpha diversity indices for the selfed individuals (N=20) and the outcrossed individuals (N=10) using the three diversity indices Shannon, Richness and Inverse Simpson. Bold horizontal lines indicate medians while box limits show first and third quartiles. Whiskers extend to the most extreme values within 1.5x the inter-quartile range and dots show outlying data points.


SHANNON
Shannon Index
2.5
2.0
1.5
1.0
Selfed
Outcrossed

RICHNESS
Richness
(Observed # of OTUs)
100
80
60
40
20
Selfed
Outcrossed

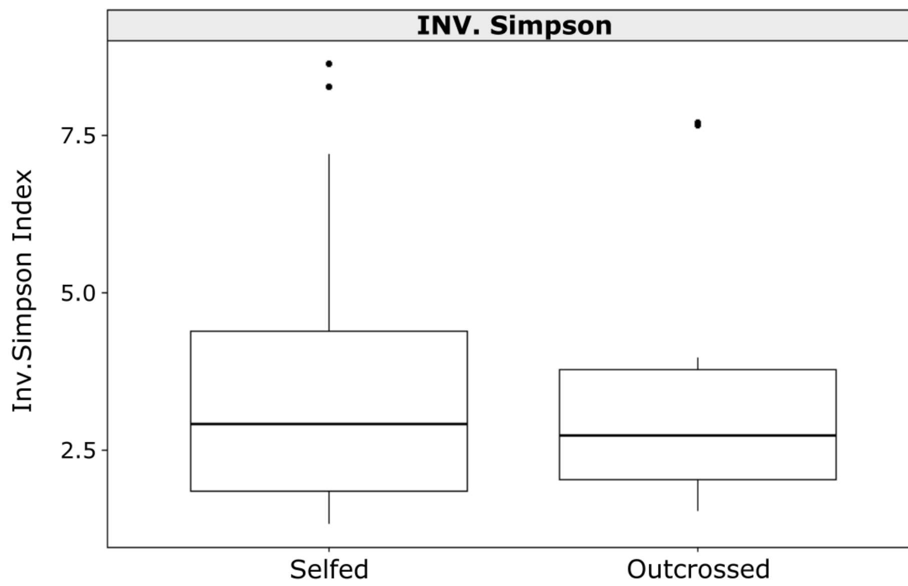

INV. Simpson
Inv.Simpson Index
7.5
5.0
2.5
Selfed
Outcrossed

Table 1: Details of the used microsatellite primers (5'-3').

Multiplex	Locus	F-primer sequence	R-primer sequence	Conc. In PCR (µM)	Size (bp)
1	214	GGCAACGATAGACCACGAAT	GTCATCTACGAAGGCGAAGC	0.2	383/414
	33	TTATTGCATCGTCAGCATCG	CGATTATGTTTTCATCACGATTG	0.2	170/176
	34	TTCCACTCAGGTCACGACAG	CCAAAAGCGATACCTTCGTT	0.2	203/209
	9	AAATTTGCAGCCCATTTGTC	ATTCAACGATCCGGACACTC	0.2	179/185
	93	GTGTGGATGCGTGAGTTTTG	CGCGTGCACCAACAATAATA	0.2	120/124
2	13	AAAGGGTTCCGGTAACCAAG	AAAGCAAACCCTTCCCTAGC	0.2	146/147
	173	CGTCGCTAGCAATCAAACTG	CAGCCTCAGTATGCTCCACA	0.3	420/474
	193	ACCTACGAGGCTGACGAGAA	CTCGGCATCGTGTTTGTATG	0.05	319
	216	GATAGCGGTGGCTTGAGTTT	TTAGTCCGCTCCGGTCTATG	0.2	219
	24	ACAAGTCAATTCTGCCCACA	CAAAAGCGAAAACGGAAGTC	0.2	140/148
3	12	TTCGGGAGTCATCCAAAAAG	TGCAGCCAAAGAGTCAAAGA	0.2	246/252
	183	AATGGCACGGTAAAATACGC	ACACCCGACTGACGATTTTC	0.2	192/198
	184	GTCATCGTCAATCGAGGCTA	CGTTGTCCACCCAATTATCC	0.2	241/250
	208	GGCTGCCGAAAGTAATGAAA	GCTCTGTAAACGGTCGCTGT	0.2	345/353
4	210	GACGTTCAGCCGTTAGTTCAG	CTATGAGGCTCCTGGTCAGC	0.05	195/224
	32	GTGGATGGATCTCGGTGAAA	TGTCTGCCATCTCCAGACAG	0.05	253/280
	75	CACGAGGTCCACCACAAAAT	CCGGTTTAGGGCAAAGAATA	0.05	250
	85	TCGGCTGATTTGCATTTCGT	AAACGATCTGCCCTGAATTG	0.2	368/374

Table 2: Summary of analysis of variance of the effect of cross and family on the three diversity indices Shannon, Inverse Simpson and Richness. The factors have the following levels: cross (selfed, outcrossed), family (1-10). Significant p-values are shown in bold.

Shannon

Source	df	MS	F	P
Cross	1	0.0002	0.0008	0.98
Family	9	0.71	3.28	**0.04**
Cross x Family	9	0.24	1.12	0.43
Error	10	0.22		

Richness

Source	df	MS	F	P
Cross	1	0.60	0.004	0.95
Family	9	490.60	2.99	0.051
Cross x Family	9	254.97	1.55	0.25
Error	10	164.00		

Inv. Simpson

Source	df	MS	F	P
Cross	1	0.05	0.01	0.91
Family	9	7.19	1.84	0.18
Cross x Family	9	3.91	1.00	0.49
Error	10	3.91		

Conclusion

The series of experiments summarized in this thesis substantiate the role of *Daphnia* as a powerful model system by providing a basis to establish it as a model for experimental aquatic host-microbe research. Essential insights into fundamental mechanisms and processes between *Daphnia* and their associated microbial community were gained, contributing to the ever-growing knowledge necessary to eventually understand host-microbe interactions in their full complexity.

We investigated how specific the association of *Daphnia* and their accompanying microbes is by reciprocally transplanting the microbiota between different host species and measuring different life history traits to quantify fitness consequences that might arise. We find that there are specific interactions between *Daphnia* and their microbial consortia, but not supporting the idea of coevolved mutualistic relationships. This is supported by the fact that being associated with a foreign microbial community does not negatively affect fitness in *Daphnia magna* and *Daphnia pulex*. This is in contrast to what Rawls et al. (2006) found, using zebrafish and mice. Although metabolic responses were stimulated in both hosts by the microbiota from either species, immune responses were induced only by their respective normal microbiota. Also Salem et al. (2013) demonstrated a high degree of host-symbiont specificity in two bug species from the Family Pyrrhocoridae where fitness of symbiont-deprived bugs could only be completely restored by the original microbiota. This discrepancy could be attributable to ecological differences of the hots, as ecology has been shown to play an important role for microbiota composition (Ferrari, West, Via, Godfray, & Al, 2011; Hird, Sánchez, Carstens, & Brumfield, 2015). While the hosts used in these and other examples (Koch & Schmid-Hempel, 2012; Lau & Lennon, 2012; Sison-Mangus, Jiang, Tran, & Kudela, 2014) differed in this regard to a greater or lesser extent, we worked with species co-occurring in the same habitat occupying overlapping niches. Further, we confirmed previous results that *Daphnia magna* suffers from negative fitness effects in the absence of microbes (Callens et al., 2016; Peerakietkhajorn, Kato, Kasalický, Matsuura, & Watanabe, 2016; Peerakietkhajorn, Tsukada, Kato, Matsuura, & Watanabe, 2015; Sison-Mangus, Mushegian, & Ebert, 2014), at the same time demonstrating the identical effect in *Daphnia pulex*. Thus, this is likely a general pattern found in *Daphnia*. From these results it becomes apparent that although there are specific interactions between *Daphnia* and their microbes that are essential for *Daphnia* in order to survive, the relationship does not support the idea of coevolved mutualism. Part of this is likely the result of the constant coexistence with and exposure to the omnipresent

microbes in the aqueous environment. At the same time, the cyclic parthenogenetic life cycle of *Daphnia* also plays an important part in contributing to this. Since no bacteria are transmitted vertically through ephippia (Mushegian, Walser, Sullam, & Ebert, 2017; Sison-Mangus, Mushegian, et al., 2014) the microbial community needs to be reestablished at the beginning of every growth season anew from the environment.

In another experiment we show that only a fraction of the microbiota gets transmitted from mothers to their offspring during asexual reproduction. This is in accordance with results from other studies showing that the microbiota of mothers and offspring are not totally identical (Inoue & Ushida, 2003; Ley, Hamady, & Lozupone, 2008). On top of this we find that the ratios of shared and unique microbes between mothers and offspring were uniform across native and foreign microbiota origins. We hypothesize that the consistent ratios are attributable to the interplay of an undirected transmission process and the anatomical features of *Daphnia*. Since the brood pouch, an open system with water circulation where the developing asexual eggs get deposited, is located in close proximity to the anal opening, it is very likely that the developing embryos get exposed to microbes transmitted through feces. The circumstance that the origins do not differ illustrates again that *Daphnia* are habituated to the coexistence with microbes per se but not with a certain community, confirming our previous notion regarding the specificity of the *Daphnia*-microbiota association.

Once established, the constant exposure of these associations to various biotic and abiotic factors imposes the challenge of keeping them in a healthy, stable state (Faith et al., 2013; Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). We kept *Daphnia magna* individuals with different microbial communities in monoclonal and mixed pairs to examine microbiota stability, defined as resistance to colonization and replacement by new microbes, and to determine how two microbiota affect each other's composition. While for instance Alpert et al. (2008) showed that between 77 to 88 % of the microbes in mice remained stable over a period of 12 months, we report that the microbial communities in *Daphnia* converged after 21 days by incorporating new microbes. A possible explanation for this finding is given by Tap et al. (2015) who observed that in humans higher microbiota richness was associated with higher microbiota stability. Based on this, knowing that microbial diversity of *Daphnia* from the field decreases upon entering the laboratory environment (Pichon, unpublished; Sullam et al., in prep.), one would expect the microbiota to become less stable. This seems to apply to our experiment at first appearance, but the microbes only intermixed and not replaced each other. Thus, instead of rating it as unstable, we suggest to classify the *Daphnia*-

microbiota association as modifiable. In addition, as the individuals with different microbial communities originated from different populations which is equivalent to different genotypes, we were able to demonstrate that host genotype did not influence the process of microbiota intermixing.

On the basis of this discovery, we attempted to investigate the influence host genotype exerts on the microbiota in more detail. For this purpose, the microbial communities of selfed and outcrossed animals were compared to link their homozygosity level to differences in their microbial diversity. Earlier studies on this topic in mice illustrated that no differences between inbred and outbred individuals with respect to their microbial diversities were detectable (Kreisinger, Cížková, Vohánka, & Piálek, 2014; Pang et al., 2012). Our results are in accordance with these findings, corroborating previous conclusions that in *Daphnia magna* the genotype does not affect the microbiota.

This thesis represents a significant advancement in understanding basic mechanisms in the *Daphnia*-microbiota system and provides fundamental knowledge essential for future research to build upon. The main conclusions are that I) beneficial microbes are most likely common in the environment, as *Daphnia* do not form coevolved mutualistic relationships with a specific community; II) the association is modifiable but not influenced by the genotype of the host and III) mothers transfer a stable portion of their microbiota vertically to their offspring during asexual reproduction while the rest is likely of environmental origin. Taken together, these results demonstrate that the *Daphnia*-microbiota relationship represents a promising and powerful tool to address basic questions about the complex interplay between hosts and microbes and to investigate how they affect important aspects of each other's life.

Outlook: Further directions of research

The experiments presented in this work represent investigations spanning a limited range of the whole spectrum of interdependencies between *Daphnia* and their associated microbes. Thus, it is certain that the true level of complexity of this relationship is underestimated and remains unexplored. In the following paragraphs we therefore discuss different aspects and limitations of our work and propose possible directions for future research.

First, we were limited to ephippial eggs as sole source for axenic animals because at the time the experiments were conducted it was not possible to reliably get viable axenic animals from asexually produced eggs. While the feature that every ephippial egg represents a

different genotype allowed us to show that the host genotype does not influence the microbiota, it made it impossible for us to detect putative genotype specific effects. Since in the meantime there is a protocol published to reliably get sterile parthenogenetic eggs (Peerakietkhajorn et al., 2015) we suggest to conduct similar experiments as described in this thesis with asexual eggs to confirm the validity of our findings on an even narrower genetic scale.

Second, the microbiota was always treated as an entity. However, the microbes might very well differ in their ways they interact with the host. The situation is similar to the previous one: looking at how the whole microbiota interacts with the host masked effects of individual microbes. Thus, we recommend focusing on individual members rather than the whole community. In combination with the possibility to now keep the genotype of the host constant by using parthenogenetic eggs, this has the potential to reveal unexpected and astonishing effects.

Last but not least, we directed our attention primarily to how the host benefits from being associated with microbes. But to what degree do microbes benefit from that? As this cannot be assessed for the whole community, we advise to use *Daphnia* with mono-associated microbes to tackle this question. Subsequently, fitness comparisons between associated microbes and their free-living forms can be carried out.

Despite all that, our work provides valuable insights into the basic mechanisms of beneficial associations between *Daphnia* and the microorganisms they coexist with. As we have just started to scratch the surface, this model system still holds many surprises waiting to be revealed.

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Part 2

Chapter 5

Temperature-dependent benefits of bacterial exposure in embryonic development of Daphnia magna resting eggs

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Author contributions: AAM designed and performed all experiments, analyzed data and wrote the paper. EB identified and cultured bacterial strains. TMMS set up the experiment involving parthenogenetic eggs. DE designed experiments and edited the paper.

Abstract

The environments in which animals develop and evolve are profoundly shaped by bacteria, which affect animals both indirectly through their roles in biogeochemical processes and also through direct antagonistic or beneficial interactions. The outcomes of these activities can differ according to environmental context. In a series of laboratory experiments with diapausing eggs of the water flea *Daphnia magna*, we manipulated two environmental parameters, temperature and presence of bacteria, and examined their effect on development. At elevated temperatures (≥26 °C), resting eggs developing without live bacteria had reduced hatching success and correspondingly higher rates of severe morphological abnormalities compared to eggs with bacteria in their environment. The beneficial effect of bacteria was strongly reduced at 20 °C. Neither temperature nor presence of bacteria affected directly developing parthenogenetic eggs. The mechanistic basis of this effect of bacteria on development is unclear, but these results highlight the complex interplay of biotic and abiotic factors influencing animal development after diapause.

Introduction

All animals evolved in an environment with an omnipresence of bacteria. Bacteria affect animals' environments from global scales (e.g. driving elemental cycles and ecosystem productivity (Howard et al. 2006; van der Heijden et al. 2008)) to extremely local (e.g. degrading polysaccharides in the gut (Martens et al. 2011)). Accordingly, animal evolution has widely featured adaptations to ecosystems shaped by bacteria (McFall-Ngai et al. 2013), as well as interactions with bacteria that affect animals' responses to other environmental factors. Bacteria can protect animals and their embryonic stages from pathogens (Gil-Turnes et al. 1989), heavy metal pollution (Senderovich & Halpern 2013; Breton et al. 2013), or toxic secondary compounds in plant diets (Kohl, Weiss, et al. 2014); conversely, they can convert xenobiotics into more harmful forms (Freeland & Janzen 1974; Zheng et al. 2013). Bacteria can provide crucial signals about the environment, as in the case of marine tubeworm larvae that use molecules from surface-associated bacteria as cues to settle and metamorphose (Shikuma et al. 2014). Presence of bacteria is an environmental factor that induces aspects of the development of the vertebrate gut epithelium (Bates et al. 2006) and immune system (Ivanov et al. 2009), influencing fat storage (Semova et al. 2012) and systemic inflammatory

response (Galindo-Villegas et al. 2012). The role of bacteria in normal animal development has been further demonstrated in mosquitoes, which failed to develop past the first larval instar without bacteria (Coon et al. 2014), and in Drosophila, which failed to develop under nutrient-poor conditions without bacteria (Shin et al. 2011). The specificity, evolutionary history, and underlying mechanistic causes of these types of interactions vary widely (Angela E. Douglas 2014).

Under changing environmental conditions, the effects of positive interspecies interactions can become dampened or more pronounced. If one or both species are stressed, the effect of each individual interaction might be altered, if the ability of one or both species to perform their functions is affected or if a particular function becomes more important for fitness (Xie et al. 2013; Kiers et al. 2010; Márquez et al. 2007). Furthermore, stressful conditions can reveal cryptic phenotypic variation among individuals, meaning the variation and net effect of the interaction on the population level might be altered. The stresses caused by increasing global temperatures are predicted to affect many insect-symbiont interactions (Wernegreen 2012), change the phenology of plant/herbivore or plant/pollinator interactions (Musolin et al. 2010), and generally affect the microbial ecology of aquatic environments.

The water flea *Daphnia*, a planktonic microcrustacean, is a model for studies of organismal responses to ecological challenges in both basic and applied research settings (Colbourne et al. 2011). Found in a geographically and ecologically wide range of environments, from the tropics to arctic regions, *Daphnia* species exhibit great phenotypic diversity and have been used to test numerous theories in evolutionary ecology (Ebert 2011; Altermatt & Ebert 2008; Lynch & Ennis 1983). In addition to being used as an environmental quality monitor under contemporary conditions, *Daphnia* also serves as a record of historical adaptation to changing environments through dormant stages archived in sediments, which can be "resurrected" and compared to modern phenotypes (Frisch et al. 2014). These resting stages, encased in chambers called ephippia, are produced by *Daphnia* in the sexual phase of its reproductive cycle, typically in response to conditions indicating environmental deterioration or the end of a season (e.g. crowding or changes in photoperiod). Development of the resting stage arrests at the onset of gastrulation, in an approximately 1000-cell stage (Baldass 1941) with the embryo contained in a protective, inflexible tertiary egg membrane in addition to the two membranes found around directly developing parthenogenetic eggs (Navis et al. 2015). These ephippial embryos can then persist for periods of days to decades and be dispersed to new habitats, surviving drying, temperature extremes, anoxia and chemical

exposure. For simplicity, we refer to the diapausing, tertiary-membrane-bound embryos as "eggs" and use "embryo" to refer to all post-diapause developmental stages until the animal reaches a freely swimming state. (Throughout this paper we use eggs that have been removed from ephippial shells in order to standardize their treatment; we emphasize this to avoid confusion arising from the fact that some literature uses "resting egg" to refer collectively to the entire ephippium and the embryos inside it.) The cues and environmental conditions allowing emergence from diapause are relatively poorly understood (Smirnov 2014; Vanvlasselaer & De Meester 2010), but the "seed bank" of resting eggs of *Daphnia* and other invertebrates is recognized as an important component of ecosystem dynamics (Hairston 1996). Resting stages may spend considerable lengths of time in varying degrees of contact with bacteria-rich sediments, and bacteria have been detected on the inside surfaces of ephippial shells (Schultz 1977). The roles of bacteria at all stages of the *Daphnia* life cycle are therefore of interest for understanding determinants of phenotype and fitness and subsequent effects on the ecosystem.

We previously found that *Daphnia magna* raised in sterile environments after emerging from surface-sterilized eggs grow more slowly, reproduce less, and die sooner than animals subjected to identical treatment but colonized with bacteria ("conventionalized" by exposure to bacteria from homogenized adult *Daphnia* during development) (Sison-Mangus et al. 2014). In the course of developing our protocols for germ-free and conventionalized animals, we serendipitously observed that under some conditions, a beneficial effect of bacteria on fitness could be observed even earlier, during embryonic development of resting eggs. In a series of experiments manipulating temperature and bacterial environment of surface-sterilized eggs in fully factorial setups, we confirmed that at temperatures of 26-28 °C, in the absence of live bacteria, embryonic development failed at higher rates than when bacteria were present in the hatching medium.

Methods

<u>Comparing hatching rates</u>

Except where noted, diapausing eggs used in these experiments were collected in a carp pond near Munich, Germany (site code DE-K2-2; coordinates = N 48.2046028°, E 011.6793556°). Ephippia were collected at this site in 2009 and have since been kept in moist conditions in the dark at 4 °C. Eggs were manually removed from ephippia under a dissecting microscope

using forceps and transferred to tissue culture plates containing artificial *Daphnia* medium (ADaM) (recipe at http://evolution.unibas.ch/ebert/lab/adam.htm). Collected eggs were stored in the dark at 4 °C overnight until experiment was set up the following day.
To manipulate temperature, we constructed a cooling device to hold six 96-well flat-bottomed tissue culture plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) under an overhead light with a cooling element under one half of each plate. The temperature in the cool half was adjusted to 20 °C (hereafter referred to as "standard" temperature) while the temperature in the uncooled half, warmed by the lamp, ranged from 26 to 28 °C (hereafter referred to as "warm" temperature).

All eggs were surface-sterilized in one batch with household bleach (≤5% sodium hypochlorite) for 5 minutes in an Eppendorf tube, which was inverted continuously to expose all sides of eggs. Bleach was removed and eggs were washed by adding and removing sterile (autoclaved) ADaM or water 3 times. Eggs were transferred into a wide, shallow dish of sterile ADaM and haphazardly placed in individual wells of 96-well tissue culture plates containing 180 µl sterile ADaM. No eggs were placed in the wells immediately alongside the temperature boundary at the center of the plate.

Alternating rows of wells were assigned to be sterile (STE) or conventionalized (CONV) (randomizing the assignment of the first row), with equal numbers of STE and CONV rows in each plate. To the CONV rows, 20 µl *Daphnia* homogenate (consisting of 10 intermediate-sized adult *Daphnia* freshly homogenized in 1.5 ml ADaM) was added. To the STE, 20 µl sterile ADaM was added. These procedures were carried out under a sterile laminar flow hood. Plates were covered and inspected with an inverted light microscope; any eggs that were visibly mechanically damaged were excluded from further analysis. Plates were then placed on the cooling device, randomizing which half of the plate was cooled.

Substantial numbers of free-swimming hatchlings were observed in the warm treatment 3 days after the experiment was set up, and in the cool treatment 1 day later, consistent with previous observations of temperature effects on development time. We checked for hatchlings daily and report the proportion of free-swimming hatchlings in each treatment combination on the fifth day after the experiment was set up, when emergence of new hatchlings in both temperature conditions had slowed or stopped. Development was analyzed as a binary variable, "success" or "failure," with "success" defined as a neonate freely swimming in the well. The "failure" category consisted of multiple outcomes, mainly divisible into i) eggs that show no signs of development visible with light microscopy and ii) hatchlings

or embryos exhibiting severe, obvious morphological abnormalities preventing them from swimming normally, such as misshapen carapaces and eyes, stunted or missing appendages or setae, or prematurely broken membranes. The failure category also included any developing embryos that had not reached a free-swimming state by the end of the experiment but did not have any obvious abnormalities, which always comprised less than 1-3% of the totals at the time points in the experiments when outcomes were reported. We used swimming vs. non-swimming as our criterion in order to be conservative in our categorization, as it was not possible for the observer to be blinded to the treatment since bacteria or *Daphnia* homogenate were sometimes visible in the wells under the microscope. Except where differences are noted, these assay procedures were repeated in all following experiments.

To test if the observed effect was specific to the Munich population, a similar experiment was carried out using ephippia collected from a rock pool in Finland. These eggs were conventionalized with a homogenate of animals originating from this population.

Effects of individual bacterial strains

To confirm that the observed effects in the bacterial treatment were due to bacteria, and not to some other component of the homogenized *Daphnia* body, we conducted an experiment using pure cultures of bacterial strains isolated from apparently healthy field-collected *Daphnia* or laboratory-grown algal food. Five strains – *Pseudomonas* sp, *Burkholderiales sp*, *Aeromonas sp*, *Brevundimonas sp* (from *Daphnia*) and *Variovorax sp* (from algae) – were arbitrarily selected from the laboratory stock collection and their effect on hatching was contrasted with germ-free conditions at 22-23 (due to technical problems with the cooling device) and 27 °C. These strains were grown for 3 days in liquid LB medium (Sigma-Aldrich) at 37 °C with shaking, without regard to the growth phase each culture would reach during this time. Culture medium was removed by decanting after centrifugation, and bacteria were resuspended in sterile ADaM and diluted in ADaM to roughly the same final OD600 (calculated to be ~0.017-0.019, except for *Burkholderiales*, the concentration of which was ~0.001 because the culture did not grow to sufficient density). Another treatment consisted of a mixture of these strains. A treatment using whole-*Daphnia* homogenate as the bacterial source was also included, but all wells with this treatment became thickly overgrown with filaments of an unidentified bacterium, preventing normally and abnormally developed animals from being accurately distinguished. This treatment was therefore excluded from

analysis. Hatching rates were reported as in the previous experiment but on the fourth day instead of the fifth.

Effect of heat-killed or low dose bacteria

To determine whether the beneficial effect on hatching could be obtained by exposure to a generic microbial signal (e.g. lipopolysaccharide), we conducted an experiment with *Pseudomonas* and *Brevundimonas* administered either live or heat-killed. Both strains were cultured for 7 days. They were then diluted to OD600 = 0.2 and half of each culture was heat-killed at 80 °C for 1 hour. 20 µl of the live or heat-killed suspensions was added to wells containing 180 µl of sterile ADaM.

To determine whether a low dose of bacteria could produce the beneficial effect, we administered *Pseudomonas* at doses of 200 or 200,000 CFU (as determined by spread-plating dilutions) per egg.

Timing of bacterial effect

We wished to see whether bacteria would still have a beneficial effect if added after 16 hours of development at the warm temperature. (This timepoint was chosen based on results of a previous pilot study.) We inoculated two separate liquid cultures of *Pseudomonas* from single colonies on LB agar plates, 16 hours apart. The first culture was washed and diluted and added to treated eggs in wells as in the previously described experiments; the second was washed and diluted in the same way 16 hours later and added to a subset of bacteria-free eggs. At this time 20 µl of sterile ADaM was added to both bacteria-free and *Pseudomonas*-treated disturbance control groups. A subset of eggs was inspected with the microscope at 16 hours to approximately determine the average developmental stage at this point, and two *Pseudomonas*-treated individuals were removed from the wells and treated with DAPI stain (VectaShield kit) to visualize bacterial presence on the egg. A standard-temperature treatment was not included in this experiment.

Effect on directly developing eggs

To examine the effect of temperature and bacteria on non-diapausing eggs, we used parthenogenetic eggs of three different *Daphnia* clones (called Mu12, T2 and T3) originating from the same Munich location as the collected ephippia.

Three isofemale lines were established by hatching ephippia and kept under standard laboratory conditions for several generations before the experiment: 400 ml jars of ADaM kept at 16:8 light:dark cycle at 20 °C and fed every other day with 50 million cells of the green alga *Scenedesmus* sp.

For the experiment, one-day-old juveniles were placed individually in 100 ml jars filled with ADaM and kept under standard laboratory conditions until they reached maturity. When the first offspring were present, the adult animals were transferred to new jars with fresh medium. Following this, the eggs from the second clutch were collected within 24 h of being deposited, by sucking them out of the brood pouch with a Pasteur pipette and transferring them to a 1.5-ml Eppendorf tube. At this stage, the asexual eggs are still encased in a chorion, similarly to diapausing eggs. The collected eggs were surface-sterilized following the protocol of Peerakietkhajorn et al (2015). In short, the eggs were incubated for 30 min in 0.25% glutaraldehyde and washed three times with sterile water before they were placed individually in the wells of a 96 well plate. Resting eggs from ephippia were surface-sterilized using the same method and included for comparison. *Pseudomonas* suspension or sterile ADaM were added as previously. Wells were checked twice daily for swimming hatchlings.

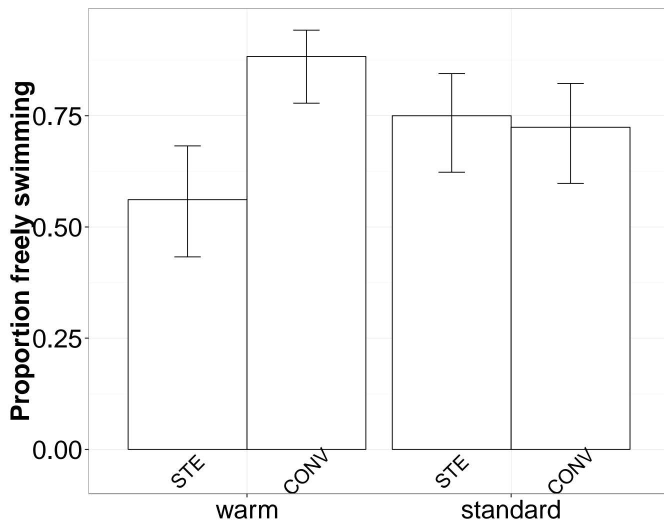
Statistical analysis

All statistical analyses were performed using the software package R 3.1.3 (R Core Team). The proportion of freely swimming hatchlings in each condition was analyzed with logistic regression (binomial error distribution with logit link function), setting warm and sterile conditions as the reference levels in each analysis. In the experiment examining directly developing eggs, these eggs were analyzed with a genotype effect included while ephippial eggs were analyzed in a separate model. Binomial confidence intervals were calculated for each treatment combination using the default Wilson method in the R package Hmisc.

Results

In a comparison of eggs exposed to bacteria-free or "conventionalizing" conditions (addition of a homogenate of lab *Daphnia* with complete microbiota), a clear interaction between temperature and bacterial treatment was observed (Figure 1A). Under standard (20 °C) conditions, bacteria-free and conventionalized eggs had similarly high rates of successful development (i.e. developing to a free-swimming state). Under warm (26-28 °C) conditions,

however, the rate of successful development of bacteria-free eggs was dramatically lower compared to conventionalized eggs. Unsuccessful development in all groups consisted of a combination of different outcomes, from eggs displaying no apparent signs of development to a variety of abnormal phenotypes lacking the ability to swim freely (Figure 1B). Observed abnormalities included malformed carapaces and eyes; broken membranes spilling yolk; and stunted appendages with missing setae. A similar difference in successful development under warm conditions was observed using eggs from a population originating from a Finnish rock pool (13/32 (41%) success in bacteria-free, 20/25 (80%) success in conventionalized, Fisher's exact test p=0.003).



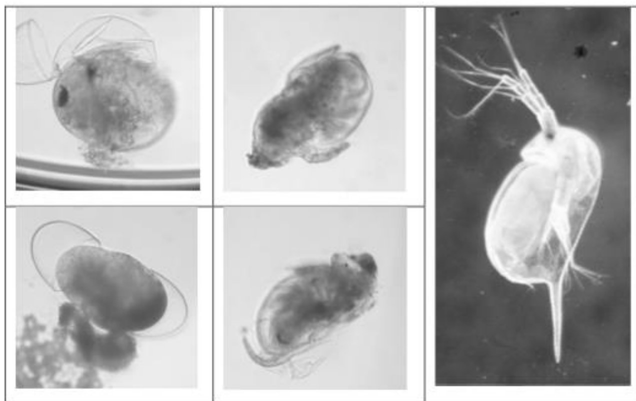


Figure 1. A (top). Proportions of resting eggs that reached a free-swimming state under warm and standard, bacteria-free (STE) and conventionalized (CONV) conditions. N=57 to 60 individuals in each treatment combination. Error bars represent 95% binomial confidence intervals. Odds ratio for CONV vs STE under warm conditions: 5.9. For logistic regression results see Table 1A. **B (bottom).** Examples of developmental abnormalities observed; photos shown are from warm, bacteria-free condition of experiment. At right, an example of a normally developed neonate; image compiled from stacked photographs of an immobilized individual. Photos have been converted to grayscale, and brightness and contrast have been adjusted.

In an experiment using single strains of lab-cultured bacteria under warm and standard temperature conditions, the bacteria-free group under warm conditions again had the lowest rate of successful development out of all treatments (Figure 2). Of the bacterial strains tested, the *Pseudomonas* sp strain resulted in the highest rate of successful development under warm conditions, significantly higher than that of the bacteria-free group. Since the *Pseudomonas* strain appeared to recapitulate the effect of *Daphnia* homogenate, further experiments aiming for more controlled conditions were conducted using this strain.

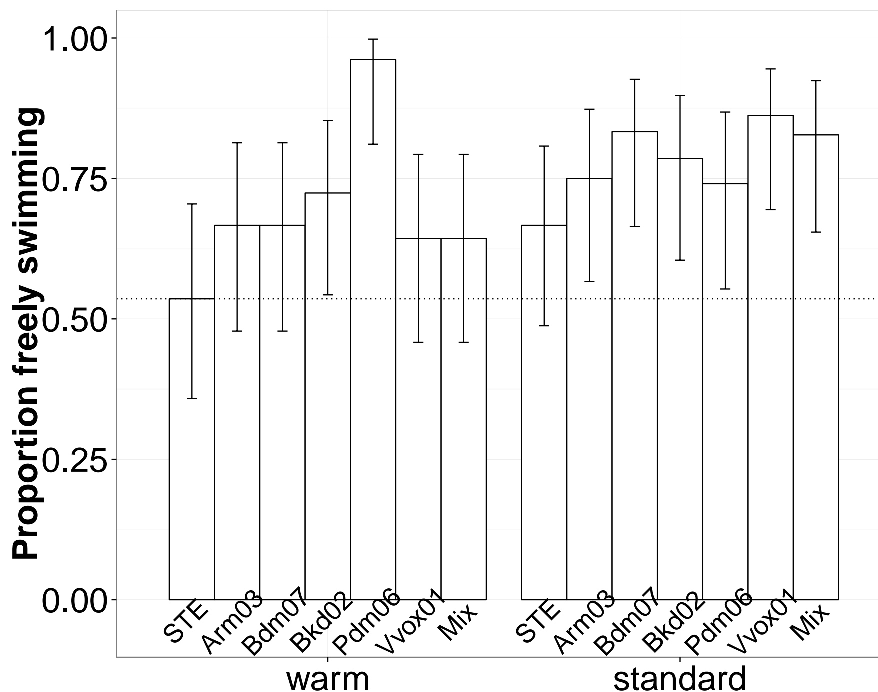


Figure 2. Proportions of resting eggs reaching a free-swimming state when exposed to different bacterial strains under warm and standard temperature conditions. STE=bacteria-free, Arm03=*Aeromonas sp*, Bdm07=*Brevundimonas sp*, Bkd02=*Burkholderiales sp*, Pdm06=*Pseudomonas sp*, Vvox01=*Variovorax sp*, Mix=mixture of these five bacterial strains. N=26 to 30 in each treatment combination. Horizontal line represents successful development at sterile warm condition, for comparison. Odds ratio for Pdm06 vs. sterile under warm condition: 21.7. Error bars represent 95% binomial confidence intervals. For logistic regression results see Table 1B.

Eggs treated with heat-killed *Pseudomonas* had rates of failure similar to bacteria-free eggs under warm conditions (Figure 3), indicating that the beneficial function of the bacterial cells was inactivated by heat. The *Brevundimonas* strain from the previous experiment was also tested in this experiment; it provided a significant improvement in hatching rates over the bacteria-free condition, but a smaller benefit than *Pseudomonas*. The effect of *Pseudomonas* was also tested at two different doses (Figure 4); the higher dose had a stronger beneficial effect than the low dose.

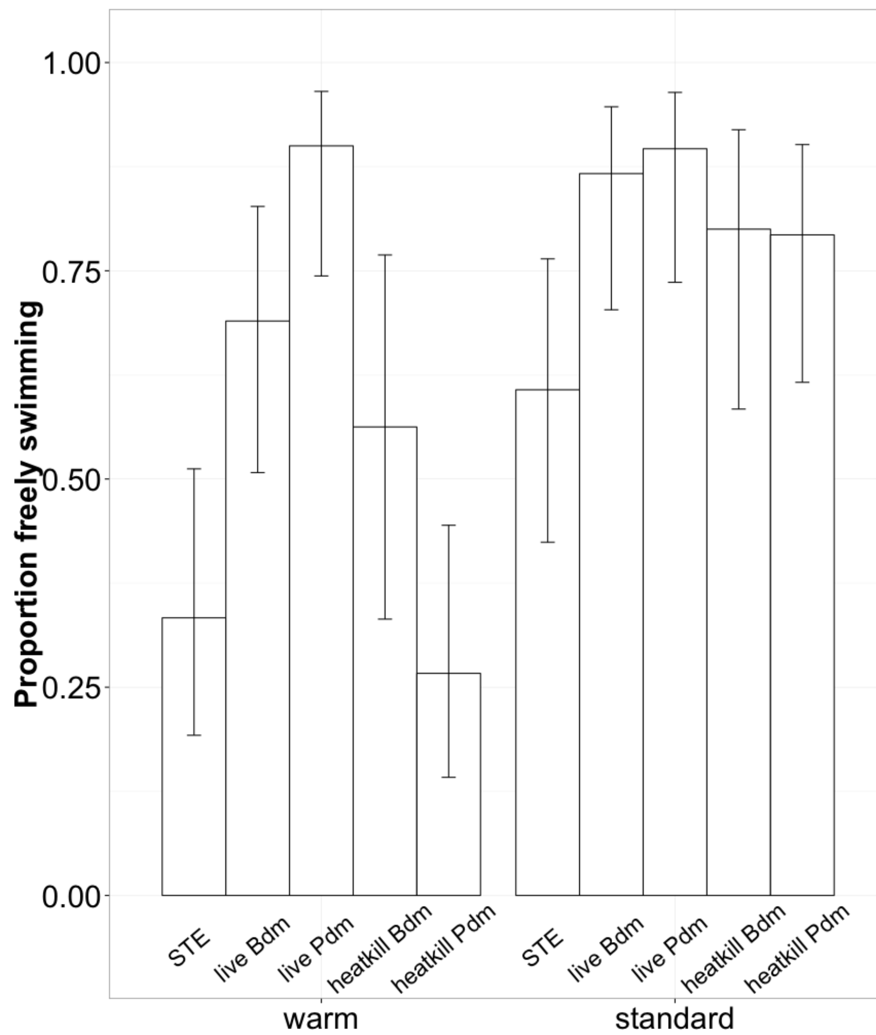


Figure 3. Proportions of resting eggs reaching a free-swimming state when exposed to live and heat-killed *Pseudomonas* (Pdm) and *Brevundimonas* (Bdm) under warm and standard temperature conditions. N=28 to 30 in each treatment combination except for heatkill Bdm/warm: n=16. Odds ratio for live Pseudeomonas vs. sterile under warm condition: 18. Error bars represent 95% binomial confidence intervals. For logistic regression results see Table 1C.

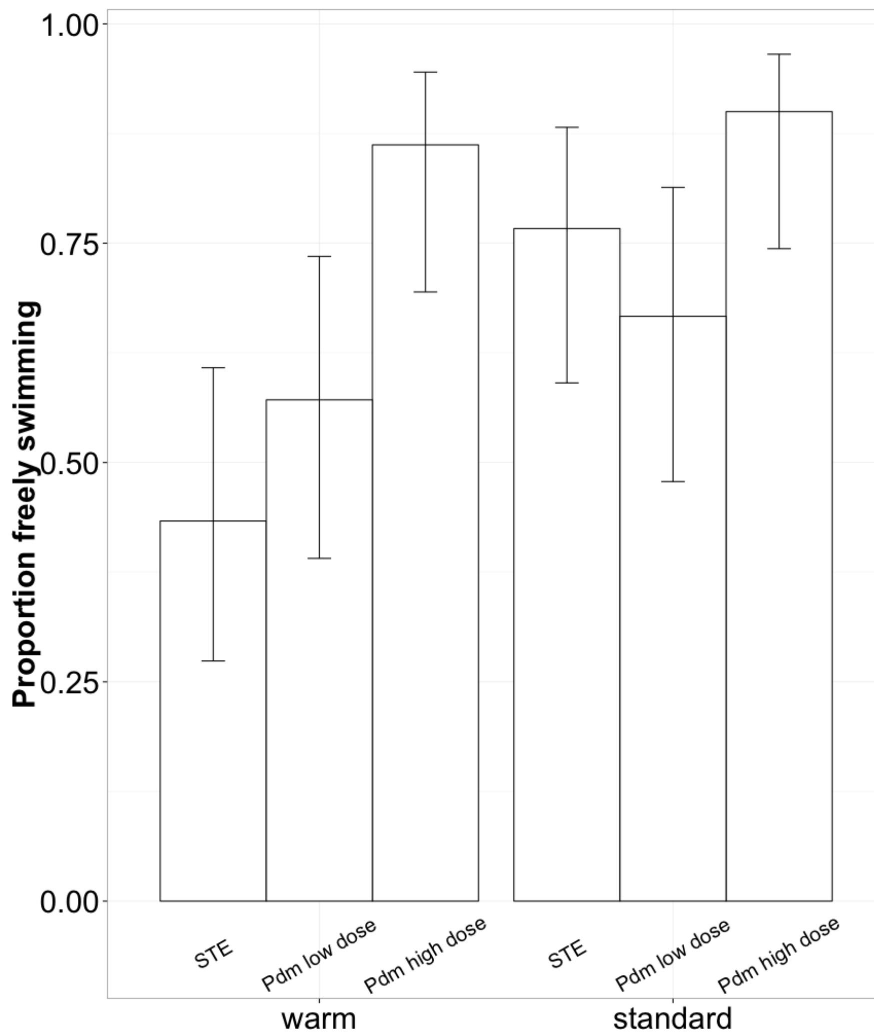


Figure 4. Proportions of resting eggs reaching a free-swimming state when exposed to different doses of *Pseudomonas* (Pdm) bacteria. N=27 to 30 in each treatment combination. Error bars represent 95% binomial confidence intervals. Odds ratio for Pseudomonas high dose vs. sterile: 8.22. For logistic regression results see Table 1D.

Adding *Pseudomonas* to bacteria-free embryos 16 hours after they had been placed under warm conditions did not improve rates of successful development over embryos that were bacteria-free for the entirety of the experiment (Figure 5). Therefore bacteria could only rescue the development of embryos if they were already present less than 16 hours after the onset of the warm temperature condition. Observation of a subset of these embryos at 16 hours showed that none of the eggs had yet shed their outer, inflexible membrane. Most of the embryos observed had begun to show some slight differentiation of segments at this point. DAPI staining of eggs exposed to *Pseudomonas* for 16 hours showed bacterial cells irregularly distributed on the surface of the egg, with no apparent pattern.

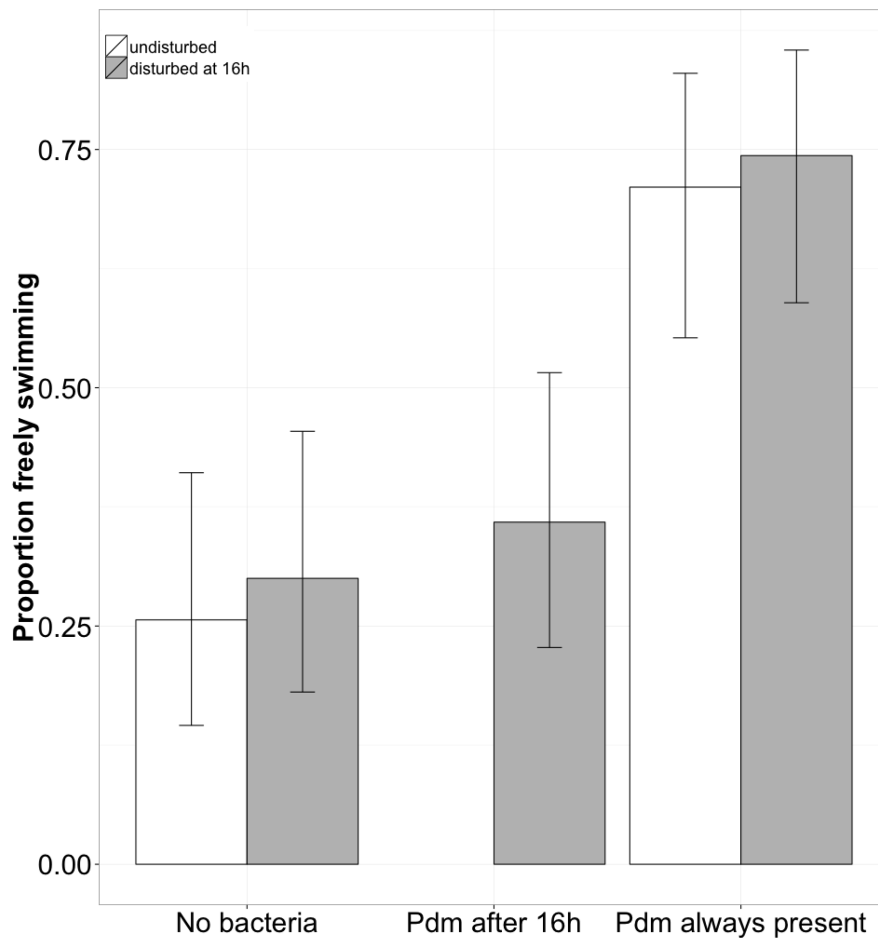


Figure 5. Comparison of successful development rates of eggs exposed to *Pseudomonas* from beginning of experiment or after 16 hours of bacteria-free development under warm conditions. Control treatments disturbed by pipetting at 16 hours are included. N=38 to 40 per treatment group. Error bars represent binomial confidence intervals. Odds ratio for Pseudomonas always present vs. no bacteria: 7.2. For logistic regression results see Table 1E.

The bacterial and temperature treatments had no effect on the development success of directly developing parthenogenetic eggs of three different *Daphnia* genotypes (Figure 6). Therefore this effect seems to be limited to resting eggs. Resting egg development showed the same pattern of bacterial and temperature effects in this experiment as in previous ones, indicating that the observed effect was not dependent on whether hypochlorite or glutaraldehyde was used for surface-sterilization.

Overall across our experiments, exposure to bacteria (either whole-*Daphnia* homogenate or *Pseudomonas* sp) increased the odds of successful development under warm conditions by ratios ranging from 4.6 to 21.7 (Table 2).

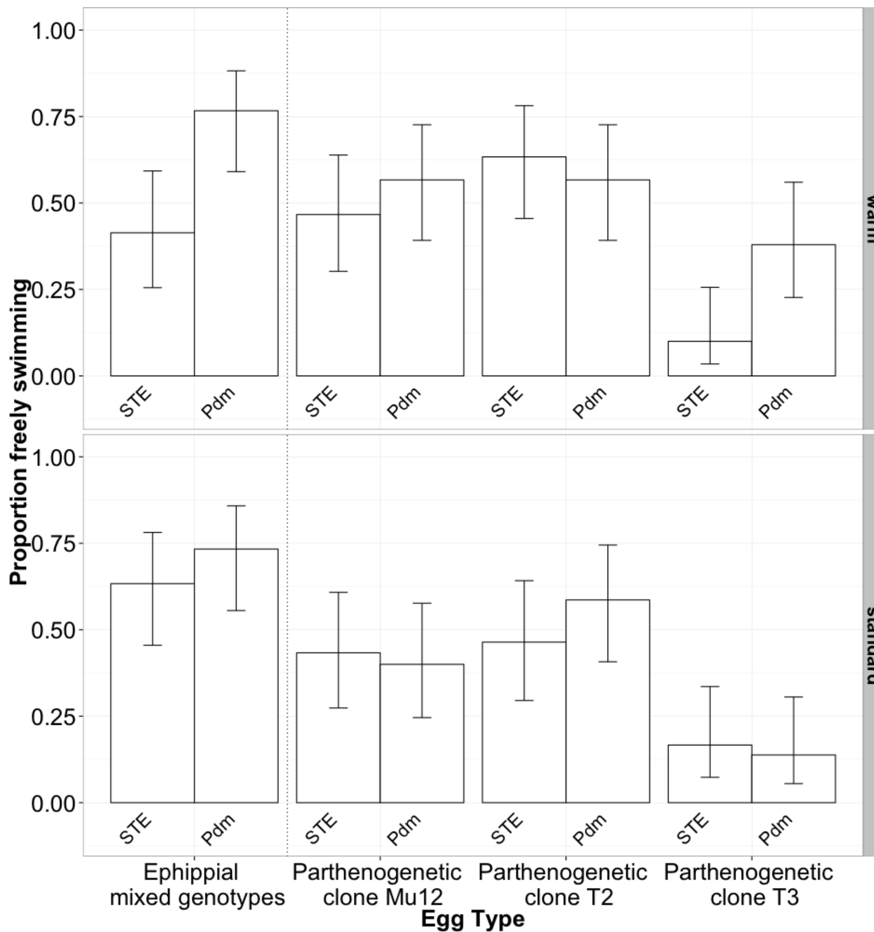


Figure 6. Effect of bacteria-free (STE) and Pseudomonas-exposed (Pdm) conditions on development at standard and warm temperature for directly developing parthenogenetic eggs of three different Daphnia clones as well as ephippial eggs. N=29 to 30 per treatment combination. Error bars represent 95% binomial confidence intervals. Separate logistic regressions were performed for ephippial and parthenogenetic eggs, setting sterile and warm condition as reference level in both. Odds ratio for ephippial eggs Pseudomonas-exposed vs. sterile under warm conditions: 4.7. For logistic regression results see Table 1F.

Table 2. Consistent effects of conventionalizing bacteria or *Pseudomonas sp* across experiments. Shown are odds ratios of successful development of the bacterial treatment significantly differing from sterile reference condition in each experiment.

	Warm condition		Standard condition	
Experiment/trial	Odds ratio	Fisher's exact test p-value	Odds ratio	Fisher's exact test p
Fig. 1	5.9	0.00014	0.87	0.83
Fig. 2	21.7	.00041	1.42	0.58
Fig. 3	18	1.1e-5	5.6	0.015
Fig. 4	8.22	0.0009	2.74	0.299
Fig. 5	7.2	9.2e-5	na	na
Fig. 6	4.7	0.0082	1.59	0.58
Mean +/- s.e.m.	**10.95 +/- 2.89**		**2.44 +/- 0.85**	

Table 1. Coefficients of logistic regressions. In all models, sterile and warm conditions are set as the reference levels unless otherwise noted. Asterisks represent p-values significant at the .05 (*), .01 (**), and .001 (***) alpha levels.

	Estimate	**Std. Error**	**z value**	**Pr(>|z|)**
A. *Effect of conventionalizing bacterial mixture (Figure 1)*				
(Intercept)	0.2469	0.2669	0.925	0.355058
CONV	1.7775	0.4827	3.683	0.000231 ***
STANDARD temp	0.8518	0.4080	2.087	0.036845 *
CONV:STANDARD	-1.9111	0.6438	-2.968	0.002995 **
B. *Effect of individual bacterial isolates (Figure 2)*				
(Intercept)	0.1431	0.3789	0.378	0.70570
Arm03	0.5500	0.5570	0.988	0.32340
Bdm07	0.5500	0.5570	0.988	0.32340
Bkd02	0.8220	0.5623	1.462	0.14381
Mix	0.4447	0.5469	0.813	0.41619
Pdm06	3.0758	1.0876	2.828	0.00468 **
Vvox01	0.4447	0.5469	0.813	0.41619
STANDARD	0.5500	0.5418	1.015	0.31004
Arm03:STANDARD	-0.1446	0.8067	-0.179	0.85776
Bdm07:STANDARD	0.3662	0.8368	0.438	0.66163
Bkd02:STANDARD	-0.2158	0.8236	-0.262	0.79327
Mix:STANDARD	0.4308	0.8312	0.518	0.60425
Pdm06:STANDARD	-2.7191	1.2352	-2.201	0.02771 *
Vvox01:STANDARD	0.6947	0.8597	0.808	0.41904
C. *Effect of heatkilled bacteria (Figure 3)*				
(Intercept)	-0.693147	0.387298	-1.790	0.07350 .
live Bdm	1.491655	0.557773	2.674	0.00749 **
heatkilled Bdm	0.944462	0.635585	1.486	0.13729
live Pdm	2.890372	0.721325	4.007	6.15e-05 ***
heatkilled Pdm	-0.318454	0.566087	-0.563	0.57374
STANDARD temp	1.128465	0.547478	2.061	0.03928 *
live Bdm:STANDARD	-0.055171	0.865624	-0.064	0.94918
heatkill Bdm:STANDARD	0.006515	0.930699	0.007	0.99442
livePdm:STANDARD	-1.166206	1.020683	-1.143	0.25322
heatkillPdm:STANDARD	1.226870	0.824822	1.487	0.13690
D. *Effect of low dose of Pseudomonas (Figure 4)*				
(Intercept)	-0.2683	0.3684	-0.728	0.46655
Pdm high dose	2.1008	0.6525	3.220	0.00128 **
Pdm low dose	0.5559	0.5306	1.048	0.29478
STANDARD temp	1.4578	0.5675	2.569	0.01021 *
Pdm high dose:STANDARD	-1.0932	0.9912	-1.103	0.27004
Pdm low dose:STANDARD	-1.0524	0.7966	-1.321	0.18647
E. *Effect of adding Pseudomonas after 16h of development under warm conditions (Figure 5). Sterile and undisturbed set as reference levels.*				
(Intercept)	-1.0516	0.3147	-3.342	0.000832 ***
Pdm added 16h	0.2792	0.4509	0.619	0.535786
Pdm always	1.9370	0.3591	5.394	6.89e-08 ***
disturbed	0.1925	0.3591	0.536	0.591799

Discussion

We have shown a consistent positive effect of exposure to bacteria on the successful development of *Daphnia magna* from resting eggs at a temperature of 26-28 °C. Under warm conditions, the rate of successful development of eggs without bacteria in their environment is much lower than that of eggs exposed to bacteria, with a higher incidence of severe morphological abnormalities resulting in fewer freely swimming neonates in bacteria-free conditions. This effect is observable both using a complete suite of *Daphnia*-associated bacteria derived from homogenizing whole adult daphnids, and with at least one individual strain (*Pseudomonas* sp) of bacteria. Since a strain with this positive effect was observed in an arbitrary selection of five bacterial strains from our collection, we assume that this property may be relatively widespread among *Daphnia*-related bacteria. This would be similar to results from studies of mosquitoes, in which a wide range of bacterial strains promoted larval development (Coon et al. 2014). Interestingly, the mixture of the five strains tested did not have the same beneficial effect as the *Pseudomonas* strain alone, indicating either that *Pseudomonas* was not present at a high enough concentration in the mixture to have an effect, or that the strains in this particular mixture had antagonistic effects on each other with respect to their effect on the embryo. It is unknown to which bacteria, and in which combinations, eggs would be exposed in natural settings. The ephippia in which eggs are deposited are derived from maternal carapaces, and bacteria have been observed on their internal surfaces (Schultz 1977). Many egg-containing ephippia collected from natural sediments are partially degraded or not completely sealed (personal observation), permitting exposure to environmental bacteria. Natural environments would almost certainly contain harmful bacteria in addition to potentially beneficial ones, making the effects of bacteria in natural settings difficult to predict.

Among the animals that failed to develop normally, abnormality appeared to arise at different developmental stages. Among those that resembled undifferentiated eggs at the end of the experiment, our methods could not distinguish whether this was due to developmental failure/death at a very early stage or due to continued diapause. Bacteria could be involved in diapause termination, analogously to bacteria that induce metamorphosis between life stages in some marine invertebrates (Shikuma et al. 2014). However, a majority of the unsuccessful outcomes consisted of visibly initiated but abnormal development, so we presume that the effect observed in this experiment is primarily one related to embryonic development in general rather than diapause termination specifically. Nonetheless, organisms with a

diapausing embryonic stage are an interesting case study on the subject of ecological dimensions of development (Gilbert & Epel 2009), since they face a unique set of challenges related to the developmental environment: they must be impervious to environmental conditions for the length of diapause, respond appropriately to cues indicating favorable conditions for emerging from diapause, and complete development in environments potentially very different from those experienced by their parents. Understanding the environmental parameters that affect successful development in these organisms could therefore be useful for understanding how these complex responses are regulated.

It is unclear whether the observed effect of bacteria is indirect or direct; e.g. whether bacteria act by modifying the chemical or physical environment around the egg, thus creating conditions more favorable for development, or whether bacteria are engaged in some kind of specific, direct molecular interaction with the developing embryo. A combination of indirect and direct effects is also possible. For example, in *Aedes aegypti* mosquitoes, bacteria were hypothesized to stimulate hatching by decreasing the dissolved oxygen concentration locally around eggs (Gillett et al. 1977), but also appeared to have a stimulating effect at high oxygen conditions (Ponnusamy et al. 2011). Such observations highlight the necessity of keeping microbial activities in mind as environmental factors that modify the effects of other environmental parameters. Normal development failed to be rescued when we added bacteria to bacteria-free embryos after 16 hours of development at the warm temperature. This could be either because this window represents a critical phase in the development of the embryo, or because it takes longer than 16 hours for the beneficial effect of the bacteria to take effect (e.g. if a bacterially produced factor must accumulate to a certain level in the water before it can benefit embryos).

The phenotypes observed in this experiment were not completely penetrant. Developmental abnormalities were diverse and occurred at many different stages. A fraction of individuals failed to develop normally in all treatments (consistent with previous observations of resting egg hatching), and a portion (usually 30-50%) of individuals successfully developed to a freely swimming stage even in the warm, bacteria-free treatment. This could reflect heterogeneity in the experimental conditions (e.g. between wells of the culture plates) or heterogeneity in the embryos. The field-collected resting eggs used in this study vary in genotype, size, length of time since deposition, and most likely maternal condition. Accordingly, there could be genetic or maternal factors that affect the extent to which an individual is sensitive to temperature and bacteria. Strong genetic variation in

responses to microbiota has been observed in *Drosophila* nutrition-related traits (Dobson et al. 2015). The outcomes observed here resemble environmental canalization (Flatt 2005), with bacteria in some way contributing to the homeostatic mechanism that stabilizes the phenotypic outcome under the elevated temperature condition. Stressful conditions reveal cryptic phenotypic variation in many organisms (Badyaev 2005); our results suggest that such conditions may reveal cryptic variation in dependency on microorganisms. Viewed another way, given that many stress responses are generalized (Feder & Hofmann 1999; Jones et al. 2015), it is possible that pathways activated by exposure to bacteria are also protective against heat. Since resting egg hatching occurs not only in spring, but also in summer when dried-out shallow pools are refilled by rain, some populations could either regularly or unpredictably experience the temperatures used in our experiments.

The development of parthenogenetic eggs of three different genotypes was unaffected by either temperature or bacterial presence in our experiment. The beneficial role of bacteria could be related to specific characteristics of resting eggs, such as the tertiary membrane. On the other hand, one study reported high rates of inviability and developmental abnormalities in the parthenogenetic eggs of microbiota-free *Daphnia* mothers under sterile conditions (Peerakietkhajorn, Kato, et al. 2015). Since gut microbiota are thought to contribute to the nutrition of adult *Daphnia* (Gorokhova et al. 2015), and resting eggs are often produced under conditions of high crowding that are accompanied by food scarcity, sensitivity to absence of bacteria could be a characteristic of eggs produced by undernourished mothers. Studies have demonstrated various effects of maternal nutritional status on disease resistance of offspring (Mitchell & Read 2005). If the effect observed here involves cross-talk between immune-related and other developmental signaling pathways, interesting connections could be made to studies in ecoimmunology investigating connections between health, disease and various ecological stressors.

Extended exposure to sodium hypochlorite of developing *Daphnia* resting embryos is toxic (Raikow et al. 2007), while brief exposure to sodium hypochlorite of uninduced resting eggs is a routine laboratory procedure (Luijckx et al. 2012) which has no apparent negative effects when eggs are hatched in conventional (nonsterile) conditions. In our experiments, eggs briefly (5 minutes) exposed to hypochlorite and then re-inoculated with bacteria had restored or elevated hatching success compared to eggs kept sterile after exposure. Therefore it is possibly worth expanding toxicological studies to investigate whether the effects of toxic compounds or other stressors on animals could be partly due to their effects on microbes in

the animals' environment. Similarly, transformation of toxicants by bacteria in the environment may be another critical parameter in determining safe exposure levels.

The molecular basis of the developmental abnormalities observed in these experiments is unknown, but some similar morphological abnormalities in *Daphnia* are reported in the ecotoxicology literature as consequences of exposure to chemicals with endocrine-disrupting properties, particularly with effects on ecdysteroids (Mu & Leblanc 2002; Flaherty & Dodson 2005). Since ecdysone signaling is also involved in processes dependent on bacteria (i.e. invertebrate immune response) (Regan et al. 2013; Rus et al. 2013), we speculate that absence of bacteria could result in hormonal dysregulation with negative consequences for development. Several studies have noted the close link between innate immune regulation and regulation of development and growth (Shin et al. 2011; McFall-Ngai 2002), and the coincident signaling pathways underlying both (McFall-Ngai et al. 2013; Hayden & Ghosh 2004). Since animal developmental programs evolved in the presence of bacteria, it is conceivable that normal development can depend on processes sensitive to bacterial presence even in early stages. It remains to be seen how relevant the effect observed here is in natural settings; however, these findings potentially have general relevance to the understanding of the complex ecological dimensions of development and of the effects of bacterial activities on other organisms in the ecosystem.

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Additional Data

Table S1 shows the results of additional trials on the effects of bacteria on development conducted as the methodology for this study was being developed and additional parameters were tested. These trials were not included in the main study due to either methodological concerns or because of redundancy with other results. The main effect – of bacteria-free embryos having the lowest rate of successful development under warm conditions – was consistent across all trials except two that had confirmed bacterial contamination. Names of bacterial strains refer to Ebert lab culture collection. Table S2 shows additional examples of developmental abnormalities observed in warm, bacteria-free conditions.

Table S1.

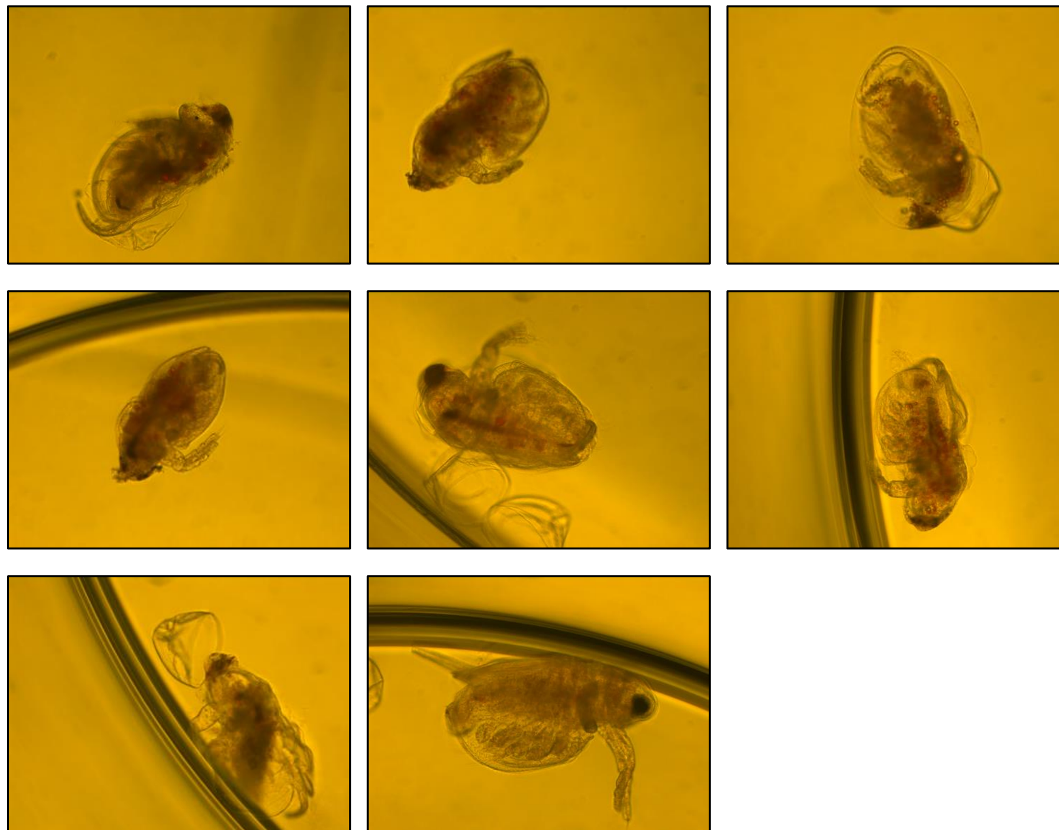
Testing	Treatments	population	Environment	Results	Comments
Basic experiment evaluating effect of bacteria	sterile	Munich K2-2	middle lab (warm)	9/43 (21%)	Possibly flawed randomization procedure
	Daph. homogenate			26/45 (58%)	
Basic experiment evaluating effect of bacteria	sterile	Munich K2-2	middle lab (warm)	11/53 (20%)	Frequently disturbed
	untreated eggs			13/55 (23%)	
	Daph. homogenate			19/50 (38%)	
Different bacterial sources	sterile	Munich K2-2	middle lab (warm)	9/33 (27%)	Possibly flawed randomization procedure
	nonsterile ADaM			8/35 (23%)	
	Daph. homogenate			26/36 (72%)	
	Ephippia/sediment homogenate			25/36 (69%)	
	sterile		climate room (cool)	33/44 (75%)	
	nonsterile ADaM			37/43 (86%)	
	Daph. homogenate			36/46 (78%)	
	Ephippia/sediment homogenate			29/42 (69%)	
Testing cooling device	sterile	Munich K2-2	cool device	13/19 (68%)	Possibly flawed randomization procedure
	Daph. homogenate			12/19 (63%)	
	sterile		warm device	5/17 (29%)	
	Daph. homogenate			14/19 (74%)	
	sterile		climate room	6/13 (46%)	
	Daph. homogenate			13/17 (76%)	
Basic experiment evaluating effect of bacteria	sterile	Munich K2-2	cool device	34/45 (76%)	PCR revealed contamination
	Daph. homogenate			36/46 (78%)	
	sterile		warm device	36/43 (83%)	
	Daph. homogenate			40/43 (93%)	
Dosage experiment	sterile	Munich K2-2	cool device	>80%	PCR and culturing revealed contamination; trial terminated
	Pdm06-high			>80%	
	Pdm06-low			>80%	
	Bdm07-high			>80%	
	Bdm07-low			>80%	
	sterile		warm device	>80%	

	Pdm06-high			>80%	
	Pdm06-low			>80%	
	Bdm07-high			>80%	
	Bdm07-low			>80%	
Evaluating effects of bacterial culture filtrate	sterile	Munich K2-2	cool device	23/29 (79%)	Bench setup was disturbed, possibly affecting temperature. A few "Filtrate" wells show evidence of bacteria still being present. Pdm culture seems to continue to grow in wells (visibly cloudy).
	sterile LB			24/28 (86%)	
	Filtrate LB			21/28 (75%)	
	Pdm06 in LB			17/28 (61%)	
	sterile		warm device	10/29 (34%)	
	sterile LB			17/30 (57%)	
	Filtrate LB			21/30 (70%)	
	Pdm06 in LB			29/30 (97%)	
Evaluating effect of supplementing with 20-hydroxyecdysone (20E)	sterile	Munich K2-2	cool device	40/48 (83%)	20E added at beginning of experiment - unclear how long it stays in medium
	Pdm06			45/50 (90%)	
	sterile + 0.5 uM 20E			38/50 (76%)	
	sterile + EtOH vehicle			39/50 (78%)	
	sterile		warm device	23/49 (47%)	
	Pdm06			46/50 (92%)	
	sterile + 0.5 uM 20E			22/49 (45%)	
	sterile + EtOH vehicle			22/47 (47%)	
Basic experiment evaluating effect of bacteria, with additional quality control: well-aerated medium, checked at 16h, no eggs in any edge wells	sterile	Munich K2-2	cool device	46/71 (65%)	
	Daph. homogenate			43/71 (60%)	
	sterile		warm device	54/72 (75%)	
	Daph. homogenate			20/72 (28%)	
Evaluating effect of supplementation with vitamin B12	sterile	Munich K2-2	cool device	30/45 (67%)	
	sterile + .01 mg/ml vit B12			38/47 (81%)	
	Pdm06			37/48 (77%)	
	sterile		warm device	23/46 (50%)	
	sterile + .01 mg/ml vit B12			19/48 (40%)	

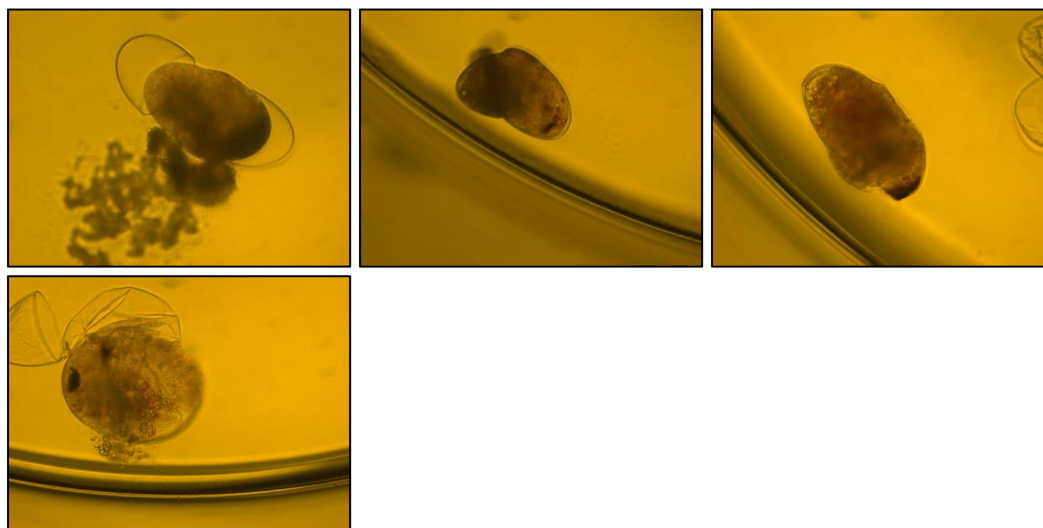
	Pdm06			40/47 (85%)	
Evaluating effect of Pseudomonas on ephippial eggs of inbred lines	sterile	clone CHH-434Inb2	cool device	16/39 (41%)	
	Pdm06			31/40(78%)	
	sterile		warm device	6/40 (15%)	
	Pdm06			22/39 (56%)	
	sterile	FAinb3	cool device	1/25 (4%)	
	Pdm06			3/25 (12%)	
	sterile		warm device	0/24 (0%)	
	Pdm06			3/24 (13%)	
Comparing effect of different strains of Pseudomonas and E. coli	Ecoli	Munich K2-2	cool device	23/28 (82%)	Strains had different growth rates and cell counts
	Pdm01			29/30 (97%)	
	Pdm02A			24/30 (80%)	
	Pdm02D			29/30 (97%)	
	Pdm06			22/29 (76%)	
	Pdm16			27/30 (90%)	
	sterile			20/29 (69%)	
	Ecoli		warm device	21/30 (70%)	
	Pdm01			25/30 (83%)	
	Pdm02A			27/29 (93%)	
	Pdm02D			25/30 (83%)	
	Pdm06			24/30 (80%)	
	Pdm16			24/29 (83%)	
	sterile			10/30 (33%)	
Evaluating effect of Pseudomonas and Sphingomonas strain; outcomes scored while blinded to treatment (neonate swimming visible to naked eye)	sterile	Munich K2-2	middle lab (warm)	3/60 (5%)	Sphingomonas did not re-grow on culture plate when sampled from experiment; later learned that Sphingomonas strains can be sensitive to 4 degrees C (used as temporary storage during setup of experiment) (Julia Vorholt, personal communication)
	Sgm02			4/60 (7%)	
	Pdm06			34/60 (57%)	

Table S2. Examples of developmental abnormalities of resting eggs developing under warm, bacteria-free conditions.

Stunted or missing appendages and setae; misshapen carapace and eye:



Eye formed but no segmentation or other morphological features; broken or "exploded" membranes:



Note: similar outcomes can be found across all treatment groups but are most frequent in warm, bacteria-free conditions. Diagnostic character for scoring outcomes is swimming ability.

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Chapter 6

The combined effect of temperature and host clonal line on microbiota composition

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Author contributions: All authors designed the study. DE and TS ran the experiment, and SP and TS collected the data. KS analyzed the data with input from SP. KS wrote the paper, and SP and DE contributed to revisions. All authors approved the final version.

Abstract

Host-associated microbiota vary across host individuals and environmental conditions, but the relative importance of nature and nurture is difficult to disentangle. We sought to experimentally determine the factors shaping the microbiota of the planktonic Crustacean *Daphnia magna* interact. We used clonal lines from a wide geographic distribution, which had been kept under standardized conditions for over 30 generations. Replicate populations were kept for three generations at 20 and 28 °C. Host clonal line, environment (*i.e.* temperature) and their interaction influenced microbiota community characteristics and the abundance of common microbial species, with the interaction of host clonal lineage and environmental effects generally explaining a high degree of variance for community composition, while microbial diversity was better explained by clonal host lineage alone. Our results highlight the prominent effects that host clonal lineage and its interaction with the environment has on host-associated microbiota.

Introduction

Environmental and genetic factors contribute to the composition of animal-associated microbiota. The environment can affect the microbial pool to which a host is exposed or alter the physiology of the host's microbes, thereby influencing its community structure (Fan *et al.* 2013; Seedorf *et al.* 2014). The genotype of a host, on the other hand, can impact its microbiota by regulating microbial recognition and selection, influence immune parameters and the biochemical conditions of tissues (Garrett *et al.* 2010; Franzenburg *et al.* 2012). The involvement of environmental factors and host genetics in controlling a host's microbiota has been demonstrated (Benson *et al.* 2010; Campbell *et al.* 2012), and we aim to investigate the degree to which a combination of both (*i.e.* host clonal lineage and environmental interactions) influence the structure of host-associated microbiota, which is considered to be a polygenic trait (Benson *et al.* 2010).

Changes in the expression of phenotypic traits due to environmental factors may be parallel or non-parallel among host genotypes. Non-parallelism is observed as when hosts with different genetic backgrounds respond differently to environmental conditions and it produces interspecific variation, from which diversification may arise (Pfennig *et al.* 2010; Moczek *et al.* 2011). Environmental components strongly affect host-associated microbiota,

including diet (David *et al.* 2013; Carmody *et al.* 2015), salinity (Schmidt *et al.* 2015), and exposure to pathogen or infection status (Cariveau *et al.* 2014; Jani & Briggs 2014). Studies that investigated genetic and environmental interactions used in bred host individuals to test the effect of diet (Parks *et al.* 2013; Carmody *et al.* 2015) because inbred lines allow replication of genotypes, but at the cost of unnaturally low levels of heterozygosity and associated inbreeding depression. Our current study avoids this problem by using natural clonal hosts reared under different environmental conditions.

We use the freshwater crustacean *Daphnia magna*, which reproduces *via* cyclic parthenogenesis, to test how different host clonal lines and temperature conditions (20 and 28 °C) influence their host-associated microbiota. *Daphnia*-associated microbiota are an integral part of their biology. Studies with germ-free *D. magna*, revealed their dependence on microbes for survival and reproduction (Callens *et al.* 2015; Peerakietkhajorn *et al.* 2015; Sison-Mangus *et al.* 2015), and certain strains of bacterioplankton increase *Daphnia* fecundity (Peerakietkhajorn *et al.* 2016). Additionally, the presence of bacteria helps resting eggs develop normally at higher temperatures, suggesting an important interaction between temperature and bacteria with functional implications (Mushegian *et al.* 2016).

Temperature has shown to disrupt normal host-microbe symbioses in a variety of animals (Prado *et al.* 2010; Fan *et al.* 2013; Lokmer & Wegner 2015). The way in which organisms respond to temperature shifts has significant implications for their health and persistence. Our study organism, *D. magna*, exhibit both local adaptation and adaptive phenotypic plasticity with regard to temperature tolerance (Yampolsky *et al.* 2014a), and genotypes differ in their gene expression levels and ability to function at high temperatures (Yampolsky *et al.* 2014a; Yampolsky *et al.* 2014b). We use *D. magna* clones that had been kept for at least five years (> 75 generations) under standardized stock center conditions, where the acquisition of microbiota through an open culture system was possible. All these clones had been established from planktonic females collected in the field, and no attempts had been made to alter or clear their natural microbiota. These clones had been collected from in a wide range of climatic conditions, allowing us to further test to what degree their conditions at the site of origin may have influenced their microbiota. Our analysis highlights the role that host clonal line and its interaction with the environment has in shaping a host's microbiota.

Methods

Daphnia magna is a planktonic crustacean, found in small to large sized ponds and lakes with a distribution across Eurasia, North America and parts of Africa. Under laboratory conditions animals can be kept clonal for many years. Animals are 0.6 to 5 mm in length and reach maturity at about 8 to 15 days at 20 °C. The animals used in this study were treated identically to those detailed in Yampolsky *et al.* (2014a), in which a geographically diverse set of *D. magna* clones, originating from locations with varying temperature regimes, were used to study local adaptation and phenotypic plasticity to temperature tolerance. The *D. magna* clones included in this study were a subset of the clones used by Yampolsky *et al.* (2014a). For the current analysis, we focused on 16 host clones (Table 1). We excluded 6 clones from the Yampolsky *et al.* (2014a) study because (i) clone KE-1-1 was later suspected to be mixed up with another clonal line, (ii) clones DE-Iinb-1 and FI-Xinb-3 are inbred lines, (iii) clone FI-FAT-1-6 was excluded from the analysis due too low 16S rDNA sequencing coverage, and (iv) clone FI-FSP1-16 and FI-FHS2-11 were split among the two extraction groups (discussed below).

Before the experiment, all clones had been kept for at least five years under laboratory conditions identical to the experimental conditions at 20 °C in the same walk-in climate chamber. For the experiment, clonal lines were acclimated for at least three generations to constant temperatures of either 20 or 28 °C. We choose these two temperature conditions because one is close to the physiological optimum of *Daphnia* (20 to 23 °C) and the other is at the upper extreme where reproduction for all clones was still possible (28 °C). Although this temperature range is perhaps broader than would be expected by long-term ecological change (climate change), seasonal temperature variation can easily include this temperature range. In particular, populations in the Southern part of the species distribution are exposed to extreme summer temperatures, and populations also live in very small water bodies that change quickly due to strong solar radiation. Thus, we believe that our experimental conditions are within the natural range of what our study species experiences.

Six independent replicate populations per clone were kept in separate jars for each temperature. Before and during the experiment, all animals were kept in open jars filled with non-sterile medium (ADaM, Klüttgen *et al.* 1994) and fed non-sterile food (chemostat grown green algae *Scenedesmus* sp.). These conditions allowed for *Daphnia* to pick up microbiota from surrounding environmental sources such as the food and medium. The 28 °C treatment

was chosen as the upper limit at which all *D. magna* clones are able to reproduce and complete their life cycle based on a pilot experiment.

From each temperature and clone line treatment, three adult *D. magna* from the third generation were collected from 3 of the 6 replicate populations. Individual *Daphnia* were rinsed twice with sterile medium under a laminar flow hood in a 24 well-plate then placed into separate Eppendorf tubes. Animals were then immersed in 200 µL of TE 1X buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) and kept at -20 °C until DNA extraction.

<u>Amplicon sequencing of Microbiota</u>

DNA was extracted from *D. magna* using a cetyl trimethylammonium bromide (CTAB)-based isolation protocol. Animals were ground with a sterile pestle in a lysozyme solution, followed by incubation at 37 °C for 45 min during which extraction was mixed at 850 rpm. Proteinase K was added to achieve a 300 µg/mL solution, which was then mixed at 850 rpm for 1 h at 55 °C. After the addition of 15 µL of 20 mg/mL RNase A solution, 300 µL of CTAB 2X, 12 µL of 0.2% β-mercaptoethanol and 60 µL of 20% PVP were added, and then samples were mixed for 1 hr at 300 rpm and 65 °C. We then added 1 volume of chloroform isoamyl alcohol (CIA) to the samples, followed by centrifugation for 8 minutes at 12,000 rpm at 15 °C and collection of the aqueous phase. The CIA and centrifugation steps were repeated, and approximately 1 volume of CIA was added. The nucleic acids were then precipitated from the aqueous phase with 50 µL of sodium acetate 3M pH 5.2 and 900 µL of cold isopropanol. After an overnight incubation, samples were centrifuged for 30 min at 14,000 rpm at 4 °C. Pellets were rinsed with 70 % v/v cold ethanol twice and then were dried using a Speed-Vac for 10 min at 45 °C prior to resuspension in 30 µL of TE buffer.

The V3-V5 region of the eubacterial 16S rDNA gene (positions 327-969) was amplified with PCR and sequenced using 454 LibL sequencing technology (Roche, Switzerland) from two to three replicates per host genotype per acclimation temperature (N = 89). Denoised reads of average Phred quality score above 25 over a window of 25 bp and of length between 400 and 480 bp were retained (*i.e.* encompassing the V3 and V4 variable regions). Singletons and chimeras identified with UCHIME (Edgar *et al.* 2011) were removed. Filtered reads were then clustered into operational taxonomic units (OTUs) at 97% sequence identity level using USEARCH v8.0.1623 (Edgar 2010). Taxonomic assignment was done using BLAST (Altschul *et al.* 1990) against the Greengenes database v13_8 (McDonald *et al.* 2012) with default settings. The most abundant sequence in each OTU cluster was chosen as the representative

sequence of that OTU and sequences were aligned with PyNAST (Caporaso *et al.* 2010a). The sequences belonging to the genus *Pasteuria* were filtered from the DNA libraries because it is a known bacterial pathogen of *Daphnia* that is regularly worked with in the same laboratory. It appeared that the *Pasteuria ramosa* reads found were from PCR contamination, which was corroborated with additional testing. Due to negative blank extractions, we believe the contamination was restricted only to *Pasteuria* and not other bacterial species. A phylogenetic tree was then built using RAxML v8.1.20 (Stamatakis 2014) for computation of weighted UniFrac distances (Lozupone *et al.* 2007). OTU count tables were built in QIIME (Quantitative Insights Into Microbial Ecology) software package v1.9.0 (Caporaso *et al.* 2010b).

Data analysis

Data were rarefied to 2,650 reads per sample prior to analyses. All extractions done in this study were performed either between 9 March and 12 April 2013 (early extraction group) or between 9 December 2013 and 31 January 2014 (late extraction group). An analysis of the data determined that the date (early vs. late extraction group) at which we extracted the DNA from the samples had a very strong effect on the microbiota composition of *Daphnia magna* (Figure S1). The early extraction group clearly separated along from the late extraction group in multivariate analyses. Because most samples from the same clone were extracted in the same period, we separated our analyses into two extraction groups, , both of which include 8 clones in the study, for all further analysis.

For each of the two extraction groups, which were analyzed separately, we used the vegan package (Oksanen *et al.* 2015) in R v3.1.2 (R Core Team, 2014) to analyze whole community structure using relative abundances projected on the non-metric multidimensional space (NMDS). For a more detailed analysis we focused on the 10 most abundant OTUs that were dominant in both extraction groups. These 10 OTUs made up the majority of the relative abundance of both libraries, comprising 76.1% of the early extraction group's library and 68.3% of the late extraction group's library. Within each extraction group, we performed type III ANOVAs to determine the effect of clone, temperature and the interaction of clone and temperature on the arc-sin transformed relative abundance. Levene's test was used to check for homoscedasticity of variances.

In addition to Bray-Curtis distances, we used weighted UniFrac distances (Lozupone *et al.* 2007), which incorporates microbial phylogenetic relatedness into abundance-based

distance measurements. The effects of acclimation temperature, *D. magna* genotype, and their interaction on microbial community structure were analyzed by permutational multivariate analysis of variance (adonis function in vegan with 99,999 permutations) using both Bray-Curtis and weighted UniFrac distance matrices. Within QIIME, MetagenomeSeq (Paulson *et al.* 2013) was employed to determine which OTUs significantly differed between treatments across all OTUs present in our samples.

Results

Effects of genotype, environment and their interaction on microbiota

Based on Bray-Curtis distances, the composition of microbiota differs due to the interaction of host clonal line and acclimation temperature, while weighted UniFrac reveals differences across acclimation temperature and host clonal line with no interaction effect. The two measures of alpha diversity (microbial phylogenetic diversity and Chao1) show differences across host genotype (Table 1).

The 10 most abundant OTUs that were present in both extraction batches made up 76.6% of the early extraction group dataset and 70.2% of the late extraction group dataset, and they were predominantly composed of members from the family Comamonadaceae. Comamonadaceae accounted for 5 of the top 10 most abundant OTUs with the other 5 OTUs composed of diverse bacterial orders. We tested for interaction effects of host clonal line and environmental effects to see if host-associated microbial communities exhibit parallel response to temperature treatment. Of the 10 most abundant OTUs, 5 show significant non-parallel changes (host clonal line × environmental effect, Fig. 2) across both extraction groups (Table 2). After accounting for multiple comparisons 3 of the non-parallel host clonal line × temperature interactions remained significant, and when combined those OTUs accounted for a relative abundance of 46.2% of the *Daphnia* microbiota. The differential abundance analysis that we ran also showed 4 OTUs (Table 3) that were significantly different across the two temperature treatments.

Discussion

We use clonal host individuals to investigate the effect of host clonal line, temperature, and their interaction on host-associated microbiota. We found that host clonal line × temperature interactions affect the community structure and the relative abundance of half of the dominant members of *D. magna* microbiota. We also found that diversity of microbial community is more affected by host clonal background rather than the temperature treatment.

The *D. magna* host lines used in this study were kept as monoclonal populations for at least five years in the laboratory after being cloned from females collected in their native habitats. Lab conditions were identical for all *D. magna* in this study including the culture room, medium, food quality and quantity, feeding schedule, temperature and day/night rhythm. The homogenous lab environment apparently did not eradicate the differences of the ancestral microbiota. However, we cannot determine if the strong clonal line effects and interaction effects between host clonal line and the environment we see are due to a carry-over effect of the microbiota each host brought into the lab or due to the genetic influence in determining which microbes establish within each host given an array of possible environmental bacterial that they encounter. Distinguishing between these hypotheses requires further experiments.

Five of the ten most abundant OTUs are assigned to the family Comamonadaceae (OTUs 1, 3, 4, 8, and 12) and account for the most abundant family within *D. magna* microbiome, making up 70.0% of the microbiota. Overall three of the four most abundant Comamonadaceae OTUs show considerable host clonal line × temperature interactions, and these three OTUs accumulatively account for 46.2% of the sequenced microbiota. A number of previous studies have noted the prevalence and stressed the importance of Comamonadaceae on *Daphnia*, in particular bacteria in the genus, *Limnohabitans* (Qi et al. 2009; Freese & Schink 2011). *Limnohabitans* has been implicated in important ecosystem processes such as the transfer of dissolved organic carbon in lake food webs (Eckert & Pernthaler 2014). Our results suggest that the genotype of *Daphnia* impacts the relative abundance of *Limnohabitans* OTUs. In particular, the representative sequence of OTU 3, which is the second most abundant OTU and strongly influenced by host clonal line, matches 100% to a *Limnohabitans* OTU that was suggested to play an important role in the food web of a large pre-alpine lake (Lake Zurich) (Eckert & Pernthaler 2014) (Genbank Accession #: HF968601).

Our results suggest that such food web effects might be influenced by the composition of the *Daphnia* population and thus may have broader implications for ecosystem processes.

Temperature has mixed effects on different systems, including no change (Lokmer & Wegner 2015) or an alteration in microbial diversity (Wilkins *et al.* 2015). The difference in relative abundance of OTUs between the two acclimation temperatures may be due to a modification in the host's selectivity for those particular microbes at different temperatures, a result of the physiological limitations of the bacteria or due to a difference in competitive ability at the different temperatures (Nishiguchi 2000; Webster *et al.* 2008).

The significant host clonal line and environmental interaction effect for half of the abundant OTUs, suggests that the host may be mediating the effect of temperature. There are also significant interaction effects across community composition of the *D. magna* microbiota as a whole when considering the PCoAs from Bray-Curtis, but not weighted UniFrac distances. The difference between these two metrics is most likely due to the microbes that differ between host clone lines. The prevalence of host clonal line × temperature effects may indicate that the genetic variation that exists in *D. magna* may enable diverse responses of their microbiota to a change in temperature. Although we cannot surmise if these patterns in microbiota would provide a selective advantage from the experiment at hand, the presence of marked host clonal line × temperature effects shows that responses of microbiota to environmental change are specific to the clonal line.

Conclusion

Overall, the consistent effect of host clonal line × temperature on the relative abundance of many of the most dominant OTUs suggests that *D. magna* clonal background has a non trivial influence on regulating its microbiota. Environmental main effects are weaker than host clonal background effects, but they are often specific to particular host clone lines and bacterial species, resulting in significant interaction effects. Based on this study, the specialization of microbes to habitats and host species (Mariadassou *et al.* 2015) appears to extend to host clonal lines. The effect of host clonal line is further underlined by the strong clonal differences found after animals were kept for at least 30 generations under identical, open-culture, laboratory conditions and exposure to the same microbiota in medium, food, and air. This is even more surprising as *D. magna* does not transmit microbes with their eggs, although the rearing of the eggs in an open maternal brood pouch allows for transmission from maternal microbes to the offspring (Ebert 2013; Sison-Mangus *et al.* 2015). Thus, all

microbes have to colonize the developing host de novo every generation. The presence of microbes from the common laboratory environment seems not to have had a lasting impact on this, but it is still feasible that each population seeds its tank with specific microbiota. A number of microbial community constituents seem largely resilient to temperature-induced changes in microbiota, and those that shift due to temperature-induced changes, do so in a way that is mediated by the host clonal line. How each host clonal line responds to environmental change, however, is difficult to predict until the ultimate genetic cues dictating microbial differences across host clonal lines are deciphered.

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Figures

Fig. 1: Analyses of microbiota from 16 host clones at 20 and 28 °C, showing (A) 8 clones that were in the early extraction group (stress = 0.21) and (B) 8 clones that were in the late extraction group (stress = 0.20). Data are shown with non-metric multidimensional scaling, where distance between points represents ecological distance between communities (based on Bray-Curtis distances).

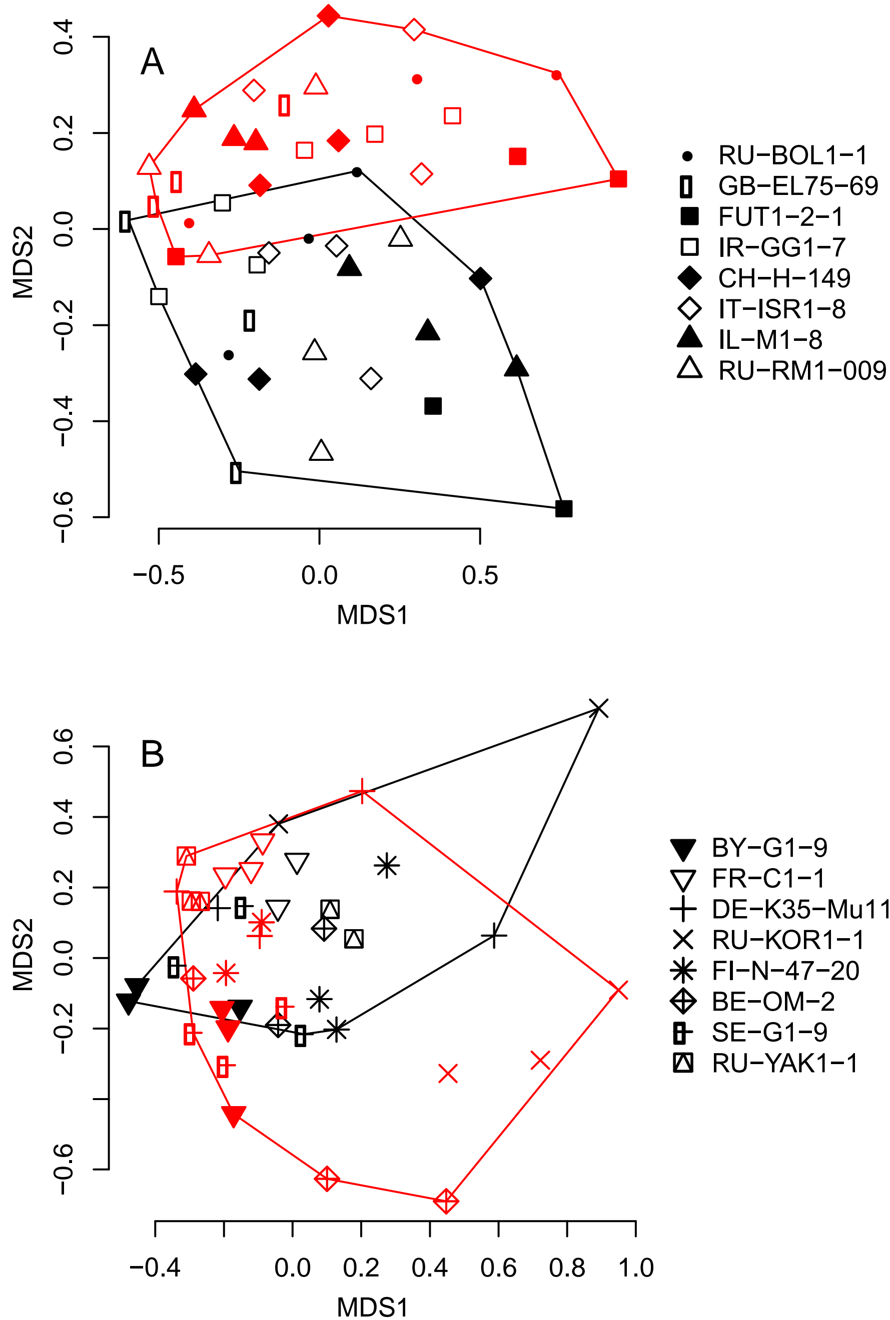
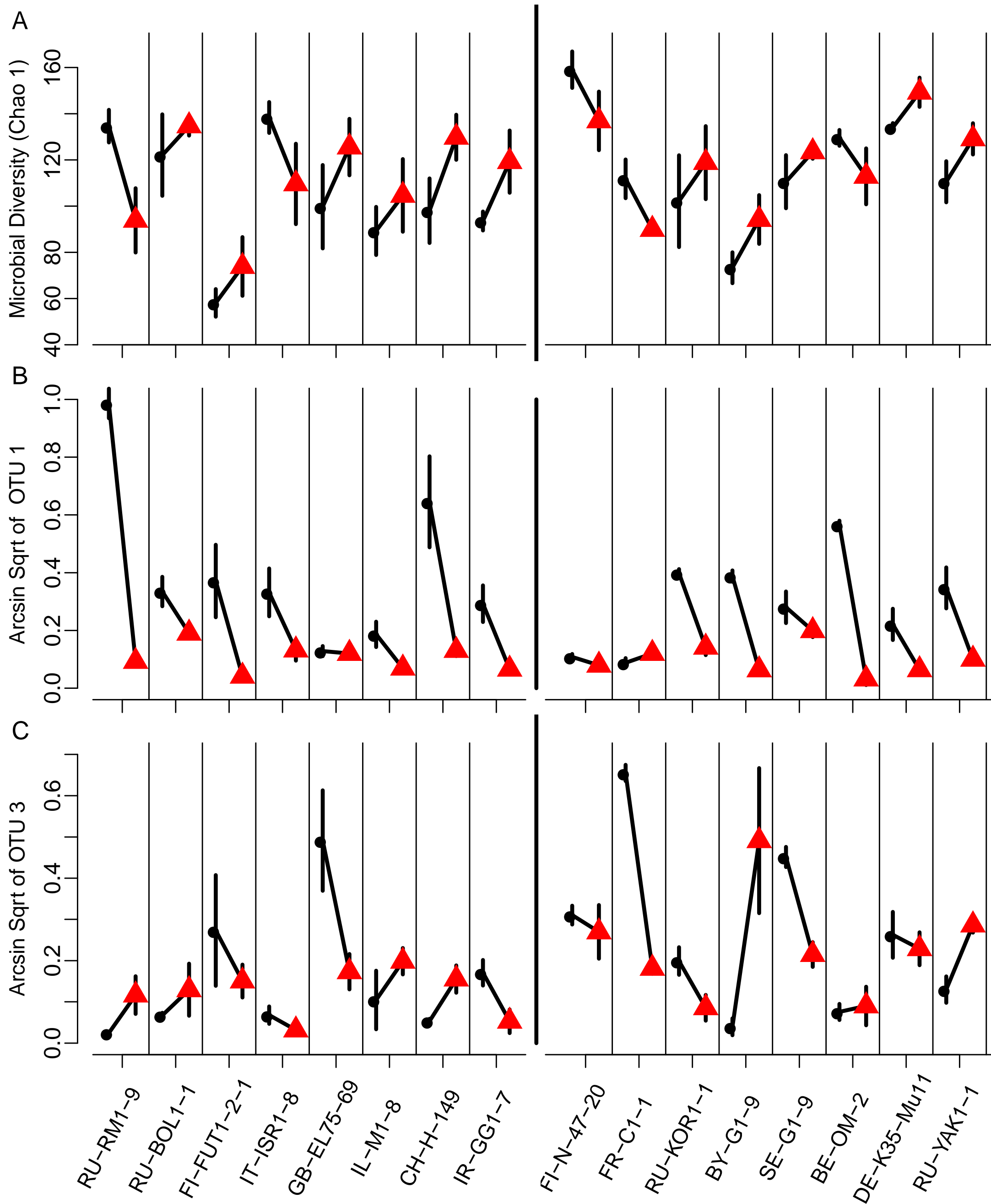


Fig. 2: Microbiota community changes across 16 host clonelines and two temperatures, including (A) change in diversity (B) changes in OTU 1 (family Comamonadaceae) and (C) changes in OTU 3 (family Comamonadaceae). Both OTU 1 and OTU 3 as well as OTU 4, 8, and 12 (not pictured) all have significant shows significant interaction effects based on different reaction norms across temperatures for different host clone lines, which are listed along the x-axis. The 8 clones shown in the left panels were in the early extraction group while the 8 clones in the rights panels were in the late extraction group.



Supplementary Figure 1: separation of *Daphnia magna* microbiota A) according to clone identity and B) according to date of extraction. Data are shown with non-metric multidimensional scaling, where distance between points represents ecological distance between communities (based on Bray-Curtis distances). Clone identity, temperature and extraction date all were significant within an Adonis analysis ($p < 0.001$). Due to confounding of extraction date and clone, the early extraction group (extracted between 6 March 2013 – 12 April 2013) and the late extraction group (extracted between 9 December 2013 – 31 January 2014) were separated during further analysis. Within the late and early extractions, extraction date no longer had a significant effect within Adonis analyses.

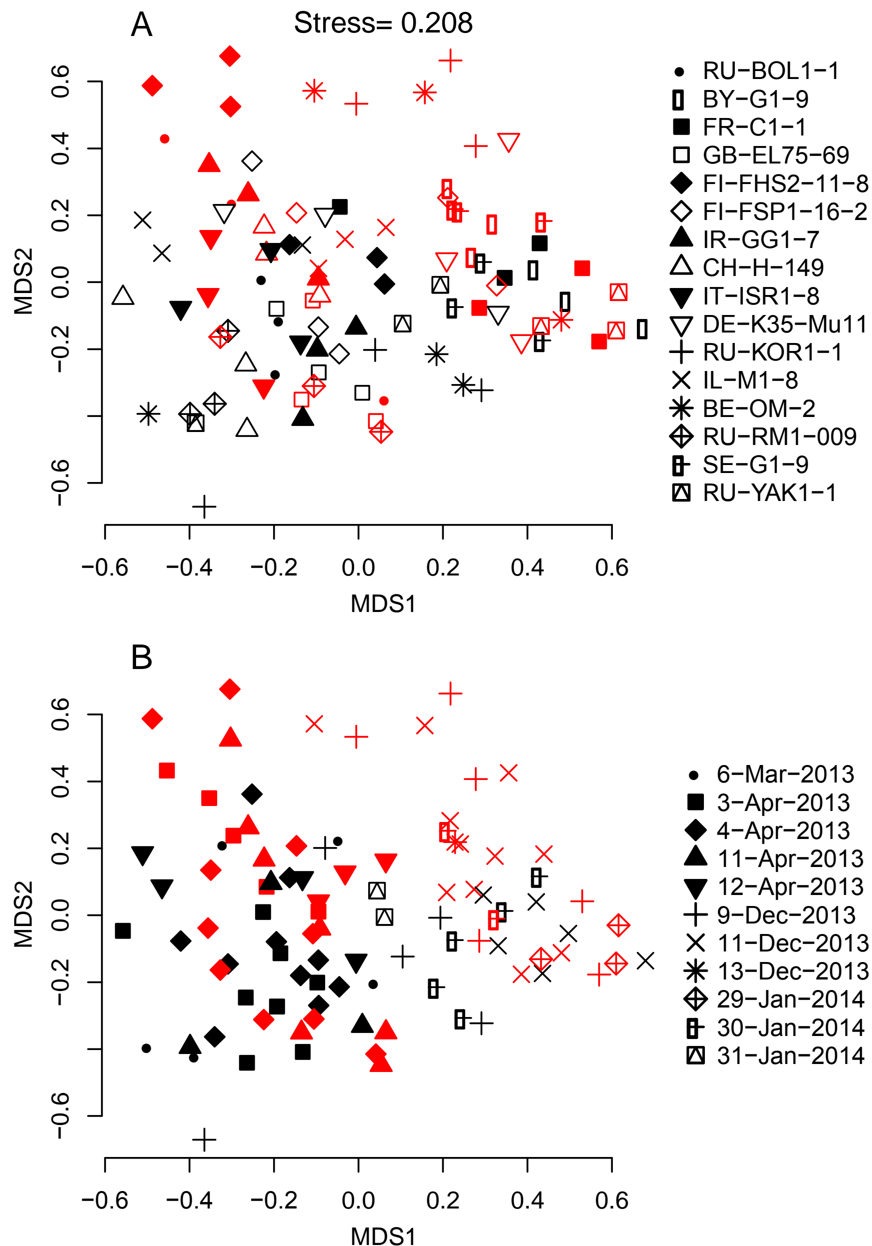


Table 1: Effects of host genotype, acclimation temperature, and their interaction on microbial community composition of *Daphnia magna*. Differences in Bray-Curtis distances and weighted UniFrac distances were tested with Adonis (99999 permutations). The differences in bacterial diversity, including Phylogenetic Diversity and Chao1 indices, are calculated using an ANOVA (type III Sum of Squares). Significant p-values are in bold.

	Early Extraction Group		Late Extraction Group	
a) Adonis (Bray-Curtis)				
Host clone line	$F(7,31) = 2.695$	**$p < 0.001$**	$F(7,26) = 2.832$	**$p < 0.001$**
Acclimation temperature	$F(1,31) = 6.438$	**$p < 0.001$**	$F(1,26) = 5.806$	**$p < 0.001$**
Host clone line x acclimation temperature	$F(7,31) = 2.840$	**$p < 0.001$**	$F(7,26) = 3.125$	**$p < 0.001$**
b) Weighted UniFrac				
Host clone line	$F(7,31) = 2.233$	**$p = 0.001$**	$F(7,26) = 2.055$	**$p < 0.012$**
Acclimation temperature	$F(1,31) = 3.696$	**$p = 0.006$**	$F(1,26) = 4.634$	**$p < 0.005$**
Host clone line x acclimation temperature	$F(7,31) = 1.491$	$p = 0.062$	$F(7,26) = 1.259$	$p = 0.210$
c) Phylogenetic Diversity				
Host clone line	$F(7,31) = 2.405$	**$p = 0.043$**	$F(7,26) = 3.538$	**$p = 0.008$**
Acclimation temperature	$F(1,31) = 0.528$	$p = 0.473$	$F(1,26) = 3.659$	$p = 0.067$
Host clone line x acclimation temperature	$F(7,31) = 1.804$	$p = 0.122$	$F(7,26) = 1.388$	$p = 0.252$
d) Microbial Diversity (Chao1)				
Host clone line	$F(7,31) = 2.233$	**$p = 0.019$**	$F(7,26) = 4.020$	**$p = 0.004$**
Acclimation temperature	$F(1,31) = 3.696$	$p = 0.054$	$F(1,26) = 1.897$	$p = 0.180$
Host clone line x acclimation temperature	$F(7,31) = 1.491$	$p = 0.137$	$F(7,26) = 1.251$	$p = 0.312$

Table 2: Effects of host clonal line, acclimation temperature, and their interaction on the most abundant OTUs of *Daphnia magna*. The realtive abundace of the OTU across combined extraction groups is shown next to the lowest taxonomic classification. Differences in OTU realtive abundance are calculated using an ANOVA (type III Sum of Squares) for each extraction group. Significant p-values are in bold and values with asterisks remain significant after adjustment for multiple comparisons using the Benjamini & Hochberg procedure. Levene's Test was used to test homogenity of variance, which only OTU 36 of the late extraction group failed.

	Early Extraction Group		Late Extraction Group	
OTU 8 (Family Comamonadaceae, 19.4%):				
Host clone line	F(7,31) = 1.978	p = 0.091	F(7,26) = 3.989	**p = 0.004** *
Acclimation temperature	F(1,31) = 6.439	**p = 0.016**	F(1,26) = 0.782	p = 0.385
Host clone line x acclimation temperature	F(7,31) = 4.065	**p = 0.003** *	F(7,26) = 5.537	**p < 0.001** *
OTU 3 (Family Comamonadaceae, Genus Limnohabitans, 19.2%):				
Host clone line	F(7,31) = 6.033	**p < 0.001** *	F(7,26) = 6.309	**p < 0.001** *
Acclimation temperature	F(1,31) = 0.514	p = 0.479	F(1,26) = 0.191	p = 0.666
Host clone line x acclimation temperature	F(7,31) = 3.065	**p =0.0143** *	F(7,26) = 6.255	**p < 0.001** *
OTU 4 (Family Comamonadaceae, 14.7%):				
Host clone line	F(7,31) = 1.985	p = 0.089	F(7,26) = 0.831	p = 0.571
Acclimation temperature	F(1,31) = 1.640	p = 0.210	F(1,26) = 0.042	p = 0.839
Host clone line x acclimation temperature	F(7,31) = 2.192	p = 0.063	F(7,26) = 1.207	p = 0.334
OTU 1 (Family Comamonadaceae, 7.6%)				
Host clone line	F(7,31) = 16.711	**p < 0.001** *	F(7,26) = 17.121	**p < 0.001** *
Acclimation temperature	F(1,31) = 2.256	p = 0.143	F(1,26) = 0.372	p = 0.547
Host clone line x acclimation temperature	F(7,31) = 8.568	**p < 0.001** *	F(7,26) = 12.414	**p < 0.001** *
OTU 11 (Order [Saprospirales], 2.5%):				
Host clone line	F(7,31) = 6.034	**p < 0.001** *	F(7,26) = 0.674	p = 0.692
Acclimation temperature	F(1,31) = 0.012	p = 0.915	F(1,26) = 10.479	**p = 0.003** *
Host clone line x acclimation temperature	F(7,31) = 3.674	**p = 0.005** *	F(7,26) = 1.662	p = 0.163
OTU 6 (Family Halomonadaceae, 2.4%):				
Host clone line	F(7,31) = 1.243	p = 0.310	F(7,26) = 1.698	p = 0.105 *
Acclimation temperature	F(1,31) = 0.017	p = 0.898	F(1,26) = 3.419	p = 0.076
Host clone line x acclimation temperature	F(7,31) = 1.760	p = 0.132	F(7,26) = 2.863	**p = 0.024** *
OTU 26 (Order Rhizobiales, 2.4%):				
Host clone line	F(7,31) = 6.735	**p < 0.001** *	F(7,26) = 3.989	p = 0.452
Acclimation temperature	F(1,31) = 4.780	p = 0.036	F(1,26) = 0.782	**p < 0.001** *
Host clone line x acclimation temperature	F(7,31) = 4.896	**p < 0.001** *	F(7,26) = 5.537	**p = 0.045**
OTU 7 (Genus Rhodobacter, 1.8%):				
Host clone line	F(7,31) = 2.008	p = 0.086	F(7,26) = 1.933	p = 0.153
Acclimation temperature	F(1,31) = 0.467	p = 0.499	F(1,26) = 0.506	p = 0.483
Host clone line x acclimation temperature	F(7,31) = 1.778	p = 0.127	F(7,26) = 2.497	**p = 0.042**
OTU 12 (Family Comamonadaceae, Genus Hydrogenophaga, 1.8%):				
Host clone line	F(7,31) = 2.646	**p = 0.029**	F(7,26) = 3.989	p = 0.210
Acclimation temperature	F(1,31) = 4.281	**p = 0.046**	F(1,26) = 0.782	p = 0.738 *
Host clone line x acclimation temperature	F(7,31) = 2.763	**p = 0.024**	F(7,26) = 5.537	**p = 0.026**
OTU 36 (Genus Flavobacterium, 1.6%):				
Host clone line	F(7,31) = 0.437	p = 0.871	F(7,26) = 3.504	**p = 0.009**
Acclimation temperature	F(1,31) = 0.036	p = 0.851	F(1,26) = 2.643	p = 0.116
Host clone line x acclimation temperature	F(7,31) = 0.411	p = 0.888	F(7,26) = 1.188	p = 0.344

Table 3: OTUs identified by a differential abundance analysis who's relative abundance changes between two temperature regimes in both extraction groups (P > 0.05 after accounting for multiple comparisons)

OTU ID	adjusted p-value (EG)	adjusted p-value (LG)	Direction	Taxonomy
OTU 45	0.0029	p > 0.0001	decrease at high temperature	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Methylophilales; f__Methylophilaceae
OTU 23	0.0039	p > 0.0001	decrease at high temperature	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Xanthomonadales; f__Sinobacteraceae; g__Nevskia;
OTU 1	0.0001	0.0019	decrease at high temperature	k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__Burkholderiales; f__Comamonadaceae
OTU 353	0.0024	0.0205	increase at high temperature	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhizobiales