Experimental investigations of diverse interactions between an aquatic crustacean and associated environmental bacteria

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Summary

All animals spend their lives in contact with diverse communities of microorganisms termed their microbiota, and the full range of effects these interactions have on animal ecology and evolution is only beginning to be appreciated. This thesis presents a series of experiments investigating the relationship between the water flea *Daphnia magna* and the environmental microbes with which it naturally coexists. These experiments lay a foundation for further investigations into host-microbiota interactions in aquatic settings.

The Introduction (Chapter 1) gives a brief overview of the conceptual issues raised by current studies of host-microbe interactions and introduces the ecological model organism *Daphnia*. In Part I of the thesis, I use newly developed methods for raising bacteria-free *Daphnia* to investigate the roles of bacterial microbiota in animal functioning. First, we examined the effect of bacteria-free conditions on basic *Daphnia* life history traits. We found that absence of microbiota has consistent, strong negative effects on *Daphnia* survival, growth and reproduction (Chapter 2). The effects of microbiota were generally robust to experimental conditions, but variation in the responses observed prompted further investigation into environment-specific benefits of these bacteria. We find that the magnitude of the beneficial effect of microbiota depends on diet (Chapter 3). In addition, we find that bacteria have a positive effect on embryonic development of resting eggs under warmed temperature conditions (Chapter 4). These results indicate a diversity of beneficial effects of *Daphnia*-associated bacteria.

In Part 2 of the thesis, I investigate how *Daphnia*-microbiota associations are formed in light of specific ecological characteristics of the host, namely diapause and genetically variable sediment browsing behavior. We find that diapausing stages of *Daphnia* are associated with beneficial bacteria even after years of dormancy, and use next-generation sequencing of bacterial taxonomic markers to characterize these bacterial communities (Chapter 5). We also investigated the effect of behavior on the composition of host-associated microbiota (Chapter 6), concluding that differences in microbiota diversity between host genotypes may be partially determined by genetic variation in behavior.

In Chapter 7, I argue that the evolution of host-microbe associations cannot be understood without attention to the effect of the interaction on the microbial symbiont community, and furthermore encourage re-framing the effects of complex microbiota as questions of community ecology and ecosystem function, rather than as a simple mutualism between two entities. I conclude with a list of specific research hypotheses raised by my work, and suggest approaches for answering them (Chapter 8).

Taken together, these results suggest that bacteria play fundamental, often cryptic roles in *Daphnia* biology, and that these relationships arose as a result of the omnipresence of bacteria throughout the history of *Daphnia* evolution.

Acknowledgments

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1. Introduction

   Since the early days of microbiology, humans have been intrigued by the idea that the invisible multitudes of microbes that surround us can influence our lives in unseen ways. From the early soil scientists, who transformed our view of soils from that of a dead substrate to a material teeming with living processes (Krasilnikov 1961), to the father of immunology, Elie Metchnikoff, who toward the end of his life became misguidedly convinced of the life-extending properties of yogurt (Mackowiak 2013), scientific history is filled with both successful and unsuccessful attempts to incorporate microbial activities into our understanding of fundamental life processes.

   Microbial interactions with higher organisms have been found to be responsible for a staggering variety of natural phenomena. Microbes are constantly transforming the organic material of the biosphere, fundamentally shaping the environments in which we live (Burgin et al. 2011). They carry out localized energy conversion processes such as large-scale fermentation of plant matter in the guts of termites and ruminants (Brune & Dietrich 2015; Poulsen et al. 2014; Mackie 2002), and photosynthesis in lichens, sponges and coral reefs (Thompson et al. 2015; Venn et al. 2008). They synthesize diverse metabolic products, like vitamins and antibiotics (Snyder & Rio 2015; Kaltenpoth & Engl 2014). They mediate traits we thought were "ours," like social odors or individual variation in drug metabolism (Theis et al. 2013; Nicholson et al. 2005). And they facilitate complex, subtle, unpredictable effects on myriad animal physiological systems, from the immune system to the nervous system (Lee & Brey 2013; Fischbach & Segre 2016).

   The sheer diversity of such effects has prevented many generalizations from being made about what, exactly, is the meaning of microbial interactions for our understanding of life. In one sense, this is appropriate; despite the number of reviews on the broad topic of "the role of microbiota in eukaryote evolution and/or human health," it is not necessarily sensible to assume that an entire domain of life would have a particularly unified effect on another. But the ubiquity and diversity of microbial interaction also make it a worthwhile exercise to examine how higher organisms would function in the complete absence of any bacterial influences. This allows us to interrogate some of the basic assumptions we have about how living systems function. To this end, a crucial experimental tool has been the development of axenic and gnotobiotic animal systems (Smith et al. 2007; Rawls et al. 2004; Erkosar et al. 2013). In early studies of nutrition, the goal of developing methods to raise animals in the absence of microbiota was to see what the "true" nutritional and metabolic capabilities of the study animals were, in the absence of "noise" from microorganismal activity (Dougherty 1956). Today's researchers are more interested in the reality of the combined effects of hosts and microbiota, and use the axenic condition as a

counterfactual. These studies have revealed that, far from being exclusively agents of disease, microorganisms have diverse beneficial effects on eukaryote hosts.

1.1 Types of beneficial host-microbe interactions

"Beneficial" here is defined simply as the host having higher fitness or a normal phenotype in the presence of the microbe as opposed to in its absence. (As will be discussed below, whether this kind of dependency is a universal "benefit" can be debated.) A large category of microbial benefits — and the focus of much of the classical research on mutualism — is the repertoire of additional primary and secondary metabolic functions that microbes can add to the functional capabilities of their host, thus providing a ready-made evolutionary novelty (Douglas 2014; Feldhaar 2011). Primary metabolic capabilities include microbial fermentation of plant material (Mackie 2002), production of amino acids missing in unbalanced diets (Wilkinson & Ishikawa 2000), and recycling of nitrogenous metabolic waste products (Sabree et al. 2009). Secondary metabolic capabilities include production of luminescence (Wollenberg & Ruby 2012), antibiotics (Harris et al. 2009), vitamins (Sudakaran et al. 2015), pigments (Barbieri et al. 2001), or odors (Wada-Katsumata et al. 2015). How cooperative sharing of these products is regulated and stabilized over evolutionary time is an ongoing area of study (Douglas 2008). The recognition that multiple microbial goods and services are required in many systems further complicates our understanding of the evolution of these systems, because multiple mutualists can have non-additive effects on host fitness (Afkhami et al. 2014). Furthermore, some symbionts themselves have symbionts, meaning that interspecies interactions can be interpreted as interactions between communities (Ferrari & Vavre 2011).

Another category of microbial benefits, less straightforwardly explained in terms of the sum of host and microbial metabolism, involves microbes influencing the regulation and performance of host functions; Moran calls this type of effect "addiction to infectious agents," while Douglas refers to it as "the symbiotic basis of health" (Moran 2002; A. E. Douglas 2014b) For example, in *Drosophila*, microbiota enhance the host's digestive capabilities not through the addition of microbial digestive enzymes to the host's, but because presence of microbiota stimulates host digestive enzyme production (as well as basal expression of a number of other essential genes) (Erkosar et al. 2015; Broderick et al. 2014). In many other systems, normal functioning of the immune system is "primed" or "educated" by the microbiota (Chung et al. 2012; Gollwitzer et al. 2014). These types of effects reflect the fact that wild-type animal phenotypes evolved in the context of microbial interaction. Such dependency on microbial presence for normal host gene regulation can arise in two ways. First, microbes can provide reliable cues about environmental conditions that hosts can use in regulating their functions; examples include metabolites from certain growth phases of gut microbes signaling host satiety (Breton et al. 2016), or marine microbes providing diffusible cues that allow planktonic larvae to settle and metamorphose on particular substrates (Winkler et al.

2015; Shikuma et al. 2014). Another, somewhat counterintuitive type of dependency on microbes arises as a consequence of ancient conflict (A. E. Douglas 2014b). If infection rates are very high in an ancient population, then host mechanisms to tolerate or compensate for the effect of the microbe can evolve to become constitutively expressed; these mechanisms and any resulting pleiotropic effects (for example, due to conserved signaling pathways between immune-related and other developmental processes) then become part of the "normal" phenotype, which can then be inappropriate or deleterious in the microbe's absence. An example is suppression of iNKT cells by *Bifidobacteria* (An et al. 2014); while initially this manipulation probably served the interests of the bacterium, the host's evolution to compensate for this effect means that in the absence of bacteria, excessive inflammation and deleterious health effects occur. Another way of stating this idea is that omnipresence of microbes during host evolution can lead to accumulation of mutations that are only deleterious in the absence of these microbes; this can be the case whether the microbes are initially beneficial or harmful.

In other words, there are some beneficial symbioses that enable adaptation to particular environments, and others that are themselves part of the environment that the host adapts to. These scenarios are not mutually exclusive. Microbial metabolites can serve simultaneously as environmental signals and usable goods; and any exchange of microbial goods and services can be accompanied by costs or conflicts that require tolerance or compensation mechanisms. Near-ubiquitous infection rates can result from selection favoring hosts carrying microbes, which then in turn creates selection for regulatory mechanisms to limit the costs of hosting these symbionts. A combination of "reliable cue" and "ancient conflict" effects arises when microbes serve as a signal for stressful conditions, and generalized stress responses are triggered; for this reason, for example, exposure to microbes can make a host better equipped to cope with subsequent chemical stress (Jones et al. 2015). The involvement of microbiota in obesity in mammals appears to result from a combination of certain microbes being more efficient energy harvesters and also influencing host regulation of fat storage through host-genotype-specific immune-related insulin signaling pathways (Turnbaugh et al. 2006; Bäckhed et al. 2004; Tremaroli & Bäckhed 2012). But making distinctions on the mechanistic basis of beneficial effects of microbiota is important because it deeply influences the evolution of the relationship by affecting the balance between fitness costs and benefits for each party involved. If the goods or services being produced are based on byproducts of other functions, they can be cost-free (Douglas 2008; Bronstein 2009). In situations where benefits arise as a side effect of ancient conflict, there is often little to no alignment between host fitness and symbiont fitness, yet the interaction can in theory stably persist. Likewise, the degree of species-specificity might be different for different types of effects — mutualisms based on exchange of costly products usually require evolution of specific mechanisms for maintaining partner fidelity or partner choice (Archetti et al. 2011), while host evolution to respond to microbially-based environmental cues requires recognition based on a microbial traits reflecting

adaptation to the environment, not necessarily to the host. In sum, all of these types of effects might be expected to have different degrees of specificity, stability, and long-term adaptive value.

1.2 Effects on adaptation

As a result of all the possible effects described above, beneficial symbioses can have a variety of effects on the adaptive trajectory of the host. The most obvious effect is niche expansion through novel adaptations: for example, carrying amino-acid-synthesizing endosymbionts allows aphids to live on a nutrient-poor food source (Moran 2006; Henry et al. 2013; Kiers & West 2015). However, these adaptations can also constrain niches via tradeoff effects: insects are often limited in their temperature tolerance due to the narrow temperature tolerance of their nutritional symbionts (Moran & Yun 2014; Wernegreen 2012; Nougué et al. 2015). For this reason, "mutualism meltdown" is a concern in the context of global change ecology: any environmental change has the possibility to affect organisms not only directly, but also through deleterious effects on other organisms with which it has interdependencies (Kiers et al. 2010). This becomes an even more complex problem when networks of multiple interacting species are involved. The relationship between diversity and stability of multispecies ecosystems is a long-standing problem in ecology (Ives & Carpenter 2007; Girvan et al. 2005). Simulations of cooperative and competitive networks of interactions in microbiota have suggested that some degree of competition between microbial community members is necessary for optimal stability in the face of perturbation, because it creates redundancy and lessens the "domino effect" that perturbation creates in highly cooperative systems (Coyte & Schluter 2015).

In relation to symbiont-mediated host gene regulation, Soen sketched out the idea that microbiota could contribute to stabilization of host phenotypes, due to adaptation of hosts and microbiota to their environment and to each other ("coordinated adaptation") (Soen 2014); the microbiota are a part of the frequently encountered environment of the sort that allows for evolution of deeply canalized developmental programs (Flatt 2005; Waddington 1942). This idea was supported empirically by the observation that removing microbiota in the parental generation increased the variability of development time among mutant *Drosophila* lines (Elgart et al. 2016). As well in *Drosophila*, different host genotypes have different responses to absence of microbiota, and a genome-wide association study showed that microbiota can mask the phenotypic effects of variability of some genetic loci, or conversely mediate the effects of variability at others (Dobson et al. 2015).

1.3 Conditional outcomes

Many beneficial interactions between hosts and microbes can vary depending on environmental conditions (Bronstein 1994). One reason for this is that under different conditions, the partners might be more or less able to perform the service, due to changes in ecological stress or resource availability. Environmental effects on the beneficial phenotype can act through effects on individuals (e.g. stressed individuals being less productive) or on the population. The magnitude of some benefits depends on the abundance of partners performing it, so conditions affecting population size of symbionts can also lead to a change in the level of benefit (Cunning & Baker 2014; Prado et al. 2010). In some cases, sufficiently stressful conditions can lead to a complete change in functional roles: noting the commonalities between immune and digestive physiology, and the fact that microbiota can make some animals more likely to survive starvation, Broderick proposed that among other things, host-associated microbiota can be a reserve food source for some hosts (Broderick 2015). Likewise, ecological conditions may exacerbate whatever costs symbiosis carries, by analogy to environmentally contingent host-pathogen interactions (Wolinska & King 2009).

The relative benefit compared to a symbiont-free condition can also be environmentally contingent because particular goods or services are more required under some conditions than others; for example, defensive symbionts would not provide any benefit in the absence of pathogens. Differences based on changes in requirements can be related to stress-mediated changes in performance because stressful conditions can reveal weaknesses in particular adaptations; for example, under high temperature, the cobalamine-independent methionine synthesis pathway of *Chlamydomonas* algae is repressed, meaning that external input of cobalamine produced by bacteria is required for methionine synthesis via a different pathway (Xie et al. 2013). Within a species, stress (defined as conditions far outside the norm typically experienced by the organism) can reveal cryptic phenotypic variation among individuals (Badyaev 2005), which could result in different requirements and different abilities to perform functions, resulting in a different average cost or benefit on a population level.

Understanding environmental conditionality of host-microbe interactions is the first step in elucidating the role of microbes in local adaptation of their hosts. If the magnitude of a beneficial effect of microbiota varies between environmental conditions, it suggests that microbiota may be involved in traits related to adaptation to that environment. A more specific prediction is that the relative benefit from a symbiosis based on exchange of goods will be strongest in the ancestral environmental condition, because this is the condition in which the good or service is required. In contrast, relative benefits due to symbiont-dependent physiological regulation should be most visible (or most variable) under unusual or stressful conditions, because this would contribute to further decanalization of microbiota-dependent traits. Thus, experiments evaluating the magnitude of the effect of microbiota under different environmental conditions are essential both for providing clues as to the functional roles of the

microbiota, and for understanding how they influence adaptation under natural conditions.

1.4 Parasitism, commensalism and mutualism

The literature on beneficial symbionts has frequently investigated the connections between beneficial symbionts and parasites. Why do some microbes that live on animals cause illness while others are necessary for health? It has frequently been stated that relationships between hosts and symbionts can fall along a "continuum" from mutualism to parasitism, but what this means is not always clearly defined. It is hypothesized that the outcome of an interaction between a particular host and a particular parasite will vary depending on environmental conditions, as discussed above. This has been shown to be true to some extent in many systems (Chamberlain et al. 2014), but a full parasitism-mutualism spectrum with the same partners has rarely been demonstrated; more often environmental conditions change the extent of harm or benefit, moving the organism closer to the "commensal" center of the continuum, but do not change a parasite into a mutualist or vice versa (Regus et al. 2014). Another type of "continuum" is over evolutionary time — it has often been assumed that beneficial relationships most often begin as parasitism and then proceed through a process of loss of virulence and eventually to benefit. However, the evidence for this is scarce and the conceptual foundation of this idea is shaky. Loss of parasite virulence over time is only inevitable if transmission is strictly vertical, because a strictly vertically transmitted parasite negatively affecting host reproductive success would eventually go extinct. Strictly vertical transmission is not found in most host-symbiont systems (see section on transmission modes below); in most other cases, the trajectory of the relationship will depend on factors such as the mechanistic connection between virulence and transmission (i.e. does making the host sick affect the parasite's ability to be transmitted to a new host?) and the non-host-related factors affecting the evolution of microbial traits (Ebert 2013; Levin 1996). In a Proteobacteria-wide study of microbes with known effects on hosts, it was found that mutualistic lifestyles evolved repeatedly from both parasitic and free-living ancestors (Sachs et al. 2014). Furthermore, experimental studies have shown that mutually beneficial growth can arise as a natural consequence of two organisms having complementary functions in an environment where both functions are required, without the need for any previous coevolutionary history (Hom & Murray 2014). On the other hand, it has been demonstrated that ordinarily benign bacterial communities can be detrimental to hosts with compromised immunity due to inbreeding, hybridization or age (Brucker & Bordenstein 2013; Clark et al. 2015). This demonstrates that the evolution of immunity – and the optimization of its costs – is indeed a constant challenge in a world filled with both friend and foe microbes. From the point of view of human health – and with the recognition that what we experience as illness is caused not just by parasite activity, but by our immune response – this has led

to the formulation of the "damage-response framework," which does not attempt to categorize symbionts at all but rather classifies the functional result of the host-symbiont interaction (Casadevall & Pirofski 2015).

Another important connection to the world of parasites is through the evolution of tolerance. Tolerance is defined as a mechanism of coping with disease not by attempting to reduce or eliminate (resist) parasites, but by limiting or compensating for the damage caused by parasites (Medzhitov et al. 2012). As discussed above, if infection rates are very high in a population in which tolerance rather than resistance is the favored strategy, then tolerance mechanisms and any resultant pleiotropic effects might evolve to be expressed constitutively, resulting in a sub-optimal phenotype in the absence of the parasite. Tolerance is fundamentally different from disease resistance in an evolutionary sense because, by its nature, it does not result in selection on parasites to evolve counter-adaptations because by definition it does not reduce parasite fitness. Tolerance mechanisms have traditionally been understudied in animals compared to plants (Baucom & De Roode 2011). The reasons for this are partly historical, but could also be biological, for example if animals have inherently less need for tolerance mechanisms than sessile plants. It is unknown whether there is natural variation in, or tradeoff costs to, tolerance in animals (Raberg et al. 2009); one proposal is that a certain "equilibrium" level of tolerance is fixed in animal populations, and thus not observable. The cryptic dependencies on microorganisms revealed by experiments with axenic animals might be informative for evaluating these questions — different degrees of fitness "loss" experienced by different hosts in a bacteria-free state, for example, may reflect differences in the tolerance strategies and tradeoffs that have evolved in their different lineages. This could be why the effects of microbiota on *Drosophila* phenotypic traits can vary so widely depending on host genotype (Dobson et al. 2015).

1.5 Modes of transmission

From an evolutionary standpoint, one of the most important distinctions is between symbionts that are transmitted vertically (from parents to offspring) versus horizontally (from the environment or unrelated individuals). Vertical transmission couples the evolutionary success of host and symbiont, and makes symbionts into an additional form of heritable non-genetic variation in the host. Horizontal transmission refers to acquisition of symbionts from other individuals or from the environment. Most microbiota exhibit mixed-mode transmission, resulting in frequent but not perfect alignment of host and microbe evolutionary interests (Ebert 2013).

Strictly vertical (transovarial) transmission results in a number of unusual genomic features due to frequent population bottlenecking and lack of recombination, including genomic erosion which in turn leads to further dependency on the host (Bennett & Moran 2015). Bennett and Moran point out that in these systems, hosts tend to become dependent on the symbionts beyond the original benefit they derive from it, and that this level of codependence entails risks and vulnerabilities as the symbiont

experiences irreversible mutation accumulation. In vertically transmitted symbionts that are transmitted extracellularly, even rare opportunities for recombination tend to prevent these genomic consequences (Salem et al. 2015), but the potential for host "addiction" still exists, since there is the equivalent selection for potentially maladaptive immune and other physiological factors in order to maintain the immediate benefits provided by the symbionts. Vertical transmission, however, is not the only way to ensure reliable presence: some symbionts can independently be very common in host environments, and thus also contribute to selection for host dependency. Symbionts being ecologically widespread (locally or globally) is sometimes an alternative explanation for patterns that can also be explained by vertical transmission (Faith et al. 2014; Zamborsky & Nishiguchi 2011); while this might seem to be a less interesting scenario to study, its biological importance should not be underestimated, and is an underlying assumption for scenarios such as adaptive gene loss due to "leaky" functions in ecological communities (D'Souza et al. 2014; Estrela et al. 2015). Furthermore, transmission from parents to offspring sometimes occurs as a result of seemingly adaptive traits (e.g. stereotyped behaviors such as egg smearing or egg capsule consumption, or specialized reproductive processes such as bacteriocyte sequestration) (Salem et al. 2015; Damiani et al. 2010; Vigneron et al. 2014), whereas in other cases, it appears to result as an inevitable consequence of processes such as vaginal delivery in mammals, parental care, or parent-offspring proximity (Dominguez-Bello et al. 2010; Spor et al. 2011). The latter set of processes may not have been selected to maintain particular microbiota, but can nevertheless result in particular distinctive patterns of microbiota composition, affecting subsequent host evolution. Zeng et al performed simulations examining vertical and horizontal transmission (and transmission from hosts into environments, changing the environmental species pools) as neutral sampling processes, and showed that even in the absence of competition between hosts and microbes, the contribution of microbiota by parents and previous generations to offspring and offspring environments, respectively, shapes the microbial community structure experienced by subsequent generations (Zeng et al. 2015).

Despite the seeming favorability of vertical transmission for maintaining beneficial symbionts, evidence suggests that vertical transmission is frequently imperfect. In plants, fungal endophytes can fail to be transmitted at any of multiple life-stage transitions. In a subset of species, failure to transmit the symbiont to seeds results in failure of those seeds to germinate, whereas in others transmission of the endophyte did not affect germination probability (Afkhami & Rudgers 2008). In *Drosophila*, despite the considerable protection against disease provided by *Spiroplasma*, the defensive symbiont has not reached 100% prevalence in any studied population due to imperfect maternal transmission (Jaenike et al. 2010), possibly due to environmental factors such as temperature-sensitivity of the transmission process. Therefore, the physical route of transmission, the availability of alternate sources of transmission, and

the environmental factors affecting both are relevant aspects to understanding the evolutionary ecology of a particular host-symbiont system.

A potentially important feature of adaptation via symbiosis is the possibility of a host's symbiont community changing within a generation in response to environmental conditions and then being transmitted to the next generation in its modified form. Although this possibility has been characterized as "neo-Lamarckian" (Rosenberg & Zilber-Rosenberg 2011), a more conservative way to describe it would be as a parental effect, i.e. a way in which an organism's phenotype can be affected by the environment experienced by the individual's parents (Badyaev & Uller 2009). In the framework for discussing parental effects proposed by Badyaev and Uller, transfer of symbionts and transfer of modified environmental factors are examples of "somatic tissue-to-somatic tissue" parental effects, and as such are in a position to function towards transferring novel adaptive variation and facilitating short-term adaptation. From this perspective, the question in any particular system is whether transmission of symbionts from parents to offspring serves largely to "reconstruct the parental developmental niche" or to modify it. Both alterations to the microbiota, and parental effects as a whole, have been suggested as mechanisms through which adaptive evolution could occur in ecological time and allow persistence through stressful periods, giving host genetic adaptation time to "catch up" (Räsänen & Kruuk 2007; Rosenberg & Zilber-Rosenberg 2011). It is worth noting that intergenerational transfer of modified bacterial communities might be a particularly efficient mechanism for anticipatory parental effects, since bacteria selected by a particular environmental condition could be both a reliable indicator of information about that environment and a source of functions important in that environment.

Although several well-known obligate symbioses are maintained through strictly horizontal transmission (Nussbaumer et al. 2006; Kikuchi et al. 2007), horizontally transmitted symbioses are often considered to be a source of variability rather than consistency of phenotypes within host lineages. If horizontally transmitted symbionts are considered as a type of environmental factor when it comes to host phenotypic variation, then host genetic factors that modify or select horizontally transmitted microbiota can be considered traits that modify the environment (Wong et al. 2015), subsequently modifying the phenotype. From this standpoint, it also makes sense to make another distinction between types of microbial phenotypic effects: those that act independently of host genotype (Koch & Schmid-Hempel 2012), versus those that mediate a particular genotype-phenotype connection (i.e. a host genotype selects for microbes that perform a certain function) (Chaston et al. 2015). In the former case, studying the host genome for clues to particular adaptations outside of environmental context would likely miss the fact that the "real" cause of these adaptations is the symbionts, whereas in the latter case, genotype effects and microbiota effects on the phenotype would be equivalent (but potentially still dependent on a local environment where the right microbiota are available). Horizontally transmitted symbionts could also

be considered a type of social or community resource (Lombardo 2007; Henry et al. 2013; Koch & Schmid-Hempel 2011), particularly if the abundance of microbes available to transmit to others is a function of host quality (i.e. if hosts are able to significantly "overproduce" microbes). The concentration of bioluminescent *Vibrio* in patches of open ocean depends strongly on the presence of bobtail squid in that area, since squid grow populations of these microbes in their light organ and expel them daily (Lee & Ruby 1994). A relevant question, therefore, is what percentage of the microbes in an environmental reservoir is potentially beneficial to hosts (Salem et al. 2015), and whether this depends on host densities or genotypes.

1.6 Units of selection

The dual observations that symbionts can profoundly influence host phenotypes and can frequently be transmitted between generations has led to the holobiont/hologenome concept, which posits that the primary unit of selection in evolution is the host with all of its associated microbiota and with microbes acting analogously to genes or alleles (Zilber-Rosenberg & Rosenberg 2008). This idea has generated a great deal of discussion, with well-considered criticisms and rebuttals from a number of sub-disciplines (Moran & Sloan 2015; Bordenstein & Theis 2015; Theis et al. 2016; Douglas & Werren 2016).

The discussions about the relevance of hologenomes as evolutionary units has much in common with an older discussion about the concept of "niche construction" as an evolutionary process. Host effects on populations of microbes and subsequent effects of these changed microbial communities on hosts can be considered niche construction under some formulations, so it is instructive to examine the concepts side-by-side. Both niche construction theory and hologenome theory attempt to incorporate clearly important non-genetic ecological processes —without which the organism would frequently be unrecognizable — into the conception of the organism (or its developmental process, or its evolutionary trajectory) itself. The precise definitions of these concepts have themselves evolved, resulting in ongoing confusion.

While some formulations (e.g. Dawkins's "extended phenotype") limited the definition of niche construction only to organismally-mediated environmental changes that are adaptive to the organism (Dawkins 2004), later work attempted to expand this idea to include to any changes in the environment caused by a particular phenotype that in turn affect the evolution of subsequent generations. This urged a focus on reciprocal organism-environment feedbacks as explanations for concordance between phenotypes and environments (Odling-Smee et al. 2013; Laland et al. 2015). In a similar shift, the earliest formulation of the hologenome theory of evolution focused on the fact that symbionts could be directly transmitted between generations and thus serve as an additional form of heritable variation, but more recent literature proposes doing away with the distinction between vertical and horizontal transmission and conceptualize "community heritability" of hologenomes as the tendency for certain host-symbiont

gene combinations to repeatedly reoccur in a way that co-varies with host relatedness, regardless of the underlying reason for this concordance (Theis et al. 2016). In both of these conceptual discussions, the utility and coherence of the concept is limited by a lack of clarity about the boundaries of the phenomena in question. A clearly defined boundary for the definition of genetic individuality is passage through a single-cell gamete bottleneck; this is where the variation that is acted upon by selection ultimately originates. It is clear that many important biological phenomena require relaxing this definition somewhat; the literature on parental effects, for example, arises out of the observation that phenotypes are frequently affected not only by an individual's genotype and environment, but by the phenotype and environment of its parents, which can transmit phenotypically important non-genetic factors between generations. This is a useful and necessary addition to our understanding of continuity of phenotypes but it is still ultimately tied to an understanding of the factors in question, i.e. to the information value or direct resource value of parental phenotypic factors. In contrast, a criticism of the niche construction theory formulated in the "extended evolutionary synthesis" view would be that it generalizes to the point of triviality the observation that organisms change environments and vice versa. In practice, every researcher delineates the boundaries of their research question, and studies of particular organism-environment feedbacks in specific systems tend to advance understanding more than abstract attempts to formulate niche construction as a new paradigm.

A similar requirement to specifically delineate meaningful boundaries is present in the discussion of hologenome theory, and in this case, the shift in focus away from the fraction of the holobiont that is directly vertically transmitted between generations is a step in the wrong direction. Even if, as proponents suggest, horizontally transmitted symbionts can be incorporated into the hologenome concept by analogy to genetic recombination or horizontal gene transfer, the question remains what the holobiont actually *is,* other than the subset of bacteria that is captured when an animal individual is sampled. When a host is not a culture flask, with a clearly defined inside and outside, self and non-self, and is not lineage of periodically bottlenecked cells or cellular components, then the question becomes difficult to narrow down – in practice, it is not always clear where a holobiont ends and the environment begins. A definition of a selectable individual based on statistical clustering of animal and microbial genes could just as easily be applied to units that don't reproduce, like a series of ponds containing many interacting, functionally interconnected species. After all, in pond metacommunities there could also be a degree of concordance between animal population genetic structure and bacterial community composition with certain predictable phenotypic consequences, but it would not follow that ponds are selectable units. The way selection acts on animal individuals is clear; the way it acts on poorly defined assemblages is not.

The understanding that multi-lineage interaction networks with certain collective phenotypes are pervasive in nature is certainly a major scientific advance. The question

is whether it advances understanding to conceptualize these phenomena in terms of a new kind of unit of selection, analogous to an organism. In a discussion of philosophical issues in microbial ecology (O'Malley 2014), O'Malley suggests that the viewing these collectives as an organism-like individual unit is not always appropriate, suggesting instead that additional understandings of fitness might be developed for microbial ecology, based for example on longevity and persistence of assemblages rather than reproduction of individuals. More saliently, O'Malley points out that microbial ecology in general and host-microbiota interactions specifically are still in a phase where they need to be understood on a case-by-case basis, and that any particular instances of adaptation may have resulted from selective processes acting on different units.

Thus, while part of the goal of any research program is to lead to generalizable insights, elucidating the specific features of a particular study system is a valuable goal in and of itself. With this in mind, my goal in this thesis was to investigate the relationship between an animal and its microbiota under multiple environmental conditions as the first step in specifying the functional roles that microbiota might fill, with attention to specific ecological features of the model in question. Using the experimental construct of *Daphnia magna* raised under bacteria-free conditions, I attempted to understand the ways in which microbes influence the function, fitness, and ecology of this environmentally important organism. The focus was thus on general and environment-specific effects of bacteria on *Daphnia*, rather than on the *Daphnia* microbiome as a study subject in itself.

1.7 *Daphnia magna* in the field and laboratory

The aquatic, filter-feeding microcrustacean *Daphnia* is one of the oldest model organisms in ecology and evolution (Lampert 2011). Its natural ecology is quite well-studied compared to many experimental models. *Daphnia* is found worldwide in limnetic habitats ranging from temporary rock pools to eutrophic lakes, where it is frequently a dominant zooplankter; for this reason, it has become a model for questions relating to local adaptation and environmental health (Ebert 2011). In applied settings, *Daphnia* is commonly used in ecotoxicology tests. Studies of adaptation are relevant here as well because there is considerable variability between genotypes in sensitivity or resistance to particular pollutants, potentially affecting interpretation of tests (Baird et al. 1991; Coors et al. 2009).

Experiments evaluating *Daphnia* adaptations have a number of simple read-outs based on life history characteristics such as survival, growth and reproduction. Genotype effects are straightforward to evaluate in *Daphnia* because their clonal parthenogenetic reproduction allows for the production of many genetically identical replicates. Parthenogenetic eggs are directly developing and complete embryonic development in their mother's brood chamber, which is located under the carapace but exposed to circulating water. After being released from the brood chamber, animals go through 4-6 juvenile instars before producing first clutches of eggs. In natural

populations, *Daphnia* population densities can fluctuate over several orders of magnitude over the course of a season, with density-dependent population dynamics due to competition for food and transmission of parasites (Ebert 2005).

Under deteriorating environmental conditions or during peak density phases, *Daphnia* can begin to produce males and reproduce sexually. The products of sexual reproduction are long-lasting diapausing embryos, enclosed in cases called ephippia (singular ephippium). These can survive multiple environmental stresses and resume development under more favorable conditions; they are the major source of *Daphnia* recruitment at the beginning of a season (Hairston 1996). The ability to store ephippia for long periods after sampling from geographically and temporally diverse locations makes them another useful experimental feature. Surprisingly, despite the large amount of research into this diapausing stage, only the process of inducing ephippia production is reasonably well-understood; the evidence about the precise cues and conditions allowing exit from diapause is considerably more equivocal (Vanvlasselaer & De Meester 2010; Allen 2010). Thus, ephippial embryos are both convenient for experimental manipulation, and also interesting biologically because of their relation to questions about acclimation and adaptation after dispersal or environmental change.

Studies of *Daphnia*-microbe interactions have a long history, due primarily to studies of microbes as food and as parasites for *Daphnia*. Informal recommendations among hobbyists advise against using sterile water for *Daphnia* husbandry, as bacteria in the water appear to result in better population health. Despite this, it has been unclear exactly what roles bacteria can play in *Daphnia* ecology and evolution. Early attempts at sequencing the *Daphnia* genome revealed the extent of bacterial association even under long-term laboratory conditions (Qi et al. 2009). Later sequencing projects showed that *Daphnia*-associated microbiota have a distinct community structure from the surrounding water and sediment (Freese & Schink 2011; Samuel Pichon et al unpublished). Nevertheless, there is not currently any evidence that *Daphnia* microbiota comprise a distinct category from "environmental" bacteria on any basis other than their immediate physical association with the animal. The goal of the experiments in this work was to evaluate the types and consequences of coexistence with these bacteria on *Daphnia*, rather than assuming any particular co-evolutionary scenario. Interestingly, one of the most abundant bacterial taxa in aquatic settings, comprising various strains of *Limnohabitans* (Comamonadaceae), is largely found in association with *Daphnia* and is most active in the uptake of dissolved organic carbon in that state (Eckert & Pernthaler 2014), meaning that *Daphnia* bodies serve as a site for a major transformation in the aquatic carbon cycle. In general, in water bodies with little physical structure and oligotrophic nutrient conditions, animals may serve as higher-density nexuses of microbial activity. The microenvironmental changes occurring around these clusters could be ecologically important both for the organism and for the larger environment.

This thesis had the following specific aims: i) Develop methods to raise bacteria-free *Daphnia*; ii) Evaluate the fitness and function of bacteria-free *Daphnia* compared to those with conventional microbiota; iii) Evaluate how the effect of microbiota varies under different environmental conditions, manipulating factors such as diet or abiotic environment, in order to identify phenotypic traits that might be affected by microbiota; iv) Determine how *Daphnia* forms beneficial associations after diapause, and whether vertical transmission of microbiota through diapause exists; v) Evaluate how genetic variation in a quantitative host trait (browsing behavior) can influence horizontal acquisition of microbiota from the environment. Taken together, these studies provide insights into the types of beneficial interactions between *Daphnia* and microbes might exist in natural settings.

2. Water fleas require microbiota for survival, growth and reproduction

Marilou P. Sison-Mangus, Alexandra A. Mushegian, and Dieter Ebert

Abstract

Microbiota play diverse roles in the functioning of their hosts; experiments using model organisms have enabled investigations into these functions. In the model crustacean *Daphnia*, little knowledge exists about the effect of microbiota on host well-being. We assessed the effect of microbiota on *D. magna* by experimentally depriving animals of their microbiota and comparing their growth, survival and fecundity to that of their bacteria-bearing counterparts. We tested *Daphnia* coming from both lab-reared parthenogenic eggs of a single genotype and from genetically diverse field-collected resting eggs. We showed that bacteria-free hosts are smaller, less fecund, and have higher mortality than those with microbiota. We also manipulated the association by exposing bacteria-free *Daphnia* to a single bacterial strain of Aeromonas sp., and to laboratory environmental bacteria. These experiments further demonstrated that the *Daphnia*-microbiota system is amenable to manipulation under various experimental conditions. The results of this study have implications for studies of *D. magna* in ecotoxicology, ecology and environmental genomics.

2.1 Introduction

All eukaryotes spend their lives associated with communities of microorganisms, known as microbiota. While some microbes are parasites that can cause disease, many others lie on the spectrum between commensalism and mutualism and may significantly influence their hosts' nutrition (Dethlefsen et al. 2007), development (Bates et al. 2006) and disease resistance (Macpherson & Harris 2004; Koch & Schmid-Hempel 2011). The use of model organisms such as the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, the house mouse *Mus musculus*, and the zebrafish *Danio rerio* has facilitated understanding of the mechanisms by which certain biological functions of the hosts are modulated by their microbiota (Erkosar et al. 2013; Rawls et al. 2004; Cabreiro & Gems 2013; Turnbaugh et al. 2006). As interest in environmental genomics emerges, the roles of microbiota in the ecology and evolution of an increasing number of non-model organisms are being investigated, revealing a high diversity in the types of effects observed (Fraune & Bosch 2010; Engel et al. 2012; Koch & Schmid-Hempel 2012; Brucker & Bordenstein 2013). Here we present the first experiments addressing the role of microbiota in a crustacean model, *Daphnia*.

Organisms across multiple taxa appear to generally suffer fitness consequences when raised without bacterial associates, but the nature and magnitude of these consequences varies strongly by taxa and environmental conditions. For example, germ-free mice and rats have marked deficiencies in gastrointestinal and immune development (Chung et al. 2012; Ivanov et al. 2009) and are leaner than conventional mice (Bäckhed et al. 2004), but can survive and reproduce under laboratory conditions if provided with a chemically defined diet (Pleasants et al. 1986). Bacteria-free zebrafish exhibit visible degeneration of intestinal tissues by Day 8 post-fertilization and have

100% mortality by Day 20 unless bacteria are re-introduced (Rawls et al. 2004). Bacteria-free *Drosophila* larvae have slowed or arrested development depending on dietary conditions (Erkosar et al. 2013; Shin et al. 2011), and germ-free adult flies have been reported to have reduced lifespan compared to conventional flies (Brummel et al. 2004). The development and lifespan of the nematode *C. elegans*, on the other hand, is twice as long when cultured in axenic conditions (Houthoofd et al. 2002), while addition of live *E. coli* in the diet restores its normal life history (Lenaerts et al. 2008). The disparate nature of these effects in the small number of model systems examined, and the complex interactions between hosts, microbiota, and environment, prevent many generalizations from being made across taxa.

The planktonic crustacean *Daphnia* is a widely used model in ecology and ecotoxicology, as well as in population and quantitative genetics, environmental genomics, the evolution of sex, and host-parasite interactions (Ebert 2011). Since microbiota can be a cryptic source of environmental and phenotypic variation among animals (Bleich & Hansen 2012; Koch & Schmid-Hempel 2012), understanding the influence of microbiota on *Daphnia* biology is crucial. *Daphnia* reproduces both clonally and sexually, has a short life cycle, and has sequenced genomes and other genomic tools available (Colbourne et al. 2011). These features provide opportunities for identifying the influences of bacterial symbionts on *Daphnia* physiology at the molecular level and these findings can be placed in an ecologically relevant framework. The microbiota of three species of *Daphnia* have been described, and despite the inter-continental distribution of these species, they harbor diverse but similar bacterial communities, a hint that *Daphnia* and their microbiota may have established a stable relationship (Qi et al. 2009). At present, the contribution of microbiota to *Daphnia* health is unknown and the dynamics of the interaction are uncharacterized.

We used the species *Daphnia magna* to investigate the influence of microbiota on the animal's life history. We provide the first report that *D. magna* can be rendered bacteria-free and provide experimental evidence that the microbiota play a major role in host fitness. We demonstrate that bacteria-free *D. magna* grow more slowly, are less fecund, and have higher mortality than those with microbiota. We conducted our experiments with *D. magna* raised from a lab-reared parthenogenic clone and from field-collected resting (sexual) eggs. While the former controls for the genetic background of the host, the latter confirms that the observed effects are not limited to a single host genotype.

2.2 Materials and methods

Animals

Animals were reared from both parthenogenetic and resting eggs of *Daphnia magna*. In the study using parthenogenetic eggs, the *D. magna* clone Xinb3 was used because its genome has been sequenced and other genomic tools (such as genetic map,

EST library, QTL-panel) have been developed (Routtu et al. 2010). The clone originated from a rock pool population in southwestern Finland and was selfed three times after initial collection to create an inbred line, which has been maintained in the laboratory for several years. The resting eggs used in the other experiments came from a sediment sample collected in a carp-breeding pond (labeled K2-2) close to Ismaning, near Munich, Germany. Resting eggs are sexually produced and are encased in a protective shell called an ephippium. They can be kept for years under cold and dark conditions before hatching is stimulated with light at room temperature (Davison 1969; Pancella & Stross 1963).

Daphnia from parthenogenetic eggs

Growth and fecundity experiment: Female *D. magna* of clone Xinb3 were synchronized and standardized to constant conditions to reduce variation in egg stage, cohort and quality caused by maternal status (i.e., maternal effects), that can subsequently impact offspring performance (Lynch & Ennis 1983). Same clutch progenies of a single *Daphnia* mother were grown in the same culture conditions for 4-5 generations until a large cohort of animals of the same size, age and reproductive stage was produced. Eggs (within 24 hours after eggs were released from the ovary) from 200 females were carefully removed from the mothers' brood chambers and washed 3 times with autoclaved 0.2 μm filtered artificial *Daphnia* medium, ADaM (see recipe at http://evolution.unibas.ch/ebert/lab/adam.htm). Eggs were randomly assigned into four groups, one of which was left untreated. The remaining three groups of eggs were treated with a combination of three antibiotics, Ampicillin (Applichem #A0839) at 1 mg mL-1, Kanamycin (Fluka Biochemika #60615) at 50 μg mL-1 and Tetracycline (Fluka Biochemika # 87128) at 50 μg mL-1, until hatching (2 days). Prior to conducting this experiment, we also tested the sterilizing agents mercuric chloride, sodium hypochlorite and PVP-Iodine, but these chemicals caused very high mortality in parthenogenetic eggs.

After antibiotic treatment, hatchlings from all groups were washed twice, including the untreated group. Each individual hatchling was placed in an experimental jar containing 80 mL ADaM and 59 million cells of axenic algae (see below) and closed with a 0.2 μm membrane screw cap (Duran #1088655, Mainz, Germany) that allowed for air exchange but prevented bacterial contamination. The three antibiotic-treated groups were grown in the following conditions: 1) ADaM alone (BacFree), 2) ADaM with triple antibiotics (BacFree+AB), 3) ADaM supplemented with bacteria (Bac-Suppl). Hatchlings from the untreated group (4) were grown in ADaM. The bacterial supplement in the Bac- Suppl group was a suspension of bacteria from the pooled bodies of the mothers of the harvested eggs, which were crushed and the homogenate filtered with a UV-bleached 7.0 μm mesh filter. The filtrate was washed once by centrifugation at 3000g for 1 min and diluted in 6-mL ADaM, and 100 μL of this

bacterial suspension was dispensed per jar. All procedures necessitating sterile conditions were carried out under a UV-sterilized laminar flow hood.

Jars from all four treatment groups were randomly positioned in a 20°C temperature-regulated incubator room with 16:8 light:dark photoperiod and carefully shaken once a day to resuspend algae, which would otherwise sediment to the bottom of the jar. The jars were repositioned every other day. *Daphnia* (n=8-10 replicates per treatment) were measured for body length at Day 4 and another set of replicates were measured at Day 10 (destructive harvesting, as animals were no longer axenic after measurements were taken). A third set of animals was monitored for fecundity until Day 25. Five to ten egg-bearing individuals from this set were sacrificed to count the number of first clutch eggs. Two animals from each treatment group were used for PCR screening of bacteria at the egg stage, Day 4, and Day 10.

Mortality experiment: A second experiment with *Daphnia* from parthenogenetic eggs was performed to determine the mortality of bacteria-free animals. A similar set-up was performed as above with the following modification: only 2 treatments were compared (BacFree versus Bac-Suppl) and hatchlings in Bac-Suppl treatment were only exposed to bacteria for 24 hours before placing them in experimental jars. Five eggs in 2-mL sterile round bottom Eppendorf tubes were allowed to develop in triple antibiotic solution for 48 hours. Hatchlings were rinsed twice with ADaM to remove antibiotics and those intended for Bac-Suppl treatment were exposed to bacterial suspension for 24 hours. The bacterial suspension was prepared as above but without the 7.0 µm mesh filtration. Prior to transferring to experimental jars, Bac-Suppl hatchlings were washed once to remove unattached bacteria that might serve as an uncontrolled food resource for the *Daphnia*.

Ninety-three jars with individual *Daphnia* hatchlings per treatment were prepared at Day 1 and monitored daily for mortality. *Daphnia* were fed twice (at Days 1 and 16) with 37 million cells of axenic live algae per feeding.

Daphnia with a single bacterial strain: To determine if the growth of *Daphnia* exposed to a single bacterium differs from the growth of *Daphnia* that is exposed to a bacterial mixture, a third experiment was carried out using the same set-up as for the mortality experiment. Eggs were allowed to develop in ADaM with double antibiotic solution (Ampicillin and Kanamycin at 1 mg mL-1 and 50 µg mL-1, respectively) for 48 hours and then washed once with ADaM before being separated into three groups: Bac-Suppl, BacFree and Aeromonas- treated. The Aeromonas sp. strain (Xinb3-6, Genbank accession no. KF924766) was previously isolated from the *D. magna* Xinb3 clone, and cultured in Luria-Bertani medium. Bacteria from the homogenized mothers and the Aeromonas culture were washed once via centrifugation at 3000g for 5 minutes, resuspended in ADaM and adjusted to the same OD600 (0.63 – 0.65) with an Eppendorf Biophotometer (Eppendorf AG, Germany) before adding 100 µL of the bacterial suspension to bacteria-free hatchlings. After 24 hours, individual hatchlings

were rinsed with ADaM and grown in experimental jars for 6 days for body size measurement (n=8 to 9 individuals per treatment).

PCR screening of animals: In all experiments, PCR screening of bacteria on *Daphnia* sampled before and after the experiment was carried out. *Daphnia* and bacterial DNA were extracted with the modified Hotshot Method (Montero-Pau et al. 2008) and 16s rDNA was amplified using 327F (5-ACACGGYCCARACTCCTAC-3') and 936R (5'-TTGCWTCGAATTAAWCCAC-3') primer pair targeting the conserved sequences flanking the V3-V6 hypervariable regions. PCR conditions were as follows: 94°C for 2 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and extension of 72°C for 10 min. The extracted DNA of an adult *Daphnia* with normal microbiota and nuclease-free PCR water were used as positive and negative controls for 16s rDNA PCR amplification, respectively. *Daphnia* 18s rRNA screening was also carried out in tandem with the bacterial screening using the primers H18S_F (5'-CTGAATATCGCAGCATGGAAT- 3') and H18S_R (5'-TCGGACAGGGAGAGTGAAAC-3'). Positive amplification of 18s rRNA verifies that DNA extraction was successful, indicating that negative 16S rDNA amplification results (especially for bacteria-free samples) were not due to failed DNA extraction.

Bacteria-free algae: Axenic algae were obtained by treating *Scenedesmus obliquus* culture with triple antibiotics (as above) for three culture passages. Axenicity of the algae was verified with three combined methods: PCR screening for 16s rDNA with bacterial primers 327F and 936R, bacterial culturing in four media (Luria-Bertani, Muller-Hinton, MacConkey and Mannitol Salt Phenol Red Agar) and visual inspection of bacteria by phase contrast microscopy. In one of the axenicity trials (out of five), the PCR in one out of three samples amplified 16S rDNA. Sanger sequencing revealed that the PCR product was caused by algal chloroplast amplification and not bacterial 16s rDNA amplification. Further tests carried out using the other two methods failed to detect bacteria as well. Antibiotics treatment of algae followed by axenicity screening were always carried out prior to using axenic algae in each experiment. Antibiotics from the axenic algal food were removed by centrifugation at 3000 g for 5 min and the resuspension of algal pellet in ADaM.

Daphnia from Resting Eggs

We also looked at the effect of microbiota manipulation in *D. magna* at the population level using sexually produced diapausing eggs from ephippia. As resting eggs are very tolerant of chemical treatment (Vizoso et al. 2005; Luijckx et al. 2012), we used household bleach (sodium hypochlorite) instead of antibiotics to remove the bacteria from the egg surfaces. We also used autoclaved algae instead of axenic live algae as alternative food to the *Daphnia*.

Mortality and fecundity experiment: Ephippia were collected from a sediment sample and manually opened with forceps under a dissecting microscope. Resting eggs immersed in ADaM were refrigerated overnight until experimental treatment. Three

treatments were carried out: 1) E-Untreated, 2) E-BacFree and 3) E-Bac-Suppl (E-indicating "ephippial source"). A set of six eggs in Eppendorf tubes from E-BacFree and E-Bac-Suppl groups were exposed to 500 μL of 5% sodium hypochlorite solution for 5 minutes, inverting tube gently 10 times followed by rinsing twice with ADaM. Eggs for E-Untreated group were not surface-sterilized with bleach but were also rinsed twice with ADaM. Each set of eggs was placed into a separate jar with ADaM until hatching. Hatching jars of the E-Bac-Suppl group were supplemented with 100 μL of a bacterial suspension obtained from one homogenized adult *D. magna* in 500 μL ADaM. The bacterial sources came from *Daphnia* conventionally raised from the same batch of ephippia. Bacterial exposure of hatchlings in hatching jars lasted <24 hours. One hatchling was transferred to each experimental jar, ensuring independence of replication. *Daphnia* (n=11 to 15 individuals per treatment) were fed every 3-4 days with 50 μL suspension of autoclaved *Scenedesmus* algae (298 million cells ml-1). Hatching jars and experimental jars were kept in the incubator room and maintained as in the parthenogenetic *Daphnia* experiment. Mortality and reproduction were monitored daily until termination of experiment at Day 21.

Growth experiment: The same procedure was followed as the mortality and fecundity experiment with a minor modification. The E- Bac-Suppl group in this experiment was supplemented with bacteria from a *D. magna* lab clone originating from the same Munich population. Moreover, a modified ADaM was used in this experiment, with the sodium bicarbonate reduced by 25% to lessen precipitation during autoclaving. *Daphnia* were fed every 1-2 days and measured at Day 6 (n= 8 individuals per treatment), before mortality reduced the number of surviving animals in the E-BacFree treatment too much.

Daphnia *with environmental bacteria*: A third experiment was conducted to see if ephippial eggs exposed to bacteria from a non-*Daphnia* source would exhibit similar growth as those supplemented with *Daphnia* microbiota or bacteria-free *Daphnia*. Since we have previously cultured many species of bacteria from lab-prepared ADaM, we used non-sterilized ADaM from the standing laboratory stock as a source of bacteria. The same procedure as in the growth experiment was followed except that in this experiment, we used ADaM diluted 1:1 with Milli-Q water before autoclaving, and the resting eggs were bleach-sterilized in a single batch, subdivided into 3 groups and hatched in a 24-well sterile plate in the following media: sterile ADaM (E-BacFree), non-sterile ADaM (E-Bac-ADaM), and ADaM supplemented with bacteria from homogenized adults (E-Bac- Suppl). Eggs were hatched under constant light without climate control; later experiments suggested that higher temperature (~26oC) reduces the hatching rate of axenic eggs (unpublished data). After emergence, hatchlings were transferred to experimental jars with diluted sterile ADaM and were fed every 1- 2 days. Sizes of 5-7 individuals per treatment were measured at Day 6.

PCR screening of bacterial 16s rDNA of two individuals from each treatment was carried out at the egg stage and on 6-day old animals. The universal bacterial primers

27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-CGGYTACCTTGTTACGACTT-3') (Weisburg et al. 1991) were used. DNA was extracted with the modified HotShot method (Montero-Pau et al. 2008).

Statistical analysis

      Data were analyzed with the statistical software package JMP 10.0. Size data of the parthenogenetic *Daphnia* were tested for normality of distribution and equality of variances prior to analysis with ANOVA. The dataset was then fitted with the model: Size = Treatment + Day + Treatment * Day. Data that did not satisfy assumptions of normal distribution or equal variances were tested instead with the non-parametric Kruskal-Wallis test. Post-hoc comparison of means was carried out using Tukey HSD for datasets analyzed by ANOVA and the non-parametric Steel-Dwass test for the dataset analyzed by Kruskal-Wallis. Means and standard errors are reported. Differences in survival rates between treatments were tested with the Mantel Cox log-rank test.

2.3 Results

      PCR screening of bacterial 16s rDNA from *Daphnia* individuals at different sampling points in the experiments confirmed the absence of bacteria in BacFree treatments and the presence of bacteria in the Bac-Suppl *Daphnia* from both parthenogenetic and resting eggs. From both Untreated and E-Untreated *Daphnia*, to which bacteria had been neither added nor chemically removed, we obtained mostly positive but occasionally negative PCR results. We surmised that bacteria adhering to the surface of the eggs from these samples might have been occasionally reduced to undetectable levels during the washing steps; the initial abundances of bacteria adhering to surfaces of parthenogenetic and ephippial eggs are unknown. Hatching rate of parthenogenetic eggs exposed to triple antibiotic solution during egg development was between 98-100%. Hatching success of resting eggs was typically between 30 to 70% of eggs, which is consistent with typical observations in other experiments on resting egg hatching (De Meester and Dejager 1993).

Bacteria-free Daphnia are smaller than bacteria-treated Daphnia

      Body sizes were significantly different among BacFree, Untreated and Bac-Suppl *Daphnia* from parthenogenetic eggs ($F_{7, 67} = 20.67$, $p<0.0001$). There was no significant interaction between effects of treatment and day of measurement. We did not see a significant difference in the sizes of *Daphnia* between Untreated and Bac-Suppl treatment, but the *Daphnia* from these two treatments were significantly larger than *Daphnia* from BacFree and BacFree+AB treatments (Figure 1a). *Daphnia* in the BacFree treatment were significantly larger than *Daphnia* from BacFree+AB, suggesting some harm caused by the long-term application of the antibiotics in addition to the harm caused by the lack of bacteria.

Similarly, *Daphnia* from resting eggs exhibit significant differences in size at Day 6 (Kruskal Wallis, $\chi2= 9.68$, $p < 0.008$) (Figure 1b). E-Bac-Suppl animals are significantly larger than E-BacFree and E-Untreated *Daphnia*; the latter two groups are not significantly different in size. Due to high mortality previously observed in bacteria-free ex-ephippial animals, we did not measure body sizes of animals later than Day 6 in this experiment.

Bacteria-free Daphnia have low fecundity and survival

All (26/26) Bac-Suppl *Daphnia* carried eggs in their brood chamber at Day 11 while only 10 of 24 *Daphnia* in the Untreated treatment carried eggs at Day 11 (Figure 2a). Five more egg-bearing *Daphnia* in the Untreated groups were observed at Day 17, bringing the total rate to 58%. Egg-bearing *Daphnia* from the BacFree and BacFree+AB treatments were first seen at Day 13, reaching 26% in both treatments at the end of the experiment at Day 25 (BacFree-AB, 5/19; BacFree, 5/19). This strong effect on fecundity was further supported by the observation from the separate mortality experiment using parthenogenetic eggs, where 97% of the *Daphnia* in the Bac-Suppl group produced eggs as compared to only 5% (5/93) in the BacFree treatment.

Bac-Suppl *Daphnia* had significantly more eggs in their first clutch than *Daphnia* from Untreated, BacFree and BacFree+AB treatments (Figure 2b) (Kruskal Wallis, $\chi2= 24.97$, $p < 0.0001$). In addition, the remaining Bac-Suppl females (n=16) successfully produced hatchlings (5.1 ± 0.8) and second clutch eggs (10 ± 0.6) while the remaining individuals from the other three treatments had not produced any hatchlings or second clutch eggs when the experiment was terminated at Day 25.

None of the animals in the E-BacFree group survived to reproduction. Animals in the E-Untreated group either died before reproducing, or still had not reproduced when the experiment was terminated at Day 21. Most E-Bac-Suppl *Daphnia* (13/15) produced eggs starting at Day 9 and released hatchlings 3-4 days later (mean = 3.7 ± 0.5).

a.

Daphnia from Parthenogenetic eggs

b.

Daphnia from Resting eggs
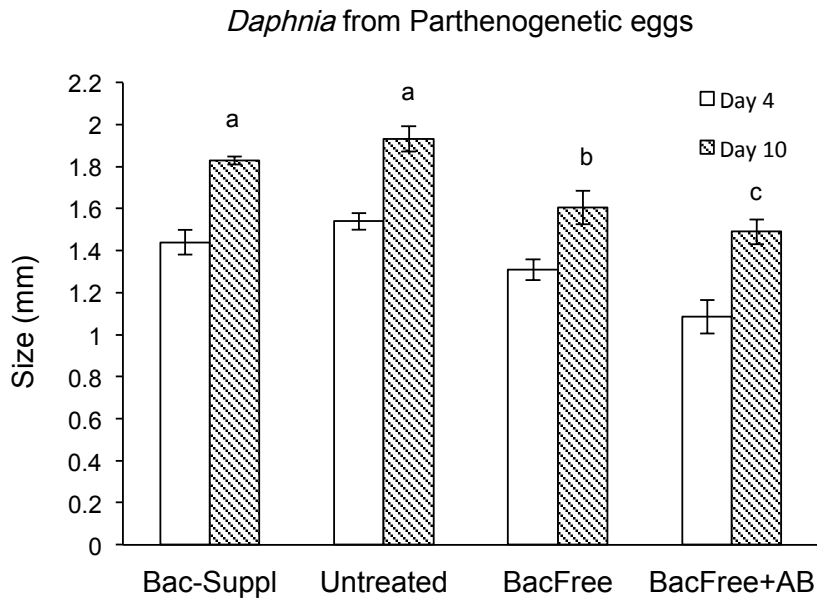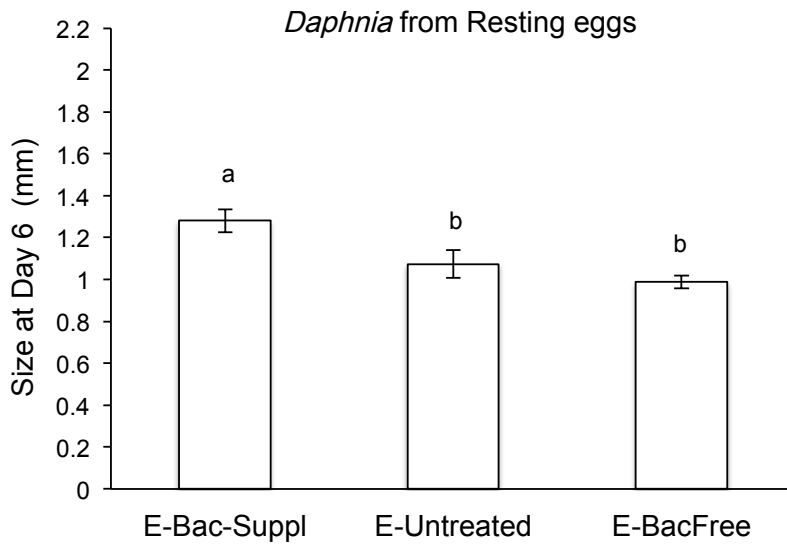
Figure 1. Size comparisons of *Daphnia* from (a) parthenogenetic and (b) ephippial eggs with and without microbiota. a) Bacterial treatment has a significant effect on sizes of parthenogenetic *Daphnia* at Day 4 and 10 (one-way ANOVA, $p < 0.0001$). Group means were compared with Tukey HSD test. b) Bacterial treatment has a significant effect on sizes of ex-ephippial *Daphnia* at Day 6 (Kruskal-Wallis test, $p < 0.008$). Steel-Dwass test was used for pairwise comparisons of groups. Groups not connected by same letter are significantly different ($p < 0.05$). Means and standard errors are shown.

a.
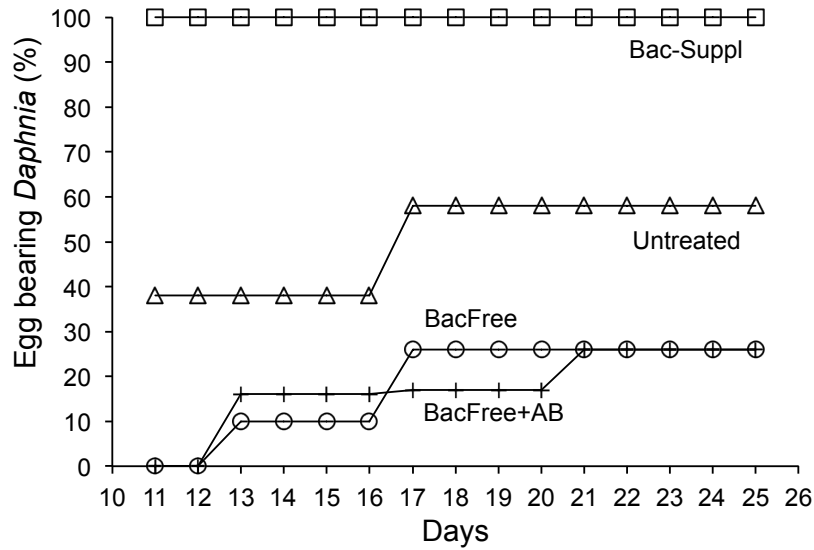
b.
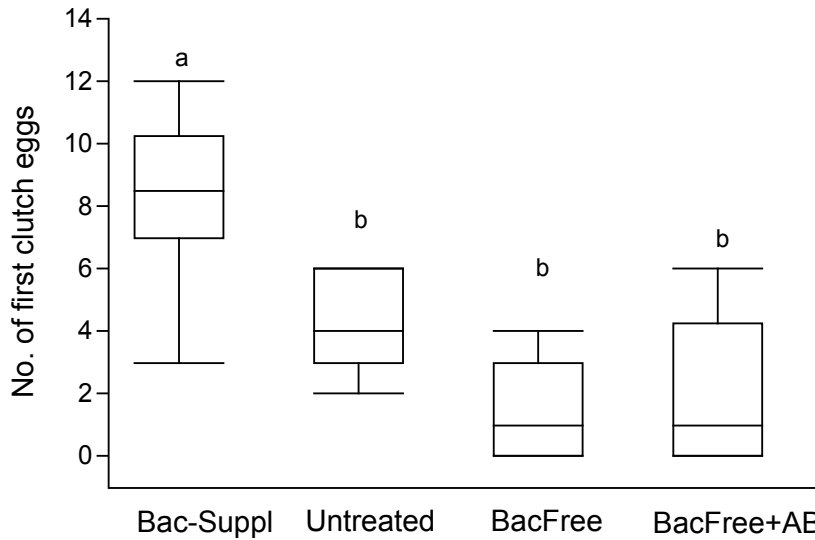
Figure 2. Fecundity of *Daphnia* raised from parthenogenetic eggs under different treatments. a) Percentage of egg-bearing *Daphnia* between Days 11 and 25 for the 4 treatment groups. b) Size of first clutch (median, lower and upper quartile, range) in 4 treatment groups. Bacterial treatment has a significant effect on the number of first clutch eggs produced (Kruskal-Wallis test, $p < 0.0001$) and comparison of the groups show that Bac-Suppl produced significantly higher number of eggs than Untreated, BacFree and BacFree+AB (Steel-Dwass tests, all $p < 0.01$). Groups not connected by same letter are significantly different.

In a separate experiment, we tested the mortality rate of BacFree *Daphnia* compared to Bac-Suppl *Daphnia* coming from parthenogenetic eggs. Mortality of *Daphnia* was significantly higher in BacFree treatment as compared to the Bac-Suppl treatment (Mantel Cox log-rank test, χ^2= 95.2, p< 0.0001). At Day 33, all *Daphnia* in the BacFree treatment had died, while 49% of Bac-Suppl *Daphnia* were still alive at this time. In a similar experiment with *Daphnia* hatched from ephippial eggs, the E-Bac-Suppl *Daphnia* also survived significantly longer than E-BacFree or E-Untreated *Daphnia* (Mantel Cox log-rank test, χ^2= 20.7, p< 0.001), with 86.7% still alive when the experiment was terminated at Day 21, versus 38.5% alive in the E-Untreated treatment group and none alive in the E-BacFree treatment group (Figure 3).
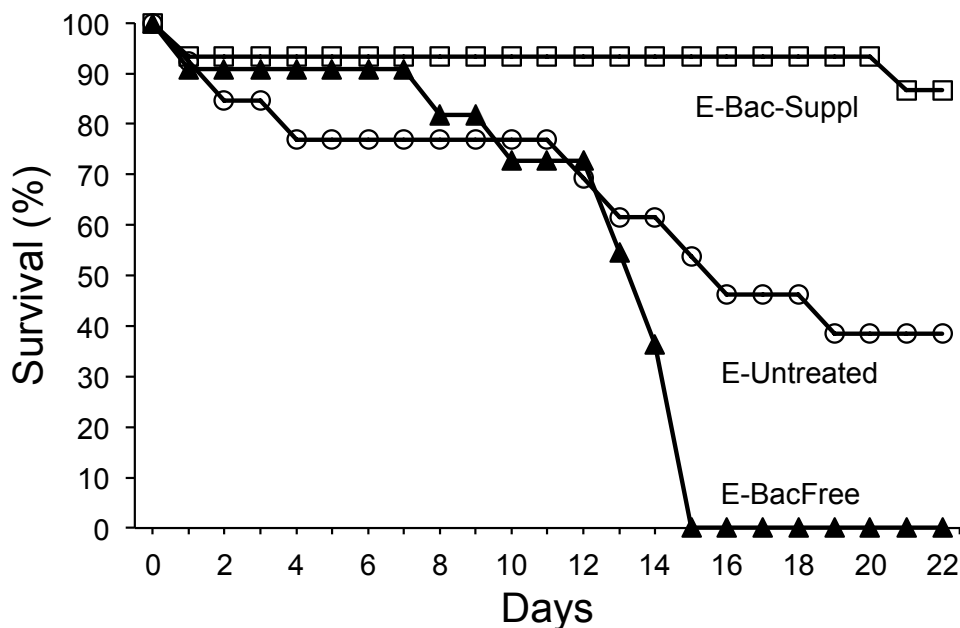
Figure 3. Survival curves of bacteria-supplemented (E-Bac-Suppl), untreated (E-Untreated) and bacteria-free (E-BacFree) *Daphnia* hatched from ephippial eggs. Mantel Cox Log rank test indicates that bacteria-supplemented *Daphnia* lived longer than untreated and bacteria-free *Daphnia* (p< 0.001).

Growth of Daphnia with a single bacterium and Daphnia with environmental bacteria

  Daphnia from parthenogenetic eggs exposed to the *Daphnia*-derived bacterium Aeromonas sp. were similar in size to the *Daphnia* supplemented with microbiota suspension derived from their mothers (Steel-Dwass test, p= 0.22, Figure 4a), and *Daphnia* from both of these treatments were significantly larger than bacteria-free *Daphnia* (Steel-Dwass test, both p= 0.002). Furthermore, *Daphnia* from ephippial eggs exposed to bacteria from a non-*Daphnia* source (E-Bac-ADaM) reached the same size at

Day 6 as those supplemented with bacteria from a *Daphnia* source (E-Bac-Suppl, Steel-Dwass test, p= 0.79, Figure 4b). *Daphnia* in both treatments were significantly larger than BacFree *Daphnia* (Steel-Dwass test, p= 0.02).

a

b
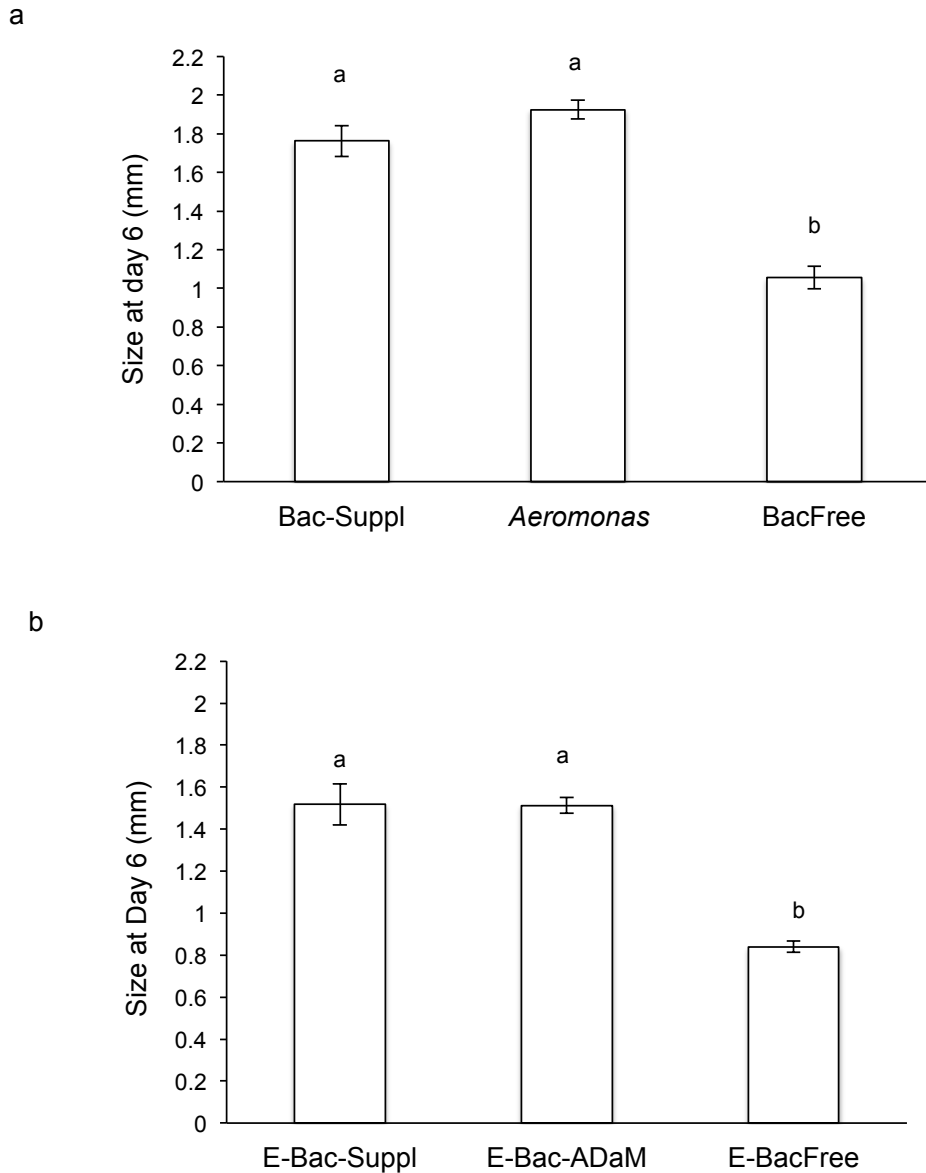
Figure 4. Body size at Day 6 of *Daphnia* treated with different sources of bacteria. a) *Daphnia* from parthenogenetic eggs associating either with a supplement containing diverse bacteria from the mother (Bac-Suppl), a single bacterium (*Aeromonas* sp.) or bacteria free (BacFree). Bac-Suppl and *Aeromonas* treatments do not differ significantly, but they significantly differ from the BacFree treatment (Steel-Dwass test, p<0.002). b) *Daphnia* from ephippial eggs exposed to bacteria from a non-*Daphnia* source (E-Bac-ADaM; non-sterile ADaM) had the same body size at Day 6 as *Daphnia* exposed to bacteria from a *Daphnia* source (E-Bac-Suppl). Both *Daphnia* groups are significantly bigger than E-BacFree *Daphnia* (p<0.02). Groups not connected by same letter are significantly different.

2.4 Discussion

This is the first study to report that the fitness of *D. magna* is compromised without bacterial associates and that the *Daphnia*-microbiota association can be experimentally manipulated. Our two experimental approaches showed similar overall effects on *D. magna* even though the methods differ in host genetic backgrounds (clonal versus mixed genotypes of *Daphnia*), diet (bacteria-free live algae versus autoclaved algae) and chemicals used to render the animals bacteria-free (triple antibiotics versus sodium hypochlorite), suggesting that our results hold true under diverse conditions. The restoration of normal functioning by adding bacteria to germ-free eggs (in the Bac-Suppl, Bac-Aeromonas, E-Bac-Suppl and E-Bac-ADaM treatments) shows that the low fitness observed in germ-free *Daphnia* is due to lack of bacteria and not due to the antimicrobial substances used in the treatments. Our results are consistent with observations of fitness reductions in arthropods when reared without microbiota (Shin et al. 2011; Salem et al. 2013). The negative PCR results for surface-sterilized eggs suggest that *Daphnia* do not transmit bacterial symbionts inside the eggs. However, bacteria are present on the surface of parthenogenetic eggs and are able to partially rescue host fitness in the absence of additional bacteria. In contrast, fitness of ex-ephippial *Daphnia* from the untreated groups was not rescued, with host fitness being comparable to that of bacteria-free animals. The relative importance to host functioning of bacteria acquired during development in the brood chamber, retained in ephippia during diapause, and acquired from the environment upon hatching is of considerable interest.

Compared to bacteria-bearing *Daphnia*, bacteria-free animals showed reduced growth, fecundity and survival. Furthermore, they were more transparent ("ghost like"), the gut hardly contained food, and the animals contained very few of the yellow-red lipid droplets typically observed around the gut and the ovaries of conventional *Daphnia*. We also observed differences in the amount of algae left over in the jars, with the bacteria-supplemented *Daphnia* having much less leftover algae in their jars than the bacteria-free animals. The latter either consumed less algae, or the algae passed through the gut without being digested. Similar observations were seen in experiments using autoclaved algae. Hence, the symptoms we observed in microbiota-free *D. magna* could be attributed to reduced food intake or energy uptake or both. This suggestion is consistent with observations of reduced fat reserves in microbiota-free mice and zebrafish (Semova et al. 2012; Bäckhed et al. 2004) and to findings from abalone and sturgeons relating the presence of different bacteria to enhanced digestive enzyme activities (Askarian et al. 2011; Zhao et al. 2012). Although *D. magna* has digestive enzymes used for breaking down food such as proteases, amylases and lipases (Hasler 1935; Von Elert et al. 2004), the contribution of gut microbiota to these functions is unknown. It also remains to be seen whether bacteria affect the development and maturation of the *Daphnia* gut, as they do in the development of vertebrate gut epithelia (Rawls et al. 2004; Hooper et al. 2001; Bates et al. 2006), or whether bacteria promote

growth factor signaling and intestinal stem cell activity as in Drosophila (Shin et al. 2011; Storelli et al. 2011).

Bacteria can also serve as food for *Daphnia*, forming a minor part of a diet dominated by algae (Urabe & Watanabe 1991). Bacteria alone cannot meet the nutritional requirements of *Daphnia* because they lack sterols and poly-unsaturated fats that are required by *Daphnia* for somatic growth and reproduction (Martin-Creuzburg et al. 2011; Martin-Creuzburg et al. 2005; Taipale et al. 2012). In our study, *Daphnia* from all treatments were fed equal amounts of *Scenedesmus*, an alga that typically sustains growth and reproduction in *Daphnia*. Our results demonstrate that in the absence of bacteria, a diet of algae alone is not sufficient for normal *Daphnia* functioning. Bacteria are required either as nutritional supplements or functional partners or both, perhaps providing essential dietary components, assisting with digestion, or modulating other physiological processes.

Long-term exposure to antibiotics over the *Daphnia* lifespan can augment the negative impact of germ-free state on animal growth compared to short-term exposure (48 hours during early development; Figure 1). Long-term maintenance of germ-free animals by mixing antibiotics with food should be used, therefore, with caution.

2.5 Conclusion

Consistent with experiments from other animal taxa, we showed that *Daphnia* suffers significant losses in fitness when deprived of bacteria and that these losses are prevented when bacteria are restored or replaced. Bacteria on egg surfaces do not appear to be sufficient for normal *Daphnia* fitness, though they appear to have some partial fitness benefits. Our findings and the methods developed here offer the opportunity to incorporate microbiota as a factor in research on environmental health. *Daphnia* has been one of the most studied organisms in ecological and ecotoxicological research for over a century and is a model system for environmental health genomics. In studies of immunity, ecotoxicology and ecology where growth and fecundity of *Daphnia* are commonly used as measures of health (Lampert 2011), the impact of microbiota as a crucial environmental factor should be taken into consideration.

3. Presence of microbiota reverses the relative performance of *Daphnia magna* on two experimental diets

Alexandra A. Mushegian and Dieter Ebert

Abstract

The outcomes of host–symbiont interactions may differ according to environmental context, and symbioses may enable host adaptation to diverse environments. We find that the effects of two different experimental diets, algae and yeast, on the water flea *Daphnia magna* depend on whether the animals possess microbiota, suggesting that the presence of microbiota determines which diet is superior. Our study hints at both diet-dependent and diet-independent effects of microbiota on *Daphnia* fitness.

3.1 Introduction

In many host–symbiont systems, the extent of the fitness benefit provided by symbiosis compared to a non-symbiotic state varies depending on environmental factors. One reason for this is that environmental conditions might affect how well partners are able to perform their function, thus changing the absolute benefit of symbiosis. Another reason is that particular services might be more or less necessary depending on the environment, thus changing the relative benefit for the host. Likewise, the costs that symbionts exact on hosts may have different fitness consequences in different contexts. Accordingly, experiments with microbiota-free hosts aiming to elucidate the function of the microbiota in host fitness should be conducted under diverse conditions (Chamberlain et al. 2014).

The water flea *Daphnia magna* Straus is an aquatic crustacean that has long been a model for diverse host–microbe interactions. In natural settings, *Daphnia* is colonized with gut bacteria and epibionts (Qi et al. 2009; Eckert & Pernthaler 2014). Several studies comparing bacteria-free and symbiotic *Daphnia* specimens have independently shown strong fitness costs of the absence of bacteria on *Daphnia* life history (Sison-Mangus et al. 2015; Peerakietkhajorn, Tsukada, et al. 2015; Callens et al. 2015), but the exact fitness consequences have varied between experiments. Callens et al. (Callens et al. 2015) explicitly investigated the effect of food availability on the magnitude of the benefit provided by microbiota. In contrast to studies in fruit flies and earthworms (Shin et al. 2011; Lund et al. 2010), where the growth-promoting benefits of symbiosis are most pronounced under nutrient-poor conditions, Callens et al. (Callens et al. 2015) found that microbiota conferred greater benefits (in the form of increased host growth and survival) over a bacteria-free state as food abundance increased. Thus, in *Drosophila*, the food × microbiota interaction appears to result from more severe costs of poor nutrition for germ-free than symbiotic animals, whereas in *Daphnia*, increasing food abundance results in more sharply increasing benefits in symbiotic than in bacteria-free conditions. The pattern in *Daphnia* is consistent with the explanation that one of the roles of their microbiota is to facilitate extraction of nutritional benefits from a plant-based diet. Germ-free daphnids that are unable to efficiently utilize this diet might not benefit from its greater availability.

We hypothesized that there would be strong diet-dependent effects of microbiota on *Daphnia* under qualitatively as well as quantitatively different diets because of the different functional roles that microbiota would be required to fulfill in each dietary condition. To test this, we fed germ-free and conventionalized animals with *Scenedesmus* algae and baker's yeast.

3.2 Materials and methods

Detailed methods for *Daphnia magna* husbandry can be found in Sison-Mangus et al. (2015) (Sison-Mangus et al. 2015). Briefly, genetically diverse diapausing eggs from a natural population of *D. magna* were decapsulated and surface-sterilized, allowed to develop in either sterile medium or medium supplemented with bacteria, and then were transferred into sterile medium and fed sterile food.

Experimental diets in this study were the green alga *Scenedesmus obliquus*, produced in a chemostat, and commercially available active dry baker's yeast re-activated in *Daphnia* medium (ADaM). Solutions of 14 mg/ml fresh weight algae or yeast per volume ADaM were prepared. Both diet suspensions were autoclaved, frozen for storage, and vortexed to re-suspend before feeding. We prepared bacteria-free and conventionalized animals by surface-sterilizing diapausing eggs as described in Sison-Mangus et al. (2015). Surface-sterilized eggs were placed singly in wells containing either sterile ADaM or ADaM supplemented with homogenized adult *Daphnia* (previously raised under standard laboratory conditions and fed with *Scenedesmus)* as a bacterial source. Plates were placed in an incubator at 20 °C for hatching with a light:dark cycle of 16:8 h.

Newly hatched neonates from both groups were each transferred into 1 ml sterile ADaM to dilute carryover of unattached bacteria in the conventionalized group, and then transferred into bottles (1 animal/bottle) containing 80 ml autoclaved ADaM as in the previous experiment. Animals were transferred into fresh sterile bottles immediately before feeding every 2–3 days in order to reduce the effect of uncontrolled bacterial proliferation due to uneaten food in the bottles. Each animal was fed 600, 650, 700, 800, 900, and 900 µl of the corresponding food solution on days 1, 4, 6, 9, 11, and 13 of the experiment, respectively, increasing the food amount as the animals grew in size. Eight random animals from each treatment combination were removed at 7 days old for body size measurement; these replicates were then removed from the experiment. The remaining animals (6–8 per treatment combination) were monitored for reproduction. The experiment was terminated on day 15.

Statistical analyses were carried out using the software package R (R Core Team n.d.). Log-transformed size data were analyzed by two-way analysis of variance (Table 1).

3.3 Results and discussion

Consistent with earlier studies (Sison-Mangus et al. 2015; Callens et al. 2015), germ-free animals were significantly smaller than conventionalized animals at day 7 ($p < 0.0001$). However, germ-free and conventionalized animals had opposite responses to the experimental diets, with a highly significant microbiota × diet interaction (Fig. 1, Table 1, $p = 0.001$). Conventionalized animals had better health as determined by size (Fig. 1) and fecundity on the algae diet than on the yeast diet. 5 out of 6 of the algae-fed conventionalized animals remaining in the experiment produced live neonates by two weeks old, while only 1 out of 5 of the remaining yeast-fed animals did; furthermore, the single reproducing yeast-fed individual produced only 2 neonates, while the reproducing algae-fed individuals produced on average 11.4 neonates (s.e.m. ± 4.1) (Kruskal–Wallis rank sum test including zeros: $p = 0.035$). The algae diet thus appeared to be superior for conventionalized hosts. In contrast, bacteria-free animals grew larger on the yeast than the algae diet (Fig. 1, Tukey's Honest Significant Difference: $p = 0.03$), but none of the bacteria-free animals reproduced.
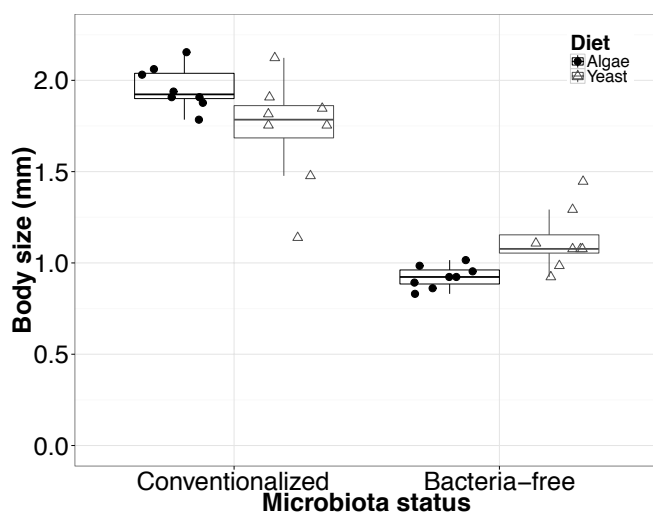
Figure 1. Body size of 7-day-old germ-free and conventionalized Daphnia fed algae or yeast. N=8 per treatment combination. Box plots show median and 25th and 75th percentile; whiskers represent points falling within 1.5x interquartile range.

Table 1. Analysis of variance results for log-transformed body size at day 7.

	Df	F value	Pr(>F)
Microbiota	1	169.833	2.08e-13
Diet	1	0.307	0.584
Microbiota: Diet	1	13.158	0.00113
Residuals	28		

The two diets likely differed in a number of parameters: particle size, cell wall composition, and macro- and micronutrient composition. The fact that their relative benefit for daphnids was reversed between bacteria-free and conventionalized animals

suggests that one of these diets is not inherently superior to the other, but rather that the limiting factor for *Daphnia* growth on each diet varies depending on the presence of microbiota.

A plausible explanation for our results, consistent with previous studies (Gorokhova et al. 2015; Callens et al. 2015), is that the benefits of an algal diet are only accessible to animals associated with bacteria, for example because bacteria are involved in specialized digestive processes. It may be particularly relevant that the source of the microbiota used in this experiment were algae-fed animals, in which the microbiota may have been pre-adapted to this diet. However, alternative explanations are also possible; for example, the yeast diet might encourage enhanced growth of harmful bacteria, leading to dysbiosis and decreased fitness in the conventionalized animals. Furthermore, the presence of bacteria in the gut or in the diet may change nutritional stoichiometry and thus energy allocation. Considering the long-term risks and challenges of maintaining interspecies associations, we might expect different effects of microbiota – including different degrees of dependency – in populations adapted to different dietary conditions. A related result has been found in *Artemia* specimens, which were able to survive at lower salt conditions than usual when fed on a yeast diet because they were no longer dependent on their salinity-sensitive gut bacteria (Nougué et al. 2015).

Our observation that germ-free animals were much smaller than the corresponding conventionalized animals on both diets (Tukey's Honest Significant Difference test: $p < 10^{-7}$ for algae diet; $p < 10^{-6}$ for yeast diet) indicates that the main beneficial effect of the microbiota is independent of the diets tested. This could be due to microbiota providing services such as metabolic waste recycling, or due to systemic effects on gut integrity, growth factor signaling, or nutrient allocation. We previously found that under some conditions, bacteria can have a positive effect on *Daphnia* even during embryonic development (Mushegian et al. 2016), suggesting that a multitude of processes may be sensitive to bacterial presence.

While our experiments used the extreme state of bacteria-free animals, the combination of diet-dependent and diet-independent effects suggests that not merely the presence of bacteria, but natural variation in the bacterial community may affect *Daphnia* phenotype and fitness, for example if different bacterial strains are responsible for different general and diet-specific effects. Further experiments using chemically defined diets and compositionally defined microbiota will be necessary to work out the mechanism responsible for these effects. Environmental context-sensitive benefits of symbiosis could point toward ways in which microbiota mediate local adaptation or phenotypic plasticity of their hosts.

3.4 Additional Data

The previously presented experiment on microbiota-specific effects of diets included the following key methodological steps: i) equalizing the diets by fresh weight ii) rinsing animals after conventionalization treatment iii) frequent transferring of animals into sterile medium to control the accumulation of excess food and proliferation of external bacteria iv) tracking animals until reproductive age. However, two earlier variants of the experiment lacking some of these steps also showed similar results of germ-free and conventionalized animals trending in opposite directions in a comparison of yeast and algae diets. In addition, an experiment comparing axenic fresh algae with autoclaved algae showed that the difference in quality between these two diets also depended on the presence of microbiota.

Trial 1: Algae and yeast diets

In this experiment, diets consisted of equal cell counts algae and yeast (100 million cells/ml), autoclaved and fed at 800 µl at the beginning of the experiment. Animals were not transferred into new media during this time, and body size was measured on day 6. Results are shown in Figure S1.
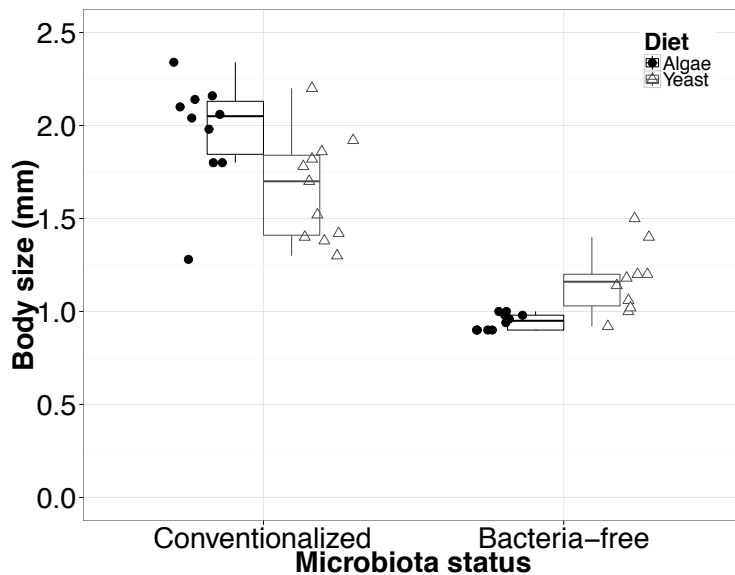
Figure S1.

Trial 2: Algae, yeast and Spirulina

This experiment was intended to test whether the cyanobacterium *Spirulina* sp, a nutritional supplement sometimes used by hobbyists in *Daphnia* husbandry, improves bacteria-free *Daphnia* health. Suspensions of 72 mg/ml food in ADaM were prepared; in diets containing Spirulina, it was present in an approximately 0.5x ratio to the algae or yeast. Suspensions were autoclaved and fed at volumes of 100, 100, and 200 µl on days 1, 3, and 5 of the experiment. Body size was measured on day 8 of the experiment. Results are shown in Figure S2.
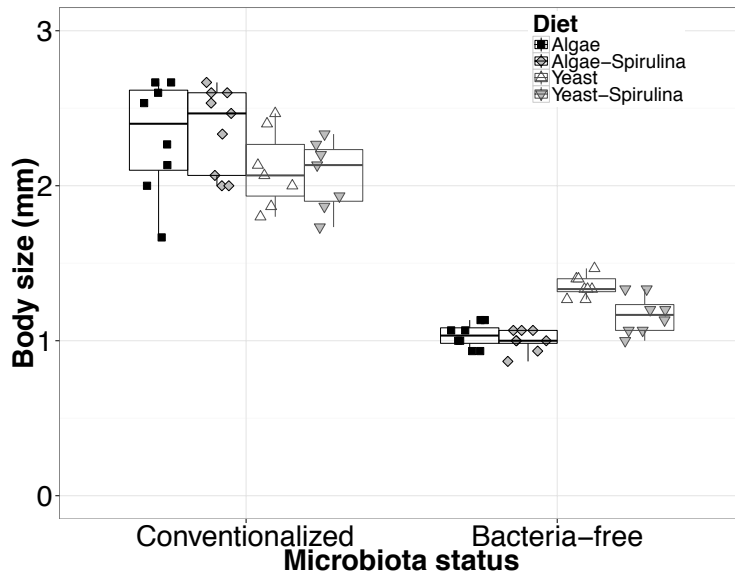
Figure S2.

Trial 3: Autoclaved and fresh axenic algae

    While the context-dependency of the effect of microbiota is itself of interest, it is still relevant to verify whether experimental conditions resulting from technical requirements are unusually stressful. Therefore, we tested whether autoclaved algae was a poor-quality food source compared to the fresh algae on which animals are typically reared in the lab. A culture of axenically produced algae (treated with antibiotics, then serially cultured in sterile media) was divided, and half was autoclaved. Equal volumes of these suspensions were fed to bacteria-free and conventionalized animals and body size was measured at 7 days old. Results are shown in Figure S3. Conventionalized animals grew larger on autoclaved algae, while axenic animals were equally small on both diets.

    It is worth noting that axenically grown fresh algae may also meaningfully differ from conventional algae. Bacteria-free algae appear to grow slower than expected, and bacterial strains such as *Variovorax* isolated from conventional laboratory algae are related to ones that have been shown to have growth-promoting effects on plants (Maignien et al. 2014). Nutrient limitation and other conditions can affect the thickness and digestibility of *Scenedesmus* cell walls (Van Donk et al. 1997). Therefore, it is likely that experiments with bacteria-free conditions will always have to have additional "unnatural" parameters.
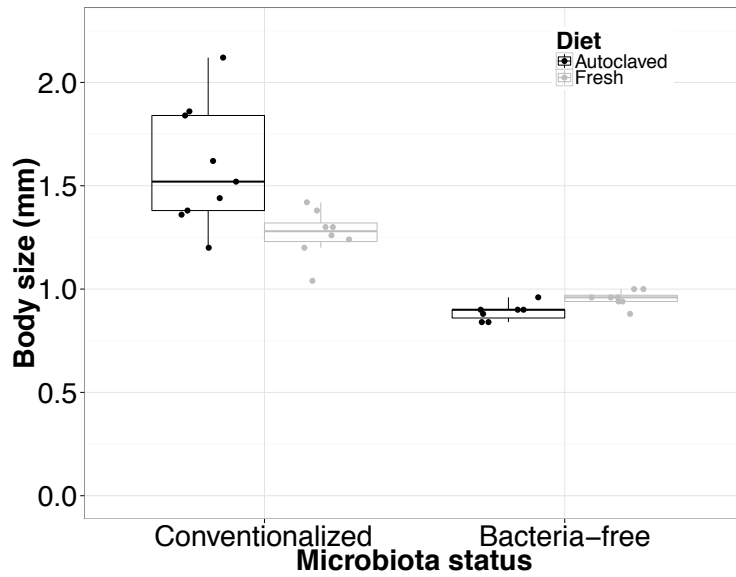
Figure S3.

From all of these observations, we conclude that the extent of the benefit conferred by microbiota can vary depending on diet, with presence of microbiota modifying *Daphnia* response to dietary differences.

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

Alexandra A. Mushegian, Elodie Burcklen, Tobias M.M. Schär, and Dieter Ebert

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.

4.1 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 (A. E. Douglas 2014b).

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. 2015). 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.

4.2 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.

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.

4.3 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).
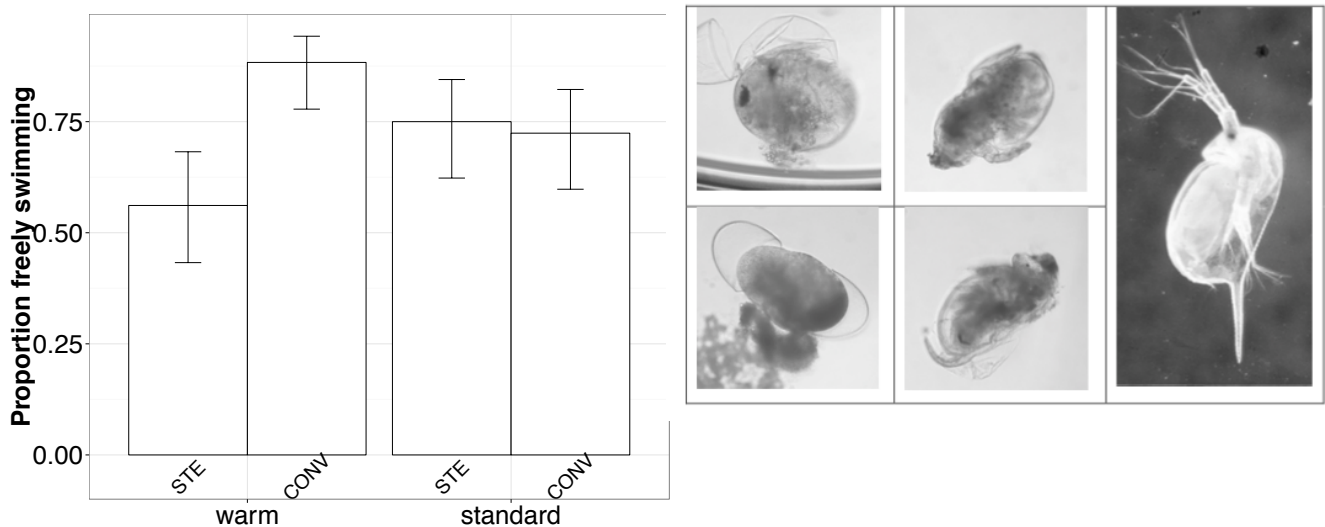
Figure 1. A (left). Proportions of resting eggs that reached a free-swimming state under warm and standard, bacteria-free (STE) and conventionalized (CONV) conditions. N=57 to 60 individuals in each treatment combination. Error bars represent 95% binomial confidence intervals. Odds ratio for CONV vs STE under warm conditions: 5.9. For logistic regression results see Table 1A. **B (right).** 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.
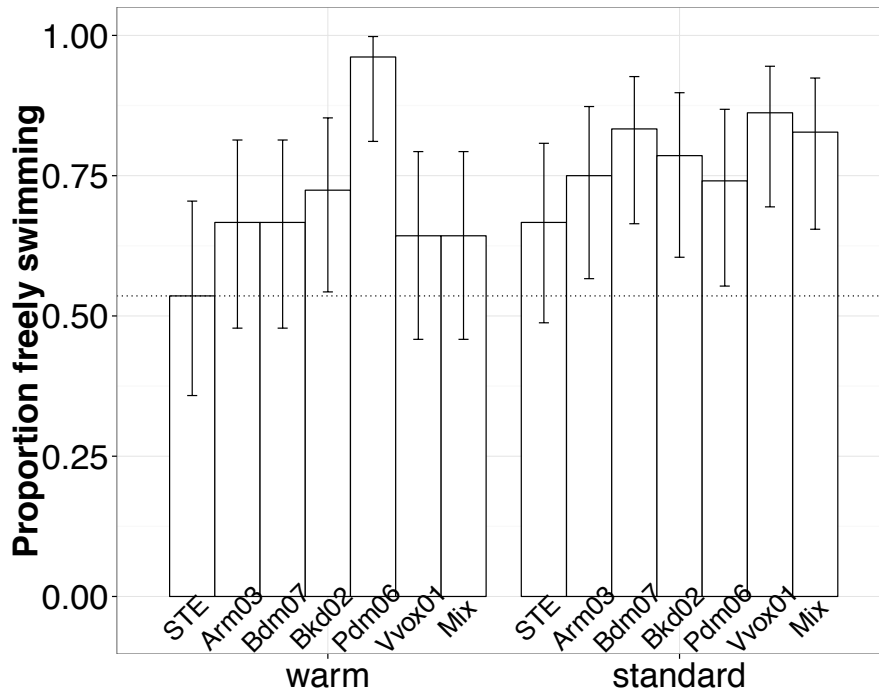
Figure 2. Proportions of resting eggs reaching a free-swimming state when exposed to different bacterial strains under warm and standard temperature conditions. STE=bacteria-free, Arm03=*Aeromonas sp*, Bdm07=*Brevundimonas sp*, Bkd02=*Burkholderiales sp*, Pdm06=*Pseudomonas sp*, Vvox01=*Variovorax sp*, 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.
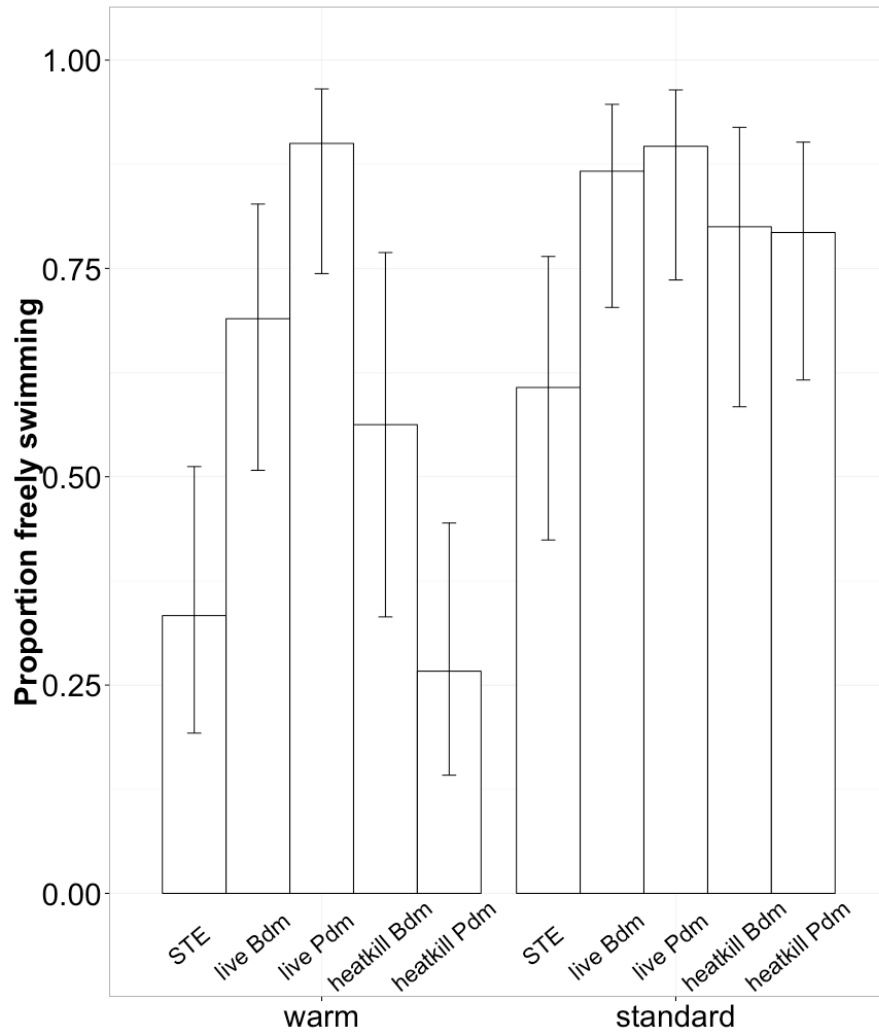
Figure 3. Proportions of resting eggs reaching a free-swimming state when exposed to live and heat-killed *Pseudomonas* (Pdm) and *Brevundimonas* (Bdm) under warm and standard temperature conditions. N=28 to 30 in each treatment combination except for heatkill Bdm/warm: n=16. Odds ratio for live Pseudeomonas vs. sterile under warm condition: 18. Error bars represent 95% binomial confidence intervals. For logistic regression results see Table 1C.
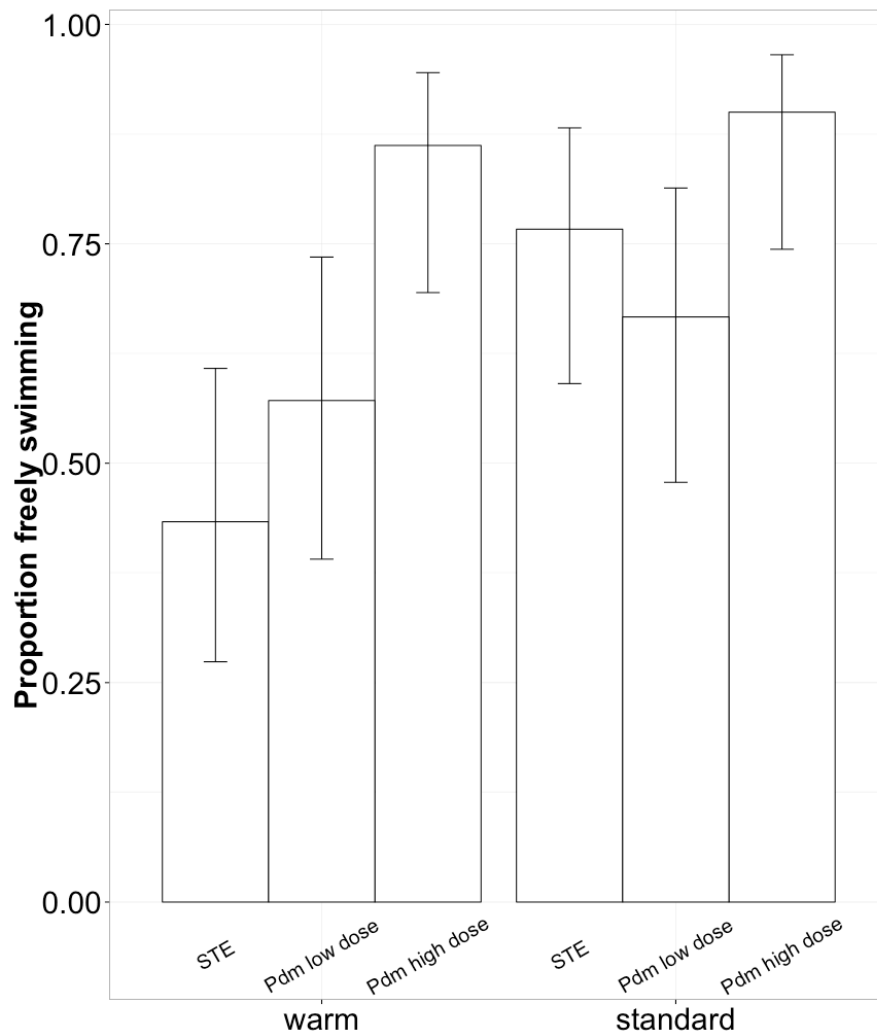
Figure 4. Proportions of resting eggs reaching a free-swimming state when exposed to different doses of *Pseudomonas* (Pdm) bacteria. N=27 to 30 in each treatment combination. Error bars represent 95% binomial confidence intervals. Odds ratio for Pseudomonas high dose vs. sterile: 8.22. For logistic 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.
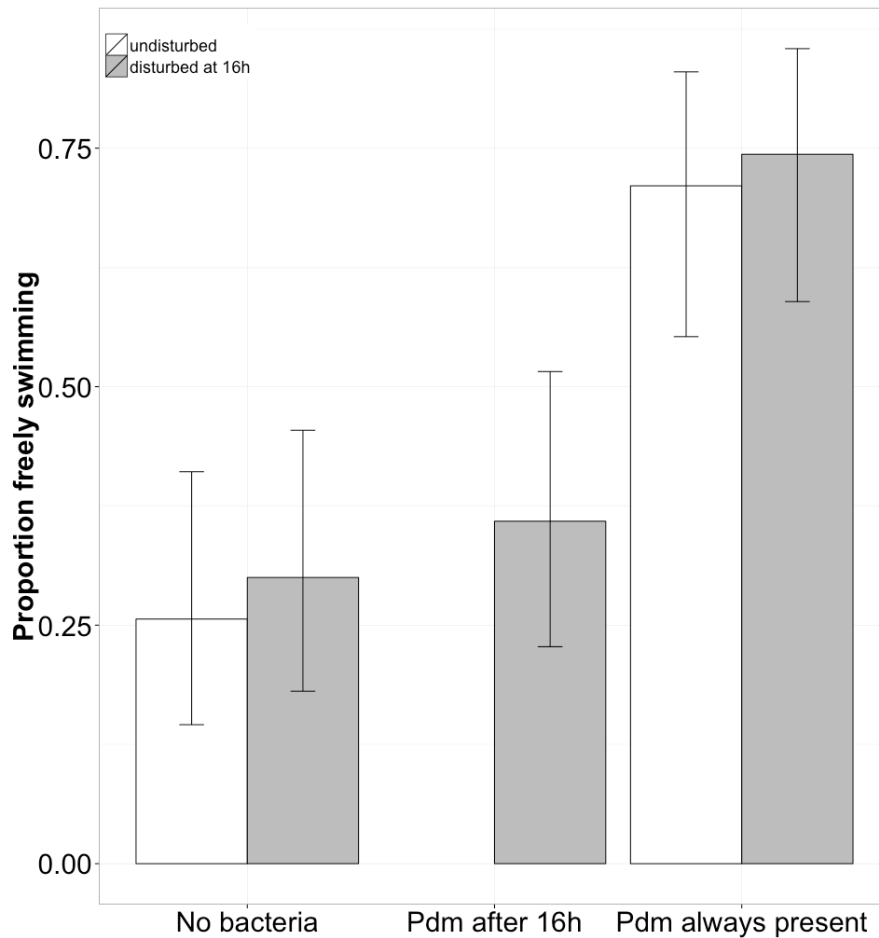
Figure 5. Comparison of successful development rates of eggs exposed to *Pseudomonas* from beginning of experiment or after 16 hours of bacteria-free development under warm conditions. Control treatments disturbed by pipetting at 16 hours are included. N=38 to 40 per treatment group. Error bars represent 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).
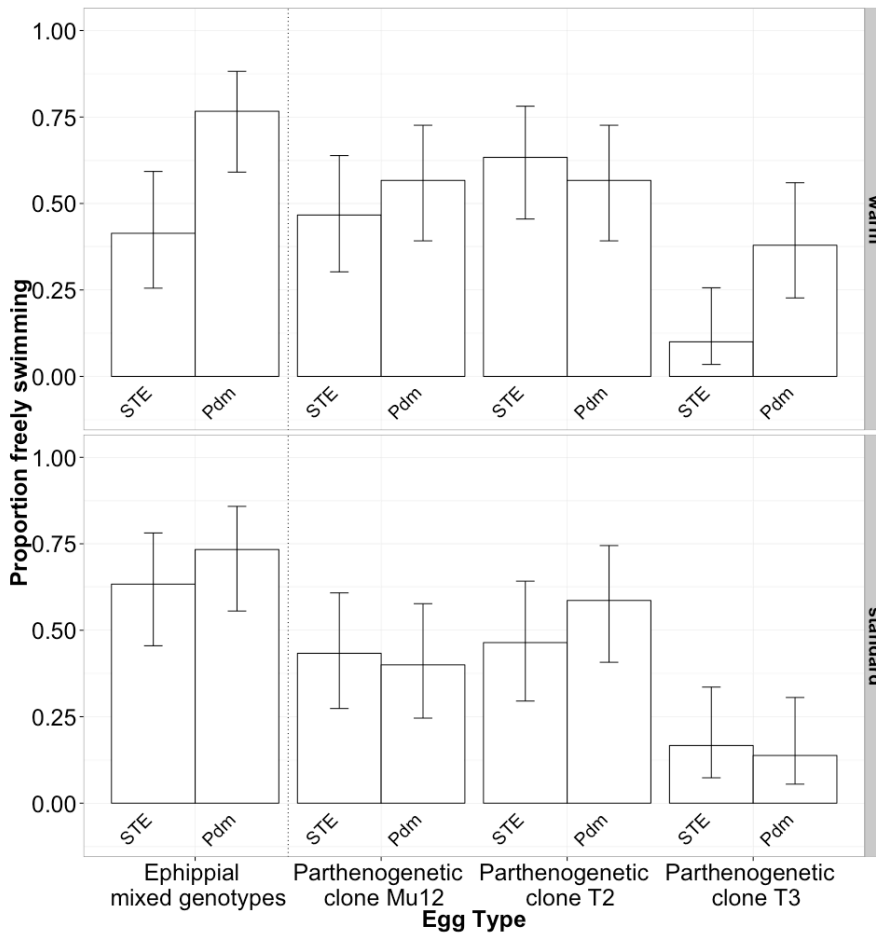
Figure 6. Effect of bacteria-free (STE) and Pseudomonas-exposed (Pdm) conditions on development at standard and warm temperature for directly developing parthenogenetic eggs of three different Daphnia clones as well as ephippial eggs. N=29 to 30 per treatment combination. Error bars represent 95% binomial 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

4.4 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.

4.5 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, no microscopy)	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. 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.

Stunted or missing appendages and setae; misshapen carapace and eye:
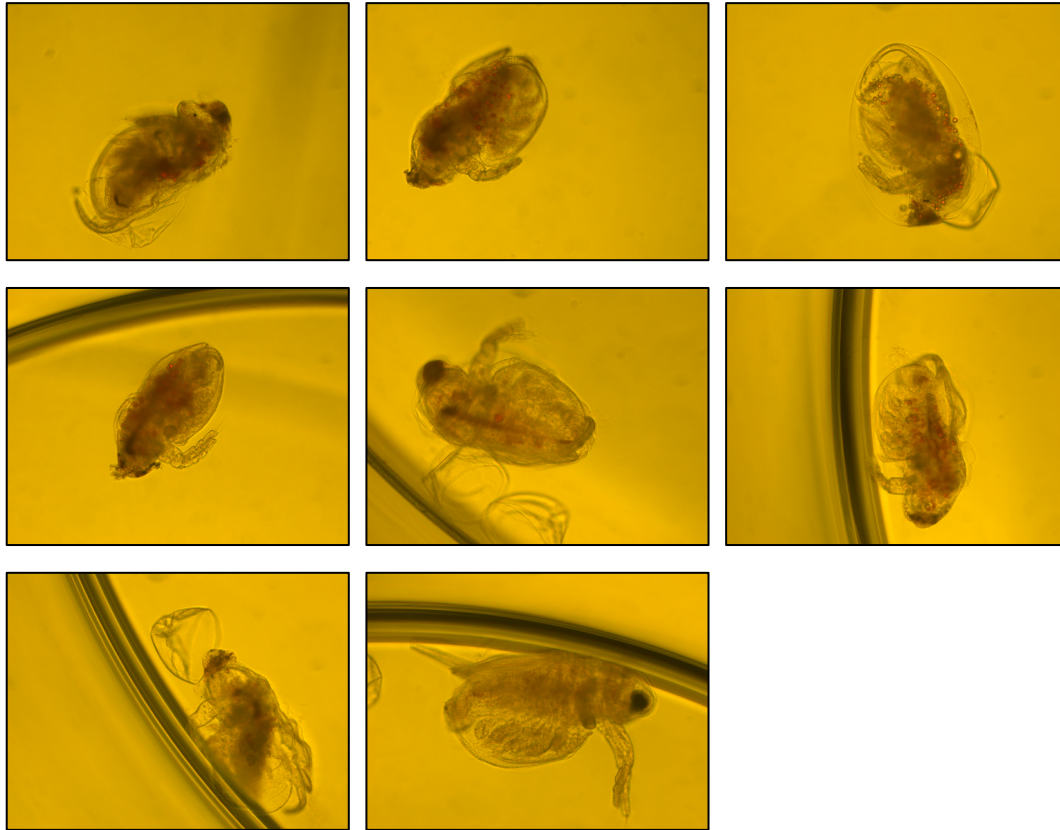
Eye formed but no segmentation or other morphological features; broken or "exploded" membranes:
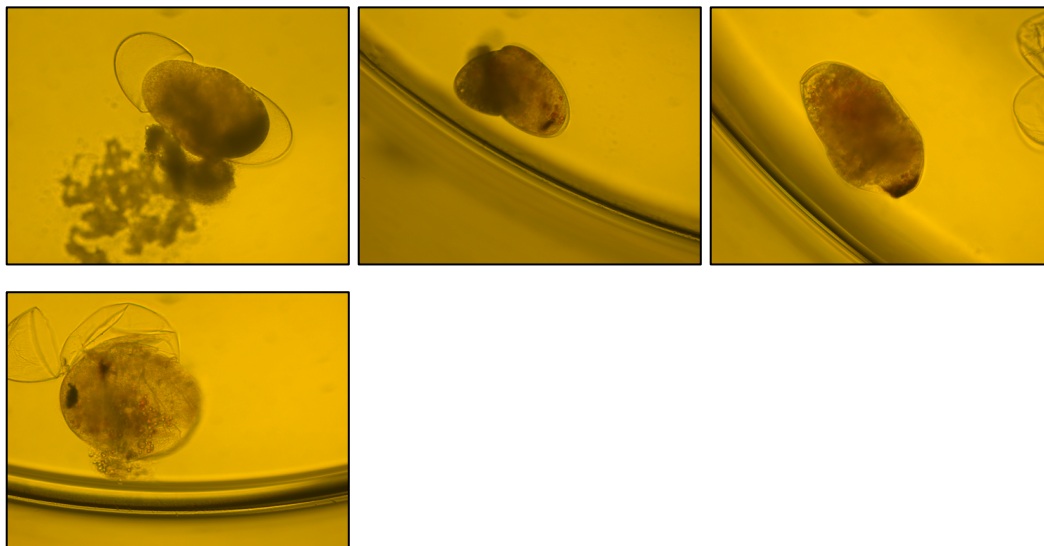
5. The microbiota of diapause: How host-microbe associations are formed after dormancy in an aquatic crustacean

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Abstract

1. A critical question in symbiosis research is where and how organisms obtain beneficial microbial symbionts in different ecological contexts. Microbiota of juveniles are often derived directly from their mother or from the immediate environment. The origin of beneficial symbionts, however, is less obvious in organisms with diapause and dispersal stages, such as plants with dormant seeds and animals in ephemeral or strongly seasonal habitats. In these cases, parents and offspring are separated in time and space, which may affect opportunities for both vertical and horizontal transmission of symbionts.

2. The planktonic crustacean *Daphnia* produces long-lasting resting eggs to endure winter freezing and summer droughts and requires microbiota for growth and reproduction. It is unknown how hatchlings from resting stages form associations with microbial consorts after diapause.

3. Using natural samples of *D. magna* resting eggs after several years of storage, we show that the total bacterial community derived from both the exterior and interior of the eggs' ephippial cases is sufficiently beneficial to ensure normal *Daphnia* functioning in otherwise bacteria-free conditions. We do not find direct evidence that the required bacteria are of maternal origin, though sequencing reveals that the resting stage is accompanied by bacterial taxa previously found in association with adult animals.

4. These findings suggest that while *Daphnia* are strongly dependent on environmental bacteria for normal functioning, host-bacteria associations are somewhat general and availability of specific bacteria is not a strong constraint on host ecology. Nevertheless, animals and microbes may be ecologically linked through co-dispersal.

5.1 Introduction

Eukaryotes serve as habitats for communities of microorganisms. Experimental studies show that these communities often provide important benefits to the organism hosting them (Moran 2007; A. E. Douglas 2014b). One of the most salient questions from an evolutionary and ecological standpoint is whether beneficial symbionts are acquired from an individual's parents (vertical transmission), or from the environment or unrelated individuals (horizontal transmission). Frequently, both modes of transmission are observed (Ebert 2013), and empirical studies have shown the presence of both vertically and horizontally transmitted symbionts (of the same and different species) within the same host (Salem et al. 2015; Ferrari & Vavre 2011). The relative importance of differently transmitted members of a diverse symbiont community to host performance has not been well quantified in any system and might depend a great deal on the ecology of the particular system, e.g. population density, social structure, behavior, or diet. Variation in these factors might affect the way a host organism interacts with potential sources of microbiota.

A distinctive ecological feature of many plants and animals is the presence of dormant or diapausing stages (e.g. seeds, cysts or resting eggs) that allow survival through inhospitable environmental conditions and often serve for dispersal to new habitats. While in many systems, some vertical transmission of microbiota can be assumed due to physical proximity of parents and offspring (Salem et al. 2015; Dethlefsen et al. 2007), this assumption may not hold in the same way for diapausing organisms, which can be extremely separated in both time and space from their parents (Hairston 1996). Microbial communities may be altered during diapause; furthermore, the spatial autocorrelation between host genotypes and pools of microbes available for horizontal acquisition can be weakened or lost. Therefore it is of particular interest to understand how an organism emerging from diapause establishes essential microbiota.

Beneficial microbes from the parent might persist in association with the resting stage, perhaps entering a dormant state themselves. Alternatively, the host organism might be required to find beneficial microbes in its new environment immediately after emerging from diapause. Both scenarios have been observed in plants: some plants transmit fungal symbionts into seeds, albeit with imperfect maintenance, while others form new associations after germination (Hodgson et al. 2014; Afkhami & Rudgers 2008; Rodriguez et al. 2009). The two scenarios have different implications. If the necessary beneficial microbiota are transmitted by the parent into the resting stage, then the microorganisms can be considered a form of parental effect or a source of heritable non-genetic variation in their host (Badyaev & Uller 2009; Zilber-Rosenberg & Rosenberg 2008). The occurrence of microbial transmission through resting stages would also support the often untested assumption that the microbial symbionts are deriving a benefit from the association (Mushegian & Ebert 2016), since host and symbiont reproduction would be linked across host generations. Where the host is dependent on acquiring beneficial microbes from the environment after dormancy, we would expect low taxon-specificity in the host-microbe association, because selection would disfavor reliance on specific microbes whose presence is not guaranteed in the environment where hatching occurs. To the extent that the host does have specific requirements of microbial associates, the availability of bacteria able to fulfill these requirements (or the availability of some other source of the services these symbionts provide) would then be a factor determining the suitability of a new habitat for colonization after dispersal (Pringle et al. 2009).

Dormancy is widespread among aquatic organisms living in temporary water bodies such as rock pools and bromeliad leaves, as it provides one of the only mechanisms for aquatic organisms to survive the disappearance of the habitat. In this study, we investigated which sources of bacteria were sufficient to ensure the normal functioning of a planktonic crustacean emerging from diapause. The water flea *Daphnia magna* produces long-lasting resting eggs in response to cues indicating deteriorating environmental conditions (e.g. changes in photoperiod or crowding). These eggs are enclosed in a case called an ephippium derived from the mother's carapace (Figure 1).

The eggs inside the ephippia can survive in pond sediments for up to hundreds of years, tolerating drying, hypoxia and various types of chemical stress, but often hatch as soon as the environmental conditions become favorable again, e.g. after the winter. (The resting stage is actually an embryo arrested in a ~1000-cell early gastrula stage (Baldass 1941), but we use the term "resting egg" following conventional practice. Note also that we use "egg" to refer to only the embryo, not to the entire ephippial structure; see Figure 1.)
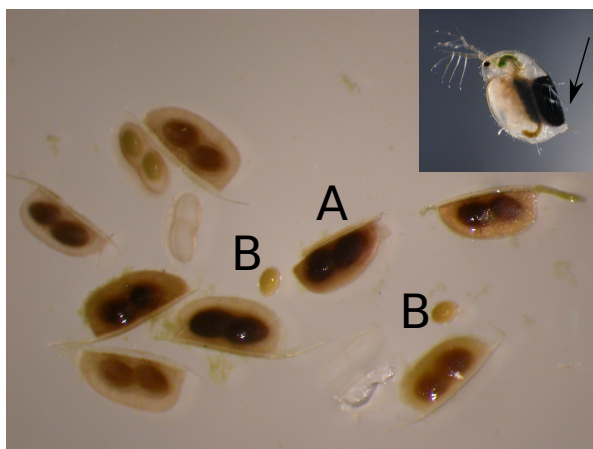
Figure 1. Inset: *D. magna* carrying ephippium (indicated with arrow). Ephippia typically house one or two resting eggs and are shed when mother molts. A) Shed ephippium. B) Decapsulated resting eggs.

Apart from occasional vertically transmitted microsporidian parasites (Sheikh-Jabbari et al. 2014), our previous studies did not detect any intracellular symbionts in resting eggs. We found a strong requirement for extracellular microbiota in *Daphnia* fitness (Sison-Mangus et al. 2015), but we did not explicitly investigate the relative importance of various plausible natural sources of microbiota in providing this benefit.

Here, we set out to answer three questions: i) Which, presumably vertically acquired, bacteria are present inside the closed ephippium? ii) Is this set of bacteria sufficient to ensure normal functioning of the animal after emergence from diapause? iii) If the bacteria inside the resting egg case are not sufficient, what additional bacteria are required? We focused on identifying the minimal amount of bacterial exposure that results in normal health of *Daphnia* hatchlings from resting eggs. To do so, we compared animals emerging into sterile environments from surface-sterilized ephippia to animals exposed to environments with different sources of bacteria immediately upon emergence. Our results show that animals whose only source of bacteria was from inside of their ephippium failed to grow normally or reproduce, whereas the additional presence of bacteria on the outside surface of the ephippial shell was sufficient for the restoration of normal performance. This suggests that, while an animal's external environment is a crucial source of beneficial bacteria, the bacterial component of the environment is readily found in association with the resting/dispersing stage.

5.2 Methods

The ephippia used in this study were collected in bulk sediment samples in a carp pond near Munich, Germany in 2009 (coordinates: 48.206630, 11.705730). They have since been stored under moist, dark conditions at 4 °C. For our experiments, we selected ephippia that appeared to be completely sealed by manually sorting through them and selecting those that did not open when gentle pressure was applied with a metal forceps to the round edge. Ephippia selected for the experiment were stored in 96-well tissue culture plates filled with ADaM and refrigerated until sufficient ephippia were collected for the experiment to be set up; no attempt was made to maintain aseptic conditions during the collection phase.

<u>Experiment 1</u>

Experiment 1 consisted of 6 overlapping treatments representing increasingly diverse and abundant sources of bacteria (summarized in Table 1). The STE (sterile) treatment consisted of eggs removed from ephippia (decapsulated; Figure 1) and directly treated with 5 % hypochlorite (household bleach) for 5 minutes, as in previous studies (Sison-Mangus et al. 2015; Retnaningdyah & Ebert 2016), then rinsed and placed in wells containing sterile artificial *Daphnia* medium (ADaM, (Ebert et al. 1998)). In the VRT (vertical) treatment, eggs were left inside ephippia, but ephippia were surface-sterilized with 1.25 % hypochlorite for 2 minutes and placed in sterile ADaM. This milder sterilization treatment was used to avoid the ephippium opening, but it nevertheless appeared to effectively remove bacteria from the outer surface of the ephippia while preserving those inside (see sections on sequencing). In the EPH (nonsterile ephippium) treatment, ephippia were not surface-sterilized but were rinsed by flushing with sterile water and placed in sterile ADaM. In the NST (non-sterile environment) treatment, water-rinsed ephippia were placed in nonsterile ADaM, which had been exposed to the laboratory environment for one week. In the OCC (previously occupied medium) treatment, water-rinsed ephippia were placed in ADaM that had been previously occupied by live *Daphnia* for a week. Finally, in the DAPH (*Daphnia* microbiota) treatment, water-rinsed ephippia were placed in wells supplemented with a homogenate of whole adult *Daphnia* with a normal microbiome (as in (Sison-Mangus et al. 2015)).

All procedures necessitating sterile conditions were carried out under a laminar flow hood. All ephippia were rinsed with sterile ADaM in one batch, then sequentially divided into six groups, which were then randomly assigned to treatments. Ephippia assigned to treatments EPH, NST, OCC and DAPH were placed individually into wells of 96-well tissue culture plates containing 200 µl of the corresponding hatching medium. Ephippia assigned to the VRT treatment were treated with 1.25 % hypochlorite (diluted household bleach) and rinsed by adding and removing sterile ADaM twice, then distributed to wells containing sterile ADaM. Ephippia assigned to the STE treatment

65

were briefly soaked in 5% hypochlorite to cause the ephippia to open; the eggs were then extracted and directly exposed to 5% hypochlorite for 5 minutes followed by washing 3 times. All of plates in which ephippia were hatched contained all of the treatments in randomly assigned rows. Plates were placed under a fluorescent lamp with daylight spectrum to induce hatching.

After approximately 60 hours, a timepoint at which 17-31% of ephippia or eggs had hatched in each treatment group, 15-20 randomly selected newly emerged neonates from each treatment group were transferred from hatching plates into individual sterile rearing jars containing 80 mL autoclaved ADaM and previously shaken to aerate. Jars were kept at 20 °C with a 16:8 hour light:dark cycle. Each jar was fed 8.75, 6.13, 8.75, and 25 million cells of sterile *Scenedesmus obliquus* algae on the first, third, sixth, and 14th day respectively, produced by treatment with antibiotics according to the protocol in our previous study (Sison-Mangus et al. 2015) and kept frozen until use. In addition, animals were fed autoclaved algae due to insufficient supply of antibiotic-treated algae. A solution of 173 million algae cells/mL was autoclaved and kept frozen; on days 10, 12, and 19 jars were fed 50, 50, and 150 μl of this solution respectively. At all times, there were excess algae present in all jars, meaning daphnids were feeding essentially *ad libitum*. Jars were gently shaken daily to resuspend food particles that settled at the bottom, and neonates produced by the experimental animals were removed. Mortality and fecundity were monitored for 22 days, and body size of the surviving individuals was measured on the last day.

At five days old, three individuals from each treatment were used to estimate abundance of culturable bacteria. In the STE treatment group, where mortality was high, the animals that were used for culturing were ones that had died on that day; in all other groups live animals were collected. Each individual was transferred to an Eppendorf tube, washed with 500 μl ADaM, and homogenized with a plastic pestle in 320 μl TE buffer. From each individual, two samples of 30 μl each were plated on R2A medium plates and placed at 30 °C. As controls, 30 μl of TE buffer and 30 μl of ADaM from a jar containing sterile ADaM and food, but no animals, were also plated. Two to three additional animals per treatment were removed at this stage with the intent to be used for sequencing, but were not ultimately used for this purpose due to poor overall DNA yield.

Experiment 2

In Experiment 1, the greatest increase in fitness compared to bacteria-free animals occurred when animals were exposed to unsterilized ephippia. To confirm this, and to eliminate the possibility that the chemical sterilization treatment itself contributed to the fitness loss, we performed Experiment 2 (summarized in Table 2). Here, all eggs used were decapsulated and surface-sterilized, and placed in either sterile ADaM or ADaM supplemented with bacteria. The bacteria used were either from a suspension of whole ephippia crushed in ADaM, or one of two bacterial isolates:

Acidovorax sp isolate ei77 (betaproteobacteria, Comamonadaceae, Genbank accession number MF138148) or *Arthrobacter sp* isolate ei2 (Actinobacteria, Micrococcaceae, Genbank accession number MF138147), previously isolated from neonates newly emerged from ephippia and identified by Sanger sequencing of 16S rDNA. Another treatment consisted of a mixture of *Acidovorax* and *Arthrobacter*. In parallel, decapsulated eggs were washed with sterile water and placed in sterile ADaM. All eggs were decapsulated manually using forceps under a dissecting microscope and surface-sterilized with 5% hypochlorite for 5 minutes, followed by three rinses with sterile water. Untreated control eggs were only washed with water. Eggs were haphazardly distributed to individual wells of flat-bottomed 96-well tissue culture plates containing 180 µl sterile ADaM, and rows of wells were randomly assigned different bacterial treatments. To each well, 25 µl of the corresponding bacterial suspension (or 25 µl sterile ADaM in germ-free treatments) was added.

The bacterial treatments were prepared as follows: *Acidovorax* and *Arthrobacter* cultures were grown in 6 mL liquid LB medium at 30 °C with shaking for three days. Culture medium was removed by decanting after centrifugation and bacteria were resuspended in sterile ADaM and diluted to an OD600 of 0.2. The ephippial bacterial treatment was prepared by collecting approximately 200 ephippia from a sediment sample, rinsing them with sterile water, and crushing them with a plastic pestle in 1.8 mL sterile ADaM. The resulting supernatant was used as the bacterial suspension. The numbers of bacterial cells in each treatment were not quantified, but 25 µl samples of each bacterial suspension were plated on LB agar and incubated at 28 °C to confirm that viable bacteria were present. The suspension from crushed ephippia showed a notable diversity of colony morphologies present, as would be expected from a typical soil sample (Figure S1).

A total of six plates of eggs were produced, with 9-10 eggs in each treatment in every plate. To test the effect of these bacteria on a previously studied microbiota-sensitive trait, hatching success under elevated temperature conditions (Mushegian et al. 2016), plates were placed on a device we constructed that cooled one half of each plate to 20 °C and kept the other half at 26-27 °C. We counted the number of freely swimming neonates visible to the naked eye (while blinded to the bacterial treatment) three and four days later and report the total proportion observed across these two observation points. On the fourth day, we randomly selected 12 neonates from each bacterial treatment, from the cool hatching condition only, to continue the experiment. Each neonate was first transferred into a tube containing 800 µl of sterile ADaM to dilute carryover of unattached bacteria. Then each neonate was transferred into a sterile rearing jar as in the previous experiment. Food in this experiment was prepared by autoclaving and freezing a solution of *Scenedesmus* algae containing 100 million cells/ml. Animals received 300, 100, 200, 200 and 300 µl of this solution on the first, second, sixth, ninth, and eleventh day of the experiment. On day 11, when many individuals had released offspring, all experimental animals were transferred into new

sterile jars before feeding. Mortality and fecundity were monitored as described before. The experiment was terminated on day 15 and surviving animals were measured.

<u>Characterizing ephippia-associated microbiota</u>

The bacterial community associated with ephippia was characterized by high-throughput sequencing of amplicons of the V3-V4 variable region of the 16S ribosomal RNA gene on the Illumina MiSeq platform (Caporaso et al. 2012). We prepared surface-sterilized and water-rinsed ephippia (n=12 each) in the same manner as in experiment 1. As controls to demonstrate the efficacy of surface-sterilization and the preservation of bacterial DNA inside closed, surface-sterilized ephippia, we also included ephippia that had been opened and sterilized on both the inner and outer surface, as well as opened, untreated ephippia. After treatment, ephippia were frozen at -20 °C for several days to improve DNA extraction efficacy. Total genomic DNA was extracted from individual ephippia using the MoBio PowerSoil DNA extraction kit. We amplified the V3 variable region of the bacterial 16S ribosomal RNA gene with primers 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGA-3') and 785R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGA-3') with Illumina adapter sequences and 0-3 bp random frameshifts, using NEBNext High Fidelity PCR Master Mix (New England Biolabs catalog#M0541L) for 28 cycles. For each sample, four 12.5-µl PCR reactions were performed and pooled. PCR product was purified twice with Ampure beads, amplified for 8 cycles with Nextera XT v2 indexing primers, and purified again. Libraries were normalized, pooled, and sequenced on the Illumina MiSeq (reagent kit v3, 300 bp paired-end reads).

Raw reads were quality controlled with FastQC v.0.11.4 (Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc, Babraham Institute, UK). Paired reads were merged (FLASH v1.2.9), primers trimmed (Cutadapt v1.5), and quality filtered (PRINSEQ-lite v0.20.4). OTU clustering including abundance sorting and chimera removal was performed using the UPARSE workflow (Edgar 2013). Only those OTUs represented by 5 or more reads in the global dataset were included. Taxonomic assignment was performed using UTAX against the Greengenes v13/5 database. Further analyses were performed using the software package R 3.1.3 (R Core Team n.d.), the Bioconductor library phyloseq 1.14.0 (McMurdie & Holmes 2013), vegan (Oksanen 2013), and ggplot2 (Wickham 2009).

Since individual ephippia contain very low bacterial biomass, we paid special attention to the issue of reagent contamination with bacterial DNA (Salter et al. 2014). We divided samples from all treatments evenly amongst two DNA extraction batches and performed PCRs all in the same batch. We sequenced four reagent-only negative controls from the DNA extraction step (two per day on which extractions were performed) as well as a reagent-only control from the PCR step. Negative controls clustered separately from samples in NMDS ordination based on Bray-Curtis sequences

(Figure S2). Bacterial sequences found in the five blanks were regarded as possible contaminants, and OTUs from which more than 12 reads total were found amongst all the blanks were excluded from the sample data set. This criterion may be too stringent, since contaminating bacterial DNA may be very similar on a 16S sequence level to genuinely common environmental bacteria; for example, we eliminated several *Pseudomonas sp* OTUs from the data set based on their prevalence in negative controls, despite being able to culture *Pseudomonas* from the ephippia and ex-ephippial animals. (*Pseudomonas* is a large genus in which lack of resolution based on 16S sequences is known (Ait Tayeb et al. 2005)). Nevertheless, sequences belonging to the excluded OTUs made up on average 5.6 % of sample reads and no more than 16.5 % in any of the samples. ADONIS analysis using Bray-Curtis distances showed that there was no significant difference in community composition between batches either before or after removing contaminant OTUs ($p >0.6$ for all).

We also compared opened, surface-sterilized ephippia (sterilization controls) with the extraction blanks to see if any bacterial taxa from the ephippial surface were not completely removed by the surface-sterilization treatment. The sterilization controls clustered with extraction blanks in NMDS ordination (Figure S2). DESeq analysis (see below) did not reveal any taxa that were significantly (adjusted p-value <0.05) overrepresented in sterilization controls compared to DNA extraction controls, so we assume that incomplete surface-sterilization of closed ephippia does not affect the interpretation of our results.

We used the implementation of DESeq2 v1.10.1 (Anders & Huber 2010) in phyloseq to normalize counts and identify bacterial taxa that were significantly (adjusted p-value <0.01) overrepresented in "natural" ephippia as opposed to surface-sterilized ones (McMurdie & Holmes 2014); this list represents one set of candidates for bacterial taxa possibly responsible for major *Daphnia* fitness effects. For a conservative estimate of OTUs that were present in a majority of ephippia, we rarefied communities to an equal sampling depth of 26307 reads (sampling without replacement; seed = 10; one sample with less than 4000 reads was excluded from this analysis) and listed those OTUs that were present at more than one read in 50% or more of surface-sterilized ephippia. (These were also present in over half of natural ephippia, as expected, except for OTU#14, which was found in 4/11 natural ephippia). These sets of candidates were compared with a subset of a previous microbiota sequencing dataset of long-term laboratory-reared *Daphnia* (Sullam et al. 2017). Representative OTU sequences from our study and the previous study were aligned with PyNAST (Caporaso et al. 2010) against the Greengenes reference set implemented in MacQIIME v. 1.9.1 and then filtered using the Greengenes lanemask. A tree was constructed using RaXML (v8.1.21) from the filtered alignment and an archaeon, *Methanobrevibacter smithii* (Genbank accession # CP000678) was used to root the tree.

Table 1.
Treatments in Experiment 1.

Treatment	Ephippium	Hatching medium	Assumed bacterial sources	Culturable bacteria recovered from three 5-day-old animals*
STE	Ephippium removed; eggs surface-sterilized	Sterile	none	0, 0, 0
VRT	Ephippium surface-sterilized	Sterile	Inside ephippium (presumed maternal origin)	+, +, 0
EPH	Ephippium washed with water	Sterile	Inside + outside ephippium	+++, +, ++
NST	Ephippium washed with water	Nonsterile medium (open container in lab)	Inside + outside ephippium + general laboratory environment	+, +, +
OCC	Ephippium washed with water	Nonsterile medium previously occupied by *Daphnia*	Inside + outside ephippium + general laboratory environment + released by *Daphnia*	+, +++, +
DAPH	Ephippium washed with water	Supplemented with homogenized *Daphnia*	All above + all *Daphnia*-associated bacteria	+, +, +

*Symbols represent colony abundance categories, from the average of two technical replicate plates for each individual, with results from individual animals separated by commas. 0: <1 colony; +: 1-150 colonies; ++: 151-300 colonies; +++: >300 colonies

Table 2.
Treatments in Experiment 2.

Treatment	Embryo treatment	Hatching medium	Culturable bacteria recovered from two 5-day-old animals
Untreated (untr)	Decapsulated, washed with water	Sterile	no
Bacteria-free (STE)	Decapsulated, surface-sterilized	Sterile	no
Acidovorax (Avx)	Decapsulated, surface-sterilized	Supplemented with *Acidovorax*	yes
Arthrobacter (Arb)	Decapsulated, surface-sterilized	Supplemented with *Arthrobacter*	no
Mixture (Avx+Arb)	Decapsulated, surface-sterilized	Supplemented with *Acidovorax* + *Arthrobacter* mixture	yes
Ephippial bacteria (EphBac)	Decapsulated, surface-sterilized	Supplemented with supernatant of crushed ephippia	yes

5.3 Results

In Experiment 1, totally germ-free animals (STE) had the highest mortality, consistently with previous experiments (Sison-Mangus et al. 2015; Callens et al. 2015); all had died within a week. Animals from surface-sterilized ephippia (VRT) had higher survival but only one individual had reproduced by 21 days old (normally one would expect the first brood around 8 – 12 days), and animals were significantly smaller than the remaining treatment groups (Figure 2; Table 3A).
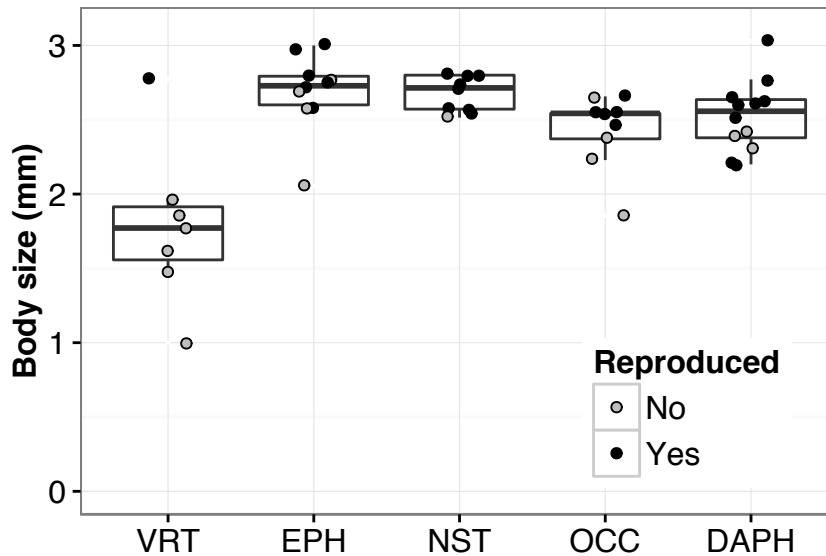
Figure 2. Experiment 1: Size and reproductive success of animals surviving to 21 days after treatment with different bacterial sources. STE=Sterile, VRT=Vertical, EPH=nonsterile ephippia, NST=Nonsterile medium, OCC=Daphnia-occupied medium, DAPH=Daphnia microbiota; see table 1 for detailed description of treatments. None of the STE animals were alive at this point. Average number of free-swimming offspring of individuals who reproduce: VRT=6; EPH=9.7, s.e. 2.7; NST=5.1, s.e. 0.85; OCC=5.6, s.e. 1.5; DAPH=7.3, s.e. 3.1. Analysis of variance of size data: Treatment p=1.22e-6. Tukey's Honest Significant Difference test reveals significant difference (p<.001) between VRT and all other groups; no other contrasts are significant.

The remaining treatment groups, which all had in common the presence of bacteria on the outer surface of the ephippium from which they emerged, had similar rates of growth, survival and reproduction (analysis of variance on fecundity data including zeros: df=4, F=0.96, p = 0.44). Culturing bacteria from homogenized five-day-old animals revealed considerable variation in the number of culturable bacteria present between individuals, but no strong pattern of differences in abundance between nonsterile treatments (Table 1). Dead germ-free animals were confirmed to have no culturable bacteria present, except for one fungus-like colony on one technical replicate plate. In the VRT treatment group, one out of the three biological replicates had zero culturable bacteria present. We also plated a sample of the culture medium ADaM from an empty (animal-free) bottle to which we had added algal food in parallel to the

experimental bottles as a control. This bottle contained a high concentration of a bacterium identified by Sanger sequencing of 16S rDNA as similar to *Methylobacterium radiotolerans,* a common airborne contaminant. Since this was not detected in the bacteria-free animal samples, we think it is likely that this bacterium represents chance contamination of the single control bottle.

Table 3. Analysis of variance results for size data

A. Experiment 1 (Figure 2)

	Df	F value	Pr(>F)
Treatment	4	12.12	1.22e-06 ***
Residuals	42		

B. Experiment 2 (Figure 4; STE and Untreated individuals excluded)

	Df	F value	Pr(>F)
Treatment	3	41.08	5.21e-10 ***
Residuals	26		

To confirm that ephippia-associated bacteria were sufficient for restoration of a normal microbiota-carrying phenotype, we performed a second experiment (Table 2). The overall performance of all groups in this experiment was better than in Experiment 1 (animals in Experiment 1 took a week longer to achieve comparable body sizes and reproductive output to those observed in Experiment 2), but the patterns of differences between treatments were consistent with the first (Figure 4; Table 3B), with the total bacteria-associated community restoring normal daphnid functioning. In Experiment 2, we standardized the treatment of eggs by decapsulating and surface-sterilizing eggs in all treatments, and then exposed them to a suspension obtained by crushing ephippia or to pure cultures of two bacterial strains isolated from ex-ephippial neonates. These strains were identified by Sanger sequencing as *Arthrobacter sp*, which matched one of the taxa overrepresented on the outside of ephippia (100% sequence match to OTU#19), and *Acidovorax sp*, which was present in a majority of surface-sterilized ephippia (97.4% sequence match to OTU#27, though the representative OTU from the Illumina sequencing was classified as *Rhodoferax*) (see Table 5). Both of these strains also had >98% sequence identity with strains that had previously been isolated in culture from lab- or field-collected *Daphnia* (unpublished data). We also included an "untreated" group in which eggs were decapsulated but not surface-sterilized or re-exposed, only rinsed with sterile water and hatched in sterile medium. The germ-free and untreated animals had similarly low fitness, failing to reach reproductive age, as in our previous study (Sison-Mangus et al. 2015). There were also no culturable bacteria recoverable from the untreated animals at 5 days old on either LB or R2A medium (Table 2). Exposure to *Acidovorax* improved survival and growth over germ-free conditions, but the animals exposed to this bacterium either singly or in combination with *Arthrobacter* were ~30 % smaller than animals exposed to ephippial bacteria, and only two individuals (both in the *Acidovorax + Arthrobacter* exposed group) had produced eggs

(but no neonates) by the end of the experiment. Exposing animals to the suspension of bacteria from whole ephippia restored fitness, including improving rates of hatching from ephippia (Figure 3; Table 4). All animals except for one individual had produced first clutch eggs by 9 days old and had released one or two clutches of live neonates by the time the experiment was terminated at day 14.
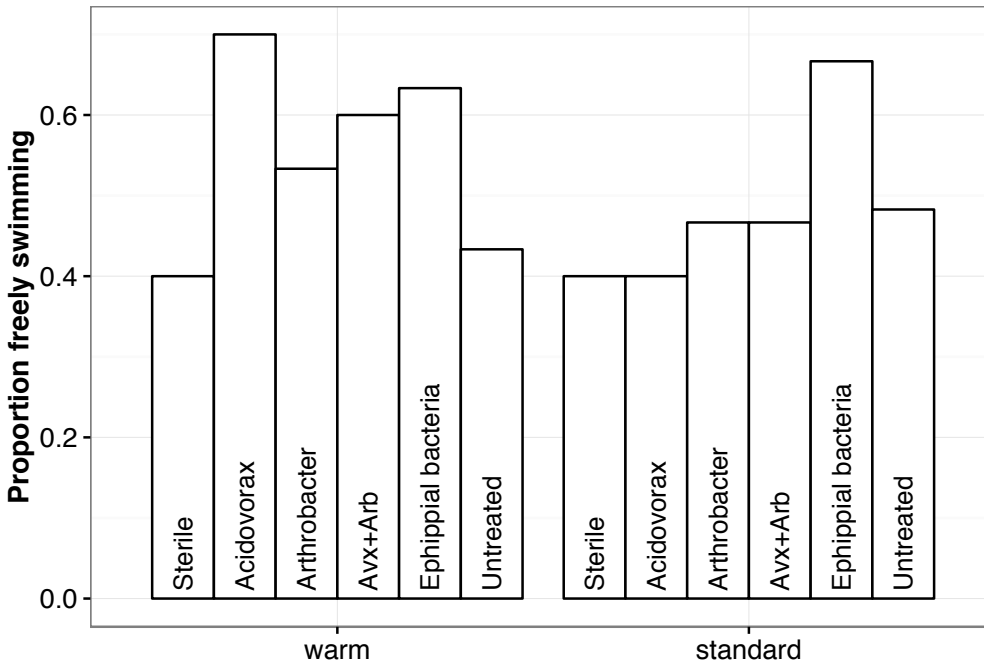
Figure 3. Experiment 2: Effects of ephippial bacteria on hatching of resting eggs at standard and elevated temperature. N=29 to 30 per treatment combination. For statistical analysis see Table 4.

Table 4. Logistic regression coefficients for hatching data of experiment 2 (Sterile, warm reference levels):

Coefficients:

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-0.2639	0.2840	-0.929	0.35274
Acidovorax	0.6092	0.3708	1.643	0.10038
Arthrobacter	0.4075	0.3699	1.102	0.27055
Avx+Arb	0.5418	0.3703	1.463	0.14345
Ephippial bacteria	1.0297	0.3788	2.719	0.00656 **
Untreated	0.2343	0.3721	0.630	0.52884
Cool temp	-0.2873	0.2144	-1.340	0.18031

We focused our sequencing efforts on surface-sterilized versus "natural" ephippia and provide two lists of candidate taxa for future experiments: those consistently found across a majority of ephippia even after surface-sterilization, and those over-represented on the outer surface (Figure 5; Table 5). Both lists contain a number of common soil bacterial genera, such as Acidobacteria, as well as taxa previously found at high abundances in adult *Daphnia* (Figure 5; Qi et al. 2009), such as various strains of the Comamonadaceae family.
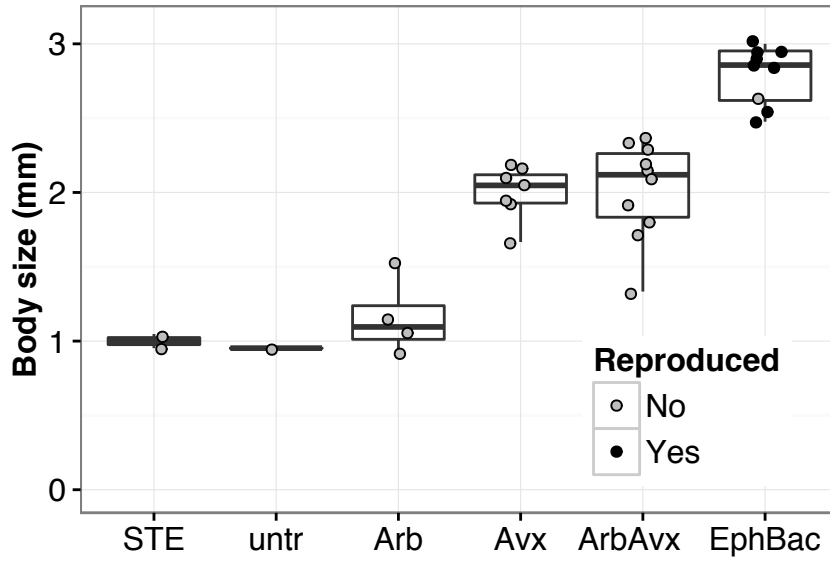
Figure 4. Experiment 2: Size and reproductive success of animals surviving to 14 days, treated with different bacterial sources. STE=Sterile, untr=untreated, Arb=*Arthrobacter,* Avx=*Acidovorax,* Arb+Avx=*Arthrobacter* and *Acidovorax* mixture, EphBac=suspension of crushed ephippia. Average number of free-swimming offspring of reproducing individuals in group treated with ephippial bacteria: 10.38, s.e. 1.33. Analysis of variance of size data was performed with sterile and untreated individuals excluded; Treatment p=5.2e-10.
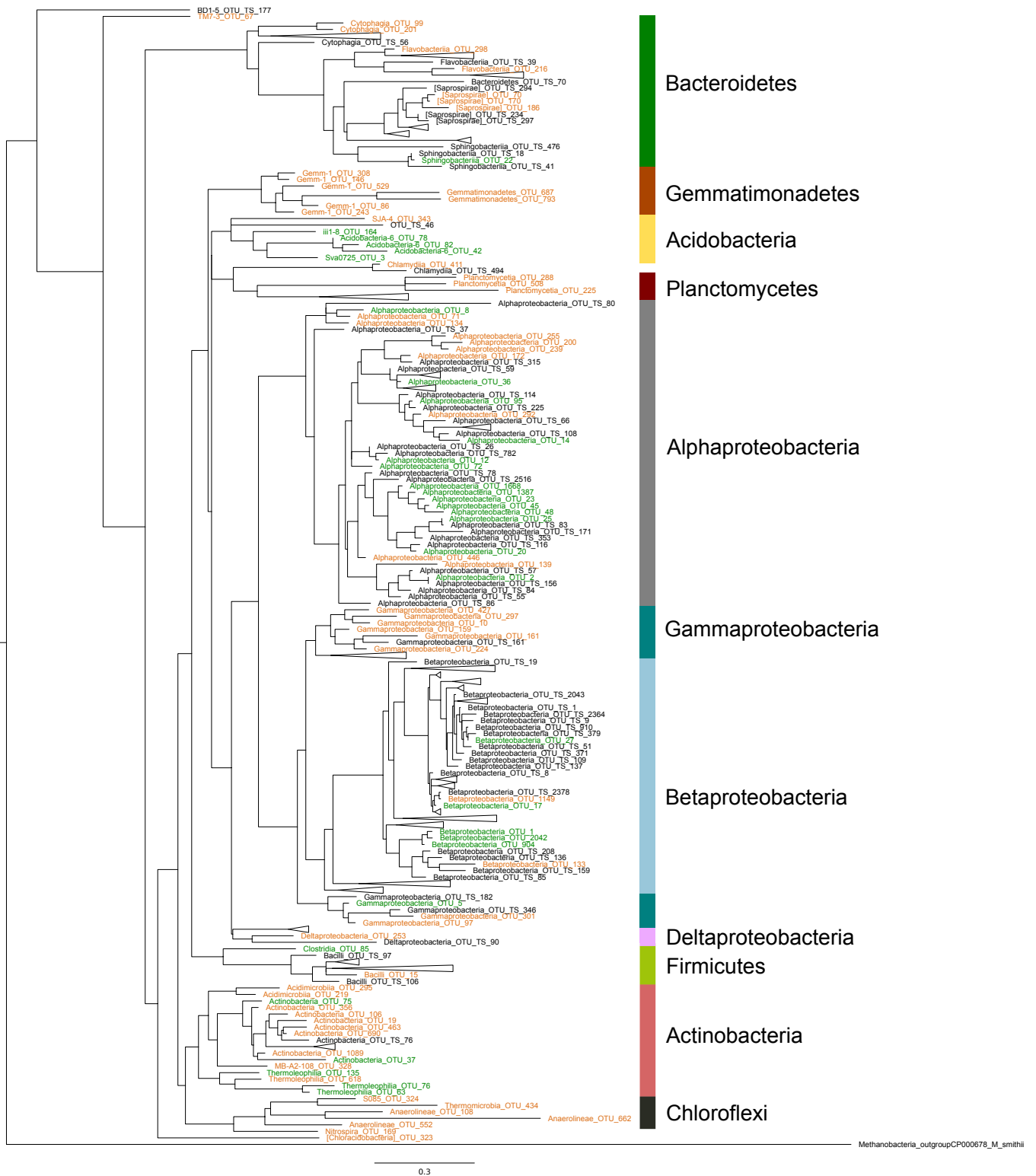
Figure 5. Relationships of ephippia-associated bacteria to bacteria found in lab-reared adult *Daphnia* in a previous study (OTUs shown in black font). Green text represents OTUs from surface-sterilized ephippia (Table 5A) while orange text represents strains overrepresented on outer ephippial surfaces (Table 5B).

Table 5. Sequencing results. Consensus 16S sequences for these lists of candidate taxa can be found in the Dryad depository https://doi.org/10.5061/dryad.c57t1.

A) Bacterial OTUs (defined by 97% sequence similarity) detected in at least half of surface-sterilized ephippia. All listed taxa were assigned with a confidence level of at least 0.9 to GreenGenes taxonomy; lower taxonomic levels that could not be assigned with this level of confidence were omitted.

Phylum	Class	Order	Family	Genus	OTU IDs
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Mycoplana	OTU_2
	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae		OTU_20
	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Rhodoplanes	OTU_23,OTU_45,OTU_4, OTU_1387
	Alphaproteobacteria	Rhizobiales	Rhizobiaceae		OTU_12
	Alphaproteobacteria	Rhizobiales			OTU_72,OTU_25
	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae		OTU_8
	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	OTU_95
	Alphaproteobacteria	Sphingomonadales			OTU_14
	Alphaproteobacteria				OTU_1668
	Betaproteobacteria	Burkholderiales	Comamonadaceae	Rhodoferax	OTU_27
	Betaproteobacteria	Burkholderiales	Comamonadaceae		OTU_17
	Betaproteobacteria	Hydrogenophilales	Hydrogenophilaceae	Thiobacillus	OTU_1,OTU_2042, OTU_904
	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Lysobacter	OTU_5
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae		OTU_22
Actinobacteria	Actinobacteria	Actinomycetales	Nocardioidaceae		OTU_37
	Thermoleophilia	Gaiellales	Gaiellaceae		OTU_135
	Thermoleophilia	Solirubrobacterales			OTU_76,OTU_63
Acidobacteria	Acidobacteria-6	iii1-15			OTU_42,OTU_82,OTU_78
	Sva0725	Sva0725			OTU_3

B) Bacterial OTUs overrepresented on outside surfaces of ephippia, as determined by DESeq analysis of surface-sterilized versus natural ephippia. All listed taxa were assigned with a confidence level of at least 0.9; lower taxonomic levels that could not be assigned with this level of certainty were omitted.

Phylum	Class	Order	Family	Genus	OTU IDs
TM7	TM7-3				OTU_67
TM6	SJA-4				OTU_343
Proteobacteria	Alphaproteobacteria	Rhizobiales			OTU_446
	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae		OTU_200,OTU_172,OTU_239,OTU_255
	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae		OTU_71,OTU_134
	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae		OTU_292
	Alphaproteobacteria				OTU_139
	Betaproteobacteria	Burkholderiales	Comamonadaceae		OTU_1149
	Betaproteobacteria	MND1			OTU_133
	Deltaproteobacteria				OTU_253
	Gammaproteobacteria	Marinicellales	Marinicellaceae		OTU_297
	Gammaproteobacteria	Chromatiales			OTU_10
	Gammaproteobacteria	Thiotrichales	Piscirickettsiaceae		OTU_427,OTU_159,OTU_224
	Gammaproteobacteria	Xanthomonadales	Sinobacteraceae		OTU_161
	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae		OTU_97,OTU_301
Planctomycetes	Planctomycetia	Pirellulales	Pirellulaceae		OTU_225,OTU_508,OTU_288
Nitrospirae	Nitrospira	Nitrospirales	Nitrospiraceae		OTU_169
Gemmatimonadetes	Gemm-1				OTU_243,OTU_529,OTU_86,OTU_308, OTU_146
	Gemmatimonadetes				OTU_793,OTU_687
Firmicutes	Bacilli	Bacillales	Planococcaceae	Sporosarcina	OTU_15
Chloroflexi	Anaerolineae	Anaerolineales			OTU_108
	Anaerolineae	Caldilineales	Caldilineaceae	Caldilinea	OTU_552
	Anaerolineae	SBR1031	A4b		OTU_662
	S085				OTU_324
	Thermomicrobia				OTU_434
Chlamydiae	Chlamydiia	Chlamydiales			OTU_411

Phylum	Class	Order	Family	Genus	OTU IDs
Bacteroidetes	Saprospirae	Saprospirales	Chitinophagaceae		OTU_186,OTU_170,OTU_70
	Cytophagia	Cytophagales	Cytophagaceae		OTU_99,OTU_201
	Flavobacteriia	Flavobacteriales	Cryomorphaceae		OTU_298
	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Aequorivita	OTU_216
Actinobacteria	Acidimicrobiia	Acidimicrobiales			OTU_295,OTU_219
	Actinobacteria	Actinomycetales	Cellulomonadaceae		OTU_690
	Actinobacteria	Actinomycetales	Micrococcaceae		OTU_19
	Actinobacteria	Actinomycetales	Micromonosporaceae		OTU_106
	Actinobacteria	Actinomycetales			OTU_463,OTU_356
	Actinobacteria				OTU_1089
	MB-A2-108	0319-7L14			OTU_328

5.4 Discussion

We asked the question: how is a hatchling from a resting stage, removed in space and time from its mother, able to acquire essential symbionts for normal development and function? The conclusion from our study is that field-collected resting stages, including their exterior and interior, are associated with sufficient beneficial bacteria for normal host growth and development, even after years of storage. We also found that the assumed vertically transmitted fraction of this bacterial community is insufficient for normal hatchling fitness. Further sources of environmental bacteria beyond those associated with the ephippium do not provide additional benefits. It remains unclear what the identity and ultimate sources of the host essential microbial taxa are, though we provide some candidates.

The inconsistent presence of culturable bacteria in five-day-old animals emerging from surface-sterilized resting stages (ephippia) both confirms previous observations that ephippia at least occasionally have bacterial cells on their inner surfaces (Schultz 1976), while also hinting that some ephippia might have few or no viable bacteria inside, despite the presence of bacterial DNA as revealed by PCR and sequencing. This situation could be comparable to plants occasionally losing seed-transmitted fungal endophytes (Afkhami & Rudgers 2008). The combined evidence from this and our previous study (Sison-Mangus et al. 2015) suggests that sufficient bacteria do not reliably adhere to the surfaces of decapsulated resting eggs and that whatever bacteria are present inside the ephippium are apparently lost when the egg is removed from the ephippium. Researchers who wish to avoid using harsh chemicals while investigating the effects of microbiota on *Daphnia* might consider using decapsulated resting eggs washed with sterile water and raised in sterile medium as their model for bacteria-deficient animals, though more tests would be required to evaluate the variability resulting from this procedure. Sampling and plating water from the bottles of animals exposed to ephippia-associated bacteria (Experiment 2) showed that the bacteria initially carried on the animals could also persist in the external medium, suggesting that the bacteria associated with ephippia are not necessarily dependent on *Daphnia* but are rather in flux with the environment, similarly to observations in *Drosophila* (Wong et al. 2015; Blum et al. 2013)

We had previously observed that bacteria can be beneficial for embryonic development of resting eggs at elevated temperature (Mushegian et al. 2016), but did not know whether any bacteria found in the natural environment of resting eggs would have this effect. The marginally significant effect of exposure to *Acidovorax* on hatching rates and the improvement in survival and growth *Acidovorax*-exposed animals suggest that bacteria internal to ephippia may be beneficial to *Daphnia* even if they are not sufficient on their own for normal functioning or always viable inside ephippia after a long diapause. Similar OTUs (96-100% sequence identity) have been detected in association with *Daphnia* parthenogenetic embryos and adults (unpublished data). The

other bacterial strain we tested, *Arthrobacter*, was not recovered in culture from the bodies of two five-day-old animals; this group did not exhibit any significant fitness benefits over the bacteria-deficient groups. This suggests that *Arthrobacter* failed to stably colonize the animals in this experiment, despite matching an OTU (OTU 19) that is overrepresented on the outer ephippial surfaces. The mixture of *Acidovorax* and *Arthrobacter* was less effective than *Acidovorax* alone at enhancing hatching rates but equally effective at promoting survival and growth. In an additional parallel to other animal systems, the two strains tested here showed a similar difference in efficacy to broadly taxonomically related strains used in an experiment with larvae of the mosquito *Aedes atropalpus*. In that study, a Micrococcineae strain isolated from mosquitoes failed to rescue survival of mosquitoes to adulthood, while a Comamonadaceae strain had a rescue effect equivalent to nonsterile rearing (Coon et al. 2016). It would be interesting to see if there are conserved patterns of interactions between aquatic invertebrates and broad taxa of aquatic bacteria.

Both the inner and outer surfaces of ephippia are associated with genera of bacteria commonly identified in studies of the microbiota of adult *Daphnia* (Figure 5). Of particular interest in both lists are sphingolipid-producing bacteria, *Sphingobacterium* (Bacteroidetes) and *Sphingomonas* (Alphaproteobacteria). Sphingolipids are ubiquitous structural and signaling molecules among eukaryotes but are only found in these two clades of bacteria, where their functions are poorly understood. Sphingolipids of host-associated Bacteroidetes modulate mammalian immune systems (An et al. 2014) and influence multicellular development in choanoflagellates (Alegado et al. 2012). Sphingobacteria also affect the morphological development of macroalgae by an unknown mechanism (Marshall et al. 2006). Sphingomonads are mostly known as ubiquitous environmental bacteria but also protect plants against pathogens, possibly by priming the immune system (Innerebner et al. 2011). It would be interesting to see whether these taxa are involved in providing signals related to growth and development in environmentally sensitive aquatic crustaceans.

We initially assumed that the bacteria enclosed in surface-sterilized ephippia would be those acquired from the mother at the time that the ephippium was deposited, while the outer surface would contain primarily environmental bacteria acquired while the ephippia were resting in sediment or being handled in the laboratory. Our sequencing results show that this distinction is not so clear-cut. One of the most abundant bacterial taxa detected in surface-sterilized ephippia was *Thiobacillus sp,* a sulfur-oxidizing bacterium that is not commonly associated with *Daphnia* or other hosts. This suggests either that ephippia are permeable to bacteria after deposition, or that the ephippium captures a sample of pond water as it is deposited, in which the relative abundances of different taxa subsequently change. If seemingly closed ephippia are partially permeable, it is possible that our surface-sterilization treatment could have partially altered the internal community in addition to the alterations that the internal

community undergoes after deposition. Given these issues, we cannot say with certainty whether the beneficial microbiota associated with untreated natural ephippia are primarily of maternal or environmental origin. Microbial source tracking and visualization technology – deployed over months or years, to approximate the ecological challenges undergone by these organisms – would be helpful for more precisely answering this question.

Overall, our findings suggest that it is unlikely that ephippia function as a place to preserve specific beneficial maternal microbiota, especially since we observe that ephippia collected from natural settings are frequently incompletely closed or partially degraded. While some bacteria on the outer surface of the ephippium are similar to those found in adult *Daphnia* and could potentially originate from the mother, they are also exposed to ecological interactions (including competition, predation, and physical disturbance) with microbes from the environment. The dormant *Daphnia* embryo is unlikely to exert much influence to regulate or maintain them, as supported by the considerable variation in the dominant bacterial taxa of individual ephippia. The availability of maternal microbiota in ephippia might, therefore, vary depending on factors such as length of diapause and burial in different environments. This might then provide the opportunity to acquire new, potentially better-adapted microbiota in a new environment. Thus, given that the diapausing stage is a recurring phase of the life cycle of *Daphnia* during which there is potential for host and essential symbionts to become decoupled, we think that the process by which *Daphnia* benefit from bacteria is better understood as an open system (Wong et al. 2015), in which genetic traits of *Daphnia* might influence the composition of the environmentally acquired microbiota at some life stages, and, conversely, possibly influence the microbial ecology of the larger environment (Degans et al. 2002; Eckert & Pernthaler 2014) rather than as a "holobiont" in which host and microbial genes are selected as a unit. For the latter to be the case, reliable co-transmission of hosts and symbionts between generations would be required; covariance of host genotype and microbial community, which is often used as a proxy, is not enough to conclude a holobiont as a higher-level unit of selection (Douglas & Werren 2016).

Nevertheless, we identify the ephippial propagule as a link between the diapausing organism and the microbial world, as the first and sufficient point of contact with demonstrably beneficial bacteria. It would be interesting to see whether the ephippial substrate under natural circumstances tends to select for bacteria that are beneficial to *Daphnia*. The carriage of bacteria in or on ephippia might have ecological consequences, even if it does not result in the maintenance of specific partnerships – *Daphnia* and bacteria from a source environment might co-disperse, resulting in occasional patterns of co-occurrence that could be detected with methods being developed in microbial biogeography (Falush et al. 2003). Co-dispersal could also possibly result in alterations in the microbial ecology of the new habitat that subsequently affect host survival, for example by "seeding" the new environment with

food bacteria (Stallforth et al. 2013), horizontally transmissible symbionts (Frank et al. 2009), or pathogens novel to the native inhabitants (Vilcinskas et al. 2013). Such effects could potentially be considered "niche construction" according to some formulations (Laland et al. 2015). Co-dispersal of multiple organisms, even if not driven by specific host adaptations selected to maintain this co-dispersal, has been implicated in successful biological invasions (Simberloff & Von Holle 1999).

Taken together, our results show that bacteria are crucial for normal *Daphnia* growth and development but are readily available even to hosts emerging in unfamiliar circumstances from a long diapause, despite the apparent absence of parental mechanisms for guaranteeing transmission of specific bacteria through this long separation. We cautiously speculate that strong dependence on relatively nonspecific bacteria in the environment might be particularly common amongst aquatic animals. Experimental studies have shown that the absence of bacteria is lethal (i.e. animals do not survive to reproductive stage or fail to reproduce) in larval zebrafish (Pham et al. 2008), *Daphnia* (Sison-Mangus et al. 2015; Callens et al. 2015; Peerakietkhajorn, Tsukada, et al. 2015), *Hydra* (Rahat & Dimentman 1982), and the aquatic larvae of mosquitoes (Coon et al. 2014; Coon et al. 2016). In contrast, fitness effects of germ-free status in *Drosophila* and mice are comparatively less drastic (Erkosar et al. 2013; Smith et al. 2007). Part of this difference undoubtedly has to do with the limited range of conditions under which experiments have been conducted; technical changes in experimental conditions can alter the severity of these effects, for example in zebrafish (Rendueles et al. 2012), and it is difficult to make direct comparisons across systems. But we also wonder whether the constant, unstructured, unavoidable exposure to bacteria in aqueous environments may have resulted in aquatic animals evolving to function dramatically better in the context of omnipresent bacterial influences, as a result of constitutive expression of traits involved in tolerance of bacteria and resultant pleiotropic effects (A. E. Douglas 2014b). Such dependency would ordinarily be cryptic because it would only be evident in the unnatural and extreme environment of totally bacteria-free conditions, but the accumulation of mutations that are only deleterious in the absence of bacteria may have unexpected long-term evolutionary consequences (examples from transovarially transmitted endosymbiont systems can be found in (Bennett & Moran 2015; Flores et al. 2015)). From the standpoint of experimental studies specifically in *Daphnia*, these results show that special efforts to maintain microbiota in laboratory conditions are likely unnecessary, but that standardization of microbiota may help to reduce experimental variation.

We see, however, that not all strains associated with ephippia have equal fitness-restoring effects – *Arthrobacter* failed to stably colonize *Daphnia*, and animals exposed to this species did not receive any benefit compared to germ-free animals. Similarly, closely related strains of *Limnohabitans* bacteria appear to vary in their colonization ability and effects on *Daphnia* fitness (Peerakietkhajorn, Kato, et al. 2015). The

beneficial effects of microbiota on *Daphnia* likely extend beyond general improvement of health and additionally encompass specific services like degradation of dietary proteins and polysaccharides (Callens et al. 2015; Gorokhova et al. 2015) or detoxification and waste recycling (which might also be particularly important in aquatic settings, where exposure to toxic metabolic waste products is not easily avoided). Possibly, all or most habitats theoretically able to support *Daphnia* also support microbes able to perform such services. Several studies of mycorrhizal systems have shown that plants are often able to readily form productive partnerships with the fungi found in a new habitat, either because the relationship is generalist (Peay et al. 2015; Bruns et al. 2002) or because the fungal strains used by the more specialist taxa are independently geographically widespread (Davis et al. 2015; Davison et al. 2015; Ogura-Tsuita & Yukawa 2008). Further work should examine the relationships between *Daphnia* dispersal and adaptation and local microbial ecology.

5.5 Supplemental figures

Figure S1. Colony diversity of culturable bacteria from crushed ephippia.

Figure S2. Community similarity of bacterial sequences from negative and positive controls compared to ephippial samples. Gray points represent samples; colored points represent controls. NMDS stress=0.104.
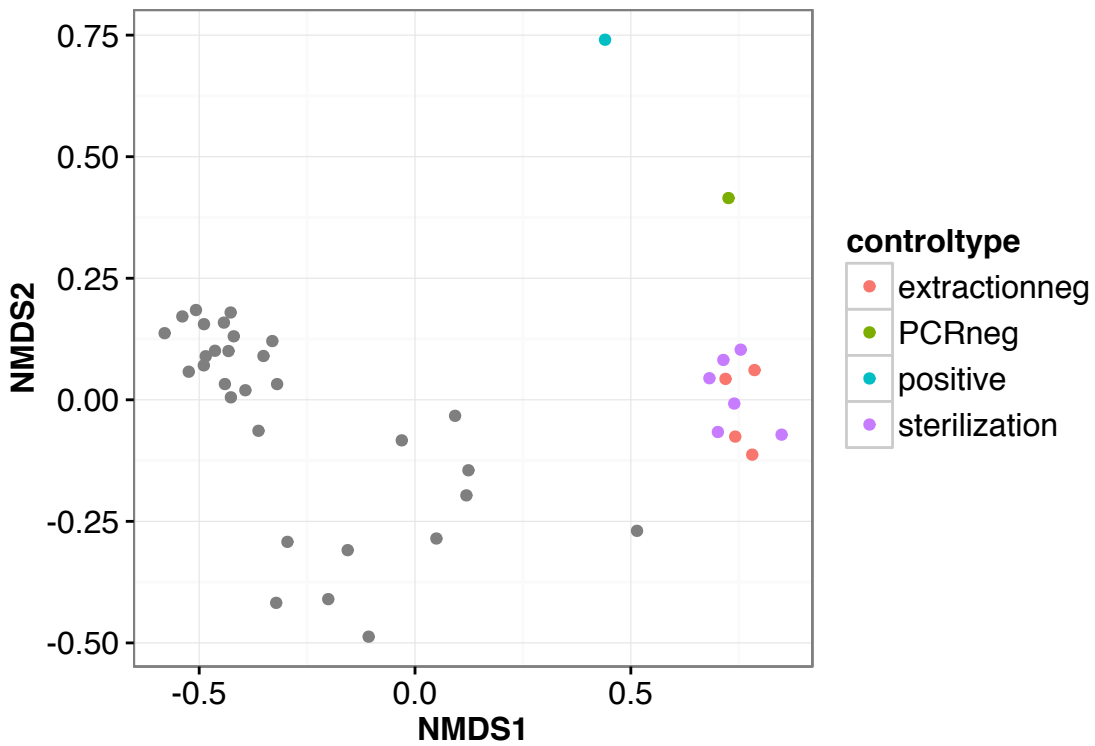
6. Environmental sources of bacteria and genetic variation in behavior influence host-associated microbiota

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Abstract

In many organisms, host-associated microbial communities are acquired horizontally after birth. This process is believed to be shaped by a combination of environmental and host genetic factors. We examined whether genetic variation in animal behavior could affect the composition of the animal's microbiota in different environments. The freshwater crustacean *Daphnia magna* is primarily planktonic, but exhibits variation in the degree to which it browses in benthic sediments. We performed an experiment with clonal lines of *D. magna* showing different levels of sediment-browsing intensity exposed to either bacteria-rich or bacteria-poor sediment or whose access to sediments was prevented. We find that the bacterial composition of the environment and genotype-specific browsing intensity together influence the diversity and composition of the *Daphnia*-associated bacterial community. Exposure to more diverse bacteria did not lead to a more diverse microbiome, but greater abundances of environment-specific bacteria were found associated with host genotypes that exhibited greater browsing behavior. Our results indicate that individual behavior can mediate genotype-by-environment interaction effects on microbiome composition.

6.1 Introduction

Every multicellular organism is colonized by a community of microorganisms: its microbiota (McFall-Ngai et al. 2013). The host provides a habitat for a complex and dynamic consortium of microorganisms, many of which have fundamental influences on the host's well-being (Zilber-Rosenberg & Rosenberg 2008). A central concern in both infectious disease epidemiology and in studies of host-associated microbial community ecology is the transmission of microbes between host individuals and between hosts and the environment. Many bacterial assemblages are transmitted from host mother to offspring (Funkhouser & Bordenstein 2013; Salem et al. 2015), but the diversity of microbiota typically changes over time depending on the microbes available in the environment (Rajilic-Stojanovic et al. 2009; Shin et al. 2015). In some cases, environmentally acquired microbes are even essential for the completion of postembryonic development (e.g. Cheesman et al. 2011; Diaz Heijtz et al. 2011). Thus microbes from the environment can be co-opted as part of the microbiota, or can affect host health during a transient occupation (Voss et al. 2015).

Environmental effects on microbiota community structure have been extensively documented (Fan et al. 2013; Seedorf et al. 2014) and studies on model organisms have started to shed light on the relative importance of environmental and host genetic factors in determining microbiota composition (Campbell et al. 2012; Spor et al. 2011). Recently, the focus has been moving towards a better understanding of the mechanisms of bacterial aqusition from the environment. Host genetics have been shown to play a role in the establishment of microbial associations through microbial recognition, immune selection, and determination of the biochemical niche (Spor et al. 2011).

Importantly, these processes select microbes after the host has come in contact with bacterial communities in the environment. The initial encounter may be a key phase of the host's colonization by microbes. If host genetics influence interaction with the environment, for example through the expression of behavioral variation, it may influence the initial encounters with environmental bacteria and thus affect the composition of the host microbiota.

Many animals utilize different habitats according to behavioral strategies collectively termed habitat selection. If habitats differ in their microbial communities, host behavior influencing habitat choice and the microbiome may influence each other. Hosts may have evolved strategies to ensure or avoid encounter with beneficial and pathogenic microorganisms. Avoidance behaviors of harmful bacteria are well documented, and behavior is considered one of the first lines of defense against infectious disease. For example, the nematode *Caenorhabditis elegans* actively avoids pathogenic bacteria and the genetic determinants of this behavior have been worked out (Meisel & Kim 2014). The opposite case, where a host's behavior is involved in the acquisition of beneficial bacteria from the environment, has received less attention, despite speculation about the role of human behaviors such as outdoor play in preventing autoimmune diseases (Rook 2013). The overall effects of host habitat choice behavior on microbiome composition have not, to our knowledge, been explored in any system. An analysis of natural genetic variation in behavioral traits potentially influencing microbiota acquisition is therefore timely (Ezenwa et al. 2012). If variation in behavior affects the composition of the host's microbial community, then behavior could underlie some genotype-environment interaction effects on microbiota. The goal of this study was to examine the effect of genetic variation in host behavior on microbiota composition in different environments using the freshwater planktonic crustacean *Daphnia magna*.

Recently, it has been shown that *D. magna* microbiota play a major role in host fitness (Sison-Mangus et al. 2015), that both host clonal line and environmental factors are determinants of microbiota community structure (Sullam et al. 2017), and that genotype-specific microbiomes can mediate daphnids' adaptive traits (Macke et al. 2017). However, little is known about the mechanisms by which the host acquires microbiota from the environment. A specific behavior, termed sediment browsing, mediates the interaction between *D. magna* and bottom sediments of ponds and lakes (Horton et al. 1979; Decaestecker et al. 2002). During browsing, the animals swim along the sediment surface, stirring up particles, and then ingest the particles by filter feeding. Besides representing valuable food reservoirs, sediments are likely important environmental sources of bacteria. Therefore, the physical contact with the sediments resulting from browsing might present both disease risks and benefits from increased contact with bacteria. Previous work found evidence of genetic variation for the levels of browsing activity in *D. magna* (Arbore et. al. 2016).

We performed a laboratory experiment in which we analyzed the browsing behavior and microbiota of 12 genetically distinct *D. magna* clones allowed to browse in sediment. The animals were exposed to three different treatment conditions, where they had access to either previously autoclaved (i) or untreated ("natural" and therefore microbe-rich) sediments (ii), or where their access to natural sediment was prevented (iii) (Figure 1A). We hypothesized that *D. magna* clones exhibiting more intense browsing behavior would have more diverse microbiota in conditions where they had access to bacteria-rich sediment. In this experiment, we made no assumptions about whether bacteria found in the sediments were beneficial, harmful, or neutral for the host, nor whether they colonized *Daphnia* stably or transiently; therefore, the patterns observed here could be applicable to studies of disease, microbiota, or general environmental microbial community dynamics. Our analysis illustrates how a behavioral trait can mediate the interplay between genetic and environmental variation in the establishment of host-microbe associations.

6.2 Methods

Overview of the experiment

In this study, we combined the analysis of animals with constitutive (genetic) differences in browsing behavior with manipulations of the environment that affected animals' access to the sediments. Animals were either exposed to natural sediment, to autoclaved sediment, or to natural sediment blocked by a permeable net barrier (Figure 1A).
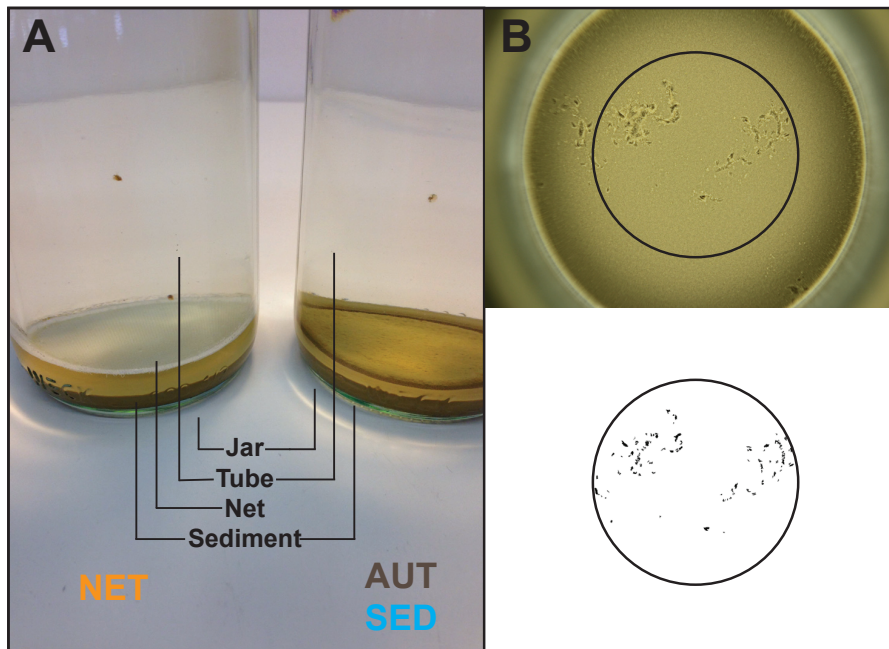
Figure 1: Experimental set-up and browsing behavior assay. A: Jars used in the experiment contained a layer of fine loess and contained two animals each. The animals were prevented from browsing on untreated sediments by a net placed 5 mm above the sediment surface (NET, right) or were allowed to

browse on autoclaved sediments (AUT) or untreated sediments (SED) (left). B: Traces left by one animal browsing on a sediment surface for 30 minutes and the same picture after processing for quantification of the browsing behavior.

In order to analyze both the browsing behavior of the animals and their microbiota, we placed two animals in each jar; of these pairs of animals, after 6 days of exposure to the different treatments, one animal was used to assay browsing behavior while the other was used for microbiota analyses.

Experimental animals

D. magna reproduces by cyclical parthenogenesis. Clonal populations can be generated and propagated in the laboratory through asexual reproduction. Here, we refer to such genetically identical individuals as "replicates" or "animals" while we refer to different genetic lines as "clones." In this study we used 12 *D. magna* clones from our stock collection, originating from different populations (Table 1). The animals were propagated from stock cultures maintained in the laboratory in standardized conditions and without any effort to modify their microbiota. The browsing behavior of these clones has been assessed before (Arbore et al. 2016) and was shown to differ among genotypes.

All animals used in this study were females. Prior to the experiment, every clone was propagated in individual replicates for three generations in order to minimize variation due to maternal effects. These animals were kept individually in 100-ml glass jars filled with 80 ml of ADaM (*Daphnia* medium (Kluttgen et al. 1994)) randomly distributed within trays in incubators with a 16:8 light/dark cycle and constant temperature of 20 °C. To establish every generation, the animals were isolated at 4 days old and fed daily with chemostat-grown green algae *Scenedesmus* sp: 1 x 10^6 algae cells/animal until day 5, 2 x 10^6 until day 8, 2.5 x 10^6 until day 10, 3 x 10^6 until day 12, and 5 x 10^6 onwards. The animals were transferred to fresh medium when they were 12 days old and thereafter every day.

Table 1. Names, number of individual replicates included in the microbiota analyses in the three treatments (AUT, NET and SED) and origin information of the 12 *Daphnia magna* clones used in this study. AUT: Exposure to autoclaved sediment; NET: prevented exposure to untreated sediment; SED: exposure to untreated sediments.

Clone ID	N (AUT)	N (NET)	N (SED)	Country	Latitude, N	Longitude E/W	Source	Description
BE-OHZ-T10	4	5	3	Belgium	50°50'00"N	4°39'00"E		
CZ-N1-1	8	8	8	Czech Rep.	48°46'31.14"N	16°43'24.70"E		
CZ-N2-6	6	7	6	Czech Rep.	48°46'31.14"N	16°43'24.70"E		
DE-K35-Mu10	4	3	4	Germany	48°12'23.93"N	11°42'34.98"E	*D. magna* Diversity panel	A geographically diverse collection of clones maintained asexually in the laboratory since 2012
DE-KA-F28	8	7	7	Germany	50°56'02"N	6°55'41"E		
ES-DO1-1	7	6	7	Spain	36°58'42.1"N	6°28'39.5"W		
TR-EG-1	5	7	8	Turkey	39°49' 25"N	32°49' 50"E		
BE-WE-G59	7	8	7	Belgium	51°04'04"N	3°46'25"E		
No-V-7	4	3	2	Norway	67°41'13.06"N	12°40'19.09E		

Clone ID				Description			Source	Description
IXF1	5	8	7	F1 clone			*D. magna* QTL panel	An intercross F2 recombinant panel maintained asexually in the laboratory since 2006/2007
F2-82	7	7	4	F2 clone				
F2-918	5	6	6	F2 clone				

For the experiment, we used animals from the 4th generation of each of the 12 clones. These animals were kept in groups of 8 siblings belonging to one clutch of one mother. At 4 days old (± 1 day), 6 animals from every clutch were randomly assigned in pairs to individual jars divided into the three different treatments (split brood design); each such jar containing a pair of animals was an experimental replicate. In total, we included in the experiment 540 animals (270 pairs) corresponding to 15 pairs of clone BE-OHZ-T10, 18 pairs of clones DE-K35-Mu10 and NO-V-7, 21 pairs of clone F2-918, and 24 pairs of each of the remaining clones. Variation in replicate numbers resulted from differences in availability of female offspring at the time that the treatments were established.

Experimental design

The experiment was conducted in cylindrical glass jars (height = 20 cm; diameter = 6.5 cm) (Fig. 1A) kept in cardboard boxes on shelves in a climate room (16:8 light/dark cycle at 20 °C), loosely covered with transparent plastic film and top-illuminated with neon lights. In this way, light only entered the jars from the top. All the experimental jars were first filled with 400 ml of medium. 15 ml of a suspension of loess (fine silt) was then carefully deposited on the bottom using a serological pipette. The loess was previously collected from a soil stock, suspended in water, passed through a 200 μm filter and washed to remove very fine particles. After two days of sedimentation, the loess formed a 1 cm layer at the bottom of the jar. Then, an acrylic tube (height = 21 cm; diameter = 5 cm) was inserted into the jars and kept in position with a plastic ring fixed to the opening of the jar, so that its lower end was positioned close to the sediment surface. In one treatment (NET), the acrylic tube was closed with a 500 μm net at the lower end (suspended 5 mm above the sediment surface) preventing animals from direct contact with the sediment (Fig.1A left). In the other two treatments (AUT and SED), the acrylic tubes had no net so that the animals had free access to the sediment (Fig.1A right). In the AUT treatment, the loess was previously autoclaved while in the SED and the NET treatment the loess was left untreated ("natural"). (After autoclaving, AUT sediment was handled in the same way as natural sediment, i.e. exposed to nonsterile media and laboratory environment.) After inserting the tubes, the jars were left undisturbed for two days before the animals were introduced in order to allow the sediment to settle. Immediately before the experiment, the sediments of three jars of each of the SED and the AUT treatment were sampled and frozen at -20 °C; these sampled jars were not used further.

Two animals from the same clutch were carefully introduced into the inner tube of each jar. The 264 jars, each containing one pair of animals, were evenly distributed among the treatments and their positions in the incubator room were randomized. The animals remained in the experimental jars for 6 days. During this time, the animals were carefully fed twice daily with 2.5×10^6 algal cells. At day 6, all animals were collected and one member of every pair was assigned to the behavioral assay (see below)

and the other was frozen for later DNA extraction. 32 pairs of animals were lost or damaged during the experiment and were excluded from further analyses. At the end of the experiment, 3 sediment samples from the NET treatment and 3 sediment samples from the AUT treatment were collected and frozen at -20°C.

Behavioral analysis

The animals for the behavioral assay were transferred individually from the sediment jars to 100-ml glass jars filled with medium and kept in an incubator and fed daily with 5×10^6 algal cells. The behavior assay was conducted over two days when the animals were 12 to 14 days old with all replicates for the different clone by treatment combinations evenly distributed across time. The behavior assay was performed as described in Arbore et al. (2016). Briefly, we measured the traces left by individual replicate animals on a smooth surface layer of sediment (loess) at the bottom of tall cylindrical glass jars (20 cm tall, 6.5 cm diameter; Fig. 1B) during 30 minutes. The sediment surface was photographed before animals were released (time 0), using a ring light to ensure uniform illumination. The jar was then transferred into a cardboard tube and illuminated from the top with a neon light and one animal was introduced in each jar. After exactly 30 minutes, the animal was removed and the sediment surface was again photographed (time 1), in the same position and under the same light conditions. Using the software ImageJ (http://rsb.info.nih. gov/ij/), the pictures were converted to grey scale and a central circular area was cropped to exclude shadows from the edge of the jar (Fig. 1B). Pictures were processed such that the browsing traces of the animals on the sediment surface resulted black areas against a white background. Then the number of black pixels was quantified. Pictures taken at time 0 were used to correct the values calculated for the browsing traces when irregularities on the sediment surface were detected (i.e. in cases the picture of time 0 was not entirely white). The pixel values were then log-transformed ($[\log_{10}(X+1000)]$; 1000 corresponds approximately to the number of pixels of one individual browsing trace). During the assay, four animals were accidentally damaged while handling and were excluded from the analyses. The body lengths of the animals used for behavior analysis were measured after the behavioral assay.

The adjusted intra-class correlation coefficient for the browsing behavior (equivalent to broad sense heritability) was calculated with a linear mixed effect (LMM) model, with treatment as a fixed effect and clone as a random effect (R software package rptR developmental version; (Nakagawa & Schielzeth 2010)). Confidence intervals and statistical significance were calculated using parametric bootstrapping with 5000 iterations and a randomization procedure with 5000 permutations.

DNA extraction, library preparation and sequencing

The animals assigned to the microbiota analysis were transferred individually from the sediment treatment jars to 40 mL of autoclaved ADaM for about 2 hours to dilute carryover of unattached bacteria. Then, the animals were transferred into 1.5 ml Eppendorf tubes, the ADaM was removed and the tubes were stored at -20 °C until DNA extraction.

DNA was extracted from single animals using a cetyltrimethylammonium bromide (CTAB)-based protocol. The animals were ground with a sterile pestle in 1.5 ml Eppendorf tubes in a 10 mg/ml lysozyme solution and mixed at 850 rpm and 37 °C for 45 minutes. Then, a 20 mg/ml solution of proteinase K was added and the tubes mixed at 850 rpm and 55 °C for 1 hour. After an RNase treatment (20 mg/ml) at room temperature for 10 minutes, a preheated 2X solution of CTAB was added and the tubes mixed at 300 rpm and 65 °C for 1 hour. After two rounds of chloroform isoamyl alcohol (CIA) purification (1 volume CIA; 8 minutes centrifugation at 12,0000 rpm and 15 °C), a solution of sodium acetate 3M pH 5.2 and isopropanol were added to the DNA solution and the tubes were stored overnight at -20 °C. The following day, DNA was purified by two rounds of 70 % ethanol precipitation and suspended in water. The extractions were then incubated at 4 °C overnight and then stored at -20 °C.

All DNA extractions were conducted over a period of 6 days with samples from the different clone by treatment combinations randomly distributed between the days and one reagent-only negative control extraction included every day. DNA from the sediment samples and from one negative control was extracted on a different day using a commercial kit (PowerSoil® DNA Extraction kit; MO BIO Laboratories, cat. 12888-100).

We sequenced amplicons of the V3-V4 variable region of the bacterial 16S rRNA gene using the Illumina MiSeq platform. Amplicons were generated using NEBNext High Fidelity PCR Master Mix (New England Biolabs catalog#M0541L) for 27 cycles in 25 µl reactions containing 3% DMSO. The primers used were 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGA-3') and 785R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGA-3') with Illumina adapter sequences and 0-3 bp random frameshifts. PCR product was purified with Ampure beads at a 0.6x ratio of beads to PCR product, amplified for 9 cycles with Nextera XT v2 indexing primers, and purified again. Libraries were quantified with Qubit and quantitative PCR, normalized, and pooled, followed by additional bead purification to remove remaining short fragments before sequencing on the Illumina MiSeq (reagent kit v3, 300 bp paired-end reads). The same library pool was used for two MiSeq runs; after checking that there was no statistical difference in community composition between the runs (Adonis analysis of Bray-Curtis dissimilarity between samples, p=0.394), the data from the two runs were merged.

Raw reads were quality controlled with FastQC (Babraham Institute, UK). Paired reads were merged (FLASH v1.2.9), primers trimmed (Cutadapt v1.5), and quality filtered (PRINSEQ-lite v0.20.4). OTU clustering including abundance sorting and chimera removal was performed using the UPARSE workflow (Edgar 2013). Only those OTUs represented by 5 or more reads in the run were included. Taxonomic assignment was performed using UTAX against the GreenGenes v13/5 database. We analyzed samples with more than 5000 total reads. This left 214 samples; numbers of replicates for each combination of variables are reported in Table 1.

Since individual *Daphnia* contain low bacterial biomass, we considered the issue of reagent contamination with bacterial DNA (Salter et al. 2014). Samples were processed in haphazard order, so erroneous sequences originating from reagent contamination were expected to be distributed randomly and not confounded with any treatment or genotype. For our research question, we were interested in patterns of diversity and changes in composition in response to experimental factors rather than in the presence or absence of any particular strain. For all analyses, we first tested for processing batch effects and stratified the main analysis by batch if they were significant.

For statistical analyses in which host clone was a fixed effect, we excluded clone NO-V-7, since it did not have at least 3 replicates in each treatment; we included this clone in analyses where clone was treated as a random effect. We examined the effects of experimental factors on both overall diversity and the community composition of each animal's microbiota using standard ecological diversity indices and ordination methods. To evaluate the effect of animal behavior on microbiota, we used as proxies for individual behavior either the mean browsing intensity index of the clone or the browsing intensity of the individual co-housed with the sequenced individual in the same jar ("jar-mate").

Analyses were carried out in R (3.4.3), using the packages phyloseq (1.22.3), vegan (2.4.6), plyr (1.8.4), dplyr (0.7.4), DESeq2 (1.18.1), nlme (3.1.131), lme4 (1.1.15), metacoder (0.2.0.9012), and ggplot2 (2.2.1).

6.3 Results

Browsing intensity, animal microbiota, and sediment bacteria

Consistently with previous studies (Arbore et al. 2016), browsing behavior intensity varied among *Daphnia* clones (Fig. 2). Clone and treatment, but not their interaction, had a significant effect on browsing behavior (analysis of variance: clone F=12.717, df=11, p<0.0001; treatment F=4.100, df=2, p=0.018; clone*treatment F=1.274, df=22, p=0.193). The average browsing intensity of animals from the NET treatment was lower than that of animals in the SED and AUT treatments (Fig. S1). The total phenotypic variance for browsing behavior explained by clone, after controlling for

the treatment effect, corresponded to 36.5% (95% CI = [13.2, 55.3%], p = 0.0002). Clone but not treatment had a significant effect on body size, so we assume that access to (and type of) sediment did not substantially affect nutrition and growth over the timeframe of the experiment (analysis of variance: clone F=8.08, df=11, p < 0.001; treatment F=2.01, df=2, p=0.137; clone*treatment F=1.43, df=22, p=0.103). Individual body size was uncorrelated with behavior (analysis of variance: F=0.346, df=1, p=0.56; Fig. S2).
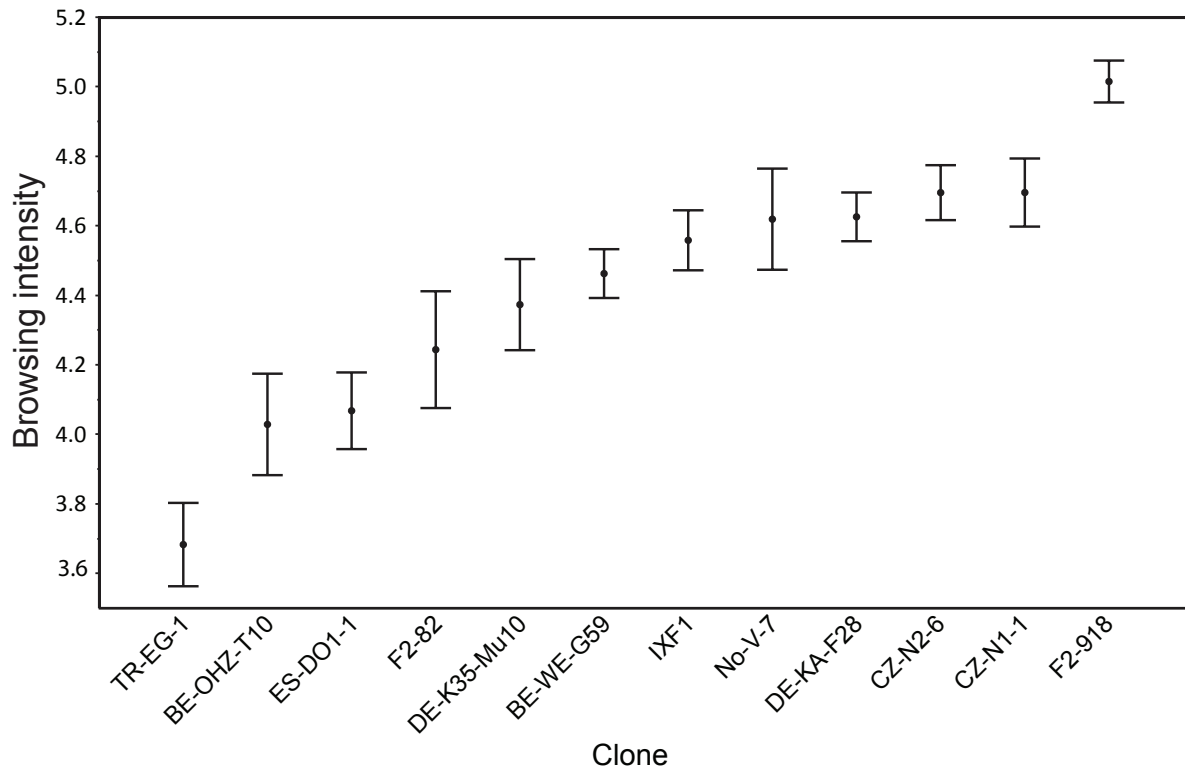
Figure 2: Browsing intensity of 12 *D. magna* clones (mean and SE). Browsing intensity was defined as the Log_{10} of the area of the browsing traces left by individual replicate animals browsing on a sediment surface for 30 minutes (see Fig. 1).

A total of 370 OTUs were found among the animal samples; of these, 318 were found in less than 10% of samples. (See Fig. S3A-C for taxonomic heat trees of OTUs with presence/absence information) (Foster et al. 2016). Consistently with multiple previous studies of *Daphnia* microbiota (Peerakietkhajorn, Kato, et al. 2015; Qi et al. 2009; Callens et al. 2015; Eckert & Pernthaler 2014), the most abundant bacterial species was a single strain (OTU_1) of *Limnohabitans* sp (Betaproteobacteria, Comamonadaceae), with a mean relative abundance across all clones of 0.39 (s.e.m. 0.02). Interestingly, a second strain of *Limnohabitans* (OTU_2) was a dominant strain only in the three clones originating from clones bred in the laboratory as part of a genetic breeding design (QTL panel; 0.32 mean relative abundance among individuals of clones IXF1, F2-82, F2-918; 0.0016 mean relative abundance in remaining clones).

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As expected, the sediment originating from the SED treatment had much higher bacterial species richness than that originating from the AUT treatment (Fig. S4).

Effects of treatment and clone on alpha diversity

Both *Daphnia* clone and treatment, but not their interaction, had significant effect on the Shannon and inverse Simpson alpha diversity indices (Table 2). For further analyses, we focused on the Shannon index, because it takes into account not only species richness but also evenness (with additional species given more weight as they become more abundant). The Shannon index displayed no significant effect of processing batch (df=5, F=1.42, p=0.22). Shannon diversity estimates for the 12 clones arranged in order of increasing average browsing intensity and the three groups (AUT, NET and SED) are shown in Fig. 3 (species richness and inverse Simpson index are shown in Fig. S5A-B). Unexpectedly, the highest average alpha diversity in most clones (9/12) was observed in the AUT treatment group, despite their exposure to less-diverse sediment than the SED group. Therefore, diversity of animal microbiota does not directly reflect diversity of bacteria in the environment.
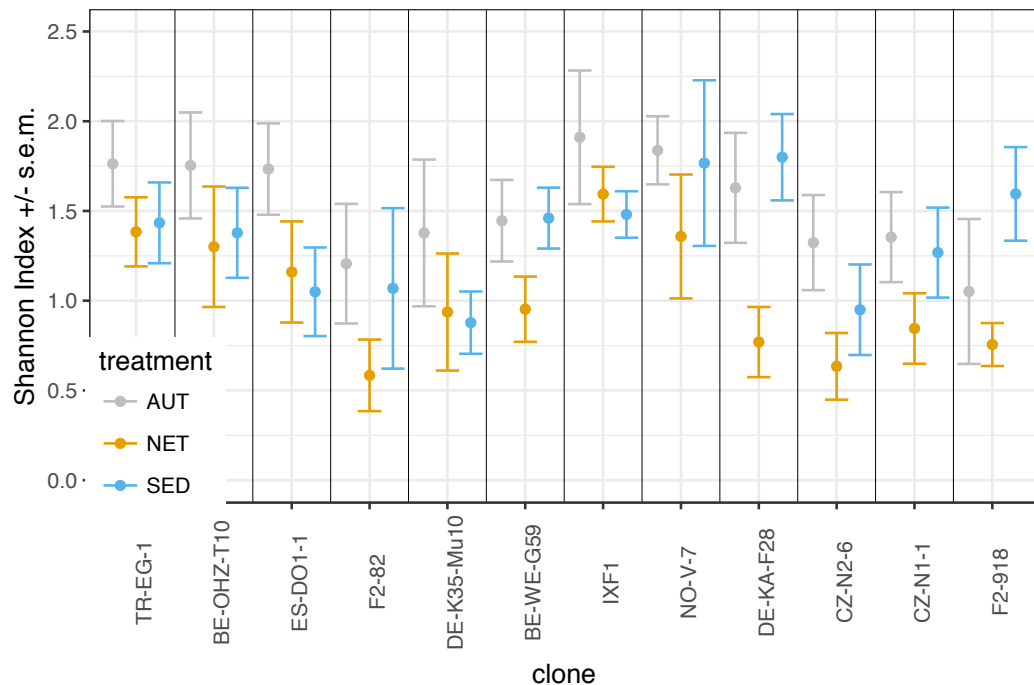
Figure 3: Microbiota diversity (Shannon index) of *Daphnia* clones under three different treatment conditions (AUT, NET and SED). AUT: Exposure to autoclaved sediment; NET: prevented exposure to untreated sediment; SED: exposure to untreated sediments. Error bars represent standard error of the mean. Clones are arranged left-right by increasing average clone browsing intensity.

Table 2. Results of analyses of variance of different alpha diversity indices. All treatments are included; clone NO-V-7 is excluded.

	Richness	Shannon	Inverse Simpson
Clone	F=0.40, df=10, **p=.944**	F=2.43, df=10, **p=.00997***	F=1.98, df=10, **p=.0383***
Treatment	F=4.91, df=2, **p=.00842***	F=12.21, df=2, **p<.0001***	F=13.25, df=2, **p<.0001***
Clone:Treatment	F=0.75, df=20, **p=.770**	F=0.78, df=20, **p=.734**	F=0.65, df=20, **p=.8124**
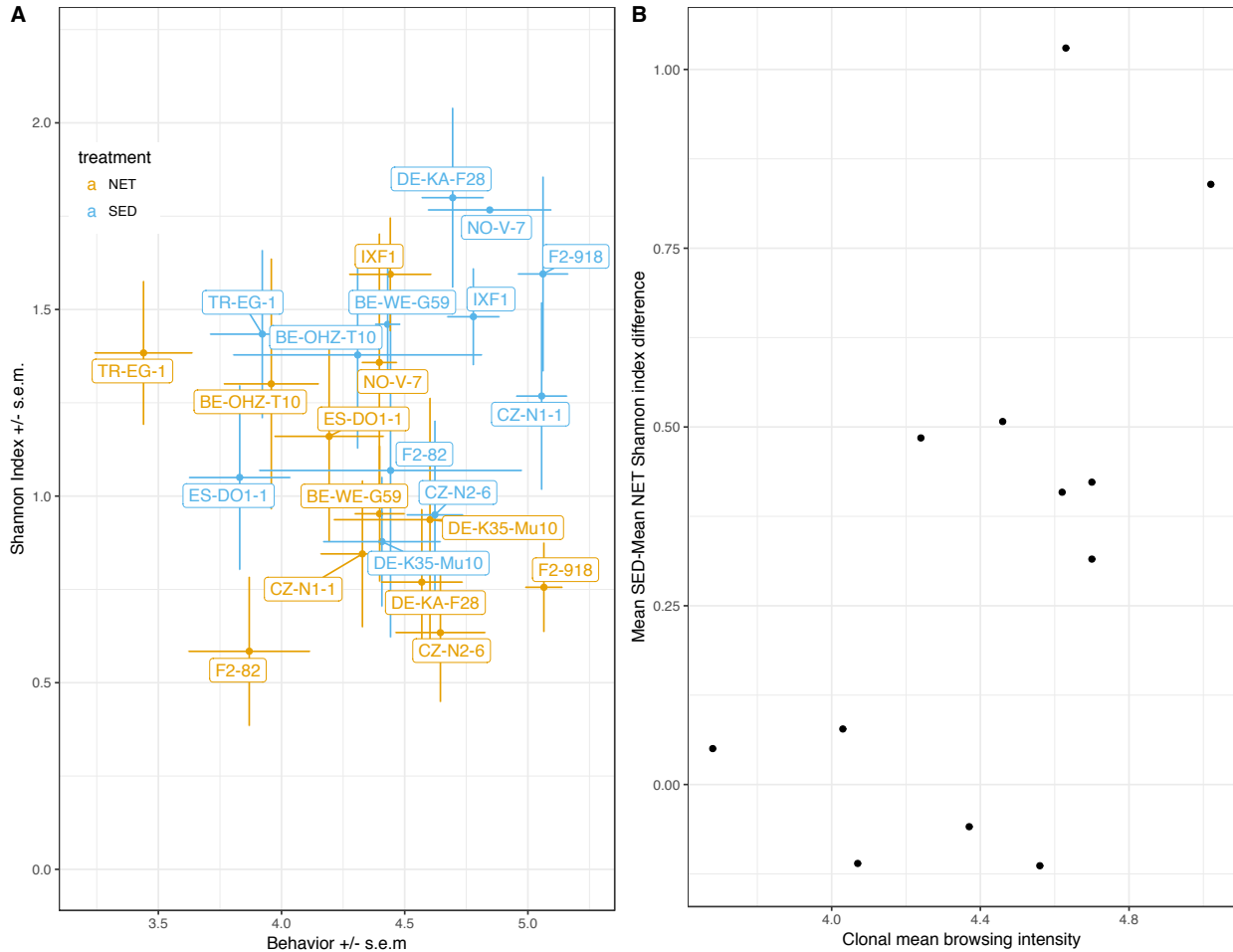
Figure 4: Average browsing intensity and average microbiota diversity in the NET and SED treatments. A: average clone browsing intensity and average clone microbiota diversity in the NET and SED treatments. Average browsing intensities were calculated based on samples whose jar-mates passed the sequence quality control (N=214, Table 1). B: average clone browsing intensity and the difference between average Shannon diversity in the SED treatment and average diversity in the NET treatment. Here, average browsing intensities were calculated based on the complete set of samples (N=228, i.e. all assayed jar-mates). Error bars represent standard error of the mean.

To specifically investigate the effect of direct access to the same bacteria-rich sediment, we compared the NET and SED treatment groups' diversity as a function of clonal average behavior in each group (Fig. 4A). The difference in mean Shannon

diversity between SED and NET animals was greatest at the highest average clonal level of browsing intensity (Fig. 4B; linear regression p=0.055). A similar tendency could be seen when the browsing intensity of each individual's jar-mate was used as the proxy for individual behavior (Fig. S6). Shannon diversity significantly depended on the interaction between treatment and clonal average browsing intensity in a linear mixed-effects model with clone included as a random effect (Table 3); the same was true when treatment-specific clonal average behavior was used as the behavior proxy, but not when individual jar-mate behavior was used (Table S1).

Table 3. Effect on Shannon index. NET and SED treatments only, all clones included. Linear mixed-effects model with treatment, clonal average browsing intensity and clonal average size as fixed effects and clone as random effect.

	numDF	denDf	F-value	p-value
(Intercept)	1	129	270.12	<.0001
Treatment	**1**	**129**	**12.26**	**0.0006 ***
Clone average behavior	1	9	0.275	0.613
Clone average size	1	9	2.568	0.144
Treatment:Clone average behavior	**1**	**129**	**4.677**	**0.0324 ***
Treatment:Clone average size	1	129	0.0980	0.755

Community composition and acquisition of bacteria from sediment

To examine shifts in bacterial community composition in response to environmental treatments, we Hellinger-transformed the bacterial abundances by taking the square root of the relative abundance of each taxon in each sample to reduce the influence of rare taxa, and then calculated pairwise Bray-Curtis distances between samples. The average distance to the centroid (dispersion) was lower in the NET group than in the AUT and the SED groups (Fig. 5), suggesting that access to sediment increases variability of microbiota regardless of the composition of the sediment. To see whether the different sediments resulted in systematically different microbiota composition, we excluded the NET group and performed principal coordinates analysis (PCoA) (Fig.6). Adonis analysis stratified by processing batch showed that both treatment and clone had a significant effect (treatment: R^2=0.05, p=0.001; clone: R^2=0.16, p=0.001), but not their interactions (treatment:clone p=0.63). However, clones also showed significant differences in dispersion (p<0.001).
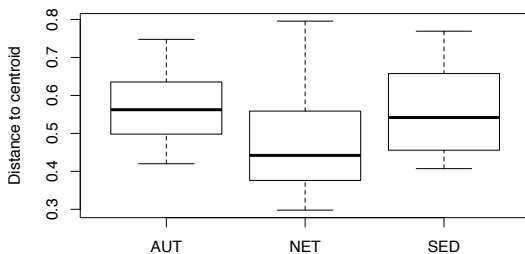
Figure 5: Within-group dispersion of community similarity. The median distance to the centroid is lower in the NET treatment group than in the others (Permutation test of multivariate dispersion p<0.0001, 999 permutations), meaning that NET communities are less variable than AUT or SED microbiotas.

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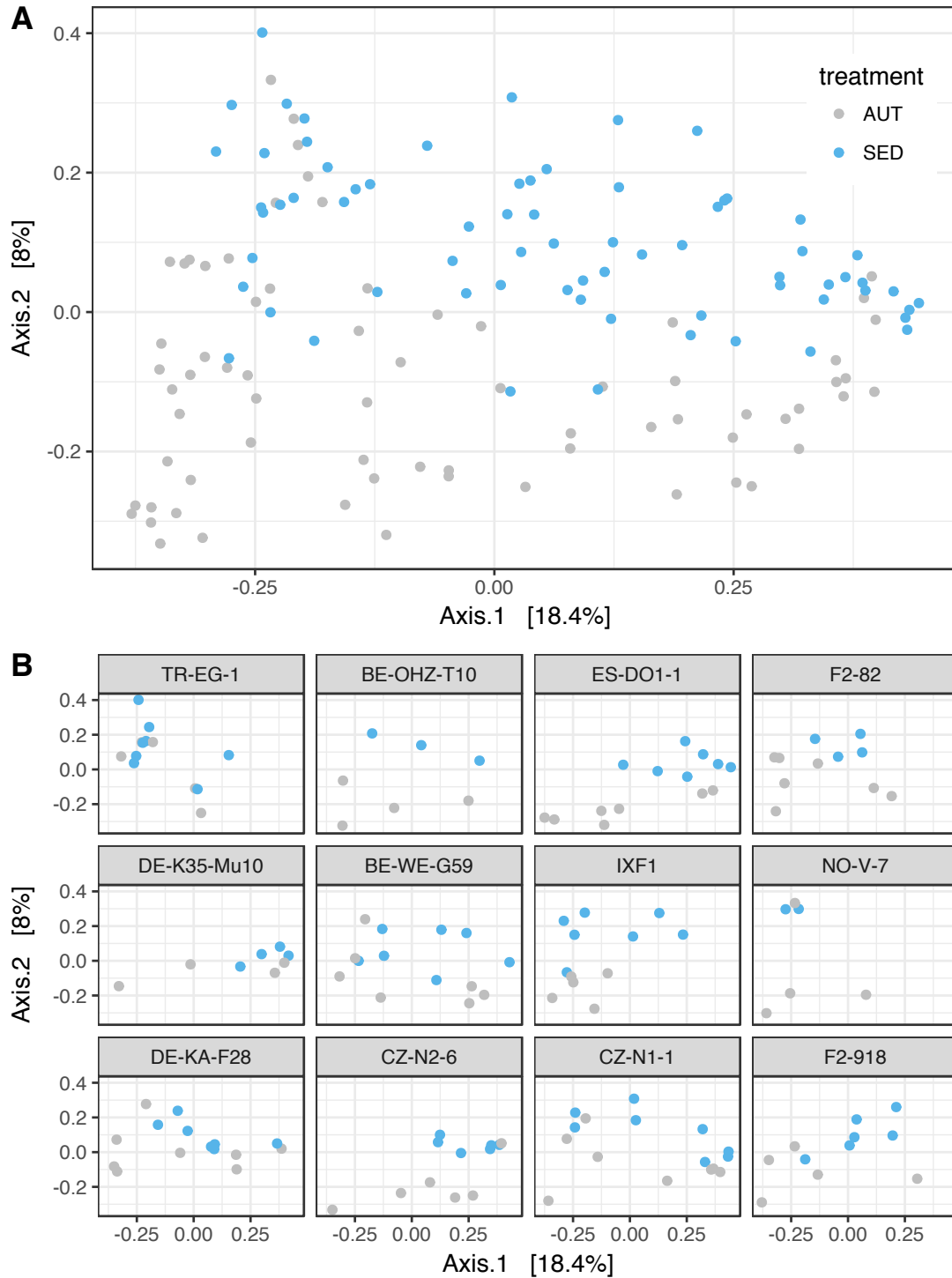
Figure 6: Similarity of bacterial community composition in the AUT and SED treatments.
A: first and second axis of a principal coordinates analysis (PCoA) of bacterial community composition based on Hellinger-transformed Bray-Curtis dissimilarities. B: first and second axis of a principal coordinates analysis (PCoA) of bacterial community composition by *Daphnia* clone.

Having confirmed that differences in the sediment environment resulted in differences in animal microbiota composition, we next explored the extent to which

environment-specific bacteria contributed to these differences. We used DESeq2 to determine which bacteria were significantly more present in natural sediment than autoclaved sediment (n=3 each). 115 OTUs were calculated to be significantly differentially present between the two sediment types; of these, 48 had at a log2-fold increase of at least 8 in natural sediment compared to autoclaved sediment. We refer to these as natural-sediment-derived taxa. The 8-fold threshold was chosen based on inspecting the data; similar results were seen when sediment-derived bacteria were defined by a log2-fold change of 5 or 10; see Fig. S7. Only one of these OTUs was found in a majority of animals, and the median number of animals in which a given OTU was found was 6.5. We therefore concluded that animals likely acquired environmental bacteria randomly rather than selectively from the environment. Accordingly, we looked at the total relative abundance of all natural sediment–derived bacteria in each individual.

The relative abundance of natural-sediment-derived bacteria was generally low in both the AUT and NET treatment groups, and increased with browsing intensity in the SED treatment group (Fig. 7), with a significant interaction effect between treatment and clonal average browsing intensity (Table 4). Treatment-specific clonal average behavior showed the same significant interaction effect with treatment, but the interaction effect was not significant when jar-mate behavior was used as the behavior proxy. Among the set of clones examined here, an appreciably high relative abundance of sediment-derived bacteria was detectable mainly in clones with a browsing intensity index higher than mean 4.4 (clones IXF1, NO-V-7, DE-KA-F28, CZ-N2-6, CZ-N1-1, F2-918). The mean relative abundance of sediment-derived bacteria in the SED treatment in the pooled animals from these clones was 0.14 (s.e.m. 0.026), whereas it was 0.048 (s.e.m. 0.0097) in the lower-browsing clones. Across all clones in the AUT and NET treatment groups, the average relative abundance of sediment-derived bacteria was 0.051, nearly identical to that of the low-browsing clones in the SED conditions.

Table 4. Effect on relative abundance of sediment-derived bacteria. All treatments and all animals included. Linear mixed-effects model with treatment and clonal average browsing intensity as fixed effects and clone as random effect.

	numDF	denDf	F-value	p-value
(Intercept)	1	198	53.999	<.0001
Treatment	**2**	**198**	**6.500**	**0.0018***
Clone average behavior	1	10	0.490	0.4998
Treatment:Clone average behavior	**2**	**198**	**4.185**	**0.0166***
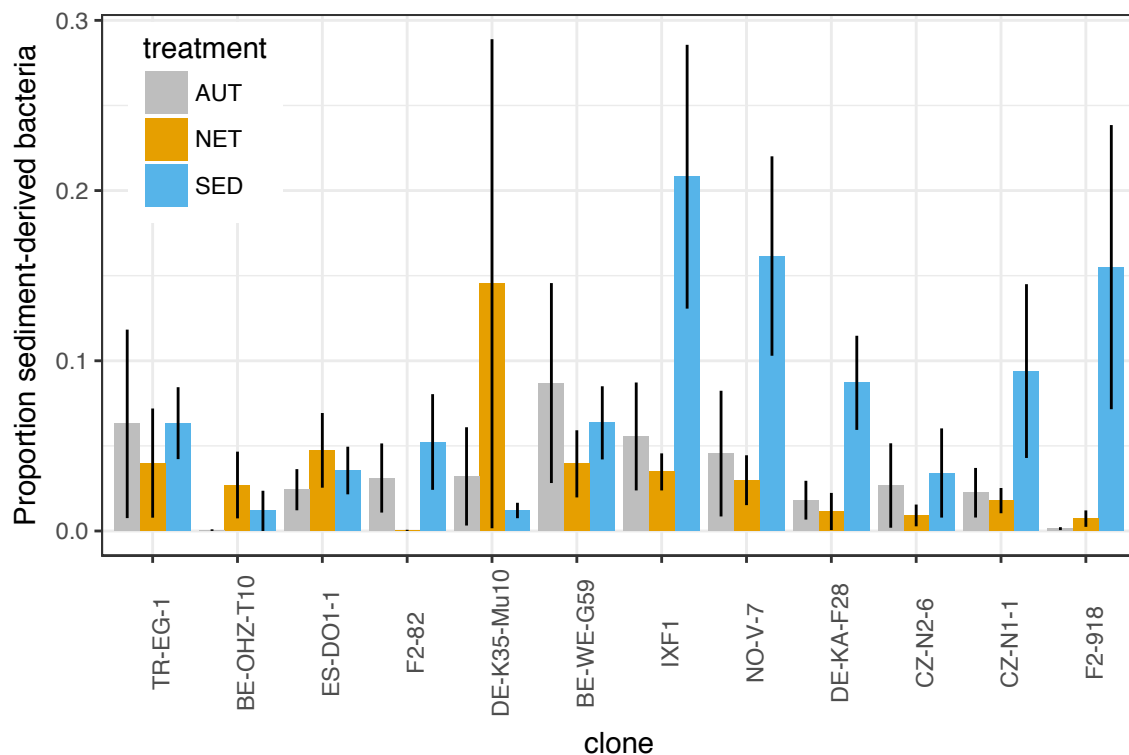
Figure 7: Analysis of sediment-derived bacteria. Proportion of sediment-derived bacteria in the microbiota of animals from AUT, NET and SED treatments. Sediment-derived bacteria were identified by comparing autoclaved and untreated sediment samples (log2-fold increase of at least 8 in natural sediment compared to autoclaved sediment). Error bars correspond to standard errors of the mean. Clones are arranged left-right by increasing average clone browsing intensity.

6.4 Discussion

Our results have several implications for studies of animal-associated microbiota in diverse environmental settings. First, we confirm the intuition that environmental sources of bacteria affect the diversity of animal microbiota, but not because more diverse environments always create more diverse microbiota; rather, the animals we exposed to the less species-rich autoclaved sediments had higher overall diversity in their microbiota than those exposed to untreated, bacterial-species-rich sediment. We hypothesize that this might be due to competitive interactions between *Daphnia* microbiota and the particular microbes found in these sediments. The untreated sediments may contain bacteria that can outcompete multiple strains of "native," preexisting *Daphnia* microbiota. If this were the case, then browsing in sediment could have multiple opposing effects on overall microbiota diversity: on the one hand, it would bring daphnids into contact with more diverse bacteria, but on the other hand those bacteria could reduce existing microbiota diversity. In the NET treatment, animals might be exposed to some sediment-derived bacteria in the water column but lack access to the full diversity of bacteria in the sediment. We also saw that having access to either sediment increased the variability of community composition as measured by multivariate dispersion. These results suggest that having access to multiple habitats

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with different bacterial communities can affect the diversity and composition of an animal's microbiota. Therefore, fine-scale heterogeneity in a host's habitat might be a relevant aspect to take into account when examining effects of environment on animal microbiota. This is especially important when considering ecological immunology, because disease-causing bacteria in the environment may cause short-term risk but also long-term fitness benefit via processes like immune priming (Kaltenpoth & Engl 2014; Olszak et al. 2012).

Our data further suggest that the diversity of *Daphnia*-associated microbiota in a particular environment may to some extent be mediated by genotype-specific sediment browsing intensity. This was apparent as the net barrier made the greatest difference in microbial alpha diversity in high-browsing host clones. However, this effect may be partially obscured by several factors: the hypothesized competitive exclusion effects we allude to above, and also non-behavior-related host genotype effects on microbiota diversity. While host genotype had an effect on microbial diversity, the highest- and lowest-browsing clones in our study had similar microbial alpha diversity overall. It was only in evaluating the difference between presence and absence of the barrier that an effect of browsing on diversity could be seen. We conclude that the effect of environmental bacteria on host-associated microbiota is not additive. The clearest effect of environmental bacteria on host-associated microbiota was not on alpha diversity, but relative abundances of certain taxa.

Clones with low average browsing intensity had no greater amount of sediment-specific bacteria than animals exposed to autoclaved sediment or prevented from browsing, whereas those with high browsing intensity could reach over 60% of reads from environment-derived bacteria in some individuals. While many studies of animal microbiota rightly concern themselves with distinguishing between truly "host-associated" microbiota versus "transient environmental" microbiota, these results raise the possibility that the amount of environmental microbes found in association with an animal could itself be a host-genotype-specific feature of the microbiome.

A key question for understanding symbiosis that future studies should address is whether browsing behavior affects community composition by simple exposure to more colonizing bacteria, or by more frequent replenishment of bacterial taxa that would not otherwise persist in association with the host (Blum et al. 2013). For example, browsing frequently enough may replenish bacteria that would otherwise be lost when the animal molts. In this study, we made no assumptions about the type of the interactions between the sediment-associated bacteria and the *Daphnia*, but still were able to see that presence of sediment-associated bacteria affected the bacteria that *Daphnia* were carrying.

It would also be interesting to investigate whether carriage of bacteria on *Daphnia* from the sediment into the water column affects bacterial dynamics in the larger environment; previous studies have shown that movement of *Daphnia* between benthic and limnetic environments represents a mechanism of bacterial dispersal in the

environment (Grossart et al. 2010). Studies using classification methods more sensitive than 16S-based taxonomy may be necessary to unambiguously distinguish and assign sources to different bacterial strains.

6.5 Conclusion

We show that at least some characteristics of host-associated microbial community composition result from genotype-by-microhabitat interactions, specifically ones resulting from genotype-specific variation in behavior. Behavior may thus be considered a genetic factor that shapes microbial exposure in a given environment. Overall, these results provide further evidence that environment, behavior, genetics, disease risk, and microbial community composition are interrelated in potentially complex ways. Our observations indicate a need for more integrative eco-immunology studies, in which the interfaces between behavioral ecology, microbial community ecology and evolution of immune function are explored. Studies can take advantage of the experimental tractability of the *Daphnia*-microbiota system to further investigate these relationships in mechanistic detail.

6.6 Supplemental figures and table

Supplementary Figure 1. Browsing intensity index by environmental treatment.
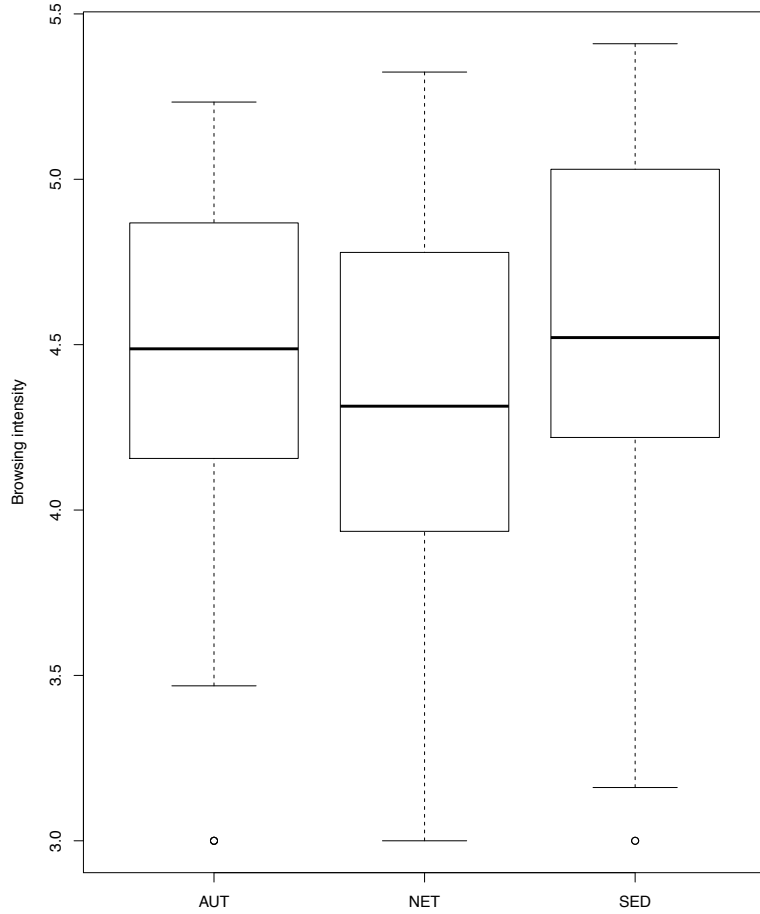
Supplementary Figure 2. Clonal average size and clonal average browsing intensity are uncorrelated.
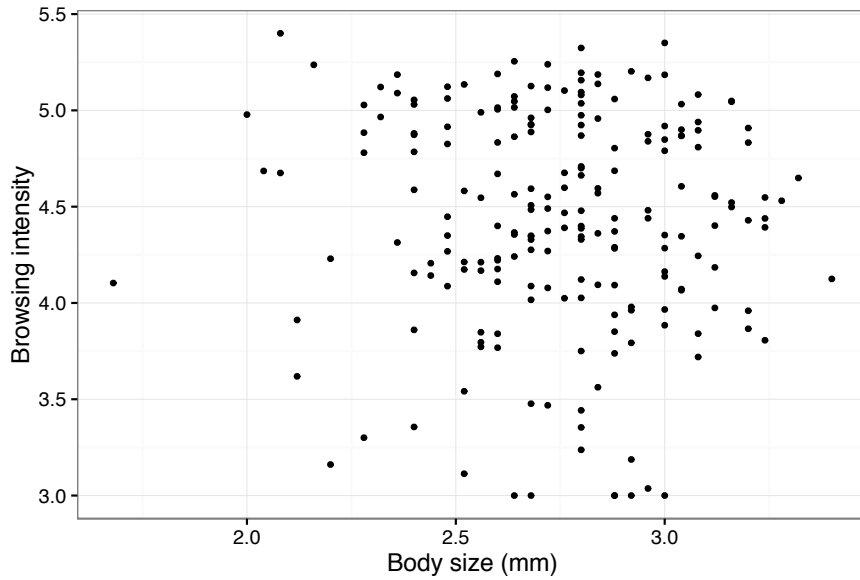
Supplementary Figure 3: Taxonomic trees of OTUs found in sequenced animals, highlighting presence/absence and relative abundance in AUT (A), NET (B) and SED (C) treatment groups. Node size represents the number of samples in the treatment group in which a given taxon is found, whereas node color represents the median relative abundance of the taxon among samples in the treatment group.

(A)

(B)
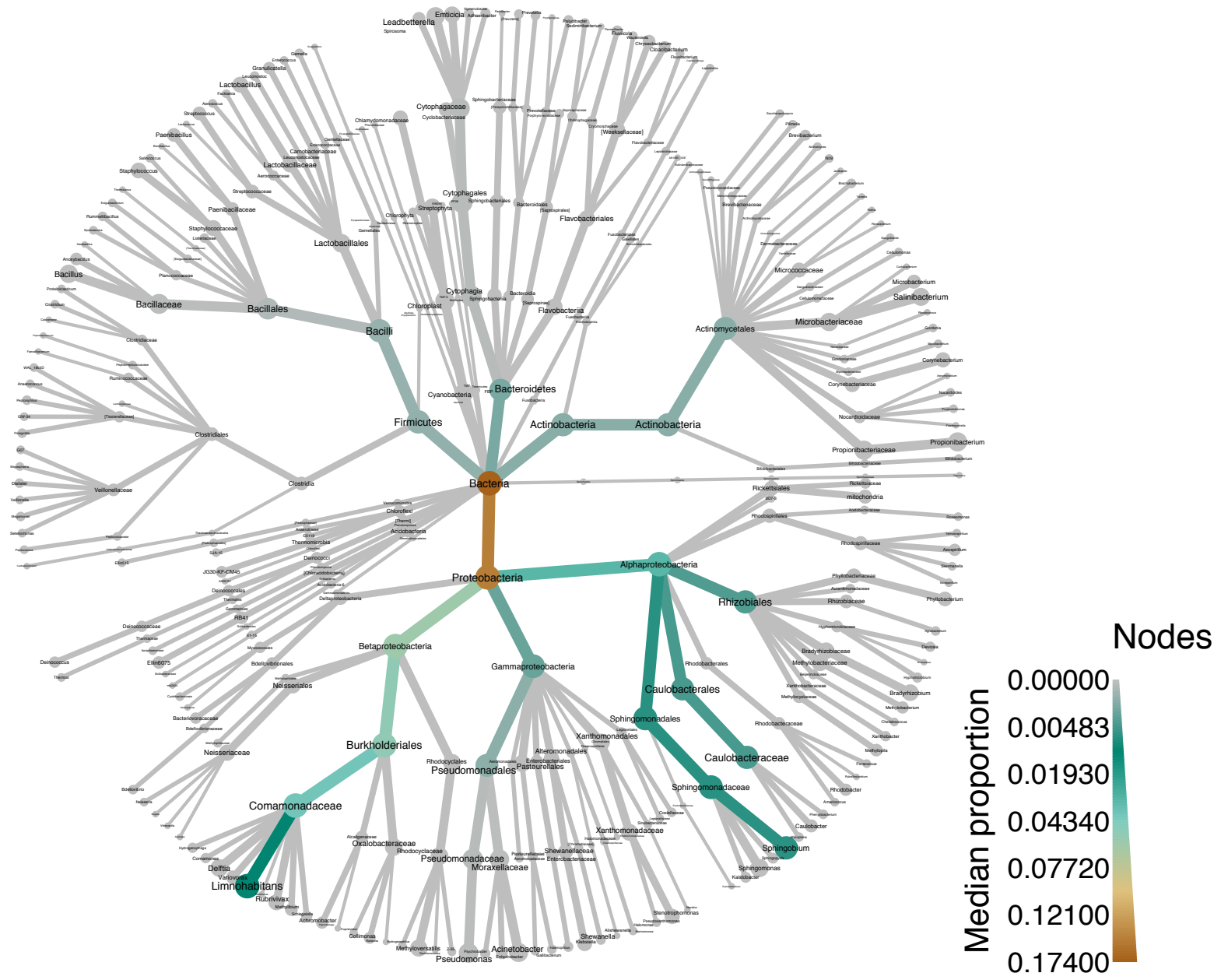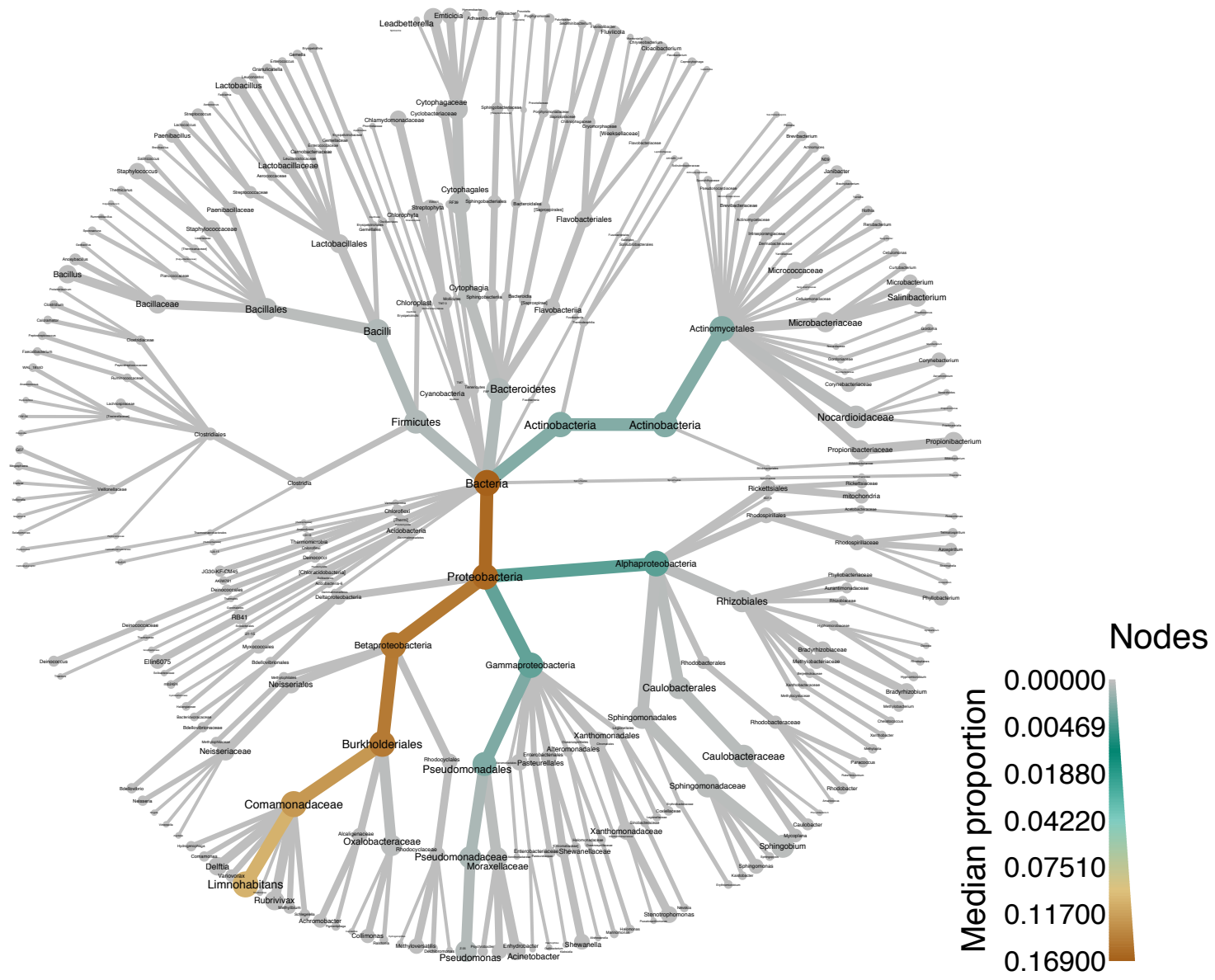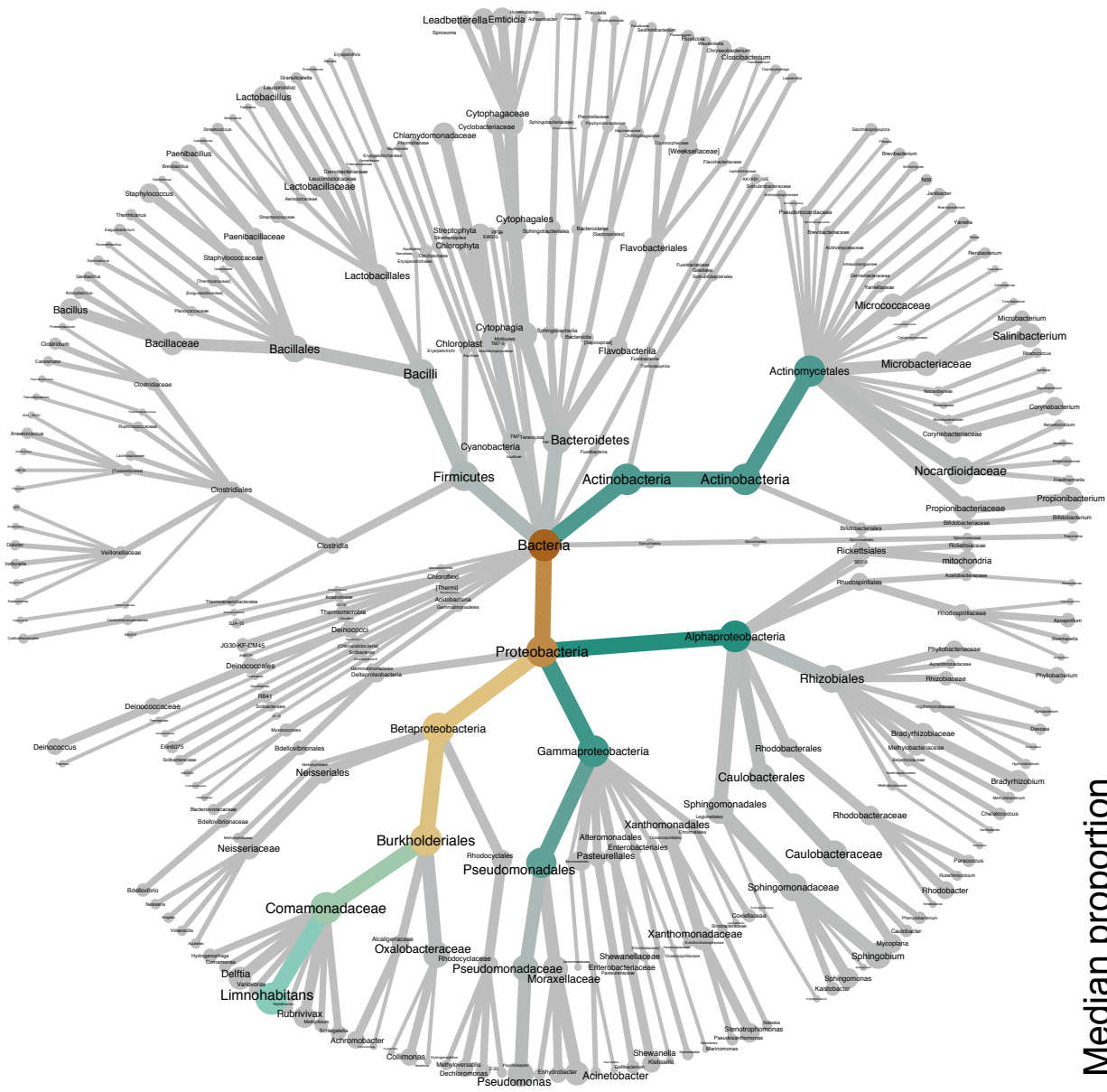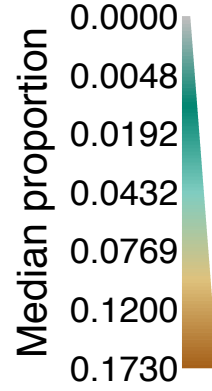
(C)
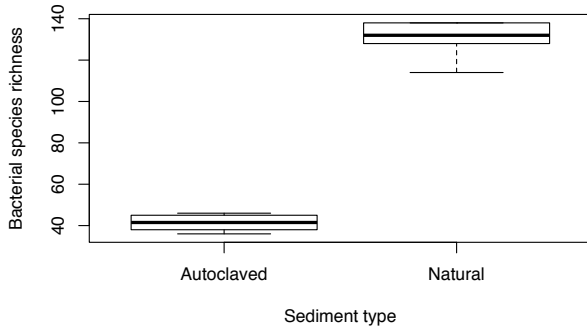
Supplementary Figure 4: Bacterial species richness of autoclaved and untreated sediment. N=6 samples per treatment, 3 each collected at beginning and end of experiment.

Supplementary Figure 5. Average microbiota diversity by clone and treatment using species richness after rarefying to an even sampling depth (A) or inverse Simpson index (B).
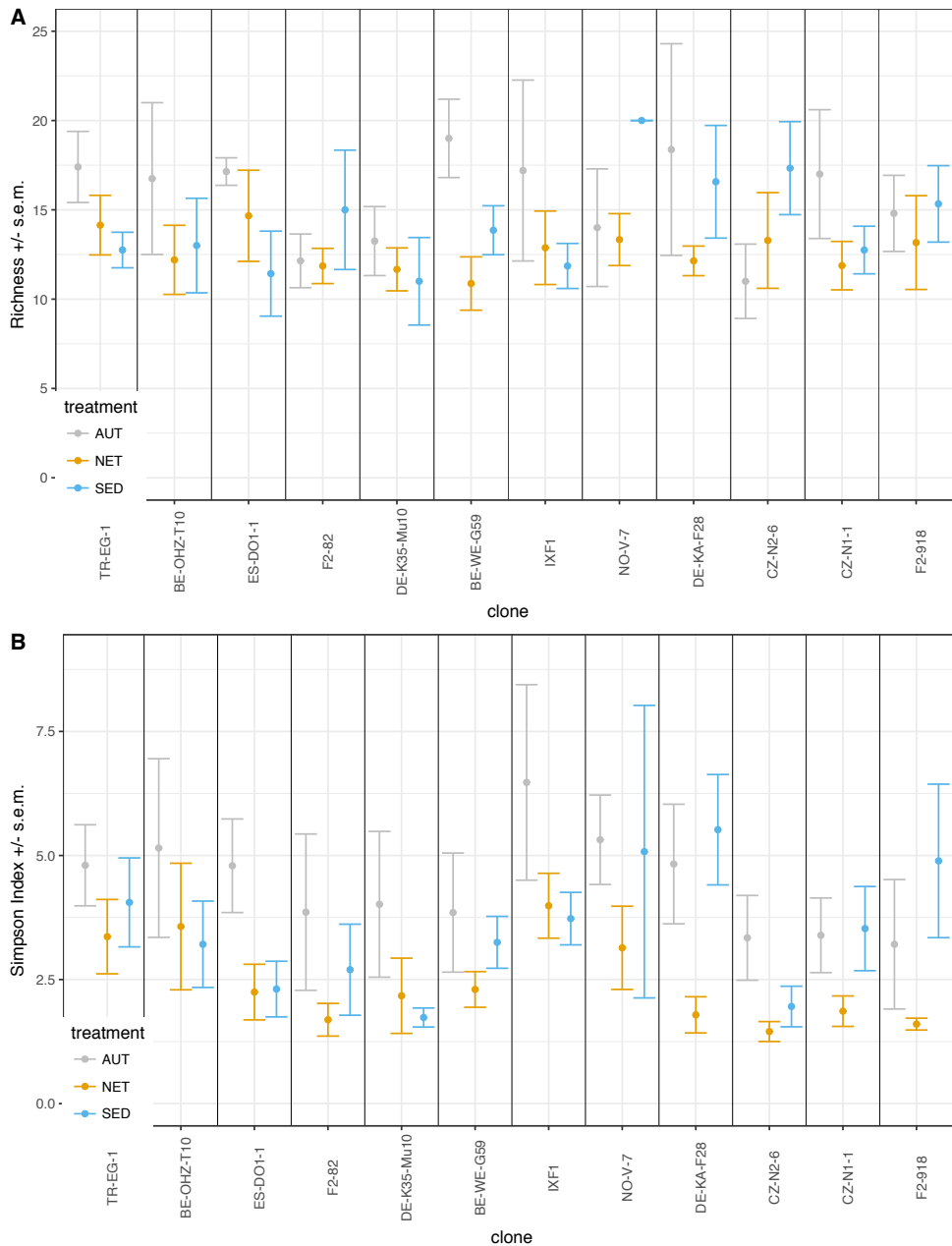
Supplementary Figure 6. Shannon diversity index as a function of the browsing intensity calculated for each individual's jar-mate. Lines represent linear regression; shaded area represents 95% confidence interval.
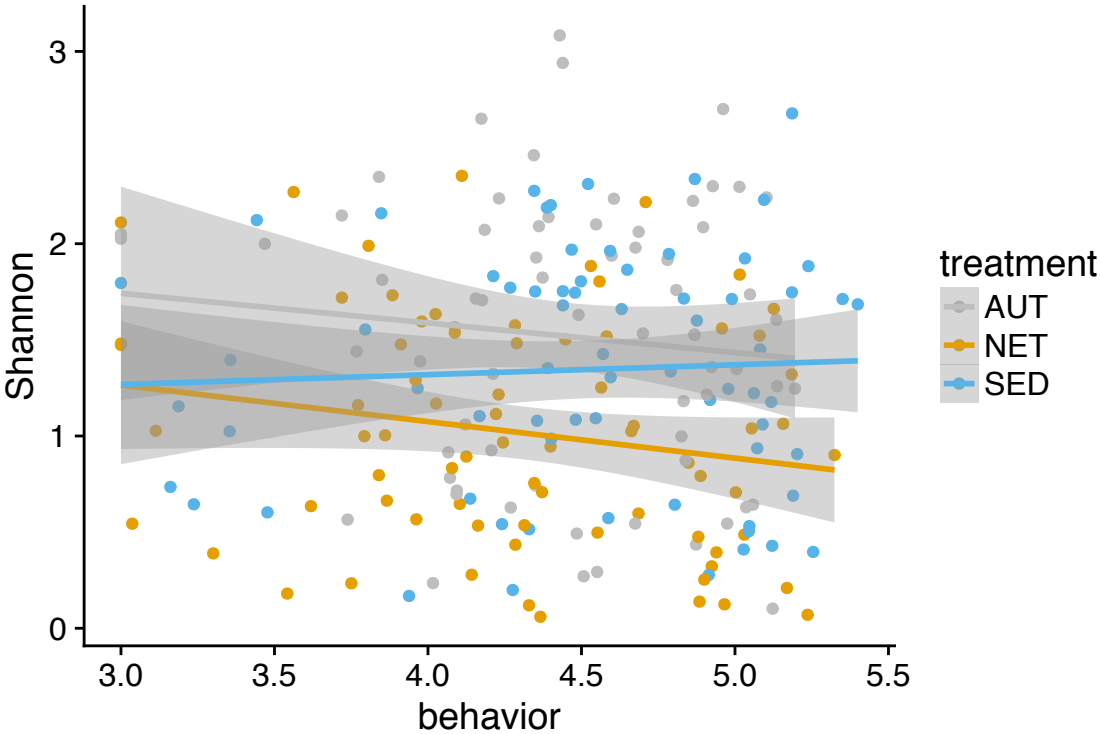
Supplementary Figure 7. Relationship between browsing intensity and proportion of sediment-derived bacteria in the microbiota using jar-mate's browsing intensity as behavior proxy and different thresholds for defining sediment-derived bacteria. (A) Sediment-derived bacteria defined by log2-fold change of 5 or more between autoclaved and natural sediment samples (B) Sediment-derived bacteria defined by log2-fold change of 8 or more between autoclaved and natural sediment samples (C) Sediment-derived bacteria defined by log2-fold change of 10 or more between autoclaved and natural sediment samples.

(A)
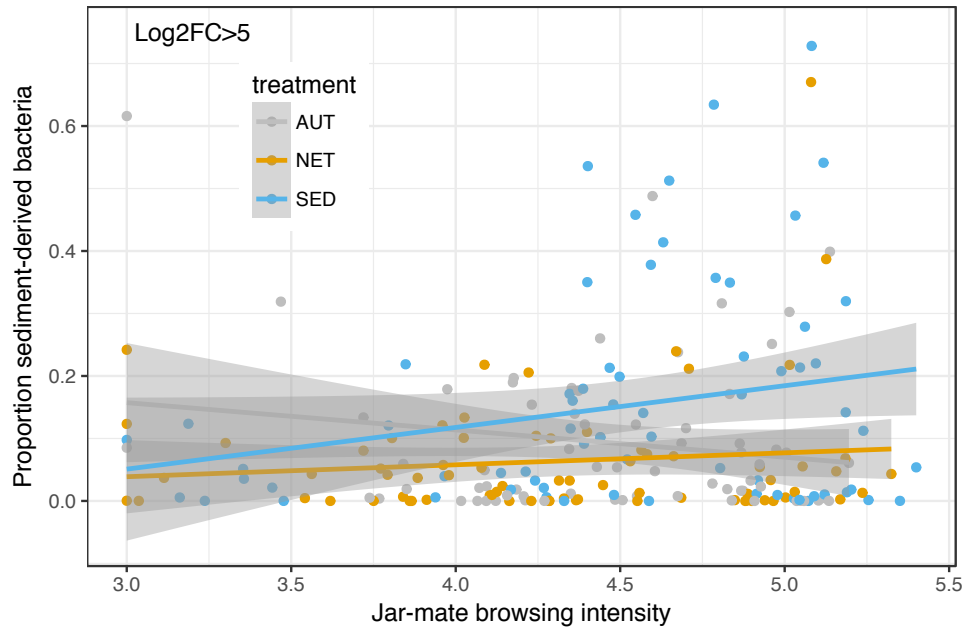
(B)
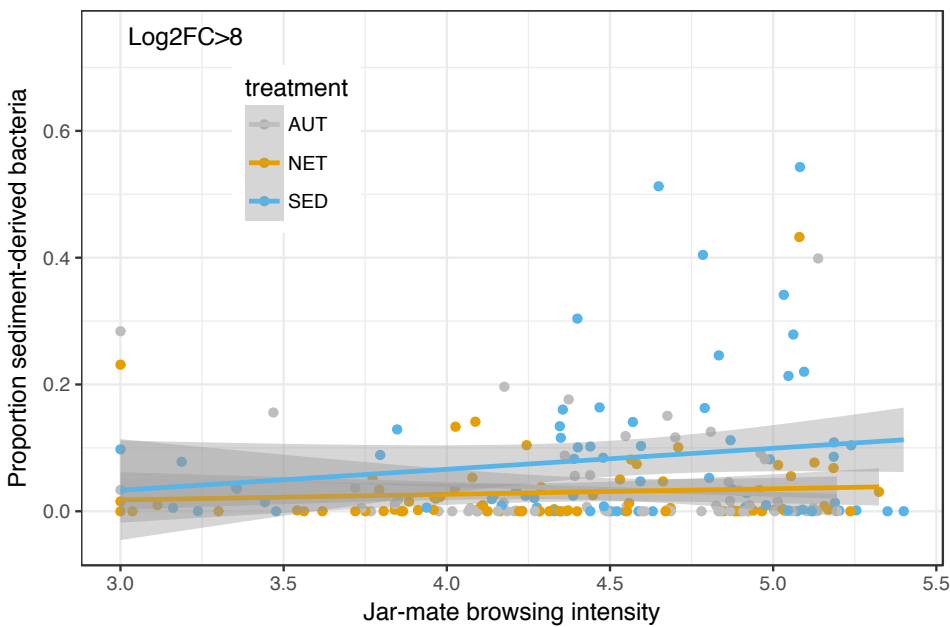
(C)
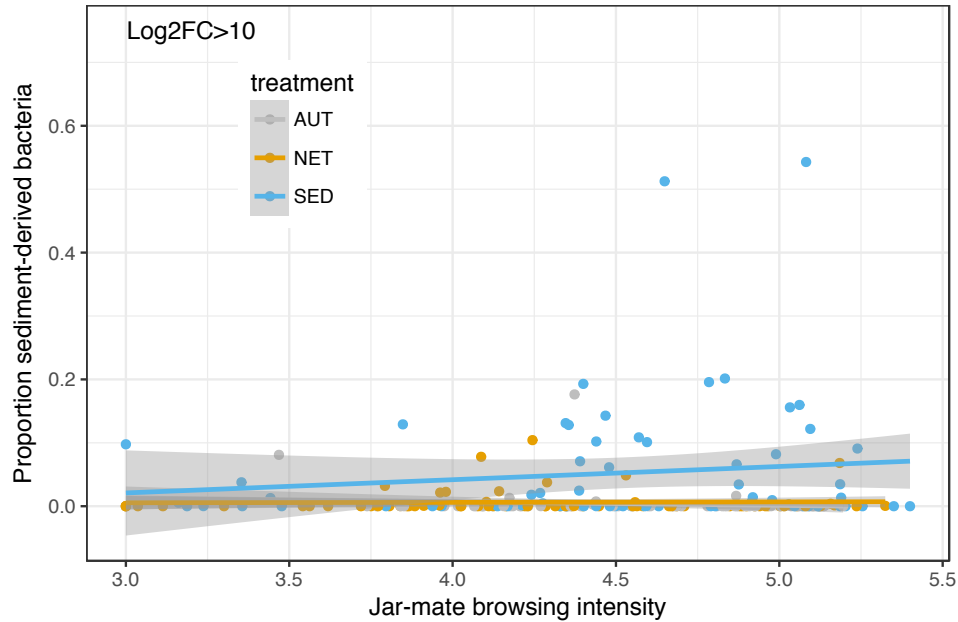
Supplementary Table 1. Effect of treatment (NET or SED) and behavior on Shannon diversity index, using treatment-specific clonal average behavior (A) or jar-mate behavior (B) as behavior proxy.

A. Linear mixed-effects model with clone as random effect

	numDF	denDF	F-value	p-value
(Intercept)	1	124	262.61367	<.0001
treatment	1	124	11.76313	0.0008
trtbehavior	1	124	0.05191	0.8201
cloneavgsize	1	9	2.07427	0.1837
treatment:trtbeh	1	124	4.62296	0.0335
treatment:cloneavgsize	1	124	0.01471	0.9036

B. Analysis of variance

	Df	Sum Sq	Mean Sq	F value	p-value
treatment	1	3.85	3.845	10.490	0.0015 **
jar-mate behavior	1	0.27	0.267	0.727	0.3953
treatment:behavior	1	0.72	0.722	1.969	0.1628
Residuals	138	50.58	0.367		

7. Rethinking "mutualism" in diverse host-symbiont communities

Alexandra A. Mushegian and Dieter Ebert

Abstract

While examples of bacteria benefiting eukaryotes are increasingly documented, studies examining effects of eukaryote hosts on microbial fitness are rare. Beneficial bacteria are often called "mutualistic" even if mutual reciprocity of benefits has not been demonstrated and despite the plausibility of other explanations for these microbes' beneficial effects on host fitness. Furthermore, beneficial bacteria often occur in diverse communities, making mutualism both empirically and conceptually difficult to demonstrate. We suggest reserving the terms "mutualism" and "parasitism" for pairwise interactions where the relationship is largely independent of other species and can be verified by measuring the fitness effect experienced by both partners. In hosts with diverse microbial communities, we propose re-formulating some of the essential questions of symbiosis research – e.g. concerning specificity, transmission mode, and common evolutionary fates – as questions of community ecology and ecosystem function, allowing important biological interactions to be investigated without making assumptions about reciprocity. Understanding the fitness of host-associated bacteria is a crucial component of investigations into the role of microbes in eukaryote evolution.

7.1 Introduction

Serious attention to symbiosis began with the observation by nineteenth-century botanists that lichens were composed of fungi and algae living together and interacting (Sapp 1994). From the beginning, there was disagreement about what kind of relationship the lichen symbiosis represented: some researchers compared the association to a farmer with crops or a master with slaves, and others posited that fungus and alga were providing each other with reciprocal ecological benefits. The latter idea – of two species forming a mutually beneficial partnership – came to be known as mutualism. Mutualistic relationships such as legume-rhizobium interactions have been intensively studied with respect to their emergence, maintenance and co-evolution, but the difficulty of demonstrating a true mutualism – in which effects of the relationship are evaluated for both partners – has been noted repeatedly (Douglas & Smith 1989; Meyer 1924; Wooldridge 2010).

An increasing number of studies provide experimental evidence of animals benefiting from relationships with microbes. Such relationships are often explicitly or implicitly treated as mutualisms despite the fact that in most cases the benefit has only been demonstrated for the host, and is merely assumed for the microbial consorts (Garcia & Gerardo 2014). Many microbes that associate with eukaryote hosts are also found, at least transiently, in a non-host-associated state, for example *E. coli* (human gut as well as soil and water), acetic acid bacteria (diverse insects as well as fruits, flowers and fermented foods), and bioluminescent *Vibrio* (sepiolid squids and seawater) (Blount 2015; Salem et al. 2015; A. E. Douglas 2014a; Wollenberg & Ruby 2012). The only microbial taxa for which the existence of a free-living state can be nearly definitively ruled out are host-dependent, strictly vertically transmitted endosymbionts, which appear to be an exception among the multitude of symbiotic microbes (Ebert 2013). The factors influencing the fitness of beneficial microbes in their free-living versus host-associated state have rarely been quantified and compared. This is understandable given the difficulty of directly observing microbial activities, but correctly

identifying the nature of a host-microbe relationship is required in order to interpret findings with regard to the relationship's evolution. For example, it seems premature to ask how a host-microbiota system prevents the emergence of microbial "cheaters" if it is not yet known whether and how the microbe profits from the association in the first place.

Those systems in which a truly mutual fitness benefit has been demonstrated between animals and microbes, like the squid-*Vibrio* system (Wollenberg & Ruby 2012), tend to be ones involving a relationship between one animal species and one microbial species performing one particular function. In contrast, an increasing amount of research on beneficial effects of microbial symbionts has examined the role of entire host-associated communities, consisting of diverse species, on the host's phenotype and fitness (Chu et al. 2013; Bäckhed et al. 2004; Coon et al. 2014; Broderick et al. 2014; Koch & Schmid-Hempel 2012). We argue that defining the nature of a relationship between an animal host and a diverse microbial community as mutualism, commensalism or parasitism poses not only empirical, but also conceptual challenges. We propose approaching this question in the larger framework of questions in community ecology and the context-dependency of species interactions. Understanding the fitness of microbes in symbiotic and free-living states will help contextualize the numerous studies showing host factors shaping their microbiota composition (Rawls et al. 2006; Bevins & Salzman 2011) by helping to identify whether these patterns represent adaptive partner choice mechanisms or a different kind of community assembly process, and to clarify areas of conflict and cooperation between animals and diverse microbes. This type of information is crucial for understanding the role of biotic community interactions in animal evolution. We highlight the diverse types of interactions that can result in hosts benefiting from microbial communities and the importance of comparing host-associated and non-host-associated lifestyles of microbes.

7.2 Fitness and function

Broadly, investigations into symbiotic relationships can focus on function or fitness. Functional investigations identify and quantify the specific goods and services being exchanged between the partners (for example, by tracing nutrient fluxes or distinctive behaviors), but do not necessarily quantify the resulting fitness increase of each of the partners as a result of the functional exchange, and therefore do not judge the exchange's adaptive significance. In some cases, the fitness benefit of a symbiotic activity might be reasonably assumed from the observation of a function – for example, nectar produced by plants is metabolically costly, yet appears to serve no other function besides rewarding pollinators. Since we do not expect interspecies altruism, it is reasonable to conclude that attracting pollinators increases the plant's evolutionary success. In other cases, the fitness effect of a demonstrated function might not be so obvious. Demonstrating an interaction's net fitness benefit would then require experiments comparing the fitness of organisms with and without a symbiotic partner. Understanding the functional exchange may be necessary to design the correct experiment for evaluating fitness. Studies explicitly investigating fitness effects are much more strongly dependent on the specific ecological context of the system in question, because the fitness benefit of a symbiotic function directly depends on how crucial

the function is in a particular environment. Systems differ in the extent to which this can plausibly vary (Chamberlain et al. 2014; Bronstein 1994), which in turn affects the design of experimental conditions. Whether a demonstration of reciprocal fitness benefits is necessary to call a relationship a "mutualism," or whether demonstrating reciprocal functional exchanges is sufficient to earn that label, can vary depending on the research question being asked; but an understanding of the fitness effects on both partners engaged in a functional interaction is required for posing questions related to the evolution of the relationship.

Both functional and fitness-based investigations into the benefits microbes receive from hosts can be difficult, although technological advances are improving the ability to detect functional exchanges. The existence of host-associated microbes is enough to demonstrate contact and coexistence, but a fitness benefit for the microbial symbiont cannot easily be demonstrated without contrasting the symbiotic state with an alternative lifestyle. Traditionally, discussions have focused on the host as a source of nutrients or of a competition-free environment for microbes, but symbiotic microbes do not universally receive these benefits, and furthermore receiving these benefits does not automatically translate to increased fitness (Garcia & Gerardo 2014). However, animal hosts might also provide other services with potential effects on fitness, such as increased dispersal ability (as vectors) or increased opportunities for genetic recombination. Therefore, at the very least a greater understanding of the ecology and natural history of microbes in their natural habitats—knowledge of the sort that is often intuitive and well-established about their eukaryote hosts—is required to guide investigations into the evolution of these relationships.

Theoretical predictions about the evolution of host-microbe interactions considered broadly – e.g. about the probabilities of transitions between parasitism and mutualism – often hinge on the extent to which the evolutionary fates of host and microbe are tied together. Typically, determining the strength of this association is done through studies of symbiont transmission. Persistent vertical transmission of microorganisms (between parents and offspring) is typically seen as the host and symbiont having aligned evolutionary interests, while horizontal transmission (between unrelated hosts) is considered to have more potential for host-symbiont conflict. The growing understanding that most symbionts, beneficial and harmful, are transmitted both vertically and horizontally has complicated many models of symbiosis evolution (Ebert 2013). The predictions are further complicated when a diverse community of microbial symbionts is considered, because different members of the community might have different fitness effects on each other and on the host, and different dominant modes of transmission. Furthermore, the fitness of symbionts in a free-living state is an important parameter that is often overlooked. Symbiont evolution that occurs in the environment is often only considered relevant insofar as it affects the probability of re-infecting a new host. But in fact the activities and sources of selection on microbes in non-host environments are quite relevant when considering the scenarios in which host-benefiting microbes might have evolved.

7.3 Mutualism is not the only explanation for beneficial microbes

<u>Microbes as very local environmental factors</u>

The term "commensalism" is usually used to refer to situations where bacteria neither harm nor benefit the host, but it could also refer to a situation where bacteria benefit a host but are neither helped nor harmed by it compared to an alternate free-living state. Such a situation could be expected if the benefit a microbe provides is a byproduct of a function that evolved for a purpose unrelated to the host. This is especially plausible in protective symbioses based on secondary metabolic functions, such as detoxification of heavy metals or plant toxins (Senderovich & Halpern 2013; Kohl, Weiss, et al. 2014) or production of defensive compounds against other microbes (Gil-Turnes et al. 1989), which are likely to be beneficial to bacteria regardless of whether they are living with a host or in a non-host environment. The benefit to the host could be coincidental and may not affect the microbe's fitness. The importance of byproduct mutualisms is well recognized (Douglas 2008), but we wish to emphasize that the benefits of byproducts need not be part of reciprocal exchanges (McNally & Brown 2015). This scenario is analogous to the coincidental evolution hypothesis for virulence, which posits that some "virulence factors" are the result of microbial adaptations to non-host environments such as soils, and their effect on eukaryote health is accidental and selectively neutral for the microbe (Levin 1996).

More broadly, beneficial microbes can be seen as being involved in facilitation. This term encompasses a spectrum of beneficial interactions, from commensal chance encounters to specifically co-adapted mutualisms. Facilitation, in this context, means that microbial activities somewhere in the host's internal or external habitat modify aspects of the environment, such as availability of nutrients or concentration of toxins, in a way that improve living conditions for an unrelated species, which is independently evolving in response to the altered environment. Examples of organisms modifying environments in ways that make them more suitable for the survival of others are numerous and fundamentally important, starting with processes as basic and generalized as photosynthetic carbon fixation. A relevant question is whether the facilitative effect of an organism's activity is under selection independently of its original function (e.g., whether protecting one's host against fungal infection results in fitness increases beyond those attained by producing antifungal compounds for one's own purposes). But this question cannot be addressed in the absence of information about variations in fitness of all players involved, particularly obviously about the organism performing the facilitation (Bronstein 2009). Its answer certainly cannot be assumed based on the relative physical location of the host and microbe.

In addition to specific facilitative activities, the omnipresence of bacteria in diverse environments might mean that bacteria are a general feature of environments to which proper physiological function is adjusted (Gilbert 2002; A. E. Douglas 2014b). This idea is supported by the observation that gut and immune system differentiation is induced by host exposure to bacteria, sometimes including nonspecific bacterial components such as lipopolysaccharide (Bates et al. 2006), and underlies a version of the "hygiene hypothesis" that human immune dysregulation results from insufficient contact with environmental bacteria (Macpherson & Harris 2004). These kinds of mechanisms may be of crucial biological importance in the evolution of animals, but do not necessarily require bacteria to experience greater fitness with

the host than without it. This is especially true if the host is responding to the microbes as a developmental cue providing information about environmental conditions (A. E. Douglas 2014b). On the other hand, in some cases there are functional hints that mutualistic exchanges might indeed be occurring in the course of microbe-mediated developmental processes, for example the widespread foraging on host-derived glycans by some beneficial bacterial taxa (Schwartzman et al. 2014) and the presence in human breast milk of specific compounds indigestible by humans but digestible by bacteria that promote gut development (Koropatkin et al. 2012). Depending on whether animals are responding to bacteria in their developmental environment or modifying their environment by increasing the fitness of another organism, the genetic basis of developmental processes would be conceived of differently. In the latter case the evolution of the animal phenotype would be driven by selection on functionally linked animal and microbial genes, whereas in the former case selection would only be acting on the animal genes governing the animal's response to the bacteria in the environment. Therefore measurements of bacterial fitness are required for characterizing units of selection and boundaries of extended phenotypes.

Our conception of host environments as inherently beneficial for microbes may originate with a bias towards thinking about mammalian guts, which are warm, nutrient-rich, relatively long-lasting habitats. The assumption that these are generally preferable conditions needs to be empirically tested, but it is already obvious that the same characteristics are not true of all eukaryotic host taxa. The effects of phylogenetically diverse eukaryote host environments on microbial fitness might be a relevant aspect to study in order to understand the role of microbes in eukaryotic evolution and diversification. For example, it has been speculated that one evolutionary benefit of endothermy is that it provides a thermally stable environment for microbiota (McFall-Ngai et al. 2013). Quantifying the fitness benefit for microbes associated with hosts at the evolutionary transition between modes of thermal regulation would be informative for evaluating this hypothesis.

Microbes as unwilling participants

Another scenario in which hosts benefit from microbes involves hosts capturing and parasitizing or "enslaving" microbial symbionts. Microbes might be expected to be able to evolve means of avoiding or escaping such exploitation relatively quickly, due to their short generation times compared to that of hosts; on the other hand, such avoidance adaptations might not provide a significant selective advantage for microbes if the risk of capture is small in a given environment, as would be the case if the overall encounter rate in the microbial population were low. Therefore, information about generation times and population densities of both microbes and hosts in natural environments would be helpful for informing epidemiological predictions and determining whether modified host-parasite evolutionary models might be appropriate. Indirect evidence of beneficial bacteria suffering a fitness cost from living in a host comes from *Drosophila*, which were found to require an external source of bacteria to sustain their population of defensive symbionts (Blum et al. 2013). That is, the population of beneficial bacteria did not reproduce at replacement rate when associated with the host. A positive population growth rate of bacteria within a host may therefore be an important basic parameter to ascertain.

Shifts between symbiosis and predation may also be common (Lindquist et al. 2005). An endosymbiont of cereal weevils provides specific metabolic products in the early life of its host and is transmitted vertically between generations (a presumed fitness benefit for the bacterium), but the majority of the dense bacteriocyte-associated bacterial population is autophagously digested by the host later in life (presumably benefiting the host as a nutritional source) (Vigneron et al. 2014). Over evolutionary time, there is a one base pair difference between a food bacterium "farmed" by the slime mold Dictyostelium and a non-edible but beneficial strain of the same bacterium – and both strains are typically carried by dispersing hosts (Stallforth et al. 2013). In the same way that human facilitation of agricultural crops can be seen as an evolutionary victory or defeat for the plant lineage, the ultimate effect of this kind of host-association on a bacterium is not obvious. The long-term evolutionary pitfalls of dependence on another species have been noted for both hosts and symbionts (Bennett & Moran 2015; Douglas & Smith 1989; Levin 1996). Understanding the precise short-term costs and benefits for both partners is essential for making predictions about the long-term fate of the relationship.

7.4 A single host and a community of symbionts

Thus far we have seen how few assumptions can be made about relationships even between one host and one beneficial symbiont. When a single host is home to a diverse community of symbionts, characterizing the relationship as parasitic, commensal or mutualistic with the appropriate attention to both host and microbial sides of the interaction becomes even more difficult, if not impossible. The host becomes a site of a whole ecosystem of interspecies interactions. A community might contain detrimental microbial species whose presence is "tolerated" as a side effect of allowing beneficial species to flourish. Even among the beneficial fraction of the community, the fitness benefit might consist of several different functions (e.g. nutritional, defensive, immune-modulating) performed by several different species, the effects of which are frequently non-additive ((Newell & Douglas 2014); see (Afkhami et al. 2014) and references therein). This is important, because the fitness benefit of each microbial function to the host can be dependent on which other microbially-mediated functions are also present, and both conflict and synergy between the effects of different community members are possible. Furthermore, a single function benefiting the host could be the result of the combined activities of multiple bacterial species, for example when a number of different bacteria create an odor profile in a host that affects social or sexual signaling (Theis et al. 2013). As an additional complication, the community might consist of a mixture of species that provide specific benefits (e.g. synthesizing particular metabolites) and nonspecific ones (e.g. preventing infections by competitively excluding pathogens, a function that could be carried out by a wide range of bacteria). Finally, the beneficial effects of some strains might be strongly dependent on their abundance (Cunning & Baker 2014).

Thus, the overall positive fitness effect experienced by a host may come from a number of different species forming a community consisting of members with varying functions and levels of functional redundancy, but their net effect is nevertheless straightforwardly evaluated by measuring the fitness increase experienced by the single host. But there is no

equivalent way of measuring the net fitness benefit experienced by the bacterial community as a result of host-association, since the specific community in question can only be a unit of selection when it is associated with a host, and it does not exist as such independently. One could examine each member of the community individually, measuring the fitness increase it obtains from the host compared to a free-living state, but a conclusion of mutualism or parasitism for that particular microbe might then be inaccurate from the point of view of the host, because the effect on host fitness of a single type of symbiont might be completely different from the effect of the total community. In addition, focusing on each microbial community member in isolation risks ignoring one of the most important and potentially costly challenges on the host's side: the need to discriminate between harmful and beneficial microbes.

Furthermore, the benefit of host-association to any particular bacterial strain might depend on the other bacterial species present, in which case the cost or benefit of host-association cannot be strictly attributed to the host. Sharing a host might be beneficial because it brings different species of cross-feeding bacteria into physical proximity (Russell 2001). On the other hand, host-association might bring a bacterium into close proximity with a strong competitor that it would not otherwise encounter. The nature of the relationship might change drastically with the addition or subtraction of another member of the community (Hay et al. 2004). The ecological concept of "facilitation cascades," wherein a positive relationship between two species is mediated by a third (Bell et al. 2014), may be relevant. Likewise, outside the host, microbes do not occur alone. What should be considered the reference state? An estimated 80% of free-living environmental bacterial species have metabolic dependencies on other organisms, including other bacteria (D'Souza et al. 2014); in other words, community context is important outside the host as well (Zelezniak et al. 2015). Furthermore, possible alternatives to living in a host might include surfaces, particles, or other host species, in which new microbe-eukaryote or microbe-microbe interactions might occur (Frommlet et al. 2015) – how should these be included in the fitness comparison? Finally, the requirements of living in a host versus in the external environment might be so different that the bacterium adopts different phenotypes or "life history" strategies in both (e.g. entering dormant phases), raising the question of which phenotypes or strategies should be compared and over which timescales.

Studies examining specific pairs of hosts and microbes are valuable in their own right for detecting functions and exchanges. But in cases where we observe a bacterial community whose aggregated activities affect host fitness, what we are observing is a community-level phenomenon that can often not be approximated by quantifying the fitness effects of isolated pairwise interactions. This does not necessarily mean that community-level selection is the main driving force behind these phenomena. Ecological communities can have reproducible dynamics and predictable outcomes for their members without being the result of Darwinian selection on the overall trajectory (Dawkins 2004). The "hologenome theory of evolution" (Zilber-Rosenberg & Rosenberg 2008) elucidated the key insight that microbes can serve as a form of heritable adaptive variation in host phenotypes, but this does not mean, as the authors imply, that "holobionts" (combined hosts+symbiotic communities) are necessarily the most relevant unit of selection. First, transmission of microbial communities between host

generations is required for this conception to be valid. But even if this condition is met, we must examine whether selection on a host in fact also selects the host-associated bacteria, or whether bacteria can persist and reproduce in their free-living state regardless of what happens to the host. Put another way: How often is transmission of microbial genes accompanied by transmission of host genes? In the case of strictly vertically transmitted symbionts, this is obvious – in the absence of a free-living state, selection on endosymbionts is embedded in selection on the hosts. In other taxa, the impact of higher-level selection on microbes can only be assessed by quantifying the strength of selection in both the free-living and the host-associated state of each microbial community member.

These considerations bring back the fundamental question of the extent to which the evolutionary fates of host and multiple species of microbe are interrelated. Selection on the host should act to maximize the beneficial effect of its symbionts on itself (Dethlefsen et al. 2007). A consistently beneficial microbial community can be maintained either through selection on host traits that select beneficial bacteria from the environment, or by selection on mechanisms of vertical transmission that directly pass beneficial symbionts to the host's offspring (Sanders et al. 2014). Both processes have been shown to operate, sometimes on different microbial taxa, in diverse eukaryote species (Funkhouser & Bordenstein 2013; Ebert 2013; Koch et al. 2013). These processes can conflict or not with the best interests of the individual microbial species involved. A crucial point is that multiple bacteria in a community might have a common evolutionary fate while they are associated with a host – if they are co-transmitted to the next generation of the host – but have entirely independent forms of selection acting on them when they are outside the host or on different hosts. Therefore there is no meaningful way in which a collective microbial community can be considered a unit of selection both inside and outside the host, and therefore the beneficial community cannot be said to be "mutualistic."

7.5 Communities within communities

We propose that the terms "mutualism" and "parasitism" should be restricted to pairwise interactions between a single host and single microbe in which the outcome of the interaction for both host and microbe is both i) experimentally verified and ii) not strongly influenced by the community context under ecologically realistic conditions. Individual strains of bacteria within a microbiome might well prove to fit this description after appropriate experimentation, but a whole microbiome does not. For understanding complex host-microbiota interactions, many of the questions that have traditionally interested symbiosis researchers can be addressed while remaining agnostic as to the reciprocity of the interaction.

Instead, host-microbiota symbioses should be treated as special cases of ecological communities within the larger ecological community of whatever ecosystem they inhabit, in which the activities of all organisms may be experienced by others as either ecosystem services or environmental challenges. This allows one more fully to address the ways in which microbes and animals affect each other's fitness and subsequent evolution. Approaches can be borrowed from ecosystem ecology addressing how the composition of a community affects some aspect of ecosystem functioning. Importantly, the aspect of interest might, for example,

be proliferation of host or microbial biomass or genes, or activation of some physiological pathway of the host; researchers can focus on their organism of interest with an understanding that they are part of a multifactorial system. Experimental studies will necessarily involve simplifications, but we believe that the appropriate simplifications for the purpose of understanding fitness in complex communities involve controlling environmental variables or focusing on a limited number of parameters. Neither reducing communities to pairwise interactions nor treating an entire microbial community as a single unit is a universally appropriate approach.

Research questions can be asked afresh from the point of view of community ecology and ecosystem functioning:

Diversity-function relationships – which functions and properties of the host-microbe community are influenced by the species richness, diversity, and composition of the microbial community? Are certain combinations of host and microbial species more stable or long-lasting than others?

Approaches: Experimental manipulation of microbial communities, with quantification of effects on parameter of interest (e.g. host or microbe biomass). Long-term field studies of populations of hosts; sampling microbiota both over an individual's lifetime and in a host population over time. Relevant examples: (Maurice et al. 2015; Bell et al. 2005; Kane et al. 2011)

Specificity and redundancy of ecosystem functions – to what degree can certain functions be only performed by certain organisms? Which costs or benefits of organismal activities accrue only to particular other species? Is this specificity the result of selection on one or multiple organisms?

Approaches: Identify goods and services being produced by one organism and used by others; evaluate their specificity and patterns of utilization within the community (e.g. consumer-resource networks). Look at patterns of selection on genes related to the interaction. How do they differ in different settings? Relevant examples: (Dorrestein et al. 2014; Lee et al. 2013; Hom & Murray 2014; Baker et al. 2015)

Resilience after disturbance – are certain combinations of hosts, microbes, and their functions reliably restored after drastic environmental changes?

Approaches: Experimental perturbations of communities (both host-associated and environmental), with "natural" pools of inputs available to restore them, and comparison of ecosystem functioning before and after disturbance. Relevant examples: (Antonopoulos et al. 2009)

Quantification of vertical and horizontal transmission – which microbes are inherited by host offspring? How stable or reliable is this inheritance in different settings? Which symbionts are meaningful sources of heritable variation in host phenotype?

Approaches: Examine transfer of microbes between host parents and offspring as well as transmission into the environment. Particular attention to long-distance dispersal propagules may be informative for evaluating strength of association between several species. Identify environmental factors that might affect transmission (e.g. premature separation of

parents and offspring). Experimentally evaluate effects of differently transmitted symbionts on host phenotype. Related examples: (Wornik & Grube 2010; Henry et al. 2013)

Population genetic structure and speciation of host-associated versus free-living microbes – Is there genetic differentiation between the free-living and host-associated fraction of a particular bacterial species in the same habitat?

Approaches: Sample similar bacteria from hosts and environments; use population genetics methods to evaluate whether they form distinct populations or phylogenetic lineages. Relevant examples: (Luo et al. 2011)

Source-sink dynamics of symbionts – Do particular microbes colonize hosts from the environment and decline in number within hosts, or do they proliferate mainly inside hosts and disperse into the environment?

Approaches: Use population genetic methods of tracking lineages, experimentally "tag" microbes to track their dispersal and division, or examine the effect of introducing a host with microbiota into a sterile environment. Relevant examples: (Sokurenko et al. 2006; Wong et al. 2015)

Common goals and fates – how strongly do changes in environmental conditions affecting hosts consequently affect the microbial population, and vice versa?

Approaches: Evaluate how composition of both non-host-associated and host-associated microbial communities changes with alterations in the environment. Relevant examples: (Wong et al. 2015)

Limiting factors – are the factors that limit the ecological niche of hosts and microbes the same, or different? What are the consequences of being limited to the ecological range of one's partner?

Approaches: Identify limiting factors for symbiotic and free-living hosts and microbes. Evaluate the transmission success and environmental persistence of microbiota from ecologically stressed hosts; conversely, assess performance of hosts under conditions limiting to microbiota. Relevant examples: (Wernegreen 2012; Kohl, Amaya, et al. 2014)

These questions are already being addressed by researchers who study symbioses. The examples given here are only a tiny fraction of the diverse approaches to studying symbiosis that currently exist. We believe that formulating these questions explicitly in the context of communities, paying particular attention to microorganisms both in and out of host-association, provides ways of avoiding unfounded assumptions and leaves open more avenues for understanding the consequences of the relationships of diverse organisms.

7.6 Conclusions and outlook

The field of symbiosis research has come a long way since the first symbiotic interactions were described in the second half of the 19th century. The focus traditionally was on pairwise interactions: one host and one symbiont species. In the cases studied, the functional relationship between the two partners was either so strong or so clearly definable that associations with other species were either not influencing the picture in a significant way, or were doing so in a way that was clearly distinguishable from the effect of the primary symbiont. While the symbiont was generally assumed to receive a net benefit from the

association, the hosts may have had a net cost (parasitism) or a net benefit (mutualism). Here we suggest limiting the use of the terms mutualism and parasitism to these sorts of cases: species pairs with evidence for the costs or benefits in both partners.

The advent of new technologies changed the picture drastically, as hosts – with or without a dominant primary symbiont – were found to harbor up to thousands of species of microbes associated with them. Communities with overall beneficial effects on their host were called mutualistic, even as it was neither clear what proportion of the microbes in the community were providing the benefits, nor which of the microbial community members benefited from being host-associated. While it is possible to study individual members of such a microbial community and apply the traditional concepts for symbiotic relationships, it may not be meaningful, given the many possible interactions one microbe species has with the many other microbes, the host, and the non-host environment.

We suggest applying the toolbox from community and ecosystem ecology, which is well equipped to deal with such complex scenarios. Crucial to these goals will be reconsidering the diverse effects experienced by hosts from the microbes' point of view. How unique or desirable are different hosts as habitats? Which microbes experience consequences, positive or negative, for the effects they have on other species? How important is host survival and reproduction for the persistence of different microbial lineages performing different activities? If we are to seriously take on the task of evaluating the role of microbes in host evolution, we cannot avoid delving into the rich ecosystem of diverse interactions taking place at the host-microbe interface. In turn, the replication across time, space, and taxa provided by host-associated microbial community datasets can provide powerful tools for testing principles in community ecology, for example about community assembly rules, ecosystem stability, and niche construction. Host-microbiome associations may not be mutualisms, but they are certainly dynamic models for understanding eco-evolutionary feedbacks in biological communities.

8. Conclusion

The work in this thesis provides a foundation for understanding the full complexity of the types of host-microbe interactions that can occur in aquatic settings. The key conclusions are: i) bacteria can benefit *Daphnia* through multiple physiological pathways, the effects of which are relevant in different environmental conditions, and ii) bacteria that are beneficial to *Daphnia* are most likely common and widespread, as befits a host with recurring dormant stages. Taken together, these results suggest that microbial features of the environment might be relevant to take into account when considering factors influencing local adaptation. Manipulation of *Daphnia*-microbiota relationships can provide a powerful tool for investigating basic questions about the implications of living in constant exposure to bacterial influences.

8.1 Results in context

We provide the first report of the consequences of raising *Daphnia magna* in the complete absence of bacteria. Across multiple experiments, we show that axenically grown *Daphnia* suffer from slowed growth, early mortality, and low reproduction. This was later confirmed by additional studies using independently developed methods (Callens et al. 2015; Peerakietkhajorn, Tsukada, et al. 2015; Peerakietkhajorn, Kato, et al. 2015). While the main effect of absence of bacteria is consistent, differences in the observed effects between experimental conditions are instructive. Responses to differences in dietary conditions provide the first clue to the adaptive roles of bacteria. In both Callens et al and our study, the same food source (*Scenedesmus*) is used, and absence of microbiota affects all of the life history traits examined; in particular, bacteria-free animals barely grow larger than neonates, regardless of the amount of *Scenedesmus* they are fed. Bacteria-free animals grow larger on a diet of yeast, but this is not because yeast is an inherently superior diet, as evidenced by the fact that conventionalized animals show the opposite pattern. Taken together, these results suggest the following interrelated conclusions: i) the primary beneficial effect of bacteria is probably not solely because bacteria are food, because in this case we would expect that bacteria would provide the greatest relative benefit at low food levels, rather than the strong non-additive effects we find; ii) the limiting factor for growth on any diet depends on the presence of microbiota, and there is apparently a particularly strong need for microbiota on a diet of *Scenedesmus* and iii) a strong effect on growth exists that is independent of the diets tested. A different set of studies by Peerakietkhajorn et al, using a diet of *Chlorella*, found no effects of microbiota on body size or survival, but a strong effect on reproductive success, with high rates of inviable and malformed embryos under bacteria-free conditions. There are two subtly different interpretations of this finding: either bacteria-free conditions make animals unable to produce viable offspring, or bacteria-free conditions make animals produce offspring that are themselves highly sensitive to bacteria-free conditions. This distinction acquired new

relevance with our observation that bacteria positively affect the viability of resting embryos under elevated temperature conditions.

In contrast to standard temperature conditions, where bacteria had little to no effect on embryonic development, resting eggs developing under warmed temperature conditions exhibited a strong difference in successful development from gastrulae into free-swimming neonates depending on whether bacteria were present. Absence of bacteria resulted in a lower proportion of resting eggs successfully developing, with a correspondingly higher rate of developmental abnormalities arising at different stages. Live bacteria administered at a sufficiently high dosage were required to have this protective effect, and there appeared to be some difference in efficacy between different bacterial strains. Since there was no organic material present to sustain microbial metabolic activity other than the embryos themselves in these experiments, we think it is somewhat more likely that the effect is caused by a direct interaction between bacteria and embryos than by bacterial modification of the environment independent of the embryo, though either or both scenarios might be relevant in ecological settings. Given the diversity of phenotypes seen among unsuccessfully developing eggs, we speculate that the effect of bacteria is to stabilize regulatory processes, possibly because of the conserved signaling pathways underlying both immune processes and other aspects of development; regulatory hormones such as ecdysone may be involved. Although the immune system has not yet developed at this stage of *Daphnia* development, the common biochemistry underlying immunity and regulation of development may mean that basic developmental processes are sensitive to the presence of bacteria, and become more so in the presence of stress; this may be relevant in multiple animal systems. We did not find an effect of bacteria or temperature on the development of parthenogenetic eggs of three different genotypes, meaning the effect is either related to the process of diapause itself, or that it is that it is significantly lessened in eggs produced by healthy, well-fed, lab-reared conventional parents in general or these genotypes specifically. Given the range of genotype-specific responses of *Drosophila* phenotypes to bacteria, all of these possibilities deserve further investigation.

Based on all these observations, we hypothesize that there are at least three, possibly distinct, mechanisms by which bacteria benefit *Daphnia*: i) homeostatic regulation during embryonic development ii) assistance with digestion of plant material, and iii) promotion of growth and general health during the juvenile stage through adulthood. We further hypothesize that i) is a type of effect related to conservation of protective stress responses, ii) is related to exchange of goods and services, and iii) might be a result of a combination between beneficial microbial activities and host physiological responses to microbes.

These experiments were conducted using the extreme state of totally bacteria-free conditions. However, the observation that not all bacteria have equivalent effects coupled with the observation of both general and environment-specific effects of bacterial absence suggests that not only absence of bacteria, but variation in bacterial

community can influence *Daphnia* phenotype. How selection acts on this phenotypic variation depends on modes of transmission.

The experiments in Part I were conducted without regard to the real transmission dynamics of microbiota in natural settings, instead simply directly exposing animals to the bacteria of interest. Part II focused on special cases of vertical and horizontal transmission related to special ecological features of *Daphnia*: diapause and browsing behavior. The first study attempted to determine whether beneficial bacteria could be vertically transmitted through diapausing stages. We found that although some beneficial bacteria (that improve resting egg development and juvenile growth) appear to be vertically transmitted, they are not sufficient on their own to restore a normal phenotype compared to a bacteria-free state, but the addition of external bacteria associated with the resting stage was sufficient. Thus, although we do not find evidence that *Daphnia* and specific beneficial microbiota are evolutionarily linked through reproduction over long timescales, they may be ecologically linked through co-dispersal, with the animals likely influencing the composition of the bacterial community after emergence from diapause. Parallels to this scenario are found in plant systems. As a first attempt to evaluate the mechanisms by which *Daphnia* genetic variation can affect bacterial exposure and microbiota acquisition, we conducted an experiment using *Daphnia* clones exhibiting genetically variable sediment browsing behavior. The effect of preventing access to bacteria-rich sediments was strongest in genotypes typically exhibiting high browsing activity, suggesting that genetic variation in behavior could mediate some genotype and genotype-by-environment interaction effects on the bacterial microbiota to which an animal is exposed over the course of its life.

Across experiments, sources of beneficial bacteria included adult *Daphnia*, the nonsterile laboratory environment, pure cultures of isolated *Daphnia*-associated bacteria, and bacteria associated with the shells of long-term refrigerated resting stages. Thus, although not every bacterial strain has an equivalent effect on *Daphnia* fitness, we conclude that beneficial bacteria for *Daphnia* are not rare, nor are they necessarily dependent on *Daphnia*. A high prevalence of potential bacterial associates is the most plausible explanation for *Daphnia*'s high dependency in the apparent absence of reliable transmission mechanisms; furthermore, given the types of effects observed, the dependency might in fact *result* from the high historical prevalence of bacteria that interact with *Daphnia* and the accumulation of mutations that are deleterious in their absence. Studies of the effects of *Daphnia* on bacterial fitness and function are required to complete the evolutionary picture.

8.2 Limitations of the study system

Axenic animals are a powerful tool for exploratory studies of host-microbiota interactions. However, they have important limitations that should be taken into account when designing studies.

First, in order to maintain axenic conditions, numerous additional factors that might affect animal physiology must be altered. Food must be autoclaved or grown axenically, both of which affect its nutritional quality. Autoclaved medium may precipitate or require additional aeration, and animals must be kept in closed containers with air exchange through a small, membrane-covered opening. All of these factors may meaningfully affect animal functioning independently of bacterial influences, and this might need to be taken into account depending on the question being asked.

Second, although experiments control for the effects of antibacterial agents by re-infecting antibiotic-treated animals with bacteria, any off-target effects of antibiotic treatment might still complicate interpretation of results. Bacterial effects that compensate for off-target antibiotic effects are biologically valid bacteria-mediated benefits, but may make it more difficult to isolate other beneficial functions. The observation that decapsulated resting eggs tend to have very few bacteria associated with them and display a similar microbiota-free phenotype in sterile conditions may be useful in this regard.

Third, the differential mortality and asynchronous development between bacteria-free and conventionalized animals means that detailed comparisons can only be made between very young (and accordingly very small) animals. This and the previous factors make it a crucial priority to identify the functional roles of *Daphnia* microbiota in order to more directly manipulate microbiota-related traits of interest and assay more specific phenotypic traits.

8.3 Outlook: "Further research is needed"

Daphnia promises to be a powerful model for investigating how not only resistance to infection, but also tolerance of and cooperation with microbes are fundamental processes that shape animal development and evolution. In particular, resting eggs both provide a useful experimental feature and provide a great opportunity to examine effects on genetically diverse populations, ask questions about regulation of embryonic development in diverse ecological contexts, and relate animal and bacterial biogeographic patterns. Studies examining and manipulating microbial communities can add an additional dimension to the established understanding of *Daphnia* phenotypic and ecological diversity. The experiments presented here represent a starting point and have generated several salient research questions.

Functional studies

Effect of bacteria-conditioned medium on embryonic development. Can the beneficial effect of bacteria on development under warm conditions be achieved by exposing eggs to medium previously "conditioned" by occupation with bacteria and eggs? If so, a secreted bacterial factor may be responsible and could be identified.

Effect of microbiota on gut tissue integrity. Adapt the "Smurf" assay used in tests of *Drosophila* gut permeability (Vijendravarma et al. 2015) to see if presence of bacteria affects the development of the *Daphnia* gut ephithelium.

Bacteria and water quality. Test whether living in a sterile chemostat or in water containing a charcoal filter affects survival of bacteria-free *Daphnia*, in order to determine whether microbiota are involved in e.g. nitrogenous waste recycling.

Effect of maternal nutritional status on sensitivity of embryos to bacterial status. Induce ephippia production in well-fed and starved mothers using hormonal cues. Test whether these eggs differ in their hatching rates under warm conditions in the presence and absence of bacteria. If possible, perform the same experiment with parthenogenetic eggs from well-fed and undernourished mothers.

Ecological studies

Habitat suitability and local microbial ecology. Do habitats with abiotic characteristics suitable for *Daphnia* (pH, salinity, etc) tend to harbor bacterial communities that have a positive effect on *Daphnia*? Conversely, do unsuitable environments tend to have unsuitable bacteria?

Effect of microbiota-Daphnia association on ecosystem processes. How do bacteria affect nutrient cycling or other environmental changes when they are free-living versus associated with *Daphnia*? How does the microenvironment around a microbiota-carrying *Daphnia* differ from that of an axenic *Daphnia*? What feedbacks exist between *Daphnia*-mediated and bacteria-mediated changes in the environment?

Evolutionary studies

Sex-specific effects of microbiota on health. In *Drosophila*, the effects of microbiota on various nutrition-related traits are sex-dependent. *Daphnia* offers the advantage of a system in which sexes are genetically identical, and it has also been hypothesized that immune traits should vary between the sexes. Test whether the magnitude of the beneficial effect of microbiota differs between males and females.

Effect of microbiota on embryonic development in heat-adapted populations. We predict that the effect of microbiota on regulatory and developmental processes will be strongest under conditions different from ones to which the population is adapted. Therefore, it would be interesting to see whether the beneficial effect of microbiota on development under stressful conditions is lessened in populations that regularly experience elevated temperatures (e.g. in shallow temporary pools.)

Evolution in response to the activities of microorganisms is both a unifying theme of biology, and also contributes to the diversity of species, functions, phenotypes and adaptations that we see in natural settings. *Daphnia* and the microorganisms with which it interacts in diverse aquatic environments provide a useful case study these diverse and dynamic consequences.

Appendix 1: Methods and considerations for experiments with bacteria-free *Daphnia*

Raising bacteria-free *Daphnia magna* from resting eggs

1. Keep ephippia in the dark at 4 degrees C until needed.
2. Collect resting eggs from ephippia. This can be done up to a day before setting up the experiment. Use two pairs of forceps to open ephippia under a dissecting microscope. Use a Pasteur pipette or very gentle handling with the forceps to transfer eggs to a tissue culture plate filled with chilled ADaM. Placing one egg per well allows you to keep track of how many eggs you've collected.
3. Wrap the plate of eggs in aluminum foil and place it at 4 degrees C until ready to treat the eggs.
4. UV-irradiate the sterile cabinet for 30 minutes.
5. Using a Pasteur pipette, place eggs with liquid into 1.5 or 2 mL Eppendorf tubes, as many eggs per tube as you want to treat in one batch.
6. Place tubes at 4 degrees C until ready to treat; lay tubes horizontally so that eggs are in one layer in the liquid rather than piled on top of each other.
7. In the sterile cabinet, use a sterile Pasteur pipette to remove storage liquid from tube of eggs. A small amount of liquid may remain; better to leave this rather than risk damaging the eggs with the pipette or accidentally discarding them.
8. Immediately add 500-1000 ul of household bleach (5% sodium hypochlorite).
9. Invert tube gently at least 10 times to make sure all sides of all eggs are exposed to bleach. Expose for 5 minutes.
Shorter exposure times and lower sodium hypochlorite concentrations will almost certainly also be effective at surface-sterilizing resting eggs; the high concentration and time used here is intended to be conservative with respect to ensuring axenicity.
10. Carefully remove bleach from tube with a sterile Pasteur pipette.
11. Immediately add 1 mL sterile ADaM or water and invert tube to rinse.
12. Remove ADaM with a sterile Pasteur pipette and repeat washing step for a total of 2-3 washes.
13. If all eggs in the treatment batch are to be transferred to the same container for hatching, use a sterile Pasteur pipette to transfer them directly from the tube into the container (bottle, Eppendorf tube, or tissue culture plate). If the eggs are to be divided into different locations (e.g. if they are to be placed in individual wells of a 96-well plate) it is easier, and carries less risk of damaging eggs, to first transfer all eggs into a wider shallow container of sterile ADaM (e.g. a well of a 6-well tissue culture plate). From there eggs can be transferred to desired locations with a sterile Pasteur pipette.
14. Add to hatching containers any bacteria to which you wish to expose developing eggs, either from a suspension of homogenized adult Daphnia, or from a pure culture.
15. Place eggs in hatching location. It is difficult to reliably guarantee hatching of resting eggs; the exact cues required may vary between different populations, and hatching success may vary according to factors such as age or quality of the eggs. However,

exposing eggs to strong light appears to be an important cue in inducing hatching. Presence of bacteria can affect hatching success; generally this effect is reduced at 20 degrees.

16. Once the hatchlings are freely swimming in their hatching container (the length of development time varies, but is typically around 3 days at 20 degrees C), transfer them with sterile Pasteur pipettes to whatever container they will be reared in, e.g. bottles closed with a 0.2 micron filter lid containing 80 mL autoclaved ADaM. Feed with sterile algae, either fresh or autoclaved. If using autoclaved algae, vortex the autoclaved algae suspension first for ~15 minutes to disaggregate clumps of cells formed during autoclaving. Avoid using a repeat pipettor to feed multiple jars, as splashing from jars onto the pipette tip is a route of cross-contamination.

17. Add more algae to the jars in the course of experiment as needed. Open jars for feeding in the sterile cabinet. Frequent transfers into fresh sterile jars helps reduce the effect of uncontrolled bacterial growth on uneaten food in jars, but may not be feasible depending on sample sizes.

18. A frequent problem is the introduction of an unknown lab pest (a filamentous bacterium thought to be *Sphaerotilus* sp) in supplements of homogenized lab *Daphnia*. Large populations of this bacterium can grow even from *Daphnia* from apparently uninfested cultures. Thoroughly aerating ADaM by shaking prior to the experiment may reduce this risk, but this has not been systematically tested.

Appendix 2: Miscellaneous observations

Reversibility of bacteria-free *Daphnia* phenotypes

We wished to determine whether *Daphnia* living without a microbiome for a period of time would regain normal function once restored to nonsterile conditions, or whether the microbiota-free phenotypes became permanent after some time period. Bacteria-free and conventionalized animals were set up as described in previous studies, kept in sterile jars and fed sterile food. On the first, third, sixth, ninth and fifteenth day of the experiment, a fraction of the bacteria-free and conventionalized animals were transferred to jars filled with nonsterile ADaM and exposed to the laboratory environment. Animal body sizes were measured on the day that they were transferred to the nonsterile condition; all animals in the nonsterile condition were measured at these time points as well to keep track of growth.
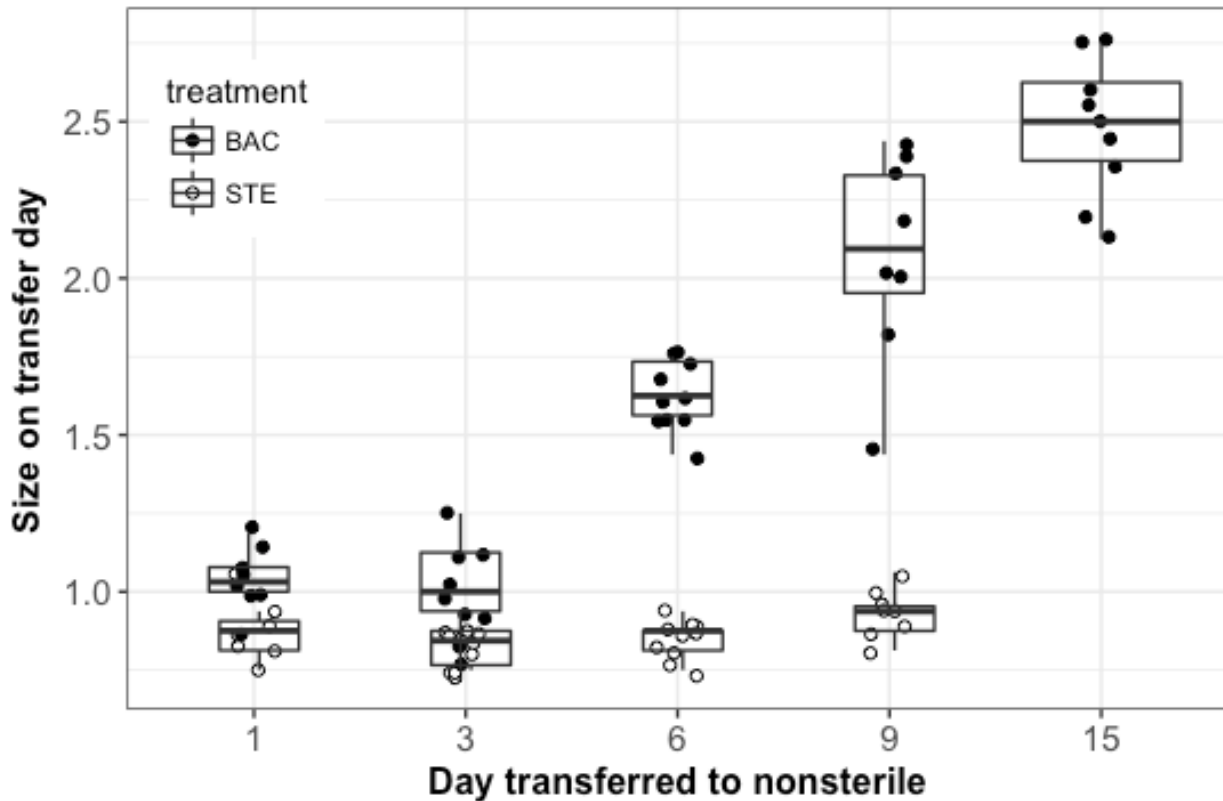
Figure A1. Body sizes (in mm) of bacteria-free (STE) and conventionalized (BAC) animals on the day of transfer from sterile to nonsterile conditions. The body sizes shown for animals transferred on day 1 were actually measured on day 3 due to fragility of neonates. No bacteria-free animals remained alive in sterile conditions to transfer on day 15.

As expected, the conventionalized animals grew larger as the experiment went on, whereas the bacteria-free animals barely grew, consistently with previous observations. However, bacteria-free animals transferred into nonsterile conditions prior to day 9 – and thus presumably re-colonized with native microbiota – rapidly regained the ability to grow.
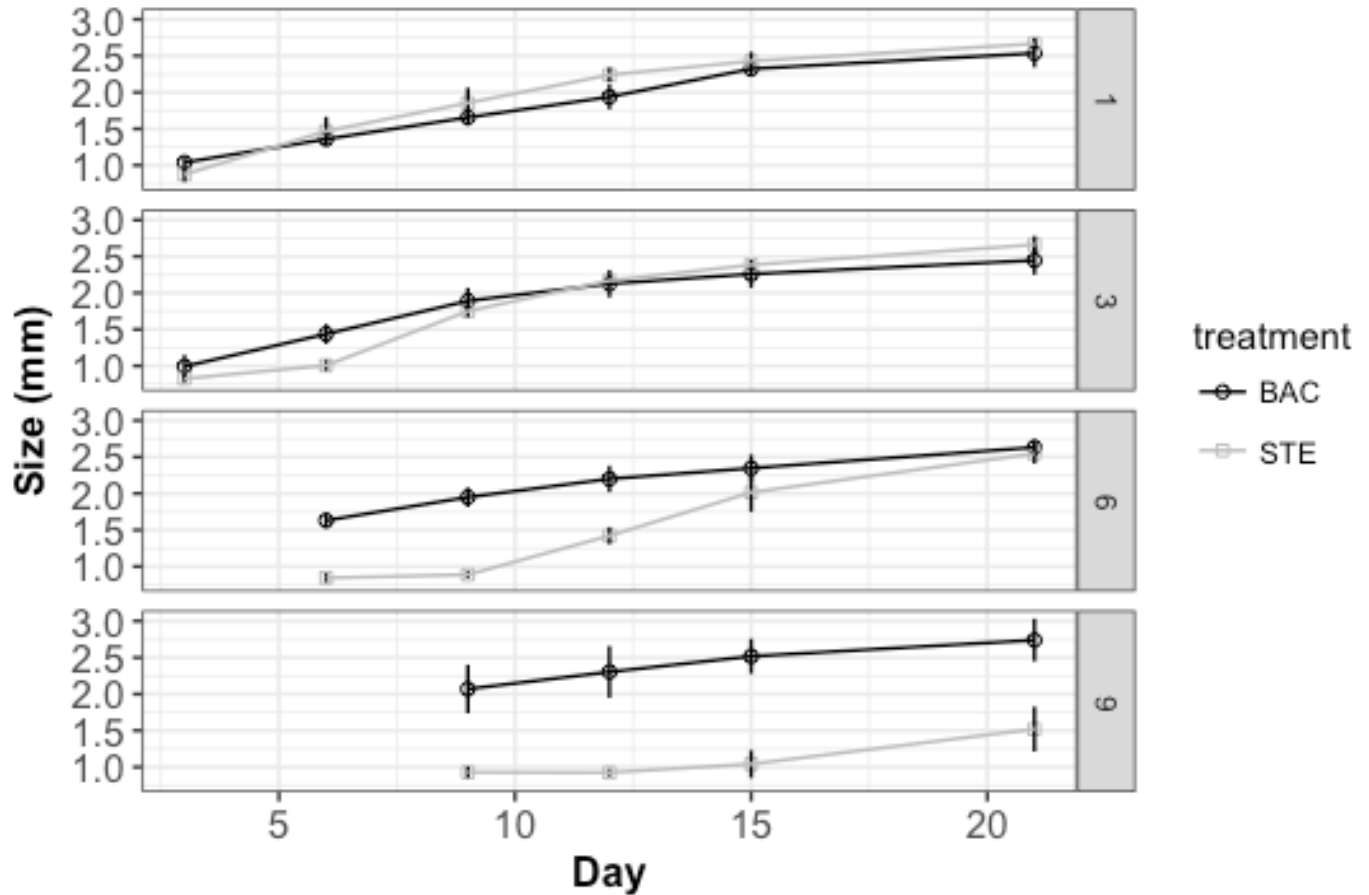
Figure A2. Body sizes (in mm) of bacteria-free (STE) and conventionalized (BAC) animals over time of being kept in nonsterile conditions. Panels represent cohorts transferred to the nonsterile condition on days 1, 3, 6, and 9. Error bars represent standard error of the mean.

By day 21, animals that had been kept bacteria-free for up to 6 days were indistinguishable from their conventionalized counterparts. The growth rate of bacteria-free animals transferred to nonsterile conditions on day 9 had appeared to increase by day 21, but it is unknown whether they would eventually reach maturity.

From these results we conclude that the effect of bacteria-free rearing is to some extent reversible, supporting the idea that bacteria provide services such as food digestion or cues such as growth signals. It also supports the idea that bacteria support growth through a distinct mechanism from their effect on embryonic development under temperature stress, because that effect is irreversible after 16 hours of bacteria-free development.

Tolerance of heavy metals among *Daphnia*-associated microbes

A study of chironomids in polluted streams found that the animals were protected from heavy metal toxicity by detoxifying microbes found in association with the eggs and larvae (Senderovich & Halpern 2013). The authors showed that many microbes isolated from these larvae were able to grow in the presence of heavy metals, and a subset of these were able to remove metals from liquid culture; manipulating the microbial community of the larvae directly showed that these microbes improved larval survival in the presence of the metal. Microbes have also shown a protective effect against heavy metal toxicity in mice and plants. *Daphnia* are often used in tests of ecotoxicity and as indicators of environmental quality, but there is surprisingly little standardization with respect to variability among animals, and studies have shown that *Daphnia* clones can vary substantially in their resistance to toxicity (Baird et al. 1991). Differences in microbiota can be an additional source of variation among individuals, or they can mediate genotype-specific resistance. Even if microbes themselves do not change the pollution-resistance of the host, host and microbe pollution-resistance are expected to be correlated in *Daphnia*, since *Daphnia* depend on microbiota for normal functioning and any evolution of resistance in *Daphnia* would have to be accompanied either by evolution of independence from bacteria, or parallel evolution of resistance in bacteria. Accordingly, it would be interesting to see whether differences in *Daphnia* sensitivity to heavy metal exposure are either caused by or correlated with the heavy metal sensitivity of their microbiota.

The following are preliminary results from trials screening for metal-resistant bacteria among the cultivable microbiota of *Daphnia*. Tests were conducted using homogenates of multiple laboratory clones of Daphnia as well as previously isolated bacteria. These methods and questions lend themselves to adaptation for use in undergraduate research projects, so priority was placed on identifying fast-growing species that could be used in the timeframe of a course.

From each clone, 3 replicate individuals were used. Each individual was homogenized in 250 μl sterile ADaM and 30 μl each of this homogenate was plated on plain LB medium and each of the metal-supplemented plates. Stock solutions of metals were prepared by dissolving K_2CrO_4, $Pb(NO_3)_2$, $CuSO_4$, or $ZnSO_4$ in water and filter-sterilizing the solution, which was then added to the autoclaved culture medium before preparing plates.

Table A1. (+) indicates that multiple colonies were observed after culturing for 3 days at 37 °C.

Daphnia Clone	Replicate	LB medium	LB + 5 mM Zn	LB+ 5 mM Cr
Mu10	1	-	-	-
	2	+	-	
	3	+	-	-
HO2	1	+	-	-
	2	+	-	+
	3	-	-	-
Xinb3	1	+	-	-
	2	+	+	+
	3	-	-	-
Iinb1	1	+	-	-
	2	-	-	-
	3	+	-	-

This trial showed that clones HO2 and Xinb3 at least occasionally had some metal-resistant bacteria. However, it also showed that LB medium does not always support growth of *Daphnia* microbiota, consistent with previous observations. Further trials were conducted using Reasoner's 2 (R2A) medium with incubation at 30 °C.

Table A2. Growth after 5 days of culturing.

Daphnia Clone	Replicate	R2A medium	R2A + 2.5 mM Cd	R2A+ 2.5 mM Cr	R2A + 2.5 mM Zn
Xinb3	1	+	-	+	+
	2	+	-	+	+
Iinb1	1	+	-	+	+
	2	+	-	+	+
Mu10	1	+	-	-	+
	2	+	-	-	+

These results suggest that the presence and absence of metal resistance may differ among the microbiota of different clones. Later 16S sequencing of some of the metal-resistant isolates showed that they are primarily species of *Sphingomonas* and *Sphingopyxis* (data not shown).

As another approach, bacterial colonies isolated on metal-free R2A medium were re-streaked on metal-supplemented R2A plates.

Table A3. Number of tested colony isolates that grew when patched onto metal-supplemented media (1/3 = one out of three tested colonies showed growth). All colonies were initially isolated on R2A. From each individual, colonies with different morphologies were selected. These plates were incubated at 30 °C.

Isolated from	Animal Replicate	R2A + 5 mM Cd	R2A + 5 mM Cr	R2A + 5 mM Pb
Xinb3	1	0/3	1/3	0/3
	2	1/3	0/3	0/3
	3	0/3	0/3	0/3
Mu10	1	1/3	1/3	0/3
	2	1/3	1/3	1/3
	3	1/3	0/3	0/3

Isolated from	Animal Replicate	LB medium	LB+ 2.5 mM Cd	LB +2.5 mM Cr
Iinb1	1	2/2	0/2	1/2
	2	3/3	0/3	0/3
	3	1/1	1/1	1/1
Xinb3	1	3/3	0/3	0/3
	2	3/3	1/3	0/3
	3	1/1	1/1	1/1

Table A4. Screening of previously isolated and identified Daphnia-associated bacteria for metal-resistance. All isolates shown were able to grow on metal-free media. Isolates were chosen either because they are known to be beneficial to Daphnia, or because they were found to be metal-resistant in the Senderovich and Halpern study. *E. coli* from a TOPO TA cloning kit was used as a comparison. LB plates were incubated at 37 °C while R2A plates were incubated at 30 °C.

Bacterial ID	Isolate	Isolated from	LB+ 5mM Zn	LB+ 5mM Cr	R2A+ 5mM Cd	R2A+ 5mM Cr	R2A+ 5mM Pb
Aeromonas04	Arm04/BK26	clone Xinb3	-	+	-	-	-
	Arm04/DF058A	field Daphnia	-	+	-	-	-
Exiguobacterium02	Exb02/AL021	lab algae	-	+	-	+	-
	Exb02/DLIb-14	clone Iinb1	-	+	-	+	-
	Exb02/DLIb-15	clone Iinb1	-	+	-	+	-
Pseudomonas16	Pdm16/DLt1n8F1	lab Daphnia	-	+	-	-	-
	Pdm16/DLt2n14NF1	lab Daphnia	-	+	-	-	-
	Pdm16/Dlt2n19NF10	lab Daphnia	-	+	-	-	-
Pseudomonas17	Pdm17/DLt1n8F3	lab Daphnia	-	+	-	-	-
Limnohabitans sp		lab Daphnia			-	-	-
E. coli					-	-	-

Overall, these results show that a number of Daphnia-associated bacteria exhibit resistance to heavy metals. However, both the bacterial resistance phenotype and the total amount of culturable bacteria can depend on the bacterial culture media used. Quantitative methods such as qPCR should be used to relate bacterial culturing results to absolute bacterial abundances before attempting to quantify differences in the level of metal resistance found amongst the microbiota of different animals.

Detection of an a vertically transmitted intracellular parasite in bacteria-free and conventionalized Daphnia

The goal of this attempted experiment was to see how the effect of a transovarially transmitted pathogen differed between bacteria-free and conventionalized *Daphnia,* in order to better understand the interacting effects of perfectly vertically transmitted pathogens and exogenously acquired beneficial microbiota. To do so, we used our standard conventionalization treatment procedure on a population of resting eggs (called HA1-1) collected in Tvaerminne, Finland, where resting eggs were known to be infected with the microsporidian parasite *Hamiltosporidium tvaerminnensis* at a rate of approximately 50% (E. Sheikh-Jabbari, unpublished data). We confirmed this in our sample of resting eggs using PCR (*H. tvaerminnensis*-specific primers targeting the beta-tubulin gene). Eggs surface-sterilized with bleach also had clearly detectable *H. tvaerminnensis,* no differently than water-rinsed eggs, confirming that the parasite is indeed transmitted intracellularly through embryos.

Unfortunately, several attempts at this experiment had to be terminated because conventionalized animals would become thickly overgrown with a "cloud" of an unknown filamentous bacterium (thought to be *Sphaerotilus sp.*), a frequently observed laboratory pest. Although the cultures of Daphnia used for the conventionalization treatment appeared to be free of this bacterium, it emerged in young conventionalized animals, suggesting that it might persist at low levels in Daphnia lab cultures. Both bacteria-free and conventionalized treatments thus had high mortality, presumably for different reasons. Dead animals were collected for PCR screening for the parasite.

After terminating one trial of the experiment at day 9, we checked for the prevalence of *Hamiltosporidium* in a few animals to evaluate its detectability in adults. Unexpectedly, we detected it in only 1/30 bacteria-free animals, and 7/21 conventionalized animals. Not only was this unexpectedly low given previous reports that the parasite does not affect hatching of resting eggs, but it was also significantly different between treatments (Fisher's exact test p=0.006). Multiple additional trials gave ambiguous evidence that presence of the parasite might bias hatching in the presence of the bacterial removal and supplementation treatments. However, this could not be definitively confirmed. In one large trial (n=510), we attempted to evaluate in a full factorial setup the effects of different methods of surface-sterilization (water vs. bleach) and bacterial supplementation on hatching success and parasite prevalence (screening both successfully and unsuccessfully hatched embryos). While all four treatment combinations had hatching rates of 70-85%, we were not able to evaluate the prevalence of the parasite in bacteria-free versus conventionalized or successfully vs. unsuccessfully developing embryos. The primary technical problem arose from the fact that while the PCR screen unambiguously detected the parasite in eggs and adults, results were extremely ambiguous in small neonate animals or partially developed post-

diapausing embryos. Using standard PCR conditions, bands corresponding to PCR product produced from neonates were faint and difficult to discern, despite clear PCR results when *Daphnia* DNA was amplified. Somewhat clearer results could be obtained using NEBNext High Fidelity PCR Master Mix (New England Biolabs), but this PCR protocol resulted in approximately 1/3 of samples having ambiguous results due to multiple PCR product bands or smeared bands. With further optimization, possibly of a quantitative PCR protocol, it would be interesting to see whether the parasite and external bacteria have an interacting effect on hatching success or if the parasite loads falls to an undetectable level in certain conditions.

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