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ORIGINAL ARTICLE

Water fleas require microbiota for survival, growth and reproduction

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Microbiota have 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 *Daphnia 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 parthenogenetic 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.
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Introduction

All eukaryotes spend their lives associated with communities of microorganisms, known as microbiota. Although 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 and Harris, 2004; Koch and 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 (Rawls *et al.*, 2004; Turnbaugh *et al.*, 2006; Cabreiro and Gems 2013; Erkosar *et al.*, 2013). 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 and Bosch, 2010; Engel *et al.*, 2012; Koch and Schmid-Hempel, 2011; Brucker and 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 vary strongly by taxa and environmental conditions. For example, germ-free mice and rats have marked deficiencies in gastrointestinal and immune development (Ivanov *et al.*, 2008; Chung *et al.*, 2012) 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), and germ-free adult flies have been reported to have reduced lifespan compared with 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), whereas addition of live *Escherichia coli* 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.

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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). As microbiota can be a cryptic source of environmental and phenotypic variation among animals (Bleich and Hansen, 2012; Koch and 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, http://server7.wfleabase.org/). 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 intercontinental 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 have a major role on 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 parthenogenetic 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.

Materials and methods

Animals

Animals were reared from both parthenogenetic and resting eggs of *D. 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; Colbourne *et al*., 2011). 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 (Pancella and Stross, 1963; Davison, 1969).

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 and 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 h after eggs were released from the ovary) from 200 females were carefully removed from the mothers' brood chambers and washed three 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, Darmstadt, Germany) at 1 mg ml^{-1}, kanamycin (Fluka/Biochemika #60615, Buchs, Switzerland) at 50 μg ml^{-1} and tetracycline (Fluka/Biochemika #87128) at 50 μg ml^{-1}, until hatching (2 days). Before 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 ultraviolet-bleached 7.0 μm mesh filter. The filtrate was washed once by centrifugation at 3000 *g* 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 ultraviolet-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 re-suspend 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 killed 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 setup was performed as above with the following modification: only two treatments were compared (BacFree versus Bac-Suppl) and hatchlings in Bac-Suppl treatment were only exposed to bacteria for 24 h 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 h. Hatchlings were rinsed twice with ADaM to remove antibiotics and those intended for Bac-Suppl treatment were exposed to bacterial suspension for 24 h. The bacterial suspension was prepared as above but without the 7.0 μm mesh filtration. Before 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 setup 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 h 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 (Sigma, St Louis, MO, USA). Bacteria from the homogenized mothers and the *Aeromonas* culture were washed once via centrifugation at 3000 *g* for 5 min, resuspended in ADaM and adjusted to the same OD_{600} (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 h, 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′-ACACGGYCCA RACTCCTAC-3′) and 936R (5′-TTGCWTCGAATTAA WCCAC-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′-CT GAATATCGCAGCATGGAAT-3′) and H18S_R (5′-TC GGACAGGGAGAGTGAAAC-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 before 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'). Sets 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 min, 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 individually 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 h. One hatchling was transferred to individual experimental jar, ensuring independence of replication. *Daphnia* ($n=11$–15 individuals per treatment) were fed every 3–4 days with 50 µl suspension of autoclaved *Scenedesmus* algae (298 million cells per ml).

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 with 50 µl of autoclaved algae (100 million cells per ml). and measured at day 6 ($n=8$ individuals per treatment), before mortality reduced the number of 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*. As 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 three 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 ($\sim 26\,^{\circ}$C) 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′-AGAGTTTGATC MTGGCTCAG-3′) and 1492R (5′-CGGYTACCTTGTT ACGACTT-3′) (Weisburg *et al*., 1991) were used, as this primer pair was available to the worker at the time of the study. 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 (Cary, NC, USA). Size data of the parthenogenetic *Daphnia* were tested for normality of distribution and equality of variances before analysis with analysis of variance. The data set 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 data sets analyzed by analysis of variance and the non-parametric Steel–Dwass test for the data set 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.

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 30–70% 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, $\chi^2 = 9.68$, $P < 0.008$) (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 with only 5% (5/93) in the BacFree treatment.

Bac-Suppl *Daphnia* had significantly more eggs in their first clutch than *Daphnia* from untreated,

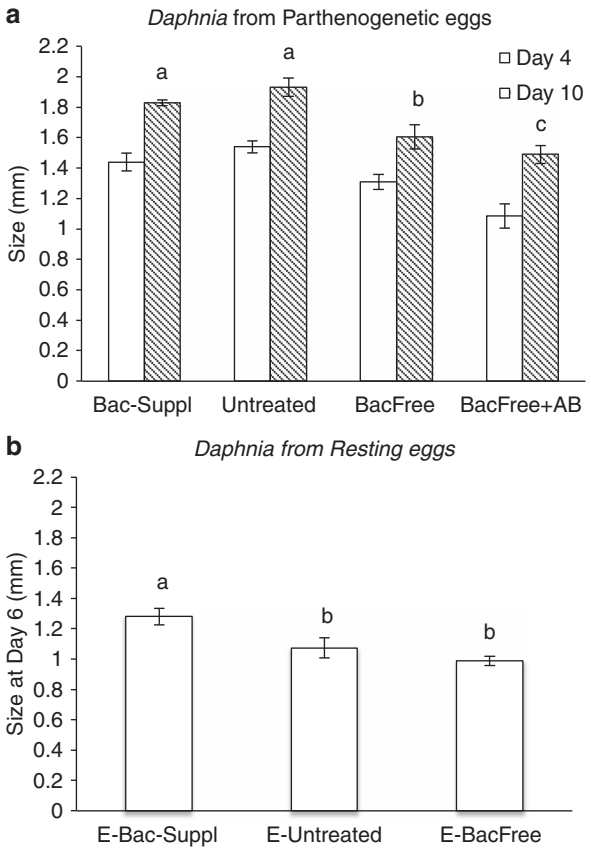

Figure 1 Size comparisons of *Daphnia* from (**A**) parthenogenetic and (**B**) ephippial eggs with and without microbiota. (**A**) Bacterial treatment has a significant effect on sizes of parthenogenetic *Daphnia* at day 4 and 10 (one-way analysis of variance, $P < 0.0001$). Group means were compared 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 s.e. are shown.

BacFree and BacFree + AB treatments (Figure 2B) (Kruskal–Wallis, $\chi^2 = 24.97$, $P < 0.0001$). In addition, the remaining Bac-Suppl females ($n = 16$) successfully produced hatchlings (5.1 ± 0.8) and second clutch eggs (10 ± 0.6), whereas 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).

In a separate experiment, we tested the mortality rate of BacFree *Daphnia* compared with Bac-Suppl *Daphnia* coming from parthenogenetic eggs. Mortality of *Daphnia* was significantly higher in BacFree treatment as compared with the Bac-Suppl treatment (Mantel Cox log-rank test, $\chi^2 = 95.2$,

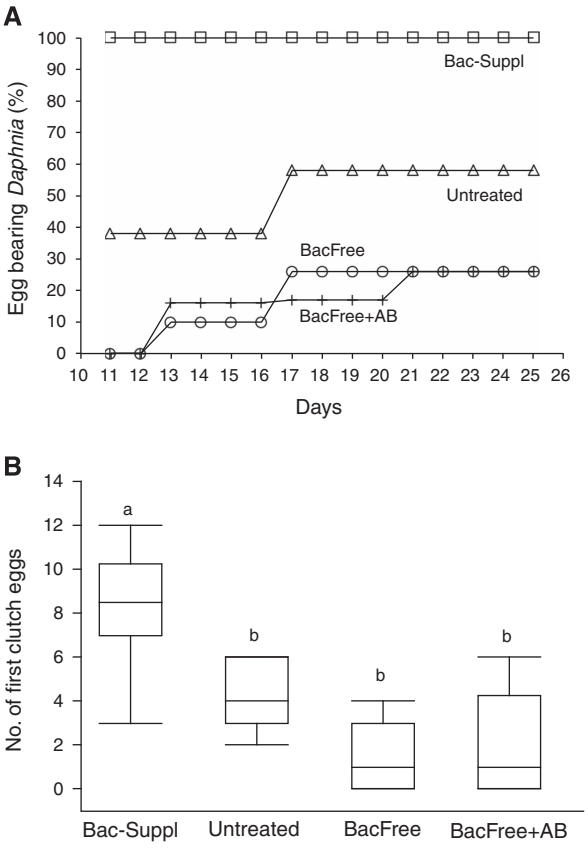

Figure 2 Fecundity of *Daphnia* raised from parthenogenetic eggs under different treatments. (**A**) Percentage of egg-bearing *Daphnia* between days 11 and 25 for the four treatment groups. (**B**) Size of first clutch (median, lower and upper quartile, range) in four treatment groups. Bacterial treatment 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.

$P<0.0001$). At day 33, all *Daphnia* in the BacFree treatment had died, whereas 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, $\chi^2 = 20.7$, $P<0.001$), 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).

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,

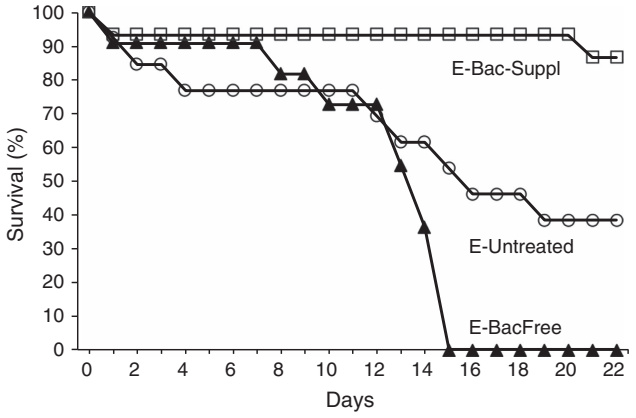

Figure 3 Survival curves of bacteria-supplemented (E-Bac-Suppl), untreated (E-untreated) and bacteria-free (E-BacFree) *Daphnia* hatched from ephippial eggs. Mantel Cox log-rank test indicates that bacteria-supplemented *Daphnia* lived longer than untreated and bacteria-free *Daphnia* ($P<0.001$).

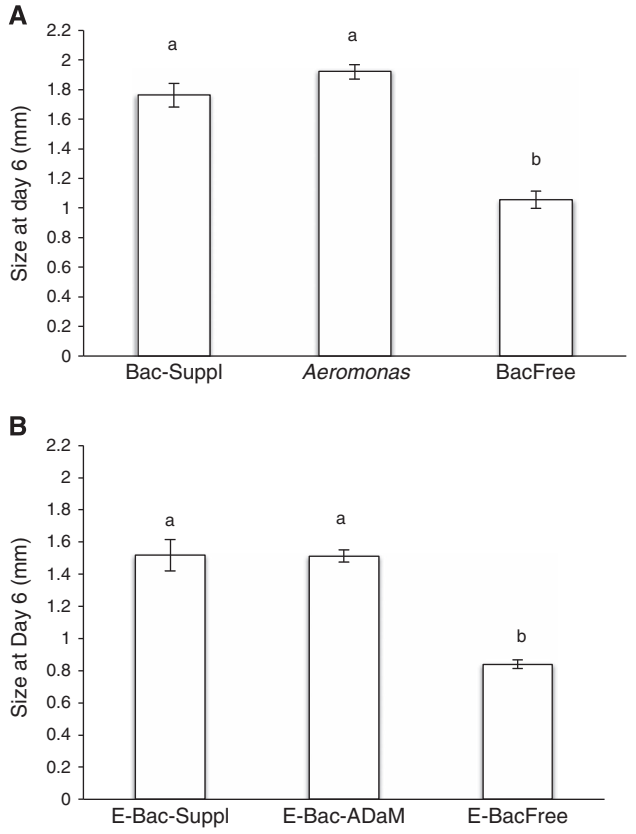

Figure 4 Body size at day 6 of *Daphnia* treated with different sources of bacteria. (**A**) *Daphnia* from parthenogenetic eggs associating either with a supplement containing diverse bacteria from the mother (Bac-Suppl), a single bacterium (*Aeromonas* sp.) or bacteria free (BacFree). Bac-Suppl and *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.

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, $P = 0.02$).

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 the 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 (Douglas, 1996; 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 with 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 leftover 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 (Bäckhed *et al.*, 2004; Semova *et al.*, 2012) 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 (Hooper, 2004; Rawls *et al.*, 2004; Bates *et al.*, 2006), or whether bacteria promote growth factor signaling and intestinal stem cell activity as in *Drosophila* (Shin *et al.*, 2011).

Bacteria can also serve as food for *Daphnia,* forming a minor part of a diet dominated by algae (Urabe and Watanabe, 1991). Bacteria alone cannot meet the nutritional requirements of *Daphnia* because they lack sterols and polyunsaturated fats that are required by *Daphnia* for somatic growth and reproduction (Martin-Creuzburg and Von Elert, 2004; Martin-Creuzburg *et al.*, 2008, 2009, 2011; 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 by aiding digestion, synthesizing essential dietary components 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 with short-term exposure (48 h during early development, Figure 1). Long-term maintenance of germ-free animals by mixing antibiotics with food should be used, therefore, with caution.

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, although 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 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.

Conflict of Interest

The authors declare no conflict of interest.

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