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RESEARCH ARTICLE

# 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 role in biogeochemical processes and directly through 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 with eggs with bacteria in their environment. The beneficial effect of bacteria was strongly reduced at 20°C. Neither temperature nor the 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.

**KEY WORDS: Water flea, Bacteria, Diapause, Environment**

## INTRODUCTION

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 scales (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 and Halpern, 2013; Breton et al., 2013), or toxic secondary compounds in plant diets (Kohl et al., 2014); conversely, they can convert xenobiotics into more harmful forms (Freeland and 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). The 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 (Douglas, 2014).

Under changing environmental conditions, the effects of positive interspecies interactions can become dampened or more pronounced. If one or both species are stressed, the effect of each individual interaction might be altered if the ability of one or both species to perform their functions is affected or if a particular function becomes more important for fitness (Xie et al., 2013; Kiers et al., 2010; Márquez et al., 2007). Furthermore, stressful conditions can reveal cryptic phenotypic variation among individuals, meaning the variation and net effect of the interaction on the population level might be altered. The stresses caused by increasing global temperatures are predicted to affect many insect–symbiont interactions (Wernegreen, 2012), change the phenology of plant–herbivore or plant–pollinator interactions (Musolin et al., 2010), and generally alter 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 and Ebert, 2008; Lynch and 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 with 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, at the ∼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 study, we used eggs that had 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

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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 and 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* Straus 1820 raised in sterile environments after emerging from surface-sterilized eggs grow more slowly, reproduce less and die sooner than animals subjected to identical treatment but colonized with bacteria ('conventionalized' by exposure to bacteria from homogenized adult *Daphnia* during development) (Sison-Mangus et al., 2014). In the course of developing our protocols for germ-free and conventionalized animals, we serendipitously observed that under some conditions, a beneficial effect of bacteria on fitness could be observed even earlier, during embryonic development of resting eggs. In a series of experiments manipulating the 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.

## MATERIALS AND METHODS

### Comparison of hatching rates

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 the 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 min in an Eppendorf tube, which was inverted continuously to expose all sides of the 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 centre of the plate.

Alternating rows of wells were assigned to be sterile or conventionalized (randomizing the assignment of the first row), with equal numbers of sterile and conventionalized rows in each plate. To the conventionalized wells, 20 µl *Daphnia* homogenate (consisting of 10 intermediate-sized adult *Daphnia* freshly homogenized in 1.5 ml ADaM) was added. To the sterile wells, 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 analysed 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 showed 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 total at the time points in the experiments when outcomes were reported. We used swimming versus 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 because bacteria or *Daphnia* homogenate were sometimes visible in the wells under the microscope. Except where noted, these assay procedures were repeated in all subsequent experiments.

To test whether 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.

### Effect of individual strains of bacteria

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) (GenBank accession numbers KU577468, KU577466, KU577467, KU577465 and KU577464, respectively) – were arbitrarily selected from the laboratory stock collection and their effect on hatching was contrasted with germ-free conditions at 22–23°C (because of 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 $OD_{600}$ (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-



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*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 $OD_{600}$=0.2 and half of each culture was heat-killed at 80°C for 1 h. 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 h of development at the warm temperature. (This time point 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 h 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 h 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 h 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 on a 16 h:8 h light:dark cycle at 20°C, fed every other day with 50 million cells of the green alga *Scenedesmus* sp.

For the experiment, 1 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, similar 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 was added as above. Wells were checked twice daily for swimming hatchlings.

### Statistical analysis

All statistical analyses were performed using the software package R 3.1.3 (R Core Team). The proportion of freely swimming hatchlings in each condition was analysed 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, the data were analysed with a genotype effect included while ephippial eggs were analysed in a separate model. Binomial confidence intervals were calculated for each treatment combination using the default Wilson method in the R package Hmisc.

## RESULTS

In a comparison of eggs exposed to bacteria-free (sterile) or conventionalizing conditions (addition of a homogenate of lab *Daphnia* with complete microbiota), a clear interaction between

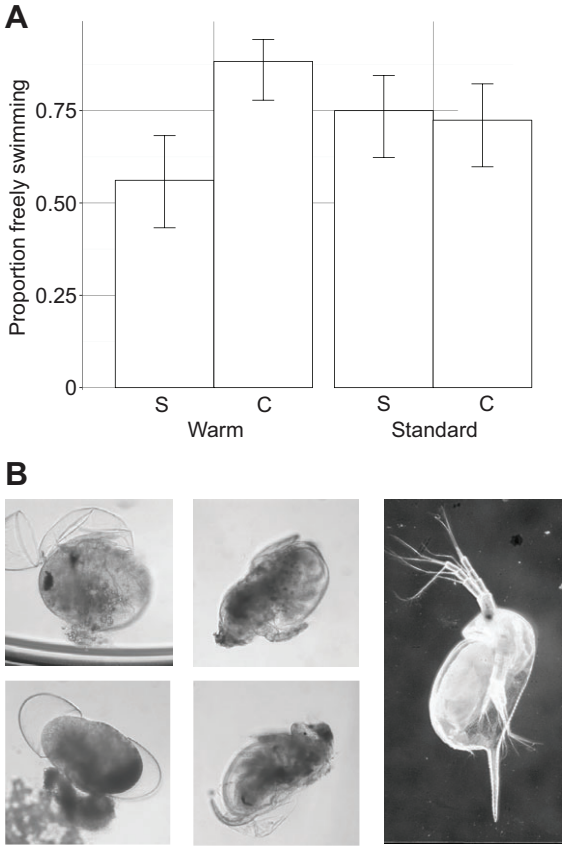


**Fig. 1. Effect of temperature and bacterial treatment on *Daphnia* development.** (A) Proportion of resting eggs that reached a free-swimming state under warm and standard, bacteria-free (sterile, S) and conventionalized (C) conditions. *N*=57–60 individuals in each treatment combination. Error bars represent 95% binomial confidence intervals. Odds ratio for conventionalized versus sterile under warm conditions: 5.9. For logistic regression results, see Table 1. (B) Examples of the developmental abnormalities observed; photos shown are from warm, bacteria-free conditions. Right, an example of a normally developed neonate; the image was compiled from stacked photographs of an immobilized individual. Photos have been converted to greyscale, and brightness and contrast have been adjusted.

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**Table 1. Effect of conventionalizing bacterial mixture (Fig. 1)**

| | Estimate | s.e. | *Z*-value | Pr(>\|*Z*\|) |
|---|---|---|---|---|
| Intercept | 0.2469 | 0.2669 | 0.925 | 0.355058 |
| Conventionalized | 1.7775 | 0.4827 | 3.683 | 0.000231*** |
| Standard | 0.8518 | 0.4080 | 2.087 | 0.036845* |
| Conventionalized:standard | −1.9111 | 0.6438 | −2.968 | 0.002995** |

Coefficients of logistic regressions are presented. Sterile and warm conditions are set as the reference levels. Asterisks represent significance (*$P$=0.05, **$P$=0.01 and ***$P$=0.001).
For a description of conditions, see Materials and methods.

temperature and bacterial treatment was observed (Fig. 1A, Table 1). 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 than that of 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 (Fig. 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 conditions, 20/25 (80%) success in conventionalized conditions; Fisher's exact test $P$=0.003].

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

Eggs treated with heat-killed *Pseudomonas* had rates of failure similar to bacteria-free eggs under warm conditions (Fig. 3, Table 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 (Fig. 4, Table 4); the higher dose had a stronger beneficial effect than the low dose.

Adding *Pseudomonas* to bacteria-free embryos 16 h 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 (Fig. 5, Table 5). Therefore, bacteria could only rescue the development of embryos if they were already present less than 16 h after the onset of the warm temperature condition. Observation of a subset of these embryos at 16 h 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 h showed bacterial cells irregularly distributed on the surface of the egg, with no apparent pattern.

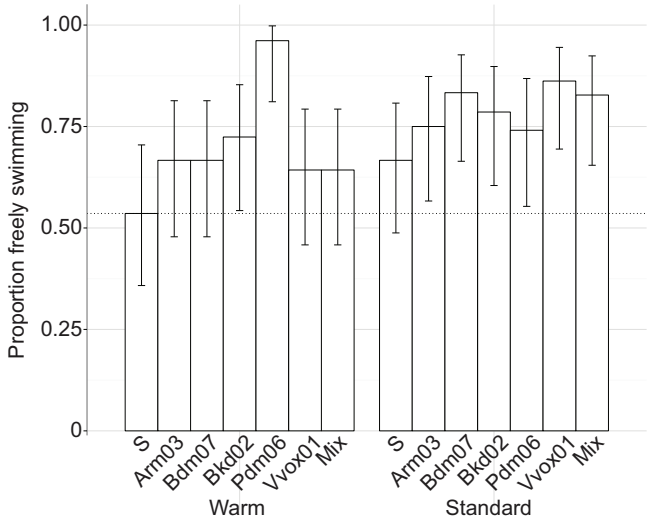


**Fig. 2. Proportion of resting eggs reaching a free-swimming state when exposed to different bacterial strains under warm and standard temperature conditions.** Sterile (S), bacteria-free; Arm03, *Aeromonas* sp.; Bdm07, *Brevundimonas* sp.; Bkd02, *Burkholderiales* sp.; Pdm06, *Pseudomonas* sp.; Vvox01, *Variovorax* sp.; Mix, mixture of these five bacterial strains. $N$=26–30 in each treatment combination. Dotted line is shown to emphasize rate of development in warm, sterile conditions. Odds ratio for Pdm06 versus sterile under warm conditions: 21.7. Error bars represent 95% binomial confidence intervals. For logistic regression results, see Table 2.

**Table 2. Effect of individual bacterial isolates (Fig. 2)**

| | Estimate | s.e. | *Z*-value | Pr(>\|*Z*\|) |
|---|---|---|---|---|
| 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 |

Coefficients of logistic regressions are presented. Sterile and warm conditions are set as the reference levels. Asterisks represent significance (*$P$=0.05 and **$P$=0.01).
For definitions of bacteria abbreviations, see Fig. 2.

**Table 3. Effect of heat-killed bacteria (Fig. 3)**

| | Estimate | s.e. | *Z*-value | Pr(>\|*Z*\|) |
|---|---|---|---|---|
| Intercept | −0.693147 | 0.387298 | −1.790 | 0.07350 |
| Live Bdm | 1.491655 | 0.557773 | 2.674 | 0.00749** |
| Heat-killed Bdm | 0.944462 | 0.635585 | 1.486 | 0.13729 |
| Live Pdm | 2.890372 | 0.721325 | 4.007 | 6.15e−05*** |
| Heat-killed 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 |
| Heat-killed Bdm:standard | 0.006515 | 0.930699 | 0.007 | 0.99442 |
| Live Pdm:standard | −1.166206 | 1.020683 | −1.143 | 0.25322 |
| Heat-killed Pdm:standard | 1.226870 | 0.824822 | 1.487 | 0.13690 |

Coefficients of logistic regressions are presented. Sterile and warm conditions are set as the reference levels. Asterisks represent significance (*$P$=0.05, **$P$=0.01 and ***$P$=0.001).



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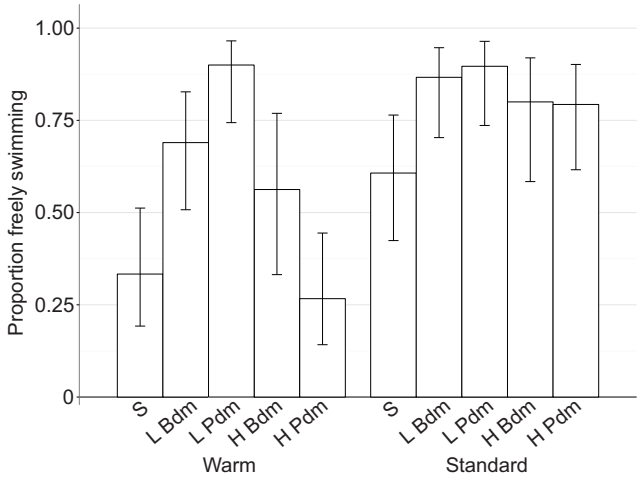


**Fig. 3. Proportion of resting eggs reaching a free-swimming state when exposed to live (L) and heat-killed (H) *Pseudomonas* (Pdm) and *Brevundimonas* (Bdm) under warm and standard temperature conditions.** *N*=28–30 in each treatment combination except for heat-killed Bdm+warm: *N*=16. Odds ratio for live *Pseudeomonas* versus sterile under the warm condition: 18. Error bars represent 95% binomial confidence intervals. For logistic regression results, see Table 3.

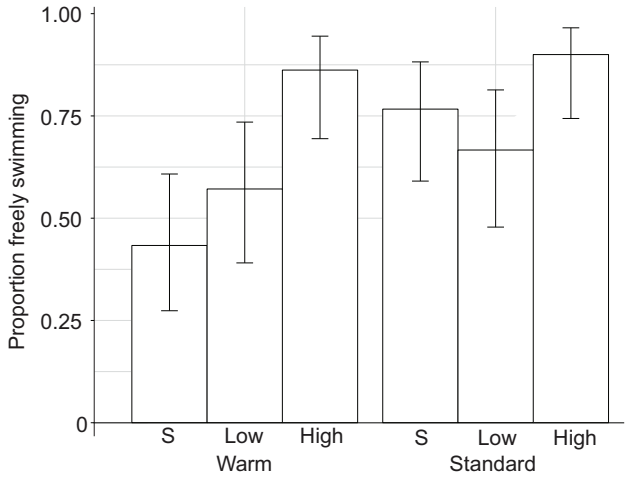


**Fig. 4. Proportion of resting eggs reaching a free-swimming state when exposed to different doses of *Pseudomonas* bacteria.** *N*=27–30 in each treatment combination. Error bars represent 95% binomial confidence intervals. Odds ratio for *Pseudomonas* high dose versus sterile: 8.22. For logistic regression results, see Table 4.

The bacterial and temperature treatments had no effect on the development success of directly developing parthenogenetic eggs of three different *Daphnia* genotypes (Fig. 6, Table 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 4.7 to 21.7 (Table 7).

## DISCUSSION

We have shown a consistent positive effect of exposure to bacteria on the successful development of *D. 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 was 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 was 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. As 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 (A.A.M., 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. For 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 to continued

**Table 4. Effect of a low dose of *Pseudomonas* (Fig. 4)**

| | Estimate | s.e. | *Z*-value | Pr(>\|*Z*\|) |
|---|---|---|---|---|
| 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 | 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 |

Coefficients of logistic regressions are presented. Sterile and warm conditions are set as the reference levels. Asterisks represent significance (**P*=0.05 and ***P*=0.01).

**Table 5. Effect of adding *Pseudomonas* after 16 h of development under warm conditions (Fig. 5)**

| | Estimate | s.e. | *Z*-value | Pr(>\|*Z*\|) |
|---|---|---|---|---|
| Intercept | −1.0516 | 0.3147 | −3.342 | 0.000832*** |
| Pdm added at 16 h | 0.2792 | 0.4509 | 0.619 | 0.535786 |
| Pdm always present | 1.9370 | 0.3591 | 5.394 | 6.89e−08*** |
| Disturbed | 0.1925 | 0.3591 | 0.536 | 0.591799 |

Sterile and undisturbed were set as reference levels. Asterisks represent significance (****P*=0.001).
Coefficients of logistic regressions are presented.

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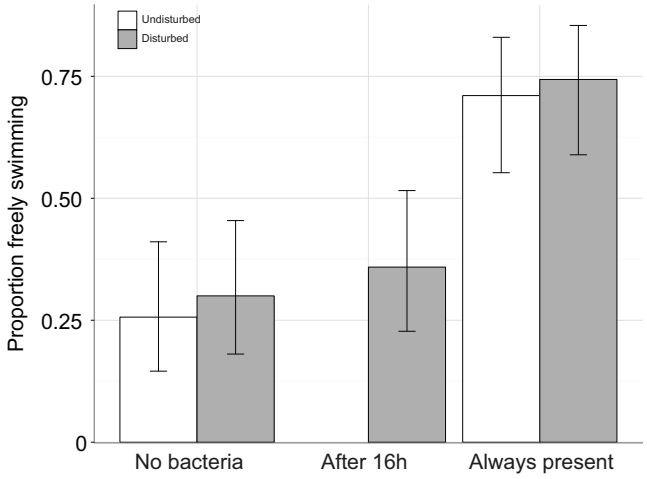


**Fig. 5. Comparison of successful development with *Pseudomonas* bacteria added at different times.** Eggs were exposed to *Pseudomonas* from the beginning of the experiment or after 16 h of bacteria-free development under warm conditions. Control eggs disturbed by pipetting at 16 h are included. *N*=38–40 per treatment group. Error bars represent binomial confidence intervals. Odds ratio for *Pseudomonas* always present versus no bacteria: 7.2. For logistic regression results, see Table 5.

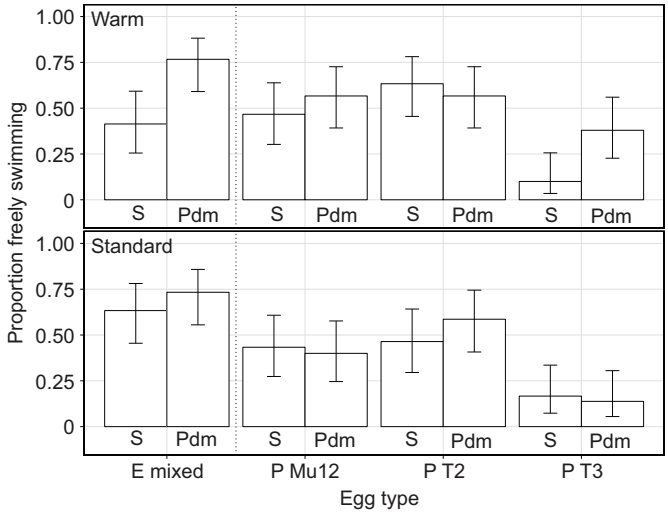


**Fig. 6. Effect of bacteria-free and *Pseudomonas*-exposed conditions on development at standard and warm temperature for directly developing parthenogenetic eggs and ephippial eggs.** The parthenogenetic eggs were from three different *Daphnia* clones. Egg type: P, parthenogenetic; E, ephippial; mixed, mixed genotype; Mu12, T2 and T3, clones. *N*=29–30 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 the reference level in both. Odds ratio for *Pseudomonas*-exposed versus sterile ephippial eggs under warm conditions: 4.7. For logistic regression results, see Table 6.

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, the 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 and Epel, 2009), as 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 favourable 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, i.e. whether bacteria act by modifying the chemical or physical environment around the egg, thus creating conditions more favourable 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 h of development at the warm temperature. This could be either because this window represents a

**Table 6. Effects on ephippial and parthenogenetic eggs (Fig. 6)**

| | Estimate | s.e. | *Z*-value | Pr(>\|*Z*\|) |
|---|---|---|---|---|
| Ephippial eggs | | | | |
| Intercept | −0.3483 | 0.3770 | −0.924 | 0.35559 |
| Pdm | 1.5379 | 0.5731 | 2.683 | 0.00729** |
| Standard | 0.8949 | 0.5345 | 1.674 | 0.09410 |
| Pdm:standard | −1.0728 | 0.8016 | −1.338 | 0.18075 |
| Parthenogenetic eggs | | | | |
| Intercept | −0.1335 | 0.3660 | −0.365 | 0.71520 |
| Pdm | 0.4018 | 0.5193 | 0.774 | 0.43910 |
| Standard | −0.1347 | 0.5193 | −0.259 | 0.79529 |
| Clone T2 | 0.6801 | 0.5268 | 1.291 | 0.19668 |
| Clone T3 | −2.0637 | 0.7101 | −2.906 | 0.00366** |
| Pdm:standard | −0.5390 | 0.7378 | −0.731 | 0.46504 |
| Pdm:clone T2 | −0.6801 | 0.7409 | −0.918 | 0.35868 |
| Pdm:clone T3 | 1.3030 | 0.8868 | 1.469 | 0.14177 |
| Standard:clone T2 | −0.5549 | 0.7462 | −0.744 | 0.45708 |
| Standard:clone T3 | 0.7225 | 0.9381 | 0.770 | 0.44117 |
| Pdm:standard:clone T2 | 1.3087 | 1.0533 | 1.243 | 0.21405 |
| Pdn:standard:clone T3 | −1.3889 | 1.2614 | −1.101 | 0.27086 |

Sterile and warm conditions are set as the reference levels. Asterisks represent significance (**$P$=0.01).

**Table 7. Consistent effects of conventionalizing bacteria or *Pseudomonas* sp. across experiments**

| | Warm condition | | Standard condition | |
|---|---|---|---|---|
| Experiment/trial | Odds ratio | *P*-value | Odds ratio | *P*-value |
| Fig. 1 | 5.9 | 0.00014 | 0.87 | 0.83 |
| Fig. 2 | 21.7 | 0.00041 | 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 | n.a. | n.a. |
| Fig. 6 | 4.7 | 0.0082 | 1.59 | 0.58 |
| Mean±s.e.m. | 10.95±2.89 | | 2.44±0.85 | |

Odds ratios of successful development of the bacterial treatment that significantly differed from the sterile reference condition in each experiment are shown. *P*-values were calculated using Fisher's exact test.

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critical phase in the development of the embryo, or because it takes longer than 16 h for the beneficial effect of the bacteria to begin (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 varied in genotype, size, length of time since deposition, and probably 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 expose 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 and Hofmann, 1999; Jones et al., 2015), it is possible that pathways activated by exposure to bacteria are also protective against heat. As 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. However, one study reported high rates of inviability and developmental abnormalities in the parthenogenetic eggs of microbiota-free *Daphnia* mothers under sterile conditions (Peerakietkhajorn et al., 2015). As 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 the absence of bacteria could be a characteristic of eggs produced by undernourished mothers. Studies have demonstrated various effects of maternal nutritional status on the disease resistance of offspring (Mitchell and Read, 2005). If the effect observed here involves cross-talk between immune-related and other developmental signalling 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) that has no apparent negative effects when eggs are hatched in conventional (non-sterile) conditions. In our experiments, eggs briefly (5 min) exposed to sodium hypochlorite and then re-inoculated with bacteria had restored or elevated hatching success compared with 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 and LeBlanc, 2002; Flaherty and Dodson, 2005). As ecdysone signalling is also involved in processes dependent on bacteria (i.e. invertebrate immune response) (Regan et al., 2013; Rus et al., 2013), we speculate that the 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 signalling pathways underlying both (McFall-Ngai et al., 2013; Hayden and Ghosh, 2004). As animal developmental programmes evolved in the presence of bacteria, it is conceivable that normal development can depend on processes sensitive to bacterial presence even in early stages. It remains to be seen how relevant the effect observed here is in natural settings; however, these findings potentially have general relevance to the understanding of the complex ecological dimensions of development and of the effects of bacterial activities on other organisms in the ecosystem.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

A.A.M. designed and performed all experiments, analysed the data and wrote the paper. E.B. identified and cultured the bacterial strains. T.M.M.S. set up the experiment involving parthenogenetic eggs. D.E. designed the experiments and revised the paper.

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