MOLECULAR ECOLOGY

Molecular Ecology (2016) **25**, 4930–4945 doi: 10.1111/mec.13798

Effects of host genetics and environment on egg-associated microbiotas in brown trout (*Salmo trutta*)

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Abstract

Recent studies found fish egg-specific bacterial communities that changed over the course of embryogenesis, suggesting an interaction between the developing host and its microbiota. Indeed, single-strain infections demonstrated that the virulence of opportunistic bacteria is influenced by environmental factors and host immune genes. However, the interplay between a fish embryo host and its microbiota has not been studied yet at the community level. To test whether host genetics affects the assemblage of egg-associated bacteria, adult brown trout (*Salmo trutta*) were sampled from a natural population. Their gametes were used for full-factorial in vitro fertilizations to separate sire from dam effects. In total, 2520 embryos were singly raised under experimental conditions that differently support microbial growth. High-throughput 16S rRNA amplicon sequencing was applied to characterize bacterial communities on milt and fertilized eggs across treatments. Dam and sire identity influenced embryo mortality, time until hatching and composition of egg-associated microbiotas, but no link between bacterial communities on milt and on fertilized eggs could be found. Elevated resources increased embryo mortality and modified bacterial communities with a shift in their putative functional potential. Resource availability did not significantly affect any parental effects on embryo performance. Sire identity affected bacterial diversity that turned out to be a significant predictor of hatching time: embryos associated with high bacterial diversity hatched later. We conclude that both host genetics and the availability of resources define diversity and composition of egg-associated bacterial communities that then affect the life history of their hosts.

Keywords: bacterial diversity, host genetics, microbiota, *Salmo trutta*, salmonid embryo

Received 11 May 2016; revision received 25 July 2016; accepted 5 August 2016

Introduction

Mortality in fish is typically highest at early developmental stages (Llewellyn *et al.* 2014). Egg-associated bacteria play a crucial role here ranging from being the immediate cause of mortality (Wedekind 2002; Neff & Pitcher 2005; Wedekind *et al.* 2010) to acting as a first line of defence against pathogens (Boutin *et al.* 2012; Liu *et al.* 2014). Much is known about the interaction of fish embryos and their associated bacterial pathogens from single-strain infections (e.g. von Siebenthal *et al.* 2009; Aykanat *et al.* 2012; Clark *et al.* 2014). However, single-strain infections represent a strong simplification. Recent findings based on high-throughput sequencing show a considerable diversity of egg-associated microbiotas (Liu *et al.* 2014; Wilkins *et al.* 2015a, b; Stephens *et al.* 2016), and at the community level, the interactions of hosts and their associated bacteria are still not well understood (Llewellyn *et al.* 2014; Waldor *et al.* 2015). Here, we specifically concentrate on the role of host genetics and environment during this interaction.

Analyses based on uncontrolled outbreaks of *Pseudomonas fluorescens* (an opportunistic fish pathogen) showed that not only virulence factors but also hatching time can be affected (Wedekind 2002) and that host susceptibility depends on immune genes (Wedekind *et al.* 2004). Subsequent experimental incubations confirmed these first findings (e.g. von Siebenthal *et al.* 2009;

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Aykanat *et al.* 2012; Clark *et al.* 2014). It could be demonstrated that during early development, the embryo relies on antimicrobial compounds that the mother allocated to the eggs before spawning (Hanif *et al.* 2004; D'alba *et al.* 2010). At later developmental stages, the importance of zygotic gene expression becomes crucial for the interaction with associated bacteria (Finn 2007; Clark *et al.* 2013a, 2014). Host genetic effects could be revealed for important embryo life-history traits such as mortality in *Oncorhynchus tshawytscha* (Aykanat *et al.* 2012), *Salmo trutta* (Jacob *et al.* 2010; Clark *et al.* 2013b) or *Coregonus palaea* (von Siebenthal *et al.* 2009; Clark & Wedekind 2011; Clark *et al.* 2014) and time until hatching (Clark *et al.* 2013a, 2014), size at hatching (Clark *et al.* 2014, 2016) and immune gene expression (Clark *et al.* 2013a; Wilkins *et al.* 2015c) in *C. palaea*. The interplay of fish embryos with their associated bacteria also depends on environmental factors, which, for example, can turn opportunistic bacteria into virulent fish pathogens (Jacob *et al.* 2010; Wedekind *et al.* 2010). However, the factors that influence the assemblage and dynamics of fish embryo-associated bacterial communities are still poorly understood.

In adult fish, the composition of associated bacterial communities has been correlated with environmental factors, such as sampling location (Roeselers *et al.* 2011; Sullam *et al.* 2015), salinity (Schmidt *et al.* 2015), diet (Wilson *et al.* 2008; Bolnick *et al.* 2014a; Sevellec *et al.* 2014; Miyake *et al.* 2015; Smith *et al.* 2015) and temperature (Wilkins *et al.* 2015a). For bacterial communities in the guts, there is accumulating evidence that host genetics affects the composition of fish-associated microbiotas (Ghanbari *et al.* 2015). Specific bacterial groups could be linked to host phenotype (Sun *et al.* 2009; Li *et al.* 2013; Star *et al.* 2013), candidate loci in the host genome (Boutin *et al.* 2014) and alleles of the major histocompatibility complex (MHC; Bolnick *et al.* 2014b). Fish gut microbiotas are not only significantly different among different host species (Li *et al.* 2012; Larsen *et al.* 2013; Givens *et al.* 2014) but also between populations (Skrodenyte-Arbaciauskiene *et al.* 2006) and even families (Navarrete *et al.* 2012). In contrast to these distinct fish microbiotas, bacterial communities on fish embryos are expected to reflect mostly the surrounding water (Ghanbari *et al.* 2015) because in the aquatic environment bacteria can migrate freely between habitats and hosts (Llewellyn *et al.* 2014). Nonetheless, recent characterizations of naturally spawned salmonid eggs have shown that there are egg-specific microbiotas that do not correspond to the surrounding water environment (Wilkins *et al.* 2015a, b). Furthermore, bacterial diversity decreased during embryogenesis suggesting a specific role of the host at this early developmental stage (Wilkins *et al.* 2015b).

Here, we investigated host genetic effects on embryo-associated bacterial communities using salmonid eggs. In salmonids, fertilization is external and females provide large numbers of eggs. Full-factorial breeding designs can therefore be applied where every female is crossed with every male ('North Carolina II design', Lynch & Walsh 1998). These designs allow for the disentanglement and estimation of environmental, dam, sire and dam × sire interaction effects. Dam effects combine maternal genetic and maternal environmental effects (Royle *et al.* 1999; von Siebenthal *et al.* 2009; Aykanat *et al.* 2012) while sire effects directly reveal additive genetic variance for a trait because fathers only contribute genes to their offspring (Lynch & Walsh 1998). Accordingly, additive genetic effects can be estimated through paternal effects in this system. We sampled brown trout (*S. trutta*) from a natural subalpine river and collected their gametes for full-factorial in vitro fertilizations. Resulting embryos were raised individually under controlled conditions. To vary the host–microbiota environment, we added different concentrations of nutrient broth to the embryos. Nutrient broth provides resources for bacterial growth but is not harmful to embryos on its own (Wedekind *et al.* 2010). Naturally occurring bacteria on the eggs were characterized and quantified with a bacteria-specific, next-generation 16S rRNA amplicon sequencing protocol. We addressed the following objectives: (i) Are there genetic host effects on the diversity and composition of egg-associated bacteria? (ii) Which core bacterial taxa and functional pathways are affected? (iii) Is there a link between associated microbiota and host life history? (iv) Are these interactions dependent on the availability of bacterial resources?

Materials and methods

Sample acquisition and experimental protocol

Adult brown trout (*Salmo trutta*) were caught with electrofishing at their natural spawning grounds in the river Kiese (7°37′11,27″; 46°50′55,85″). They were kept at a hatchery for some days until the fish could be stripped of their gametes. Milt from each individual sire (40 μl) was immediately frozen in liquid nitrogen for later bacterial characterization. The remaining gametes were used for full-factorial in vitro fertilizations following the methods described in Jacob *et al.* (2007). Eight females were crossed with seven males in all possible combinations (56 families). After distributing fertilized eggs singly to 24-well plates (Falcon, BD Biosciences, Allschwil, Switzerland) in a block-wise design, eggs were incubated in a 6.5 °C climate chamber in 2 mL of standard water. 'Standard water' was chemically

standardized according to the OECD guidelines (OECD 1992). It had been autoclaved, temperated and oxygenated before use. Once embryos had reached the late-eyed developmental stage (45 days after fertilization), healthy-looking embryos were redistributed singly to new 24-well plates. Now, they were incubated at different concentrations of nutrient broth or they were sham-treated with standard water. Undiluted nutrient broth (NB) consisted of 3 g meat extract and 5 g bactopeptone (Sigma-Aldrich, Buchs, Switzerland) in 1 L of standard water. Two different nutrient broth concentrations (1:1000 and 1:500 dilution in the well) and sham treatment were applied to 15 replicates each per family and treatment (n = 2520 singly raised embryos).

Fourteen days after treatment, five eggs per family and treatment were sampled and stored at −80 °C for later characterization of their associated bacterial communities. All other eggs (n = 10 per family and treatment) were transferred to new 24-well plates with 2 mL of standard water per well.

In a subsample of 20 wells per treatment, we measured oxygen concentrations (Firesting O_2, Pyroscience, Aachen, Germany) as well as pH (bench pH meter FiveEasy® Plus, Life Sciences, Basel, Switzerland) every 3 days after the start of the treatment at a distance of 4 mm away from the egg membrane. Figure S1 (Supporting information) summarizes the experimental design.

Statistical analyses of embryo performance

Embryo mortality was analysed as a binary response variable (dead before hatching or hatched) with logistic mixed-effect models by treating each embryo as an independent replicate. Treatment (nutrient broth concentrations) and bacterial alpha diversity on the eggs 14 days after incubation with nutrient broth (phylogenetic distance, see below for its calculation) were entered as fixed effects. Parental origin was entered as random dam, random sire and random dam × sire interaction effects. While dam effects encompass both genetic and maternal environmental effects, sire effects represent one-quarter of the additive genetic variation, assuming that epistatic effects are negligible (Lynch & Walsh 1998). Separate models were fitted to investigate the interaction of treatment with dam or sire effects, or bacterial diversity, respectively. To test for the significance of an effect, a reduced model omitting the variable of interest was compared to the reference model. The goodness of fit of the different models is given by the logarithm of the approximated likelihood (ln L) and by the Akaike's information criterion (AIC). To test whether models differ in their goodness of fit, the models were compared with likelihood ratio tests (LRT). The LME4 package version 1.1.7 for logistic mixed-effect model analyses (Bates & Sarkar 2007) was used in R version 3.1.3 (R Development Core Team 2015). Time until hatching was analysed as a continuous response variable in analogous linear mixed models (LMM).

DNA extraction and preparation for sequencing

Five fertilized eggs per family and treatment were pooled for bacterial DNA extraction. Milt was analysed individually for each sire. This resulted in 175 samples (56 families × three treatments and seven milt samples) (for detailed DNA extraction protocols and PCR conditions, see Supplementary Information). Cleaned PCR products were sent in equimolar amounts for MiSeq illumina sequencing (Nextera protocol; Microsynth, Balgach, Switzerland) using four libraries of 48 individually tagged samples each (combined with samples from another project in the fourth library) in one full sequencing run (Fig. S1, Supporting information).

Bacterial diversity on fertilized eggs

The following analyses were done in the QIIME version 1.8.0 framework (Caporaso *et al.* 2010a). After a stringent quality control pipeline for raw next-generation sequencing reads, operational taxonomic units (OTUs) of bacteria were inferred. Different steps of the quality control pipeline and OTU-picking algorithms are described in detail in the Supplementary Information. An open BLAST search was applied (Lan *et al.* 2012) with the RDP classifier version 2.2 (Wang *et al.* 2007) and the GREENGENES reference database version 12.13 (McDonald *et al.* 2012). This approach enables the identification of previously undescribed bacterial sequences in a data set (Caporaso *et al.* 2010a). A unique sequence is assigned a new OTU ID at the lowest taxonomic level possible (see Material and Methods, Supporting information). Before inferring alpha and beta diversities, the OTU table was normalized with respect to inferred 16S rRNA gene copy numbers according to Paulson *et al.* (2013; with the script 'normalize_table.py' and using the 'CSS' algorithm) although the sequencing depth was comparatively high and the discrepancy between the samples with the lowest and highest number of sequences was only a factor of two (Fig. S2, Supporting information). To graphically represent bacterial community composition of different groupings, the non-normalized data set after quality control was used.

Phylogenetic distance within bacterial communities on the eggs was calculated as alpha diversity measure that estimates the total descending branch length for all OTUs in a sample (Gotelli & Chao 2013). A LMM using the LME4 package in R was applied to investigate the

effects of nutrient broth treatment and the effects of dam and sire identity on bacterial diversity associated with fertilized brown trout eggs (14 days after treatment). Prior to this analysis, alpha diversity measures were tested for their normal distribution, homogeneity of variances and homoscedasticity of the error terms. There were no deviations from the assumption of equal variances ($F_{2,7} = 0.18$, $P = 0.9$). Significance of different explanatory variables was inferred analogously to the analysis on embryo performance above. The interaction effect of dam × sire was not analysed due to low sample sizes of replicates for this term. The relationship between bacterial diversity on the eggs and embryo performance (mortality and hatching time, respectively) was investigated with Pearson's product moment correlation (r) using mean values of brown trout families. As mortality might bias hatching time, the same analysis was also applied to a subset of families with no mortality. The results on timing of hatching and on bacterial diversities were verified using two other alpha diversity measures that do not rely on an underlying phylogenetic tree but instead on the rarity of bacterial species (Chao 1) and the crude number of observed bacterial (observed number of species) taxa after quality control and normalization.

Bacterial composition on fertilized eggs

Pairwise UniFrac distances (Lozupone & Knight 2005) were calculated to quantify the differences in bacterial composition among samples. UniFrac distances quantify the fraction of unique branch lengths against the total branch length between pairs of bacterial communities from a common phylogenetic dendrogram (Lozupone & Knight 2005), where 0 indicates that two samples are identical and 1 indicates that two samples have no bacterial species in common. To build a phylogenetic dendrogram, OTUs were aligned using the PYNAST algorithm version 1.2.2 (Caporaso *et al.* 2010b). A phylogenetic tree was built using FASTTREE version 2.0 (Price *et al.* 2008; using default parameters). Quantitative measures (i.e. weighted UniFac) are appropriate for revealing community differences that are due to changes in relative taxon abundance (e.g. when a particular set of taxa grow well because a limiting nutrient source becomes abundant). Qualitative measures (i.e. unweighted UniFrac) are most informative when communities differ primarily by what can live in them (e.g. at high temperatures), partially because abundance information might obscure significant patterns of variation in which taxa are present (Lozupone & Knight 2007). Different nutrient broth concentrations were expected to affect mostly bacterial taxa abundance and not composition on fertilized eggs. Hence, weighted UniFrac measures were used for the analysis of embryonated eggs. However, different bacterial taxa might be found on the gametes of individual, natural spawners. Accordingly, unweighted UniFrac distances were applied for the analysis of bacterial communities on milt. Adonis pairwise permutation tests (R package 'vegan', Oksanen *et al.* 2013; with the functions 'vegdist' and 'Adonis') were applied with 999 permutations to analyse whether treatment, dam and sire effects explain a significant part of the variation in bacterial composition on the eggs. This analysis was performed in the R environment and is further explained in the Supplementary Information. These analyses of variance methods are nonparametric, but they assume equal variances among groups of samples and they are most reliable with balanced designs (Anderson & Walsh 2013). The R package 'Permdisp' was applied to test for equal variances of bacterial composition among categorical predictor variables (Anderson 2006). Significance and P-values were obtained by permutation ($n = 999$). P was considered significant if ≤0.05.

Principal coordinate analysis (PCoA) plots (two dimensional) were created to visualize the permutation tests using EMPEROR version 0.9.3 (Vazquez-Baeza *et al.* 2013). Bacterial communities on embryos in the high-nutrient broth treatment were compared pairwise among sires using two-sided Student's two-sample t-tests in the QIIME pipeline. Nonparametric P-values were obtained by permutation ($n = 999$; see Supplementary Material and Methods). They were considered significant at <0.05 with a Bonferroni multiple testing correction controlling the FDR.

To calculate a core microbiome on fertilized eggs, bacterial taxa were identified that are present in 90% of all samples (90% was chosen arbitrarily as a threshold after visual inspection of the data; Fig. S3, Supporting information). We used the QIIME script 'compute_core_microbiome.py' for this selection and a closed BLAST search had to be adopted (Lan *et al.* 2012). Based on these core microbiomes, a synthetic metagenome was generated with PICRUST version 1.0.0 (Langille *et al.* 2013) using the online Galaxy version, analogous to Wilkins *et al.* (2015a). The OTU table was normalized and then used to predict bacterial metagenomes. Predicted metagenomes were analysed with STAMP version 2.1.2 to visualize presumable functions of the synthetic metagenomes in the three different treatment groups (Parks *et al.* 2014). Bacterial pathways were derived from the KEGG database (Kanehisa & Goto 2000; Kanehisa *et al.* 2016). To test for significant differences, we used Kruskal–Wallis H-tests, a nonparametric method for testing whether or not the medians of more than two groups are equal. This test was used to compare (i) the bacterial composition (OTUs) on fertilized eggs

among different sires and (ii) bacterial composition (OTUs) and bacterial gene pathways between the high-nutrient broth treatment and the control group. These tests were based on relative bacterial abundance in our data set. Only bacterial taxa were included that were present in all comparison groups and that met the assumptions of this distribution-free approach; that is the distribution of bacteria in each group was similarly shaped with differences in their medians (Segata *et al.* 2011). We applied a Benjamini–Hochberg multiple testing correction controlling the FDR, and the threshold was set as FDR value <0.05. The R package 'PHYLOSEQ' version 1.7.12 (McMurdie & Holmes 2013) was used to build a heat map of the most variable bacterial taxa among the offspring of different sires and treatments.

Bacterial composition on milt

To disentangle paternal environmental effects (i.e. bacterial composition on milt) and paternal genetic effects, the bacterial composition was contrasted between milt before fertilization and fertilized eggs. Bacterial communities on milt before fertilization were summarized with a heat map of the 40 most abundant core bacterial taxa, analogously to the bacterial composition on fertilized eggs above. Analyses of variance were performed to compare bacterial communities on milt and fertilized eggs in the same ways as the analyses above using 'vegan' and 'Adonis'. Student's two-sample *t*-tests of pairwise means were calculated to determine whether means were significantly different from each other. Conservative nonparametric *P*-values using 999 Monte Carlo permutations of the raw data are reported instead of parametric *P*-values from a *t*-distribution. Principal coordinate analysis (PCoA) plots (2-dimensional) were created to visualize the permutation tests. LEfSe (Segata *et al.* 2011) was used to discover discriminatory bacterial taxa between milt and fertilized eggs using the online Galaxy version. First, a pairwise Wilcoxon test was applied to identify features that differ in distribution between the two groups. Then, their effect sizes were calculated using LDA scores (Segata *et al.* 2011).

Bacterial composition of milt before fertilization, of fertilized eggs 14 days after treatment according to their mother's identity and according to their father's identity, respectively, were represented in bar plots at the class, order, family and genus level separately. These bacterial compositions were graphically represented after quality control and before normalization of the OTU table to capture most of the underlying diversity. Biological significance of the discriminatory taxa was inferred with a search in Web Of Knowledge™ version 5.12 (Thomson Reuters) or according to Austin & Austin (2007).

Results

Embryo mortality

Embryo mortality increased with elevated bacterial resources (Fig. 1a, Table 1) and was different among the offspring of different mothers and fathers (Table 1). We found significant effects of dam (but not sire) identity on the susceptibility to the stress treatment (models 5 and 6 in Table 1). Bacterial diversity and possible dam × sire interactions did not affect embryo mortality (models 2, 7 and 8 in Table 1).

Time until hatching

Time until hatching was not significantly different among treatments (Fig. 1b, Table 2). However, embryos differed in their average hatching time depending on their mother and their father (Table 2). Time until hatching could be predicted by the bacterial diversity on the eggs 14 days after treatment (Table 2, Fig. 2), but there were no significant interaction effects either between treatments and parental origin or between dams and sires (Table 2). These results did not change when another alpha diversity measure was used (Chao 1 and observed number of species, respectively), with the exception of a significant alternative alpha diversity measure × treatment interaction term (Table S1, Supporting information).

Oxygen and pH

pH measurements were not significantly different among treatments ($F_{2,59} = 0.05$, $P = 0.98$; Fig. S4, Supporting information). Oxygen concentrations decreased steadily during embryogenesis, reached a minimum before hatching and recovered after hatching. Oxygen concentrations were not significantly different among treatments ($F_{2,59} = 1.5$, $P = 0.19$).

Bacterial diversity on fertilized eggs

MiSeq sequencing of the four libraries resulted in a total of 45 588 998 reads before quality control. After the splitting of reads according to primer sequences and barcodes, a total of 19 347 049 reads could be retained ($\mu = 105\,722 \pm 51\,880$ per sample; Table S2 and S3, Supporting information). After quality control, 8916 790 reads remained ($\mu = 48\,726 \pm 24\,295$ per sample) including a mean of 1874 (±747) OTUs per sample. Rarefaction curves of alpha diversities before normalization are shown in Figure S2 (Supporting information). Bacterial diversities on fertilized eggs were not significantly affected by the nutrient broth treatment

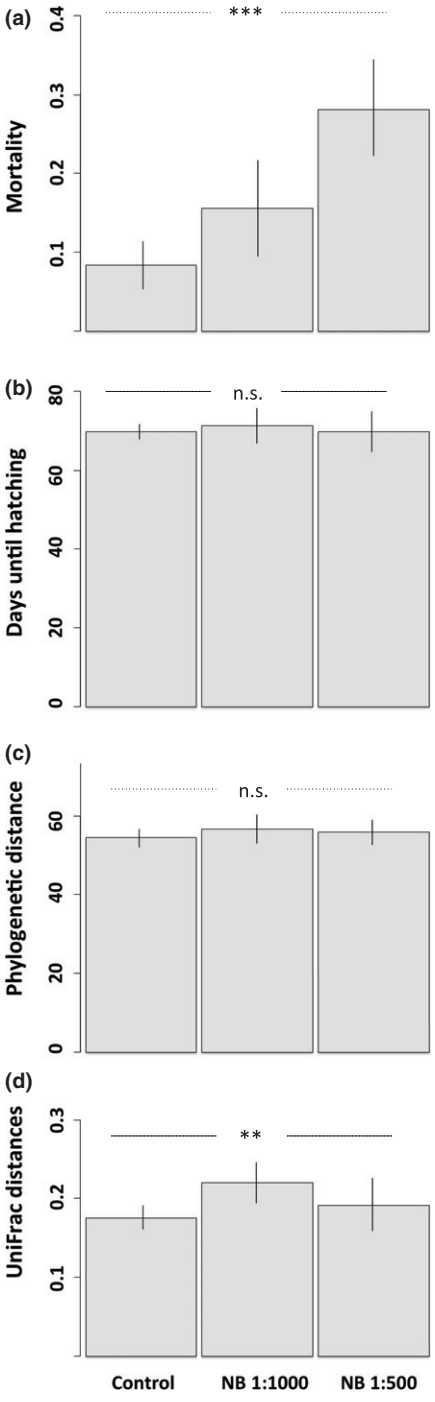

Fig. 1 Effects of treatment on embryo life history and associated microbiota. Effects of different dilutions of nutrient broth (NB; control = sham-treated) on embryo mortality (a), time until hatching (b), egg-associated bacterial diversity (c; measured as phylogenetic distance) and bacterial community distances (d; weighted UniFrac distances). Means and 95% confidence intervals across all half-sib families; n.s. = not significant; ** $P < 0.01$; *** $P < 0.001$; see text for statistics.

(Fig. 1c, Table 3 and S4, Supporting information). Bacterial diversity was significantly different among the offspring of different sires while maternal effects played no significant role (Table 3). These results did not change qualitatively when another alpha diversity measure was used (Chao 1 and observed number of species, respectively; Table S5, Supporting information).

We found no significant relationship between bacterial diversity on the eggs and embryo mortality within the three treatments (control: $r = 0.02$, $t_{54} = 0.15$, $P = 0.88$; NB 1:000: $r = 0.14$, $t_{54} = 1.02$, $P = 0.31$; NB 1:500: $r = 0.21$, $t_{54} = 1.52$, $P = 0.14$). However, bacterial diversity correlated with time until hatching ($r = 0.53$, $t_{54} = 4.6$, $P < 0.0001$; Fig. 2). This was still true when only families were analysed with no mortality ($r = 0.61$, $t_{28} = 4.4$, $P < 0.0001$; Fig. S5, Supporting information).

Bacterial composition on fertilized eggs

Bacterial compositions on trout embryos grouped according to their mother's or father's identity and within the different nutrient broth concentrations are summarized in Figure S6 and S7 (Supporting information). Bacterial communities on fertilized eggs differed significantly in composition among the three nutrient broth concentrations ($F_{2,55} = 10.4$, $R^2 = 0.07$, $P < 0.001$; Fig. 1d). We found 27 bacterial taxa that were significantly enriched in the high-nutrient broth treatment (Fig. S8a, Table S6, Supporting information). Bacteria that mostly increased in abundance included Alphaproteobacteria, *Pseudomonas*, *Caulobacter*, Rhodoclyclaceae, Caulobacteraceae, *Janthinobacterium* and Oxalobacteraceae (all >20% and $P < 0001$; for OTU IDs see Table S6, Supporting information). We also found significant parental effects on bacterial community compositions (dam: $F_{7,6} = 7.3$, $R^2 = 0.18$, $P < 0.001$; sire: $F_{6,7} = 9.4$, $R^2 = 0.21$, $P < 0.001$; Fig. 3, Table 4). Variances of bacterial composition among categorical variables (dam, sire and treatment, respectively) were not significantly different (F_2 always <2.2, P always >0.15). Figure 4 and Table S7 (Supporting information) show the differential composition of trout embryo-associated microbiotas among the offspring of different sires, including 52 characteristic bacterial taxa determined to the lowest taxonomic level possible. The variance in bacterial abundance among sires was highest in the families of Alphaproteobacteria, Hyphomonadaceae, Oxalobacteraceae, Comamonadaceae and Caulobacteraceae and in the genera of *Devosia*, *Paucibacter*, *Mycoplana*, *Arcicella*, *Caulobacter* and *Pseudomonas*. Pairwise comparisons between bacterial communities on the offspring of different sires revealed three significant

Table 1 The influence of treatment, dam and sire effects, and bacterial diversity on embryo survival

		Model parameters				Likelihood ratio tests[a]		
Model	Effects	Fixed	Random	AIC	ln L	δAIC	χ^2	*P*
Reference		**T, A**	**D, S**	**1115.7**	**−551.9**			
1	T	A	D, S	1176.3	−584.1	60.6	64.5	<0.0001
2	A	T	D, S	1113.7	−551.9	2	0.01	0.91
3	D	T, A	S	1226.4	−608.2	110.7	112.7	<0.0001
4	S	T, A	D	1124.9	−557.4	9.2	11.1	<0.001
5	T × D	T, A	S	1112.1	−545.1	3.6	13.8	0.02
6	T × S	T, A	D	1125.2	−551.6	9.5	0.5	0.99
7	T × A	T, A	D, S	1117.1	−550.6	1.4	2.6	0.27
8	D × S	T, A	D, S	1160.3	−540.2	44.6	23.4	0.91

Four main logistic mixed-effect models were compared to a reference model (in bold) to test whether treatment (T), dam (D) and sire (S) effects, and bacterial alpha diversity (A) on the eggs explain a significant part of the variance in embryo survival. Four additional models were fitted to investigate the interaction terms of treatment with dam, sire or bacterial alpha diversity, respectively, as well as the interaction of dam and sire effects. Significant effects are highlighted in grey.
[a]Degrees of freedom = 1.

Table 2 The influence of treatment, dam and sire effects, and bacterial diversity on time until hatching

		Model parameters				Likelihood ratio tests[a]		
Model	Effects	Fixed	Random	AIC	ln L	δAIC	χ^2	*P*
Reference		**T, A**	**D, S**	**5609.5**	**−2798.8**			
1	T	A	D, S	5608	−2799	1.5	0.51	0.47
2	A	T	D, S	5611.5	−2800.2	2	3.1	0.04
3	D	T, A	S	5663.1	−2826.6	53.6	55.6	<0.0001
4	S	T, A	D	5669	−2829.5	59.5	61.5	<0.0001
5	T × D	T, A	S	5613.4	−2798.8	3.9	0.09	0.95
6	T × S	T, A	D	5611.9	−2797.9	2.4	1.65	0.43
7	T × A	T, A	D, S	5610.4	−2798.2	0.9	1.1	0.3
8	D × S	T, A	D, S	5647.1	−2783.6	37.6	30.4	0.65

Different linear mixed-effect models were compared to a reference model (in bold) as in Table 1. Significant effects are highlighted in grey.
[a]Degrees of freedom = 1.

differences after correcting for multiple testing (Fig. S9, Table S8, Supporting information): The bacterial composition on the offspring of sire 2 was significantly different from sire 5 ($t = 6.6$, $P < 0.001$), sire 6 ($t = 5.34$, $P < 0.001$) and sire 7 ($t = 4.78$, $P < 0.001$).

We found 95 core bacterial taxa on fertilized eggs 14 days after treatment (Fig. S3, Supporting information) that were used to contrast differences in the putative, functional potential of bacterial communities with increasing nutrient broth concentrations (Table 5). Forty-eight significantly overrepresented bacterial pathways in the high-nutrient broth treatment relative to the control were found. The most prominent ones included bacterial toxins (effect size: 0.251, $P < 0.0001$), aminoacyl-tRNA (0.152, $P < 0.0001$), lipopolysaccharide biosynthesis (0.223, $P < 0.0001$), inositol phosphate metabolism (0.421, $P < 0.0001$) and the transcription machinery (0.189, $P < 0.0001$).

Bacterial composition on milt

Bacterial communities on fertilized eggs (Fig. S6, S7 and S8a, Supporting information) and milt (Fig. S8b and S10, Supporting information) were significantly different from each other (Adonis: $P < 0.001$, $R^2 = 89.2\%$, $F_{1,6} = 13.9$; two-sided Student's two-sample *t*-test: $t_1 = 8.9$, $P < 0.001$). Ordering the axes according to bacterial community distances of milt vs. fertilized eggs explained 57% of the variance in bacterial composition in the PCoA (Fig. 3). Thirty bacterial taxa may be used

as biomarkers for bacterial communities on milt (all with LDA scores log10 > 4; Fig. S11, Supporting information) and only two for fertilized eggs 2 weeks after treatment. Six putatively pathogenic bacterial taxa could be found on milt and fertilized eggs according to a search in Web Of Knowledge™ version 5.12 (Thomson Reuters) or according to Austin & Austin (2007): *Aeromonas* spp., *Lactococcus garvieae*, *Streptococcus iniae*, *Pseudomonas fluorescens*, *Stenotrophomonas maltophilia* and several species of the genus *Staphylococcus*.

Discussion

We found that embryo genetics defines egg-associated bacterial diversity and composition. When we tested for potential paternal carry-over effects, we discovered a great diversity of bacteria in milt that did, however, not correspond to egg-associated bacterial communities at later developmental stages. Biomarker analysis resulted in an extensive list of bacterial taxa that accounted for the difference between the two habitats. Several of the gamete-associated bacteria have been shown previously to build a tight relationship with their fish host, such as *Enterobacter hormaechei* (Sullam *et al.* 2012), *Citrobacter*, *Lactococcus* (Smith *et al.* 2015) and *Leuconostoc* (Liu *et al.* 2014). They might represent symbionts of the male spawners.

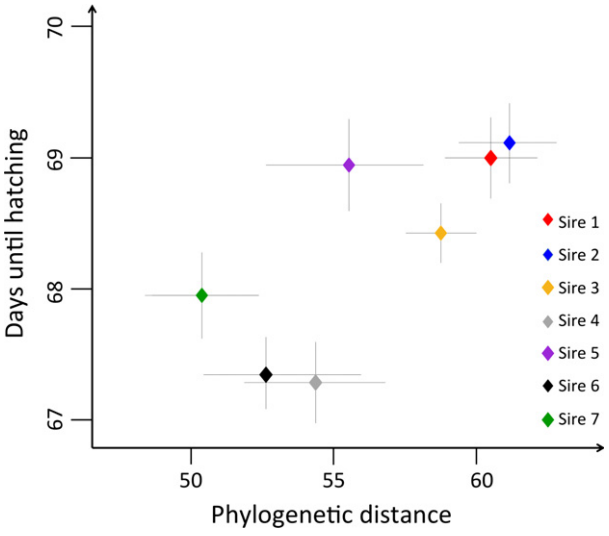

Fig. 2 Relationship of bacterial diversity and hatching time. Bacterial alpha diversity was estimated as phylogenetic distance. Means per sire and 95% confidence intervals.

Bacterial diversity on the fertilized eggs was typified predominantly by environmental bacteria and correlated with time until hatching. This correlation could be driven by attributes of the egg-associated bacteria or by consequences of the switch in host life history, for example longer time until hatching allowed for a greater diversity of associated bacteria. These two hypotheses are nonexclusive and empirical data support both: evidence for the first hypothesis includes pathogen effects that caused changes in life-history traits of salmonid embryos, for example. on the timing of hatching in particular, that have been observed in similar experimental set-ups (Thorpe *et al.* 1998; Hale 1999; Pompini *et al.* 2013; Clark *et al.* 2014). In our experimental set-up, individual bacteria on the offspring of different sires could be making the difference in timing of hatching due to variations in chorion degradation capacity (Hansen & Olafsen 1999). In contrast, host effects on bacterial diversity in salmonid embryos have been shown in field studies (Wilkins *et al.* 2015a, b). Notably, naturally spawned whitefish (*Coregonus* spp.) eggs showed a decrease in bacterial diversity during embryogenesis suggesting specific interactions between the embryo host and its associated bacteria (Wilkins *et al.* 2015b). Moreover, simultaneously collected water samples surrounding the whitefish spawning places turned out to be significantly different in bacterial

Table 3 The influence of treatment, dam and sire effects on bacterial diversity

		Model parameters				Likelihood ratio tests[a]		
Model	Effects	Fixed	Random	AIC	ln L	δAIC	χ^2	*P*
Reference		**T**	**D, S**	**1225.9**	**−606.95**			
1	T	—	D, S	1222.3	−607.17	3.6	0.42	0.81
2	D	T	S	1225.3	−607.66	0.6	1.41	0.23
3	S	T	D	1240.5	−615.27	14.6	16.64	<0.0001
4	T × D	T	S	1227.4	−606.72	1.5	0.56	0.75
5	T × S	T	D	1227.7	−606.85	1.8	0.29	0.86

Different linear mixed effect models were compared to a reference model (in bold) as in Table 1. Significant effects are highlighted in grey.
[a]Degrees of freedom = 1.

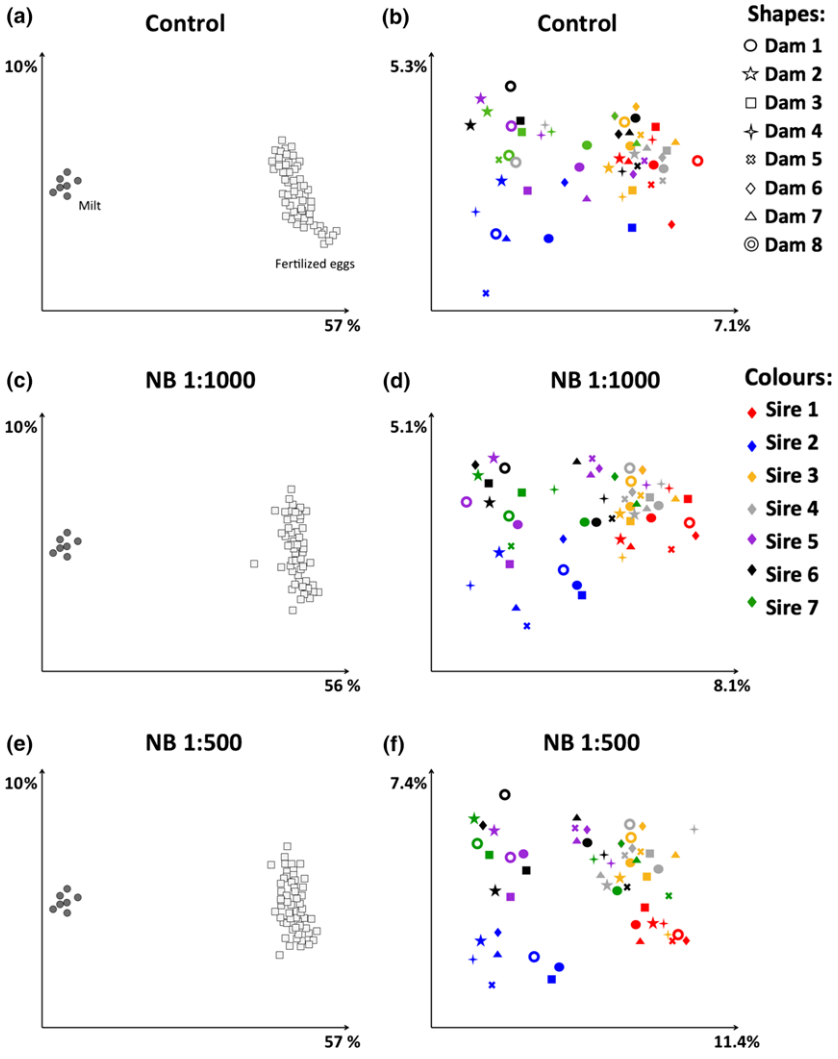

Fig. 3 Bacterial community distances on brown trout milt and fertilized eggs. Two-dimensional principal coordinate analysis showing the two axes that explain most of the variance in bacterial composition (percentages explained are given at each axis) on milt (circles) vs. on fertilized eggs (squares; a, c, e) and on embryonated eggs (b, d, f). Sham controls (panels a, b) and nutrient broth at a concentration of 1:1000 (c, d) and of 1:500 (e, f). Colours correspond to the ones used in Fig. 2.

composition. This discrepancy between bacterial composition on the host and its environment has been observed in other field studies that revealed host selection for associated bacterial composition (e.g. Llewellyn *et al.* 2014; Maignien *et al.* 2014).

Embryo mortality

Embryo mortality increased with elevated bacterial resources, confirming previous findings in *Coregonus palaea* (Wedekind *et al.* 2010; Clark *et al.* 2013a) and *S. trutta* (Jacob *et al.* 2010). Mortality could not be directly linked to oxygen depletion or to a change in acidity in the wells. Moreover, oxygen levels in the wells never reached a lower limit known to obstruct embryo development (Wedekind & Müller 2004). This supports the hypotheses that either a change in bacterial life history or in bacterial community composition has detrimental effects on embryo hosts. Contrary to previous observations in whitefish (Clark *et al.* 2013a, 2014), we did not find any treatment effects on the timing of hatching.

Environmental effects

We found that the composition of bacterial communities and their putative functional pathways were dependent on the availability of bacterial resources. Most of the bacterial taxa that were significantly more abundant in the high-nutrient broth treatment compared to the control could not be identified at the species level, including bacterial sequences in the groups of Alphaproteobacteria, Rhodocyclaceae, Caulobacteraceae, Oxalobacteraceae and Comamonadaceae. Several taxa could be assigned to genera, such as *Pseudomonas*, *Caulobacter*, *Janthinobacterium*, *Rhodoferax*, *Paucibacter* and *Acidvorax*. At the species level, there were *Methylotenera mobilis* and *Pseudomonas veronii* enriched, while

Blastomonas natatoria, *Flavobacterium succinicans* and *Sphingopyxis alaskensis* were under-represented. Increased host mortality at elevated nutrient concentrations suggests that changes in microbial communities affected bacterial virulence. This could be due to an increase of virulent bacteria or due to indirect effects; that is essential bacteria for normal embryonic development are outcompeted when the resource conditions change. Bacteria with a pathogenic potential could be found on milt, as well as on fertilized eggs. Some of the bacterial taxa that markedly increased with elevated bacterial resources have been shown in other studies to harm their salmonid hosts. For example, *Aeromonas* spp. can cause septicaemia or ulcer disease in many freshwater fishes (Austin & Austin 2007), and several studies on various salmonids have reported pathogenic effects of infection by *Lactococcus garvieae* and *Streptococcus iniae* (Eldar & Ghittino 1999), *Pseudomonas fluorescens* (von Siebenthal *et al*. 2009; Clark *et al*. 2013a), *Stenotrophomonas maltophilia* (Looney *et al*. 2009) or species of the genus *Staphylococcus* (Craig & Pilcher 1966; Gil *et al*. 2000). However, changes in bacterial abundance in our data set are only relative within the same data set and cannot be taken as direct estimates of bacterial abundance because we did not estimate cell counts for any bacterial group.

Nutrient broth might affect bacterial communities by creating competition for increased resources (Rasche *et al*. 2011). Bacterial species have shown to differ in their efficiencies of converting nutrients into growth (Weintraub *et al*. 2007). A change in density of particular groups of bacteria could lead to a transition in their life-history strategies that turns benign bacteria into virulent ones (Diggle *et al*. 2007; Wedekind *et al*. 2010). Superior *r*-strategist from freshwater ecosystems that were enriched in the high-nutrient broth treatment included *Caulobacter* spp. (Amon 1998), *Janthinobacterium*, particularly at low water temperatures (Alonso-Saez *et al*. 2014), and *Paucibacter* (Lymperopoulou *et al*. 2012). Lymperopoulou *et al*. observed a simultaneous increase of *Paucibacter* with sequences of Alphaproteobacteria and Sphingomonadaceae.

Another strategy that some bacteria use to outcompete others is the production of antibiotic compounds (Cordero *et al*. 2012). Antibiotic production comes with a cost that may only be affordable if resources are prevalent (Morlon 2012). Antimicrobial compounds or their metabolites can cause harm to the embryo hosts as a side effect (Hill *et al*. 2005). We can only hypothesize why this community dynamics increased embryo mortality. Experimental manipulations of the trout egg-associated bacterial community are necessary to infer causalities (Smith *et al*. 2015; Waldor *et al*. 2015).

We did not find evidence for an interaction between environmental effects and host genetics but changes in bacterial community composition were accompanied by switches in the bacteria's functional potential. Putative functional gene pathways of bacteria that were enriched at high-nutrient broth concentrations included, for example, the biosynthesis of bacterial toxins. The involvement of this pathway aligns well with competition among bacteria and detrimental effects to the embryo host (Chow *et al*. 2014; Evans & Wallenstein 2014). Further harmful combinations for the embryo host comprised gene pathways of pertussis, cancer, influenza or toxoplasmosis. This list of putative bacterial gene pathways can be used for future hypothesis testing and experimental designs using the salmonid embryo host system.

Table 4 Analyses of variance in bacterial community distances 14 days after treatment

Model terms	d.f.	SS	*F*	R^2	*P*
Treatment	2	0.22	10.36	0.07	<0.001
Dam	7	0.53	7.33	0.18	<0.001
Sire	6	0.59	9.37	0.21	<0.001
Treatment × dam	14	0.18	1.22	0.06	0.09
Treatment × sire	12	0.11	0.91	0.03	0.66
Residuals	126	1.31		0.44	
Total	167	2.94		1	

The effects of treatment, parental origin and their interactions were tested in one multivariate analysis of variance using weighted UniFrac distance matrices among bacterial communities on eggs as the dependent variable with permutation tests for significance (function 'Adonis' in R package 'vegan'). Significant effects are highlighted in grey.

Parental effects

Because our experimental design was fully crossed, that is each female was crossed to each male, and each cross

Fig. 4 Differences in bacterial community composition among the offspring of different sires. Bacterial taxa (OTUs: operational taxonomic units) that differed significantly in abundance among the offspring of different sires irrespectively of treatment. The abundance of OTUs is given in shades of grey (dark = high abundance, light = low abundance). Samples are ordered according to multidimensional scaling (Rajaram & Oono 2010). *P*-values refer to a significant difference in abundance of bacteria according to the stamp analysis (a Benjamini–Hochberg multiple testing correction was applied controlling the false discovery rate). Effect sizes of medians among the offspring of different sires are shown.

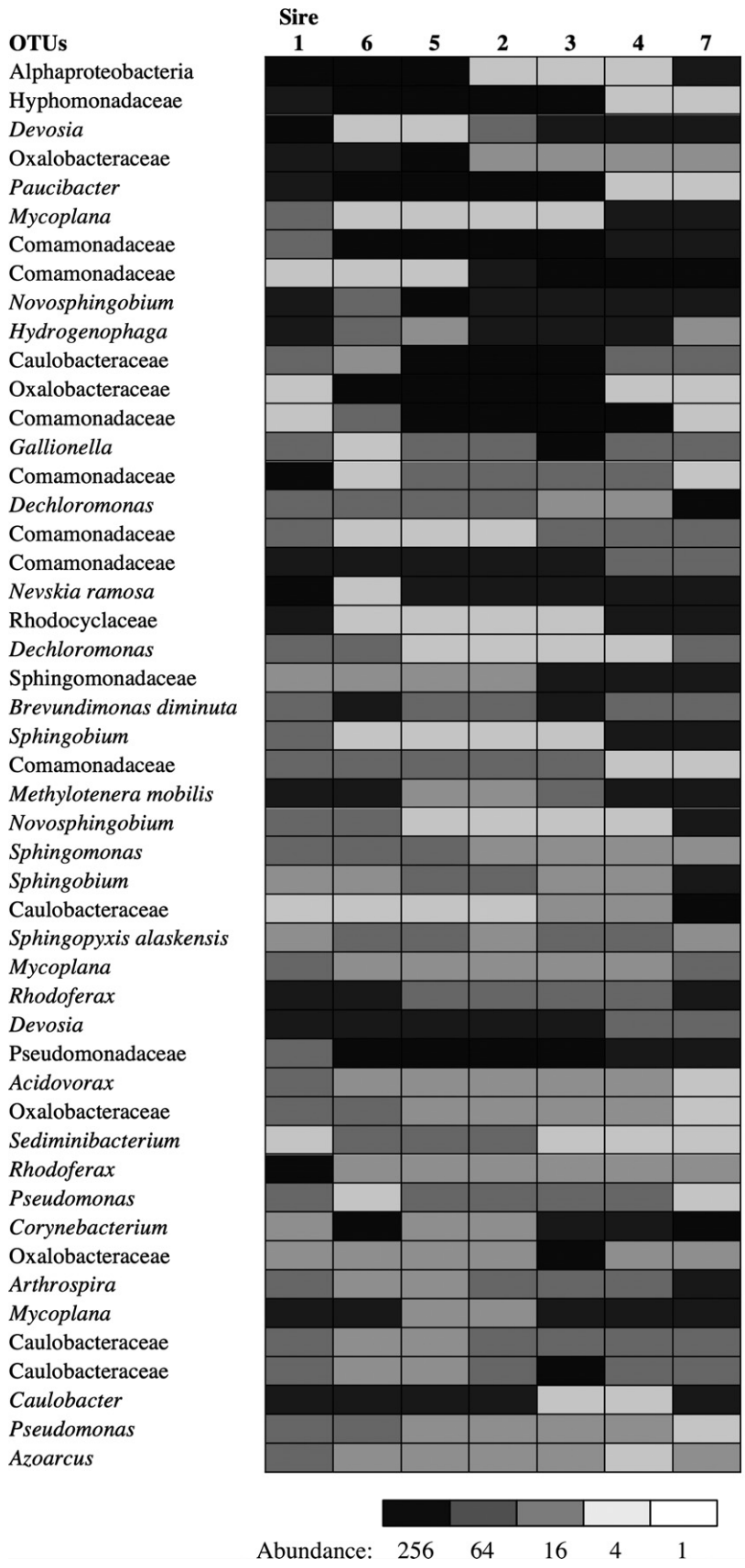

Table 5 Differences in predictive, functional pathways of core bacterial communities on fertilized eggs among different treatment groups

Observed pathway	*P*-values (corrected)	Effect size
Bacterial toxins	<0.0001	0.251
Aminoacyl-tRNA biosynthesis	<0.0001	0.152
Lipopolysaccharide biosynthesis proteins	<0.0001	0.223
Inositol phosphate metabolism	<0.0001	0.421
Transcription machinery	<0.0001	0.189
Lysine biosynthesis	<0.0001	0.161
Homologous recombination	<0.0001	0.126
Lipopolysaccharide biosynthesis	<0.0001	0.165
Alanine, aspartate and glutamate metab.	<0.0001	0.222
Pertussis	<0.0001	0.128
Glutamatergic synapse	<0.0001	0.21
Riboflavin metabolism	<0.0001	0.324
Pathways in cancer	<0.0001	0.22
Translation factors	<0.0001	0.122
Plant–pathogen interaction	<0.0001	0.138
Isoquinoline alkaloid biosynthesis	<0.0001	0.178
Biosynthesis of siderophore group	<0.0001	0.185
Lipoic acid metabolism	<0.0001	0.14
Protein export	0.0001	0.117
Progesterone-mediated oocyte maturation	0.0001	0.117
Antigen processing and presentation	0.0001	0.117
DNA replication	0.0002	0.114
Peptidoglycan biosynthesis	0.0002	0.11
Lipid biosynthesis proteins	0.0002	0.109
Biotin metabolism	0.0002	0.115
D-Glutamine and D-glutamate metabolism	0.0002	0.114
Amyotrophic lateral sclerosis (ALS)	0.0003	0.108
Glycerophospholipid metabolism	0.0004	0.102
Phosphonate and phosphinate metabolism	0.0005	0.099
Glycosyltransferases	0.0008	0.094
DNA replication proteins	0.001	0.092
Two-component system	0.001	0.087
Mismatch repair	0.001	0.087
Amino sugar and nucleotide sugar metabolism	0.002	0.08
Nucleotide excision repair	0.002	0.082
Phenyltransferases	0.002	0.081
Fatty acid biosynthesis	0.003	0.077
p53 signalling pathway	0.005	0.071
Viral myocarditis	0.005	0.071
Influenza A	0.005	0.071
Toxoplasmosis	0.005	0.071
Tropane, piperidine and pyridine biosynthesis	0.008	0.064
One carbon pool by folate	0.02	0.053
Cyanoamino acid metabolism	0.02	0.054
Pentose and glucuronate interconversions	0.02	0.053
Cell cycle—Caulobacter	0.03	0.045
Phenylpropanoid biosynthesis	0.03	0.047

'Observed pathways' were derived from the KEGG database using STAMP version 2.1.3 (Parks *et al.* 2014). Effect sizes of medians between the high-nutrient broth treatment (1:500) and the control group as well as corrected *P*-values are shown (a Benjamini–Hochberg multiple testing correction was applied controlling the false discovery rate, and the threshold was set as <0.05). Effect sizes are given as difference in median relative frequencies.

experienced all treatments, we were able to estimate significance and strength of all individual effects. Dam effects were found for treatment-induced mortality, time until hatching and bacterial community composition. Dam effects represent a mix of maternal genetic and maternal environmental effects (Royle *et al.* 1999; von Siebenthal *et al.* 2009; Aykanat *et al.* 2012). For example, maternal effects can include the expression of specific zygotic genes as well as supplements, such as proteins or mRNA that the mother allocated to the eggs before spawning (D'alba *et al.* 2010).

Sire effects were found for embryo mortality and time until hatching; that is, there was additive genetic variance for viability under our experimental conditions. Such genetic sire effects are estimated without the need of genomic sequencing, and they can be interpreted as the immune competence of the embryo (Evans *et al.* 2010; Clark *et al.* 2013a; Wilkins *et al.* 2015c), its genetic load or overall genetic variability (Neff & Pitcher 2005) and its genetic quality (Neff & Pitcher 2005; Wedekind *et al.* 2008; Jacob *et al.* 2010). We also found significant sire effects on egg-associated bacterial diversity and composition. The offspring of different sires varied with regard to bacterial composition and abundance of particular bacterial groups. This included many environmental bacteria with known origins in freshwater systems, such as *Brevundimonas diminuta* (Han & Andrade 2005), members of the family of Caulobacteraceae (Newton *et al.* 2011), taxa in the genera of *Comamonas*, *Rhodoferax*, *Hydrogenophaga*, *Limnohabitans*, *Delftia* and *Devosia* (Madigan *et al.* 2010; Zhang *et al.* 2012), as well as *Flavobacterium* (Newton *et al.* 2011), and the family of Hyphomonadaceae (Madigan *et al.* 2010; Newton *et al.* 2011). Differences in bacterial community composition were mostly pronounced in the high-nutrient broth treatment. When we compared different sires within this treatment in a pairwise manner, not all bacterial communities were

markedly different from each other. Bacteria on the offspring of sire 2 were the most distinct. Assuming that these embryos only inherited genes from their father, it seems that this father was the most genetically distinct sire. The offspring of this father may either actively influence their associated bacteria through immune gene expression (Finn 2007; Clark *et al.* 2013a; Wilkins *et al.* 2015c) or passively through secondary metabolites (Milligan-Myhre *et al.* 2011; Stephens *et al.* 2016) and surface receptors coded by the host genotype (Llewellyn *et al.* 2014). Hatchery managers may be advised to use a large number of sires for their breeding protocols in order to minimize the detrimental genetic effects of particular individuals. Moreover, they should keep organic pollution at a minimum.

In conclusion, our experiment demonstrates that the environmental effects and host genetic factors characterize the fish egg–bacteria ecosystem. Assessing the causal role of host genetic variation on bacterial community dynamics will help us understand the mechanisms of colonization and the correlation to specific functions of host-associated bacterial communities.

Acknowledgements

We thank S. Blaser, G. Brazzola, E. Clark, M. dos Santos, P. Engel, C. Heiniger, A. Liesch, L. Marques da Cunha, Y. Poirier, M. Pompini, T. Reusch, N. Reusser, A. Rogivue, J. van der Meer and F. Witsenburg for assistance and/or discussion; U. Gutmann, B. Bracher and M. Schmid (Fischereistützpunkt Reutigen) for catching the fish and stripping them, C. Küng (Fisheries Inspectorate Berne) for permissions; and G. Stone and three reviewers for comments. This study complied with the relevant ethical regulations imposed by the University of Lausanne and the canton in which it was carried out. Additional authorizations from the cantonal veterinary offices were not required as all manipulations on embryos were performed prior to yolk sac absorption. The Swiss National Science Foundation provided funding for this study. L. Wilkins was supported by a fellowship in Life Sciences (Faculty of Biology and Medicine, University of Lausanne).

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L.F., C.W. and L.W. designed the project. L.W. and C.W. executed the experiment. L.W. performed the molecular genetic and the statistical analyses. All authors participated in the discussion of the results. L.W. and C.W. wrote the manuscript that was then critically revised L.F.

Data accessibility

Data on embryo performance, OTU abundance and identity for each sample, R-scripts and a map file as used for the QIIME analysis are deposited on the Dryad repository doi: 10.5061/dryad.jg33 m. Raw sequencing data are archived at the NCBI sequence read archive (SRA) under BioProject number PRJNA329047 and BioSample number SAMN05390601. Individual samples are deposited under reference SRP079355.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 The influence of treatment, dam and sire effects, and alternative bacterial diversity measures on time until hatching.

Table S2 Number of reads after the different steps of quality control.

Table S3 Samples and their barcodes used in this study.

Table S4 Summary of mean bacterial diversities.

Table S5 The influence of treatment, dam and sire effects on alternative bacterial diversity measures.

Table S6 Differential abundance of bacteria in the high nutrient broth treatment.

Table S7 Differential abundance of bacteria among the offspring of different sires.

Table S8 Pairwise comparisons of bacterial communities among the offspring of different sires in the high nutrient broth treatment.

Fig. S1 Experimental procedures.

Fig. S2 Rarefaction curves of alpha diversity measure.

Fig. S3 Number of bacterial taxa that could be found at different fractions of samples.

Fig. S4 Average oxygen concentrations and pH measurements in a subsample of experimental cells across treatments.

Fig. S5 Relationship of bacterial diversity and hatching time in brown trout embryos.

Fig. S6 Characterization of microbiotas on fertilized brown trout eggs in the cold room 14 days after treatment grouped according to mother's identity.

Fig. S7 Characterization of microbiotas on fertilized brown trout eggs in the cold room 14 days after treatment grouped according to father's identity.

Fig. S8 Heatmap of core bacterial taxa on fertilized brown trout eggs and milt.

Fig. S9 Pairwise comparisons of bacterial communities among the offspring of different sires in the high nutrient broth treatment.

Fig. S10 Characterization of microbiotas on brown trout milt before fertilization.

Fig. S11 Histogram of the LDA scores computed for features differentially abundant between brown trout milt before fertilization and fertilized eggs.