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Additive Genetic Effects on Embryo Viability in a Whitefish (Salmonidae) Influenced by the Water Mould *Saprolegnia ferax*

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Abstract

Animals and plants are associated with symbiotic microbes whose roles range from mutualism to commensalism to parasitism. These roles may not only be taxon-specific but also dependent on environmental conditions and host factors. To experimentally test these possibilities, we drew a random sample of adult whitefish from a natural population, bred them *in vitro* in a full-factorial design in order to separate additive genetic from maternal environmental effects on offspring, and tested the performance of the resulting embryos under different environmental conditions. Enhancing the growth of symbiotic microbes with supplemental nutrients released cryptic additive genetic variance for viability in the fish host. These effects vanished with the concurrent addition of the water mould *Saprolegnia ferax*. Our findings demonstrate that the heritability of host fitness is environment-specific and critically depends on the interaction between symbiotic microbes.

Keywords: *Coregonus* sp.; *Saprolegnia* sp.; Symbiotic microbe; Fish embryo; Cryptic genetic variation

Introduction

Opportunistic microbial pathogens are ubiquitous in the aquatic environment, and naturally spawned eggs are typically associated with microbial communities that can be benign or virulent depending on host characteristics or environmental conditions [1,2]. The interactions within these communities, as well as between them and their hosts, are likely to be complex and dynamic [3]. On the one hand, microorganisms typically live in rich communities where different species or even different life-history forms of same strains influence each other [4-7]. On the other hand, amphibian and fish embryos have diverse defenses against microbial pathogens. Egg characteristics and maternally transmitted substances seem to play a major role [8], especially so at early developmental stages. At late developmental stages, embryos also develop an immune competence based on their own genetic constitution, as demonstrated in half-sib breeding experiments in amphibians [9] and fish [10,11,12].

Even though hosts are often likely to be challenged with different sorts of microorganisms at once, few studies have examined the role of microbial ecology on infection [13] and those that have been conducted have primarily been confined to mice [14]. Whitefish embryos (*Coregonus* sp., Salmonidae) and their symbiotic microbes offer an excellent opportunity to study the interplay between microbes and host factors. Whitefish are external fertilizers, have no parental care, and generate large numbers of gametes. This allows for full-factorial *in vitro* fertilizations and within-family comparisons [15]. Here we used such an experimental approach to examine whether changes in the environmental conditions, especially with the addition of the water mould *Saprolegnia ferax*, can affect fitness and the heritability of fitness-relevant traits within a host population.

If virulence is defined as the severity of a disease and measured by the reduction of host fitness [16], simple microhabitat changes like, for example, changes in the availability of resources can be sufficient to turn opportunistic into virulent fish pathogens [2,17]. This link between resource availability and virulence of microbial symbionts can be a very direct one, as experimentally demonstrated by Wedekind et al. [2] enhanced growth of microbes can induce host mortality before potential secondary stressors like, for example, oxygen deficiency could possibly harm fish embryos, and treatment with antibiotics and fungicides cancels the effects of resource availabilities. While these previous results demonstrate the importance of environmental conditions on the performance of whitefish embryos, the possible interaction between these environmental conditions and additive genetic effects on the host's side have not yet been explored. We therefore bred whitefish *in vitro* and challenged the resulting embryos by adding supplemental nutrients as in Wedekind et al. [2] and by further adding the oomycete *Saprolegnia ferax*, i.e. a ubiquitous aquatic microbe and opportunistic pathogen [18] that has previously found associated with whitefish eggs [19]. We used an experimental breeding design and a full-factorial treatment that allowed us to separate sire from dam effects on embryo performance. Because whitefish embryos receive only genes from their fathers, sire effects on embryo survival directly reveal additive genetic variance for fitness. The aims of the present study were (i) to determine whether host survival varied across different environments, and (ii) to estimate the importance of embryo genetics on survival in each ecological context.

Materials and Methods

Coregonus palaea (a large-type lake whitefish) were caught with gill nets from their spawning grounds in Lake Geneva, Switzerland. Twelve females and 12 males were haphazardly selected and stripped of their gametes. Gametes were used for artificial fertilizations in a full-factorial breeding design [20] yielding 144 sib groups. Fertilizations and egg washing were conducted as described in von Siebenthal et al. [12]. The water used here and subsequently to raise the embryos had been chemically standardized according to the OECD guideline

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203 (OECD 1992), aerated, and tempered before use. Each sib group was divided among three Petri dishes, each with 50 ml of standardized water. Fertilized eggs were stored in a climate room at 6.5°C, and left undisturbed for two weeks. Water was then exchanged and embryos were examined to determine fertilization success and viability. Sib groups with very low fertilization success were discarded as they contained an insufficient number of eggs for experimental work. Fertilization success was not used as a variable in the analysis as it is prone to reflect differential handling efficiency of the experimenter. In order to keep close to a full-factorial design while maintaining a large sample size, the design was reduced to 10 dams and 8 sires resulting in 78 sib groups. Seven embryos were selected from each sib group and placed with sterile Pasteur pipettes into 24-well plates (Falcon, Becton Dickinson) filled with 2 ml of sterile standardized water per well. Embryos were placed one per well in a block-wise design, so that four plates contained one replicate set of all parental combinations. This resulted in 28 plates with 546 embryos. After egg distribution, plates were placed into cooling units at 2.5°C to retard development, providing time for further preparations (see below). Eggs were left undisturbed until the late eyed stage (218 degree days), except for weekly controls. During the controls, embryos were examined on a light table (Hama professional, LP 555) and with a stereo zoom microscope (Olympus SZX9) at 6.5°C. No mortality or developmental abnormalities were observed during this period of the study.

S. ferax (DSMZ 1489) was cultivated on potato dextrose agar (PDA). Agar blocks were routinely cut from areas of thick hyphal growth and inverted onto new PDA plates. Zoospores were prepared as described in Bly et al. [21], with the following modifications. Agar plugs with *S. ferax* growth were cut from PDA plates and transferred to 10 50ml conicals, each filled with 20ml of PG-1 broth. Conicals were placed on a shaker (120 rpm) and left at 22°C for three days. After, the media was removed from the conicals and mycelia were rinsed three times with sterile-filtered pond water. Mycelia were placed into Petri dishes, each with 20 ml of sterile-filtered pond water. Plates were left at 22°C overnight. Ten milliliters were pipetted off the top of the Petri dishes and pooled. Zoospores concentrations were determined using a Neubauer hemocytometer. This method generally yielded ~ 2000 cysts/ml.

The *S. ferax* solution was diluted such that an inoculation with 90µl per well resulted in a concentration of 100 cysts/ml. Prior to inoculation, Tryptic Soy Broth (TSB) (Sigma Aldrich, Buchs, Switzerland), a general microbial growth medium, was added to the solution, resulting in a concentration of 1:20,000 TSB per well. Fifty days post fertilization, 234 eggs (3 replicates per family combination) were exposed to 90µl/well of the oomycete. The remaining eggs were inoculated with 90µl of, respectively, sterile standardized water (control) or TSB in sterile standardized water (resulting in a 1:20,000 dilution per well). Following inoculation, plates were kept at 6.5°C and left undisturbed for one week. Mortality or hatching was then recorded daily until all embryos had either hatched or died.

Each embryo was treated as an independent replicate. Embryo mortality was entered as a binary response variable in generalized linear mixed models (GLMM). Treatments were entered as fixed effects, and parental identity as random sire and dam effects. Sire and dam interaction effects were not included in the model due to low full-sibship replicate numbers per treatment. Moreover, in previous studies, sire and dam interaction effects were not found to have a significant effect on pathogen-induced embryo mortality [22,12]. To determine whether fixed or random effects explained a significant amount of variation in embryo mortality, a reference model including all effects was compared to a reduced model lacking the effect that was tested. Likelihood ratio tests were then used to compare "goodness of fit" between models. All analyses were done with the R software [23] including the lme4 package [24].

Results

Adding TSB increased mortality in comparison to the control (Figure 1 & Table 1a), but this effect was largely cancelled when TSB was applied together with *S. ferax* (Table 1b, c). Embryo mortality was influenced by dam effects in all treatment groups (Table 2). Sire effects were only significant in the high-stress group "TSB" (Table 2 & Figure 1). No significant sire effects could be found in the control or in the "*S. ferax*" treatment.

Adding supplemental nutrients, either with or without *S. ferax*,

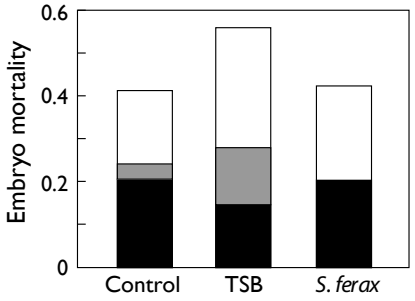

Figure 1: Average embryo mortality at elevated resource availability ("TSB") and in combination with *S. ferax*. The amount of variance that is explained by dam and sire effects is indicated in black and gray shading, respectively (see also Table 2).

Model*	Effect tested	AIC	X^2	d.f.	p
a) Control vs. TSB					
t + d + s		409.3			
d + s	Treatment	415.9	8.6	1	0.003
t + d + t|s	Treatment x sire	411.9	1.4	2	0.51
t + t|d + s	Treatment x dam	404.2	9.1	2	0.01
b) TSB vs. *S.ferax*					
t + d + s		503.2			
d + s	Treatment	509.0	7.8	1	0.005
t + d + t|s	Treatment x sire	502.2	5.0	2	0.08
t + t|d + s	Treatment x dam	506.3	0.9	2	0.63
c) Control vs. *S.ferax*					
t + d + s		502.4			
d + s	Treatment	500.6	0.2	1	0.65
t + d + t|s	Treatment x sire	505.8	0.6	2	0.74
t + t|d + s	Treatment x dam	498.1	8.3	2	0.02

*t = treatment; d = dam; s = sire, t|d = dam and dam x treatment interaction; t|s = sire and sire x treatment interaction

Table 1: Likelihood ratio tests on mixed model logistic regressions on embryo mortality (based on a binomal distribution). Different models are compared to determine whether a treatment by itself or in interaction with parental effects caused changes in embryo mortality. "TSB" = addition of supplementary nutrients to induce growth of microbial pathogens, "*S.ferax*" = addition of TSB plus *S. ferax*. The Akaikes information criterion (AIC) indicates the quality of fit of a model. P-values were obtained from comparisons between a model and its reference model (t+d+s).

Model*	Effect tested	AIC	X^2	d.f.	p	σ^2 (% of total)
a) Control						
d + s		199.3				
d	Sire	198.5	1.1	1	0.28	0.03 (8.0)
s	Dam	217.0	19.7	1	<0.001	0.21 (52.5)
b) TSB						
d + s		211.5				
d	Sire	215.7	6.2	1	0.01	0.09 (23.7)
s	Dam	214.7	5.2	1	0.02	0.10 (26.3)
c) *S.ferax*						
d + s		305.2				
d	Sire	303.2	0.0	1	1.0	0.0 (0.0)
s	Dam	326.0	22.8	1	<0.001	0.17 (47.2)

*d = dam, s = sire

Table 2: Likelihood ratio tests on mixed model logistic regressions on embryo mortality within the three treatments. Different models are compared to determine whether sire and dam (random factors) cause significant variance in embryo mortality (binary response variable). The Akaikes information criterion (AIC) indicates the quality of fit of a model. P-values were obtained from comparisons between a model and the respective reference model (s+d) within each treatment.

changed the maternal effects on embryo viability (Table 1a,c), even if, overall, the *S. ferax* treatment did not significantly increase embryo mortality. This means that the *S. ferax* treatment had different effects on different maternal sib groups, and that these effects cancelled each other out when averaged over the whole study population. None of the possible treatment by sire interactions was statistically significant (Table 1). No treatment by parent interaction was found in the model including "TSB" and "*S. ferax*" (Table 1b), suggesting that the oomycete does not interact with host factors when nutrient levels are high.

Discussion

We found that elevated resource availability negatively impacted host survival. A recent experiment done under very similar conditions demonstrated that addition of nutrients directly increases bacterial numbers and fungal biomass in a concentration-dependent manner, and thereby affects the survival of whitefish embryos [2]. Moreover, enhanced nutrient concentrations could be used to experimentally induce pathogen-related stress in another salmonid [17]. As in these previous studies, the increased mortality we observed in our experiments is likely to stem from a shift in the symbiotic microbial communities from benign to virulent. However, we also found that the detrimental effect of additional nutrients could be largely negated through the addition of a third-party microbe. Co-inoculation with *S. ferax* significantly reduced embryonic mortality under resource-rich conditions, i.e. the oomycete mitigated the virulence-enhancing effects of the nutrient treatment. Because we based our experiments on Wedekind et al.'s [2] protocol, it is unlikely that the observed patterns in mortality resulted from a secondary stressor like reduced oxygen concentrations. Moreover, should oxygen have been depleted with increased bacterial growth, it should have been equally consumed in the presence of *S. ferax* resulting in comparable mortality.

Parental effects on embryo viability could be found in all treatment groups. These parental effects were largely due to dam effects, which can be a mixture of maternal environmental and additive genetic effects. Significant sire effects could only be found in the TSB treatment that caused the highest embryo mortality. If epistatic effects were negligible, the additive genetic variance for embryo viability in this treatment group would be 0.95 (4 x the sire component of variance, see Lynch and Walsh [20]. However, such quantitative estimates should be taken with care. First, previous analyses on whitefish suggest that epistatic effects are not negligible [25], i.e. that the additive genetic variance is likely to be overestimated here. Second, it remains unclear whether heritability estimates taken from such laboratory studies can be representative for natural situations. Third, in the present study we could not determine possible non-additive genetic effects. However, previous studies on the interaction between microbial pathogens and embryos in late developmental stages suggest that non-additive genetic effects on host resistance/tolerance are negligible in whitefish [22,12].

No significant additive genetic variation could be observed in the other treatment groups. This supports the hypothesis that environmental stress releases cryptic genetic variation, i.e. that some genetic variance for fitness-relevant traits persists but is not apparent under some environmental (usually benign) conditions [26-28].

Oomycetes of the genus *Saprolegnia* can be important pathogens of fish and amphibia [18]. However, their virulence is typically context-specific, depending, for example, on host population density [29], microbial competitors [8,14], or on the host's developmental stage and immune competence [8,9,31-32]. Within the context of our experiment, *S. ferax* showed even probiotic characteristics, i.e. it conferred a beneficial effect to the host by reducing the virulence of the ambient microbial community [33,34]. *S. ferax* may not be equally beneficial in other contexts (i.e. without addition of supplemental nutrients). Here, its overall positive effects were significant when growth of microbial communities was experimentally enhanced. However, we also found a significant dam x treatment effect when comparing embryo mortality in the control to the *S. ferax* treated groups. Different maternal sib groups reacted differently to changes in their microbial communities, i.e. *S. ferax* seems to confer positive effects to some types of hosts (with their specific microbial symbionts) and be virulent to others.

The question remains as to how *S. ferax* quelled the virulent effect of supplemental nutrients. As we did not find significant host by treatment interactions in the resource-rich environments, the positive effect of *S. ferax* seems to be mainly due to competitive interaction with other microbes. It is possible that *S. ferax* releases compounds, e.g. antibiotics that suppress bacterial growth. However, extracts from a *S. ferax* culture were not found to prevent bacterial proliferation (data not shown). Alternatively, the oomycete may have become dominant in the environment, utilizing the nutrients for its own growth and development, thus impeding more virulent egg pathogens. Previous studies examining antagonism between fungi and bacteria have suggested similar trophic processes between competing microbes [35].

Despite the relative ease with which fish embryos can now be studied under natural or controlled laboratory conditions, our current understanding of fish egg-associated microbial communities and their relationship with disease is still weak. The taxa that are frequently isolated from healthy eggs of freshwater species (e.g. *Pseudomonas* spp, *Flavobacterium* spp. *Aeromonas* spp, and *Saprolegnia* spp) are paradoxically also believed to be the causative agents of infection. Notably, most of our information concerning the embryonic microbiota comes from culture-based microbiological methods, which may only reveal a fraction of the total microbiota [36,37]. Next-generation sequencing technology will provide a better understanding of the complex compositions of microbial communities of salmonid embryos.

In conclusion, adding nutrients to enhance growth of symbiotic

microbes increased embryo mortality and released cryptic genetic variation for viability in a natural population of whitefish. Addition of *Saprolegnia ferax* reduced host mortality in nutrient-rich conditions, probably due to competitive interactions between different microbes. These beneficial effects had a strong effect on the pattern of expressed genetic variation in the host population.

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