Effects of resource availability on evolution of virulence and competition in an environmentally transmitted pathogen

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Abstract

Understanding ecological and epidemiological factors driving pathogen evolution in contemporary time scales is a major challenge in modern health management. Pathogens that replicate outside the hosts are subject to selection imposed by ambient environmental conditions. Increased nutrient levels could increase pathogen virulence by pre-adapting for efficient use of resources upon contact of a nutrient rich host or by favouring transmission of fast-growing virulent strains. We measured changes in virulence and competition in Flavobacterium columnare, a bacterial pathogen of freshwater fish, under high and low nutrient levels. To test competition between strains in genotype mixtures, we developed a quantitative real-time PCR assay. We found that the virulent strain maintained its virulence and outcompeted less virulent strains independent of the nutrient level and resource renewal rate, but a less virulent strain further lost virulence in flow-through chemostats under low nutrient level and in long serial culture under high nutrient level. Our results suggest that increased outside-host nutrients might maintain virulence in less virulent strains and increase their contribution in epidemics in aquaculture. The results highlight a further need to study the role of resources in the outside-host environment in maintaining strain diversity and driving evolution of virulence among environmentally growing pathogens.

Key words: bacterium, Flavobacterium columnare, fish disease, interference competition, qPCR, resource competition
Background

Evolution can have a significant impact on properties and responses of organisms within ecological time scale over a few tens or hundreds of generations (Geerts, et al. 2015, Stuart, et al. 2014, Yoshida, et al. 2003). These effects can be especially pronounced in organisms with short generation times, such as microbes (Hiltunen, et al. 2014). Evolutionary changes resulting in more serious disease and epidemics are presenting major challenges for health management (Griette, et al. 2015), and emphasize the importance of understanding the ecological and epidemiological factors underlying the evolutionary changes for disease control (Bull and Lauring 2014, Cressler, et al. 2016, Kennedy, et al. 2016, Lively, et al. 2014).

Hosts and the nutrition they offer are among the fundamental selective environments encountered by pathogens. However, in pathogens capable of environmental growth and replication, the outside-host environment might be equally important by favouring phenotypic properties that increase fitness in that particular environment (Brown, et al. 2012) and selecting for genotypes conferring these properties (New, et al. 2014). Thus outside-host conditions can be important for ecological and evolutionary trajectories for environmentally replicating opportunistic pathogens.

Microbial competitive ability in a particular resource environment is mainly governed by resource utilization traits such as growth rate (Litchman, et al. 2015). For environmentally growing bacteria, fast growth is essential in fighting against competing microbes, but will also increase the probability of being the first to infect a host upon contact. High resource availability may therefore increase the share of fast growing strains in host infections.

Resource environment may also direct selection on resource utilization traits (Litchman, et al. 2015) and induce diversification in resource use (Cooper and Lenski 2000, Jasmin and Kassen 2007, Szappanos, et al. 2016). Resource utilization traits can be traded off against each other, such that adaptation to one resource environment may decrease the competitive ability of a microbe on alternative resources (Cooper and Lenski 2000, Litchman, et al. 2015). On the other hand, growth in matching outside-host resources might shorten the lag-phase in growth and speed up the ability

Intensive farming is suggested to favour increase in pathogen virulence for example via increased contact rates, fast turnover of hosts and strain competition (Kennedy, et al. 2016, Mennerat, et al. 2010, Pulkkinen, et al. 2010). However, in addition to epidemiological factors, intensive farming environments contrast the natural environment with regard to ambient conditions such as resource availability for environmentally growing microbes. Nutrient enrichment is increasingly being connected with increase in infectious and parasitic diseases (Aalto, et al. 2015).

Recent work has also shown the potential of higher resource availability in the outside-host environment to induce increased virulence on environmentally growing opportunistic pathogens (Kinnula, et al. 2017b, Penttinen, et al. 2016, Wedekind, et al. 2010).

Increased virulence at fish farms over the course of last 40 years has been suggested for *Flavobacterium columnare*, an environmentally growing bacterial pathogen of freshwater fish (Kunttu, et al. 2012, Suomalainen, et al. 2006a), potentially due to selection for certain genotypes of the bacterium (Pulkkinen, et al. 2010, Sundberg, et al. 2016). Apart from diseased fish, *F. columnare* can be frequently isolated from lake water and biofilms (Kunttu, et al. 2012), and the bacterium can efficiently transmit to a new host also from dead fish (Kunttu, et al. 2009b). Higher ambient nutrient concentration leads to increased virulence in fish challenge experiments (Kinnula, et al. 2017b, Penttinen, et al. 2016). In aquaculture, high fish densities increase water nutrient levels due to fish excretion, faeces and uneaten fish feed (Lalonde, et al. 2015). A positive association between growth rate and virulence (Pulkkinen, et al. 2010) could indicate potential for selection towards higher virulence under high nutrient availability for this environmentally growing pathogen.

Here we examined the hypothesis that high nutrient levels in the outside-host environment in fish farms select for more virulent strains of *F. columnare* by 1)
increasing the overall virulence of the strains towards fish hosts and/or 2) favouring virulent strains over less virulent strains in competition. To study the first question, we first cultured a virulent and a less virulent strain under low and high nutrient level in monocultures. After evolving under certain nutrient level, we tested if the overall virulence in the population had changed in comparison to ancestral strains (fish challenge tests in vivo). In order to study the second question on the effect of nutrient level on competition between the pair of a virulent and a less virulent strain, we initiated co-cultures with a 1:1 ratio of each strain and followed genotype frequencies using real-time quantitative PCR (qPCR) assay developed for this purpose. We also tested growth inhibition between the strains, because interference (direct) competition is common for *F. columnare* growing on surfaces (Ashrafi, et al. 2017, Sundberg, et al. 2016).

We performed two separate experiments, each with the same virulent but a different less virulent bacterial strain. The two experiments differed in the rate of resource renewal. In the first experiment, bacteria were inoculated into batch cultures and only the small volume of culture removed upon sampling was replaced with fresh medium (low rate resource renewal). In the second experiment, high rate resource renewal was executed at high nutrient level with daily serial transfer of a small aliquot of bacterial culture to fresh growth medium in test tubes and at low nutrient level by constant flow-through of the medium in chemostats (Velicer and Lenski 1999). In the high rate renewal experiment, the co-cultures initiated from ancestral strains were terminated after three weeks, when the virulent strain had outcompeted the less virulent strain. While the monocultures were maintained as they were, another set of co-cultures was initiated using bacteria evolved at the respective nutrient level in monocultures in order to study if potential adaptation to a nutrient level changes the competition outcome. For the high rate resource renewal experiment we also tested if biofilm formation of the strains explain competition outcome.

We expected that cultivation in high nutrient level would pre-adapt the bacteria for fast exploitation of nutrient rich host and therefore increase their virulence in fish challenge tests. Concerning competition, we expected that the virulent strain would grow faster (Pulkkinen, et al. 2010) and outcompete a less virulent strain under high nutrient level. However, under low nutrient level, the slow-growing less virulent
strain was expected to win because the replication of the virulent strain should be restricted by the high nutrient requirements of a high growth rate (Hibbing, et al. 2010).

Methods

Bacterial strains and culture conditions

The virulent strain B402 was isolated from a diseased fish at a fish farm. It was compared pairwise in two separate experiments with a less virulent strain (B407 or B398) isolated from river water (Kunttu, et al. 2012). The virulence of these strains had been tested previously in fish challenge experiments (Kunttu, et al. 2012). Strains were grouped in genotypes based on ARISA (automated ribosomal intergenic spacer analysis) (Kunttu, et al. 2012), and more recently on MLSA (multilocus sequence analysis) (Ashrafi, et al. 2015).

All strains were preserved as stock cultures containing 10% foetal calf serum and 10% glycerol at -80°C. The stocks were revived by incubation in 5 mL of modified Shieh medium (from now on Shieh medium) (Song, et al. 1988) at room temperature and constant shaking (120 RPM) for 24 hours and subsequent renewal in 1:10 for another 24 hours.

Bacterial cultures were grown either under low or high nutrient level. For each nutrient level, the Shieh growth medium was adjusted to the desired concentration with sterile water. Water, rather than nutrient-free saline buffer was used, because of low tolerance of *F. columnare* to salt water (Suomalainen, et al. 2006a). Bacterial densities used in inoculation were determined by measuring the optical density of the revived culture at 595 nm and comparing to a previously determined relationship between optical density and colony forming unit (CFU mL⁻¹) counts. During the experiment, the bacterial growth in the cultures as CFU mL⁻¹ was quantified by serial dilution and plate cultivation on Shieh-agar.

Batch culture experiment (low rate resource renewal)
In batch cultures, one-tenth of culture volume was replaced with fresh medium upon sampling. Bacteria (a virulent strain B402 and a less virulent strain B407) were grown in 5% (low nutrient level) and 50% (high nutrient level) Shieh medium. The total culture volume was 30 mL in 50 mL plastic test tubes, three replicates per strain or co-culture. The estimated starting number for bacteria was $1.0 \times 10^4$ CFU mL$^{-1}$ for the monocultures and $0.5 \times 10^4$ CFU mL$^{-1}$ for each of the two strains (1:1 ratio) for the co-cultures. The batch cultures were kept in a shaker incubator under constant agitation (120 RPM) at 25 °C.

Samples were taken from all cultures on days 0-5, 7, 9, 11, 16, 21 and 35 (see supplementary Table S1). Each sampling day, 3.1 mL samples were taken from each tube and replaced with fresh medium. One hundred µL was used for plate cultivation for both mono- and co-cultures. For co-cultures, in order to determine the proportions of two bacterial strains in the sample, 3 mL was used for DNA extraction for qPCR (see below). On day 21, samples collected from the bacterial populations evolved in monocultures were tested for virulence in a fish challenge experiment in vivo and compared to ancestral isolates (see below).

**High rate resource renewal experiment**

High rate resource renewal was executed with continuous supply of low nutrient level (2%) Shieh medium in chemostats (a flow-through microcosm) or with daily serial transfer in high nutrient level (20%) Shieh medium in test tubes (Velicer and Lenski 1999). The concentration of Shieh medium at the low nutrient level could not be kept at 5% used in the batch experiment because it blocked the chemostat tubes, but was decreased to 2%. Consequently, the concentration in the high nutrient level was decreased from 50% to 20%, in order to keep the relative difference between low and high nutrient level the same, i.e. 10 x differences. The strains used were B402 (virulent) and B398 (less virulent). The chemostat volume was approximately 455 mL. The chemostats were assembled aseptically and magnetic stirrer bars were placed at the bottom of the chemostats. A constant flow of fresh 2% Shieh medium was supplied from sterile 5 L flasks to the bottom of the chemostat with a Gilson peristaltic pump at dilution rate 0.21-0.24 day$^{-1}$. During the experiment, the medium bottle was replaced at ca. 20 day intervals. The overflow from the top of the
chemostat was collected via tubing to a separate waste bottle and discarded. For high
nutrient level, the total culture volume was 30 mL in 50 mL plastic test tubes. In order
to maintain the bacterial population in exponential growth phase, a 50 µL sample was
transferred to a new tube containing 30 mL of fresh medium each day. The culture
tubes were kept under constant agitation (120 RPM), in the same room as the
chemostats, with temperature adjusted at 22.6 ± 0.1°C. Each tube culture was
replicated three times and each chemostat twice.

The monocultures were maintained continuously for 53 days (Table S1). Samples
collected from the evolved populations on days 24 and 53 were stored at -80°C as
described above and later tested for virulence in a fish challenge experiment *in vivo* in
comparison to ancestral isolates (see below). Co-cultures were started with ancestral
strains simultaneously with the monocultures, however, when the virulent strains
became dominant during the first two weeks at both nutrient levels in all replicates
and no further change was seen within the next week (see Fig. 2B), co-cultures were
discarded at day 20. A second set of co-cultures were initiated with samples taken
from monocultures on day 25 and, for one chemostat - due to a fail in first inoculation
- on day 31 (see below, Table S1). The idea of the second set of co-cultures was to
study if the competitive outcome would change after the strains had already been
adapting to a certain nutrient level. The second set of co-cultures was maintained for
28 days.

The estimated starting number for bacteria for the batch monocultures was 1.0x10⁴
CFU mL⁻¹ and 0.5x10⁴ CFU mL⁻¹ for each of the two strains (1:1 ratio) in co-
cultures. Chemostat monocultures were inoculated with 1.0x10⁶ CFU mL⁻¹ and the
first co-cultures with 0.5x10⁶ CFU mL⁻¹ of each of the two strains. The second co-
cultures were inoculated with bacteria taken from the monocultures and diluted to
desired bacterial concentration using the data from last preceding CFU-
measurements. The concentration of bacteria in one of the monocultures used for
inoculation was so low that one co-culture had to be started with lower concentration
than aimed, with a total concentration of 5.0x10⁴ CFU mL⁻¹ (2.5x10⁴ CFU mL⁻¹ of
each strain). Another co-culture started at the same time crashed and was re-started 6
days later with a total concentration of 1.0x10⁶ CFU mL⁻¹ (0.5x10⁶ CFU mL⁻¹ of each
of the two strains). In comparison of growth and proportion of strains in competition in co-cultures, the days since inoculation were used (see Table S1).

For tube cultures, the plate counting was done from the culture remaining after the daily transfer of 50 µL to fresh medium and for chemostats from samples taken with a sterile needle and syringe through two ports near the top and bottom of the chemostat (total sample volume approximately 40 mL) mixed in equal proportions. For co-cultures the remaining culture in the tube and chemostat sample were used for DNA-extraction and subsequent qPCR (see below).

Biofilm formation in the high rate resource renewal experiment

In order to evaluate if differences in relative amount of biofilm formation between the virulent and less virulent strain in the high rate resource renewal experiment affected their growth and competition, we quantified the relative amount of biofilm formed during 48 hour incubation in low or high nutrient level. Bacterial cultures (strains B402 and B398) were inoculated into 200 µL of 2% or 20 % Shieh medium (1.0x10^6 and 1.0x10^4 CFU mL^{-1}, respectively) in eight replicate wells per strain x nutrient level on 96 well spectrophotometer plates. After 48 h, the wells were emptied, rinsed with distilled water, and the biofilm bound to the walls of the wells were dyed with 200 µL of 1% crystal violet solution. After 10 min, the wells were emptied with swift shaking of the overturned plate and rinsed by filling with distilled water. Rinsing and emptying was repeated three times and after the last rinse, the wells were visually confirmed to be dry. 96% ethanol was added to dissolve the dye. After 24 h, the optical density was measured with a spectrophotometer with 570 nm wavelength.

Assessment of genotype frequencies in co-cultures

To evaluate the frequency of virulent and less virulent strain in co-culture we developed a quantitative PCR (qPCR) assay including primer design and tests of primer specificity and efficiency. We used F. columnare trpB (tryptophan synthase subunit B) sequences (Ashrafi, et al. 2015) to design specific primers that amplify genotype C DNA only, and universal primers that amplify all five genotypes found in Finland (genotypes A, C, E, G and H). The proportions of two bacterial strains in co-
culture were then determined using ΔCq (Briand, et al. 2008). In short; standard
curves were prepared using samples containing known proportion of the virulent
strain (B402, genotype C) DNA mixed together with the less virulent strain (B407,
genotype G, or B398, genotype A) DNA and by plotting the proportion of the virulent
strain against the ΔCq value of the sample (ΔCq = Cq_{specific} – Cq_{universal}).
The PCR-reactions with specific and universal primers were always run within the
plate and the ΔCq values were calculated using the mean of three or sometimes two
of the three replicates for analysis. The unknown samples were run similarly,
together with a positive control containing DNA of the strains in 1:1 proportion. The
positive control was used to normalize the ΔCq values of the samples before
determining the proportion of the virulent strain in a sample using the constructed
standard curve. For further details, including DNA extraction and qPCR assay
validation, see supplementary methods, tables S2-S3 and figures S1-S3.

**Interference competition**

To evaluate the potential for interference competition via excreted products in
explaining the outcome of competition in co-cultures, we studied the growth
inhibition between the pairs of strains used in the experiments with the double layer
method (Sundberg, et al. 2016). Shortly, an aliquot of bacterial culture was
centrifuged (17,000 g, 3 min) and five μL of the supernatant was pipetted on top of
Shieh agar plate, where 300 μL of the other bacterium strain had been poured after
mixing with soft Shieh agar (0.7%). Three separate replicates were prepared for each
strain. Plates were checked after 48 h to detect if the supernatant had inhibited the
growth of the underlying strain.

**Fish challenge experiments**

Prior challenge experiments, ancestral strains were revived from frozen stock cultures
and all bacterial samples from different nutrient levels were subcultured in 100%
Shieh as described above (basic culture conditions). Thus the outcome of the
challenge tests were likely not affected by the nutrient levels added to the fish
containers.
Challenge experiments were performed with rainbow trout fingerlings obtained from a fish farm using ground water, ensuring that the fish had no previous exposure to *F. columnare*. The fish were maintained in the laboratory in aerated well water in glass aquaria at 12:12 L:D cycle at 17°C and fed with commercial pellets. The water temperature was increased gradually to 25°C (1-1.5 °C per day) during ten days before the bacterial challenge. The fish were transferred individually into transparent plastic containers with 0.5 L of aerated well water at 25°C. The containers were randomly assigned to different treatments (10 replicates per treatment) and the cultures, adjusted to 1.5E+03 CFU ml⁻¹ final concentration, were added to the containers in 0.1mL of 100% Shieh according to continuous challenge method (Kinnula, et al. 2015). The fish were monitored at one hour interval for signs of bacterial infection and morbidity. Due to fast development of disease symptoms, the diseased fish were mostly not responding to external stimuli and were killed by cutting the spinal cord with scissors leading to instant death. A bacterial sample was taken from the gills with a sterile loop and spread on agar plates containing modified Shieh medium and tobramycin (Decostere, et al. 1997). Fish weight in mg was taken down. The fish surviving until the end of experiments were euthanized with an overdose of MS-222, sampled for bacteria on gills and weighed.

Columnaris disease is a threat especially to young salmonids, with a rapid progress of the disease at freshwater fish farms. Challenge experiments were performed with rainbow trout fingerlings (*Oncorhynchus mykiss*) (age 0+) with a mean weight 2.10±0.69 g when testing bacteria from the batch experiment and 0.39±0.12 g when testing bacteria from the high rate resource renewal experiment. For this size range, previous studies have confirmed a qualitatively similar progress of infections (Kinnula, et al. 2015, Laanto, et al. 2014, Sundberg, et al. 2014). The experiments were ended after 45 h (batch experiment) and 49 h (high rate resource renewal experiment). The fish challenge experiments were conducted according to the Finnish Act on the Use of Animals for Experimental Purposes under the license number ESAVI/10184/04.10.07/2014 granted to Jouni Taskinen by the National Animal Experiment Board of the Regional State Administrative Agency of Southern Finland.
Statistical analyses

Competition and growth

The data was analysed separately for the batch experiment and the high rate resource renewal experiment. For the high rate resource renewal experiment the data was divided in two parts according to the duration of the two sets of co-cultures (days 0-24 and 25-53). The data was analysed in R version 3.4.3 with the package MCMCglmm (Hadfield 2010) with Bayesian mixed models using Markov chain Monte Carlo estimation. To study the outcome of competition in co-cultures using qPCR-data, ratio of the virulent strain in competition was analysed by using nutrient level (low vs. high) as a fixed factor and day since inoculum as a continuous factor.

The non-independence of multiple observations from the same replicate was accounted for by including replicate identity as a random factor. The bacterial growth (CFU ml⁻¹) was compared between monocultures and co-cultures using nutrient level (low vs. high) and strain or strain combination (virulent, less virulent, co-culture) as fixed factors. Day since inoculum was included as a continuous covariate and replicate identity as a random factor. The models for studying competition from qPCR data were fitted with Gaussian distribution and the effect of nutrient level on growth were fitted using Poisson distribution to account for overdispersion of the data. As model selection with DIC criteria might not be reliable for non-Gaussian data, the models were fitted including all main effects and 2-way interactions. For part 1 (days 0-24) in high rate resource renewal experiment also 3-way interactions were included. Inverse-Wishart priors with a low degree of information were used for both fixed and random effects (Hadfield 2010). Diagnostic tools from the Coda package (Plummer, et al. 2006) were used for determining the number of the iterations (600 000), length of thinning (130) and burn-in period (100 000) of the models. Gelman-Rubin test and visual inspection of traces on three chains were used for checking model convergence. The potential scale reduction factor was close to 1 among chains in all analyses.

Differences between factors are considered significant if the 95% credible intervals for the posterior means do not overlap with zero.

In order to evaluate if the outcome of competition is affected by differences in growth between the strains in different nutrient levels in the beginning of the experiments
(days 0-1), initial population growth was calculated for each monoculture as
\[
\ln\left(\frac{N_{\text{day}1}}{N_{\text{day}0}}\right)/1 \text{ day (Lenski, et al. 1991). } N_{\text{day}0} \text{ was the initial inoculation density and } N_{\text{day}1} \text{ the population density on day 1 determined by plate counting. These values were compared between the virulent and less virulent strain within each nutrient level separately for the batch experiment and the high rate resource renewal experiment with ANOVA.}

**Biofilm formation**

For evaluation of the effect of biofilm formation on the outcome of growth and competition in the high rate resource renewal experiment, the optical density values obtained from the separate biofilm experiments between the virulent and less virulent strain under low and high nutrient level were compared with ANOVA after log-transformation.

**Fish challenge tests**

For analysing data from *in vivo* fish challenge experiments, generalized linear models with binomial distribution were used to study the effect of bacterial strain identity and treatment (ancestral isolate, evolved under low or high nutrient level) on the morbidity of fish. The analyses were performed separately for the batch experiment and for the high rate resource renewal experiment. Strain and treatment were included as fixed factors. Fish weight was included as a continuous covariate to control for the effect of surface area and respiration rate on infection risk of a fish. Model selection was based on Akaike information criteria, performed with stepAIC function from package MASS in R (Venables and Ripley 2002), starting from the full model including all 3-way interactions. In the challenge experiment for bacteria evolved in the high rate resource renewal experiment, samples collected from the cultures on day 24 and day 52 were considered independent of each other and included as fixed treatment factors (ancestor, high nutrient day 24, high nutrient day 52, low nutrient day 24, low nutrient day 52).
Results

Fish challenge experiments

Batch culture experiment

Culturing bacteria in batches with low rate resource renewal at low or high nutrient levels did not change the virulence of the bacteria towards rainbow trout fingerlings in the challenge experiment as compared to the ancestral isolates. The best model explaining the outcome of the challenge experiment according to the AIC criteria included only the effect of strain (Table 1). The virulent strain B402 caused higher morbidity in rainbow trout than the less virulent strain B407 in all treatments (Fig. 1A, Supplementary Fig. S4A).

High rate resource renewal experiment

Populations evolved under high rate resource renewal in low or high nutrient levels for different lengths of time (24 days or 52 days) were compared to ancestral isolates in their ability to induce morbidity in fish. The best model according to AIC criteria included interaction between the strain (virulent, less virulent) and the treatment (ancestral, low nutrient at day 24 or 52, or high nutrient at day 24 or 52). The morbidity caused by the virulent strain B402 was higher than that caused by the less virulent strain B398. The nutrient level did not affect the morbidity caused by the virulent strain B402. However, for the strain B398, which was originally less virulent, morbidity in fish decreased after culturing as compared to that caused by ancestral strain. This was especially evident after culturing under low nutrient level but also after culturing at the high nutrient level for the longer time period (Table 2, Fig. 1B, Supplementary Fig. S4B).

Competition and growth
**Batch experiment**

The proportion of the virulent strain determined with the qPCR assay did not differ between high and low nutrient level (MCMCglmm model: the 95% credible intervals for the posterior means for the low nutrient level and interaction between low nutrient: day overlapped zero; Table S4, Fig. 2A). The proportion of the virulent strain increased during the experiment as indicated by the positive deviance of day from zero (posterior mean for day 0.003, 95% CI:0.001-0.005).

The bacterial concentrations of the virulent strain in monoculture, the less virulent strain in monoculture and the competition treatment did not differ from each other, they did not change during the experiment and the nutrient level did not affect the concentrations (Table S4, Fig. 3A-B). This is shown by the posterior distribution credible intervals which overlapped with zero for all variables considered in the MCMCglmm model.

The initial population growth during the first 24 h showed different patterns among the strains in the monocultures in the low and high nutrient levels (ANOVA: strain F\(_{1,8}\) = 59.139, p< 0.001, nutrient F\(_{1,8}\) = 89.048, p< 0.001, nutrient x strain effect F\(_{1,8}\) = 28.010, p< 0.001). The initial population growth was higher in the monocultures of virulent strain than for the less virulent strain in the high nutrient level, but there was no difference between the strains in the low nutrient level (pairwise comparisons, supplementary Table S7). Both strains had higher initial growth when grown in high nutrient level than under low nutrient level (Table S7).

**High rate resource renewal experiment**

When the co-cultures were started from ancestral strains, the proportion of the virulent strain did not differ between high and low nutrient level (MCMCglmm model: 95% CI for the posterior means overlapped zero for the low nutrient level and the interaction between low nutrient level: day; Table S5, Fig. 2B). The proportion of the virulent strain increased from the initial 50% to approximately 100% during the experiment as indicated by the positive deviance of day from zero (MCMCglmm model: 0.020, 95%CI: 0.010-0.030).
The concentration of the less virulent strain in monocultures at the low nutrient level was lower than the concentration of the virulent strain during the first three days (Fig 3C). This is indicated by negative posterior mean -5.479 and 95% CI not overlapping zero (-9.029 – -1.662) for less virulent strain: low nutrient level interaction effect. The concentration of the co-culture did not differ from the concentration of the virulent strain monoculture at low nutrient level (posterior mean 0.722; 95% CI: -3.067 – 4.257 for co-culture: low nutrient interaction; Table S5). However, after 6 days the monocultures of the less virulent strain at low nutrient level reached almost the same concentration as the virulent strain monocultures and the co-culture (Fig. 3C), as suggested by a significant positive deviance from zero for the posterior mean for the 3-way interaction between less virulent strain: low nutrient level: day (0.353, 95% CI: -0.007 – 0.670; Table S5, Fig. 3C). At high nutrient level the growth of the less virulent strain monocultures, the virulent strain monocultures and the co-cultures did not differ from each other (95% CI of less virulent strain B398 and co-culture overlapped zero; Table S5, Fig. 3D).

The initial population growth during the first 24 h differed between the strains in the low and high nutrient levels in the monocultures (ANOVA: strain $F_{1,6} = 88.258$, $p< 0.001$, nutrient level $F_{1,6} = 279.167$, $p< 0.001$, nutrient level x strain effect $F_{1,6} = 87.872$, $p< 0.001$), but to opposite direction as compared to the low resource renewal experiment. The virulent and the less virulent strain had similar initial growth in the high nutrient level, but in the low nutrient level the initial growth was higher for the virulent strain than for the less virulent strain (pairwise comparisons, Table S7). The initial population growth was higher at high nutrient level than under low nutrient level for both strains (Table S7).

In the second part of the experiment testing whether adaptation to a particular resource level in monocultures changed the competition outcome (Fig. 2, 2. co-culture), the proportion of the virulent strain in the competition treatment increased during the experiment (MCMCglmm model: posterior mean for day 0.029, 95% CI: 0.021 – 0.037, Table S6, Fig. 2C). However, the two chemostat (low nutrient level) co-cultures behaved very differently, thus nutrient level was not included in the statistical analysis. The inoculations taken from the monocultures at day 24 did not
result in equal proportion of the less virulent and virulent strains in co-cultures, but
the proportion of the virulent strain was lower, ca. 30% for the high resource
treatment and the other one of the chemostats and only a few percent for the other
chemostat. In spite of lower proportion at the start, and remaining at very low level
for 7-10 days in the high nutrient level co-cultures and the other chemostat, the
virulent strain eventually outcompeted the less virulent strain at both nutrient levels
(Fig. 2C).

The concentrations of the virulent and less virulent strain monocultures in the second
part of the high rate resource renewal experiment (Fig. 3, 2. co-culture) were higher
than those of the co-cultures, because the monocultures were continuously maintained
since the beginning of the first part and co-cultures were initiated with a lower
concentration (MCMCgllmm model: posterior mean -3.645, 95% CI: -5.020 – -2.228
for co-culture). However, the concentration of the co-culture increased during the
experiment (co-culture:day -0.170, 95% CI: 0.098 – 0.239; Fig. 3C-D). The bacterial
concentrations were lower at low nutrient level (posterior mean -1.640, 95% CI: -
3.013 – -0.153) and they decreased during the experiment as compared to the high
nutrient level (posterior mean -0.239, 95% CI: -0.293 – -0.179 for low nutrient
level:day interaction, Table S6, Fig. 3C-D). There was no difference in growth
between the less virulent and the virulent strain (95% CI for the posterior mean for
B398 less virulent strain overlapped zero, Table S6).

Biofilm formation

In the experiment evaluating differences in relative amount of biofilm formation
between the virulent and less virulent strain in the high rate resource renewal
experiment, the less virulent strain B398 formed more biofilm than the virulent strain
B402 (ANOVA: F_{1,23} = 6.65, p=0.02). There was no interaction between the strain
and resource level (F_{1,23} = 2.33, p=0.14).

Interference competition
In the experiment evaluating the potential for interference competition via excreted products between the strains, the virulent strain B402 inhibited the growth of the less virulent strain B407 used in the batch experiment in all 3 replicates, but no inhibition was observed vice versa. There was no inhibition between the virulent strain B402 and the less virulent strain B398 used in the high rate resource renewal experiment.

Discussion

Both the within host and the outside host environment affect the ecology and evolution of environmentally growing opportunistic pathogens (Brown, et al. 2012). The two environments may select for different properties for example in resource use, but on the other hand growth in one environment may also pre-adapt for utilisation of resources in the other environment (Ketola, et al. 2016, New, et al. 2014). Environmental resources may thus affect pathogen virulence by inducing changes in host utilisation rates. Here we tested the hypothesis that higher nutrient availability in the outside host environment increases the virulence of an environmentally growing opportunistic pathogen of fish, *Flavobacterium columnare*. We expected that evolving in high nutrient environment would increase virulence due to selection for fast resource use. As virulent strains of *F. columnare* have higher growth rate than less virulent strains (Pulkkinen, et al. 2010), we also expected that high nutrient conditions would favour virulent strains in competition.

Increased resource availability in the outside-host environment could increase virulence in *F. columnare* by increasing bacterial population size, and thus the dose encountered by the host (Kinnula, et al. 2017b, Kinnula, et al. 2015), or by facilitating host invasion via virulence factor activation (Kinnula, et al. 2017b, Penttinen, et al. 2016), such as increased expression of tissue-degrading enzymes (Penttinen, et al. 2016). In the current experiments, however, immediate nutrient environment did not affect the virulence, as we sub-cultivated all bacteria in 100% Shieh medium and adjusted the dose prior the fish challenge. Therefore any changes in virulence were expected to be due to adaptation in the nutrient environment where the strain was evolving. In contrast to our expectations, we did not detect increase in virulence after evolving in the high nutrient level for either the virulent or the less virulent strains as...
compared to ancestor.

Instead, the virulent strain maintained its virulence at the same high level independent of the nutrient level and resource renewal rate. These results suggest that increased resources in the outside-host environment do not affect virulence in highly virulent strains. However, the less virulent strain decreased in virulence in cultivation at constant low nutrient supply in chemostats or in long serial cultivation in tubes in the high rate resource renewal experiment. Loss of virulence has been commonly observed in pathogenic micro-organisms upon successive sub-cultivation in vitro (Ford, et al. 2002, Gonzalez-Carrillo, et al. 2016, Moody, et al. 1990, Songe, et al. 2014), pointing to trade-offs between long-term survival in outside-host and within-host environment (Ferenci 2016) or relaxed selection for virulence in the outside-host environment (Mikonranta, et al. 2012). Increased supply of resources might therefore maintain virulence at higher level in less virulent strains in the outside-host environment.

In F. columnare, virulence is associated with rhizoid colony morphology, with a loss of virulence upon change into two other morphs (rough or soft) (Kunttu, et al. 2011, Kunttu, et al. 2009a). Maintenance of F. columnare for 5 months in stationary starvation conditions led to diversification in colony morphology and virulence such that rhizoid morph became more virulent and rough morph less virulent than the ancestral strain (Sundberg, et al. 2014). The strains used in the current experiment expressed originally rhizoid colonies upon cultivation on agar plates and no change was observed during cultivation in batches. Bacteria from chemostats, however, formed colonies that were intermediate between rhizoid and soft morphs (Supplementary Fig. S5). The appearance of cells giving rise to soft colonies might thus have decreased the overall virulence of the chemostat population. Resources in the outside-host environment might contribute to the virulence of F. columnare via diversification of cell types forming different colony morphologies, which might be related to trade-offs in acquisition of nutrients in outside-host and within-host environments. The mechanisms behind the different colony morphologies and their virulence in F. columnare are not clear, but virulence is associated with gliding motility of the cells, which in turn is affected by nutrient availability (Laanto, et al. 2014).
In direct competition over resources, the competitor with the fastest turnover rate of resources into biomass is expected to win. In *F. columnare*, individually assessed growth rates have been found to have a good agreement with the outcome of competition in liquid culture (Ashrafi, et al. 2017). However, in current experiments, outcome of competition in co-culture could be explained only in two cases with differences in the initial growth rates of the strains measured in monocultures at respective nutrient levels. Instead, we found that the virulent strain was superior in competition both at high and low nutrient level regardless of the resource renewal rate. The virulent strain outcompeted the less virulent strains in co-cultures in a few days even after adaptation to prevailing nutrient level and after starting from a very low proportion (see Fig. 2C). Similar genotype based dominance has been detected previously (Kinnula, et al. 2017a) and more studies are needed to clarify whether the traits harboured by the dominant strains are common to all virulent genotypes of *F. columnare*.

Apart from direct competition over resources, microbes compete with each other with various indirect mechanisms, including chemical interference (Hibbing, et al. 2010). For *F. columnare*, interference competition is common (Ashrafi, et al. 2017, Kinnula, et al. 2017a, Sundberg, et al. 2016). Here we confirmed growth inhibition of the less virulent strain B407 by the virulent strain B402, which might explain the competition outcome in the batch experiment. However, the competitive interactions via toxin production have been detected for colonies growing on surfaces, and it is not clear whether they play a role for planktonic growth (Cornforth and Foster 2013). The pair used in the high rate resource renewal experiment did not inhibit each other’s growth, offering no explanation for the competition outcome in this experiment.

In addition to resource level, resource renewal rate can affect different traits in growth (Finkel 2006, Velicer and Lenski 1999). Even though we expected that bacteria grown under high nutrient level would be selected for fast growth (Frank 2010, Litchman, et al. 2015, Velicer and Lenski 1999), it is possible that conditions in the batches with low rate renewal of resources selected similar traits under both low and high nutrient level. Without continuous renewal of nutrients, the bacteria might have been adapted for fast growth before reaching carrying capacity well prior to the next resource renewal. This saturation phase might then select for survival of bacterial
cells that are capable of remaining viable in starvation conditions (Arias, et al. 2012) or capable of saprophytic usage of their conspecific cells. *F. columnare* has been shown to survive and remain infective for several months when maintained in water without added nutrients (Arias, et al. 2012, Kunttu, et al. 2009b, Sundberg, et al. 2016), and this feature has been attributed to a saprophytic capacity in the bacterium, supported by experiments showing replication in fish carcasses (Kunttu, et al. 2009b), (but see (Arias, et al. 2012) for an opposing view). It is also possible that nutrients were depleted in the tube cultures with high rate resource renewal despite of the high dilution aimed at maintaining the cultures at exponential growth stage prior daily serial culture, and then these conditions might have selected for survival rather than for high growth rate as well (Velicer and Lenski 1999).

On the other hand, the conditions in flow-through chemostats, favouring cells that can grow and multiply at lowest nutrient level, were expected to favour the less virulent strain (Gresham and Hong 2015, Litchman, et al. 2015, Velicer and Lenski 1999). In the high rate resource renewal experiment, the less virulent strain had a propensity to form biofilm on chemostat walls, and its higher relative biofilm forming capacity as compared to the virulent strain was confirmed in a separate experiment on 96-well plates at both nutrient levels. As no inhibition was detected between the pair used in this experiment, the competition outcome in chemostats was possibly driven by the less virulent strain diverting part of the population growth into the biofilm instead of liquid culture. In high nutrient level, however, the daily transfer might have prevented formation of biofilm for the less virulent strain, as no differences in concentration between strains were seen. However, adhesion capacity has not been found to be connected with virulence in *F. columnare* (Kunttu, et al. 2009a, Suomalainen, et al. 2006b). In fish farming conditions, tendency to form biofilm could give at least short-term competitive advantage or a possibility to survive also for less virulent strains in situations where the entire water mass of a fish tank is renewed.

In addition to fish farms, *F. columnare* can be commonly isolated from lake water and biofilms at shore (Kunttu, et al. 2012). The selection regime for survival and infectivity might greatly differ between these two environments. In the lake water the resource level is still much lower than the lowest resource level used in this study (2% Shieh medium). In a fish farming environment, excess nutrients are released to the
water from fish excretion, faeces and uneaten feed in varying concentrations depending for example on the fish species, biomass, water exchange rate, feeding rate and temperature (Lalonde, et al. 2015). Thus the resource quality available in the outside host environment matches closely the resources provided by the host and could pre-adapt the bacteria for faster host exploitation. Our results suggest that the outside-host nutrient environment has less impact on strains that are highly virulent, but rich resources in the outside-host environment might have significance in maintaining virulence in less virulent strains and increase the contribution of less virulent strains in columnaris epidemics in aquaculture. As inter-strain interactions of the co-infecting strains might determine the outcome of infection (Kinnula, et al. 2017a), further studies are needed on the role of outside-host resources in maintaining strain diversity and driving evolution of virulence among environmentally growing pathogens.

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Table 1. Model selection and the summary of the best fitting model on the morbidity risk of the rainbow trout in the fish challenge experiment testing the strains used in the batch experiment. Model selection is based on Akaike information criteria (AIC). The best fit model estimating morbidity risk of the host (rainbow trout) within time is marked with bold. P value indicates the significance of the term removed from the higher model based on log-likelihood test (LRT).

<table>
<thead>
<tr>
<th>Model a</th>
<th>AIC</th>
<th>DF</th>
<th>LRT</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>S+T+W+S:T+W+S:T+W</td>
<td>302.65</td>
<td>128</td>
<td>0.255</td>
<td>0.88</td>
</tr>
<tr>
<td>S+T+W+S:T+W+S:T+W</td>
<td>298.91</td>
<td>130</td>
<td>0.178</td>
<td>0.67</td>
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<tr>
<td>S+T+W+S:T+W+S:T+W</td>
<td>295.28</td>
<td>132</td>
<td>0.374</td>
<td>0.83</td>
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<tr>
<td>S+T+W+S:T+S:T+W</td>
<td>294.93</td>
<td>132</td>
<td>0.026</td>
<td>0.99</td>
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<tr>
<td>S+T+W+S:T+S:T+W</td>
<td>293.10</td>
<td>133</td>
<td>0.170</td>
<td>0.68</td>
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<tr>
<td>S+T+W+S:T+S:T+W</td>
<td>291.30</td>
<td>134</td>
<td>0.832</td>
<td>0.83</td>
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<tr>
<td>S+W+S:T+W</td>
<td>288.45</td>
<td>136</td>
<td>1.149</td>
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<tr>
<td>S+T+W+S:T+W</td>
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<td>135</td>
<td>0.130</td>
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<tr>
<td>S+W+S:T+W</td>
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<td>S+W+W+S:T+W</td>
<td>293.28</td>
<td>138</td>
<td>0.943</td>
<td>0.33</td>
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<tr>
<td>S</td>
<td>285.63</td>
<td>138</td>
<td>8.626</td>
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Summary of the best fitting model

<table>
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<th>SE</th>
<th>z value</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Intercept) b</td>
<td>-2.9</td>
<td>0.13</td>
<td>-23.607</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Strain(Less virulent)</td>
<td>-0.53</td>
<td>0.18</td>
<td>-2.988</td>
<td>0.003</td>
</tr>
</tbody>
</table>

a S, strain (virulent B402, less virulent B407); T, treatment (ancestor, low nutrient level, high nutrient level), W, fish weight; +, main effect; colon, interaction. b Intercept includes the effect of the virulent strain.
Table 2. Model selection, summary of the best fitting model and the significance of the variables included in the best fitting model on the morbidity risk of the rainbow trout in the fish challenge experiment testing the strains used in the high rate resource renewal experiment. Model selection is based on Akaike information criteria (AIC). The best fit model estimating morbidity risk of the host (rainbow trout) within time is marked with bold. P value indicates the significance of the term removed from the higher model based on log-likelihood test (LRT).

<table>
<thead>
<tr>
<th>Model</th>
<th>AIC</th>
<th>DF</th>
<th>LRT</th>
<th>p</th>
</tr>
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<tr>
<td>S+T+W+S:T+W+T:S:W</td>
<td>424.52</td>
<td>200</td>
<td></td>
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<tr>
<td>S+T+W+S:T+S:W+T:W</td>
<td>419.59</td>
<td>204</td>
<td>3.064</td>
<td>0.547</td>
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<tr>
<td>S+T+W+S:T+S:W</td>
<td>412.58</td>
<td>208</td>
<td>0.991</td>
<td>0.911</td>
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<tr>
<td>S+T+W+S:T</td>
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<td>209</td>
<td>0.913</td>
<td>0.339</td>
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<tr>
<td>S+T+S:T</td>
<td>410.97</td>
<td>210</td>
<td>8.947</td>
<td>0.062</td>
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Summary of the best fitting model

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<th>Estimate</th>
<th>SE</th>
<th>z value</th>
<th>p</th>
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<tbody>
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<td>-3.17</td>
<td>0.32</td>
<td>-9.805</td>
<td>&lt; 0.001</td>
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<td>Strain(Less virulent)</td>
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<td>0.47</td>
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<td>0.2808</td>
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<td>High day 24</td>
<td>-0.04</td>
<td>0.37</td>
<td>-0.104</td>
<td>0.9175</td>
</tr>
<tr>
<td>High day 52</td>
<td>-0.01</td>
<td>0.37</td>
<td>-0.030</td>
<td>0.9761</td>
</tr>
<tr>
<td>Low day 24</td>
<td>0.03</td>
<td>0.40</td>
<td>0.076</td>
<td>0.9396</td>
</tr>
<tr>
<td>Low day 52</td>
<td>-0.10</td>
<td>0.40</td>
<td>-0.254</td>
<td>0.7996</td>
</tr>
<tr>
<td>Less virulent: high day 24</td>
<td>-0.69</td>
<td>0.57</td>
<td>-1.223</td>
<td>0.2212</td>
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<tr>
<td>Less virulent: high day 52</td>
<td>-1.39</td>
<td>0.32</td>
<td>-2.267</td>
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<tr>
<td>Less virulent: low day 24</td>
<td>-1.82</td>
<td>0.72</td>
<td>-2.515</td>
<td>0.0119</td>
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<tr>
<td>Less virulent: low day 52</td>
<td>-1.11</td>
<td>0.64</td>
<td>-1.732</td>
<td>0.0833</td>
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Significance of variables included in the best fitting model

<table>
<thead>
<tr>
<th>Source</th>
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<td>Null</td>
<td>182.32</td>
<td>78.62</td>
<td>103.71</td>
<td>&lt;0.001</td>
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<tr>
<td>Bacterial strain</td>
<td>1</td>
<td>3.81</td>
<td>99.90</td>
<td>0.432</td>
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<td>Treatment</td>
<td>4</td>
<td>8.95</td>
<td>90.95</td>
<td>0.062</td>
</tr>
</tbody>
</table>

a S, strain (virulent B402, less virulent B407); T, treatment (ancestor, low nutrient level day 24 sample, low nutrient level day 52 sample, high nutrient level day 24 sample, high nutrient level day 52 sample), W, fish weight; +, main effect; colon, interaction. b Intercept includes the effect of the ancestral virulent strain.
Figure captions

Fig. 1 The predicted cumulative mortality of rainbow trout (*Oncorhynchus mykiss*) fingerlings in continuous challenge experiments with *Flavobacterium columnare*. A) In fish challenge with bacteria cultivated in monocultures in the batch experiment, the risk depended only on the strain identity of the bacterium, but not whether it was ancestral isolate or cultured under low or high resource level. B) In fish challenge with bacteria from the monocultures from the experiment with high rate resource renewal, culturing in different nutrient environments did not change the virulence in the virulent strain (high = high nutrient level, low = low nutrient level). The less virulent strain decreased in virulence after cultivation at low nutrient level and after the longer cultivation period (52 days) at high nutrient level.

Fig. 2 The proportion of the virulent strain in co-cultures (mean ± SE): A) in the experiment with low resource renewal, B) in the experiment with high resource renewal started from ancestral isolates (1. co-culture) and C) in the experiment with high resource renewal after starting the co-cultures with bacteria from the monocultures after adaptation to a particular nutrient level (2. co-culture). In panel C, the proportion of the virulent strain is shown separately for both low nutrient level replicates due to large differences between replicates. Note that due to technical reasons (see Supporting information), the calculated proportion of the virulent strain may slightly exceed 1. The two values below the x-axis in C) denote for the corresponding days for monocultures in comparison to Fig. 3C-D.

Fig. 3 The bacterial concentrations (CFU ml⁻¹) in monocultures of the less virulent strain (Less virulent), monocultures of the virulent strain (Virulent) and co-cultures (L+V). A) Batch experiment (low resource renewal) at nutrient levels 5% (low nutrient) and B) 50% Shieh medium (high nutrient). C) The experiment with high rate resource renewal at 2% Shieh medium (low nutrient) and D) 20% Shieh medium (high nutrient). In the beginning of the high rate resource renewal experiment the co-cultures were started from ancestral isolates and discarded at day 20 (1. co-culture). New co-cultures were started (2. co-culture) with bacteria from the monocultures after
adaptation to certain nutrient level. Monocultures were maintained continuously for the whole duration of the high rate resource renewal experiment.