Comparative susceptibility of turbot, halibut, and cod yolk-sac larvae to challenge with *Vibrio* spp.

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ABSTRACT: In intensive aquaculture systems, high mortalities are frequently observed during the early life stages of marine fish. The aim of this study was to investigate differences in the susceptibility of turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus* and cod *Gadus morhua* to various strains of *Vibrio anguillarum* (serotypes O1, O2α and O2β), *V. salmonicida* and *V. splendidus*. The bath challenge experiments were performed using a multidish system, with 1 egg well^{-1}. Unchallenged eggs and larvae were used as controls. Larvae in challenged groups that suffered high mortality rates were examined by immunohistochemistry. The overall results with respect to mortality showed that the O2α serotype was pathogenic to all 3 species, while the O1 serotype was pathogenic to halibut and cod. The immunohistochemical examinations revealed differences in histopathology. The O1 serotype produced more severe and highly developed infections than the O2α serotype. In larvae exposed to the O1 serotype, necrosis and bacterial cells were seen in the dermis, gastrointestinal tract, brain and eye area, while in larvae exposed to the O2α serotype, bacteria were usually limited to the gastrointestinal tract. These results suggest either that there are undetermined species differences in host immunity or that these pathogens are host-specific even in the early life stages of fish. The O2β strain did not cause an increased mortality to halibut and turbot.

KEY WORDS: Yolk-sac larvae · Bath challenge experiment · Immunohistochemistry · *Vibrio anguillarum* · Vibriosis

INTRODUCTION

Turbot *Scophthalmus maximus*, halibut *Hippoglossus hippoglossus* and cod *Gadus morhua* are increasingly becoming important species in European aquaculture. However, the production of these species has been limited by unstable production of juveniles due to high mortalities during the larval stages (reviewed by Bricknell & Dalmo 2005, Samuelsen et al. 2006). Vibriosis has been, and still is, one of the major disease problems of the aquaculture industry. *Vibrio anguillarum*, *V. salmonicida*, *V. ordalii* and *V. vulnificus* are among the pathogens that lead to the greatest losses in aquaculture all over the world (reviewed by

Toranzo et al. 2005). Good vaccines developed in the late 1980s and early 1990s have reduced the problem in salmonid farming, but vaccines developed for cod still do not provide sufficient protection (reviewed by Sommerset et al. 2005, Bricknell et al. 2006, Samuelsen et al. 2006); thus, vibriosis is still a cause of serious financial losses.

Vibrio salmonicida is the causative agent of 'coldwater vibriosis', which is a disease that commonly occurs during the winter or at temperatures below 15°C (Egidius et al. 1986). It is known to be a problem in the farming of salmonid fish, especially the salmon *Salmo salar,* but little information is available concerning *V. salmonicida* in marine fish species (reviewed by

Bricknell et al. 2006). *V. splendidus* has been described as being pathogenic to fish (Thomson et al. 2005, Bergh & Samuelsen 2007) and bivalves (Gómez-León et al. 2005, Sandlund et al. 2006).

The greatest losses to vibriosis in the aquaculture industry are caused by *Vibrio anguillarum*, which is a species that has 23 known O serotypes (Pedersen et al. 1999). Individual serotypes are often associated with certain species of fish and not all are believed to be pathogenic. Serotypes associated with disease in farmed fish are O1, O2 and O3 (reviewed by Toranzo et al. 2005). The O2 serotype has been divided into 2 subserotypes (O2α and O2β). While O2α has been isolated from both salmonid and marine fish, O2β is usually isolated from cod and other nonsalmonids (Mikkelsen et al. 2007). In turbot farming, O1 is the dominant serotype (Larsen et al. 1994), while both O1 and O2 cause disease in halibut (Bergh et al. 1997, Bricknell et al. 2000, Hoare et al. 2002). The O3 serotype is rare in comparison with the other 2 and is usually isolated from eels (reviewed by Toranzo et al. 2005).

Despite all the published work on the bacteria listed above, little is known about their capacity to infect fish during their early life stages. Various aspects of differences in host susceptibility to pathogenic agents are also of interest. As new species emerge in the aquaculture industry, it is likely that pathogens will be transferred between species. Although species may not develop the disease, they may act as reservoirs of the pathogen. The objective of this work was to perform a challenge study of turbot, halibut and cod larvae, comparing their susceptibility to different serotypes of *V. anguillarum*, an atypical strain of *V. salmonicida* and a *V. splendidus* strain.

MATERIALS AND METHODS

Broodstock, eggs and larvae. Eggs of turbot, halibut, and cod were obtained from broodstock at the Institute of Marine Research, Austevoll Research Station (Storebø, Norway), and incubated as described by Bergh et al. (1997) and Bergh (2000).

Bacterial strains. The bacterial strains and serotypes used are shown in Table 1. Strain HI-610 has previously been used in a range of challenge experiments with cod and halibut (Samuelsen & Bergh 2004, Skjermo & Bergh 2004, Vik-Mo et al. 2005, Selje-stokken et al. 2006, Engelsen et al. 2008, Sandlund & Bergh 2008). Strain HI-618 has previously been described by Wiik et al. (1989) (annotated as HI-4791). Strain H-644 was isolated by O. M. Rødseth and K. Andersen (unpubl. data). *V. salmonicida* strain HI-651 (Wiik et al. 1995) was from the Institute of Marine

Table 1. Bacterial strains used in the study

Designation	Bacterial strain/serotype
HI-610	*Vibrio anguillarum* strain 610 serotype O2α
HI-618	*Vibrio anguillarum* strain 618 serotype O2β
HI-644	*Vibrio anguillarum* strain 644 serotype O1
HI-651	*Vibrio salmonicida* strain 651
HI-1576	*Vibrio splendidus* strain 1576

Research and *V. splendidus* strain HI-1576 was originally provided by the National Veterinary Institute, Norway. Strains HI-610 and HI-618 were originally isolated from diseased cod, HI-644 from diseased turbot, and HI-651 and HI-1576 from diseased halibut fry. Following primary isolation and characterisation, all bacterial strains had been stored in the culture collection of the Institute of Marine Research at –80°C in a 20% glycerol/marine broth (MB, Difco 2216, Difco) stock. They were incubated at 15°C and grown on Petri dishes with Difco 2216 marine agar (MA) for 48 h. Colonies of the bacteria were transferred to Erlenmeyer flasks with MB (Difco 2216) and shaken at 80 rpm in a shaking incubator (INFORS AG CH-4103) for 48 h at 10°C. It should be noted that bacterial suspensions used for challenging turbot larvae were grown at 15°C. The bacterial cultures were harvested by centrifugation (Heraeus Sepathec Megafuge 1.0 R) at $2772 \times g$ for 10 min at 4°C, washed twice with phosphate-buffered saline (PBS) and suspended in PBS.

All 3 species of fish were challenged with all 5 bacterial strains. Two different challenge doses were used, equivalent to a bath challenge concentration in wells of approximately 10^6 (high) and 10^4 CFU ml^{-1} (low). Unchallenged larvae were used as controls. Larval mortality was recorded daily. The challenge protocols were modified from Bergh et al. (1992, 1997).

The eggs are fragile and some were damaged during handling; thus, the challenge groups contained slightly different numbers of larvae.

Challenge experiments. The temperatures used during the experiments were chosen so as to provide near-optimum conditions for culturing each fish species.

The turbot eggs and larvae were incubated as described by Bergh et al. (1997), in 24-well dishes at 15°C in an airconditioned room. The various challenge groups contained either 72 (control, high-challenge dose of HI-618 and low-challenge dose of HI-610), 71 (high-challenge doses of HI-610 and HI-1576 and low-challenge doses of HI-618 and HI-1576), 70 (high- and low-challenge dose of HI-644) or 69 (high- and low-challenge dose of HI-651) larvae, as larvae damaged through treatment were killed and discarded. The eggs were challenged 1 d before hatching. The exper-

iments lasted for 5 d post-hatch (dph) until the end of the yolk-sac stage.

The challenge experiments with halibut larvae were performed in darkness in an airconditioned room at 6°C as described by Bergh et al. (1992, 1997). The eggs were challenged 4 d before hatching. One day after hatching, the wells were washed, the water and eggshell remains were removed, and 10 ml of sterile seawater was added. These challenge groups contained either 60 (control larvae, larvae challenged with HI-610 or HI-576) or 58 (larvae challenged with HI-618, HI-644 or HI-651) larvae. The halibut larvae were only exposed to the highest challenge dose. Halibut larvae have a long yolk-sac stage and this experiment lasted for 23 dph.

Cod eggs and larvae were incubated and challenged as described by Bergh (2000). In this experiment, 24-well dishes were used, with 2 ml sterile seawater and 1 egg well^{-1}. The eggs were incubated at 6°C. All challenge groups contained 72 larvae except the group challenged with a low dose of HI-610 that contained 69 larvae, and were challenged 7 d before hatching. The experiments lasted for 10 dph until the end of the yolk-sac stage. Two separate larval batches were challenged in order to verify the reproducibility of the experimental protocol.

Antisera. Antisera were made for *Vibrio anguillarum* strains HI-610 and HI-644 according to the method described by Oeding (1957) and the standard protocol of the University of Bergen, Laboratory Animal Facility (the Vivarium), in which formaldehyde-killed, washed bacteria were administrated by intravenous injection to rabbits. The polyclonal antisera were absorbed as described by Knappskog et al. (1993), in order to minimize the possibility of cross-reaction. The anti-*V. anguillarum* serum was absorbed against the other serotypes used in this experiment; i.e. the O2α serotype serum was absorbed against serotype O2β and serotype O1 strain HI-644. Each of the absorbed antisera was tested for cross-reaction with other *V. anguillarum* strains and tissue samples prior to immunohistochemistry. No cross-reaction or background staining was observed. The dilution used on tissue samples was determined after testing a range of antiserum dilutions on tissue samples.

Immunohistochemistry. In order to provide material for immunohistological examinations, additional multiwell dishes were set up within the different treatments. Two or 3 larvae were taken from each treatment group daily during periods of mortality throughout the experiment. Larval samples were fixed in 4% phosphate-buffered formaldehyde, dehydrated in ethanol, cleared in xylene and embedded in paraffin. They were sectioned at 3 µm (Leica Jung Biocut 2035), incubated at 58°C for 30 min, dewaxed in xylene, rehy-drated in a series of ethanol baths and washed in running tapwater. The absorbed polyclonal antisera, anti-*V. anguillarum* 610 and 644 were diluted in Tris buffer with 2.5% BSA. In order to prevent nonspecific antibody binding, sections were blocked by using 5% BSA in Tris for 20 min. Biotinylated swine anti-rabbit immunoglobulin was used as a secondary antibody (Dako A/S), and Avidin-biotin-peroxidase-complex (ABComplex/HRP) reaction kit (Dako A/S) and AEC+ Substrate Chromagen (Dako) were used to stain all samples. Positive bacterial staining was indicated by red coloration. Shandon's haematoxylin was used for counterstaining, coloring the tissue blue. Unchallenged larvae were used as negative controls. Bacterial smears on microscope slides were used as positive controls. This control procedure has previously been used with different *Vibrio* spp. at our laboratory (Sandlund et al. 2006). The antiserum against strain HI-610 has previously been tested in cod larvae (Engelsen et al. 2008). All incubations were performed at room temperature (20°C) in a humidity chamber in a fume hood.

Statistical analyses. Since the survival and mortality data were not normally distributed, nonparametric tests were used. A 2×2 contingency table ($p < 0.01$) with Bonferroni correction for multiple independent tests (implemented using Statistica v 7.0, StatSoft) was used to test for mortality differences among the treatment and control groups. Since multiple independent tests were used to test for differences in mortality rate among all the challenged larval groups and the 3 larval control groups, a Bonferroni correction was applied in order to minimize the possibility of making a type II error (Rice 1989). We thus tested for 5 bacterial strains, and the p-value was corrected by a factor of 5 ($p = 0.05/5 = 0.01$) (see Rice 1989). Yates' correction was used since there was only 1 df.

RESULTS

Challenge experiments

All undamaged eggs hatched normally. The cumulative mortality and statistical analysis showed that turbot larvae suffered the highest mortality when challenged with *Vibrio anguillarum* serotype O2α (Fig. 1, HI-610). Mortality was found to be significantly different from that in the control group at 2 dph in both high- and low-challenge dose groups ($p < 0.01$ Bonferroni correction, Fig. 1). Towards the end of the experiment (4 and/or 5 dph), the larval groups challenged with low doses of *V. splendidus* (HI-1576) and *V. anguillarum* serotype O1 (HI-644) respectively, suffered mortality that was significantly different from that of the control group (Fig. 1).

Halibut larvae were most susceptible to *Vibrio anguillarum* serotype O2α (HI-610) and serotype O1 (HI-614) (Fig. 2). Statistical analysis showed that mortality in these challenge groups was significantly dif-
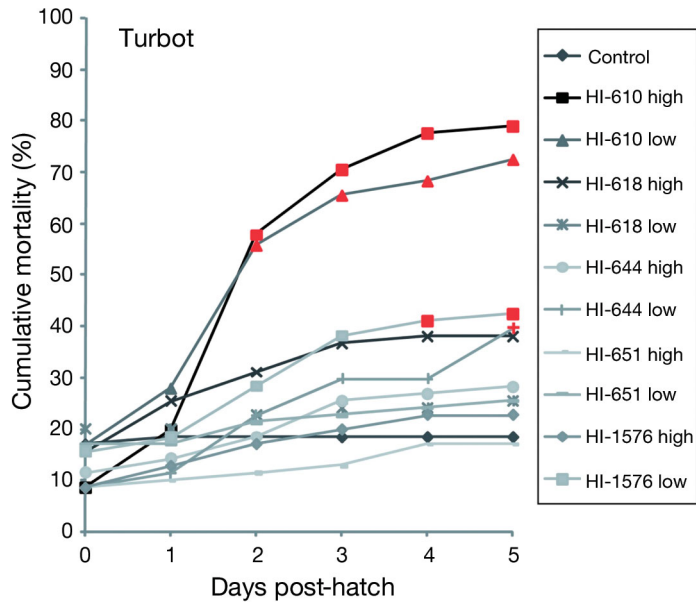

Fig. 1. *Scophthalmus maximus.* Cumulative percentage mortality of turbot larvae challenged with the bacterial strains HI-610, HI-618, HI-644, HI-651, and HI-1576 (see Table 1 for bacterial strains used. High: challenge dose 10^6 CFU ml^{-1}, low: challenge dose 10^4 CFU ml^{-1}, control: unchallenged larvae. Day 0: day of hatching. Red symbols: mortality rates significantly different from the control ($p < 0.01$ Bonferroni correction)
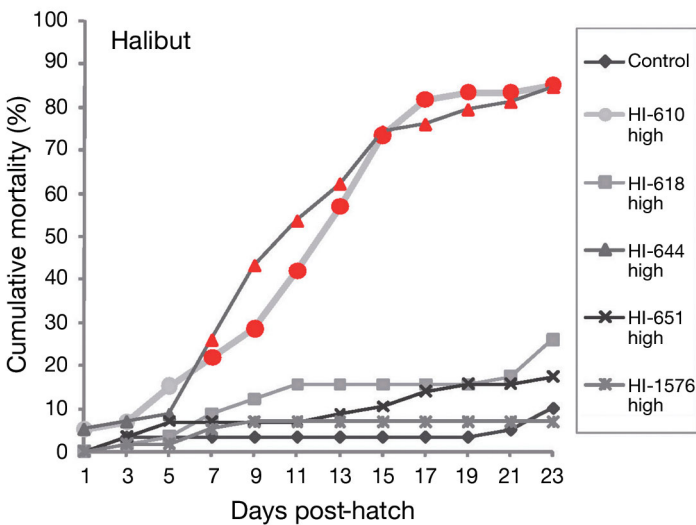

Fig. 2. *Hippoglossus hippoglossus.* Cumulative percentage mortality of halibut larvae challenged with the bacterial strains HI-610, HI-618, HI-644, HI-651, and HI-1576 (see Table 1 for bacterial strains used). High: challenge dose 10^6 CFU ml^{-1}, control: unchallenged larvae. Red symbols: mortality rates significantly different from the control ($p < 0.01$ Bonferroni correction)

ferent from that of the control group from 7 dph onward ($p < 0.01$ Bonferroni correction, Fig. 2).

The cumulative mortalities in the 2 separate groups of cod larvae were similar (data not shown), so the cumulative mortality and statistical analysis are shown for only one of the groups (Fig. 3). Cod larvae showed equal susceptibility to the high-challenge dose of *Vibrio anguillarum* serotype O1 (HI-644) and O2α (HI-610). Statistical analysis showed that the mortalities of these challenge groups were significantly different from that of the control group starting at 3 and 4 dph, respectively ($p < 0.01$ Bonferroni correction). In the low-challenge dose groups, the only observed significant difference in mortality, which was between the control and the group challenged with *V. anguillarum* serotype O2α started from 7 dph onward. Although the cumulative mortality reached 35% in the high-challenge dose group of *V. anguillarum* serotype O2β (HI-618), this was significantly different from that in the control only at 7 and 8 dph ($p < 0.01$ Bonferroni correction).

Immunohistochemistry

Immunohistochemical examinations were used for verification instead of re-isolation of bacteria, and were therefore performed on the larval groups that suffered the highest mortality rates compared to the control group. No control larvae were positively immunostained by either of the antisera (Fig. 4a).
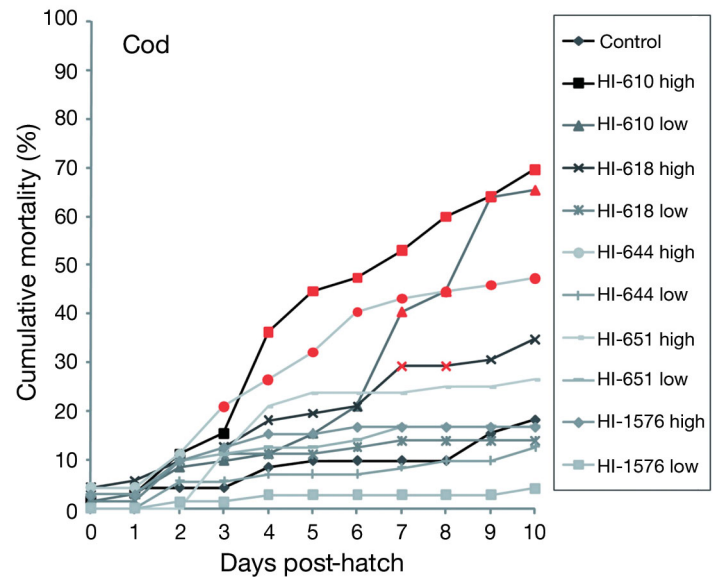

Fig. 3. *Gadus morhua.* Cumulative percentage mortality of cod larvae challenged with the same bacterial strains and doses as in Fig. 1. Day 0: day of hatching. Red symbols: mortality rates significantly different from the control group ($p < 0.01$ Bonferroni correction)

The immunohistochemical findings in turbot larvae challenged with *Vibrio anguillarum* serotype O2α (HI-610) demonstrated the presence of bacteria in the gastrointestinal tract, abdominal cavity, gallbladder (Fig. 4b) and kidney.

Differences in histopathology were observed in halibut larvae challenged with *Vibrio anguillarum* serotype O1 (HI-644) and O2α (HI-610) (Fig. 4c,d). Tissue damage was mostly found in larvae challenged with the O1 serotype. Large quantities of bacteria sur-
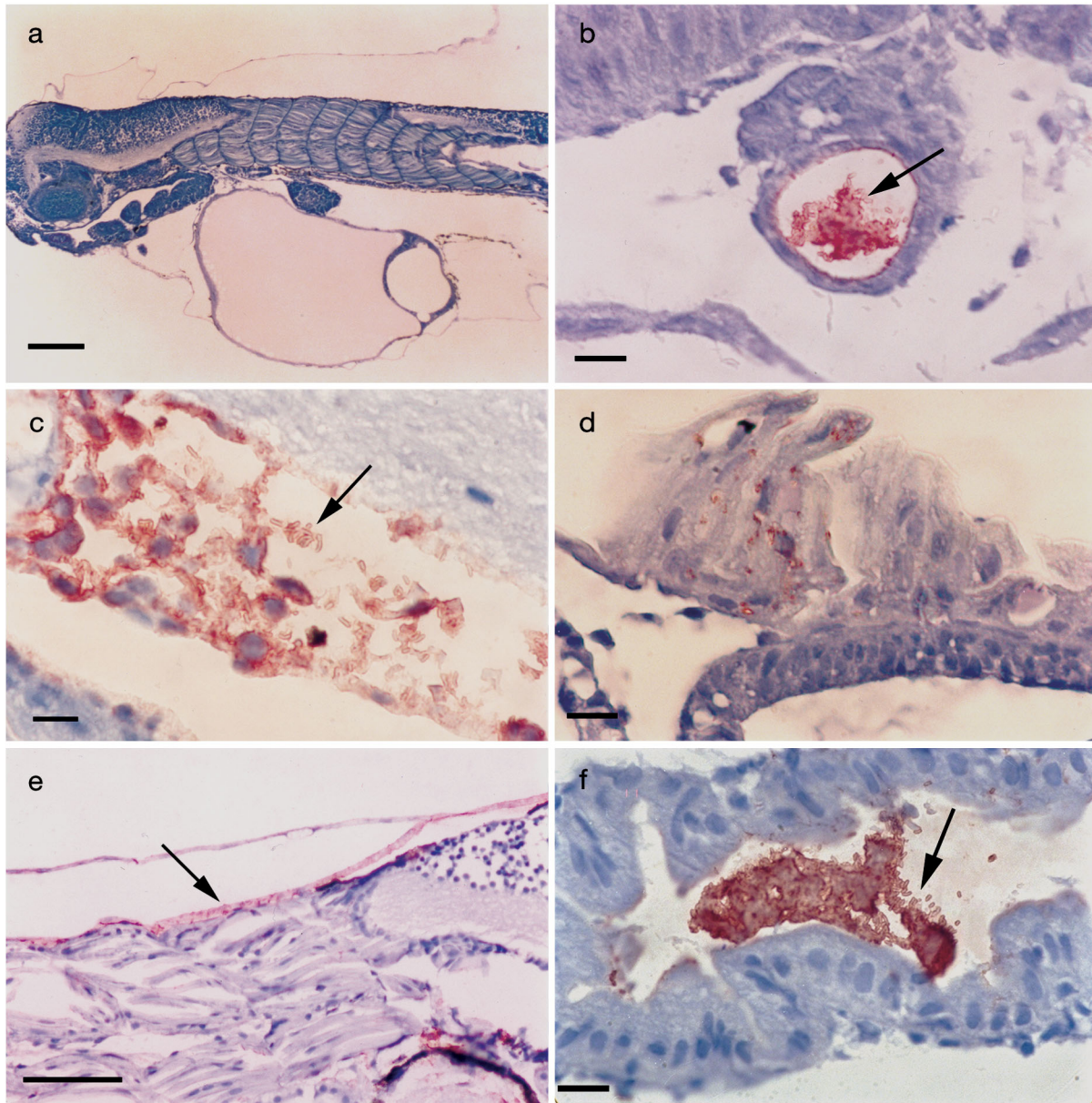

Fig. 4. *Scophthalmus maximus*, *Hippoglossus hippoglossus*, *Gadus morhua*. Immunohistochemical staining of paraffin sections from larvae using the Avidin-biotin method, rabbit anti-*Vibrio anguillarum* HI-610 and *V. anguillarum* HI-644 sera, and Shandon haematoxylin counterstain. Positive immunohistochemistry is visualized by red coloring of the bacteria. Counterstaining gives tissue different tones of blue. (a) Turbot *S. maximus* control larva, showing no positive immunostaining. Magnification: 100×; scale bar: 100 µm. (b) Turbot challenged with *V. anguillarum* HI-610, showing positive immunostaining of bacteria in gallbladder (arrow). Magnification: 1000×; scale bar: 10 µm. (c) Halibut *H. hippoglossus* challenged with *V. anguillarum* HI-644, showing brain tissue with necrosis and free bacteria (arrow). Magnification: 1000×; scale bar: 10 µm. (d) Halibut challenged with *V. anguillarum* HI-610, showing positive staining of bacteria associated with sensory cells on the dorsal side of the head. Magnification: 1000×; scale bar: 10 µm. (e) Cod *G. morhua* challenged with *V. anguillarum* HI-644, showing positive immunostaining in dermis and muscle tissues (arrow). Magnification: 400×; scale bar: 50 µm. (f) Cod challenged with *V. anguillarum* HI-610, showing positive immunostaining of bacteria in intestine (arrow). Magnification: 1000×; scale bar: 10 µm

rounding necrotic cells were found in the dermis and in brain (Fig. 4c), and liquefactive necrosis was seen in striated muscle cells of muscle tissue (data not shown). Necrotic tissue was infiltrated with bacteria, and unattached bacteria were also observed in the yolk-sac area, gastrointestinal tract and the area around the eyes (data not shown). In halibut larvae challenged with the O2α serotype (HI-610), histopathology was limited to a few necrotic cells in the dermis and positive staining of bacteria in early developed sensory cells on the dorsal side of the head (Fig. 4d). Bacteria were also observed in the gastrointestinal tract (data not shown).

In cod larvae challenged with the O1 serotype (HI-644), bacteria were observed in the dermis and the head region (Fig. 4e). In larvae challenged with the O2α serotype (HI-610), bacteria were only observed in the gastrointestinal tract (Fig. 4f). All the cod larvae examined displayed few signs of pathology.

DISCUSSION

The results revealed differences in susceptibility among turbot, halibut and cod larvae to various serotypes of *Vibrio anguillarum*. The high-challenge dose of *V. anguillarum* serotypes O2α and O1 had a significantly negative effect on halibut and cod larvae. Turbot larvae were mostly affected by the O2α serotype. The difference in mortality observed at the end of the experiment for the turbot larval group challenged with the low-challenge dose of the O1 serotype strain, could be an artifact as this is at the end of the non-feeding yolk-sac period. If this bacterial strain had been pathogenic to these larvae, the same mortality should have been observed in the high-challenge dose group as well. This suggestion is supported by the immunohistochemical findings that showed no pathology in examined larvae. The fact that the O1 serotype seemed to affect cod and halibut the most would suggest that there are differences in the immune system among the 3 species and/or that the bacterium has a preference for one specific host. The nonspecific immune system has been studied in cod (Lange et al. 2004, reviewed by Falk-Petersen 2005) and halibut larvae to a certain extent (Lange et al. 2006, reviewed by Falk-Petersen 2005). In cod larvae, the complement component C3 was detected at 1 dph and was found in most organs at 15 dph (Lange et al. 2004). In halibut, the C3 factor was found at 30 dph (Lange et al. 2006); however, this was the earliest sampling in that particular study. Patel et al. (2009) found that the lymphoid organs were not morphologically well developed until the late metamorphic stage and that immunoglobulin M (IgM) proteins were detected from Day 94 onward.

Similarly in turbot, the spleen and thymus appear at the end of the yolk-sac stage (reviewed by Falk-Petersen 2005), indicating that the specific immune response can be expected after this stage. A recent study by Corripio-Miyar et al. (2007) of IgM development in the haddock *Melanogrammus aeglefinus*, suggests that the immune system starts to develop at ~25 to 29 dph. In view of the present knowledge on the development of the specific immune system in cod and halibut (Schrøder et al. 1998, Lange et al. 2006, Patel et al. 2009), it should be emphasized that the present experiment was conducted at developmental stages at which the specific immune system was immature or even undeveloped. Preference for a particular host species has been found among *V. anguillarum* strains that were tested on different species of fish mucus (Larsen et al. 2001). The same study showed an overall preference for rainbow trout mucus over mucus from cod, common bream *Abramis brama* and flounder *Platichtys flesus*. Differences in host susceptibility to *Mycobacterium marinum* have also been found (Wolf & Smith 1999). The study showed great differences in the inflammatory response between striped bass, *Morone saxatilis*, and the tilapia hybrid *Oreochromis niloticus* × *O. mossambicus* × *O. aureus*. The striped bass that experienced the highest mortality also displayed the most severe clinical signs (granulomas with necrosis and inflammation). Differences in virulence have also been shown in experiments involving various species of bivalves that were exposed to different *Vibrio* strains (Nicolas et al. 1992, 1996, Luna-González et al. 2002).

As previously described, *Vibrio anguillarum* serotype O2β is the strain that is most frequently associated with disease in cod and nonsalmonid fish (Mikkelsen et al. 2007). The O2β isolate used in this study did not have negative effects on turbot and halibut larvae but in the cod larval experiments the mortality results were not as straightforward. In the cod experiments, significant differences in mortality were observed on Days 7 and 8 post-hatch but not at the end of the experiment, indicating that this isolate could not be highly pathogenic. This is also supported by the immunohistochemical examinations. A difference in virulence between the O2α and the O1 serotype was also verified in terms of challenge dose. The low-challenge dose (tested with cod and turbot only) of the O2α serotype was pathogenic to turbot and cod larvae, while the low-challenge dose of the O1 serotype only seemed to have a minor negative effect, if any, on turbot larvae. Differences in the virulence of *V. anguillarum* strains have previously been reported (Larsen et al. 1988, reviewed by Toranzo & Barja 1990, Lemos et al. 1991, Pedersen et al. 1997), but the mechanisms involved are often debated. The presence of iron-uptake mechanisms is regarded as essential for virulence (reviewed by Johansson & Cos-

sart 2003), although nonpathogenic *V. anguillarum* strains are known to possess similar systems (Lemos et al. 1991). Pedersen et al. (1997) compared the virulence of *V. anguillarum* serotype O1 isolates to the presence of virulence plasmids, outer membrane proteins and siderophore production. They found that all virulent strains carried a specific 67 kbp virulence plasmid and produced the outer membrane protein OM2. However, some strains that were carrying the virulence plasmid and producing both the membrane protein and siderophores proved to be nonvirulent; hence, the authors suggested that other factors might also be involved in virulence. All these studies emphasize the complexity of virulence studies and that it is likely that more than one factor need to be present, or that different strains use different mechanisms of virulence.

The increase in mortality started at different days post-hatch in the 3 species (Figs. 1 to 3). In the halibut group, mortality increased at 7 dph, while in the turbot and cod groups, it started at 2 dph and 3 to 4 dph, respectively. This may be partly related to rearing temperature. Our halibut and cod were reared at 6°C (compared to 15°C for turbot), which may slow down bacterial growth and delay infection. In addition, it must be emphasized that ontogenetic development occurs far more rapidly in turbot than in halibut, while cod has an intermediate development rate.

The immunohistochemical examinations revealed differences in the infection caused by the *Vibrio anguillarum* serotypes O1 and O2α. The infection caused by *V. anguillarum* serotype O1 was more extensive (Fig. 4c,e) than that caused by the O2α serotype (Fig. 4b,d,f), which usually took the form of bacteria in the gastrointestinal tract. These findings are similar to those observed by Engelsen et al. (2008), who found that cod yolk-sac larvae challenged with the *V. anguillarum* O2α strain experienced high mortality. Engelsen et al.'s histopathological observations were few and suggested excretion of bacterial toxins as a possible cause of death. The histological findings in the present study suggest that different bacterial strains use different host interactions and mechanisms of infection. This was also suggested by Larsen et al. (1988), based on haemagglutination tests and hydrophobic interaction chromatography of various serotype O1 and O2 isolates.

The portal of entry and preferred adherence and attachment sites of *Vibrio anguillarum* are currently debated. The suggestion that *V. anguillarum* may have a stronger tendency to adhere to intestinal than to skin mucosa was a result of studies performed on turbot (Oisson et al. 1996) and rainbow trout *Oncorhynchus mykiss* mucus (O'Toole et al. 1999). These studies were performed using the O1 and O2α serotypes, respec-

tively. The skin has been suggested to be the major attachment and proliferation site in ayu *Plecoglossus altivelis* and rainbow trout (Kanno et al. 1989, Spanggaard et al. 2000, respectively). The gills have also been suggested as an infection site in rainbow trout *Salmo gairdneri* (Baudin Laurencin & Germon 1987). On the other hand, it has been reported that *V. anguillarum* serotype O2 does not adhere to mucus in the same way as the O1 serotype but rather to other components of the epithelial surface (Knudsen et al. 1999). These authors suggested that *V. anguillarum* O2 might employ an infection strategy involving adhesion to mucus. The above references all illustrate the complexity of and variables involved in these kinds of studies, the use of different species and bacterial strains, but more importantly, they suggest the possibility that different strains of *V. anguillarum* have different host infection strategies.

The *Vibrio splendidus* and *V. salmonicida* strains that we tested did not cause a rise in mortality compared to the control group. Turbot larvae exposed to *V. splendidus* experienced a significantly higher mortality than the controls only at the end of the experiment, probably due to the lack of feeding as the yolk-sac stage was ending. As reviewed by Thompson et al. (2004), *V. splendidus* was originally described as a nonpathogenic strain that was isolated from the aquatic environment. However, Reid et al. (2009) recently demonstrated mortality of cod larvae in challenge experiments with *V. splendidus*, following oral administration via live feed. Avirulent strains of *V. splendidus* have previously been reported in challenge experiments performed on halibut larvae (Verner-Jeffreys et al. 2003) and turbot (60 g) (Farto et al. 1999). *V. salmonicida* is associated with mortality at low temperatures and is regarded as nonpathogenic to salmon at temperatures >10°C (Enger et al. 1991). The temperatures used in the halibut and cod experiments should therefore be optimal for virulence studies that use this bacterium. In the turbot study performed at 15°C, the temperature may have been too high to cause disease. Following the findings of Reid et al. (2009), it could also be hypothesized that *V. splendidus* is dependent upon oral administration to cause mortality.

In conclusion, the different strains of *Vibrio anguillarum* caused different mortalities to the 3 different species of fish larvae. Thus, species-specific interactions between bacteria and host may well be present at the yolk-sac stage of fish. We further conclude that immunohistochemistry with absorbed polyclonal antisera is a powerful tool for investigating bacterial infections in fish larvae, including demonstrating the presence of bacteria and pathological alterations in various tissues.

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Sandlund et al.: Susceptibility of fish yolk-sac larvae to *Vibrio* spp.

37

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