Changes in Behaviour of Atlantic Halibut (*Hippoglossus hippoglossus*) and Turbot (*Scophthalmus maximus*) Yolk-Sac Larvae Induced by Bacterial Infections

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Eggs of Atlantic halibut (*Hippoglossus hippoglossus*) and turbot (*Scophthalmus maximus*) were exposed to *Flexibacter ovolyticus* and pathogenic *Vibrio* sp. strains prior to, and during hatching. Activity, buoyancy and mortality of the yolk sac larvae were monitored from hatching until time of first feeding. The halibut larvae showed reduced activity and increased mortality in response to the challenge of bacteria, compared to uninfected control groups. In addition, the infected halibut larvae showed increased specific density compared to the uninfected larvae. These responses were not found for turbot. However, turbot larvae infected with *Vibrio anguillarum* had lower activity than larvae infected with *F. ovolyticus*. The reduced activity of halibut larvae occurred 1–2 weeks prior to the increased mortality, allowing infections to be detected at an early stage. The results suggest that the behaviour of fish larvae is influenced by bacterial infection.

Les oeufs de flétan (*Hippoglossus hippoglossus*) et de turbot (*Scophthalmus maximus*) ont été exposés à *Flexibacter ovolyticus* et des souches pathogènes de *Vibrio* sp., avant et pendant l'éclosion. L'activité, la flottabilité et la mortalité des larves vésiculées ont été contrôlées de l'éclosion au premier repas. Par comparaison à leurs groupes témoins non infectés, les larves de flétan avaient une activité réduite et une mortalité accrue du fait qu'elles étaient exposées aux bactéries. En outre, les larves de flétan infectées étaient plus denses que les larves non infectées. Ces réponses n'ont pas été observées chez les larves du turbot. Toutefois, parmi ces dernières, les larves qui étaient infectées par *Vibrio anguillarum* étaient moins actives que les larves infectées par *F. ovolyticus*. L'activité réduite des larves de flétan a été observée une à 1–2 sem avant que la mortalité ne s'accroisse; il est ainsi possible de détecter les infections très tôt. Les résultats donnent à penser que le comportement des larves de poisson est influencé par les infections bactériennes.

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Regulating buoyancy and activity is the means by which pelagic fish larvae influence their spatial distribution (Pittman et al. 1990b), which eventually influences their potential to survive. Infections by different pathogens are often associated with characteristic changes in host behaviour (Dobson 1988, Dawkins 1990 and references therein). Microorganisms are intimately associated with the mucosal surfaces of the skin and intestine of fish larvae (Yoshimizu et al. 1980; Sugita et al. 1982; Campbell and Buswell 1983; Olafsen 1984; Muroga et al. 1987; Sugita et al. 1988; Hansen et al. 1992 a, b; Olafsen and Hansen 1992). In the case of halibut larvae, some of these microorganisms have been shown to be opportunistic pathogens (Bergh et al. 1992).

If pathogens cause significant changes in the buoyancy and behaviour of marine fish larvae, they may indirectly influence the spatial distribution of the larvae in their natural environment as well as in aquaculture systems. In aquaculture, a pathogen-induced shift in behaviour might provide a suitable way of detecting infections at an early stage, thereby making it possible to discard infected larvae. In the natural environment such a possible mechanism would constitute a hitherto unrecognized indirect pathway by which pathogens influence the potential for survival of their hosts. The purpose of the present study was to investigate whether opportunistic pathogens are capable of changing the behaviour of their hosts.

Materials and Methods

Bacteria

The following bacterial strains were used: *Flexibacter ovolyticus* NCIMB 13127 (National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland), isolated from halibut eggs (Hansen et al. 1992a); *Vibrio* sp. HI-10448, isolated from halibut; and *Vibrio anguillarum* HI-11360, isolated from turbot (O.M. Rødseth, Institute of Marine Research, Bergen, Norway personal communication). *Flexibacter ovolyticus* was used to infect both halibut and turbot; *Vibrio* sp. HI-10448 was used in the experiments with halibut; and *V. anguillarum* HI-11360, with turbot. All bacteria were cultured on Difco 2216 Marine Broth

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(Difco, Detroit, USA) with 1.5% Bacto agar (Difco) added, and suspended in 25 ppt autoclaved seawater immediately before infection.

Halibut Experiments

The halibut eggs were reared in 250-L upwelling incubators, at 7–8°C until three days before hatching. They were then transferred to polystyrene multidishes (Nunc, Roskilde, Denmark). Each multidish consisted of 6 identical wells, 35 mm in diameter and 18 mm high. Each well contained 11 mL autoclaved 25 ppt seawater. One egg was transferred to each well. The infection and further incubation was performed according to Bergh et al. (1992). Two days before hatching the eggs were infected with the various bacteria. Groups infected with *F. ovolyticus* were denoted FA, FB and FC. The groups infected with *Vibrio* sp. HI-10448 were denoted VA, VB, and VC. Each infected group consisted of 60 larvae, each in an individual well. To provide enough larvae to allow for simultaneous video recordings of infected groups and control groups, the control group consisted of 180 larvae, each in an individual well. Three different concentrations of each bacterium were used, approximately $1.0 \cdot 10^6$ (Groups FA and VA), $5.0 \cdot 10^5$ (Groups FB and VB) and $1 \cdot 10^5$ (Groups FC and VC) bacteria $\cdot$ mL^{-1}, as verified by epifluorescence counting. One day after hatching, 10 mL of water was removed from each well and 10 mL autoclaved 25 ppt seawater was immediately added.

The eggs/larvae were incubated in darkness in a temperature-controlled room at 5°C. Mortality was recorded until 34 d after hatching.

The larvae were filmed on days 3, 9, 13, 24, and 33 after hatching. They were filmed under red light with an intensity of 2.5 lux with a Philips L DH 670 video camera. One multidish of the control group, one multidish of the *F. ovolyticus*-infected group and one multidish of the *Vibrio* sp. HI-10448-infected group were filmed simultaneously for 30 min, thus 18 larvae were filmed simultaneously. The camera was mounted approximately 1 m above the multidishes. All wells of the 3 multidishes were included in the viewfield of the camera. After 30 min, the 3 multidishes were replaced by other multidishes, until all had been filmed. Activity was measured from the video recordings. In this study, activity is defined as the percentage of the time the larvae showed any movement that was visible on the video recordings during the last 15 min of the 30-min recording period for each well. In addition, the percentage of larvae that showed any movement during the 15-min observation time was measured.

For buoyancy measurements, two different batches of eggs were used, one infected with *F. ovolyticus* and one infected with *Vibrio* sp. HI-10448. The challenge doses were approximately equal to VA and FA (above), and each group consisted of 60 larvae. In addition, one control group of 60 larvae was used for each batch of eggs.

Turbot Experiment

The turbot eggs were obtained from a commercial hatchery (Øye havbruk, Øye, Norway). The eggs were brought to the laboratory one day before hatching and immediately transferred to polystyrene multidishes (Nunc, Roskilde, Denmark). These multidishes had 24 identical wells, each 15.8 mm in diameter and 18.0 mm high. Each well contained 2 mL autoclaved 25 ppt seawater. One egg was transferred to each well. The eggs were divided into four groups. Each group consisted of 144 larvae. Two groups were infected with *F. ovolyticus*, one (*F. ovolyticus* 1) immediately after transfer to the polystyrene dishes, and one (*F. ovolyticus* 2) immediately after all eggs had hatched. One group was infected with *V. anguillarum* HI-11360, immediately after transfer to polystyrene dishes. A fourth group served as a control. The concentrations of bacteria were approximately $1 \cdot 10^6$ bacteria $\cdot$ mL^{-1} in the wells for each of the three infected groups. Seventy-two of the 144 larvae from each group were checked daily for mortality, and filmed on days 1, 3 and 5. The remaining 72 larvae from each group were used for buoyancy measurements.

The eggs/larvae were incubated and filmed in white light at 117.4 lux. Mortality was recorded until 6 d after hatching. Each multidish was filmed for 20 min on days 1, 3, and 5 after hatching. Four multidishes were filmed simultaneously, one control, and one of each of the three infected groups. After 20 min, the 4 multidishes were replaced by others, until three multidishes from each group (72 larvae) had been filmed. The camera was mounted approximately 1 m above the multidishes, and all wells of the four multidishes were included in the viewfield. Activity was measured as the percentage of time that the larvae showed any visible movement, during the last 10 min of the 20-min video recording period. In addition, the percentage of larvae that showed any movement during the 10-min observation time was measured.

Measurements of Buoyancy

Larval buoyancy was measured for both species by their position in a buoyancy column, according to Coombs (1985). The column consisted of a glass cylinder of 45-mm internal diameter and a height of 70 cm with a salinity gradient and internal standards. For buoyancy measurements, 10 larvae from each group were anaesthetized in their wells by addition of 100 μL of a 1 g $\cdot$ L^{-1} stock solution of methomidate chloride (Hypnodil™ Janssen Pharmaceutica, Belgium) in distilled water. After transfer to the buoyancy column, the specific density of the larvae was calculated from their position relative to the standards, by a computer programme developed by Dr. A. Mangor-Jensen, Austevoll Aquaculture Research Station. No larvae were used repeatedly for this purpose. Buoyancy measurements were carried out on days 7, 9, 14, 17, 21, 23, and 28 (halibut infected with *Vibrio* sp. HI-10448); days 7, 10, 14, 16, 21, and 23 (halibut infected with *F. ovolyticus*); and days 1–6 (turbot experiment).

Results

Mortality

All groups of infected halibut larvae had significantly higher numbers of mortalities during the experimental period than the control group (Fig. 1; $q > 11.62$, $p < 0.001$, Tukey-type multiple comparison of proportions, Zar 1984), with the exception of group VC ($q = 4.327$). A dose-response relationship was found for the groups infected with *Vibrio* sp. HI-10448. No such dose-response was found for the groups of larvae infected with *F. ovolyticus*; the group exposed to the highest concentration of bacteria showed the lowest mortality.

In the experiment with turbot larvae, *F. ovolyticus* did not cause mortality significantly different from that in the control group (Fig. 2). The group infected with *V. anguillarum* HI-11360, however, showed a significantly higher mortality than all other

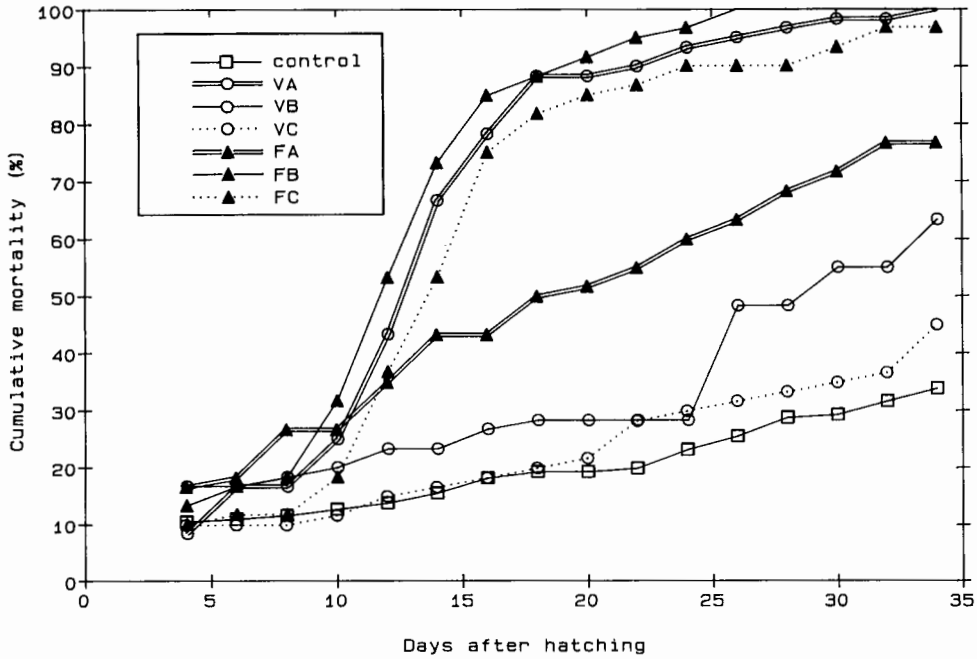

FIG. 1. Cumulative mortality (%) of halibut larvae. Groups infected with *Flexibacter ovolyticus* are denoted FA, FB and FC, whereas the groups infected with *Vibrio* sp. HI-10448 are denoted VA, VB, and VC. Each group consisted of 60 larvae each in individual wells, except the control group that consisted of 180 larvae each in individual wells. Three different concentrations of each bacterium were used, approximately $1.0 \cdot 10^6$ (Groups FA and VA), $5.0 \cdot 10^5$ (Groups FB and VB) and $1.0 \cdot 10^5$ (Groups FC and VC) bacteria $\cdot$ mL^{-1}.

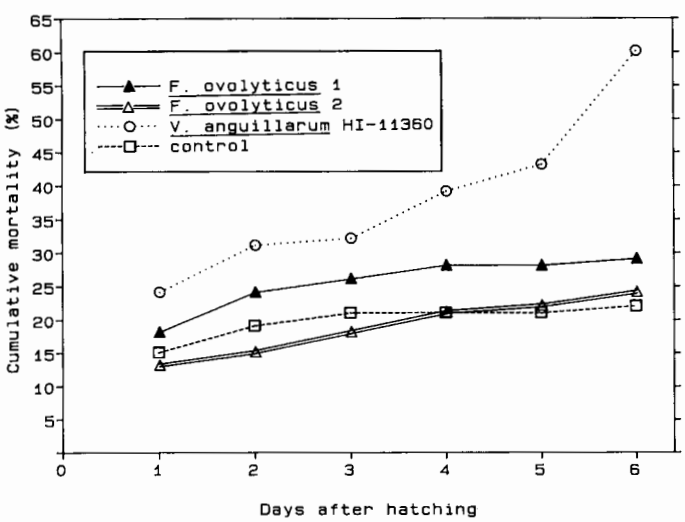

FIG. 2. Cumulative mortality (%) of turbot larvae. Group *F. ovolyticus* 1 was infected immediately after transfer to polystyrene dishes, whereas group *F. ovolyticus* 2 was infected immediately after all eggs had hatched. One group was infected with *V. anguillarum* HI-11360, immediately after transfer to polystyrene dishes. A fourth group served as a control. The concentrations of bacteria were approximately $1 \cdot 10^6$ bacteria $\cdot$ mL^{-1} in the wells for each of the three infected groups. Each group in this part of the experiment consisted of 72 larvae.

groups at the end of the experiment ($q > 6.58$, $p < 0.001$, Tukey-type multiple comparison of proportions, Zar 1984).

Activity

Those halibut larvae in the control group that survived throughout the experiment showed an activity approximately one order of magnitude higher than all groups that were infected with varying concentrations of *Vibrio* sp. HI-10448 or *F. ovolyticus* (Fig. 3a). Activity was significantly lower in all these groups compared to the control group, at day 3 ($p < 0.001$, Robust *t*-test of medians, Hoaglin et al. 1983). In addition, the fraction of infected larvae that showed any measurable activity (Fig. 3b) was significantly lower than that in the uninfected group ($\chi^2 = 59.98$, $p < 0.001$). Of all the larvae that showed any measurable activity at day 3, 30.9% died during the experimental period, compared to 89.0% mortality among those that did not show any measurable activity at day 3. The difference is significant ($\chi^2 = 37.15$, $p < 0.001$).

The *F. ovolyticus*-infected larvae could not be significantly separated from the *Vibrio*-infected larvae with respect to activity. After day 13, however, the activity of the remaining larvae in the *F. ovolyticus*-group was zero. The characteristic decrease in activity at day 8 which was found for both the control group and the *Vibrio*-infected group is typical of halibut larvae kept in darkness (Skiftesvik et al. unpublished data).

No difference in mean activity could be found for turbot (Fig. 4a). However, at day 1, significant differences in the fraction of the active larvae were found ($\chi^2 = 17.33$, $p < 0.001$), as a higher fraction of both groups of *F. ovolyticus*-infected larvae were active (Fig. 4b). At day 5, differences in the fraction of active larvae were found ($\chi^2 = 13.05$, $p < 0.005$) as a significantly lower fraction of the *V. anguillarum* HI-11360-infected group was active than in the *F. ovolyticus*-infected groups

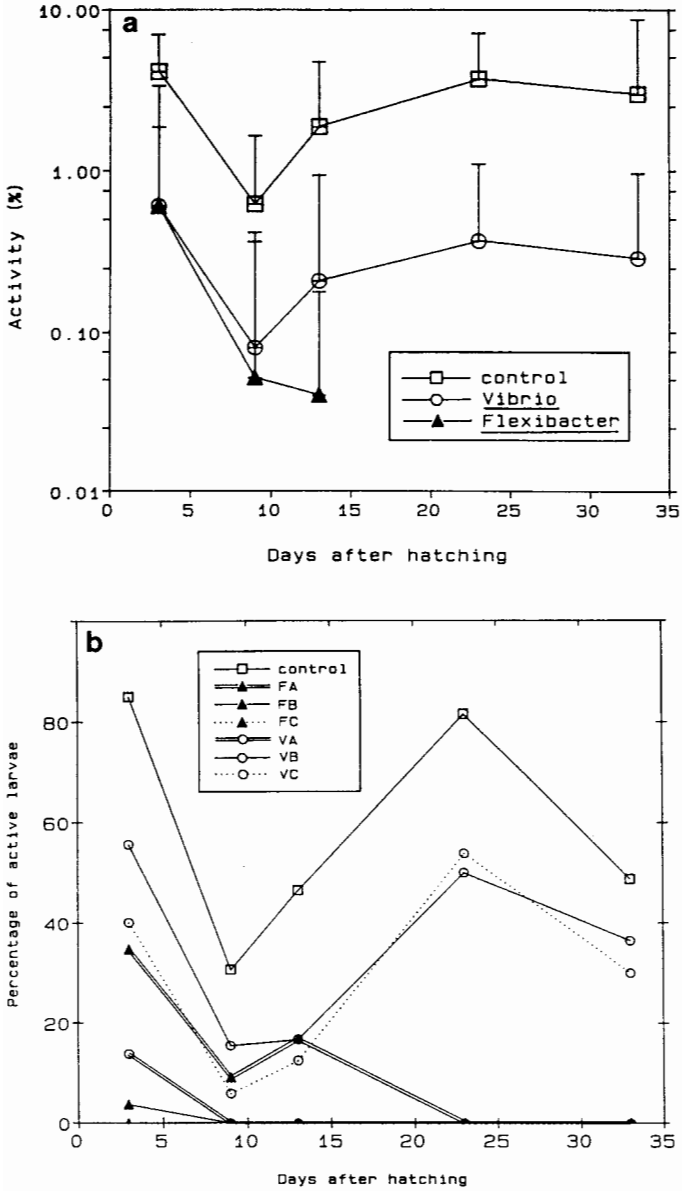

FIG. 3. a) Mean activity of the halibut larvae (percentage of time that movement was detected). In the control group, only those larvae that survived throughout the experiment are included; b) Percentage of active halibut larvae. All larvae that were alive at the time of sampling in each group are included.

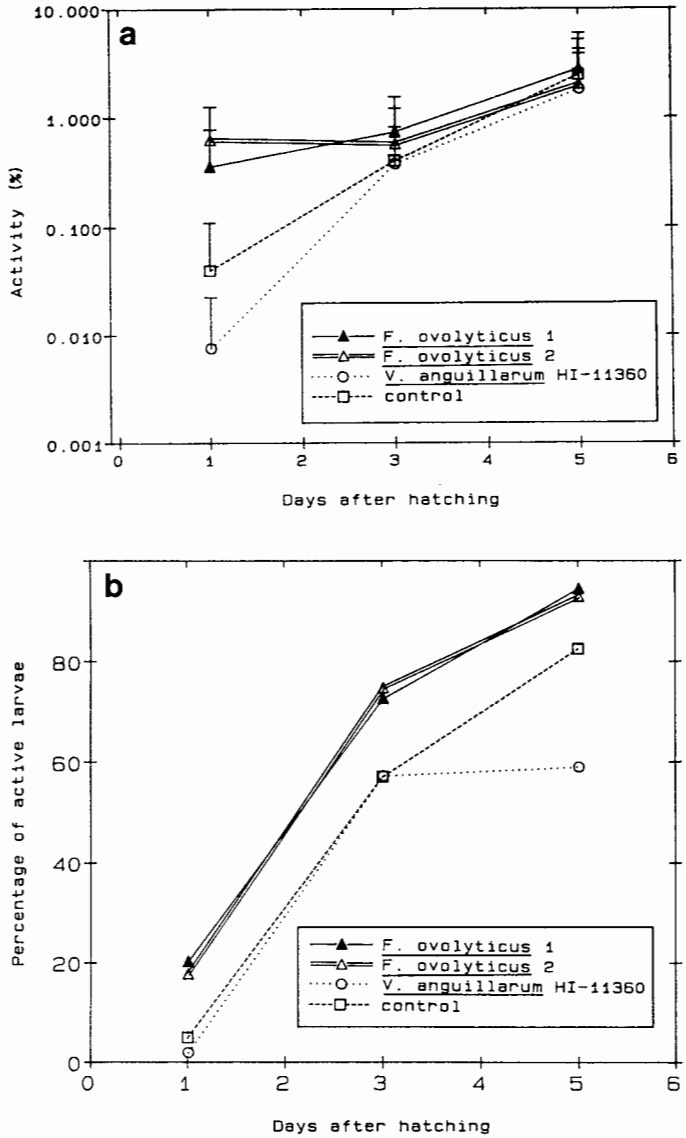

FIG. 4. a) Mean activity of the turbot larvae (percentage of time that movement was detected). All larvae that were alive at the time of sampling in each group are included; b) Percentage of active turbot larvae. All larvae that were alive at the time of sampling in each group are included.

($q \geq 4.6$, $p < 0.01$ Tukey-type multiple comparison of proportions, Zar 1984). However, the control group could not be significantly separated from either of the infected groups.

Density

The specific densities of halibut larvae infected with *Vibrio* sp. HI-10448 and those infected with *F. ovolyticus* (Fig. 5) were not significantly different from their respective control groups at the beginning of the experiment. However, from day 9 to day 17 the *Vibrio* sp. HI-10448-infected group had significantly ($p < 0.048$, Student's *t*-test) higher specific density than its control group (Fig. 5a). A similar significance ($p < 0.011$, Student's *t*-test) was found only at day 14 for the *F. ovolyticus*-infected group (Fig. 5b). No significant differences were found thereafter.

The specific density of the turbot larvae was approximately 1.020 $g \cdot cm^{-3}$ at the beginning of the experiment, slowly increasing to the range 1.021–1.025 at day 6. The group infected with *V. anguillarum* HI-11360 had significantly ($p < 0.0035$, Robust *t*-test of medians, Hoaglin et al. 1983) lower specific density at day 2, but otherwise, the control group could not be significantly separated from any of the infected groups (results not shown).

Discussion

The present results demonstrate a behavioural response to bacterial infections in halibut and turbot larvae that has so far not been recorded in research on fish larvae. Activity is a reflection of metabolic state and influences the energy requirements of larval fish (Laurence 1977). As we have shown that activity was affected by bacterial infections, such infections might influence the results from measurements of activity, and thus the energy

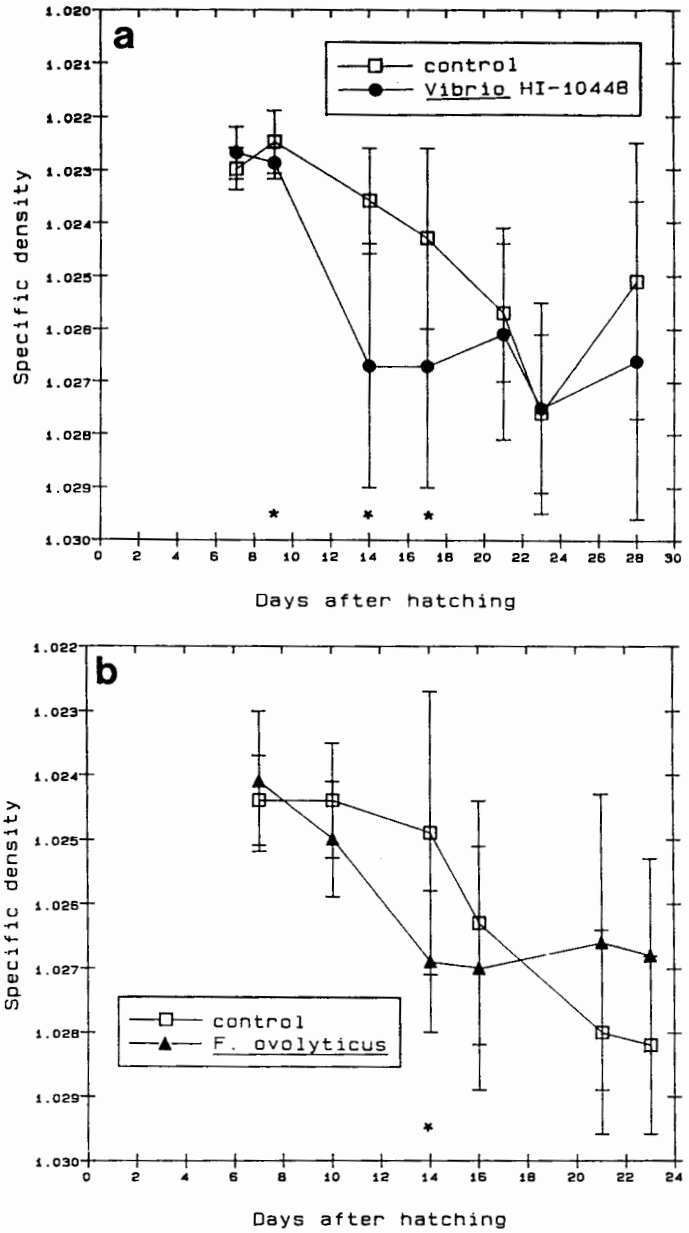

FIG. 5. Mean specific density ($g \cdot cm^{-3}$) of halibut larvae in the experiment with *Vibrio* sp. HI-10448 (a) and with *Flexibacter ovolyticus* (b) and their respective control groups, with standard deviation. The differences were significant at the level of $p < 0.05$ (Student's *t*-test) at the samplings marked with an asterisk.

requirements of fish larvae. This may also have been the case in earlier studies in which the behaviour of these larvae was investigated (Naas and Mangor-Jensen 1990; Pittman et al. 1990b; Van der Meeren, 1991). The level of activity is partially governed by the need to avoid predators and, from time of first-feeding, the need to search for food (Skiftesvik 1992). Thus, changes in the level of activity may alter both the susceptibility of larvae to predation, and their ability to search for and catch food items.

The increased specific density of the infected halibut larvae shows that bacterial infections might also cause alterations in the spatial distribution. Changes in buoyancy cause alterations in the vertical distribution of the larvae. Thus, both the reduced activity and the increased specific density would eventually alter the susceptibility of the larvae to predation in nature. This could constitute an indirect mechanism by which pathogens alter the survival potential of their hosts, in addition to the direct mortality caused by bacterial infection.

The mortality curve for the halibut larvae supports the conclusion of Bergh et al. (1992) that both *F. ovolyticus* and *Vibrio* sp. HI-10448 may cause substantial mortality throughout the yolk sac stage. Compared to the experiments described by Bergh et al. (1992), in which *F. ovolyticus* caused high mortalities prior to hatching, the time from infection until hatching was considerably shorter in the present study, allowing the bacteria less time to proliferate on the egg surface. We found no direct relationship between the dose of *F. ovolyticus* and mortality of the halibut larvae. A rapid decrease in viability, probably caused by bacteriophages, is typical of dense cultures of *F. ovolyticus* (Hansen et al. 1992a), although the factors responsible for lysogenic induction are unknown. Phage-mediated lysis of a dense culture could possibly explain the decreased pathogenicity of the highest concentration of *F. ovolyticus*.

The *Vibrio* sp. HI-11360 strain was pathogenic for turbot larvae. In contrast to the results from the halibut experiments, *F. ovolyticus* caused no significant mortality to turbot larvae, indicating that this bacterium is either nonpathogenic to turbot, or at least less pathogenic to turbot than to halibut. This might be due to the differences in the rearing temperatures of the two species. The only significant differences in behaviour among the groups in the turbot experiment were in the activity measurements and the fraction of active larvae between the groups infected with *F. ovolyticus* and the group infected with *Vibrio* HI-11360. Although the control group could not be significantly separated from the infected groups with respect to behavioural parameters, the differences among the infected groups show that the behaviour of turbot larvae might be affected by exposure to bacteria. It could be hypothesized that a nonpathogenic bacterium, as in this case *F. ovolyticus* may well be, could outcompete opportunistic pathogens present, and thus be beneficial to these larvae compared to the control group.

The interspecific difference in the buoyancy response could be related to the differences in habitat of the two species. Halibut spawn at 300–700 m depth (Haug 1990) and virtually nothing is known about the yolk sac larvae of this species in nature. Turbot larvae occur in the upper 10 m of the water column (Nellen and Hempel 1970; Russel 1976). All measurements of specific density of turbot imply that these larvae are distributed in the uppermost part of the water column. Thus, while small changes in the buoyancy of halibut larvae might cause large vertical movements, this would not necessarily be the case for turbot. In addition, halibut hatch at a primitive ontogenetic stage (Pittman et al. 1990b) compared to turbot (Russel 1976). This may explain the interspecific variation in the susceptibility of the larvae to different pathogens. The mortality in the infected groups of halibut larvae was probably highly selective, as the control groups tended to have higher buoyancy toward the end of the experiment.

Different species of the genus *Vibrio* are known to cause systemic infections in various fish species (Egidius 1987). Ultimately, the bacteria may infiltrate muscular tissue including the heart, as demonstrated by Totland et al. (1988) for Atlantic salmon (*Salmo salar*) infected by *V. salmonicida*. From studies on halibut larvae, infiltration of heart tissue and blood vessels has been shown, as well as the presence of large amounts of bacteria among the gill arches (Bergh et al. 1992). Reduced

activity and problems with buoyancy regulation could be viewed as a response to such pathological changes.

To counteract larval sinking and avoid the accumulation of bacteria, large-scale rearing of halibut yolk-sac larvae has so far been based on upwelling incubators of the types described by Pittman et al. (1989, 1990a), although high rates of flow may cause decreased survival and yolk-sac utilization compared to low rates (Opstad and Bergh 1993). The results from our buoyancy measurements show that infected halibut larvae tend to be less buoyant; thus they may sink to the bottom of the incubators, close to the water inlet through which dead larvae are removed. Thus, the inlet water will pass through a zone with dead and infected larvae, a probable source of continuous release of bacteria, as has been shown for Atlantic salmon (*Salmo salar*) smolts infected with *Aeromonas salmonicida* subsp. *salmonicida* (Enger et al. 1992). Consequently, the upwelling water may become contaminated before reaching the healthy larvae. This implies a mechanism by which pathogens, once established in an incubator, might proliferate rapidly.

The reduced activity of the infected halibut larvae occurred early in the experiment, well in advance of the increased mortality. This may provide a means by which infected larvae could be detected at an early stage. In contrast to complicated histological and microbiological techniques, behavioural responses are relatively easy to recognize. Thus, the measurement of such responses constitutes a possible means by which infected larvae could be sorted out. The increased specific density of the halibut larvae should make it possible to sort out the infected larvae at the bottom of the incubators, thereby reducing the infectious load of pathogens in the incubators.

We conclude that pathogenic bacteria are capable of influencing the activity and positioning in the water column of their hosts. Thus, any investigation of the behaviour of larval fish should take into account the potential effects of bacterial infection.

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