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 uninoculated 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 œufs 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'élosion. L'activité, la flottabilité et la
mortalité des larves yolk-sac ont été contrôlées de l'élosion au premier repas. Par comparaison à leurs groupes
témoin 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 de 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
déteindre 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
(Pettman 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). Micro-
organisms are intimately associated with the mucusous surfaces
of the skin and intestine of fish larvae (Yoshitama et al. 1980;
Sugita et al. 1982; Campbell and Buswell 1983; Olafsen 1984;
Maroga 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. 1990a); Vibrio sp. HI-10448 isolated from
halibut; and Vibrio anguillarum HI-11360, isolated from turbot
(O.M. Redsted, 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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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 polyester multisheets (Norsk, Roskilde, Denmark). Each multisheet consisted of 6 identical wells, 35 mm in diameter and 18 mm high. Each well contained 11 mL autoclaved 25% seawater, which 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 F. ovaliculatus. Groups infected with F. ovaliculatus were denoted PA, 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. Three different concentrations of each bacterium were used, approximately 1.0 - 10³ (Groups PA and VA), 5.0 - 10³ (Groups FB and VB) and 1.0 - 10⁴ (Groups VC and 'C') bacteria/mL, as verified by epifluorescence counting. One day after hatching, 10 mL of seawater was removed from each well and 10 mL autoclaved 25% 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 LD LH70 video camera. One multisheet of the control group, one multisheet of the F. ovaliculatus-infected group and one multisheet 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 multisheets. All wells of the 3 multisheets were included in the viewfield of the camera. After 30 min, the 3 multisheets were replaced by other multisheets, until all had been filmed. Each larvae 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. ovaliculatus 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 (Dye harbor, Dye, Norway). The eggs were brought to the laboratory one day before hatching and immediately transferred to polyester multisheets (Norsk, Roskilde, Denmark). These multisheets had 16 identical wells, each 18.9 mm in diameter and 18.9 mm high. Each well contained 2 mL autoclaved 25% seawater. One egg was transferred to each well. The eggs were divided into four groups. Each group consisted of 114 larvae.

Two groups were infected with F. ovaliculatus, one (F. ovaliculatus 1) immediately after transfer to the polystyrene dishes, and one (F. ovaliculatus 2) immediately after all eggs had hatched. One group was infected with V. angularis HI-11360, immediately after transfer to polystyrene dishes. A fourth group served as a control. The concentrations of bacteria were determined by inoculation of 100 mL of conditioned seawater to 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, one of each of the three infected groups. After 28 min, the 4 multisheets were replaced by others, until three multisheets (72 larvae) had been filmed. The camera was mounted approximately 1 m above the multisheets, and all wells of the four multisheets 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 mL of a 1 g·L⁻¹ stock solution of methohexitone (Hypnodil™, Pharmacia, Uppsala) 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, 16, 17, 21, 23, and 28 (halibut infected with Vibrio sp. HI-10448), days 7, 10, 14, 16, 21, and 23 (halibut infected with F. ovaliculatus) 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; y > 11.62, p < 0.001, Tukey-type multiple comparison of proportions, Zar 1984), with the exception of group VC (y = 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. ovaliculatus; the group exposed to the highest concentration of bacteria showed the lowest mortality.

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


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

Activity

Those habitus 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 *V. alginolyticus* (Fig. 3a). Activity was significantly lower in all these groups compared to the control group, at day 3 ($p < 0.001$), Robust tests of medians, *Houglum 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 ($X^2 = 59.98, p < 0.001$). Of all the larvae that showed any measurable activity at day 3, 30% 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 ($X^2 = 37.15, p < 0.001$).

The *V. alginolyticus*-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 *V. alginolyticus* 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 habitus larvae kept in darkness (Shifrin et al., unpublished data).

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

The specific densities of halibut larvae infected with Vibrio sp. HI-10448 and those infected with _F. psychrophilum_ (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 < 0.013, Student's t-test) was found only at day 14 for the _F. psychrophilum_-infected group (Fig. 5b). No significant differences were found thereafter.

The specific density of the turbot larvae was approximately 1.020 g 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, Houing et al. 1983) lower specific density at day 6, 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.
The susceptibility of the larval to predation in nature. This could possibly indicate an indirect mechanism by which pathogens alter the survival potential of their hosts, in addition to the direct mortality caused by bacterial infection.

The decrease in the halibut larvae supports the conclusion of Bergh et al. (1992) that both F. ovoviviparum 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. ovoviviparum caused high mortalities prior to hatching, the time from infection until hatching is 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. ovoviviparum and mortality of the halibut larva in our study. A rapid decrease in viability probably caused by bacteriophages, is typical of diverse cultures of F. ovoviviparum (Hansen et al. 1992a), although the factors responsible for the observed decrease are unknown. A decrease of a dense culture could possibly explain the decreased pathogenicity of the highest concentration of F. ovoviviparum.

The Vibrio sp. HI-1156 strain was pathogenic for larval turbot. In contrast to the results from the halibut experiments, F. ovoviviparum 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 turbott experiments were in the activity measurements and the fraction of active larvae between the groups infected with F. ovoviviparum and the group infected with Vibrio HI-1156. 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 larva might be affected by exposure to bacteria. It could be hypothesised that nonpathogenic bacterium, as in this case F. ovoviviparum may well be, could cope more

inter-specific competition and pose a threat to the studied species. However, the differences observed in the present study may not necessarily be the case for turbot. In addition, halibut hatch at a prerequisite ontogenetic stage (Pitman et al. 1990b) compared to turbot (Russe 1976). This may explain the inter-specific variation in the susceptibility of the larvae to different pathogens. The mortality of the infected groups of halibut larvae was probably highly selective, as the control groups tended to have higher mortality towards the end of the experiment.

Different species of the genus Vibrio are known to cause systemic infections in various fish species (Eigdiz et al. 1987). Although the first two groups of bacteria were identified with the help, as demonstrated by Todtmann et al. (1938) 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 benthic, large-scale rearing of halibut yolk-sac larva has so far been based on upwelling incubators of the types described by Pettersen 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 incubated 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 mor-
tality. This may provide a means by which infected larvae could have been detected at an early stage. In contrast to complicated historical and microbiological techniques, behavioural responses are relatively easy to recognize. Thus, the measure-
ment of such responses constitutes a possible means by which infected larvae could be sorted out. The increased specific dexterity of the halibut larva should make it possible to sort out the infected larva 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 behavioural

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