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Vaccination improves survival of Baltic salmon (*Salmo salar*) smolts in delayed release sea ranching (net-pen period)

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Abstract

Baltic salmon (*Salmo salar*) of the Finnish Iijoki stock were hatched and reared in freshwater in a salmon hatchery on the Danish island of Bornholm in the Baltic sea. Salmon parr were divided in three groups each comprising 22 000 fish. One group was vaccinated by intraperitoneal injection with a non-mineral oil-adjuvanted vaccine consisting of formalin killed *Aeromonas salmonicida*, *Yersinia ruckeri* and *Vibrio anguillarum* (serotype O1 and O2). A second group was vaccinated by 1 h bathing in a corresponding vaccine without adjuvant. A third group was left as untreated control. Subsequently, presmolt groups were transferred to three identical net-pens located next to each other in the Baltic Sea (salinity 8 ppt), 500 m from the north-eastern coast of Bornholm, where they were studied for four months until tagging and release for restocking purposes. Mortality during this period in the ip vaccinated group was minimal (0.02%, RPS (relative per cent of survival) 99.80) and significantly lower compared to 10.13% mortality in the control group and 2.51% mortality (RPS 75.2) in the bath vaccinated group. Specific disease outbreaks were not observed during the four months. Growth was significantly enhanced in the injection vaccinated group compared to both the unvaccinated control and the bath vaccinated group. The humoral antibody response to the various bacteria was significantly elevated in the injection vaccinated group showing 4–5 fold titre increases three and four months after immunization. In contrast, no increase of titres was seen in the bath vaccinated and untreated groups.

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Marked cellular reactions in the abdominal cavity of injected fish were registered. A total of 3000 fish have been tagged and released to evaluate the effect of vaccination on the recapture rate. The implications of immunoprophylactic measures in the restocking programme with Baltic salmon are discussed. © 1997 Elsevier Science B.V.

1. Introduction

During the last five decades, hatchery reared salmon have constituted an increasingly important part of the Baltic salmon stock (Ackefors et al., 1991). Thus, it is estimated that only 10% of the stock is based on natural spawning in rivers with outlet to this largest brackish sea in the world. From an original annual smolt production of 10 million, this value is presently 400 000 smolts and the hatchery reared smolts amount to 4.3 million a year (Christensen, 1992). The decrease in natural reproduction is caused by the destruction of natural spawning grounds due to power plant constructions in the Swedish and Finnish rivers. In addition, pollution of important salmon rivers in other Baltic countries has eliminated salmon stocks. The release of smolts in the Baltic by Danish funds were formerly based on buying Swedish salmon and subsequent release in Danish and Swedish waters. During the latest years, a newly constructed salmon hatchery on the Danish island of Bornholm has hatched and reared Baltic salmon (Finnish Iijoki stock) to the smolt size and the present annual production reached 200 000 smolts in 1996. The stocking programme operates with 1 year old fish from the hatchery. These are transferred to net cages in the Baltic sea near the coast line of northeast Bornholm where they are kept for four months prior to release. In this way, they are imprinted on this locality and are expected to return to this area after the foraging period in the central Baltic (Glüsing and Rasmussen, 1996). As the salmon parr in the salmon hatchery are kept under strict hygienic conditions, it could be postulated that these smolts after release into a natural environment are more vulnerable to pathogens in the environment compared to the natural salmon stock or salmon from less hygienic production plants. Thus, several studies have documented the presence of a number of bacterial and parasitic pathogens in the Baltic (Larsen and Mellergaard, 1981; Dalsgaard, 1986; Buchmann, 1987, 1989; Buchmann et al., 1993; Dalsgaard et al., 1994). We have conducted an investigation to answer this question. The health conditions in the freshwater parr period in the hatchery and the smolt period in the netcages have been monitored. In addition, the possible prophylactic effect of two vaccination regimes have been tested. Following, the net cage period subsamples of the three groups have been tagged in order to evaluate later if the vaccination has an effect on recapture.

2. Materials and methods

2.1. Hatchery

The Salmon Hatchery is located in Nex, Bornholm. The rearing system is based on recirculation of tap water using biofilters, ozone-treatment and mechanical separation of

sludge-particles. Fish are kept in fibre-glass tanks with continuously monitored levels of oxygen and pH.

2.2. Net-pens

Three identical cylindrical net-pens (diameter 8 m, depth 5 m, mesh size 5 mm) were placed in the Baltic Sea on a position 500 m from the north-eastern coast of Bornholm near the fishing village of Tejn.

2.3. Fish

The experiments were conducted by using the Iijoki-stock of Baltic salmon (*Salmo salar*). Eyed eggs were imported in January 1995 from Finland and hatched on Bornholm. The salmon fry and parr were kept in the freshwater system until stocking in net-pens in April 1996. A total of 66 000 salmon were used: 22 000 were injection vaccinated (ip), 22 000 were bath vaccinated, and 22 000 were kept as untreated controls.

2.4. Growth

The mean body length and weight of the salmon parr at April 29 (stocking of net-pens) were 15 cm and 36 g, respectively. These values were obtained from large scale grading of fish in the fish tanks. At the end of the growth period (August 27 and 28) 50 fish from each net-pen were measured (Table 1).

2.5. Feed

Commercial pelleted trout feed (Aller Mølle, Denmark) was applied during the entire study period. Automatic feeders supplied feed which varied from 5–25 kg per net-pen per day in the first month, 20–25 kg per net-pen per day in June and July and to 25–45 kg per pen per day in August.

2.6. Temperature

The temperature in the hatchery was kept between 7 and 11°C through the entire freshwater period (continuous electronic recordings). The sea temperature was 3.5°C on

Table 1
Body length and weight of salmon smolts after 4 months in the net-pens[a]

	Number	Length (cm) mean (sd)	Weight (g) mean (sd)
Injection vaccinated	50	27.9 (1.9)	187.4 (46.4)
Bath vaccinated	50	24.6 (2.4)	124.1 (40.7)
Un-vaccinated control	50	25.8 (2.3)	143.2 (37.0)

[a] The length and weight of the injection vaccinated fish were significantly higher than in the other two groups ($p < 0.05$, t-test).
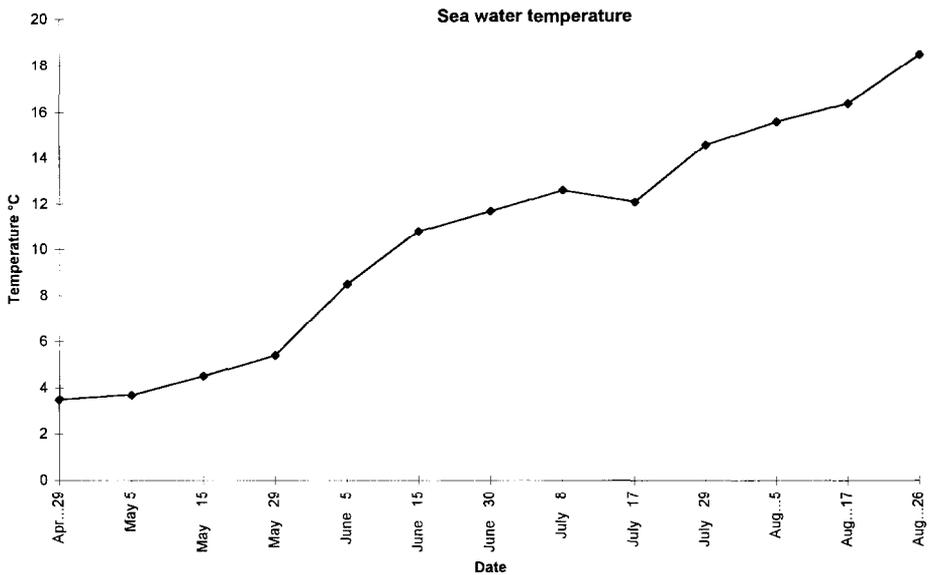
Fig. 1. Temperature recordings at the net-pen site during the investigation period.

April 29, 1996, when the salmon were transferred to the net-pens. Therefore, the water in the transport fish tanks was cooled by 1 h inflush of cold sea water to acclimatize the salmon before stocking the pens. The temperature on the net-pen location in the Baltic was recorded through the entire sea-period by the use of a hand thermometer (Fig. 1).

2.7. Salinity

The salinity in the surface water east of Bornholm is 8 ppt (brackish water) (Buchmann, 1994).

2.8. Vaccine

The injection vaccine used for vaccination of 22 000 salmon on March 27, 1996 was Aquavac™ Multivac E (Aquaculture Vaccines, Essex, UK), containing formalin-in-activated *Aeromonas salmonicida, Vibrio anguillarum* (serotype O1 and O2) and *Yersinia ruckeri* and a non-mineral oil adjuvant (Montanide ISA 7 11) (0.1 ml vaccine per fish). The bath vaccine used was Aquavac Triple (Immersion) containing the same pathogens except that *V. anguillarum* (serotype O2) was replaced by *Vibrio ordalii* possessing antigens cross-reacting with *V. anguillarum* serotype O2. Fish were immersed 1 h according to the manufacturer's recommendations (April 2, 1996). After vaccination, fish were kept until April 29, when they were transported in oxygenated fish tanks to the net-pens.

2.9. Mortality in net-pens

Daily, or (in windy periods) every few days, mortality was registered by emptying the dead fish collector at the bottom of the net-pen. The total mortality and the relative per

Table 2
Mortality and relative per cent of survival (RPS) in the three net-pens in the period from April 29 until August 28, 1996[a]

	Mortality (total number of dead fish)	Mortality (%)	RPS
Injection vaccinated salmon	5	0.02	99.8
Bath vaccinated salmon	551	2.51	75.2
Un-vaccinated control	2228	10.13	–

[a] Mortality of both injection vaccinated and bath vaccinated salmon was significantly lower than in un-vaccinated fish ($p < 0.05$, χ^2-test).

cent of survival (RPS) (1 − (vaccinate mortality/control mortality) × 100) (Ellis, 1988) were calculated (Table 2).

2.10. Bacteriology

2.10.1. Water

2.10.1.1. Freshwater. Monthly, water samples (1 l) were taken in sterile bottles from the fish tank in the hatchery. Total viable bacterial counts were determined on blood agar base (BA, Gibco) with 5% citrated calf blood after 10-fold dilutions and incubated at 20°C for 48 h and on tryptone yeast agar (TYA) after incubation at 15°C for 10 d. Colonies on TYA, suspected as *Flavobacterium psychrophilum*, were subcultured and identified (Dalsgaard, 1993).

2.10.1.2. Brackish water. The water samples (1 l) were taken in sterile bottles from the middle net-pen containing injection vaccinated fish. Total viable bacterial counts were determined on BA as above but in addition thiosulphate-citrate-bile salts-sucrose (TCBS) Agar (Difco) was used for detection of *Vibrio*-species (48 h at 20°C and 24 at 37°C).

Biochemical and serological tests of bacteria suspected as *V. anguillarum* were conducted according to Larsen (1983) and Sørensen and Larsen (1986).

2.10.2. Fish

A total of 15 parr were taken monthly from October 1995 until April 1996 from the freshwater rearing tanks. From the individual net-pens 10 smolts were taken monthly from May until August (30 fish per month).

Samples from kidney, spleen and brain were inoculated on BA (Gibco) with 5% citrated calf blood (20°C for 48 h), (TYA) (15°C for 10 d) and TCBS Agar (Difco) (20°C for 48 h).

Gills, skin and intestinal content of 3 fish from each net-pen were screened for bacteria (9 fish per month). Two gill arches (the second from each side) were excised and transferred to 10 ml physiological saline. Likewise, 2 cm^2 of skin was swabbed with a sterile cotton swab, which subsequently also was transferred to saline. In the same way, 2 cm of the rectum (cut 1 cm from the anal opening) was inoculated in saline. After whirlmixing, ten-fold dilutions were made and spread on BA and TCBS. Plates were incubated at 20°C for 48 h, whereafter colonies were counted.
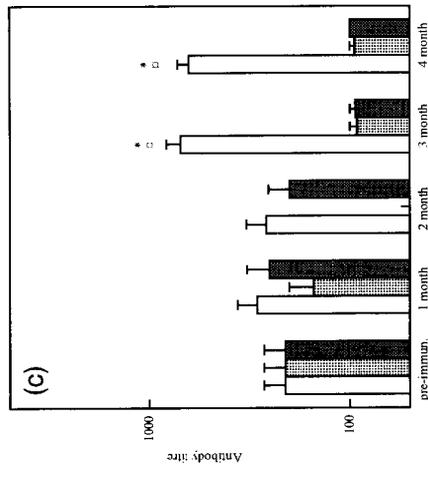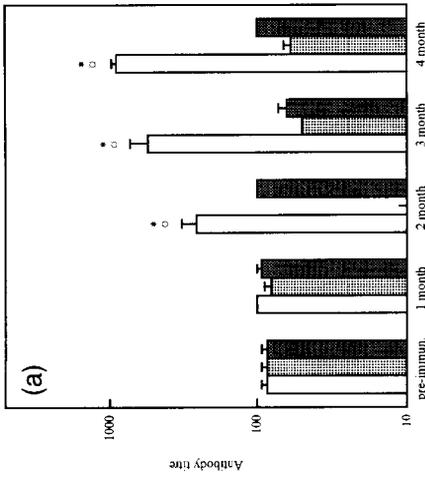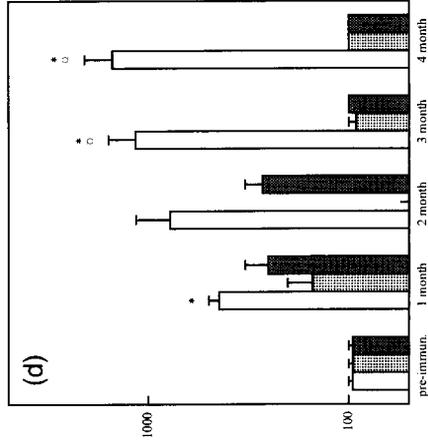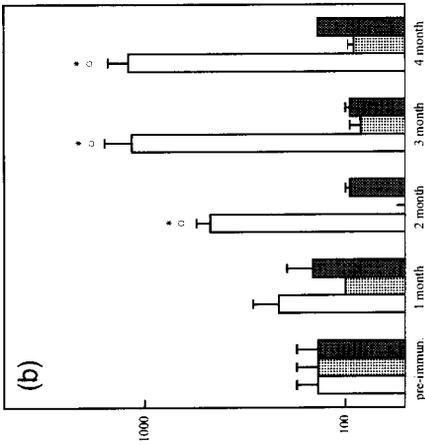
Colonies on TCBS, suspected as *V. anguillarum*, were subcultured and identified as described by Larsen (1990) and Aalbæk and Pedersen (1992). Serotyping and plasmid profiling (Pedersen et al., 1996) were then conducted as described above to characterize this species.

2.11. Parasitology

From October 1995, monthly samples of 10 fish from the hatchery and from May 1996 10 fish from each net-pen were taken and subjected to a full parasitological examination as described by Buchmann et al. (1995).

2.12. Virology

On two occasions, samples of 15 parr (freshwater period, December 1995) and 30 smolts (brackish water period, July 1996) were examined for virus (VHS, IPN, IHN) infections (Danish Veterinary Laboratory, Aarhus).

2.13. Blood sampling

Blood samples were drawn from the caudal vein from 10 fish on March 25 (two days pre-immunization), 3×10 fish (10 from each group) in April (1 month post-immunization, still freshwater) and subsequently, 10–14 blood samples were taken each month (May–August) from each of the net-pen groups. Fish were anaesthetized in 50 mg 1^{-1} benzocaine before blood sampling with heparinized syringes, blood was centrifuged at 5000 RPM for 6 min, whereafter plasma was recovered and frozen at $-20°C$.

2.14. Humoral immune response

An ELISA-procedure was established and all steps were performed at room temperature. Formalin-killed whole cells of each of the four pathogens *A. salmonicida, Y. ruckeri, V. anguillarum* serotype O1 and O2 were used as coating antigen. Nunc-96 wells immunoplates (Nunc, Roskilde, Denmark) were coated (1 h) with a 200 µl suspension of 1×10^8 cells of one of the bacterial species per ml in coating buffer (4.29 g $Na_2CO_3 \cdot 10H_2O$ and 2.93 $NaHCO_3$ in 1 l (pH 9.1)). Uncoated sites were blocked with 0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 h. Between each of the following steps, wells were rinsed by 3×5 min wash (PBS with

Fig. 2. Measurements of humoral response of salmon in the three groups before (pre-immun.) and after vaccination. (*): significant difference from pre-immun., (○): significant difference from bath vaccination and control groups. Pre-immun. refers to pre-immune fish in freshwater (two days before immunization). 1 month refers to 1 month post-immunization in freshwater. Month 2–4 refers to the net-pen period in the Baltic. White columns: Injection vaccinated, light-gray columns: bath-vaccinated, dark columns: un-vaccinated control. (a) Anti-*A. salmonicida* titres (mean and SEM); (b) Anti-*V. anguillarum* serotype O1 titres (mean and SEM); (c) Anti-*V. anguillarum* serotype O2 titres (mean and SEM); (d) Anti-*Y. ruckeri* titres (mean and SEM).

Table 3
Adhesions and macroscopic visible patological changes in the three groups

	April	May	June	July	August
Injection vaccinated	1	1	1	2–3	3–4
Bath vaccinated	0	0	0	0	0
Un-vaccinated control	0	0	0	0	0

0.05% Tween 20). Antigen-coated plates were incubated with salmon plasma (diluted 1:50, 1:100, 1:500, 1:1000, 1:4000 and 1:8000 in PBS) for 1 h, washed and incubated with rabbit anti-salmon Ig (1:1000) (Buchmann and Pedersen, 1994) for 1 h and finally incubated for 1 h with alkaline phosphatase conjugated goat anti-rabbit Ig (Sigma 3687) (1:1000). Plates were developed with p-nitrophenylphosphate (Sigma 2765) as substrate. The reaction was stopped by adding 50 μl 3 M NaOH to each well, whereafter OD was read on a Multiscan RC, Type 351 ELISA-reader (Labsystems, Finland) at 405 nm. Positive reaction was set at an OD of 2 × zero-sample (PBS) (Fig. 2a–d).

2.15. Intra-abdominal lesions

10 fish from each net-pen were examined monthly to register macroscopic pathological lesions in the abdominal cavity (Table 3). The lesions were ranked according to the following scale, which is slightly changed in relation to the ranking of Midtlyng et al. (1996):

0: No adhesions or visible pathological changes.

1: Few fibrin-strings present. Easy to loosen.

2: Several fibrin-strings present in a larger area, some well developed but easily loosened.

3: Marked adhesions between organs and abdominal wall. Easy to loosen.

4: Extensive adhesions in a large area with numerous fibrinous adhesions. Demand considerable efforts to loosen.

5: Organs in abdomen more or less assembled in a package. Difficult to loosen. Often combined with melanosis and hyperaemia.

6: As 5, but even more extensive. Open wounds are registered when the adhesions are loosened. Excessive melanin encrustment and hyperaemia.

2.16. Statistics

The Student's t-test was applied to evaluate differences of means (body size of fish and antibody titres). The χ^2-test was used to assess the differences in mortality between groups. Differences were accepted as significant when $p < 0.05$.

3. Results

3.1. Water analysis

3.1.1. Freshwater

Total viable bacterial counts in the freshwater fish tanks ranged from 5.1×10^2 to 4.6×10^5 colony forming units (cfu) ml^{-1} (BA) and from 0.9×10^2 to 5.5×10^5 cfu

ml^{-1} (TYA). During the seven months from October 1995 until April 1996, a total of 12 yellow pigmented Gram-negative strains unable to utilize carbohydrates and grow on blood agar but actively proteolytic were identified as *F. psychrophilum*.

3.1.2. Brackish water

No seasonal variation was detected in viable bacterial counts in brackish water (net-pen period in the sea). This parameter ranged from 3.4×10^2 to 7.3×10^4 cfu ml^{-1} (BA). The total *Vibrio* spp. counts (TCBS, 20°C for 48 h) varied from 0 in April to 27 cfu per 10 ml in August 28. Further identification showed that 14 of 17 *Vibrio*-isolates were *V. anguillarum* (serotypes O3, O7 and O9).

3.2. Fish analysis

3.2.1. Infections with bacteria

No infections with bacteria was detected in parr in the freshwater phase.

In July, *V. anguillarum* serotype O2A were isolated from 2 fish (injection vaccinated). In August, the same serotype and a non-typeable *V. anguillarum* were isolated from 2 bath vaccinated fish.

3.2.2. Bacteria from skin, gills and intestine

The mean bacterial counts (BA) varied from 4.1×10^2–1.1×10^5 cfu ml^{-1} (gills), 2.5×10^1–1.2×10^4 (mucus) and 1.5×10^1–2.4×10^4 (intestine) from the March sample to the July sample. TCBS-colonies were not recovered until July. These *Vibrio*-counts increased from July to August as 1.3×10^1 to 1.5×10^3 cfu ml^{-1} (gills), and as 2 to 6.1×10^2 cfu ml^{-1} (mucus). In the intestine, the count decreased from 5.4×10^3 (July) to 4.5×10^2 (August) cfu ml^{-1}. This was caused by a very high count $(5.4 \times 10^4$ cfu $ml^{-1})$ in one fish in July. The majority of the counts on TCBS were *V. anguillarum*. Serotyping of a number of strains showed a high diversity. Three strains belonged to serotype O1, but none of them carried the 67 kb virulence plasmid.

3.2.3. Parasites and virus

No parasites or virus were detected on any occasion throughout the investigation.

3.3. Humoral immune response

The antibody-titre did not increase in the un-injected control fish or the bath-vaccinated salmon. In contrast, the injection vaccinated fish showed a significant humoral immune response towards all the antigens (Fig. 2). One month post-immunization the anti-*Y. ruckeri* titres were already significantly increased and two months after vaccination the fish responded to *A. salmonicida* and *V. anguillarum* serotype O1. The response to *V. anguillarum* serotype O2 was weaker showing increased levels only after three months. For all pathogens, the humoral immune response reached its highest levels in July and August.

3.4. Growth

The injection vaccinated fish showed (when measured in August) a significantly ($p < 0.05$) higher body weight and length compared to both the bath immunized fish and the control group (Table 1).

3.5. Mortality

Salmon vaccinated by intraperitoneal injection experienced a very low mortality. Only 5 of 22 000 fish died in this group. This was significantly different from the other two groups. Especially the control group suffered from significant mortalities which also were higher than in the bath vaccinated group (Table 2).

3.6. Intra-abdominal lesions

No adhesions or pathological changes were recorded in control fish or bath vaccinated fish. In the injection vaccinated fish a slight response (class 1) was seen after one month. Similar changes occurred until June whereafter the lesions became more severe and increased (class 3–4) (Table 3) until tagging and release in August. Vaccine residuals (white drops) were still seen in the body cavity in August.

4. Discussion

The delayed release technique, applying accommodation of salmon smolt in net pens in specified coastal positions before final release, has been adopted as a valuable method to increase the stock of salmon in the Baltic Sea. The commercial fishery will hereby be concentrating on hatchery-reared fish in desired locations, saving wild salmon for natural spawning in the rivers. Environmentally sound measures to improve the survival of these fish are therefore highly desirable. Aquacultured rainbow trout in net-pens in the Baltic have suffered from vibriosis (Thorburn, 1987) and the causative organism *V. anguillarum* is widespread in this brackish water zone (Buchmann et al., 1993, Tiainen et al., 1994) representing a threat to marine fish farming. Vaccination of fish has been demonstrated to offer farmed salmonids a significant protection against several bacterial pathogens (Larsen, 1988, 1990; Lillehaug, 1990, 1991; Lillehaug et al., 1992; Press and Lillehaug, 1995). In the present investigation, we have demonstrated a significant increase of survival in the net-pen period after intra-peritoneal vaccination with a triple vaccine containing formalin killed *V. anguillarum* (serotype O1 and O2), *A. salmonicida* and *Y. ruckeri*. Bath vaccination using a vaccine containing comparable antigens improved the survival to a lesser, but still significant, extent. Whether these vaccination procedures also will result in higher recaptures of tagged salmon during the following three years will be investigated.

The investigations in the salmon hatchery showed that the salmon parr were reared under hygienic conditions. The freshwater organism *F. psychrophilum*, which is able to infect fish under certain conditions, was on only few occasions isolated from the water.

However, no infections in fish were detected and the rearing system evidently is operating with a low infection pressure. Thereby, the exposure of the fish immune system to pathogens is low. Whether this untriggered immune system represents a negative or positive characteristic when salmon are transferred to the marine environment is still not clear. In any case, it was previously demonstrated that fish from the same cohort were fully capable at the age of 6 months of mounting a humoral immune response against DNP–HSA (Nielsen and Buchmann, 1997).

It was clearly demonstrated that the marine environment contained *V. anguillarum*, a potential pathogen of marine fishes. Although a number of different serotypes were isolated from the surface and intestinal content of the net pen fish, the virulence plasmid (67 kb) were not found in any of the isolates. The presence of the plasmid in *V. anguillarum* serotype O1 makes this particular organism extremely infective (Larsen et al., 1994; Pedersen et al., 1996). If the virulent form of *V. anguillarum* serotype O1 had occurred a serious outbreak of vibriosis might have caused a much stronger difference between groups. However, the virulent form of this serotype was not isolated from any sample despite its prevalence in fish from the Baltic Sea (Tiainen et al., 1994).

The high survival of injection-vaccinated fish was associated with significant increases of antibody titres 1–2 months after vaccination. Neither control fish nor bath vaccinated fish exhibited any increase in specific antibody production. The ability of the Atlantic salmon (Norwegian strains) to produce specific antibodies against a number of injected bacterial antigens has been reported previously (Håvarstein et al., 1990; Bøgwald et al., 1991; Lund et al., 1991, 1995). However, studies on the humoral immune response of the isolated Baltic salmon stock are sparse (Nielsen and Buchmann, 1997). Therefore, the present study is relevant to the future management of the stocking experiments in the Baltic Sea.

No major disease outbreaks were recorded in the net-pens and the mortalities occurred continuously. Due to the practical design of the net-pen rearing, only live fish were examined and some infections in the dead fish may have been over-looked.

The absence of parasitic infections in these net-pen salmon in the studied area is interesting. It has been documented that salmonids in this locality often are infected with helminths (Buchmann, 1987, 1989). However, many of those are dependent on transfer by intermediate hosts as pulmonate snails, copepods or gammarids. As the salmon were located 500 m from the snail colonized rocky shore and were fed pelleted feed, the helminth infection pressure was minimized.

Although no viral or parasitic infections were found, neither during the freshwater phase nor during the brackish water period, it is likely that the general arousal of the immune system after vaccination will produce some non-specific protection. Thus, it was clearly seen that fish injected with the triple-vaccine produced macroscopic reactions in the abdominal cavity. The cellular infiltrations probably reflected macrophage and other cellular activity. However, whether this unspecific activation in turn increased the resistance of the fish to other pathogens is yet to be demonstrated.

Several studies have indicated a negative effect on weight gain following vaccination (Lillehaug et al., 1992) or merely injection (Nielsen and Buchmann, 1997). This could be explained by the growth depressing effect of cortisol, a hormone liberated for more than one month following fish stressing (Nielsen et al., 1994; Nielsen and Buchmann,

1997). The sterile peritonitis elicited by the oil-adjuvants could play a role in this connection as well, although Midtlyng et al. (1996) did not record any growth retardations in vaccinated fish with lesions. In the present investigation, the injection vaccinated fish showed a superior growth compared to both bath vaccinated and unvaccinated control fish. It could be hypothesized that the 4 month long sea period had normalized the cortisol levels in injected fish and that the cortisol production in bath vaccinated and control groups also rose during transportation and stocking in net-pens. This would level out the differences in cortisol concentrations between groups and if the health status further increased in the injection vaccinated fish, this would result eventually in better growth.

At the end of the net-pen period a total of 3000 salmon were tagged with Carlin-tags and released in the Baltic. The effect of vaccination on recapture will probably be known in 3 years as the main part of recaptured Baltic salmon are caught within such a time period following tagging (Glüsing and Rasmussen, 1996). An increase in recapture of bath-vaccinated steelheads was registered by Amend et al. (1980). The vaccination using a *V. anguillarum* bacterin resulted in a significantly higher return rate to a river in California after migration in the Pacific Ocean. The superior effect of the injection vaccine as compared to bath vaccine allows a hope for an even better result in the Baltic.

Acknowledgements

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