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Association Between Immunisation, Reduced Weight Gain and Plasma Cortisol Concentrations in Juvenile Baltic Salmon *(Salmo salar)*

By *M. Engelbrecht Nielsen*[1] and *K. Buchmann*[2]

[1]Marine Biological Laboratory, University of Copenhagen, Helsingør, and [2]Section of Fish Diseases, Department of Veterinary Microbiology, The Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

Engelbrecht Nielsen, M., and K. Buchmann: Association between immunisation, reduced weight gain and plasma cortisol concentrations in juvenile Baltic salmon (*Salmo salar*). Acta vet. scand. 1997, 38, 275-282. – The changes in plasma cortisol levels, immune response parameters and growth of juvenile Atlantic salmon (*Salmo salar*) were monitored during a 50 days period following a DNP-HSA (di-nitrophenyl human serum albumin) immunisation program. Antibody titers rose significantly after a single immunisation. An increased plasma cortisol concentration was observed in association with injection of both antigen and saline. A single injection had a significant negative effect on growth of fish and fish subjected to 2 injections with a 25 days interval had an even larger growth reduction. The plasma cortisol concentration and the specific antibody response were compared at an individual level but no correlation was found. Total serum protein increased during the experimental period independently of immunisation. In contrast the total serum immunoglobulin 50 days after the first immunisation was clearly connected to antigen exposure. The observations are discussed in relation to immunophysiological changes during immunisation and stress induction.

fish; immune response; stress; DNP-HSA.

Introduction

Production in teleosts of a specific antibody response following antigen exposure is well documented through numerous investigations (*Buchmann et al.* 1991 and 1992, *Woo* 1992, *Leiro et al.* 1993, *Kofod et al.* 1994, *Höglund & Pilström* 1995). The Atlantic salmon (*Salmo salar*) is no exception to this (*Håvarstein et al.* 1990, *Lund et al.* 1991, *Magnadottir & Gudmundsdottir* 1992) but only limited knowledge of these issues have been obtained from the Baltic race of this teleost species. Likewise, a wealth of work has been conducted on the physiological changes in fishes during stress and the subsequent elevation in plasma cortisol levels (*Schreck* 1982, *Pickering* 1990). *Lillehaug* (1991) suggested that reduced growth in injected salmon compared with non-injected salmon is connected to the release of cortisol due to stress induced by the injection although no evidence was presented in favour of this view. As very few attempts have been made to associate between reduced growth and the stress induced by injection/vaccination of fish we elucidate in this paper the connection between the humoral response of Atlantic salmon in relation to the plasma cortisol concentration following

an immunisation with HSA-DNP (Di-nitrophenyl human serum albumin).

Materials and methods

Fish

Juvenile 1+ parr Atlantic salmon, Baltic race from the Finnish river Iijoki, (n = 600, mean mass 38.4 ± 1.3 g; mean fork length 14.9 ± 0.2 cm) reared by Bornholms Salmon Hatchery. The salmon were distributed equally in 6 1000 l round indoor fibreglass tanks and acclimated for 1 week prior to the experiment. The fish were fed twice daily with commercial dry pelleted feed. The fish received 2% of their start body weight per day. Each tank was supplied with 200 l/h. Water temperature during the experimental period was within the range 12-16 °C and the photo period was 12L:12D.

Immunisation

At the start of the experiment fish from 2 of the tanks were immunised with 0.1 ml DNP-HSA (Sigma Chemicals, A-6661) at a concentration of 5 mg/ml, diluted in phosphate buffered saline pH 7.2 (PBS). Furthermore fish from 2 other tanks were injected with PBS pH 7.2 and used as controls. The fish from the 2 remaining tanks were non-injected controls. After 25 days half the groups were immunised again as described above.

Sampling Procedure

At each sampling (0 and 50 days and 0, 25, and 50 days for fish injected once and twice, respectively) 12 salmon from each tank were rapidly netted, anaesthetised and killed in an overdose of Benzocaine (Sigma Chemicals, E-1501). In this manner all fish were unconscious within 15 s. Blood was collected via the caudal vessel in heparinized syringes and dispensed into 1.5 ml micro centrifuge tubes. Netting and blood sampling of all fish were carried out within 10 minutes. After centrifugation (5 min×16,000 g) plasma was stored at –20 °C until further analysis.

Antibody titer determination using ELISA

Polystyrene microtitre plates (Sero-Well, UK.) were coated with 200 µl coating buffer (4.29 g $Na_2CO_3 \cdot 10H_2O$ and 2.93 g $NaHCO_3$ diluted to 1l with deionised water, pH 9.6) containing 0.02 mg of the antigen (DNP-HSA) pr ml for 12h at 4 °C. Unbound antigen was removed by 5 successive washings with a washing buffer (phosphate buffered saline, PBS pH 7.2 with 0.05% Tween-20) whereafter antigen-uncoated sites were blocked with 200 µl blocking buffer (PBS pH 7.2 containing 0.5% BSA and 0.01% sodium azide) for 15 min at room temperature. Between each of the following steps the wells were washed 5 times with washing buffer. From each plasma sample a standard series was made 1:64, 1:128, 1:256, 1:512, 1:1,024, 1:2,048, 1:4,096, 1:8,192 and 1:16,384 with dilution buffer (PBS pH 7.2 containing 0.1% bovine serum albumin and 0.01% sodium azide). In duplicates 200 µl of each concentration of the standard series was added to the coated wells. The microtitre plate was then incubated during gentle shaking for 2 h at room temperature. Hereafter 200 µl rabbit anti-salmon Ig (diluted 1:2000 in dilution buffer) was added to the wells and the plate was incubated with gentle shaking for 2 h at room temperature. Each well was then supplied with 200 µl goat anti-rabbit Ig alkaline phosphatase conjugate (Sigma Chemicals, A-3687) (diluted 1:2000 in dilution buffer) and incubated with gentle shaking for 90 min at room temperature. Finally 200 µl enzyme substrate (Sigma Chemicals, N-2765) was added to the wells which were incubated for 30 min at room temperature. The enzyme reaction was stopped by adding 50 µl of sodium hydroxide (3M). The resulting colour reaction was measured spectrophotometrically at 405

nm using a microplate reader (Multiscan RC, type 351, Labsystems, Finland).

Total immunoglobulin determination

The total concentration of immunoglobulin was determined using a modification of the above described ELISA method. The ELISA procedure was modified by first coating wells with a monoclonal antibody against *Rainbow trout* Ig (*Thurander et al.* 1990), then applicating plasma samples and finally using rabbit anti salmon Ig and phosphatase conjugated goat serum. Standard series and curve was produced from purified salmon Ig.

Cortisol assay

Plasma cortisol was measured by a commercial ELISA (BioChem ImmunoSystems GmbH, Freiburg, Germany). The limit of detection of the ELISA was 6.5 ng/ml. The intraassay and interassay coefficient of variation was 2.6-5.4% and 4.3-6.7% (min.-max.), respectively.

Protein assay

The concentration of protein in each sample was measured in triplicate by a commercial Coomassie Protein Assay (Pierce Chemical Company, U.S.A., No. 23200) according to the manufacturers recommendations.

Growth parameters

Fork length (cm) and wet weight (g), were measured at 0 and 50 days (fish injected once) and at 0, 25 and 50 days (fish injected twice) . Furthermore the condition factor ($100*w/l^3$) was calculated for all sampling times.

Statistical analysis

Student's *t* test was applied. Significant differences were accepted at a probability level of 0.05. Data are given as arithmetic means ± SEM.

Results

At day 0 all investigated parameters were at a similar level in all the 6 groups ($p>0.05$). During the experiment no mortality was observed.

The juvenile Baltic salmon immunised once with DNP-HSA experienced a significant increase of anti-DNP-HSA titers after 25 days. A second immunisation 25 days after the first did not improve the response significantly. However, also salmon merely injected with saline responded although the titers was significantly lower than in the DNP-HSA immunised fish. Non-injected control fish exhibited no titer increase (Fig. 1).

The total serum protein concentration rose significantly in all groups from day 0 to day 50 in the experiment (Fig. 2).

In contrast, the total immunoglobulin concentration did not increase during the first 25 days and only in the injected groups the IgM concentration increased 50 days post immunisation (Fig. 3).

The plasma cortisol level in the juvenile salmonids was significantly elevated 25 days after the first injections both with injection of antigen and with saline. The second injection increased the plasma cortisol level significantly in the fish subjected to 2 injections compared with the fish subjected to one injection. Those subjected to one injection did not show any significant elevation in plasma cortisol level after 50 days. (Fig. 4). The effect of sampling on plasma cortisol level was tested with a regression analysis. Residuals from this analysis were plotted against sampling sequence of individual (1 to 12) and there was no indication of a trend in the residual plots that could imply a sampling sequence effect (data not shown).

Growth parameters. The 3 groups measured at 25 days showed a significant increase in weight from 0 to 25 days with a tendency towards a larger weight increase in the non-injected control group compared to the groups injected with

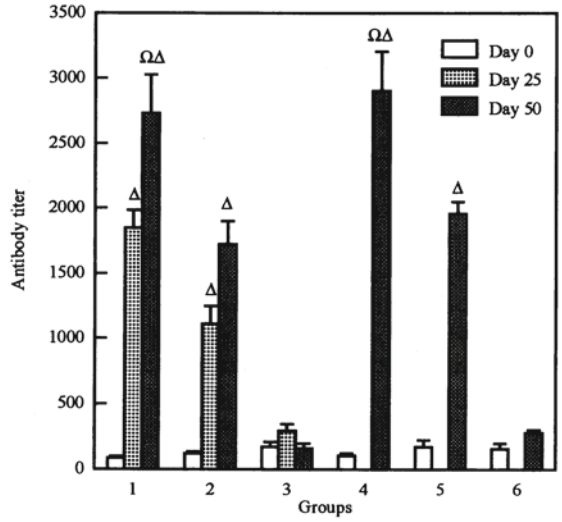

Figure 1. Antibody titer (mean ± SEM); 1 = DNP-HSA injected twice; 2 = PBS injected twice; 3 = Non-injected controls for group 1 and 2; 4 = DNP-HSA injected once; 5 = PBS injected once and 6 = non-injected controls for group 5 and 6; (Δ) = $p<0.05$ (Different from day 0); (Ω) = $p<0.05$ (Different from day 25); (n =12) for all groups.

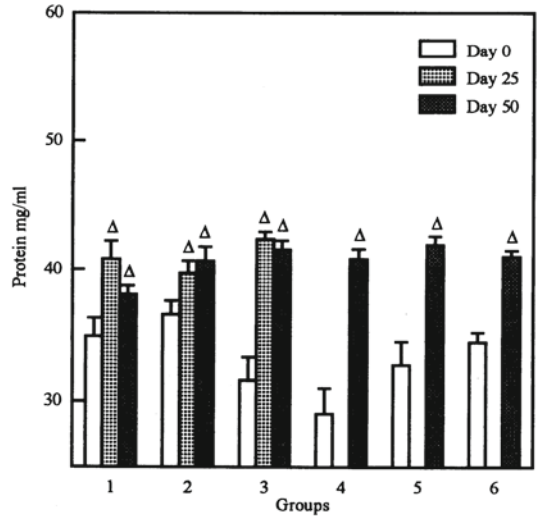

Figure 2. Total protein concentration (mean ± SEM); 1 = DNP-HSA injected twice; 2 = PBS injected twice; Non-injected controls for group 1 and 2; 4 = DNP-HSA injected once; 5 = PBS injected once and 6 = non-injected controls for group 5 and 6; (Δ) = $p<0.05$ (Different from day 0); (n =12) for all groups.

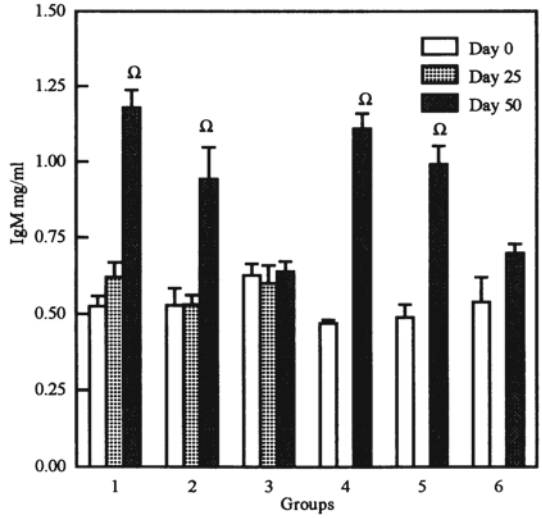

Figure 3. Total IgM concentration (mean ± SEM); 1 = DNP-HSA injected twice; 2 = PBS injected twice; Non-injected controls for group 1 and 2; 4 = DNP-HSA injected once; 5 = PBS injected once and 6 = non-injected controls for group 5 and 6; (Ω) = $p<0.05$ (Different from day 0); (n =12) for all groups.

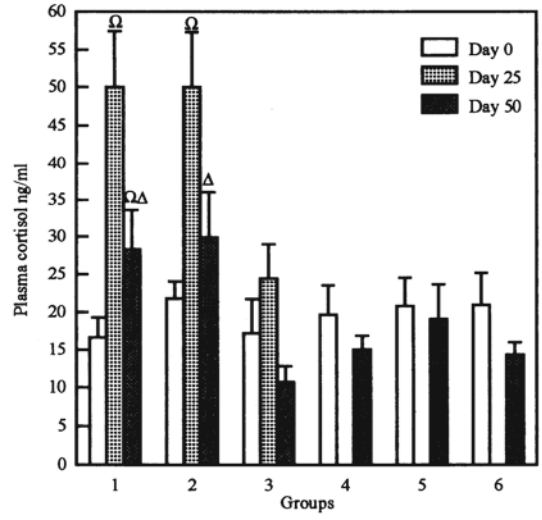

Figure 4. Total plasma cortisol concentration (mean ± SEM); 1 = DNP-HSA injected twice; 2 = PBS injected twice; Non-injected controls for group 1 and 2; 4 = DNP-HSA injected once; 5 = PBS injected once and 6 = non-injected controls for group 5 and 6; (Ω) = $p<0.05$ (Different from day 0 and 50); (Δ) = $p<0.05$ (Different from day 50 in the fish subjected to one injection); (n =12) for all groups.

Table 1. Growth parameters from fish immunised twice. (*) $p<0.05$ (Different from values at 0 days); (Ω) $p<0.05$ (Different from both DNP and PBS at 50 days) (n = 12).

	0 days			25 days			50 days		
2 Injection	DNP	PBS	Con.	DNP	PBS	Con.	DNP	PBS	Con.
Length (cm)	14.79 ± 0.3	15.00 ± 0.2	15.00 ± 0.3	16.21 ± 0.3 *	16.46 ± 0.2 *	16.13 ± 0.3 *	16.25 ± 0.4 *	16.00 ± 0.4 *	17.17 ± 0.5 *Ω
Weight (g)	35.43 ± 1.4	37.04 ± 1.0	37.18 ± 1.4	48.90 ± 1.7 *	47.04 ± 1.6 -*	50.08 ± 1.4 *	48.75 ± 2.7 *	46.44 ± 2.7 *	55.40 ± 2.8 *Ω
Cond.fact. (w/l^3*100)	1.1 ± 0.05	1.1 ± 0.02	1.1 ± 0.03	1.2 ± 0.03	1.1 ± 0.03	1.2 ± 0.04	1.1 ± 0.04	1.1 ± 0.02	1.0 ± 0.03

DNP-HSA and PBS (Table 1). All 6 groups experienced a significant increase in body weight from day 0 to day 50, although the injected group exhibited an inferior weight gain.

Discussion

Juvenile Baltic salmon is able to mount a marked humoral response against the DNP-HSA antigen even when the antigen is injected without adjuvans. The capability of Atlantic salmon to mount an antibody production has been demonstrated by numerous authors previously (*Magnadottir & Gudmundsdottir* 1992, *Lund et al.* 1991, *Håvarstein et al.* 1990). It is noteworthy that fish injected with saline alone showed an increase in anti DNP-HSA titers, this could be explained by the occurrence of natural antibodies against DNP which is well documented from vertebrates including fish (*Marchalonis & Warr* 1978). The manipulation and injection of salmon is likely to arouse the immune system of the salmon non-specifically and the subsequent immunoglobulin production seems to involve the production of antibodies with DNP-specificity.

The titer increase was also correlated partly to the rise in the concentration of total immunoglobulin, which however was highest 50 days post immunisation. In addition, the antigen exposed fish contained more immunoglobulin, a fact stressing the importance of the humoral immunity in salmon. Although this ability to produce specific immunoglobulin in fish following antigen injection is of immense importance in aquaculture it should be noted that the manipulation and injection retards growth significantly. This reduction is correlated to the cortisol production after handling and is at least partly connected to the growth depressing effect of this hormone (*Pickering* 1990). A decreased growth as a side effect to injection of fish is previously seen in the Atlantic salmon

Table 2. Growth parameters from fish immunised once. (*) $p<0.05$ (Different from values at 0 days); (Ω) $p<0.05$ (Different from both DNP and PBS at 50 days) (n=12).

	0 days			50 days		
1 Injection	DNP	PBS	Con.	DNP	PBS	Con.
Length (cm)	14.63 ± 0.4	14.88 ± 0.2	14.83 ± 0.2	17.17 ± 0.3 *	17.00 ± 0.4 *	17.25 ± 0.4 *
Weight (g)	39.30 ± 1.5	36.04 ± 1.0	35.18 ± 1.7	55.68 ± 1.9 *	54.92 ± 2.7 *	57.82 ± 3.0 *Ω
Cond.fact. (w/l^3*100)	1.2 ± 0.06	1.2 ± 0.05	1.2 ± 0.04	1.1 ± 0.03	1.1 ± 0.05	1.2 ± 0.02

(*Lillehaug* 1991) with growth reductions as high as 4.4%. The explanation for this growth depression was suggested to be the catabolic effects of steroid hormones as cortisol. However, no experimental evidence has not uptil now been presented to support this view.This suggestion is well in accordance with the results of the present study where injections twice elevate the plasma cortisol level in the fish. The low cortisol responce 50 days post injection in fish subjected to only one injection compared to fish injected twice should probably be explained by the long period of recovery after injection.

Despite the reduced growth in injected fish all groups showed significantly elevated plasma protein concentrations from start to end of the experiment. This could be ascribed to the protein rich diet available.

Cortisol is known to affect the immunity of vertebrates including salmonid fishes (*Chilomonczyk* 1982, *Pickering* 1984, *Maule et al.* 1989, *Carlson* 1993), and a correlation between plasma cortisol levels and the measured humoral immune response in individual fishes was to be expected but was not found. This is in accordance with *Nichols & Weisbart* (1984) who found very large individual variation in plasma cortisol levels in higher vertebrates as well as teleosts. Thus, it is known that plasma cortisol levels in fishes fluctuate faster and more extensively (*Mazeaud & Mazeaud* 1981) than the prolonged activation of β-lymphocytes in the humoral immune response proces. Therefore, no clear cut correlation between plasma cortisol levels and titer increase could be found in individual fishes.

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Sammendrag

Sammenhæng mellem immunisering, vækst og cortisol i Østersølaksen (Salmo salar).

Ændringer i plasmacortisolniveauer, immuntitre og vækstparametre, som følge af en immunisering med DNP-HSA (di-nitrophenyl human serum albumin), blev målt gennem en 50 dages periode. Antistoftitre var signifikant forhøjede efter en enkelt immunisering. Plasmacortisol niveauet steg signifikant som følge af injektion med enten antigen eller saltvand. En enkelt injektion havde en negativ indflydelse på væksten og 2 injektioner med 25 dages interval havde en yderligere negativ effekt på væksten. Plasmacortisolresponset og antistoftitre blev sammenlignet på individ niveau, men ingen korrelation blev fundet. Den totale mængde af serumprotein steg gennem hele eksperimentet i alle forsøgsgrupper. Den totale mængde af immunglobulin i serum havde derimod efter 50 dage en klar sammenhæng med præsentationen af antigen. De fundne resultater er diskuteret ud fra immunofysiologiske ændringer under en immunisering og den efterfølgende stresssituation.

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Reprints may be obtained from: M. Engelbrecht Nielsen, Marine Biological Laboratory, University of Copenhagen, Denmark. E-mail: engelbrecht@inet.uni-c.dk, fax: +45 49 26 11 65, tel: +45 49 21 16 33 - 321.