

ASSOCIATION BETWEEN IMMUNISATION AND PLASMA CORTISOL
IN JUVENILE BALTIC SALMON (*SALMO SALAR*).

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Abstract

The changes in plasma cortisol levels, immune response parameters and body size of juvenile Atlantic salmon (*Salmo salar*) were monitored during a 50 days period following a DNP-HSA (Di-nitrophenyl human serum albumin) immunisation programme. Antibody titers rose significantly after a single immunisation. An increased plasma cortisol concentration was observed in association with injection of both antigen and saline. Injection had a significant negative effect on growth of fish. The fish subjected to two injections with a 25 days interval had an even larger growth reduction. The plasma cortisol concentration and the specific antibody responses 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 immuno physiological changes during immunisation and stress induction.

Introduction

Production in teleosts of a specific antibody response following antigen exposure is well documented through numerous investigations (Leiro *et al.* 1993; Buchmann *et al.* 1991; Höglund and Pilström 1995). The Atlantic salmon (*Salmo salar*) is no exception to this (Lund *et al.* 1991; Håvarstein *et al.* 1990) 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 (Pickering 1990; Schreck 1982). However, very few attempts have been made to associate between these parameters with growth and the stress induced by injection/vaccination of fish. In this paper we elucidate the connection between the humoral response of Atlantic salmon in relation to the plasma cortisol concentration as a reaction to an immunisation with HSA-DNP (Di-nitrophenyl human serum albumin).

Materials and methods

Fish. Juvenile 1+ parr Atlantic salmon, Baltic race from the Finish river Iioki, ($n = 600$, mean mass 38.4 ± 1.3 g; mean fork length 14.9 ± 0.2 cm) reared Bornholms Lakseklækkeri. The salmon were distributed equally in six 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 $200 \text{ L} \cdot \text{h}^{-1}$. Water temperature during the experimental period was within the range $12\text{-}16^\circ\text{C}$ and the photo period was 12L : 12D.

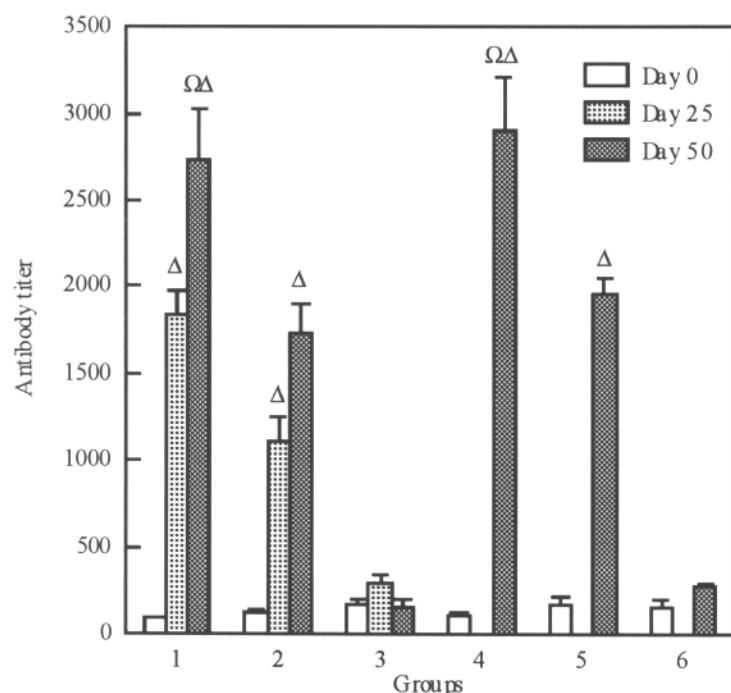


Figure 1. Antibody titer (mean \pm SEM); 1 = DNP-HSA injected twice; 2 = PBS injected twice; 3 = Non-injected controls for group 1 and 2; 4 = DNP-HSA injected once; 5 = PBS injected once and 6 = non-injected controls for group 5 and 6; (Δ) = $P < 0.05$ (Different from day 0); (Ω) = $P < 0.05$ (Different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

Immunisation. At the start of the experiment fish from two 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 two other tanks were injected with PBS pH 7.2 and used as controls. The fish from the two remaining tanks were non-injected controls. After 25 days half the groups were immunised again as described above.

Sampling Procedure. At each sampling (0, 25 and 50 days for group 1, 2 and 3 and 0 and 50 days for group 4, 5 and 6) 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 \times 16,000 g) plasma was stored at -20°C for further analysis.

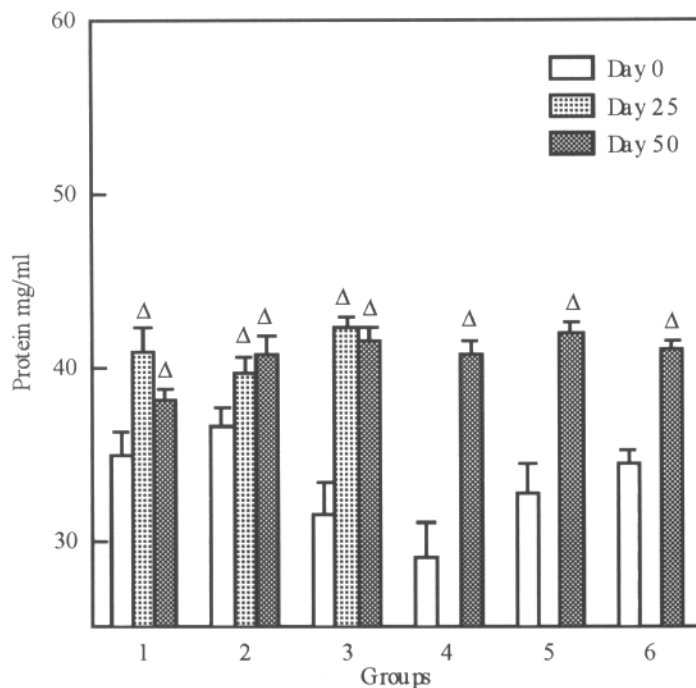


Figure 2. Total protein concentration (mean \pm SEM); 1 = DNP-HSA injected twice; 2 = PBS injected twice; Non-injected controls for group 1 and 2; 4 = DNP-HSA injected once; 5 = PBS injected once and 6 = non-injected controls for group 5 and 6; (Δ) = $P < 0.05$ (Different from day 0); (n = 12) for all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

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⁻¹. The intraassay and interassay coefficient of variation were 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 Commassie Protein Assay (Pierce Chemical Company, U.S.A.) according to the manufacturers recommendations.

Growth parameters. Fork length and wet weight were measured at 0, 25 and 50 days in group 1, 2 and 3 and at 0 and 50 days in group 4, 5 and 6. Furthermore the condition factor 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 \pm SE.

Antibody titer determination (ELISA). Polystyrene microtitre plates (Sero-Wel, UK.) were coated containing 0.02 mg of the antigen (DNP-HSA). Secondary antibody rabbit anti-salmon Ig. Tertiary antibody goat anti-rabbit Ig alkaline phosphatase conjugate (Sigma Chemicals, A-3687). Enzyme substrate (Sigma Chemicals, N-2765). Colour reactions 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 ELISA. Although with the following differences: The wells were coated with monoclonal antibodies against immunoglobulin from salmon and a standard series was made from purified immunoglobulin from Salmon.

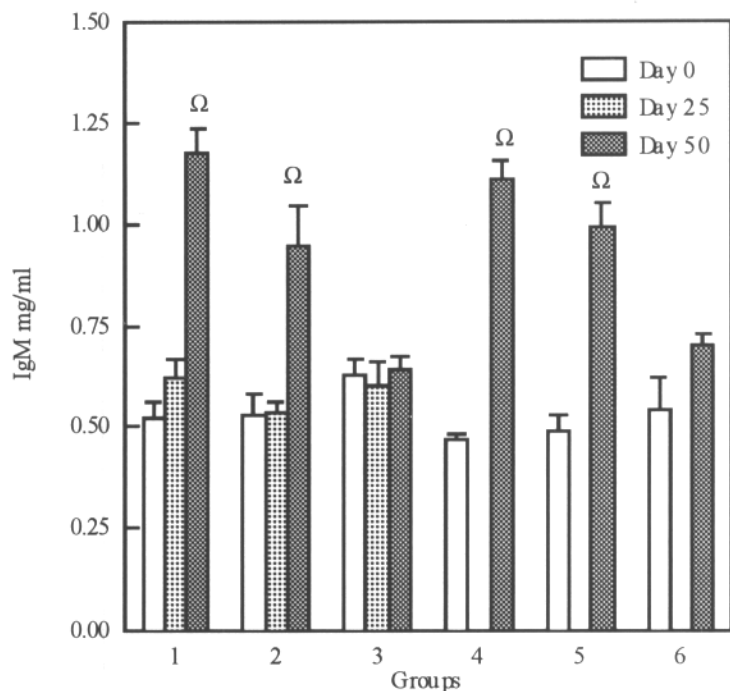


Figure 3. Total IgM concentration (mean \pm SEM); 1 = DNP-HSA injected twice; 2 = PBS injected twice; 3 = Non-injected controls for group 1 and 2; 4 = DNP-HSA injected once; 5 = PBS injected once and 6 = non-injected controls for group 5 and 6; (Ω) = $P < 0.05$ (Different from day 0); (n = 12) for all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

Results

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

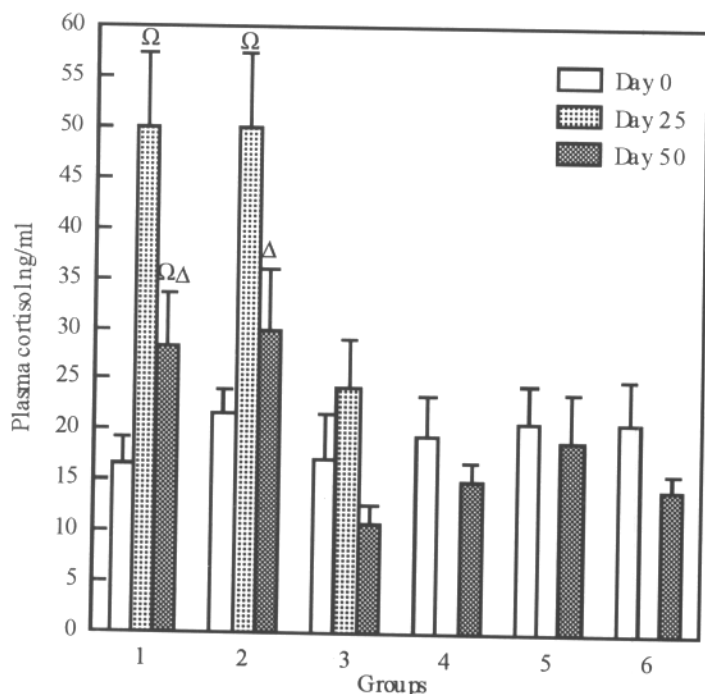


Figure 4. Total plasma cortisol concentration (mean \pm SEM); 1 = DNP-HSA injected twice; 2 = PBS injected twice; 3 = Non-injected controls for group 1 and 2; 4 = DNP-HSA injected once; 5 = PBS injected once and 6 = non-injected controls for group 5 and 6; (Ω) = $P<0.05$ (Different from day 0 and 50); (Δ) = $P<0.05$ (Different from day 50 in the fish subjected to one injection); (n=12) for all groups.

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 two injections compared with the fish subjected to one injected at 50 days. The fish 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 individuals (1 to 12) and there was no indication of a trend in the residual plots that could imply a sampling sequence effect.

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 were 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 increase did not experience an elevation during the first 25 days. The IgM concentration was significantly increased 50 days post immunisation compared with day 0 and 25 in all injected groups. (Fig. 3).

The plasma cortisol level in the juvenile salmonids increased significantly 25

	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	
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	

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

1 Injection	0 days			50 days		
	DNP	PBS	Con.	DNP	PBS	Con.
Length (cm)	14.63 ±0.4	14.88 ±0.2	14.83 ±0.2	17.17 ±0.3 *Ω	17.00 ±0.4 *Ω	17.25 ±0.4 *
Weight (g)	39.30 ±1.5	36.04 ±1.0	35.18 ±1.7	55.68 ±1.9 *Ω	54.92 ±2.7 *Ω	57.82 ±3.0 *
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

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

Growth parameters. Three groups showed a significant increase in weight from 0 to 25 day with a tendency towards a larger increase in weight in the non-injected control group compared to the groups injected with DNP-HSA and PBS (Table 1). All six groups experienced a significant increase in body weight from day 0 to day 50. At day 50 the non-injected control fish showed a significantly larger weight gain compared with the injected fish.

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 (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. Occurrence of natural antibodies against DNP is well documented from vertebrates including fish (Marchalonis and 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 specific immunity in salmon. Although this potential ability to produce specific immunoglobulin in fish following antigen injection is of importance in aqua culture it should be noted that the manipulation and injection retards growth significantly. This reduction is correlated to the cortisol production after handling and is probably connected to the growth depressing effect of this hormone (Pickering 1990).

Cortisol is known to affect the immunity of vertebrates including salmonids fishes (Carlson 1993; Maule *et al.* 1989; Pickering 1984; Chilomonczyk 1982), and a correlation between plasma cortisol levels and the measured humoral immune response was to be expected but was not found. This is in accordance with Nichols and Weisbart (1983) who found very large individual variation in plasma cortisol levels in higher vertebrates as well as teleosts. This individual variation might be the explanation for the lack of correlation between cortisol and the observed humoral immune response

References

- Buchmann, K, Pedersen, LØ, and Glamann, LØ 1991 Humoral immune response of European eel, *Anguilla anguilla*, to a major antigen in *Anguilla crassus* (Nematoda). *Diseases of Aquatic Organisms* 12, 55-57 p.
- Carlson, RE, Anderson, DP and Bodammer, JE 1993 *In vivo* administration suppresses the *in vitro* primary immune response of winter flounder lymphocytes *Fish and Shellfish Immunology*, 3, 299-312 p.
- Chilomonczyk, S 1982 Rainbow trout lymphoid organs: cellular effects of corticosteroids and anti-thymocyte serum. *Developmental and comparative immunology* 6, 271-280 p.

- Höglund, J and Pilström, L 1995 Mechanical isolation and characterization of antigens from adult *Anguillicila crassus*. *Fish and Shellfish Immunology* **5**, 51-60 p.
- Håvarstein, LS, Endersen, C, Hjeltnes, B, Christie, KE and Glette, J 1990 Specific immunoglobulins in serum from Atlantic salmon, *Salmo salar* L., immunised with *Vibrio salmognicida* and infectious pancreatic necrosis virus. *Journal of Fish Diseases*, **13**, 101-111 p.
- Leiro, J, Estévez, J, Santamarina, MT and Ubeira, FM 1993 Humoral immune response of turbot, *Scophthalmus maximus* (L.), to antigens from *Tetramicra brevifilum* Matthews & Matthews, 1980 (Microspora). *Journal of Fish Diseases* **16**, 557-584 p.
- Lund, V, Jørgensen, T, Holm, KO and Eggset, G 1991 Humoral immune response in Atlantic salmon, *Salmo salar* L., to cellular and extracellular antigens of *Areomonas salmonicida*. *Journal of Fish Diseases* **14**, 443-452 p.
- Marchalonis, JJ and Warr, GW 1978 Phylogenetic origins of immune recognition: naturally occurring DNP-binding molecules in chordates sera and hemolymph. *Developmental comparative immunology*, **2**, 443-460 p.
- Maule, AG, Tripp, RA, Kaattari, SL and Schreck, CB 1989 Stress alters immune function and disease resistance in Chinook salmon, *Oncorhynchus tshawytscha*. *Journal Endocrinology* **120**, 135-142 p.
- Pickering, AD 1984 Cortisol induced lymphocytopenia in brown trout, *Salmo trutta* L. *General and Comparative Endocrinology* **48**, 269-274 p.
- Pickering, AD 1990 Stress and the suppression of somatic growth in teleost fish. *Progress in Comparative Endocrinology* 473-471 p. Wiley-Liss, Inc.
- Schreck CB 1982 Stress and Rearing of Salmonides. *Aquaculture*, **28**, 241-249 p.