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The effect of sea bream (*Sparus aurata*) broodstock and larval vaccination on the susceptibility by *Photobacterium damsela* subsp. *piscicida* and on the humoral immune parameters

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Abstract

Sea bream broodstock were immunised 1 or 2 months before spawning with a novel photobacteriosis vaccine. Sixty-seven-day-old larvae (mean weight 22.3 mg) originating from immunised and non-immunised parents were experimentally infected with the *Photobacterium damsela* subsp. *piscicida* (*Phdp*). Larvae from immunised fish showed delayed onset and lower mortality (66.67%) compared with larvae from control fish (80%). Eighty-nine-day-old larvae (mean weight 162.2 mg) from both groups were bath vaccinated with *Phdp* and *Escherichia coli* lipopolysaccharides (LPS) and larval samples were collected for measurement of humoral parameters. Larvae vaccinated with *Phdp* and LPS showed significantly higher anti-protease activity, lysozyme activity and total immunoglobulin compared to the controls. One-hundred-and-twenty-day-old larvae (mean weight 297.85 mg) from both parental groups were challenged with (LD_{70}) virulent *Phdp* bacterial cells. Vaccinated larvae from both groups showed significantly less mortality compared to the respective controls. The RPS values of larvae from immunised parents vaccinated with *Phdp* and LPS was 95.83% and 72.22%, respectively. The RPS values of larvae from non-immunised parents vaccinated with *Phdp* and LPS was 62.5% and 70.83%, respectively. Results are discussed with respect to the beneficial effect of broodstock immunisation prior to spawning and the immunisation of larvae on their survival against photobacteriosis.

Keywords: Sea bream larvae; Vaccination; Photobacteriosis

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1. Introduction

Photobacteriosis, caused by *Photobacterium damsela* subsp. *piscicida* (*Phdp*) is a disease causing heavy losses in the sea bream industry. The disease mainly affects larvae causing mortality of 50–100%, but it can also affect juveniles with mortality of up to 50% [1].

Throughout the last 20 years, there have been a variety of studies analysing the effectiveness of immunisation in preventing photobacteriosis [2]. Although some protection was achieved with vaccine preparations during the 1980s in Japan, the results were not reproducible. In Europe, it was reported [3] that better protection against photobacteriosis was obtained with an ECP-enriched bacterin, achieving relative percent survival (RPS) values higher than 75% when vaccination of fish occurred between 0.5 and 2 g. Further studies demonstrated that vaccination at the larval stage (50-day-old fish) is also effective, with RPS values of 84–90% [4].

Phdp cells grown in novel media and ECPs were used for bath, oral and intraperitoneal vaccination of sea bass and good protection (RPS 90–100%) was achieved [5,6]. Significant increase of the survival in Atlantic halibut (*Hippoglossus hippoglossus* L.) larvae was reported after exposure to 50 and 100 μg ml^{-1} of lipopolysaccharide (LPS) from day 20 to 96 and throughout the yolk sac period as compared to untreated larvae [7].

However, vaccination of fish at too young an age may give an undesired effect of immunosuppression because major lymphoid organs like kidney, spleen and thymus are not developed completely [8]. So the right age for vaccination and the immunisation route have to be estimated for different fish species like sea bream. It is reported that sea bream larvae may not be immunocompetent earlier than 77 days of age and vaccination at too early an age could create problems [8].

This study presents data on the humoral immune response in juvenile sea bream. The effects of a new *Phdp* vaccine formulation and LPS were studied on sea bream larvae derived from immunised and non-immunised parents. The measured parameters included some innate and acquired humoral responses, protection against live pathogen, and fish growth.

2. Materials and methods

2.1. Bacteria

A *Phdp* isolate (Sk7) isolated from Greece during a natural photobacteriosis outbreak in sea bream in the summer of 1999 was used throughout this study. The primary isolate was kept at −70 °C on cryobeads (Protect, England). For the initiation of a culture a microbead was added directly to a small volume of growth medium.

2.2. Media and culture conditions

The following materials were used for the preparation of culture media: brain heart infusion broth (BHIB), yeast extract (Oxoid), bacto-peptone and fish-peptone (Difco), ethylene diamino diacetic acid (EDDA), glucose, NaCl, $CaCl_2$, KH_2PO_4, $FeSO_4$, $MgSO_4$ and $MnSO_4$ (Sigma). The bacteriological media combinations that were used for culturing the pathogen were the following: BHIB + 2% NaCl, YPE + O. salts, YPE + O. salts + 0.15 mM EDDA + 2% glucose and YFP + O. salts + 0.15 mM EDDA + 2% glucose. The latter media formulations are described elsewhere [9]. The culture, harvest and processing of *Phdp* cells was performed as previously described [5].

2.3. Broodstock vaccination and conditions

Forty fish were divided into two groups, having 5 males and 15 females in each group (mean weight 2 kg). Fish were anaesthetised in a solution (0.01%) of 2-phenoxyethanol (Merck) and were injected intraperitoneally (i.p.) with a dose of 1.0 ml of formalin (1%) inactivated *Phdp* SK7 (10^8 cells ml^{-1}) grown in YPE+O. salts+EDDA+glucose, 2× YPE+O. salts and 2× YFP+O. salts+EDDA+glucose (bacterial cells were cultured separately in each medium and then suspensions, prepared as described above, were mixed in equal volumes). Control fish were i.p. immunised with 1.0 ml sterile phosphate buffered saline (PBS, 0.1 M, pH 7.2). Fish were vaccinated twice on 31 July and 29 August. Immunised and control (non-immunised) fish were kept in separate tanks and supplied with conditioned sea water, at the hatchery facilities of Selonda SA, Epidavros, Greece.

2.4. Larvae rearing conditions

After hatching larvae were reared in Selonda's hatchery facilities. The completion of yolk sac absorption was achieved very early at day 10 and first feeding with live *Artemia nauplii* began at day 5. Feeding on dry pellets alone was started at day 40. After weaning they were transferred to our facilities at day 52 of age.

2.5. Aquaria systems

Larvae were kept in a re-circulated, UV-treated (2× 55 W UV-C lamps) marine water tank system. The water temperature was maintained at 12–17 °C, with salinity at 35‰, oxygen 5 ppm and pH 7.9. The water was aerated with the aid of an air pump with capacity of 1 m^3 h^{-1}. Each tank had 700 l holding capacity, the total capacity of the system was 8 m^3 and the water exchange occurred every 1 h. The levels of ammonia, nitrites and nitrates were monitored daily. Larvae were fed four times per day with pellets from a commercial feed at 1% of body weight.

Vaccinated larvae were kept in 24×5 l re-circulating seawater bucket system consisting of a 1 m^3 h^{-1} pump (having a canister filled with sand-activated carbon sieving material) which elevated the water from a 0.5 m^3 sum tank through a 15 W UV-C unit to the bucket system.

Experimentally infected larvae were kept in a static water aquaria system and fed four times a day. All other conditions were as described previously.

2.6. Collection of larvae after vaccination and preparation of homogenates

Every third day post-larvae vaccination (see below), two larvae from each group were collected for analysis of body weight, total proteins, anti-proteases activity, lysozyme activity, total immunoglobulins, specific antibody titre (against *Phdp* and *E. coli* LPS), and lectins. Larval homogenates were prepared as described previously [9].

2.7. Calculation of the LD_{70} dose and susceptibility tests

A virulent bath challenge was used to evaluate differences in protective immunity in larvae from immunised and non-immunised mothers when larvae were 57 days old. *Phdp* SK7 cells prepared in YFP+O. salts+EDDA+glucose were finally suspended at OD 1 (10^8 cells ml^{-1}, calculated after plate count assays) at 610 nm in sterile sea water. Infectivity trials of larvae originating from non-immunised (NIM) and immunised (IM) brood fish groups were carried out with different concentrations of bacteria. Triplicate groups of larvae were immersion infected for 1 h with dilutions of 10^6, 10^4 and 10^2 bacterial cells ml^{-1} (Groups A, B and C) in sea water. Non-infected control (NIC) larvae group (Group D) received

exactly the same treatment with sterile sea water. Thirty larvae were used in each sub group. Mortalities were monitored for a period of 10 days and fish were tested with immunohistochemistry using specific anti-*Phdp* monoclonal antibodies [10] to confirm the cause of morbidity. An LD_{70} dose was calculated on the basis of mortality in all groups.

The above experiment was repeated in 67-day-old larvae in respect of its conditions using only the LD_{70} dose on triplicate groups of larvae originating from both parental groups. The cumulative mortalities were recorded for 10 days and the final cumulative percent mortality was calculated.

2.8. Bath vaccination experiments of larvae from immunised and non-immunised fish

Eighty-nine-day-old larvae from both parental groups were bath immunised with either whole *Phdp* SK7 bacterial cells (10^6 ml^{-1}) or *E. coli* LPS (50 μg ml^{-1}) (Sigma). Bacterial cells were prepared as previously described for the broodstock immunisations. Larvae were bath vaccinated for 1 h in the vaccine solutions under constant aeration. Control non-immunised larvae (CNI) received exactly the same treatment but in sterile sea water. The larvae were then held in clean sea water for a further 1 h to remove any unattached bacterial cells and they were then transferred to their holding tanks. Fifty larvae were used for each group and the experiment was run in triplicate. Vaccinated and control larvae were tested for protection against photobacteriosis 30 days post immunisation when the larvae were 120 days old. Larvae were challenged by bath exposure as described above with the previously calculated LD_{70} dose. Thirty larvae were used in each group and the experiment was run in triplicate. Mortality was recorded for 10 days and the relative percent survival (RPS) was calculated: RPS=(1−% mortality of immunised larvae/% mortality of control non-immunised larvae)×100. The schedule of experiments is shown in Fig. 1.

2.9. Protein concentration

A protein assay kit, based on the Bradford method [11] from Bio-Rad was used. Bovine serum albumin (BSA) was used as the standard protein. All the samples collected were analysed in triplicate.

2.10. Weight of larvae

Samples from the different larval rearing were collected in a net, rinsed with distilled water, and their wet body weight was measured and recorded. Weights were recorded in triplicate groups.

2.11. Anti-protease activity

A modification of the method described by Ellis [12] was used [13]. Briefly, 20 μl of samples (larval homogenates) were incubated with trypsin. To this, PBS and azocasein (Sigma) were added and further incubated, prior to the addition and incubation with trichloroacetic acid (TCA). The supernatant of this mixture was transferred to a 96-well microtray containing NaOH. The OD was read at 450 nm. For a 100% control, buffer replaced the larval homogenate and for a negative control, buffer replaced both larval homogenate and trypsin. The percentage inhibition of trypsin activity by each sample was calculated by comparing it to the 100% control sample. All the samples collected were analysed in triplicate.

2.12. Lysozyme activity

The lysozyme activity of samples ((larval homogenates) was measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus* [14]. On a 96-well microtray, larval homogenates four two-fold serial dilutions in PBS were mixed with a 0.4 mg ml^{-1} suspension of *M.*

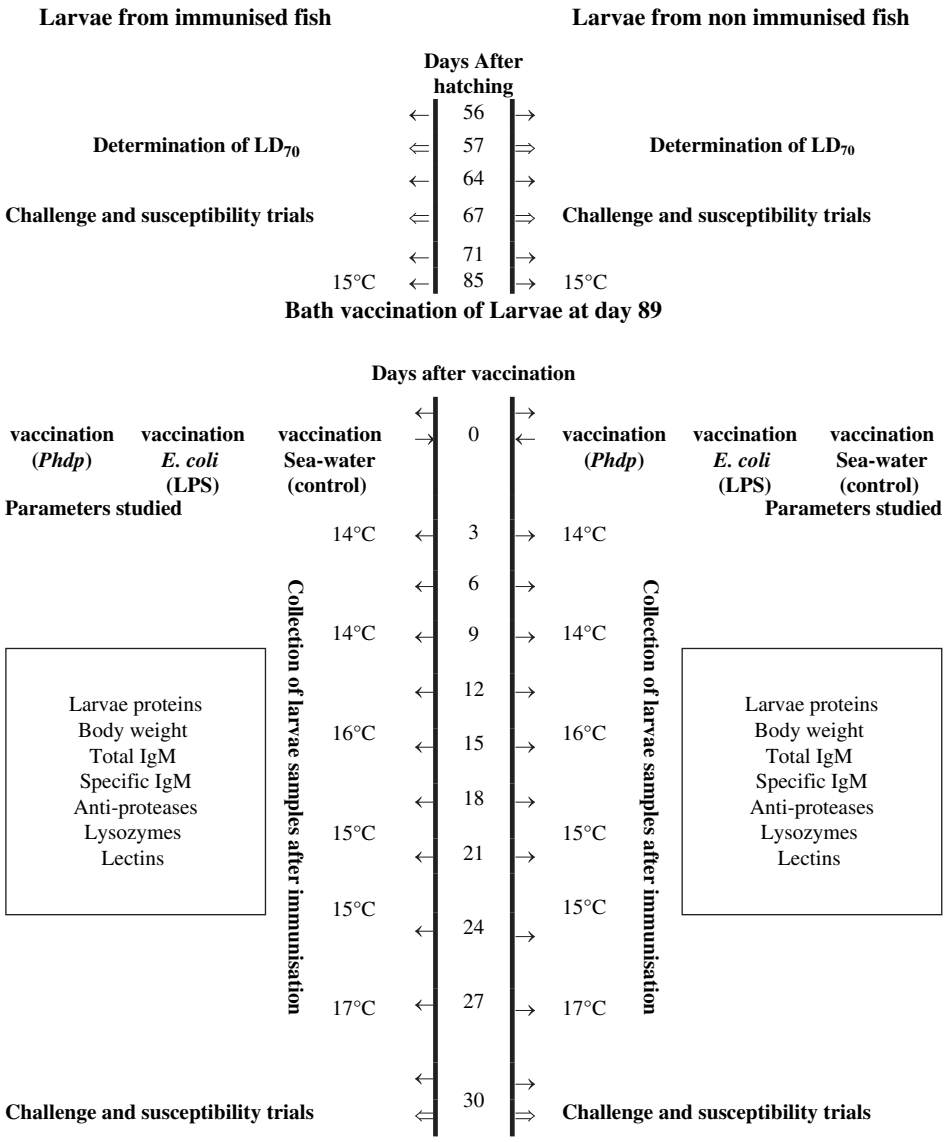

Fig. 1. Flow diagram for the experiment concerning vaccination by bath exposure of 89-day-old larvae from immunised and non-immunised broodstock for 1 h with formalin (1%) treated *Phdp* (10^6 cells ml^{-1}) and lipopolysaccharide (50 μg ml^{-1}) from *E. coli* (LPS).

lysodeikticus (Sigma) in PBS. The microtray was incubated and the OD was read at 450 nm at 0, 15, 30 and 60 min. For a positive control, homogenates were replaced by hen egg white lysozyme (serial dilutions starting at 1.6 μg ml^{-1}) and for a negative control, buffer replaced larval homogenate. A unit of lysozyme activity was defined as the amount of serum causing a decrease in the OD reading of 0.001 min^{-1}.

2.13. Enzyme linked immunosorbent assay (ELISA) for determination of total immunoglobulins

The samples were assessed with ELISA using rabbit anti-sea bream IgM (obtained from Dr Donatella Volpatti, University of Udine, Italy) by the method of Bakopoulos et al. [15] with slight modification.

Samples and reactive agents were added in the following sequence: (a) 100 μl of antigens (serially diluted standard from 0.007 to 1.00 μg ml^{-1} of purified sea bream serum IgM, or dilutions, 1:16 for larval extracts). For the calibration test of the ELISA, 100 μl of serial dilutions of larval extracts (1:4–1:512) were added in each well (triplicate) and 100 μl of serial dilutions of purified serum IgM (from 0.007 to 1.00 μg ml^{-1}) were used as a reference. Sea bass serum, eggs and PBS were used as negative controls. (b) 100 μl of a rabbit anti-sea bream IgM diluted 1:100 in LSWB-10% goat serum. (c) 100 μl goat anti rabbit IgG-biotin conjugate diluted 1:2000 in LSWB–10% goat serum. (d) 100 μl extravidin-HRP diluted 1:1000 in LSWB–10% goat serum. Colour development was performed for 15 min at 22 °C. All samples were placed in triplicate on the plates and the mean and standard deviation (SD) were calculated for each sample IgM concentration. Data were expressed as μg g^{-1} of wet body weight in larvae.

2.14. Enzyme linked immunosorbent assay (ELISA) for determination of specific immunoglobulins against Phdp and E. coli LPS

The samples were assessed with ELISA using rabbit anti-sea bream IgM by the method of Bakopoulos et al. [15] with slight modification.

Samples and reagents were added in the following sequence: (a) *Phdp* SK7 cells (OD 1 at 610 nm) 100 μl $well^{-1}$ were added to the wells (*E. coli* cells at OD 1 were used as negative control). For the determination of specific immunoglobulins against *E. coli* LPS, the latter antigen replaced *Phdp* cells at a concentration of 100 μg ml^{-1}. (b) 100 μl of samples or dilutions, 1:2 for larval extracts. For the calibration test of the ELISA, 100 μl of serial dilutions of immunised serum (1:4–1:512), were added in wells (triplicate) and used as a standard reference. (c) 100 μl of a rabbit anti-sea bream IgM diluted 1:100 in LSWB–10% goat serum. (d) 100 μl goat anti rabbit IgG-biotin conjugate diluted 1:2000 in LSWB–10% goat serum. (e) 100 μl extravidin-HRP diluted 1:1000 in LSWB–10% goat serum. Colour development was performed for 15 min at 22 °C. The samples were placed in triplicate on the plates and the mean and standard deviation (SD) were calculated for each sample. A standard curve was constructed with sera from immunised fish. The titre of these sera was set to be 100 units [16]. The specific antibody titre (units) of the samples were assigned by comparison with the standard plasma.

2.15. Specific antibody titre mg^{-1} IgM

Specific antibody titre units mg^{-1} IgM was calculated for the larval extracts of all groups [16]. The specific antibody titre units mg^{-1} IgM represents the ratio between specific antibody titre and amount of total immunoglobulins present in samples from both groups.

Specific antibody titre units mg^{-1} IgM was calculated as follows: specific antibody titre units mg^{-1} IgM = value of specific antibody titre (units)/IgM (mg g^{-1} of wet body weight) in larval extracts.

2.16. Haemagglutination assay for lectins

Serial twofold dilutions of the larvae extracts from all groups were performed using phosphate buffered saline (PBS, pH 7.2) in U-shaped (96 wells, Greiner, Microlon) bottom microtitre wells to which an equal volume of freshly prepared 2% erythrocyte suspension (sheep or rabbit in PBS) was added [17]. The activity was expressed as titre, i.e. the reciprocal of the highest dilution showing complete agglutination.

2.17. Statistical analysis

Data were evaluated using one-way analysis of variance (ANOVA). Groups were considered to be significantly different if $P<0.05$. When a significant F value was obtained for ANOVA the differences between all groups were tested by using Tukey's HSD multiple comparisons test.

3. Results

3.1. Determination of an LD_{70} and susceptibility tests of sea bream larvae from immunised and non-immunised broodstock

The larvae originating from immunised parents showed delayed onset and reduced mortality rates in comparison to the larvae originating from the non-immunised parental group (data not shown). The challenge experiment was repeated once more and similar results were obtained. On the basis of both trials mean cumulative mortalities were obtained and an LD_{70} was calculated to be 7.2×10^4 cells ml^{-1}.

The challenge performed using the LD_{70} with larvae from immunised and non-immunised groups indicated a cumulative mortality of 66.67 and 80% (Fig. 2), respectively. The mortality rate was again different between larval groups, with the mortalities of larvae originating from immunised parents beginning between days 2 and 8 (maximum rate at days 5–7) while for the offspring of the non-immunised parents mortalities started from days 1 to 8 (maximum rate at days 3–6).

3.2. Total proteins

The total protein contents were measured in larval extracts of all groups (data not shown). Until day 30 of the experiment, no significant difference was found in protein levels for all groups (Table 1). In larvae from immunised (IM) parents vaccinated with *Phdp* cells (IM P) or with LPS (IM LPS) and from

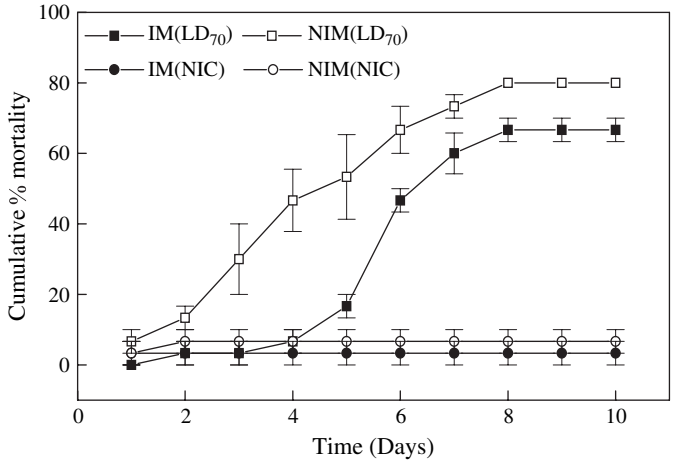

Fig. 2. Cumulative percent mortality due to *Photobacterium damsela* subsp. *piscicida* with LD_{70}. Larvae from immunised (IM) and non-immunised (NIM) broodstock were infected by virulent bath challenge with LD_{70} (7.2×10^4 cells ml^{-1}) of *Photobacterium damsela* subsp. *piscicida*. Non-infected control (NIC). Each data point represents the mean (±SE) of triplicates.

Table 1
Difference tested by one-way ANOVA in larvae from immunised (IM) and non-immunised (NIM) broodstock after immunisation by bath exposure for 1 h with formalin (1%) treated *Phdp* 10^6 cells ml^{-1} (P) and lipopolysaccharide (50 μg^{-1}) from *E. coli* (LPS)

Groups	Total proteins	Body weight	Anti-protease activity	Lysozymes	Total immunoglobulins
IM (P)	A	A	A	A	A
IM (LPS)	A	A	AB	AB	AB
IM (CNI)	A	A	B	BC	BC
NIM (P)	A	A	C	BC	BC
NIM (LPS)	A	A	C	CD	BC
NIM (CNI)	A	A	C	D	C

	Specific antibody titre against *Phdp*	Specific antibody titre (units mg^{-1} IgM)	Specific antibody titre against LPS
IM (P)	A	A	A
IM (LPS)	B	B	B
IM (CNI)	B	B	A
NIM (P)	A	A	A
NIM (LPS)	B	B	B
NIM (CNI)	B	B	A

	Lectins	Cumulative % mortality with LD_{70}
IM (P)	A	A
IM (LPS)	A	AB
IM (CNI)	A	BC
NIM (P)	A	AB
NIM (LPS)	A	AB
NIM (CNI)	A	C

Control non-immunised (CNI). The differences between all groups were tested by using Tukey's HSD multiple comparisons test. Identical letters indicate no significant difference between groups, different letters indicate a significant difference ($P<0.05$).

non-immunised (NIM) parents vaccinated with *Phdp* cells (NIM P) or with LPS (NIM LPS) and larvae used as control, the total protein contents ranged between 15.85 and 16.40 mg g^{-1}.

3.3. Body weight of larvae

The wet body weight of larvae was recorded in both groups from day 0 to day 30 (data not shown). No significant difference was found among the body weights of all groups (Table 1) until day 30 of the experiment. In larvae from immunised (IM) parents vaccinated with *Phdp* cells (IM P) or with LPS (IM LPS) and from non-immunised (NIM) parents vaccinated with *Phdp* cells (NIM P) or with LPS (NIM LPS) and larvae used as control, the body weight ranged between 135.52 and 332.00 mg.

3.4. Anti-protease activity

The anti-protease activities (% of trypsin inhibition) were measured in larval extracts of all groups (Fig. 3). From day 0 to day 30 an increase in anti-protease activities was observed. Significant difference ($P<0.05$) was found among activities in the IM P and IM CNI groups, regarding the IM larvae, and these groups showed significant difference ($P<0.05$) among activities in comparison to the NIM larvae. The NIM larvae groups did not show significant difference among activities (Table 1).

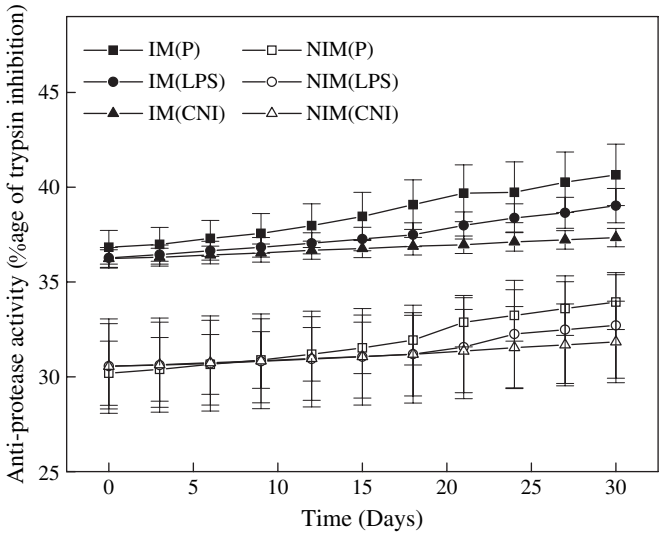

Fig. 3. Total anti-protease activity level changes (% age of trypsin inhibition) in larvae from immunised (IM) and non-immunised (NIM) broodstock after immunisation by bath exposure for 1 h with formalin (1%) treated *Photobacterium damsela* subsp. *piscicida* 10^6 cells ml^{-1} (P) and lipopolysaccharide (50 μg ml^{-1}) from *E. coli* (LPS). Control non-immunised (CNI). Each data point represents the mean (±SE) of triplicates.

3.5. Lysozyme activity

The lysozyme activities (units g^{-1}) were measured in larval extracts of all groups (Fig. 4). From day 0 to day 30 an increase in lysozyme activities was observed. Significant difference ($P<0.05$) was found among activities in the IM P, IM CNI and NIM CNI and between IM LPS and NIM LPS groups (Table 1).

3.6. Calibration assay and total immunoglobulin

A standard curve for purified sea bream IgM and a dose response curve for larval extracts with twofold dilutions were calibrated (data not shown). A suitable dilution factor for larval extracts was 16, corresponding to an IgM concentration of 0.13 (μg g^{-1}) for larval extracts. This concentration corresponded to a linear portion of the curve of the OD response of the IgM and was the most suitable for calculating the IgM concentrations of the different samples. Total amount of IgM in larval extracts (μg g^{-1})=0.13 (IgM known standard)×16 (calibrated larval extracts dilution).

The total immunoglobulin levels were measured in larval extracts of all groups (Fig. 5). Significant difference ($P<0.05$) was found among immunoglobulin levels in the groups IM P, IM CNI, NIM P, NIM LPS (immunoglobulin levels between the last three groups did not differ statistically) and NIM CNI while IM LPS levels differ from NIM CNI (Table 1).

3.7. Specific antibody titre and value of specific antibody titre (units per mg IgM) against Phdp

The specific immunoglobulin titre (units) (Fig. 6) and the specific antibody titre (units per mg IgM) were calculated in larval extracts of all groups (data not shown). In larvae from immunised parents (IM), the IM P or IM LPS and IM CNI larvae groups had specific antibody units of 0.00–9181.96±1103.79 units mg^{-1} IgM and no specific antibody units, respectively. In larvae from non-immunised parents (NIM), the NIM P

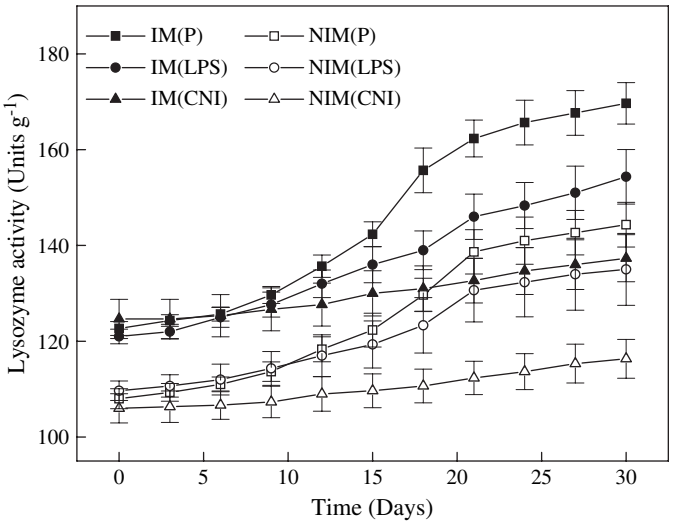

Fig. 4. Total lysozyme level changes (units g^{-1} wet body weight) in larvae from immunised (IM) and non-immunised (NIM) broodstock after immunisation by bath exposure for 1 h with formalin (1%) treated *Photobacterium damsela* subsp. *piscicida* 10^6 cells ml^{-1} (P) and lipopolysaccharide (50 μg ml^{-1}) from *E. coli* (LPS). Control non-immunised (CNI). Each data point represents the mean (±SE) of triplicates.

or NIM LPS and NIM CNI larvae groups had specific antibody units of 0.00–9230.94±1125.99 units mg^{-1} IgM and no specific antibody units, respectively. The first detectable specific antibody titre was noted on day 6 in both groups. From day 6 to day 24, an increase in specific immunoglobulin levels was observed but from day 24 to day 30 the titre levels remained almost constant (140.00±14.42 units and 122.67±14.53

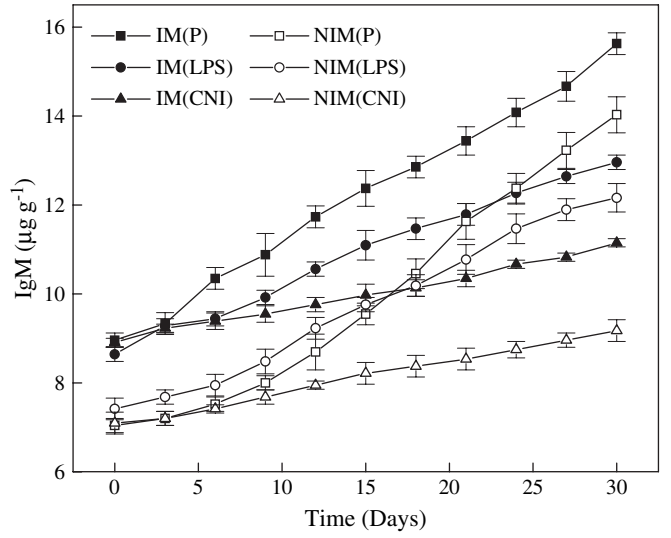

Fig. 5. Total IgM level changes (μg g^{-1} wet body weight) in larvae from immunised (IM) and non-immunised (NIM) broodstock after immunisation by bath exposure for 1 h with formalin (1%) treated *Photobacterium damsela* subsp. *piscicida* 10^6 cells ml^{-1} (P) and lipopolysaccharide (50 μg ml^{-1}) from *E. coli* (LPS). Control non-immunised (CNI). Each data point represents the mean (±SE) of triplicates.

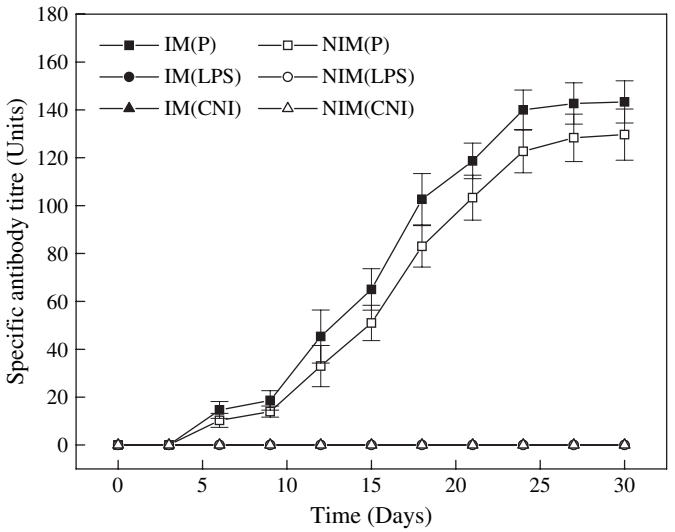

Fig. 6. Specific antibody titre level changes (units against *Phdp*) in larvae from immunised (IM) and non-immunised (NIM) broodstock after immunisation by bath exposure for 1 h with formalin (1%) treated *Photobacterium damsela* subsp. *piscicida* 10^6 cells ml^{-1} (P) and lipopolysaccharide (50 μg ml^{-1}) from *E. coli* (LPS). Control non-immunised (CNI). Each data point represents the mean (±SE) of triplicates.

units) in both groups (Fig. 6). Higher levels of specific antibody titre were noted in the IM P group but no significant difference ($P<0.05$) was found among specific immunoglobulin titre levels in comparison to the NIM P group (Table 1).

3.8. Specific antibody titre against LPS from E. coli

Specific antibody titre against LPS was measured in larval extracts of all groups (Fig. 7). From day 6 to day 24, an increase in specific immunoglobulin levels was observed but from day 24 to day 30 the titre levels remained almost constant in both groups. Higher levels of specific antibody titre against LPS was noted in the IM LPS group but no significant difference was found among specific immunoglobulin titre levels in comparison to the NIM LPS group (Table 1).

3.9. Haemagglutinin activity

Haemagglutinin activity was observed in larval extracts of all groups from the day 0 indicated by titre values of 1:2. This value remained the same until day 9, whereas from day 12 until day 30 a titre value of 1:4 was noted in all groups and the difference was not significant among groups (Table 1).

3.10. Protection studies and relative percent survival

Thirty days post immunisation larvae were subjected to bath challenges using the LD_{70} (Fig. 8). The cumulative % mortalities were recorded in all groups. The larvae originating from the immunised parents showed better protection levels in comparison to similar larvae from non-immunised parents. Significant difference ($P<0.05$) was found between the % mortality levels in IM P, IM CNI and NIM CNI groups (Table 1).

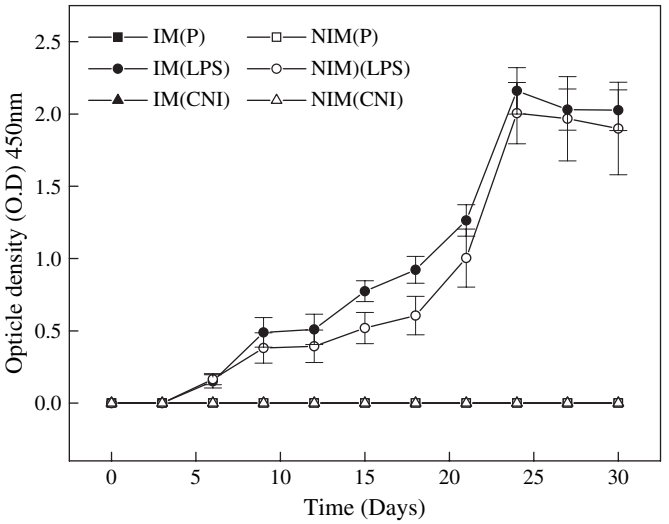

Fig. 7. Specific antibody titre level changes (optical density against *E. coli* LPS) in larvae from immunised (IM) and non-immunised (NIM) broodstock after immunisation by bath exposure for 1 h with formalin (1%) treated *Photobacterium damsela* subsp. *piscicida* 10^6 cells ml^{-1} (P) and lipopolysaccharide (50 μg ml^{-1}) from *E. coli* (LPS). Control non-immunised (CNI). Each data point represents the mean (±SE) of triplicates.

In larvae from immunised parents (IM), the IM P and IM LPS larvae groups had a relative percent survival of 95.83±7.22 and 72.22±25.46, respectively. In larvae from non-immunised parents (NIM), the NIM P and NIM LPS larvae groups had a relative percent survival of 62.50±54.49 and 70.83±14.43, respectively. No significant difference was found among relative percent survival of all groups.

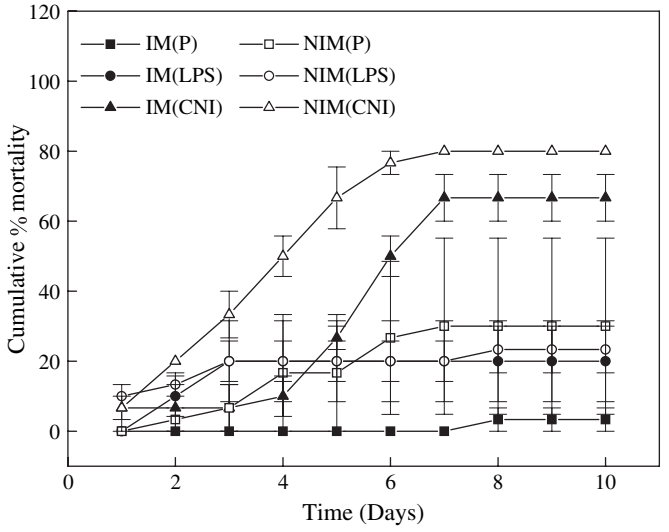

Fig. 8. Cumulative mortality with LD_{70} infection of *Photobacterium damsela* subsp. *piscicida* in larvae from immunised (IM) and non-immunised (NIM) broodstock after immunisation by bath exposure for 1 h with formalin (1%) treated *Photobacterium damsela* subsp. *piscicida* 10^6 cells ml^{-1} (P) and lipopolysaccharide (50 μg ml^{-1}) from *E. coli* (LPS). Control non-immunised (CNI). Each data point represents the mean (±SE) of triplicates.

4. Discussion

Sea bream broodstock were vaccinated with a novel vaccine mixture as previously described [6]. Larvae originating from immunised and non-immunised parents were subjected to experimental infectivity trials with *Phdp* at day 57 post-hatch. The challenge experiments revealed a better response to *Phdp* infection from larvae originating from the immunised parental group. The difference between larval groups was more evident, when the bacterial infectious pressure was declining, as it was evident from the 1st and 2nd challenges. Thus, the beneficial effect of parental vaccination prior to spawning was more evident when the challenge pressure was 10^4 and 10^2 cells ml^{-1} (data not shown). Furthermore, the larvae originating from immunised parents showed delayed onset and reduced mortality rates in comparison to the larvae originating from the non-immunised parental group. The present results are comparable with other studies on transfer of maternal immunity in some fish species. In the guppy [18] and tilapia [19], transfer of specific protection against various pathogens has been reported. The low susceptibility in rainbow trout fry against *Vibrio anguillarum* was described [20] until the age of 4 weeks by bath challenge. The ability of larvae originating from immunised parents to respond better to infection was clearly seen in this study, but this ability should be coupled with reasonable infectious loads for better demonstration. After the determination of the LD_{70} for larvae, subsequent experiments always revealed a difference in favour of about 13.3–20% in cumulative mortalities in the larvae originating from immunised parents in comparison to larvae originating from the non-immunised group (Fig. 2). The mechanism underlying the transfer of maternal immunity and protection in offspring by immunisation of fish prior to spawning season needs further studies. However, the present study has clearly demonstrated that the immunisation of broodstock before spawning may serve to reduce the early mortality in newly hatched larvae.

In order to assess the ability and the levels of a response from these larvae against *Phdp* and a T-cell independent antigen, the larvae were vaccinated via immersion with *Phdp* bacterial cells or *E. coli* purified LPS at day 89 (162.2 mg). The following 30 days larval extracts were evaluated for the presence of non-specific and specific humoral immune parameters and at day 120 they were subjected to experimental infectivity trials with *Phdp* virulent bacterial cells in order to assess the effect of the above vaccinations on the protection of larvae.

The earliest age at which different fish species can produce antibodies varies greatly and is dependent on water temperature, size, antigen used and route of administration. No oviparous teleosts have demonstrated the ability to mount an immune response within 1 week post-hatch. Rainbow trout is able for antibody production against *Aeromonas hydrophila* when exposed at 3 weeks post-hatch [21] and to dinitrophenol-keyhole limpet haemocyanin when exposed at 1 month post-hatch [22], but not before these ages. Fish larvae are very small in size and fragile at the early stages of their life cycle and vaccination by injection is unfeasible. Immersion and oral (when the problems associated with antigen denaturation and effective absorption by the gut will be solved) vaccination is an effective and practical method for mass vaccination of fish larvae.

Breuil et al. [23] suggested that the immune system of the sea bass larvae is probably competent for antibody production at 50 days. Clinical observations show that sea bass fry are highly sensitive to bacterial diseases during this period [24]. It was reported that mortalities mainly occurred in the early stages of the sea bream growth cycle and recommended to start vaccination in 50–60 days old fish [4].

The total protein contents measured in larval extracts and their body weights were similar and no significant difference was found in all groups (data not shown).

Significantly ($P > 0.05$) higher anti-protease activity levels were found in the group that belonged to the immunised parents and the IM P group activities differed significantly from the IM CNI group (Fig. 3) (Table 1). Fish plasma contains a number of protease inhibitors, principally α1-anti-protease, α2-anti-plasmin and α2-macroglobulin (α2M) which may play a role in restricting the ability of bacteria to invade and grow in vivo [26]. However, pathogenic bacteria have evolved evasive mechanisms. For example, the

highly toxic serine protease produced by *Aeromonas salmonicida* is resistant to α1-anti-protease. This is regarded as a universal serine protease inhibitor and represents over 80% of the anti-protease activity in salmonid plasma. Nevertheless, the *A. salmonicida* serine protease is inhibited by α2M [26]. The ability of salmonid plasma to inhibit this bacterial protease has been correlated with between species differences in susceptibility to furunculosis [27,28]. No report is available to compare the anti-protease activity levels in fish larvae after vaccination as reported in this study. However, there is evidence that vaccination of IM larvae with *Phdp* resulted in a significant increase in anti-protease activities in comparison to NIM larvae.

Higher lysozyme levels were found in the groups that belonged to the immunised parents (Fig. 4). In larvae from each group (immunised and non-immunised parents), significant difference ($P<0.05$) was found among activities in the IM P, IM CNI and NIM CNI and between IM LPS and NIM LPS groups (Table 1). Lysozyme has been found in fish mucus, serum and ova [29–32] and cells involved in this activity are peritoneal macrophages and blood neutrophils [30,33]. There are several reports of lysozyme isolated from fish serum and ova, being bactericidal even for important fish pathogens like *A. salmonicida* and *A. hydrophila* [34–35]. No report is available, however, to compare the lysozyme levels in fish larvae after immersion vaccination with this study.

Higher total immunoglobulin levels were found in the groups that belonged to the immunised parents (Fig. 5). In larvae from each parental group (IM and NIM), significant difference was found among immunoglobulin levels (Table 1). Atlantic salmon *Salmo salar* L. fry were fed *A. salmonicida* lipopolysaccharide (LPS)-coated feed for 62 days. Fry receiving LPS-coated feed showed an increase in total immunoglobulin levels compared with fry receiving control feed [35].

In larvae deriving from both parental groups bath vaccinated with *Phdp* cells, specific antibody titre levels against *Phdp* were observed, whereas in larvae bath vaccinated with *E. coli* LPS and larvae used as control, exhibited no specific antibody titre against *Phdp* (Fig. 6). No significant difference ($P<0.05$) was observed in specific antibody titre of both groups (Table 1). Mughal and Manning [22] reported that carp (*Cyprinus carpio*), 4 weeks old, had already developed an immune response and memory formation after priming by injection or direct immersion with *A. salmonicida*. Similar results were also obtained for rainbow trout [36]. Mughal et al. [37] found that carp immunised at 4 weeks old with *A. salmonicida* developed memory, while they became tolerant after immunisation with human gamma globulin.

The value of specific antibody titre per mg IgM represents the ratio between specific immunoglobulin and total immunoglobulin contents in all samples. It was observed that specific antibody titre per mg IgM against *Phdp* was similar in larvae deriving from IM and NIM parents, indicating that a common feature of specific immunity exists in both groups with specific proportion. So on the basis of these results (data not shown), it is conceivable that the immune system of larvae from both groups is equally efficient at this age (90 days) to respond against antigen.

In larvae deriving from both parental groups bath vaccinated with lipopolysaccharide from *E. coli,* specific antibody titre levels were observed, where as in larvae bath vaccinated with formalin treated whole *Phdp* SK7 bacterial cells and larvae used as control exhibited no specific antibody titre against lipopolysaccharide from *E. coli*. The first peak of antibody titre was observed at day 6 then this specific titre gradually increased until day 24 and then remained constant until the end of the experiment at day 30 (Fig. 7). No significant difference was observed in specific antibody titre of both groups (Table 1). There are several reports detailing the vaccination of fish, both fry and juveniles, by whole bacterial cells and LPS [20,38–42]. In these studies, the antigens were administered either intraperitoneally or by immersion. A specific immune response was demonstrated, and in some cases, an enhanced protection against challenge by the same microorganism was found. Ellis [43] claimed that rainbow trout B cells may be stimulated by T-independent antigens (i.e. LPS) when the fry is 4 weeks of age (0.13 g). Development of memory has been demonstrated in fry older than 8 weeks (0.26 g), but long-term memory responses in salmonid fish may not be expected before the fry is above 4 g. Guttvik et al. [35] reported that in Atlantic salmon fry fed with *A. salmonicida* lipopolysaccharide (LPS) coated feed, no specific antibody titre was found.

The same haemagglutinin activity was found in both parental groups bath vaccinated with both types of antigen and non-vaccinated control. No significant difference was observed in larval extracts of all groups (Table 1). Lectins have been found in salmon eggs [44], serum [45] and mucus [46]. These lectins are Ca^{2+}-dependent and can agglutinate a number of fish bacterial pathogens. Their role in defence is still unclear but in mammals they can have opsonic and complement-activating properties [46]. No report is available to compare the lectins titre levels in fish larvae after immersion vaccination with formalin treated bacteria or lipopolysaccharides.

Thirty days post-immunisation, as previously described, both IM and NIM larvae groups were subjected to bath challenges using the LD_{70} dose. RPS values were calculated for larvae from both groups (data not shown) and although an identifiable trend of better protection was noted for the IM P larvae, no significant difference was observed among the groups (Table 1). The presence of specific antibodies against *Phdp* was detected in sea bream larvae from both groups vaccinated with the novel *Phdp* vaccine mixture. The values of specific antibody titre were the same in both groups. So it is evident that the immune system of sea bream larvae is efficient at this age (160 mg) to respond against antigens. It is concluded that (a) larvae can be vaccinated successfully as early as 90-days post-hatch (160 mg) and (b) that these larvae can be protected against *Phdp* after heavy challenge with extremely high survival rates (more than 96%).

The order in which the major lymphoid organs developed in sea bream are kidney at day 54, spleen and thymus at 77 days. Based on the information on the relationship between morphology of lymphoid organs and their functional maturation it was suggested that sea bream may not be immunocompetent earlier than 77 days of age and vaccination at too early age could create problems [8].

The minimal size at which a response was detected in sockeye (*Oncorhynchus nerka*), pink (*O. gorbuscha*), chum, coho, Chinook salmon (*O. tshawytscha*) and rainbow trout (*Oncorhynchus mykiss)* was 1.0–2.5 g [25]. Size was also shown to be a better indicator of immunological maturation than age in *Sebastiscus marmoratus* [25]. Whereas in the present study immune response was detected in sea bream larvae when their size was smaller as compared to the other studies and the body weight was 157–166 mg. This disagreement may be due to the inter species difference among teleosts or better immunostimulatory effect of the novel vaccination mixture in sea bream larvae.

This work also involved stimulation and assessment of the humoral non-specific defence of the sea bream larvae by means of lipopolysaccharide. It was observed that larvae from both groups (immunised and non-immunised) bath vaccinated with lipopolysaccharide from *E. coli* exhibited good protection against *Phdp* when compared with the groups immersion vaccinated with the *Phdp* novel vaccine mixture. Moreover, when the relevant percent survival (RPS) was calculated in all groups, although a certain trend in favour of the IM larvae vaccinated with *Phdp* cells was observed, no significant difference was found. This may be due to stimulation of non-specific defence mechanisms by LPS because no specific antibodies against *Phdp* were present in the groups vaccinated with LPS. This stimulation was evident by the significant increase in anti-protease and lysozyme activity in the same groups. Furthermore, the presence of specific antibodies against LPS were also detected which indicates that the immune system of sea bream larvae can initiate an effective immune response against antigens at this weight. The results of this study on the beneficial effect of non-specific stimulation can be compared with a previous study [7]. The latter study showed that the RPS for LPS-treated larvae was 11% (25 μg LPS ml^{-1}), 32% (50 μg LPS ml^{-1}) and 18% (100 μg LPS ml^{-1}).

Eighty-nine-day-old larvae vaccinated with *Phdp* cells or LPS showed significant increase in anti-protease activity, lysozyme activity and total immunoglobulins compared to the controls. Specific antibodies against *Phdp* and LPS were present in the respective vaccinated groups of IM and NIM larvae. The presence of specific immunoglobulins represents the efficient onset of specific immune response of sea bream larvae at the age of day 89 (160 mg). Vaccinated larvae from both groups showed significantly less mortality compared to the respective controls. The results of this study have shown that the novel vaccine mixtures and LPS performed well as immunostimulants in sea bream larvae. In addition to the specific

immune response, non-specific defence mechanisms may be important to withstand infection, especially in the early phases of the infection. So immersion vaccination can be used in larvae at the age of 90 days (160 mg) when the immune system can respond well against antigens.

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