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Quantitative and qualitative analysis of sea bream, *Sparus aurata* (L.), humoral immune response, vaccinated with commercial and experimental vaccines against vibriosis and photobacteriosis

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The specific humoral immune response of sea bream, Sparus aurata *(L.), against* Vibrio anguillarum *O1 and* Photobacterium damselae *subsp.* piscicida *(Phdp) after immunization with commercial and experimental bacterins was analysed quantitatively and qualitatively. Specific anti-*V. anguillarum *O1 and anti-Phdp levels provoked by the adjuvanted commercial vaccine reached higher levels in comparison to the aqueous commercial and experimental bacterins. Infection of vaccinated fish with* V. anguillarum *O1 bacterial cells acted as a boost of the humoral immune response, except for the sera of the group vaccinated with the adjuvanted vaccine. Infection with Phdp acted as a boost of the humoral immune response mainly for the group vaccinated with a monovalent Phdp bacterin and to a lesser degree for the group vaccinated with the aqueous commercial vaccine. Western blot analysis of the sera against* V. anguillarum *O1 whole cell antigens revealed strong reactions to only a few antigens below 54 kD and above 15 kD and weak reactions to other antigens. Similar reactions were observed from the sera isolated from the controls. Western blot analysis of the sera against Phdp whole cell antigens revealed strong reactions to only a handful of antigens below 20.7 and below 6.4 kD. Sera from the control group, as in the case of* V. anguillarum *O1, reacted with Phdp whole cell antigens. No differences regarding antigen reactions between monovalent and bivalent formulations were noted, in contrast to the adjuvanted and aqueous bacterins.*

Keywords: *Vibrio anguillarum* O1, *Photobacterium damselae* subsp. *piscicida*, sea bream, humoral immune response, vaccines

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INTRODUCTION

Intensive mariculture of sea bream (*Sparus aurata*, L.) and sea bass (*Dicentrarchus labrax* L.) in the Mediterranean has contributed to the development of serious disease outbreaks caused by viruses, bacteria and parasites (Athanassopoulou & Bitchava, 2010). Due to the limitations imposed by the type of intensive mariculture practised (i.e. offshore floating cages) on the isolation and eradication of these outbreaks, the majority of these pathogens have become endemic to the marine environment causing serious mortalities every year, if left unattended. Two Gram negative bacteria, *Photobacterium damselae* subsp. *piscicida* (Bakopoulos *et al.*, 1995) and different serotypes of *Vibrio anguillarum* (Sorensen & Larsen, 1986; Toranzo & Barja, 1990) are the infectious agents of the most important bacterial diseases of sea bass and bream, photobacteriosis and vibriosis, respectively. The development of serious outbreaks of these diseases during the mariculture of both species is prevented by general hygiene measures and, most important, by vaccination.

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Vaccines against either photobacteriosis or vibriosis have been commercially available for quite some time. Woo & Bruno (1999) have reported good protection of these vaccines against vibriosis, while efficacy studies have been inconclusive, especially in the field, for photobacteriosis (Nakai *et al.*, 1992; Le Breton, 1999, 2009; Romalde, 2014). The humoral response of sea bream against *Ph. damsela* subsp. *piscicida* (Phdp, hereafter) monovalent formulations has been described before both quantitatively and qualitatively (Arijo *et al.*, 2004; Hanif *et al.*, 2005), while the humoral response of sea bream to monovalent commercial vaccines of *V. anguillarum* is not well documented. Individual administration of vaccines (i.e. via intraperitoneal injection) causes stress and injuries to the fish that lead to mortalities and is labour intensive. For these reasons, vaccination using this type of administration is practised only once during the on-growing phase of both fish species (lasting 15–18 months) to the expense of better protection through anamnestic administrations. For similar reasons mariculture companies prefer the use of multivalent commercial products containing various combinations of antigens of the same bacterial species, such as *V. anguillarum* serotype O1 and O2 (Angelidis *et al.*, 2006), or different bacterial species, such as *V. anguillarum* and Phdp or even combinations of different bacterial species and virus (i.e. one or two serotypes of *V. anguillarum* plus Phdp

plus b-nodavirus), with the latter products being in the experimental phase. The obvious benefit of multivalent vaccines from the companies' perspective is 'single injection – multiple protection'. However, published scientific data on the assessment of such products is limited or inconclusive (i.e. Gravningen *et al.* (1998), regarding photobacteriosis) and the situation is similar with the analysis of the humoral responses caused by such multivalent products. Interestingly, the work of Nikoskelainen *et al.* (2007) with rainbow trout, has indicated that multiple antigens in polyvalent vaccines may interfere with the specific responses obtained quantitatively as well as in respect to the biological functions of specific antibodies.

Thus, the aim of this study was to investigate and compare both the quantitative (specific antibody titres for either pathogen) and qualitative (antigens recognized for either pathogen) aspects of the humoral immune response of sea bream after vaccination with multivalent (*V. anguillarum* and Phdp) or monovalent, for Phdp only, commercial and experimental vaccines. And, more specifically, to evaluate if the inclusion of adjuvant effects the repertoire of antigens recognized and if the presence of multiple antigens in polyvalent vaccines interfere with the immune responses towards each different pathogen.

MATERIALS AND METHODS

Fish

The fish used in the experiments were healthy, non-vaccinated gilthead sea bream that were donated by a local hatchery (Selonda S.A., Loutra hatchery, Lesvos, Greece). The initial average weight of the fish was 2.3 g and by the end of the experiment was 30.3 g. Fish were fed daily 2% of their biomass with a commercial fish feed. Fish handling (weighing, vaccination, blood collection, infections) were performed under anaesthesia using 0.2%, 2-phenoxyethanol.

System

On arrival to the experimental facilities of the Department of Marine Sciences, University of the Aegean, fish were placed in a seawater tank system comprising eight cylindro-conical tanks each one with a holding capacity of 2 m^3. The tanks were connected in a closed, fully recirculating water circuit, with a seawater pump having a 9 $m^3\ h^{-1}$ capacity and two air pumps (150 $l\ h^{-1}$) which provided air in the tanks through airstones. Finally, the water passed through a sand filter and a biological filter. The system was also connected to a water sterilization unit 110 W UV. Fish remained there until they reached an average weight of 20 g, they were then vaccinated, separated into five experimental groups and placed in separate tanks until the end of the experiment.

The infectious experiments were performed in 50-l aquariums. They were operated in a static way and the water renewal was partially (1/2) performed on a daily basis. Each aquarium had its own continuous air supply provided by two air pumps (90 $l\ h^{-1}$) and airstones.

Both systems were cleaned of fish faeces and remaining food while water parameters were monitored daily. Temperature ranged between 18.5°C in spring to 26°C in summer. Salinity of water was 32‰, oxygen levels ranged between 4.6 and 6.8 $mg\ l^{-1}$, total ammonia nitrogen never exceeded 0.5 $mg\ l^{-1}$ and pH ranged between 7.7 and 7.9.

Bacteria

The bacteria used in this study were: Phdp isolated in South Evoia in 2012, kindly donated by Dr Papanna, Nireus SA; *V. anguillarum* serotype O1, isolated in the Ionian Sea, kindly donated by the Fish Diseases Reference Laboratory, Central Veterinary Laboratory, Ministry of Agriculture, Greece. Both isolates were kept in −85°C using the cryobeads storage system.

Commercial and experimental vaccines

The commercial vaccines used were: an adjuvanted vaccine (AJ) which is an injectable emulsion containing mineral oil and formalin-inactivated bacterial cultures of *V. anguillarum* (serotype O1) and Phdp; an aqueous non-adjuvanted vaccine (AV) which is an aqueous suspension for injection containing formalin-inactivated cells of two serotypes (O1 and O2) of *V. anguillarum* and Phdp. More information cannot be provided because of its proprietary nature. The efficacy of both these products against infection with Phdp has been described before (Bakopoulos *et al.*, 2015a) and field data support the efficacy of both products against Vibriosis. Both these products were purchased and kindly donated by the company Cephalonian Aquaculture for the purposes of this study.

The experimental vaccines used were both non-adjuvanted aqueous solutions: one formulation was monovalent against photobacteriosis (Eph) and contained 6.24×10^7 formalin-inactivated Phdp bacterial cells ml^{-1}; the other formulation was bivalent and contained 5.36×10^9 formalin-inactivated bacterial cells (Phdp+*V. anguillarum* O1) ml^{-1} (Evip). These experimental vaccines were briefly prepared as follows: a bead from each −85°C stored bacteria was placed in brain heart infusion broth (BHIB) with 2% NaCl and were cultured with progressive addition of fresh sterile medium for 72 h at 22°C. Cultures were then centrifuged at 500 ***g*** for 1 h at 4°C. The bacterial pellet was washed twice in sterile 2% NaCl and after their last wash they were formalin-inactivated as described previously (Bakopoulos *et al.*, 2003b).

Vaccination

A total of 740 fish were vaccinated with a single intraperitoneal (i.p.) injection: 200 fish with the AV vaccine, 200 with the AJ vaccine, 200 with the experimental bivalent Phdp – Vibrio (Evip) bacterin, 140 with the experimental monovalent Phdp (Eph) bacterin. Finally, another 700 fish were injected with a sterile solution of 2% NaCl (controls).

The volume of each injection was 100 µl $fish^{-1}$, following the instructions of the commercial vaccine manufacturers. Each different experimental group was placed in a separate tank. Fish were monitored daily for any adverse effects or the development of disease signs and their health status was mainly assessed by their feeding and reaction to stimuli behaviour.

Experimental infections

Two infections were performed during the course of this study in order to collect serum samples from surviving fish for immunological analysis. The first was performed on day 53 post-vaccination with i.p. injection of 100 µl of a bacterial

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suspension of *V. anguillarum* O1 at 7.1×10^5 cells ml^{-1} per fish for each experimental group. The second was performed on day 65 post-vaccination, similarly as above and the bacterial suspension contained 9.2×10^4 Phdp cells ml^{-1}. Both bacteria were cultured in BHIB 2% NaCl and isolated as described above. In both infections, 60 fish from each vaccine group and from the controls, except from the monovalent experimental vaccine Eph group (only for the infection with *V. anguillarum* O1), were used. For each infection and vaccinated group, infected fish were distributed randomly into three aquaria (20 fish each). The fish were monitored daily for any development of disease signs and mortalities and these fish were discarded, after confirmation of the cause of disease with microbiological sampling.

Blood sampling

After light anaesthesia, blood was collected from the caudal vein. Blood was allowed to clot for 10 min at room temperature and was refrigerated (4°C) overnight. It was then centrifuged at 200 ***g*** for 10 min and the serum was carefully isolated. Finally the sera were stored at −20°C until used. Sera were collected from three randomly selected fish of each vaccine group and were stored separately. Blood and serum were similarly isolated during the experimental infections of fish.

ELISA analysis

Specific antibody levels in the isolated sera from both vaccinated and vaccinated/infected fish against either *V. anguillarum* O1 or Phdp were measured using a simple indirect ELISA (Bakopoulos *et al.*, 1997). Briefly, plates were coated with either *V. anguillarum* O1 or Phdp for 2 h at room temperature. Free unbound sites in the wells were blocked with 10% bovine serum albumin, overnight at 4°C. Fish sera were added at 1:100 dilution for 1 h. Negative controls included triplicate wells with the diluent of fish sera. Wells were then filled with anti-sea bream IgM MAbs (Aquatic Diagnostics Ltd – ADL, UK) and were incubated for 1 h. Anti-mouse IgG HRP (Sigma) diluted 1:1000 in 5% goat serum, was then added for 1 h, prior to the addition of chromogen (tetramethylbenzidine). The reaction was terminated with the addition of 2 M H_2SO_4. Reactions were read with a MR-96A (MINDRAY) microplate photometer at 450 nm.

Electrophoresis and WB analysis

Whole bacterial cells of either *V. anguillarum* O1 or Phdp were harvested from liquid culture in BHIB 2% NaCl, as described above; their concentration was made 10^9 bacterial cells ml^{-1}, they were spun down and the resulting bacterial pellet was resuspended in 500 µl of electrophoresis sample buffer (ESB) (Nomura & Aoki, 1985). Electrophoresis was performed on polyacrylamide gels, 4% stacking and 12% separating, according to the discontinuous method of Laemmli (1970).

The SDS–PAGE gels were transblotted onto PVDF membranes (BIORAD) according to Towbin *et al.* (1979) and the instructions of the manufacturer. Success of transblotting was assessed by Coumassie blue staining of the gels after the transfer.

Blots were WB analysed using the method of Wiens *et al.* (1990) as modified by Bakopoulos *et al.* (1997). Briefly, blots were incubated for 1.30 h at room temperature (r.t.) with pooled fish sera samples from each collection date and experimental group at a dilution of 1:100 in Tween 20 – TBS, pH 8. Negative controls were incubated with only the diluent of fish sera. Blots were then washed prior to the addition of anti-sea bream IgM MAbs (ADL, UK), diluted 1:300 in T-TBS and were incubated for 1 h. After another wash, blots were incubated with anti-mouse IgG conjugated with biotin (Sigma) at a dilution of 1:1000 for 30 min, washed again and extravidin-HRP (Sigma) was added at a dilution of 1:500 for a further 30 min. After a final wash, the chromogen solution was added and reactions were allowed to develop for 30 min after which they were stopped by immersing the blots in tap water.

All incubations were performed under shaking at r.t., except the first incubation with the sea bream serum samples, which was performed using a multi-screening WB apparatus (BIORAD). Blots were then dried and immediately scanned.

Statistical analysis

Statistical analysis was performed with the statistical calculation tool R and using the tests ANOVA and LSD. Comparisons were considered significantly different when $P < 0.05$.

Ethics

The work presented in the article has been carried out in an ethical way and according to Directive 2010/63/EU on the protection of animals used for scientific purposes.

RESULTS

Specific anti-*V. anguillarum* antibody titres of vaccinated sea bream

Triplicate serum samples from each vaccinated group and controls and from each sampling date were analysed with an indirect ELISA and the results are shown in Figure 1.

As shown in Figure 1 (left side), a rise of the specific anti-*V. anguillarum* antibodies was detected from D7 post-immunization for all experimental groups. The highest values of specific antibodies against *V. anguillarum* were measured in the sera of the bivalent commercial vaccine AJ group. Lower specific anti-*V. anguillarum* antibodies were measured in the sera of fish groups immunized with the aqueous commercial and experimental bacterins (AV and Evip). The control group sera did possess some low anti-*V. anguillarum* specific antibody activity.

Statistical comparison of specific antibody titres of the experimental groups sera against *V. anguillarum*, for the entire period, revealed that these titres in the immunized groups were significantly higher than the controls (*P* values: AV *vs* C = 0.012×10^{-7}; AJ *vs* C = 0.002×10^{-13}; Evip *vs* C = 0.02×10^{-5}). Specific anti-*V. anguillarum* O1 titres of the sera isolated from the AJ group were significantly higher in comparison to the other immunized groups (*P* values: AJ *vs* AV = 0.017×10^{-8}; AJ *vs* Evip = 0.013×10^{-4}). In contrast, the aqueous commercial vaccine AV had no statistical

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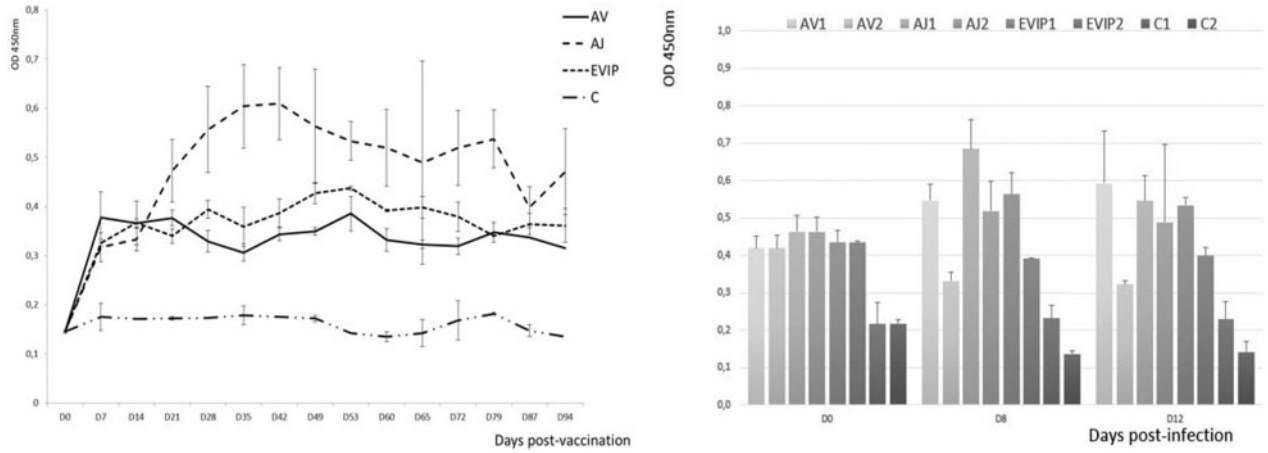

Fig. 1. Left: Development of specific anti-*V. anguillarum* sea bream antibodies over time post-vaccination. Means and standard deviation. AV, aqueous commercial vaccine, *Vibrio anguillarum*-Phdp; AJ, adjuvanted commercial vaccine, *Vibrio anguillarum*-Phdp; Evip, aqueous experimental vaccine, *Vibrio anguillarum*-Phdp; C, control, sterile 2% NaCl. Right: Development of specific antibody titres against *V. anguillarum* in vaccinated fish (samples with number 2) after infection (samples with number 1) with *V. anguillarum*. Means and standard deviation. AV, aqueous commercial vaccine, *Vibrio anguillarum*-Phdp; AJ, adjuvanted commercial vaccine, *Vibrio anguillarum*-Phdp; Evip, aqueous experimental vaccine, *Vibrio anguillarum*-Phdp; C, control, sterile 2% NaCl.

difference compared with the aqueous experimental bivalent vaccine Evip ($P = 0.174$).

Specific anti-*V. anguillarum* antibody titres of vaccinated sea bream infected with *V. anguillarum*

Vaccinated sea bream were infected with *V. anguillarum* on day 53 post-vaccination and triplicate serum samples from each infected group and controls and from each sampling date (days 8 and 12 post-infection only, due to mortality that reached 55% in the controls) were analysed with an indirect ELISA. The results are shown in Figure 2. For comparison, serum samples from days 60 and 65 post-vaccination of vaccinated but not infected fish were analysed in the same ELISA. Thus, the day zero (D0) corresponds to day 53 (D53) of the vaccinated-only fish.

Significantly higher responses (Figure 1, right side) were achieved from the vaccinated/infected (AV1, Evip1, C1) fish groups as compared to the vaccinated-only (AV2, Evip2, C2) groups (*P* values: $P = 0.03 \times 10^{-3}$, $P = 0.006 \times 10^{-2}$, $P = 0.03 \times 10^{-3}$, respectively) but this was not the case for the AJ groups where although higher specific titres were measured in the infected fish sera, these were not significantly different from the non-infected fish ($P = 0.1527$). Statistical comparisons of specific anti-*V. anguillarum* O1 reactions among the infected groups showed that significantly higher reactions were achieved from all the groups that were previously immunized (AV1, AJ1, Evip1) in comparison to the non-immunized control (C1) (*P* values: 0.0085×10^{-4}, 0.023×10^{-10}, 0.0075×10^{-5}, respectively) and that these reactions were significantly higher for the AJ1 group in comparison to the AV1 group ($P = 0.0114$) but there was no statistical difference between the AJ1 and the Evip1 and the AV1 and the Evip1 groups (*P* values: 0.0633 and 0.667, respectively).

Western Blot analysis of sea bream sera against *V. anguillarum*; vaccinated fish groups

Pooled serum samples from each blood collection date and for each experimental group (except the Eph fish group) were utilized in WB analysis against electrophoresed *V. anguillarum* O1

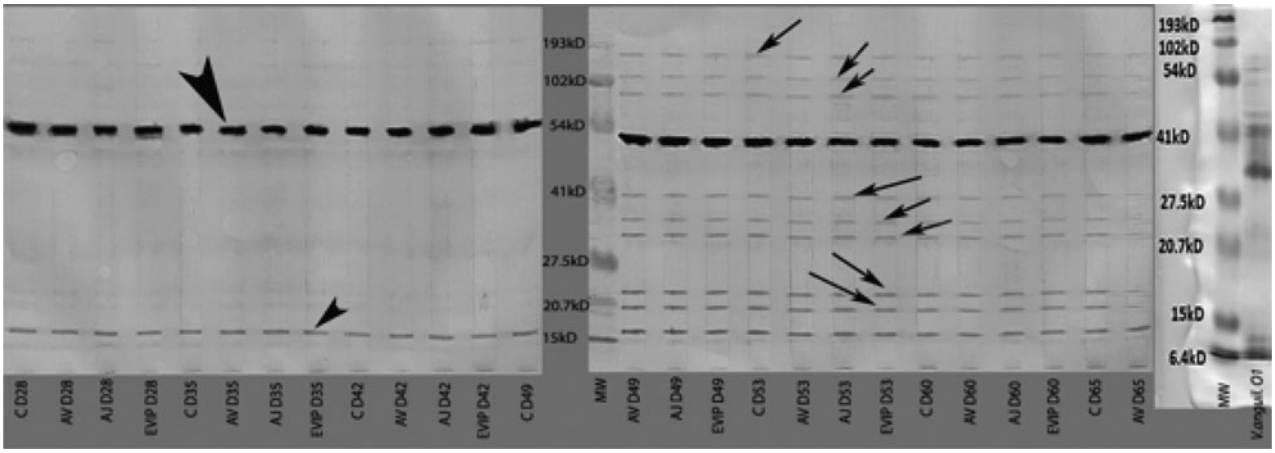

Fig. 2. Representative WB analysis of sera from vaccinated fish groups against *V. anguillarum* O1. Right of figure the electrophoresis of *V. anguillarum* O1 whole cells (Coomassie blue staining).

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whole cells. Interestingly, even from Do and from all experimental groups, including the control fish, sera reacted strongly with only two *V. anguillarum* O1 whole cell antigens at just below 54 kD (thick arrowhead, protein) and at just above 15 kD (thin arrowhead, lipopolysacharide) (Figure 2). Weak reactions with various other antigens (thin arrows, Figure 3) were noted at below 193 kD, 102 kD (2–3 antigens), between 41 kD and 27.5 kD (2–3 antigens) and at 20.7 kD (2 antigens). These reactions were directed against protein antigens (102, 41–27.5 kD, 20.7 kD) with the 193 kD reaction to be directed to a lipopolysaccharide antigen. This reaction motif was observed from Do to D94 post-immunization for all groups with the difference being the strength of reactions which were higher during D65 to D87 post-immunization and culminated to only the <54 kD and the >15 kD antigens in the last samples (D94). Negative control tests did not show any reactions.

Western Blot analysis of specific sea bream anti-*V. anguillarum* sera; vaccinated/infected fish groups

Pooled serum samples from each blood collection date and from each experimental group after infection of vaccinated fish were utilized in WB analysis against electrophoresed *V. anguillarum* O1 whole cells (Figure 3). Until D8 post-infection and for all experimental groups, except the AJ group, sera reacted with the two aforementioned major reacting antigens of *V. anguillarum* O1 whole cells. The AJ group reacted also with protein antigens between 41 and 27.5 kD (thick arrowhead) and this reaction was noticed for both the AJ and AV groups on D12 post-infection.

Specific anti-Phdp antibody titres of vaccinated sea bream

Triplicate serum samples from each vaccinated group and controls and from each sampling date were analysed with an indirect ELISA. The results are shown in Figure 4 (left side).

Specific Anti-Phdp antibody titres rose sharply at the same levels for all vaccinated fish groups from D7 post-vaccination and remained at the same levels until D14. From D21, specific anti-Phdp antibody levels of the AJ group increased further, peaked on D49 and remained higher than all the other experimental groups until the end of monitoring (D94). The specific anti-Phdp antibody titres for the other experimental groups of aqueous vaccines after the initial increase at D7 post-vaccination remained at the same levels with minor fluctuations showing a reduction tendency towards the last serum samplings. Some low anti-Phdp antibody reactions were noted from sera collected from the control group for the whole duration of the study.

The statistical comparison of the specific anti-Phdp antibody values obtained from the sera analysis of all groups (*P* values of comparisons shown in Table 1) revealed significantly higher antibody levels from all experimental groups when compared with the controls. The adjuvanted AJ vaccine group developed significantly higher antibody levels when compared with all the other vaccine groups. There was no statistical difference between the antibody levels reached by the aqueous bivalent commercial vaccine AV and the aqueous bivalent Evip and monovalent Eph experimental vaccines, while there was a small statistical difference between the two latter vaccine groups, with the Evip group specific anti-Phdp reactions being higher.

Specific anti-Phdp antibody titres of vaccinated sea bream infected with Phdp on day 65 post-vaccination

Vaccinated sea bream were infected with Phdp on day 65 post-vaccination and triplicate serum samples from each vaccinated/infected group and controls and from each sampling date (days 8, 12, 20 and 28 post-infection, since the cumulative mortality of controls reached 25%) were analysed with an indirect ELISA. The results are shown in Figure 6. For comparison reasons, serum samples from days 72, 79, 87 and 94 post-vaccination of vaccinated-only fish were analysed in the same ELISA. Thus, the day zero (Do) corresponds to day 65 (D65) of the vaccinated-only fish.

As is evident from Figure 4 (right side), specific anti-Phdp levels in the sera isolated from the vaccinated-infected fish fluctuated in higher or lower levels in comparison to the sera of the vaccinated fish during the assessment period. When the specific immune responses between vaccinated/infected and vaccinated-only fish groups were statistically compared no statistical difference was found for the AJ1 *vs* AJ2 groups ($P = 0.5514$) and the Evip1 *vs* Evip2 groups ($P = 0.06345$), while the rest of the comparisons showed that the specific reactions were significantly higher for the infected groups (AV1 *vs* AV2, $P = 0.029$; Eph1 *vs* Eph2, $P = 0.001896$; C1 *vs* C2, $P = 0.01775$). These differences were more pronounced between the monovalent Eph vaccinated/infected fish and the vaccinated-only fish.

Table 2 provides the *P* values of the statistical comparison of the specific anti-Phdp antibody levels developed in the vaccinated/infected groups of fish against this pathogen.

The AJ group sera had significantly higher anti-Phdp antibodies compared with all other groups, as did the AV, Eph and Evip groups sera compared with the controls. There was no statistical difference in the comparison of groups AV with Eph, AV with Evip and Evip with Eph.

Western Blot analysis of specific sea bream anti-Phdp sera; vaccinated fish groups

Pooled serum samples from each blood collection date and for each experimental group were utilized in WB analysis against electrophoresed Phdp whole cells. Interestingly, even from Do until D14 and from all experimental groups, including the control fish, sera reacted strongly with a Phdp whole cell lipopolysaccharide antigen below 20.7 kD, and at and below 6.4 kD with protein and lipopolysaccharide antigens, as can be concluded by the Coomassie blue stained electrophoretic profile of Phdp whole cells. Weak reactions with various other protein antigens were also noted in the whole MW range. From D21 to D35 post-immunization the only difference noted between groups was that sera from the AJ group reacted, in addition to the aforementioned antigens, with whole cell material below 102 kD, above 41 kD and at 20.7 kD (two antigens). The most complete reactions were noted from D42–D60 (Figure 5) and especially for the AJ group sera and, summarizing, they were as follows: strong reactions below 20.7 and at 6.4 kD and below (arrowheads,

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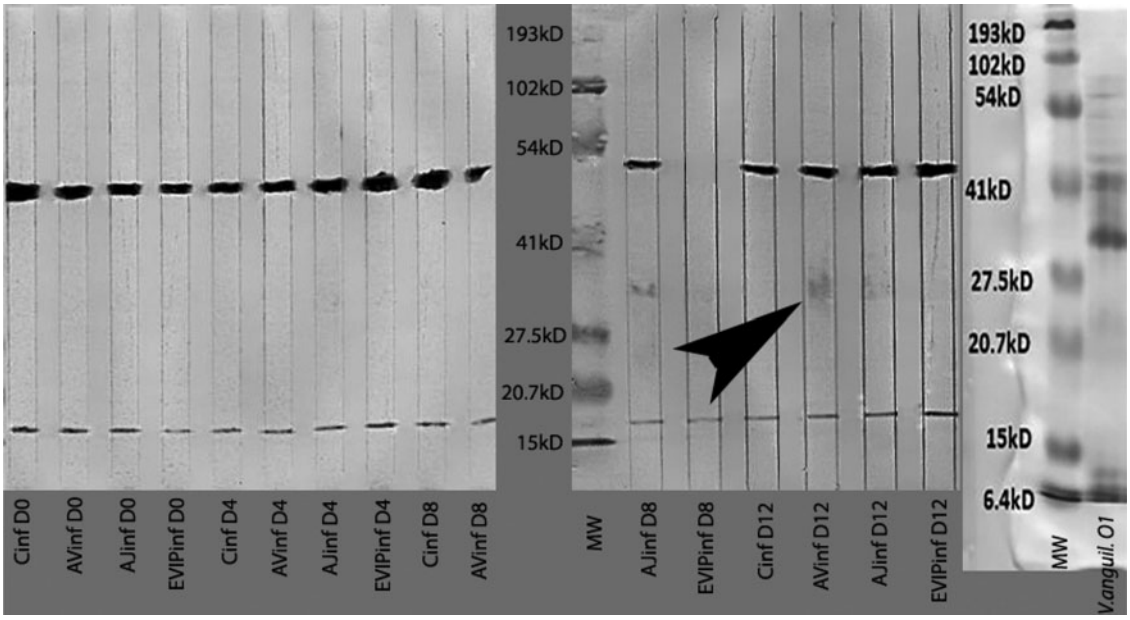

Fig. 3. Representative WB analysis of sera from vaccinated/infected fish groups against *V. anguillarum* O1. Right of figure shows the electrophoresis of *V. anguillarum* O1 whole cells (Coomassie blue staining).

Figure 5). Weak reactions (thin arrows) against many other whole cell material between 193 and 102 kD, between 102 and 54 kD, at 54 kD, above, at and below 41 kD, at 27.5 kD, above and at 20.7 kD, between 15 and 6.4 kD. And although these weak reactions were shared among all experimental groups including the controls, including the strong reaction below 20.7 kD, the major difference between groups was the reaction of the AJ sera at and below 6.4 kD, apparently unique for this group. In the following days, D65 to D94 post-immunization, the superiority of the reactions of the AJ sera both quantitatively and qualitatively remained. As is evident, no differences between monovalent and bivalent formulations were noted, in contrast to the adjuvanted and aqueous bacterins. No reactions were observed in the negative control.

Western Blot analysis of specific sea bream anti-Phdp sera; vaccinated/infected fish groups

Pooled serum samples from each blood collection date and for each experimental group after infection of vaccinated fish were utilized in WB analysis against electrophoresed Phdp whole cells (Figure 6). For the whole monitoring period, Do–D28 post-infection, and for all experimental groups, sera reacted to similar Phdp whole cell antigens, as described for the WB analysis with the sera from the vaccinated fish (Figure 5) with the AJ sera reacting strongly with antigens below 15 kD at the area of 6.4 kD of Phdp whole cells. A strong reaction of sera from the control/infected group was noted at 20.7 kD and only at D16 post-infection. This

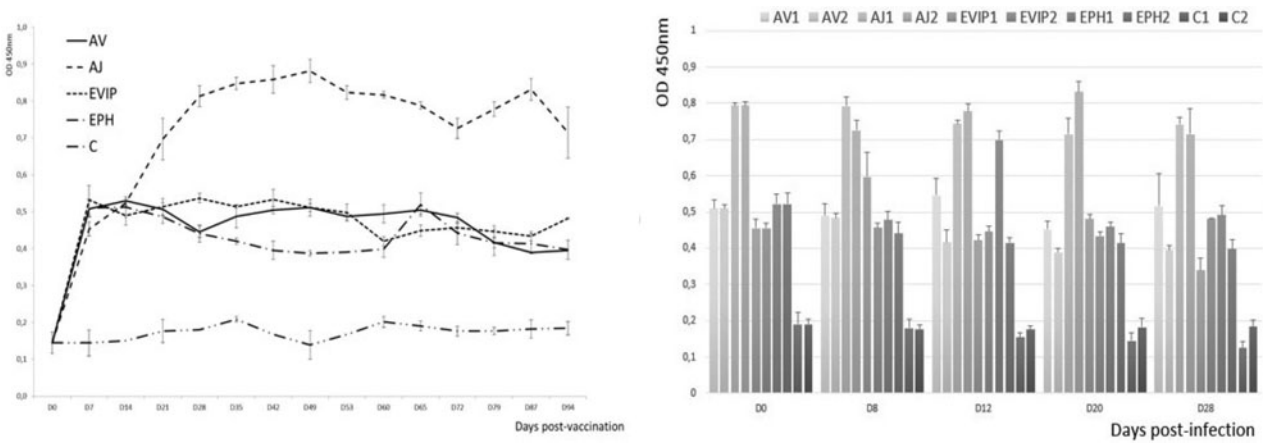

Fig. 4. Left: Development of specific anti-Phdp sea bream antibodies over time post-vaccination. Means and standard deviation. AV, aqueous commercial vaccine, *Vibrio anguillarum*-Phdp; AJ, adjuvanted commercial vaccine, *Vibrio anguillarum*-Phdp; Evip, aqueous experimental vaccine, *Vibrio anguillarum*-Phdp; Eph, monovalent aqueous experimental vaccine; C, control, sterile 2% NaCl. Right: Development of specific antibody titres against Phdp in vaccinated fish (samples with number 2) after infection (samples number 1) with Phdp. Means and standard deviation. AV, aqueous commercial vaccine, *Vibrio anguillarum*-Phdp; AJ, adjuvanted commercial vaccine, *Vibrio anguillarum*-Phdp; Evip, aqueous experimental vaccine, *Vibrio anguillarum*-Phdp; Eph, monovalent aqueous experimental vaccine; C, control, sterile 2% NaCl.

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Table 1. Statistical differences of specific antibody titres against Phdp in vaccinated fish.

Fish groups	C	AV	AJ	Evip
AV	0.002×10^{-13}	–	–	–
AJ	0.002×10^{-13}	0.002×10^{-13}	–	–
Evip	0.002×10^{-13}	0.104	0.002×10^{-13}	–
Eph	0.002×10^{-13}	0.426	0.002×10^{-13}	0.034

reaction was unique for this group and for this day post-infection, since it disappeared in the following days.

DISCUSSION

The aim of this study was to analyse and compare the quantitative and qualitative aspects of the humoral immune response of sea bream after vaccination with multivalent (*V. anguillarum* and Phdp) or monovalent, for Phdp only, commercial and experimental vaccines. And, more specifically, to evaluate if the inclusion of adjuvant affects the repertoire of antigens recognized and if the presence of multiple antigens in polyvalent vaccines interfere with the immune responses towards each different pathogen. This research was prompted by the scarce information on the elucidation of specific immune responses raised by commercial multivalent vaccines and by studies (Nikoskelainen *et al.*, 2007) indicating, in other fish species, that multiple antigens in polyvalent vaccines may interfere with the specific responses obtained both quantitatively as well as in respect to the biological functions of specific antibodies.

Serving the aim of this study, sea bream were i.p. immunized against *V. anguillarum* O1 and Phdp with two polyvalent commercial vaccines, one containing a mineral oil adjuvant (AJ) and the other being an aqueous solution (AV), one laboratory-made aqueous polyvalent solution (Evip) and another one monovalent (against Phdp) aqueous solution (Eph). To these four fish groups, a control group was added which received an i.p. injection of sterile 2% NaCl. All fish groups were infected at certain time points during the study with either *V. anguillarum* O1 or Phdp. Sera were collected from immunized and immunized/infected fish and were analysed for specific antibodies against the two pathogens both quantitatively and qualitatively.

In this study, specific antibody levels against either of the pathogens and for all experimental groups (excluding controls) increased from D7 post-vaccination and in both occasions, specific antibody levels continue rising for the adjuvanted AJ vaccine group, which reached a plateau from D35 to D49 post-vaccination. For all the groups that were vaccinated with the aqueous formulations, after the initial rise of specific antibodies titres, a fluctuation of these titres was observed around similar levels for the whole duration of the study. A similar increase 2 weeks post vaccination of specific anti-*V. anguillarum* antibodies was measured by Coeurdacier *et al.* (1997) in sea bass, a week later in comparison to our study, that reached a plateau after D50 and elevated levels lasted for longer than 10 months. In our study and for the aqueous formulations this plateau was reached very quickly and fluctuated around the same levels for about 100 days post-immunization. In another study, specific anti-Phdp antibody levels increased by D9 post-immunization (Bakopoulos *et al.*, 2015b). Santarem & Figueras (1994) in turbot (*Scophthalmus maximus*) and Arijo *et al.* (2004) in sea bream showed that specific anti-Phdp antibodies against O-antigen or whole bacterial cells, respectively, peaked at D28 post-immunization and a similar pattern was observed in our study for both pathogens regarding the sera collected from the AJ group (with the week to week differences attributed to the formulations and the physical water parameters of each different experiment).

Table 2. Statistical difference between the specific antibody levels developed against Phdp in vaccinated/infected fish groups.

Fish groups	C1	AV1	AJ1	Evip1
AV1	0.0014×10^{-12}	–	–	–
AJ1	0.002×10^{-13}	0.002×10^{-13}	–	–
Evip1	0.0066×10^{-10}	0.884	0.002×10^{-13}	
Eph 1	0.005×10^{-11}	0.298	0.002×10^{-13}	0.352

Specific antibody titres against Phdp produced by all the experimental groups were higher when compared with the specific anti-*V. anguillarum* antibody titres, in respect to the magnitude of reactions in the ELISA, despite plates being coated with a similar number of bacterial cells. This indicates that Phdp antigens cause a higher stimulation of the humoral immune response. Similar magnitude of reaction observations were made in a previous study with sea bass (Bakopoulos *et al.*, 2015b). Furthermore, Schroder *et al.* (2009) reported that specific antibodies against *V. anguillarum* antigens are directed towards a limited number of antigens, such as primarily to LPS, as was observed in this study as well.

Specific antibodies titres raised against either of the pathogens were significantly higher from the experimental group that was immunized with the adjuvanted commercial vaccine (AJ). This is in agreement with previous studies (Bakopoulos *et al.*, 2015a, b) and the presence of adjuvant has been shown to have a profound effect on the efficacy of adjuvanted *vs* aqueous formulations (Gravningen *et al.*, 1998; Lund *et al.*, 2003; Nikoskelainen *et al.*, 2007; Bakopoulos *et al.*, 2015a). This effect is due to stimulation of non-specific immune factors; it has been shown adjuvants induce a strong attraction of different types of leukocytes in the area of vaccination and can have a prolonged effect due to slower release of antigen (Afonso *et al.*, 2005). The monovalent formulation against Phdp (Eph) did not cause the production of higher specific anti-Phdp titres in comparison to the other aqueous formulations (both commercial and experimental). As a matter of fact anti-Phdp titres caused by the Eph formulation were not statistically different from the titres caused by the polyvalent AV commercial vaccine and were statistically lower than the titres caused by the experimental Evip formulation. This has been noted from similar studies with sea bass (Bakopoulos *et al.*, 2015b), and there are studies (Hoel *et al.*, 1997, 1998) suggesting that when antigens from Vibrio species (such as *V. salmonicida*) are included in multivalent bacterins (i.e. *V. salmonicida* plus *Aeromonas salmonicida*) these may play an adjuvant role.

Interestingly, some low but measurable reactions from the sera collected from the control group, even from Do post-immunization, were noted against both *V. anguillarum* O1 and Phdp. These results usually remain unnoticed, not mentioned or not discussed in similar studies, but in view of the

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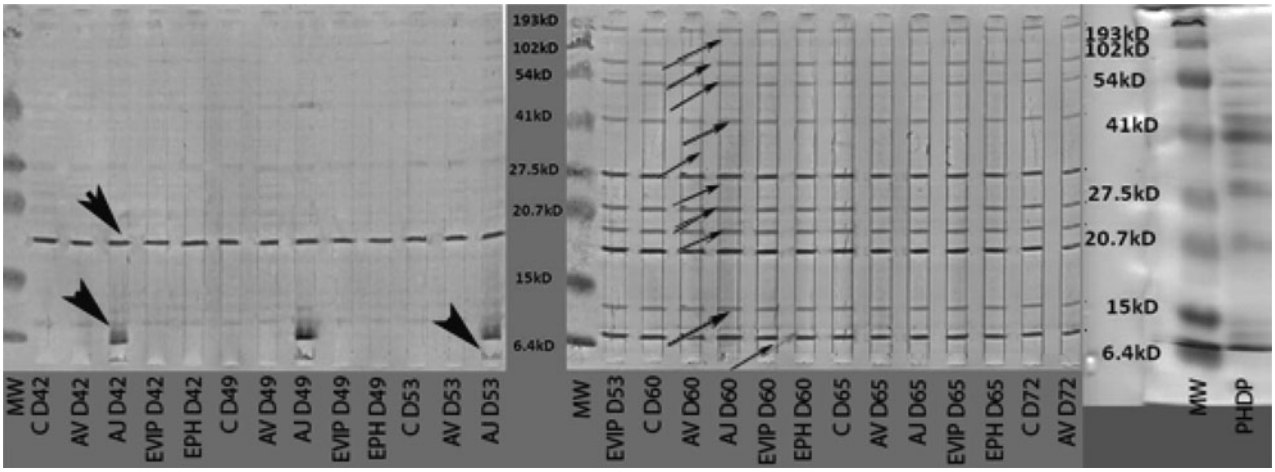

Fig. 5. Representative WB analysis of sera from vaccinated fish groups against Phdp. Right of figure shows the electrophoresis of Phdp whole cells (Coomassie blue staining).

WB analyses performed in this study, deserve more attention. Reactions of sera collected from control sea bream groups towards the outer membrane and the O-antigen of Phdp were found by Arijo *et al.* (2004) and, similarly, Nikoskelainen *et al.* (2007) found that control rainbow trout sera reacted with *A. salmonicida* and *Flavobacterium psychrophilum*, but not *V. anguillarum* O1, whole bacterial cells. In all these cases, similar to this study, fish that were not previously vaccinated against the various pathogens were used. Furthermore, the fish used in this study originated from a hatchery facility using water from wells and, therefore, never encountered the pathogens in their culture environment. These reactions have been attributed to natural 'general purpose' antibodies (Strømsheim *et al.*, 1994) reacting with antigens of pathogens, as suggested by Ardó *et al.* (2010) in common carp against *A. hydrophila* and by Sinyakov *et al.* (2002) in carp (*Carassius aurata*) against *A. salmonicida*.

All vaccinated experimental groups, including the controls, were infected with low doses of either *V. anguillarum* O1 or Phdp in order to investigate if these infections could act as immune response boosters in the surviving fish, as might well happen regularly in the field. Specific anti-*V. anguillarum* O1 antibody titres from D8 post-infection and for all experimental groups were significantly higher, except for the AJ group sera, compared with vaccinated-only fish during the same period (D60 and D65 post-vaccination) and compared with D7 and D14 post-vaccination.

Specific anti-Phdp antibody titres from D0 to D28 post-infection showed fluctuation in respect to the magnitude of responses as compared with the vaccinated-only fish for the same period (D65 to D94). Statistical evaluation of the measured antibody levels showed that infection acted as a boost of the humoral response for only the AV and the Eph groups, with the latter group's specific reactions showing the highest statistical difference from the vaccinated-only fish.

Despite the low numbers of samples collected and analysed from the fish infected with *V. anguillarum* O1 and the fluctuation in specific antibody levels measured from the sera collected after infection with Phdp, the early response to infection was an increase of specific antibody levels on both

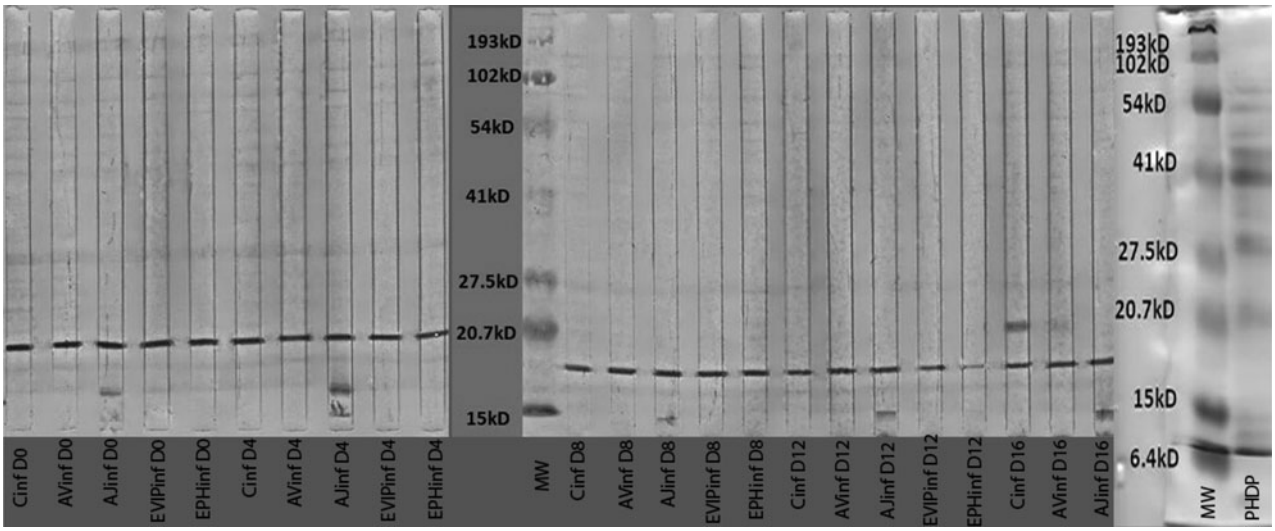

Fig. 6. Representative WB analysis of sera from vaccinated/infected fish groups against Phdp. Right of figure shows the electrophoresis of Phdp whole cells (Coomassie blue staining).

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occasions. Also, on both occasions the sera collected from the AJ group did not show significantly different specific antibody titres from the vaccinated-only fish, a result that is probably due to the already higher specific antibody titres evidenced for these sera against both of the pathogens.

The infection did act as a booster of the immune response early post-vaccination for the aqueous bacterins and this was more pronounced for the monovalent anti-Phdp Eph formulation. This is an indication of interference of multiple antigens in polyvalent products on the magnitude of the secondary humoral immune response.

No remarks can be made as to whether infection can act as a boost for the adjuvanted bacterin (AJ) since the high specific antibody titres may interfere with the immune stimulation of the infection and infection further away from the initial vaccination could very well result in different findings. Coeurdacier *et al.* (1997) did show an increase of specific anti-*V. anguillarum* serum antibodies in sea bass boosted with inactivated cells of the pathogen, which in the long term was not statistically significant in comparison to fish immunized only once and a similar result was seen earlier in turbot by Estevez *et al.* (1994). On the other hand, Arijo *et al.* (2004) did show an increase of specific anti-Phdp titres in sea bream which were vaccinated with an inactivated Phdp whole cell bacterin and then boosted with the same bacterin, with our results being in agreement with the latter study. More specifically, these authors found significant increases of serum antibody titres to the bacterin, ECPs, OM and LPS but, in contrast, the serum antibody titres for other antigenic fractions, such as O antigen, were not higher than those obtained in the first immunization.

In order to illustrate against which antigens of *V. anguillarum* O1 specific antibodies in the sera collected from the experimental groups are directed and to determine if there are any differences between groups, sera from each collection date post-vaccination were used in WB analysis of *V. anguillarum* O1 whole bacterial cells. Interestingly, sera from all experimental groups reacted strongly with only two antigens at <54 and at >15 kD and weakly with a range of other antigens. No changes were noted regarding the repertoire of antigens recognized in relation to time post-vaccination, except for the strength of reaction that became higher as the immune response was maturing and especially for the sera collected from the AJ group. The latter was due to the higher levels of specific anti-*V. anguillarum* O1 titres measured at the ELISA analysis. To our knowledge there is no published information regarding the antigens of *V. anguillarum* O1 recognized by sea bream immunized with either experimental or commercial vaccines. An article published by Schroder *et al.* (2009) concerning the homologous serum reaction of Atlantic cod (*Gadus morhua*) against *V. anguillarum* O1 bacterial cells revealed, in agreement to our results, recognition of few antigens at 75, 25 and 20 kD. The 20 kD antigen maybe the same with the >15 kD antigen recognized in this study. Differences in antigen recognition by immune sera between different animal species are to be expected and it has been illustrated before (i.e. Bakopoulos *et al.*, 1997). Sera collected from vaccinated fish that were infected with *V. anguillarum* O1, at D8 and D12 and from the AJ and AV groups, reacted with additional antigens of the pathogen indicating that boosting the immune response may give a better qualitative result regarding the repertoire of antigens recognized.

Similar WB analysis was performed with Phdp whole bacterial cells. Again, the antigens recognized by the sera from the various experimental groups were limited to an antigen at <20.7 kD and at and below 6.4 kD, against which reactions were strong and a number of other antigens against which reactions were weaker. The stronger, more complete reactions were noted from the sera collected from the AJ group which reacted with a higher range of antigens in comparison to sera collected from the other groups. The sera isolated from the experimental group immunized with the monovalent formulation (Eph) did not recognize any different Phdp antigens. It is evident that the inclusion of an adjuvant to the formulation which induces a strong attraction of different types of leukocytes in the area of vaccination (Afonso *et al.*, 2005) not only enhanced the production of specific anti-Phdp levels, but also had a positive effect on the repertoire of antigens recognized. This has positive implications on the efficacy of the vaccine, as has been previously recognized (Gravningen *et al.*, 1998; Bakopoulos *et al.*, 2015a). In the study of Hanif *et al.* (2005) sera from sea bream broodstock immunized twice with inactivated whole Phdp bacterial cells reacted with Phdp antigens at the range of 117–34.7 kD, contradicting the reactions we saw in this study. These differences may be due to the age of fish and the media used for the cultivation of Phdp for the production of the bacterin (Bakopoulos *et al.*, 1997, 2003a) which influence the phenotypic expression of antigens. The reactions seen in the study of Hanif *et al.* (2005) may well correspond to some of the weak reactions of the sera seen in this study. The WB analysis of sera from all fish groups that were infected with Phdp, did not reveal any differences in antigen recognition among groups, with the reactions seen from the AJ group remaining stronger and towards more antigens, as for the vaccinated-only fish.

An interesting finding of the WB analyses performed against both *V. anguillarum* O1 and Phdp is that the control groups (even at Do), which were not immunized against any of the pathogens, reacted with the same antigens of *V. anguillarum* O1 and Phdp cells with which the immune sea bream sera reacted, with the exception of the reactions of the sera from the AJ group at low molecular weight against Phdp. These reactions correspond to the presence of antibodies against both pathogens in the control sera that were analysed with ELISA and a similar phenomenon has been noted by other researchers. For example, Coeurdacier *et al.* (1997) noticed a drop in antibody titres in sea bass after immunization with inactivated *V. anguillarum* cells and the decrease of IgM level after injection was explained as the result of consumption of IgM, with paratopes close to the injected antigen epitope, which were mobilized to compensate for the lack of the specific antibodies. Arijo *et al.* (2004) did find, but did not discuss, reactions of control sea bream sera against the OM and O antigen of Phdp. Furthermore, Schroder *et al.* (2009) showed that sera from Atlantic cod immunized against the O1, O2, O2a and O2b serotypes of *V. anguillarum*, including the controls (injected with saline), all reacted with the same antigens of *V. anguillarum* O1, with no difference between heterologous, homologous and control sera. These reactions could be attributed to the presence of natural antibodies with low specificity against specific antigens (Sinyakov *et al.*, 2002; Ardó *et al.*, 2010) or to specific antibodies against *V. anguillarum* O1 or Phdp that were produced as a response of the fish contact with low

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numbers of these, endemic to the marine environment, fish pathogens before their arrival to the laboratory. However, the latter is not the case for the fish used in this study, since they never came into contact with these pathogens.

Concluding, the adjuvanted formulation caused the production of higher specific antibody levels against both pathogens due to the previously described local inflammatory effects caused by the adjuvant. This attraction of large numbers of leukocytes at the site of injection is beneficial for the magnitude of the humoral immune response.

The monovalent Phdp bacterin formulation did not cause the production of higher specific anti-Phdp antibody levels in comparison to the other aqueous bivalent formulations tested, indicating that the combination of *V. anguillarum* O1 and Phdp whole cell antigens does not influence the magnitude of the specific humoral immune response against Phdp. Infection of vaccinated fish with low doses of either of the pathogens resulted in boosting the humoral response in respect to its magnitude, evident only for the aqueous formulations in this study. Therefore, when adjuvanted vaccines are used, an anamnestic vaccination should be performed only when specific antibody titres have dropped. Otherwise, a positive effect on the magnitude of the humoral immune response cannot be expected.

When fish immunized with the monovalent Phdp formulation were infected with Phdp, a higher magnitude secondary humoral immune response was measured indicating a negative effect of multiple antigens in the vaccines, at least in respect to anamnestic immunizations.

Sea bream specific anti-*V. anguillarum* O1 antibodies recognized only a handful of antigens and there was no difference between the formulations used, except for the strength of reaction noted by the adjuvanted vaccine group. These same antigens were recognized by sera originating from the control group. Sea bream specific anti-Phdp antibodies, similarly, recognized only a handful of Phdp antigens and there was no difference between the aqueous bivalent and monovalent formulations and the controls, in contrast to the adjuvanted formulation. Sera from the latter group reacted more strongly and with a higher repertoire of antigens. The inclusion of adjuvants not only increased the magnitude of the humoral immune response but also had a positive effect on the repertoire of antigens recognized by the immune sera.

Further research is required towards the improvement of the repertoire of antigens recognized, with the adjuvanted formulations being superior in this respect for the time being. In addition, since polyvalent products need to be produced (i.e. to include antigens from fish nodaviruses or other emerging bacterial pathogens), the complex interactions between these antigens (i.e. immunodominance, immune suppression) need to be evaluated.

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