EFFICACY TESTING OF AN IMMERSION VACCINE AGAINST *AEROMONAS SALMONICIDA* AND IMMUNOCOMPETENCE IN BALLAN WRASSE (*LABRUS BERGYLTA,* ASCANIUS)

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Abstract

The development of effective vaccines is a critical step towards the domestication of emerging fish species for aquaculture. However, traditional vaccine delivery through intraperitoneal (i.p.) injection requires fish to reach a minimum size and age and therefore cannot provide protection at early developmental stages when infection may occur. This study investigated the effectiveness of immersion vaccination with respect to immunocompetence in a cleaner fish species (ballan wrasse, *Labrus bergylta*, Ascanius) used in Atlantic salmon farming as an alternative means to control sea lice. The species is susceptible to atypical strains of *Aeromonas salmonicida* (a*As*) at early life stages (<15 g), when i.p. vaccination is not applicable. While immersion vaccination is currently used in commercial hatcheries, the optimal fish size for vaccination, and efficacy of the vaccine delivered by this route has not yet been established. Importantly, efficacy depends on the capability of the species immune

system to recognise antigens and process antigens to trigger and produce an adaptive immune response, (process known as immunocompetence). In this study, the efficacy of a polyvalent autogenous vaccine administered by immersion in juvenile ballan wrasse and the subsequent immune response induced was investigated after prime and booster vaccination regimes. In addition, temporal expression (0 - 150 days post hatch) of adaptive immune genes including major histocompatibility complex (MHC II CD74 molecule) and immunoglobulin M (IgM) was assessed using quantitative PCR (qPCR). Prime and/or boost vaccination by immersion of juvenile ballan wrasse (0.5 g and 1.5 g corresponding to 80 and 170 days post hatch (dph), respectively) did not provide significant protection against a*As vapA* V after bath challenge under experimental conditions. Despite no evident protection > 80 dph, MHC II and IgM transcripts were first reported at 35 and 75 dph, respectively, suggesting a window of immunocompetence. The results provide important new information on the onset of adaptive immunity in ballan wrasse and highlight that immersion vaccination in the species for protection against a*As* should be performed at later developmental stages (>1.5 g) in the hatchery.

Key words: ballan wrasse, immersion vaccination, *Aeromonas salmonicida,* adaptive immunity, IgM, MCH II.

1. Introduction

Atypical strains of *Aeromonas salmonicida* (a*As*) are currently the most significant cause of mortalities in hatchery and post cage deployment of farmed ballan wrasse (*Labrus bergylta*, Ascanius) [1]. The bacterium is highly heterogenous infecting a variety of fresh and marine fish species and host specificity of strains has been reported [2]. Recently, partial sequencing of the paracrystalline surface array protein gene (*vapA* gene) has successfully been used to distinguish typical and atypical *As* strains to 23 *vapA* types [3, 4]. Ballan wrasse is susceptible to a*As vapA* type V and VI in Scotland and Norway, respectively [4]. The successful development and application of vaccines to regulate bacterial challenges is increasingly important for ballan wrasse, as an emerging species, however such developments are hampered by the lack of basic understanding of immunity and immunocompetence in the species [5].

Vaccination is the most common disease prevention strategy in the finfish aquaculture industry [6]. Vaccines can be administrated by either injection (intraperitoneally (i.p.) or intramuscularly (i.m.), immersion (dipping or bathing) and in some cases orally in feed [7]. Each practise has advantages and disadvantages. For instance, i.p. injection is the most commonly used method for juvenile fish (>15 g) generating greater protection than immersion especially facilitated by

including effective adjuvants, and provides a platform for standardised dosing of vaccine for each individual fish [6, 8]. However, immersion is the preferred method for small fish (0.5 – 25 g) as it is less invasive, therefore minimising handling stress, is often more affordable and less labour intensive [6, 8]. A combination of these practises is usually applied in marine fish which are immunised initially by immersion (prime and booster may be required) at an early life stage and then i.p. injection vaccinated before being deployed to sea cages for on growing or, in the case of cleaner fish, for sea lice control [9].

Following immersion vaccination, immune related cells in the mucosa-associated lymphoid tissue (MALT) uptake the antigen [10]. Several studies have investigated the efficacy of immersion vaccination and antigen uptake however our understanding of the mucosal immune response in fish still remains limited [11]. A prerequisite for successful vaccination is a fully developed functional immune system able to develop immunological memory. The ontogeny of the immune system has been studied in various teleost fish species, showing species specific windows ranging from 8 days pre-hatching in rainbow trout (*Oncorhynchus mykiss*, L.) up to 5 and 10 month post hatch for carp (*Cyprinus carpio*, L.) and zebrafish (*Danio rerio*, Hamilton), respectively [12]. The anterior kidney and thymus are considered to be fully developed prior to hatching in rainbow trout (*Oncorhynchus mykiss*, L.) and Atlantic salmon [13], while immunocompetence (presence of IgM) occurs later during alevin development and is size dependant (fish of 20 – 30 mm in length) [14, 15]. In marine fish species, like sea bass (*Dicentrarchus labrax*, L.), spotted wolffish (*Anarhichas minor*, L.) and Atlantic cod (*Gadus morhua*, L.) studies have shown IgM positive lymphocytes between 1 and 10 weeks after hatching [15]. There are three main immunoglobulin (Igs) isotypes in teleost fish including IgM, IgD and IgT. Expression of functional Igs gives an indication of immune competence this would facilitate the identification of an immersion vaccination window prior to i.p. injection and after immunocompetence has been achieved [16]. IgM is the first antibody produced in teleost fish and it consistently appears at the mucosal sites in contrast with IgT that is not found in every fish species [17]. Thus, IgM is broadly used as a marker for immune competence. Another important component of the adaptive immune system in fish is the major histocompatibility complex (MHC) which is responsible for the recognition and presentation of antigen epitopes on T cell receptors (TCRs) [18]. The MHC genes produce two membrane glycoproteins class I and II [19]. MHC II express glycoproteins which are present only in antigen presenting cells (APCs) such as B cells and trigger the production of specific antibodies against exogenous antigens [20]. The binding with T helper cells (Th) activates B cells and memory cells differentiation [20]. Literature on ballan wrasse immunity is very limited and the ontogeny of the adaptive immune response has not been studied to date. Haugland, et al. [21] previously characterised the cellular innate response, and Bilal, et al. [22], [23] have reported adaptive

immune responses with regards to the presence of IgM, T cell receptors (TCRs) and somatic mutations of lymphocytes. In addition, the relative expression of antibodies (IgM, IgT and IgD) and TCR genes has been studied in the gut of ballan wrasse [24].

In an attempt to address disease challenges due to a*As (vapA* type V), juvenile ballan wrasse in commercial Scottish hatcheries have typically been bath vaccinated at ca. 0.5 g, followed by booster vaccination at 1.0 – 1.5 g (1:10 dilution for 3 – 4 h) and then administered an i.p. injection booster between 10 and 15 g with a polyvalent autogenous vaccine (Ridgeway Biologicals, DDT Germany). In recent experimental trials, the injectable version of this commercial vaccine offered high protection to ballan wrasse (25 – 50 g) when fish were challenged with heterologous and autologous strains of a*As vapA* type V (RPS 79 % and 91 %, respectively) [25]. However, it became apparent that the species can encounter and succumb to bacterial disease in commercial hatcheries at earlier developmental stages (prior to i.p. vaccination) [26] and immunisation at earlier developmental stages is therefore needed. The efficacy of the autogenous immersion vaccine currently used in wrasse hatcheries has not been assessed and the size at which fish can be successfully vaccinated by immersion remains to be determined.

The aims of this study were to 1) assess the efficacy of the immersion autogenous vaccine vaccination against a*As* to evaluate if the current commercial strategy of bath vaccination was effective for the autogenous vaccine and 2) to determine the ontogeny of the farmed ballan wrasse adaptive immune system using temporal gene expression analysis of immune genes for antigen presentation (MHC II) and antibody production (IgM). Results will ultimately help to optimise the vaccination strategy for farmed juvenile ballan wrasse.

2. Materials and Methods

2.1. Immersion vaccination and challenge trial

2.1.1. Fish acclimation

Two batches of juvenile ballan wrasse (0.3 ± 0.15 g at 60 days post hatch (dph) and 1.0 ± 0.2 g at 150 dph) produced from eggs collected from a common broodstock population were obtained from a joint venture hatchery owned by MOWI (Scotland, UK) and Scottish Sea Farms and transported to the Centre for Environment, Fisheries and Aquaculture Science (Cefas, Weymouth UK). Fish were kept in aerated aquaria (150 L) during an acclimation period of three weeks at a constant temperature of 15 ± 1 °C and photoperiod of 20 h light: 4 h dark. Water flow was set at 4.0 L / min and dissolved oxygen (DO) maintained at 8.0 ± 0.5 mg / L. Fish were fed with Otohime Fish Diet B2 & C1 (Biokyowa, Japan) at 7 – 8 % of the body weight for 7 h / day using automatic feeders.

2.1.2. Fish health assessment

The health status of the fish was assessed prior to transportation as described in a previous study [25]. Briefly, whole larvae were screened for pathogens including common isolated bacteria (a*As* and Vibrionaceae) as well as amoebic gill disease (AGD) by histology, bacteriology and molecular techniques [27-29]. In addition, fish health was assessed upon arrival to Cefas facilities for biosecurity and precautious experimental measures and to confirm that fish were not carrying any notifiable viral diseases as listed in the OIE manual of diagnostic tests for aquatic animals [30]. Furthermore, whole fish samples were fixed in 10% neutral buffered formalin to confirm by histology that fish did not have any pathology prior to challenge.

2.1.3. Immersion vaccination and sampling

The two fish size cohorts post acclimation (small (S) - 0.5 g ± 0.2 g at 80 dph and large (L) - 1.5 ± 0.4 g at 170 dph) herein designated as small and large, were bath vaccinated with a formalin inactivated multivalent autogenous vaccine provided by Ridgeway Biologicals Ltd (IDT Biologika, Germany) in static sea water for 3 h at 15°C. The isolates contained in the vaccine were recovered from diseased ballan wrasse sampled at commercial hatcheries in Scotland and characterised as part of a previous study [26]. The vaccine used contained 9 bacterial strains, 4 of which were a*As* (3 strains of *vapA* type V; and 1 strain of *vapA* type VI;) and 5 isolates belonging to the *Vibrionaceae* family (Table 1). The vaccine was diluted 1:100 and the final vaccination doses are given in Supplementary Table 1. Mock vaccinated fish were exposed to sterile sea water (33 ppt). Tissues (liver (L), spleen (S) and head kidney (HK)) were sampled from vaccinated and mock vaccinated groups (n= 6 per group) at pre-vaccination (0 h) and 24 h post prime and booster vaccination. Samples were preserved in RNA stabilisation buffer [31] for later immune genes expression analysis.

Table 1. Bacterial isolates in the autogenous immersion vaccine.

Isolate ID	Bacterial species	Year of isolation	*vapA* type and /or isolate #
TW3/14	*Aeromonas salmonicida*	2014	V
TW4/14	*Aeromonas salmonicida*	2014	V
TW187/14	*Aeromonas salmonicida*	2014	V
TW184/16*	*Aeromonas salmonicida*	2016	VI
TW242/16	*Aliivibrio logei*	2016	Isolate 1
TW322/16	*Aliivibrio salmonicida*	2016	Isolate 1

TW130/16	*Vibrio splendidus*	2016	Isolate 1
TW319/16	*Vibrio ichthyoenteri*	2016	Isolate 1
TW138/16	*Photobacterium indicum*	2016	Isolate 1

(*) isolated from lumpsucker.

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160 **2.1.4. Bacteria preparation for bath challenge**

161 From previous studies, the a*As vapA* type V strain (TW 4/14) isolated from infected farmed
162 ballan wrasse in Scotland and used in the formulation of the vaccine, caused mortalities > 60
163 % in fish exposed by bath and i.p. injection under experimental conditions [25, 32]. This
164 homologous strain was used for the bath challenge to assess the efficacy of the immersion
165 vaccine and it was grown in Tryptone Soya Broth (TSB) at 22°C for 48 h with continuous
166 shaking at 150 rpm. The bacteria were centrifuged at 2,000 x g for 10 min, bacterial pellets
167 were then washed with phosphate buffered saline (PBS) and re – suspended in sterile sea
168 water to an OD_{550} 0.8. Colony forming unit (CFU) per mL were counted by the 10 – fold dilution
169 method.

170 **2.1.5. Bath challenge and sampling**

171 Fish were challenged as described in an established challenge model for the species [32].
172 Briefly, all groups were starved for 24 h prior to challenge. Triplicate groups of vaccinated and
173 mock vaccinated fish were bath challenged with a*As vapA* type V strain (TW 4 / 14) after 654-
174 degree days (DD) post prime immunisation and after 495 DD post booster in small aerated
175 aquaria of 5 L for the small cohort and 8 L for the large cohort (50 fish / aquarium). The bacteria
176 were adjusted to an OD_{550} 0.8 (10^8 - 10^9 CFU / mL in 5 or 8 L of static sea water) and fish were
177 challenged for 4 h at 15 °C, as shown in Supplementary Figure 1. Control groups were
178 exposed to sterile sea water. The fish were then allocated to 30 L holding aquaria each with
179 water flows of 0.5 L / min, while all other parameters remained the same as outlined prior. The
180 fish were monitored for up to 30 days post infection (dpi). Ballan wrasse with obvious signs of
181 disease were considered as moribund and were sacrificed using an overdose of MS-222 (40
182 ppm; Tricaine methane sulphonate, Sigma). Head kidney swabs were sampled from moribund
183 fish (n= 5 per tank) and plated onto tryptone soya agar (TSA) to confirm specific mortality due
184 to a*As*. Suspected a*As* colonies were confirmed with the agglutination test (Mono-AQUA
185 BIONOR™, BIONOR). In addition, liver, spleen and head kidney tissue samples (n= 299; 100
186 from L, 99 from S and 100 from HK) from all moribund fish were fixed in 100% ethanol for
187 bacterial species / type confirmation with previously published conventional PCRs and qPCR
188 as described below [33, 34]. Tissues samples (L, S and HK; n= 6 per treatment for vaccinated,

mock vaccinated and control groups) were fixed in RNA stabilisation buffer after termination of the challenge trial for gene expression analysis.

2.1.6. DNA extraction and quality check

DNA was extracted from preserved tissues using a salt precipitation method as described in Khanam, et al. [35]. The extracted DNA was re-suspended in 5 mM Tris, concentration measured and standardised at 100 ng / µL using NanoDrop® ND-1000 (Thermo Fisher Scientific). Sample integrity was validated by gel electrophoresis. The samples were stored at - 20°C until use.

2.1.7. Conventional PCRs and Quantitative PCR (qPCR) for *Aeromonas salmonicida*

The presence of bacterial DNA was assessed on the V3-V4 hypervariable region of the *16S* rRNA gene – 16S PCR; if samples were negative no further testing was carried out as bacterial load in the samples was considered below the assay detection limit. If positive, samples were then screened with a species-specific PCR (*vapA*) for presence of *As* DNA using a previously published PCR protocol targeting the hypervariable region of *vapA* gene – *vapA* PCR [4]. An a*As vapA* type V specific PCR (*aAs* type V specific PCR) previously developed by our group [36] was used to determine the presence or absence of a*As vapA* type V. The primer pairs used for the tissue samples screening with conventional PCRs are listed in Table 2. The relative molecular weight of the amplicons was compared against a 100 bp gene ruler (Thermo Scientific) on 1 % agarose gel. *Aeromonas salmonicida* bacterial loads were assessed on tissue samples that were confirmed positive for *As* DNA with the conventional PCRs using a modified protocol [34] as described in [36]. The primers and qPCR conditions are listed in Table 1. Any samples above 35 Cross point were considered negative.

Table 2. Primers used for amplification of broad-range bacterial DNA, *Aeromonas salmonicida* and atypical *Aeromonas salmonicida vapA* type V specific with conventional PCR and quantitative PCR.

Gene	Oligo sequence	Annealing (°C)	Product size (bp)	Publication
Bacterial *rRNA* 16S	F341: CCTACGGGNGGCWGCAG R805: GACTACHVGGGTATCTAATCC	54.0	465	[37]
***vapA* partial**	F2: CTGGACTTCTCCACTGCTCA R3: ACGTTGGTAATCGCGAAATC	53.0	626	[4]
***vapA* partial**	Vspec–F: CAACGGTTTCTGGAGTAATAACTTT Vspec – R: TGCATCAGCAACAGCGGTAGT	57.0	254	[33]

vapA	F: ACTGTCTGTTACCCTGCCA R: GCTACTTCACCCTGATTGG	60.0	[34]

2.1.8. Immune gene expression

2.1.8.1. RNA extraction and cDNA synthesis

RNA was extracted from samples preserved in TRI Reagent (Sigma, UK) following the manufacturer's instructions. The quality and quantity of the RNA was assessed by electrophoresis and was measured with a Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific), respectively. The RNA pellet was eluted in 15 µL of nuclease-free water prior to cDNA synthesis with QuantiTect® Reverse Transcription kit (Qiagen) following the manufacturer's protocol. Samples were stored at -20 °C until use.

2.1.8.2. Primers design and specificity

Primers were designed for two target genes (MHC II - CD74 molecule and IgM) to assess the effect of bath vaccination on juvenile ballan wrasse adaptive immune responses. Furthermore, a primer pair was also designed for a housekeeping gene (β-actin), while previously published elongation factor α (ELFα) primers [24] were used as the second housekeeping gene for expression normalisation. The primers used for qPCR are provided in Table 3. Primers were designed based on sequencing similarity of > 88 % using NCBI Primer – Blast software (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/) [38] and following good primer design criteria [39]. Validation of the newly designed primers was carried out and amplification products sent for sequencing. In brief, separate PCR amplifications were performed for all the primers pairs in a total reaction of 10 µL each including; 1µL (1/20 cDNA synthesised of 250 ng of total RNA from whole larvae, HK, L, S, 0.1 uM of each primer, 1 x Q5® Hot start high fidelity Master mix (2x) (NEB biolabs) and nuclease free water to reach the desirable final volume. The following thermal cycling conditions were used in a Biometra, TAdvanced: 1 cycle of initial denaturation at 98 °C for 1 min, 30 cycles at 95 °C for 15 sec (denaturation), 56 °C for 15 sec (annealing) and 72 °C for 15 sec (extension), followed by final extension 1 cycle at 72 °C for 2 min and a cooling step at 4 °C. The relative molecular weight of the amplicons was calculated against a 100 bp marker (Thermo Fisher Scientific). Resulting PCR products were sequenced by Eurofins to confirm identity.

Table 3. Primer pairs used for quantitative PCR (qPCR) to assess ballan wrasse adaptive immune responses.

Target gene	Oligo name	Sequence	Annealing (°C)	Accession number	Source
Major histocompatibility complex II (MHC II)	MHC II F_5	CCAATGTTGCGTCTCCGATG	56	XM_020645324.2	This study
	MHC II R_5	CACTGACTACAGCGTCCTGC			
Immunoglobulin M (IgM)	IgM F1	AGTCCCGTATGTCCTGTGGT	56	KX688616.1	This study
	IgM R1	CCCTTGCTCGTCTGTCCAAT			
Beta actin β-actin	β-actin F4	GCCAGAAGGACAGCTACGTT	56	XM_029281812.1	This study
	β-actin R4	TCGATGGGGTACTTCAGGGT			
Elongation factor alpha (ELF- α)	ELFα F	ATTGATGCCCCTGGACAC	56		[24]
	ELFα R	CCTCAAACTCACCGACACC			

2.1.8.3. Quantitative PCR (qPCR)

The expression of target genes; MHC II and IgM and housing keeping genes; β-actin and ELFα was measured on a LightCycler®480 instrument (Roche Diagnostics). Target gene expression was normalised against the β-actin and ELFα housekeeping genes. Every reaction contained a final volume of 10 µL, containing 1µL of cDNA (either sample or negative control; nuclease free water), 5 µL of Luminaris Color HiGreen q PCR MasterMix (Thermo Fisher Scientific), 0.5 µL of each forward and reverse primers, and 3.0 µL of nuclease free water. Cycling conditions consisted of an initial activation of DNA polymerase at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing at 56 °C for 30 sec, and extension at 72 °C for 30 sec. The assay included duplicates of each sample, non-template control and a serial dilution (from 10 to 10^8 copies per 1 µl) of a linearised plasmid construct pGEM-T Easy vector (Promega, France) which incorporated a PCR fragment of the target gene. Melt curves were checked to ensure specific amplification. Crossing points (Cp) values were calculated employing the second derivative maximum method in the LightCycler software (Supplementary Table 2).

2.2. Ontogeny of the adaptive immune system

Whole ballan wrasse larvae were sampled and fixed in RNA stabilisation buffer from the same production tank at nine time points from 0, 15, 30, 60, 90, 115, 130 and 150 (dph) (0.3 ± 0.1 g at 60 dph and 1.0 ± 0.2 g at 150 dph) from two commercial hatcheries (site A and B) in Scotland (n= 20 per time point per hatchery). The samples were stored at - 20 °C until processing.

Whole larvae were processed for times 0 – 60 dph. The head, tail, and dorsal area (dorsal fin and muscle) were removed prior to RNA extraction for larvae from 90 - 150 dph to enrich transcripts associated with target lymphoid organs and gut regions as shown in Figure 1. The ontogeny of immune response was assessed with qPCR as described below.

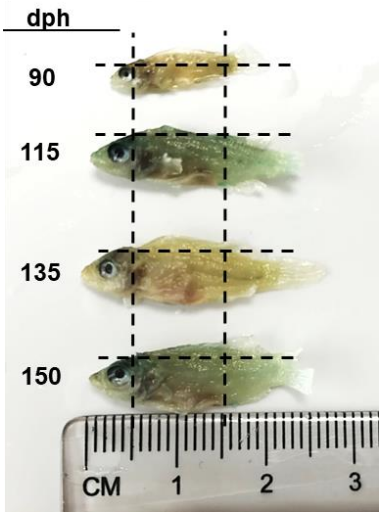

Figure 1. Juvenile ballan wrasse from > 90 days post hatch (dph) used in ontogeny study. Dashed line shows the regions where the head, tail, and dorsal area (dorsal fin and muscle) were removed from the larvae prior to RNA extraction to enrich transcripts associated with target lymphoid organs and gut.

2.3. Statistical analysis

The geometric mean was calculated for housekeeping gene concentration and used for normalisation of target genes concentration (MHC II –CD 74 and IgM). The mean and the standard error were calculated for each target gene. MHC II–CD 74 and IgM expression was assessed and visualised on a histogram graph using Sigma Plot (SYSTAT inc Software, Inc., San Jose California USA). Statistical analysis was conducted using Minitab software (Minitab18). Gene expression transcript concentrations were assessed with General Linear Model (GLM) for normality and homogeneity of variance and data were normalised using Log_{10} or natural log (LN). Determination of whether a difference in expression occurred for either of the genes during the ontogeny and challenge trial studies was tested by ANOVA with Tukey's post hoc test (significance level; $p < 0.05$). The results from qPCR for *As* bacterial load in tissues samples from challenged fish in this experiment were tested using Mood's median non-parametric test (significance level; $p < 0.05$). Statistical analysis on bath challenge data were performed in statistical software R [40] for Kaplan – Meier survival curves, log-rank non-

parametric tests test (significance level; $p < 0.05$) [41, 42] and pairwise comparison test (significance level; $p < 0.05$) [43].

2.4. Ethics statement

Animal procedures for bacterial infection and vaccination were performed under UK Government Home Office project licence PC5CD1B81 following approval by the Animal Welfare and Ethical Review Body (AWERB) at the Centre for Environment, Fisheries and Aquaculture Science (Cefas) and University of Stirling. Ballan wrasse were treated in accordance with the Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039).

3. Results

3.1. Immersion vaccine efficacy trial

3.1.1. Cumulative mortalities

Morbidities in the small cohorts for both the mock and vaccinated groups started at 8 dpi and 10 dpi, respectively, while morbidities in the large cohorts for prime vaccinated groups were noted at 9 dpi (mock) and 13 dpi (vaccinated) (Figure 2). Morbidities were first recorded in the booster vaccinated group from the large cohort at 8 dpi followed by the mock vaccinated at 9 dpi except from a single tank (T08 – 34) for which morbidities started earlier at 3 dpi even though these fish received a similar infection dose than the others from the same group. The survival rate from this tank was the lowest from all groups reaching 33.3 % (Figure 2). The mean cumulative mortalities for the large fish challenged after booster vaccination were calculated with and without tank T08 – 34 (Table 4). Mean cumulative mortalities (%) for vaccinated and unvaccinated groups (either prime or booster) for both cohorts (small and large) were not statistically different (Table 4). Survival rates (%) did not differ between vaccinated and mock vaccinated groups (Figure 2). Control fish from both prime and booster vaccinated groups in the large population experienced 4 and 2 % mortalities, respectively, but bacteriology and molecular analysis showed that they were not caused by a*As.* No mortalities were recorded in the control fish from the small cohort.

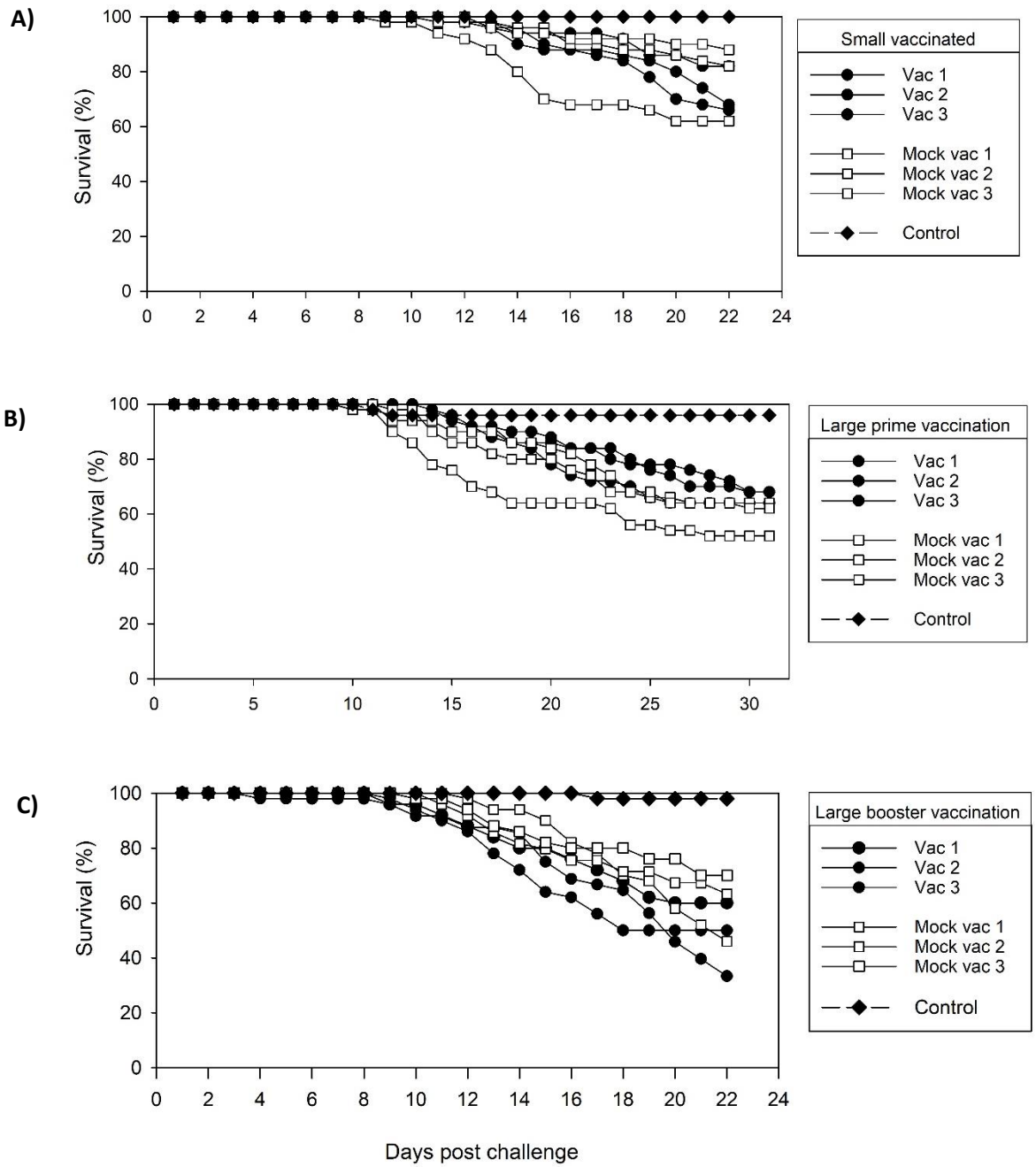

Figure 2. Survival curves for juvenile ballan wrasse bath challenged with atypical *Aeromonas salmonicida vapA* type V upon prime (both cohorts) and booster (large cohort only) immersion vaccination with an autogenous vaccine at two developmental stages. Small (0.5 g ± 0.2 g or ca. 80 days post hatch; dph) (A) and large cohort (1.5 ± 0.4 g or ca. 170 dph) (B) following prime vaccination and large cohort (C) upon booster vaccination. The vaccinated group is presented as Vac (1-3) and mock vaccinated as Mock vac (1-3) on the legend.

Table 4. Challenge dose (CFU / mL) of atypical *Aeromonas salmonicida vapA* type V and mean cumulative mortalities (%) with standard deviation (±SD) for ballan wrasse juveniles (n= 50). Small and large cohorts were prime vaccinated, and only a group of fish from the large cohort received booster vaccination. The immunisation period for prime and booster groups were 645 and 420 DD, respectively. No mortalities were recorded for control groups. Non-specific mortalities were recorded in one of the replicates from the vaccinated group of the large cohort (T08-34). The mean cumulative mortalities were calculated with and without this tank. No statistical difference ($p < 0.05$) was observed between the different groups or cohorts.

Cohort	Vaccination status	Mean CFU / mL	Mean cumulative mortalities (%)
Small	Vaccinated	2.98E+09	28.0 ± 8.7[a]
	Mock vaccinated	5.23E+09	23.0 ±13.6[a]
Large	Vaccinated	5.65E+08	34.0 ± 2.0[b]
	Mock vaccinated	9.95E+08	37.0 ± 7.0[b]
	Booster vaccinated	3.77E+09	30.0 ± 7.0 (*54 ± 15.7)[c,d]
	Mock booster vaccinated	5.11E+09	40.0 ± 12.4[c,d]

*Mean cumulative mortalities taking into account T08 – 34.

a,b,c,d statistical difference at trial termination $p= 0.29$, $p= 0.232$, $p= 0.232$, $p= 0.691$ without (T08 – 34), respectively.

3.1.2. Bacteria presence and load in morbid fish

Head kidney swabs from moribund fish plated on TSA had small pin head creamy a*As* like colonies and were positive with the agglutination test, except for three samples from tank T08-34 between 3 and 8 dpi. The *As* qPCR loads were similar for all tissue samples (HK, L and S) from both vaccinated and mock vaccinated fish with Cp value ranging from 21 to 35 (Table 5). The conventional PCR specific for a*As* type V confirmed the presence of a*As* type V bacteria in the same samples as qPCR. Large moribund fish (n= 3) from tank T08-34 which had been

booster vaccinated and challenged with a*As* type V were negative for all 3 screening methods and bacteria did not grow on TSA plates.

Table 5. Bacterial load in tissues samples (head kidney, liver and spleen) from small and large cohort ballan wrasse (n= 15 per treatment 10 %) challenged with atypical *Aeromonas salmonicida vapA* type V following vaccination (prime and booster; only large) with a polyvalent autogenous vaccine or mock vaccination. Load was assessed by quantitative PCR and expressed as mean cross point (Cp), Cp values > 35 considered negative. No statistically significant differences between treatments were observed..

Cohort	Vaccination status	Mean bacterial load (Cp) in tissue		
		Kidney	Liver	Spleen
Small	Prime vaccinated	27.64 ± 4.71	25.87 ± 4.47	29.64 ± 5.79
	Prime mock vaccinated	27.90 ± 4.21	26.92 ± 4.73	26.88 ± 3.14
Large	Prime vaccinated	29.14 ± 2.55	28.74 ± 4.22	28.15 ± 3.31
	Prime mock vaccinated	27.62 ± 5.06	25.17 ± 3.10	24.50 ± 2.32
	Booster vaccinated	29.18 ± 3.77	27.94 ± 3.51	27.51 ± 3.72
	Booster mock vaccinated	27.69 ± 2.11	25.60 ± 3.20	28.39± 3.70

3.1.3 Gene expression at pre- and post- vaccination and post- challenge

Overall, gene expression (MCH II –CD 74 and IgM) was not significantly different in tissues or between groups (pre - and post- vaccination and vaccinated and mock vaccinated) after challenge with a*As vapA* type V (Figures 3 and 4) with a few exceptions. The relative expression of both target genes was statistically significantly higher in head kidney and spleen of both small and large booster control groups in comparison with challenged, mock and vaccinated fish of the same cohorts.

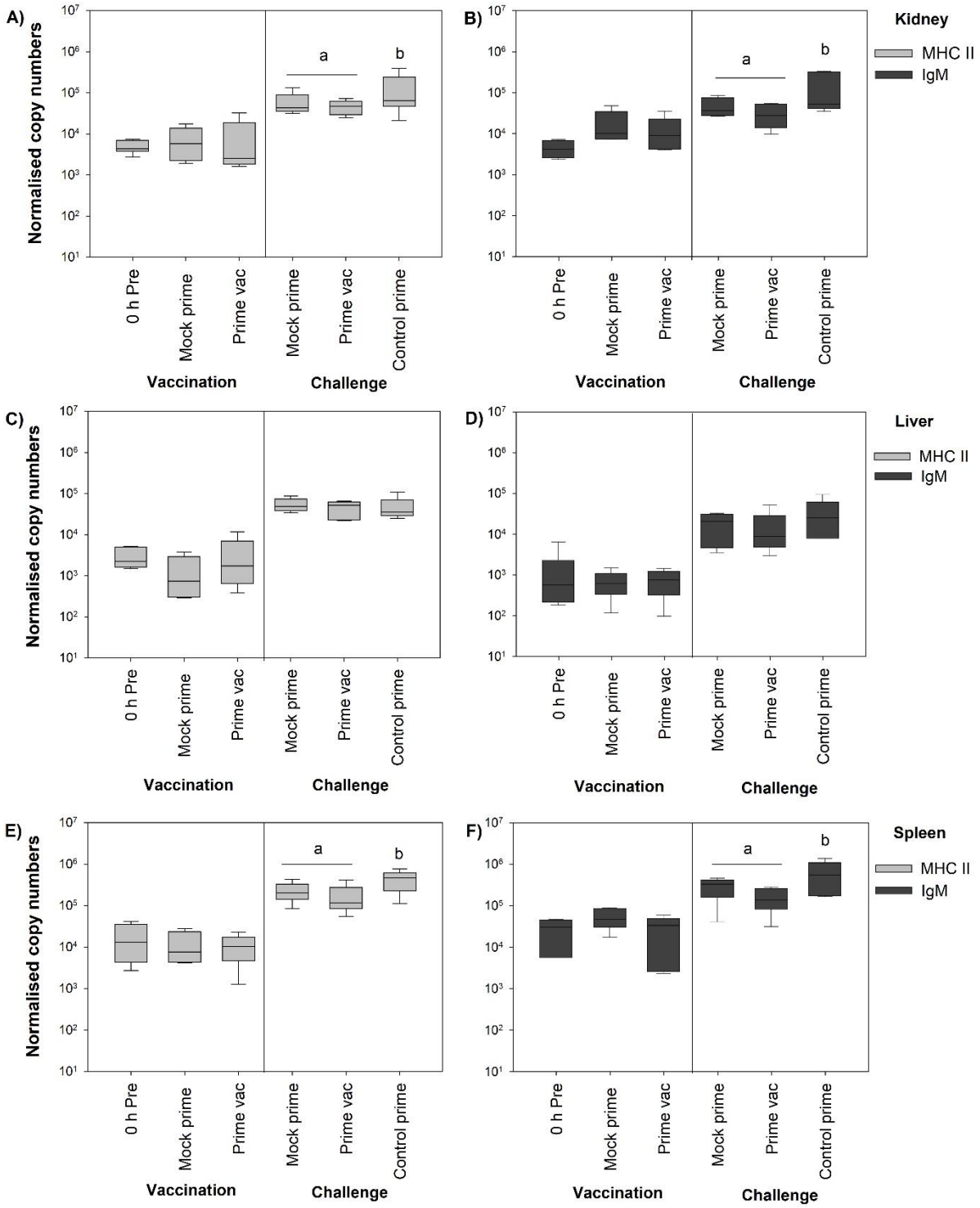

Figure 3. Box and whisker plots showing the normalised number of MHC II and IgM transcripts obtained by qPCR from head kidney (A, B), liver (C, D) and spleen (E, F) from small cohort ballan wrasse at pre- and post- prime (24 h) vaccination by immersion and post – bath challenge with atypical *Aeromonas salmonicida vapA* type V (n= 6 for each group). Pre-vaccinated and vaccinated groups are presented as pre and vac, respectively and mock vaccinated (vaccination control) as mock in the legend. Boxes represent the interquartile

range, the line inside the box shows the median value and the whiskers, outside the boxes, relate to the maximum and minimum values. Statistically significant differences (Tukey's test, $p < 0.05$) between groups are represented by superscripts.

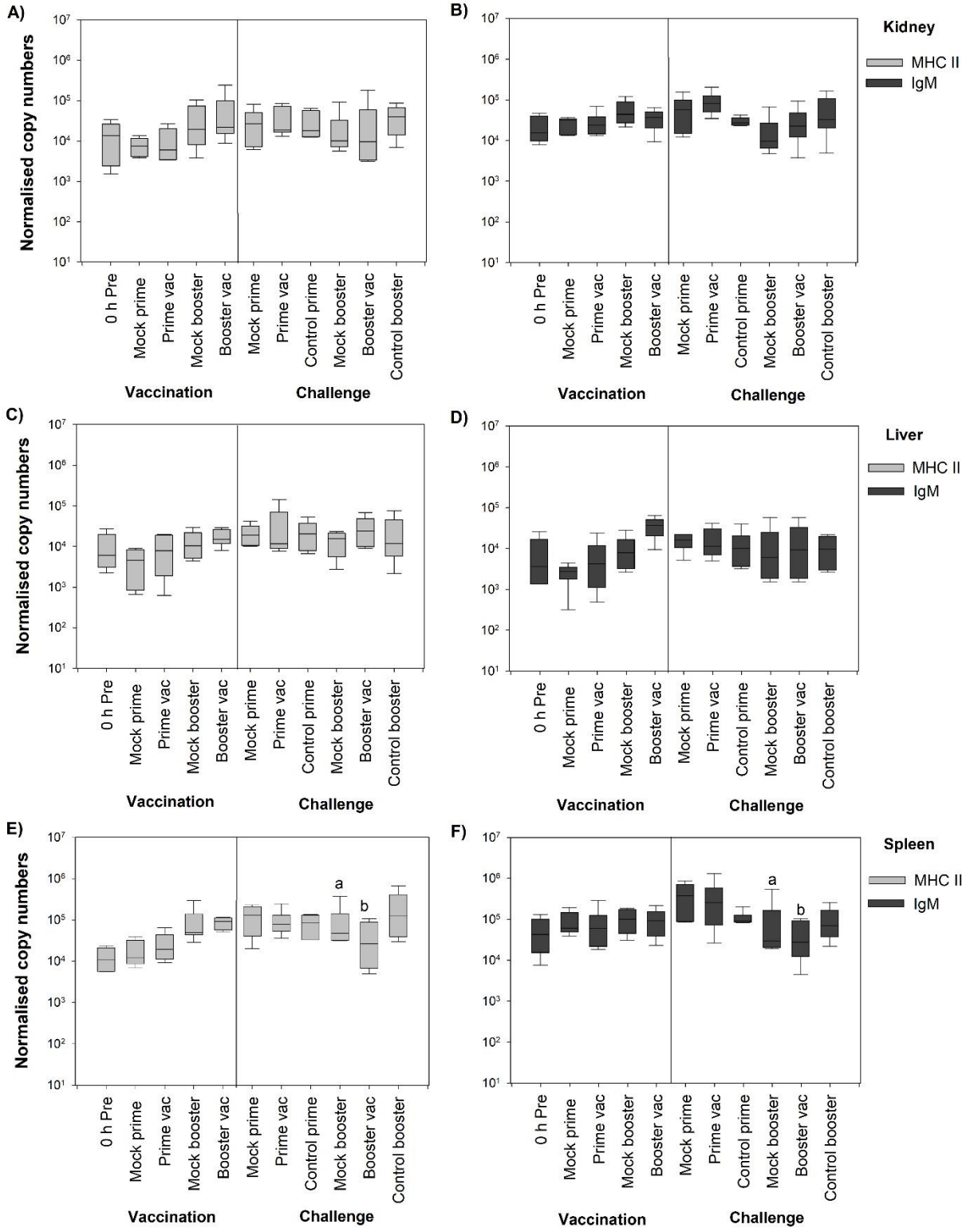

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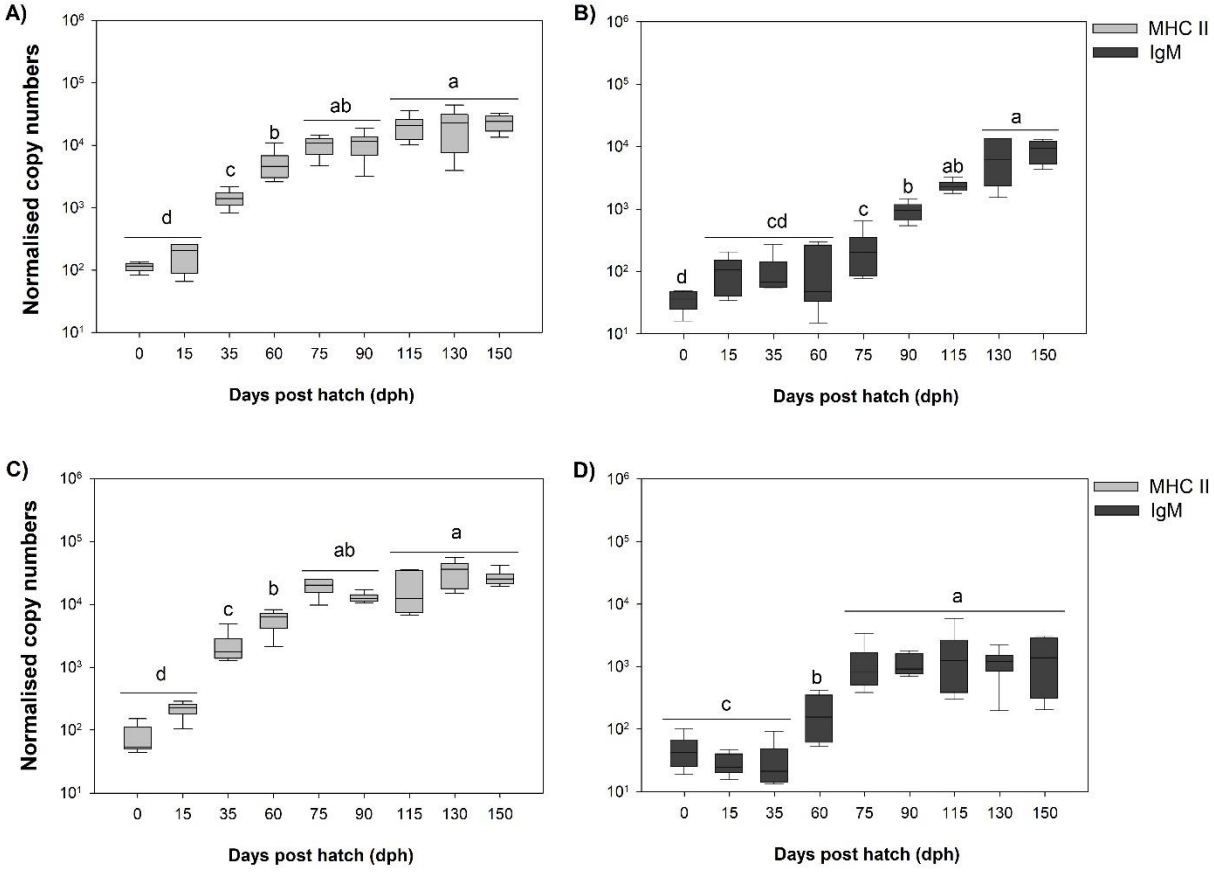
Figure 4. Box and whisker plots showing the normalised number MHC II and IgM transcripts obtained by qPCR from head kidney (A, B), liver (C, D) and spleen (E, F) tissues of large cohort ballan wrasse at pre- and post- prime (24 h) vaccination by immersion and post – bath challenge with atypical *Aeromonas salmonicida vapA* type V (n= 6 for each group). Boxes represent the interquartile range, the line inside the box shows the median value and the whiskers, outside the boxes, relate to the maximum and minimum values. Pre-vaccinated and vaccinated groups are presented as pre and vac, respectively and mock vaccinated (vaccination control) as mock in the legend. Statistically significant differences (Tukey's test, $p < 0.05$) between the groups are represented by superscripts.

3.2. Ontogeny

A significant increase in expression of both MHC II and IgM was noticed in both surveyed hatchery site populations. Expression levels of MHC II were significantly higher than basal levels (0 dph) from 35 dph (Figure 5 A). Temporal gene expression continued to increase until 115 dph when expression remained unchanged until the end of the study (150 dph). Additionally, the IgM expression levels significantly increased by 75 dph in comparison to earlier time points and continued to increase significantly until 130 dph (Figure 5 B). Similar trends were noticed both for MHC II and IgM expression for site B (Figure 5 C and D).



388

389 **Figure 5.** Box and whisker plots showing the normalised number of MHC II and IgM transcripts
390 obtained by qPCR from whole ballan wrasse larvae (n= 6 for each group) from 0 to 150 days
391 post hatch (dph) at site A (A, B) and B (C, D). Boxes represent the interquartile range, the line
392 inside the box shows the median value and the whiskers, outside the boxes, relate to the
393 maximum and minimum values. Statistically significant differences (Tukey's test, $p < 0.05$)
394 between the time points are represented by superscripts.

395 ## 4. Discussion

396 This study aimed to assess the efficacy of an immersion autogenous vaccine (Ridgeway
397 Biologicals LTB) used for early juvenile developmental stages (<15 g) in ballan wrasse
398 hatcheries in Scotland and then determine the ontogeny of key representative components of
399 the adaptive immune system of the species. This would ultimately assist the establishment of
400 a vaccination timeframe for farmed ballan wrasse for protection against a*As*. The results
401 showed no significant differences in the mean cumulative mortalities for immersion vaccinated
402 juvenile ballan wrasse (both small cohort and large cohorts) challenged with a*As* by bath
403 inoculation post immunisation. The expression of MHC II (CD74 molecule) and IgM transcripts
404 were first identified at 35 and 75 dph, respectively and were observed to increase until

termination of the study at 150 dph which suggests initiation towards maturation of adaptive immunity.

In the present study all vaccination groups (prime small (ca. 0.5 g) and large (ca. 1.5 g), and booster large) along with mock vaccinated fish were challenged against a*As* type V post immunisation (prime at 654 DD and booster at 495 DD) using a challenge model we developed previously [32]. Morbidities between vaccinated and mock vaccinated fish were not statistically different for any of the three groups indicating that the vaccine did not confer protection in the fish under the conditions used in this study. This confirms previous reports in which immersion vaccination was shown to have low potency [9]. Chakraborty, et al. [14] reported no protection, but an apparent delayed infection (ca. 3 weeks) in juvenile lumpsuckers (ca. 2.9 g) vaccinated with a commercial polyvalent vaccine against *Vibrio anguillarum* following a full vaccination regime (prime and booster bath and i.p. injection). Vaccinated fish showed higher bacterial loads in some cases, however no significant differences were found in the present study between vaccinated and mock vaccinated fish. Importantly, it must be acknowledged that while qPCR detected the bacterial load within the tested tissues, it could not distinguish between live and dead cells.

The vaccine protection was also evaluated in prime and booster immunised fish after challenge with a*As* by profiling the relative expression of adaptive immune related genes (MHC II – CD74 molecule and IgM). No statistically significant differences were noted between treatments (mock and vaccinated fish) post challenge (up to 30 dpi) for any of the three groups. The relative expression levels of both target genes were higher in the head kidney and spleen sampled from control fish in both the small and large cohorts in comparison to the challenged mock and vaccinated fish of the same cohort. MHC II (CD74 molecule) expression was higher in the spleen of all groups but not in other tissues at the end of the challenge period. MHC II expression was higher in liver tissues for all cohorts post infection in this study, suggesting a functional role of MHC II in this tissue. These results agree with a previous report for miiuy croaker, *Miichthys miiuy* [44]. Antigen presentation of MHC II molecules may occur in other organs like the liver, which very recently has been considered to have an immunological role in mammals [45, 46]. Yang, et al. [47] and Causey, et al. [48] suggested a similar role of the liver in teleost fish as lymphocytes are present in the organ. In the present study, the total IgM expression levels were similar in the lymphoid organs for both mock and vaccinated groups suggesting no vaccine-associated immunity against a*As*. In a previous study, expression of membrane IgM (mIgM) was located in different tissues in fish challenged with *Photobacterium damselae subsp. piscicida* prior (spleen and gut) and post vaccination (only in spleen) [49]. Thus, comparative analysis of gene expression with ELISA for specific antibody presence (IgM

and IgT) was undertaken previously by the authors and confirmed that vaccination resulted in a systemic IgM response while the challenge resulted in a local IgT response only [49]. Despite several attempts to collect blood samples using a capillary tube, blood isolation was not successful in the present study due to the small size of the fish, and as such serology tests could not be performed although we previously demonstrated enhanced serum IgM levels in i.p. vaccinated wrasse that correlated with protection [25].

While the organogenesis in ballan wrasse has been described previously [50, 51], the onset of the expression of immune related genes in tissues of ballan wrasse species has not been described to date and the immune system of *L. bergylta* is currently only partially characterised [21-23]. In this study, the temporal expression of MHC II (CD74) and IgM transcripts was investigated in newly hatched ballan wrasse larvae (0 dph) until 150 dph using qPCR in two commercial hatcheries in Scotland. MHC II and IgM expression levels significantly increased at 35 and 75 dph, respectively, and continued to do so in larvae originating from site A. MHC I and II are responsible for antigen recognition and presentation and play a significant role in activation of adaptive immune response and memory [44]. Hence, the detection of MHC II gene expression prior to IgM in the study was expected considering the role that MHC II plays in immunological processing in phagocytes [45]. Saravanan, et al. [52] were the first to document expression of MHC II in Rohu (*Labeo rohita*) at 28 dph. Earlier expression of MHC II molecules has been observed for sea bass (*Dicentrarhus labrax*) at 4 dph [47] and common carp (*Cyprinus carpio*) at 28 dpf [48]. IgM mRNA transcripts were first detected at 5 dph with a significant increase observed at 35 dph for olive flounder*, Paralichthys olivaceus* [49]. In teleost fish maternal immune elements have a significant role in survival of eggs and newly hatched larvae. Innate (lysozyme and antibacterial peptides) and adaptive (IgM) immune elements can pass from broodstock to offspring [15, 53, 54]. However, maternal immunity has only a short duration of several weeks post hatch (3 – 4) [55]. For example, IgM decreased in striped trumpeter (*Latris lineata*) by 100 dph [56]. In the present study, the temporal expression profile observed for MCH II and IgM in ballan wrasse from 35 dph and 75 dph, respectively continued to increase suggesting a relationship with the animal's size and immune responsiveness appeared therefore to not be derived from maternal transfer. Differences in expression patterns and relative gene expression were observed for MHC II (CD 74) and IgM between the two hatcheries. This could be explained by the fact larvae from site B were mixed with larvae from other developmental stages (earlier and later) during husbandry procedures (*i.e.* grading) in contrast with larvae from site A whereby the same batch and tank of fish were sampled during the entire study. Thus, the results from site A are considered more reliable.

The ontogeny results indicated that expression of MHC II and IgM should have been initiated at least for the large cohort used in the immersion vaccination study prior to vaccination (ca. 170 dph at prime vaccination). Indeed, the overall relative expression of MHC II and IgM was higher in the tissues (HK, L and S) of both fish sizes at vaccination in comparison with ontogeny fish samples, which included multiple pooled organs including gut tissue. Nevertheless, the mean cumulative mortality and the gene expression results of the immunised and bath challenged fish in this study indicates that the vaccine did not elicit protection. It is important to mention that the detection and expression of these adaptive immune genes only provides an indication of the onset of immunocompetence but not full immunological maturity [57]. Similarly, previous studies have demonstrated that presence of lymphoid organs and initiation of IgM gene expression does not guarantee immunocompetence [58-61] which may explain the result of the immersion vaccination. In addition, there are various factors apart from immunocompetence that need to be considered for a successful vaccine *e.g.* the vaccine formulation, vaccine dose, the vaccination route, vaccination time for immersion vaccines, the use of an adjuvant, and the challenge trial including pathogen pressure / load, exposure time and virulence of the pathogen [62-64]. In addition, multivalent vaccines need rigorous testing and careful selection of strains or different species included in the formulation as combinations of antigens could interact synergistically or antagonistically and stimulate, cross-react, compete and lead to immunosuppression [65]. Nikoskelainen, et al. [66] reported that different multivalent vaccines composition against *A. salmonicida subsp.salmonicida*, *Listonella anguillarum* and *Flavobacterium psychrophilum* had an effect in antibody production in rainbow trout (*Oncorhynchus mykiss*). The results obtained from this study further illustrate the challenges of immersion vaccination against typical and atypical strains of *As,* for which historically immersion vaccination has proven challenging and only few experimental successes have been reported with demonstrated protection to fish [2, 67-69].

Overall, the results of the vaccine trial conducted in this study suggested that immersion vaccination for farmed ballan wrasse should be applied at a later stage of the production cycle (*e.g.* size > 1.5 g), at least for the a*As* component of the vaccine. In addition, we reported for first time, the onset of adaptive immune genes expression for ballan wrasse (MHC II – CD74 at 35 dph and IgM at 75 dph), which is critical for commercial vaccination strategies. Further research is needed to better understand the adaptive immune system of juvenile ballan wrasse including, its maturation and uptake and processing of antigens for ballan wrasse juveniles. All of the above will help to optimise vaccine formulation and vaccination regimes in hatcheries and mitigate the high mortalities occurring during early developmental stages. Protection at this early stage is vital to boost the cleaner fish sector productivity to supply the increasing

demand from salmon producers for healthy, disease free and effective delousing ballan wrasse.

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7. Conflict of interest Statement

The authors declare no conflict of interest.

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Supplementary Tables

Supplementary Table 1. Vaccination doses (mL) are given below including calculated and actual amounts for small and large cohorts.

Size (g) at vaccination	Vaccination status	Fish number	Biomass at vaccination (kg)	Tank (L)	Actual vaccine volume (mL)
0.5	Vaccinated	207.0	0.10	141.0	39.0
0.5	Mock	272.0	0.14	159.0	44.0
1.5	Vaccinated	207.0	0.31	165.0	46.0
1.5	Mock	272.0	0.41	151.0	42.0
2.5	Booster vaccinated	210.0	0.32	155.0	43.0
2.5	Booster mock	275.0	0.41	151.0	42.0

Supplementary Table 2. Standard curves quality testing on transformed plasmids containing target and housekeeping genes (*IgM*, *MHC II*, *β-actin* and *ELFα*). Table contains the Cp upper and lower limit, efficiency and slope of the each assay.

Target gene	Cp lower / upper limit	Efficiency	Slope
MHC II	10.51 / 33.7	1.958	-3.437
IgM	9.99 / 34.1	1.929	-3.504

β-actin	10.4 / 34.6	1.976	-3.381
ELFα	10.5 / 32.3	1.924	-3.519

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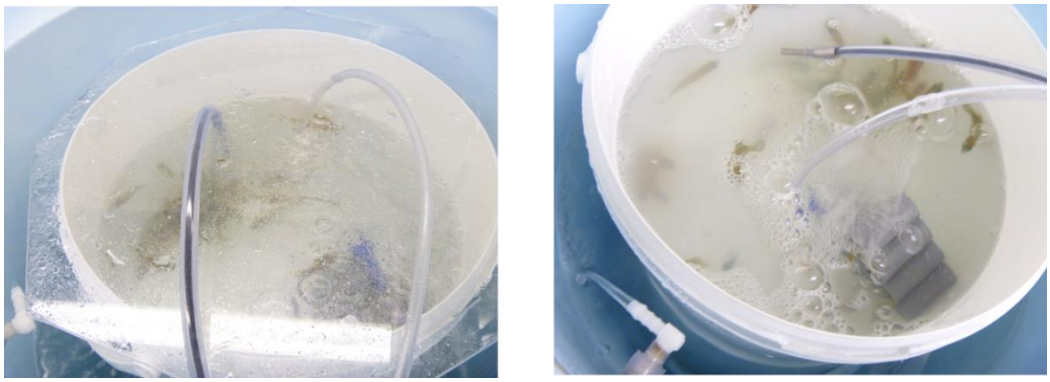
Supplementary Figures



Supplementary Figure 1. Challenge of ballan wrasse juveniles with atypical *Aeromonas salmonicida vapA* type V in small aerated aquaria (8 L).

Highlights

- Autogenous vaccine offers no protection in farmed ballan wrasse at 0.5 g - 1.5 g against atypical *Aeromonas salmonicida* (a*As*) *vapA* V.
- Immersion vaccination for a*As* should be performed at bigger sizes > 1.5 g.
- Onset of adaptive immune genes responses reported for first time.
- MHC II and IgM transcript were first reported at 35 and 75 days post hatch.