Studies on the antibody response and side effects after intramuscular and intraperitoneal injection of Atlantic lumpfish (*Cyclopterus lumpus* L.) with different oil-based vaccines.

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Abstract

Atlantic lumpfish (*Cyclopterus lumpus* L.) is used as a biological delousing agent for sea lice (*Lepeophtheirus salmonis* K.) infestations in Norwegian aquaculture. Here we present a study on the antibody response and vaccine side effects after intramuscular and intraperitoneal injection of lumpfish with two vaccines. Both vaccines contained bacterial antigens from atypical *Aeromonas salmonicida* A-layer type V and VI, *Vibrio anguillarum* serotype O1 and *Moritella viscosa* sp., but one vaccine contained a vegetable oil-based adjuvant while the other contained a mineral oil-based adjuvant. Intramuscular injection of the mineral oil-based vaccine caused a high acute mortality of fish within 48 hours after immunization. Intraperitoneal injection of the mineral oil-based vaccine resulted in a lower severity of intra-abdominal side effects than the vegetable oil-based vaccine. Intramuscular injection of the mineral oil-based vaccine resulted in a significantly higher antibody response against *A. salmonicida* when compared to controls and the vegetable oil-based vaccine group. The antibody response was poor against *V. anguillarum* and *M. viscosa* for all groups. Our results indicate that intramuscular injection of oil-based vaccines might be feasible for providing immunological protection for Atlantic lumpfish against bacterial diseases, especially atypical *A. salmonicida*, but more work is required to identity optimal adjuvants.

Keywords: Atlantic lumpfish, intramuscular injection, antibody response, oil adjuvant, side effect, vaccination.

Introduction

Atlantic lumpfish, *Cyclopterus lumpus* L., is a marine fish commonly used as "cleaner fish" for biological delousing of salmon louse, *Lepeophtheirus salmonis* (Krøyer), infestations on farmed Atlantic salmon, *Salmo salar* L., in Norway. Commercial production of lumpfish has increased rapidly, from just above 430.000 individuals in 2012 to nearly 12 million individuals in 2015 (Norwegian Directorate of Fisheries, 2016). Disease after sea transfer is currently a major challenge, especially bacterial infections caused by atypical *Aeromonas salmonicida*, *Vibrio anguillarum*, *Vibrio ordalii*, *Pseudomonas anguilliseptica*, *Pasteurella* sp. and *Tenacibaculum* spp. (Hjeltnes et al., 2016). Such bacterial infections may lead to mass mortality of the lumpfish, and a national survey conducted in 2013 reported a total mortality of 48 % among the localities included, where 75 % were caused by bacterial infections (Nilsen et al., 2014). Similar findings were also reported in a study of increased mortality of lumpfish during autumn/late summer of year 2015, were bacterial agents was confirmed in nearly 80 % of the case materials and atypical furunculosis and vibriosis was considered to be the most important causes of mortality (Bornø et al., 2016). The authors suggested improved vaccines and vaccination strategies as an important step in reducing future mortality events of lumpfish.

Lumpfish have previously been immunized with *Vibrio ordalii*, *Vibrio anguillarum*, two strains of atypical *Aeromonas salmonicida*, and a *Pasteurella*-like bacteria, showing a higher presence of specific antibodies (IgM) in immunized fish, except for fish immunized with the *V. ordalii* agent (Ronneseth et al., 2015). Currently, there are only four commercially available vaccines for lumpfish against bacterial diseases (reviewed in Johansen et al. (2016)), two regular and two autogenous. The autogenous vaccines consists of a dip vaccine, containing *Vibrio splendidus*, *Vibrio logei*, *Vibrio ordalii* and *Vibrio anguillarum* O1 antigens, and an injection vaccine containing two isolates of atypical *Aeromonas salmonicida*,

Vibrio anguillarum O1 and, in some instances, *Moritella viscosa* sp. antigens. Similarly, the two regular vaccines consists of a dip vaccine, containing *Vibrio anguillarum* O1 and O2a and b antigens, and an injection vaccine, ALPHA MARINE® micro 4, containing *Vibrio anguillarum* O1 and O2a and b and atypical *Aeromonas salmonicida* antigens. ALPHA MARINE® micro 4 was recently pulled from the market, and replaced by the injection vaccine AMARINE® micro 4-2, containing atypical *Aeromonas salmonicida* (A-layer type 3 and 6), in addition to *Listonella anguillarum* (syn. *Vibrio anguillarum*) serotype O1 and O2a antigens (Ø.B. Vågnes 2016, personal communication, April).

Despite of more than 60 % of the lumpfish being vaccinated when transferred to sea, either by injection or dip vaccine, or a combination of these two, several investigators request improved vaccines or vaccination methods for cleanerfish (Bornø et al., 2016). The pelvic fins of lumpfish form suction discs which they use to strongly anchor themselves to rocks or other surfaces (Davenport, 1985). This can potentially be a problem during administration of injectable vaccines, which are normally deposited into the abdominal cavity, causing more handling of the fish (Vestvik, 2014). One other alternative could be to inject the vaccine at a different and more easily accessible administration site, such as the dorsal musculature.

Up to now, the vaccine strategy includes intraperitoneal injection of vaccines. Since it may be favorable to immunize fish through intramuscular injection, we hypothesized that lumpfish immunized via intramuscular injection are well tolerated, and induce comparable antibody response compared to intraperitoneal immunization.

Materials and methods

Fish

Atlantic lumpfish (*Cyclopterus lumpus* L.) with mean weight of 5 and/or 10 grams was provided by Norsk Oppdrettsservice AS in Flekkefjord. The fish were kept in the rearing facilities at Havbruksstasjonen i Tromsø AS. The fish were fed Amber Neptun, obtained from Skretting AS, and fed ad libitum throughout the experiment. The fish were kept in circular 140 L tanks with continuous flow of sea water. The water temperature was about 9 °C four days prior to the start, and was gradually heated to and kept at 10 °C (± 1 °C) throughout the experiment. The fish were kept under constant 24:0 hour light:dark conditions, and water temperature, fish appetite, behavior and mortality was checked daily, while O_2 saturation was checked weekly. The fish displayed no signs of disease or mortality at the start of the experiment. Prior to the start of the experiment, the fish were sorted in two different size groups to ensure that each individual had a minimum body weight of either 10 g (± 0,5 g) in size group 1, or 5 g (± 1,0 g) in size group 2. Feeding was withheld 48 hours prior to immunization, to reduce the risk of accidental injection into the internal organs e.g. a faeces-filled intestine, and was resumed 24 hours after immunization. The experiment was approved by the Norwegian Animal Research Authority (NARA).

Vaccine antigens

Two experimental vaccines containing different oil adjuvants were specifically produced and kindly donated by Vaxxinova Norway AS for this study. Both vaccines contained four formalin-inactivated bacterial antigens: *Aeromonas salmonicida* A-layer type V, *Aeromonas salmonicida* A-layer type VI, *Vibrio anguillarum* serotype O1 and *Moritella viscosa* sp.

Vaccine 1 was prepared using a vegetable oil-based adjuvant (unspecified), while vaccine 2 was prepared using a mineral oil-based adjuvant (paraffin).

Vaccine formulation

The bacterial isolates was initially manually mixed to the desired concentration (unspecified) in PBS containing 1,5% NaCl. Further mixing was conducted by using an IKA T18 Ultraturrax homogenizer until both vaccines contained a final formulation of 70% oil and 30% water (w/o). Any further information regarding the adjuvants and bacterial isolates used for the vaccines has been withheld due to competitive considerations.

Immunization/vaccination of lumpfish

The fish were anesthetized with Finquel vet. (Western Chemical Inc., USA), individually weighed and tagged using a Visible Implant Fluorescent Elastomer kit (Northwest Marine Technology Inc., USA) prior to immunization. The fish were immunized by intraperitoneal (ip.) or intramuscular (im.) injection of 0,05 ml per fish of vaccine 1 (VO) or vaccine 2 (MO), whereas control groups received phosphate-buffered saline (PBS). The im. injection was done on the left side of the dorsal hump, just vertically for the pectoral fins, and the ip. injection was done in the ventral midline, in the central area between the vent opening and the caudal edge of the suction disc. Injection was performed using a Microfish 0,05 ml hand-operated reusable syringe (Kaycee Veterinary products LTD, UK), and 0,5 x 4 mm and 0,5 x 3 mm vaccination needles (Unimed SA, Switzerland) for size group 1 and size group 2, respectively. Groups of 62 fish were injected im. and groups of 25 fish were injected ip. Due to high acute mortality observed after im. injection of size group 1 lumpfish with the mineral oil-based vaccine, size group 2 lumpfish were not im. injected with this vaccine. After injection, the fish

were kept in an anesthetic-free tank until recovery, before being transferred back to their original holding tanks for the rest of the experiment. Fish to be sampled on the same day as immunization was kept in a separate 50 L plastic tank for recovery and kept there until the time of sampling.

Scoring of vaccine side effects

Lumpfish immunized via im. and ip. injection were evaluated for possible vaccine side effects. Weight and length was measured in both groups and the number of dead fish was registered during the full length of the study. Ip. injected fish were scored according to the Speilberg scoring system (Midtlyng et al., 1996). The scoring system was modified to fit lumpfish. In short, the following modifications were done; a separate scoring scale for melanisation of abdominal organs and abdominal wall, and a separate scoring scale for vaccine residues, as described by Aunsmo et al. (2008) and Fredriksen & Grip (2012).

Sampling from immunized lumpfish

Parallel series of 4 fish were randomly collected 1 h, 4 h, 1 d, 2 d, 4 d, 7 d and 28 d post immunization (dpi.) and series of 10 fish were collected 14 d and 42 d post immunization. The remaining fish were collected at 84 dpi. The fish were killed by a sharp blow to the head, immediately followed by blood sampling. Peripheral blood was collected from vena caudalis of the lumpfish into clot activator tubes (BD, USA), and kept on ice until serum preparation for ELISA. Individual body weight and body length was registered, and vaccine side effects (Speilberg score) was evaluated for ip. immunized fish prior to organ sampling. From im. immunized fish, samples were dissected from head kidney, spleen, gills, and the integument and underlying muscular tissues surrounding the injection site. From ip. immunized fish, organ samples were dissected from head kidney, gills, spleen and the gastro-intestinal tract, including the pyloric caeca. All samples were put on 10% formalin. Due to the decision to not

im. inject size group 2 lumpfish with the mineral oil-based vaccine, only samples from fish collected in size group 1 was used for the rest of the study.

Enzyme-linked immunosorbent assay (ELISA)

A whole cell/competitive ELISA was used to determine serum reactivity to formalin-inactivated bacterial antigens (*Aeromonas salmonicida* A-layer type V, *Aeromonas salmonicida* A-layer type VI, *Vibrio anguillarum* serotype O1 and *Moritella viscosa* sp.). All antigens were kindly donated by Vaxxinova Norway AS. Briefly, microtiter plates (MaxiSorp, Nunc) were coated for 5 minutes with 100 µl of a 0,1% poly-L-lysine solution (Sigma) in coating buffer. Further, 100 µl ultrasonicated or whole cell bacteria (10^8 cellmL^{-1}) in 50 mM carbonated-bicarbonate buffer (pH 9,6) were added per well before the plates were centrifuged and incubated at 4 ^{o}C over night. Thereafter, all plates were saturated with 5% dry milk in PBST (phosphate buffered saline with Tween 20, pH 7,3) and incubated at room temperature for 2 hours. Lumpfish sera were diluted 1:100 and 1:200 in 0,5% dry milk in PBST, 100 µl well^{-1}, and incubated over night at 4 ^{o}C. Bound antibodies were detected by incubations at room temperature with polyclonal rabbit-anti-lumpfish IgM (produced according to Bilal et al.(2016)), (2h), followed by incubation with goat-anti rabbit IgG conjugated with alkaline phosphate (Sigma) in 0,5% dry milk in PBST (1h). Finally, the substrate p-nitrophenyl phosphate (Sigma) in substrate solution (0,1 M glycine, pH 10,4, with 1 mM $MgCl_2$ and 1 mM $ZnCl_2$), followed by incubation for 30 min at room temperature in dark. After the final incubation the optical density (OD) of the plates were immediately analyzed with a spectrophotometer (VersaMax Absorbance Microplate Reader; Molecular Devices, LLC, USA) at a wavelength of 405 nm.

Preparation of samples for histology and immunohistochemistry

Sections of muscle tissue from the injection site of lumpfish 1 h, 4 h, 1 day, 2 days, 7 days, 21 days and 42 days after im. injection were fixed in 10% formalin for 24 h at room temperature, transferred to 70% EtOH and thereafter embedded in paraffin. Sections (3 µm) were cut and stained with haematoxylin and eosin (HE) for histology. Immunohistochemistry (IHC) for *V. anguillarum* serotype O1 was performed on dewaxed and rehydrated paraffin sections. The primary antibody, raised in rabbit was a gift from Nofima AS, Tromsø. Staining was performed using a goat-anti rabbit IgG conjugated with alkaline phosphate (Sigma), fast red visualization system and haematoxylin counterstaining.

Slides were microscopically analyzed by using an Axio Lab.A1 microscope (Carl Zeiss Microscopy GmbH, Germany). Images were acquired by using a Moticam 5.0 MP camera (Motic®, USA), and were further processed using the software Motic Images Plus 2.0 (Motic, USA).

Statistical analysis

All collected data were treated and analyzed statistically by using the software Prism version 7.02 (GraphPad Software Inc., USA). Group means were compared by using one-way ANOVA with Tukey Multiple Comparison test, and differences were considered significant with a p-value less than 0,05 ($p < 0,05$).

Results

Mortality was monitored daily during the course of the experiment and were used to calculate accumulated mortality rates for all fish groups. Within 48 hours after immunization a total of fifteen lumpfish, im. injected with the mineral oil-based vaccine, died (30,6% mortality) (Fig. 1). The majority of these fish died within the first 8 hours after vaccination. A total of four lumpfish im. injected by the vegetable oil-based vaccine died (8,2% mortality); the last mortality was registered at 63 dpi. (not shown). No mortalities were registered for the control group (PBS) or the ip. injected fish.

It has been shown for several aquacultured fish species that vaccination may result in a decreased growth (Berg et al., 2006a). As such, we analysed the lumpfish growth following im. and ip. immunization by measuring body weight at the start of immunization on day 0, followed by 21 and 42 dpi. At day 42 our results showed an average of 27% reduction in mean weight of in the VO and MO group compared to the control group (PBS) after im. injection (Fig. 2). However, the difference between vaccine injected and PBS injected fish were not significant (p=0,07). At day 84 the VO group showed a 59% significant reduction compared to the MO group (p=0,03). At day 21 there were no differences in mean weight. For ip. injected fish our results demonstrated a significant reduction (p=0,02) of 37% in mean weight for the VO group at day 21 and a non-significant reduction (p=0,39) of 17% for the MO group compared to the PBS group, (Fig. 3). At day 42 there were a significant reduction (34%) (p=0,03) of mean weight for the VO group and a non-significant reduction (p=0,26) of 19% for the MO group compared to the fish that received PBS. Finally, at day 84 both the VO and the PBS group showed a significant reduction of 38% (p=0,04) and 39% (p=0,04), respectively, compared to the MO group.

The Speilberg score is a well established tool for evaluating side effects after ip. injection of oil-based vaccines in Atlantic salmon (Berg et al., 2006a). In our study we assessed the degree

of adhesions between viscera and abdominal wall, visible melanin pigmentation of viscera and abdominal wall/fillet, and vaccine residues of ip. injected lumpfish at 21, 42 and 84 dpi. by using a modified Speilberg score. The highest severity of intra-abdominal adhesions was seen in the VO fish at all sampling times, significantly different compared to the MO group at 21 ($p=0,04$) and 42 ($p<0,05$) dpi. (Fig. 4). At day 84 the mean scores had reached its highest point at 5,2 and 4,6 for the VO and the MO group, respectively, the results were, however, not significantly different ($p=0,27$). No melanisation of viscera or abdominal wall/fillet was observed (Table 1). Both vaccinated groups showed a reduction in mean score for vaccine residues over time, where the MO group were significantly lower than the VO group at 21 ($p<0,05$) and 42 ($p<0,05$) dpi. (Table 1). At day 84 the MO group showed a higher mean score than the VO group, but the difference was not significant ($p=0,30$). No intra-abdominal adhesions, melanisations or residual vaccines were observed in the control group (PBS).

The Atlantic lumpfish possess an adaptive immune system and produce specific antibodies upon immunization (Ronneseth et al., 2015), but whether im. immunization induces robust antibody response is an open question. We measured the specific IgM response of pooled and individual serum samples from both im. and ip. injected lumpfish against atypical *A. salmonicida* A-layer type V and VI, *V. anguillarum* serotype O1 and *M. viscosa* sp. at 21, 42 and 84 dpi. by ELISA. Our results suggested a robust IgM response against *A. salmonicida* type V and VI for both im. and ip. injected fish with the mineral oil-based vaccine at 42 and 84 dpi. (Fig. 6). However, the IgM response was low against *V. anguillarum* O1 and *M. viscosa* sp., and close to the same level displayed by the control fish (PBS) after both im. and ip. injection (results not shown). Fish immunized by the vegetable oil-based vaccine showed generally low IgM responses after both im. and ip. injection, except for one group on 84 dpi. (Fig. 5). This group showed a high IgM response against *A. salmonicida* type V after ip. injection of the vegetable oil-based vaccine.

ELISA was used to measure the individually specific IgM responses of ten fish sampled from each group at 42 dpi, against atypical *A. salmonicida* A-layer type V and VI, *V. anguillarum* serotype O1 and *M. viscosa* sp. For lumpfish immunized by the mineral oil-based vaccine, our results indicated significantly high IgM responses for both im. and ip. injected fish against *A. salmonicida* type V and VI ($p<0,05$) when compared to the control groups (PBS) (Fig. 8). The im. injected fish showed less variation between high and low IgM responders than the ip. injected fish. Lumpfish immunized by the vegetable oil-based vaccine demonstrated low IgM responses against all antigens mentioned, except for a single individual fish in the ip. injected control group (PBS) against both *A. salmonicida* type V and VI (Fig. 7).

On gross examination of im. injected lumpfish we observed residual vaccine around the injection site, and along the dorsal side of the fish during early sampling (results not shown). As such, we performed histological evaluation of transversal sections of the skin and muscular tissue. In vaccinated fish, local reactions in the tissue around the injection site were noted, primarily inflammation of the connective tissue between the dermis and muscular layer of the skin. Granuloma-like structures were noted in some sections. The majority of local reactions were observed as early as 48 hours post immunization, while no clear distinction between VO and MO groups could be established (results not shown).

Immunohistochemistry was used to detect residual vaccine antigens of *V. anguillarum* serotype O1 in cross sections of skin and muscle tissue from im. injected lumpfish sampled at 42 d.p.i. The antigen was present in sections from fish injected with both the vegetable oil-based (Fig. 9b) and the mineral oil-based vaccine (Fig. 9c), and was mostly detected in the connective tissue between the dermis and muscular layer of the skin. In addition, signs of inflammation could be observed in several sections. No antigen was detected in any sections from the control fish (PBS) (Fig. 9a).

Discussion

In this study we have immunized Atlantic lumpfish with two injection vaccines containing different oil adjuvants. To the best of our knowledge, this is the first study to examine antibody response and fish welfare after intramuscular injection of oil-based vaccines in Atlantic lumpfish.

A total of 10 lumpfish died within 8 hours, with the first fish within 1,5 hours, after intramuscular injection of the mineral oil-based vaccine. From 8 to 48 hours another 5 fish died. The fish that survived the first 48 hours showed no signs of adverse reactions or changed behavior. The mortality was much lower for fish injected with the vegetable oil-based vaccine, were 3 fish died within 48 hours after injection. No mortalities were registered throughout the experiment for the control group (PBS) and ip. injected fish. To our knowledge, acute mortality after im. injection of oil-based vaccines in fish have not been previously reported. Treasurer and Cox (2008) investigated whether the dorsal median sinus (dms), the adipose tissue running under the dorsal fin of the fish, could be used as an alternative route to intraperitoneal vaccination of Atlantic salmon. They noted that dead Atlantic salmon having received dorsal median sinus injection of oil-based vaccines showed signs of muscular damage at the injection site. They concluded that the vaccine had been administered too deep, and that this was the most probable cause of the observed cumulative mortality of 0,5-1% for the dms group. Ip. injected fish in the same experiment reached a mortality level of 1-5%, but this appeared to be primarily caused by *Saprolegnia* infections. Fløgum (2016) reported that the vegetable oil-based vaccine used in vaccination of ballan wrasse did not appear to cause increased mortality. However, although these reports indicated that adjuvant formulation might affect post-immunization mortality after im. injection, the exact cause of the observed acute mortality in our study is unclear.

Fish ip. injected by the vegetable oil-based vaccine exhibited a significantly higher score of intra-abdominal adhesions, than fish injected with the mineral oil-based vaccine. This was in contrast to what was expected, since non-mineral oils are considered to be well tolerated and produce less side effects than traditional mineral oils (Aucouturier et al., 2001, Tafalla et al., 2013). Low or moderate adherence formation after ip. injection of non-mineral oil-based vaccines have been observed in several fish species, such as Atlantic salmon (*Salmo salar* L.) (Midtlyng et al., 1996), Atlantic halibut (*Hippoglossus hippoglossus* L.) (Bowden et al., 2003), turbot (*Psetta maxima* L.) (Sitja-Bobadilla et al., 2008) and ballan wrasse (*Labrus bergylta* L.) (Fløgum, 2016). However, our results are in agreement with the observations of Rønsholdt and McLean (1999), who reported adhesion scores of up to grade 6 in rainbow trout (*Oncorhynchus mykiss* L.) at seven weeks post ip. injection with a monovalent vaccine containing a metabolizable oil adjuvant. Poppe and Breck (1997) reported in a case study high adhesion scores appearing several months after vaccination, where Atlantic salmon were ip. injected with a multivalent vaccine adjuvanted with a mixture of vegetable and animal oils. Although the authors listed several possible factors to explain the results, the exact cause for the development of intra-abdominal lesions seen in our study is unknown.

None of the vaccinated fish displayed any signs of melanisation, which results in dark pigmentation of abdominal organs or the abdominal wall. We have also noted the same results up to 18 weeks post vaccination in an earlier immunization trial using a divalent oil-based injection vaccine for lumpfish (Erkinharju 2015, personal observation, May 12). Melanisation after intraperitoneal vaccination has been observed in other fish species, such as Atlantic salmon (Midtlyng et al., 1996, Berg et al., 2006b), rainbow trout (Ronsholdt and McLean, 1999) and Atlantic cod (Mutoloki et al., 2008). However, according to Maira et al. (2008) melanin formation does not seem to be present at any degree in Atlantic cod and Fløgum

(2016) observed no signs of pigmentation in vaccinated ballan wrasse. Our results are in agreement with these observations.

Our results demonstrated a high specific antibody (IgM) response against atypical *A. salmonicida* A-layer type V and VI for lumpfish at 42 and 84 d.p.i. This was confirmed by statistical analysis of individual samples collected at 42 d.p.i., were vaccinated fish had significantly higher mean values than the controls (PBS). Successful vaccination of other fish species, such as Atlantic salmon and rainbow trout, against atypical variants of *A. salmonicida* have proven to be very difficult and given highly variable results, as reviewed by Gudding (2014). However, other researchers have shown that it is possible to provide protection and strong antibody response for both spotted wolfish (*Anarchicas minor* O.) (Lund et al., 2002), ballan wrasse (Biering et al., 2016) and Atlantic lumpfish (Ghebretnsae, 2015) against atypical variants of *A. salmonicida* through vaccination, and are in accordance with our observations.

Lumpfish immunized by the vegetable oil-based vaccine displayed a low specific IgM response against all antigens at every sampling point, except for ip. injected fish against *A. salmonicida* A-layer type V at 84 d.p.i. This was confirmed by statistical analysis of individual samples collected at 42 d.p.i., were vaccinated fish was in general not significantly different from the controls (PBS). Very few studies have examined the antibody responses after vaccination of fish with non-mineral oil-based adjuvants. However, our results are in agreement with the observations of Bowden et al. (2003), who reported low antibody titers of Atlantic halibut up to several months after vaccination. Sitja-Bobadilla et al. (2008) detected low numbers of serum antibodies in turbot injected with a multivalent vaccine prior to challenge. A vegetable oil-based vaccine was also used for vaccination of ballan wrasse in the study reported by Fløgum (2016); however, the specific antibody response was not examined.

The specific IgM response against *V. anguillarum* serotype O1 and *M. viscosa* sp. was lower than for both the A-layer types of *A. salmonicida*, and did not increase over time. Similar results have been observed for Atlantic cod, where *V. anguillarum* appeared to induce a lower and more varied antibody response when compared to *A. salmonicida* (Lund et al., 2006, Schroder et al., 2009). We have also observed a low specific IgM response in lumpfish during an earlier immunization trial, by using ip. injection of a divalent vegetable oil-based vaccine containing *Vibrio salmonicida* antigens (Erkinharju 2015, personal observation, July 14). However, Ghebretnsae (2015) demonstrated a near similar antibody response for Atlantic lumpfish against both *A. salmonicida* and *V. anguillarum* after ip. injection. However, vaccination of Atlantic salmon have also resulted in significant protection against *V. anguillarum* and *M. viscosa* (Colquhoun and Lillehaug, 2014).

There was no significant differences in the specific IgM responses between im. and ip. injected fish for all bacterial antigens. To our knowledge, no studies have compared the immune responses of teleost fish after im. and ip. injection of vegetable oil-based or mineral oil-based vaccines. However, our results are in agreement with the observations by Treasurer and Cox (2008) on Atlantic salmon, who reported no significant differences between the antibody responses of dms and ip. injected fish. Haugland et al. (2005) investigated the expression profiles of inflammatory and immune-related genes of Atlantic salmon after both im. and ip. injection of a multi-component vegetable oil-based vaccine, and discovered highly variable individual responses to vaccination. However, no further analysis of the immune response was reported.

Our analysis of serum samples from fish sampled at 42 d.p.i. displayed an apparent individual variation between high and low specific IgM responders against atypical *A. salmonicida* A-layer type V and type VI, after both ip. and im. injection of the mineral oil-based vaccine. In a different study, Grontvedt and Espelid (2004) reported significant antibody response and

protection after vaccination and challenge of spotted wolfish with atypical variants of *A. salmonicida*, although the individual responses could vary from none to high. However, more work is needed to investigate whether the immunization described in our study would provide sufficient protection towards development of disease in Atlantic lumpfish.

Immunohistochemical analysis on cross sections of skin and muscle tissue from im. injected lumpfish detected the presence of *V. anguillarum* serotype O1 antigens, confirming residual vaccine was still present in the tissues at 42 d.p.i. Several inflammatory cells was observed near the encapsulated vaccine antigens in sections from fish injected with both vaccines. This indicates that an inflammatory response had been triggered, which is necessary for the proper development of an immune response in teleost fish after injection of oil-based vaccines (Berg et al., 2006a).

To conclude, our study indicates that intramuscular injection of oil-based vaccines might be feasible for providing immunological protection for Atlantic lumpfish against bacterial disease, especially against atypical *A. salmonicida*, but more work is required to identify adjuvants that provide optimal fish welfare in addition to immunological protection.

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Figure legends

Figure 1. Percentage accumulated mortality of intramuscularly injected fish. Number at the start was 49 fish for all groups. VO=vegetable oil group, MO=mineral oil group, PBS=phosphate buffered saline group.

Figure 2. Comparison of mean weight (g) ± S.D. for intramuscularly injected fish at the start of vaccination on day 0 (n=62), and at 21 days (n=10 for VO and PBS, n=4 for MO), 42 days (n=10) and 84 days (n=2 for VO, n=4 for MO and n=5 for PBS) post vaccination. Combination of different letters (a, b and c) indicates significant ($p < 0,05$) differences between groups. VO=vegetable oil group, MO=mineral oil group, PBS=phosphate buffered saline group.

Figure 3. Comparison of mean weight (g) ± S.D. for intraperitoneally injected fish at the start of vaccination on day 0 (n=25), and at 21 days (n=10), 42 days (n=10) and 84 days (n=5) post vaccination. Combination of different letters (a, b and c) indicates significant ($p < 0,05$) differences between groups. VO=vegetable oil group, MO=mineral oil group, PBS=phosphate buffered saline group.

Figure 4. Comparison of mean Speilberg score of adherences ± S.D. at day 21 (n=10), day 42 (n=10) and day 84 (n=5) post vaccination for intraperitoneally injected fish. Combination of different letters (a and b) indicates significant ($p < 0,05$) differences between groups. VO= vegetable oil group, MO=mineral oil group, PBS=phosphate buffered saline group.

Table 1. Mean score of melanisation of abdominal organs and abdominal wall/fillet, and mean score of vaccine residues for intraperitoneally injected fish at day 21 (n=10), day 42 (n=10) and day 84 (n=5) post vaccination. VO=vegetable oil group, MO=mineral oil group, PBS= phosphate buffered saline group.

	Day 21			Day 42			Day 84		
	VO	MO	PBS	VO	MO	PBS	VO	MO	PBS
Melanisation of abdominal organs	0	0	0	0	0	0	0	0	0
Melanisation of abdominal wall/fillet	0	0	0	0	0	0	0	0	0
Vaccine residues	2	0,7	0	1,6	0	0	0,4	0,8	0

Figure 5. Comparison of specific antibody (IgM) responses against *Aeromonas salmonicida* V (a) and *Aeromonas salmonicida* VI (b), from lumpfish im. and ip. injected with the vegetable oil-based vaccine. Values are presented as ELISA readings (mean O.D. at 405nm) of pooled serum samples diluted 1:100. Number of fish (n) for IM groups: day 21 (n=10), day 42 (n=10) and day 84 (n=2 for VO and n=5 for PBS). Number of fish (n) for IP groups: day 21 (n=10), day 42 (n=10), day 84 (n=5). VO=vegetable oil group, PBS=phosphate buffered saline group, IM=intramuscular, IP=intraperitoneal.

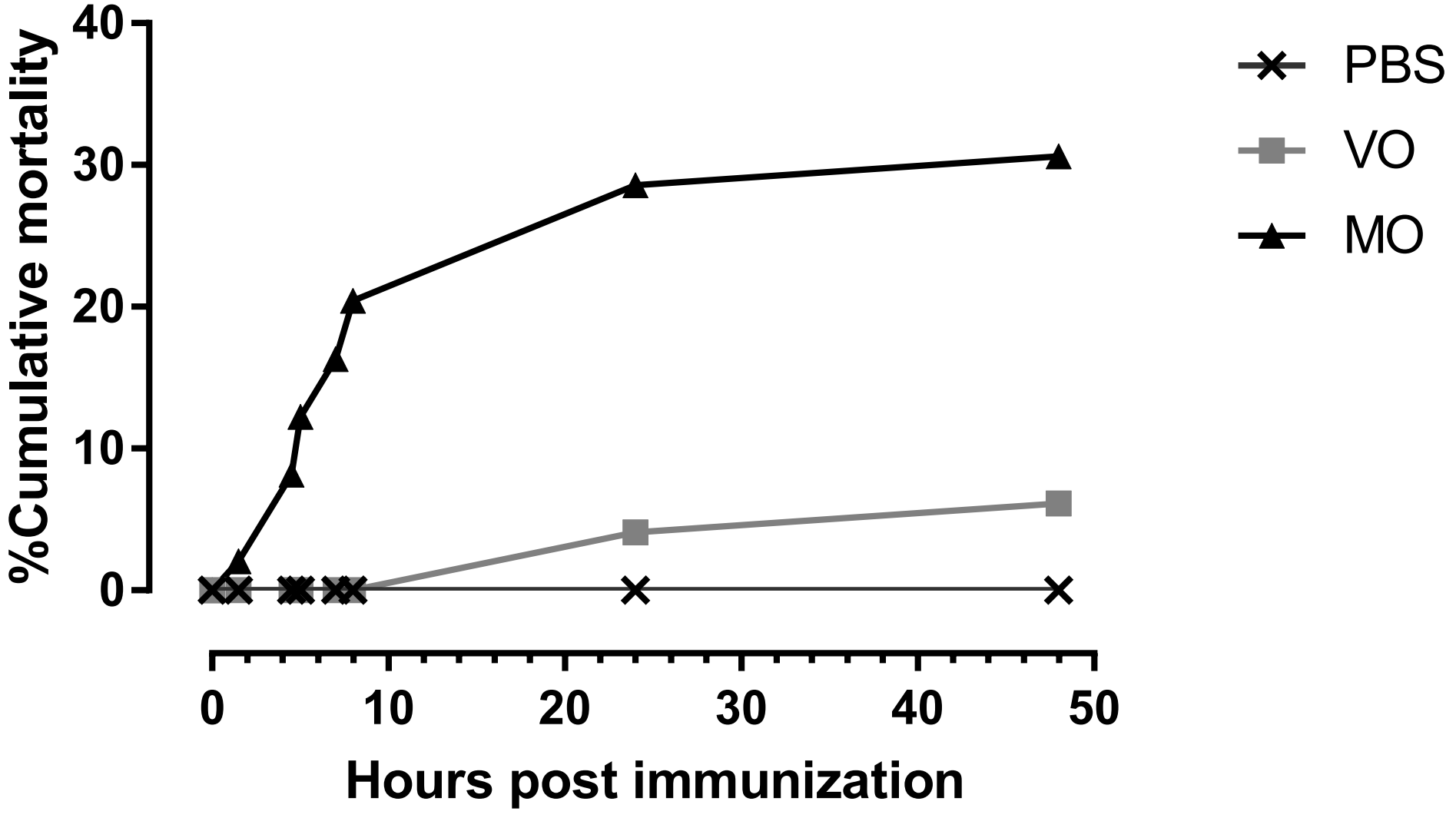
Figure 6. Comparison of specific antibody (IgM) responses against *Aeromonas salmonicida* V (a) and *Aeromonas salmonicida* VI (b), from lumpfish im. and ip. injected with the mineral oil-based vaccine. Values are presented as ELISA readings (mean O.D. at 405nm) of pooled serum samples diluted 1:100. Number of fish (n) for IM groups: day 21 (n=10 for PBS and n=4 for MO), day 42 (n=10) and day 84 (n=4 for MO and n=5 for PBS). Number of fish (n) for IP groups: day 21 (n=10), day 42 (n=10), day 84 (n=5). MO=mineral oil group, PBS=phosphate buffered saline group, IM=intramuscular, IP=intraperitoneal.

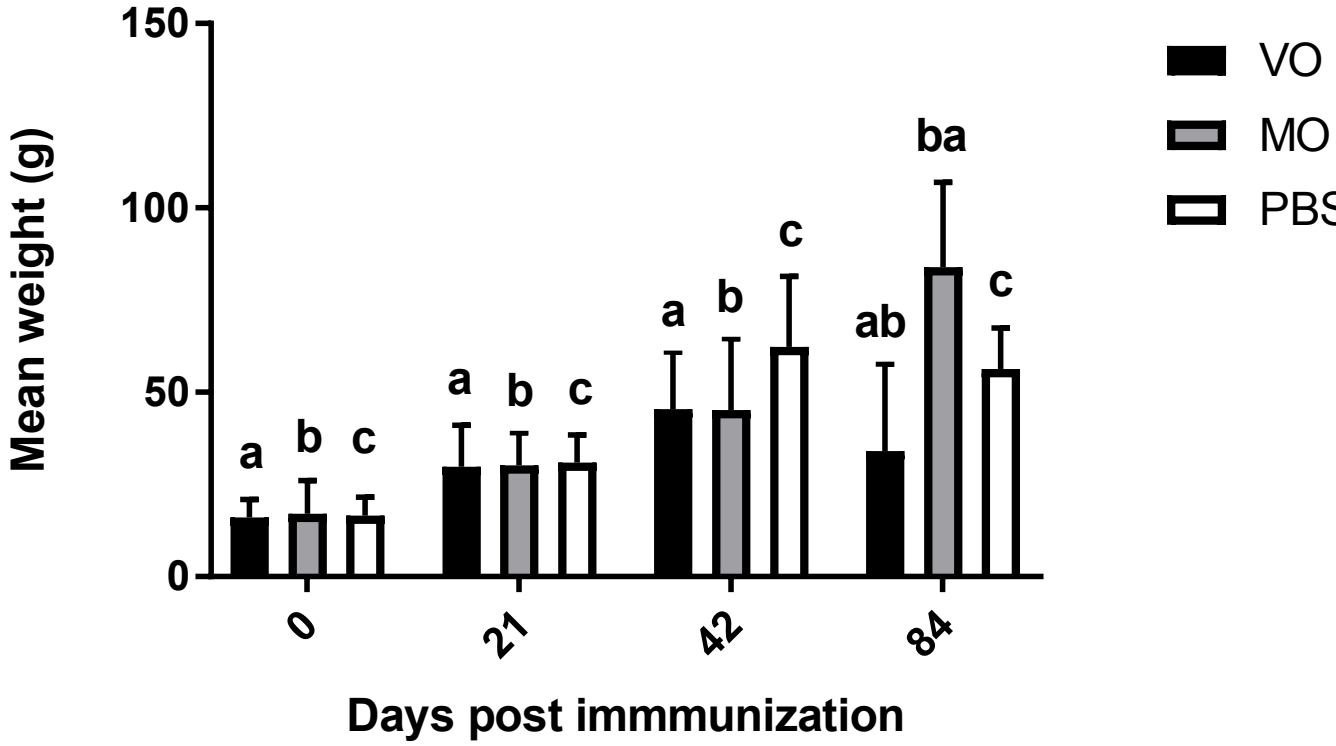
Figure 7. Comparison of specific antibody (IgM) responses against *Aeromonas salmonicida* V (a) and *Aeromonas salmonicida* VI (b), from lumpfish im. and ip. injected with the vegetable oil-based vaccine, at 42 days post vaccination. Values are presented as ELISA readings (mean O.D. ± S.D.) of individual serum samples diluted 1:100 (n=10). Combination of different letters (a, b and c) indicates significant ($p < 0,05$) differences between groups. VO=vegetable oil group, PBS=phosphate buffered saline group, IM=intramuscular, IP=intraperitoneal.

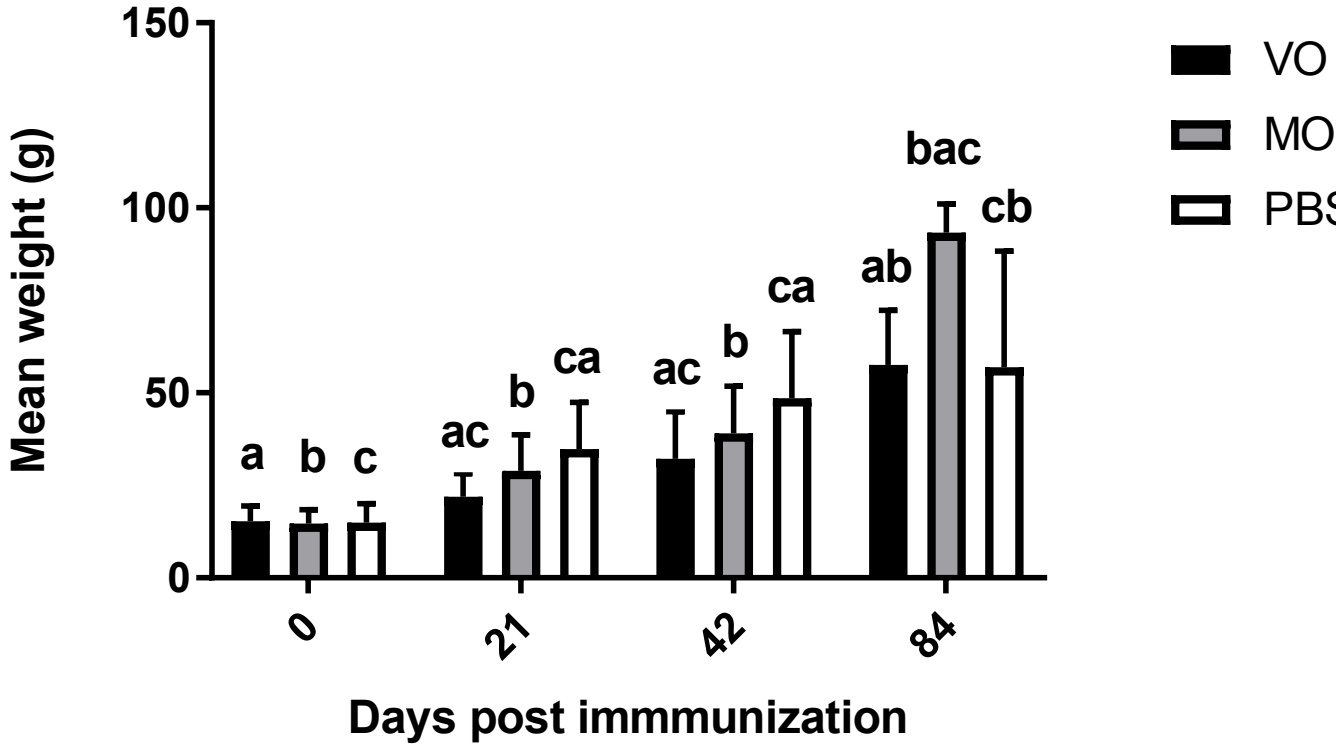
Figure 8. Comparison of specific antibody (IgM) responses against *Aeromonas salmonicida* V (a) and *Aeromonas salmonicida* VI (b), from lumpfish im. and ip. injected with the mineral oil-based vaccine, at 42 days post vaccination. Values are presented as ELISA readings (mean O.D. ± S.D.) of individual serum samples diluted 1:100 (n=10). Combination of different letters (a, b and c) indicates significant ($p < 0,05$) differences between groups. VO=vegetable oil group, PBS=phosphate buffered saline group, IM=intramuscular, IP=intraperitoneal.

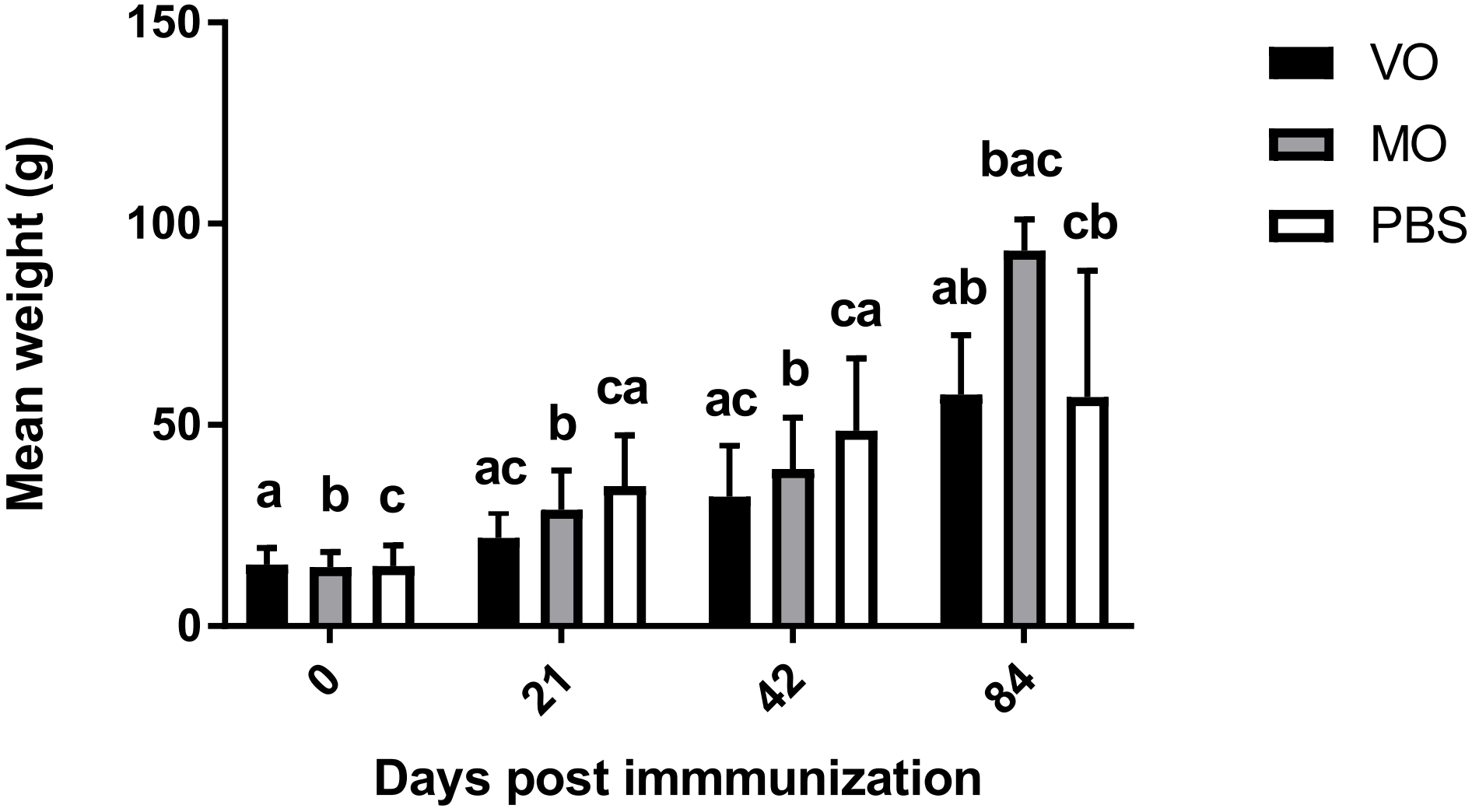
Figure 9. Immunohistochemistry images of lumpfish intramuscularly injected with PBS (a), vegetable oil-based vaccine (b) and mineral oil-based vaccine (c) at 42 days post

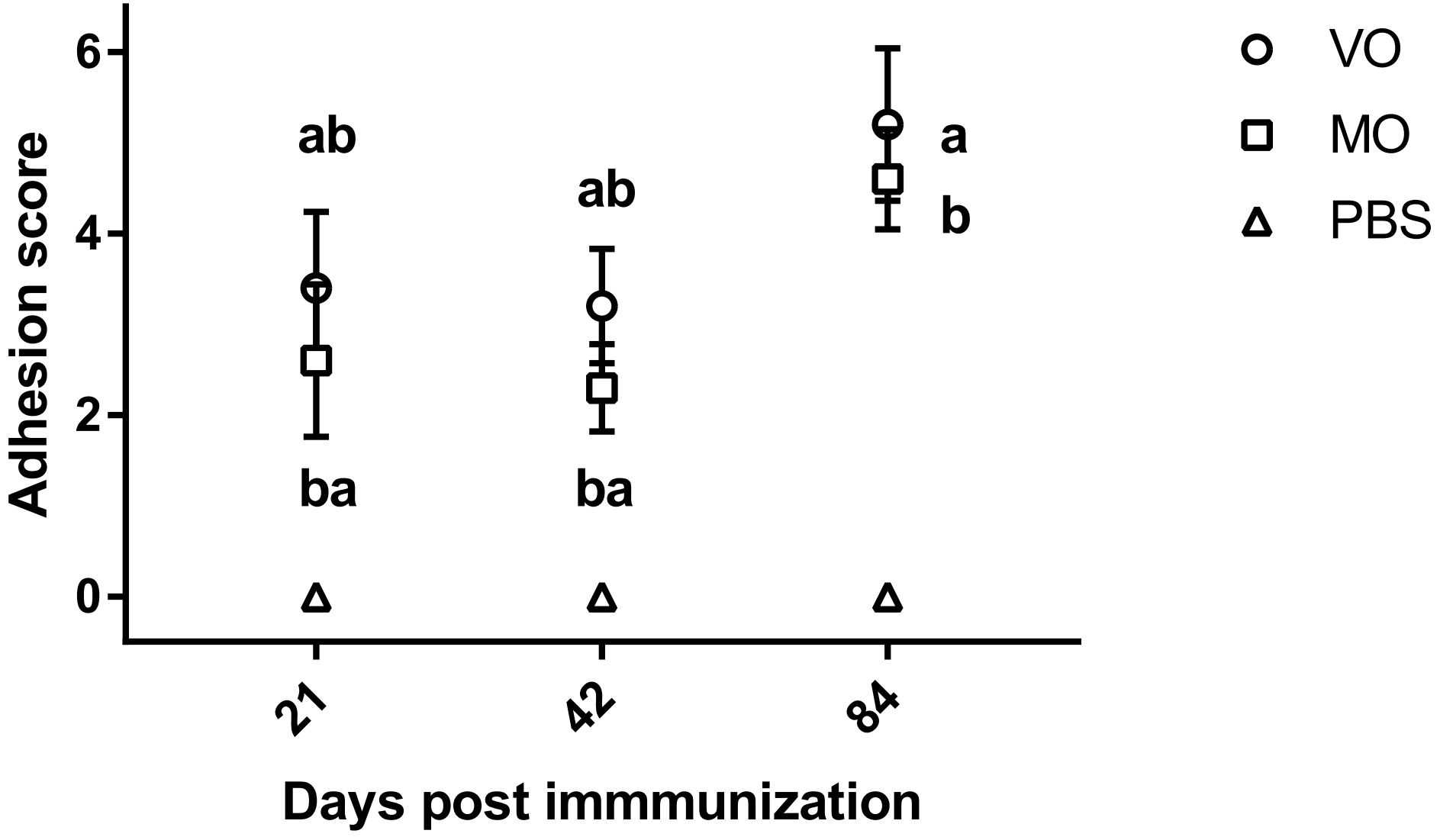
immunization. Note the presence of red stained *V. anguillarum* O1 antigens in the vaccinated fish. Scale bar = 100 μm. Image shown at 10 x magnification.


%Cumulative mortality
40
30
20
10
0
0
10
20
30
40
50
Hours post immunization
PBS
VO
MO

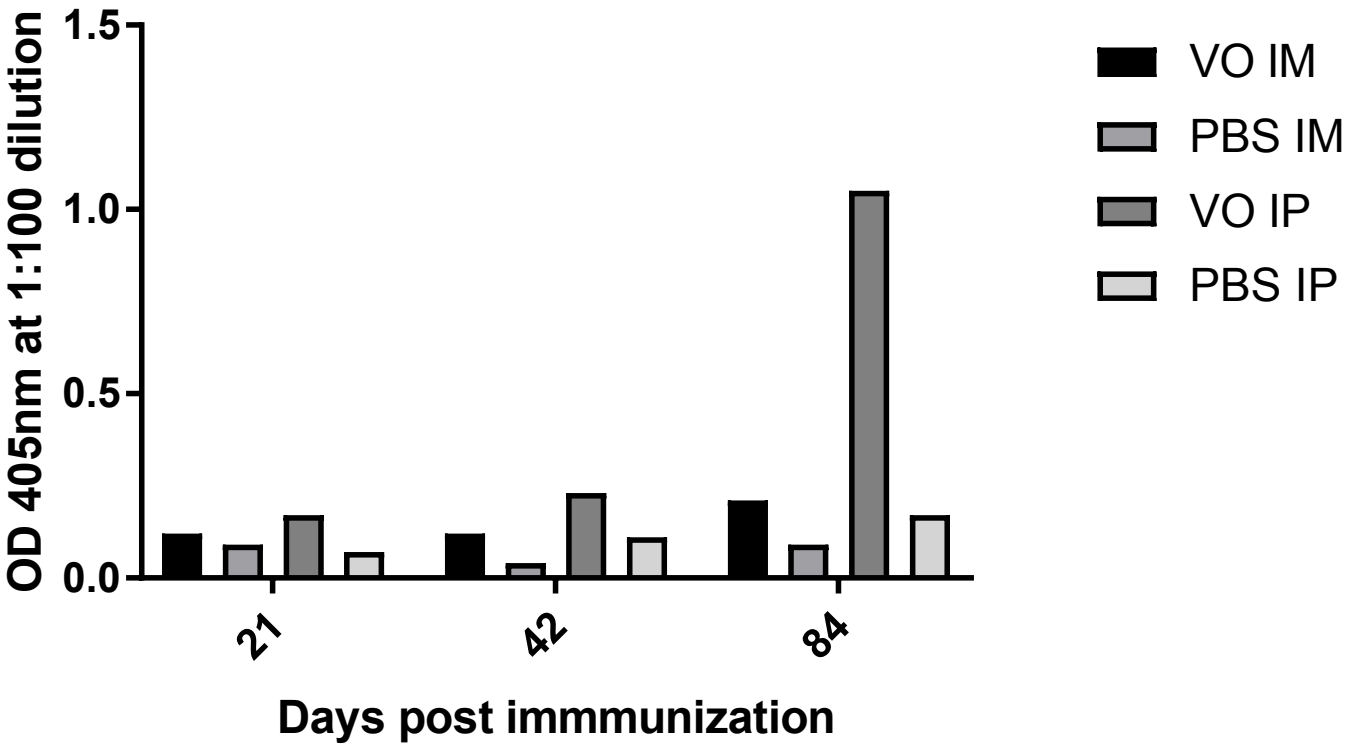

150
100
50
0
Mean weight (g)
a
b
c
a
b
c
a
b
c
ab
ba
c
0
21
42
84
Days post immmunization
VO
MO
PBS


150
100
50
0
Mean weight (g)
a
b
c
ac
b
ca
ac
b
ca
ab
bac
cb
0
21
42
84
Days post immmunization
VO
MO
PBS

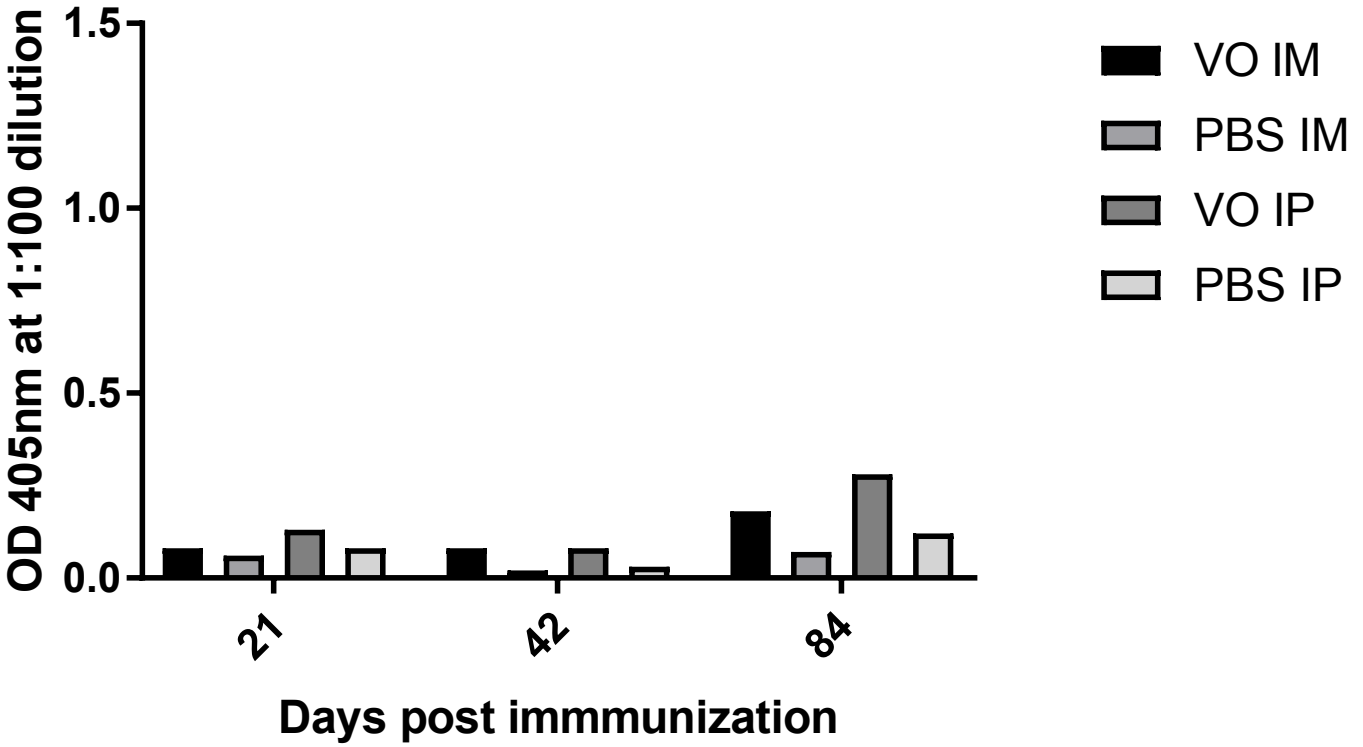

150
100
50
0
Mean weight (g)
a
b
c
ac
b
ca
ac
b
ca
ab
bac
cb
0
21
42
84
Days post immmunization
VO
MO
PBS


Adhesion score
6
4
2
0
ab
ab
a
b
ba
ba
21
42
84
Days post immmunization
VO
MO
PBS

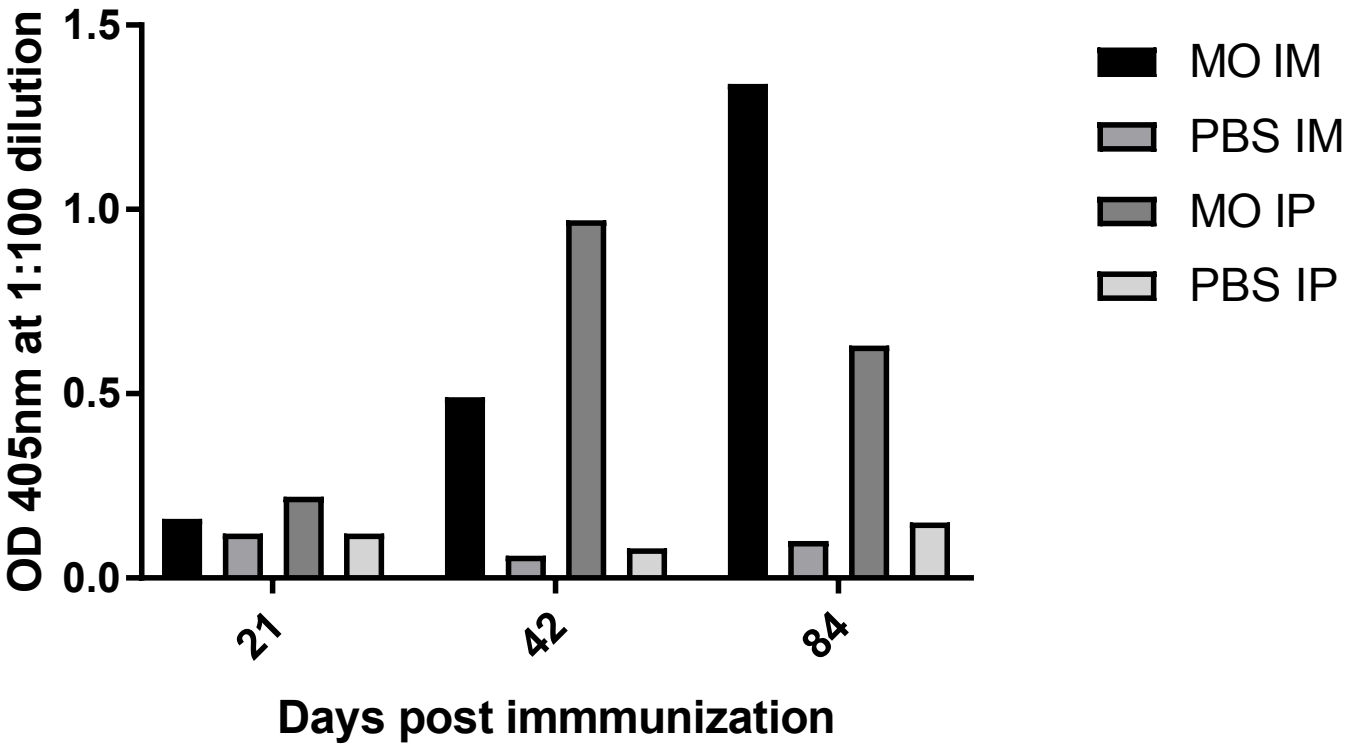
a)


OD 405nm at 1:100 dilution
1.5
1.0
0.5
0.0
21
42
84
Days post immmunization
VO IM
PBS IM
VO IP
PBS IP

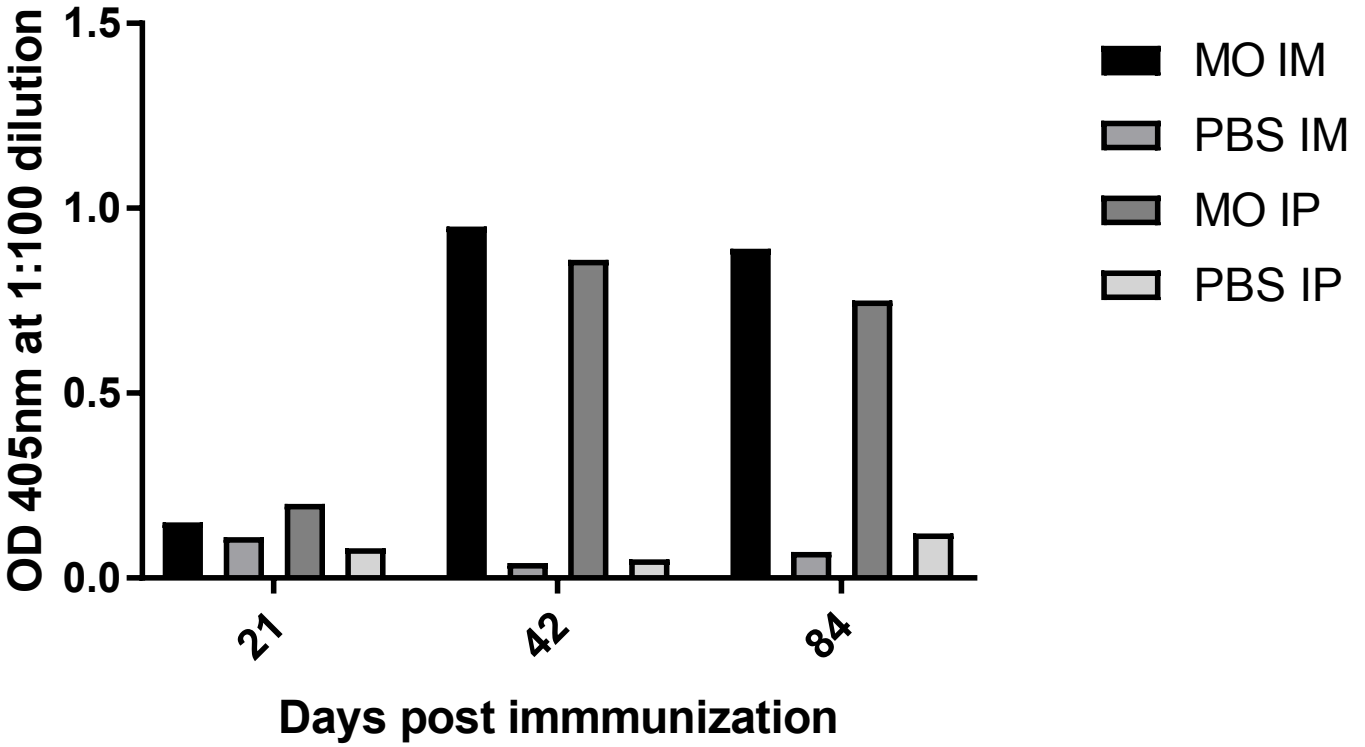
b)

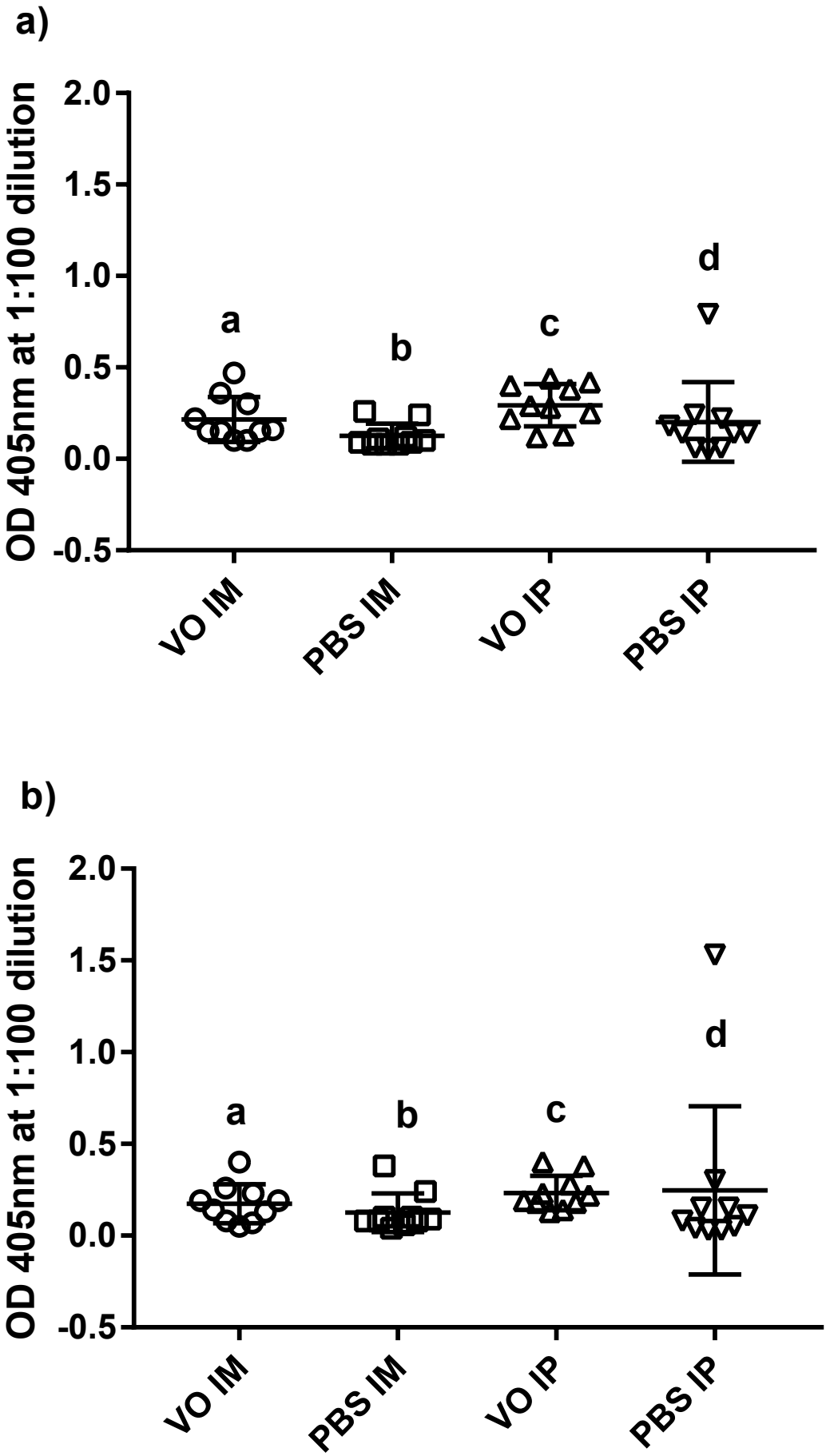

OD 405nm at 1:100 dilution
1.5
1.0
0.5
0.0
21
42
84
Days post immmunization
VO IM
PBS IM
VO IP
PBS IP

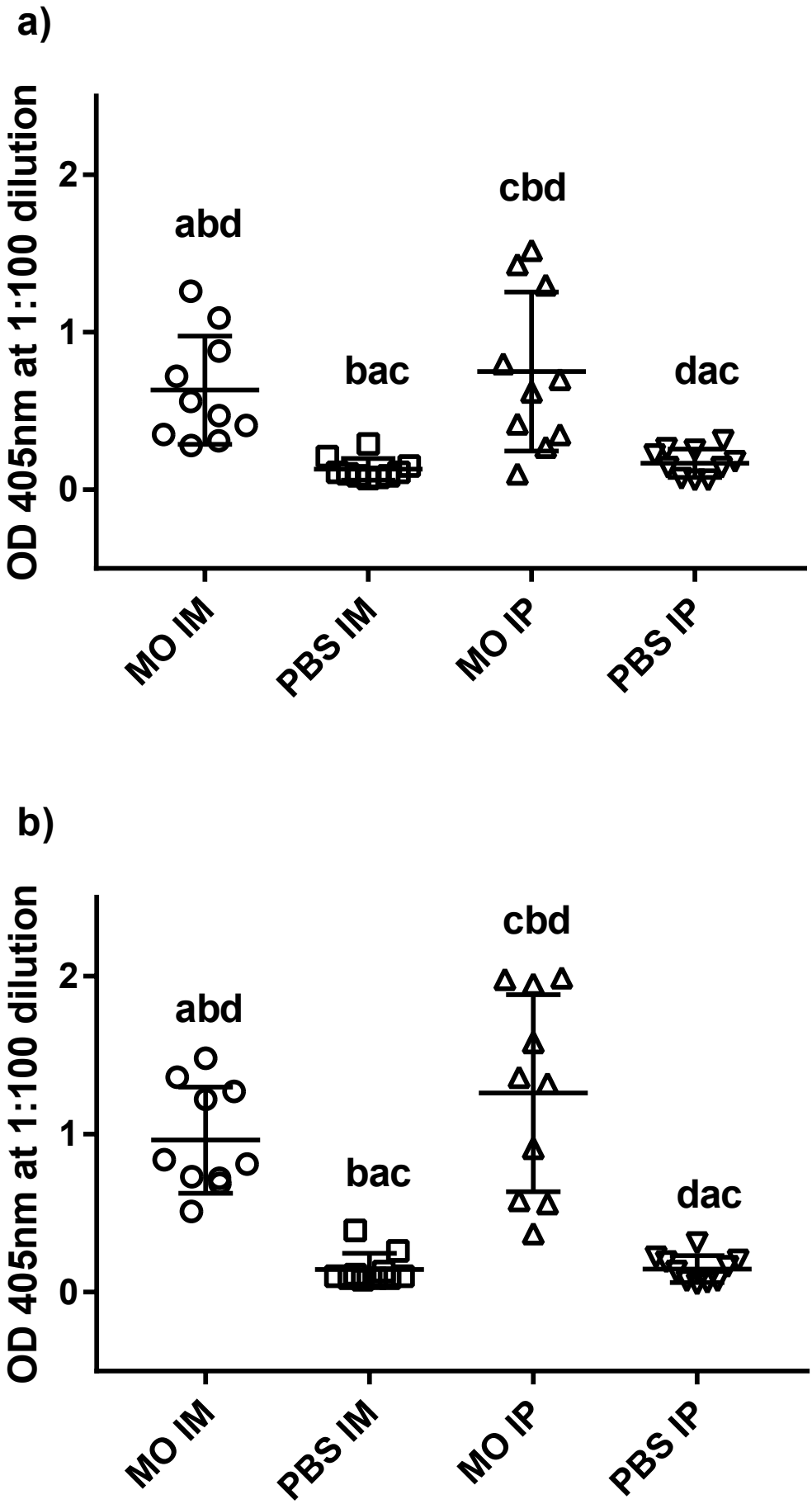
a)

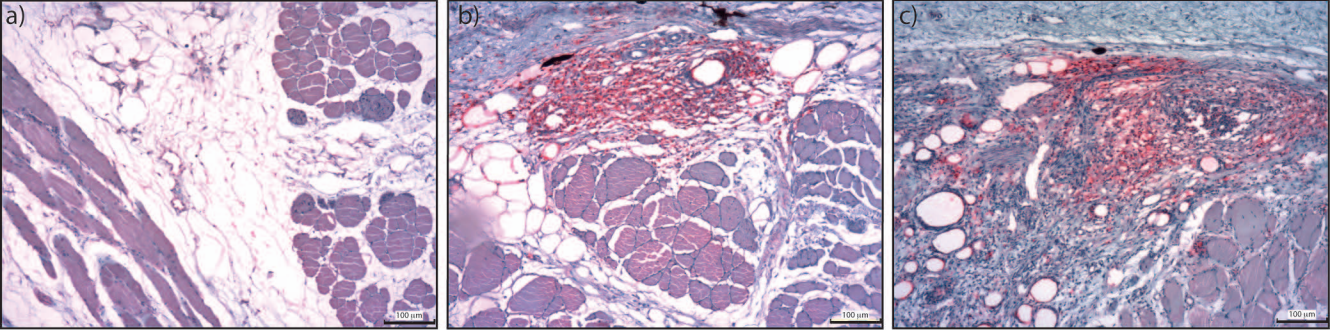

OD 405nm at 1:100 dilution
1.5
1.0
0.5
0.0
21
42
84
Days post immmunization
MO IM
PBS IM
MO IP
PBS IP

b)


OD 405nm at 1:100 dilution
1.5
1.0
0.5
0.0
21
42
84
Days post immmunization
MO IM
PBS IM
MO IP
PBS IP


a)
OD 405nm at 1:100 dilution
2.0
1.5
1.0
0.5
0.0
-0.5
a
b
c
d
VO IM
PBS IM
VO IP
PBS IP
b)
OD 405nm at 1:100 dilution
2.0
1.5
1.0
0.5
0.0
-0.5
a
b
c
d
VO IM
PBS IM
VO IP
PBS IP


a)
OD 405nm at 1:100 dilution
2
1
0
abd
bac
cbd
dac
MO IM
PBS IM
MO IP
PBS IP
b)
OD 405nm at 1:100 dilution
2
1
0
abd
bac
cbd
dac
MO IM
PBS IM
MO IP
PBS IP


a)
100 µm
b)
100 µm
c)
100 µm