Vaccination of Atlantic lumpfish (*Cyclopterus lumpus* L.) at a low temperature leads to a low antibody response against *Aeromonas salmonicida*.

Toni Erkinharju[1], Roy A. Dalmo[1], Øyvind Vågsnes[2], Ivar Hordvik[3], and Tore Seternes[1]

1 Norwegian College of Fishery Science, University of Tromsø, Tromsø, Norway

2 Vaxxinova Norway AS, Bergen, Norway

3 Department of Biology, University of Bergen, Bergen, Norway

Keywords: Atlantic lumpfish, vaccine, Aeromonas salmonicida, temperature, immune response, side effect

Abstract

We present a study on the effect of water temperature on immunization of Atlantic lumpfish. 360 fish were vaccinated with either 50 µl of an oil-based injection vaccine (VAX), with *Aeromonas salmonicida* and *Vibrio salmonicida* antigens, or PBS. Fish were vaccinated at three different water temperatures, 5 °C, 10 °C and 15 °C, and sorted into 6 groups (N=60). Lumpfish were weighed every three weeks after vaccination, sampled at 3, 6, 9 and 18 weeks post immunization (wpi), and evaluated by modified Speilberg score, ELISA and immunoblotting. Vaccinated fish showed low antibody response against *V. salmonicida*. Fish vaccinated at 5 °C showed significantly lower antibody response against *A. salmonicida* throughout the study. At higher temperatures, vaccinated fish showed significantly increased antibody responses, at 18 wpi for 10 °C and at 6 and 18 wpi for 15 °C. Immunoblotting demonstrated specific response against the LPS antigen of *A. salmonicida* in the 10 °C and 15 °C VAX groups. Mean body weight increased in all groups throughout the study. Vaccinated fish had low Speilberg scores with no melanisation of abdominal tissue. Our results show that vaccinating lumpfish at a lower water temperature may lead to a low antibody response against *A. salmonicida*.

Introduction

Atlantic lumpfish (*Cyclopterus lumpus* L.) and different species of labrid fish (mostly goldsinny wrasse, *Ctenolabrus rupestris* L., corkwing wrasse, *Symphodus melops* L., and ballan wrasse, *Labrus bergylta* L.) are used as "cleanerfish" for delousing of sea lice in aquaculture production of salmonid fish. The number of cleanerfish put into cages with Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* L.) in Norway has increased exponentially in the last few years, and reached 36.1 million individuals during 2016 (Norwegian Directorate of Fisheries (2017)). Lumpfish was the most commonly used species, with 15.8 million individuals sourced from both wild caught fisheries and farming.

Wild populations of lumpfish have a wide geographic distribution and are located in both the Western and Eastern part of the North Atlantic Ocean. In the Eastern part records have shown that observations of lumpfish occur along the entire Norwegian coastline and as far north as the Svalbard archipelago (J Davenport (1985)). According to hydrographical measurements, the water temperature in the upper layers of Norwegian coastal waters vary throughout the year, and can be below 5 °C over several months during winter time in Northern Norway (Nordland, Troms and Finnmark county), and be as high as 16 °C during summer time in Southern Norway (Institute of Marine Research (2014)). As such, lumpfish have a broad temperature range and a recent study has shown that lumpfish continue to feed and grow at temperatures close to 4 °C and 16 °C (Nytro et al. (2014)). Wrasses are more temperature sensitive and enter a state of reduced physiological activity (torpor) at temperatures below 5-7 °C (Espeland et al. (2010); Sayer and Reader (1996)). Therefore, lumpfish is regarded as a more suitable cleanerfish species for the northernmost salmon farms.

Studies have shown that environmental temperature can affect the immune system of several teleost fish species, especially antibody levels and antibody related functions (Huang, Ma, and Wang (2011); Lillehaug, Ramstad, Bækken, and Reitan (1993); Lorenzen et al. (2009);

Magnadottir et al. (1999); Martins, Xu, Shoemaker, and Klesius (2011); Varsamos, Flik, Pepin, Bonga, and Breuil (2006)). To our knowledge there are only a few studies published on the immune system of Atlantic lumpfish. Haugland et al. (2012) isolated leukocytes from the peripheral blood, head kidney and spleen of wild caught lumpfish males, and analyzed the non-specific immune response by the use of flow cytometry. The authors successfully identified several different leukocytes and measured high phagocytic capacity and high respiratory burst activity, indicating a possible first step in the antigen presentation route of the lumpfish immune system. In another study, Rønneseth et al. (2015) isolated IgM+ B cells from peripheral blood, head kidney and spleen, which demonstrated both high phagocytic abilities and high proportion among blood phagocytes. However, the highest phagocytic capacity was observed in monocytes and in some uncharacterized IgM- cells. Lumpfish were also immunized with *Vibrio ordalii*, *Vibrio anguillarum*, two strains of atypical *Aeromonas salmonicida*, and a *Pasteurella*-like bacteria, eventually showing a greater presence of specific antibodies in immunized fish, except for fish immunized with the *V. ordalii* agent. Finally, lumpfish antibodies have been further studied by Bilal et al (2016), who compared the IgM of lumpfish and five species of wrasse. For lumpfish the IgM concentration in serum was estimated to 1-2,6 mg/ml, or 3% of the total protein concentration, near the same level as Atlantic salmon.

Further knowledge of the lumpfish immune system is important to develop effective vaccines against disease. A number of different bacterial agents associated with infectious disease in other fish species, have been detected in lumpfish over the last few years. This includes reports of *Pasteurella* sp., *Vibrio anguillarum*, *Vibrio ordalii*, *Aeromonas salmonicida*, *Pseudomonas anguilliseptica*, *Moritella viscosa* and *Tenacibaculum maritimum* (reviewed in Powell et al. (2017)). Immunization and vaccination studies have shown that it is possible to induce development of specific antibody responses and immunological protection against

bacterial diseases in lumpfish (Erkinharju et al. (2017), Ronneseth et al. (2015), Ronneseth, Haugland, Colquhoun, Brudal, and Wergeland (2017)). However, little is known on the possible effects of low water temperatures on the development of antibody response in Atlantic lumpfish.

To investigate if temperature modulates the lumpfish antibody response, we hypothesized that vaccinating lumpfish at three different water temperatures (5 °C, 10 °C and 15 °C) induces different antibody responses. To study this we used an experimental vaccine with two bacterial antigens, *Aeromonas salmonicida* and *Vibrio salmonicida*, known to cause furunculosis and vibriosis, two significant diseases affecting both cultured and wild-living marine fish (Jansson and Vennerström (2014)). As far as the authors are aware, this is the first study to examine lumpfish antibody response against bacterial antigens after immunization at a low water temperature.

Materials and methods

Fish

Atlantic lumpfish (*Cyclopterus lumpus* L.) were kindly donated by Akvaplan-niva AS (Tromsø). The fish were kept in the rearing facilities at the Aquaculture Station in Tromsø, in circular 140 L tanks with continuous flow of seawater. The fish were fed Amber Neptun (Skretting AS, Norway) ad libitum throughout the experiment. The fish were kept under constant 24:0 hour light:dark conditions where water temperature, fish appetite, behavior and mortality was checked daily, while O_2 saturation was checked weekly. Two months prior to the start of the experiment several lumpfish displayed wounds/lesions on the tail, and all lumpfish were treated 2 x with formalin (1:3 000 dilution) by the fish health personnel. However, the fish displayed no signs of disease or mortality at the start of the experiment. Prior to the start of the experiment, the lumpfish were sorted into three different water temperature groups, kept in separate 140 L tanks. The water temperature was gradually adjusted to and kept at 5 ^{o}C (± 1 ^{o}C), 10 ^{o}C (± 1 ^{o}C) or 15 ^{o}C (± 1 ^{o}C) for the different groups. Feeding was withheld for 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), under ID number 6843.

Vaccine

An experimental vaccine was specifically produced and provided by Vaxxinova Norway AS for this study. The vaccine contained two formalin-inactivated bacterial antigens: *Aeromonas salmonicida* and *Vibrio salmonicida*. The vaccine was prepared by using a vegetable oil-based

adjuvant (unspecified). Any further information regarding the adjuvant and bacterial isolates used for the vaccine has been withheld due to competitive considerations.

Immunization/vaccination of lumpfish

A total of 360 lumpfish were used for this study. Three groups of 60 fish were immunized by intraperitoneal (ip.) injection of 0.05 ml per fish of the vaccine (VAX), whereas three groups of 60 fish received 0.05 ml phosphate-buffered saline (PBS). The 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 Socorex® self-refilling syringe and 0.7 x 7 mm vaccination needles (Unimed SA, Switzerland). After immunization the lumpfish were kept in six separate 140 L tanks of 60 fish in each, according to water temperature and vaccine/control groups; 5 ^{o}C VAX, 5 ^{o}C PBS, 10 ^{o}C VAX, 10 ^{o}C PBS, 15 ^{o}C VAX and 15 ^{o}C PBS.

Scoring of vaccine side effects

Lumpfish were evaluated for possible vaccine side effects. Body weight and length was measured in all groups and the number of dead fish was registered during the full length of the study. The side effects were evaluated and given score using a modification of the Speilberg scoring system adopted to lumpfish as described previously (Erkinharju et al., 2017).

Sampling from immunized lumpfish

Series of 10 fish were randomly collected from all groups at 3 weeks post immunization (wpi.) and series of 20 fish were collected from the 5 ^{o}C VAX, 5 ^{o}C PBS, 15 ^{o}C VAX and 15 ^{o}C PBS groups at 6 wpi., and from the 10 ^{o}C VAX and 10 ^{o}C PBS at 9 wpi. The remaining

fish in all groups were collected at 18 wpi. Fish were collected according to this regime to compare results from the different temperature groups at early immunization (3 wpi), at the end of the immunization study (18 wpi) and at 630 degree days post immunization (ddpi.) (Table 1). The fish were sacrificed by a sharp blow to the head and blood sampling performed immediately after. 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 recorded, and vaccine side effects (modified Speilberg score) was evaluated prior to organ sampling. Organ samples were dissected from skin, heart, head kidney, gills, spleen, liver and the gastro-intestinal tract, including the pyloric caeca. All samples were put in 10% formalin.

At 5 wpi., several lumpfish in the 15 °C groups displayed restless behavior with fast breathing at the water surface and loss of appetite. Five of these fish were collected, three from the 15 °C VAX and two from the 15 °C PBS group, killed by a sharp blow to the head and checked for possible fish health related problems. No specific pathogen was detected, except for a few *Gyrodactylus* sp. parasites in the gills. Water temperature for both groups was at 15,2 °C, and the oxygen (O_2) saturation was 95% for 15 °C VAX and 94% for 15 °C PBS, respectively. Based on this observation and by recommendation from fish health personnel in charge at the research facility, all lumpfish groups were treated with 2 x formalin (1:3 000 dilution). At the end of treatment, the lumpfish had resumed natural swimming and feeding behavior, which remained unchanged for the rest of the study. Mortality was very low; one dead fish in the 15 °C VAX was registered during the formalin treatment.

Enzyme-linked immunosorbent assay (ELISA)

A whole cell/competitive ELISA was used to determine serum reactivity to formalin-inactivated bacterial antigens (*Aeromonas salmonicida* and *Vibrio salmonicida*). Vaxxinova Norway AS provided all antigens. Serum pools was prepared by mixing 20 µl serum from each sampled fish per group. Pools and individual samples of lumpfish sera were diluted 1:100 and 1:200 and analyzed as described previously (Erkinharju et al., 2017). In addition analysis of specific serum reactivity against preparations of A-layer protein and lipopolysaccharide (LPS) from *A. salmonicida* (purified as described in Bjørnsdottir et al (1992) and Dalmo et al (1998)) was performed. By using stepwise incubations with polyclonal rabbit anti-lumpfish IgG (1h) (produced according to Bilal et al. (2016)), a goat-anti-rabbit Ig conjugated with alkaline phosphatase (1h) (Sigma) and p-nitrophenyl-phosphate as substrate (30 min) (Sigma), bound antibodies were detected by reading the colour reaction at a wavelength of OD_{405} nm with a spectrophotometer (VersaMax Absorbance Microplate Reader; Molecular Devices, LLC, USA).

In addition, analysis of specific serum reactivity against preparations (1 µg/ml) of A-layer protein and lipopolysaccharide (LPS) from *A. salmonicida* (purified as described in Bjørnsdottir et al (1992) and Dalmo et al (1998)) was performed. Individual lumpfish sera, and pooled sera from four lumpfish of each group (5 ^{o}C VAX, 5 ^{o}C PBS, 10 ^{o}C VAX, 10 ^{o}C PBS, 15 ^{o}C VAX and 15 ^{o}C PBS) sampled at 18 wpi, were evaluated by ELISA as described above.

Western blotting

Specific antibodies in pooled sera from four lumpfish of each group (5 ^{o}C VAX, 5 ^{o}C PBS, 10 ^{o}C VAX, 10 ^{o}C PBS, 15 ^{o}C VAX and 15 ^{o}C PBS) sampled at 18 wpi, were evaluated qualitatively by Western blotting, using purified LPS from *A. salmonicida*. LPS (2.25 µg) in 1x LDS with 20 mM DTT were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (4–12% NuPAGE; Invitrogen), followed by transfer to a

0.45 μm pore size polyvinylidene difluoride membrane (Millipore). The membrane was washed with TBS (20 mM Tris and 137 mM NaCl, pH 7.6) and then blocked in TBS with 0.1% (v/v) Tween-20 (TTBS) with 5% (w/v) non-fat dry milk. After blocking, the membrane was washed three times in TTBS and incubated for 24 hours at 4 °C with serum diluted 1:20 in TTBS with 5% (w/v) non-fat dry milk added to the TTBS. The membrane was washed three times, followed by incubation with primary antibody diluted 1:1000 overnight at 4 °C. After three washes with TTBS, the membrane was incubated for 1 h with secondary antibody (goat anti-rabbit IgG (H+L) conjugated with HRP, Invitrogen). Antibodies were diluted in TTBS with 5% (w/v) non-fat dry milk. The membrane was washed three times in TTBS and detection was performed by using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). The size of the antigen was estimated using the MagicMark Western protein standard (Invitrogen).

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 two-way and one-way ANOVA with Tukey Multiple Comparison test, and differences were considered significant with a p-value less than 0.05 ($p < 0.05$). For growth, vaccine side effects and specific antibody response (IgM) data, all vaccinated groups (VAX) were compared to their respective control groups (PBS). In addition, for vaccine side effects and specific antibody response (IgM) data, the different vaccine and control groups were compared to each other.

Results

Fish growth

Vaccination of fish with injectable oil-adjuvanted vaccines may cause side-effects affecting the animals' growth, health and welfare (Poppe and Koppang (2014)). Thus, we analyzed lumpfish growth following ip. immunization by measuring body weight at the start of immunization on day 0, followed by 3, 6, 9, 12, 15 and 18 wpi (Fig. 1). At the end of the study at 18 wpi, all fish demonstrated a significant increase in mean weight ($p < 0.05$) when compared to the mean weight at the start on day 0 (320% for 5 °C VAX, 300% for 5 °C PBS, 381% for 10 °C VAX, 503% for 10 °C PBS, 396% for 15 °C VAX and 425 for 15 °C PBS). The average weight of the fish in the different vaccine groups was lower than the weight of control fish, but only the 10 °C groups were statistically different. Fish vaccinated at 10 °C demonstrated a significant reduction by 19 % at both 15 ($p=0.02$) and 18 wpi ($p=0.01$) when compared to the control fish. Vaccinated fish in the 5 °C and the 15 °C group demonstrated a weight reduction of 7 % ($p=0.96$) and 8 % ($p=0.82$) at 15 wpi, and 9 % ($p=0.87$) and 11 % ($p=0.33$) at 18 wpi, respectively.

Side effect score

As immunisations may induce other side effects than decreased growth, we evaluated side effects using a modified Speilberg score following ip. injections of vaccine. The degree of adhesions between viscera and abdominal wall, visible melanin pigmentation of viscera and abdominal wall/fillet, and vaccine residues of lumpfish at 6, 9 and 18 wpi was evaluated (Table 2). Immunised fish showed low to moderate adherence scores at every sampling point, with no statistical significant differences between the different temperature levels ($p > 0.05$) at 6, 9 and 18 wpi. Melanisation of viscera or abdominal/fillet was not observed in any vaccinated fish. All vaccinated groups showed a reduction in mean score for vaccine residues

over time, where the 5 °C group was significantly lower than the both the 10 °C and the 15 °C group ($p < 0.05$) at 18 wpi. No intra-abdominal adhesions, melanisations or residual vaccines were observed in the control fish injected with PBS.

On gross examination of vaccinated fish we observed fibrinous strands between abdominal organs (typically liver and gastro-intestinal tract) or between organs and the abdominal wall (Fig. 2). Residual vaccine was registered as either free-floating or varying degree of encapsulated droplets. No melanisation of viscera or abdominal/fillet was observed. No clear distinction could be established between the different temperature groups.

Antibody response against A. salmonicida and V. salmonicida

Antibody response has been described to be water temperature dependent (Makrinos & Bowden, 2016). Thus, the specific IgM response of serum samples from immunized lumpfish against *A. salmonicida* and *V. salmonicida* was measured by ELISA at 3, 6, 9 and 18 wpi, and at 105, 210, 315, 630, 1260 and 1890 ddpi. The antibody contents of pooled serum samples from vaccinated fish suggested a robust IgM response against *A. salmonicida* at 6 and 18 wpi. for fish vaccinated at 15 °C, and at 9 and 18 wpi. for fish vaccinated at 10 °C (Fig. 3a). However, the IgM response against *A. salmonicida* was low for fish vaccinated at 5 °C and close to the same level as in the control fish serum samples. The vaccinated fish in both the 10 °C and 15 °C groups showed high IgM responses at 630 ddpi., and at 1260 ddpi for the 10 °C group and at 1890 ddpi for the 15 °C group (Fig. 3b). The response was low for the 5 °C group.

ELISA was also used to analyze specific individual IgM response against *A. salmonicida* and *V. salmonicida*. Sera from fish sampled at 3, 6, 9 and 18 wpi. were subjected to analysis. Fish vaccinated at 10 °C and 15 °C showed significant increase in the IgM response against *A. salmonicida* at 18 wpi. ($p < 0.0001$), and at 6 wpi. for the 15 °C group ($p=0.004$), when

compared to the mean IgM response at 3 wpi. (Fig. 4). Fish vaccinated at 5 °C showed non-significant IgM response at both 18 wpi. ($p=0.43$) and 6 wpi. ($p=0.86$) when compared to the mean response at 3 wpi. No significant increase in IgM response was registered in control fish kept at the same temperature throughout the study ($p > 0.05$).

Fish vaccinated at 10 °C demonstrated a specific IgM response against *A. salmonicida*, which was significant when compared to control fish at 18 wpi. ($p < 0.0001$), but not at 3 wpi. ($p > 0.99$) and 9 wpi. ($p=0.10$) (Fig. 4). The IgM response for fish vaccinated at 15 °C was significant when compared to control fish at both 18 wpi. ($p=0.04$) and 6 wpi. ($p=0.02$), but not at 3 wpi. ($p=0.99$). Fish vaccinated at 5 °C showed no significant differences from the control fish ($p > 0.05$).

It has been shown that for several fish species the antibody levels may be reduced at low water temperatures and increased at higher water temperatures (Makrinos & Bowden, 2016). As such, we compared the specific IgM response of vaccinated fish against *A. salmonicida* at 3 wpi., 18 wpi. and 630 ddpi. (Fig. 4). At three weeks post immunization there was no significant difference between vaccinated fish in any temperature group ($p > 0.05$). Fish vaccinated at 10 °C and 15 °C both showed significant IgM response at eighteen weeks post immunization, compared to fish vaccinated at 5 °C ($p=0.0032$ and $p=0.03$, respectively). The 10 °C and 15 °C groups were not significantly different compared to each other ($p=0.68$). At 630 degree days post immunization only fish vaccinated at 15 °C showed a significant IgM response, compared to fish vaccinated at 5 °C ($p=0.04$).

Lumpfish sera were also analysed for specific IgM response against *V. salmonicida* by ELISA. All vaccinated groups at the different temperatures demonstrated low levels of IgM response against *V. salmonicida*, close to the level of the fish in the control groups, except for two individual high responders detected at 6 wpi. (or 630 ddpi.) in the 15 °C group (Fig. S1).

Antibody response against purified LPS and A-layer

It has been shown that the fish antibody response to *A. salmonicida* are mainly directed against the A-layer protein and the LPS O-antigen (Gudmundsdottir & Bjornsdottir, 2007). As such, we tested the IgM response of five individual lumpfish sera from each group (except for three from the 5 °C and two from the 5 °C PBS group at 3 wpi. against purified preparations of the A-layer protein and LPS from *A. salmonicida* by ELISA at 3, 6, 9 and 18 wpi. Only the sera from one fish, vaccinated at 5 °C and sampled at 6 wpi., showed a specific response against the A-layer protein, while the majority were considered to be low or non-responders (Fig. 5a). Similar results were observed against the LPS, where the sera of two fish only, vaccinated at 10 °C and sampled at 18 wpi., showed specific anti *A. salmonicida* LPS immunoreactivity (Fig. 5a). The majority of sera from control fish (PBS) all demonstrated low responses against both antigens (Fig. 5b).

The sera from four vaccinated lumpfish in each temperature group, showing high specific antibody response *against A. salmonicida* in the ELISA assay, were pooled and further analysed by ELISA and Western blotting. Sera from four control fish (PBS) were also pooled and included in the assays. When analysed by ELISA, vaccinated lumpfish in all groups demonstrated low antibody response against the A-layer, and only vaccinated lumpfish at 10 °C showed a high response against the LPS antigen (Fig. S2a). Control fish demonstrated low antibody response against both antigens (Fig. S2b). In the Western blotting lumpfish sera were analysed for specific antibodies against the LPS antigen. Sera from vaccinated fish at 10 °C and 15 °C showed binding to LPS in the 50-75 kDa region, with a strong band in the 10 °C VAX group and a weak band in the 15 °C VAX group (Fig. 6). No band was seen in the 5 °C VAX group. Sera from control fish showed no bands against LPS (not shown).

Discussion

In this study, we have vaccinated Atlantic lumpfish with a two-component oil-based injection vaccine at water temperatures of 5 °C, 10 °C and 15 °C. We have studied possible vaccine related side effects by evaluating records of body weight and side effect score data from a modified Speilberg scoring system. We have also analyzed the specific antibody response (IgM) against *A. salmonicida, V. salmonicida* and purified LPS and A-layer protein after immunization, with ELISA and Western blotting assays.

Juvenile lumpfish kept in captivity, both vaccinated and unvaccinated, has shown rapid growth rates and increase in body mass in earlier studies (Imsland et al. (2016); Nytro et al. (2014)). In our study, only lumpfish vaccinated at 10 °C demonstrated a significant reduction in mean weight at 15 and 18 wpi., when compared to the control fish. Although growth retardation after vaccination have been observed in several studies of salmonid fish (Berg, Rodseth, Tangeras, & Hansen, 2006; Midtlyng & Lillehaug, 1998; Oppedal, Berg, Olsen, Taranger, & Hansen, 2006), reports on side effects following immunisation with adjuvanted vaccines in other farmed fish species are few. Magnadottir et al. (2001) observed no differences in growth rate between Atlantic cod (*Gadus morhua* L.) immunised with PBS, haptenated or non-haptenated protein antigen. In a recent field study of lumpfish immunized with a trivalent injection vaccine, Sæbjørnsen (2017) reported no differences in specific growth rate between vaccinated and non-vaccinated fish. The exact cause of the reduced mean weight at 10 °C in our study is therefore difficult to explain.

In the present study, vaccinated lumpfish demonstrated low to moderate adhesion scores with no significant differences between the different temperature levels. For Atlantic salmon water temperature is an important factor affecting the development of intra-abdominal side effects after vaccination with oil-based injection vaccines. Berg et al (2006) observed faster development and higher levels of intra-abdominal lesions in fish that were vaccinated at the

highest water temperature. In addition, all vaccinated groups showed increased adhesion scores after sea transfer, when the temperature increased during summer and autumn. However, it is difficult to compare our results with other aquaculture fish species, since oil-based adjuvanted vaccines are seldom used outside of salmonid aquaculture and there are very few reports of vaccine-related side effects in non-salmonid species (Poppe & Koppang, 2014). Magnadottir et al (2001) described fusion of inner organs in Atlantic cod immunised with two different protein antigens, mixed with 1:1 with Freunds complete adjuvant (FCA) and kept at a water temperature of either 4 or 9 °C, but no further description of temperature-related differences was reported. Sæbjørnsen (2017) observed low to moderate levels of intra-abdominal adhesions in vaccinated lumpfish kept in sea cages with salmon, where the ambient water temperature was roughly 6-8 °C at the first recording, and then dropped to 2-7 °C towards the final recording. However, there were some uncertainty to the scoring due to a low number of recorded individuals.

Melanisation at the injection site of the vaccine is common in Atlantic salmon and is used as an important criteria when evaluating injection site reactions by the "Speilberg scoring system". Extensive melanisation may lead to downgrading of salmon carcass quality and economic loss for the producer (Poppe & Koppang, 2014). We observed no signs of dark pigmentation, caused by melanisation, in any of the vaccinated lumpfish. Similar results were noted in an earlier study, where lumpfish were vaccinated with two different oil-based multivalent vaccines through intramuscular and intraperitoneal injection (Erkinharju et al., 2017). Sæbjørnsen (2017) also registered no melanisation of abdominal organs or muscle fillet after vaccination with a trivalent vegetable oil-based injection vaccine. Absence of melanin formation after vaccination has also been reported from other marine species, such as Atlantic cod and ballan wrasse (*Labrus bergylta* L.) (Fløgum (2016); Maira C. (2008)). Melanin and melanin-containing cells have been described in Atlantic lumpfish skin (J.

Davenport & Bradshaw, 1995), but to the best of our knowledge there are no reports of melanin-associated side effects occurring in lumpfish after vaccination. As such, melanisation should not be used as part of the evaluation criteria when examining vaccine-related side effects in lumpfish. However, additional work is needed to further investigate the role of melanin in vaccination of teleost fish.

There are indications in the scientific literature that fish may rely more on specific immune responses at higher water temperatures, such as up-regulation of antibody related functions (Makrinos and Bowden (2016)). Our results demonstrated a significantly high antibody (IgM) response against *A. salmonicida* for vaccinated fish at 10 °C and 15 °C, when compared to control fish at 6 and 18 wpi. In addition, at 18 wpi. vaccinated fish at both 10 °C and the 15 °C had significantly higher antibody response than fish at 5 °C. A few recent studies have observed high antibody levels against atypical strains of *A. salmonicida* after injection with oil-based vaccines at temperature levels of 10 °C (Erkinharju et al., 2017; Nordstrand, 2017) and 12 °C (Ronneseth et al., 2017). The lumpfish vaccinated at 15 °C reached a peak in the antibody response against *A. salmonicida* at six weeks post immunization, while lumpfish vaccinated at 10 °C reached the highest antibody response levels at eighteen weeks post immunization. Similar kinetics in the antibody response against *A. salmonicida* have been reported from studies on Atlantic salmon (Eggset, Mikkelsen, & Killie, 1997; Romstad, Reitan, Midtlyng, Gravningen, & Evensen, 2012). However, the duration of high antibody levels was different, were Romstad et al. (2012) observed a significant decrease from 9 wpi to 12 wpi, and Eggset et al. (1997) noted high levels at both 9 and 18 wpi. Our results are in agreement with Eggset et al. (1997).

Lumpfish vaccinated at a water temperature of 5 °C did not demonstrate a significant antibody (IgM) response against *A. salmonicida* at any sampling point, when compared to control fish immunised with PBS. Our results are in agreement with other vaccination studies

on Atlantic salmon, were fish reared and vaccinated at 2 °C (Eggset et al., 1997) and 3 °C (Romstad et al., 2012) showed very low antibody responses against *A. salmonicida* after injection. This is also supported by studies on other fish species immunised with different antigens at different water temperatures, were the lower temperature levels lead to either low or delayed specific immune responses (Lillehaug et al. (1993); Lorenzen et al. (2009); Martins et al. (2011); Rijkers, Frederixwolters, and Vanmuiswinkel (1980); Xu, Sheng, Xing, and Zhan (2011)). However, it is uncertain whether the results observed in our study is due to low production of circulating antibody levels, or the result of a delay in the specific immune response.

In the present study, we examined the specific antibody response against *A. salmonicida* for lumpfish at different degree-days post vaccination. For fish vaccinated at 5 °C we observed a low response up to 630 ddpi., while fish vaccinated at 10 °C and 15 °C demonstrated a high response at 1260 and 1890 ddpi., respectively. At 630 ddpi., only fish vaccinated at 15 °C showed significant antibody response when compared to control fish. Although vaccine companies rarely recommend water temperature levels for successful vaccination of fish, they often provide a minimum amount of degree-days after vaccination for immunity to develop. For oil-adjuvanted vaccines for Atlantic salmon, this is often 500 degree-days from vaccination to sea transfer (Lillehaug, 2014). Immunological protection against *A. salmonicida* has been shown in studies of Atlantic salmon immunised at different water temperature levels, although the specific antibody response was reduced at low temperatures (Eggset et al. (1997); Eggset, Mortensen, and Loken (1999); Romstad et al. (2012)). However, in our study we have only examined the specific antibody responses, and a follow up challenge study is needed to determine if vaccinated lumpfish are protected against disease.

In the present study the specific antibody response (IgM) of vaccinated lumpfish against *V. salmonicida* was lower than for *A. salmonicida* in all temperature groups. We have observed

similar results in a recent immunization study after vaccinating lumpfish with multivalent vegetable and mineral oil-based injection vaccines, were the IgM response against *Vibrio anguillarum* serotype O1 and *Moritella viscosa* sp. were lower than for two strains of atypical *A. salmonicida* (Erkinharju et al., 2017). There are few published studies examining the antibody response after vaccination with *V. salmonicida* in other fish species. Several studies on Atlantic salmon have shown that it is possible achieve a specific immune response*:* by vaccinating with monovalent vaccines (Hoel, Reitan, & Lillehaug, 1998), through injection with non-adjuvant containing vaccines (Steine, Melingen, & Wergeland, 2001), or with multivalent oil-adjuvanted vaccines (Berg et al., 2006), and when vaccinating at high water temperature levels (Eggset et al., 1997; Lillehaug et al., 1993). However, it is not known what is the exact cause of the low antibody response observed in our study. Rønneseth et al (2015) demonstrated significantly higher antibody response against *V. anguillarum*, but not *Vibrio ordalii*, after immunizing lumpfish three times with formalin-inactivated bacterial suspensions. It is unknown whether including additional immunisation steps in our study would have resulted in a higher antibody response against *V. salmonicida*.

Both the A-layer protein and LPS are considered the most immunoreactive structures of *A. salmonicida* when immunizing Atlantic salmon and rabbit Lund, Jorgensen, Holm, and Eggset (1991). By preparing pooled serum samples from four fish in each group, we detected a high antibody response against purified LPS in the 10 °C VAX group by ELISA, in addition to a strong band in the 10 °C VAX and a weaker band in the 15 °C VAX groups by Western blotting. Our results differ from Grøntvedt and Espelid (2004), who found no specificity against LPS in sera from vaccinated wolffish (*Anarhichas minor* Olafsen) by Western blotting or ELISA. Marsden et al (1995) found LPS to be a stronger immune stimulant of rainbow trout leukocytes than the A-layer at temperature level of 14-16 °C. However, it is uncertain to what magnitude LPS may have affected the specific antibody responses observed in our

study. Lumpfish sera in our study were also analysed for specific antibodies against purified A-layer protein by Western blotting, but no conclusive results were obtained. In addition to LPS and the A-layer, other cellular- or extracellular *A. salmonicida* antigens might also have stimulated the immune response (Gudmundsdottir & Bjornsdottir, 2007). This was confirmed by analysing individual lumpfish sera against the A-layer and LPS by ELISA, were only a few individuals showed specific antibody response against the antigens.

To conclude, our study indicates that vaccinating lumpfish at low water temperatures lead to a reduced antibody response against *A. salmonicida*. Lumpfish tolerate vegetable oil-based injection vaccines well, and vaccinating lumpfish over a wide temperature range give low vaccine side effects. These findings are crucial in order to optimize vaccine production and vaccination procedures of Atlantic lumpfish. It would be beneficial to follow up with a challenge study to determine if vaccinated lumpfish are protected against disease from *A. salmonicida*, and whether the vaccine is of any commercial use.

Acknowledgements

The authors thank Vaxxinova Norway AS for their helpful advice and support during the conduct of this immunization trial. We also wish to thank Martin R. Lundberg and Guro Strandskog for their technical support during these experiments. The study was funded by the University of Tromsø – The Arctic University of Norway and the Research Council of Norway (grant no. 239140).

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585

586

587 **Figure legends**

588 **Table 1.** Distribution of degree days post immunization for 5 °C, 10 °C and 15 °C water
589 temperature groups at 3, 6, 9, 12, 15 and 18 weeks post immunization. Degree days of similar
590 length are underlined.

Weeks post immunization	Degree days post immunization at 5 °C	Degree days post immunization at 10 °C	Degree days post immunization at 15 °C
3	105	210	315
6	210	420	630
9	315	630	945
12	420	840	1 260
15	525	1 050	1 575
18	630	1 260	1 890

591

592 **Figure 1.** Comparison of mean weight (g) ± S.D. for lumpfish immunized at 5 °C (a), 10 °C
593 (b) and 15 °C (c) at the start of vaccination on day 0 (n=30), and at 3 weeks (n=10), 6 weeks
594 (n=30), 9 weeks (n=30, except for n=25 for 15 °C VAX and n=26 for 15 °C PBS), 12 weeks
595 (n=30, except for n=28 for 10 °C VAX, n=24 for 15 °C VAX and n=26 for 15 °C PBS), 15
596 weeks (n=30, except for n=28 for 10 °C VAX, n=23 for 15 °C VAX and n=27 for 15 °C
597 PBS) and 18 weeks (n=30, except for n=28 for 10 °C VAX, n=23 for 15 °C VAX and n=27
598 for 15 °C PBS) post vaccination. ANOVA was used for statistical analysis. Combination of
599 different letters (ab and ba) indicates significance between groups ($p < 0.05$), were
600 a=vaccinated fish and b=control fish. VAX=vaccinated fish, PBS=control fish.

Table 2. Mean score of adherences, melanisation of abdominal organs and abdominal wall/fillet and mean score of vaccine residues for immunized lumpfish at week 6, week 9 and week 18 post vaccination. VAX=vaccinated fish, PBS=control fish.

Weeks post immunization	Group	Number of fish	Mean adherence score (0-6) ± S.D.	Mean melanisation score (0-3) ± S.D.	Mean residual vaccine score (0-3) ± S.D.
6	5 °C VAX	20	1,45 ± 0,60	0	1,9 ± 0,45
	5 °C PBS	20	0	0	0
	15 °C VAX	20	1,8 ± 0,77	0	1,85 ± 0,49
	15 °C PBS	20	0	0	0
9	10 °C VAX	20	1,45 ± 0,69	0	1,5 ± 0,51
	10 °C PBS	10	0	0	0
18	5 °C VAX	30	1,53 ± 0,82	0	0,5 ± 0,51
	5 °C PBS	10	0	0	0
	10 °C VAX	28	1,46 ± 1,04	0	1 ± 0,00
	10 °C PBS	10	0	0	0
	15 °C VAX	23	1,39 ± 0,84	0	1,02 ± 0,18
	15 °C PBS	10	0	0	0

Figure 2. Vaccinated lumpfish in the 10 °C group at 18 weeks post immunisation showing fibrinous strands between the abdominal wall and ventral parts of the liver (arrow), and an encapsulated vaccine droplet (circle). Note the absence of melanin deposits (dark pigment). The left operculum has been removed to show the gills. No clear distinction could be established between the different temperature groups.

Figure 3. Comparison of specific antibody (IgM) responses against *Aeromonas salmonicida* at 5 °C, 10 °C and 15 °C from immunized lumpfish at 3, 6, 9 and 18 wpi. (a) and at 105, 210, 315, 630, 1260 and 1890 ddpi. (b). Values are presented as ELISA readings (mean O.D. at 405nm) of pooled serum samples diluted 1:200. Number of fish (n) pooled: week 3 (n=10 for VAX and PBS), week 6 (n=20 for VAX and PBS), week 9 (n=20 for VAX and PBS), week 18 (n=20 for VAX and n=10 for PBS). VAX=vaccinated fish, PBS=control fish.

Figure 4. Comparison of specific antibody (IgM) responses against *Aeromonas salmonicida* at 5 °C (a), 10 °C (b) and 15 °C (c) from immunized lumpfish. Values are presented as ELISA

readings (mean O.D. at 405nm) of individual serum samples diluted 1:200. Number of fish (n) for 5 °C groups: week 3 (n=9 for VAX, n=10 for PBS), week 6 (n=19 for VAX, n=20 for PBS) and week 18 (n=20 for VAX, n=10 for PBS). Number of fish (n) for 10 °C groups: week 3 (n=8 for VAX, n=10 for PBS), week 9 (n=20 for VAX and PBS) and week 18 (n=19 for VAX, n=10 for PBS). Number of fish (n) for 15 °C groups: week 3 (n=10 for VAX and PBS), week 6 (n=20 for VAX and PBS) and week 18 (n=20 for VAX, n=10 for PBS). 630 ddpi is at 18 wpi for 5 °C groups, at 9 wpi for 10 °C groups and at 6 wpi for 15 °C groups. ANOVA was used for statistical analysis. Asterisk (*) indicates significance between groups: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ns=no significance. VAX=vaccinated fish, PBS=control fish.

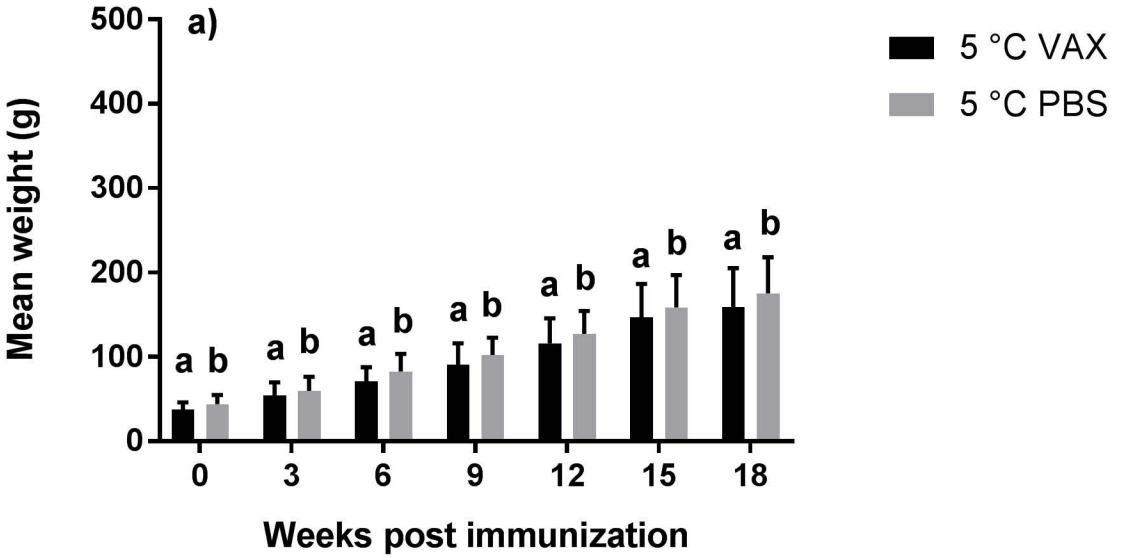
Figure 5. Comparison of specific antibody (IgM) responses against the A-layer protein and LPS of *A. salmonicida* at 5 °C, 10 °C and 15 °C from vaccinated fish (a) and control fish (b) at 3, 6, 9 and 18 wpi. Values are presented as ELISA readings (mean O.D. at 405nm) of individual serum samples diluted 1:200. Number of fish (n) is 5 for all groups, except for vaccinated fish (n=3) and control fish (n=2) at 3 wpi.

Figure 6. Western blotting showing specific antibody responses of vaccinated lumpfish (VAX) at 5 °C, 10 °C and 15 °C against the LPS antigen of *A. salmonicida*. Sera were pooled from four lumpfish in each group.

Supporting information

Figure S1. Comparison of specific antibody (IgM) responses against *Vibrio salmonicida* at 5 °C, 10 °C and 15 °C from immunized lumpfish at 3, 6, 9 and 18 wpi. (a) and at 630 ddpi. (b). 630 ddpi is at 18 wpi for 5 °C groups, at 9 wpi for 10 °C groups and at 6 wpi for 15 °C groups. Values are presented as ELISA readings (mean O.D. at 405nm) of pooled (a) and individual (b) serum samples diluted 1:200. Number of fish (n) pooled: week 3 (n=10 for VAX and PBS), week 6 (n=20 for VAX and PBS), week 9 (n=20 for VAX and PBS), week 18 (n=20 for VAX and n=10 for PBS). Number of fish (n) at 630 ddpi: 5 °C (n=20 for VAX and n=10 for PBS), 10 °C (n=20 for VAX and PBS) and 15 °C (n=20 for VAX and PBS). VAX=vaccinated fish, PBS=control fish.

Figure S2. Comparison of specific antibody (IgM) responses against purified A-layer protein and LPS of *A. salmonicida* at 5 °C, 10 °C and 15 °C from vaccinated fish (a) and control fish (b). Values are presented as ELISA readings (mean O.D. at 405nm) of pooled serum samples of four fish diluted 1:200. The antibody response of each group against the *Aeromonas salmonicida* bacteria is included for reference.


a)
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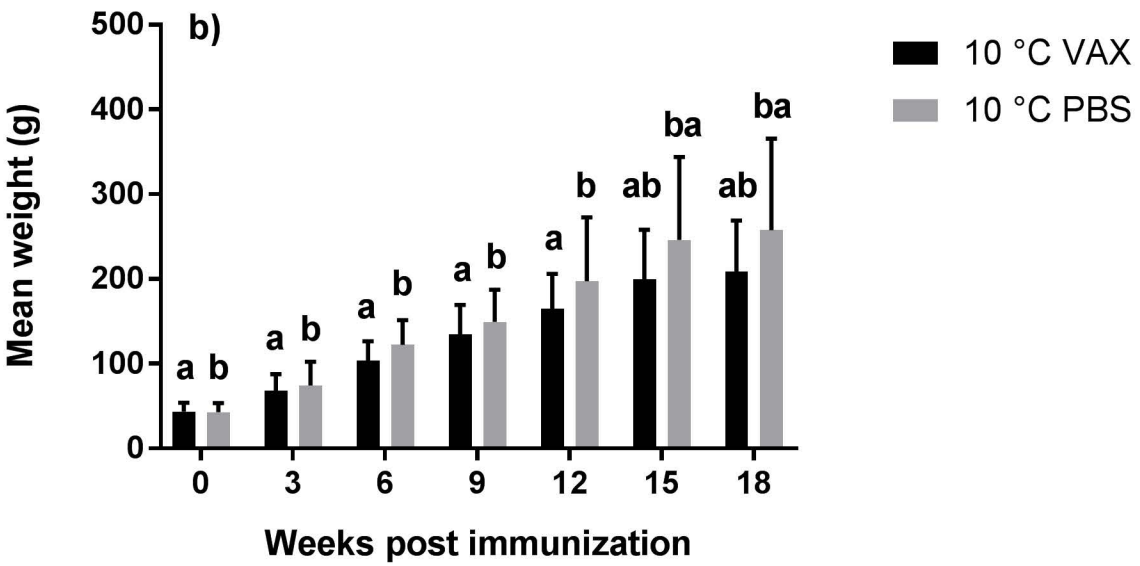
Weeks post immunization
5 °C VAX
5 °C PBS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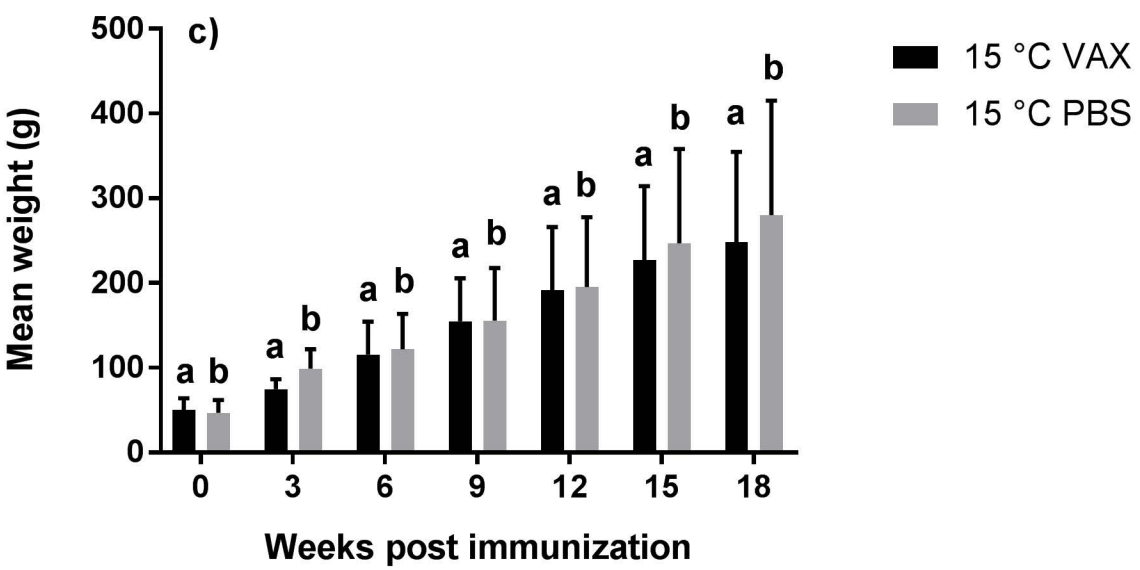
Weeks post immunization
10 °C VAX
10 °C PBS


c)
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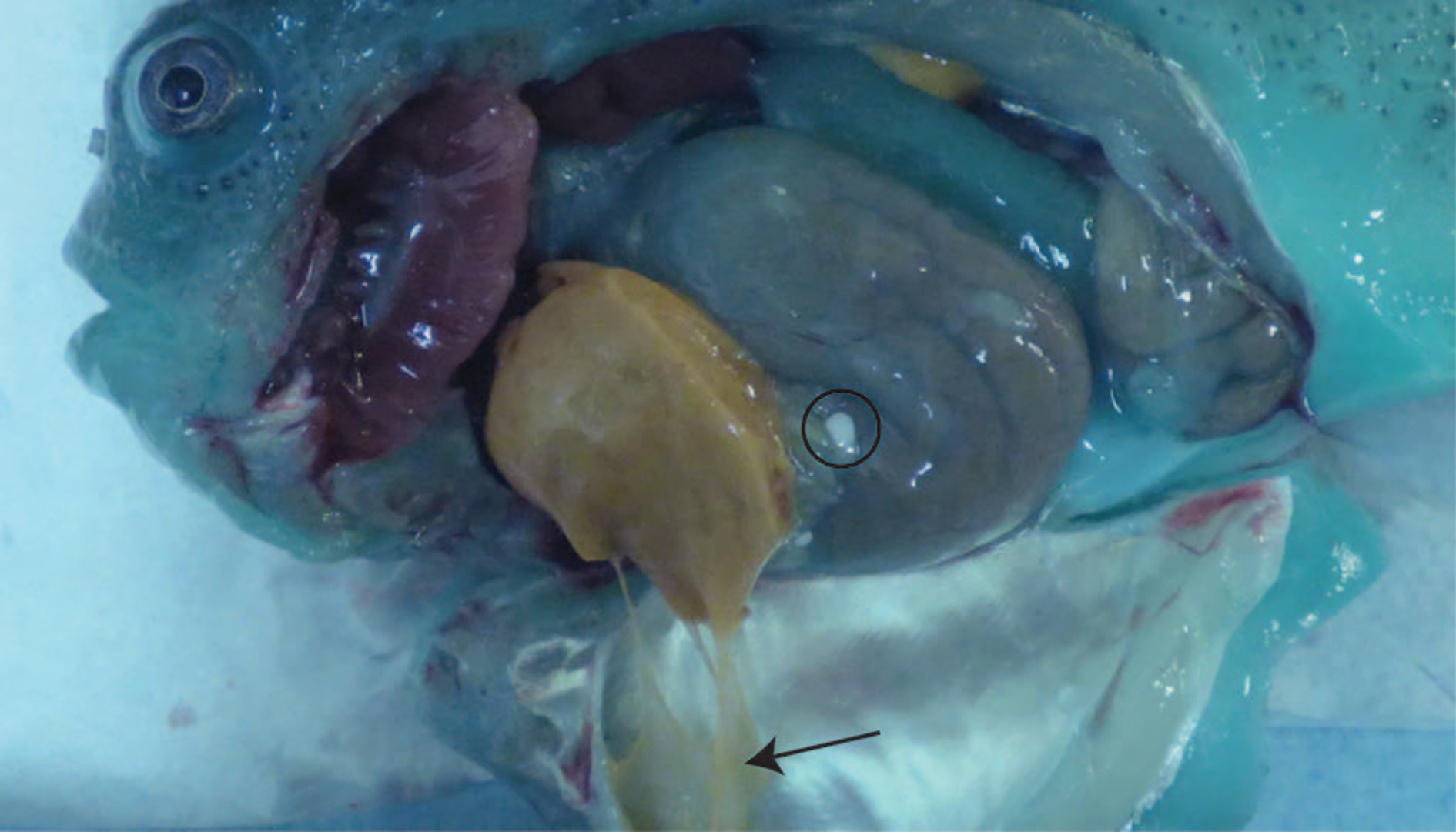
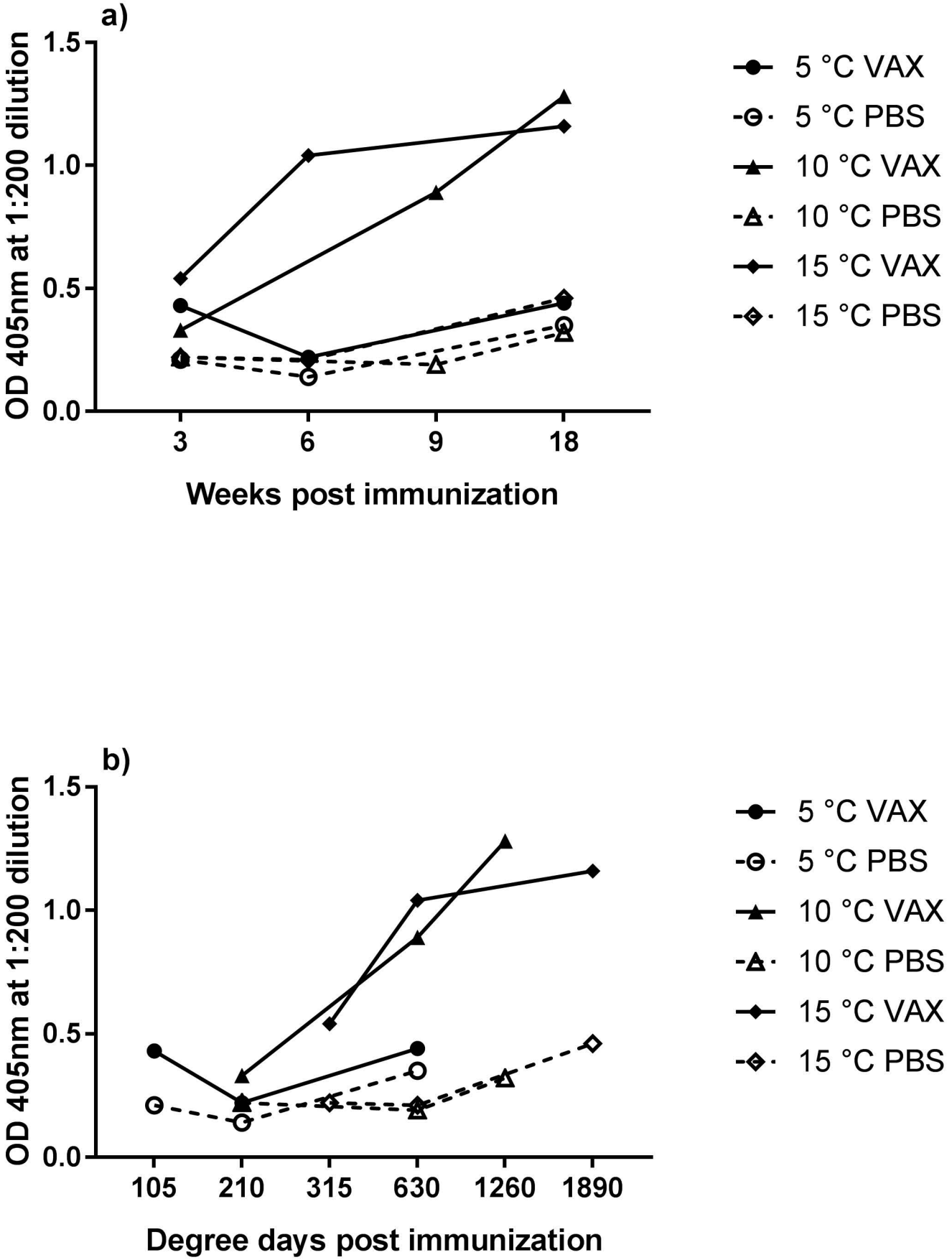
Weeks post immunization
15 °C VAX
15 °C PBS




a)
OD 405nm at 1:200 dilution
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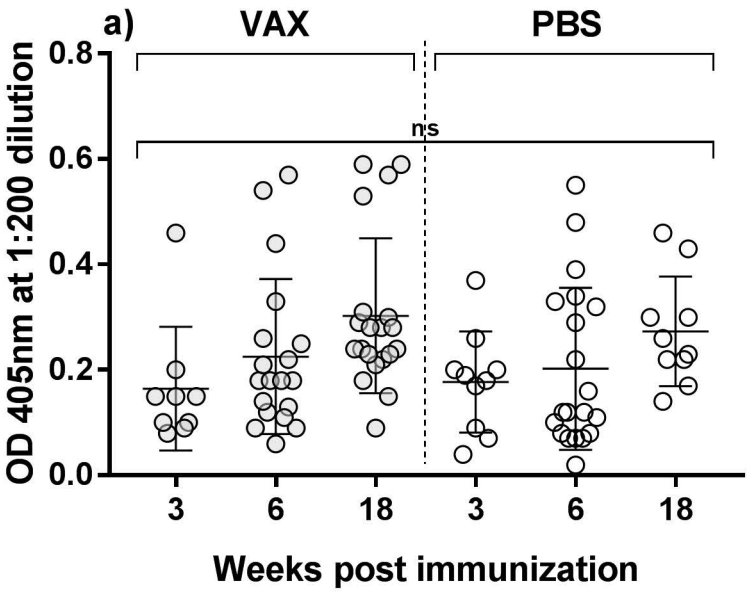
1.0
0.5
0.0
3
6
9
18
Weeks post immunization
5 °C VAX
5 °C PBS
10 °C VAX
10 °C PBS
15 °C VAX
15 °C PBS
b)
OD 405nm at 1:200 dilution
1.5
1.0
0.5
0.0
105
210
315
630
1260
1890
Degree days post immunization
5 °C VAX
5 °C PBS
10 °C VAX
10 °C PBS
15 °C VAX
15 °C PBS


a)
VAX
PBS
OD 405nm at 1:200 dilution
0.8
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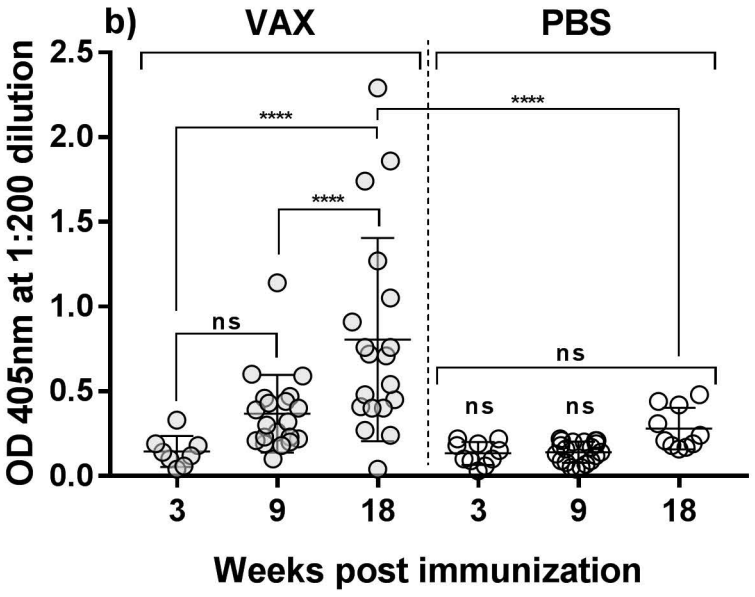
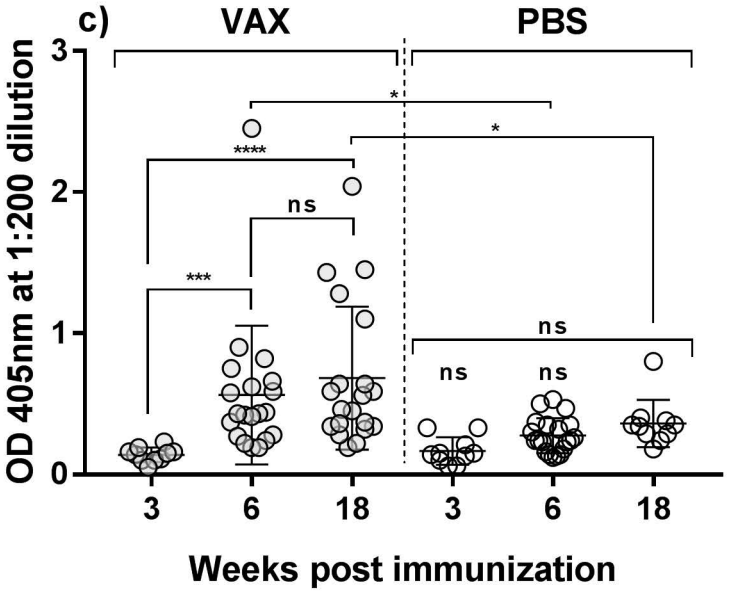
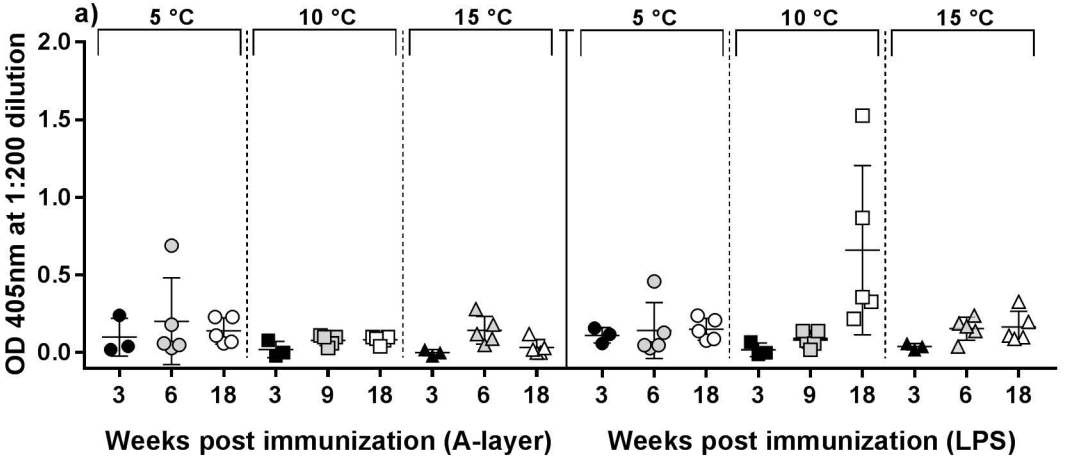
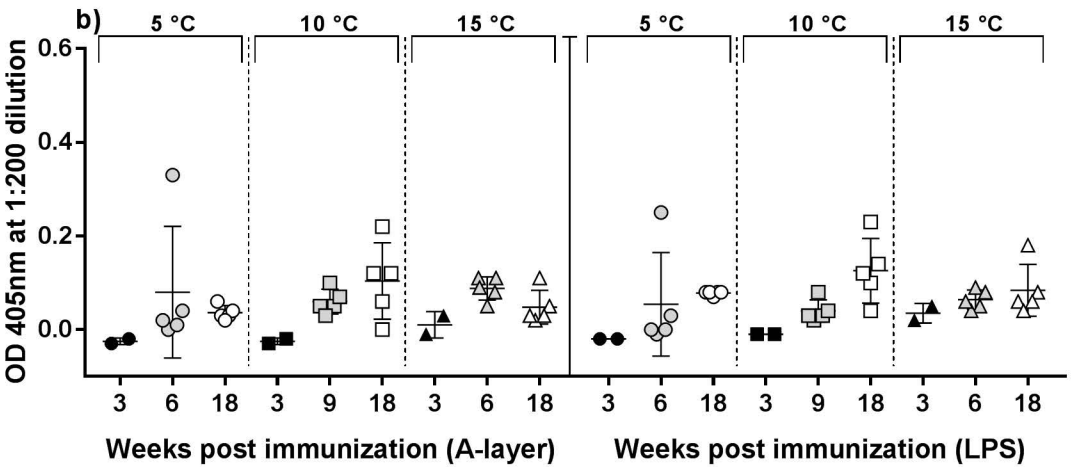
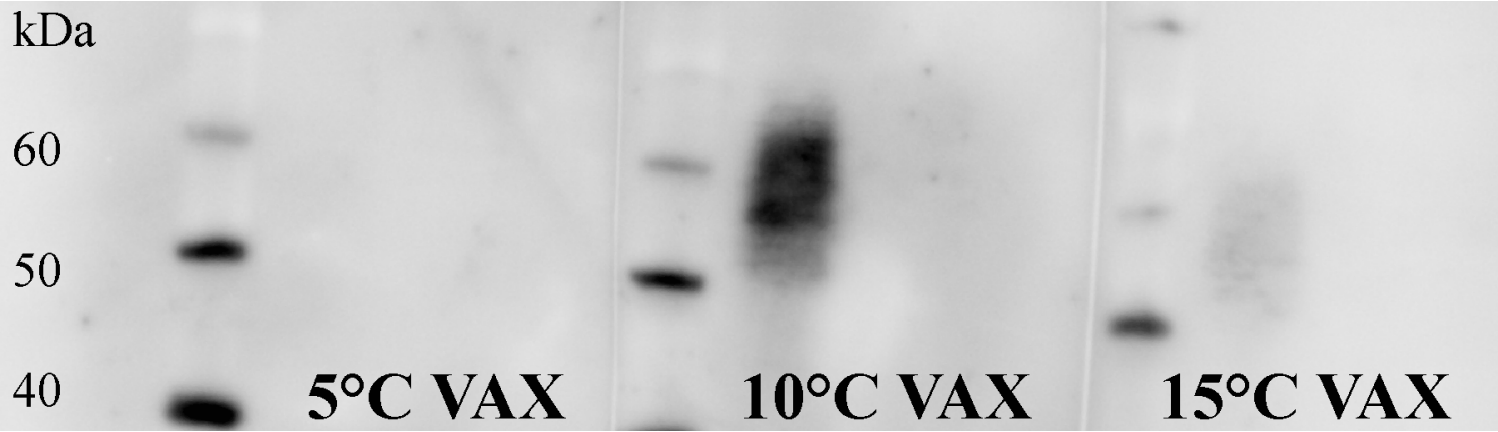
3
6
18
3
6
18
Weeks post immunization

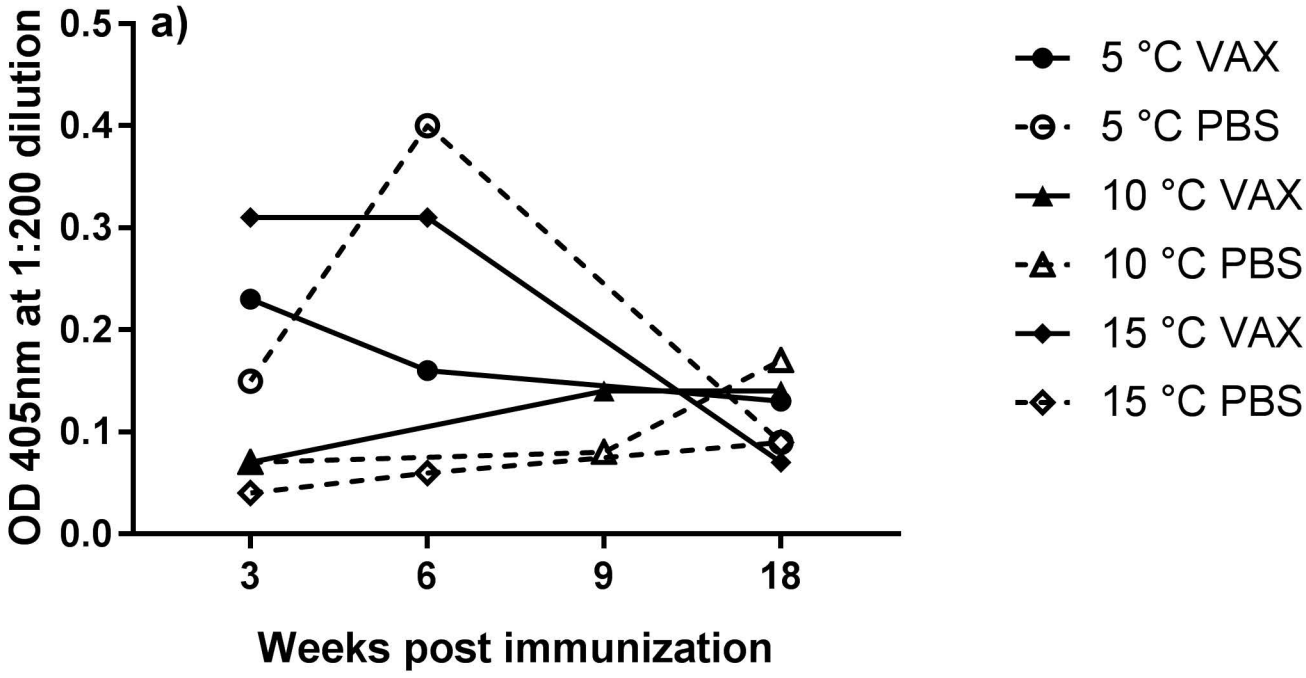

b)
VAX
PBS
OD 405nm at 1:200 dilution
2.5
2.0
1.5
1.0
0.5
0.0
****
****
****
ns
ns
ns
ns
3
9
18
3
9
18
Weeks post immunization

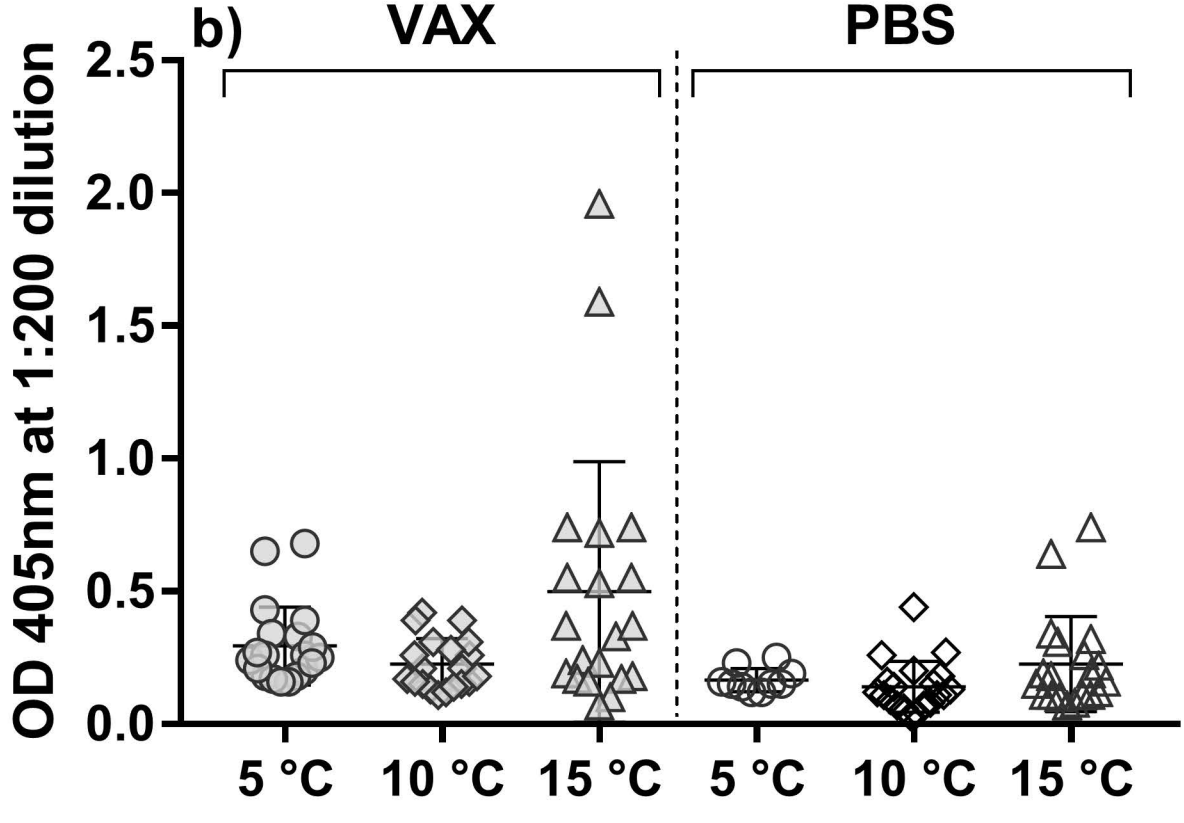

c)
VAX
PBS
OD 405nm at 1:200 dilution
3
2
1
0
*
*
****
ns
***
ns
ns
ns
3
6
18
3
6
18
Weeks post immunization

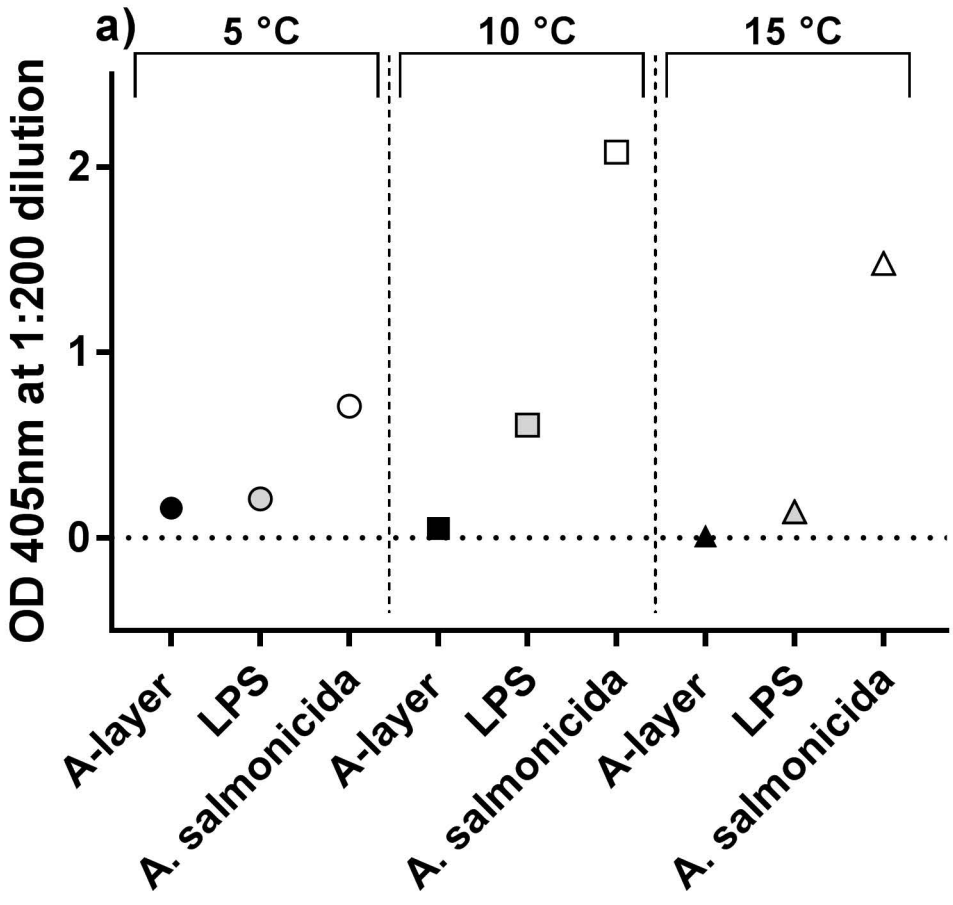

a)
5 °C
10 °C
15 °C
5 °C
10 °C
15 °C
OD 405nm at 1:200 dilution
2.0
1.5
1.0
0.5
0.0
3 6 18 3 9 18 3 6 18 3 6 18 3 9 18 3 6 18
Weeks post immunization (A-layer)
Weeks post immunization (LPS)

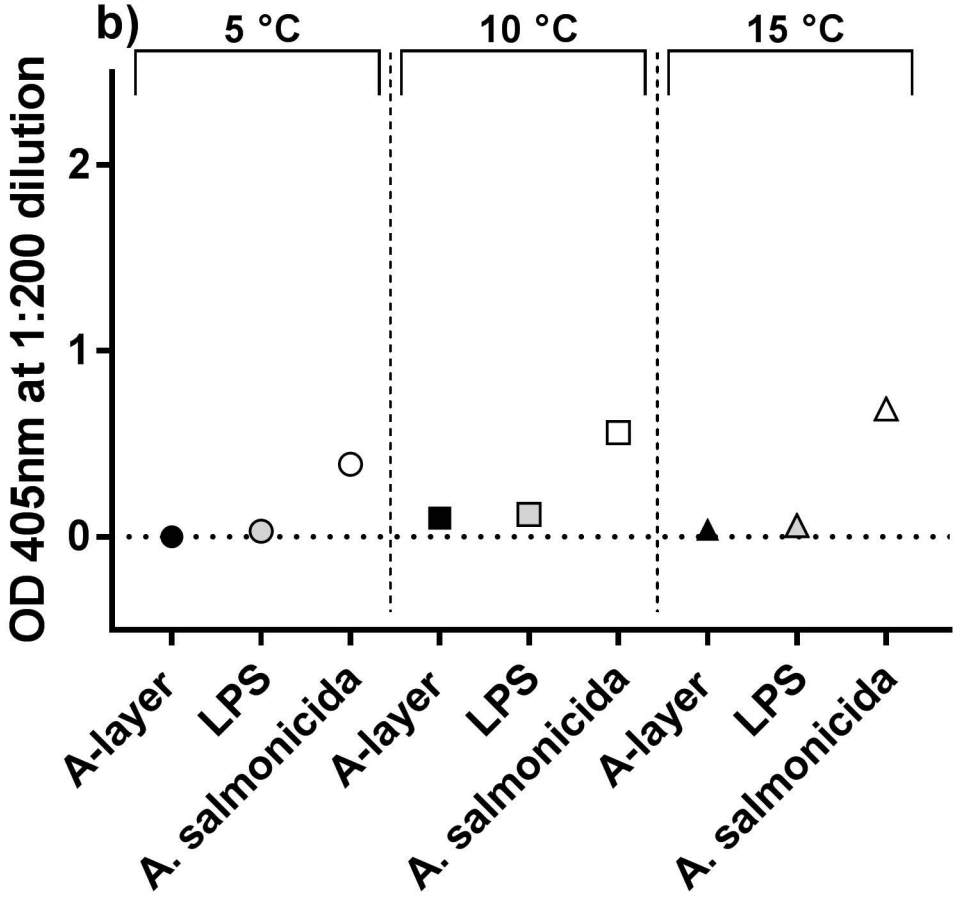

b)
5 °C
10 °C
15 °C
5 °C
10 °C
15 °C
OD 405nm at 1:200 dilution
0.6
0.4
0.2
0.0
3 6 18 3 9 18 3 6 18 3 6 18 3 9 18 3 6 18
Weeks post immunization (A-layer)
Weeks post immunization (LPS)


kDa
60
50
40
5°C VAX
10°C VAX
15°C VAX


a)
OD 405nm at 1:200 dilution
0.5
0.4
0.3
0.2
0.1
0.0
3
6
9
18
Weeks post immunization
5 °C VAX
5 °C PBS
10 °C VAX
10 °C PBS
15 °C VAX
15 °C PBS


b)
VAX
PBS
OD 405nm at 1:200 dilution
2.5
2.0
1.5
1.0
0.5
0.0
5 °C
10 °C
15 °C
5 °C
10 °C
15 °C


a)
5 °C
10 °C
15 °C
OD 405nm at 1:200 dilution
2
1
0
A-layer
LPS
A. salmonicida
A-layer
LPS
A. salmonicida
A-layer
LPS
A. salmonicida


b)
5 °C
10 °C
15 °C
OD 405nm at 1:200 dilution
2
1
0
A-layer
LPS
A. salmonicida
A-layer
LPS
A. salmonicida
A-layer
LPS
A. salmonicida