Title

Production of a monoclonal antibody specific to sablefish (*Anoplopoma fimbria*) IgM and its application in ELISA, western blotting, and immunofluorescent staining

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Keywords: monoclonal antibody, IgM, immunoglobulin, ELISA, western blot, sablefish, *Aeromonas salmonicida*

Abstract

Sablefish (*Anoplopoma fimbria*) are an emerging aquaculture species native to the continental shelf of the northern Pacific Ocean. There is limited information on both innate and adaptive immunity for this species and new tools are needed to determine antibody response following vaccination or disease outbreaks. In this paper, a monoclonal antibody, UI-25A, specific to sablefish IgM was produced in mice. Western blotting confirmed UI-25A recognizes the heavy chain of IgM and does not cross react to proteins or carbohydrates in serum of four other teleost species. An ELISA was developed to measure *Aeromonas salmonicida* specific IgM in the plasma of sablefish from a previous experiment where fish were immunized with a proprietary *A. salmonicida* vaccine. UI-25A was used in western blot analyses to identify immunogenic regions of *A. salmonicida* recognized by this specific IgM from vaccinated sablefish. Immunofluorescent staining also demonstrated the ability of UI-25A to recognize membrane-bound IgM and identify IgM+ cells in the head kidney. These results demonstrate the usefulness of UI-25A as a tool to improve the understanding of antibody-mediated immunity in sablefish as well as to provide valuable information for vaccine development and expansion of aquaculture efforts for this fish species.

1.0 Introduction

The sablefish (*Anoplopoma fimbria*), also known as black cod, is one of the most valuable species in the Pacific commercial fishing industry [1]. It currently has an average wholesale price of $12.25 per lb in the Alaskan market [2], and there are well developed markets for this species in Japan, North America, and the UK. However, wild stocks have declined in recent decades, and this has raised questions about the sustainability of commercial harvesting [3]. As a result, investment into sablefish aquaculture has increased, and this industry has significant potential due to this species's value and existing market [4]. Sablefish have been farmed in North America since the 1970s, though usually on a small scale, and generally incorporated into existing salmonid farm infrastructure. Improvements in culture techniques over the past decade include

the production of all-female monosex stocks through direct and indirect sex control [5] with growth significantly faster as compared to males. Further, the use and optimization of formulated diets with reduced fish meal and fish oil levels have been explored [6], and captive breeding programs have been successful in improving larval survival [7,8]. The latter has increased the availability of juvenile fish for commercial grow out operations, which has been a longstanding obstacle to the growth of sablefish aquaculture.

Even with a steady supply of juvenile fish, diseases remain a significant barrier to increasing production of many aquaculture species [9]. Disease prevention is crucial to the development of sablefish as a commercial aquaculture species. Sablefish are particularly susceptible to infections with atypical *Aeromonas salmonicida* strains [10], and this gram negative bacterial pathogen is routinely isolated during disease outbreaks in culture settings [11–13]. Prevention of disease caused by this pathogen is considered vital to expansion of commercial scale operations. Several studies have demonstrated the effectiveness of *A. salmonicida* vaccines in sablefish with respect to increased survival following pathogen challenge [12,13]. However, the lack of a species-specific monoclonal antibody (mAb) capable of recognizing sablefish IgM has limited the study of their adaptive immune responses. With mAbs, tools such as enzyme-linked immunosorbent assays (ELISA), western blots, and immunofluorescent microscopy, can be used for evaluating the immune response of sablefish after vaccination and disease outbreak, or the effect of various abiotic conditions on their immune function and disease resistance. A polyclonal antibody to sablefish IgM was used to develop a direct sablefish-specific ELISA assay measuring IgM and to examine how chronic hypoxia affects adaptive immune function [14]. However, given that mAbs contain only one antibody form for a specific epitope, they rarely cross react with non-target proteins that may be present in ELSIA assays [13].

Vaccines work by stimulating a highly specific, and long lasting, adaptive immunity through a cell-mediated response involving T cells and humoral immunity characterized by B cell activities. In teleosts, the humoral immune response is comprised of antigen specific antibodies (Ab), or membrane-bound immunoglobulins (Igs) produced by B cells [15]. To date, several different types of Igs, all sharing the basic structure of 2 heavy (H) chains and 2 light (L) chains, have been described in fish including IgM, IgD, and IgT/IgZ [15]. IgT is believed to be strongly associated with mucosal immunity in fish [16], while IgM is the predominant systemic Ig that is commonly detected in teleost serum. IgM can exist as two physical forms; a membrane-bound Ig

on the surface of B cells including plasmablasts or plasma cells, and the secreted antibody form most often found in a tetrameric structure [17]. The two forms of IgM protect the host against pathogens through multiple mechanisms, including neutralization, complement pathway activation, opsonization, and phagocytosis [15]. Measuring the production and defensive capabilities of IgM in fish is an important tool to characterize humoral immunity. This is achieved through the development of mAbs in model species, such as mice, rabbits, and chickens, that bind to species-specific IgM molecules and can be used with other widely available reagents in serological assays. Many of these mAbs have been developed in recent years for species that include sea bass (*Lateolabrax japonicus*) [18], muskellunge (*Esox masquinongy*) [19], bighead catfish (*Clarias macrocephalus*) [20], Nile tilapia (*Oreochromis niloticus*) [21], large yellow croaker (*Larimichtys crocea*) [22], smallmouth bass (*Micropterus dolomieu*) [23], gibel carp (*Carassius gibelio*) [24], and hybrid snakehead (*Channa maculate*♀ × *Channa argus*♂) [25]. These antibody-specific mAbs have been used in various applications including ELISA, western blotting, and immunofluorescent microscopy [18,19,21]. These mAbs are critical for developing non-lethal sero-diagnostic assays to evaluate immune responses following infection, vaccination, or even feeding of various immunostimulants [24].

The goal of the present study was to produce mAbs specific to sablefish IgM in mice through standard procedures. IgM was purified from sablefish plasma for the purpose of mice immunizations, and the sensitivity and specificity of mAb candidates were characterized by ELISA and western blotting analyses. The application of the resulting anti-sablefish IgM mAb was investigated using ELISA, western blotting, and immunofluorescent microscopy applications. These results will facilitate further understanding of the characteristics and functions of sablefish humoral immunity.

2 Materials and Methods

2.1 Declaration of Conflicting Interests and Animal Care and Use

No conflicts of interest are declared by the authors. Live animal work with burbot and rainbow trout was approved by the University of Idaho's Institutional Animal Care and Use Committee (IACUC; #2020-33 and #2020-69). Blood collected from the sablefish vaccination experiment was approved by the University of Washington's IACUC protocol #4078-05. Blood collected at Memorial University for IgM purification was approved under IACUC protocol 16-92-KG.

Sablefish collected for immunofluorescent microscopy analysis were covered under Oregon State University's IACUC protocol 2020-0095.

2.2 IgM Purification

Sablefish sera for the purposes of IgM purification was generously donated from Memorial University of Newfoundland. IgM was purified using a Pierce IgM Purification kit with a mannan binding protein column (Thermo Fisher Scientific, Waltham, MA, USA) following methods similar to those used for other teleost species [13,26]. Briefly, pooled sera samples were dialyzed against 20 mM Tris with 1.25 M sodium chloride (pH 7.4) overnight using a 3.5 K MWCO Slide-a-lyzer (Thermo Fisher Scientific) then diluted 1:1 with IgM column binding buffer. This mixture was applied to the column in 1 mL portions and allowed to bind according to manufacturer's guidelines. The protein concentration of eluted IgM fractions was quantified with a NanoDrop 2000 Microvolume Spectrophotometer (Thermo Fisher Scientific). All fractions greater than 0.005 µg/mL were pooled and concentrated using a Pierce Protein Concentrator PES with a molecular weight cutoff of 10,000 daltons (10 K; Thermo Fisher Scientific).

A sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize the purity and molecular weight of the proteins within the concentrated sablefish IgM. Sablefish serum was diluted 1:10 and purified IgM diluted 1:2 in 2x Laemmeli sample buffer containing 5% dithiothreitol (DTT) as a reducing agent. Samples were denatured in 100 °C water for 10 minutes. After, 10 µL of samples were added to wells of a 4-20% gradient precast stain-free polyacrylamide gel (BioRad, Hercules, CA, USA), as well as an unstained protein standard ladder (BioRad). Electrophoresis was run at 90 V for 15 minutes, then 120 V for 120 minutes.

2.3 Hybridoma production

Mouse anti-sablefish IgM mAb candidates were produced by the Washington State University Monoclonal Antibody Center (WSU-MAC; Pullman, WA, USA) following standard protocols [27]. To prepare the emulsion for immunizations, purified sablefish IgM was dialyzed overnight using a Tube-O-Dialyzer (Thermo Fisher Scientific) to remove sodium azide prior to injection. Sablefish IgM was then diluted to 0.5 mg/mL in sterile saline, of which 2 mL was added to a Sigma Adjuvant System (MilliporeSigma, Burlington, MA, USA). Four BALB/c mice were injected intraperitoneally with 200 µL of emulsified mixture and boosted at 21 days. After the

first booster, blood from each mouse was collected, pooled, and allowed to clot overnight at 4 °C to collect sera which was tested to confirm presence of antibodies specific to sablefish IgM.

Mouse sera was tested for specific antibodies to sablefish IgM at the University of Idaho using a protocol provided by WSU-MAC to detect specific antibodies in mice. Briefly, high binding plates (Corning Inc., Corning, NY, USA) were coated with 100 µL of one of the following antigens: sablefish plasma, purified sablefish IgM, burbot (*Lota lota maculosa*) sera, and rainbow trout (*Oncorhynchus mykiss*) sera. Burbot and rainbow trout sera were collected from naïve juvenile fish held at the University of Idaho for use as negative controls. Serial doubling dilutions of coating antigens were made in 0.05 M Tris (pH 9.5) from 0.625 to 0.039 µg/mL for serum or plasma, and 0.125 to 0.0078 µg/mL for pure sablefish IgM. Plates were incubated overnight at 4 °C, and the following day, plates were washed once with 200 µL wash buffer (2.25% $Na_2HPO_4*7H_2O$, 0.25% $NaH_2PO_4*H_2O$, 8.76% NaCl, 1% NaN_3, 0.5% tween-20). Following this, the plates were blocked with 100 µL of 0.05 M Tris (pH 9.5) containing 0.3% bovine serum albumin (BSA), sealed, and incubated overnight at room temperature (RT). Plates were then washed twice, and 100 µL of mouse sera diluted 1:120 in phosphate buffered saline (PBS; 0.3% BSA, 0.05% tween-20, and 0.02% NaN_3) was added to all wells and incubated at 37 °C for 1 hour. After this, plates were washed four times, and 100 µL of biotinylated goat anti-mouse IgG (Thermo Fisher Scientific) diluted 1:1000 was added and allowed to incubate at 37 °C for 1 hour. Plates were again washed four times before the addition of 100 µL of streptavidin-alkaline phosphatase (BioRad) diluted 1:1000 in PBS containing 0.3% BSA and 0.01% NaN_3. After a 1 hour incubation at 37 °C the plates were washed four times, and 100 µL of p-nitrophenyl phosphate (PNPP) substrate (Thermo Fisher Scientific) mixed according to manufacturer's guidelines, was added to the wells. Finally, the plates were incubated with PNPP for 30 minutes before adding 50 µL of 0.3 M NaOH to stop color development, and the optical density (OD) of the wells was read at 405 nm using a microplate reader (Powerwave XS; BioTek, Winooski, VT, USA).

After an antibody response was confirmed, mice were boosted a third time. After 72 hours, spleen cells were collected from sacrificed mice and fused with myeloma cell line X63 AG8.653 using 50% polyethylene glycol. Selection of fused cells was performed in 96-well tissue culture plates by adding hypoxanthine-aminopterin-thymidine (HAT) media supplement (Sigma-Aldrich, St. Louis, MO, USA) after 24 hours of cell growth at 37 °C. After 7 days post cell

fusion, colonies with positive hybridoma growth were kept and their supernatant harvested for additional screening.

2.4 mAb screening

2.4.1 Indirect ELISA

Supernatants from positive hybridoma colonies, each containing a unique mAb, were screened for reactivity to sablefish IgM using methods and reagents based on an ELISA developed to quantify specific antibodies in rainbow trout [28,29]. Briefly, purified sablefish IgM diluted to 1 µg/mL in carbonate coating buffer (pH 9.5) was added to wells and incubated overnight at 4 °C. Plates were washed three times with 200 µL of potassium phosphate-buffered saline with 0.05% Tween-20 (KPBS-T) then blocked overnight at RT with 150 µL of KPBS-T containing 5% non-fat dried milk (NFDM) and 0.02% NaN_3. Plates were washed three times again before mAb candidates, diluted 1:50 in KPBS-T, were added in duplicate (100 µL/well) and allowed to incubate for 1 hour at 37 °C; a 1:200 dilution of serum from immunized mice was used as a positive control and KPBS-T as a negative control. After this, plates were washed three times, then 100 µL of goat anti-mouse IgG conjugated with horseradish peroxidase (BioRad) diluted 1:2000 in KPBS-T with 0.1% NFDM was added to the wells, and they were incubated for 1 hr at 37 °C. Finally, the plates were washed thrice again, 50 µL of ABTS peroxidase substrate (SeraCare Life Sciences Inc., Milford, MA, USA) was added, the plates were incubated for 15 minutes at RT before 50 µL of 1% sodium dodecyl sulfate (SDS) stop solution was added, and the plates read at 405 nm to measure specific binding.

This protocol was repeated using mAb candidates that demonstrated high reactivity after initial screening, with steps remaining the same unless otherwise noted. Sensitivity to pure sablefish IgM concentrations was tested using doubling serial dilutions of a 1 µg/mL coating mixture from 1:1 to 1:2048 (final lowest concentration of 4.89×10^{-4} µg/mL). To assess cross reactivity, other plates were coated with a similar series of dilutions (1:1 to 1:2048) with a 10 µg/mL starting concentration of control serum from rainbow trout and burbot, and using a final lowest concentration of 4.89×10^{-3} µg/mL. Species-specific mAbs were used as a positive control to confirm coating of rainbow trout (Warr 1.14 [30]) and burbot (manuscript in preparation [31]) IgM to ELISA plates. All mAbs were tested in duplicate at a 1:50 dilution. The top four mAb candidates were also tested at various dilutions to determine their sensitivity to sablefish IgM.

For this, wells were coated with 1 µg/mL of purified sablefish IgM and the mAb candidates were applied at dilutions of 1:100, 1:1000, 1:10000, 1:100000, 1:500000, 1:1000000, or 1:5000000 in KPBST in triplicate.

2.4.2 Western blot

Western blot analysis was used to determine the region of specificity for the top four mAb candidates to sablefish IgM. Sablefish plasma was diluted 1:10, with the conditions for SDS-PAGE the same as described in Section 2.1 apart from the use of pre-stained protein standards (BioRad) in lanes on either side of each mAb candidate. Proteins were transferred from the polyacrylamide gel to nitrocellulose paper with a Trans-Blot Turbo Transfer System (BioRad) according to manufacturer's protocols, protein standards on the nitrocellulose indicated a successful transfer. After transfer, the nitrocellulose paper was cut into sections using the pre-stained standards as a guide, with each section containing a lane of protein standard and sablefish plasma. Sections were placed in sterile 15 mL tubes and blocked with phosphate-buffered saline (PBS) containing 4% non-fat dried milk (NFDM) for 1 hour at RT under constant gentle rocking. Sections were then washed three times for 5 minutes each with PBS containing 0.02% tween-20 and 0.01% sodium azide (PBST-Az). Supernatants containing mAb candidates were applied undiluted and incubated with gentle rocking for 1 hour at RT. Sections were washed again, and alkaline-phosphatase (AP) conjugated goat anti-mouse IgG (BioRad) was diluted 1:500 in PBST-Az and the sections gently rocked for another hour at RT. After a final wash, bands were visualized with an AP color development substrate (BioRad), the reaction stopped by washing sections three times with ultrapure water for 10 seconds.

2.4.3 Cloning and mAb Characterization

Based on the ELISA and western blot results, mAb 25 was chosen for downstream applications. Cloning of the selected hybridoma line was performed at WSU-MAC using standard limiting dilution methods [27]. The three fastest growing clones (A, B, and C) were selected and screened again using the same methods from Section 2.3.1 to confirm that the desirable characteristics of sensitivity and specificity to sablefish IgM were retained. The final clone, named UI-25A, was also tested via western blotting to ensure that there was no cross reactivity to other fish species. Serum samples were collected from one fish of each of the following species: coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*), rainbow trout, and burbot. These

samples as well as sablefish plasma were diluted 1:10 in 2x Laemmli sample buffer and subjected to the same conditions as described in Section 2.3.2.

A rapid ELISA kit (Thermo Fisher Scientific) was used to determine the isotype of the antibodies produced by the cloned hybridoma. Hybridoma cells secreting UI-25A were grown in serum free media, and approximately 50 mL of the supernatant was harvested and used for subsequent testing. The concentration of this stock of UI-25A was measured via radial immunodiffusion (RID, Rockland Immunochemicals, Pottstown, PA, USA).

2.5 ELISA to measure *A. salmonicida* specific antibodies

In a previously published study [12], sablefish were vaccinated via intraperitoneal injection with a proprietary vaccine prepared in an oil-based emulsion that included the atypical *A. salmonicida* T30 isolate, control fish were injected with the oil-based emulsion without vaccine. Plasma samples from both vaccinated and control sablefish (mean weight 115.7 g; mean fork length 231 mm) were collected 8 weeks after vaccination and stored at -80 °C. These samples were used as controls for ELISA development and optimization. Detection of specific antibodies was conducted with the same equipment, reagents, and wash steps as the previously described ELISA protocol used to screen mAb candidates.

To prepare the bacterial coating antigen, 25 mL of tryptic soy broth (TSB) supplemented with 1.5% (w/v) NaCl was inoculated with a single bacterial colony of *A. salmonicida* T30. The culture was incubated at 21°C for 48 hours with gentle shaking. The broth was then centrifuged at 3,000 *g* for 10 minutes, the media was decanted, and bacterial cells washed with sterile PBS. After another centrifugation, the bacterial cells were resuspended in approximately 10 mL of sterile PBS and subjected to 10 freeze-thaw cycles. After the last thaw, the protein concentration was measured using a NanoDrop 2000 (Thermo Fisher Scientific).

Checkerboard titrations (CBT; [32]) were performed to determine optimal concentrations of coating antigen, primary antibody (mouse anti-sablefish mAb), and secondary antibody (goat anti-mouse conjugated with horseradish peroxidase [HRP]). The reagents and general steps were based on a previously developed and optimized ELISA [28,29] for the measurement of *Flavobacterium psychrophilum* specific antibodies in rainbow trout. All conditions for CBTs were run on duplicate plates, one for each positive or negative control sample, each plate also included a blank control of PBS instead of plasma. The T30 antigen was tested at 5 and 10

µg/mL. The primary antibody (UI-25A) was tested in serial dilutions from 1:50 to 1:3200, and the secondary goat anti-mouse conjugated with HRP was tested in dilutions from 1:3000 to 1:8000. Plasma from either naïve sablefish (negative control) or fish immunized against T30 (positive control) were diluted from 1:50 to 1:102400, and a blank PBS control was also used for each plate. Conditions that limited background signal in negative controls, while still maintaining high titers in positive controls, were chosen for assays.

Briefly, plates were coated overnight at 4°C, washed, then blocked with 150 µL of KPBS-T with 5% NFDM for 1 hour at RT. Plasma samples were diluted in KPBS-T and incubated for 1 hour at 15 °C. Then, 100 µL of the mouse anti-sablefish mAb dilution was added and incubated for 1 hour at RT. After another wash, 100 µL of the goat anti-mouse HRP conjugate dilution was added and incubated for 1 hour at RT. After the final wash, 50 µL of ABTS peroxidase substrate (SeraCare Life Sciences Inc.) was added and color was allowed to develop for 10 minutes, after which 50 µL of 1% SDS stop solution was added. Plates were read at 405 nm in a microplate reader (BioTek). After optimization, sablefish plasma from the Arkoosh et al. [12] study was tested to compare antibody titers between 20 vaccinated and 20 control fish. Log antibody titer was calculated as the reciprocal of the highest plasma dilution that had an optical density greater than two times the blank negative control.

2.6 Western blot analysis of *A. salmonicida* protein and LPS

The T30 isolate used as part of the previous vaccination experiment in sablefish was subjected to SDS-PAGE using both whole cell and LPS extract preparations. Western blotting was then used to determine antigenic regions of the bacteria using plasma from vaccinated fish. For the whole cell sample, the T30 isolate was grown at room temperature in TSB supplemented with 1.5% NaCl for 24 hours under gentle agitation. The culture was then centrifuged at 3,000 *g* and washed once with sterile PBS, centrifuged again, then resuspended in sterile PBS to obtain an OD_{525} of 1.6. Aliquots of 50 µL were stored at -20 °C until use. Samples were then diluted 1:1 in 2x Laemmli sample buffer containing 5% DTT , denatured for 10 minutes at 100 °C, and loaded into a 4-20% precast gradient polyacrylamide stain-free gel (BioRad). Conditions for electrophoresis and transfer to nitrocellulose membrane were identical to those described in Section 2.3.2. Once transferred, the nitrocellulose membrane was blocked for 1 hour at RT with PBS containing 4% NFDM. Plasma from sablefish immunized against T30 was diluted 1:50 in

PBS containing 0.05% Tween-20, 0.05% NaN_3, and 2% NFDM, then incubated on the membrane overnight at 15 °C with gentle rocking. The membrane was washed three times with PBS containing 0.05% Tween-20 and 0.05% NaN_3 (PBST-Az), after which UI-25A was diluted 1:100 in PBST-Az and incubated on membrane for 1 hour at RT. Following three more washes with PBST-Az, AP conjugated goat anti-mouse immunoglobulin (BioRad) was diluted 1:500 in PBST-AZ and incubated for 1 hour at RT. After three more washes, specific antigenic binding was visualized using a commercial AP conjugate substrate kit (BioRad). Each individual wash was performed for 5 minutes with gentle rocking.

For LPS extraction, bacterial cells were standardized to an OD_{525} of 0.80 in sterile PBS using the same methods as the whole cell preparation. A 1.5 mL sample of the suspension was centrifuged, and PBS was removed before solubilizing the pellet in 200 µL of lysing buffer (2% SDS, 2% ß-mercaptoethanol, 10% glycerol, 0.1 M Tris-HCl). The lysate was heated to 100°C for 10 minutes in a water bath, then cooled to RT. Protein digestion was performed by adding 3 µL of proteinase K (20 mg/mL; Thermo Fisher Scientific) to 20 µL of lysate then incubated at 60 °C for 1 hour. This sample was then diluted 1:1 in 2x Laemmli buffer and run in an SDS-PAGE like the whole cell preparation. A modified protocol from Tsai and Frasch [33] was used for silver staining. Briefly, the gel was rinsed 3 times (10 s each) with distilled water (dH_2O), and then oxidized with 0.07 g of periodic acid, 30 mL of ethanol, 10 mL of acetic acid, and 55 mL of dH_2O for 20 minutes with gentle rocking. After three rinses with dH_2O (5 min each), staining was performed with 1 g/L of silver nitrate in dH_2O for 30 minutes with gentle shaking, then thrice rinsed again. Color development solution was made fresh (3 g sodium carbonate, 0.02 mL formaldehyde, and 100 mL dH_2O) and chilled to 4 °C before being applied to the gel. Development was stopped with a solution of 10% acetic acid in dH_2O, the gel was imaged with a Gel Doc EZ system (BioRad). A western blot was done on another gel that was left unstained, using the same methods as the whole cell preparation.

2.7 Immunofluorescent staining of membrane-bound IgM

Sablefish, of approximately 100 g, held at the NOAA's Newport Research Station (NRS) Fish Disease Laboratory (Newport, OR) were euthanized and shipped overnight to the University of Idaho. The sablefish were originally obtained from NOAA's Manchester Research Station (Manchester, WA) and transferred to NRS once weaned from live feeds. At the time of

collection, the sablefish were fed a commercial dry pellet diet (Bio-Oregon, Longview, WA, USA) and reared in flow-through seawater filtered to 25 µm and UV-light treated prior to entering the lab. Head kidney tissue was aseptically collected, and a sterile scalpel blade was used to cut tissues in half. The newly exposed side of the tissue was blotted on a microscope slide (Thermo Fisher Scientific) and allowed to air dry. Cells were fixed for 5 minutes with 4% paraformaldehyde (Thermo Fisher Scientific), gently rinsed with dH_2O, and allowed to air dry again. Slides were blocked with phosphate buffered saline with 0.05% Tween-20 (PBS-T) containing 5% BSA for 1 hour at RT. Then, the UI-25A mAb was diluted 1:80 in PBS-T containing 1% BSA and the slide was incubated with UI-25A at RT for 1 hour. Slides were washed with PBS, without Tween-20, three times for 5 minutes each. A goat anti-mouse secondary antibody conjugated with Alexa Fluor 555 (Thermo Fisher Scientific) was diluted to 2 µg/mL in PBS-T with 1% BSA and incubated for 1 hour in the dark at RT. Slides were washed twice again for 10 minutes with PBS-T, before applying Fluoromount-G Mounting Medium with DAPI (Thermo Fisher Scientific) to the slides and sealing with a coverslip. This same process was repeated for head kidney tissue collected from burbot to serve as a negative control. Images were acquired using a Nikon Andor spinning disk confocal microscope with a Zyla sCMOS camera and Nikon Elements software, a 40X objective was used for imaging.

2.8 Statistical analyses

All statistical analyses were performed in R version 4.1.3 [34]. Before comparing OD_{405} values for the ELISA assays, normality and variance assumptions were evaluated with Shapiro-Wilks and Bartlett's tests, respectively. A one-way ANOVA was used to compare differences among treatments, and significant ($p < 0.05$) differences were identified with Tukey's HSD tests. Data from log antibody titer analysis was non-parametric, and thus, a Mann-Whitney U test was used for statistical comparison.

3 Results

3.2 IgM Purification

IgM isolated from sablefish plasma showed distinct bands at both ~75 kDa and ~25 kDa by SDS-PAGE (Figure 1). These are the predicted sizes of the H and L chain fragments of IgM based on other teleost species. Some banding was detected at 100 kDa, and these are intact H and

L chains that did not fully denature, whereas bands at higher molecular weights correspond to potential monomeric IgM structures.

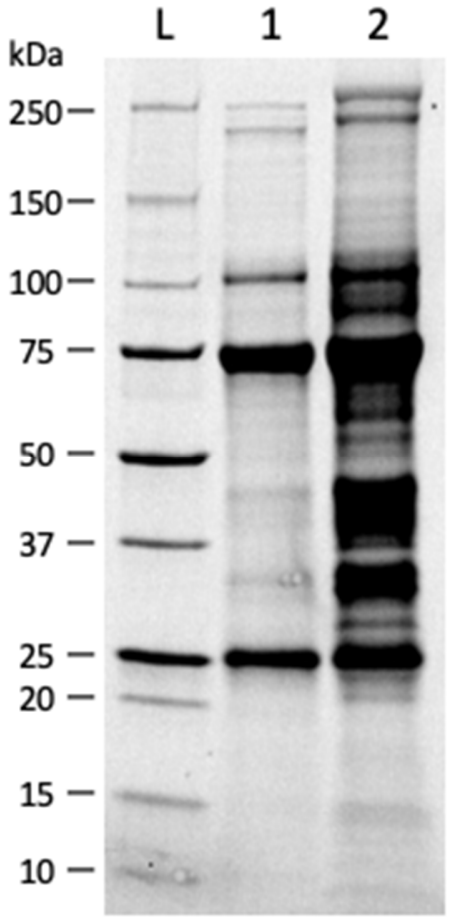


Figure 1 Purified IgM from sablefish plasma analyzed by SDS-PAGE. Lane L, protein standard ladder; Lane 1, purified sablefish IgM; Lane 2, sablefish plasma. Banding at 75 and 25 kDa represent the heavy and light chains of sablefish IgM respectively.

3.3 Hybridoma production

Wells were coated with five doubling serial dilutions of fish plasma or sera (0.625, 0.312, 0.156, 0.078, and 0.039 µg/mL) or with pure sablefish IgM at 1/5th of those concentrations (0.125, 0.062, 0.031, 0.015, 0.007 µg/mL); for simplicity these will be referred to as the A (highest) through E (lowest) coating concentrations for the results shown in Figure 2. Polyclonal antibodies from immunized mice developed significantly higher absorbance in wells coated with sablefish IgM or plasma in coating concentrations A, B, and C ($p < 0.05$). At concentration C,

absorbance from wells coated with sablefish plasma was significantly higher as compared to pure IgM ($p = 0.04$), but was not different at any other concentrations. Absorbance of wells at concentration D was different between sablefish plasma and rainbow trout or burbot serum ($p < 0.05$), there was also a significant difference between pure IgM and burbot serum ($p = 0.017$) but not rainbow trout serum. At concentration E, absorbance in wells coated with sablefish plasma was significantly higher compared to burbot serum ($p = 0.012$) but no other coating antigens.

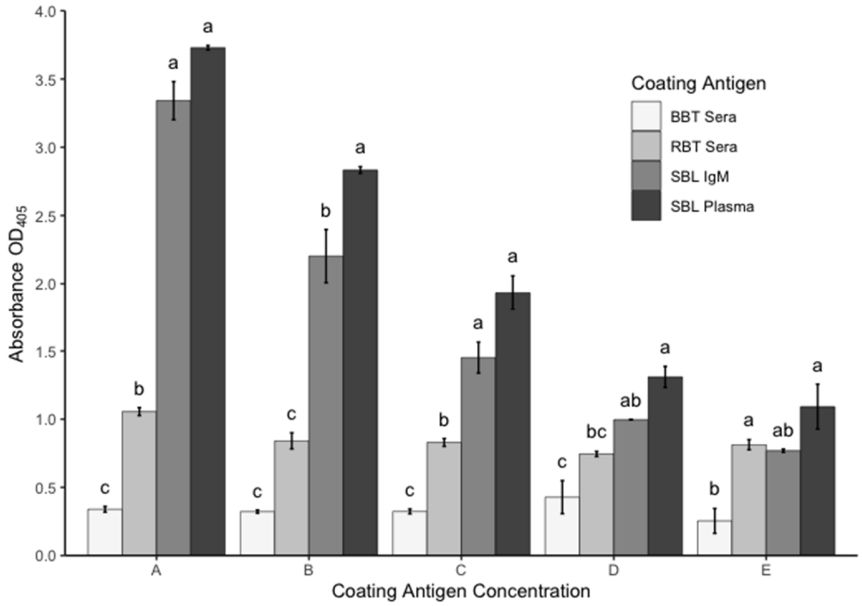
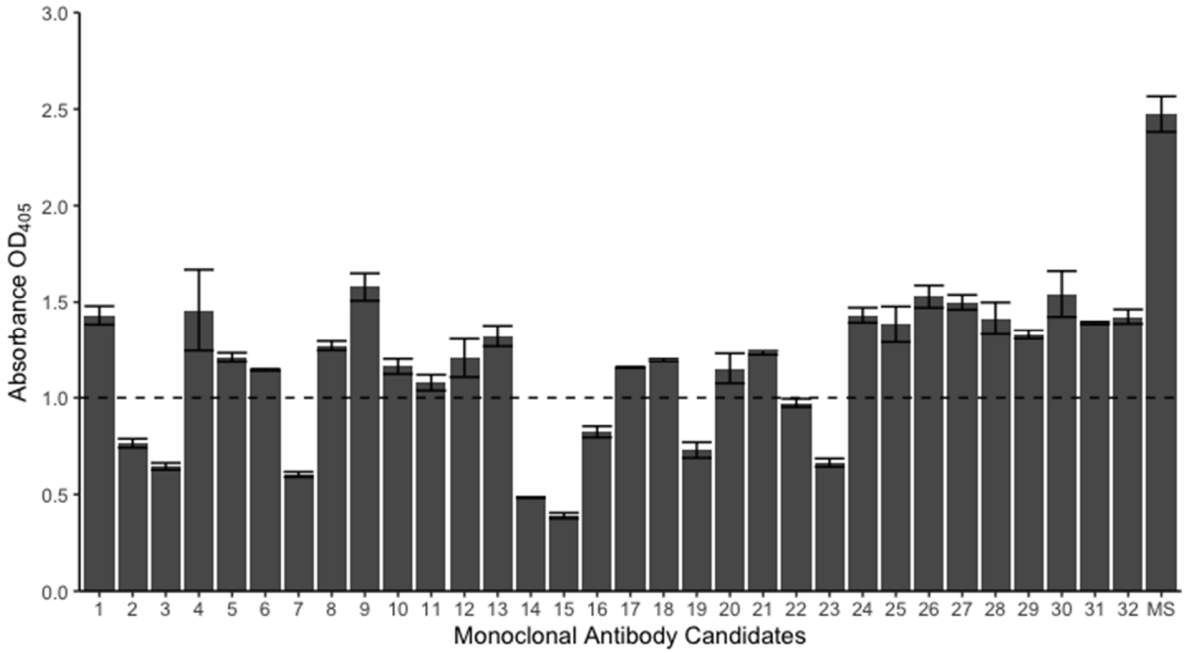


Figure 2 Screening of serum from mice immunized with sablefish IgM for specificity to various coating antigens (BBT = burbot; RBT = rainbow trout; SBL = sablefish) at different concentrations (X-axis from A to E: 0.625, 0.312, 0.156, 0.078, and 0.039 µg/mL for sera and plasma; 0.125, 0.062, 0.031, 0.015, 0.007 µg/mL for purified sablefish IgM). Values are the mean of duplicates ± SEM. Letters above bars indicate statistically significant differences among groups at specific antigen concentrations ($p < 0.05$).

3.4 mAb screening

3.4.1 ELISA

Of the 32 mAbs screened, 24 (1, 4, 5, 6, 8, 9, 10, 11, 12, 13, 17, 18, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, 32) had an OD_{405} mean absorbance value (± standard error of the mean) of 1.0 or greater in wells coated with 1 µg/mL of sablefish IgM and were kept for further analysis (Figure 3).



394

395 **Figure 3** Screening of mAb candidates produced by 32 different hybridoma colonies recognizing purified sablefish IgM. MS,
396 mouse serum positive control. Dashed line at OD_{405} of 1.0 was used as the threshold for high reactivity to the target antigen.
397 Values are mean of duplicates ± SEM. Plates were coated with 1 µg/mL of purified sablefish IgM.

398 The 24 mAbs were screened at a range of purified sablefish IgM coating concentrations, and all
399 candidates had high absorbance values in wells with a high concentration of purified IgM.
400 Whereas, no cross reactivity was observed between the 24 mAb candidates and rainbow trout or
401 burbot sera (Supplement Table 1). There were four mAbs selected for further analysis (1, 9, 25,
402 30) based on their reactivity to 0.0625 µg/mL of sablefish IgM (Figure 4).

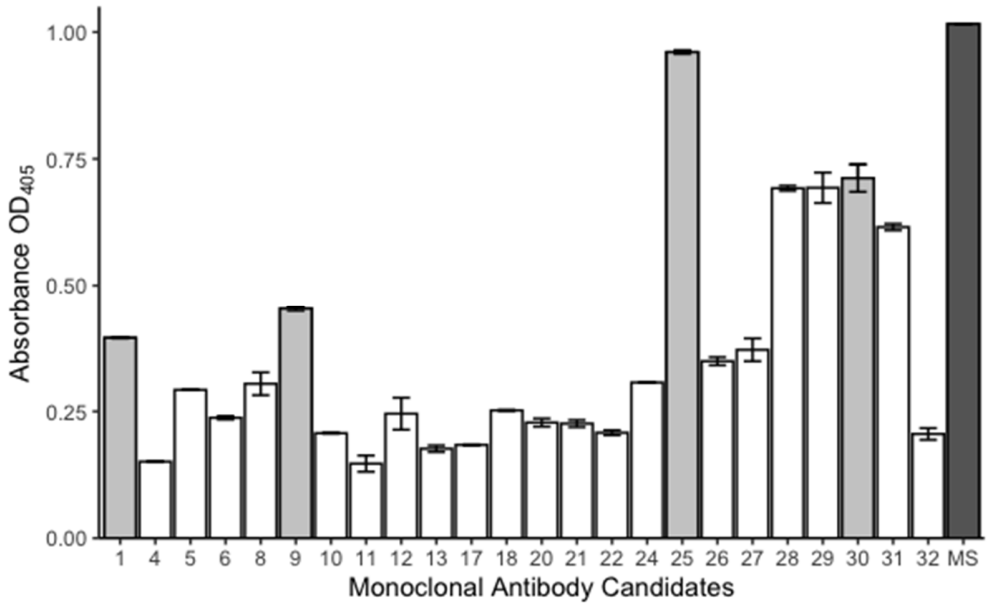
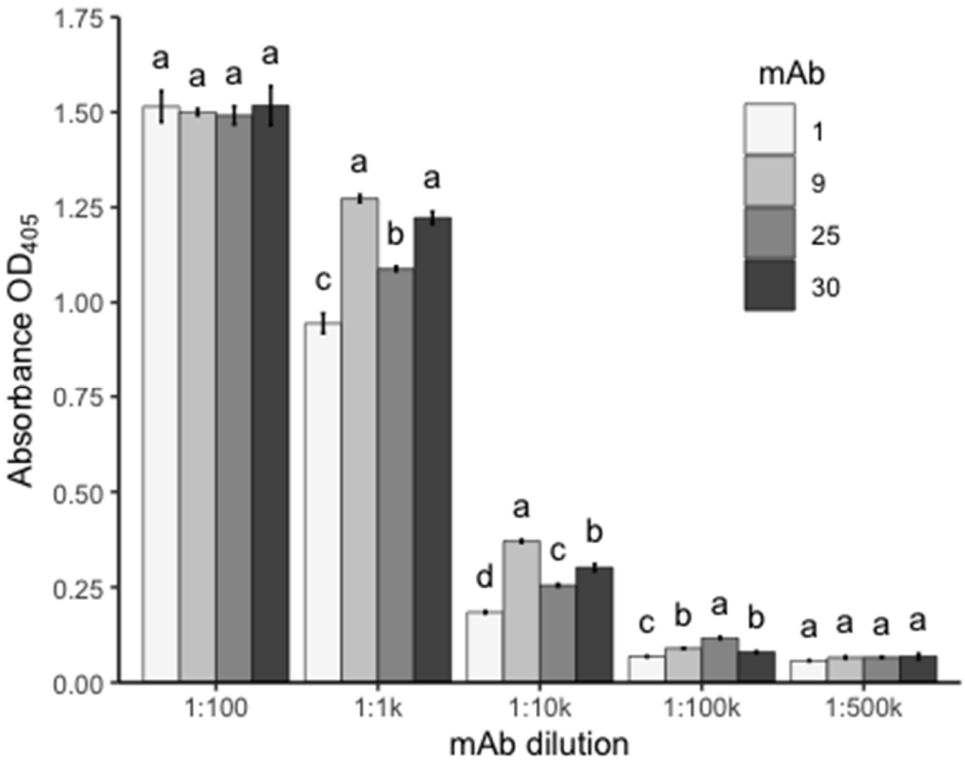


Figure 4 Screening of the top 24 mAb candidates at a 1:50 dilution and with wells coated with 0.0625 µg/mL of purified sablefish IgM. MS (dark gray), mouse serum positive control. The top four candidates (1, 9, 25, 30) are shown in light gray and were chosen for further analysis. Values are mean of duplicates ± SEM.

Various dilutions of the top four mAbs were tested for their sensitivity to 1 µg/mL of sablefish IgM coating antigen. There was no difference in absorbance among the mAbs at dilutions of 1:100 and 1:500k (Figure 5). At a 1:1k dilution, mAbs 9 and 30 had significantly higher absorbance than 1 and 25 ($p < 0.05$). At 1:10k, all were significantly different from each other with mAb 9 being the highest, followed by 30, 25, and 1 ($p < 0.05$). However, at 1:100k, mAb 25 was significantly higher than all other candidates, while 9 and 30 were only higher than mAb 1 ($p < 0.05$).

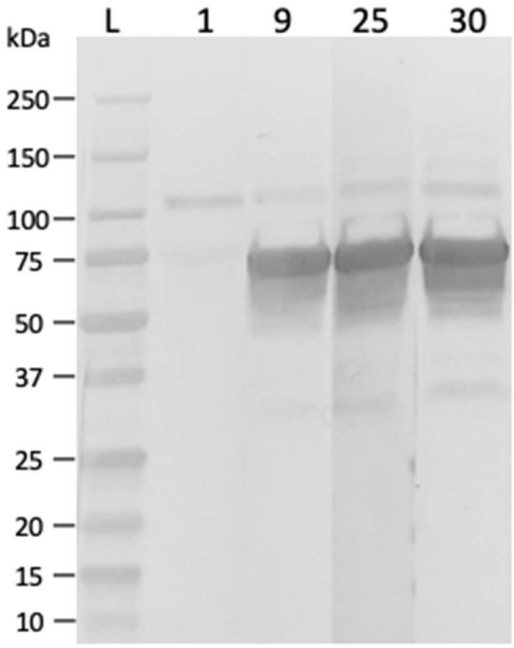


414

415 **Figure 5** Screening of the top four mAb candidates at different serial dilutions. X-axis indicates mAb dilution in KPBS-T.
416 Values are the mean of 3 replicates ± SEM. Letters above bars indicate statistical significances among groups at specific dilutions
417 ($p < 0.05$).

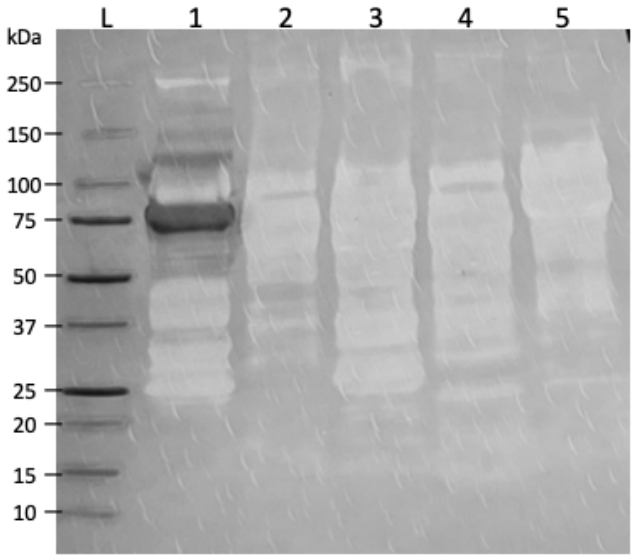
418 ### 3.4.2 Western blot

419 The four mAbs (1, 9, 25, 30) with high reactivity at low concentrations of sablefish IgM were
420 used for the western blotting analysis. All mAbs reacted with a banding at ~75 kDa, indicating
421 that they were bound to the H chain of sablefish IgM (Figure 6), though mAb 1 had faint
422 reactivity relative to other candidates. Ultimately, mAb 25 was chosen for further testing and use
423 in downstream analyses because of its high reactivity towards low concentrations of sablefish
424 IgM during the ELISA screening.



425

426 **Figure 6** Western blot analysis of mAb candidates and their binding specificity to proteins in sablefish plasma. An SDS-
427 PAGE was performed with sablefish plasma, then probed with individual mAb candidates (1, 9, 25, 30) to determine their region
428 of reactivity to sablefish plasma. Bands at 75 kDA represent binding to the heavy chain of sablefish IgM. Numbers above lanes
429 denote mAb candidate used for immunostaining. All mAb candidates were specific to the heavy chain of sablefish IgM.



430

431 **Figure 7** Western blot analysis of raw fish plasma or serum using UI-25A. An SDS-PAGE was performed on several different
432 fish species serum: Lane L, protein standard ladder; Lane 1, sablefish plasma; Lane 2, coho salmon serum; Lane 3, Atlantic
433 salmon serum; Lane 4, rainbow trout serum; Lane 5, burbot serum. White shadows represent proteins in fish plasma or serum that
434 were transferred to nitrocellulose paper, dark bands represent specific binding of UI-25A. This demonstrates the specificity of

UI-25A to the heavy chain of sablefish IgM (~75 kDa) and lack of cross reactivity to proteins found in blood circulation of other fish species.

3.4.3 Cloning and characterization

The three clones of mAb 25 behaved similarly and retained specificity and sensitivity to sablefish IgM as well as lacking cross reactivity to other teleost serum and the *A. salmonicida* T30 antigen. Clone A, referred to as UI-25A, was chosen for further downstream applications. Western blot analysis (Figure 7) demonstrates the specificity that UI-25A has to the heavy chain of sablefish IgM with reactivity at 75 kDa, and a lack of reactivity to other products in sablefish plasma as well as proteins and carbohydrates in sera from the other fish species. UI-25A was typed as an IgG2b mouse antibody, and the concentration of the stock used for all other applications described was 60 µg/mL.

3.5 Development of ELISA to measure specific antibodies in sablefish

Through standard checkerboard titrations, the optimal concentration was chosen for coating antigen, primary, and secondary antibodies. The T30 coating antigen was used at 5 µg/mL, while the primary and secondary antibodies were optimized at 1:800 and 1:5000 dilutions, respectively. Log antibody titers were significantly different between vaccinated and control fish 8 weeks after vaccination (3.86 ± 0.09 vs. 2.43 ± 0.05; $p < 0.0001$; Figure 8). This demonstrates the effectiveness of UI-25A in measuring specific circulating antibodies and differentiating immune responses following vaccination.

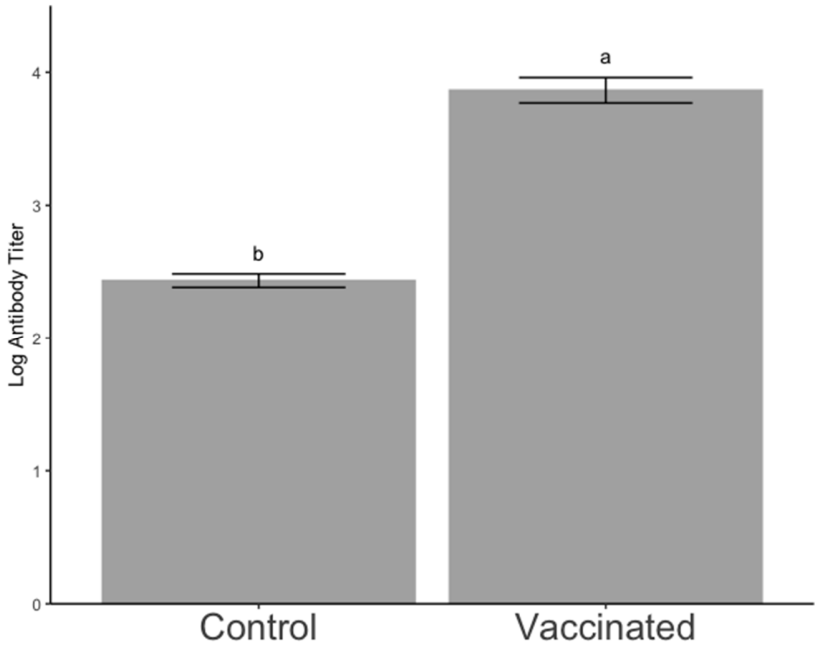
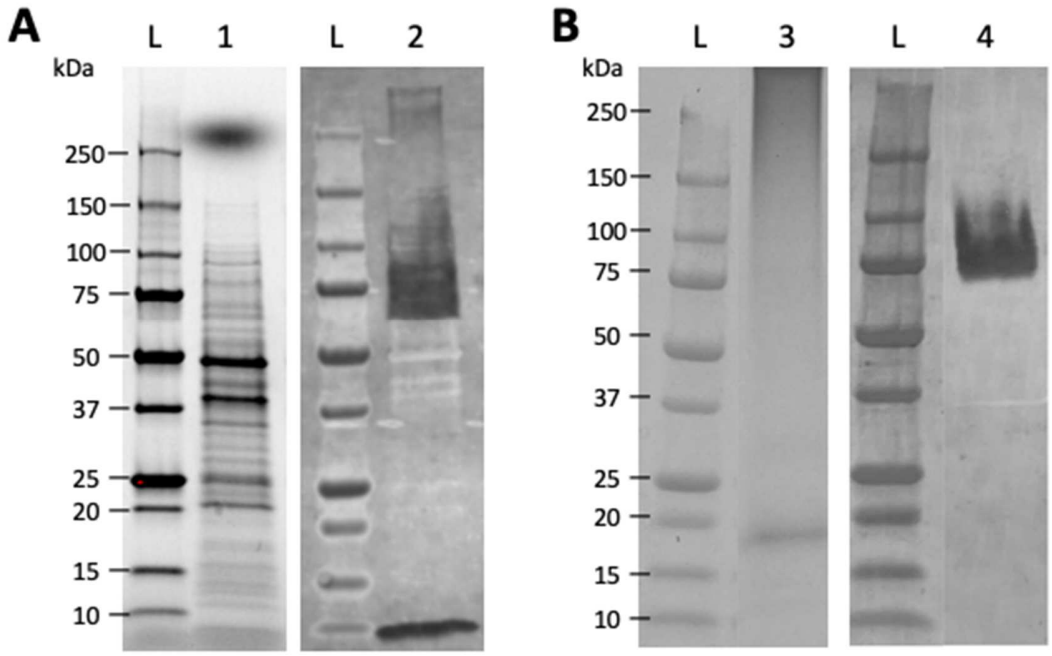


Figure 8 *A. salmonicia* specific IgM levels, expressed as log antibody titers, detected using an ELISA with plasma samples from vaccinated (Vaccinated) and naïve (Control) sablefish from a previous experiment. Letters above bars indicate a significant difference between groups ($p < 0.05$).

3.6 Western blot analysis of *A. salmonicida*

Western blotting of the T30 whole cell profile with the plasma from vaccinated sablefish showed reactivity of primary immunogenic regions at 70-80 and 10 kDa (Figure 9A). In the LPS extract, plasma was only reactive to the 70-80 kDa region (Figure 9B).

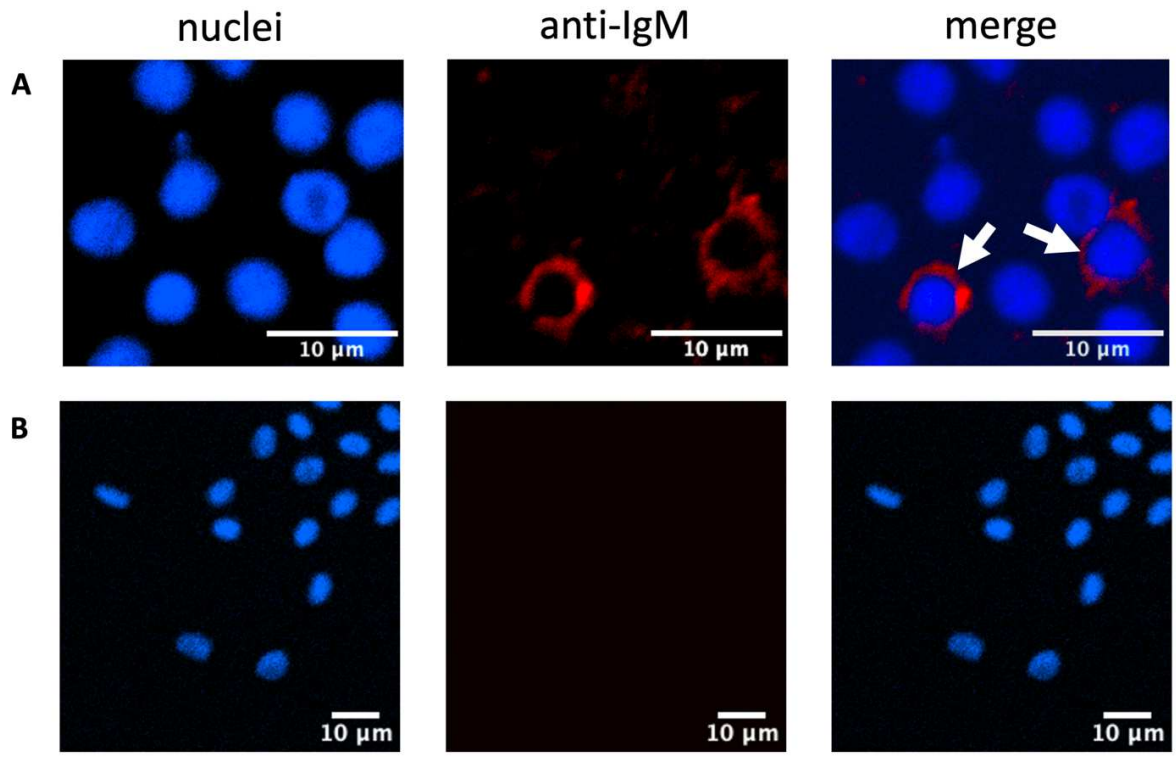


462

463 **Figure 9** Western blot images of the T30 whole cell and LPS fractions reacting with plasma from immunized sablefish and
464 visualized with UI-25A. A, whole cell profile of T30; Lane L, protein standard ladder; Lane 1, SDS-PAGE profile; Lane 2,
465 western blot profile of T30 whole cell probed with immunized sablefish plasma. B, extracted LPS profile of T30; Lane L, protein
466 standard ladder; Lane 3, silver stained SDS-PAGE profile of LPS; Lane 4, western blot profile of T30 LPS probe with
467 immunized sablefish plasma. Dark bands in lanes 2 and 4 show immunogenic regions of *A. salmonicida* isolate T30 to which
468 sablefish IgM binds to.

469 **3.7 Immunofluorescent staining of membrane-bound IgM**

470 Imaging showed red fluorescent signals on IgM+ cells collected from head kidney imprints. The
471 UI-25A did not bind to all sablefish head kidney cells (Figure 10A) and did not bind to any cells
472 from similarly prepared burbot head kidney tissue imprints (Figure 10B). This demonstrates the
473 ability of UI-25A to recognize membrane-bound IgM in sablefish in addition to secreted IgM
474 antibodies in circulation recognized by the ELISA.



475

476 **Figure 10** Immunofluorescence staining of head kidney imprints from sablefish and burbot. Nuclei were stained with DAPI
477 (blue), UI-25A anti-IgM was stained with Alexa Fluor 555 (red), the merge images show both stains overlaid. A,
478 immunofluorescence-stained sablefish with UI-25A mAb with white arrows indicating IgM+ cells; B, immunofluorescence-
479 stained burbot with UI-25A mAb.

480 **4 Discussion**

481 The development of the UI-25A antibody will benefit both researchers and the aquaculture
482 industry. Literature on fish immunology has increased along with the worldwide growth of
483 aquaculture, and reviews can be found on innate [35], cell mediated [36], and humoral immunity
484 [15]. Antibodies (polyclonal or monoclonal) specific to fish Igs are important components in the
485 development of vaccines, immune boosting treatments, and diagnostic assays that further support
486 aquaculture. They are also useful in the management and monitoring of diseases in wild fish
487 populations. In the past, these tools were reserved for economically important species such as
488 Atlantic salmon [37] or catfish [38] where demand and funding for vaccines or other diagnostic
489 assays were higher. Presently, fish immunoglobulins, specifically their structure and role in
490 immune function, are much better understood and fundamental research is useful not only in

aquaculture but also in understanding the evolution of vertebrate immunity [39]. The reduced cost and increasing availability of tools to create mAbs has led to a renaissance of increased production for niche and emerging aquaculture species.

Sablefish aquaculture attracts growers and investors as an emerging aquaculture species due to its limited commercial availability, high marketability and value, and their ability to be incorporated into existing aquaculture operations including net pens and the use of commercial salmonid diets [4]. Research of the immune system of sablefish is increasing with several recent studies characterizing blood reference intervals [40], susceptibility to pathogens [41], vaccination [12,13], and the effect of environmental factors on immune function [14].

Sablefish IgM, purified through a mannan binding protein matrix, showed two distinct bands at about 75 and 25 kDA in reduced SDS-PAGE conditions. These bands correspond to the H and L chain sizes of other Teleostei IgM such as large yellow croaker [22], Nile tilapia [21], Indian major carps [42], muskellunge [19], Atlantic salmon [37], and sea bass [18]. In the present study 32 mAb candidates were evaluated for their specificity and sensitivity to sablefish IgM using ELISA and western blot techniques. The selected mAb, UI-25A, is specific to the H chain of sablefish IgM which is a desired epitope for these tools because of its specificity to individual Ig classes, in this case IgM, whereas the L chain may share kappa or lambda chains across different Ig classes [19]. Research has shown that the constant domain of the H chain determines the Ig class and mediates the functions of an antibody [16]. Even so, cross reactivity of UI-25A among Ig classes cannot be completely ruled out until IgT and IgD have been detected and characterized in sablefish. These Ig classes have been found in many studied teleosts, but not all [16], and none have included species closely related to sablefish. The only other member of the family Anoplopomatiae, the skilfish (*Erilepis zonifer*), is not well studied except for some general information on life history, distribution, and current stock status [43]. Cross reactivity of UI-25A to skilfish IgM was not tested due to lack of available samples from the species, but there may be interest in the future to determine the extent of similarities in antibody characterization between the Anoplopomatidae family members. To our knowledge, this is the first reported mAb available for detection of sablefish IgM.

The UI-25A mAb is effective in detecting circulating antibodies in sablefish specific to atypical *A. salmonicida*, an important pathogen affecting sablefish aquaculture. Research has clearly

demonstrated that IgM levels and their specificity to pathogens are important for a well-developed humoral immune response in fish [44]. The ability of mAbs, including UI-25A, to identify specific antibodies in the blood is a powerful tool for evaluating the immune response of fish under various conditions. This study found significantly (~ 1.6-fold) higher *A. salmonicida* specific IgM titers in vaccinated sablefish compared to a control group (Figure 8), which corresponded to significantly higher survival during a pathogen challenge previously performed with these treatment groups [12].

UI-25A was also successfully used to determine the specificity of antibody responses using immunoblotting of whole cell and LPS extracts from *A. salmonicida* (Figure 9). Reactivity to immunogenic regions during western blot analysis demonstrated that sablefish antibodies were specific to both carbohydrate and protein antigens of *A. salmonicida* isolate T30 after vaccination. Similar responses have been observed in other species [45], and future studies may use these techniques to further elucidate the antibody response of sablefish to a variety of *A. salmonicida* isolates. This technique will also be useful in screening important target antigens for vaccine formulations against *A. salmonicida* or other pathogens [46]. Western blotting may be used to evaluate the ability of formulated vaccines to induce specific antibody responses against novel or emerging isolates, and this has previously been done for other diverse bacterial pathogens [47]. Aside from measuring and evaluating specific antibody presence in the blood, UI-25A can also be used to characterize the distribution and activity of IgM+ cells. Through immunofluorescent staining, we have demonstrated that the UI-25A mAb can detect and bind to membrane-bound sablefish IgM (Figure 10). As these samples were head kidney imprints, the IgM+ cells are likely long lived plasma cells [15]. However, with the recent discovery of the phagocytic capabilities of IgM+ cells in the peripheral blood [48], this type of staining with UI-25A may be applied to other assays, such as flow cytometry, to quantify the activity of IgM+ B cells [18,20].

In summary, a murine mAb specific to the H chain of sablefish IgM was developed. A number of potential mAb candidates were screened by ELISA and western blotting before selecting UI-25A as the optimal clone for downstream applications. UI-25A was successfully applied in an ELISA and western blotting to characterize the immune response of sablefish vaccinated against atypical *A. salmonicida*. In addition, UI-25A was used in immunofluorescent microscopy to identify IgM+ cells. This study, and the characterization of UI-25A, provides a valuable tool for the study

of immunity in sablefish and has implication for the development of disease prevention strategies and advancing the culture of this species.

Acknowledgements

This work was funded by a grant from the Western Regional Aquaculture Center (Award No. 2020-38500-32561). We would also like to acknowledge the funding for previous work by Arkoosh et al. [12] that was used in this manuscript; specifically, Rick Goetz, Graham Young, Mary Beth Rew-Hicks, Wendy Olson, Washington Sea Grant, University of Washington, and funding from the National Oceanic Atmospheric Administration (Award No. NA14OAR4170078). We thank Victoria Hulubei for her assistance and expertise in the development of monoclonal antibodies at WSU. We also thank Benjamin LaFrentz for providing comments and feedback on the manuscript. Sablefish plasma at Memorial University of Newfoundland was collected as part of a project that was funded through the Ocean Frontier Institute (Module J) and the Canada First Research Excellence Fund. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement.

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