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ORIGINAL ARTICLE

Biology

Dynamics of serum IgM level during the growth of juvenile Japanese amberjack *Seriola quinqueradiata*

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Abstract

Immunoglobulins (Igs), also termed antibodies, orchestrate host-acquired immune responses against foreign antigens, including invasive pathogens. In fish, IgM, which is present predominantly circulating in the blood, is particularly important for humoral systemic immunity and protecting the host from pathogens. The efficacy of inactivated vaccines, a major type of vaccine commonly used worldwide in fish, is directly linked to the serum antibody level; however, the timing of the appearance of systemic IgM circulating in the blood has not been determined in fish. In the present study, we examined the dynamics of serum IgM levels in juvenile Japanese amberjack *Seriola quinqueradiata*, using a highly sensitive sandwich enzyme-linked immunosorbent assay (ELISA) that we developed for IgM. We found that serum IgM concentration in young fish up to 72 days post-hatching (d.p.h.) (mean ± standard error of the mean [SEM]; body weight: 5.73 ± 0.38 g, standard length [S.L.]: 72.2 ± 1.94 mm) was sustained at a low level, but that the level significantly increased from 79 d.p.h. onward, reaching a mean of 84.76 ± 9.23 μg/mL at 85 d.p.h. (body weight: 14.05 ± 0.92 g, S.L.: 101.1 ± 2.07 mm). These results suggest that systemic immunity mediated by IgM is only partially matured in the early growth stage of juveniles. The present findings could help establish effective vaccination programs for infectious diseases in young fish.

Keywords Antibody · IgM · Serum antibody · Humoral immunity · Juvenile · Japanese amberjack *Seriola quinqueradiata*

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Introduction

Antibodies, alternatively referred to as immunoglobulins (Igs), are soluble proteins involved in the elimination of foreign antigens that have invaded the body, such as pathogens. The proteins induced by infection are exclusively produced by B-lymphocytes (B cells) to induce an antigen–antibody reaction that involves specific binding to the antigen. Teleost fish only possess three different Ig heavy chain isotypes, namely, IgM, IgD, and IgT/Z (Salinas et al. 2011). IgM is the most abundant Ig in plasma and the principal Ig involved in systemic immunity in teleost fish (Xu et al. 2013). The protective role of plasma Igs against pathogens has been demonstrated by a passive immunization study in several fish species (Rajan et al. 2017). Inactivated vaccines, the main type of vaccine used for cultured fish, function by imitating infection by certain pathogens and induce Ig production to protect against the pathogens (Gudding et al. 1999; Munang'andu and Evensen 2019). Indeed, Munang'andu and Evensen (2019) reported that serum IgM titer in vaccinated fish is correlated with protective immunity. The timing of antibody emergence has been studied in several fish species, with the results showing that the first appearance of IgM-bearing cells generally occurs 1 month after hatching, although this depends on the fish species (Scapigliati et al. 1999). IgM is then secreted from the cells and confers a systemic antibody response (Piazzon et al. 2016). However, the dynamics of secreted IgM in the plasma of teleost fish remain unknown.

Japanese amberjack *Seriola quinqueradiata* and other related species, including greater amberjack *Seriola dumerili* and yellowtail amberjack *Seriola aureovittata*, are the predominant species in the Japanese aquaculture industry, accounting for more than one half of the total production of finfish aquaculture in Japan (Matsuura et al. 2019). In addition, Japanese amberjack has been designated an important export product in Japan due to increased overseas demand in addition to domestic demand. Infectious diseases are thus an obstacle to increased production in aquaculture, and the use of vaccines is an effective solution. Recently, an urgent need has arisen for effective vaccines for juvenile Japanese amberjack due to increased disease occurrence in young fish. To develop vaccines for young fish, information regarding the dynamics of Igs, particularly the timing of the first appearance of Igs, is essential. However, no such studies have been performed to date in amberjack species due to the lack of research tools for Ig quantification.

In the present study, we report the development of a sandwich enzyme-linked immunosorbent assay (ELISA) capable of quantifying trace amounts of amberjack IgM. Using this newly developed method, we examined the dynamics of serum IgM at each growth stage of Japanese amberjack juveniles.

Materials and methods

Ethics statement

All experiments using animals in this study, including handling, husbandry, and sampling, were carried out in accordance with the policy designated by the Institutional Animal Care and Use Committee of the Fisheries Technology Institute and were approved by the committee (no. 22003).

Fish

Japanese amberjack juveniles cultured at the Kamiura Field Station, Fisheries Technology Institute, were used for serum sampling at 46, 51, 57, 64, 68, 72, 79, 85, and 96 days post-hatching (d.p.h.). Juveniles were maintained in 60-kL tanks and reared in running water with aeration at 20−23 °C. All fish were fed dry pellets once each day. The serum used for purification of amberjack IgM was collected from juveniles approximately 30 cm in standard length (S.L.). These fish were maintained in a 0.5-kL round tank in running water at 23−25 °C with aeration and fed dry pellets twice each day except on the day before and the day of blood collection. The body weight and S.L. of the juveniles were recorded before each sampling.

Serum preparation

Blood was collected for serum preparation by the following methods, depending on the size of the juvenile, after recording the body weight (g) and S.L. (mm). For small juvenile fish up to 57 d.p.h. (body weight: 0.95 ± 0.08 g, S.L.: 38.9 ± 1.29), exuded blood from the caudal peduncle of fish severed with a scalpel (FEATHER Safety Razor; FEATHER Safety Razor Co., Ltd, Gifu, Japan) was collected by immersion in phosphate buffer solution (Electronic Supplementary Material [ESM] Fig. S1) because the amount of exuded blood was too small to collect directly. For fish between 64 and 96 d.p.h. (body weight: 3.19 ± 0.20−25.0 ± 1.20 g, S.L.: 59.5 ± 1.43 to 119.4 ± 1.83 mm), blood exuding from the severed caudal peduncle was collected using capillary tubes. For fish used for IgM purification, blood was collected using a syringe (Terumo Corp., Tokyo, Japan). The collected blood was incubated for 1 h at room temperature to promote coagulation and then centrifuged at 1000 *g* for 5 min at 4 °C. The supernatant was collected as serum and used for subsequent analyses. The amount of protein in the crude serum was determined by the Bradford method, with bovine serum albumin (TakaRa, Shiga, Japan) used as the

standard. All fish were anesthetized using 2-phenoxyethanol (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) before blood collection.

Purification of Japanese amberjack IgM

Serum prepared from two Japanese amberjack approximately 30 cm in S.L. was mixed and diluted fivefold in phosphate-buffered saline (PBS). Ammonium sulfate was added to the mixture to a final concentration of 35%, and the mixture incubated with agitation at room temperature for 1 h. The supernatant was collected by centrifugation at 8000 *g* for 5 min at 4 °C and then mixed with ammonium sulfate to a final concentration of 50% and incubated at room temperature for 1 h. After agitation, the precipitate was harvested by centrifugation at 8000 *g* for 5 min at 4 °C. The precipitate was washed twice with saturated ammonium sulfate in PBS by centrifugation at 8000 *g* for 5 min at 4 °C and then suspended in 1 mL of 10 mM Tris–HCl (pH 7.4). The suspension was subjected to gel filtration chromatography using Sephacryl S-300 (Cytiva, Tokyo, Japan) for crude purification, followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue (CBB) staining of the gel to confirm the fractions containing IgM. The IgM-containing fractions by gel filtration chromatography were collected, mixed, and added to an anion exchange column (HiTrap Q HP column; Cytiva, Marlborough, MA, USA). Protein fractionation was performed by gradient elution using 10 mM Tris–HCl (pH 7.4) buffer with varying NaCl concentration (0–1 M NaCl). The fractions containing IgM were confirmed by SDS-PAGE and CBB staining of the gel. The purified product was concentrated by ultrafiltration, and the purity was confirmed by SDS-PAGE followed by CBB staining. The protein was confirmed as Japanese amberjack IgM by western blotting using a mouse monoclonal antibody against IgM (mAb, IgG1; Matsuyama et al. 2016). The purified IgM was stored at − 30 °C until use after adding glycerol to a final concentration of 50%. Protein amount was determined by the Bradford method, and bovine normal IgG (Nippon Bio-Rad Laboratories K.k., Tokyo, Japan) was used as the standard.

Generation of polyclonal antibody against Japanese amberjack IgM

The sandwich ELISA requires an antibody pair specific for a different epitope of the antigen; this pair contributes to the high sensitivity and specificity of the method (Gan and Patel 2013). We therefore produced another antibody specific to Japanese amberjack IgM, as required for the method, in addition to a mouse mAb against the IgM established by Matsuyama et al. (2016). A Japanese white breed rabbit was immunized 4 times at 1- to 4-week intervals with 0.1−0.3 mg of purified IgM. Serum from the immunized rabbit was prepared from whole blood collected from the heart 1 week after the last immunization. The titer of the serum antibody was confirmed to be sufficiently elevated by ELISA before whole blood collection. Protein G Sepharose gel (Cytiva) was then mixed with the serum overnight at 4 °C using a rotator to purify total rabbit IgG. The mixture was packed in an open column and washed with PBS at 10-fold the volume of the gel. After washing, an elution buffer containing 0.1 M glycine–HCl (pH 2.7) was added to the column to elute the whole rabbit IgG bound to the gel. The elution was performed until protein elution could no longer be confirmed. The buffer of the eluate, pH-adjusted using 2 M Tris–HCl (pH 7.4), was exchanged for PBS by dialysis, and glycerol was added to the purified IgG to a final concentration of 50% for storage at − 30 °C until use. The amount of IgG protein was determined by the Bradford method, and bovine normal IgG (Nippon Bio-Rad Laboratories K.k.) was used as the standard. This purified IgG was used as a polyclonal antibody (pAb) against Japanese amberjack IgM.

Western blotting

Western blotting using the pAb and mAb as primary antibodies was performed to confirm the antigen specificity. Protein samples were boiled for 2 min in 2× SDS sample buffer (ATTO, Tokyo, Japan), followed by separation in a 15% SDS-PAGE gel. The protein was then transferred onto a polyvinylidene difluoride (PVDF) membrane (ATTO). The membrane was incubated in blocking reagent (StartingBlock T-20; Thermo Fisher Scientific Japan, Tokyo, Japan) for 1 h at room temperature, followed by reaction with 1.0 μg/mL of each antibody overnight at 4 °C. After the reaction, the membrane was washed 5 times with Tris-buffered saline including 0.1% Tween-20 (TBS-T; FUJIFILM Wako Pure Chemical Corp., Richmond, VA, USA) and probed for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-mouse or rabbit antibody (Agilent Technologies, Santa Clara, CA, USA) diluted 1:10,000. The signal of protein labeled with the peroxidase-conjugated antibody was visualized using Western Lightning ECL (PerkinElmer Inc., Waltham, MA, USA) and Hyperfilm ECL (Cytiva) after five washings with TBS-T.

Development of a sandwich ELISA for quantification of amberjack IgM

The pAb against the IgM was used as the capture antibody. In brief, the pAb diluted to 1 μg/mL was coated onto the wells of a Nunc MaxiSorp plate (Thermo Fisher Scientific Japan) at 4 °C overnight. The treated plate was blocked with 5% skim milk (FUJIFILM Wako Pure Chemical Corp.) in

TBS-T for 1 h at room temperature, and then appropriately diluted serum samples were added to the wells. After incubation for 1 h at room temperature, the mAb against Japanese amberjack IgM (Matsuyama et al. 2016) was loaded onto the plate as the detection antibody. The mAb, conjugated with peroxidase using a Peroxidase Labeling Kit-NH2 (DOJINDO LABORATORIES, Kumamoto, Japan) according to the manufacturer's protocols and guidelines, was diluted to 1 µg/mL before use. The plate was then washed 5 times with TBS-T after a 1-h incubation at room temperature. Substrate solution including 3,3′,5,5′-tetramethylbenzidine (TMB; Seracare Life Sciences, Milford, MA, USA) was loaded onto the plate, and the plate was incubated for 10 min at room temperature for color development. To stop the enzyme reaction, 1 M phosphoric acid was added to the plate. The colorimetric signal was measured at 450 nm using a microplate reader (PerkinElmer Inc.). The amount of IgM protein was calculated based on a calibration curve using a twofold serial dilution of the purified IgM. Indirect ELISA using either pAb or mAb was also performed to compare the sensitivity of each ELISA. The twofold serial dilution series of purified IgM coated on the ELISA plate was detected using the mAb or pAb followed by probing with anti-mouse or rabbit IgG (H + L) secondary antibody conjugated with HRP (Agilent Technologies), respectively.

Results

Generation of a pAb against IgM

The first step in the sandwich ELISA was purification of both the endogenous IgM used for pAb generation and the protein standard. Crude protein of Japanese amberjack serum was purified by ammonium sulfate precipitation followed by two steps of chromatography, including gel filtration and anion exchange (ESM Fig. S2), and high-purity IgM-like protein that exhibited a molecular weight (MW) of > 205 kDa under non-reducing conditions was obtained (Fig. 1a). Under reducing conditions, the protein migrated as double bands with MW of approximately 70 and 25 kDa (Fig. 1b), corresponding to the heavy and light chains of fish IgM, respectively, as reported by Uchida et al. (2000). The previously obtained mAb reacted with the protein having a MW of > 205 kDa under non-reducing conditions (Fig. 1c), indicating that the purified protein was IgM. Under reducing conditions, the mAb reacted with a protein of approximately 25 kDa (Fig. 1d), in agreement with Uchida et al. (2000). A rabbit pAb against the IgM was then generated to develop the new tools for IgM quantification. The pAb was confirmed to react with the protein, which exhibited a MW of > 205 kDa under non-reducing conditions (Fig. 1e). The

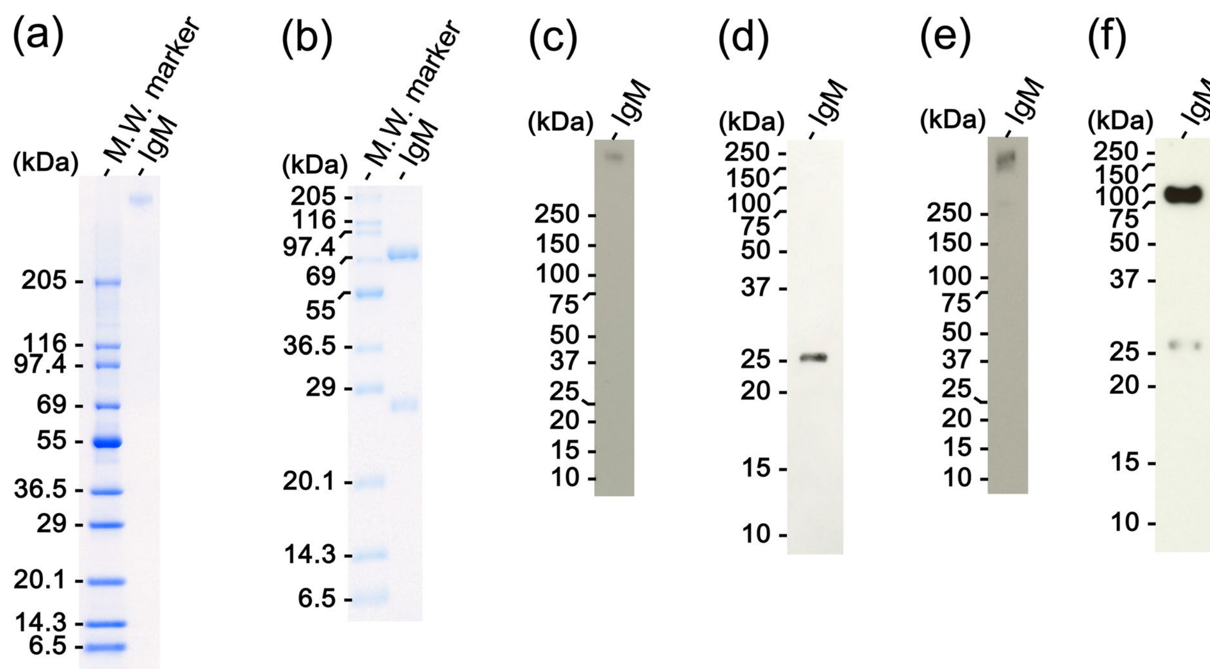

Fig. 1 Validation of a polyclonal antibody (pAb) developed against IgM of Japanese amberjack *Seriola quinqueradiata*. Purified IgM was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions (**a**, **c**, **e**) or reducing conditions (**b**, **d**, **f**) followed by staining with Coomassie brilliant blue (**a**, **b**) or western blotting probed with monoclonal antibody (mAb, **c**, **d**) or pAb (**e**, **f**). *IgM* Immunoglobulin M, *MW* molecular weight

pAb reacted strongly with a protein of approximately 70 kDa and weakly with a protein of approximately 25 kDa under reducing conditions (Fig. 1f). These results indicated that the pAb recognized both the light chain and heavy chain of IgM, whereas the mAb recognized only the light chain of IgM.

Development of a sandwich ELISA for IgM

A sandwich ELISA was constructed using the pAb newly established in the present study as the capture antibody and the mAb as the detection antibody. Dilution series of purified IgM were analyzed using this method, and the results showed good linearity at IgM concentrations ranging from 0.12 to 3.91 ng/mL ($R^2 = 0.9996$, $p < 0.0001$; Fig. 2a). Another combination of the mAb and pAb as the capture and detection antibodies, respectively, resulted in a decrease in sensitivity, ranging from 0.49 to –7.81 ng/mL ($R^2 = 0.9980$, $p < 0.0001$; ESM Fig. S3). A conventional indirect ELISA was also performed to compare the sensitivity of each method. The detection range of the indirect ELISA using the pAb and mAb was 0.24–7.81 ng/mL ($R^2 = 0.9952$, $p < 0.0001$; Fig. 2b) and 0.98–15.63 ng/mL ($R^2 = 0.9994$, $p < 0.0001$; Fig. 2c), respectively.

Dynamics of serum IgM amount associated with fish growth

To investigate the dynamics of serum IgM levels in *S. quinqueradiata* juveniles, we measured the amount of serum IgM using the established quantification method. The minimum size of sampled fish was 30.8 ± 0.98 mm (S.L.), with a weight of 0.38 ± 0.03 g at 46 d.p.h. (mean ± standard error of the mean [SEM]). Both the S.L. and body weight were significantly correlated with growth stage (d.p.h.) ($p < 0.0001$, Spearman's rank correlation coefficient and the Pearson correlation coefficient, respectively; Fig. 3a, b); in addition, S.L. and body weight were significantly correlated ($p < 0.0001$, Spearman's

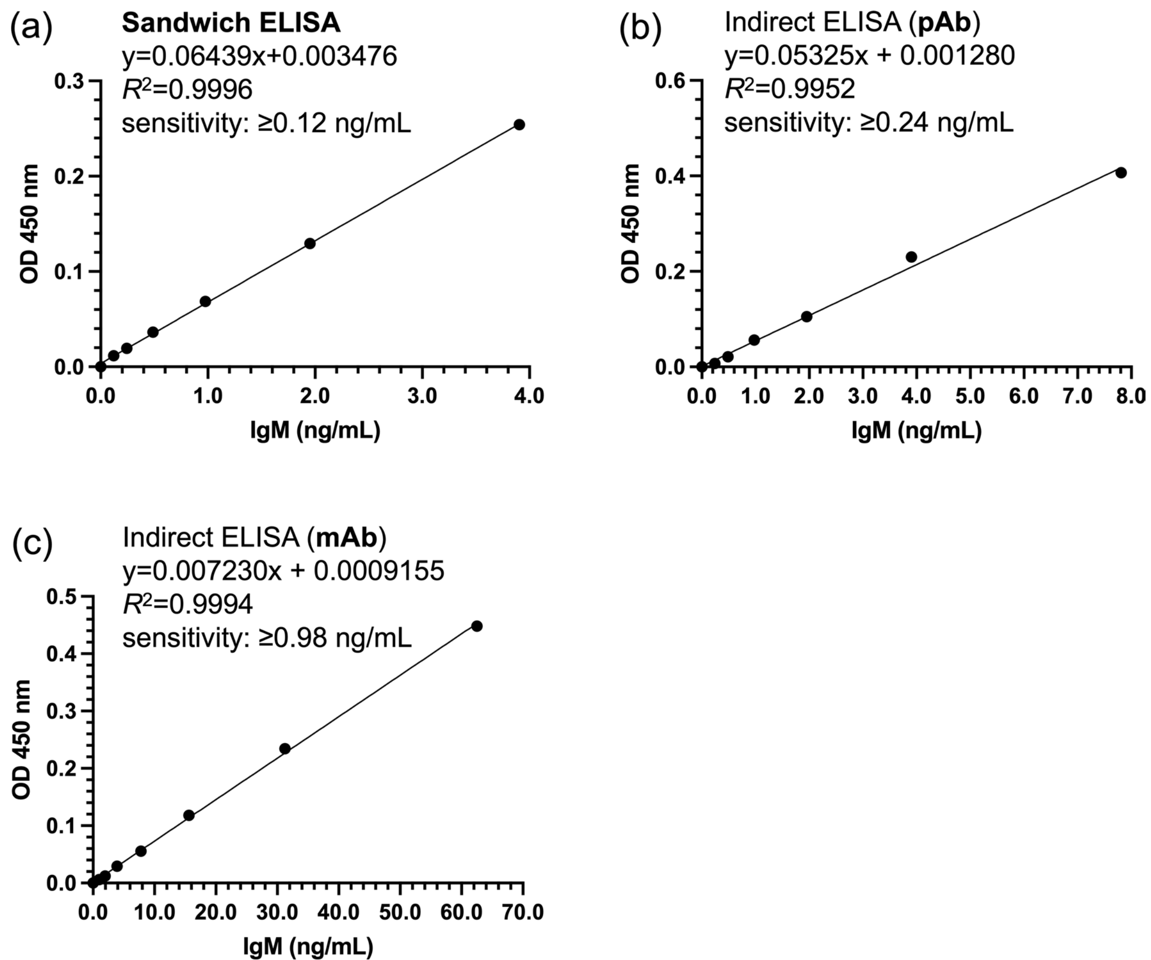

Fig. 2 Validation of a sandwich ELISA for Japanese amberjack IgM. Standard curves of three different types of ELISA for Japanese amberjack IgM. Two-fold serial dilution series of IgM were analyzed by sandwich ELISA (**a**), indirect ELISA using the pAb (**b**), and indirect ELISA using the mAb. *ELISA* Enzyme-linked immunosorbent assay, *mAb* monoclonal antibody, *OD* optical density

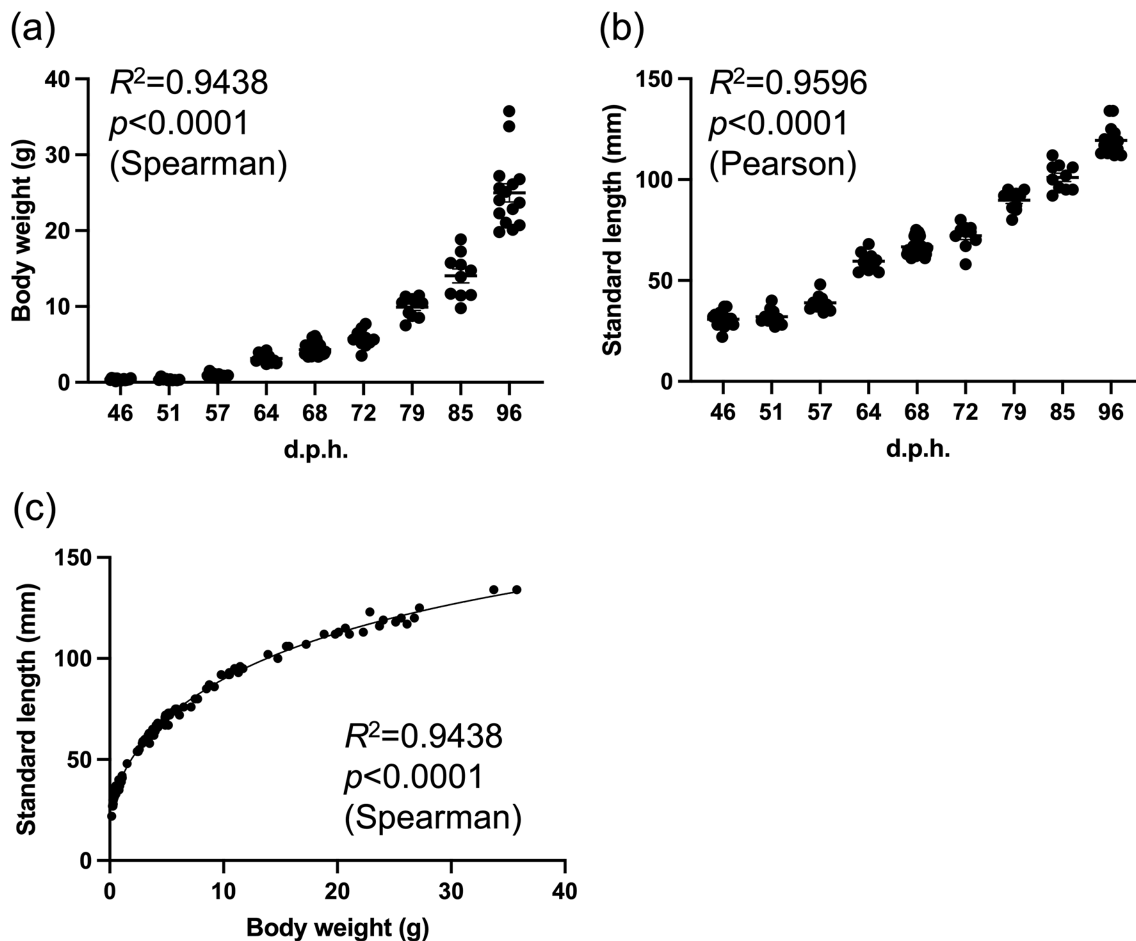

Fig. 3 Dynamics of growth in juvenile Japanese amberjack. **a**, **b** Changes in body weight (**a**) and standard length (S.L.) (**b**) of juvenile fish during growth; $n \geq 10$. Data points in **a** and **b** represent the mean ± standard error of the mean. **c** Correlation between body weight and S.L. of Japanese amberjack juveniles. *d.p.h.* Days post hatching

rank correlation coefficient; Fig. 3c). For fish at 46 to 57 d.p.h., blood could be sampled, but the volume of exuded blood was not sufficient to collect using capillaries. Consequently, the amount of serum IgM in small juvenile fish between 46 and 57 d.p.h. could not be quantified due to random dilution of the collected blood by the immersion method (ESM Fig. S1). Blood collection using capillaries and subsequent quantification of IgM was successful after 64 d.p.h. Serum IgM levels in fish rapidly increased within the 13 days from 72 to 85 d.p.h. and were 33.06 ± 2.99, 77.65 ± 11.68 and 84.76 ± 9.23 μg/mL at 72, 79, and 85 d.p.h. (mean ± SEM), respectively (Fig. 4a). To estimate the IgM level in serum of fish younger than 57 d.p.h., the relative amount of IgM normalized to total serum protein was measured. Serum IgM was detected in juveniles at 46 d.p.h., but the ratio was low before 68 d.p.h., except for a temporary increase at 64 d.p.h. The ratio started to increase from 72 d.p.h. onwards, peaking at 85 d.p.h. (Fig. 4b). No substantial change in the amount of total serum protein associated with growth was detected and, consequently, there was no correlation between body weight and total protein amount (Fig. 4c; $p = 0.99$, Pearson correlation coefficient).

Correlation between serum IgM level and fish size

The correlation between serum IgM level and fish size was analyzed to investigate the maturation of IgM-associated immunity with fish growth. A moderate positive correlation was observed between serum IgM level and fish size (body weight and S.L.) in the fish at 64 to 96 d.p.h. (Fig. 5a, b; $R^2 = 0.3573$ and 0.4642, respectively; $p < 0.0001$, Pearson correlation coefficient). The correlation between relative IgM level and fish size was also analyzed for fish younger than 57 d.p.h. In these samples, the

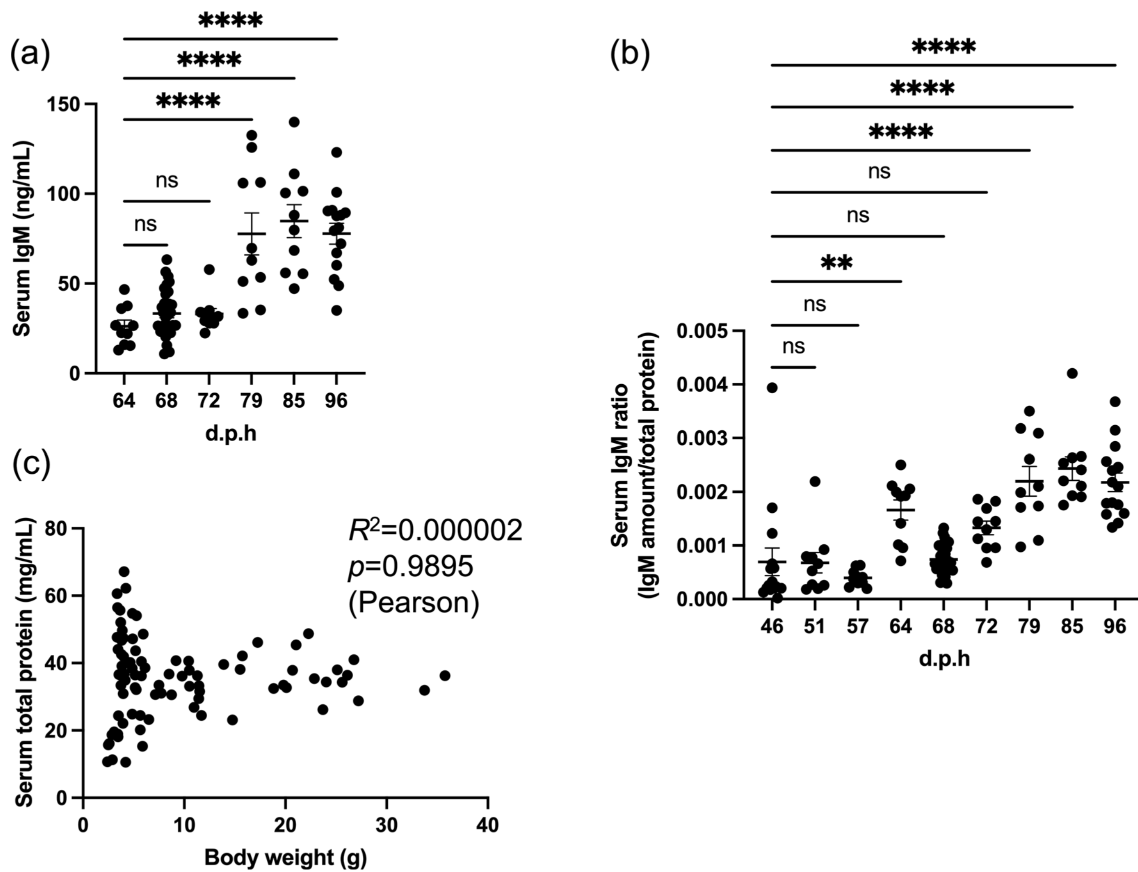

Fig. 4 Dynamics of serum IgM levels during fish growth. **a**, **b** Changes in absolute serum IgM level (**a**) and relative serum IgM level normalized to total serum protein (**b**) during growth of Japanese amberjack juveniles. Statistical significance was determined using one-way analysis of variance, followed by Tukey's multiple comparisons tests. Asterisks indicate significant difference at $^{**}p < 0.01$ and $^{****}p < 0.0001$; sample size: $n \geq 10$. Data points in **a** and **b** represent the mean ± standard error of the mean. **c** Correlation between the amount of total serum protein and size of Japanese amberjack juveniles based on Pearson correlation coefficients and linear regression analysis

relative IgM level was also correlated with body weight and S.L. (Fig. 5c, d; $R^2 = 0.3536$ and 0.4167, respectively; $p < 0.0001$, Pearson correlation coefficient).

Discussion

In the present study, we established a high-sensitivity quantification method for IgM in Japanese amberjack. A sandwich ELISA using a newly developed pAb against IgM enabled precise quantification of trace amounts of protein (low nanogram/milliliter range). To investigate the ontogeny of systemic acquired immunity, we examined the dynamics of serum IgM in juvenile Japanese amberjack using the sandwich ELISA. The level appeared to be low in fish until 72 d.p.h. (mean ± SEM; body weight: 5.73 ± 0.38 g, S.L.: 72.2 ± 1.94 mm) and then significantly increased from 79 to 96 d.p.h. (body weight: 9.91 ± 0.42−25.0 ± 1.20 g, S.L.: 89.8 ± 1.58−119.4 ± 1.83 mm). These dynamics suggest that IgM-related systemic immunity is only partially matured during the early growth stage of juveniles.

A sandwich ELISA developed using the pAb and mAb as capture and detection antibodies, respectively, achieved highly sensitive quantification of Japanese amberjack IgM, with sub-picogram sensitivity (> 0.12 ng/mL), making it possible to quantify serum IgM levels in small juvenile fish. Our method is far superior to previously established methods, including both sandwich and indirect ELISAs for use in rainbow trout *Oncorhynchus mykiss*, Nile tilapia *Oreochromis niloticus*, and sea bass *Dicentrarchus labrax*, which can be used to measure IgM at ≥ 200 ng/mL (Castillo et al. 1993), ≥ 20 ng/mL (Velazquez et al. 2021), and ≥ 10 ng/mL (Breuil et al. 1997), respectively. The sensitivity of sandwich ELISAs is generally superior to that of direct or indirect ELISAs, which can be performed without a capture antibody (Aydin 2015). In our study, the sensitivity of the sandwich ELISA was approximately two- to ninefold greater than that of indirect ELISAs using

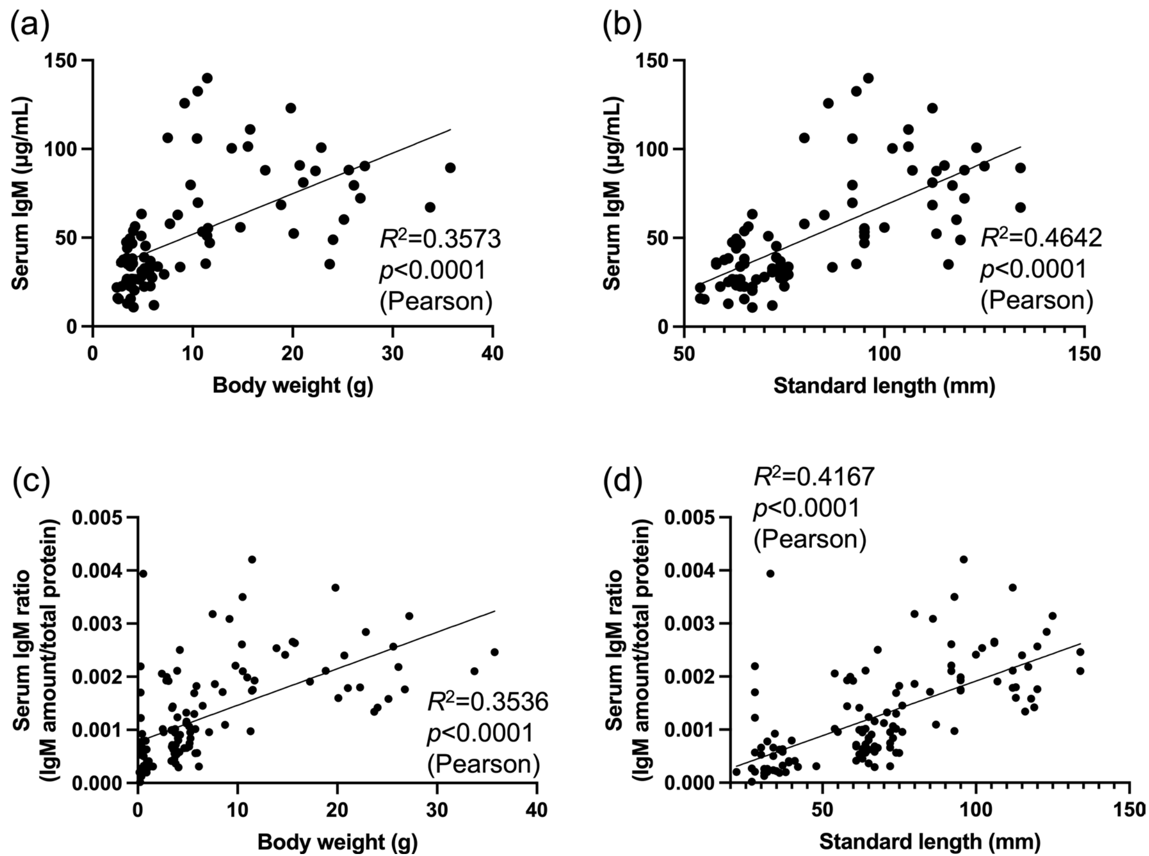

Fig. 5 Correlation between absolute serum IgM level (**a**, **b**) or relative serum IgM level normalized to total serum protein amount (**c**, **d**) and fish size in terms of body weight (**a**, **c**) or standard length (**b**, **d**). Data were evaluated using Pearson correlation coefficients and linear regression analysis

the mAb or pAb (Fig. 2a–c). The highly sensitive method was also suitable for analyzing diluted samples; thus, the method is effective for examining samples from small fish, which can be difficult to prepare in sufficient volume for analysis.

For further validation of the ELISA described herein, we analyzed a portion of each serum sample rather than the purified IgM. The serum IgM levels determined using the sandwich ELISA and indirect ELISA using the pAb were similar (mean ± SEM; 29.2 ± 3.15 and 30.8 ± 3.34 μg/mL, respectively), whereas the indirect ELISA with the mAb overestimated the serum IgM concentration by approximately 1.8-fold compared with the other ELISAs (52.9 ± 6.04 μg/mL) (ESM Fig. S4). No binding of the antibodies to unrelated proteins was detected (ESM Fig. S5), suggesting that the difference in the results was not caused by non-specific reaction of the antibodies. Although the reasons for this discrepancy remain unknown, it is important to assume such an effect can occur when dealing with results obtained using different ELISA methods.

Both the body weight and S.L. of Japanese amberjack juveniles used in this study rapidly increased between 46 and 96 d.p.h. as the fish grew, with a strong correlation between both parameters (Fig. 3a, b, c). In contrast to fish growth, the IgM level rapidly increased from 72 d.p.h. onward, peaking at 85 d.p.h. (Fig. 4a), suggesting that maturation of systemic immunity occurs within a short period. The average serum IgM concentration in adult fish has been reported to be > 1.0 mg/mL (Hordvik 2015; Bilal et al. 2021), whereas serum IgM in juveniles of Japanese amberjack younger than 96 d.p.h. was < 0.1 mg/mL. In comparison, the level in more mature fish at 122 d.p.h. (mean ± SEM; body weight: 56.1 ± 1.99 g, S.L.: 156.9 ± 2.01 mm) was found to be dramatically higher, at 1.29 ± 0.17 mg/mL, which was significantly higher than that of juveniles at 96 d.p.h. (ESM Fig. S6) and similar to levels found in other fish species. These findings suggest that IgM levels in younger juveniles (before 96 d.p.h.) have not yet begun to markedly increase. In rainbow trout, Sano (1960) investigated the albumin/globulin (A/G) ratio in serum at different growth stages instead of the amount of serum IgM. The A/G ratio in the serum of young fish tended to decrease as the fish aged, in agreement with our present findings (Fig. 4b). In Japan, several polyvalent vaccines, including formalin-killed *Lactococcus garvieae*,

have been approved for amberjack species, and these vaccines are considered to induce antibody-mediated immunity (Ooyama et al. 1999; Nakajima et al. 2014). A passive immunization trial using fish serum antibodies raised against killed bacteria suggested that Igs conferred protection against the disease (Ooyama et al. 1999). The minimum size of individual fish certified as effectively protected from *Lactococcus* by vaccination was 10 g (Matsuura et al. 2019); thus, the IgM level in fish smaller than 10 g (younger than approximately 80 d.p.h.) may be inadequate for protection.

The level of serum IgM in fish younger than 72 d.p.h. and weighing < 5.73 ± 0.38 g appeared to be low (Fig. 4a), suggesting that the systemic antibody response in fish of this size is immature. Bakopoulos et al. (2003) reported that an inactivated vaccine against pasteurellosis (infection by *Photobacterium damselae* subsp. *piscicida*) that confers protection through enhancement of the specific antibody titer in adult sea bass did not confer any protection in small-sized fish weighing 1.5−2.0 g (size similar to that of the fish in our study at 57−64 d.p.h.; Fig. 3a) via the intraperitoneal route. The lack of protective efficacy in small fish agrees well with the low levels of serum IgM detected in small fish in the present study. Correlation analyses demonstrated that fish size measured according to both body weight and S.L. was correlated with serum IgM level (Fig. 5a, b); thus, our data could be used to predict the optimal timing of vaccination.

For fish younger than 57 d.p.h., we determined the relative rather than absolute amount of IgM. The amount of total serum protein was used for normalization of the IgM level because it tended not to fluctuate with fish growth (Fig. 4c). Furthermore, only a trace amount of IgM was detected in the immersion fluid of the tail fin, which was not cut, as was done in juvenile fish sampled at 57 d.p.h. (ESM Fig. S7). Thus, mucosal IgM did not affect the results, and the sampling method is considered to be sufficiently reliable. The determination of relative IgM levels suggested that at 46 d.p.h. (mean ± SEM; body weight: 0.38 ± 0.03 g, S.L.: 30.8 ± 0.98 mm), the fish already harbor serum IgM, but the levels remain low until 68 d.p.h. (body weight: 4.31 ± 0.14 g, S.L.: 66.7 ± 0.77 mm), except for a transient increase at 64 d.p.h. Breuil et al. (1997) reported that the IgM level in whole juvenile sea bass increases and is correlated with body weight beginning in the early growth stage (approx. 30 d.p.h.). However, the increase in IgM level observed in that study corresponded to the maturation of IgM-producing cells before the transition to systemic immunity, unlike the case with serum IgM, and thus transition of mature IgM from producing cells to systemic immunity may be delayed for dozens of days. Further studies, including analyses of the dynamics of IgM-producing cells in whole fish, are needed to characterize the dynamics of this process.

The transfer of maternal IgM to offspring reportedly differs by species, including both freshwater and seawater fishes, such as Mozambique tilapia *Oreochromis mossambicus* (Takemura 1993), rainbow trout (Castillo et al. 1993), red seabream *Pagrus major* (Kanlis et al. 1995), sea bass (Breuil et al. 1997), and others. However, maternal IgM is thought to disappear during completion of the yolk absorption process (Swain and Nayak 2009). Thus, IgM found in fish in the larval or later stages is produced by these fish rather than by the maternal fish.

Igs are one of the most important molecules involved in vaccine-induced protective immunity (Li et al. 2014), and serum IgM is widely accepted as the dominant Ig isotype related to systemic immunity (Piazzon et al. 2016; Yu et al. 2020). Our report is the first to describe the dynamics of serum IgM in small juvenile fish, although a previous study examined the dynamics of IgM using whole fish (Castillo et al. 1993). The dynamics of serum IgM is important for predicting the timing of the development of immunity mediated by systemic antibodies; thus, our findings should facilitate the development of effective vaccine programs to maintain fish health in aquaculture. Further studies are needed to examine the ontogeny of antibody responses induced by vaccination.

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Declarations

Conflict of interests The authors have no competing interests to declare that are relevant to the content of this article.

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