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Fish size matters – Variable food allergen profiles in farmed and wild Malabar red snapper (*Lutjanus malabaricus*)

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ABSTRACT

Allergic reactions to fish pose complex food safety challenges, driven by species diversity and underexplored intraspecies variability. This study comparatively examines allergen profiles in Malabar red snappers (*Lutjanus malabaricus*, $n = 39$) across body sizes, anatomical regions and production origins using SDS-PAGE, immunoblotting and quantitative mass spectrometry. Protein profiles varied greatly by fish size and muscle region, but not by origin. Smaller fish contained higher levels of major allergen parvalbumin and creatine kinase, while larger fish exhibited elevated levels of heat-labile allergens enolase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase. Parvalbumin levels were highest in head, followed by belly, dorsal, and tail. Greatest variation was observed for the three heat-stable allergens – parvalbumin, tropomyosin, and collagen. Minimal origin-dependent differences affected 2 of 11 registered fish allergens. We established an integrated proteomics workflow for systematic allergenicity assessments to uncover intraspecies variability and provide foundational knowledge for understanding intraspecies variability to improve food safety strategies.

1. Introduction

Fish poses major food allergy risks to sensitised consumers, which can only be mitigated through challenging diagnosis and management strategies (Davis et al., 2020). Allergic reactions occur upon ingestion or inhalation of specific fish proteins (allergens), recognised by Immunoglobulin E (IgE) antibodies. While more than 1000 different fish species are consumed globally, our knowledge about species- and specimen-specific allergenicity remains limited (Ruethers, Raith et al., 2018; Dijkema et al., 2022).

Parvalbumin is the major fish allergen for which limited species- and patient-specific cross-reactivity has been demonstrated with most

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affected consumers tolerating at least one fish species (Leung et al., 2024; Sørensen et al., 2017; Xepapadaki et al., 2021). Species- and tissue-specific allergenicity has been investigated in previous studies with a focus on parvalbumin levels (Kobayashi et al., 2016). Furthermore, parvalbumin levels and immunoreactivity are affected by physicochemical properties and food processing methods such as heating, fermentation, and enzymatic treatments (Costa et al., 2021; Taki et al., 2023). Apart from parvalbumin, ten additional fish allergens are registered in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature database (www.allergen.org): enolase, aldolase, tropomyosin, collagen, creatine kinase, triosephosphate isomerase (TPI), pyruvate kinase, lactate dehydrogenase (DH), glucose-6-phosphate isomerase (G-6-PI), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Kalic et al., 2021). Among all 11 registered fish allergens, only parvalbumin, tropomyosin and collagen are highly heat-stable (Kalic et al., 2020; Ruethers et al., 2021).

Intraspecies variability based on fish sizes, however, has not been thoroughly investigated. The impact of production origin also remains to be elucidated. Protein compositions and allergen contents may vary between wild and farmed sources as shown for crustaceans previously (Dorney et al., 2024). Previous studies comparing allergen profiles between wild and farmed fish are limited to parvalbumin: A significant parvalbumin overexpression was reported in farmed sea bream (*Sparus aurata*) compared to wild counterparts (Piovesana et al., 2016). A similar trend was initially reported for European sea bass (*Dicentrarchus labrax*) in 2005; however, subsequent findings from the same research group contradicted this observation in 2018 (Chiozzi et al., 2018; Monti et al., 2005).

Red snappers are key aquaculture species in the Asia-Pacific region and are highly traded globally. This study investigates intraspecies variability in protein composition and allergen abundance in Malabar red snapper (*Lutjanus malabaricus*), focusing on fish size, body part differences and comparison between differentially farmed and wild-caught specimens. Allergens, primarily parvalbumin, have been previously described for other snapper species and closely related emperors, though no snapper or emperor allergens are currently listed in the WHO/IUIS Allergen Nomenclature Database (Sharp et al., 2015; Ruethers, Taki et al., 2018, 2020; Liang et al., 2021). We hypothesised that fish size, body part, and/or production origin affect the abundance of fish allergens in Malabar red snapper muscle tissue. This is the first study to resolve a comprehensive allergen repertoire in a snapper/emperor species and compare protein profiles and allergen abundances in fish of different sizes, as well as assess the impact of origin on the complete allergen repertoire.

2. Materials and methods

2.1. Sample collection

Thirty-nine deceased whole Malabar red snappers were purchased from farmers and retailers to analyse intraspecies protein variabilities. Morphological species identifications were confirmed by polymerase chain reaction and Sanger sequencing (DIN, 2019). Standardised skeletal muscle tissues were collected from 4 distinct and predetermined anatomical body regions: head, dorsal, tail, and belly (Supplementary Fig. S1) and immediately stored at −80 °C. Dorsal muscle tissues were used to analyse protein variations across fish sizes and origins.

Allergen profile variations associated with fish size were assessed in 24 healthy snappers of the same age, which were harvested on the same day (4th May 2023). Size was measured in total length, which ranged from 212 to 413 mm (195–1330 g in weight; refer to Supplementary Table S1 for details). The corresponding fingerlings originated from Malaysia and were collectively reared under identical conditions in one floating cage along Singapore's Johor Strait for 9 months.

To investigate the impact of origin on allergen profiles, a subset of 5 specimens (300–357 mm, 440–730 g) was selected and compared with 5 size-matched market specimens (290–360 mm, 424–754 g) from each of 3 different production origins: another sea-cage farm in Singapore, a sea-cage farm in Malaysia, and wild-caught fish from Indonesian waters (detailed in Supplementary Table S2).

2.2. Protein extraction

Protein extraction procedures were optimised and standardised based on previously established methods (Ruethers et al., 2019, 2022). Tissues (8 cm^3 cubes) were pulverised under liquid nitrogen. Proteins were extracted in phosphate buffer saline (PBS, pH 7.4) and quantified using the Pierce™ Dilution-Free™ Rapid Gold BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Total protein profiling

Protein profiles were resolved by Sodium Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) using a double wide multicaster kit and a mini vertical double-wide 20 cm × 16 cm system (CBS Scientific, San Diego, CA, USA). Protein bands were visualised by Coomassie Brilliant Blue R250 (Bio-Rad Laboratories, Hercules, CA, USA) staining and scanned with an Odyssey M® imaging system (LI-COR Biosciences, Lincoln, NE, USA). Densitometric analysis was performed using the GelAnalyser software (version 23.1.1). Bands containing fish allergens were identified based on their molecular weight and mass spectrometric analyses after in-gel tryptic digestion in previous studies (Kalic et al., 2020; Ruethers et al., 2019, 2021).

2.4. Detection of the major fish allergen parvalbumin and recognition by human IgE

Bands containing parvalbumin or other IgE-binding proteins were detected by immunoblotting as established previously (Ruethers et al., 2022). Proteins were separated by SDS-PAGE followed by transfer onto a 0.2 μm nitrocellulose membrane using a Trans-Blot® semi-dry transfer cell (Bio-Rad Laboratories). The membrane was incubated with a monoclonal anti-parvalbumin antibody PARV-19 (Sigma-Aldrich, St. Louis, MO, USA), in-house polyclonal antibody generated against parvalbumin from Asian seabass (Sharp et al., 2015) or a pool of six sera from paediatric subjects with clinically confirmed IgE-mediated allergy (Ruethers et al., 2021) followed by monoclonal anti-human IgE antibody (sc-53,346 by Santa Cruz Biotechnology, Dallas, TX, USA). Ethical approval was obtained from the Sydney Children's Hospitals Network (LNR-14/SCHN/185) and written informed consent was provided by all parents. Antibody-binding was visualised after incubation with immunofluorescence secondary antibody DyLight 800 goat anti-mouse/rabbit (H + L) 4× PEG (Invitrogen, Carlsbad, CA, USA) using the above-mentioned Odyssey M system with subsequent densitometric analyses using GelAnalyser software.

2.5. Mass spectrometric analysis

The protein composition of all extracts was analysed by mass spectrometry after tryptic digestion, based on previously described methods (Chin et al., 2024; Jerry et al., 2024; Ruethers et al., 2023). In brief, tryptic digestion was performed utilising the S-Trap™ micro spin column (ProtiFi, Farmingdale, NY, USA), following the manufacturer's protocol. Samples were analysed on a Dionex UltiMate 3000 RSLC system (Thermo Fisher Scientific) coupled to the nanoelectrospray interface of an Orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). Tryptic peptides were first trapped on an Acclaim Pepmap nano trap column (Dinoex-C18, 100 Å, 75 μm × 2 cm) before separation on an Acclaim Pepmap RSLC analytical column (Dinoex-C18, 100 Å, 75 μm × 50 cm). Peptide elution was performed with dimethyl sulfoxide, acetonitrile and formic acid. MS1 spectra were acquired over the range

m/z 350–1400 at a resolution of 120,000 with a maximum injection time of 50 ms and a normalised AGC target of 250%. Peptide ion fragmentation was carried out using data-independent acquisition (DIA) mode with 50 × 14.7 *m/z* windows from *m/z* 360.5–1046.5 with a 1 *m/z* overlap between adjacent windows. Ions were activated using higher-energy collisional dissociation with a normalised collision energy of 30%, a maximum injection time of 55 ms and a normalised automatic gain control target of 2000%. MS2 analysis was performed at a resolution of 30,000 over the *m/z* range 200–2000.

Proteins were identified with Spectronaut (v19.1.240806.62635; Biognosys, Schlieren, Switzerland) using Direct DIA with default parameters against a Malabar red snapper protein database, generated from an in-house genome assembly and annotation (Liang et al., 2025). Reproducibility was demonstrated by comparing the number of identified proteins in each sample (detailed in Supplementary Table S3). Abundances were expressed by intensity-based absolute quantification (iBAQ). Proteins were further grouped manually and the relative abundance of all fish allergens was calculated as iBAQ% by dividing the total iBAQ value of grouped protein allergens over the summed iBAQ values of all proteins.

2.6. Statistical validations

Statistical analyses were performed using linear regression, two-way ANOVA and Tukey's post-hoc test in GraphPad Prism 10.4.1. Results are reported as means ± standard deviation unless otherwise specified. The Coefficient of Variation (CV) was calculated by dividing the standard deviation by the mean to measure the relative dispersion or variability of a probability distribution.

3. Results

3.1. Size-dependent total protein profiles

The fish length was used as the primary, most standardised measurement to categorise fish sizes in this study; fish length correlated positively with body weight ($p < 0.0001$ with $R^2 = 0.89$, Supplementary Fig. S2). Total protein profiles exhibited distinct differences, with 20 prominent protein bands showing unique patterns that clearly varied across fish sizes (Fig. 1). Most notable differences in protein band intensities were observed at molecular weights of 11, 15, 27 and 31–61 kDa. The abundance of the minor parvalbumin band (11 kDa) showed a reduction over size with higher levels in smaller fish compared to larger fish. A similar trend was observed for a 15-kDa band. In contrast, the intensity of most bands at 31–61 kDa, predominantly containing heat-labile fish allergens, increased with fish size. Technical replicates, performed for fish specimen 21, displayed consistent protein patterns, validating the reproducibility of the extraction method.

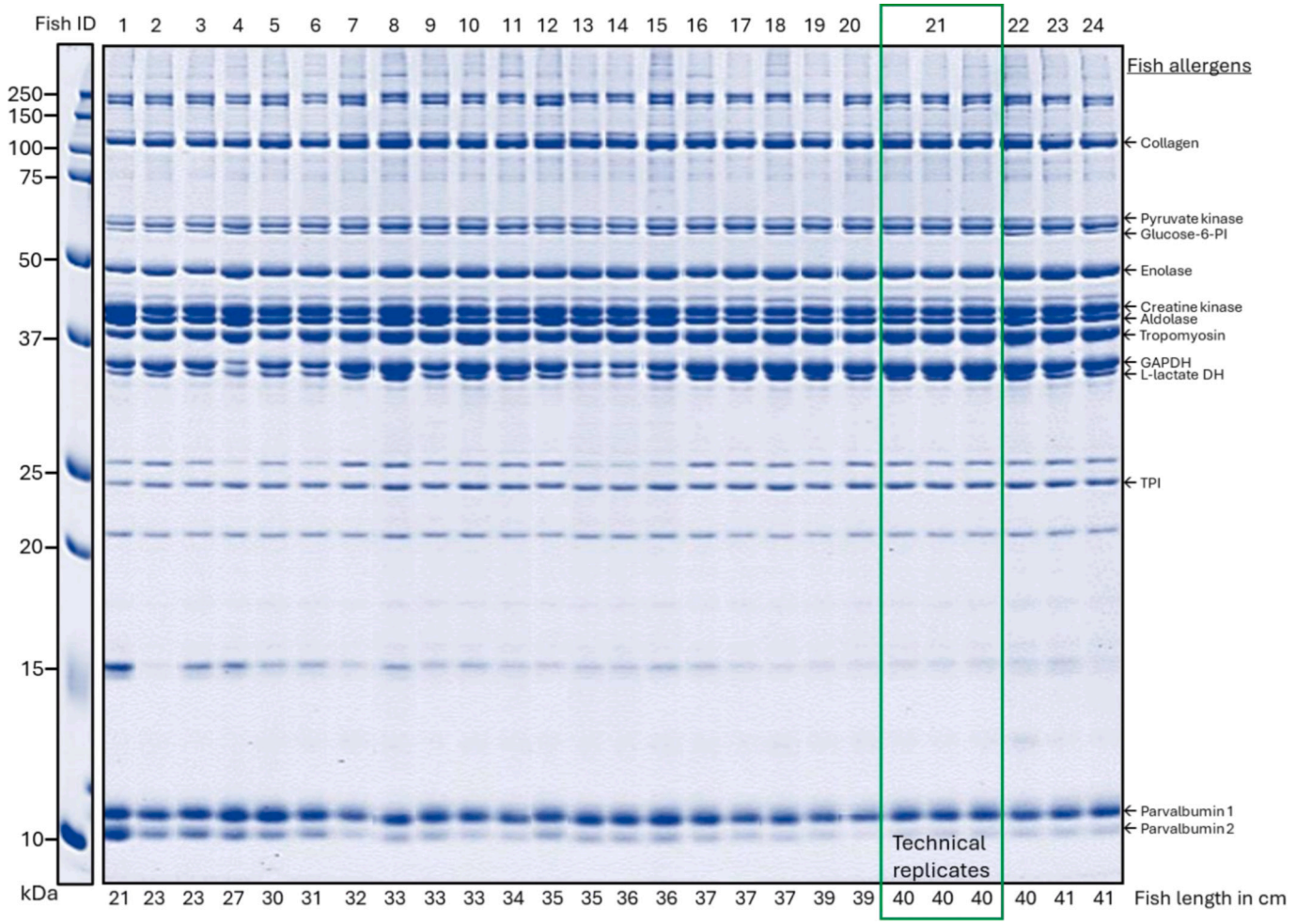

Fig. 1. Differential SDS-PAGE protein profiles of extracts from muscle tissue collected from red snapper of different sizes, with the smallest fish measuring 212 mm (1) and the largest measuring 413 mm (24) as detailed in Table S1. Technical replicates were incorporated in the analysis for samples from fish 21. Prominent differences were observed at 15, 27, 35 and 36 kDa. The bands primarily containing the 11 fish allergens are indicated with an arrow. Glucose-6-PI, glucose-6-phosphate-isomerase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; DH, dehydrogenase; TPI, triosephosphate isomerase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Smallest and largest fish differed in the abundance of allergen bands

Densitometric analysis was performed to compare the intensities of allergen-containing protein bands in extracts from the smallest and largest fish (Fig. 2a), which highlighted 6–92% changes in band intensities (refer to Supplementary Table S4 for detailed analyses of intensity peak differences). Bands containing collagen, pyruvate kinase, G-6-PI, enolase, creatine kinase, GAPDH, L-lactate DH and TPI showed a trend of increasing abundance with fish size. In contrast, the abundance decreased for bands containing parvalbumin aldolase and tropomyosin. The intensity of the 11-kDa parvalbumin band was reduced by 76% from fish 1 to fish 24, while it was only a 18% decrease for the 12-kDa parvalbumin band.

3.3. Differential immunological detection of the major fish allergen parvalbumin

Immunoblotting was conducted to confirm the presence of the major fish allergen parvalbumin at 11 and 12 kDa (Fig. 2b). Antibody-binding intensity was 2-fold higher in the smallest fish (#1, 212 mm) compared to the largest fish (#24, 413 mm). Based on SDS-PAGE profiles, both 11- and 12-kDa parvalbumin bands were equally abundant in the smallest fish, whereas the 12-kDa band was 4-fold more abundant than the 11-kDa band in the largest fish. However, in immunoblotting analyses, the signal intensity to both parvalbumin bands was comparable in both small and large fish, indicating a differential composition of parvalbumin isoforms.

3.4. Decreasing parvalbumin abundance over increasing fish size

Among 9 parvalbumin isoforms found in the annotated genome, 3 predominant parvalbumin isoforms were identified in all 24 fish with decreasing abundance over increasing fish size based on mass spectrometry (Fig. 3). Parvalbumin-3 (12 kDa) was the most abundant isoform, followed by two parvalbumin-beta-like isoforms (11 kDa). The relative abundance (iBAQ%) of the 12-kDa parvalbumin-3 isoform decreased from ~8% to ~4% as the fish length increased. Similarly, the average abundance of the parvalbumin beta-like isoform I reduced from ~4% to ~3% with increasing fish size. For parvalbumin beta-like isoform II, a 10-fold reduction in abundance was observed from the smallest to the largest fish. The reduction in the abundance of the three parvalbumin isoforms was significant ($p < 0.001$).

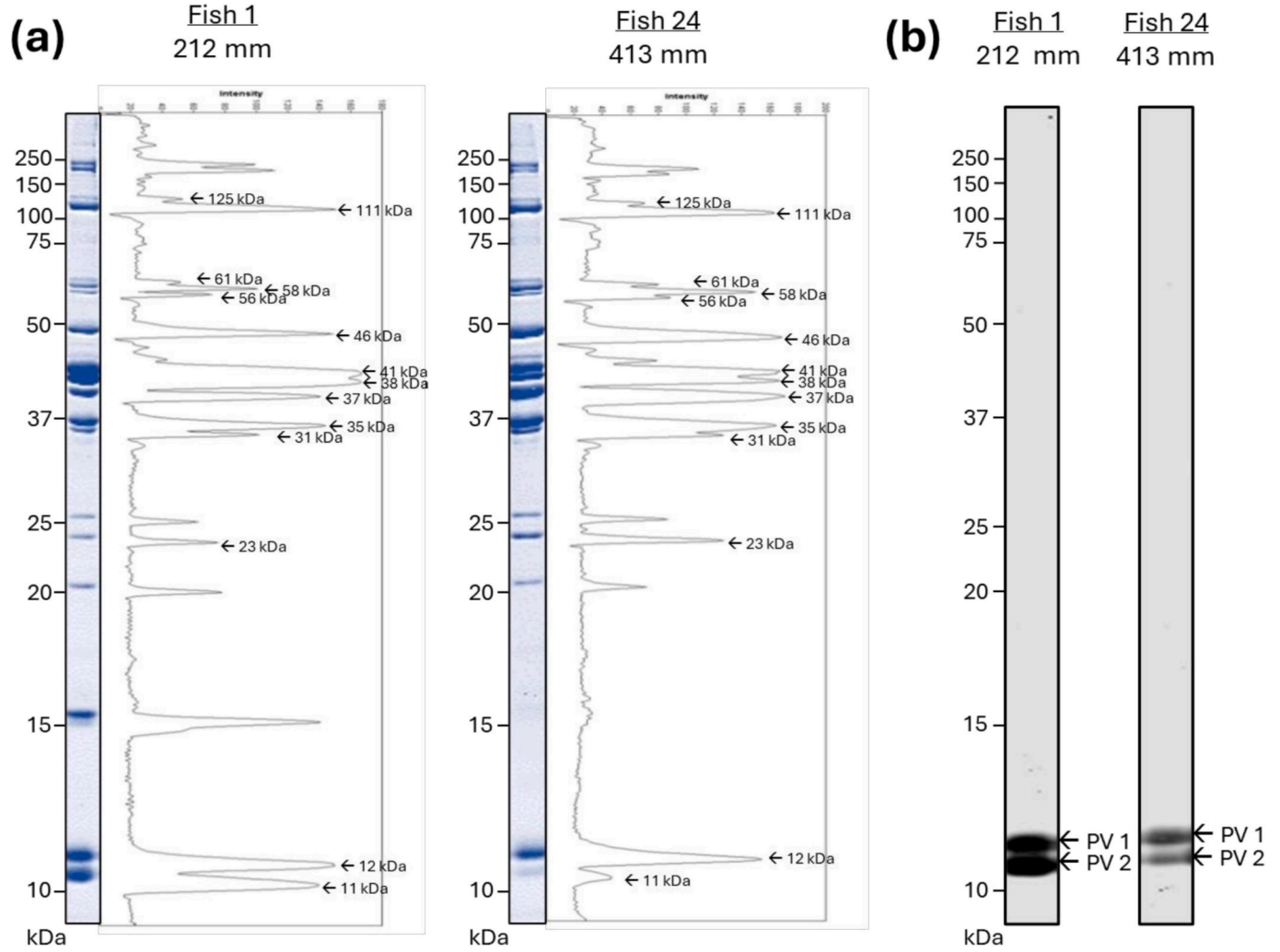

Fig. 2. Comparative analysis of the protein profiles of extracts from the two most differential-sized fish specimens. (a) Densitometric analysis of the intensity of 14 protein bands in extracts from the smallest fish 1 (212 mm) and the largest fish 24 (413 mm). The bands indicated with arrows predominantly contain the 11 fish allergens as detailed along with densitometric analyses in Supplementary Table S4. The larger fish exhibited increased protein band intensities at 125, 111, 61, 58, 56, 46, 41, 35, 31, and 23 kDa, and decreased protein band intensities at 38, 37, 12, and 11 kDa. (b) The corresponding membrane was incubated with a parvalbumin (PV)-specific antibody. PV was detected in the 11- and 12-kDa bands. The signal intensity was about 50% lower for bands in the extract from the largest as compared to the smallest specimen.

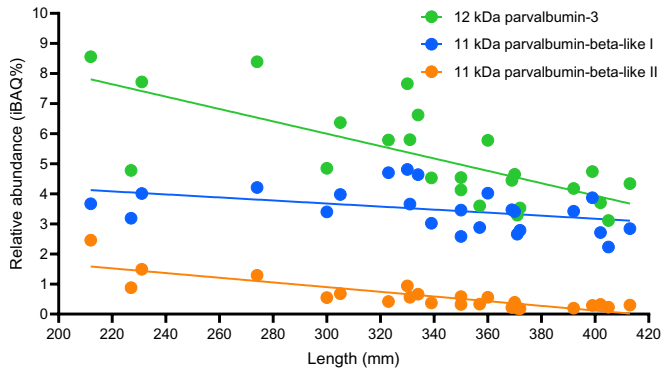

Fig. 3. Decrease in the relative abundance of 3 parvalbumin isoforms over increasing fish size ($p < 0.001$) as determined by mass spectrometric analysis after in-solution tryptic digestion. Refer to Supplementary Fig. S4 for corresponding data on all other fish allergens.

3.5. Differential size-dependent abundance of other fish allergens

The reproducibility of mass spectrometric analyses was evidenced by highly similar allergen abundances across technical triplicates (an average CV of 4.6% for all allergens; Supplementary Fig. S3). Size-dependent trends in the relative abundance of the 11 fish allergens were observed, which were most prominent for parvalbumin, aldolase and enolase (Supplementary Fig. S4). Mass spectrometric data for the 5 smallest (249 ± 37 mm) and largest (402 ± 8 mm) specimens were compiled to demonstrate differential size-dependent relative abundances for all fish allergens (Fig. 4): Creatine kinase was the most abundant allergen (23% and 21%) followed by aldolase (8% and 12%), parvalbumin (12% and 7%), GAPDH (7% and 11%), enolase (4% and 7%), TPI (3% and 4%), pyruvate kinase (2% and 4%), L-lactate DH (2% and 3%), G-6-PI (0.6%), tropomyosin (0.5% and 0.6%), and collagen (0.1%). Significant differences between the smallest and largest fish were noted for 5 allergens. The relative abundance of parvalbumin and creatine kinase was significantly higher ($p < 0.0001$ and $p < 0.001$, respectively) in the smallest as compared to the largest fish. Conversely, the glycolytic heat-labile enzymes enolase ($p < 0.01$), aldolase and GAPDH (both $p < 0.001$) were significantly more abundant in the largest fish. Differences in the abundance of tropomyosin, collagen, TPI, pyruvate kinase, L-lactate DH and G-6-PI were statistically not significant. The total abundance of fish allergens was higher in the largest as compared to the smallest fish (69% ± 1% and 63% ± 4%, respectively). Parvalbumin was almost twice as abundant in the smallest (15% ± 3%) as compared to the largest fish (7% ± 1%).

3.6. Differential protein profiles and parvalbumin detection in different body regions

Total protein profiles and antibody-binding patterns were analysed to compare 4 different muscle regions in 5 large fish (392–413 mm, fish 20–24 in Supplementary Table S1). SDS-PAGE protein profiles were distinct for tissues from each of the different anatomical body regions (Supplementary Fig. S5a). The two most varying protein bands were

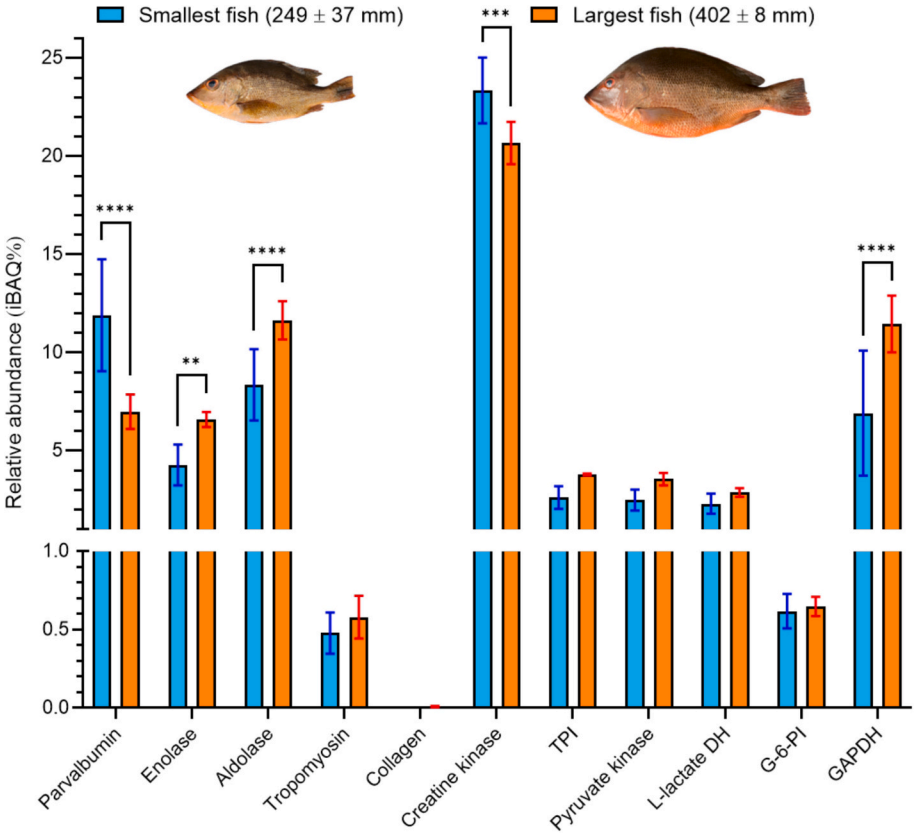

Fig. 4. Variable relative abundance of 11 fish allergens in protein extracts from the 5 smallest (249 ± 37 mm) and 5 largest specimens (402 ± 8 mm) of red snapper, determined after tryptic digestion with subsequent mass spectrometric analyses. The major fish allergen parvalbumin and creatine kinase were significantly more abundant in the smallest fish compared to the largest fish as determined by Tukey's multiple comparisons test. In contrast, the glycolytic enzymes enolase, aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were significantly more abundant in the largest fish. The other allergens, tropomyosin, collagen, triosephosphate isomerase (TPI), pyruvate kinase, L-lactate dehydrogenase (DH), and glucose-6-phosphate isomerase (G-6-PI), did not exhibit significant differences in abundance between the two fish groups. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

observed at 15 kDa across all muscle regions, with the highest abundance in the head, followed by the belly, dorsal, and tail regions. A similar pattern was noted for the two parvalbumin bands (11 and 12 kDa), where the head region showed notably higher parvalbumin levels compared to all other muscle tissues with the lowest abundance in the tail. Among all 11 allergens, the highest variability was observed for parvalbumin and creatine kinase (Supplementary Table S5). Immunoblot analysis highlighted that the head (rostral) region contained higher parvalbumin amounts compared to the tail (caudal) region (Supplementary Fig. S5b). The lowest level of parvalbumin and antibody reactivity was found in the tail muscle tissue compared to all other body regions. Densitometric analysis highlighted significantly ($p < 0.0001$) stronger band and signal intensities for parvalbumin 1 (12 kDa) as compared to parvalbumin 2 (11 kDa) across all body regions (Supplementary Fig. S6). Both parvalbumin bands and corresponding signal intensities were significantly higher in the head than in other investigated body regions ($p < 0.0001$).

3.7. Differential relative abundance of allergens in different body regions

Detailed proteomic analyses were conducted for body regions of fish 21 (399 mm), representing the largest fish group. The relative abundance of all 11 fish allergens was determined across the 4 body regions (Fig. 5). Less than 0.1% of proteins were collagens which demonstrated the highest CV with 67%. Parvalbumin was most abundant in the head (10%) followed by belly (8%), dorsal (6%) and tail (4%) muscle region, which is consistent with the observed intensity of parvalbumin bands and antibody-binding (CV: 38%). Among all fish allergens, the content of tropomyosin varied the third most (CV: 36%) and was the lowest in the dorsal and most abundant in the tail tissue. All the other allergens demonstrated a comparably lower variability (CV < 20% for creatine kinase, TPI, pyruvate kinase, lactate DH and G-6-PI; CV < 10% for enolase, aldolase and GAPDH).

3.8. Minor origin-dependent differences in protein profiles and parvalbumin detection

The protein profiles of dorsal muscle tissue from specimens of 4 origins were highly similar with only minor differences in the abundance of protein bands at 11, 15 and 27 kDa between origins (Supplementary Fig. S7a). No considerable differences were observed in the abundance of prominent allergen-containing bands between origins. Parvalbumin was detected in the 11- and 12-kDa bands of all samples with similar intensity across sub-samples, regardless of their origin (Fig. S7b).

3.9. Minor origin-dependent difference in fish allergen abundances

The relative abundance of 9 of the 11 fish allergens did not show significant differences between the different origins and across the 20 specimens (Fig. 6). Collagen was the least abundant allergen, followed

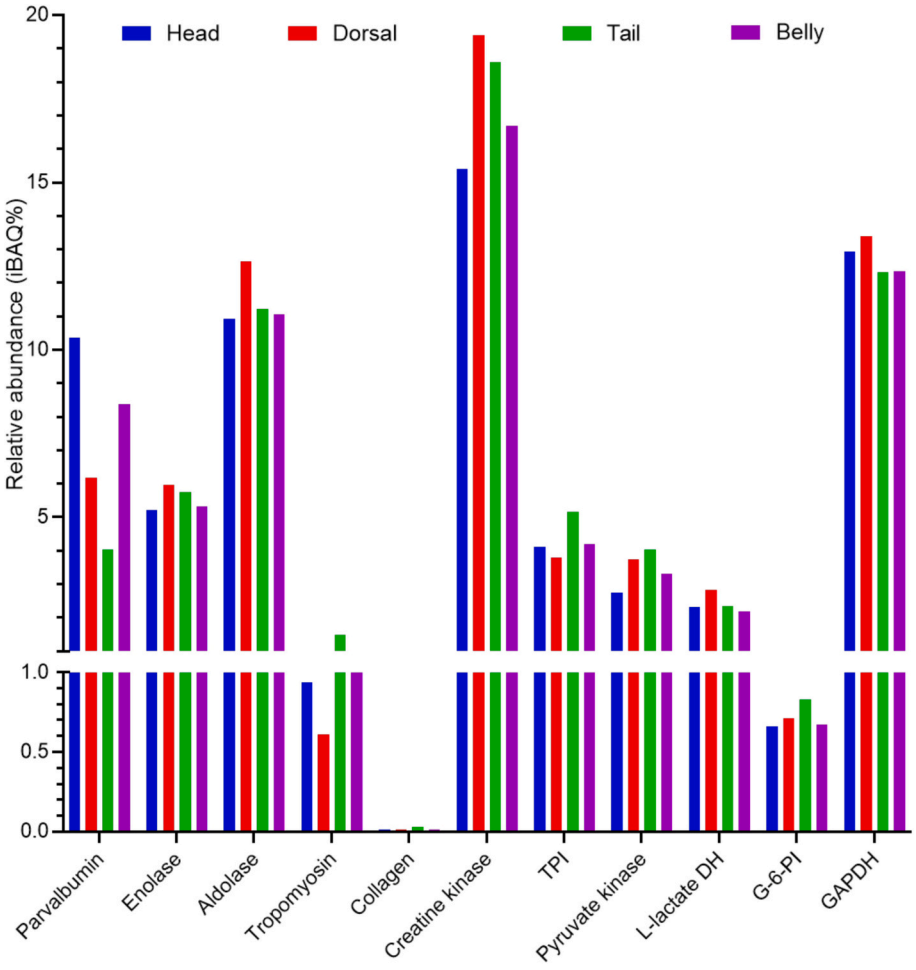

Fig. 5. Variable relative abundance of the 11 fish allergens in protein extracts from the head, dorsal, tail, and belly muscle of red snapper fish 21 (refer to Supplementary Table S1 for details) after tryptic digestion with subsequent mass spectrometric analyses. The highest variation was observed for parvalbumin, creatine kinase and tropomyosin. TPI, triosephosphate isomerase; DH, dehydrogenase; G-6-PI, glucose-6-phosphate-isomerase; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

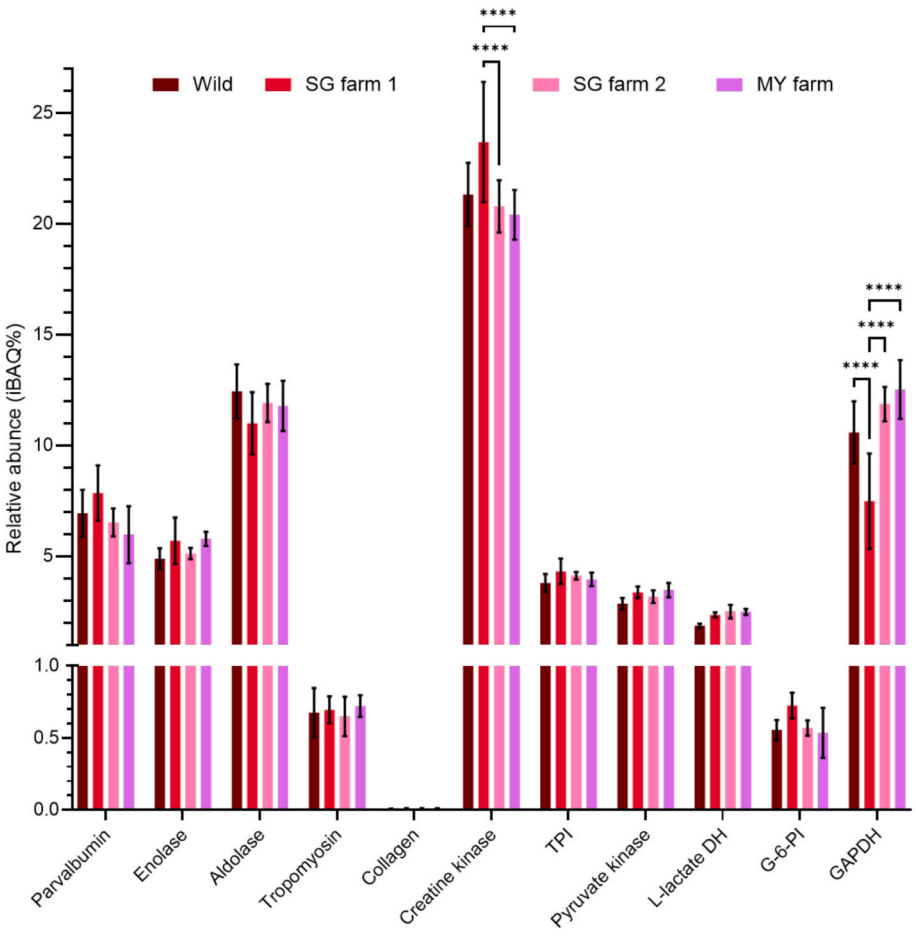

Fig. 6. Comparable relative abundance of the 11 fish allergens in protein extracts from wild-caught and farmed Malabar red snapper (refer to Supplementary Table S2 for details; 5 specimens per origin) after tryptic digestion with subsequent mass spectrometric analyses. Creatine kinase was the most abundant allergen, and a significantly higher abundance was observed for fish from Singapore (SG) farm 1 based on Tukey's multiple comparisons test. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was significantly lower abundant in the same samples. The relative abundance of the other fish allergens parvalbumin, enolase, aldolase, tropomyosin, collagen, triosephosphate isomerase (TPI), pyruvate kinase, L-lactate dehydrogenase (DH), and glucose-6-phosphate isomerase (G-6-PI) did not differ significantly. MY, Malaysia; **** $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by G-6-PI and tropomyosin, as also observed for the differentially sized fish. Creatine kinase was the most abundant allergen with significantly higher levels in samples from Singaporean farm 1 as compared to samples from the other two farms ($p < 0.0001$). In contrast, GAPDH was significantly less abundant in samples from Singaporean farm 1 than in samples from any of the other origins ($p < 0.0001$). The average relative overall abundance of all fish allergens was 67% with parvalbumin accounting for 10% of it (Supplementary Table S6).

3.10. Differential (IgE) antibody binding across fish sizes, body regions and origins

Immunoblotting patterns using pooled serum from fish-allergic subjects and a polyclonal anti-parvalbumin antibody demonstrated similar binding patterns to monoclonal PARV-19 (Fig. 7). The two major parvalbumin bands at 11 and 12 kDa showed the strongest binding. The monoclonal antibody PARV-19 and IgE antibodies showed stronger binding to the 12 kDa band, while the polyclonal antibody detected both isoforms with similar intensity. Consistent with the relative abundances and anti-parvalbumin antibody detection results, IgE-binding intensity was significantly higher in the pooled extract from the 5 smallest compared to the 5 largest fish (approximately 2-fold difference based on densitometric analysis). IgE-binding to any other bands was very faint. The prominent 15-kDa band, which showed notable variability in its abundance and very weak IgE-binding was identified by mass spectrometry as predominantly containing nucleoside-diphosphate kinase, a protein not currently registered as a fish allergen.

4. Discussion

This study established an integrated proteomics workflow for systematic allergenicity assessments and investigated associations of allergen abundances with fish size, body regions and origin within Malabar red snapper. Previous studies on size-dependent intraspecies variability in protein profiles were limited to comparing overall protein abundances and investigated wild barbel and snakehead with a lack of standardisation (Gam et al., 2006; Huriaux et al., 1997). In contrast, we analysed farmed specimens of the same age that were all raised in the same floating cage for 9 months to enable identical feed availability and environmental conditions, with fish length as the only variable.

The pronounced differences in protein abundances between small and large specimens are likely attributable to variations in muscle physiology and energy metabolism demands associated with fish growth and development (Nemova et al., 2021). Smaller red snappers contained higher levels of calcium-binding parvalbumin, likely to support muscle contraction-relaxation processes required for faster swimming and more critical escape responses compared to larger fish. Similar patterns have also been described for rainbow trout (*Oncorhynchus mykiss*), bluegill

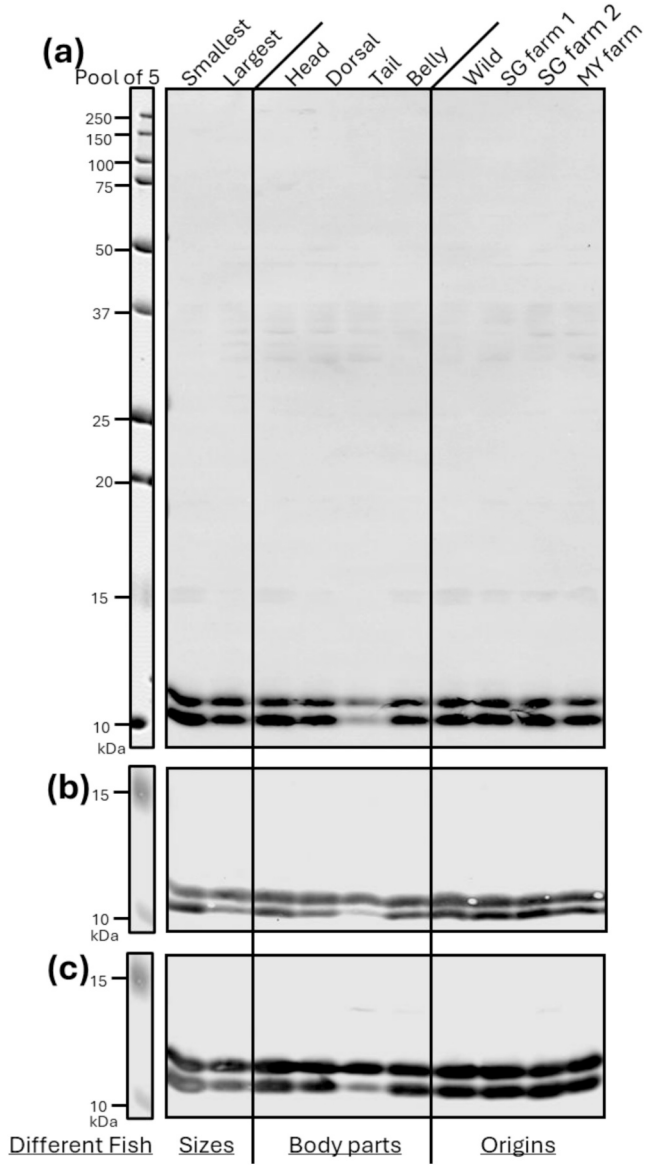

Fig. 7. Differential immunoblotting patterns for pooled samples of (i) the 5 smallest and 5 largest specimens to explore impact of fish size, (ii) head, dorsal, tail and belly samples from 5 specimens to investigate differences between body parts, and (iii) samples from 5 specimens each from different production origin. (a) A 2-fold higher IgE-binding capacity was revealed for smallest compared to largest fish using a serum pool. Binding patterns of both IgE and (b) polyclonal anti-parvalbumin antibody demonstrated similar binding patterns to (c) monoclonal PARV-19. Refer to Figs. 1, S5 and S7 for corresponding protein profiles.

(*Lepomis macrochirus*), largemouth bass (*Micropterus salmoides*) and common carp (*Cyprinus carpio*) (Brownridge et al., 2009; Campion et al., 2012; Coughlin et al., 2007). Since parvalbumin is the major fish allergen, smaller fish may be more allergenic than larger fish, especially for consumers primarily or exclusively sensitised to parvalbumin, despite an overall lower relative abundance of all fish allergens. However, higher parvalbumin content does not automatically equate to proportionally greater clinical reactivity, as individual threshold doses vary considerably among fish-allergic patients (Vera-Berrios et al., 2025). Studies on eliciting doses for fish allergens are limited, and whether a 2-fold difference in parvalbumin content would exceed clinical reaction thresholds for sensitised individuals remains to be determined. Densitometric analyses of parvalbumin bands and antibody binding indicated differential compositions of parvalbumin isoforms in small and large fish. Mass spectrometric analyses revealed a 10-fold higher abundance of parvalbumin beta-like isoform II in the smallest compared to the largest specimens. Consequently, the observed binding of the anti-parvalbumin antibody PARV-19 to the 11-kDa band can be attributed primarily to parvalbumin beta-like I rather than beta-like II. This highlights a major drawback of antibody-based detection of allergens and allergen sources: when samples contain predominantly parvalbumin beta-like II or similar isoforms, some commercially available antibodies, including the PARV-19 used here, may fail to detect them, with serious implications for food safety assessments. We previously demonstrated a lack of reliability for commercial fish enzyme-linked immunosorbent assay (ELISA) kits based on limited cross-reactivity among fish species (Ruethers et al., 2020).

Larger fish contained significantly more of the other heat-labile allergens enolase, aldolase and GAPDH (glycolytic enzymes), likely to reflect their increased energy metabolism demands. Hence, larger fish would pose higher allergy risks to consumers primarily sensitised to fish allergens other than heat-stable parvalbumin, tropomyosin and collagen. Increased aldolase, creatine kinase, pyruvate kinase, and GAPDH levels were previously associated with higher body mass and body length of differently aged yellow perches (*Perca flavescens*) (Reddish et al., 2008). Glycolysis is the primary pathway for glucose catabolism in fish muscle tissues, leading to the formation of pyruvate and generating energy. The enhanced expression of these glycolytic enzymes suggests a higher rate of glucose metabolism and Adenosine triphosphate (ATP) production necessary for sustained growth.

Creatine kinase was the most abundant allergen in Malabar red snapper. Previous IgE binding analysis with serum from 32 to 42 fish-allergic subjects identified major allergens at 51 kDa in both golden snapper (*Lutjanus johnii*) and mangrove red snapper (*Lutjanus argentimaculatus*), along with additional major allergens at 48 kDa in golden snapper and 42 kDa in mangrove red snapper, identified as creatine kinase by mass spectrometric analyses (Rosmilah et al., 2005). Creatine kinase was significantly more abundant in smaller fish, making it more allergenic for sensitised consumers. This may be due to higher metabolic rates in smaller fish, which require greater ATP turnover and thus elevated creatine kinase expression, an enzyme responsible for cellular energy homeostasis and ATP regeneration.

Our observations regarding parvalbumin contents in different body regions are consistent with previous studies, which demonstrated a higher abundance of parvalbumin in muscle tissues located near the rostral region compared to the caudal region of different species of bony fishes (Coughlin et al., 2007; Kobayashi et al., 2016; Lee et al., 2012). The high parvalbumin content in the head and dorsal regions can be explained by the higher concentration of fast-twitch muscles used to burst muscle movement in feeding responses and swimming. This protein distribution pattern reflects specific movement functions across different body regions. Muscles near the head contain a high abundance of specific proteins to support rapid movements required for feeding and respiration. Specifically, high parvalbumin content in the head or rostral region correlates with faster muscle contraction rates, while the tail or caudal region shows slower rates. The tail region contained less of the 11-kDa parvalbumin compared to the head region – it primarily functions in propulsion and steady swimming movement, which seem associated with 12-kDa parvalbumin-3. Importantly, our study extends beyond previous work, which was limited to parvalbumin, by examining the distribution of all 11 fish allergens. We revealed high variation in the content of collagen and tropomyosin, as well as allergens involved in glycolysis and metabolism, including creatine kinase, TPI, pyruvate kinase, lactate DH and G-6-PI, in the four body regions analysed. These variations reflect the predominant activities in white muscle tissue, particularly glycolysis and phosphocreatine hydrolysis, which support anaerobic metabolism for sudden movements (Nemova et al., 2021). The differential expression patterns, especially between anterior and posterior body regions, may influence overall tissue-specific

allergenicity.

The allergen profiles of fish from 4 different origins demonstrated only minor variation, with 9 out of 11 fish allergens showing no considerable difference in their abundance. Fish from Singaporean farm 1 contained significantly higher levels of creatine kinase and lower levels of GAPDH as compared to fish from the other origins, which might be due to a differential diet. Recent studies have shown that extreme changes in fish diet can correlate with changes in protein abundances (De Magalhães et al., 2020; Schrama et al., 2022; Zhong et al., 2023). Further research is warranted to investigate how factors such as habitat, environmental conditions, and aquaculture practices affect the allergen profile of this and other fish species.

Overall, our findings align with Costa et al. (2021), who reviewed physicochemical properties shaping allergen potency in animal proteins. Parvalbumin's greater abundance in certain anatomical body regions has been positively correlated with increased allergenic potential. Our observation that smaller fish contain higher parvalbumin levels, resulting in higher IgE-binding capacity, extends this relationship to include fish size as an additional factor influencing allergenicity within a species. However, the relationship between allergen abundance and clinical reactivity is complex, influenced by protein structure, thermal stability, digestibility, and individual patient sensitisation profiles.

5. Conclusion

In conclusion, this study underscores the critical role of fish size and body region in determining allergenic potential. Through a robust proteomics workflow, we provide foundational knowledge for understanding intraspecies variability in a red snapper species. Our analyses revealed that smaller red snappers contain higher levels of the major allergen parvalbumin, suggesting an increased risk of severe allergic reactions after ingestion. Notably, differing isoform distributions are posing potential challenges for allergen detection. However, larger fish exhibited higher levels of other allergens such as glycolytic enzymes, possibly related to growth, adding complexity as these allergens present a higher potential risk for consumers sensitised to a broader range of fish allergens. Different body regions exhibited distinct allergen profiles, with the most pronounced variations in the abundance of the 3 heat-stable allergens parvalbumin, tropomyosin and collagen. Anterior regions contained higher levels of these stable allergens, suggesting variable food allergy risks across different fish portions. These findings highlight the importance of considering both size and body region when evaluating allergen profiles across fish species. While fish from different origins showed only minor differences in allergen abundances, additional research with more species and using IgE-binding assays and controlled challenge studies with allergic patients is needed to confirm the allergenic potential of different fish sizes and body regions. Translating these observations to clinical recommendations remains challenging as region- and species-specific component-resolved diagnostic are not routinely available to provide individual comprehensive sensitisation profiles. Furthermore, many fish-allergic patients react to multiple fish allergens, making it difficult to predict whether consuming smaller or larger fish would reduce their allergic risk. From a food safety perspective, however, these findings suggest that risk assessments and risk mitigation strategies for allergic consumers should consider fish size in addition to body region and species. Understanding the native variability in allergen abundances and isoforms can for example assist with targeted cell line selection and culture conditions for cultivated cellular aquaculture products (cellular agriculture) with reduced allergenicity.

CRediT authorship contribution statement

Thimo Ruethers: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Afifah Sean:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Shaymaviswanathan Karnaneedi:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation, Conceptualization. **Renee Chin:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Michael G. Leeming:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Vu Nguyen:** Writing – review & editing, Visualization, Project administration, Methodology, Formal analysis, Data curation. **Roger Huerlimann:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Roni Nugraha:** Writing – review & editing, Supervision, Methodology, Investigation. **Patrizia Bade:** Methodology, Formal analysis, Data curation. **Shubha Vij:** Resources, Investigation, Funding acquisition. **Xueyan Shen:** Validation, Investigation, Funding acquisition, Data curation. **Vachiranee Limviphuvadh:** Investigation, Funding acquisition, Data curation, Conceptualization. **Nicholas A. Williamson:** Validation, Resources, Project administration, Methodology. **Jose A. Domingos:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Dean R. Jerry:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Sebastian Maurer-Stroh:** Validation, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Anand K. Andiappan:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Andreas L. Lopata:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.147950.

Data availability

Data will be made available on request.

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