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Article type : Original Article: Food Allergy and Gastrointestinal Disease

Expanding the allergen repertoire of salmon and catfish

Short title: Allergen repertoire of salmon and catfish

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ACKNOWLEDGEMENTS

TR and SK are holder of a full-time PhD scholarship from the Centre for Food and Allergy Research and James Cook University, Australia. SDK is a National Health and Medical Research Council (NHMRC) Peter Doherty Early Career Research Fellow (GNT1124143). The study was financially supported by an Australian Research Council fellowship to AL and an NHMRC grant (APP1086656) to AL and DC. The research work was partially supported by a grant from Research and Technology Transfer Affairs, Khon Kaen University (KKU), Thailand. Icons from Biorender have been used for the generation of figures. We would like to acknowledge Berit Bang from The Arctic University of Norway, Tromsø, Norway, for providing tissue samples from Atlantic cod.

AUTHOR CONTRIBUTIONS

TR performed the study and wrote the manuscript. TR, AT, HB, DC and AL designed the study. DD, SM and DC recruited the patients. AT, SK, SN, TK, ML, SD, NW and SK contributed to the generation and/or analyses of the data. All authors critically reviewed the manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no relevant conflicts of interest.

ABSTRACT

Background: Diagnostic tests for fish allergy are hampered by the large number of under-investigated fish species. Four salmon allergens are well-characterized and registered with the WHO/IUIS while no catfish allergens have been described so far. In 2008, freshwater-cultured catfish production surpassed that of salmon, the globally most-cultured marine species. We aimed to identify, quantify and compare all IgE-binding proteins in salmon and catfish.

Methods: Seventy-seven pediatric patients with clinically confirmed fish allergy underwent skin prick tests to salmon and catfish. The allergen repertoire of raw and heated protein extracts was evaluated by immunoblotting using five allergen-specific antibodies and patients' serum followed by mass spectrometric analyses.

Results: Raw and heated extracts from catfish displayed a higher frequency of IgE-binding compared to those from salmon (77% versus 70% and 64% versus 53%, respectively). The major fish allergen parvalbumin demonstrated the highest IgE-binding capacity (10-49%), followed by triosephosphate isomerase (TPI; 19-34%) in raw, and tropomyosin (6-32%) in heated extracts. Six previously unidentified fish allergens, including TPI, were registered with the WHO/IUIS. Creatine kinase from salmon and catfish was detected by IgE from 14% and 10% of patients, respectively. Catfish L-lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and glucose-6-phosphate isomerase showed IgE-binding for 6-13% of patients. In salmon, these proteins could not be separated successfully.

Conclusions: We detail the allergen repertoire of two highly farmed fish species. IgE-binding to fish tropomyosins and TPIs was demonstrated for the first time in a large patient cohort. Tropomyosins, in addition to parvalbumins, should be considered for urgently needed improved fish allergy diagnostics.

HIGHLIGHTS:

- Raw and heated extracts from catfish demonstrated a higher prevalence of IgE-binding as compared to those from salmon (77% vs 70% and 64% vs 53%, respectively).
- Tropomyosin was the second most abundant protein, after parvalbumin, in heated extracts and up to 36% of patients with clinically confirmed fish allergy (*n*=77) demonstrated IgE-binding.
- Twelve new catfish and three new salmon IgE-binding proteins were registered with the WHO/IUIS, including three tropomyosin and two triosephosphate isomerase isoforms.

KEYWORDS

Fish allergy diagnosis, *Pangasianodon hypophthalmus*, *Salmo salar*, triosephosphate isomerase, tropomyosin

1 BACKGROUND

Fish allergy is associated with high rates of anaphylaxis[1] and affected patients often suffer for a lifetime[2,3]. The prevalence is region-specific and has been reported to be as high as 3% in the general population.[4] A higher prevalence of up to 8% has been reported among fish-processing workers.[5,6] In countries with high seafood consumption, fish is the second most common trigger of food allergy, following crustaceans.[7]

The production and consumption of fish are continuously increasing,[8] making adverse reactions to fish, including IgE-mediated fish allergy, a growing health burden with a negative impact on the quality of life.[9] However, diagnostics and management of species-specific fish allergy are hampered by the lack of studies on the large number and variety of under-investigated fish species[10] and the current limited availability of *in vivo*[11] and *in vitro*[12,13] diagnostic tests,[14] as well as reliable detection methods.[15] The increasing demand for fish as a valuable protein source[16] can only be satisfied by sustainable aquaculture,[17] and therefore heavily farmed fish species require evaluation of their allergen content.

The most commonly cultured marine fish species is the Atlantic salmon (*Salmo salar*).[8] Four well-investigated salmon allergens are registered with the World Health Organisation and International Union of Immunological Societies (WHO/IUIS; www.allergen.org): β-parvalbumin (Sal s 1), β-enolase (Sal s 2), aldolase A (Sal s 3), and collagen alpha (Sal s 6). Four additional salmon and other fish proteins with reported IgE-binding are listed by other databases (i.e. www.allergome.org), suggesting a broader repertoire of salmon allergens with

potential clinical relevance. Recently, we underlined the importance of salmon collagen, tropomyosin and aldolase as IgE-binding proteins in commercial skin-testing preparations.[11]
Since 2008, the freshwater *Pangasius*/catfish surpasses salmon in global production, and since 2007, each of the two species surpass Atlantic cod (Figure S1). Two previous case reports described IgE-binding proteins in *Pangasius*/catfish, referred to as pangasius.[18,19] One patient showed IgE-binding possibly to parvalbumins, in contrast to the other patient who was not parvalbumin-sensitized, however, none of the IgE-binding proteins were identified.
The aim of this study was to identify candidates for urgently needed component-resolved diagnostics (CRDs). We therefore investigated the detailed repertoire and abundance of IgE-binding proteins in both the Atlantic salmon and *Pangasius*/catfish.

2 METHODS

2.1 In-house extracts

Whole specimen of Atlantic salmon (*Salmo salar*) and *Pangasius*/catfish (*Pangasianodon hypophthalmus*), referred to as catfish here, as well as for experimental controls, Asian seabass (*Lates calcarifer*), Atlantic cod (*Gadus morhua*), Nile tilapia (*Oreochromis niloticus*), and yellowfin tuna (*Thunnus albacares*), were sourced from local fishermen, retailers or fellow researchers. Muscle tissue samples were taken from the center of the fillets for consistency and stored at -80°C until further use.
Proteins were extracted as previously described.[11] In short, tissue was homogenized, extracted overnight in phosphate-buffered saline and after filtration stored at -20°C (raw protein extracts). For the heated extracts, tissue was heated at 95-100°C in PBS for 20 min before extraction in the same buffer.
The catfish preparation for skin prick testing (SPT) was generated by homogenizing minced raw muscle tissue with one part (w/v) Hanks' Balanced Salt Solution (Gibco®, ThermoFisher Scientific) as above. Aliquots were stored at -80°C until single use.
Collagens were extracted as experimental controls from the muscle tissue of Asian seabass, Atlantic salmon, and yellowfin tuna as described previously with modifications.[20] In short, muscle tissues were washed with water, 0.1 M NaOH, and 10% butyl alcohol, followed by extraction with 0.5 M acetic acid.

2.2 Patients

Seventy-seven children (1-18 years, interquartile range (IQR) 6-13 years) with clinically confirmed allergy and history of IgE-mediated symptoms after ingesting fish underwent

allergy skin prick tests (SPT) with commercial salmon and an in-house catfish preparation, as previously described.[11] Serum from all patients was obtained for *in vitro* analyses (*n*=77), while sIgE levels were determined for the available commercial salmon ImmunoCAP (Thermo Fisher Scientific, f41) for 43 patients (see Table S1 for demographic and clinical data). Parents gave written informed consents, and ethical approval was obtained from the Sydney Children's Hospitals Network (LNR-14/SCHN/185). Sera from two non-atopic and two atopic fish-tolerant donors were used as negative controls.

2.3 Protein concentration and SDS gel-electrophoresis

The protein concentration for all extracts was estimated using the Pierce™ BCA Protein Assay kit (Thermo Scientific) with bovine serum albumin as standard. All whole protein extracts were diluted to the same total protein concentration.

Proteins were separated according to their molecular weights using a Criterion™ SDS-PAGE system (Bio-Rad) or Dual Double Wide Mini Vertical System (C.B.S. Scientific). Proteins were visualized by Coomassie Brilliant Blue R-250 (CBB) staining and identified by subsequent immunoblotting with allergen-specific antibodies or patient serum IgE.

2.4 Immunoblotting

The separated proteins were transferred onto a nitrocellulose membrane. Subsequently, the fish allergens, parvalbumin,[21] aldolase,[22] tropomyosin,[23,24] and collagen[25] as well as patients' IgE-binding were detected as described previously.[11] In brief, membranes were blocked with casein and incubated with in-house generated polyclonal antibodies raised in rabbits against parvalbumin from Atlantic salmon and catfish and tropomyosin from shrimp,[26,27] commercial antibodies raised against rabbit aldolase (100-1141 by Rockland Immunochemicals) and tuna collagen (ab23730 by Abcam), and patients' sera. Patient blots were further incubated with a monoclonal mouse anti-human IgE antibody (sc-53346 by Santa Cruz) before all blots were developed with a corresponding infra-red-labelled antibody (DyLight anti-mouse/rabbit 4xPEG by Thermo Scientific or IR-Dye anti-goat by LI-COR®).

The Surf-Blot Antibody Screening System by Idea Scientific was used to investigate serum IgE-binding from all patients to the same extract. Densitometric analyses were conducted utilizing Image Studio Version 5.2 (LI-COR®), which allows sensitive and semi-quantitative evaluation of signals. The densitometric analyses utilizing this system is independent of background, contrast or other settings often used for best visualization of the immunoblot.

Antibody-binding intensities were determined in comparison to negative controls and other patients as well as signals to other proteins.

2.5 Mass spectrometry analysis

Whole protein extracts, as well as IgE-binding bands, were digested with trypsin and analyzed by mass spectrometry as described previously.[11,28] Results were analyzed using both Mascot (v. 2.4) search engine and MaxQuant (v. 1.6.2.3), against an NCBI database containing amino acid sequences of all salmon or catfish proteins (July 2019). The relative protein abundance is expressed in relative intensity-based absolute quantification (iBAQ%) value.[29] Identified protein groups with at least 1 unique peptide and a minimum of 2 razor/unique peptides were included in the analysis.

3 RESULTS

3.1 SDS-PAGE and the detection of previously recognized fish allergens

The protein composition of raw and heated extracts from both salmon and catfish was compared by SDS-PAGE and subsequent densitometric analyses (Figure 1). While the protein concentrations were adjusted for all extracts, the raw and heated extracts from catfish showed a higher number of protein bands than those obtained from salmon. In both raw and heated extracts, the most abundant protein bands were between 35-50 kDa and 11-12 kDa.

Using allergen-specific antibodies, the four WHO/IUIS-registered fish allergens parvalbumin, aldolase, tropomysoin, and collagen could be identified in most extracts (Figure 2). Two parvalbumin bands were detected for each species, with a higher signal intensity in the heated extracts as compared to the raw extracts. The anti-salmon parvalbumin antibody detected the 12 kDa band in salmon with the highest intensity, followed by an 11 and 12 kDa band in catfish, and the weakest intensity to an 11 kDa band in salmon. The anti-catfish parvalbumin antibody detected both 11 and 12 kDa bands in catfish with equally high intensity, while the 11 and 12 kDa band in salmon demonstrated a much lower binding capacity.

Aldolase was detected with higher intensity at 40 kDa in catfish (raw) as compared to the 37 kDa band in salmon (raw). No aldolase was detected in any heated extracts.

Tropomyosin was detected with similar intensity in heated extracts from salmon (at 37 kDa), catfish (at 35 and 36 kDa) and tilapia (at 36 kDa). A very weak and weak signal was observed for the corresponding band in the raw extract from salmon and catfish, respectively.

Collagen was detected only in salmon heated extract; however the corresponding antibody demonstrated binding to purified collagen from salmon, seabass, and tuna.

3.2 Patient characterization and *in vivo* reactivity

Twenty patients had a history of an allergic reaction to salmon (26% of cohort) and eight to catfish (10%) (Table S1). Among all 77 pediatric patients with a convincing clinical history of IgE-mediated fish allergy, the median wheal diameters for salmon and catfish were 4.5 mm (IQR; 0-6.5 mm) and 9.5 mm (5.5-14.5 mm) with 69% and 88% of patients with a SPT ≥3 mm to salmon and catfish, respectively. Lessof *et al.*[30] and Peters *et al.*[31] suggested a higher threshold to reduce the number of possible false-positive results. 43% and 78% had a positive skin reaction to salmon and catfish based on a threshold of ≥5 mm, respectively (Figure 3A). Five of the 20 salmon-allergic patients (25%) had a salmon SPT result of <3 mm, while the median for the remaining 15 patients was 7 mm (IQR; 5-8 mm). Among eight catfish-allergic patients, the median catfish SPT results was 7 mm (IQR; 4-9 mm). In summary, patients seem to demonstrate larger SPT wheal diameter to catfish, while over 10% had negative SPT results.

The median sIgE level for salmon was 3.2 kU/l (n=43; IQR; 0.5-10.7 kU/l), while four patients had <0.01 kU/l. Eighty-seven percent of patients had a sIgE level of above 0.1 kU/l, of whom 40% had a low-moderate level (ImmunoCAP class I-II) and 47% a high-very high level (class III-V) (Figure 3B). For eight salmon-allergic patients, the median sIgE level was 5.7 kU/l (IQR 0.5-23.4 kU/l); all but one patient had an elevated sIgE level. In summary for salmon, an overall positive correlation between SPT and sIgE level was observed (r_s=0.74, p<0.0001), while the SPT was negative in 18 patients (<5 mm) with elevated sIgE levels.

3.3 Serum IgE-binding of salmon and catfish proteins

Serum from all 77 patients and controls (n=4) was analyzed for IgE–binding to heat-labile and heat-stable salmon and catfish proteins (Figure S2). All IgE-binding protein bands, with at least five patients, are indicated by an arrow and the corresponding molecular weight in Figure 1: Seven and 12 bands in raw, and two and five bands in heated salmon and catfish extracts, respectively (Table S2). In addition, IgE-binding to bands with less than five patients was observed (Table S3) and their identity has not been further investigated. Nineteen IgE-binding bands were evaluated for protein identity and relative abundance by advanced mass spectrometric analyses (Table S4 and S5). The majority of detected peptides (73-100%) corresponded to up to three major isoforms of one protein each in 18 bands (Table S4 and S5). In 17 bands, other proteins with valid hits had a relative abundance of up to 9%, but were more abundant in other bands not showing IgE-binding by the same patient. This enabled us to exclude these proteins. We therefore associated IgE-binding to one protein each

for 17 IgE-binding bands. The other two analyzed IgE-binding bands were from salmon raw extract and contained considerable amounts of multiple proteins. The 37 kDa band contained both aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), while pyruvate kinase and glucose-6-phosphate isomerase were detected in the 65 kDa band. Table 1 summarises the prevalence of IgE-binding to all these proteins along with their respective allergen names where appropriate. Three salmon and 12 catfish proteins were registered as new fish allergens with the WHO/IUIS.

Parvalbumins (Sal s 1 and Pan h 1) were the only proteins binding IgE in both raw and heated extracts, and the proteins with the highest IgE-binding capacity (49% each) followed by triosephosphate isomerase (TPI; 34% to Sal s 8.0101, 14% to Pan h 8.0101) in raw extracts and tropomyosin (13% to Sal s 4.0101, 6% to Pan h 0201, and 32% to Pan h 4.0101) in heated extracts. Among all 77 patients, 70% and 77% showed IgE-binding to the raw extract from salmon and catfish, respectively, which decreased to 53% and 64% for the corresponding heated extracts.

No IgE-binding (or only a very faint signal) was observed in serum from the control patients nor in seven fish-allergic patients, who were therefore excluded from further analyses. Five of those patients had a clinical history of an allergic reaction to salmon; three had a salmon SPT result of 0 mm while the other two had 3 and 7.5 mm.

The remaining 70 fish-allergic patients were grouped based on the species implicated in the reported clinical allergic reaction and their salmon sIgE level (Table S6). Species-specific IgE-binding to parvalbumin was observed in eleven patients (salmon 9% and catfish 7%). Two of eight patients with a history of allergic reaction to catfish showed IgE-binding to catfish parvalbumin, but not to salmon parvalbumin. Monosensitivity to only one of the two catfish parvalbumins was observed in 39% of the 70 patients, while 6% (n=70) showed IgE-binding exclusively to parvalbumins. In contrast, 37% (n=70) showed no IgE-binding to parvalbumin, but to other proteins.

Forty-nine and 53 of 70 patients showed IgE-binding to parvalbumin, tropomyosin and/or TPI from salmon (70%) and catfish (76%), respectively (Figure 4). Five and seven patients (7% and 10%, respectively), demonstrated IgE-binding only to aldolase, enolase and/or GAPDH. All but two patients demonstrated IgE-binding to any of these six proteins or creatine kinase from salmon and/or catfish or pyruvate kinase from catfish. The IgE from the remaining two patients showed binding only to a 28 kDa band in the heated extract or a 30 kDa band in the raw extract from catfish. These two patients had no clinical history of an allergic reaction to salmon or catfish but to croaker and white fish, respectively.

3.4 The relative abundance of IgE-binding proteins

The relative abundance of the above described major IgE-binding proteins was evaluated by mass spectrometric analyses and totaled to 74-86% (Figure 5). Raw extracts showed a higher diversity of proteins as compared to heated extracts. Parvalbumins were the most abundant proteins in both raw (30 and 36%) and heated extracts (54 and 57%) from both species (salmon and catfish, respectively). The second most abundant proteins in raw salmon and catfish extracts were enolase (14%) and creatine kinase (12%), respectively. Tropomyosin was the second most abundant protein in heated extracts from both salmon and catfish (24 and 9%, respectively). The relative protein abundance was 80- and 46-fold higher compared to the raw extract. Collagen demonstrated low abundance (4%) in the salmon heated extract as compared to less than 0.5% in the other three extracts.

4 DISCUSSION

This is the first study to analyze the detailed allergen repertoire of two highly consumed fish species – Atlantic salmon and *Pangasius*/catfish. The latter is one of the most consumed freshwater fish species, traded worldwide under many names including pangasius, pangas, basa, catfish, swai, tra, sutchi, haiwels, cobbler, grey sole, Pacific dory, iridescent shark or freshwater fillet.

Based on the current study, the WHO/IUIS now lists twelve *Pangasius*/catfish proteins and three additional Atlantic salmon proteins (www.allergen.org) as allergens, including six fish allergens registered for the first time. However, the exact molecular properties and clinical relevance of these IgE-binding proteins require further investigations. The clinical relevance could be clarified with cell-based assays and basophil activation tests.

This study describes the identification of novel fish allergens using a well characterized cohort of fish-allergic pediatric patients by investigating the sensitization patterns to salmon and catfish. Fish allergy is a life-long condition and often starts in the early stages of life, and our patient cohort addressed this age group. A caveat of this study was the lack of comparative analysis of sensitization patterns with fish-allergic adults. To our knowledge, only two studies directly compared sensitization patterns between numerous fish-allergic children and adults. James et al. reported similar IgE-binding to parvalbumins from catfish, cod, and snapper (exact species unknown) for five children and five adults.[32] Similarly, Sharp et al. investigated IgE binding to Asian seabass parvalbumin among six children and ten adults.[33] Further comparative analysis with larger fish-allergic cohorts of different age-groups are required to investigate the role of specific fish allergens in early age sensitization.

We aimed to expand our understanding of the allergen repertoire in fish and identify suitable candidates for much-needed CRDs. The *in vitro* IgE-binding to raw and heated protein extracts from salmon and catfish depended on the presence of specific allergens and differed between patients and fish species. Importantly, the salmon sIgE level (ImmunoCAP) was not a good indicator for IgE-binding to extracts generated in-house, except for patients with a high to very high sIgE level (*n*=20) of which 95% showed IgE-binding to parvalbumin from salmon and/or catfish. The majority of patients with a negative or moderate salmon sIgE level demonstrated IgE-binding to proteins other than heat-stable parvalbumin and tropomyosin, suggesting that heat-labile proteins are under-represented in the utilized salmon ImmunoCAP.

However, there was a positive correlation between SPT results and sIgE level for salmon. Furthermore, we demonstrated a positive correlation in the SPT outcomes for salmon and catfish. It is noteworthy that the wheal diameter was overall greater for catfish compared to salmon and many patients with a negative salmon SPT had a positive catfish SPT, which may result from different procedures in generating the corresponding SPT preparations.[11] Currently, there are no commercial SPT preparations available for catfish and many other highly consumed fish species. Parvalbumin, the well-recognized major fish allergen,[9] was the protein with the highest IgE-binding capacity, possibly also due to its abundance in all extracts. However, the prevalence of IgE-binding to any salmon or catfish parvalbumin was only 57%, while in comparison previous studies state prevalences of 70-95% among fish-allergic patients.[34] This highlights the importance of additional fish allergens as also suggested previously for fish SPT diagnostics.[11] The observed limited IgE-binding of both salmon and catfish parvalbumins can partially be explained by amino acid sequence differences. Salmon parvalbumin Sal s 1.0101 has a rather low sequence identity of 66% and 57% with catfish parvalbumins Pan h 1.0101 and Pan h 1.0201, respectively. The latter two are only 57% identical, possibly resulting in the different IgE-binding observed (44% versus 10%). Similarly, differences in amino acids sequences and IgE-binding capacity of parvalbumin isoforms were previously demonstrated for Asian seabass.[33]

Tropomyosin was the second most abundant protein after parvalbumin in heated extracts. We demonstrated for the first time IgE-binding to fish tropomyosin in a large patient cohort. Previous reports of IgE-binding to fish tropomyosin are rare and include two case reports[24,35], a description of 19 patients with undefined adverse reactions after fish intake,[36] and one study with ten presumably fish-allergic patients who additionally suffered from inflammatory bowel disease or shrimp allergy.[23] We demonstrated IgE-binding to one salmon tropomyosin

and two catfish tropomyosins in 6% to 32% of our patients. The differential IgE-binding capacity of the three fish tropomyosins can be explained to some extend by amino acid sequence differences. Catfish tropomyosin Pan h 4.0101 demonstrated the highest IgE-binding capacity and shares 83% and 80% of its sequence with catfish tropomyosin Pan h 4.0201 and salmon tropomyosin Sal s 4.0101, respectively. The latter two are 93% identical. All three tropomyosin are 82-95% identical with the only other WHO/IUIS-registered tropomyosin Ore m 4.0101 from tilapia. Future research should focus on clinical cross-reactivity between various tropomyosin isoforms.

Recently, we identified heat-stable collagens as novel allergens in three fish species, including salmon.[37] In this study, however, only low quantities of collagen were detected in the PBS-based fish extracts. Collagen is generally insoluble in neutral aqueous solutions, resulting in subsequent underrepresentation in extracts, as recently demonstrated in for commercial SPT preparations.[11]

While most fish are consumed after heat-treatment, heat-labile allergens seem to be of considerable importance as demonstrated for aldolase and enolase - their implementation in CRDs can be useful.[38] In the current study, we reported IgE-binding to both allergens in catfish and registered their full sequence (Pan h 3.0101 and Pan h 2.0101, respectively). The utilization of these and other heat-labile allergens in CRDs could lower the rate of false-negative test results.

In addition, we demonstrated an even higher prevalence in our cohort for IgE-binding to TPI, which is a glycolytic enzyme found in nearly every organism and a registered allergen in arthropods[9]. IgE-binding fish TPI is distinguishable from other heat-labile proteins by its low molecular weight of 25 kDa and was previously reported in amago salmon,[39] mackerel,[40] silverside,[41] sole,[42] and swordfish.[43] It is to note that salmon TPI (34%; Sal s 8.0101) showed more frequent IgE-binding compared to catfish TPI (19%; Pan h 8.0101), possibly associated with the low sequence identity of 85% and different protein abundances.

The enzyme GAPDH was identified as an IgE-binding protein in catfish (Pan h 13.0101), but not in salmon as it was not distinguishable from aldolase. IgE-binding GAPDH has previously been reported in pilchard.[44]

We registered heat-labile creatine kinases from salmon and catfish as novel IgE-binding proteins, Sal s 7.0101 and Pan h 7.0101, respectively. IgE-binding to fish creatine kinase has previously been associated with occupational allergy[45] and allergy to bream[46] and tuna[47] but creatine kinase was not characterized and registered as an allergen.

To our knowledge, this is the first report of IgE-binding to catfish glucose-6-phosphate isomerase (Pan h 12.0101) and L-lactate dehydrogenase (Pan h 10.0101), and the second report for fish pyruvate kinase (Pan h 9.0101)[43]. However, these proteins were not separated successfully or of low abundance in the salmon raw extract. All three allergens are now listed on www.allergen.org.

In summary, this study details the repertoire of IgE-binding proteins from two highly farmed and consumed fish, marine Atlantic salmon and freshwater *Pangasius*/catfish, and demonstrated more IgE-binding allergens in catfish compared with salmon. Future research should provide additional information on clinical cross-reactivity and the implementation of parvalbumins as well as tropomyosins and selected heat-sensitive allergens in CRDs.

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TABLE 1: *In vitro* IgE-binding capacity of salmon and catfish proteins in raw and heated extracts.

Raw salmon

MW	Protein(s) in band	IUIS name	*n=77*
65	Pyruvate kinase G-6-PI	-	8%
60	n.d.	-	6%
48	beta-enolase	Sal s 2	34%
43	creatine kinase	Sal s 7.0101	14%
37	aldolase A GAPDH	Sal s 3.0101 -	26%
25	TPI	Sal s 8.0101	34%
12	PV	Sal s 1	**49%**
any other band			1%
Patients reactive to any band above			**70%**

Raw catfish

MW	Protein(s) in band	IUIS name	*n=77*
65	Pyruvate kinase	Pan h 9.0101	6%
60	G-6-PI	Pan h 11.0101	8%
50	beta-enolase	Pan h 2.0101	21%
43	creatine kinase	Pan h 7.0101	10%
40	aldolase A	Pan h 3.0101	21%
36	GAPDH	Pan h 13.0101	6%
34	L-lactate DH	Pan h 10.0101	13%
30	n.d.	-	14%
27	n.d.	-	6%
25	TPI	Pan h 8.0101	19%
12	PV 2	Pan h 1.0201	10%
11	PV 1	Pan h 1.0101	**42%**
any other band			17%
Patients reactive to any band above			**77%**

Heated salmon

MW	Protein(s) in band	IUIS name	*n=77*
37	TM	Sal s 4.0101	13%
12	PV	Sal s 1	**49%**
any other band			12%
Patients reactive to any band above			**53%**

Heated catfish

MW	Protein(s) in band	IUIS name	*n=77*
36	TM 2	Pan h 4.0201	6%
35	TM 1	Pan h 4.0101	32%
28	n.d.	-	21%
12	PV 2	Pan h 1.0201	14%
11	PV 1	Pan h 1.0101	**44%**
any other band			13%
Patients reactive to any band above			**64%**

Note: The proteins refer to the bands in Figure 1. Results are based on IgE immunoblots (Figure S2) and conforming densitometric analyses (Table S2). The identity of the proteins in the band corresponds to mass spectrometric analyses (Table S4 and S5). The WHO/IUIS name refers to the corresponding database accessible under www.allergen.org. Abbreviations: MW, molecular weight in kDa; n.d., not determined; G6-PI, glucose-6-phosphate isomerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DH, dehydrogenase; TPI, triosephosphate isomerase; PV, parvalbumin; TM, tropomyosin.

FIGURE 1: SDS-PAGE profiles and densitometric analyses of raw (A, B) and heated (C, D) extracts from salmon (A, C) and catfish (B, D). Bands with IgE-binding by at least five fish-allergic patients are indicated with an arrow and their observed molecular weight is provided.

FIGURE 2: Detection of registered fish allergens in raw (R) and heated (H) extracts from salmon (S) and catfish (C). Proteins were separated by SDS-PAGE and allergens identified by immunoblotting using antibodies specific to the respective fish allergens. Parvalbumin was detected using antibodies raised against parvalbumin (PV) from salmon (A) and catfish (B). The raw extracts from Atlantic cod containing Gad m 3.0101 and yellowfin tuna containing Thu a 3.0101 were loaded as a reference for the detection of aldolase (C). The reference for tropomyosin was heated extract from Nile tilapia, a closely related species to Mozambique tilapia, the only fish species for which tropomyosin (Ore m 5.0101) was recognized by the WHO/IUIS at the time of the study (D). Purified collagens from salmon, tuna and seabass were used as a reference for collagen detection (E).

FIGURE 3: Comparison of *in vivo* reactivity of 77 fish-allergic patients to salmon and catfish (A). The Skin Prick Test (SPT) wheal diameter is given in mm. For 43 patients, the salmon sIgE level and corresponding ImmunoCAP class were determined and compared with the corresponding SPT result (B). The sIgE level was below 0.1 kU/l (class 0) for six patients who had SPT of 0 mm. A positive correlation is indicated by a curve of best fit.

FIGURE 4: IgE-binding of 64 fish-allergic patients to two heat-stable allergens (parvalbumin (PV) and tropomyosin (TM)) and four heat-labile allergens (triosephosphate isomerase (TPI), enolase (Eno), aldolase (Ald) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) from salmon (A) or catfish (B).

FIGURE 5: Relative protein abundance in raw and heated extracts from salmon and catfish. The extracts were digested with trypsin and analyzed by mass spectrometry. The iBAQ%

value is an indicator of the relative abundance of each protein including several isoforms and is based on analyses with MaxQuant. Note: The relative abundance is only given for proteins for which IgE-binding with at least five patients was demonstrated. The WHO/IUIS-name is based on the corresponding database accessible under www.allergen.org. TPI=triosephosphate isomerase, DH=dehydrogenase, G6-PI=glucose-6-phosphate isomerase, GAPDH=glyceraldehyde-3-phosphate dehydrogenase.

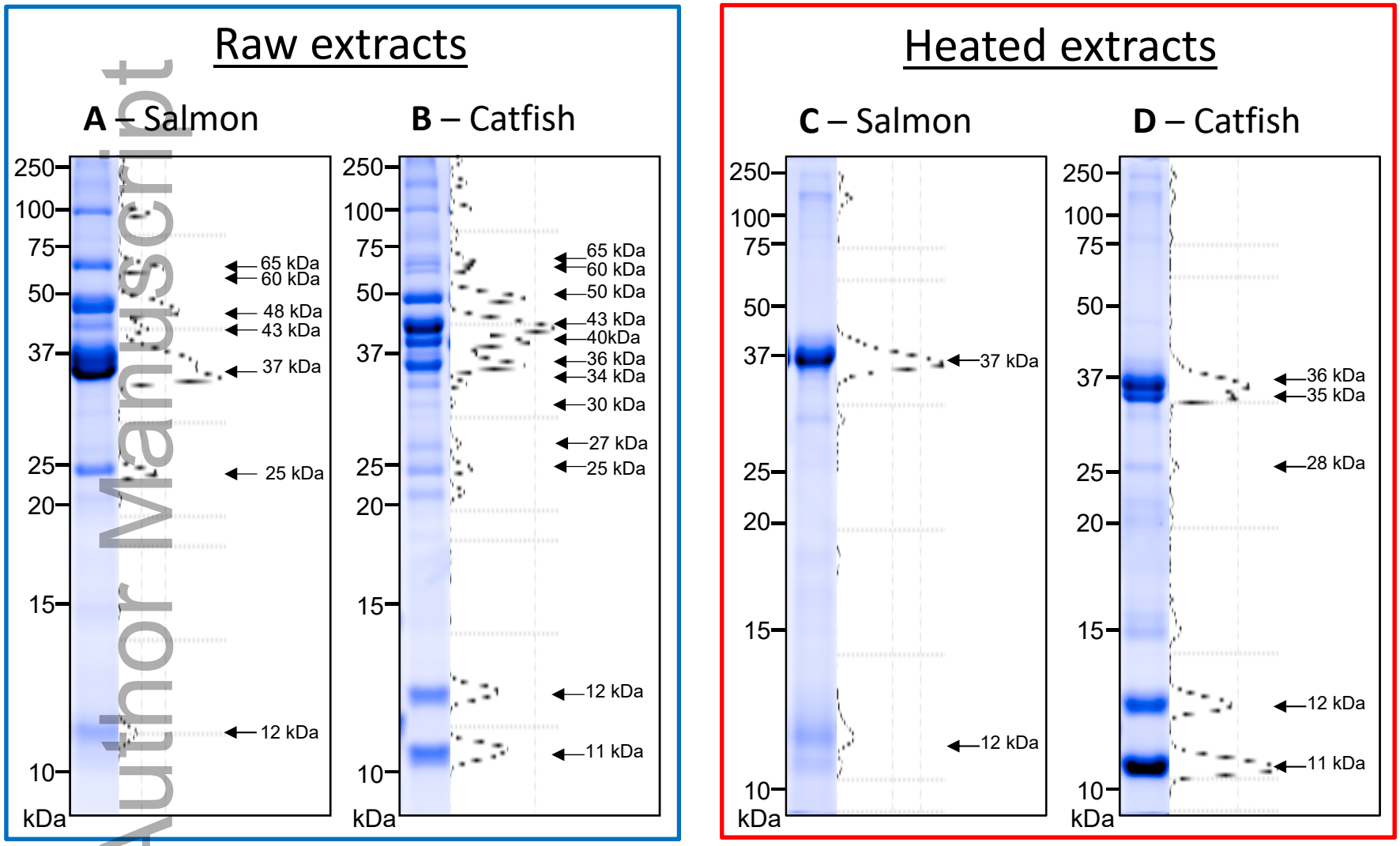

FIGURE 1

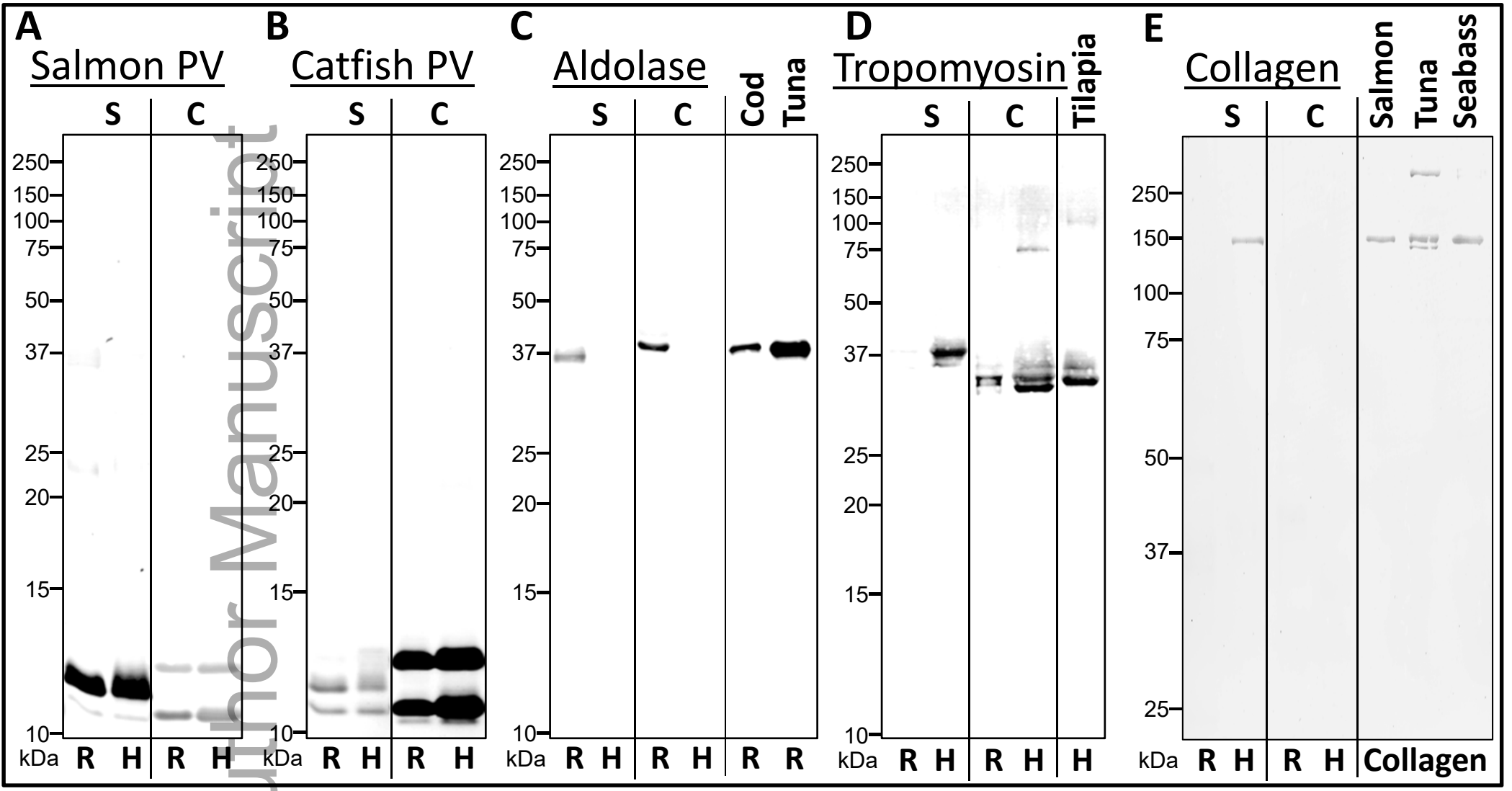

FIGURE 2

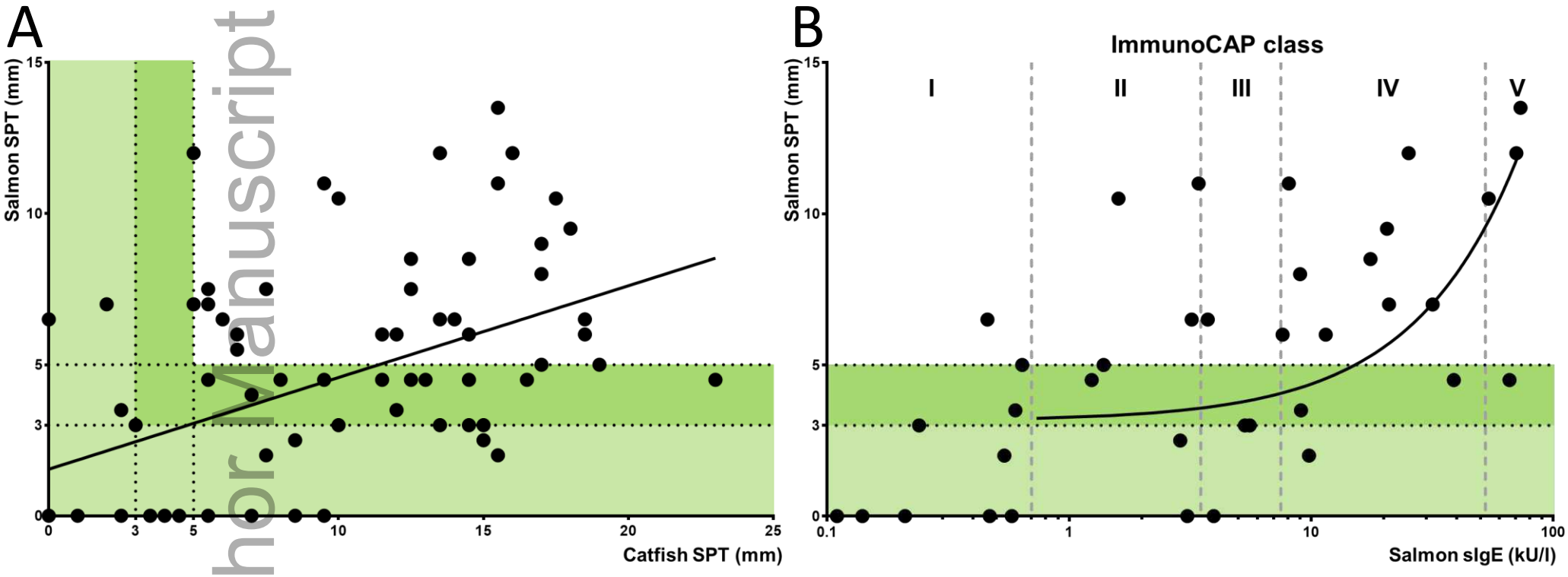

FIGURE 3

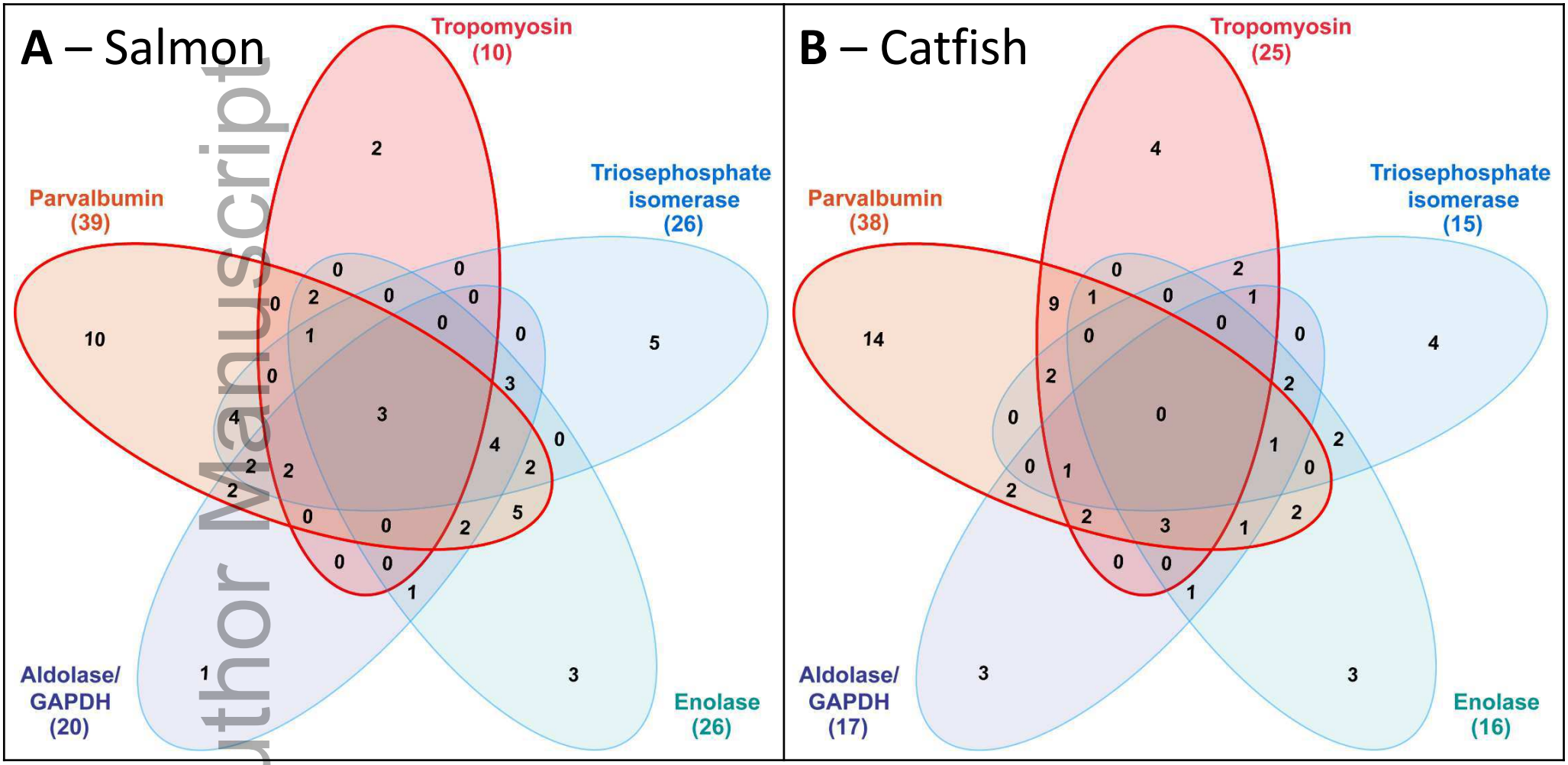

FIGURE 4

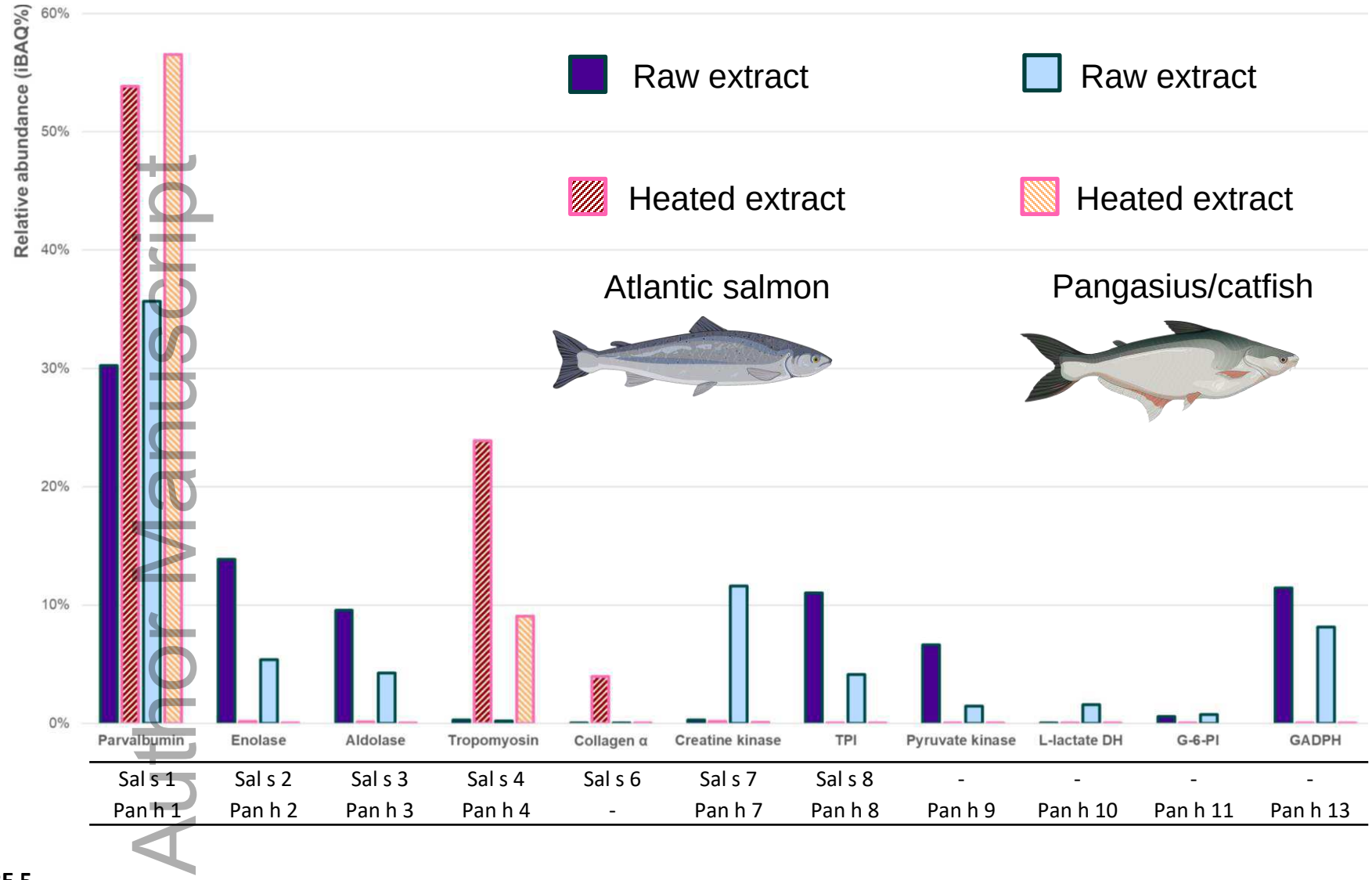

FIGURE 5

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Title:
Expanding the allergen repertoire of salmon and catfish

Date:
2021-05

Citation:
Ruethers, T., Taki, A. C., Karnaneedi, S., Nie, S., Kalic, T., Dai, D., Daduang, S., Leeming, M., Williamson, N. A., Breiteneder, H., Mehr, S. S., Kamath, S. D., Campbell, D. E. & Lopata, A. L. (2021). Expanding the allergen repertoire of salmon and catfish. Allergy, 76 (5), pp.1443-1453. https://doi.org/10.1111/all.14574.