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Thermal stress alters the allergen and proteome profile of the redclaw crayfish (*Cherax quadricarinatus*)

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ABSTRACT

Rising global temperatures and growing demand for sustainable protein are concurrent challenges driving the expansion of aquaculture, including farming of shellfish such as the Australian Redclaw Crayfish (*Cherax quadricarinatus*). Although environmental stressors alter animal physiology, their effects on food protein allergenicity remain unexplored, particularly in shellfish, a leading cause of food-induced anaphylaxis. This study investigated how rearing temperature affects protein and allergen expression in redclaw reared at either 17 °C, 28 °C, or 32 °C. Raw and cooked protein extracts were analysed using SDS-PAGE and immunoblotting with crustacean-allergic individuals (n = 19). Whole extracts were examined by liquid-chromatography mass spectrometry (LC-MS) to identify and quantify proteins, assess differential expression, and perform gene ontology (GO) analysis across temperatures. Significant temperature-dependent variation in protein and allergen profiles was observed. IgE-binding intensity increased at 17 °C and decreased at 32 °C in raw and cooked extracts, indicating increased allergenicity under cold-stress. At 17 °C, allergens including heat shock protein 70 (HSP70) and fatty acid-binding protein (FABP) were upregulated, alongside GO categories related to protein folding and metabolic/catabolic processes (e.g., triosephosphate isomerase (TPI), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase, and fructose-bisphosphate aldolase (FBA)). GO expression profiles between 28 °C and 32 °C were largely similar. Allergens tropomyosin (TM), myosin light chain (MLC), and troponin C (TnC) were unchanged across temperatures. Environmental temperature alters the allergen/protein profiles of redclaw and clinically relevant IgE-antibody reactivity. These findings highlight the impact of environmental and farming conditions when assessing food allergen exposure risks in a changing climate and highlight the need for further validation in other aquaculture species.

1. Introduction

Temperature-based changes have been widely documented in the field of aquaculture as playing a crucial role in shaping the physiology, metabolism, and overall health of cold-blooded organisms [1–4]. However, limited investigation has been performed to determine the impact of temperature-based changes in the field of food allergy. In aquaculture, research into temperature responses is vital to ensure optimal growth of aquatic stock, reduce stressors and mortalities, and allow for the translocation and farming of suitable species outside of their native habitat [1,3,5–7]. Understanding the influence of water temperature on aquaculture species is especially vital due to climate change and the increasing demand for sustainable aquaculture production [8]. Increasing global temperatures are disrupting food production, with climate change expected to warm global average temperatures by 1.5 °C by 2050 and 2-4 °C by 2100 [9]. To meet the increasing demand for safe food sources that are both nutritious and a sustainable source of protein, aquaculture is proposed to be instrumental

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in meeting the challenges of global food security, with seafood consumption from aquaculture species alone projected to increase to 21.3 kg per capita by 2032 [10–12]. Already over 50% of seafood consumed globally comes from aquaculture, which is the world's fastest-growing food production sector [13]. A major increasing aquaculture sector is the farming, translocation, and consumption of shellfish, which are the second most valuable animal-based aquaculture food group after fish [14]. A shellfish species with increasing aquaculture potential is the Australian Redclaw Crayfish *(Cherax quadricarinatus*; redclaw hereafter), the second-most translocated crayfish species globally, having been widely translocated to an estimated 67 countries (Supplementary Figure A1) [15]. Redclaw growth is primarily influenced by water temperature, with optimal growth being approximately 30 °C and lethal/stress temperatures documented at very low temperatures of 10 °C and very high at 35 °C [15,16].

The increase in production and consumption of shellfish has also coincided with increasing reports of shellfish-induced food allergy [17,18]. Shellfish allergy, which affects approximately 3% of the global population [19,20], is one of the leading causes of food-induced anaphylaxis globally [21]. Shellfish allergy occurs due to a hypersensitive immune response following exposure to shellfish allergenic proteins often present in edible muscle tissue [22]. There are currently 14 shellfish proteins officially registered in the WHO/IUIS database as allergens in approximately 30 species of shellfish including, *Penaeus monodon, Litopenaeus vannamei, Procambarus clarkii,* and more. Many characterised allergens are often either structural muscle proteins (tropomyosin (TM), troponin C (TnC), myosin light chain (MLC)) [23–25], enzymes (arginine kinase (AK) [26], triosephosphate isomerase (TPI) [27]) or stress-related proteins (heat shock protein 70 (HSP70) [28]) involved in processes such as muscle contraction or part of metabolic pathways. As animal growth and general metabolism have been widely described to be influenced by temperature changes [6,7], differential expression of proteins is likely to also occur. Given that key allergenic proteins, including TM, AK, and HSP70 are involved in muscle structure and stress responses, understanding the influence on allergenicity is important in determining future risk in food allergy. The current study aims to elucidate the role of climate-driven temperature variations in altering shellfish allergenicity, with implications for sustainable aquaculture practices, food safety and public health in an increasingly changing climate.

2. Materials and methods

2.1. Redclaw acclimation

To determine allergenomic and proteomic differentiation of redclaw (*Cherax quadricarinatus*), 16 live male redclaw (average age – four months, average weight – 30g, age and weight-matched) were sourced from the Australian Redclaw Hatchery (ACH) in Townsville, Queensland. Redclaw were initially reared at ambient temperature for two weeks in a 5000L freshwater recirculating aquaculture system, before being separated ($n = 4$) into three temperature groups: 17 °C, 28 °C (considered as the baseline), and 32 °C. Redclaw were housed individually in recirculating 50L tanks containing hides and air-stones, with four tanks assigned to each temperature treatment. Within each temperature group, tanks were interconnected to allow water flow between them, and all groups were connected to shared sumps containing an active bio-filter. The ambient room temperature was set to 17 °C to chill the 17 °C tanks, while heaters set to 28 °C and 32 °C were placed into the sumps servicing the respective temperature groups. Redclaw were assessed daily for mortality and moulting and fed daily with a mix of commercial prawn and spirulina pellets (multiple commercial sources), supplemented with peas and corn. Water quality was maintained with a flow-through rate of 2 L^{min}-1, a pH of approximately 7.0, and ammonia and nitrate concentrations at 0 ppm, with weekly monitoring. Temperature groups were reared at respective temperatures for four weeks before being harvested, euthanised, and stored on ice in preparation for protein extraction.

2.2. Preparation of redclaw Protein extracts

To generate protein extracts, redclaw muscle tissue from the abdomen (tail) was prepared according to a previously described method [29] to extract muscle protein samples. Redclaw were deshelled, and muscle was minced and weighed before being homogenised using a T10 basic ULTRA-TURRAX disperser (IKA, Humboldtstraβe, Königswinter, Germany) in PBS at 4 mL/g of tissue. Samples were then either placed on ice (raw) or cooked at 90 °C via boiling before being placed on ice. Raw and cooked samples were then incubated overnight (16h) at 4 °C before being centrifuged at 20,000×*g* for 20 min at 4 °C. The supernatant was vacuum filtered through a glass fibre filter and a 0.45 μM membrane filter (MicroScience, Taren Point, NSW, Australia). Pierce™ bicinchoninic acid assay (BCA) kit (Pierce Biotechnology Inc., Rockford, IL, USA) was utilised as per manufacturer's instructions to estimate protein concentration in redclaw extracts and standardise protein concentrations to 1.25 μg/μL for subsequent analysis. Pre-diluted bovine serum albumin (BSA; Pierce Biotechnology Inc.) was used as the protein standard.

2.3. SDS-PAGE

Protein extracts from each of the 16 redclaw specimens were resolved using a reducing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method [30]. Protein extracts were individually generated and then combined with PBS and 4X Laemmli buffer to reach a total protein content of 5 μL and a total volume of 16 μL. Samples were then heated for 10 min at 96 °C on a heat block, and vortexed for 30 s. Protein solutions, alongside a Precision Plus Protein Dual Color Standards molecular weight marker (BioRad, Hercules, CA, USA), were subsequently loaded onto an 18-well AnykD™ Criterion™ TGX™ Precast Midi Protein Gel (BioRad, Hercules, CA, USA) and separated using a Mini-Protean Tetra Cell electrophoresis system (Bio-Rad, Hercules, CA, USA) at 170 V for 1h. The resolved gel was subsequently stained with a solution of 40% methanol, 10% acetic acid and Coomassie Brilliant Blue dye for 1h with shaking, before being destained with the same solution, excluding Coomassie [31]. Destained gels were stored in a 5% acetic acid solution before being visualised using the Odyssey® CLx Imaging System using Image Studio v5.2 (LI-COR Biosciences, Lincoln, NE, USA).

2.4. IgE-antibody immunoblot

Crustacean-allergic sera (ethics approval was obtained from the Ethics Committee of James Cook University, Approval Numbers: H4313 and H6829) were selected based on; 1) clinical confirmation of crustacean allergy, 2) positive shrimp skin prick test (SPT), and 3) positive sIgE test on the MADx ALEX2™ to shrimp mix (f24) (Macro Array Diagnostics, Lemböckgasse, Vienna, Austria). A total of 19 shrimp-allergic individuals were chosen based on these criteria, as well as one non-allergic serum as a negative control.

Four ($n = 24$ extracts total) standardised extract replicates for each temperature, both raw and cooked, were selected, pooled ($n = 6$), and analysed for individual serum IgE-binding using the immunoblotting method (subject demographics are presented in the appendix; Supplementary Table B1). SDS-PAGE was performed on the pooled extracts, and proteins were transferred using a semi-dry transfer method to a 0.2 μM nitrocellulose membrane. To ensure protein transfer, a transfer buffer consisting of 10% Tris-Glycine, 20% methanol was used alongside extra-thick blot filter paper (BioRad, Hercules, CA, United States). Proteins were transferred using a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BioRad, Hercules, CA, USA) at 16V for 22 min. The membrane was blocked using 1X casein for 1h, before being washed

with PBS-T for 5 min, three times. The membrane was subsequently incubated with 200 μL of individual subject sera (including a negative control) diluted 1:20 with 0.5X casein using a Surf-Blot™ method [32] (Idea Scientific, Minneapolis, MN, USA) overnight at 4 °C for 16h in a moist plastic container with gentle rocking.

Following incubation, patient serum was aspirated, and the membrane was washed using 1x PBS-T two times for 5 min, and one time for 10 min. After washing, the membrane was incubated with mouse anti-human IgE antibody (Santa Cruz Biotechnology, Dallas, TX, USA) diluted 1:1000 with 0.5x casein (at RT for 1h). Antibody solution was subsequently discarded, and the membrane was washed with 1x PBS-T for 5 min, three times. The membrane was then incubated with goat anti-mouse IgE (4x PEG) (Invitrogen, Waltham, MA, USA) antibody diluted 1:10,000 with 0.5x casein at room temperature for 40 min with rocking. The membrane was again washed three times for 5 min with 1x PBS-T, before being washed with 1x PBS for 5 min, and distilled water for 5 min. The membrane was subsequently dried before visualisation as described in *2.3 SDS-PAGE*.

2.5. IgE-binding quantification

IgE-antibody binding and intensity were visualised using the Odyssey® CLx Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Nitrocellulose membranes for each temperature were scanned simultaneously to ensure quantification measures were standardised. Densitometric analysis was then performed using Image Studio v5.5 (LI-COR Biosciences, Lincoln, NE, USA) to determine the signal intensity of all IgE-binding bands in a lane. Any IgE-binding bands to membrane-transferred proteins were visualised and boxed in software Image Studio v5.2 using the Western Analysis function. Once boxed, the IgE-binding intensity could be quantified for each band using the total signal (volume). The molecular weight of proteins eliciting IgE-binding was estimated using the molecular weight marker. The IgE-binding intensity and prevalence at particular molecular weights (kDa) per temperature group was documented to determine potential protein allergenicity variations between temperatures. Band signal intensity for each molecular weight and sera were documented in Excel. Using the band intensity, heatmaps were constructed to compare IgE-binding prevalence and intensity between redclaw extracts from different rearing temperatures.

2.6. Liquid chromatography-mass spectrometry (LC/MS)

Proteins present in redclaw extracts were digested into peptides with trypsin protease in an in-solution tryptic digestion method using an S-Trap™ (ProtiFi, Fairport, New York, NY, USA) following the manufacturer's instructions [33]. Eluted peptides were subsequently analysed using LC/MS on an LTQ Orbitrap Elite (Thermo Scientific) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nanoHPLC (Dionex Ultimate 3000) (Thermofisher Scientific, Waltham, MA, USA). Label-free quantification using MaxQuant v2.2 [34] was performed to analyse and identify proteins and their abundance within each extract, using the following parameters: the specific digestion enzyme was trypsin/P, with the maximum missed cleavages being 2. Variable modifications included oxidation (M), Acetyl (Protein N-Term), and deamidation (NQ) and fixed variables included carbamidomethyl (C). The reference protein database was constructed in-house and included the proteomes of phylogenetically similar shellfish species: *C. quadricarinatus* (n = 31,476), *L. vannamei* (n = 26,427), *Homarus americanus* (n = 24,701), *P. clarkii* (n = 921), *Penaeus monodon* (n = 829), *Macrobrachium rosenbergii* (n = 553), *Cherax destructor* (n = 155), *Homarus gammarus* (n = 74), *Penaeus aztecus* (n = 25), downloaded from Uniprot.

2.7. Differential GO group expression analysis

All identified protein IDs (n = 833) in all samples, as processed and filtered by MSstats, were exported as a list before Uniprot ID mapping was performed to obtain entire protein sequences. Protein sequences were then imported into the software OmicsBox [35] for GO expression analysis. A BLAST search was performed on sequences using the database of subphylum Crustacea before functional analysis of BLAST proteins using Interpro was performed, allowing for the identification of protein families and domains. GO mapping and annotation were then performed using OmicsBox to assign GO terms and annotate proteins. Finally, the comparative MSstats protein log2-fold change (Log2FC) values for each temperature comparison were imported into OmicsBox and GO group differential expression analysis was performed to determine differentially expressed GO groups (p-value <0.05 and adjusted p-value <0.05) between temperature conditions and which proteins were major contributors to core-enrichment of certain GO groups.

2.8. In-silico proteome and allergen analysis

The generated MaxQuant results from LC/MS were subsequently imported into R v.4.3.1 [36], RStudio v.2024.12.0 [37] and analysed using the package MSstats [38]. Data was initially processed in MSstats using a log2 transformation, quantile normalisation, and a Tukey's Median Polish (TMP) summarisation. 'Imputation' was set to false to ensure condition-level protein presence and 'absence' was documented. A comparison matrix was then constructed comparing conditions (e.g., 28 °C versus 17 °C, etc.) before a group comparison was performed to identify differentially abundant proteins across the three temperatures using a p-value and an adjusted p-value (using the Benjamini-Hochberg Correction), which corrects data for multiple comparisons at a false discovery rate (FDR) of 5%. All proteins were plotted in a volcano plot using the Ggplot2 package [39], and differentially abundant proteins at each temperature (p-value <0.05 and adj.p-value <0.05) were identified using Log2FC. Sample size analysis was then performed using the MSstats 'designSampleSize' function [38] to calculate the minimal number of replicates required to achieve higher statistical power for future experiments investigating temperature-based changes.

3. Results

3.1. Temperature-based variations in allergic-subject IgE-Binding to raw and cooked redclaw extracts

When comparing shrimp-allergic sera IgE-binding (Fig. 1) between both raw (Fig. 1A) and cooked (Fig. 1B) redclaw tail extracts reared at different temperatures (17 °C, 28 °C, and 32 °C) showed distinct differences in IgE-binding pattern and intensity can be identified, as demonstrated by immunoblot. When crustacean-allergic sera were incubated against raw tail extract (Fig. 1A), positive IgE-binding was identified in 16 subjects (84%) at all temperatures. IgE bound specifically to two bands, an upper band at ~36 kDa and a lower band at ~35 kDa (Fig. 1A).

IgE-binding between rearing temperatures of cooked extracts (Fig. 1B) demonstrated increased IgE-binding to a much greater variety of protein bands. Across all temperatures, 17 subject sera (89%) displayed positive IgE-binding. Distinct IgE-binding regions can be seen at ~75 – 100 kDa, 25 – 40 kDa, and 18 – 20 kDa, respectively, with a total of 13 IgE-binding bands subsequently identified in these regions (Fig. 1B).

Heatmaps (Fig. 2) were generated to quantify the IgE-binding intensity of each subject sera to determine IgE-binding intensity change in response to rearing temperature, in which significant variations were identified (intensity data; Supplementary Table C1). To raw redclaw extract (Fig. 2A), two major IgE-binding bands (~37 kDa and ~36 kDa) showed the highest signal intensity at 17 °C in 12/19 (63%) subject sera,

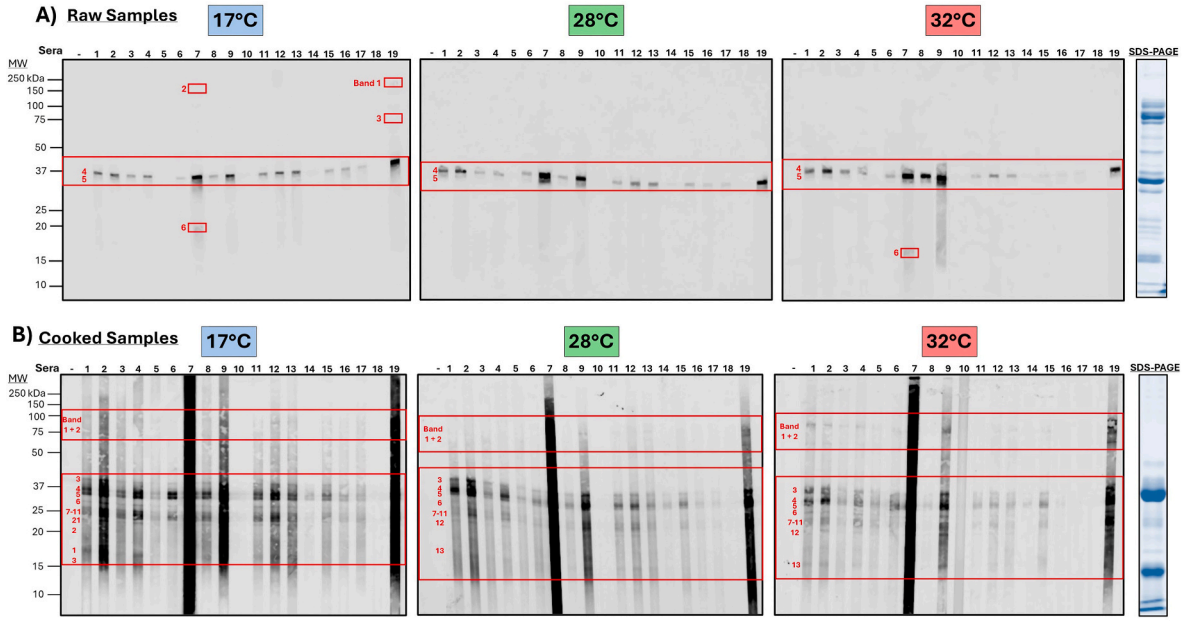

Fig. 1. Redclaw raw (A) and cooked (B) muscle immunoblot against shellfish-allergic subject sera and corresponding SDS-PAGE (aligned to immunoblot MW). To raw extracts, **A)** A total of six IgE-binding bands was identified at various MWs (~16 – 100 kDa) for all redclaw rearing temperatures (**boxed in red**). To cooked, **B)** A total of 13 IgE-binding bands was identified at various MWs (~16 – 100 kDa) for all rearing temperatures, 17 °C, 28 °C and 32 °C (**boxed in red**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

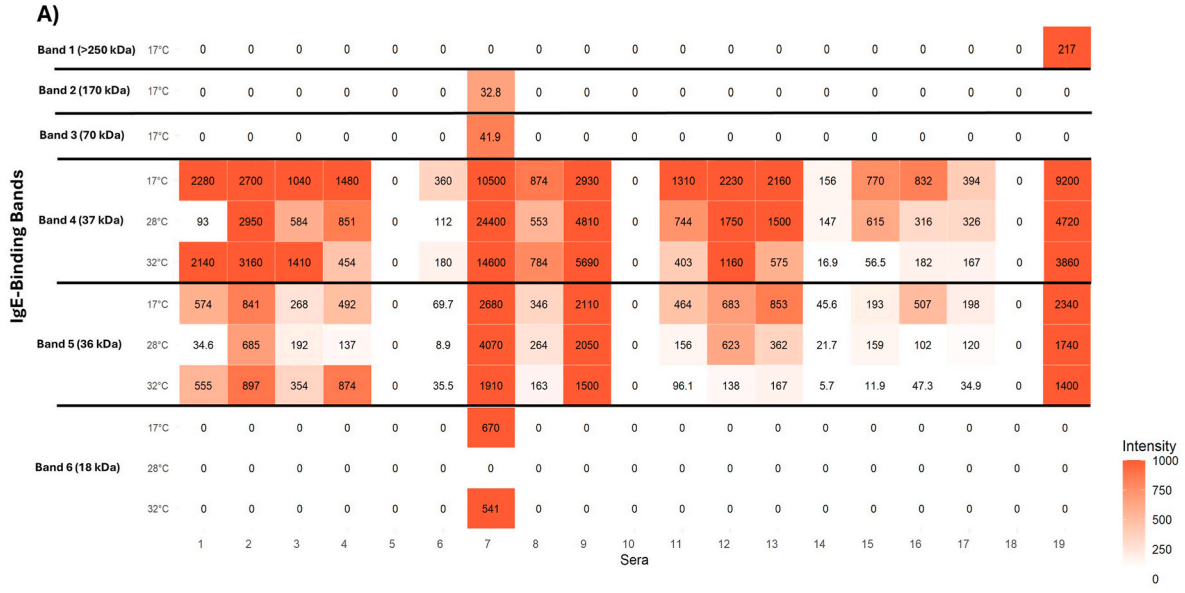

Fig. 2. A) Heatmaps comparing sera IgE-binding band signal intensities to A) raw and B) cooked redclaw muscle extract at each temperature. Band number and molecular weight (kDa) reflect boxed bands identified in each temperature immunoblot for sera numbers 1-19 (excluding the negative control sera; -). Higher IgE-binding resulted in higher signal intensity, ranging from no IgE binding (~0 intensity) to intense IgE binding (>1000 intensity).

while 9/19 (47%) and 11/19 (58%) were the lowest at 32 °C. The trend was particularly evident in serum 7-19, which displayed increased binding at 17 °C and less at 32 °C to raw protein extracts. The subjects with the most intense IgE-binding were subjects 7, 9 and 19, also displaying IgE-binding to additional bands at 17 °C (Fig. 2A). Subject 7 bound to additional bands at ~18 kDa at 17 °C and 32 °C, and ~170 kDa at 17 °C. Sera from subject 19 elicited IgE-binding at 17 °C to an additional band at ~70 kDa and >250 kDa (Fig. 2A).

Cooked redclaw protein extracts (Fig. 2B) demonstrate increased IgE-binding to a much greater variety of protein bands between temperatures. Across all temperatures, 17 subject sera (89%) displayed positive IgE-binding. Distinct IgE-binding regions can be seen at ~75 – 100 kDa, 25 – 40 kDa, and 18 – 20 kDa, respectively, with a total of 13 IgE-binding bands subsequently identified in these regions. IgE-binding varied across the 13 identified bands, but a general trend of increased signal intensity at 17 °C and decreased intensity at 32 °C was observed (Fig. 2B). Bands 1 (~100 kDa), 2 (~75 kDa), 4 (~37 kDa), 5 (~36 kDa), 6 (~34 kDa), 8 (~31 kDa) and 9 (~30 kDa) displayed the most pronounced increases in IgE-binding intensity at 17 °C, with 68-89% of sera demonstrated to have higher binding intensity. Corresponding decreases

Fig. 2. (*continued*).

in signal intensity were also identified at 32 °C, with 57%-79% of sera displaying decreased intensity. At lower molecular weights (bands 11 (~28 kDa), 12 (~26 kDa) and 13 (~18 kDa)), a similar pattern was also identified, with 68-89% of sera eliciting stronger binding signal at 17 °C and lower at 32 °C (Fig. 2B).

3.2. Temperature-based differential expression of GO groupings

Comparison of GO annotations at 17 °C versus 28 °C (Fig. 3A) and 32 °C versus 17 °C (Fig. 3C) both display significant FDR q-value scores (<0.05), indicating significant GO group enrichment. However, the 32 °C versus 28 °C (Fig. 3B) comparison displayed no significant FDR q-value scores, indicating that GO groups between 32 °C and 28 °C are

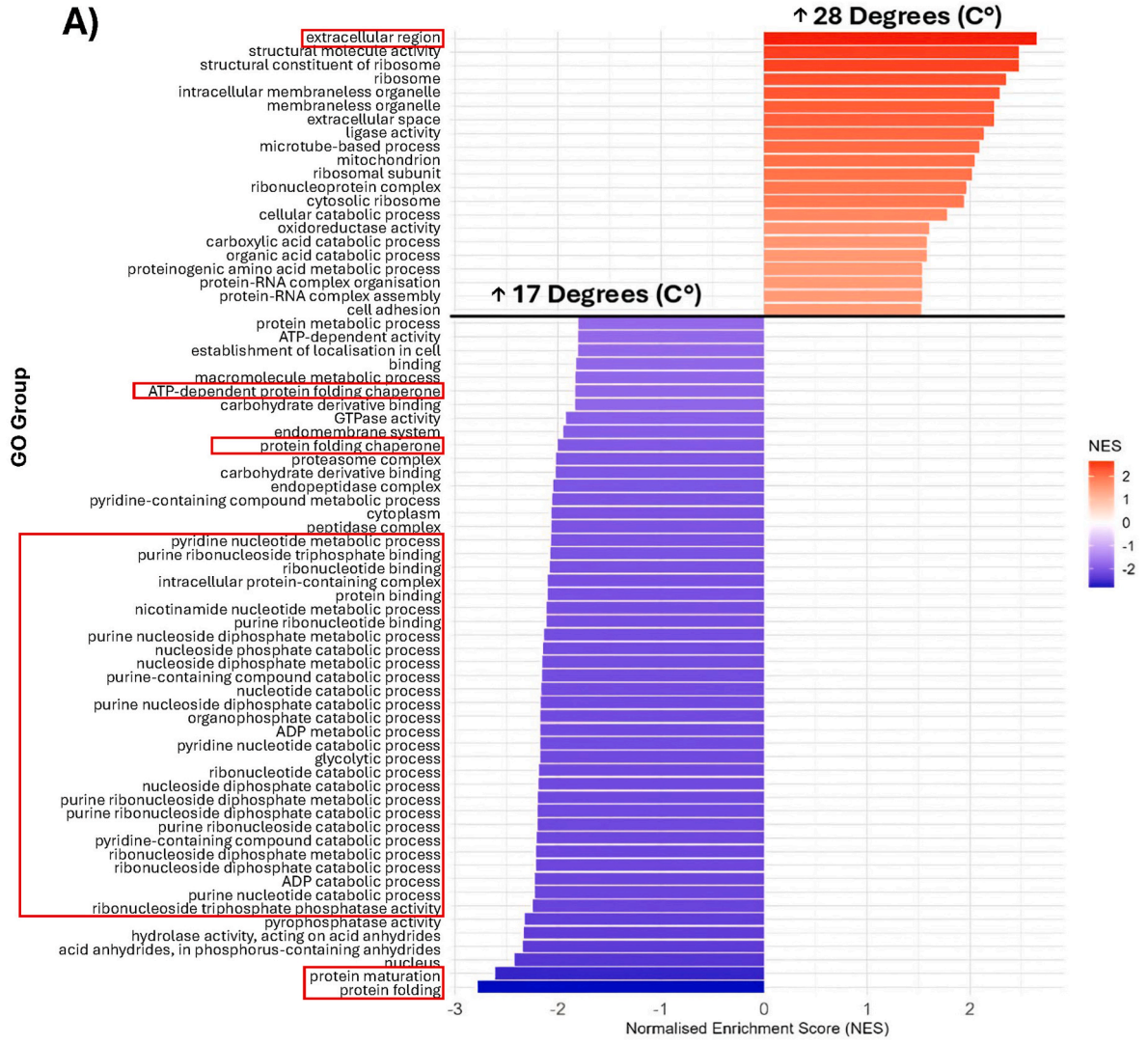

Fig. 3. Significantly enriched GO groups (FDR q-value < 0.05) between A) 17°C and 28°C, B) 28°C and 32°C and C) 17°C and 32°C. Protein GO groups are ranked by normalised enrichment score (NES). A negative NES indicates increased enrichment at the lower temperature in the comparison (as seen in blue) and a positive NES indicates increased enrichment at the higher temperature (seen in red). The higher the positive or negative NES value, the more enriched the GO group is at the higher or lower temperature, respectively. GO groups containing allergens are boxed in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

more similar than between other comparisons and largely statistically insignificant. Due to containing no FDR q-values under the significance threshold, upregulated GO groups in the 32 °C versus 28 °C comparison are less noteworthy than the other comparisons.

In the 17 °C versus 28 °C comparison (Fig. 3A), at 17 °C, proteins involved in folding, maturation, purine ribo/nucleoside catabolism/metabolism, etc., are greatly enriched when compared to 28 °C. Whereas at 28 °C, proteins involved in the extracellular region, structural ribosomal activity, etc., are significantly enriched. When comparing 28 °C versus 32 °C (Fig. 3B), proteins involved in system and cell development, chaperone proteins, and oxidoreductase activity are potentially enriched at 28 °C. Compared to 32 °C, which indicates an enrichment of transmembrane transport-related proteins, proteasome complex proteins, and proteins involved in supramolecular fibre, etc. However, the GO enrichments do not meet significance thresholds for the 28 °C versus 32 °C comparison. When investigating the 17 °C versus 32 °C comparison (Fig. 3C) similarly to 17 °C versus 28 °C (Fig. 3A), proteins involved in folding, maturation, and purine ribo/nucleoside catabolism/metabolism, etc., are again greatly enriched at 17 °C. However, at 32 °C, ribonucleoprotein complex proteins, membraneless organelle proteins, proteins involved in the extracellular region, etc., are significantly enriched.

3.3. Core enriched proteins present in redclaw Protein GO groups

When investigating GO group-based enrichments, specific proteins are considered as core-enriched proteins in a group, indicating a heightened significance to the functional enrichment. The top ten most significantly core-enriched proteins in the five most significantly enriched GO groups per comparison are displayed in Supplementary Tables D.1 - 3. Analysis of GO group-based enrichments revealed several allergenic proteins as core-enriched in specific functional pathways. At 17 °C (Supplementary Table D.1), the allergen HSP70 was a core-enriched protein in the protein folding and maturation GO group. Additionally, suspected allergens, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-bisphosphate aldolase (FBA), and enolase, were core-enriched in purine nucleoside diphosphate catabolic process, nucleoside diphosphate metabolic process, and

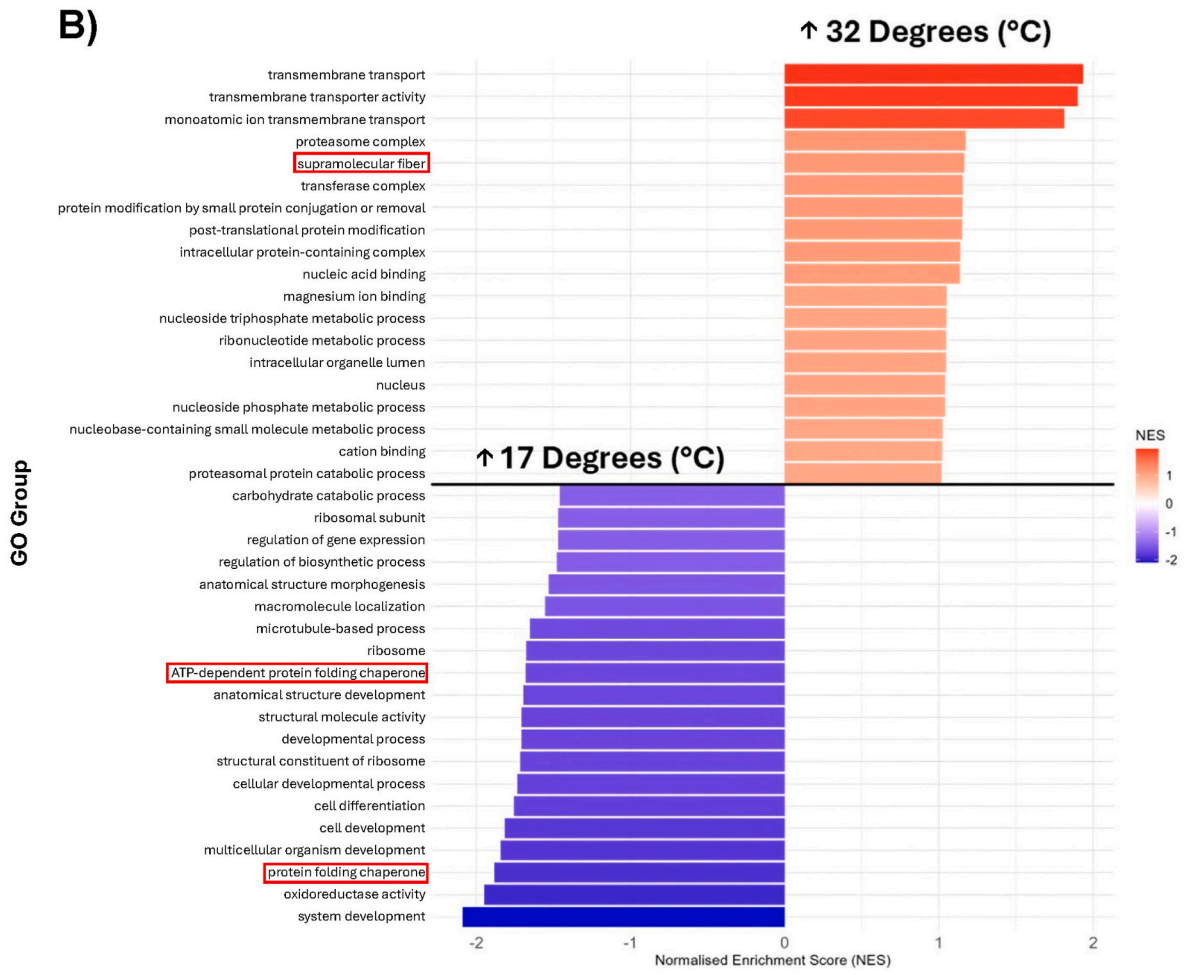

Fig. 3. (*continued*).

nucleoside phosphate catabolic process GO groups. At 28 °C, the major allergen arginine kinase (AK) was a core-enriched protein within the extracellular region GO group. No allergenic proteins were identified as core-enriched in the structural or ribosomal GO groups at this temperature.

In the GO group comparison between 28 °C and 32 °C (Supplementary Table D2), although statistical significance was lower than in other temperature contrasts, a few allergenic proteins were identified within core-enriched GO groups. At 28 °C, heat shock protein 70 (HSP70), a known allergen, was a core-enriched protein in the ATP-dependent protein folding chaperone GO group. At 32 °C, paramyosin (PM), another recognised shellfish allergen, was identified as a core-enriched protein in the supramolecular fibre GO group. No other allergens were identified as core-enriched in the remaining GO categories for this temperature comparison.

Several allergenic proteins were identified as core-enriched in GO groups between 17 °C and 32 °C (Supplementary Table D3). At 17 °C, the GO groups protein folding and protein maturation included core-enriched proteins TPI, GAPDH, FBA, all registered or suspected allergens, alongside other metabolic enzymes. At 32 °C, the extracellular region GO group included arginine kinase (AK) as the most significantly core-enriched allergenic protein. No other allergens were core-enriched at 32 °C in the remaining top GO groups.

3.4. Temperature-based differential abundance of individual redclaw proteins

A label-free quantification (LFQ) analysis was performed to determine the effect of rearing temperature on the proteome and allergome of 12 redclaw protein extracts (four per temperature). MSstats cleaned protein data identified a total of 833 proteins quantified in the LC/MS analysis. Significant differentially expressed proteins were visualised using a volcano plot comparing protein Log2FC between temperature groups. Results using p-values identify various proteins as being significantly differentially expressed in all conditions (Fig. 4). 34 proteins with significant p-values <0.05 were identified between 28 °C and 17 °C (Fig. 4A), 47 significant proteins between 32 °C and 28 °C (Fig. 4B), and 72 significant proteins were identified between 17 °C and 32 °C (Fig. 4C).

Specific proteins identified as potentially implicated in significant differential enrichment were visualised (Fig. 5). When comparing 17 °C and 28 °C, allergenic lipocalin/cytosolic fatty acid-binding proteins, HSP70, PM and FABP were enriched at 17 °C. In contrast at 28 °C, filamin A isoform X4 was the only enriched allergen (Fig. 5A). Other proteins enriched at 17 °C included DNAJ protein homolog 1, F-actin capping protein, eukaryotic translation initiation factor 6, tropomodulin, etc., which were upregulated at 17 °C versus 28 °C. Whereas proteins such as catalase, ubiquitin-like domain-containing protein, 40S ribosomal protein S3, etc., were more enriched at 28 °C.

When comparing 28 °C and 32 °C (Fig. 5B), allergenic lipocalin/cytosolic fatty acid-binding protein was also more enriched at 28 °C, alongside Filamin A isoform X4 and forms of HC. Whereas at 32 °C, the only identified enriched allergen was HSP70, though at a smaller Log2FC. Other enriched proteins included many tyrosinase copper-binding domain-containing proteins, as well as hemolymph clottable protein at 28 °C. Whereas at 32 °C, NADP-dependent oxidoreductase

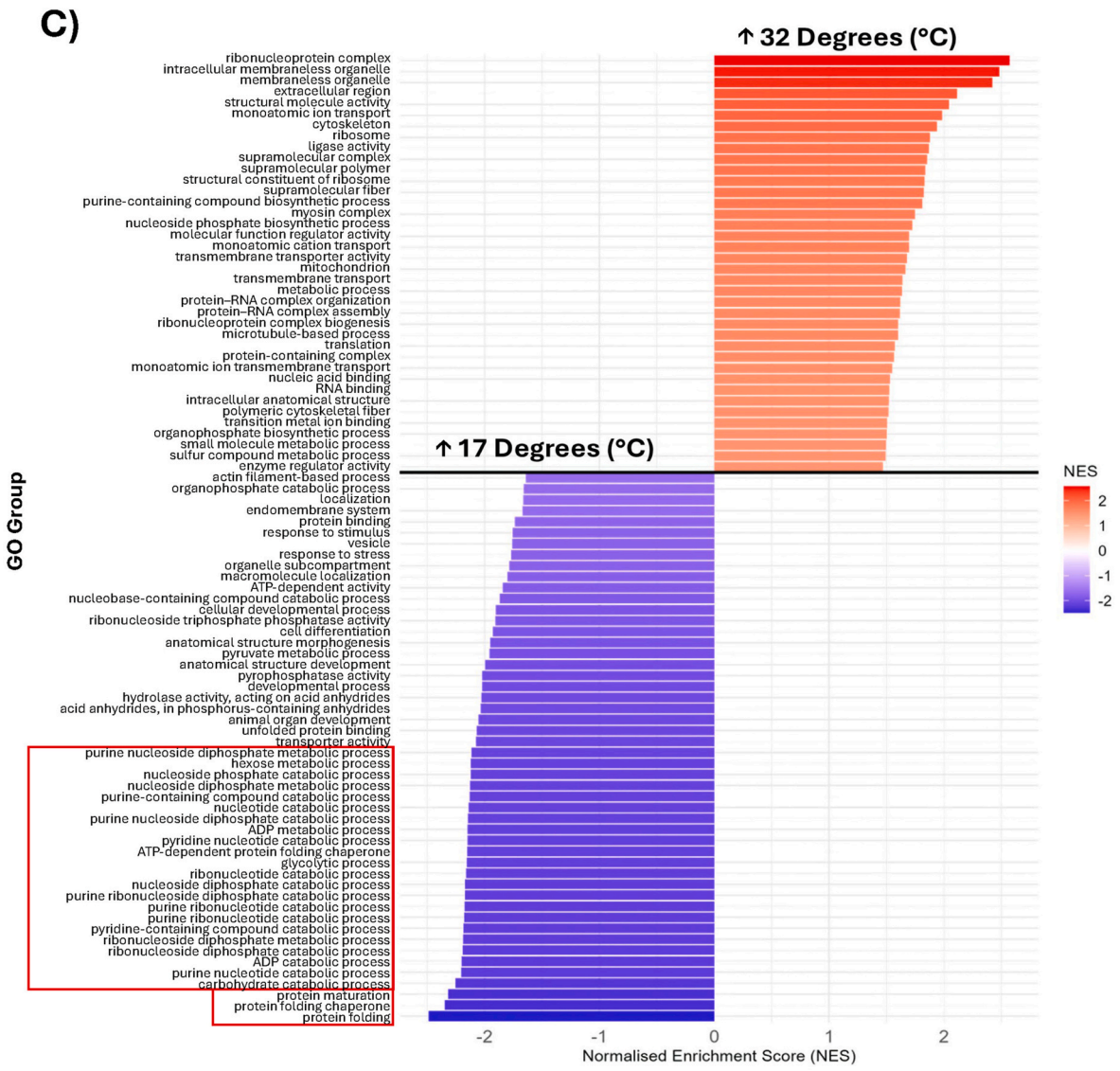

Fig. 3. (*continued*).

domain-containing protein, GAPDH, and ATP synthase subunits α and β, etc., were enriched.

The 17 °C and 32 °C comparison (Fig. 5C) identified enriched allergens at 17 °C, primarily HSP70 types and a HC subunit. Whereas at 32 °C, no allergenic proteins were found to be enriched. Other proteins enriched at 17 °C included calcyclin-binding protein, calcium-transporting ATPase, and hemolymph clottable protein-like, etc. At 32 °C, oplophorus-luciferin 2-monooxygenase non-catalytic subunit, actin, and catalase were enriched.

Multiple comparison adjustment was subsequently performed using the Benjamini-Hochberg Correction to decrease the FDR of significant differentially expressed proteins. Adjusted results identified no significantly expressed proteins after correction when comparing the acclimation temperatures, with all protein adj.pvalues calculated as being >0.05. Insignificant adj.pvalues indicate that increased statistical power is required (i.e., increased sample size, increased replicates) to effectively identify temperature-based proteome changes. The MSstats designSampleSize function was used to determine the number of biological replicates required to achieve the desired statistical power for future larger-scale analysis. As the effect temperature plays on protein expression was more subtle than hypothesised, a desired fold change of 1.5 was selected, alongside an FDR of 0.05 and a confidence power of 0.9 (false negative rate). Results indicate that approximately 33 replicates per temperature would be required in future studies.

4. Discussion

There have been few studies in the field of food allergy investigating the effect of environmental temperature on overall protein allergenicity, especially in the field of seafood allergy. The current study demonstrates that rearing temperature alters the allergen abundance, protein expression and GO group enrichment of redclaw (*C. quadricarinatus*), as well as induces differences in crustacean-allergic subject IgE-binding. These findings provide compelling evidence that rearing temperature can directly influence the allergenic potential of shellfish.

Temperature-driven changes in protein abundance, including allergen abundance and IgE-binding observed in this study, are likely due to changes in fundamental biochemical and physiological processes, as many shellfish allergens play key roles in muscle-related processes, metabolism, and stress responses. Such processes have been proposed to be influenced by changes in temperature, including altered metabolic rate, immune and stress responses, protein folding and antioxidant properties [40–43]. To our knowledge, this is the first study to investigate the impact of environmental temperature changes on the allergen

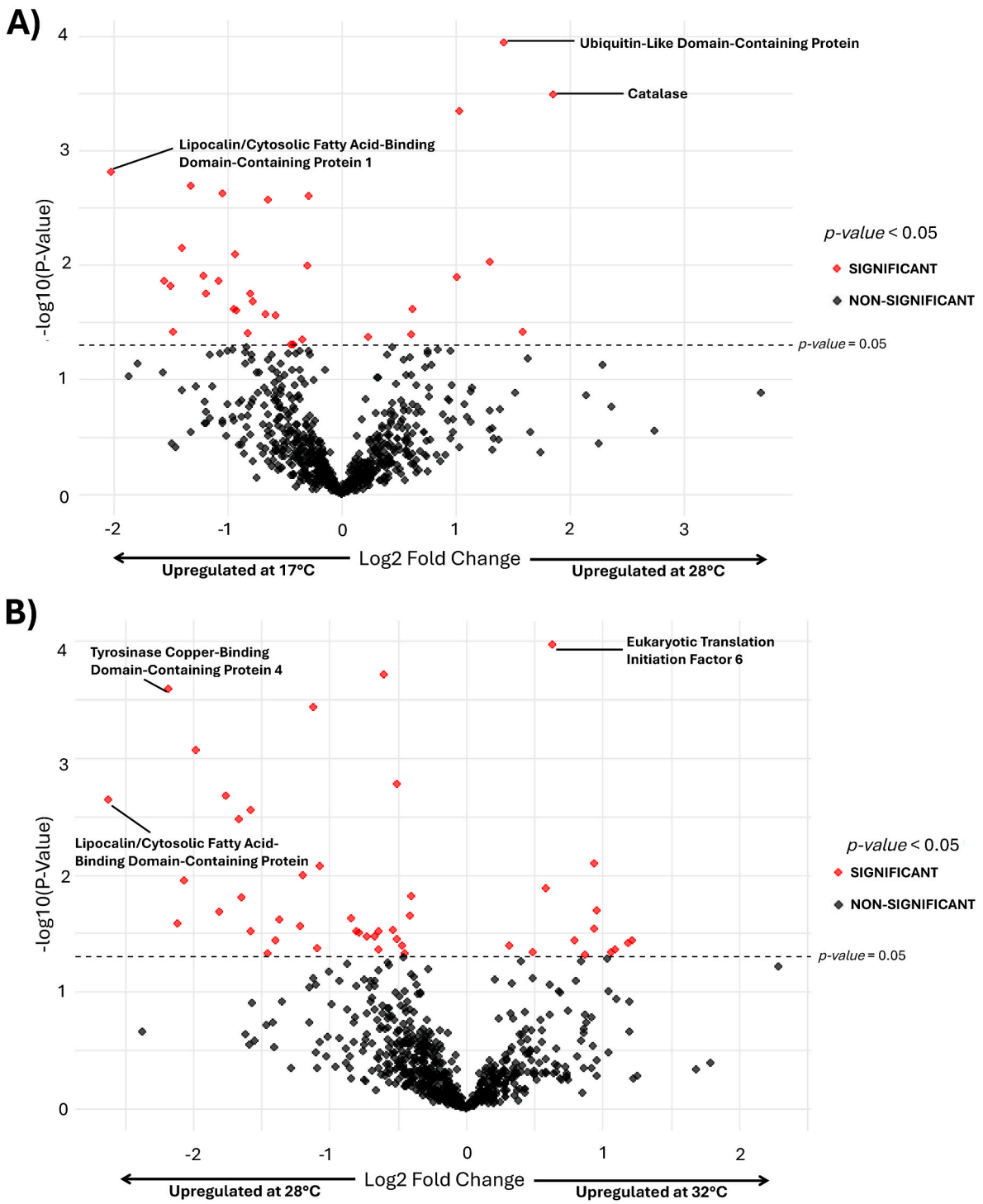

Fig. 4. Volcano plots comparing protein log2-fold changes (Log2FC) between each condition A) 28°C and 17°C, 17°C, B) 32°C and 28°C, and C) 17°C and 32°C. Proteins with a negative Log2 fold change (to the left) on the y-axis are more abundant at the colder temperature in the comparison, and a positive Log2 fold change (to the right) are more abundant at the warmer temperature. Proteins above the dashed line, the **p-value** cutoff at $-\log10^{(0.05)}$, are considered statistically significant ($p < 0.05$), and below non-significant ($p > 0.05$). Notable differential protein outliers are labelled.

and proteomic profile in an animal-derived food source.

Climate change has been shown to profoundly affect allergic disease through environmental exposures, particularly inhalant allergies via pollen and fungal spores [44]. Effects include an increase in pollen allergenicity, prolonged pollen seasons, and higher rates of allergic sensitisation, contributing to the development of conditions such as pollen-food allergy syndrome and asthma [45–48]. Climate change is also predicted to alter the geographical distribution of aeroallergens, expanding the population at risk [45]. Despite limited research currently in the field of food allergy, research has shown that climate change also has direct implications for plant food allergens. Temperature changes directly affect plant metabolism, geographical range and the induction of environmental stress-related proteins [47,49], which can potentially include allergens. In peanuts, temperature and CO_2 stress reduce flower

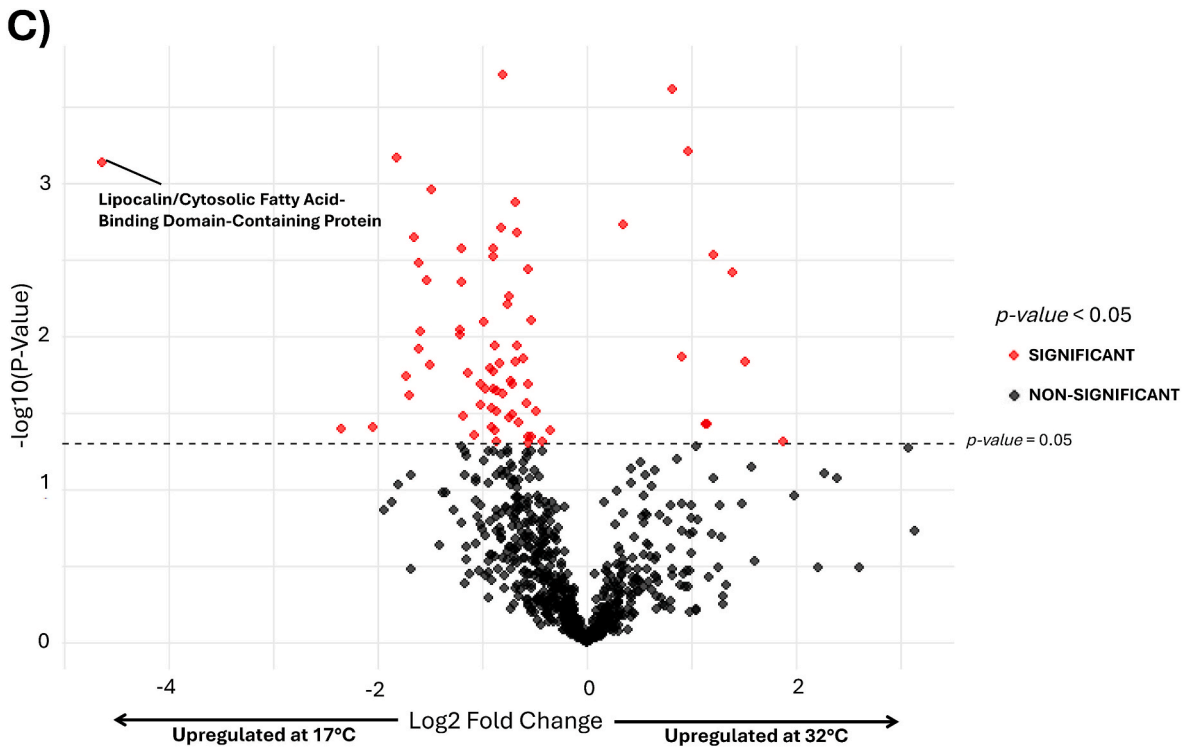

Fig. 4. (*continued*).

production, the proportion of flower-forming fruits [49,50], seed yield and pollen viability [47,49,51]. Similarly, fungal allergy research has demonstrated that environmental growth conditions influence the allergenicity of *Aspergillus fumigatus* through the differential production of allergenic proteins [52]. While current findings are largely plant-focused, the underlying principles of temperature-induced metabolic stress and stress-related protein expression are also likely applicable to ectothermic animal food sources such as shellfish.

In animal food allergy, including shellfish allergy, there has been no research outlining the effect of thermal changes on protein allergenicity. However, aquaculture research increasingly demonstrates that environmental temperature strongly influences molecular and physiological responses in aquatic species [1–4]. In the present study, major muscle-related allergens, including TM, MLC and TnC, were largely unaltered by rearing temperature. TM, MLC and TnC are accessory proteins in the actin-myosin complex, with TM and TnC regulating actin filament function, and MLC regulating myosin activity [53,54]. Their stability across temperatures suggests that core muscle contractile machinery is maintained despite thermal variation and stress. In contrast, PM, a muscle fibre protein and recognised allergen in molluscs [55–58] and mites [59], was increased at colder temperatures. PM contributes to the thick filament structure and the invertebrate 'catch' mechanism [56,60], which allows for sustained muscle tension with low ATP hydrolysis rates [56,60,61]. Increased PM abundance at 17 °C may therefore maintain and support structural stability and energy conservation under cold-induced metabolic stress in redclaw.

Allergens involved in chaperone folding (HSP70), lipid-binding (FABP), and catabolic/metabolic processes (GAPDH, TPI) were more affected by temperature. HSP70 was consistently upregulated at 17 °C, reflected in both differential protein abundance and enriched GO groups. HSP70 is a highly conserved chaperone protein responsible for maintaining protein homeostasis during periods of cold or heat stress by assisting protein folding and assembly, preventing aggregation, regulating apoptosis, and modulating immune responses [28,62,63]. Upregulation of HSP70 at lower temperatures indicates the induction of a cold-stress response and suggests reduced resilience of redclaw to colder environments when compared to warmer conditions. Similar findings have been reported in other crustaceans, including *L. vannamei*, where HSP70 was found to be upregulated in the hepatopancreas in response to acute cold stress at both a protein and transcript level [64,65]. HSP70 has also been identified as an allergen in mite and insect species [66–68] and proposed as a putative shellfish allergen [69].

Another protein of interest was FABP, a registered allergen in shellfish [70–72] and mite [71,73–75], also had an increased abundance at lower temperatures. FABPs facilitate intracellular lipid transport and play a crucial role in membrane fluidity, with membrane fluidity decreasing at low temperatures [76–78]. Increased FABP abundance at 17 °C likely reflects lipid metabolism adaptation to restore membrane fluidity during cold stress. Other studies involving redclaw have also identified a rise in FABP abundance alongside lipid levels, suggesting that FABP presence is crucial for efficient utilisation of lipids and dietary fats during periods of environmental stress [79]. Similar temperature-induced shifts in lipid metabolism have also been reported in *P. clarkii* [80], where cold exposure altered the synthesis of genes encoding long-chain fatty acid-CoA ligase 4-like, alongside an upregulation of fatty acid synthesis [80].

Additional temperature-responsive allergens included TPI and GAPDH, which are both enzymes involved in glycolysis and gluconeogenesis. TPI has been registered as a shellfish allergen in both shellfish [27,70,81–83] and mite [66,84]. GAPDH has been officially registered as an allergen in cockroach [85], and a putative shellfish allergen [69,86]. Results of the current study similarly identified an upregulation in metabolic and catabolic GO groups at 17 °C, suggesting the induction of a long-term metabolic stress response. Upregulated GO groups also included those involved in hexose metabolism, indicating an increase in sugar production to maintain energy production and fuel alternative pathways [80,87]. As well as various types of nucleoside diphosphate metabolism and catabolism, potentially indicating nucleoside breakdown and recycling in response to stress-induced damage or metabolic dysregulation [88]. Similar altered metabolic processes under cold stress have been observed in *M. rosenbergii* [89] and *Procambarus virginalis* [90], in which glycolytic and alternative energy pathways were

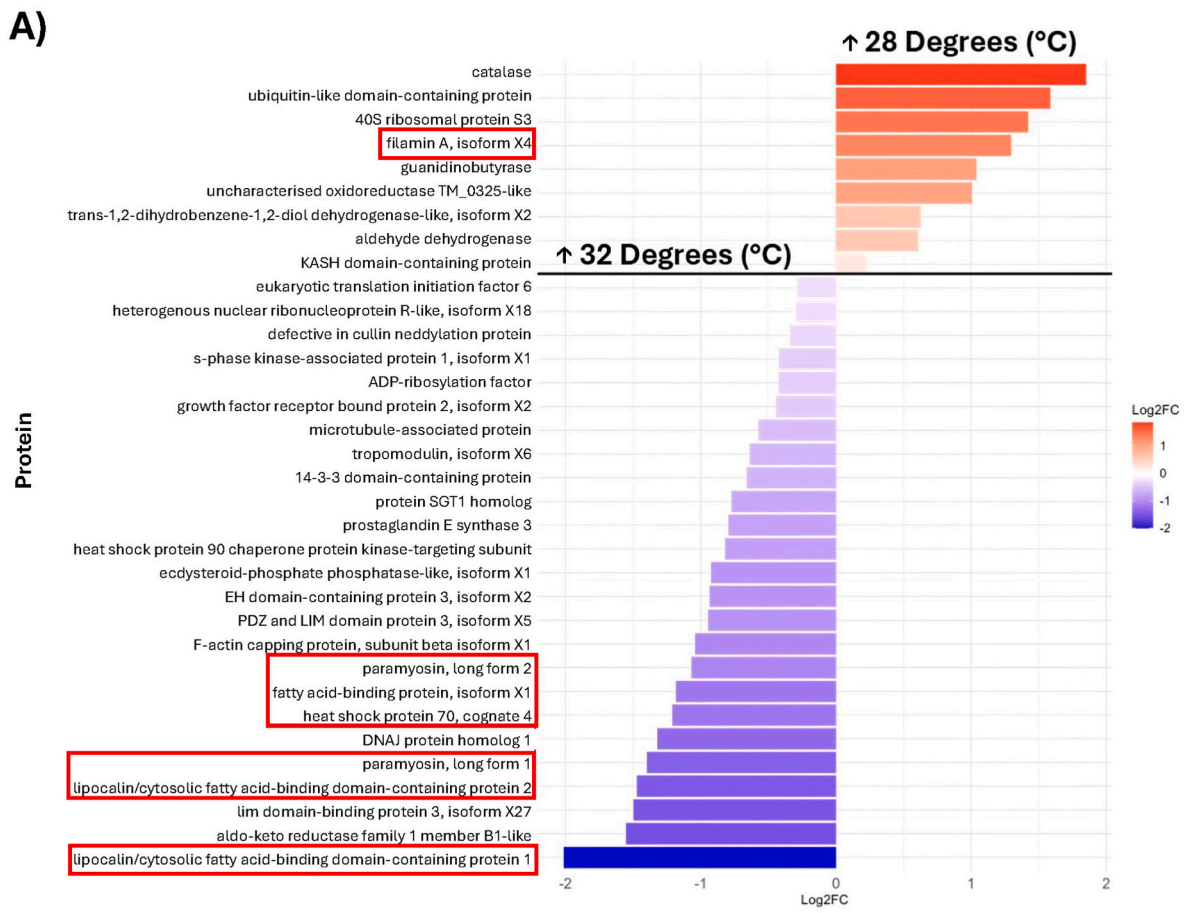

Fig. 5. Significantly abundant proteins (p-value < 0.05) between A) 17°C and 28°C, B) 28°C and 32°C and C) 17°C and 32°C. Proteins with a negative (seen in blue) Log2FC score indicate increased abundance at the colder temperature. Proteins with a positive (seen in red) Log2FC indicate increased abundance in the warmer temperature. The higher the positive or negative Log2FC, the greater increase in protein abundance at the warmer or colder temperature, respectively. Red boxes are indicative of any allergens with an increased abundance between conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

identified to be upregulated to compensate for reduced metabolic efficiency and hypoxia at low temperatures [89,90]. Therefore, under cold rearing or translocation conditions, redclaw energy production may switch to anaerobic forms such as glycolysis to counter a slower metabolism and hypoxia [90].

Despite minimal changes in major muscle allergens (TM, MLC and TnC), IgE-binding results identified an overall increase in IgE-binding to extracts from redclaw reared at 17 °C and decreased binding at 32 °C. The specific biochemical mechanism behind this overall increase in IgE-binding remains unclear, with further investigation needed to confirm or refute current findings. The altered abundance of metabolic enzymes and chaperone proteins may support current findings through the alteration of metabolic processes and protein synthesis/folding. Redclaw reared at 32 °C were able to tolerate warmer rearing temperatures much more than those reared at 17 °C, as evidenced by the increased abundance of stress-related proteins at the colder temperature. Compared to redclaw reared at 28 °C, 32 °C also seemingly improved metabolism, reduced induction of stress-related proteins and lower expression of allergens HSP70, GAPDH, TPI and FABP. Conversely, cold stress at 17 °C induced chaperone proteins such as HSP70, suggesting increased protein misfolding, protein structural changes or partial unfolding, which may increase exposure to linear allergen epitopes binding IgE-antibodies. At warmer temperatures, results identified a lack of stress-related proteins, thus, improved protein folding, potentially reducing exposure to linear allergen epitopes. Whilst most studies focus on how thermal food processing (e.g., cooking) affects protein allergenicity and allergen profiles through heat-induced unfolding [91–94], the present findings suggest that cold-stress may similarly alter allergen profiles and IgE-reactivity, particularly to enzymatic allergens, though different biochemical mechanisms.

As redclaw aquaculture expands globally, findings suggest that colder rearing conditions may enhance allergenicity. However, as redclaw are native to warm Northern Australia [15], temperature effects may be species and region-specific. Despite a lack of studies investigating temperature changes, research has been performed investigating the effect of geographic region and origin (farmed or wild caught) on shrimp species *P. monodon* [95]. Location and origin were found to affect both allergen IgE-binding and allergen diversity greatly [95]. Major allergens such as TM demonstrated up to a 13-times difference in abundance between locations and origins, whilst other allergens remained stable (e.g., MLC) [95]. In contrast, the present study identified broad changes in overall allergenicity rather than altering specific allergen levels, suggesting that temperature may physiologically drive altered allergenicity across regions, whereas geographic variation may selectively influence specific allergens. Population-dependent IgE--binding patterns have also been reported in plant food allergy, including peanut allergy, in which sensitisation profiles of allergic subjects differ greatly between regions [96]. Together with findings from the *P. monodon* study [95] and the current study, these results highlight the importance of further investigation into climate, species and regional

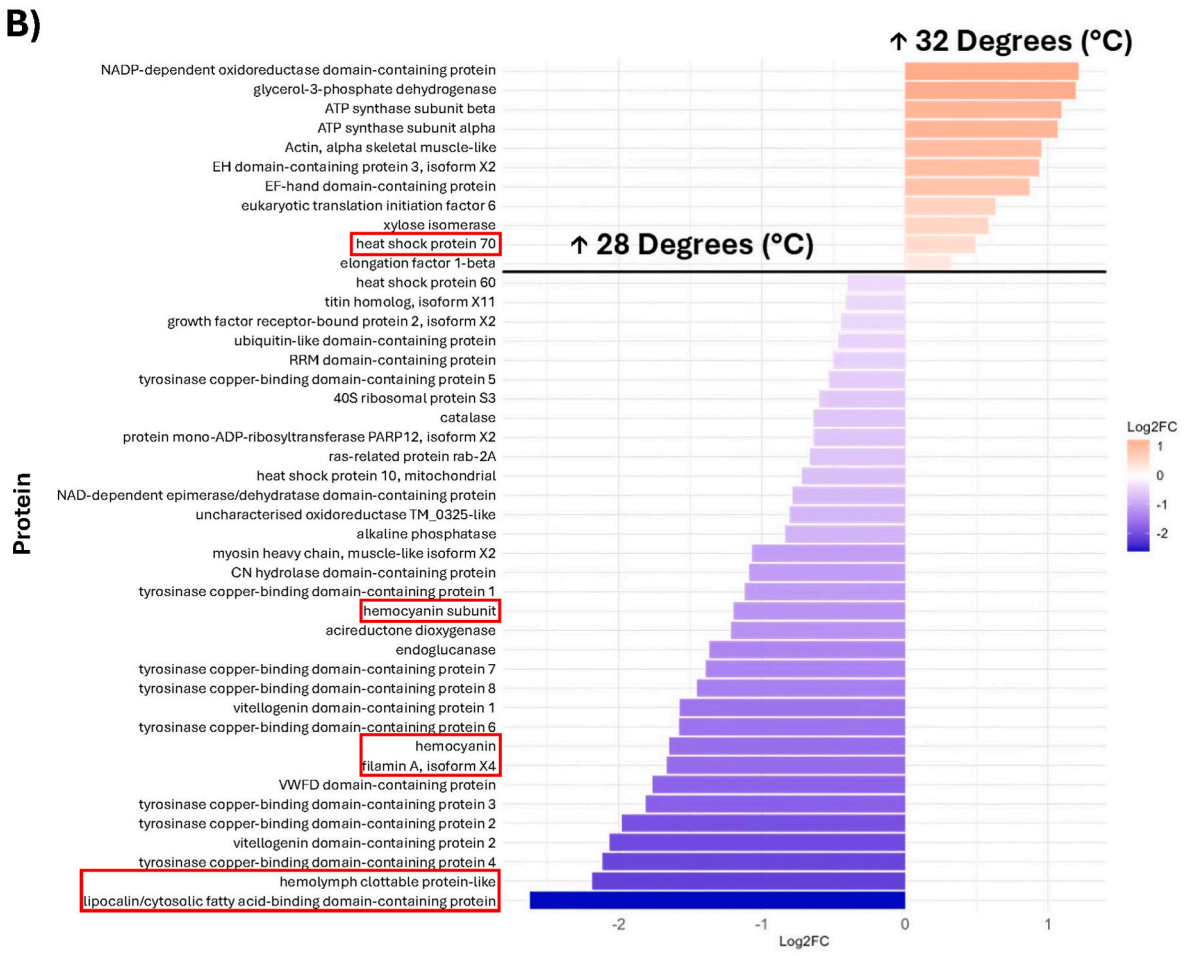

Fig. 5. (*continued*).

influences on subject IgE-patterns and protein allergenicity.

Although the current study yielded important foundational results, it was subject to limitations due to a lack of previous research and published literature on the topic. Due to a smaller effect size than anticipated, the number of replicates used was not sufficient when determining significant differences in the differential abundance of individual proteins. A larger number of replicates would be required in future studies to document individual protein differential abundance between rearing temperatures in further detail. As a result of a low replicate number in the current study, the adjusted p-value differential expression data comparing individual proteins was not significant (adj. pvalue <0.05), and raw p-values were used to determine broader scale trends in overall protein expression. Data from GO groupings were statistically significant (FDR <0.05) upon adjustment for the 17 °C versus 28 °C comparison and the 17 °C versus 32 °C comparison. However, the GO comparison between 28 °C and 32 °C did not meet significance thresholds, and thus, GO group changes between these two temperatures may not be as critical as changes present in the lower temperature comparisons. Although clear results indicate an upregulation of IgE-binding intensity at 17 °C and downregulation at 32 °C, more research needs to be performed on larger sample sizes with multiple replicates to accurately validate the initial results and demonstrate reproducibility in other crustacean species.

5. Conclusion

This study investigates how the aquaculture rearing temperature of a globally consumed crayfish species affects the expression of shellfish allergens with direct implications for allergic individuals. This study identified clear, temperature-dependent changes in IgE allergen binding, allergen abundance and protein expression, resulting in differential GO group enrichment. To our knowledge, this is the first study to examine the effects of environmental temperature on the allergenomic and proteomic profile of an animal-derived food source. This research fills a critical gap in food allergy research, particularly within the underexplored fields of seafood allergy and aquaculture. Our study emphasises the urgent need for further investigation into how climate change and species translocation may alter allergen profiles in food sources and subsequent allergenicity. As global temperatures change and aquaculture expands, understanding these interactions will be essential for allergy risk assessment, consumer protection, and the development of climate-resilient food systems.

Ethics statement

Ethics approval was not required for the conduct of this study on crustaceans by the James Cook University, Animal Ethics Committee. Serum was taken from all subjects with informed consent and sera use for this study was approved by The Ethics Committee of James Cook University, Approval Number: H4313 and H6829.

CRediT author statement

Emily Jerry: Conceptualisation, data curation, formal analysis, investigation, methodology, visualisation, writing – original draft, writing – review & editing. **Shaymaviswanathan Karnaneedi**: Methodology, writing – review & editing. **Sahel Heidari**: Methodology, writing – review & editing. **Kelly Condon**: Conceptualisation, project

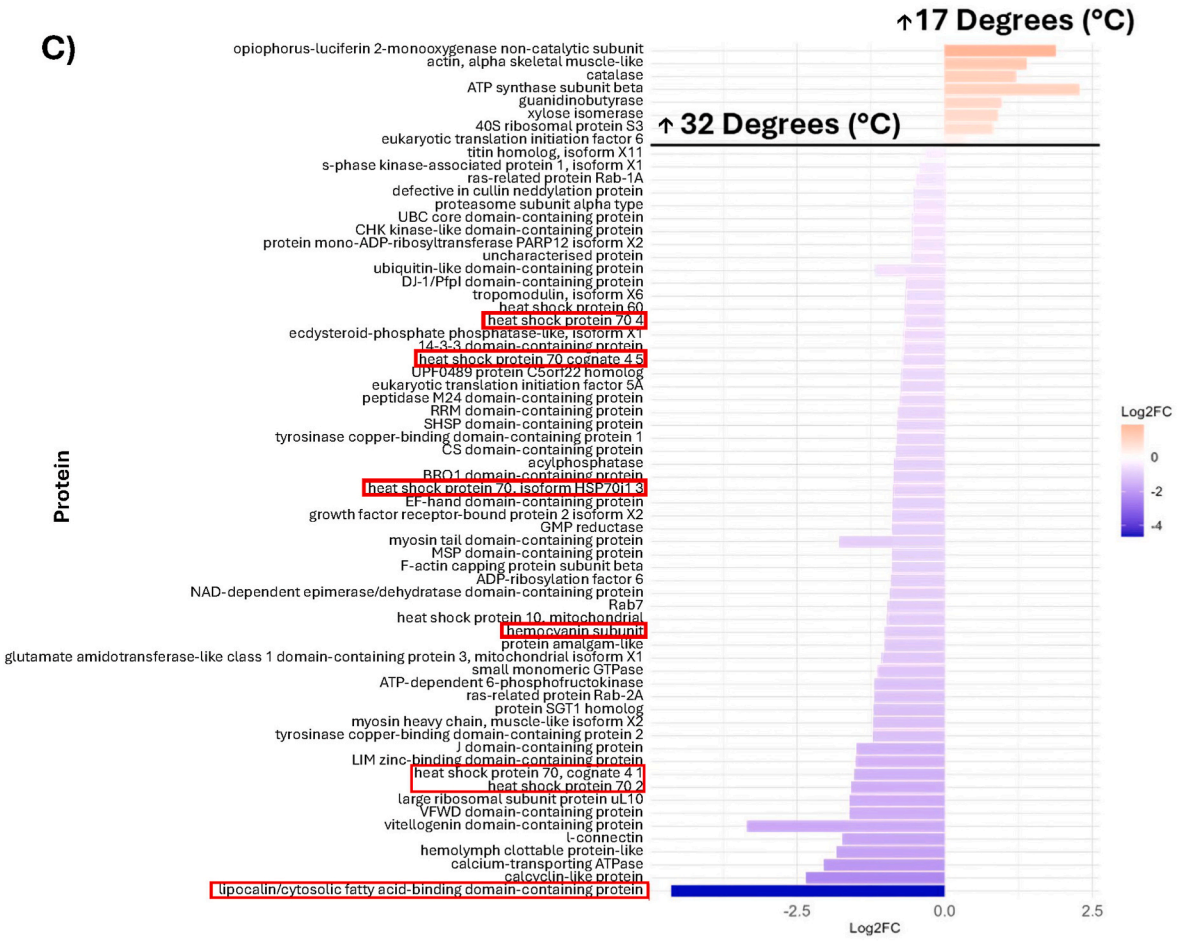

Fig. 5. (*continued*).

administration, supervision, writing – review & editing. **Andreas L. Lopata**: Conceptualisation, funding acquisition, project administration, supervision, writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2026.111289.

Data availability

Data will be made available on request.

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