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ORIGINAL ARTICLE

Rapid detection of heat stress biomarkers in Atlantic salmon (*Salmo salar*) liver using targeted proteomics

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Abstract

Most fish are ectothermic; therefore, their physiology is significantly affected by temperature. Aquaculture fish have limited ability to avoid elevated water temperatures, with impacts increasing as a result of climate change. To date, quantifying gene expression has been proposed to monitor heat stress in salmon liver. This study aimed to establish a faster multiplexed proteomics method to measure the abundance of thermal stress biomarkers in liver of salmon reared at 15°C or 20°C. Moreover, this study aimed to determine the effects that sample pooling, and data normalisation using housekeeping (HK) protein peptides would exert over the statistical significance of these thermal stress markers. A multiple reaction monitoring (MRM) mass spectrometry method, comprised 45 peptides derived from thermal stress markers and 10 peptides from HK proteins, was applied to measure these markers in liver of salmon reared at 15°C or 20°C. When samples were processed individually, 34 peptides were significant between salmon livers at 15°C or 20°C. In pooled samples, this decreased to five significant peptides. Peptides hprt1_HYADDLDR (hypoxanthine phosphoribosyl transferase) and gapdh_VPTPNVSVVDLTVR (glyceraldehyde-3-phosphate dehydrogenase) were the most stable and unstable HK protein peptides, respectively. When data was normalised with hprt1_HYADDLDR, 16 peptides were significant in individual samples and 13 in pooled samples. Significant peptides serpinh1a_ADLSNISGK, SerpinH1_TNSILFIGR, ela2_VVGGEDVR and gapdh_VPTPNVSVVDLTVR were common regardless of data strategy. A fast and reliable MRM method was established to validate thermal stress markers in salmon liver, where individual samples yielded better results than pooled samples. Sample pooling was only better when combined with normalisation as it validated twice the number of markers than sample pooling alone. This method could be applied to monitoring stress response in experiments involving feeding additives designed to mitigate thermal stress or in selective breeding programs to help understanding family variance in thermal tolerance.

KEYWORDS
climate change, protein, salmon, stress

1 | INTRODUCTION

Increased water temperatures as one effect of climate change directly affects marine and aquaculture fish beyond their optimal temperature range with detrimental effects for physiology and homeostasis (Neuheimer et al., 2011). Salmon grows fastest in seawater temperatures in the 16–18°C range (Calado et al., 2021; Johansson et al., 2006). In southern Australia, summer heatwaves have increased seawater temperature beyond salmon's optimal range, leading to reduction in feed intake, compromised osmoregulation, flesh decolouration, condition factor and hepatosomatic index in farmed salmon (Nuez-Ortín et al., 2018; Wade et al., 2019). Several studies have employed gene expression (Akbarzadeh et al., 2018; Houde et al., 2019), multigene expression array (Krasnov et al., 2020), transcriptomics (Beemelmanns et al., 2021b; Olsvik et al., 2013) and bottom-up proteomics (Nuez-Ortín et al., 2018) and identified markers that respond to heat stress in salmonids exposed to high temperature. A transcriptomic study in the liver of salmon exposed to temperature increase and hypoxia reported several genes involved in heat shock response, stress response, immune response, and cellular metabolism. Some of these markers included heat shock proteins (HSP), serpin and scavengers of reactive oxygen species (ROS) (Beemelmanns et al., 2021b). Serpin and HSP (HSP90 and HSP70) have also been reported as biomarkers for heat stress in gill of sockeye salmon in two independent thermal stress experiments (Akbarzadeh et al., 2018). A different study that used proteomics identified 276 proteins with differential abundance between salmon reared at 15°C and salmon at 21°C and determined that transcription, translation, protein degradation and cytoskeletal components were compromised in salmon at high temperature (Nuez-Ortín et al., 2018). Such studies have been valuable to identify the impacts of increased temperatures on Atlantic salmon, with increased or decreased expression of several candidate genes proposed as biomarkers of thermal stress.

Normalisation using housekeeping (HK) genes is commonly used in studies of gene expression to remove variation derived from sample preparation and instruments. Selection of a suitable gene for normalisation will depend on the gene stability across the conditions of the experiment, meaning that abundance between controls and treatments samples should not statistically differ. Currently, the suitability of a gene can be determined through several methods including NormFinder (NF) (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004) and ΔCt (Silver et al., 2006) wherein algorithms use sample variation at different levels (standard deviation, coefficient of variance [CV]) to select the most suitable gene. In mass spectrometry (MS)-based proteomics data, biases derived from sample preparation and instrumentation are often corrected by total ion current (TIC) (Deininger et al., 2011; Wulff & Mitchell, 2018) and data transformation (Callister et al., 2006; Chawade et al., 2014; Graw et al., 2020; Karpievitch et al., 2012; Sticker et al., 2020; Välikangas et al., 2016; Wang & Yang, 2019). Instrument performance is often monitored with QC injections comprised of tryptic digested beta-galactosidase peptides (Schilling et al., 2012). In proteomics, the use of HK proteins for data normalisation, as occurs in gene expression, remains largely unexplored.

Sample pooling strategies are employed to reduce biological variation, when the amount of initial material is limited or to rationalise the total number of samples in large experiments (Diz et al., 2009; Molinari et al., 2018). This is particularly true for aquaculture experiments where hundreds of samples are collected across experiments or pooling within tank to eliminate pseudo-replication (Lazic et al., 2020). Although sample pooling has been shown to be a valid procedure (Diz et al., 2009), other studies have demonstrated that pooling procedures can lead to a false selection of biomarkers in top-down proteomics (Molinari et al., 2018).

Here, we established a rapid multiplexed MS-based method to quantify and validate the abundance of thermal stress biomarkers in liver of salmon reared at 15°C and 20°C using equivalent protein peptides, from here referred to only as peptides. The effects of sample pooling and data normalisation using HK peptides were also investigated.

2 | MATERIALS AND METHODS

All experimental procedures were approved by the CSIRO Animal Ethics Committee (QAEC 2021-11) and conducted according to the Australian code for the care and use of animals for scientific purposes (NHMRC, 2013). Thirty post-smolt Atlantic salmon (*Salmo salar*) were weighed (average 106.8 ± 22.1 g) into each of eight replicate 500 L tanks, four tanks of each maintained as part of two independently recirculated systems. Four replicate tanks were reared at 15°C for 6 weeks as controls. In the other system, after 1 week at 15°C, the temperature was slowly increased at the rate of 0.5°C every 3–4 days until 20°C. All salmon were reared in a 12L:12D photoperiod and fed a commercial diet that was consumed at ~1% body weight per day. At the end of 6 weeks, four fish per tank (16 per treatment) were randomly selected and humanely euthanised by overdose in anaesthetic Aqui-S (0.2 mL/L) before liver samples from the distal tip of the large posterior lobe (Dutta, 1996) were collected, snap frozen on dry ice then stored at −80°C prior to further protein analyses. Performance data is calculated using the average of four fish per tank and statistical significance attributed using the average of four tank replicates.

Liver (50 mg) was place in 300 μL (50 mM dithiothreitol; 2% sodium dodecyl sulfate; 100 mM Tris–HCl pH 7.8) and mechanically disrupted for 30 s at 5000 rpm in a bead beater (Precellys 24, Bertin technologies). Protein homogenates were centrifuged (14,000 × *g* for 30 min at

4°C) and clear supernatants transferred into new tubes. Total protein was quantified by Bradford reagent. Individual samples were prepared using 200 μg of total protein derived from the liver of each independent fish. Pooled samples were prepared using 50 μg of total protein per fish liver from a particular tank for a final total protein amount of 200 μg. Protein reduction, alkylation and protein digestion using trypsin were carried out in 10 kDa filters as described in (Wiśniewski et al., 2009). Resulting tryptic peptides were vacuum dried before resuspension in 50 μL of 0.1% formic acid for an approximate concentration of ~4 μg/μL. Tryptic peptides (3 μL) were chromatographically separated on a Shimadzu Nexera UHPLC and analysed on a 6500 QTRAP mass spectrometer (SCIEX) equipped with a TurboV ionisation source operated in positive mode. The MS parameters were ionspray voltage (IS) 5500 V, curtain gas 35, GS1 35 and GS2 set to 40, source temperature 500°C, declustering potential set to 80 and entrance potential set to 10. Relative quantitation was achieved using scheduled multiple reaction monitoring (MRM) scanning experiments using a 30 s detection window for each MRM transition with retention time as determined in the preliminary MRM experiment and a 0.3 s cycle time. Injections of beta-galactosidase (UniProt: P00722) GDFQFNISR peptide were used to monitor instrument performance. Analyst 1.7.1 software was used for data acquisition. Skyline software was used to develop the MRM transition method as indicated in the tutorial 'Skyline Targeted Method Editing' using protein sequences for previously published heat stress biomarkers (Beemelmanns et al., 2021a; Beemelmanns et al., 2021b; Nuez-Ortín et al., 2018) downloaded from UniProt (Table 1). The *S. salar* UniProt protein database (downloaded:20201120) and salmon liver data-dependent acquisition acquired information (Rusu et al., 2022) were used to build the MRM transition method. A repository of common protein contaminants, normally incorporated during sample preparation, named the common repository of adventitious proteins (cRAP) database was appended to the main salmon database. Peptide transition peaks were integrated in Skyline software. Data wrangling and visualisation of CV and histograms were carried out in R software using the Tidyverse packages. Principal component analysis (PCA) and partial least-square discriminant analysis (PLS-DA) and peptide fold-change analysis were determined in SIMCA 16. Where applicable at least two transitions per peptide were summed to represent relative peptide abundance. Peptides derived from candidate heat stress biomarkers and HK proteins are described in Table 1. Details of peptide transitions for stress and HK proteins are indicated in Table S1.

3 | RESULTS

Final weight of sampled fish in the high- and low-temperature treatments was 153 ± 15.8 and 158 ± 12.3 g, respectively. *T*-test indicated no significant differences between the two groups in performance metrics such as final weight, weight gain, condition factor or specific growth rate (data not shown). Measurement of beta-galactosidase GDFQFNISR peptide transitions reported a CV ~10% for instrument performance (Table S2).

3.1 | Effect of temperature over the abundance of stress markers measured in liver of Atlantic salmon

After acquisition of mass spectral datasets and integration of peak areas, analysis of variance by PCA using all peptide information showed a trending separation between fish reared at 15°C and 20°C (Figure 1a). PLS-DA variance analysis showed enhanced sample separation and tighter clustering in both individual and pooled fish samples (Figure 1b). In both figure panels, reduced biological variation was observed across pooled fish samples for both temperature treatments. PLS-DA also revealed that 34 peptides were statistically significant ($p \leq 0.05$) between individual fish (IR-15°C vs. IR-20°C), whereas only five peptides were identified as significant between pooled fish samples (PR-15°C vs. PR-20°C) (Table 2). The level of significance was higher at individual fish level compared with pooled fish.

3.2 | Stability of HK protein peptides

The peptides hprt1_HYADDLDR (hypoxanthine phosphoribosyl transferase) and gapdh_VPTPNVSVVDLTVR (glyceraldehyde-3-phosphate dehydrogenase) were identified as the most stable and unstable HK protein peptides, respectively, given their exerted effect upon CV dispersion (Figure 2a). Further effect over CV distribution between normalised and raw data can be observed in Figure 2b where normalisation using peptide gapdh_VPTPNVSVVDLTVR increased CV distribution beyond the level of raw data. The synergistic effect of sample pooling and normalisation was apparent in PN data where CV dispersion was significantly reduced to 13% from an initial 32.5% in IR data (Figure 2c).

Our strategy to determine reference protein stability was compared with algorithms and approaches routinely employed in gene expression analysis (Table 3). All results were consistent in identifying peptide gapdh_VPTPNVSVVDLTVR as the most unstable candidate, whereas hprt1_HYADDLDR was the most stable candidate identified by DeltaCT (Silver et al., 2006) and second best when NF (Andersen et al., 2004) was used (Table 3). For NF, peptide gapdh_GASQNIIPASTGAAK was the most stable candidate, whereas BestKeeper (Pfaffl et al., 2004) and DeltaCT ranked it second (Table 3) and third using our approach (Figure 2a).

3.3 | Effect of data normalisation in individual and pooled samples and its impact on abundance of stress markers in liver of Atlantic salmon

Following data normalisation using the most stable HK peptide (hprt1_HYADDLDR), PCA and PLS-DA analysis also showed treatment separation and reduction of biological variation in normalised pooled samples (Figure 3a,b). One outlier in each treatment cohort was observed (Figure 3a,b) but retained in further analyses as we expect outliers to exist in field experiments. PLS-DA analysis indicated that 16

TABLE 1 Peptides measured by multiple reaction monitoring (MRM) in liver of Atlantic salmon (*Salmo salar)* reared at 15 versus 20°C.

Accession	Protein name	Gene_target peptide
C0HAB6	Heat shock protein 90-alpha 1	hs90a_DQVANSAFVER
C0HAB6	Heat shock protein 90-alpha 1	hs90a_GVVDSEDLPLNISR
XP_014070438	Serpin H1-like	serpinH1_TNSILFIGR
XP_014070438	Serpin H1-like	serpinH1_SVLSADALK
ACN60208	Myeloperoxidase precursor, partial	MPO_EFLPFTNLK
ACN12619	Superoxide dismutase, mitochondrial precursor	sod2_GDVTAQVSLQPALR
ACN12619	Superoxide dismutase, mitochondrial precursor	sod2_DFGSFQALK
P49946	Ferritin heavy subunit	FerHS_IFLQDVK
ACN12259	Ferritin	FerMS_ILLQDIK
NP_001133774	Catalase	cat_EYPLIPVGR
NP_001133774	Catalase	cat_TFFTEVLNEEER
ACI69297	Glutathione S-transferase theta 1a	gstt1a_DGDFILTESVAILK
ACI69297	Glutathione S-transferase theta 1a	gstt1a_AIVPIITGSDVPK
ACI69603	Glutathione S-transferase P	GSTp_ATCVFGQLPK
ACI69603	Glutathione S-transferase P	GSTp_AFLDSDAYK
NP_001133440	Serpin H1a precursor	serpinh1a_IYSPSSVNFADAFVK
NP_001133440	Serpin H1a precursor	serpinh1a_ADLSNISGK
B5DFX7	Heat shock cognate 70 kDa protein	hspa8_DAGTISGLNVLR
B5DFX7	Heat shock cognate 70 kDa protein	hspa8_GTLDPVEK
NP_001134295	Peroxiredoxin-1	prdx1_ATAVVDGQFK
NP_001134295	Peroxiredoxin-1	prdx1_DIQLSDYIGK
ACI68294	Peroxiredoxin-6	prdx6_LSLLYPATTGR
ACI68294	Peroxiredoxin-6	prdx6_NFDEILR
ACI67534	Peroxiredoxin-4	prdx4_DYGVFLEDAGHTLR
ACI67534	Peroxiredoxin-4	prdx4_LVQAFQYTDK
ADM15936	Thioredoxin-depen peroxide reductase, mitoch precursor	trdx_DYGILLEGPGIALR
BT072408	Caspase-8 precursor putative mRNA, pseudogene cds	casp_IIDTYSR
BT072408	Caspase-8 precursor putative mRNA, pseudogene cds	casp_GDDILNILTQVNR
A0A1S3R063	Apolipoprotein D	apod_DPSEPAK
A0A1S3R063	Apolipoprotein D	apod_ESTLSEEK
XP_014045541	Transcription factor jun-D-like	jun-d_TQNTELASTASVLR
XP_014045541	Transcription factor jun-D-like	jun-d_EQVAQLK
XP_014027024	Tyrosine-protein kinase JAK2	jak2b_DILVER
XP_014027024	Tyrosine-protein kinase JAK2	jak2b_NILVESELR
A0A1S3QSD1	Heat shock 70 kDa protein	hsp1a1_VEIIANDQGNR
A0A1S3QSD1	Heat shock 70 kDa protein	hsp1a1_NSLESYAFNMK
A0A1S3KZA2	Eosinophil peroxidase	epx_IANVFATAAYR
A0A1S3KZA2	Eosinophil peroxidase	epx_NEAELGVVLNNR
P35031	Trypsin-1	try1_LGEHNIK
P35031	Trypsin-1	try1_VTEGSEQFISSSR
A0SEG1	Alpha-amylase	amy_YQPISYNLCSR
A0SEG1	Alpha-amylase	amy_LIDMGVAGFR
XP_013984108.1	Bile salt-activated lipase	bsal_TYSYLFSEPSR
XP_013984108.1	Bile salt-activated lipase	bsal_YLEINANMNK

(Continues)

TABLE 1 (Continued)

Accession	Protein name	Gene_target peptide
A0A1S3MF78	Chymotrypsin-like elastase family member 2A	ela2_VVGGEDVR
B5XG76[a]	Glyceraldehyde-3-phosphate dehydrogenase	gapdh_GASQNIIPASTGAAK
B5XG76[a]	Glyceraldehyde-3-phosphate dehydrogenase	gapdh_VPTPNVSVVDLTVR
B5DFU2[a]	Hypoxanthine phosphoribosyltransferase	hprt1_HYADDLDR
B5DFU2[a]	Hypoxanthine phosphoribosyltransferase	hprt1_SYCNDQSTGEIK
B5DFX8[a]	Phosphoglycerate kinase	pgk1_ACADPAAGSVILLENLR
B5DFX8[a]	Phosphoglycerate kinase	pgk1_ATQEQIDSFR
B5DG78[a]	ATP synthase subunit alpha	atp5a1_TGTAEVSSILEEK
B5DG78[a]	ATP synthase subunit alpha	atp5a1_TAIAIDTIINQK
B5DG94[a]	Peptidyl-prolyl cis-trans isomerase	ppia1_VYFDITIGDTPAGR
B5DG94[a]	Peptidyl-prolyl cis-trans isomerase	ppia1_VVEGLNIIASMEK

[a]Housekeeping proteins.

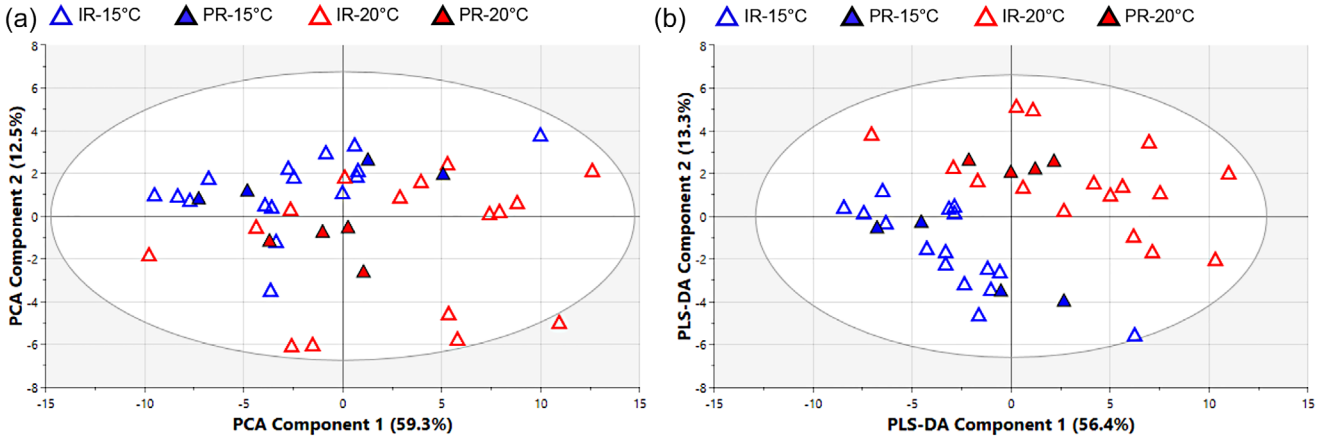

FIGURE 1 Analysis of variance by principal component analysis (PCA) and partial least-square discriminant analysis (PLS-DA): (a) PCA analysis of raw data and (b) PLS-DA analysis of raw data. Line ellipse in PCA and PLS-DA plots represents Hotelling's 95% confidence interval. IR, individual raw data; PR, pooled raw data.

peptides were significant ($p \leq 0.05$) between normalised individual fish samples at different temperatures (IN-15°C and IN-20°C) (Table 2). In the case of normalised pooled fish sample, 13 peptides were detected with significantly different abundances between temperatures (PN-15°C and PN-20°C). As occurred with non-normalised data, the level of significance was higher at individual level compared with pooled samples (Table 2).

Overall abundance of peptides reported in Table 2 indicated equivalent mean abundance between individual and pooled data (Figure 4a). Tables S3 and S4 showed no difference between individual and pooled samples in raw or normalised data. To increase the stringency of our results, when a twofold change in peptide abundance and *p*-value of 0.05 (SIMCA 16) was implemented. Results showed that peptides serpinH1_TNSILFIGR, serpin1a_ADLSNISGK and ela2_VVGGEDVR were highly significant between salmon reared at 15°C and 20°C regardless of data treatment (Figure 4b), with their abundance pattern remaining as a true effect of thermal stress (Figure 4c,f). Peptide gapdh_VPTPNVSVVDLTVR was also selected as an important marker as its fold-change was over 2 in PR and PN data, and 1.98 in IN data.

4 | DISCUSSION

4.1 | Biomarkers to measure heat stress in salmon

Here, we used MRM, a highly specific and sensitive high throughput MS technology, to rapidly measure and validate previously published biomarkers for heat stress in Atlantic salmon. A total of 34 of the 45 putative gene markers were validated on individual fish using a highly specific proteomics panel. These biomarkers included serpinh1a_ADLSNISGK and SerpinH1_TNSILFIGR as the most effective and responsive biomarkers to quantify the impact of heat stress. Additionally, we propose ela2_VVGGEDVR and gapdh_VPTPNVSVVDLTVR as novel biomarkers for heat stress.

TABLE 2 Targeted monitoring of stress markers in liver of Atlantic salmon (*Salmo salar)* reared at 15°C versus 20°C.

	Liver from salmon maintained at 15°C vs. 20°C			
Gene-peptide	**IR-15°C vs. IR-20°C**	**PR-15°C vs. PR-20°C**	**IN-15°C vs. IN-20°C**	**PN-15°C vs. PN-20°C**
serpinh1a_ADLSNISGK	*5.68E – 11*	*5.84E – 04*	*1.27E – 11*	*2.76E – 04*
serpinH1_TNSILFIGR	*2.08E – 11*	*9.15E – 04*	*1.80E – 11*	*6.39E – 04*
ela2_VVGGEDVR	*2.27E – 08*	*4.15E – 04*	*3.55E – 11*	*1.75E – 03*
gapdh_VPTPNVSVVDLTVR	*1.83E – 03*	*3.20E – 03*	*4.74E – 09*	*2.13E – 04*
hsp1a1_VEIIANDQGNR	*9.27E – 05*	*2.31E – 02*	*3.57E – 02*	*1.60E – 03*
bsal_YLEINANMNK	7.96E – 04	–	3.82E – 03	2.54E – 02
FerMS_ILLQDIK	1.35E – 03	–	1.06E – 02	3.88E – 02
prdx6_NFDEILR	1.29E – 03	–	1.47E – 02	3.78E – 03
mpx_EFLPFTNLK	1.37E – 02	–	3.01E – 02	1.67E – 02
hspa8_GTLDPVEK	3.33E – 03	–	3.73E – 02	5.37E – 03
prdx6_LSLLYPATTGR	6.33E – 04	–	2.01E – 02	–
mpx_IANVFATAAYR	6.77E – 03	–	2.16E – 02	–
amy_LIDMGVAGFR	4.17E – 03	–	4.47E – 02	–
hsp1a1_NSLESYAFNMK	–	–	1.93E – 03	–
gapdh_GASQNIIPASTGAAK	–	–	1.69E – 02	–
apod_DPSEPAK	–	–	3.54E – 02	–
gstt1a_AIVPIITGSDVPK	2.53E – 03	–	–	1.14E – 04
hspa8_DAGTISGLNVLR	2.94E – 02	–	–	3.70E – 02
prdx1_ATAVVDGQFK	3.10E – 02	–	–	2.22E – 03
amy_YQPISYNLCSR	2.26E – 03	–	–	–
atp5a1_TAIAIDTIINQK	5.63E – 03	–	–	–
atp5a1_TGTAEVSSILEEK	1.15E – 02	–	–	–
bsal_TYSYLFSEPSR	1.39E – 02	–	–	–
casp_GDDILNILTQVNR	1.50E – 02	–	–	–
gstp_ATCVFGQLPK	1.74E – 02	–	–	–
hprt1_HYADDLDR	1.99E – 02	–	–	–
hs90a_DQVANSAFVER	1.99E – 02	–	–	–
hs90a_GVVDSEDLPLNISR	2.47E – 02	–	–	–
jak2b_DILVER	2.52E – 02	–	–	–
junD_TQNTELASTASVLR	2.56E – 02	–	–	–
mpx_NEAELGVVLNNR	2.57E – 02	–	–	–
pgk_ACADPAAGSVILLENLR	2.87E – 02	–	–	–
ppia1_VVEGLNIIASMEK	2.93E – 02	–	–	–
ppia1_VYFDITIGDTPAGR	3.64E – 02	–	–	–
sod2_GDVTAQVSLQPALR	3.97E – 02	–	–	–
trdx_DYGILLEGPGIALR	4.21E – 02	–	–	–
try1_LGEHNIK	4.73E – 02	–	–	–

Note: Values are partial least-square discriminant analysis (PLS-DA) resulting *p*-values (≤0.05) following comparison of different temperatures and sample groups across raw or normalised (peptide hprt1_HYADDLDR) data. Italicised peptides were identified with a twofold-change difference in abundance. Abbreviations: IN, individual samples normalised (hprt1_HYADDLDR); IR, individual samples raw; PN, pooled samples normalised (hprt1_HYADDLDR); PR, pooled samples raw.

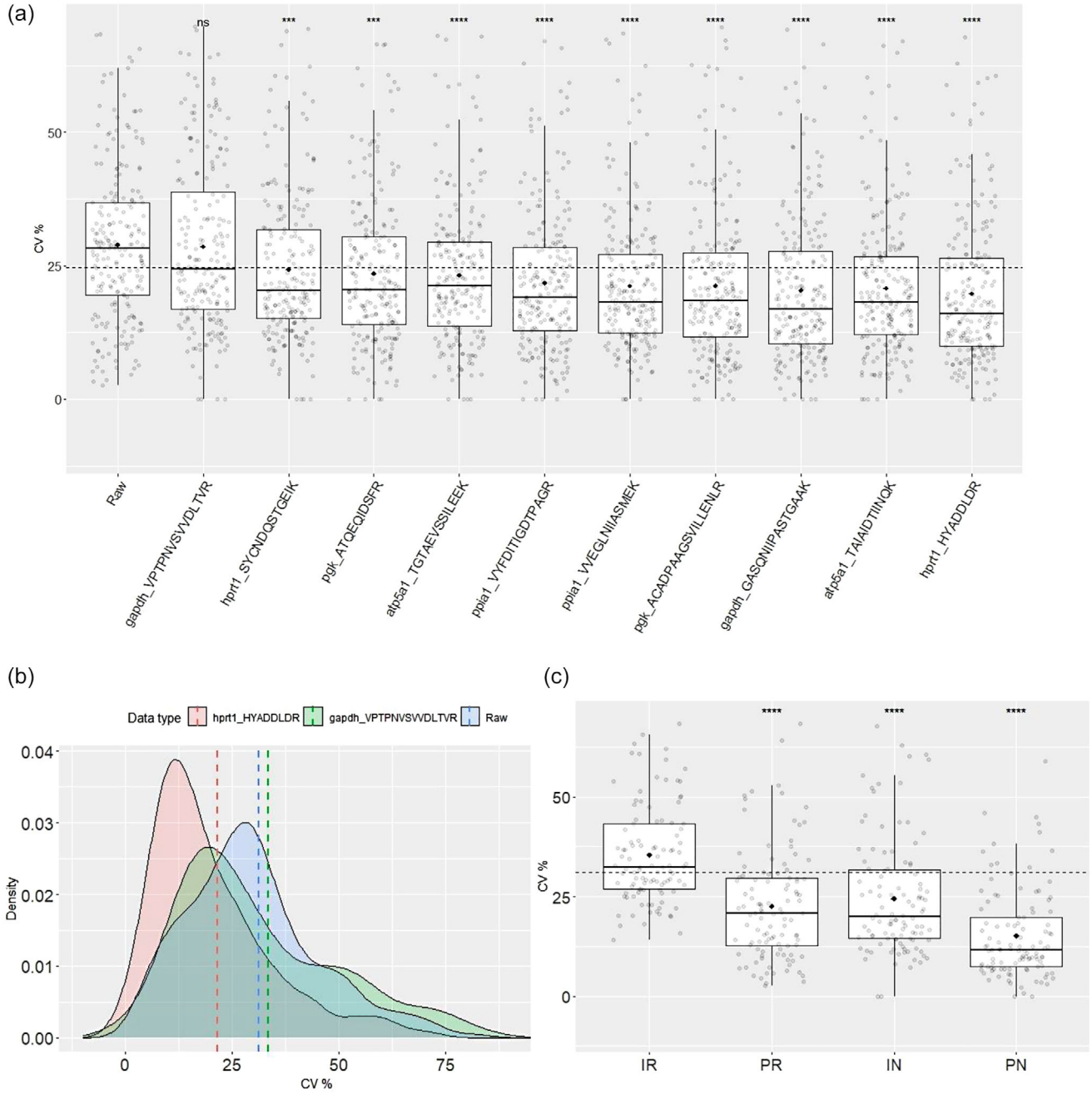

FIGURE 2 Visualisation of coefficient of variance (CV%) in raw and normalised data. (a) Spread of CV of experimental data following data normalisation with peptides derived from housekeeping proteins. Diamond inside boxplots indicate the mean CV cross all data. Grey dots represent all measured data across all peptides. Asterisks indicate level of ANOVA significance with raw data as reference group. (b) Density plot of CV for raw data and data normalised with peptides hprt1_HYADDLDR peptide and gapdh_VPTPNVSVVDLTVR. (c) Effect of pooling and normalisation with hprt1_HYADDLDR peptide, IR (individual raw data); PR (pooled raw data); IN (individual normalised data); PN (pooled normalised data). Grey dots represent all measured data across all peptides. The boxes and whiskers indicate the minimum value, the 25th percentile, the mean (diamond), the median (line), the 75th percentile and the maximum value. Asterisks in plots indicate level of significance in relation to IR data.

Serpin has recurrently been proposed as biomarker for heat stress across salmonid species using gene expression (Akbarzadeh et al., 2018; Houde et al., 2019), multigene expression array (Krasnov et al., 2020) and transcriptomics (Beemelmanns et al., 2021b; Olsvik et al., 2013). Serpin has been observed to increase as the temperature increases (Akbarzadeh et al., 2018; Beemelmanns et al., 2021a; Beemelmanns et al., 2021b; Wang et al., 2016) and known to be significantly abundant in salmonid species once the temperature rises to 16°C (Akbarzadeh et al., 2018). Consistent with previous research, our results indicated that both serpinh1a_ADLSNISGK and

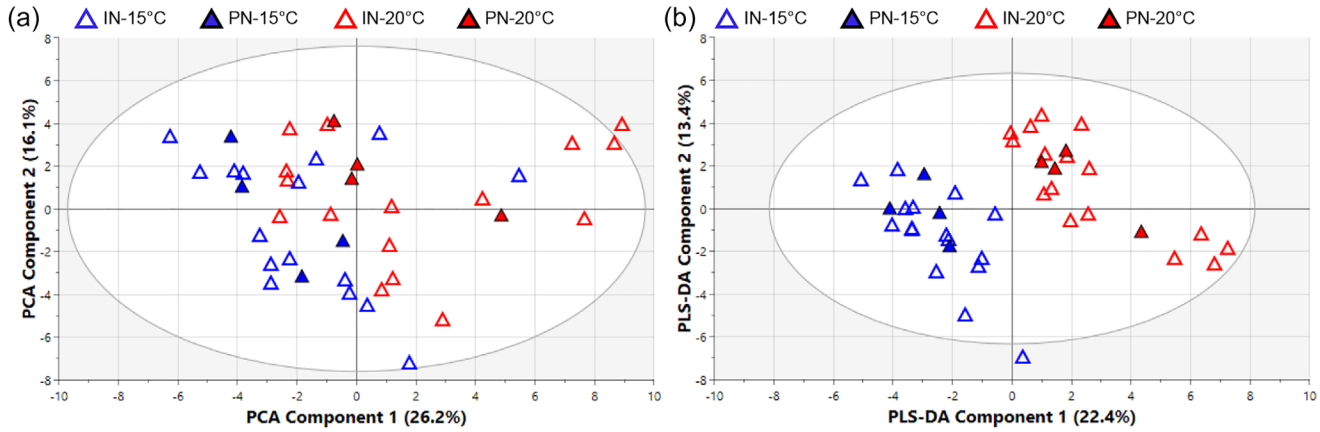

FIGURE 3 Analysis of variance by principal component analysis (PCA) and partial least-square discriminant analysis (PLS-DA): (a) PCA analysis of normalised data and (b) PLS-DA analysis of normalised data. Line ellipse in PCA and PLS-DA plots represents Hotelling's 95% confidence interval. IN, individual normalised data; PN, pooled normalised data.

TABLE 3 Ranking scores of reference housekeeping protein peptides by different approaches.

Reference protein_peptides	Delta CT[a]	NormFinder[b]	BestKeeper[c]
gapdh_VPTPNVSVVDLTVR	0.67	0.51	2.31 (0.53)
atp5a1_TGTAEVSSILEEK	0.35	0.18	1.38 (0.3)
pgk_ATQEQIDSFR	0.33	0.14	0.91 (0.19)
pgk_ACADPAAGSVILLENLR	0.31	0.15	1.41 (0.28)
atp5a1_TAIAIDTIINQK	0.30	0.13	1.41 (0.3)
ppia1_VVEGLNIIASMEK	0.29	0.15	1.55 (0.35)
hprt1_SYCNDQSTGEIK	0.29	0.12	1.34 (0.22)
ppia1_VYFDITIGDTPAGR	0.29	0.12	1.27 (0.29)
gapdh_GASQNIIPASTGAAK	0.26	0.05	0.98 (0.25)
hprt1_HYADDLDR	0.25	0.07	1.42 (0.26)

[a]Silver et al. (2006).
[b]Andersen et al. (2004).
[c]Pfaffl et al. (2004).

SerpinH1_TNSILFIGR peptides were significantly more abundant in heat stressed fish regardless of data normalisation or sample pooling. The gene *serpinh1a* is involved in collagen metabolism and has been detected at high levels in heat stressed *Danio rerio* (Lin et al., 2022), whereas *serpinH1*, also known as *HSP47*, is a molecular chaperone also associated to collagen metabolism (Ito & Nagata, 2017). Collagen has significant roles in fish muscle integrity and texture (Lin et al., 2022) and *serpinh1* provides stability to collagen molecules during thermal challenge (Akbarzadeh et al., 2018; Beemelmanns et al., 2021a). In rainbow trout, this gene has been shown to contribute to the elimination of ROS, where it was significantly more abundant in liver compared with head kidney, heart and spleen (Wang et al., 2016). Given that serpin abundance increases as temperature increases (Beemelmanns et al., 2021a; Beemelmanns et al., 2021b; Wang et al., 2016), both serpin peptides described here could rapidly be measured to monitor mitigation of chronic heat stress in salmon fed diets supplemented with specific ingredients bearing anti thermal or antioxidant properties.

A second peptide significantly induced by thermal stress was ELA2A, which in humans promotes inflammation in colonic epithelial during inflammatory bowel disease (Motta et al., 2021). Although inflammation occurs in salmonid liver following heat stress (Huang et al., 2022), it is unknown if ELA2A is involved in inflammation. Sequence alignment of the chymotrypsin-like elastase family member 2A (Cel2A) (UniProt: A0A1S3MF78) measured in this study against human protein ELA2A (UniProt: P08217) revealed a 62% sequence homology. Information about the function of elastases is scarce across fish research, however, involvement in digestive processes could be speculated from a study in Atlantic salmon where ela1 and ela2 activities were determined in salmon intestinal chyme (Krogdahl et al., 2015). Nevertheless, given the consistently increased abundance of this peptide across individual or pooled samples, this protein shows excellent promise as a biomarker of thermal stress.

GAPDH is a multifunctional protein with well-known roles in metabolism and glycolysis where it produces cytoplasmic ATP and pyruvate by anaerobic glycolysis (Nicholls et al., 2012). Previous evidence established that GAPDH is a poor performing reference gene in salmonid liver subjected to increased temperature (Ma et al., 2019). Here we observed that gapdh_VPTPNVSVVDLTVR abundance was significantly lower in heat stressed salmon, with changes in GAPDH activity and abundance associated with impaired metabolism (Lazarev et al., 2020; Peoples et al., 2018). To determine GAPDH's relevance on salmon metabolism, gapdh_VPTPNVSVVDLTVR could be monitored across a more detailed thermal profile, developing this marker as a potential reference for optimal or compromised metabolism in non-lethal salmon samples.

4.2 | Housekeeping protein normalisation in proteomics

High-throughput MS is an important tool for proteomics studies, with an increased application in aquatic animals. In its label, free modality is systematically biased. Excluding biological variation, this bias

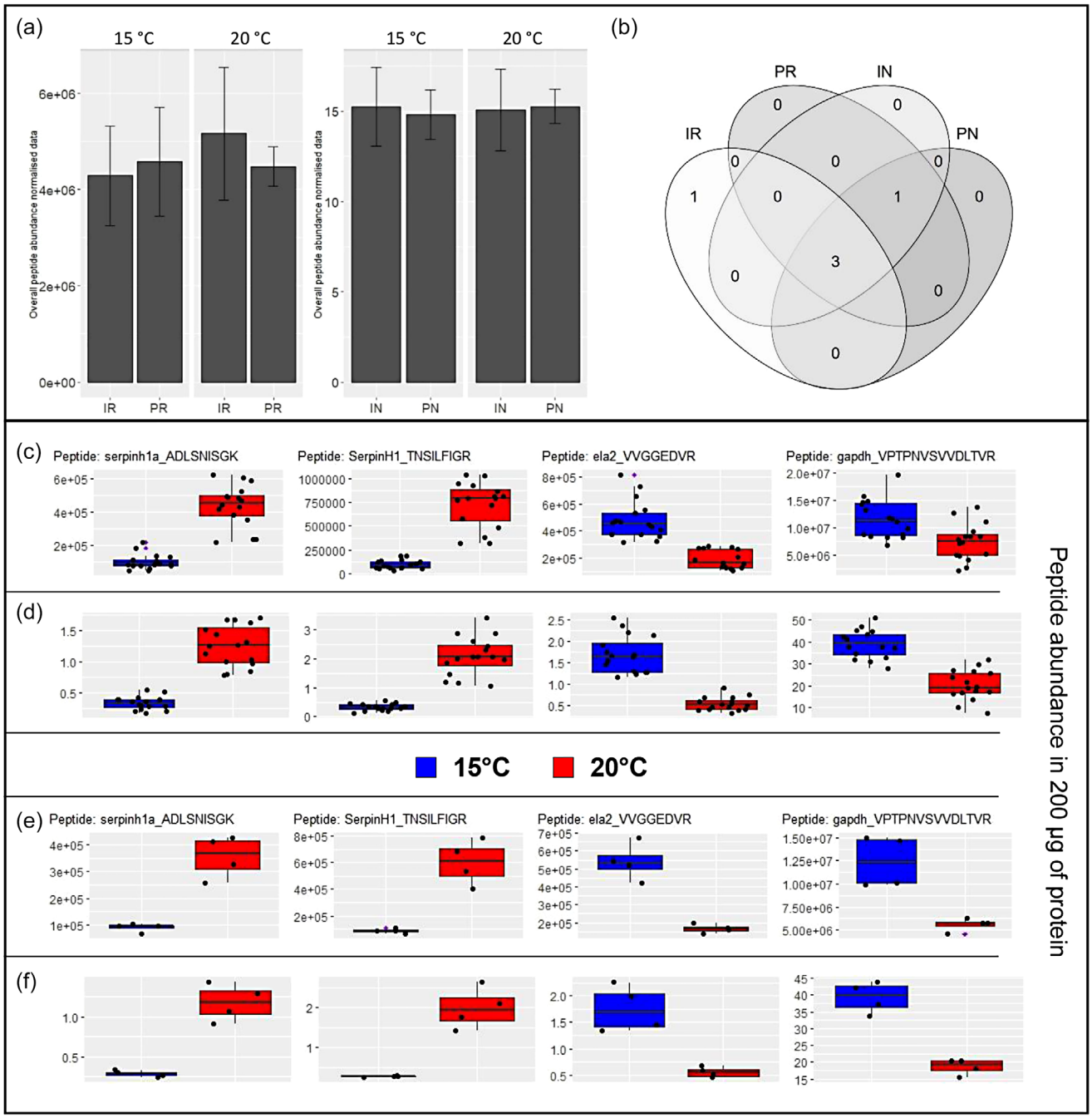

FIGURE 4 Abundance of peptides identified as biomarkers for heat stress in Atlantic salmon reared at 15°C and 20°C. (**a**) Venn diagram of peptides identified with significant abundance (twofold-change, $p < 0.05$). IN, individual normalised data; IR, individual raw data; PN, pooled and normalised data; PR, pooled raw data. (**b**) Overall peptide abundance across raw and normalised data. Bar plots are peptide abundance mean value and error bars indicate standard deviation. (**c**) Biomarker abundance in raw data (**IR**) across individual fish ($n = 16$, black circles). (**d**) Biomarker abundance in normalised data (**IN**) across individual fish ($n = 16$, black circles). (**e**) Biomarker abundance in raw data (**PR**) across pooled fish ($n = 4$, black circles). (**f**) Biomarker abundance in normalised data (**PN**) across pooled fish ($n = 4$, black circles). Purple diamonds are outliers.

derives from sample preparation and factors pertaining to instrumentation. To compensate for this, other strategies to reduce this bias have been deployed generating varying results. These strategies included central tendency, linear regression, locally weighted regression, Log2, lineal models (Loess), cyclic Loess, robust linear regression, variance stabilisation normalisation, total intensity, Median intensity, average intensity, global intensity, NF and quantile (Callister et al., 2006; Chawade et al., 2014; Graw et al., 2020; Välikangas et al., 2016; Wang & Yang, 2019). In our study, variation derived from instrument performance was consistent with previous reports (Schilling et al., 2012).

Normalisation of targeted proteomics data using HK proteins as internal references, as it is routinely applied in gene expression studies, is still under development. A couple of recent studies addressed this

topic, reaching similar conclusions regarding unsuitability of GAPDH as a reference protein for different tissues including cancer tissues (Bettencourt et al., 2020; Hu et al., 2016). In our study, abundance of peptide gapdh_VPTPNVSVVDLTVR was significantly lower in heat stressed salmon and therefore unsuitable as a reference protein peptide in agreement with previous findings (Bettencourt et al., 2020; Hu et al., 2016) and with the outputs of NF, DeltaCT and BestKeeper. In light of this result, GAPDH might be better placed as a protein of interest rather than a reference protein, however, this needs to be further investigated. GAPDH has been used as a HK gene in countless gene expression studies where at times it has been deemed stable and other times unstable. A qPCR study reported *GAPDH* as unsuitable as a reference gene following analysis on eight different tissues in Atlantic salmon (Olsvik et al., 2005) wherein *GAPDH* liver stability expression was ranked last, a result in full agreement with our analysis. A different study in Atlantic salmon evaluated the suitability of eight reference genes in distal intestine following induction of enteropathy by soybean diet supplementation indicated that *GAPDH* and *HPRTI* were amongst the most stable genes (Kortner et al., 2011). These results are partially consistent with our findings given that gapdh_VPTPNVSVVDLTVR and hprt1_HYADDLDR were the least and the most stable candidate HK peptides in our study, respectively. However, as reported before, gene stability is subjected to spatiotemporal variation in Atlantic salmon tissues where significant differences in gene stability were observed between liver and intestine (Olsvik et al., 2005). In addition to having identified HPRTI and hprt1_HYADDLDR as the most stable candidates, the work from Kortner et al. (2011) and our work are based on using the CV as a strategy to screen suitability of HK candidates. A highly comparable study evaluating reference genes by qPCR in rainbow trout under heat stress reported *GAPDH* as the most unstable reference gene using some of the algorithms included in this study (Ma et al., 2019). Other HK candidate peptides in our study showed varying degrees of stability, highlighting the importance of having more than one candidate gene or protein for data normalisation (Vandesompele et al., 2002). Although our work is based on peptide abundance, it is encouraging that variance dispersion generated results that were consistent with outputs of tools publicly available for gene expression data.

Other proteomics studies have suggested TIC as an appropriate normalising strategy (Deininger et al., 2011; Wulff & Mitchell, 2018). Here, TIC was defined as the sum of the peak area of all peptides measured by MRM for a particular sample (individual or pooled), and then the abundance of each peptide was divided by the TIC of that particular sample. This strategy successfully identified seven significant peptides in individual and pooled fish samples wherein five peptides were common (Supporting Information File 1). Compared with TIC normalisation, reference protein normalisation performed better as it identified 16 and 13 significant peptides at individual and pooled level when normalised with hprt1_HYADDLDR. TIC also identified the same four peptides validated as biomarkers for heat stress as indicated in Figure 4c,f.

4.3 | Benefit of sample pooling

There are several conditions that might influence the decision to pool, balancing the impacts of losing statistical power to identify biologically significant changes (Molinari et al., 2018), budget constraints or limited amounts of biological material. A study pioneering sample pooling in proteomics established two conditions that should be met for sample pooling to be a valid strategy (Diz et al., 2009). First, the mean expression of pooled sample should not significantly differ from individual samples and, and second, biological variance should be reduced in the pooled sample compared with individual variance. Our study demonstrated that mean peptide abundance between pooled and individual samples was not significantly different across all peptides. Diz et al. (2009) reported a 90% compliance of matched abundance between pooled and individual samples. Additionally, biological variance (CV dispersion) was not significantly different between pooled and individual samples in either raw or normalised data. Although serpinh1a_ADLSNISGK and SerpinH1_TNSILFIGR were validated as biomarkers for heat stress, the *p*-values in individual samples were of higher confidence compared with *p*-values observed in pooled samples. This decrease in statistical power between pooled and individual samples has been previously reported (Molinari et al., 2018).

5 | CONCLUSIONS

A fast targeted proteomics approach was developed to successfully validate a range of peptides as biomarkers for heat stress in the liver of Atlantic salmon, the most robust of which were serpinh1a_ADLSNISGK and SerpinH1_TNSILFIGR. Peptide ela2_VVGGEDVR is proposed as new biomarker for heat stress, although the functional role of this protein is not yet clear, whereas peptide gapdh_VPTPNVSVVDLTVR is proposed as a heat stress marker as an indicator of optimal homeostasis. This method is a tool that could be readily applied to monitor stress response over time in salmon thermal challenge or other stress experiments, including those involving feeding additives designed to mitigate thermal stress, or applied at scale in selective breeding programs to help understanding family variance in thermal tolerance. These can be valuable tools to monitor fish performance and welfare in commercial conditions, with additional development of non-destructive tissues and high-throughput methods set to aid the deployment in commercial scenarios. The pooling strategy was only improved when it was combined with normalisation as it identified more than twice the number of markers compared with sample pooling alone as well as increasing statistical significance.

AUTHOR CONTRIBUTIONS

Omar Mendoza-Porras: Conceptualisation; data curation; formal analysis; investigation; visualisation; writing—original draft; writing—review and editing. **Anca G. Rusu**: Investigation; methodology;

visualisation; writing—original draft; writing—review and editing. **Christopher Stratford**: Investigation; methodology; writing—original draft; writing—review and editing. **Nicholas M. Wade**: Funding acquisition; project administration; resources; writing—original draft; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest, or any financial or personal relationships that could inapprpriately influence this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study, if not already available in the supplementary material, are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All experimental procedures were approved by the CSIRO Animal Ethics Committee (QAEC 2021–11) and conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

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PEER REVIEW

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