Multivariate effect of salinity and temperature on survival and gene specific stress response of Bay of Fundy sourced *Lepeoptheirus salmonis* copepodites

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MULTIVARIATE EFFECT OF SALINITY AND TEMPERATURE ON SURVIVAL AND GENE SPECIFIC STRESS RESPONSE OF BAY OF FUNDY SOURCED *LEPEOPTHEIRUS SALMONIS* COPEPODITES

by

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ABSTRACT

Murray, H.M., Gallardi, D., Hobbs, K.D., Marshall, K., and Swanson, A.K. 2024. Multivariate effect of salinity and temperature on survival and gene specific stress response of Bay of Fundy sourced *Lepeoptheirus salmonis* copepodites. Can. Tech. Rep. Fish. Aquat. Sci. 3630: vii + 23 p.

The factors affecting the distribution of a parasite which contribute to its potential to cause disease within the host population are broad. These can include host availability, suitability, and in the case of many aquatic parasites, their physiological compatibility with current environmental conditions. In this study we investigated the percent survival and a gene specific transcriptomics response of the free swimming *Lepeophtheirus salmonis* infective stage (copepodite) to combinations of decreasing salinity and temperature over a trial period of 6 hours. Results demonstrated a clear effect of hyposalinity which was accentuated by lower temperatures contributing to a decline in percent survival. Additionally, transcriptomics analysis of sea lice exposed to low salinity/low temperature treatments demonstrated upregulation of categories of genes consistent with physiological stress, including those involved in protein chaperoning and DNA/chromatin stabilization. This work is consistent with similar observations of European and Pacific sourced *L. salmonis*, thus furthering our insight into host/parasite dynamics in variable estuarine environments. These initial interactions between host and parasite are important and will improve our understanding of how local environmental conditions may influence sea lice infestation success during wild smolt exposure with additional benefits for aiding in the development of new mitigation strategies for salmonid aquaculture.

RÉSUMÉ

Murray, H.M., Gallardi, D., Hobbs, K.D., Marshall, K., and Swanson, A.K. 2024. Multivariate effect of salinity and temperature on survival and gene specific stress response of Bay of Fundy sourced *Lepeoptheirus salmonis* copepodites. Can. Tech. Rep. Fish. Aquat. Sci. 3630: vii + 23 p.

Les facteurs affectant la répartition d'un parasite et contribuant à son potentiel à provoquer une maladie au sein de la population hôte sont nombreux. Ceux-ci peuvent inclure la disponibilité des hôtes, leur adéquation et, dans le cas de nombreux parasites aquatiques, leur compatibilité physiologique avec les conditions environnementales actuelles. Dans cette étude, nous avons étudié le pourcentage de survie et la réponse transcriptomique spécifique d'un gène du stade infectieux de Lepeophtheirus salmonis (copépodite) nageant librement à des combinaisons de salinité et de température décroissantes sur une période d'essai de 6 heures. Les résultats ont démontré un effet évident de l'hyposalinité qui a été accentué par des températures plus basses contribuant à une baisse du pourcentage de survie. De plus, l'analyse transcriptomique du pou du poisson exposé à des traitements à faible salinité/basse température a démontré une régulation positive de catégories de gènes compatibles avec le stress physiologique, y compris ceux impliqués dans le chaperonnage des protéines et la stabilisation de l'ADN/chromatine. Ce travail est cohérent avec des observations similaires de L. salmonis d'origine européenne et pacifique, approfondissant ainsi notre compréhension de la dynamique hôte/parasite dans des environnements estuariens variables. Ces interactions initiales entre l'hôte et le parasite sont importantes et amélioreront notre compréhension de la manière dont les conditions environnementales locales peuvent influencer le succès de l'infestation par le pou du poisson lors de l'exposition des saumoneaux sauvages, avec des avantages supplémentaires pour faciliter l'élaboration de nouvelles stratégies d'atténuation pour l'aquaculture des salmonidés.

INTRODUCTION

With the continued growth of the Atlantic salmon aquaculture industry, the challenge of dealing with aquatic health concerns is increasingly significant for the sector. Due to its global economic importance, sea lice (e.g., *Lepeoptheirus salmonis*) infection prevention and management is recognized as one of the top priorities in aquaculture health monitoring by both scientists and aquaculture practitioners (Gaffney and Lavery 2022; Forseth et al. 2017). Traditional control methods (medicinal and nonmedicinal) are available for treatment and management, including prescription drug-based therapies, as well as the application of cleaner fish technologies, hydrogen peroxide, thermal and freshwater baths, among others.

Recently, questions have been raised as to whether these parasites could be evolving tolerance to some of the routine non chemotherapeutic mitigation strategies used by salmonid farmers, including commonly applied freshwater bath treatments (Groner et al. 2019). The tolerance of sea lice to variable environmental conditions, including low salinity resistance has been explored in the past. Bricknell et al. (2006) observed during controlled laboratory based experiments that survival and host infectivity of *L. salmonis* collected from farm sites in Scotland, UK were severely compromised by short term exposure (6 hrs) to reduced salinity. Additionally, Sutherland et al. (2012) noted that during short term exposure to hyposaline conditions, dispersal stages of lice collected from farm sites in the Pacific did not always illicit large transcriptomic changes which suggested that longer term exposures in the range of days or weeks might have a more significant effect. In contrast, Crosbie et al. (2019) in a study investigating vertical distributions of nauplii and copepodites along salinity gradients observed that while the nauplii stages of *L. salmonis* collected from farm sites in Norway were particularly sensitive to low salinity conditions, whereby the infective stage copepodites showed greater resistance with a gradual decline in the proportion of individuals preferring less saline zones including salinities as low as 16-20 PSU. This raises the question as to whether different sub-populations of lice exhibit differing degrees of resistance to low salinity exposure, perhaps based on the natural environment from which they arose. It is well known that the factors that can affect the distribution of a parasite within a region, and thus contribute to its potential to cause disease within a host population, are extensive (Kołodziej-Sobocińska 2019). These can include host availability, suitability, as well as physiological compatibility with predominant local environmental conditions, which for sea lice, includes variability in both salinity and temperature (See Groner et al. 2016, Rittenhouse, Revie, and Hurford 2016, Ljungfeldt et al. 2017, and Murray and Ang 2018).

In the present study we illustrate the impact of the combined effects of decreasing salinity and temperature on the infective stage of *L. salmonis* through controlled experiments documenting % survival over time, and gene specific transcriptomic response to that environment. This work contributes to our understanding of how the local environment may affect infestation success in different source populations of parasite and will aid in the development of new mitigation strategies for control of these parasites near active salmon farms.

Additionally, this study also provides insight into how infective stages of *L. salmonis* may respond to varying environmental conditions and identifies likely

physiological stressors, thus improving the understanding of the interaction between parasite and host in estuarine areas during out migrations of spring smolts where salinity and temperatures vary greatly based on tidal and riverine flows.

MATERIALS AND METHODS

COLLECTION AND EXPERIMENTAL SET UP

Lepeophtheirus salmonis egg strings were collected from several females sourced from Atlantic salmon housed at two neighboring cage sites situated in the Bay of Fundy, New Brunswick, Canada (i.e Seeleys Cove and Grand Manan Island), and subsequently transferred on ice to the Northwest Atlantic Fisheries Centre in St. John's NL where they were randomly divided into 1 liter incubation chambers (20 individual strings per chamber) at 31 PSU/10 ^{o}C and incubated for approximately 10 days at which point hatched larvae had developed to the copepodite stage (Fig.1). Note egg strings were collected from active farm sites during harvest activities and separation of egg strings into families was not possible at that time and mixing was inevitable.

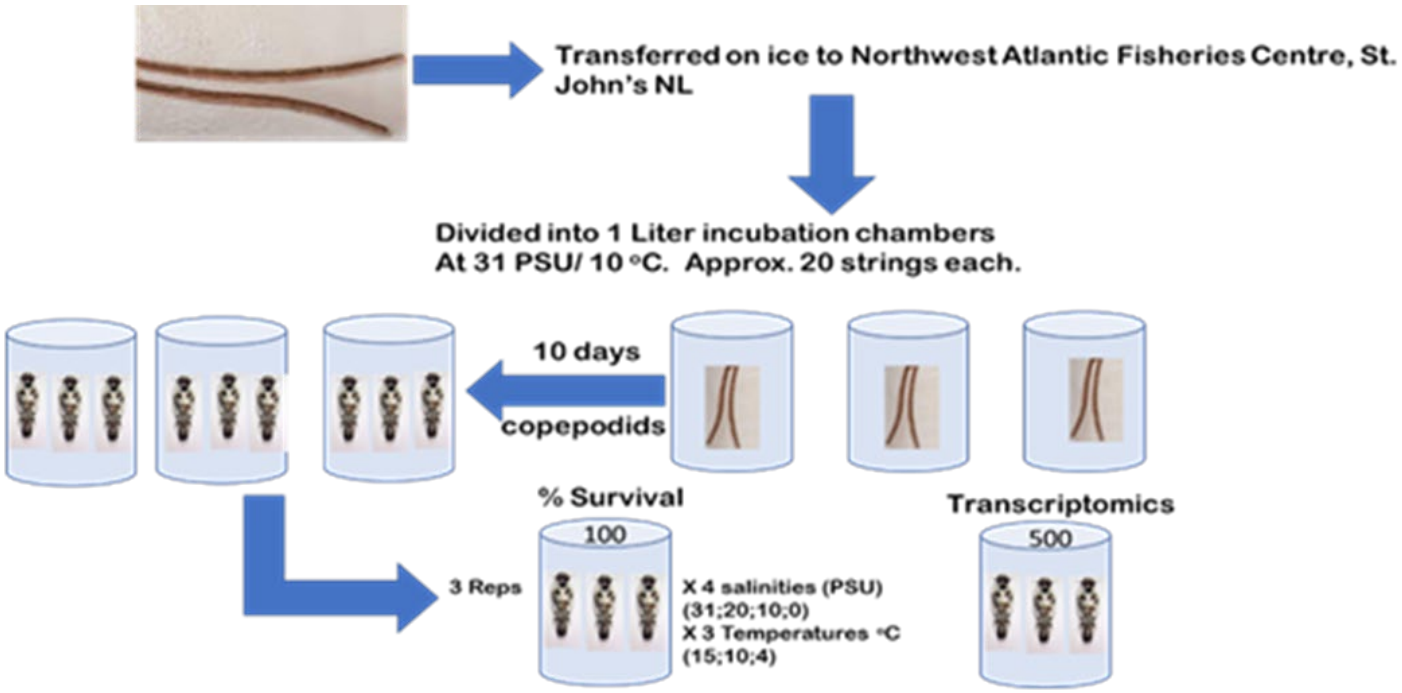

Fig. 1 Egg string collection, hatching and initial experimental setup

% SURVIVAL TRIALS

Survival trials were set up in 3 replicate 1 liter chambers for each salinity/temperature combination (i.e. salinities 30; 20; 10; and 0 PSU; 15 ºC; 10 ºC; and 4 ºC) (Fig. 1). Just prior to the beginning of the trial 100 active copepodites were transferred to each test chamber and the water adjusted to the appropriate salinity by the addition of an appropriate volume of freshwater. Accuracy of the salinity was checked with a handheld salinity meter (Ultrapen PT1, Myron L Company). Copepodite mortality was recorded hourly for each treatment over a 6 hour exposure cycle chosen as the predicted period of exposure of copepodites to reduced salinity at a river mouth during half a single tidal cycle (i.e. running from minimum to maximum salinity) as suggested by (Bricknell et al. 2006). Copepodite mortality was defined by diminished swimming behavior in the water column after a 10 min exposure to an overhead 40W daylight bulb, and a lack of physical escape response to a jet of water ejected from a pipette (Bricknell et al. 2006). Mortalities were removed from the trial containers at each time point, counted and subtracted from the total as a running count and plotted as mean % survival over time for each treatment. For analysis, mean percent survival was square root transformed and analyzed statistically using a Repeated Measures ANOVA model and plotted against treatment. Additionally, a general linear model was applied to the data in order to determine if variability in salinity and temperature were good predictors of mortality.

FUNCTIONAL TRANSCRIPTOMICS: TEMPERATURE/SALINITY RESPONSE

The functional transcriptomics response of copepodites to environmental stress was evaluated by exposing 500 individual copepodites to each of the above treatment conditions for the same 6-hour exposure period. At the end of the 6 hours, copepodites were filtered onto 47 mm nitrocellulose filters with a 8.0 um pore size (EMD Millipore) under vacuum. Membranes containing copepodites were then folded in quarters placed in a 2 ml Eppendorf tube, snap frozen on dry ice, and subsequently stored at - 80 ºC to await RNA extraction and qPCR analysis.

RNA EXTRACTION

For transcriptomics analysis, filters containing copepodites were homogenized in 800 µL of TRIzol (Invitrogen, Carlsbad, California) with stainless steel beads (5 mm; QIAGEN, Mississauga, ON) using the TissueLyser (QIAGEN, Mississauga, ON) (as modified from (Gallardi et al. 2019))

Following homogenization, copepodite RNA was extracted according to manufacturer's instructions after centrifugation at 12000xg for 5 min to remove the residual filter material. Initially, extractions were completed from half of a frozen filter except where RNA yield was low, in which case RNA was extracted from the second half of the filter and both extractions were combined. Final RNA pellets were dissolved in 30 µL RNase-free water, treated with DNase-I (QIAGEN) to degrade residual genomic DNA and purified using the RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocols. RNA concentration and purity were measured using NanoDrop spectrophotometry (NanoDrop® ND-1000, Thermo Fisher, Mississauga, ON) and RNA integrity was verified with 1% agarose gel electrophoresis. For samples with

low yield, an additional precipitation step with 3M sodium acetate was used. Only samples with an A260/280 ratio > 1.95 and an A260/230 > 1.6 were used in cDNA synthesis.

CDNA SYNTHESIS

cDNA was synthetized for each sample using 650 ng of column-purified total RNA, 1 µL of random hexamer primers (250 ng; Invitrogen), 1 µL of dNTPs (10 mM), 4 µL of manufacturer 5× Buffer, 2 µL of DTT (0.1 M) and 1 µL of M-MLV reverse transcriptase (200 U; Invitrogen) at 37 °C for 50 min in a 20 µL reaction volume as recommended in the manufacturer's instructions. The synthetized cDNA was diluted 26 times by adding 500 µL of nuclease-free water (final concentration of 1.25 ng/µL) and stored at −20 °C until further use.

qPCR ANALYSIS

Normalized relative expression of 19 genes of interest (Table 1.) was quantified by real-time quantitative polymerase chain reaction(qPCR) and analyzed based on the response of genes to the combined abiotic factors of salinity and temperature.

QPCR primers were obtained from a previously published study (Sutherland et al. 2012). Additional primers were designed in-house (Primer 3, v. 4.1.0.; https://primer3.ut.ee//); qPCR primer GenBank accession numbers, and amplicon sizes are shown in Table 1. Quality testing procedures for qPCR primer pairs, including standard curves and dissociation curves, were conducted as described by Rise et al. (2010) and Xue et al. (2015). All primer pairs were quality tested (e.g., determination of amplification efficiency, dissociation curve) using two reference templates (a pool of 6 high salinity samples (30 and 20 PSU, including 15^{o}C and 10^{o}C temperatures) and a pool of 6 Low salinity samples (10 and 0 PSU, including 10^{o}C and 4^{o}C temperatures) generated by pooling an equal quantity of each individual cDNA. The amplification efficiency (Pfaffl 2001) of each primer pair was determined using a 5-point 1:3 dilution series on technical duplicates starting with reference cDNA corresponding to 10 ng of input total RNA.

Quality controlled reactions were performed in technical duplicates using Power SYBR Green I dye chemistry in 96-well format on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The reactions contained 4 µL of diluted cDNA (10 ng input total RNA), 50 nM each of forward and reverse primer, and 1 × Power SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 13 µL. The qPCR program consisted of 1 cycle of 50 ^{o}C for 2 min, 1 cycle of 95 ^{o}C for 10 min, followed by 40 cycles of 95 ^{o}C for 15 s and 60 ^{o}C for 1 min, with the fluorescence signal data collection after each 60 ^{o}C step; a no-template control for each primer set was included. The reported efficiencies (Table 1) are an average of the values obtained from the two templates. Dissociation curves were carried out to ensure that the primer pairs amplified single products with no detectable primer dimers. In addition, qPCR primers were also tested for seven candidate normalizer genes and quality tested as above. The candidate normalizer genes were selected based on previous published studies (see Table 1) (i.e. vcl, eef1a1, tubb, 18s) or designed in house (i.e. rsp20, slc25a6, flna). One candidate normalizer did not pass quality control (18s); three candidate

normalizers (rsp20, slc25a6, vcl) showed differential expression in the QC pools and were therefore included in the study as Gene of interest; the remaining three candidate normalizers (eef1a1, tubb, flna) passed QC and were all used in the study. QPCR reactions were performed in technical triplicates using Power SYBR Green I dye chemistry in 96-well format on the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The qPCR protocol was as described earlier with an input of 4 µL of diluted cDNA (5 ng input total RNA). A no-template control for each primer set was included in each plate. Threshold cycle (Ct) values were averaged over triplicates; the normalized relative quantity (NRQ) of each transcript was determined using the method described by Xue et al. (2019), incorporating amplification efficiencies for each primer pair (Table 1). The individual with the lowest normalized expression was used as the calibrator sample (i.e., NRQ = 1.0) for each gene of interest. Graphical representations were performed with Sigmaplot 13 statistical and graphical software (Systat Software Inc., San Jose, CA). Fold upregulation for NRQ data was calculated as 2A – B, where A is the log2 transformed NRQ for each combination of temperature and salinity and B is the log2 transformed NRQ for 30 PSU in association with each temperature (15^{o}C, 10^{o}C, 4^{o}C) (Hori et al. 2012). Differences in mean fold change for each gene of interest were compared using one-way analysis of variance across all salinities and temperatures.

Table 1 Genes known to respond to environmental stressors in *Lepeophtheirus salmonis*

Gene name (symbol) Genes of interest	**GenBank Accession #**	**Primer sequence (5' -3')**	**R2**	**Amplification efficiency (%)**	**Amplicon size (bp)**	**Source**
Chromobox 1 (cbx1)	*BT121389.1*	F:TCATTGGAGCCACAGATTCC R:TCACTGTTTGAGGACATCGC	0.98	107.1	117	(Sutherland et al. 2012)
Chromobox 2 (cbx2)	*BT120793.1*	F:CAAATGCCACCAATCTCTCC R:CATCGTGATCAAATTCACCG	0.98	107.5	118	(Sutherland et al. 2012)
RB binding protein 4, chromatin remodeling factor (rbbp4)	*JP303140.1*	F:GAGAAGTGAATCGTGCTCGG R:CACGAGAACATCAGAGCTGG	0.98	104.8	80	(Sutherland et al. 2012)
Heat shock protein 90 alpha family class A member 1 (hsp90aa1)	*JP302099.1*	F:CGGGATAACTCAACTGTCGG R:CATTCTTGTCAGCATTTGCC	0.98	106.5	109	(Sutherland et al. 2012)
Chaperonin containing TCP1 subunit 6A (cct6a)	*JP313250*	F :CATGAAGGCTGCCAATAAGC R :ACTTCAAAAGCTCCAGCACC	0.99	102.5	123	(Sutherland et al. 2012)
Protein disulfide isomerase family A member 3 (pdia3)	*JP335045*	F :CCCATCTACGAGGAACTTGG R :GGAACATCATTTGCCGTAGC	0.98	105.1	83	(Sutherland et al. 2012)
Calreticulin (calr)	*XM_040714035.1*	F :CGACCCTGAAGCATCTAAGC R :CATTTACCCTTGTATGCGGG	0.98	103.3	138	(Sutherland et al. 2012)
Tumor protein p53 binding protein 2 (tp53bp2)	*XM_040711731*	F: GGACTCCTCTTCATTGTGCC R:AACCATGAAAGCCTTCCTCC	0.99	100.8	150	(Sutherland et al. 2012)

Table 1 Continued.

Gene name (symbol) Genes of interest	**GenBank Accession #**	**Primer sequence (5′ -3′)**	**R2**	**Amplification efficiency (%)**	**Amplicon size (bp)**	**Source**
Programmed cell death 4 (pdcd4)	*BT077738.1*	F: TCAATCGTAAGATGCCGTCC R:CCAGTATTCCTTGAATCGGC	0.98	109.2	77	(Sutherland et al. 2012)
Growth arrest-specific 1 (gas1)	*BT078287.1*	F:GTGAGGAACAGGAAACAAATCC R:ACAACATCCGTTTCACCTCC	0.99	94.9	106	(Sutherland et al. 2012)
Adenine phosphoribosyltransferase (aprt)	*JP304084*	F:GTTGAGGAAAAAGCATTGCC R:TTGGAACAAAAGGAACTCCG	0.97	113.5	118	(Sutherland et al. 2012)
Secretion associated Ras related GTPase 1 (sar1b)	*BT121752.1*	F:GTCCAGTTCTCATTTTGGGC R:CCTTTCCCGGTAGTTTGACC	0.99	100.6	103	(Sutherland et al. 2012)
FKBP prolyl isomerase 4 (fkpb4)	*BT121998.1*	F:ATGGTTCCCAAAGAAGAGGC R:ATCGCTCTTTGGAGTGTTCC	0.99	95.4	145	(Sutherland et al. 2012)
Myosin heavy chain, muscle (mhc)	*XM_040710562.1*	F:GGAACTCACTTATGCCACGG R:TTTGCTTCTTGTAGGAGCGG	1	97.7	101	(Sutherland et al. 2012)
Solute carrier family 31 member 1 (slc31a1)	*BT121115.1*	F:CTACAAATCCCACTGAATGCC R:AATTGAAGGACGTGCAGAGC	0.97	98.2	106	(Sutherland et al. 2012)
Glutathione S-transferase 1, isoform D (gst1d)	*BT078543.1*	F:GGAGCTCCAACAACTTCAGC R:AAGGAAGCTCTCTCGCACC	0.98	96.3	115	(Sutherland et al. 2012)
Ribosomal protein S20 (rsp20)	*BT121430.1*	F:CTGAACTGATCCGAGGAGCC R:CCATGTCTTGGAGCCCTCTC	1	95.2	122	In-house design
Solute carrier family 25 member 6 (slc25a6 alias ant3)	*BT078064*	F:ATCTTGTCTGGTGGGTGTGC R:CGTTCACCTCCCTTCTTGCT	0.99	95.1	113	In-house design

Table 1 Continued.

Gene name (symbol) Genes of interest	**GenBank Accession #**	**Primer sequence (5' -3')**	**R2**	**Amplification efficiency (%)**	**Amplicon size (bp)**	**Source**
Vinculin (vcl)	*JP310682*	F:AGATTCCAACACTGGGAACG R:CAGAGTCCATTTTTGCTCCC	0.98	98.9	78	(Sutherland et al. 2012)
Normalizers						
Eukaryotic translation elongation factor 1 alpha 1 (eef1a1)	*EF490880.1*	F:CCAAATTAAGGAAAAGGTCGACAGACGTACTG R:TGCCGGCATCACCAGACTTGA	0.99	91.2	83	(Carmichael et al. 2013)
Tubulin beta class 1 (tubb)	BT077612	F :TGCGGCTATATTTAGAGGGC R :AGGTGGAATGTCACAAACGG	0.99	90.3	136	(Sutherland et al. 2012)
Filamin A (flna)	XM_040717298.1	F:CGGGCTCTCCTTTCACAGTT R:TTCCCGGCATCTTGAAGGTC	0.98	97.9	135	In-house design

RESULTS

Percent Survival curves for *Lepeophtheirus salmonis* Copepodids

Seeley's Cove

Copepodites hatched from Seeley's Cove sourced egg strings presented decreasing % survival over time for all treatments (Fig. 2). Salinity of 30 and 20 PSU showed similar results regardless of temperature with between 70 and 80 % survival after 6 hours. In contrast, salinity treatments of 10 PSU and 0 PSU under the same temperature regimes showed steep declines in % survival following as little as one hour post initial treatment exposure. Typically, 100% mortality was observed after 6 hours at 10 PSU regardless of temperature. At 0 PSU 100 % mortality was observed after 5 hours in the 15 ^{o}C and 10 ^{o}C groups but as early as 2 hours in the 4 ^{o}C group with a significant decline in survival even after one hour.

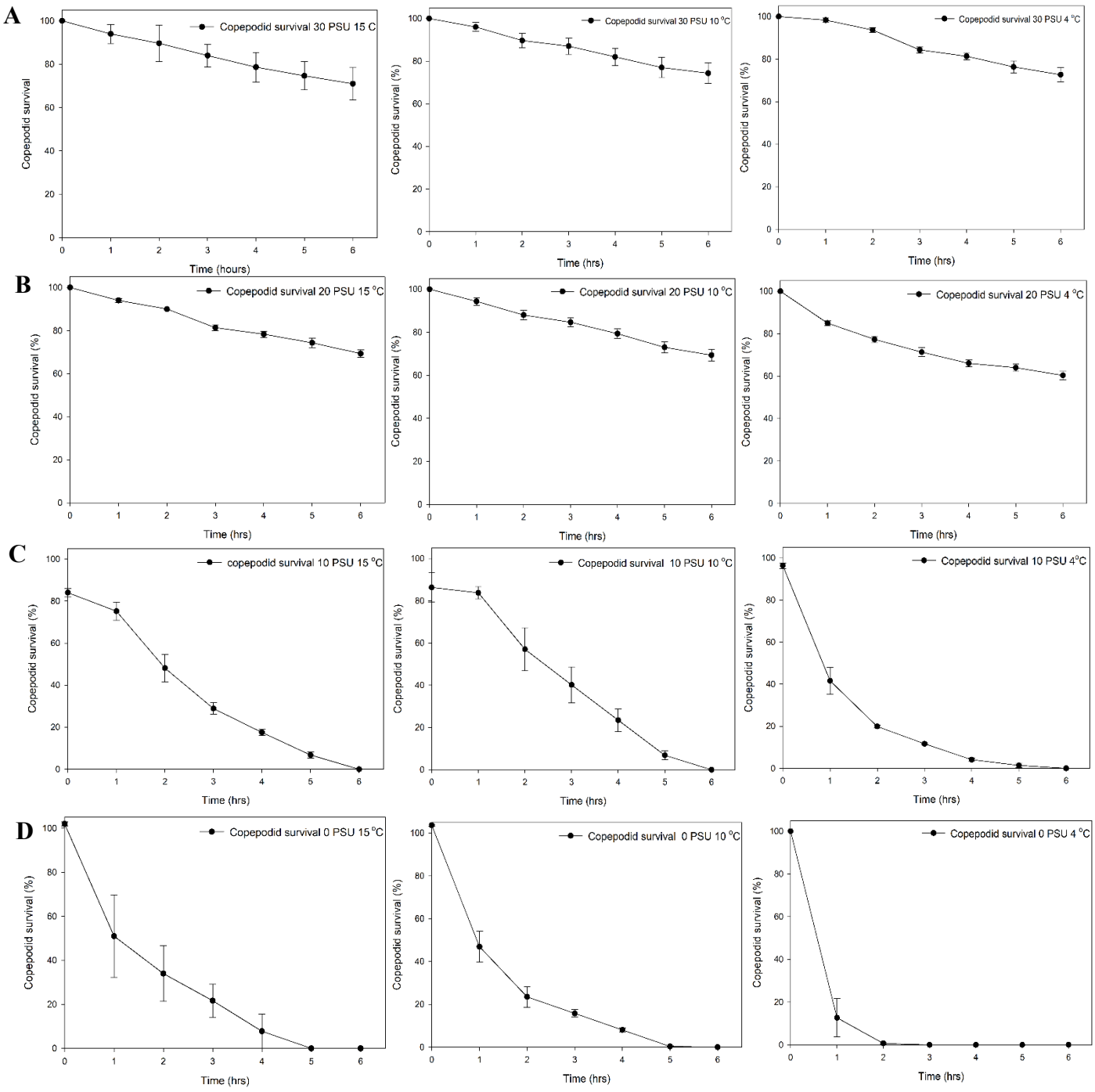

Fig. 2 Copepodite % Survival: Seeley's Cove. A 30 PSU (15 ºC, 10 ºC, 4 ºC) B 20 PSU (15 ºC, 10 ºC, 4 ºC) C 10 PSU (15 ºC, 10 ºC, 4 ºC) D 0 PSU (15 ºC, 10 ºC, 4 ºC).

Grand Manan Island

Copepodites hatched from Grand Manan Island sourced egg strings presented with decreasing % survival over time for all treatments regardless of temperature or salinity (Fig. 3). Percent survival for 30 ppt was near 80 % for the 15 ^{o}C and 4 ^{o}C groups but dropped dramatically for 10 ^{o}C with a final % survival of 50 % after 6 hours. Similarly, copepodite survival at 20 PSU was near 60 % after 6 hours for both 15 and 4 ^{o}C but dropped to below 40 % for 10 ^{o}C. Salinity at 10 PSU under the same temperature regimes showed gradual declines in % survival following initial treatment exposure. After 6 hours 40 % mortality was observed for 15 and 10 ^{o}C with 100 % mortality for the 4 ^{o}C treatment group. The 0 ppt salinity treatment at 15 ^{o}C produced 100 % mortality in 4 hours, whereas the same was observed after 6 hours for 10 ^{o}C and after 2 hours at 4 ^{o}C.

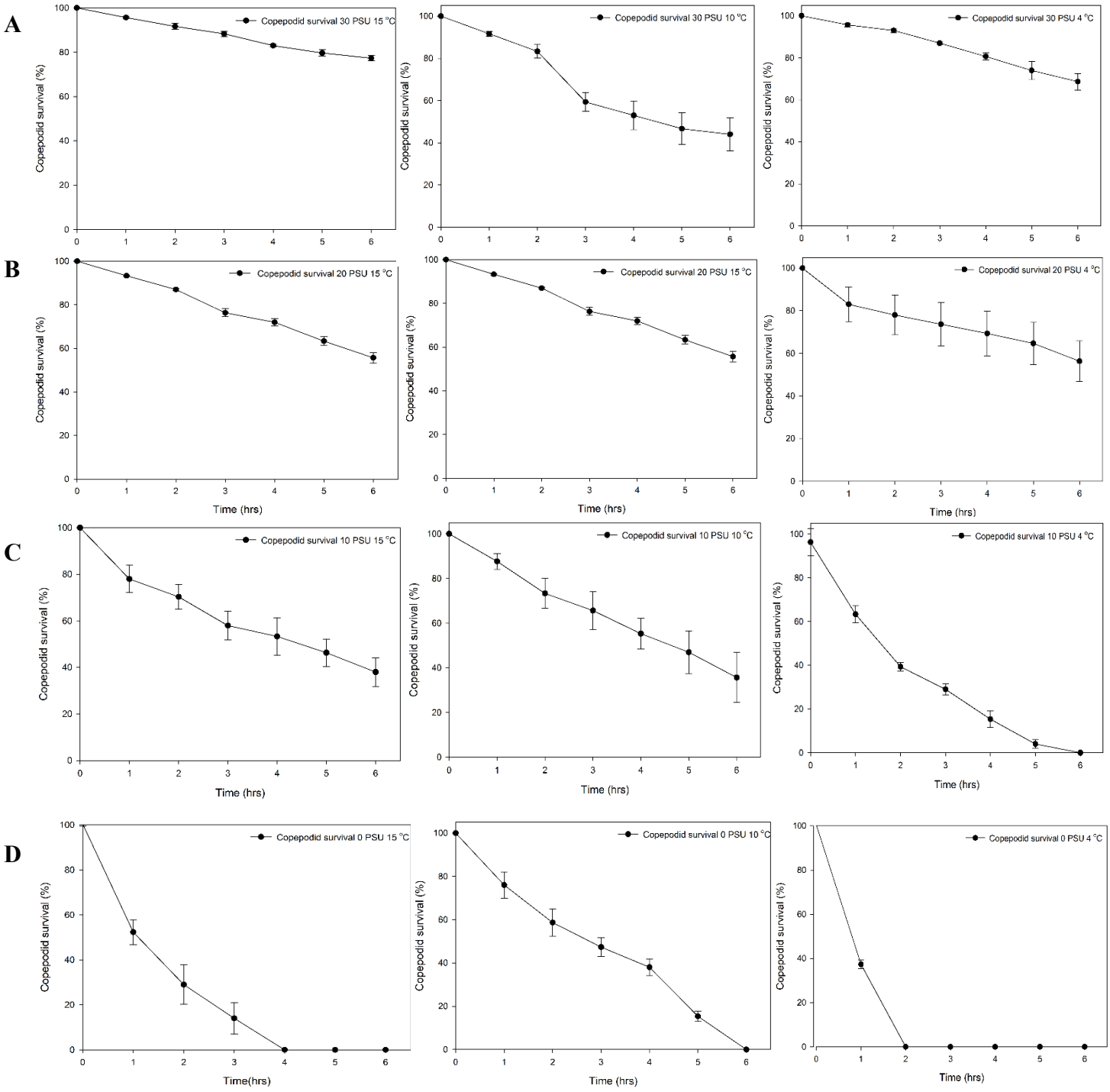

Fig. 3 Copepodites % Survival: Grand Manan. A 30 PSU (15 ºC, 10 ºC, 4 ºC) B 20 PSU (15 ºC, 10 ºC, 4 ºC) C 10 PSU (15 ºC, 10 ºC, 4 ºC) D 0 PSU (15 ºC, 10 ºC, 4 ºC).

Two-way repeated measures analysis of % survival

Seeley's Cove

Two-way repeated measures analysis of variance for copepodite % survival from Seeley's Cove sourced egg strings under different environmental combinations indicated that both salinity and temperature significantly affected % survival ($p < 0.001$). A significant interaction was observed between salinity and temperature ($p<0.001$) indicating a combined effect of both factors and as a result significance is only reported within temperature or within salinity groups. Mean copepodite % survival at 0 and 10 PSU was generally less than 50 % regardless of temperature after 6 hours (Fig. 4AB). While there was no significant difference in survival between 15 ^{o}C and 10 ^{o}C groups within 0 and 10 PSU ($p = 0.642$, $p = 0.453$ respectively), a significantly lower survival was noted at 4 ^{o}C for both salinity regimes ($p < 0.001$) (Fig. 4AB). Mean copepodite % survival at 20 and 30 PSU was generally near 80 % regardless of temperature after 6 hours with no significant difference for any temperature regime (15 ^{o}C,$p = 0.968$; 10^{o}C,$p=0.884$; 4 ^{o}C,$p= 0.484$) (Fig. 4AB). Multiple linear regression analysis indicated that regardless of salinity, both time ($p < 0.001$) and temperature ($p < 0.01$) were predictors of survival (Rsqr > 0.90).

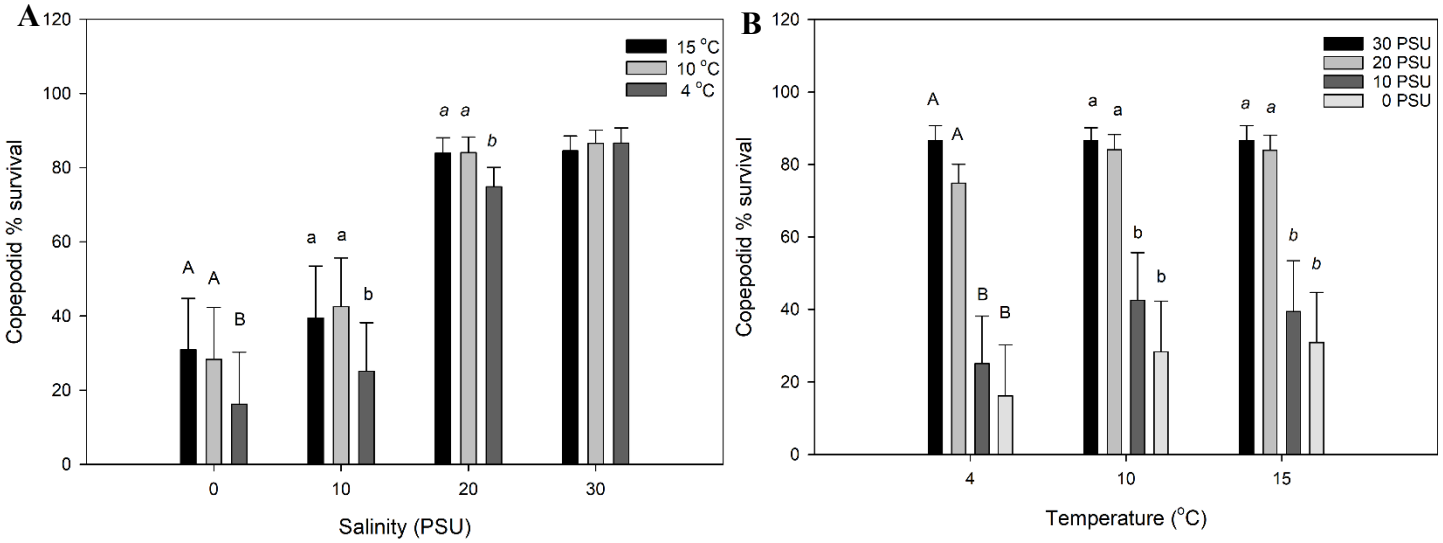

Fig. 4 Two-way repeated measures ANOVA (salinity and temperature): Seeley's Cove. A. % Survival vs Salinity with Temperature; letters represent statistical significance among temperatures within a given level of salinity B. % Survival vs Temperature with Salinity; letters represent statistical significance among salinities within a given level of temperature

Grand Manan Island

Two-way repeated measures analysis of variance for copepodite percent survival from Grand Manan Island sourced egg strings under different environmental combinations indicated that both salinity and temperature significantly affected percent survival ($p <$

0.001). A significant interaction was observed between salinity and temperature ($p < 0.001$) indicating a combined effect of both factors and as a result significance is only reported within temperature or within salinity groups. Mean percent survival for Grand Manan sourced copepodites at 0 PSU was generally less than 50 % regardless of temperature after 6 hours (Fig. 5AB). Highest percent survival at 0 PSU was observed for 10 ^{o}C at 50 %. Survival for the 15 ^{o}C group at 0 PSU was near 30 % whereas the survival for the 4 ^{o}C group at 0 PSU was approximately 20 %. There was no significant difference in percent survival after 6 hours between 15 ^{o}C and 10 ^{o}C groups at 10 PSU ($p = 0.781$; > 60 % survival) whereas both were significantly different from the 4 ^{o}C group ($p<0.001$; < 40 % survival). Mean copepodite percent survival at 20 and 30 PSU was generally over 60 % regardless of temperature after 6 hours with no significant difference between any temperature groups ($p > 0.05$) (Fig. 5AB).

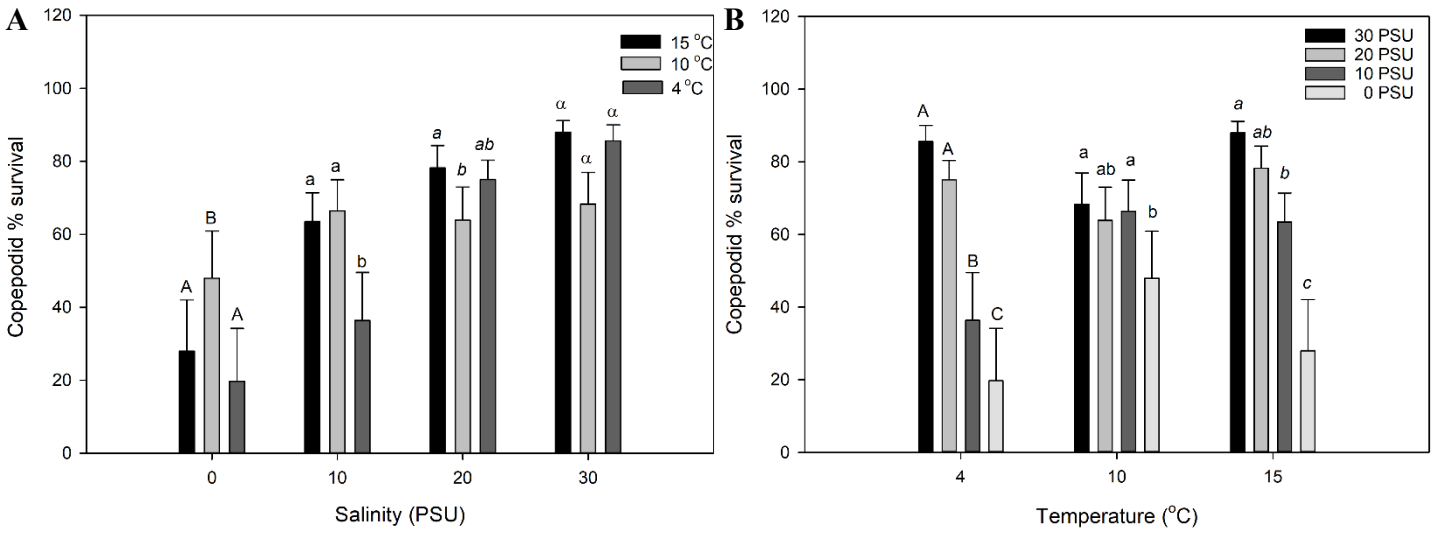

Fig. 5 Two-way repeated measures ANOVA (salinity and temperature): Grand Manan Island. A. % Survival vs Salinity with Temperature; letters represent statistical significance within temperatures at a given level of salinity. B. % Survival vs Temperature with Salinity; letters represent statistical significance within salinities at a given level of temperature

Transcriptomics Analysis

The effect of the above salinity/temperature treatments was further investigated in order to explore possible transcriptomic responses when copepodites were exposed to both factors in combination. QPCR data from Seeley's Cove and Grand Manan sites were combined for analysis based on generally similar % survival trends over all treatments. Fold change was calculated relative to the standard salinity of 30 PSU when combined with each temperature (15 ^{o}C, 10 ^{o}C or 4 ^{o}C) (control group) and consisted of the difference between the log2 transformed normalized relative quantities (NRQ) for each experimental (E) and control (C) pair (2^Log2E-Log2C). Each gene of interest was categorized based on the functional enrichment analysis completed by (Sutherland et al. 2012).

Gene expression response to salinity/temperature treatments was variable however all genes investigated did show some increase in mean fold change with reference to the baselines described above. Only four genes of the original 19 tested were found to be statistically significant ($p \leq 0.05$) for mean fold change within treatments and included those categorized as molecular chaperones within the categories of protein folding and chromatin stabilization (see Table 2; Fig. 6A-D).

All 4 of these genes showed significantly increased fold change at 4 ºC dependent on salinity. Rbbp4 showed a significant increase in mean fold change for all salinities except for 20 PSU when compared to the other temperature/salinity combinations ($p \leq 0.05$) (Fig. 6A; Table 2). Hsp90aa1 and calr both showed a significant fold change above baseline for all salinities at 4 ºC (Fig. 6BC; Table 2). Cbx1 in contrast only showed a significant fold change ($p \leq 0.05$) at 0 PSU/4 ºC when compared to the 15 ºC treatment across all salinities (Fig. 6D; Table 2)

Table 2 Mean Fold Change in expression for genes within the molecular chaperone category for *Lepeophtheirus salmonis* copepodites exposed to various salinity/temperature combinations. Bolded values indicate significance. (see Figure 6)

Treatments	Genes of Interest			
	rbbp4	*hsp90aa1*	*calr*	*cbx1*
20 PSU				
15 ºC	**0.916**	0.672	1.15	**0.952**
10 ºC	0.528	0.249	0.773	0.692
4 ºC	1.45	1.67	**2.50**	0.937
10 PSU				
15 ºC	0.578	0.469	0.778	0.666
10 ºC	0.636	0.463	1.1	0.672
4 ºC	**1.67**	**2.12**	**3.62**	**0.859**
0 PSU				
15 ºC	0.461	0.462	0.621	0.494
10 ºC	0.583	0.587	0.989	0.647
4 ºC	**1.76**	**2.37**	**4.05**	**0.935**

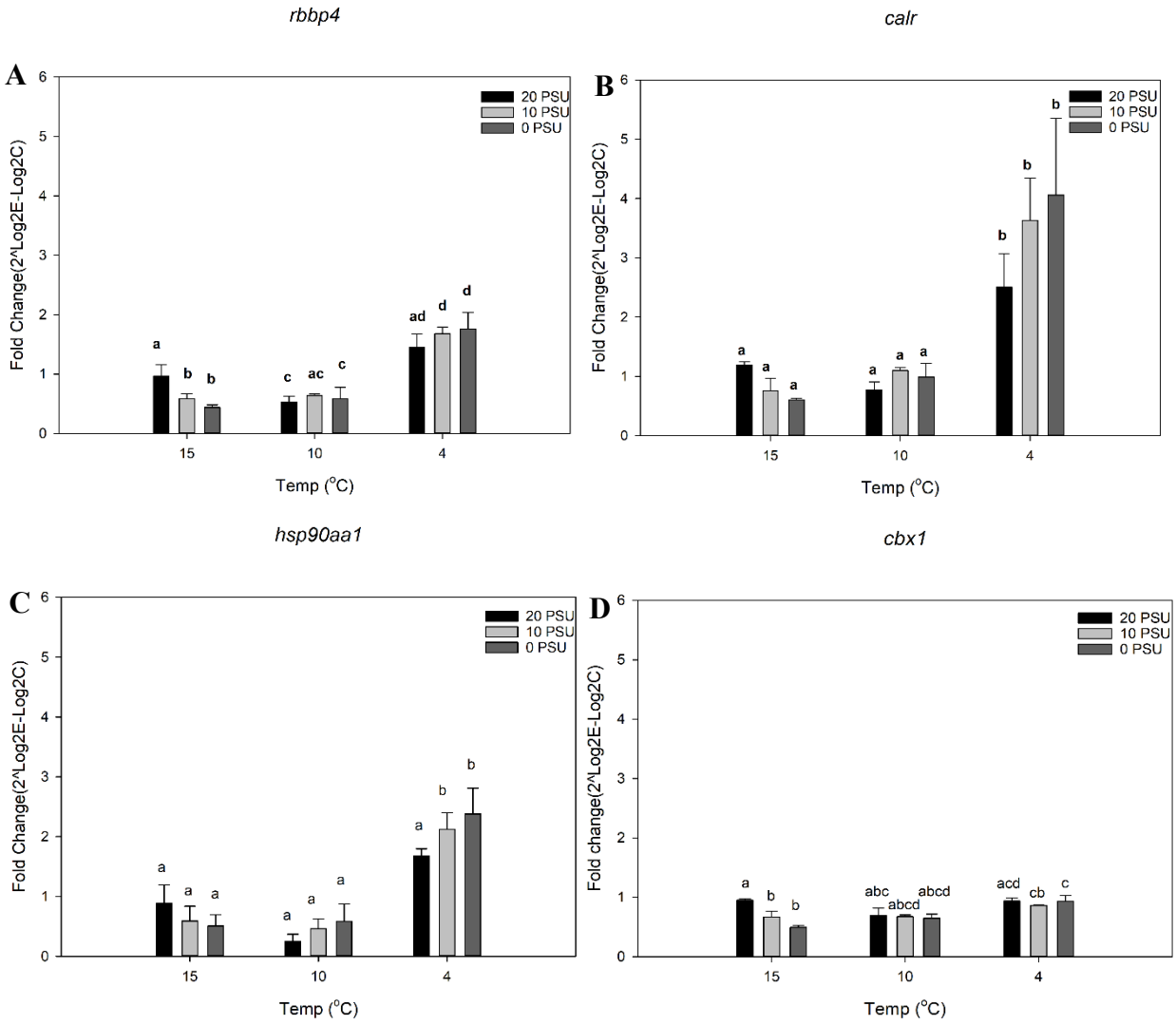

Fig. 6 Mean fold change for the molecular chaperones A. rbb4, B. calr, C. hsp90aa1 D. cbx1, following exposure of *Lepeophtheirus salmonis* copepodites to various salinity/temperature combinations. Letters represent statistical significance among combinations of salinities and temperature.

DISCUSSION

The present study demonstrates that Bay of Fundy sourced copepodites exposed to salinities of 20 ppt and above were able to attain ≥ 60 % survivability regardless of temperature after 6 hours. The observed sharp decline in survival at salinities below 20 PSU (50 % survival between 1 to 3 hours regardless of temperature) was similar to that reported by Bricknell et al. (2006), who recorded 50 % survivability below 19 PSU to be between 1 and 4 hours. In the present study, observed variability in survival between egg string sources (Seeley's Cove and Grand Manan) exposed to identical treatment conditions was interesting considering the close geographical relationship between sites. This variation could be associated with natural disparities between different

broods of lice, potentially due to inherent differences in energy reserves among individuals (Bricknell et al. 2006; Sutherland et al. 2012).

Repeated measures analysis of variance also indicated that both temperature and salinity affected survivability with a significant statistical interaction between these factors and thus a possible combined effect. Johnson and Albright (1991) made similar observations for Pacific sourced *L. salmonis* reporting that survival times were significantly higher at 30 PSU when compared to 15 PSU and that at all temperatures copepodites at low salinities were less active than those maintained at higher salinities. In the present study, the combination of low salinity and temperature also contributed to significantly lower percent survival than that of high salinity and low temperature, accentuating the impact of the combined physiological stress. From a practical sense, these data do suggest that copepodites occurring near river mouths would have reduced survival time especially during periods of cold water perhaps associated with spring thaw and/or annual snow melt. However, it seems more likely as pointed out by Crosbie et al. (2019) that the infective stages would orient near natural haloclines within the estuary and thereby be more in a position to successfully engage a potential salmonid host.

Lepeophtheirus salmonis is a stenohaline organism and that has been shown to be unable to osmoregulate effectively outside the limits of its physiological optimum (Hahnenkamp and Fyhn 1985; Johnson and Albright 1991; Tucker, Sommerville, and Wootten 2000; Brooks 2005; Bricknell et al. 2006; Crosbie et al. 2019). Hahenkamp and Fyhn (1985) reported that in low salinity or freshwater conditions hosted parasites can maintain a steady haemolymph osmolarity and chlorine ion concentration, surviving for a least one week. In contrast, free swimming infective stages (copepodites) become quickly osmotically stressed and start to die within 8 hours of exposure. Investigating this in further detail, Johnson and Albright (1991) showed that at 10 ^{o}C, newly molted copepodites survived for less than 3hrs at 5 ppt salinity and less than 1 day when exposed to 10 PSU, but at higher salinities (15- 30 PSU) and temperatures ranging between 5 and 15 ^{o}C, survival was prolonged. Similarly, Bricknell et al. (2006) and later Sutherland et al. (2012) demonstrated that salinities below 30 PSU had clear negative effects on the survival and transcriptomic response of free living copepodites from egg strings sourced from Scotland and western Canada, respectively.

From an ecological perspective, wild *L. salmonis* have only limited opportunity to interact with its Atlantic salmon host. Wild smolt out migration is thought to be an important period during which this may occur (Costelloe, Costelloe, O'donohoe, et al. 1998; Costelloe, Costelloe, Coghlan, et al. 1998). Alternatively, in regions containing Atlantic salmon aquaculture, wild adult salmon and/or smolt coming near stocked cages during annual migrations have the potential to come into contact with infective stages from farms (Vollset et al. 2023). Within a normal estuarine environment, the early infective stages of *L. salmonis* can be exposed to low salinity conditions often for prolonged periods before accessing an appropriate host. The success of any infection event will be highly dependent on the fitness of the parasite and thus its ability to tolerate physiologically challenging environmental conditions.

Acute and chronic exposures to environmental stressors have also been shown to induce various transcriptomic responses in a number of other crustacean species including *L. salmonis* (Lv et al. 2013; Lv et al. 2015; Shekhar, Kiruthika, and Ponniah 2013; Sutherland et al. 2012). Sutherland et al. (2012) reported that a short exposure of *L. salmonis* copepodites (24 hours) to a narrow range of hyposaline stress induced significant transcriptomic changes related to the extent of the stress and possibly to the time from first molt but did not explore the combined effects of temperature and hyposaline stress. In the present study we investigated the transcriptomic response of a pool of genes identified following microarray analysis by Sutherland et al. (2012) to be significant responders to hyposaline stress in *L. salmonis*. The natural exposure of copepodites to repeated low salinity fluctuations (i.e tidal cycles within estuarine environments) likely trigger genomic coping mechanisms to reduce the physiological effects of suboptimal environmental conditions reducing the metabolic cost of such cellular stress up to the point of cell death. Kultz (2005) discussed the transient cellular stress response as a mitigation strategy to reduce the damage that adverse environmental conditions can inflict on intracellular macromolecules leading to cellular metabolic impairment and/or death. The categories of genes tested in the present study, included those involved in DNA/chromatin stabilization, protein chaperoning, removal of damaged proteins and aspects of metabolic function. It is noteworthy that each individual gene from the set tested was upregulated (positive fold change) across all treatments when compared to the baseline (i.e > 0 mean fold change). These observations are generally reflective of those from Sutherland et al. (2012).

The most significant responding genes above baseline were those involved in chromatin stabilization and protein chaperoning. The significant response of most of these genes in the 4 °C treatment was interesting if not expected. Both cbx1 and rbbp4 belong to the polycomb group of proteins with a role in transcriptional repression and are known to be key epigenetic regulators of development. They also have important roles in genome defense as well as regulation of development and organismic response to environmental cues or stressors including resistance to temperature shock (Kleinmanns et al. 2017; Vyse et al. 2020). In the present study, the observed-up regulation of both of these genes in the 4 °C treatment suggests an induced requirement for genomic stabilization at temperatures below 10 °C. The lack of pronounced differences across salinity treatments suggests that this effect may be driven by temperature and not necessarily salinity.

The remaining two genes identified as molecular chaperones within the category of protein folding (hsp90aa1 and calr) are associated with a group of proteins tasked with the prevention of the misfolding and aggregation of proteins under the influence of physiological stressors including those of environmental origin (Krishna et al. 1995; Peng et al. 2016; Vabulas et al. 2010). The observed upregulation of these genes in response to the 4 ºC treatment with only minimal salinity response also suggests a partial temperature driven effect. The temperature response for all genes within this category was further enhanced by a mild increasing trend in mean fold change from 20 PSU down to 0 PSU suggesting that the combination of low temperature and

decreasing salinity created some additional physiological stress. The overall response of calr at 4 °C was near double the fold change of any other gene within the category regardless of treatment. This gene has been shown to code for an important resident protein within the endoplasmic reticulum with essential roles in molecular chaperoning and is an important responder to both temperature and salinity stress in many other crustacean species (Huang et al. 2019; Shekhar, Kiruthika, and Ponniah 2013; Visudtiphole et al. 2010). This gene's up-regulation in *L. salmonis* further accentuates its importance in the mitigation of subcellular damage due to environmental stressors like temperature and salinity shock thus acting as a key biochemical marker for environmental stress in this species among others (Visudtiphole et al. 2010).

CONCLUSIONS

The present study reports the survival and the gene specific transcriptomic response of the free-swimming infective stage of *Lepeophtheirus salmonis* sourced from the Bay of Fundy and exposed to decreasing combinations of salinity and temperature over a period of 6 hours. Cold hyposaline conditions especially those below 20 PSU (4 °C) were shown to contribute to rapid declines in percent survival and the significant up regulation of genes coding for molecular chaperones associated with protein and chromatin stabilization. Therefore, results support the general stenohaline nature of *L. salmonis* but also the possible synergistic effect of combined low temperature and hyposaline conditions. Lastly, results further the current understanding of *L. salmonis* host parasite dynamics in estuarine conditions and may contribute to the further development of mitigation strategies associated with this parasite within the aquaculture industry.

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