Systematic identification and characterization of stress-inducible heat shock proteins (HSPs) in the salmon louse (*Lepeophtheirus salmonis*)

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Abstract Salmon lice (*Lepeophtheirus salmonis*) are parasitic copepods, living mainly on Atlantic salmon and leading to large economical losses in aquaculture every year. Due to the emergence of resistances to several drugs, alternative treatments are developed, including treatment with hydrogen peroxide, freshwater or thermal treatment. The present study gives a first overview of the thermotolerance and stress response of salmon lice. Sea lice nauplii acclimated to 10 °C can survive heat shocks up to 30 °C and are capable of hardening by a sublethal heat shock. We searched in the genome for heat shock protein (HSP) encoding genes and tested their inducibility after heat shock, changes in salinity and treatment with hydrogen peroxide, employing microfluidic qPCRs. We assessed 38 candidate genes, belonging to the small HSP, HSP40, HSP70 and HSP90 families. Nine of these genes showed strong induction after a non-lethal heat shock. In contrast, only three and two of these genes were induced after changes in salinity and incubation in hydrogen peroxide, respectively. This work provides the basis for further work on the stress response on the economically important parasite *L. salmonis*.

Keywords Heat shock · Salinity · Hydrogen peroxide · Thermotolerance · Hardening · Invertebrate

Introduction

Sea lice (*Lepeophtheirus salmonis*) are parasites living on Atlantic salmon and other salmonids feeding on their blood and skin (Boxaspen 2006). Their life cycle consists of eight stages separated by molt, including two nauplius stages, one copepodid stage, two chalimus stages, two pre-adult stages and the adult stage (Hamre et al. 2013). In the first stages, they are free-living until they attach to their host during the copepodid stage. They pose a major threat to salmon farming and lead to large economic losses due to among others treatment cost and reduced fish growth (Costello 2009). The situation becomes even more severe as several of the commonly used delousing medicines lose their effect as the lice have developed resistances against them (Aaen et al. 2015). Therefore, much work is done to identify new treatment tools like vaccinations or new drugs. Treatment options besides drugs with a specific mode of action include freshwater treatment (Powell et al. 2015), treatment with hydrogen peroxide (Bruno and Raynard 1994) or thermal treatment (Grøntvedt et al. 2015). In order to facilitate development of new treatment tools, it is beneficial to gain knowledge about the stress physiology of sea lice. Molecular markers can provide insights into the cellular effects of a particular treatment even when it does not induce changes visible for the bare eye at a certain concentration.

Heat shock proteins (HSPs) are considered as “stress proteins” (Benjamin and McMillan 1998). They act as molecular chaperones refolding misfolded and denatured proteins which occur under cellular stress conditions such as after heat shock. Additionally, it has been shown in several studies that this so-called heat shock response, meaning the sudden and strong

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induction of heat shock genes, is not limited to an actual heat shock but also occurs under many other stressful conditions, such as heavy-metal stress (Karouna-Renier and Zehr 2003), osmotic stress (Spees et al. 2002) or oxidative stress (Kalmar and Greensmith 2009). Traditionally, HSPs are classified into various HSP families based on the molecular weight of the proteins (e.g., HSP40, HSP70, HSP90, small HSPs (sHSPs; 15–30 kDa according to Garrido et al. 2012). However, not all members of the HSP families are inducible by stress; some are constitutively expressed and are even essential for survival under non-stressed conditions.

To our knowledge, the cellular stress response of sea lice has not been characterized yet. In this study, we analyze the thermotolerance of sea lice nauplii and profile the expression of HSP-encoding genes after heat shock. Due to the recent release of the sea lice genome (www.licebase.org), we were able to screen virtually all sea lice genes for potential HSP-encoding genes. We have identified constitutive as well as inducible HSPs. To assess if the heat-induced genes represent general stress markers in sea lice, we additionally analyzed their expression upon osmotic and oxidative stress, altogether representing three different treatment methods for sea lice.

Methods

Animals

Two different laboratory strains of *L. salmonis* (*LsGulen* & *LsH₂O₂*) were used. *LsGulen* has been presented in detail elsewhere (Hamre et al. 2009). Animals of the *LsH₂O₂* strain show a certain resistance against hydrogen peroxide treatment. The larval stages used in the experiments were obtained by collecting egg strings from adult females. Fertilized adult females produce pairs of egg strings, each containing hundreds of eggs. Depending on mating frequency of the female, all eggs in these egg strings lead to either half or full siblings. The females carry their egg strings externally attached to their body until the Nauplius I larva hatch. For experimental purposes, the egg strings can be easily removed without harming the mother animal. They were then transferred into wells with flow-through seawater at 10 °C, where they hatched, as described in detail elsewhere (Hamre et al. 2009).

Determination of thermotolerance

As there was no information about the thermal tolerance limits for sea lice available, these limits were established in a first experiment.

Three days after hatching, the water level in the hatching wells was lowered to concentrate the animals, which were in nauplius II stage. Around 2 ml seawater, containing 50–150 animals, were carefully pipetted into plastic test tubes. The tubes were filled up

to 15 ml with seawater at different temperatures (10–35 °C) and transferred into water baths set to the corresponding temperatures. The animals were then incubated for 1 h. Afterwards the tubes were incubated for 1 h in a 10 °C cold room for recovery of the nauplii. The animals were then transferred into new hatching wells and kept at 10 °C. For every temperature, this experiment was repeated at least three times. Both lice strains were used in this experiment. On day 7 (4 days after treatment), the animals were examined with respect to the developmental stage (i.e., nauplius/copepodid) and their mobility. The ratio of copepodids to the overall number of animals in the hatching well was used as an indicator for survival. Based on the obtained data points, the LT50 value, the temperature at which 50% of the animals died, was calculated.

Hardening

A subsequent experiment was performed in order to determine if a relatively mild heat shock at a sublethal level enables the nauplii to better cope with a later, stronger nearly-deadly heat shock. This could indicate the induction of heat shock proteins. To evaluate this, nauplii of individual egg strings of the *LsH₂O₂* strain were separated into two different test tubes per egg string in approximately the same numbers. One of the tubes was kept at 10 °C as control while the other one was subjected to a heat shock of 26 °C for 1 h as described above. After recovery at 10 °C for 1 h, the animals in both tubes were concentrated by removing excess water and subsequently heat shocked at 31 °C, as described above. After the next recovery period, all animals from the different tubes were transferred to different hatching wells. On day 7 after hatching, nauplii and copepodids in each hatching well were counted. The temperatures were selected based on the outcome of the experiment to determine the thermal tolerance limits (i.e., 26 °C no effect on survival; 31 °C around 50% of the animals did not molt).

Identification of heat shock proteins

The identification of heat shock proteins was based on the recently published sea louse genome. We used the “InterProScan 5 analysis of *L. salmonis* peptides” available at www.licebase.org to search for genes encoding proteins containing characteristic domains. To detect members of the HSP70 family, we searched for the annotations IPR013126 (InterPro family) and PF00012 (Pfam domain), for HSP90 for IPR001404 and PF00183, for sHSPs for PF00011 and IPR008978 and for HSP40 for IPR001623. Regarding HSP40, we analyzed only genes that were encoding one additional HSP40-related domain like IPR009073 or IPR008971 or IPR012336. An important transcription factor involved in heat shock response (HSF1) was identified by search for IPR027725. Throughout this work, we are using Ensembl Metazoa identification numbers as gene identifiers, which can be used in Ensembl Metazoa and Licebase.

For the genes found to be heat-induced (cf. gene expression analysis), the sequence information obtained from the sea louse genome was validated by performing PCR with primers surrounding the predicted coding sequence. Primer design was conducted using Primer3 (Rozen and Skaletsky 1999). The obtained fragments were sequenced and the sequences deposited in the European Nucleotide Archive (ENA).

Gene expression analysis

To see which of the heat shock domain containing genes actually are inducible by a heat shock and to see their temporal expression pattern, a time-course experiment was performed. Three-day-old nauplii from seven to eight egg strings of the *LsH₂O₂* strain were pooled and subsequently divided into 15 test tubes. Animals in one tube were directly dried from water and transferred into RNA-later (Ambion), a buffer stabilizing RNA in tissue, preventing its degradation. The other animals were either heat shocked at 28 °C for 1 h or kept at 10 °C as control. The test tubes were then transferred to a 10 °C room where the water could cool down. After one additional hour, all animals were transferred into flow-through hatching wells. Overall, samples were taken as described, directly before the heat shock (0 h), directly after the heat shock (1 h), after the recovery (2 h) and additionally after 6, 24, 48 and 96 h after beginning of the experiment. The animals were stored in RNA-later at −20 °C. Additionally, for three egg strings of the strain *LsGulen*, the same heat shock was performed but taking only samples after 2 h (directly after the recovery phase) for heat shocked and control animals.

Osmotic stress treatment

Free-living copepodids (7 days post hatching) were divided into four groups, each incubated in different water salinity: 34.5‰, 25.8‰, 17.25‰ and 8.62‰ (100, 75, 50 and 25% seawater respectively). Water was prepared by mixing seawater with freshwater in a required proportion and the final salinity was evaluated with a salinity sensor (Cond 3110, WTW). Water temperature was maintained at 10 °C during the course of the experiment. Copepodids (around 200 per group) were placed in 50 ml test tubes and water of a specified salinity was added. Animals were incubated in stagnant water; therefore, water was changed every 4 h in order to maintain sufficient oxygen level. Animals were collected every 4 h and stored in RNAlater for subsequent total RNA extraction. Three parallels were prepared for each time point and salinity.

Hydrogen peroxide treatment

Nauplii of the *LsGulen* strain were incubated for 1 h in hydrogen peroxide (Chemco AS, Strusshamn, Norway)—seawater solutions of 500, 250 or 100 mg/l at 10 °C. Afterwards,

animals could recover for 1 h in seawater, before they were stored in RNAlater for RNA isolation. For every egg string used in this experiment, animals were treated in parallel without addition of hydrogen peroxide as a control. For each egg string, the fold changes were calculated individually.

The hydrogen peroxide concentrations used are expected to be stress-inducing for sea lice. 470 mg/l hydrogen peroxide almost completely inhibits hatching of egg strings of *L. salmonis* (Aaen et al. 2014). In adult animals, 100 mg/l for 30 min lead to around 20% immobility in a sensitive sea lice strain (Helgesen et al. 2015).

RNA isolation

RNA was isolated and purified with a combination of TRI Reagent (Sigma Aldrich) and the RNeasy Micro Kit (Qiagen). An on-column-DNase restriction was performed as described in the kit manual. RNA quantity and quality was measured on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). The ready RNA was stored at −80 °C.

Microfluidic expression measurement

To measure all identified potential HSPs as well as several reference genes at the same time, we used a microfluid chip, allowing the measurement of 48 genes in 48 samples (48.48 Dynamic Array IFC for Gene Expression; Fluidigm Corp.) on a Biomark HD machine (Fluidigm). Overall, we followed the protocol by the manufacturer “Fast Gene Expression Analysis Using EvaGreen on the BioMark or BioMark HD System”. In brief, cDNA was generated using Fluidigm Reverse Transcription Master Mix, employing 10 ng RNA. Preamplification of the target genes was performed in 11 cycles using the Fluidigm PreAmp Master Mix. Unincorporated primers were removed by Exonuclease I treatment (Thermo Fisher Scientific, Braunschweig, Germany). Preamplified samples were loaded on the primed 48.48 IFC chip and analyzed with the Biomark HD system using the manufacturer’s thermal protocol ‘GE Fast 48 × 48 PCR + Melt v2.pcl’. Detection of double-stranded DNA in the assays was done by EvaGreen (Bio-Rad Laboratories).

To analyze the data, a dilution curve for every measured gene was generated based on PCR products of known concentration and measured using conventional qPCR (see below). The primer efficiency was used to calculate the theoretical copy number of each gene based on the detected C_q value in every sample which was then normalized by the geometric mean of the two most suitable reference genes Elongation factor 1 α and Tubulin β . Their eligibility as reference gene was evaluated using the genorm module (Hellemans et al. 2007) of qBase + (Biogazelle). Fold changes were calculated for each heat shocked – control pair from each pool separately.

Primers used for this measurement are given in Electronic Supplementary Material 1.

Quantitative real-time PCR

Positive results found with microfluid technology (see above) were verified using conventional quantitative real-time PCR (qRT-PCR) using the same samples. For the temperature-related experiments, cDNA was generated based on 150 ng RNA using SuperScript II (Thermo Fisher Scientific) and cleaned up with the High Pure PCR Product Purification Kit (Roche). Five microliters was used in 12 μ l reactions using SensiFast SYBR No-ROX Kit (Bioline) on a LightCycler 96 system (Roche). For the hydrogen peroxide and salinity experiments, cDNA was synthesized using the AffinityScript qPCR cDNA synthesis Kit (Agilent Technologies) and diluted before being employed in 10 μ l reactions with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) on QuantStudio 3 qPCR machines (Applied Biosystems).

Reference genes Elongation factor 1 α (EF1 α , Frost and Nilsen 2003) and Tubulin β (TUBB, as suggested by Gallardo-Escárate et al. 2014) were used in the temperature and hydrogen peroxide experiments. As Tubulin β seemed to be unstable in the salinity trial, we used Elongation factor 1 α , filamin-A (FLNA, as suggested by Sutherland et al. 2012), and ADP/ATP carrier protein 3 (ADT3, Eichner et al. 2015), a combination recommended by the genom algorithm, embedded into the qBase + software (Hellemans et al. 2007), evaluating five potential reference genes. Primers used for this measurement are given in Electronic Supplementary Material 1.

Results

Thermotolerance

The acute thermotolerance of nauplii in salmon lice was determined to identify a suitable temperature inducing heat shock in subsequent experiments (Fig. 1). We determined the LT50-value, the temperature at which 50% of the observed nauplii did not molt to copepodids. LT50 was 30.8 $^{\circ}$ C for the *LsH₂O₂* strain and 31.3 $^{\circ}$ C for the *LsGulen* strain. Overall, the obtained curves for both strains were quite similar. While virtually all animals made the transition from nauplius to copepodid at 29 $^{\circ}$ C and below, a decline became visible starting at 30 $^{\circ}$ C. At 32 $^{\circ}$ C and above all animals remained in the Nauplius stage. Additional effects on behavior could also be observed. While copepodids that had experienced heat shocks up to 29 $^{\circ}$ C were moving actively within their hatching wells and were found in all water-depths, copepodids having experienced heat shock of 30 or 31 $^{\circ}$ C were almost exclusively lying on the ground of the hatching well showing no active

Fig. 1 Molting success after heat shock. Sea lice nauplii were incubated in water at different definite temperatures on day 3 post hatching. On day 7, the number of nauplii and copepodids was determined and the ratio of copepodids in the whole population calculated. The horizontal black line cuts the fit curves in the position at the LT50 value, the temperature at which 50% of the animals did not make the transition to copepodids

swimming movement. For those animals, some movements were only observed during counting under the microscope.

Hardening

Treating nauplii with a heat shock of 26 $^{\circ}$ C prior to a heat shock of 31 $^{\circ}$ C (hardening) led to a significantly increased ratio of copepodids in all four trials in comparison to animals of the same egg string that were directly shocked at 31 $^{\circ}$ C (Table 1). Although there were some variabilities between the egg strings, there was a clear difference ($p = 0.003$) between hardened (molting success 87–96%) and non-hardened animals (molting success 50–72%).

Identification of potential HSPs in the genome

Overall, we identified 76 putative heat shock protein encoding genes in the *L. salmonis* genome. Twenty-one of these encoded sHSPs, 42 HSP40, ten HSP70 and three HSP90. A comparison with the number of corresponding genes in other model organisms revealed roughly similar numbers (Table 2). Especially the numbers for the invertebrates were very alike or even identical (HSP90). Eye-catching was the high number of HSP-encoding genes in the zebrafish, which showed the highest number for every category, even higher numbers than the mouse, which also had slightly increased numbers of HSP-encoding genes.

Table 1 Effect of hardening on the outcome of a heat shock experiment. Nauplii were heat shocked at 31 °C, either directly or after a pre-treatment of hardening at 26 °C. Four days after the heat shock the numbers of copepodids and nauplii in each sample were counted

Egg string	Direct heat shock 10 °C → 10 °C → 10 °C → 31 °C → 10 °C			Pre-treated heat shock(hardened) 10 °C → 26 °C → 10 °C → 31 °C → 10 °C			<i>p</i> value ^a
	Copepodids (#)	Nauplii (#)	Copepodids (%)	Copepodids (#)	Nauplii (#)	Copepodids (%)	
A	49	19	72.1	44	2	95.7	0.003
B	21	21	50.0	35	4	89.7	
C	33	31	51.6	69	10	87.3	
D	46	28	62.2	55	5	91.7	

^a Paired *t* test between ratios of copepodids of directly heat shocked and pre-treated experiments

Sequence verification

The sequences of the heat-induced HSPs (cf. next section) were verified by PCR and Sanger-sequencing (Table 3). The predicted molecular mass of the sHSP family members was between 20 and 30 kDa, the mass of the heat-induced HSP40 35 kDa, HSP70 between 60 and 94 kDa, and the mass of the heat-induced HSP90 was 82 kDa. All the genes had Blast hits with heat shock proteins from various species. All sequences are deposited under the given ENA accession numbers.

Expression profiling

Overall, the gene expressions of 37 predicted genes encoding proteins with HSP domains as well as heat shock factor 1 (HSF1) were profiled in sea lice after a 1-h heat shock and subsequent recovery. The experimental overview is shown in Fig. 2. Of the analyzed genes, nine were found to be highly inducible by heat shock (Table 4). One sHSP gene (EMLSAG00000006187) was only expressed in some heat shocked animals and not at all in control animals. There was a high concordance between results from louse strain *LsH₂O₂* and *LsGulen*. All highly inducible genes in *LsH₂O₂* showed a high increase in expression in *LsGulen* as well. Of these genes, three belonged to the sHSP family, one to the HSP40, four to the HSP70 and one to the HSP90 family. All these inducible genes showed their expression maximum 6 h after beginning of the experiment. Interestingly, *LsGulen* (data obtained 2 h after experiment start only) showed fold changes that were as high or even higher (sHSP EMLSAG00000007586) than the fold changes in *LsH₂O₂* at their peak after 6 h.

For the genes with a strong heat shock induction, we validated the measurements with a conventional, non-microfluidics-based qPCR technique (Fig. 3), starting with new cDNA synthesis. Generally this method confirmed the results obtained by microfluidics described above. All nine measured genes showed a strongly increased expression after heat shock. Already directly after the heat shock (1 h), a clear increase was evident. The expression of the HSP genes increased for the next hour and in most cases until 6 h after heat shock. Twenty-four hours after

heat shock the expression of most genes returned almost to control level. In case of the sHSP genes EMLSAG00000002963 and EMLSAG00000007586, the HSP70 gene EMLSAG00000000052 and the HSP90 gene EMLSAG00000010216, an expression above control level was still apparent even after 24 h. At time points 48 and 96 h post heat shock all genes had returned to expression levels comparable to the unstressed controls.

Hydrogen peroxide induced stress

We examined the effects of hydrogen peroxide treatments on *L. salmonis* nauplii with respect to the expression of the heat-inducible HSPs (Fig. 4). Overall, the effects were non-significant for seven of nine analyzed genes (*p* > 0.05). Only at the highest H₂O₂ dose of 500 ppm the HSP40 EMLSAG00000009366 showed an upregulation around 2.5 times (*p* = 0.01) and sHSP EMLSAG00000007586 of 1.6 times (*p* = 0.03) compared to controls.

Salinity stress

Applying salinity stress, only three of the nine heat-induced HSPs showed a clear upregulation (Fig. 5). The small HSP EMLSAG00000007586 showed higher levels at salinities of 9‰ and 17‰ in comparison to the higher salinities. HSP40 EMLSAG00000009366 also showed an upregulation at 9‰, peaking at 20 h incubation time, being 2.4 times as high expressed as at the control salinity of 34.5‰. Similarly,

Table 2 Number of genes encoding HSP-specific domains, based on Ensembl Metazoa and Ensembl genome assemblies

	sHSP	HSP40	HSP70	HSP90
<i>Lepeophtheirus salmonis</i>	21	42	10	3
<i>Tribolium castaneum</i>	24	35	14	3
<i>Drosophila melanogaster</i>	23	41	13	3
<i>Caenorhabditis elegans</i>	21	33	11	3
<i>Danio rerio</i>	31	59	19	5
<i>Mus musculus</i>	28	52	13	4

Table 3 Sequence properties of heat-induced HSPs

Gene ID	Family	ENA accession	CDS length (bp)	Protein length (aa)	Predicted MW (kDa)	Best SmartBlast hit
EMLSAG00000002963	sHSP	LT844571	549	182	21	Lethal (2) essential for life, isoform A, <i>Dm</i> ^a
EMLSAG00000007581	sHSP	LT844572	744	247	28	Heat shock protein beta-1, <i>Dr</i> ^b
EMLSAG00000007586	sHSP	LT844573	786	261	30	Heat shock protein beta-1, <i>Hs</i> ^c
EMLSAG00000009366	HSP40	LT844574	951	316	35	CG5001, isoform C, <i>Dm</i> ^a
EMLSAG00000000052	HSP70	LT844575	2529	842	94	Heat shock 70 kDa protein 4 L isoform 1, <i>Hs</i> ^c
EMLSAG00000002965	HSP70	LT844576	1620	539	60	Heat shock cognate 71 kDa protein isoform 2, <i>Hs</i> ^c
EMLSAG00000010489	HSP70	LT844577	1962	653	71	heat shock cognate 71 kDa protein, <i>Dr</i> ^b
EMLSAG00000011625	HSP70	LT844578	1947	648	72	heat shock cognate 71 kDa protein, <i>Mm</i> ^d
EMLSAG00000010216	HSP90	LT844579	2145	714	82	heat shock protein 83, isoform A, <i>Dm</i> ^a

^a *Drosophila melanogaster*^b *Danio rerio*^c *Homo sapiens*^d *Mus musculus*

HSP70 EMLSAG00000011625 showed an induction in 9‰ salinity, with a maximum after 20 h of incubation and an 18 fold upregulation in comparison to the control salinity.

Discussion

The sea lice genome encodes several heat shock proteins

We identified around 70 genes putatively encoding for proteins containing HSP domains. In comparison to other animal genomes, this is within the normal range. This underlines the ancient evolutionary nature of the heat shock response (Feder

and Hofmann 1999). The only exception regarding the number of HSP-encoding genes is the zebrafish where the numbers of genes are overall about twice as high as in the other species. This can be explained by the whole-genome-duplication of teleosts around 320–350 million years ago (Glasauer and Neuhauss 2014), leading to additional paralogues for each gene. However, all the recorded HSPs are based on automatic genome annotation pipelines and prediction of protein domains. The number of sHSP-encoding genes was predicted to be roughly only half of that obtained by using the Pfam domain in comparison to the Interpro-domain. These numbers also seem to fit better to established numbers from literature (Morrow and Tanguay 2015).

Fig. 2 Experimental setup: Nauplii from several egg strings that hatched on the same date were pooled 3 days after hatching. They were distributed into different test tubes and either heat shocked at 28 °C or kept at 10 °C. Animals were sampled at the given time point

Table 4 Expression of various genes encoding heat shock proteins after heat shock

Gene ID	<i>LsH₂O₂</i>				<i>LsGulen</i>	
	Maximum average FC ^a	Time (h) ^b	<i>p</i> value ^c	Highly inducible ^d	Average FC 2h ^a	<i>p</i> value ^c
<i>sHSP</i>						
EMLSAG00000000357	2.0	96	0.08	–	1.0	0.95
EMLSAG000000002239	1.5	96	0.05	–	1.3	0.10
EMLSAG000000002377	1.3	96	0.05	–	1.1	0.73
EMLSAG000000002963	<i>24.1</i>	6	0.08	+	<i>23.6</i>	0.10
EMLSAG000000003447	1.2	2	0.02	–	1.0	0.44
EMLSAG000000006078	1.7	6	0.06	–	1.5	0.06
EMLSAG000000006187	x					
EMLSAG000000006825	2.0	6	<i>0.01</i>	–	1.8	<i>0.01</i>
EMLSAG000000007581	<i>39.7</i>	6	<i>0.00</i>	+	<i>36.6</i>	<i>0.01</i>
EMLSAG000000007586	<i>74.5</i>	6	<i>0.00</i>	+	<i>114.8</i>	<i>0.00</i>
EMLSAG000000010211	4.9	6	0.01	–	3.3	<i>0.00</i>
EMLSAG000000010559	3.2	6	<i>0.00</i>	–	2.9	<i>0.01</i>
EMLSAG000000010714	2.8	6	0.05	–	2.7	<i>0.01</i>
EMLSAG000000010793	1.5	24	0.33	–	0.7	<i>0.03</i>
EMLSAG000000012934	1.2	6	0.09	–	1.1	0.22
<i>HSP40</i>						
EMLSAG000000000116	1.5	24	0.05	–	1.2	0.08
EMLSAG0000000001013	1.1	96	0.06	–	1.0	0.71
EMLSAG0000000001361	3.2	96	0.17	–	1.5	0.29
EMLSAG0000000002291	1.3	96	0.04	–	1.1	0.29
EMLSAG0000000005749	1.5	6	0.40	–	1.6	0.17
EMLSAG0000000006084	1.6	6	0.10	–	1.5	0.09
EMLSAG0000000007075	1.6	6	0.02	–	1.7	<i>0.04</i>
EMLSAG0000000009366	<i>43.6</i>	6	<i>0.01</i>	+	<i>41.6</i>	<i>0.01</i>
EMLSAG00000000012031	<i>5.4</i>	6	0.01	–	4.1	<i>0.00</i>
<i>HSP70</i>						
EMLSAG0000000000052	<i>12.2</i>	6	<i>0.01</i>	+	<i>10.4</i>	<i>0.00</i>
EMLSAG0000000000057	1.5	2	0.03	–	1.9	0.06
EMLSAG0000000002965	<i>26.5</i>	6	0.02	+	<i>26.3</i>	<i>0.01</i>
EMLSAG0000000003639	3.9	6	0.02	–	3.6	<i>0.01</i>
EMLSAG0000000005583	1.2	24	0.24	–	1.1	0.54
EMLSAG0000000005584	1.3	96	0.32	–	0.9	0.67
EMLSAG0000000006169	1.8	6	0.06	–	1.8	<i>0.04</i>
EMLSAG0000000006538	1.9	48	0.08	–	1.2	0.46
EMLSAG00000000010489	<i>24.3</i>	6	0.01	+	<i>20.7</i>	<i>0.01</i>
EMLSAG00000000011625	<i>17.6</i>	6	<i>0.00</i>	+	<i>10.1</i>	<i>0.02</i>
<i>HSP90</i>						
EMLSAG0000000004117	1.0	96	0.93	–	1.1	0.36
EMLSAG00000000010216	<i>17.2</i>	6	0.05	+	<i>11.9</i>	<i>0.01</i>
EMLSAG00000000012414	1.4	6	0.09	–	1.2	0.27
<i>Heat shock factor</i>						
EMLSAG0000000005535	2.0	24	0.32	–	1.4	<i>0.02</i>

^a FCs >5 printed in italics^b Time, when maximum FC was reached, cf. experimental design^c Values <0.00833 printed in italics, adjusting for multiple testing per gene^d Defined by at least three time points with FC >5^e Values <0.05 printed in italics^x Only expressed in some heat shocked samples, not in control

Even though the gene numbers were similar and HSPs are evolutionary ancient, phylogenetic relationships among heat shock proteins cannot give reliable information about the actual heat inducibility, since this feature has emerged several times during evolution (Krenek et al. 2013). That is also why we profiled the gene expression upon heat shock in two sea lice strains.

Sea lice nauplii can adapt to heat shock

To get an overview over the reaction of sea lice to heat stress, we conducted experiments to assess the thermotolerance and capability of hardening in sea lice nauplii. Under the experimental conditions, *L. salmonis* nauplii acclimated to 10 °C could survive a 1-h heat shock at 30 °C to very high extent.

Fig. 3 Temporal expression profiles of heat-inducible HSP-encoding genes measured by conventional qPCR. The *different gray scales* represent animals from different egg string pools of salmon louse strain *LsH₂O₂*. At the beginning, the tubes containing the animals were filled

with 28 °C warm seawater. In the first hour, the tubes were incubated in water baths at the same temperature; in the second hour, the tubes were placed in 10 °C cold water for recovery, and after 2 h, the animals were transferred into their hatching wells

We assume that the molting capacity can be seen as an indicator of survival. Nauplii normally molt at day 5 post hatching to copepodids at 10 °C (Eichner et al. 2014). The animals were counted on day 7 post hatching to ensure sufficient time for molting. Failure to molt at this time point indicates significant developmental abnormality or death since it is unlikely that these nauplii II will molt at all. Determining the molting capacity seems to be a reliable survival indicator, as it is far easier to distinguish between nauplius and copepodid in the microscope than to assess the actual dead-or-alive status based on motility or other factors at these stages.

The capability to survive a temperature increase of 20 °C is similar to that of other aquatic organisms. Nauplii of the copepod *Tigriopus californicus*, acclimated to 20 °C, survived temperatures up to 37 °C, depending on strain (Tangwanchaoen and Burton 2014), a far higher temperature than *L. salmonis* survived in our experiments. Whereas *T. californicus* survives a higher absolute temperature than *L. salmonis* (37 vs. 31.5 °C), the latter survives a higher temperature amplitude of 21 vs. 17 °C in *T. californicus*.

The water flea *Daphnia pulex* can survive up to 35 °C (Williams et al. 2012) after acclimation to 18 to 28 °C.

Fig. 4 Gene expression of HSPs in response to three different doses of hydrogen peroxide. The nauplii were incubated in a seawater–hydrogen peroxide mixture for 1 h, were allowed to recover for 1 h in normal seawater before being sampled. Values are given as means + standard errors of the means. Only the last five digits of each gene ID are given for the clarity of the graph

Antarctic copepods have upper temperature limits at around 15 °C after direct transfer from the field at around 0 °C

(Lahdes 1995). It has been shown that heat tolerance depends on the acclimation temperature (Williams et al. 2012).

Fig. 5 Expression of heat-inducible HSPs after incubation in water of different salinities for different time spans. Values are given as means ± standard errors of the means

According to that, the determined LT50 value of 31 °C is most likely restricted to *L. salmonis* nauplii acclimated to 10 °C. In a recent study, more long-term temperature effects on sea lice were analyzed (Samsing et al. 2016). The highest temperature used in that study was 20 °C and at that temperature the lice were still reproducing. It is expected that animals acclimated to this temperature are able to survive acute exposure to higher temperatures, similar as shown in *Daphnia* (Zeis et al. 2004).

The results of our experiments indicate that nauplii affected by a moderate heat shock survive a subsequent strong heat shock to a larger extent than animals without the pre-treatment. This successful “hardening” (Bowler 2005) of the nauplii suggests the involvement of HSPs and gives a better handling of a repeated, more intense heat shock, demonstrating that the *L. salmonis* nauplii have a certain phenotypic plasticity and are able to adapt to environmental changes. For lecithotrophic larva like the salmon louse nauplii, which are completely dependent on their internal energy storage (egg yolk) for survival (Tucker et al. 2000), every production of nucleic acids and proteins reduces their energy storage. This may lead to a trade-off regarding the investment into heat shock proteins. Since the production of HSPs in response to a heat shock may lead to the depletion of the nauplius’ energy reserve necessary for other processes like molting or host finding as copepodid, it might even decrease their lifespan. On the other hand, benefits of producing HSPs were apparent in the hardening trial. Such a trade-off is generally observed between the advantage of HSP expression and costs like impact on growth and fertility (Sørensen et al. 2003). However, the costs may be even more severe when the animal is unable to refill the energy used for HSP production itself. Depleted energy storages may also explain why most animals that survived a heat shock at 31 °C and molted to copepodids showed abnormal behavior and were barely moving compared to the control animals. Overall, the results indicate that the maternal energy storage in *L. salmonis* larvae, to a large extent consisting of vitellogenins (Dalvin et al. 2011), is sufficiently large to pay the cost of expressing a wide range of genes that is only needed under detrimental environmental conditions, thereby increasing survival chances.

Nauplii can induce heat shock proteins

We analyzed the gene expression of 38 HSP-encoding genes over time upon heat shock at 28 °C. Differences between the induction levels measured by the microfluidic chip-based and the plate-based real-time qPCR approach may be due to the different techniques, such as a pre-amplification step in the microfluidic approach, which could create some bias. Additionally, all the enzymes used in the various steps after RNA isolation were produced by different companies. However, all the genes identified by microfluidics as highly inducible were confirmed by single-gene plate-based qPCR.

Nine genes were strongly induced by heat shock. The time courses obtained for the expression of the strongly heat-inducible HSP genes were very similar. This is not surprising as large parts of the heat shock response are coordinated by the transcription factor heat shock factor 1 (HSF1) (Vihervaara and Sistonen 2014; Leach et al. 2016). Our finding that the transcription levels of *hsf1* remained stable during and after heat shock, while the HSPs were strongly induced can be attributed to HSF1’s mode of action. In case of heat shock, HSF1 trimerizes and subsequently translocates into the nucleus for interaction with the DNA and thereby works as transcription factor (Vihervaara and Sistonen 2014), inducing HSP expression. To respond without delay, HSF1 must be available in the cell all the time to detect heat stress and then induce the heat shock response.

The expression profiles of *L. salmonis*’ heat-inducible HSPs also resembled the corresponding profiles in other animals. In particular, the similarities to the springtail *Orchesella cincta* (Bährndorff et al. 2009) are striking. In a very similar experiment with 1 h hardening at a high temperature and subsequent recovery phases of various lengths the maximum induction of HSP70 on mRNA level was reached 3 h after onset of the heat shock. Similar to most *L. salmonis* HSPs, the expression level returned to the control level after 24 h. The protein level increased until the end of the experiment after 50 h. In the butterfly *Melitaea cinxia*, the peak of HSP70 gene expression was reached directly after a 2-h heat shock and the levels declined with onset of a recovery phase (Luo et al. 2015). In the great pond snail *Lymnaea stagnalis*, similar patterns were found for HSP40 and HSP70 (Foster et al. 2015). In the present study, most maximum HSP expression occurred after 6 h but it has to be kept in mind that the next time point for sampling was 24 h, so a further increase could be possible. However, it is striking that for at least 5 h after the end of the heat shock and the onset of the recovery phase, the HSP mRNA levels were increasing.

A previous study characterizing effects of abiotic stress on the *L. salmonis* transcriptome revealed only very limited gene regulation by heat shock (Sutherland et al. 2012). However, the temperature increase in that study was from 10 to 16 °C, which is modest compared to the actual temperature tolerance and the 28 °C used in our experiment. Additionally, only one time point, 24 h post heat shock without a subsequent recovery phase, was examined. According to our data, most of the HSPs are almost on their base level after 24 h. We assume that the combination of a quite low heat shock temperature and a relatively late time point for gene expression measurement explain the absence of changes in the HSP expression in that study.

In the present study, we have focused on the heat shock response in free living nauplii (temperature and oxidative stress) and copepodids (osmotic stress). To get a complete overview of the heat tolerance in *L. salmonis*, all life stages

must be examined. The heat tolerance between life stages might vary and it cannot be generalized that later life stages are generally more robust than early stages (Tangwancharoen and Burton 2014). Additionally, the heat inducibility of HSPs can vary during the life cycle. One example is that 10-day-old *Drosophila* flies induce more HSPs after heat shock than *Drosophila* embryos (Leemans et al. 2000; Landis et al. 2012; Morrow and Tanguay 2015). For sea lice, it is possible that the heat shock response is limited in the free-living stages due to the restricted amount of available energy.

Implications for thermal treatment

Recently thermal treatment of sea lice infestations in aquaculture facilities has been developed. These “thermolicers” apply heated water to delouse Atlantic salmon. According to the manufacturer, the temperature in this treatment is between 30 and 34 °C depending on the seawater temperature. The LT50 values we found in our study are within this range. Hence, depending on temperature, some lice entering the thermolicer might survive the treatment. This is especially true, as the heat treatment only lasts 25–30 s to avoid damage to the fish. Therefore, it is important to prevent all lice that have entered the machine from leaving. Although we did not look in detail into differences between various lice strains, our data suggest that there might be differences between *LsGulen* and *LsH₂O₂*. It was striking that the induction of HSPs in *LsGulen* after just 2 h was as high as the induction in *LsH₂O₂* at its peak after 6 h. Additionally, induction of the sHSP gene EMLSAG00000007586 was almost twice as strong in *LsGulen* compared to *LsH₂O₂*. Furthermore, the lethal temperatures were not identical between the two strains, suggesting genetic variation for the heat shock response. If this is the case, selection towards a specific heat shock response would be possible, and machines like the “thermolicer” could contribute to shifts in heat shock response and even lethal temperature for surviving individuals. Hence, it would be interesting to compare lethal temperatures and heat shock responses from more sea louse strains to assess the variation in this trait.

Hydrogen peroxide only induces slight HSP response

Compared to heat shock, the reaction to oxidative stress caused by hydrogen peroxide treatment was almost negligible. Only two HSPs showed a significant increase, and only after treatment with the highest dose. However, it is not surprising that one of these activated genes belongs to the HSP40- or DnaJ-family because DnaJ HSPs show “the best evolutionarily conserved response to H₂O₂” (Vandenbroucke et al. 2008).

It may be considered that other HSPs that do not react to heat shock may be involved in oxidative stress instead. However, in other species, there are HSPs which react

strongly to both heat as well as oxidative stress. For example, in the abalone *Haliotis discus*, a small HSP has been identified showing rapid increase in expression upon hydrogen peroxide treatment, a higher response than for heat shock (Sun and Hu 2016). However, this was measured in the gills, a tissue with a very large surface in direct contact with the surrounding water. The salmon louse nauplii have no such tissue or direct inflow of water into the body. This may lead to less oxidative stress caused by hydrogen peroxide.

Other proteins than heat shock proteins may play important roles in response to oxidative stress. Catalase for example is known to catalyze the dissociation of hydrogen peroxide to water and oxygen (Deisseroth and Dounce 1970). Recently, it has been shown that different expression levels and enzyme activities of catalase may be responsible for different hydrogen peroxide tolerance levels in adult sea lice (Helgesen et al. 2017).

Freshwater treatment induces a slight increase in specific HSP transcripts

We analyzed the response of the heat-induced HSP genes to water with different salinities. There was no difference between the normal salinity of 35‰ and a salinity of 26‰ but some genes were induced at lower salinities. The lowest salinity of 9‰ showed the strongest response. However, this response was much smaller than upon heat shock. The strongest induced gene, HSP70 EMLSAG00000011625, increased by a factor of 19 after 20 h of incubation compared to more than 1000-fold upon heat shock after 1 h. Additionally, there was an overlap between the hydrogen peroxide and the salinity response. EMLSAG00000007586 and EMLSAG00000009366 were the only genes affected by hydrogen peroxide and both genes were also induced by a low salinity.

Sutherland et al. (2012) conducted a similar experiment with a higher resolution in salinity levels and measurements by microarray at one time point (24 h). They noticed upregulation of several HSPs upon exposure of copepodids to water with reduced salinity. The magnitude of upregulation was low, in agreement with the present results. EMLSAG00000011625 (HSP70) was the strongest regulated gene (eightfold upregulated at 25‰ in comparison to 30‰) in both studies and it was also the strongest induced gene upon heat shock. Overall, HSP70 EMLSAG00000011625 seems to be highly inducible by stressors. However, it is expected that other genes, specific to osmoregulation may be more important for salinity-induced stress and might react stronger to differences in salinities. Proteins such as sodium/potassium ATPases and carbonic anhydrases are known to be regulated by salinity transfers for many species (Havird et al. 2013). Our result showing that heat induces a stronger HSP induction than changes in salinity is in line with observations in the mud crab *Scylla*

paramamosain where a HSP70 gene was upregulated up to 2.3 times in hyperosmolarity but up to 25 times upon heat shock (Yang et al. 2013).

Conclusion

In this study, novel data on the heat shock response in the parasitic copepod *Lepeophtheirus salmonis* is obtained. We demonstrate that nauplii are able to survive 1-h long heat shocks for up to 30 °C and they actively adapt to their environment through gene expression changes. Nine genes from various HSP families are strongly heat-inducible. Their response to H₂O₂-mediated oxidative stress or to salinity-mediated osmotic stress is less pronounced. This work paves the way for future work on stress physiology in sea lice.

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