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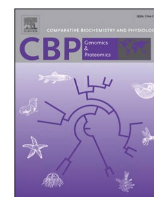

The transcriptomic response of adult salmon lice (*Lepeophtheirus salmonis*) to reduced salinity

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A B S T R A C T

Salmon lice (*Lepeophtheirus salmonis*) are marine parasitic copepods living on salmonids and are challenging for salmon aquaculture. One of several treatment methods is the application of freshwater to the fish which can lead to lice loss. However, lab experiments have shown that salmon lice, acclimated to seawater, are capable of surviving for several weeks in freshwater, when attached to a host. If not attached to a host, they die within a few hours in freshwater but can survive a longer time in brackish water. The molecular mechanisms involved in the adaptation to low salinity of the louse have not been identified yet. In this study we incubated salmon lice, being attached to a host, or detached, in seawater, brackish water and freshwater for 4 h and 1 d, sampled the animals and used RNA-Seq to identify genes involved in these mechanisms. Freshwater incubation led to a much stronger regulatory response than brackish water and a longer incubation time gave a stronger effect than a short incubation. Among the most interesting genes, upregulated in low salinity water are in addition to several transporters, several enzymes involved in amino acid metabolism and especially in the proline biosynthesis. A strong upregulation of these enzymes might lead to an accumulation of proline which is known to be used as an osmolyte in other species. While the RNA-Seq experiment was performed with female samples, qPCR showed that at least 10 genes regulated in females, were also regulated in males.

1. Introduction

Salinity is an important abiotic factor for all water-living animals (Kültz, 2015). Stenohaline animals are only capable of living in water of a certain narrow salinity range, whereas euryhaline animals tolerate a broader range. Salinity changes are challenging for the homeostasis maintenance of the blood or hemolymph and osmoregulation is necessary to deal with these changes, which is a highly cost-intensive process (Rivera-Ingraham and Lignot, 2017). Based on the way animals cope with salinity changes, they can be categorized into osmoconformers and osmoregulators (Rivera-Ingraham and Lignot, 2017). Osmoconformers maintain an inner environment with the same osmolarity as the outer medium. Osmoregulators, on the other hand, regulate the osmotic pressure of their body fluids to a specific level, independent of the environment. The greater the environmental and inner osmotic pressure differ, the more difficult and energy-demanding the process of osmoregulation gets.

The salmon louse (*Lepeophtheirus salmonis* Krøyer, 1837) is a marine obligate ectoparasitic copepod that feeds on skin, mucus and blood of their host. Hosts include a variety of salmonids, including Atlantic salmon (*Salmo salar* Linnaeus, 1758) (Boxaspen, 2006). This renders the salmon louse a huge problem for the aquaculture industry, creating huge economic damages each year (Costello, 2009).

The osmoregulation of the salmon louse has been under research for several decades. As early as 1985, a detailed study on the osmotic response of the salmon louse during the transition from sea to freshwater was published (Hahnenkamp and Fyhn, 1985). The authors measured ion concentrations and osmolarity in the hemolymph of the louse. They showed that the parasite is an osmoconformer in seawater but uses hyperosmotic regulation in low-salinity water. Additionally, they demonstrated differences in osmoregulation between brackish and fresh water as well as between lice attached to a fish or freely swimming (detached). Detached lice died after 8 h in freshwater (FW), whereas attached lice survived for more than seven days. The authors concluded that lice in brackish water (BW) use a host-independent osmoregulatory mechanism, whereas they rely on a host-dependent mechanism in FW.

Later research focused more on the general capability of salmon lice to survive in FW. An overview of the research so far is given by Wright

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et al. (2016). Adult lice attached to fish are capable of surviving a long time (several days or weeks) in FW, in contrast to lice that have been detached from their host. Copepodids on the other side, die within few hours in FW, independent on their attachment state. The salinity tolerance of copepodids has been analyzed several times. The results obtained vary broadly. Two studies found quite similar sensitivities (Bricknell et al., 2006; Sievers et al., 2019) with 50% of the copepodids dying after 8–10 h in brackish water of 23 ppt. Another study found no reduced survival in such water even after an incubation of 24 h (Andrews and Horsberg, 2020). This study also found different EC_{50}-values for salinity for two different lice populations, suggesting a genetic background of low-salinity-tolerance and delivering a possible explanation for the different outcomes of the studies. In BW it also is of importance if a copepodid is attached to a host or not (Sievers et al., 2019).

The molecular processes that occur during osmoregulation in the salmon louse have been studied to a much less extent. To our knowledge there is only one microarray-based analysis focusing on copepodids (Sutherland et al., 2012). Here, changes in salinity as well as temperature were examined. Low salinity gave a stronger regulation of gene expression than high temperature, especially when falling below a salinity threshold of 27 ppt.

In this work, we want to elucidate the molecular reactions of attached and detached adult salmon louse to brackish and freshwater through comparative transcriptomics.

2. Methods

2.1. Experimental design

Thirty-six Atlantic salmon were acclimated to four 500 l tanks (9 fish each). The fish were infected with 40 copepodids (labstrain LsGulen, first described in (Hamre et al., 2009)) each. These fish were kept in seawater (SW) at 12 °C. After 48 days, the lice had reached a relative age of 170% (Hamre et al., 2019); both females and males were in the adult stage and females were carrying egg strings. All fish from one tank were sacrificed to collect female lice which were then transferred to hatching-wells (Hamre et al., 2009) within an incubator, in which the lice could swim freely, without being attached to a host. Each incubator was supplied with running water from the bottom of the fish tanks via a thin hose. The experiment was started by lowering the water levels in the tanks to 200 l. Then, the tanks were filled with water of the desired salinity within 12 min and the water-flow continued. The three tanks and incubators were receiving (i) FW (ii) SW (iii) a mixture of FW and SW (BW) respectively. SW was taken from a depth of 105 m from the Byfjorden, outside of Nordnes, Bergen, Norway. FW was taken from the lake Svartediket, Bergen, Norway. Both water types were filtered and UV-treated. Four hours after onset of the experiment, four fish from each of the three tanks were sacrificed, the rest of the fish after 24 h. Every time, male and female lice were collected from the fish, egg strings, if present, were removed from the females, and the animals were stored in RNAlater. At the same time points (after 4 and 24 h), detached females were also transferred from their hatching-well into tubes with RNAlater. Animals in RNAlater were stored for 24 h at 4 °C and then transferred to −20 °C until RNA isolation. Tank salinity was continuously surveilled with conductivity meters (Sensortechnik Meinsberg) (Fig. 1). For the SW tank, salinity was determined to be stable at around 36 ppt, for the FW tank at 0 ppt. The BW salinity was not completely stable but had an average of 13.6 ppt when measuring from the first hour. The salinity of this water was fluctuating between 12 and 16 ppt. The detached lice in the incubators were exposed to the same water through the hose. Some spot-checks confirmed that they reached the same salinity as the tanks that were housing the fish.

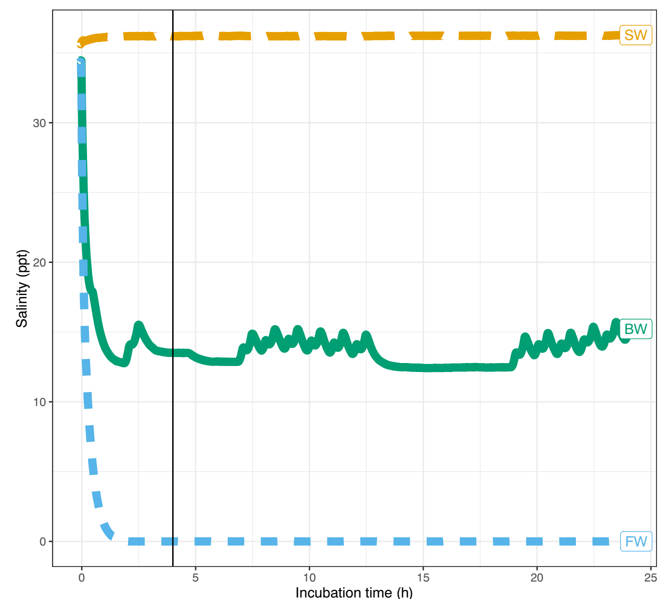

Fig. 1. Measured salinity during the experiment. The experiment was performed with three different water treatment groups (SW, Seawater; BW, Brackish Water; FW, Freshwater). The vertical line indicates the first sampling time point, the other sampling took place at the end of the measurement (24 h).

2.2. RNA-Seq

Overall, 48 samples were prepared for RNA sequencing, coming from 10 groups: “SW, attached 4h”, “SW, attached,24 h”, “BW, attached 4h”, “BW, attached,24 h”, “FW, attached 4h”, “FW, attached,24 h”, “SW, detached 4h”, “SW, dettached,24 h”, “BW, detached 4h”, “BW, detached,24 h”. For each group, between 4 and 5 samples were sequenced. RNA was isolated from whole individual adult female lice (without egg strings) from three fish per group using a combination of TRI reagent and the RNeasy Mini kit (Qiagen), following the manufacturer’s instructions, including on-column DNase-treatment. RNA quantity and purity were determined using a Nanodrop ND 1000 spectrophotometer, an Agilent 2100 Bioanalyzer and the Qubit system. Libraries were prepared using the TruSeq Stranded mRNA kit (Illumina) and quantified by qPCR employing the KAPA Library Quantification Kit. Clusters were generated using the Illumina cBot2 system, followed by sequencing using the Illumina HiSeq4000 instrument on six lanes (paired ends, 2 × 75bp).

2.3. Analysis

The obtained reads were quality checked and then pseudo-aligned to the L. *salmonis* transcriptome (based on genome assembly LSalAtl2s; available at metazoa.ensembl.org/Lepeophtheirus_salmonis) using the Salmon tool (Patro et al., 2017). Further analyses were performed in R (R Core Team, 2020) under RStudio (RStudio Team, 2020). Reads were imported into DeSeq2 (Love et al., 2014) for calculation of fold-changes (FCs) and *p*-values. Four separate Deseq2-models were generated for the different incubation times and attachment states (Attached, 4 h; Attached, 1d; Detached, 4 h; Detached, 1d). For the attached groups, comparisons for BW- and FW-treated lice in relation to SW (control) were performed, for the detached groups comparison between BW-treated lice and control (SW). Overall, this yielded six comparisons (Attached, 4 h: FW vs SW & BW vs SW; Attached, 1d: FW vs SW & BW vs SW; Detached, 4 h: BW vs SW; Detached, 1d: BW vs SW). Genes with less than 10 mapped reads were removed from the analysis. The fold-changes were shrunk with the “normal”-method of DeSeq2. A $\log_2$(FC) of 0.585 (equaling a 1.5× fold-change) and an adjusted *p*-value of 0.05

(adjusted within each analysis, we did not account for multiple testing between the comparisons) were used as limits to determine differently expressed genes (DEGs). The fold-change-threshold was taken into account for calculating the *p*-values, testing whether the $\log_2(FC)$ was greater in absolute value than the threshold (Love et al., 2014). Additionally, we calculated p-values testing for any difference from 0 (see table with all fold changes). For principal component analyses and heatmaps we used values after variance stabilizing transformation. Gene Ontology (GO) term enrichment analysis was employed using g:profiler (Raudvere et al., 2019; https://biit.cs.ut.ee/gprofiler/). We used all known genes as statistical domain scope and the g:SCS threshold method with a threshold of 0.05. Graphing was performed with the ggplot2 (Wickham Wickham, 2016) package in R. The R script, as well as information about the used packages and their versions, is given in Supplementary File 1. Throughout the manuscript we are using Ensembl stable ids (EMLSAGXXXXXXXXXXX), to unequivocally identify the genes we are referring to. These IDs should remain stable even after an update of the genome. Information connected to the individual genes can be accessed at Ensembl metazoa (http://metazoa.ensembl.org) (Howe et al., 2020).

2.4. Quantitative PCR

To validate our RNA-Seq analysis, we confirmed the gene expression in several of the original samples via qPCR. 1 μg of each RNA sample was reverse transcribed with the AffinityScript QPCR cDNA Synthesis Kit (Agilent) and the cDNA diluted 1:10. We used the same qPCR setup as described before (Borchel et al., 2019). In brief, qPCR was run with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, USA) on QuantStudio 3 qPCR machines (Applied Biosystems, Foster City, USA), with reaction volumes of 10 μl, including 2 μl of cDNA. The thermocycling parameters were as follows: initiation, 50 °C for 2 min; holding, 95 °C for 2 min; 40 cycles of 95 °C for 15 s; and then 60 °C for 1 min. Samples were run in technical duplicates and every plate contained a non-template control. Relative standard curves were run to verify a suitable reaction efficiency. Reference genes were chosen based on the RNA-Seq expression under the different treatment conditions. From overall five potential reference genes, we chose *elongation factor 1α* (*EF1A*) as well as *rps13* (previously mistakenly called *rps20*), which are established for general use in salmon lice (Frost and Nilsen, 2003), as these were least affected by the different treatments (Supplementary File 2). The geometric mean of the two reference genes was used for quantification of the target genes, as recommended (Vandesompele et al., 2002), also taking into account the reaction efficiencies. We measured ten target genes, encoding a DNA Ligase, a BCCT transporter, an amino acid transporter, a potassium channel, a histone H4, a monocarboxylate transporter, a pyrroline-5-carboxylate reductase, a Na,K-Atpase Interacting protein, a HSP70 and a delta l-pyrroline-5-carboxylate synthetase. Primers, either positioned on exon boundaries or spanning an intron, are given in Supplementary File 3.

To check gene expression in male salmon lice, we used animals that were attached to the same fish as the lice from the RNA-Seq experiment and that underwent the same treatment as the females. RNA from whole, individual males was isolated by a pure Trizol-method without a kit. RNA was then DNase-treated and cDNA synthesis and qPCR were performed as described for females, with five samples per group (attached, 1d: SW, BW, FW). To test for statistically significant effects of the water type on gene expression, we employed Kruskal-Wallis rank sum tests for each gene individually, included in the ggpubr-package (Kassambara, 2020).

3. Results

3.1. Phenotype

Detached lice did not survive for even 4 h in FW. A check of several animals under the microscope did not show any signs of life such as peristaltic or appendage movements. Therefore, samples from detached lice in FW were excluded from the analysis as it could be expected that RNA degradation was already taking place. The attached lice, on the other hand, survived the FW exposure. Even after 24 h, animals were still moving. The only observed abnormality was a lack of egg strings after 4 or 24 h in FW. Animals in BW and SW did not show any abnormalities, all detached lice survived, and lice on fish kept their egg strings attached.

3.2. Overview of RNA-Seq results

The 48 libraries sequenced obtained on average 38 ± 4 million paired-end reads. Mapping the reads to the salmon louse transcriptome using the Salmon tool yielded average mapping rates of 73 ± 2%. All RNA-Seq data related to this project are deposited at NCBI Bioproject PRJNA641271. A file containing all fold-changes and *p*-values is given in Supplementary File 4.

A principal component analysis of the RNA-Seq data showed a good separation of samples of different water treatment groups in three out of four conditions (Fig. 2). Expression patterns of attached lice after 1d-treatment clearly separated samples from SW, BW and FW. The same was true for samples from detached lice in SW and BW, with a clear separation along PC1. After short-term incubation of 4 h, the differences were less pronounced. In detached lice, no clear differences in general gene expression between SW and BW incubated lice could be observed. For attached lice, samples from BW and FW clustered together, separated from the SW-samples. Also, when performing a PCA for all samples at the same time, the separation of this different treatment groups was apparent (Supplementary File 5). For attached lice in SW, all samples were clustering close together, almost regardless of the incubation time. Detached lice in SW were separating from their attached counterparts already after 4 h and the difference was even bigger after 1d. After 4 h, samples were clustering quite close independent of water treatment, after 1d, samples from treatments with reduced salinity had a high distance from the SW samples.

In total, 498 genes were differently expressed ($|FC| \geq 1.5$, $p_{adj} < 0.05$) compared to their corresponding control under at least one condition (Fig. 3). Under all conditions, more genes were found to be up- than down-regulated. The overall highest number (more than 5× as many as the other groups) of differently expressed genes (DEGs) was found in lice that were attached to a host and incubated in FW for 1d. Many of the differently expressed genes (DEGs) were overlapping between different treatments, especially after 1d of incubation. Almost 75% of the genes that were differently expressed in BW were also differently expressed in FW. After only 4 h incubation, host-attached lice in BW had the highest number of unique DEGs, and there was a very low number of overlapping DEGs in general.

The GO-term-enrichment-analysis obtained only few significantly enriched GO-terms. After 1d attached in BW we found the GO-terms “alpha-amino acid metabolic process” (GO:1901605; $p = 0.0004$; 5 of overall 31 genes with this GO-term) and “steroid hormone receptor activity” (GO:0003707; $p = 0.03$; 3 of 18 genes) to be significantly enriched. In attached samples from FW-treated lice, we found also an enrichment of “alpha-amino acid metabolic process” (GO:1901605; $p = 0.023$; 7 of 31 genes) as well as “secondary active transmembrane transporter activity” (GO:0015291; $p = 0.011$; 9 of 53 genes). In the BW-treated detached group the GO-term “aromatic amino acid family catabolic process” (GO:0009074; $p = 0.050$; 2 of 6 genes) was significantly enriched.

When looking at a heatmap of the significantly regulated genes in attached lice after 1 d (Fig. 4), several clusters became apparent (A–F). Most DEGs were expressed lowest in SW (A,B,C). Of these, most genes were expressed on similar low levels in SW and BW and highest in FW (C). This cluster showed an enrichment for “biological regulation” (GO:0065007; $p = 0.029$; 27 of 1066 genes). Some genes less showed an

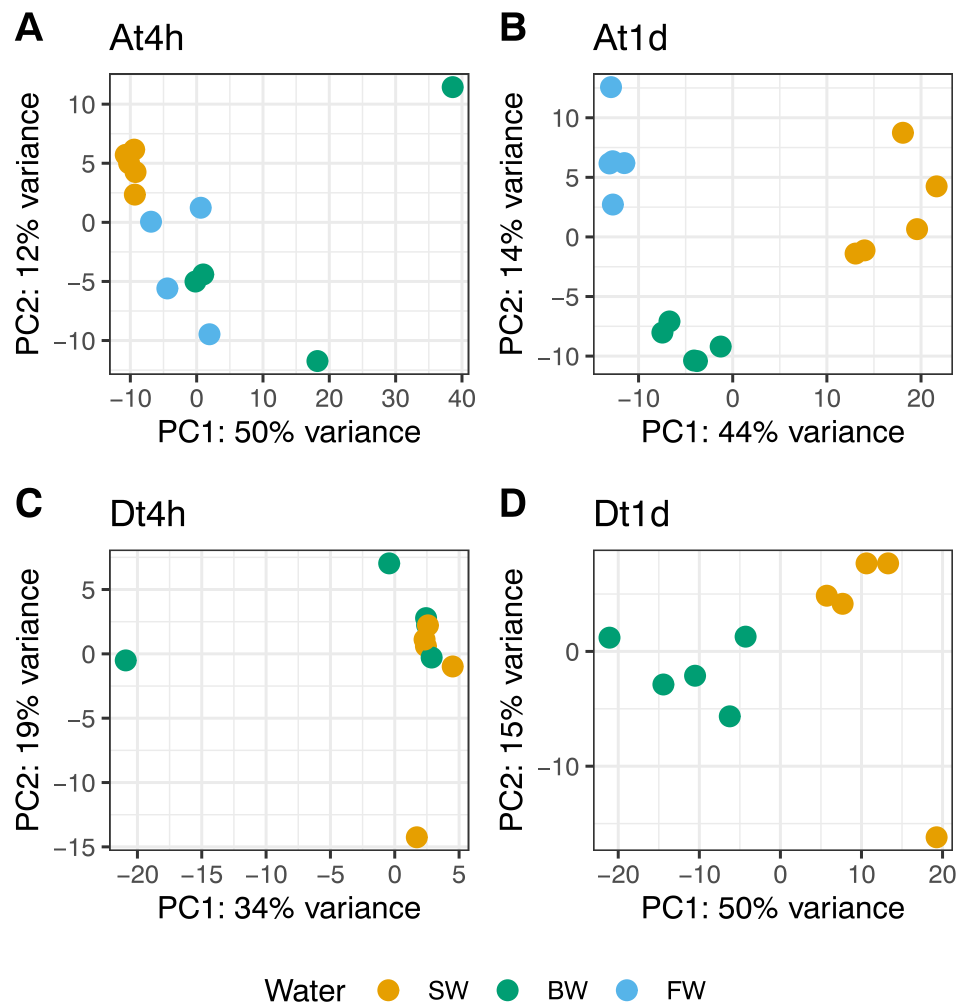

Fig. 2. Principal component analyses of the different samples (n = 4–5). Salmon lice were either attached to fish (At) or detached (Dt) and incubated in different types of water for 4 h or 1d.

increasing gene expression with decreasing salinities from SW to BW and FW (B). This cluster was enriched for "alpha-amino acid metabolic process" (GO:1901605; p = 0,029; 4 of 31 genes). Only a small fraction of genes were higher expressed in BW than in FW (A). For the down-regulated genes there were also three major clusters (D,E,F). The vast majority of these genes was only lowly expressed in FW but on somewhat comparable levels in BW and SW (F). Only three genes were downregulated exclusively in BW (E), whereas 8 genes were down-regulated in BW and FW (D). For this small cluster we found an enrichment of "sodium ion transmembrane transporter activity" (GO:0015081; 0.030; 2 of 49 genes).

Among the genes that had the highest fold-changes under various hyposaline treatments (Fig. 5) were a DNA ligase, a gene of unknown function, a heatshock protein and two genes encoding for Histone H4. The DNA ligase[1] showed a very clear pattern separating different incubation lengths and water treatment groups. The state (attached/detached) on the other hand was irrelevant. For this gene, we found a significant upregulation compared to the control (SW) in all treatments. After 4 h in BW or SW the expression was already increased 2–4-fold, after 24 h the increase was even higher. Compared to the control, it was upregulated 7-13× (BW) or even 21-fold (FW). For a gene of unknown function[2] a slight upregulation (ca 1.5-fold) was visible after 4 h; after 24 h, a strong induction (11–24-fold) was found in all groups with overlapping counts. HSP70[3] showed only a reaction in FW, but not in BW. After 4 h it was increased 3-fold, after 24 h 13-fold. Two genes encoding Histone H4[4] also showed a strong induction upon 24 h FW treatment but not BW treatment, being upregulated 23- and 9-fold, respectively.

3.3. Amino acid metabolism

Several GO-terms regarding amino acid metabolism came up within the GO-term enrichment analysis after several treatments, including genes directly or indirectly involved in proline synthesis (Fig. 6). "Proline biosynthetic process" was one of the GO-terms with lowest p-value in samples from attached 1d-BW-treated lice (GO:0006561; $p = 0.066$; 2 of 4 genes). Two upregulated genes are directly responsible for proline synthesis: a pyrroline-5-carboxylate reductase[5] (P5CR), and the only Δ-1-pyrroline-5-carboxylate synthetase[6] (P5CS) of the salmon louse. Two other P5CRs did not show a change in expression upon treatment,

[1] EMLSAG00000001767.

[2] EMLSAG00000012330.
[3] EMLSAG00000011625.
[4] EMLSAG00000005042 & EMLSAG00000004294.
[5] EMLSAG00000006000.
[6] EMLSAG00000012086.

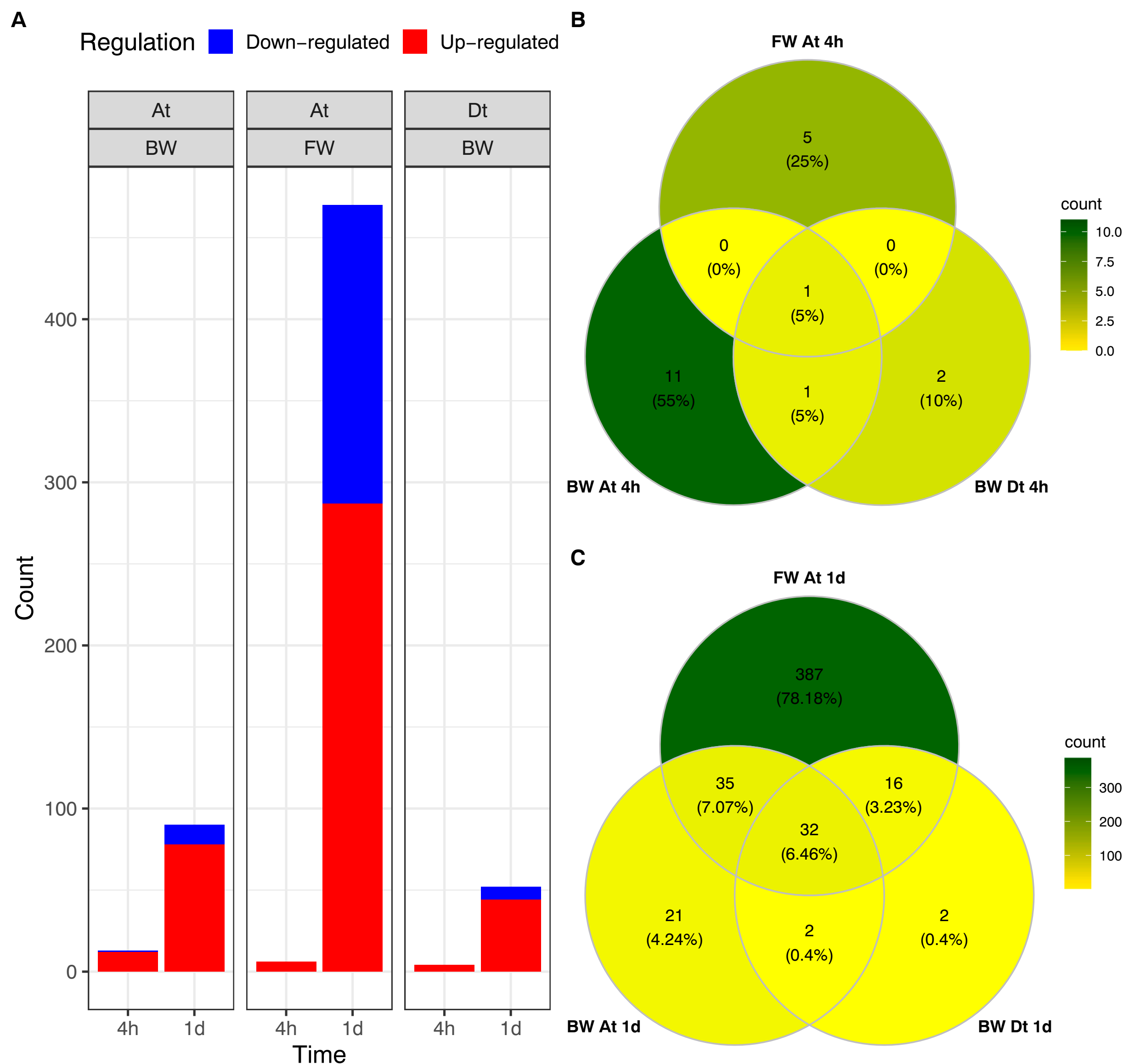

Fig. 3. Overview over DEG numbers. A: Number of up (red) and down(blue)-regulated genes after different treatments in comparison to control. Lice were incubated for different times (4 h, 1d) with different types of water water (BW, FW) and were either attached to a host (At) or had been removed from the host before the experiment (Dt). B,C: Overlap of the DEGs between the different treatments after 4 h (B) and 1d (C) incubation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

but one[5] was strongly induced after a reduction in salinity. One-day BW treatment led to an almost doubling of transcripts (attached, as well as detached), whereas FW treatment lead to an induction of almost 4-fold. A similar pattern was found for P5CS[6]. This gene was even stronger induced under hyposaline treatments. The highest upregulation was found after 1d in freshwater attached to a host (7-fold). Related to proline synthesis (see discussion) we found also that a histidine ammonia-lyase[7] (HAL) and a tyrosine aminotransferase[8] (TAT) were strongly upregulated upon hyposaline treatment (both >4-fold, 24 h FW attached).

3.4. Transporters

Overall, 33 genes with an annotation for transport were significantly regulated upon at least one treatment condition (Fig. 7). The number of up and down-regulated genes was almost identical, and genes from all clusters were among them (for clusters see Fig. 4). From the down-regulated genes, all but three genes were significantly downregulated exclusively in FW (Cluster F). Only two genes downregulated in FW were also significantly downregulated in BW (D): a transporter for neutral amino acids as well as an epithelial sodium channel. An additional gene was downregulated exclusively in BW, when attached (BCCT transporter, cluster E). Among the upregulated genes, most DEGs were upregulated in FW and roughly half of them additionally in BW. Only two genes were upregulated under all experimental conditions: A sodium-dependent phosphate transporter and an inwardly-rectifying potassium channel. Overall, several sodium-dependent transporters were regulated either up- or downwards. A number of regulated transporters were supposed to transport amino acids like glutamate, glycine and proline.

[7] EMLSAG00000007965.
[8] EMLSAG00000003315.

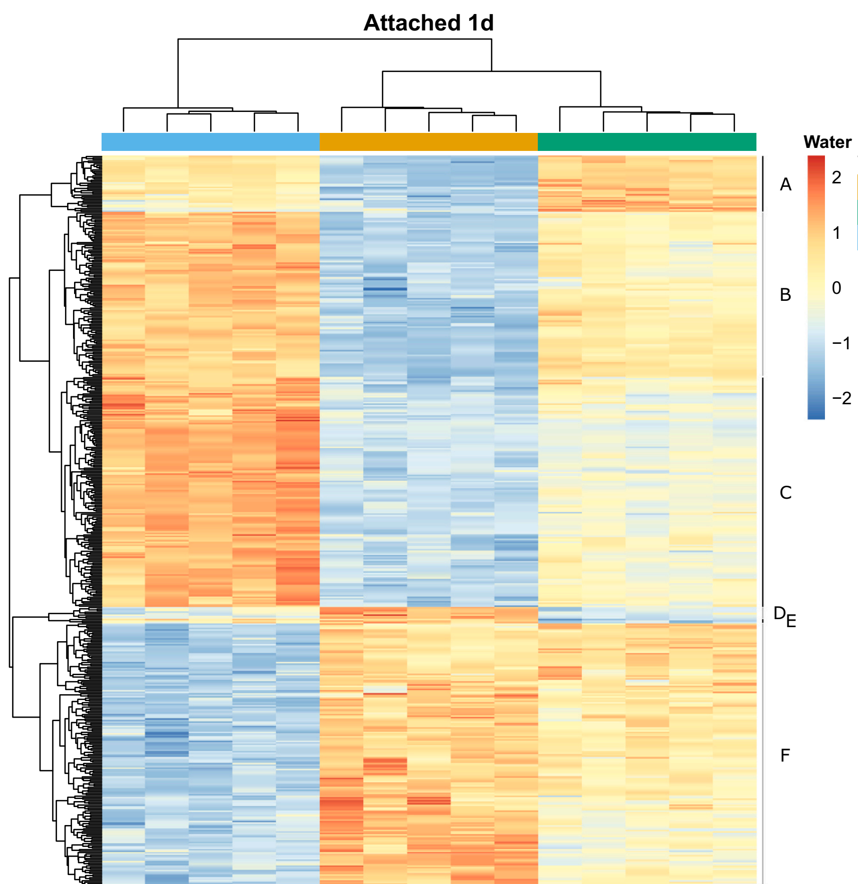

Fig. 4. Heatmap of DEGs in attached salmon lice after 1d incubation. Rows represent genes and columns indicated samples ($n = 5$). Red colors indicate a comparably high expression of a gene in a sample, blue colors a low expression. A higher-resolution version containing gene-names is given in Supplementary File 6. Some clusters are marked and labelled (A-F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Heatmap of DEGs in attached salmon lice after 1d incubation. Rows represent genes and columns indicated samples ($n = 5$). Red colors indicate a comparably high expression of a gene in a sample, blue colors a low expression. A higher-resolution version containing gene-names is given in Supplementary File 6. Some clusters are marked and labelled (A-F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

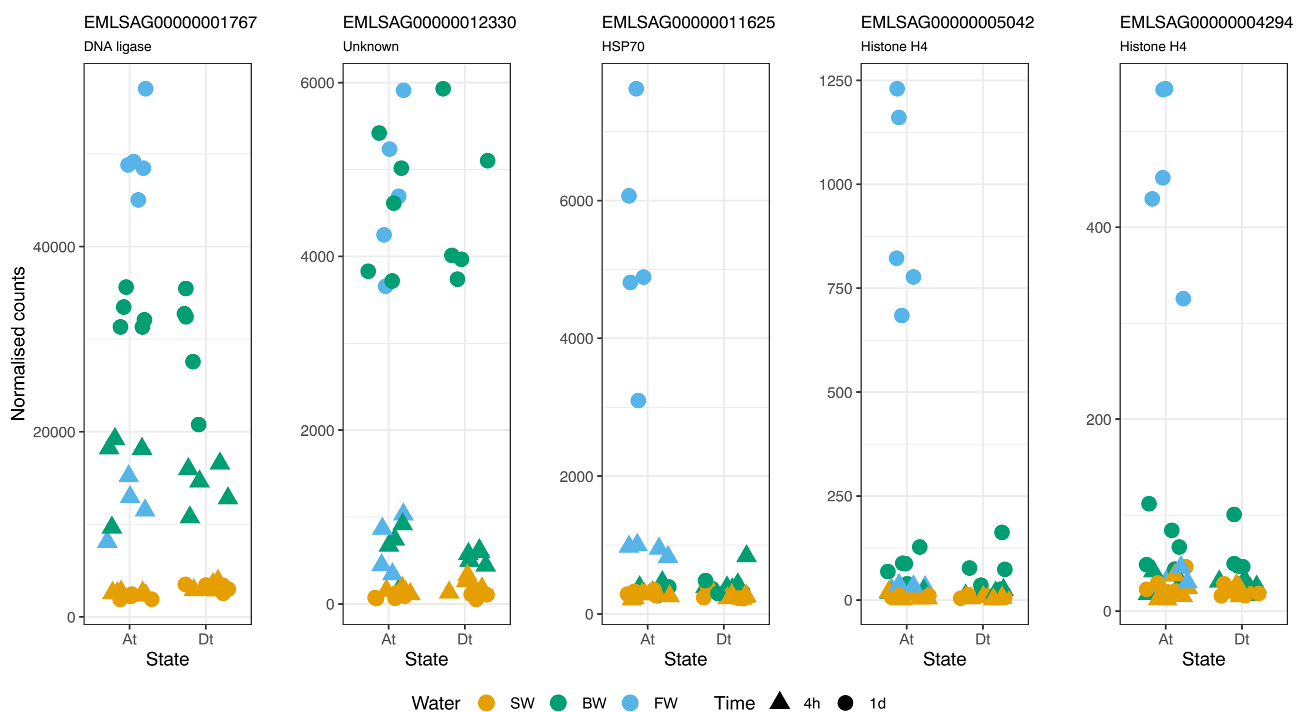

Fig. 5. Strongly regulated genes. Expression levels (as normalized counts) of some selected top differently expressed genes upon treatment of adult female salmon lice with different types of water (SW, BW, FW) for 4 h or 1d (n = 4–5). Lice were either freely swimming (detached, dt), or sitting on a host (attached, at). The normalization does not account for different gene lengths.

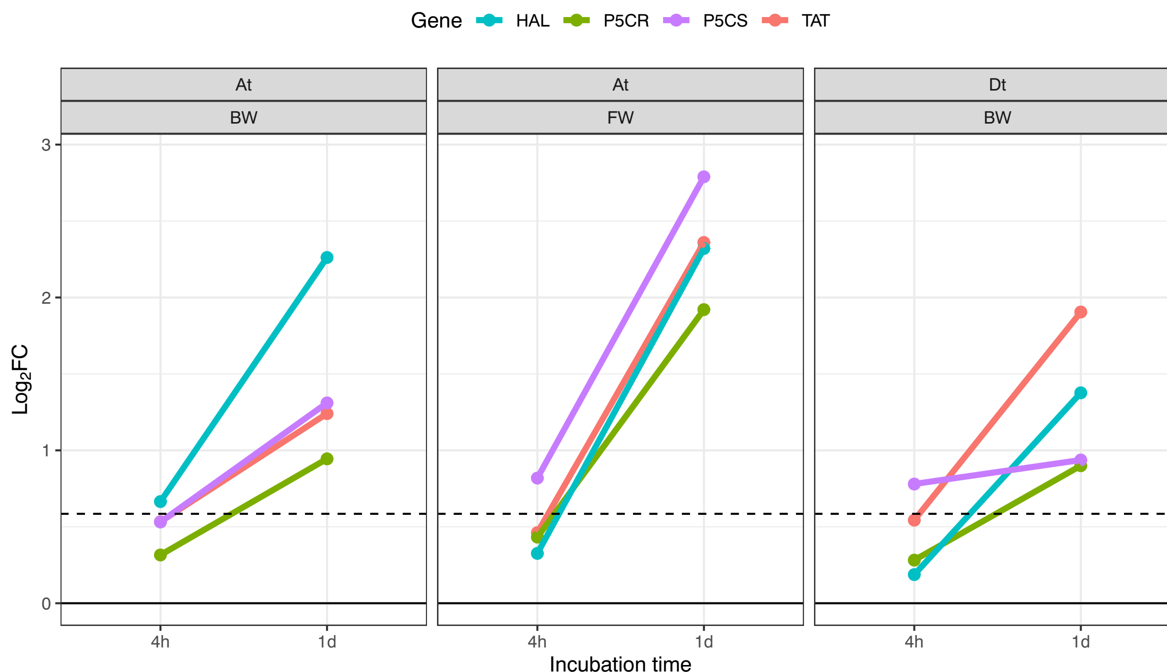

Fig. 6. Expression of selected genes of the amino acid metabolism. Salmon lice were incubated for different time periods in different types of water and were either attached (At) to or detached (Dt) from their host. Values are the mean $Log_2FC \pm SE$ in relation to the corresponding SW-control as determined by DeSeq2 analyzing the RNA-Seq data (n = 4–5). The dashed line marks an upregulation of 1.5-fold, which we used as significance threshold in this study. HAL = histidine ammonia-lyase[7], P5CR = pyrroline-5-carboxylate reductase[5], P5CS = Δ-1-pyrroline-5-carboxylate synthetase[6], TAT = tyrosine aminotransferase[8].

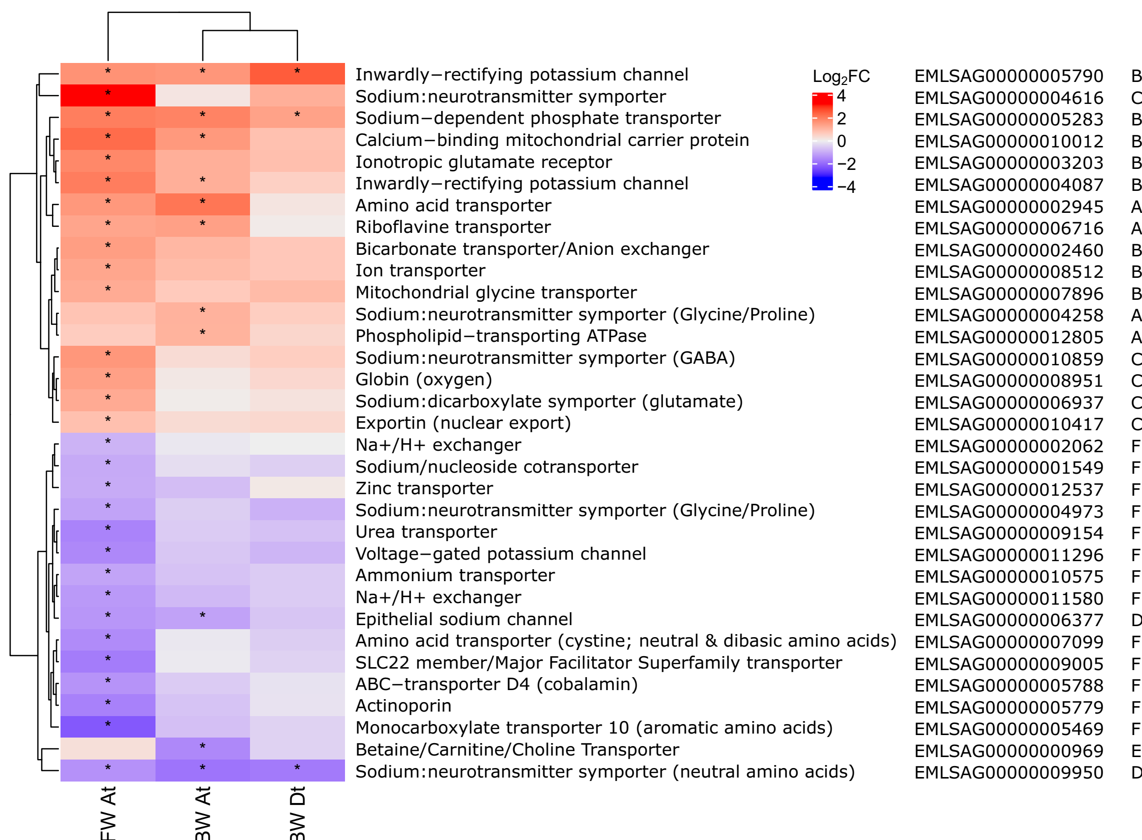

Fig. 7. Gene expression of differently expressed genes encoding proteins with the Go-term for “transporter activity” after 1 day under different conditions (n = 4–5). Asterisks mark significantly different values. Values are shown as log_2 FC compared to the seawater control. The last column refers to the clusters established in Fig. 4.

3.5. Attachment-related and water-related differences

By plotting the changes in gene expression after 24 h in BW in attached and detached lice, we got an overview over similarities and differences in the gene expression response (Fig. 8). Overall, the response between detached and attached lice was quite similar. Not one gene was significantly regulated into different directions under the two conditions. Also, most of the genes that were found to be significantly different only under one of the conditions showed a tendency to the same regulation in the other. However, a few genes showed only a clear regulation in either attached or detached state. The most prominent of these was an amino acid transporter[9] with an upregulation of 4× in attached state vs an unchanged expression in detached state (FC = 1.1). Similar differences were found for a riboflavin transporter[10] (2.6× up vs +1.1 unchanged) and a Malonate–CoA ligase[11] (2.5× up vs −1.2 unchanged). On the other side, we found a Na,K-Atpase Interacting protein,[12] being 2.2-fold downregulated in detached lice and unchanged (FC = −1.2) in attached lice. Altogether, the BW-response of attached and detached lice were thereby quite comparable.

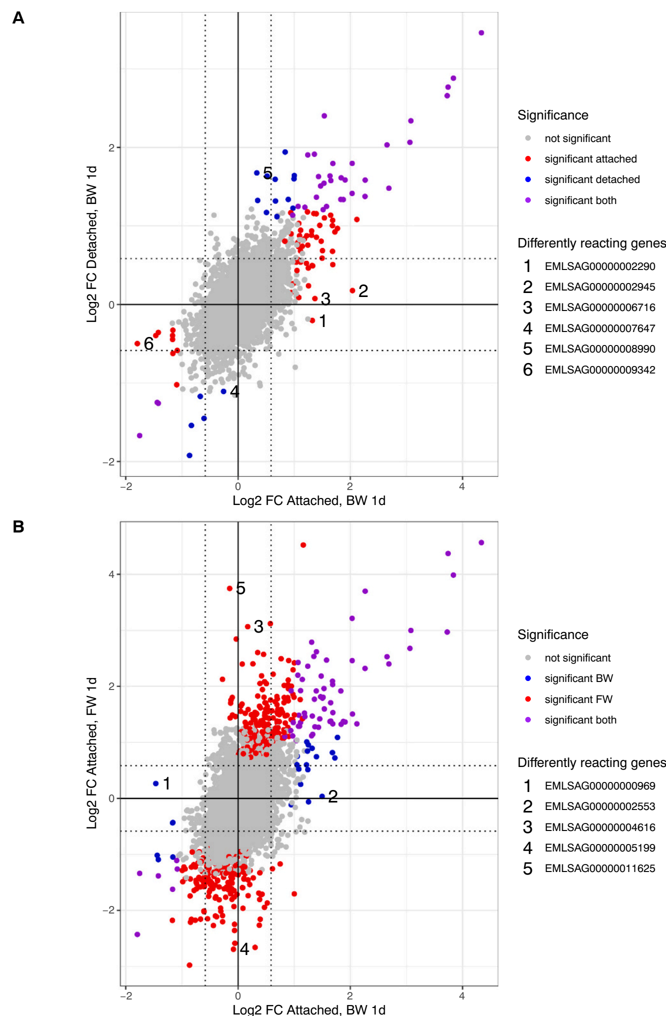

Fig. 8. Correlation of gene expression regulation under different conditions after 1 d. A: Correlation between gene regulation in BW-treated detached and attached lice. B: Correlation between gene regulation in attached FW- and BW-treated lice. Some genes, with especially low correlation were marked with numbers. Interactive versions of these plots with the possibility to select individual dots and determine the gene id are supplemented as Supplementary Files 7 & 8.

Correlation of gene expression regulation under different conditions after 1 d. A: Correlation between gene regulation in BW-treated detached and attached lice. B: Correlation between gene regulation in attached FW- and BW-treated lice. Some genes, with especially low correlation were marked with numbers. Interactive versions of these plots with the possibility to select individual dots and determine the gene id are supplemented as Supplementary Files 7 & 8.

Bigger differences were found when comparing attached lice exposed to either FW or BW. Also, in this comparison there were no genes with significant regulation in opposed directions. However, many genes were only significantly regulated under FW but not under BW. Most prominent among these were HSP70[3], which was 13× upregulated in FW, and unchanged (FC = −1.1) in BW. A similar pattern was found for a sodium-neurotransmitter symporter[13] (8,4× up in FW vs 1.1× unchanged). Another transporter for Betaine/Carnitine/Choline[14] (BCCT) was downregulated in BW (2.8× down), but not in FW (FC = 1.2).

3.6. Validation via qPCR

To validate our RNA-Seq analysis, we tested the expression of 10 genes in three samples per condition from the RNA-Seq trial in qPCR (Fig. 9). There was a very high linear correlation between fold-changes obtained by RNA-Seq and qPCR; no matter if the lice were attached or detached and in FW or BW the coefficient of determination was always high ($0.89 \le R^2 \le 0.98$). Additionally, the slope of the regression lines was very close to 1, indicating that the FC obtained by RNA-Seq was almost identical to the one obtained by qPCR.

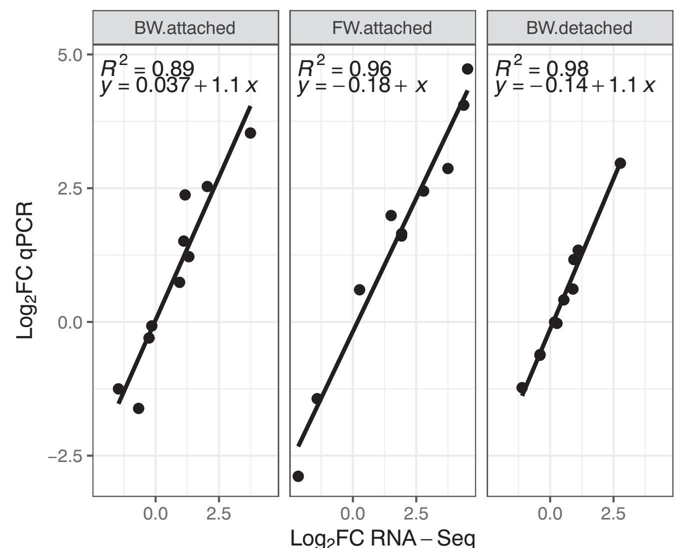

Fig. 9. Correlation between fold-changes obtained by qPCR and RNA-Seq. A subset of the RNA-Seq samples ($n = 3$) was also analyzed by qPCR, covering all conditions with 1 d treatment. Each dot represents one gene, the line shows the best fit.

[9] EMLSAG00000002945.
[10] EMLSAG00000006716.
[11] EMLSAG00000002290.
[12] EMLSAG00000007647.
[13] EMLSAG00000004616.
[14] EMLSAG00000000969.

3.7. Osmoregulation in males

Analysis of male gene expression of 10 genes that we found to be regulated in a salinity-dependent fashion in females, showed that the gene expression of basically all these genes is osmo-dependently regulated in males as well (Fig. 10). Only the apparent trend towards upregulation of HSP70 with decreasing salinity was not considered statistically significant. Strongest upregulated in the selected gene panel was the DNA ligase[1] with an upregulation of 30×, followed by a Histone[15] with a 21-fold upregulation in FW. Additionally, the genes involved in proline synthesis (P5CR, P5CS) were upregulated in males in water with reduced salinity.

4. Discussion

4.1. Response to reduced salinities

Overall, we could observe that salmon lice exposed to reduced salinities change their transcriptome in a significant and consistent manner. Apparently, this modification can be considered to be relatively slow. After 4 h, only a low number of genes had a changed their expression by more than 1.5 times. Heatshock, on the contrary, can induce heatshock proteins in lice copepodids strongly already after 1 h (Borchel et al., 2018). However, on the organismal level, it became clear, that low salinity can already have an effect within 4 h. Attached lice lost their egg strings after 4 h in FW, and detached lice died within the same period. The death of the detached lice was somewhat surprising to us, we had planned to use these animals also for RNA-Seq. In a former report it has been reported that detached female lice managed to survive in FW for 8 h (Hahnenkamp and Hahnenkamp and Fyhn, 1985). The reason for this time difference is not clear. In that study animals from the field had been used in contrast to our labstrain. The animals might therefore have a different genetic background and low-salinity-tolerance. Also, the age of the animals and other uncontrolled factors might have an impact on the osmotolerance of lice, which we did not account for.

We found that attached female lice lost their egg strings within 4 h in FW but did not lose them even after one day in BW. If this loss is due to an active mechanism controlled by the lice themselves is not known.

4.2. DNA structure and repair

EMLSAG00000001767 was the gene with the strongest and most consistent upregulation upon BW or FW treatment. It was actually the only gene that was statistically significant upregulated in every treatment, independent of water type (FW/BW) or state (attached/detached). Thereby it is of great interest. The gene encodes a DNA ligase 3. This gene, which can be found in mammals but also in a variety of lower eukaryotes (Simsek and Jasin, 2011), has been suggested to be involved in a variety of functions in mammals. Main functions are base excision repair, single strand break repair and all necessary ligations within the mitochondria (Arakawa and Iliakis, 2015). We could not find any previous reports on an upregulation of DNA ligase 3 upon osmotic or other stress. We could only find one report on another DNA ligase, DNA ligase 1, that was found to be upregulated in human cells upon UVC-irradiation and subsequent DNA damage (Montecucco et al., 1995). Potentially the observed upregulation of the salmon louse ligase might also indicate the occurrence of DNA damage. The seemingly very strong salinity-dependence of the expression of this gene might also make it a possible marker gene for the salinity that salmon lice were exposed to.

Additionally, we found three genes encoding histones to be upregulated upon treatment. EMLSAG00000005042 (upregulated under all conditions after 1d) and EMLSAG00000004294 (upregulated in FW, attached) encode Histone H4, whereas EMLSAG00000000191 (upregulated in FW & BW, attached) encodes Histone H5. Histone H5 is a so called linker histone (Lyubitelev et al., 2016), whereas histone H4 is a core histone (Hyland et al., 2005). In plants, a connection between chromatin and stress response has been established (Lämke and Bäurle, 2017). However, this is more due to posttranslational modifications than changes in transcription. Histones are considered to be expressed within the S-phase of the cell cycle only (Rattray and Müller, 2012). Outside the S-phase the accumulation of histones is toxic (Mei et al., 2017). However, different histones are involved in environmental-stress-induced DNA damage repair (Chen and Jin, 2019).

Together, the strong upregulation of transcripts of a DNA ligase and several histones might suggest that water with low salinity induces DNA damage in salmon lice which in response activates mechanisms to repair these damages.

4.3. Proline synthesis

As GO terms regarding amino acid metabolism in general and specifically proline biosynthesis came up with lower *p*-values in the GO-term enrichment analysis, we analyzed these genes more thoroughly. Proline synthesis is catalyzed by three enzymes in prokaryotes and some lower eukaryotes, and by two enzymes in plants and animals, as the functions of the two first enzymes have been adopted by one fused enzyme (Fichman et al., 2015). In the salmon louse the process is apparently using two enzymes, as we could find a bifunctional P5CS (pyrroline-5-carboxylate synthase). P5CS catalyzes the phosphorylation and subsequent reduction of glutamate to γ- glutamyl semialdehyde, which spontaneously cyclizes to Δ1-pyrroline-5-carboxylate. This intermediate is then reduced by P5CR (pyrroline-5-carboxylate reductase) to proline. At least in plants, the first step catalyzed by P5CS is the rate-limiting step of proline-synthesis (Liang et al., 2013). As we found a strong induction of P5CS and one of three P5CR-genes upon exposure to water with reduced salinity, we conclude that the capability of the lice to produce proline is increased. This is supported by the findings that two additional genes that might be important for this process also showed a high upregulation. Tyrosine aminotransferase (TAT) is the first enzyme in a pathway degrading tyrosine to fumarate and acetoacetate. In this first step L-tyrosine is converted to *p*-hydroxyphenyl-pyruvate and as a byproduct glutamate is formed (Groenewald et al., 1984). This step is considered to be rate-limiting in tyrosine catabolism (Dickson et al., 1981). An increase in specific TAT-activity was observed in the Eastern mudsnail after transfer from high to low (50%) salinity (Reed, 1971). Histidine ammonia-lyase (HAL) on the other hand catalyzes the first step of L-histidine degradation via the urocanic acid pathway. The end-product of this process is -again- glutamate. HAL is considered to be the rate-limiting enzyme of this process (Brand and Harper, 1976. As both TAT and HAL expression are strongly increased under low salinity conditions, we assume that they contribute to a higher production of glutamate, which in turn, can be metabolized further to proline.

Proline synthesis might be an important part of the adaption to a changing salinity. A modification of the proline content in response to salinity changes has been described for bacteria, plants and even some invertebrates (Fichman et al., 2015). However, in these species an increase in proline concentration is commonly detected upon transfer from low-salinity to high-salinity conditions. For example the proline concentration increased almost fourfold upon a transfer of *Tigriopus californicus* animals from 50% SW to 75% SW within 3 days (Burton, 1991). However, the nature of this increase is not understood. A later study analyzed the gene expression of P5CS and P5CR in this species, and could not detect an increase in gene expression upon hyperosmotic stress (Willett and Burton, 2002).

Thereby the increase of proline synthesis enzymes was somewhat unexpected and is seemingly in contrast to published proline concentrations in salmon lice after treatment with reduced salinity. Instead of increasing, as expected with a higher proline synthesis, proline

[15] EMLSAG00000005042.

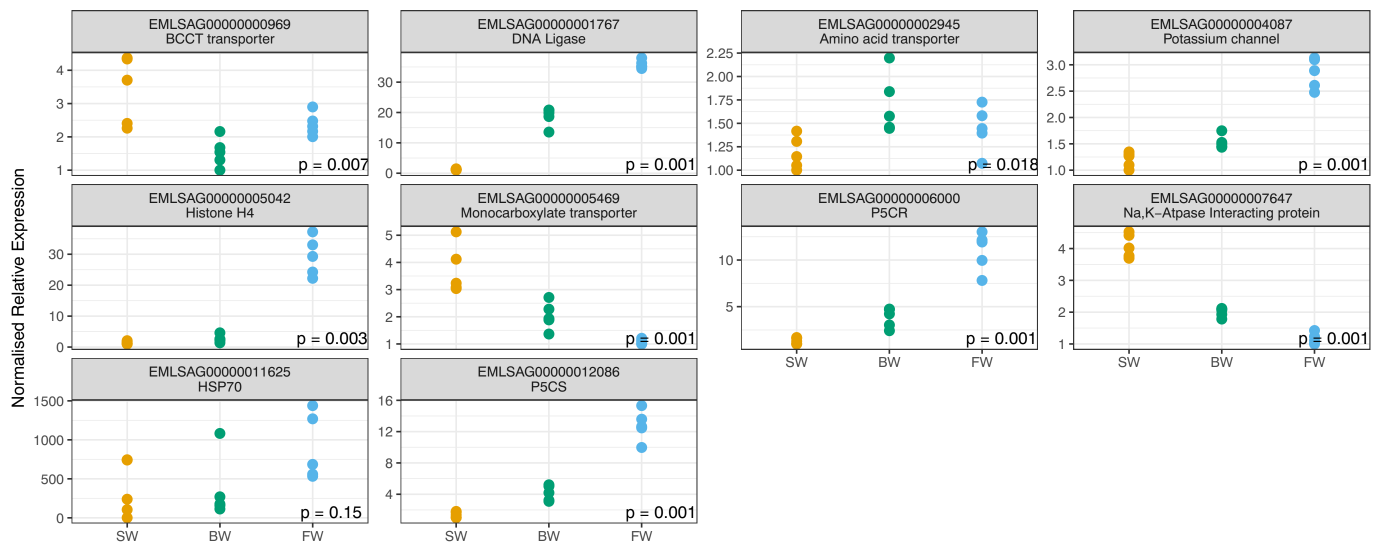

Fig. 10. Gene expression of selected genes after treatment with different water treatment groups in male salmon lice. The lice were attached to salmon, which were incubated in SW, BW or FW for 24 h. Each dot represents a measurement of an individual louse (n = 5). The lowest measured expression per gene was used for standardization and set as 1. The *p*-values are based on Kruskal-Wallis rank sum tests.

concentrations in the cephalothorax of attached salmon lice were found to be diminishing from 71 μmol/g tissue dry weight in SW to 19 μmol/g in BW and could not be measured in FW (Hahnenkamp and Fyhn, 1985). A possible explanation for this contrast might be that the louse produces proline and then secretes it from the cells into the hemolymph. Thereby, the osmolarity of the hemolymph would increase, reducing the difference between intra- and extracellular osmolarity. Such a mechanism has been suggested before for the barnacle *Balanus improvisus*, which can tolerate very low salinities in comparison to other barnacles (Sundell et al., 2019). Although just called a "working hypothesis", it is suggested that euryhaline barnacles can hyperregulate the hemolymph by producing and excreting proline from surrounding cells. This hypothesis is partly based on older works on this barnacle, suggesting that proline could accumulate in the hemolymph during hypoosmotic acclimation (Fyhn, 1976). To our knowledge, the proline content of hemolymph of salmon lice under regular conditions or hyposaline conditions has not been measured. Our findings suggest that more work on proline metabolism in salmon lice is necessary. It is also of interest whether the increased expression is taking place only within certain regions of the louse or in all cells. However, the strong and consistent upregulation of genes of this metabolic pathway and related pathways suggest that proline synthesis is playing an important role in the hyposaline osmoregulation of the louse. The importance of proline synthesis in salmon lice is also highlighted by the fact that we found P5CS in the list of upregulated genes in salmon louse copepodids exposed to water with 20 and 10 ppt respectively (Sutherland et al., 2012, probe C070R095). Increase in proline synthesis might thereby a response to low salinity, common to the different salmon louse life stages. However, P5CR, although represented on the microarray (probe C092R071) did not appear on the list of differently expressed genes.

4.4. Stress level

We detected a strong upregulation of the heat shock protein HSP70 gene[3] in the case of freshwater treatment. After 4 h the induction was 3×, after 24 h it was almost 15-fold upregulated. HSPs act as stress proteins. They are molecular chaperones supporting the refolding of misfolded and denatured proteins. The inducibility of HSPs was originally found upon heat shock, but later other stressors were identified as HSP-inducing as well. Osmotic stress is one of them. A lobster has been found to induce HSP70 expression upon transfer from SW to BW (Spees et al., 2002), and a mud crab induces HSP70 upon transfer from BW to SW (Yang et al., 2013). The HSP repertoire of salmon lice has been characterized before (Borchel et al., 2018). Overall, 37 HSPs were identified and tested for their induction upon heat shock. Nine genes were identified as strongly heat-inducible; among them the HSP70 gene[3] was identified as the strongest inducible, with an upregulation of more than 1000-fold. Additionally, this gene was found to react to hyposaline treatments in copepodids in 8.6 ppt water for 20 h with a 19-fold upregulation.

In this experiment we found an induction of the HSP70 gene only upon FW treatment but not upon BW treatment. Already 4 h after onset of the incubation, the induction was measurable and was much higher after 24 h. Among the other HSPs only one HSP40 gene[16] showed a slight induction in FW. HSP40 can modulate the HSP70 activity. Our findings suggest that FW but not BW induces a strong cellular stress response. This might also explain, why FW treatment overall leads to a much stronger change in gene expression than BW with a higher number of DEGs. Potentially some of the genes that are expressed in FW but not BW are also expressed as part of the stress response. Upon heatshock, the heatshock factor translocates into the nucleus and induces transcription of its target genes, among them HSPs. In mammalian cells, hyper- and hypo-osmotic stress can lead to the translocation of a heatshock factor (Caruccio et al., 1997). For the fruit fly, 141 target genes of the heat shock factor HF1 were identified (Birch-Machin et al., 2005). Under the assumption that a translocation of HSF into the nucleus is the reason for the increased HSP70 and HSP40 expression under FW conditions, this might also explain the high number of DEGs in this treatment. A lot of the genes changed in FW were assigned the GO-term biological regulation (cluster C). A change in the expression of such genes might modify the gene expression of other genes as well.

Also in salmon louse copepodids an upregulation of HSPs was observed upon treatment with water of reduced salinity (Sutherland et al., 2012). For this life stage, an upregulation was already observed in relatively high-saline BW (25–26 ppt). Among other HSPs, Hsp70 was upregulated 8 times at 25 ppt. This might be explained by the different FW-tolerance of the different life stages. Attached adult lice can survive in FW for days, whereas copepodids die within hours in FW.

[16] EMLSAG00000006084.

4.5. Transport

The regulation of different transporters did not give a very comprehensible picture. The number of up- and down-regulated genes was identical. Most genes were significantly changed in FW, although most genes showed a trend to a regulation in the same direction. Interestingly, two genes encoding inwardly-rectifying potassium channels were upregulated in low salinity- conditions. One of these[17] was upregulated under all BW and FW- conditions independent of attachment state, and the other[18] was upregulated in BW and FW when attached to a host. Such transporters are known to be involved in neural signaling in Drosophila (Chen and Swale, 2018). Involvement in the reaction to changed salinities was shown in eels (Suzuki et al., 1999). However, in this animal, the mRNA levels of such a potassium-transporter were found to be higher in SW than FW, in contrast to our results in the salmon louse.

Further, one amino acid transporter was upregulated, while others were downregulated. Probably, they transport different amino acids. To really uncover how the regulation of transporters influences osmoregulation, more knowledge about the function and the substrates of the different transporters is necessary. Several transporters including amino acid and calcium transporters have also been found to be differently regulated upon hyposaline treatment of salmon louse copepodids (Sutherland et al., 2012).

4.6. Attachment-related and water-related differences

The overall reaction of the salmon louse to FW is resembling the reaction to BW, but is generally stronger. As discussed, FW might pose a stressor that induces the cellular stress response in contrast to BW. This can explain the higher number of DEGs after FW treatment compared to BW treatment. The different stress levels might explain why detached lice can survive in BW but not in FW. After 4 h, detached lice die in FW, while attached lice survive and show a yet very small gene expression change. HSP70-expression was already increased slightly, but there were no signs yet of an alteration of transport mechanisms or proline synthesis or other changes. It appears unlikely that these small changes in gene expression observed after 4 h can explain the different survival of attached and detached lice. This suggests that other factors than an attachment-specific modification of gene expression are key for survival of attached lice in FW. Overall, the gene expression response of attached and detached lice to BW was very similar and even for the few identified differently reacting genes, it must be kept in mind that both groups had their individual controls. In the PCA of all samples combined, it became apparent that the general gene expression was shifted in detached control (SW) lice in comparison to attached control (SW) lice. The necessary mechanisms to survive FW treatment generally might be present in salmon lice but only operational when attached to a host. Attached lice can feed on their host and replenish lost ions from the host's blood. "The inability to compensate for the loss of body salts" (Hahnenkamp and Fyhn, 1985) has been suggested as an explanation for the death of detached salmon lice in FW. However, surviving in low salinity seems to require more action than just feeding on the host, evidenced by the strong changes in gene expression in attached lice after 24 h, suggesting that BW- and FW-exposure still require adaptation of the louse, even when attached to a host. Another explanation might be based on more physical aspects of attachment. Attached lice are to a certain degree embedded into the host's mucus and use their cephalothorax as a "suction cup" (Wagner et al., 2008). Thereby, the louse's surface in contact with the surrounding medium is minimized, potentially reducing the risk of ion loss. This might slow down the FW influx into the body long enough for the adaptation mechanisms to be activated and to prepare the louse for survival under low-salinity conditions. BW treatment might be so mild in comparison that it does not even induce a stress response and can thereby be handled by the present adaptation mechanisms in detached lice, whereas a FW treatment of these animals might have so strong effects that the activation of further adaptation mechanisms cannot be performed quickly enough for their survival.

4.7. Osmoregulation in males

Male and female salmon lice show a strong sexual dimorphism in the adult stage. Females are much bigger than males and have a different body shape. They differ also in their resistance against certain drugs (Igboeli et al., 2014) and have several genes that are expressed in a sex-biased manner in the adult stages (Poley et al., 2016). Due to these differences it was not clear if the reaction of males and females to different salinities would be similar. However, our data suggest that male and female osmoregulation are alike to a large extent. At least for the selected panel of 10 genes, the same expression patterns were found in both sexes. This suggests that our findings from female lice that we discussed here are likely to a high extent also valid for males, although we cannot exclude the possibility of differences in other genes that we have not measured via qPCR in males.

5. Conclusions

In this study, we have analyzed the transcriptomic response of adult female salmon lice exposed to brackish water (15 ppt) and freshwater (0 ppt). We showed that these treatments lead to long-term changes in gene expression, which barely start after 4 h of incubation but become stronger with a longer duration (24 h). Among the regulated genes are transporters, transcription factors and genes involved in proline synthesis, which might be a key process in adaptation to low salinities. While most of the differently expressed genes that were affected in brackish water were also differently expressed in freshwater, freshwater resulted in a change in a multitude of additional genes. Among these additional genes was HSP70, suggesting that FW but not BW induces the cellular stress response. Our results suggest that the molecular mechanisms for coping with reduced salinity are similar between adult females and males and we also found a certain overlap with the hyposalinity response of salmon louse copepodids that has been reported before (Sutherland et al., 2012). For these early stages a hyposaline response of gene expression was already detected at salinities of 25–27 ppt, which contained almost as twice as much salts as the BW used in this study. Further experiments should therefore determine the threshold level for a hyposaline response also in adult salmon lice, as this might help to understand the different low-salinity tolerance of different salmon louse life stages.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbd.2020.100778.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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[17] EMLSAG00000005790.
[18] EMLSAG00000004087.

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