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Deep transcriptome analysis of the heat shock response in an Atlantic sturgeon (*Acipenser oxyrinchus*) cell line

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ABSTRACT

Despite efforts to restore Atlantic sturgeon in European rivers, aquaculture techniques result in animals with high post-release mortality due to, among other reasons, their low tolerance to increasing water temperature. Marker genes to monitor heat stress are needed in order to identify heat-resistant fish. Therefore, an Atlantic sturgeon cell line was exposed to different heat shock protocols (30 °C and 35 °C) and differences in gene expression were investigated. In total 3020 contigs (~1.5%) were differentially expressed. As the core of the upregulated contigs corresponded to heat shock proteins (HSP), the heat shock factor (HSF) and the HSP gene families were annotated in Atlantic sturgeon and mapped via Illumina RNA sequencing to identify heat-inducible family members. Up to 6 *hsf* and 76 *hsp* genes were identified in the Atlantic sturgeon transcriptome resources, 16 of which were significantly responsive to the applied heat shock. The previously studied *hspa1* (*hsp70*) gene was only significantly upregulated at the highest heat shock (35 °C), while a set of 5 genes (*hspc1, hsph3a*, *hspb1b*, *hspb11a,* and *hspb11b)* was upregulated at all conditions. Although the *hspc1 (hsp90a)* gene was previously used as heat shock-marker in sturgeons, we found that *hspb11a* is the most heat-inducible gene, with up to 3296-fold higher expression in the treated cells, constituting the candidate gene markers for *in vivo* trials.

1. Introduction

Sturgeons are distributed in the northern hemisphere and are mostly anadromous, performing upriver migrations to spawn. They have been aquacultured for the last three decades due to the commercial value of their caviar [1], however wild populations are in severe decline [2,3] and currently the Atlantic sturgeon (*Acipenser oxyrinchus*, Mitchill, 1815) is extirpated from Europe [4].

In 1997, Germany and Poland started a project aiming to restore a self-sustaining Atlantic sturgeon population, derived from a Canadian broodstock, in the Baltic Sea. Rearing techniques have been improved in order to build an *ex-situ* broodstock locally. In addition, the Oder and Vistula river drainages, where more than 120,000 individuals have been released since 2005 [5], have been evaluated in search of possible spawning grounds [6]. The success of a restoration program depends both on politics (e.g., habitat preservation policies and regional and international cooperation) [7,8] and on biology, (eg., development of cultivation techniques adapted for restocking purposes). Aquacultured sturgeons are raised in stimulus-deprived tanks at constant temperature and photoperiod [9]; however, their distribution, abundance, and activity are determined by temperature, photoperiod and salinity fluctuations along the migration route [10,11]. An increase in water temperature, both locally due to the discharge of cooling waters or globally due to climate change, can affect swimming performance, predator avoidance, foraging behavior and shift the geographic distribution of species, especially of ectothermic animals such as fish, and result in local extinctions [12–14]. Thus, thermal adaptation is crucial for post-release survival.

As a result of physiological or chemical stress, proteins misfold and accumulate in the cytoplasm, activating a highly conserved and transient heat shock response (HSR), essential for proteostasis maintenance. HSR results from the rapid activation of the heat shock factor (HSF) by trimerization, hyperphosphorylation and translocation to the nucleus, were its DNA-binding domain (DBD) binds to the heat shock elements (HSE), located at the promoters of heat shock protein (*hsp*) genes, inducing their transcription [15]. Previous studies in *Saccharomyces cerevisiae* showed that heat-responsive gene expression attenuates within 20–40 min after induction [16]. Most vertebrates have multiple *hsf*

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genes: mammals have 4 [17], while the Spotted gar (*Lepisosteus oculatus*), sturgeon's closest living relative whose genome is sequenced, has 6 (*hsf1*, *hsf2*, *hsf3*, *hsf4*, *hsf5,* and *hsfy*).

HSPs, one of the largest and most conserved protein families, were first discovered in *Drosophila* [18] and have since then been described in all living organisms, from yeast [19] to mammals [20,21] and fish [22–24]. Its nomenclature refers to their molecular weights, however, since the human genome annotation the names have become confusing, with up to 10 different names for the same gene product. In 2009 a new nomenclature was proposed [25] based on the gene symbols that have been assigned to the HUGO Gene nomenclature committee (HGNC). This nomenclature was also used in this study, and classifies HSPs into 9 subfamilies: HspA (former Hsp70), HspH (former Hsp110), HspB (small Hsp), HspC (Hsp90), HspD (Hsp60), HspE (Hsp10) and the J domain-containing subfamilies (Hsp40) DnajA, DnajB and DnajC.

The HSP family consists of both constitutively expressed and inducible members, some of which are responsive to heat and act by binding to the exposed hydrophobic amino acid residues of the misfolded proteins preventing undesired molecular interactions. The capacity of HSPs to bind misfolded proteins is regulated through allosteric mechanisms via ATP binding and hydrolysis, with the exception of the HspB subfamily which is ATP-independent.

RNA sequencing (RNAseq) is a high-throughput tool to quantify transcriptomic changes and, unlike qPCR, is unbiased, allowing gene discovery and quantification with good correlation with qPCR when both procedures are performed well [26,27]. RNAseq has previously been used in sturgeons to study sex-related genes, developmental genes or response to infection [28–33], but never to study the heat shock response. Many studies used RNAseq to address this in teleosts [34–36], while for sturgeons only qPCR or protein analysis approaches were used [37–42]. Still, the focus has only been on *hspa1 (hsp70)* and hspc1 (*hps90)*, as the HSP family is not fully annotated in sturgeons, which are also lacking a reference genome.

Recently, the AOXlar7y cell line (***A**cipenser **o**xyrinchus* **lar**vae n°**7**, tr**y**psin-digestion) was established by whole larvae trypsin digestion [43], providing the possibility of reducing the use of *in vivo* sturgeon experiments. Still, the presence of stem cells in the culture was not confirmed so, characterization of the cell type needs to be assessed.

The objective of this study was to make an inventory of all the HSPs present in the Atlantic sturgeon transcriptome resources and identify the most heat shock-inducible family members. To this end, we annotated the complete HSP family using transcriptomic resources from Atlantic sturgeon cell line, multiple Atlantic sturgeon organs and a publicly available dataset [31] and subsequently evaluated their response to heat. Although *in vivo* validation during temperature-challenge trials is needed, the *in vitro* experiment provides a general perspective of the HSR and candidate gene markers for the selection of thermotolerant individuals with better restoration fitness.

2. Materials and methods

2.1. Cell line samples

The AOXlar7y sturgeon cell line [43] was used for the heat shock experiments. Cells from passage 22 (P22) were thawed and seeded in a 25 cm^2 cell culture flask (Corning Life Sciences, Tewksbury, MA, USA) at 25 °C, the optimal growing temperature (doubling time 110 h), in Leibovitz-15 medium supplemented with 15% FCS (fetal calf serum), 100U/mL penicillin and 0.1 mg/mL streptomycin.

Prior to the final experiment, the cells were propagated by trypsinization, homogenization and subculturing at a 1:3 ratio every 4 days, up to P29. To determine the heat tolerance of the AOXlar7y sturgeon cell line, the cells were exposed to 28 °C, 30 °C, 33 °C or 35 °C for 1, 2, 3 or 4 h and survival was inspected under the microscope after 4, 8 and 24 h of recovery. Based on this pilot experiment, the final experiment was performed as depicted in Fig. 1: 24 individual flasks were seeded at

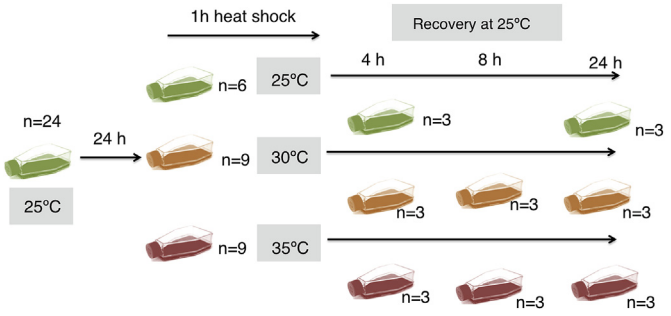

Fig. 1. Experimental design. The AOXlar7y cell line was seeded in 25 mL flasks and cultured at 25 °C for 24 h (h). Then, the cells were treated at either 30° or 35° for 1 h and then transferred back to 25 °C for recovery. RNA was harvested after recovery for 4, 8 and 24 h and sequenced with Illumina HiSeq2500.

25 °C, and after 24 h the medium was renewed at either 25 °C (n = 6), 30 °C (n = 9) or 35 °C (n = 9). The flasks were then transferred to different incubators set at 25 °C, 30 °C and 35 °C respectively and kept there for 1 h. After the heat shock all the flasks were transferred back to 25 °C to recover. After 4, 8 and 24 h of recovery, cells were lysed in QIAzol lysis reagent (Qiagen GmbH, Hilden, Germany) and stored at -80° until further processing.

2.2. Atlantic sturgeon samples

An aquaculture-reared immature female (7,700 g, 98 cm) was provided by Fischzucht Rhönforelle GmbH in Gersfeld (Germany) on July 3rd, 2015. The specimen was euthanized, and tissue samples were taken in agreement with standardized fish processing methods at a licensed and registered processing unit (Fischzucht Rhönforelle GmbH). A total of 21 samples were taken through dissection, from caudal to rostral: caudal fin, dorsal fin, pelvic fin, skin, muscle, intestine (posterior), kidney, spleen, swim bladder, gonad (2 samples), intestine (anterior), stomach, liver, gallbladder, heart, gill, brain (anterior), brain (posterior), barbel and eye. Samples were preserved in RNAlater (Qiagen GmbH, Hilden, Germany) and subsequently stored at −80 °C until further processing.

2.3. RNA extraction, Illumina library preparation, and RNA sequencing

RNA was extracted from the 21 juvenile Atlantic sturgeon tissue samples and from the 24 AOXlar7y cell line samples using the Qiagen miRNeasy Mini kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). RNA concentration and integrity were analyzed with a Bioanalyzer 2100 total RNA Nano series II chip (Agilent, Santa Clara, USA). RNA libraries were prepared from 500 ng total RNA, using the Illumina TruSeq Stranded mRNA Sample Preparation Kit according to the manufacturer's instructions (Illumina Inc., San Diego, USA) and the resulting libraries were evaluated with a Bioanalyzer 2100 DNA 1000 series II chip (Agilent, Santa Clara, USA).

All the libraries were sequenced using an Illumina HiSeq2500 instrument. Tissue libraries were sequenced as paired-end 2 × 151 nucleotides (nt) reads up to a minimum of ~20 million reads for each tissue sample, while AOXlar7y libraries were sequenced as 1 × 51nt single-reads up to a minimum of ~10 million reads for each experimental condition.

2.4. De novo transcriptome assemblies and annotation

Three reference transcriptomes were produced in the present study. Firstly, all combined juvenile sturgeon organ reads were *de novo* assembled into cDNA contigs using the De Bruijn graph-based *de novo* assembler implemented in the CLC Genomics Workbench version 4.4.1

(CLC bio, Aarhus, Denmark), resulting in an Organ assembly. Secondly, the same software was used to produce a Cell assembly with the AOXlar7y sequence reads only. Finally, all sequence reads were combined into a Cell-Organ-Embryo (COE) transcriptome, including the reads from the AOXlar7y, the juvenile sturgeon organs and a set of embryonic reads previously published by others [31].

The quality of the *de novo* assemblies was assessed by the assembly contiguity (contig N50) and the percentage of AOXlar7y mapped reads. In order to link the best assembly to zebrafish Ensembl protein identifiers, BLASTX 2.2.31 + [44] similarity searches were conducted locally against the UniProt zebrafish using an E-value cut off of 1E-5.

2.5. Gene expression and gene ontology (GO) analysis

For the gene expression analysis, Bowtie2 (version 2.2.5) [45] was used to align Illumina reads from the 24 experimental samples against the 3 *de novo* assembled reference transcriptomes (Cell, Organ and COE contigs) and against the embryonic transcriptome available at NCBI [31]. The assembly with the highest mapping percentage was used as a reference for downstream analysis. The resulting files were filtered using SAMtools (version 1.2.) [46] to exclude secondary aligned reads. Then, the aligned reads were counted from the SAM alignment files using Python package HTSeq (version 0.5.3p9) [47], and the resulting TSV files were used for identification of differentially expressed contigs (DECs) using the Bioconductor package DESeq (version 1.30.0) [48] in R software (version 3.3.0). The top 100 expressed contigs in the unstressed cells were investigated to characterize the AOXlar7y cell line. Each experimental condition (30 °C and 35 °C) and timepoint (4, 8 and 24 h) was compared to the control group, resulting in 6 DESeq pairwise comparisons. P-values were adjusted for multiple testing with the Benjamini-Hochberg procedure, which controls the false discovery rate (FDR). Contigs with an adjusted p-value (padj) < 0.05 were considered differentially expressed at each pairwise comparison between different treatments and timepoints. The set of zebrafish protein identifiers linked to these contigs was investigated in order to elucidate the broad transcriptomic changes. GO enrichment analysis was performed using the Fisher's Exact test with Bonferroni correction implemented by the PANTHER Overrepresentation test (released on 2017-12-05) using the GO Ontology database (released on 2018-07-03) [49]. As recommended by the Gene Ontology Consortium [50,51], a custom reference list containing all the top zebrafish Ensembl gene identifiers linked to the expressed COE contigs was used for the analysis. GO terms with a p-value < 0.05 were considered overrepresented in each dataset.

2.6. Annotation and expression of Hsp and Hsf genes

Spotted gar, zebrafish and human HSP and HSF protein sequences [25] were retrieved from NCBI and used as queries to find the corresponding sturgeon orthologues. Proteins were blasted against the translated COE transcriptome assembly using the CLC Main Workbench (version 7.7.3.). When full open reading frames (ORF) were not found in the COE assembly, the Organ, Cell or embryo assembly [31] were used. The human HSP protein nomenclature according to Kampinga et al. [25] was used. After the annotation of all Atlantic sturgeon *hsp* and *hsf* genes, the AOXlar7y Illumina reads were mapped to the annotated genes and those with more than 5 mismatches were filtered out in order to improve stringency. DESeq analysis was performed, following the aforementioned pipeline, in order to find candidate *hsp* markers for *in vivo* trials. Significantly upregulated transcripts (padj > 0.05) with at least 3 fold change (FC ≥ 3) between the control and at least 5 experimental conditions were considered good markers in this experiment.

The AOXlar7y and organ sequencing reads were deposited at the NCBI Short Read Archive (SRA) database under the accession numbers SRP161542 and SRP161601 respectively. The Cell, Organ, and Cell-Organ-Embryo (COE) transcriptome shotgun assembly projects have been deposited at DDBJ/EMBL/GenBank under the accession numbers GGWJ00000000, GGZX00000000 and GGZT00000000 respectively. The versions described in this paper are the first versions: GGWJ01000000, GGZX01000000, and GGZT01000000, respectively. The Atlantic sturgeon *hsp* and *hsf* ORFs were submitted to BankIT under the accession numbers MH777912-MH777987 and MH917287-MH917292 respectively.

3. Results

3.1. Heat shock treatment and RNA sequencing

The pilot experiment has shown that cells survived after being exposed to 28 °C, 30 °C or 33 °C for up to 4 h or to 35 °C for 1 h; however, acute mortality was found in cells exposed to 35 °C for 2 h or longer (data not shown). Based on this, the final experiment was performed applying a mild or a severe heat shock at either 30 °C or 35 °C for 1 h, and cells were harvested at 4, 8 and 24 h after heat shock (Fig. 1). Subsequently, high-quality RNA (average RIN value of 9.1) was isolated from all cell samples and from multiple organs of an aquacultured juvenile sturgeon specimen, and Illumina libraries were prepared and sequenced, resulting in altogether more than 1 billion reads (Table S1 in Supplementary data).

3.2. Heat shock-responsive genes in Atlantic sturgeon cell line AOXlar7y

To select the best reference for alignment of the sequencing reads, 3 Atlantic sturgeon *de novo* transcriptome assemblies were performed and evaluated. The set of reads derived from the AOXlar7y cell line was assembled to a 34.80 Mb Cell assembly containing 53,624 contigs with an N50 of 1.08 Kb. In parallel, reads from the Atlantic sturgeon organs were assembled to a 342.28 Mb Organ assembly containing 641,485 contigs with an N50 of 0.60 Kb. Finally, all sequencing reads were combined with a previously published set of ~380 million reads derived from Atlantic sturgeon embryos (SRA Accession number SRP069853) [31] and used in a *de novo* assembly that resulted in a 254 Mb Cell-Organ-Embryo (COE) assembly containing 203,131 contigs with an N50 of 1.87 Kb (Table 1).

Table 1
Overview of assemblies' statistics and read mapping. List and number of input sequence reads for each assembly, number of resulting contigs, assembly length in Megabases (Mb), contig N50 in Kilobases (Kb), maximum contig length in Kilobases (kb) and percentage of cell-derived sequencing reads mapped to each assembly.

Assembly	Cell	Organ	Embryo	COE
Input reads	cell	organs	embryo	cell + organs + embryo
n. reads (million)	364,86	639,59	380,08	1424,41
Contigs	53,624	641,485	179,564	203,131
Assembly size (Mb)	34.80	342.28	166.71	254.00
N50 (Kb)	1.08	0.60	1.94	1.87
Max (Kb)	15.63	16.64	54.44	34.02
Accession number	GGWJ01000000	GGZX01000000	GEUL01000000	GGZT01000000
Mapped reads (%)	74.0	54.0	75.0	89.6

Reads from the 24 AOXlar7y samples were aligned against the three assembled transcriptomes and the COE reference, which gave the highest overall mapping percentage (89.6%), was used for the downstream analysis (Table 1). In total 86,021 contigs (~42.3%) could be linked to zebrafish proteins (E-value < 1E-5), corresponding to 23,436 unique zebrafish proteins and 17,319 genes (Table S2 in Supplementary data). The read counts and contig lengths were used to calculate the corresponding RPKM values (Table S3 in Supplementary data). A total of 168,739 contigs (83.07%) showed expression (RPKM value > 0) in at least one of the cell samples, and 27,425 (13.50%) showed expression in all of them.

Since it was hitherto unknown what tissue type was represented by the embryonic AOXlar7y cell line, the zebrafish proteins linked to the 100 most highly expressed contigs (highest RPKM mean) were examined. In total, 34 contigs corresponded to ribosomal proteins, 5 to keratins, 5 to actin genes, 5 to tubulin and other genes were also found. Overall, keratin 4 was the most expressed contig (Table S4 in Supplementary data).

Overall, DESeq analysis showed that 3020 unique contigs (~1.5%) were differentially expressed in at least 1 of the treatments compared with the untreated cells, of which 2302 were upregulated, 714 downregulated and 4 were up or downregulated depending on the experimental condition (Table S5 in Supplementary data).

At 4 and 8 h after the 30 °C heat shock, 79 and 91 contigs were upregulated and 23 and 64 were downregulated respectively, while after 24 h only 12 were downregulated and none upregulated. After the 35 °C heat shock, respectively 1,338, 1081 and 1143 contigs were upregulated and 411, 123 and 198 were downregulated (Fig. 2).

Venn diagrams were used to determine overlaps between the sets of heat shock-responsive contigs. After the 30 °C heat shock, 140 unique contigs (0.07%) were upregulated, 30 of which at both 4 and 8 h after heat shock (Fig. 3a), while only 87 unique contigs (0.04%) were downregulated (Fig. 3b). After the 35 °C heat shock 2257 unique contigs (1.11%) were upregulated, 398 of which were upregulated at all 3 timepoints (Figs. 3c), and 664 unique contigs (0.33%) were downregulated, of which only 9 were downregulated at all timepoints (Fig. 3d).

Considering that one of our goals was to provide a list of consistently upregulated genes that need to be validated as markers *in vivo*, we examined the core set of 27 upregulated contigs (Fig. 3e). Up to 18 corresponded to 5 heat shock proteins (*hspb1, hspb11a, hsph3a, hsph2*, and *hspc1*), while 5 corresponded to 4 different proteins: clusterin (*clu*), growth factor receptor bound protein 2b (*grb2b*), atrial natriuretic peptide receptor 2-like (*npr2*) and coiled-coil domain-containing protein 17 (*ccdc17*) (Table 2 and Table S7 in Supplementary data). The remaining 4 contigs did not output any blast hit or were uncharacterized proteins.

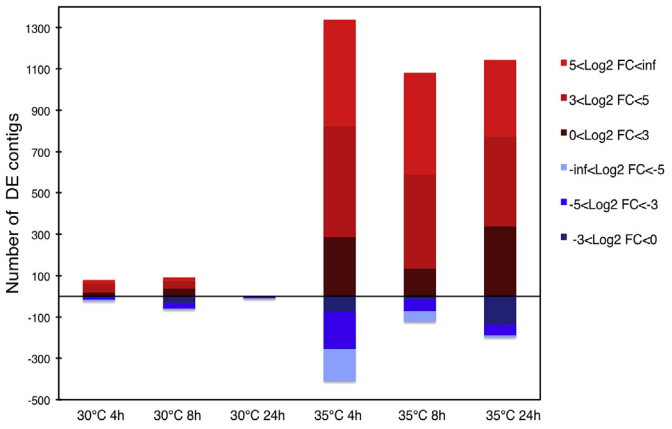

Fig. 2. Number of differentially expressed contigs (DECs) per condition. Positive values represent upregulated contigs and negative values represent downregulated contigs. Different brightness represent different fold changes (FC) ranges.

To link the differentially expressed contigs (DECs) to GO terms, the 3020 unique contigs were blasted (BLASTx) against zebrafish proteins (E-value < 1E-2), which resulted in 1383 hits (45.8%) corresponding to 1068 unique proteins and 1017 zebrafish genes (Table S6 in Supplementary data). GO enrichment analysis for biological processes (BP) and molecular functions (MF) was performed (Table S8 in Supplementary data). With respect to upregulated genes after exposure to 30 °C, cellular processes involved in the response to stimulus (e.g. 'cellular response to heat', 'cellular response to unfolded protein', 'response to temperature stimulus', 'response to unfolded protein'), protein folding (e.g. 'chaperone-mediated protein folding', 'protein refolding') and protein binding (e.g. 'heat shock protein binding', 'protein folding', 'misfolded protein binding') were overrepresented (Fig. 4), however, no overrepresented terms were found after 8 or 24 h. After the 35 °C heat shock overrepresented terms were found at all the timepoints: while GO terms related with cellular response to stimulus and stress (e.g. 'cell chemotaxis', 'leukocyte chemotaxis', 'inflammatory response') and cell migration (e.g. 'granulocyte migration', leukocyte migration', 'neutrophil migration') are overrepresented after 4 and 8 h (Fig. 5a and b), after 24 h (Fig. 5c) many more GO terms are overrepresented, being the 'response to stimulus' the predominant term (122 genes).

With respect to downregulated genes, overrepresentation of 'cell cycle' and 'mitotic cell cycle' GO terms was found only at 4 h after 35 °C heat shock.

3.3. Annotation and expression of Hsp and hsf family members in Atlantic sturgeon

Protein sequences of all known human, zebrafish and spotted gar HSP and HSF family members were retrieved from NCBI and used as queries to search for the corresponding Atlantic sturgeon orthologues in the COE, Organ, Cell or Embryo transcriptomes (Table S9 in Supplementary data).

Spotted gar HSF proteins were used to identify 6 *hsf* genes in sturgeon, which lacked the *hsf3* gene and had 2 *hsfy* genes (*hsfy1* and *hsfy2*). None of the *hsf* genes was differentially expressed at any time point after the mild and severe heat shock.

The former Hsp70 family is divided into 2 sub-families: HspA and HspA-related HspH (Hsp110). The HspA family contains 13, 8 and 7 members in humans, zebrafish and spotted gar, respectively. Nine members were found in the Atlantic sturgeon transcriptome: *hspa1, hspa5, hspa8, hspa9, hspa12a, hspa12b, hspa13, hspa14a, hspa14b*. The HspH family contains 4 members in both human and spotted gar, plus 1 duplicated gene in zebrafish. The *hsph1* member could not be retrieved in any of the available sturgeon assemblies and the *hsph3* member was found duplicated.

Humans, spotted gar and zebrafish contain the *hspb1*, *hspb2, hspb3, hspb4* (αA-crystallin), *hspb5* (αB-crystallin), *hspb7, hspb8* and *hspb9* members of the small HSP family, whereas *hspb6* is missing in spotted gar, *hspb10* is only present in humans, and *hspb11*, *hspb12* and *hpsb15* are only present in zebrafish. With the exception of *hspb3*, orthologues of all spotted gar *hspb* genes could be found in the sturgeon transcriptome, including 2 *hspb1* genes (*hspb1a* and *hpsb1b*). The *hspb11* member, absent in human and spotted gar, could also be retrieved duplicated in the sturgeon transcriptome (*hspb11a* and *hspb11b*).

The HspC family (former *hsp90*) has 5 members in humans and 4 in zebrafish and spotted gar, which lacks the *hspc2* member. The same fish members were found in the sturgeon transcriptome, with an additional *hspc3* duplication (*hspc3a* and *hspc3b*). The HspD and HspE families each contain only 1 single gene in humans, zebrafish and spotted gar, and orthologues of both of them could be found in sturgeon.

The new nomenclature for the former Hsp40 family divides it into DnajA, DnajB and DnajC families. We have found 4, 10 and 32 genes for

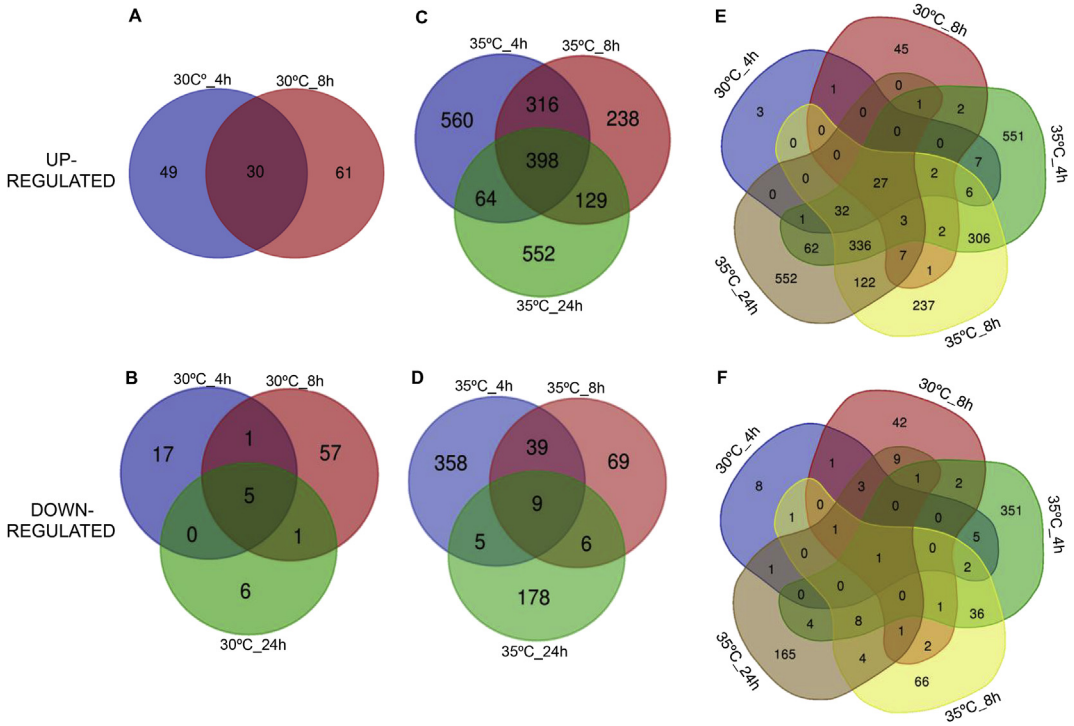

Fig. 3. Venn diagrams showing the differentially expressed contigs (DECs) overlaps per condition. The last timepoint (24 h) after the 30 °C heat shock was excluded from the diagrams as it had only 12 downregulated contigs and none upregulated. Venn diagrams at the top show the overlap of upregulated contigs at different timepoints after 30 °C (A), 35 °C (C) or both (E) treatments. At the bottom, Venn diagrams show the overlap of downregulated contigs at different timepoints after 30 °C (B), 35 °C (D) or both (F) treatments.

each family, respectively.

Upon mapping the AOXlar7y Illumina reads against the annotated sturgeon HSP genes (Table S10 in Supplementary data) and performing differential expression analysis using DESeq, we found that 16 out of 76 HSP genes (21.%) were differentially expressed between the control and some of the experimental conditions. No HSPs transcripts were found differentially expressed between the control and 24 h after the 30 °C heat shock. Within the HspA family, only *hspa1* was differentially expressed after 35 °C heat shock (Fig. 6a), being up to ~1000-fold upregulated 4 h after the 35 °C treatment.

Upregulation of 3 out of 4 HspH family members was observed (up to ~9-fold): *hsph2* and *hsph3a* were significantly upregulated at all the conditions, however, the *hsph3b* paralog was only slightly upregulated at after the 30° treatment (Fig. 6a).

In addition, 5 out of 10 HspB family members and the single HspC family member *hspc1* were upregulated after heat shock: while *hspb8* was only significantly upregulated at 4 h after the 35 °C heat shock and *hspb5* only at 35 °C, *hspb1b, hspb11a, hspb11b* and *hspc1* were consistently upregulated in all the treatments (excluding 24 h after the 30 °C treatment), with the *hspb11a* having the higher expression (as high as ~3296-fold compared to the untreated cells) (Fig. 6b). The HspE1 and HspD1 were not differentially expressed at any condition.

Within the DnajA family (Fig. 6c), only the *dnaja4* member was upregulated. Four DnajB members were upregulated: *dnajb1* and *dnajb5* were only upregulated after the 35 °C heat shock, *dnajb4* was also upregulated at 4 h after the 30 °C heat shock, and *dnajb2* was only upregulated after the 30 °C heat shock.

Although the DnajC family is the most extensive Dnaj subfamily, it contained only 1 heat shock inducible gene under our conditions, *dnajc3*, which was upregulated only at 24 h after the 35 °C heat shock.

4. Discussion

The AOXlar7y cell line is easy to maintain and propagate, providing an excellent tool for examining the effects of different stressors. Healthy AOXlar7y cells have cubic to fibroblast-like morphology, and the

Table 2
List of the core 27 up-regulated contigs and corresponding gene description, sturgeon gene name and other names present in the literature.

COE contig/s ID	Gene description	Sturgeon gene	Other names
75185, 75186, 75188	Heat shock protein beta-11	*hspb11a/b*	*hsp30*
81106, 81108	Heat shock protein beta-1	*hspb1*	*hsp27, hsp25*
68968	Heat shock 70 kDa protein 4L	*hsph3a*	*hspa4l, apg1*
66436, 3989, 4740, 133784, 161880, 133767, 63068, 79530, 69039, 69847, 169012	Heat shock protein 90 kDa alpha family class A member 1	*hspc1*	*hsp90aa1.2, hsp86, hsp90, hsp89*
113709	Heat shock 70 kDa protein 4	*hsph2*	*hspa4, apg2, hsp110*
68283, 7082	Clusterin	*clu*	*clu*
22979	Growth factor receptor-bound protein 2	*grb2*	*grb2*
166695	atrial natriuretic peptide receptor 2	*npr2*	*npr2*
64489	Coiled-coil domain-containing protein 17	*ccdc17*	*ccdc17*
46154, 75199	n.a.	n.a.	n.a.
62264, 62266	Uncharacterized protein	n.a.	n.a.

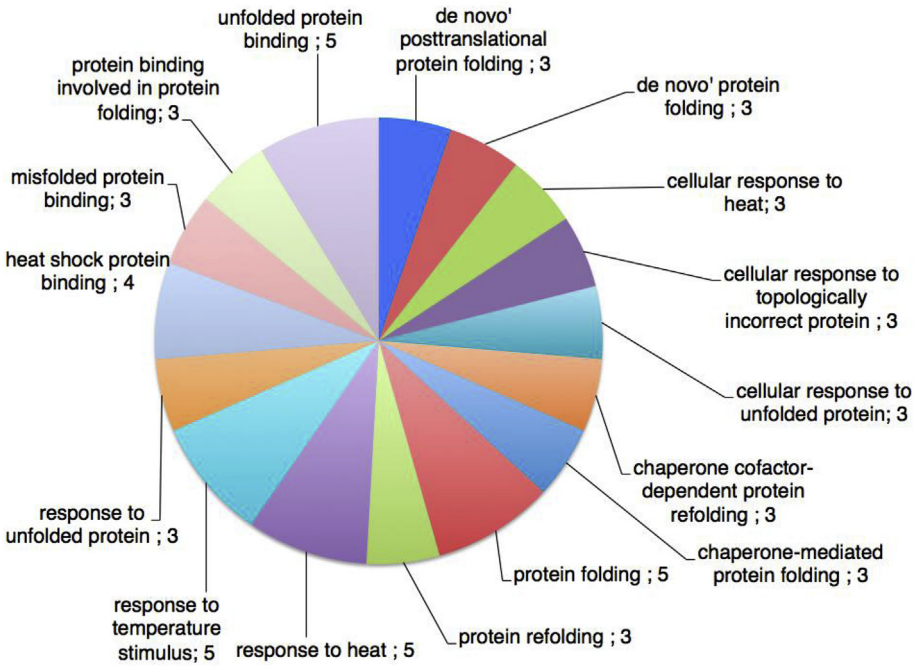

Fig. 4. Pie chart showing overrepresented gene ontology (GO) terms at 30˚. Biological process and molecular functions overrepresented at 30 °C after 4 h, including the number of differentially expressed genes in each term.

RNAseq analysis in this study shows that keratin 4 is the most abundantly expressed mRNA in this cell line, suggesting that they are epithelial cells [52]. Although both the cell line and the donor embryo have been reported to be more tolerant to cold than heat [43,53] the optimal temperature for cells is higher than for the entire animal (25 °C and 18°C-23 °C, respectively). After establishing the temperature tolerance limits, cells were exposed to a mild (30 °C) and severe (35 °C) non-lethal heat shock for 1 h and, after 4, 8 and 24 h of recovery, RNA was isolated and sequenced. By RNAseq we could get a general perspective of the heat-inducible genes, which may not truly represent the

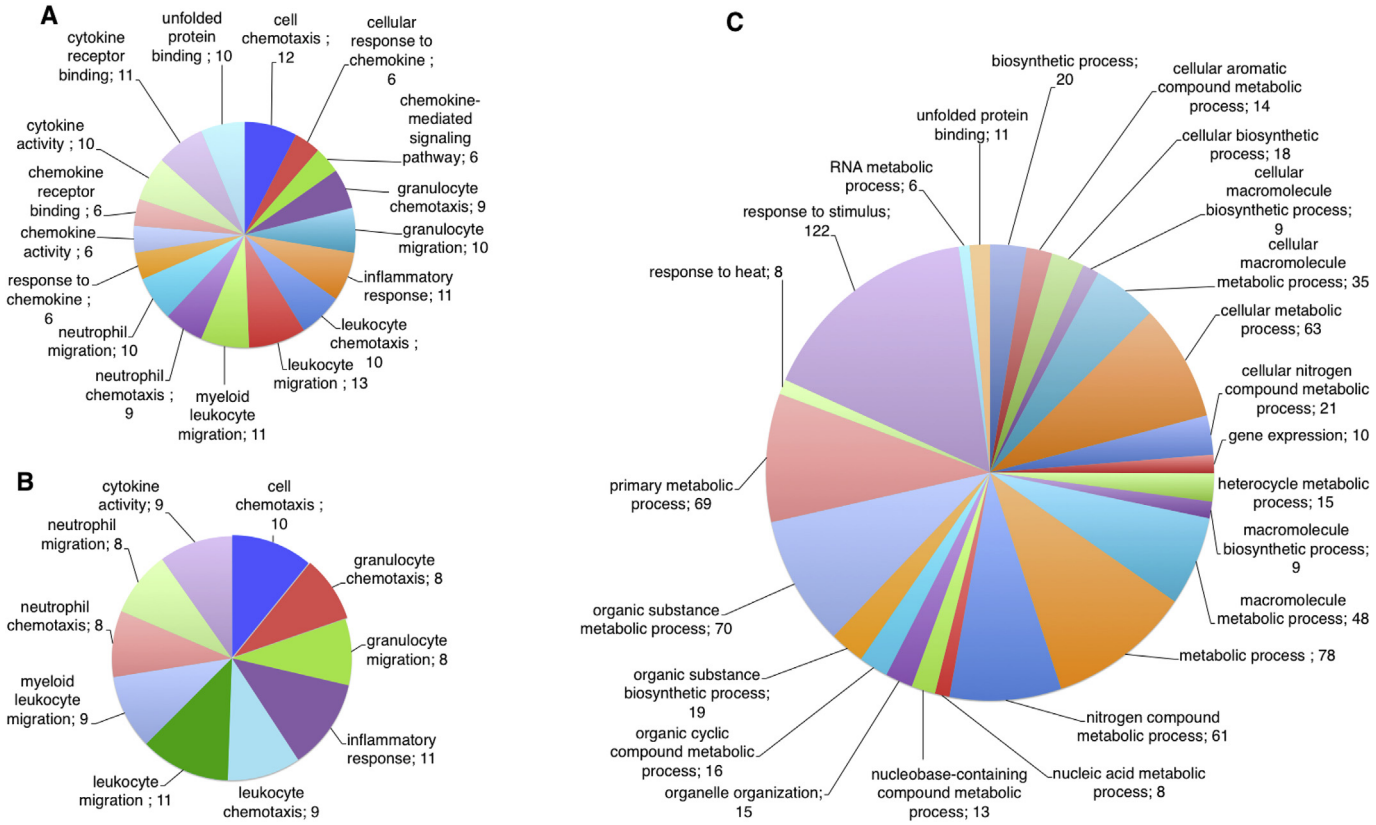

Fig. 5. Pie chart showing overrepresented gene ontology (GO) terms at 35˚. Biological process and molecular functions overrepresented at 35 °C after **A:** 4 h; **B:** 8 h; **C:** 24 h, including the number of differentially expressed genes in each term.

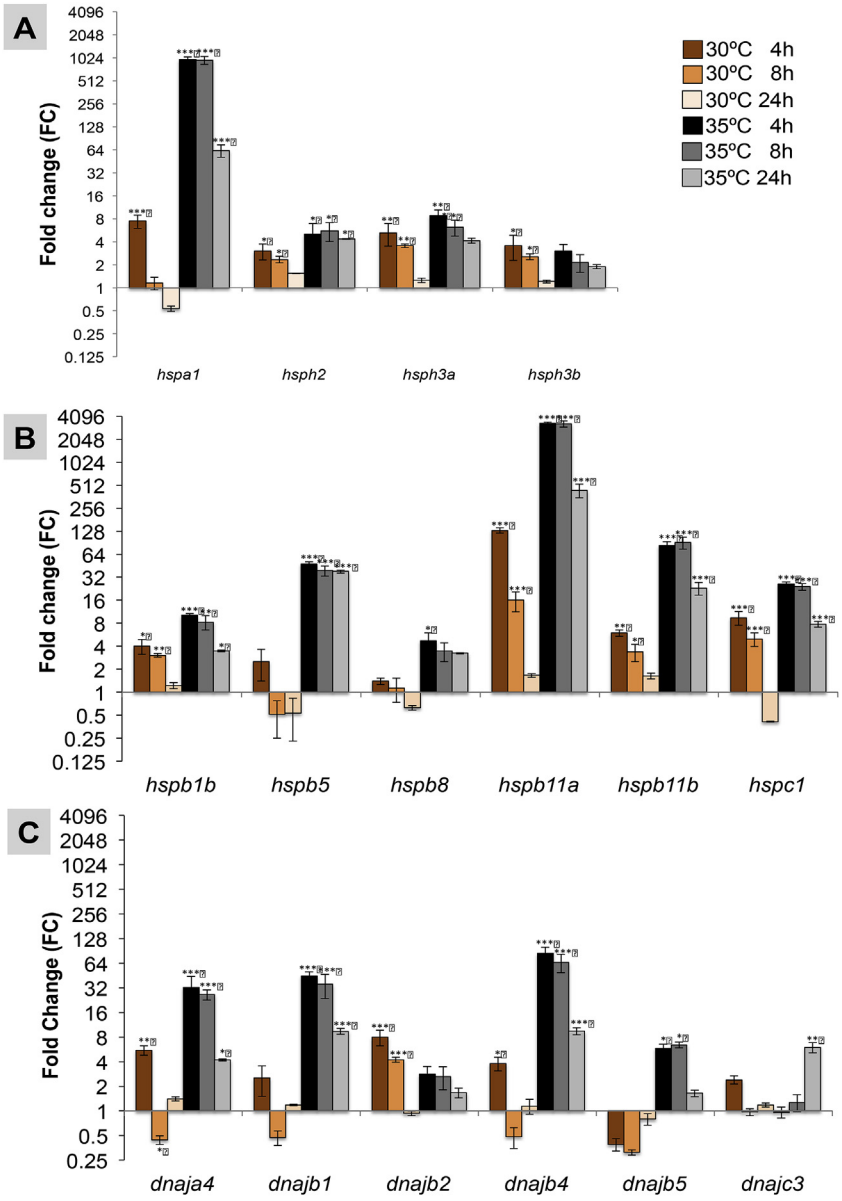

Fig. 6. Fold change (FC) of the heat shock inducible genes per condition. A: HspA and HspH genes**; B:** HspB and HspC genes; **C:** DNAJ genes. Differences are considered significant when padj < 0.05. *padj < 0.05, **padj < 0.001, ***padj < 0.0001.

metabolic state of the cells *in vivo* and therefore needs validation, but still provides the sequence of all *hsp* genes and a general perspective of which genes may be more responsive to heat.

The COE reference transcriptome had a much higher alignment rate of cellular reads (89.6%) than the transcriptomes that were assembled from the individual data sets, and was therefore selected for identification of DECs. This reference provides a considerable number of sturgeon protein sequences that contribute to sturgeon research. The set of 3020 DECs between the control and at least one of the treatments was blasted against zebrafish proteins, the most related species for which GO analysis is available. A total of 1383 DECs could be assigned to zebrafish proteins, while the remaining contigs were noncoding, sturgeon-specific, missing in zebrafish or lacked sufficient homology with their zebrafish orthologues.

The DESeq results showed a very intense response at 35 °C, initially involving energy-consuming gene upregulation and later also gene downregulation, which is an energy-saving mechanism to direct energy towards the repair of damaged molecules [36]. After a mild 30 °C heat shock there were very few transcriptional changes at early timepoints, and even zero after 24 h of recovery. The narrow temperature tolerance range between 30 °C and 35 °C where sturgeon cells switch from a mild to a severe heat shock response is in agreement with existing studies in green sturgeon (Acip*enser medirostris*) [53] and Kaluga (*Huso dauricus*) [39].

The GO analysis showed a heat shock response at 35 °C which increased with the recovery time. At 30 °C the response was more specific (overrepresentation of less GO terms); however, 24 h after heat shock the cells showed the same transcriptional activity as the untreated cells, suggesting that the cells were already fully recovered after this mild heat shock (30 °C). The bulk of the 27 contigs that were upregulated at all conditions (excluding 30 °C after 24 h) corresponded with HSP genes, and included 4 other genes: *clu, grb2b, npr2 and ccdc17*. Clusterin is a molecular chaperone [54,55] and, similarly to *npr2*, is involved in cell survival after apoptosis induction [56–58]. Coiled-coil domains are involved in the regulation of gene expression, but the specific function of *ccdc17* has not been studied in detail [59]. As both the DESeq and GO results point to the HSP genes as central players in the heat shock response, we have annotated the entire family and analyzed their

response to heat.

In total 76 Hsp and 6 Hsf genes could be retrieved in the Atlantic sturgeon transcriptomes, including 4 partial sequences and 78 full ORFs: 33 (40.24%) in the COE assembly and 45 (54.87%) in either the embryo, cell or organ transcriptomes.

In our experiment, only 16 out of 76 Hsp genes were heat-inducible, for which the coefficient of variation (CV) was lower than 30% in most of the cases (89.58%). HspB was found to be the most heat responsive family with less than 30% CV except for *hspb5*, which had high CV at all the timepoints after the 30 °C heat shock. The HspB family is ATP-independent and characterized by the presence of a conserved α-crystallin domain [60]. Their expression has been shown to enhance the post-stimulus survival of mammalian cells [61] and, besides the molecular chaperone activity, some members have additional cellular functions: *hspb1* (*hsp27*) and *hspb5*, induced by heat shock in zebrafish [62], are known to inhibit apoptosis [63–65], while *hspb8* has kinase activity [66]. Unfortunately, the human *hspb11* gene proposed by Kampinga [25], previously known as intraflagellar transport protein 24 (*ift25*), lacks the α-crystallin domain and its nomenclature hasn't been approved [67]. In fact *hspb11*, also known as *hsp30*, exists in all vertebrates except mammals [68], and doesn't share any amino acid sequence similarity with the human *ift25*. Since *hspb11* absent in both human and spotted gar genomes, the zebrafish protein sequence was used to retrieve the Atlantic sturgeon orthologue.

The few published studies on HSPs in *Acipenserids* were performed in species other than Atlantic sturgeon, focused only on *hspa1* (*hsp70)* and *hspc1* (*hsp90)* and didn't use RNAseq. Using qPCR, *hspa1* and *hspc1* were found expressed in both unstressed and heat-shocked Kaluga juvenile tissues; however, *hspa1* was found to be more inducible by cold than heat [39]. In contrast, *hspa1* showed higher expression after heat than cold stress in both white (*A. transmontanus*) and green (*A. medirostris*) sturgeon larvae [69]. Existing studies found a heat-dependent increase of deformities accompanied by an increase of HspA1 and HspA5 (Hsp78) and a decrease of HspD1 (Hsp60) protein levels [70]. If heat stress is not lethal, the accumulation of HSPs may lead to the tolerance of more severe and otherwise fatal stresses [71,72]. Some studies suggest that heat shock experienced by the parental fish or gametes could improve larvae thermotolerance, resulting in higher survival rates and lower incidence of deformities linked to high HspD1 and HspC1 protein levels [53]. After heat shock, Hsp levels were higher in a heat-adapted subspecies of doctor fish (*Garra rufa*) than in the non-adapted, indicating that Hsp levels provide thermotolerance [73].

Overall, 5 HSPs are consistently upregulated (FC ≥ 3) after all the treatments (excluding 24 h after the 30 °C heat shock) and are candidate markers for *in vivo* validation: *hsph3a*, *hspb1b*, *hspb11a*, *hspb11b* and *hspc1*. Interestingly, this list excludes *hspa1*, which is only upregulated after severe heat shock in our study. Although *hspc1*, previously found upregulated *in vivo,* is also included in the list included in the list, *hspb11a* has as much as ~3296-fold upregulation compared to the control cells, and is the best candidate marker for *in vivo* validation and trials.

Hspb11 is an intron-less gene, enabling fast expression without major splicing events [74]. Heat shock was shown to induce accumulation of hspb11 mRNA in Atlantic salmon [75], Chinook salmon (1250-fold) [76], red band trout (200-fold) [77], zebrafish [67], clawed frog [70], heat-tolerant Artcic charr [78] and killfish [79], with the latter showing more upregulation in heat-tolerant southern populations than the northern counterparts.

In conclusion, the AOXlar7y cell line provides the opportunity to reduce *in vivo* experiments on Atlantic sturgeon, an extirpated species in Europe. We have performed RNAseq on heat-shocked cells to get a general perspective of heat inducible genes, and identified and annotated 6 HSF and 76 HSP genes. Only 16 *hsp* transcripts were significantly upregulated after the applied treatment of which 5 were common to all treatments and timepoints, excluding 24 h after the 30 °C heat shock treatment. These genes had at least a 3-fold increase in expression and one of them, *hspb11a*, had as much as a 3296-fold increase. These genes are candidate markers for the selection of thermotolerant individuals and should be validated *in vivo*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.03.014.

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