Diversified regulation of circadian clock gene expression following whole genome duplication

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Abstract

Across taxa, circadian control of physiology and behavior arises from cell-autonomous oscillations in gene expression, governed by a networks of so-called 'clock genes', collectively forming transcription-translation feedback loops. In modern vertebrates, these networks contain multiple copies of clock gene family members, which arose through whole genome duplication (WGD) events during evolutionary history. It remains unclear to what extent multiple copies of clock gene family members are functionally redundant or have allowed for functional diversification. We addressed this problem through an analysis of clock gene expression in the Atlantic salmon, a representative of the salmonids, a group which has undergone at least 4 rounds of WGD since the base of the vertebrate lineage, giving an unusually large complement of clock genes. By comparing expression patterns across multiple tissues, and during development, we present evidence for strong gene- and tissue-specific divergence in expression patterns, consistent with functional diversification. Unexpectedly, we found a clear link between cortisol secretion and non-circadian changes in the expression of a subset of clock genes in the salmon gill. This regulation is linked to changes in gill function necessary for the transition from fresh- to sea-water in anadromous fish, and, in contrast to mammals, we find no evidence for coupling between cortisol and circadian expression of salmon clock genes. Overall, this analysis emphasises the potential for a richly diversified clock gene network to serve a mixture of circadian and non-circadian functions in vertebrate groups with complex genomes.

Introduction

Circadian control of metabolic physiology and behaviour is a ubiquitous characteristic across taxa [1–3]. In eukaryotes, circadian control derives from a cell-autonomous molecular oscillator, assembled from a network of transcriptional (co-)activators and (co-)repressors, chromatin modifiers and an array of post-translational regulators of protein function, often described collectively as 'clock genes'. Clock gene oscillations coordinate the transcription of multiple genes to exert effects on global cell metabolism. While the molecular clock is conserved between insects and mammals [2], the mammalian network contains many duplicated components as a consequence of both local and whole genome duplication (WGD).

Two rounds of WGD preceded the establishment of the tetrapod lineage 500 million years ago (MYA) (Figure 1A), and gave rise to the complement of clock genes seen in mammals, including multiple paralogues of *Period* and *Cryptochrome* genes. Paralogues arising from WGD are known as 'ohnologues', after Susumu Ohno, who wrote a seminal monograph hypothesising that the genetic redundancy proceeding WGD facilitates evolutionary innovation [4,5]. Nevertheless, the evolutionary importance and extent to which clock gene ohnologues are functionally divergent largely remains unclear [6–10]. Indeed the retention of multiple redundant ohnologues of core clock genes is puzzling given that the essential role of the circadian clock has not changed during the course of evolutionary history [1,2,11,12]. Conceivably, functional differences between ohnologues, achieved either by coding sequence differences or by promoter-based differences in expression level, could enable tissue-specific optimization of function, but evidence for this is sparse [11,12]. It has been suggested that preferential interactions of specific mammalian

PERIOD proteins with specific mammalian CRYPTOCHROME proteins may affect photic entrainment [13], but experimental evidence is lacking [14]. Tissue-specific functions of mammalian CKIδ/ε ohnologues in regulation of PERIOD protein stability have been suggested [15], and alterations in period (tau), amplitude and clock resetting behavior have been observed but clear distinctions of function between the ohnologues are lacking [8,16–18].

Since the ancestral tetrapod WGD events, subsequent rounds of WGD have occurred in several linages, creating highly complex genomes containing thousands of ohnologue pairs. This is exemplified by the situation found in the salmonids, which underwent two additional rounds of WGD compared to basal tetrapods, with the most recent, the salmonid-specific fourth round of duplication (Ss4R) taking place some 100 MYA (Figure 1A) [19]. The Ss4R event is a defining characteristic of the salmonid group and is theorized to have led to the evolution of anadromy; an adaptation of freshwater salmonids to spend part of their life-cycle at sea [20]. Genome-scale analysis in salmonids has begun to provide new insights into the evolutionary significance of ohnologue divergence [21,22]. Impressively, even though gene loss often occurs following duplication events (reviewed in:[23]), there remains a rich complexity of clock genes in modern salmonids with the 18 clock genes (as defined in Figure 1B) in laboratory mice having 61 counterparts in Atlantic salmon (S1 Table, Figure 1C).

To understand why so many additional copies of core clock genes are retained in the genomes of modern salmonids, we have undertaken a comprehensive analysis of clock gene expression in the Atlantic salmon, exploring temporal regulation in different tissues and responsiveness to different environmental stimuli. Here, we show diversified regulation of clock ohnologues as a result of WGD, reflecting the fundamental differences in temporal

80 organization of metabolism between tissues.

Results

Tissue-specific expression of clock gene ohnologues indicates regulatory divergence

To identify all conserved clock genes in the Atlantic salmon we extracted amino acid sequences from the highly-characterized mouse clock gene network (Figure 1B) then searched for homologous sequences in Atlantic salmon [22] and 12 other vertebrates (including five salmonids, S1 appendix). Homology relationships between protein sequences were traced back to the root of the vertebrate tree, revealing 61 canonical clock genes in the Atlantic salmon, 42 of these genes can be assigned to 21 Ss4R ohnologue pairings (referred to as Ss4R pairs from here on), while for the remaining 19 genes no extant Ss4R duplicate can be identified (Figure 1C, S1 Table, S1 appendix).

Following a WGD event the tetraploid genome incrementally returns to a diploid state (rediploidization), this process occurs at different rates at different genetic loci [24] (Figure 1C). Using published data [22] on sequence similarity in 1Mbp windows across syntenic Ss4R duplicated regions we could classify the rediploidization times for our 21 Ss4R pairs from early (approx. 87% sequence similarity) to late (>95% sequence similarity)(Figure 1C).

RNA profiling from 13 different tissues [22] demonstrated tissue-specificity of clock gene expression, and particularly highlighted the wide variety and high abundance of clock genes in the brain (S1 Figure). To assess the divergence between Ss4R pairs we calculated an expression divergence index (EDI), based on the relative expression of each member of a pair across all tissues expressed as a ratio (Figure 1D). This revealed evidence for divergent tissue-specific expression within multiple Ss4R pairs but no clear relationship to approximate time of rediploidization (Figure 1D). The Cry1-Ch12/Ch22 pair, which is linked to direct light

104 effects on circadian function in zebrafish [9] had the highest EDI, largely attributable to
105 divergent expression in the brain and gill (S1 Figure). The three Ss4R pairs of *Nr1d1* (*Rev-*
106 *erbα*) and *Nr1d2* (*Rev-erbβ*), which encode transcriptional repressors linking the circadian
107 clock to energy metabolism [25], were also highly divergently expressed genes, again due to
108 differences in the brain and gill (Figure 1D, S1 Figure). Hence tissue-specific expression
109 divergence is a feature of particular aspects of the circadian clockwork.

110

111 **Circadian and light-regulated Ss4R pair expression differs between tissues but is highly**
112 **similar within tissues**

113 To test for circadian regulation of gene expression we collected samples from fish kept in a
114 light dark cycle (diel), constant light (LL) and constant dark (DD) (Figure 2A). To avoid
115 unintended rhythmical stimuli (zeitgebers), fish were fasted from 48 hours before the first
116 sampling point and temperature was held constant. We focussed on three tissues with
117 distinctive roles in salmonid physiology: the optic tectum (OT) of the brain, because it is
118 linked to visual processing and is highly light-responsive [26,27]; the saccus vasculous (SV)
119 because it has been proposed as a mediator of photoperiodic responses [28]; and the gill
120 because it is essential for respiratory gas exchange, ion- and water balance [29]. We
121 hypothesized that expression profiles of clock genes in these three tissues would differ
122 reflecting tissue-specific differences in temporal metabolic demand. We analysed RNA
123 transcript profiles using a bespoke NanoString CodeSet which could specifically identify 46
124 clock gene targets including the 17 Ss4R pairs (S2 Table, S2A Figure & S2 appendix).

125 In diel conditions, we identified 28 oscillating transcripts (JTK-cycle adj.p<0.05, S1
126 Table) [30] (Figure 2B). Of the three tissues studied, the OT showed by far the strongest

127 oscillations in gene expression, both under diel and constant conditions (Figure 2B & C). For
128 half of the genes identified, oscillation was only observed in the OT, and even for genes
129 showing significant oscillation across tissues (e.g. Per1-Ch4) the amplitude of oscillation was
130 clearly highest in the OT (Figure 2B & D).

131 In contrast, rhythmicity in both the SV and gill was much less robust. In the SV, only
132 Nr1d1-Ch6 maintained rhythmicity and phase under DD, while in the gill only Arntl1-Ch10
133 maintained rhythmicity and phase under DD (S2B-C Figure). Hence robust circadian
134 rhythmicity is a feature of light-responsive tissue in the salmon brain, but gene expression
135 rhythms are severely dampened in the peripheral tissues we studied.

136 Although differences in absolute expression levels were widely seen within Ss4R pairs
137 - both across and within tissues, when comparing temporal dynamics of expression, within a
138 given tissue they were typically similar (Figure 2D, S1 Table, S2 appendix). This is exemplified
139 by the almost superimposable expression patterns seen for the Per1-Ch4/11 pair (Figure 2D),
140 and for the Tef-Ch3/6 pair (Figure 2D). Indeed, only two significant within-pair differences in
141 expression profile were observed (non-linear regression p-value <0.01, S1 Table, S2D-E
142 Figure): the Arntl1-Ch10/16 pairing, with Arntl1-Ch10 showing more robust and higher
143 amplitude rhythmicity than Arntl1-Ch6 in the OT (Figure 2D & S2D-E Figure), and the
144 arrhythmic Cry1-Ch12/22 pairing, with Cry1-Ch22 showing a light-induced increase in
145 expression following transfer to LL in the SV (Figure 2D & S2E Figure). Interestingly, we do
146 not observe light responses through Tef as reported in zebrafish [31].

147

148 **Regulatory divergence of clock gene ohnologues within a tissue during a developmental**
149 **transition**

The lack of circadian regulatory divergence among Ss4R pairs led us to consider whether retention of duplicates might be related to developmental changes in tissue function. One striking example of this in salmon is the transformation of gills from a salt retaining, water excreting organ in freshwater to a salt excreting water retaining organ in seawater (smoltification). This development of the anadromous lifestyle relies on hormonally-driven changes in physiology, dependent on seasonally changing day-length (photoperiod) [32]. We therefore performed a photoperiod manipulation experiment to assess the impact of photoperiod-dependent developmental changes in juvenile salmon (parr) (Figure 3A). This protocol produces a seawater-tolerant (smolt) phenotype within 4-6 weeks of return to LL (S3 Figure)(reviewed in:[32]).

We identified 30 clock genes showing significant changes in expression over the course of the experiment (FDR<0.01, S1 Table, Figure 3B); 3 clock genes were undetectable by RNA-seq, while a further 28 were present but did not change significantly over time. Amongst the differentially regulated genes we found 9 complete Ss4R pairs (18 genes), and 3 incomplete pairs (i.e. only one of the pair is differentially expressed).

Cluster analysis of the differentially expressed genes revealed 5 distinct patterns of expression over the experiment indicating that different regulatory pathways are directing the expression of particular clock genes during smoltification (Figure 3B). Based upon this analysis we identified 6 Ss4R pairs within which evidence of developmental regulatory divergence could be observed (Figure 3C). For 3 of these Ss3R pairs only one member showed a developmental change in expression (Figure 3C, Cry1-Ch12/22, Rora-Ch2/5, Rora-Ch10/16) , while for the 3 remaining Ss4R pairs the dynamics of expression, as defined by cluster analysis, differed within the pairing (Figure 3C, Nr1d2-Ch2/5, Per1a-Ch4/11, CKIδ-

Ch1/28). Therefore we see strong developmental regulation of clock genes in the gill, contrasting with the lack of circadian regulation.

Glucocorticoid signaling induces clock ohnologue expression and accounts for regulatory divergence observed in the Ss4R pair Tef-Ch3/6

While glucocorticoids play a major role in the circadian organization of mammals (reviewed in: [33]), the evidence for an analogous role in fish is unclear (References summarized in: S3 Table). Nevertheless, cortisol is a major hormonal regulator of smoltification in Atlantic salmon, steadily rising during this process [34]. We collected blood samples from fish kept in a light dark cycle (LD - 6:18) and in constant conditions (LL or DD) and found no evidence of diel or circadian rhythmicity in cortisol secretion (Figure 4A) along with weak/absent peripheral tissue clock gene oscillation (Figure 2B). We hypothesized that increasing cortisol during smoltification results in clock gene expression, and that by using a simple stress test to induce cortisol we could induce the same clock genes seen during smoltification.

To test this we conducted a 24 hour seawater (SW) challenge test in freshwater-adapted fish (Figure 4B) eliciting an osmotic stress-mediated increase in cortisol secretion (Figure 4C). Gills were collected from SW and fresh water (FW) groups. We identified 15 clock genes showing significant changes in expression in response to SW by RNA-seq (FDR<0.01, S1 Table, Figure 4D). Importantly, 87% of SW-responsive clock genes (13/15) also change in the smoltification experiment (Figure 3, S1 Table). Amongst the SW responsive genes we found 3 complete Ss4R pairs (6 genes), and 6 incomplete pairs (i.e. only one of the pair is significantly differentially expressed). To assess regulatory divergence we plotted the

fold change in response to SW for each pair which indicated 5 pairs with significant regulatory divergence (two-way ANOVA <0.01, S1 Table, Figure 4E).

To further examine if glucocorticoid signaling, via cortisol, was responsible for the induction of clock genes in the gill we used transcription factor binding site analysis [35] on clock genes induced by SW (15 genes) compared to 43 clock genes that were SW-insensitive. SW-induced circadian genes promoters were highly enriched for HSF1 (heat shock factor 1), FOXO1 (forkhead box O1), MAX1 (myc-assocated factor X1) and glucocorticoid receptor response elements (GR) (Figure 4F, S4 Table). Smoltification and responses to SW-exposure are coordinated by multiple endocrine factors including cortisol, growth hormone (GH) and IGF-1 [36,37]. HSF-1 and FOXO-1 elements are regulated by IGF1 signaling, during in stress, cellular metabolism and development [38–42]. Furthermore, the enrichment of GR implicates non-circadian glucocorticoid signaling in the induction of clock genes in the gill during smoltification and stress.

Next we wanted to experimentally test the *in-silico* association with glucocorticoid signaling and ask whether differential sensitivity to glucocorticoid signaling might account for the differential regulation observed within Ss4R pairs. We treated isolated gill arches with dexamethasone (DEX; a GR agonist) for 24h and then measured the gene expression of the 5 Ss4R pairs using qPCR (Figure 5A). We validated the experiment by assessment of a positive control gene: *Tsc22d3* (*Gilz*) [43](S4 Figure). We found that whilst some of the genes are glucocorticoid sensitive (3/10), supporting the *in-silico* association, the majority are not induced by glucocorticoids (Figure 5B-D). However, we do demonstrate that divergence in regulation due to glucocorticoid sensitivity in the Tef-Ch3/6 pair (Figure 5D).

Discussion

Divergent regulation of gene expression is a major contributor to the evolution of species diversity [44,45]. Our analysis of the circadian clock network in the Atlantic salmon reveals clear evidence of diversified expression regulation among the many retained copies of canonical clock genes that have arisen through successive WGD events. Diversified regulation is seen in tissue-specific expression patterns, and gene-specific changes in dynamic regulation within tissues. Ultimately, these differences likely reflect the uneven influence of diel factors on metabolism in central and peripheral tissues in a cold blooded fish. Altered sensitivity to cortisol emerges as one proximate cause of differences in tissue-specific expression dynamics. Overall this analysis emphasises the potential for a richly diversified clock gene network to serve non-circadian functions in vertebrate groups with complex genomes.

A striking contrast emerges between clock gene expression regulation in the OT and the gill. The OT is a brain site which is coupled to light input both through retinal afferents, photoreceptor expression, and indirect melatonin-mediated input via OT melatonin receptors [26,27,46,47]. In contrast, the gill is directly exposed to the water environment and continuously handles the osmotic and energetic challenges that this presents [48–50]. Thus while both tissues are highly metabolically active and heavily reliant on ATPase activity to maintain function, the principal environmental influence for the OT is rhythmic light dark input, while for the gill it is continuous osmotic challenge.

Given these differences in tissue function, it is not surprising to observe different complements of clock genes in the two tissues and dramatic differences in temporal dynamics. In the diel-sensitive OT a dominant diel / circadian influence on expression is seen,

while in the gill this is a much weaker influence. Nonetheless clock gene expression in the gill is dynamically regulated, both over the chronic developmental time-scales of smoltification, and acutely in response to osmotic stress. The finding that the complement of genes showing highly sensitive changes in expression in the gill is largely non-overlapping with that under light / circadian control in the OT is clear evidence for sub-functionalized expression regulation within the circadian network.

Within this picture of distinctive tissue-specific expression dynamics, the role of cortisol is of considerable interest. In mammals cortisol is a major player in circadian organization, acting as an internal zeitgeber through which the hypothalamic–pituitary–adrenal (HPA) axis can coordinate daily changes in tissue activity throughout the organism (reviewed in: [33]). Because the teleost hypothalamic–pituitary–interrenal (HPI) axis is functionally analogous to the HPA axis [51], there has been a widespread assumption that cortisol plays a similar circadian coordination function in teleosts [52], although definitive evidence for this is lacking (S3 Table)[53]. Indeed our study implies that it is unlikely that cortisol plays a circadian role in salmonids – we found no evidence for circadian or even diel changes in cortisol secretion in Atlantic salmon, and GREs are less enriched in circadian oscillating clock genes than in non-oscillating, development- and SW-sensitive clock genes. Hence while our data strongly implicate cortisol in the dynamic expression of a subset of Atlantic salmon clock genes, this seems to have nothing to do with circadian function *per se*, and more to do with a role for these genes in non-circadian influences of the HPI axis. It is interesting to speculate that this shifting relationship between glucocorticoids and clock genes could be a contributory factor for the evolution of anadromy and the regulation of its seasonal timing. Furthermore, this finding raises interesting questions about the ubiquity

and evolutionary origins of mammal-like coupling between the HPA axis and circadian function.

Much of the genetic complexity seen in the salmonid circadian clockwork arose following the Ss4R event; we therefore asked how expression patterns diverged within Ss4R pairs. Intriguingly, the answer to this question was highly dependent upon context. We saw many examples of pronounced within-Ss4R pair differences in terms of tissue-specific expression, and some 50% of identifiable Ss4R pairs showed within-pair divergence in expression during smoltification. But within-pair divergence in daily expression patterns was hardly observed. Why might this be so? We suggest this may reflect a difference in the way that selection pressures have operated on promoter regions to, on the one hand modulate tissue-specific expression and, on the other daily temporal regulation. According to this view duplication would confer freedom to diverge, thereby meeting differing tissue-specific requirements. Conversely, the daily temporal patterning may be so fundamental to cell function that any mutations leading to deviation from the ancestral dynamics were strongly selected against. This conjecture will require detailed analysis of regions of promoter conservation / divergence among Ss4R pairs.

Materials and Methods:

Evolutionary analysis

To identify gene orthologs and ohnologs we generated protein sequence homology based orthogroups using the Orthofinder pipeline [54]. For each orthogroup we used the resulting protein tree topology to manually annotate pairs of salmon ohnologs based on the following criteria: (i) salmon ohnologs should form a monophyletic clade only containing genes from other salmonid species, (ii) this monophyletic salmonid clade must have Northern pike as the sister group, and (iii) putative ohnolog pairs had to be conserved in minimum one other salmonid species. Finally, we only retained putative ohnolog if their genomic positions were defined as syntenic regions originating from the Ss4R as defined in Lien *et al*. [22].

Publically available data was used to assess the multi-tissue expression in the Atlantic salmon, these data can be found in the NCBI Sequence Read Archive (SRA): PRJNA72713 and PRJNA260929.

Ethics statement

Fish handling and euthanasia was performed by competent persons and in accordance with the European Union Regulations concerning the protection and welfare of experimental animals (European directive 91/492/CCE). The experiment was approved by the Norwegian Committee on Ethics in Animal Experimentation (ID 3630).

Animal husbandry

Juvenile Atlantic salmon (*Salmo salar*, Linnaeus, 1758) of the Aquagene commercial strand (Trondheim, Norway) were used in all experiments. Fish were held under constant light (LL; >200 lux), at 10° from hatching onwards, and kept in 500 L tanks from first feeding. The fish

were approximately 7 months old when the experiments were initiated. Up until that time the fish had been feed continuously with pelleted salmon feed (Skretting, Stavanger, Norway), from automatic feeders.

Circadian experiment I

Fish were maintained in 500L freshwater were transferred from LL to a short photoperiod (SP; 6L:18D) light schedule for 8 weeks before the start of the experiment. Two weeks before sampling, fish were distributed to two separate 150L tanks and water temperature was maintained at 14°C. The lighting and collection schedule for the experiment is represented in Figure 2A. Fish were fasted for 48 hours prior to the experiment and throughout the sampling. Collections during the dark phase were conducted under dim red light.

Circadian experiment II

Fish were maintained in 500L freshwater were transferred from LL to a short photoperiod (SP; 6L:18D) light schedule for 20 weeks before the start of the experiment. Two weeks before sampling, fish were distributed to two separate 150L tanks. The lighting and collection schedule for the experiment is represented in Figure 4A. Fish were fasted for 48 hours prior to the experiment and throughout the sampling. Collections during the dark phase were conducted under dim red light.

Smoltification experiment and seawater tests

Fish were fed continuously and in excess for eight hours a day, corresponding to the light phase of their photoperiod treatment but were fasted for 48h before sampling. Fish were maintained in 150L freshwater tanks at ambient temperatures (mean 8.5°C) were transferred from LL to a SP (8L:16:D) light schedule for 8 weeks before re-exposure to LL was

resumed. One week after return to LL, a subgroup of randomly selected fish were transferred to a 100L tank supplied with seawater (34‰ salinity). After 24h six fish were sampled from SW and FW tanks. Sampling points included in the study are represented on Figure 3A & 4A.

Sampling protocol

During sampling fish were netted out and euthanized by an overdose of benzocaine (150ppm). Weight and length were recorded, blood samples were collected from the caudal vein in heparinized vacutainers, followed by decapitation and dissection. Blood samples were centrifuged at 500 x g for 15 min to collect plasma. For RNAseq, one gill on the right side (caudal view) was collected and placed in RNAlater (Sigma-Aldrich, St. Louis, Missouri, USA). Samples were stored at 4°C for 24 hours, before being transferred to -80°C. For nanostring samples were rapidly dissected and snap frozen on dry ice before being stored at -80°C.

In-vitro Gill Culture

Juvenile Atlantic Salmon were prepared as in the smoltification experiment. Following euthanasia whole gill arches were rapidly dissected, excess mucus was removed by careful blotting onto tissue paper before the arches were transferred individually into 50 ml of pre-prepared control or treatment media. The prepared media consisted of Leibovitz L-15 (Lonza) supplemented with non-essential amino acids (1%, 100x Lonza), sodium-pyruvate (1%, 100x Lonza), 0.05 mg/ml gentamycin (Sigma) and 20% fetal bovine serum (FBS, sigma). The experimental group was supplemented with 0.1M dexamethasone diluted in DMSO (dimethyl-sulphoxide, Sigma) to a final concentration of 0.1µM. The control group contained an equivalent concentration of DMSO (0.1%). The excised gill arches were incubated for 24h

346 at 4°C, gill filaments were removed with a scalpel and snap frozen on dry ice before being
347 stored at -80°C.

348 *RNA extraction*

349 RNA extraction for RNAseq was performed using a TRIzol-based method (Invitrogen, Thermo
350 Fisher, Waltham, Massachusetts, USA), and in accordance with the manufacturers
351 recommendation. Resulting RNA concentrations and quality were checked using a NanoDrop
352 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was stored at -
353 80°C.

354 For nanostring and qPCR, SVs were extracted using QIAgen RNeasy micro kit, OT and gill
355 tissues were extracted using QIAgen RNeasy mini kit according to the manufacturers
356 instructions. RNA concentration was quantified and quality confirmed using the Experion
357 Automated Electrophoresis System (BioRad).

358 *Nanostring*

359 Custom nanostring codesets were designed by Nanostring Technologies Inc. using the
360 Atlantic Salmon reference genome (Cigene), accession numbers and target sequences are
361 shown in S2 Table. Codesets were processed by the Univerisity of Manchester Genomic
362 Technologies Core Facility. Data was processed using nSolver 4.0 software (Nanostring). Data
363 can be accessed on GEO under the project identifier GSE146530.

364 *Transcriptome sequencing and assembly*

365 Libraries were prepared using TruSeq Stranded mRNA HS kit (Illumina, San Diego, California,
366 USA). Mean library length was determined using the 2100 Bioanalyzer with the DNA 1000 kit
367 (Agilent Technologies, Santa Clara, California, USA). Library concentrations was determined

368 using the Qubit BR kit (Thermo Scientific, Waltham, Massachusetts, USA). Samples were
369 barcoded with Illumina unique indexes. The Illumina HiSeq 2500 was used to perform single-
370 end 100-bp sequencing of samples at the Norwegian Sequencing Centre (University of Oslo,
371 Oslo, Norway).

372 Cutadapt (ver. 1.8.1) was used for removal of sequencing adapters and trimming of low
373 quality bases (parameters –q 20, -O 8 -minimum-length 40). Quality control was performed
374 with FastQC software. Reads were mapped onto the references genome using STAR
375 software (ver. 2.4.2a). Read counts for annotated genes were generated using the HTSEQ-
376 count software (ver. 0.6.1p1)

377 All RNA-seq data for the smoltification experiment is available in the European nucleotide
378 archive under project number: PRJEB34224.

379 *Analysis of differentially expressed genes*

380 Analysis of differential gene expression was performed with package edgeR (ver. 3.14.0)
381 using R (ver. 3.4.2) and RStudio (ver. 1.0.153). Prior to analysis of differential expression, the
382 raw counts were filtered, setting an expression level threshold of a minimum of one count
383 per million reads (cpm) in five or more libraries, resulting in a list of 33 951 expressed genes.
384 The counts were scaled by applying trimmed means of M-values (TMM) scaling. Exact tests
385 were then performed to find genes that were differentially expressed between FW-kept and
386 24-h SW challenged fish. An ANOVA-like test was performed to find genes that were
387 differentially expressed over T1-T6 FW time-points. The test results were filtered for a false
388 discovery rate (FDR) to be less than 0.01 to identify significantly differentially expressed
389 genes. Clustering analysis was performed using Pearson correlation.

Heatmaps were generated in R using custom scripts for pheatmap. Transcription factor binding site analysis was conducted using SalmotifDB [35].

qPCR

cDNA was synthesised from sample total RNA using High capacity RNA to cDNA kit (Applied Biosystems). qPCR was performed using GoTaq Master Mix (Promega) and a 96 well thermal cycler (Applied Biosystems). Relative gene expression was quantified by the ΔΔCT method using *Ef1a* as reference gene. Primer sequences are listed in S2 Table.

Hormone Assays

Cortisol ELISA assays were performed by Stockgrand (UK).

Statistical analyses

RNAseq analysis is detailed above. Mean difference comparisons were carried out using Student's t-test (two-sided, unpaired), two-way ANOVA with post hoc tests as appropriate (Graphpad Prism 8.1.2). The expression divergence index (EDI) index was calculated as follows: EDI= abs(log2[Gene1/Gene2)].

The R package JTK cycle was used to assess rhythmicity of transcripts under LD and constant light or dark conditions [30]. For statistical comparison of gene expression between ohnologue pairs in the circadian experiment, expression was normalized to group mean then best fit sixth-order centered polynomial curves were generated by non-linear regression analysis and shared characteristics tested with extra sum of squares F test (Graphpad Prism 8.0).

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Author contributions

ACW – conceived and designed the circadian and *in-vitro* experiments, collected samples, performed RNA preparation, gill culture, qPCR, bioinformatics analysis, analysed/interpreted data, prepared the manuscript and figures. **MI–** Bioinformatic analysis, osmolality measurements, data analysis and reviewed manuscript. **EHJ –** collected samples, designed and conducted the smoltification experiment, and reviewed the manuscript. **SS –** Conducted the evolutionary analysis of clock genes and revised the manuscript. **DGH** - collected samples, analysed/interpreted data, designed and conducted the smoltification experiment, and revised the manuscript. **SHW–** conceived and designed the circadian and *in-vitro* experiments, collected samples, performed RNA preparation, gill culture, analysed/interpreted data, and prepared the manuscript.

Figure legends

Figure 1. Clock gene Ss4R duplicate pairs are divergently expressed in different tissues.

A. Phylogenetic tree highlighting WGD events and evolution of anadromy. B. The molecular clock network. C. Circos plot showing all Ss4R clock ohnologues chromosomal position and their partners indicated by the red line. Sequence similarity of the loci in a 1mb window is

584 shown on the circus plot; high similarity >95% (red), medium 95-90% (blue), low ~87%
585 (green). Sequence similarity on a genome wide level relates to approximate redipoidization
586 time of Atlantic salmon chromosomes (see grey box). D. Differential regulation of Ss4R pairs
587 in a panel of 11 different tissues. For each Ss4R pair, in each tissue, an expression divergence
588 index (EDI) index was calculated (EDI= abs(log2[Gene1/Gene2)]. The graph shows a violin
589 plot of the distribution of EDI values across all tissues. The vertical black bar represents the
590 median value. Approximate rediploidization time of the loci each pair is found on is
591 represented by a colour: red – late, blue – mid, green – early.

592 **Figure 2. The molecular clock network regulation in the Atlantic salmon.**

593 A. Diel and circadian regulation experiment design, arrows indicate tissue collection points.
594 Samples used for statistical analysis of rhythmicity under diel, LL (constant light) and DD
595 (constant dark) conditions are indicated. B. Heatmap displays all diel rhythmic genes in OT,
596 SV and gill. Overlap between tissues is shown in the venn diagram. C. Peak-phase aligned LL
597 and DD rhythmic genes in the OT. Shaded area shows 95% confidence limit. D. Example
598 duplicate comparisons from OT, SV and gill. JKTcycle (adjP<0.05*, adjP<0.01**,
599 adjP<0.001***, adjP<0.0001****).

600 **Figure 3. The molecular clock network shows ohnologue specific differences during a**
601 **photoperiodically driven developmental transition.**

602 A. Photoperiodic gene expression experiment design. LL – constant light, SP – short
603 photoperiod. Time-points for sampling indicated by arrows. B. Expression heatmap of
604 significantly photoperiodic (FDR<0.01) clock genes in the gill. Significant genes cluster into
605 five distinct expression patterns. Individual profiles are represented. Shaded area shows 95%

606 confidence limit where applicable. C. RNA-seq counts per million profiles for divergently
607 regulated ohnologue pairs.

608 **Figure 4. Stress response implicates glucocorticoid receptor signaling in clock ohnologue**
609 **regulation.** A. Diel and circadian profile of plasma cortisol. Time axis is given in continuous
610 hours since the start of the experiment, therefore 1 to 21 represent zeitgeber time (ZT) and
611 25 onwards is equivalent to circadian time (CT)1 to CT29. Due to the sampling protocol time-
612 points 1 and 5 are replotted from time-points 25 and 29. B. Sea-water stress experiment
613 design. LL – constant light, SP – short photoperiod, SW- sea-water challenge. C. Plasma
614 cortisol concentration in blood plasma in sea-water stress experiment. D. Sea-water stress
615 regulation of clock genes. Significantly regulated transcripts (FDR<0.01) are shown in red. FC
616 – fold change. E. Differential sea-water stress regulation of ohnolgue pairs. Significantly
617 different pairs (Analysis of genes where one or both genes are significantly regulated by
618 seawater (FDR<0.01), then submitted to a two-way ANOVA, with sea-water regulation and
619 interaction, p<0.05) are shown in red. F. Predicted transcription factor promoter binding
620 analysis. Both sea-water induced and not-induced gene cohorts were analysed. 50 motifs
621 were specific to the sea-water induced cohort. The top four motifs in each group are
622 displayed.

623 **Figure 5: In-vitro validation of glucocorticoid stimulated clock ohnologue expression**

624 A. Dexamethasone-dependent transcript regulation experimental design. After photoperiod
625 manipulation whole gill arches were removed and treated for 24h with glucocorticoid
626 receptor agonist dexamethasone (DEX), or dimethlysulphoxide (DMSO; control). B.
627 Comparative regulation of Cry1-Ch12/22 taken from *in vivo* sea-water stress experiment and

628 *in vitro* dexamethasone treatment. C. As in B for Nr1d2-Ch2/5 & Nr1d2-Ch14/27. D. As in B
629 for Tef-Ch1/28 & Tef-Ch3/6. Braches indicate phylogenetic relationship between gene sets.

630 **S1 Figure: Tissue specific expression of clock ohnologues**

631 A. PCA plot showing the relative tissue differences when considering clock ohnologue
632 expression. B. Heatmap showing the tissue specific expression of clock ohnologues.

633 **S2 Figure: Nanostring clock gene expression and circadian phase aligned plots**

634 A. Heatmap showing the mesor expression for each clock ohnologue in three tissues. Grey
635 indicates the gene is not expressed. B. Phase aligned plots for the gill. C. Phase aligned plot
636 for the SV. D. Arntl1-Ch10/16 comparison: plot of non-linear regression using a sixth-order
637 centered polynomial to fit the data and compare individual curves. P-value is the result of
638 extra sum-of-squares F test. E. As above for Cry1-Ch12/22.

639 **S3 Figure: Osmoregulatory capacity during the smoltification experiment**

640 A. Osmolality (mOsm kg-1) is displayed for fish in freshwater (FW – blue) and seawater (SW –
641 green). This plot show osmoregulatory capacity develops by the two latest timepoints (T5
642 and T6).

643 **S4 Figure: Gene expression of Tsc22d3-Ch3, a positive control gene for DEX treatment**

644 A. Gene expression of Tsc22dd3-Ch3 *in vivo* seawater stress experiment (RNA-seq counts per
645 million (cpm)) and B. *in vitro* dexamethasone treatment (qPCR).

646 **S1 Table: Clock genes identified in Atlantic Salmon, orthogroups, duplicates, significances**
647 **for the circadian, smoltification and seawater challenge experiments**

648 **S2 Table: Nanostring codeset design and qPCR primers**

649 **S3 Table: Summary of previous studies measuring cortisol in fish**

650 **S4 Table: SalmotifDB results – transcription factor binding site analysis**

651 **S1 appendix: Evolutionary gene trees for circadian clock genes**

652 **S2 appendix: Nanostring circadian profiles for all genes**

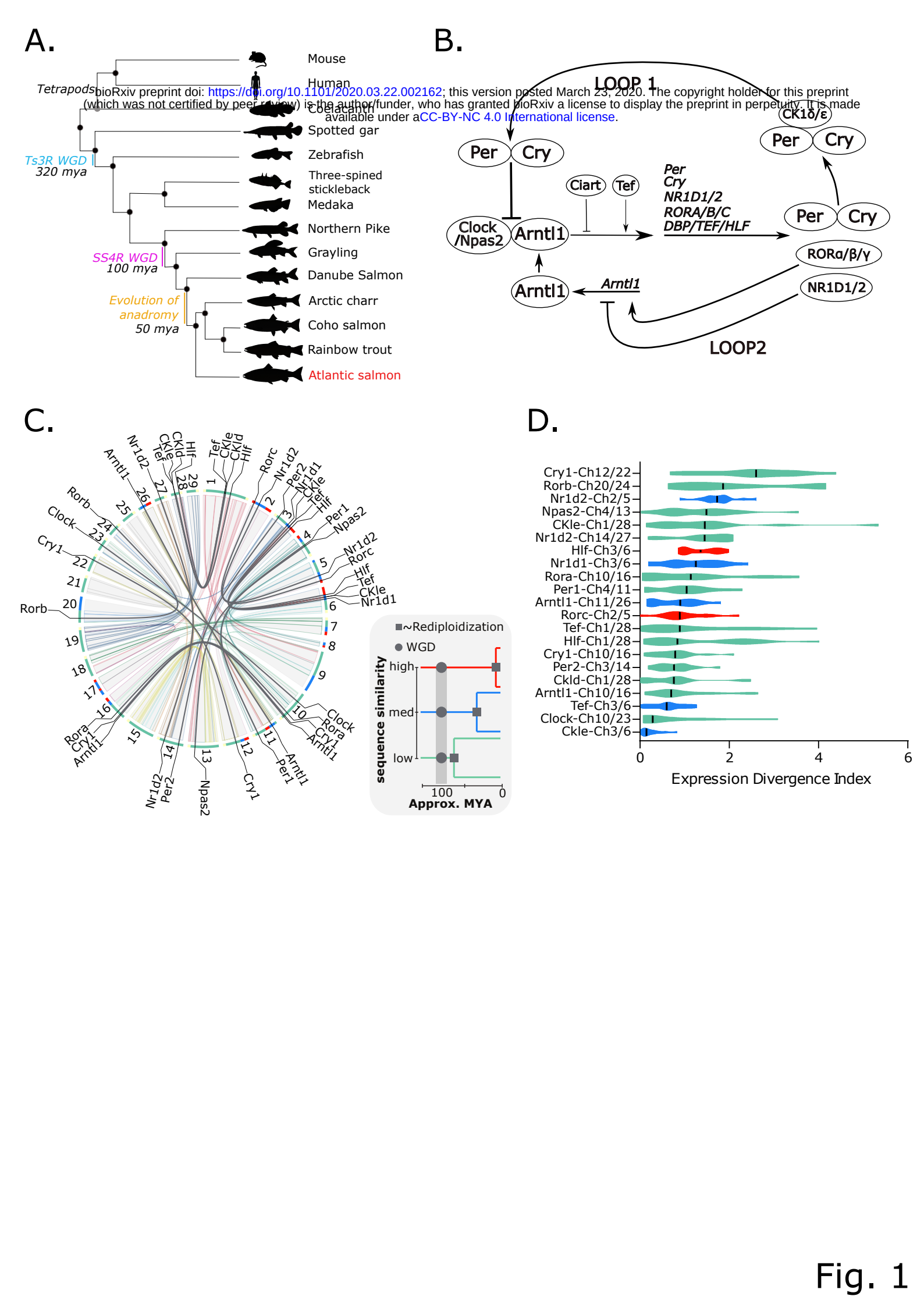


Fig. 1

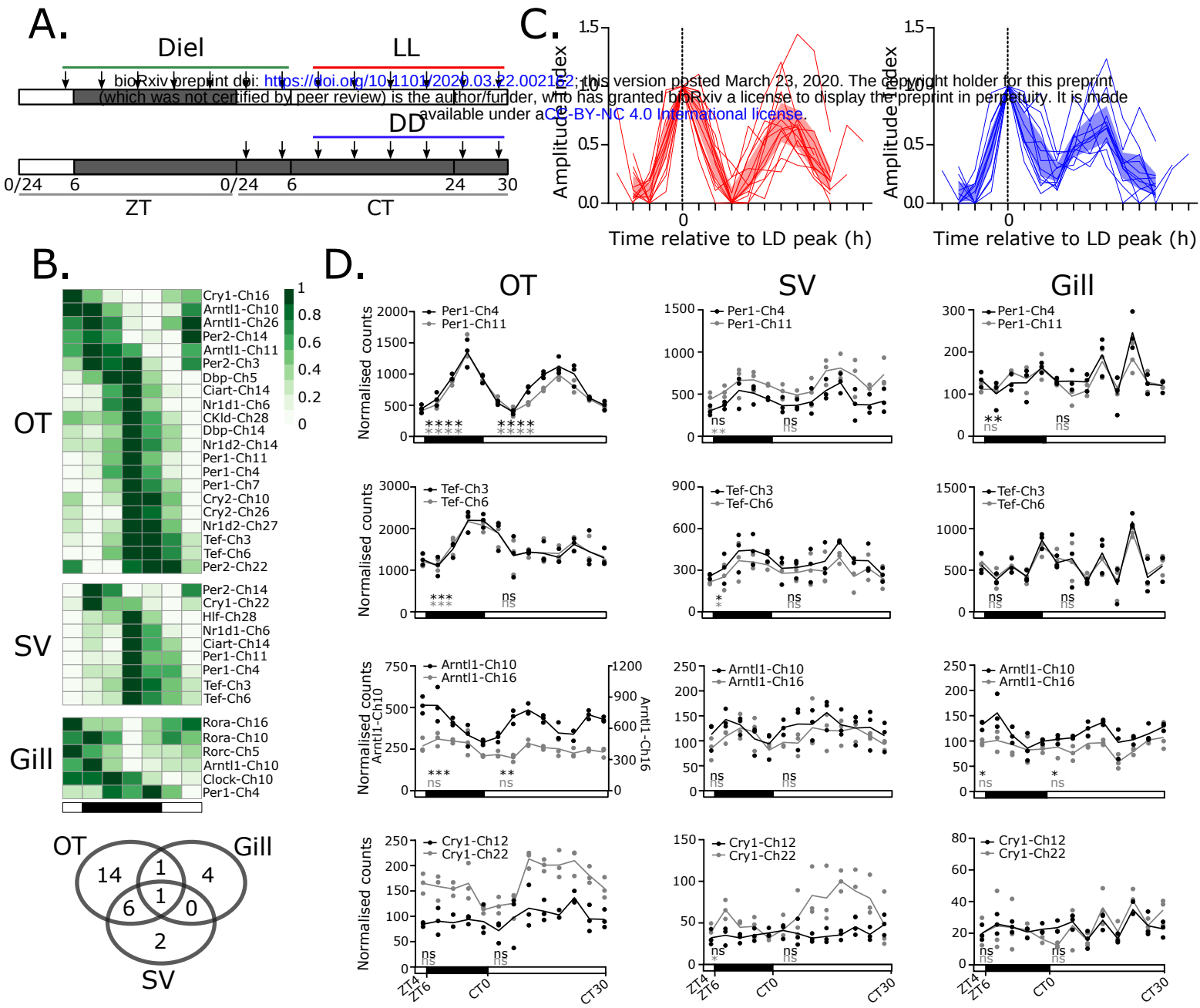


Fig. 2

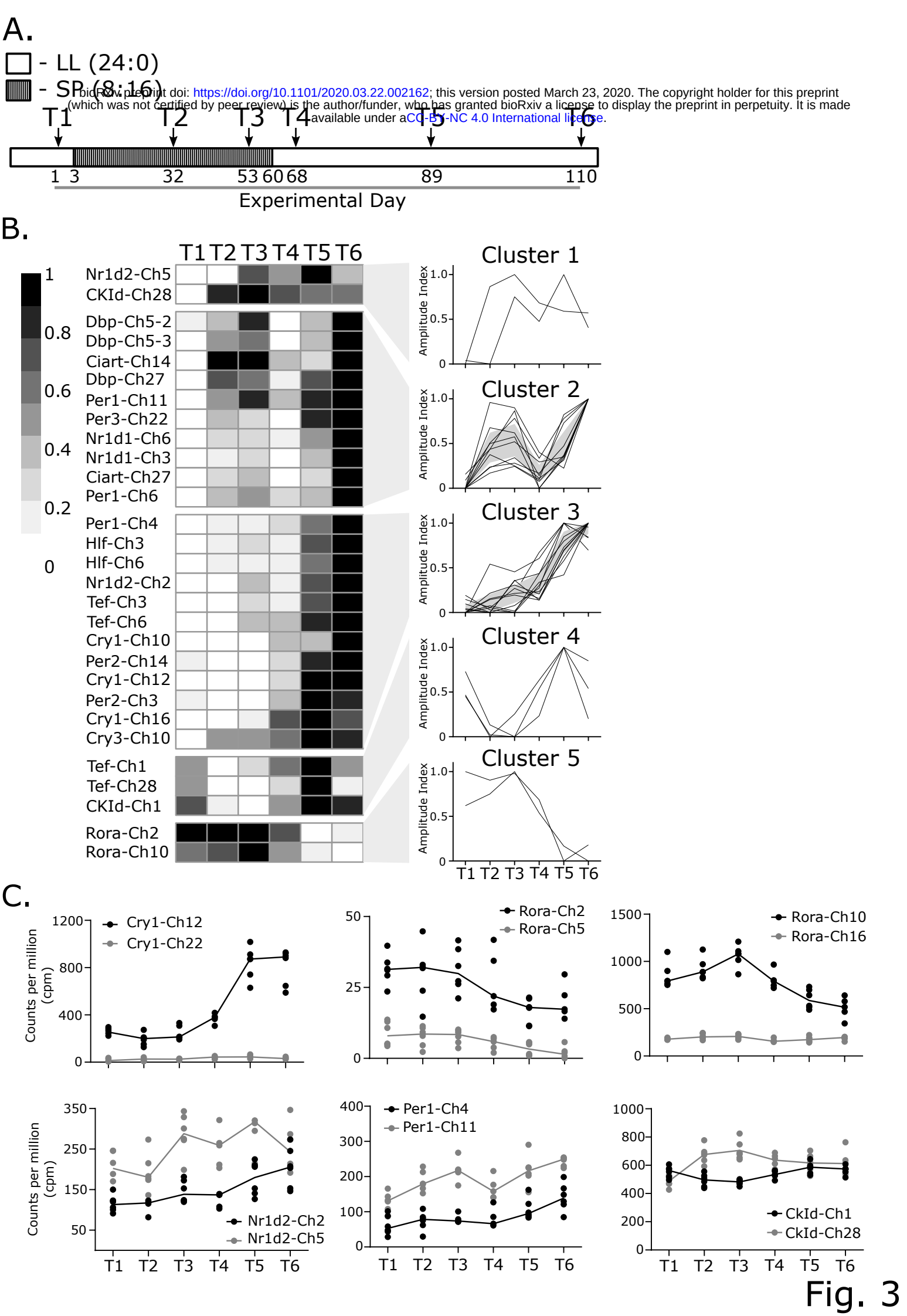
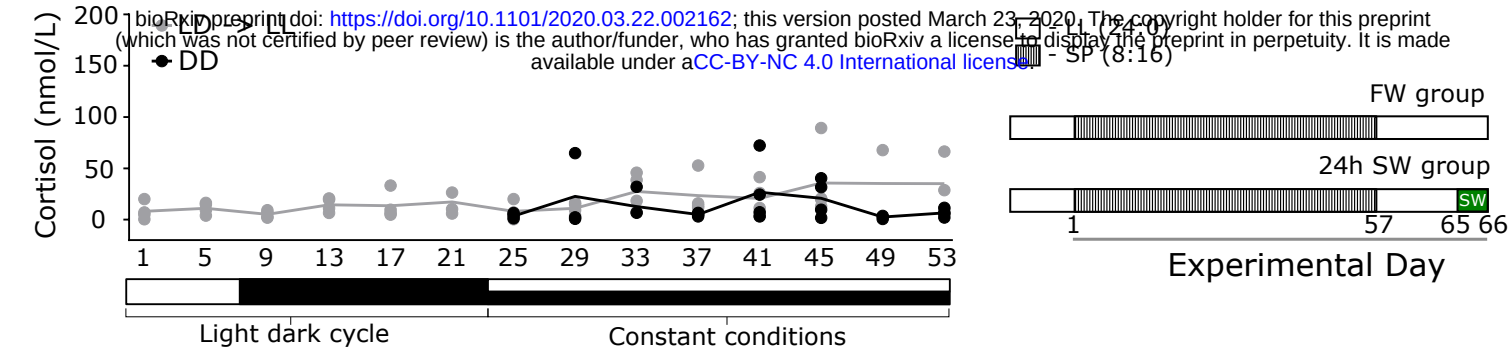
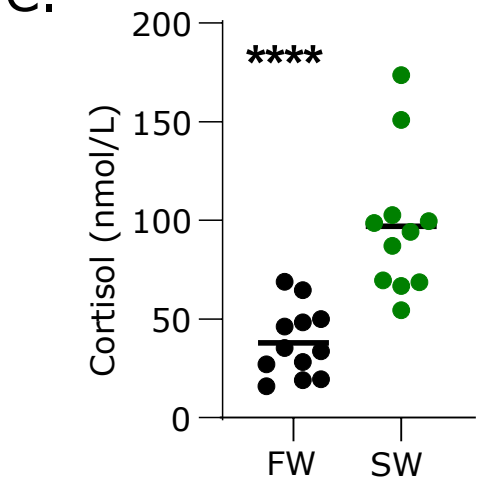
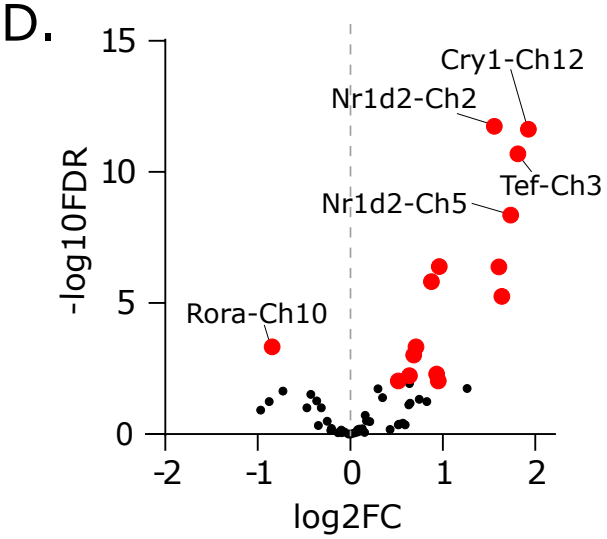
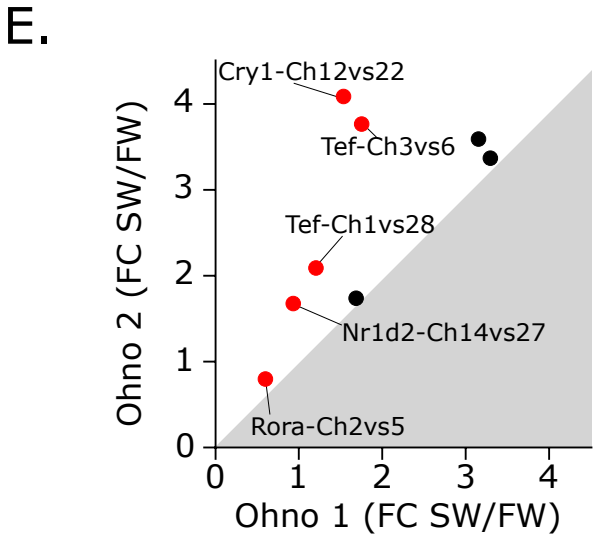


Fig. 3









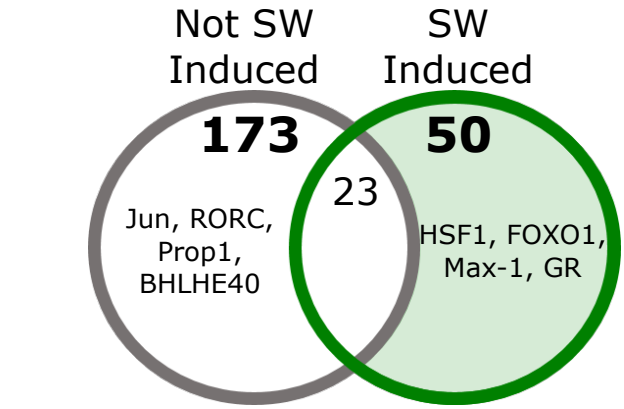


Fig. 4

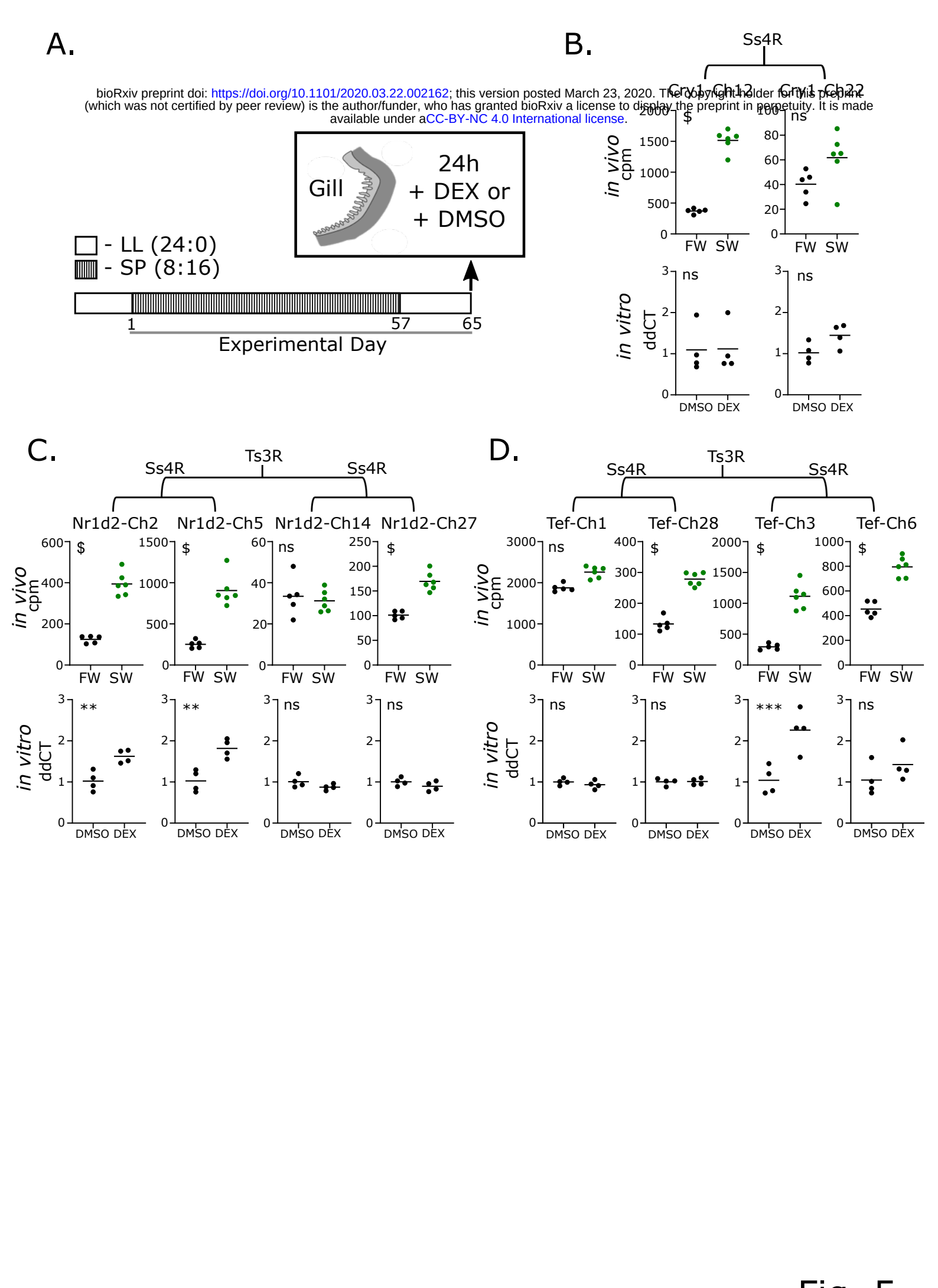


Fig. 5

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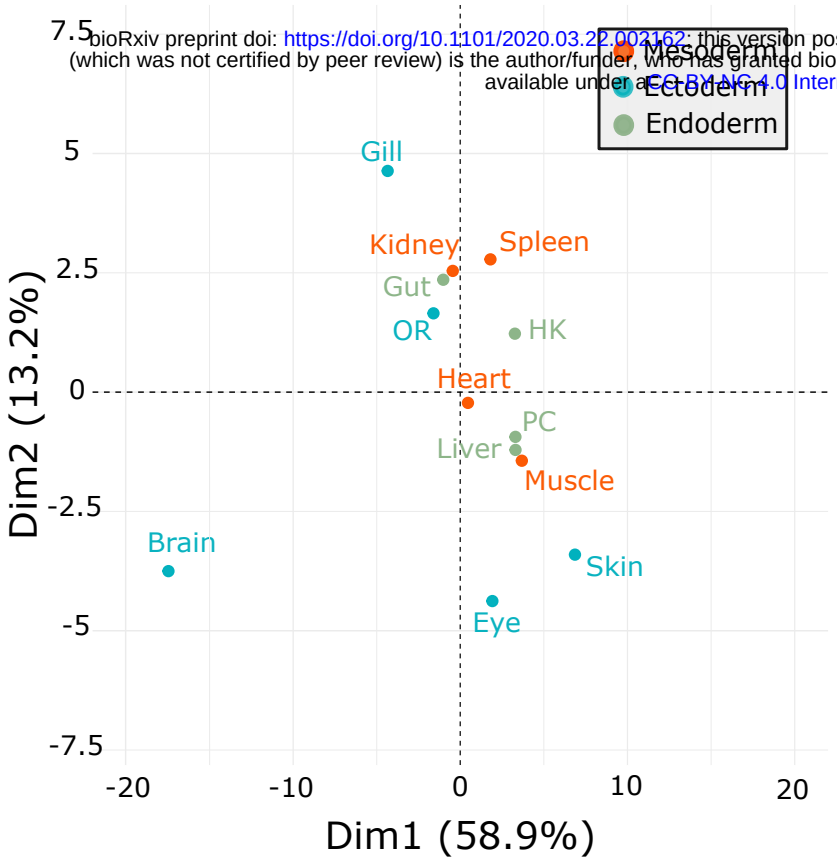

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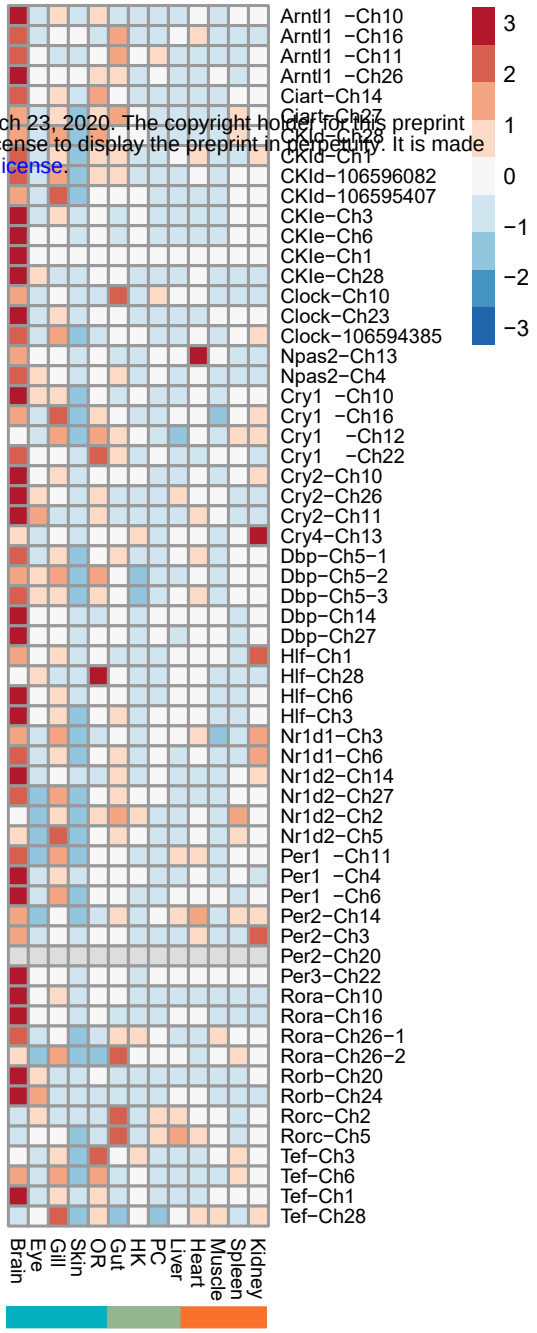
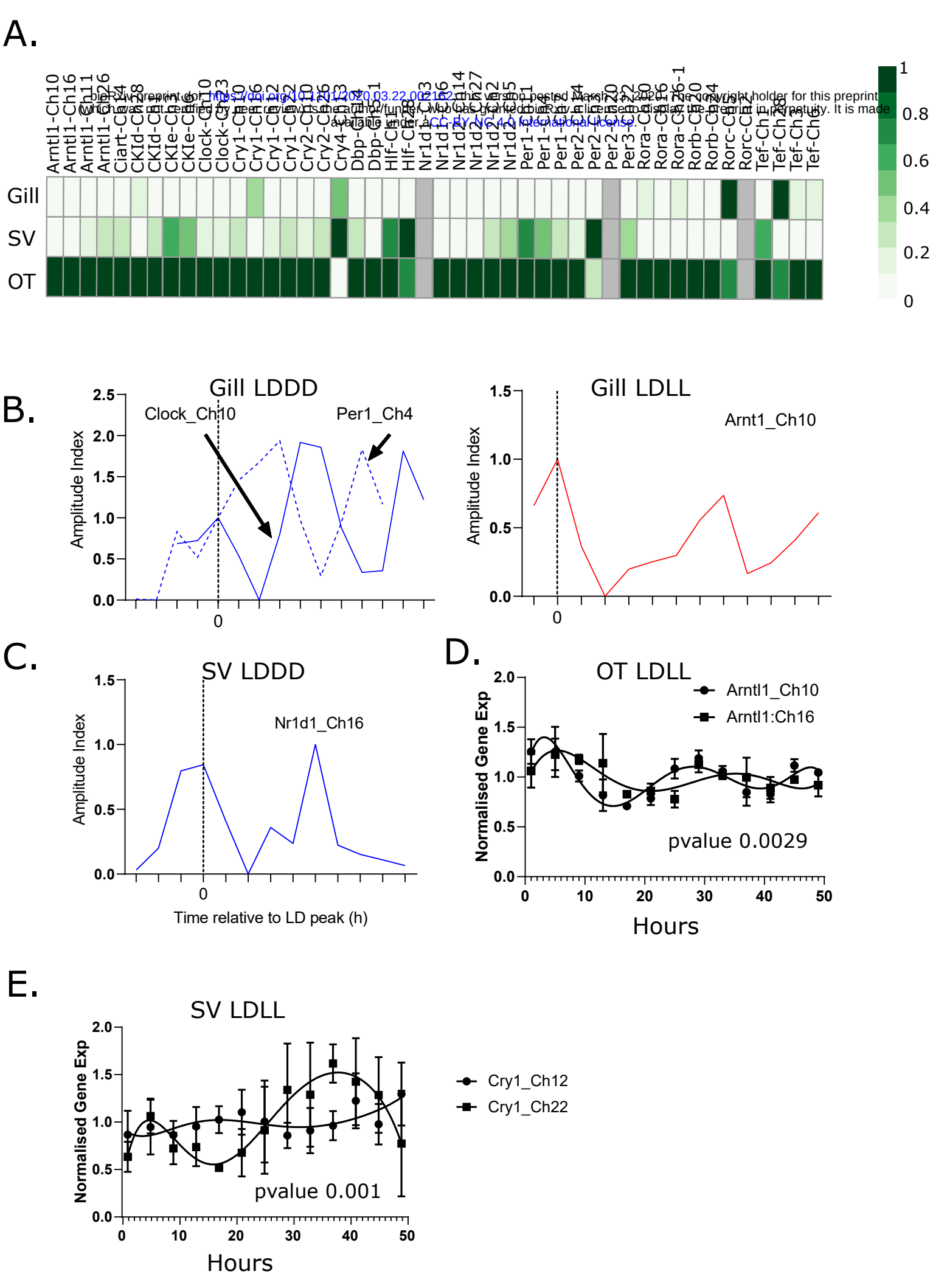
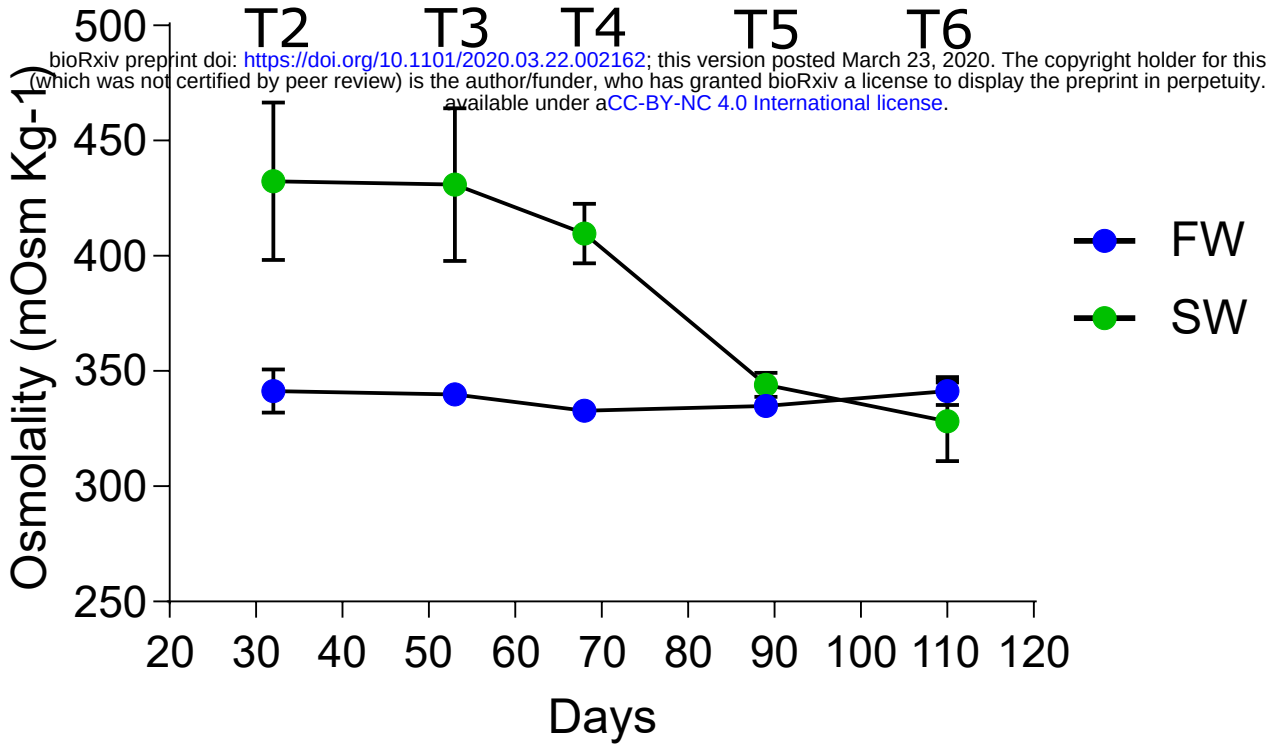


Supplementary Fig. 1

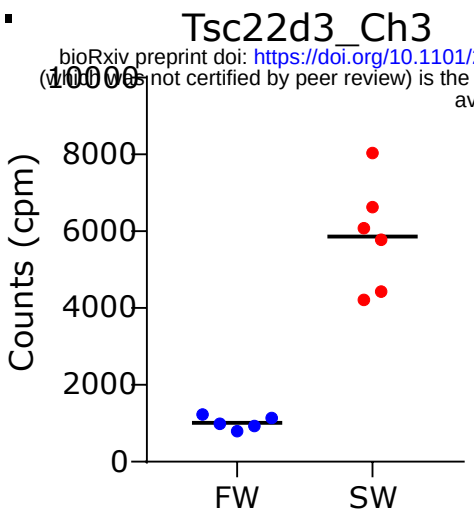


Supplementary Fig. 2



Supplementary Fig. 3

A.



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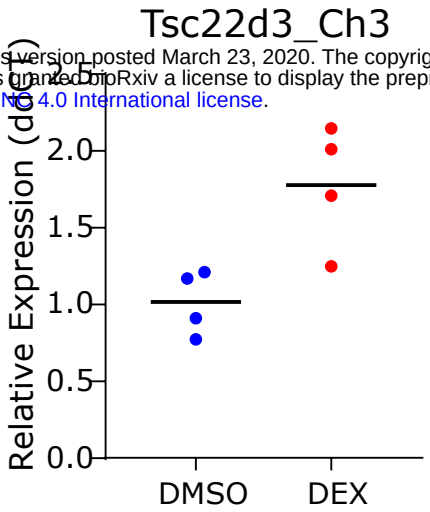

Supplementary Fig. 4