# Diversified regulation of circadian clock gene expression following whole genome duplication

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**Short title**: Circadian clock gene regulation and whole genome duplication

**Abstract**

Across taxa, circadian control of physiology and behaviour 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.

**Author Summary**

The generation of daily (circadian) rhythms in behaviour and physiology depends on the activities of networks of so-called clock genes. In vertebrates, these have become highly complex due to a process known as whole genome duplication, which has occurred repeatedly during evolutionary history, giving rise to additional copies of key elements of the clock gene network. It remains unclear whether this results in functional redundancy, or whether it has permitted new roles for clock genes to emerge. Here, based on studies in the Atlantic salmon, a species with an unusually large complement of clock genes, we present evidence in favour of the latter scenario. We observe marked tissue-specific, and developmentally-dependent differences in the expression patterns of duplicated copies of key clock genes, and we identify a subset of clock genes whose expression is associated with the physiological preparation to migrate to sea, but is independent of circadian regulation. Associated with this, cortisol secretion is uncoupled from circadian organisation, contrasting with the situation in mammals. Our results indicate that whole genome duplication has permitted clock genes to diversify into non-circadian functions, and raise interesting questions about the ubiquity of mammal-like coupling between circadian and endocrine function.

## 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

74 PERIOD proteins with specific mammalian CRYPTOCHROME proteins may affect photic
75 entrainment [13], but experimental evidence is lacking [14]. Tissue-specific functions of
76 mammalian CKIδ/ε ohnologues in regulation of PERIOD protein stability have been
77 suggested [15], and alterations in period (tau), amplitude and clock resetting behaviour have
78 been observed but clear distinctions of function between the ohnologues are lacking [8,16–
79 18].

80 Since the ancestral tetrapod WGD events, subsequent rounds of WGD have occurred
81 in several linages, creating highly complex genomes containing thousands of ohnologue
82 pairs. This is exemplified by the situation found in the salmonids, which underwent two
83 additional rounds of WGD compared to basal tetrapods, with the most recent, the salmonid-
84 specific fourth round of duplication (Ss4R) taking place some 100 MYA (Figure 1A) [19]. The
85 Ss4R event is a defining characteristic of the salmonid group and is theorized to have led to
86 the evolution of anadromy; an adaptation of freshwater salmonids to spend part of their life-
87 cycle at sea [20]. Genome-scale analysis in salmonids has begun to provide new insights into
88 the evolutionary significance of ohnologue divergence [21,22]. Impressively, even though
89 gene loss often occurs following duplication events (reviewed in:[23]), there remains a rich
90 complexity of clock genes in modern salmonids with the 18 clock genes (as defined in Figure
91 1B) in laboratory mice having 61 counterparts in Atlantic salmon (S1 Table, Figure 1C).

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

97 organization of metabolism between tissues.

98 **Results**

99 **Tissue-specific expression of clock gene ohnologues indicates regulatory divergence**

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

108 Following a WGD event the tetraploid genome incrementally returns to a diploid
109 state (rediploidization), this process occurs at different rates at different genetic loci [24]
110 (Figure 1C). Using published data [22] on sequence similarity in 1Mbp windows across
111 syntenic Ss4R duplicated regions we could classify the rediploidization times for our 21 Ss4R
112 pairs from early (approx. 87% sequence similarity) to late (>95% sequence similarity)(Figure
113 1C).

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

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

127

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

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

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

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

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

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

164

165 **Regulatory divergence of clock gene ohnologues within a tissue during a developmental**
166 **transition**

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

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

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

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

192

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

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

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

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

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

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

234 **Discussion**

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

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

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

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

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

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

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

296 **Materials and Methods:**

297 *Evolutionary analysis*

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

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

309 *Ethics statement*

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

314 *Animal husbandry*

315 Juvenile Atlantic salmon (*Salmo salar*, Linnaeus, 1758) of the Aquagene commercial strand
316 (Trondheim, Norway) were used in all experiments. Fish were held under constant light (LL;
317 >200 lux), at 10° from hatching onwards, and kept in 500 L tanks from first feeding. The fish

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

321 *Circadian experiment I*

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

328 *Circadian experiment II*

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

335 *Smoltification experiment and seawater tests*

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

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

344 *Sampling protocol*

345 During sampling fish were netted out and euthanized by an overdose of benzocaine
346 (150ppm). Weight and length were recorded, blood samples were collected from the caudal
347 vein in heparinized vacutainers, followed by decapitation and dissection. Blood samples
348 were centrifuged at 500 x g for 15 min to collect plasma. For RNAseq, one gill on the right
349 side (caudal view) was collected and placed in RNAlater (Sigma-Aldrich, St. Louis, Missouri,
350 USA). Samples were stored at 4°C for 24 hours, before being transferred to -80°C. For
351 nanostring samples were rapidly dissected and snap frozen on dry ice before being stored at
352 -80°C.

353 *In-vitro Gill Culture*

354 Juvenile Atlantic Salmon were prepared as in the smoltification experiment. Following
355 euthanasia whole gill arches were rapidly dissected, excess mucus was removed by careful
356 blotting onto tissue paper before the arches were transferred individually into 50 ml of pre-
357 prepared control or treatment media. The prepared media consisted of Leibovitz L-15
358 (Lonza) supplemented with non-essential amino acids (1%, 100x Lonza), sodium-pyruvate
359 (1%, 100x Lonza), 0.05 mg/ml gentamycin (Sigma) and 20% fetal bovine serum (FBS, sigma).
360 The experimental group was supplemented with 0.1M dexamethasone diluted in DMSO
361 (dimethyl-sulphoxide, Sigma) to a final concentration of 0.1µM. The control group contained
362 an equivalent concentration of DMSO (0.1%). The excised gill arches were incubated for 24h

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

365 *RNA extraction*

366 RNA extraction for RNAseq was performed using a TRIzol-based method (Invitrogen, Thermo
367 Fisher, Waltham, Massachusetts, USA), and in accordance with the manufacturers
368 recommendation. Resulting RNA concentrations and quality were checked using a NanoDrop
369 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was stored at -
370 80°C.

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

375 *Nanostring*

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

381 *Transcriptome sequencing and assembly*

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

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

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

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

396 *Analysis of differentially expressed genes*

397 Analysis of differential gene expression was performed with package edgeR (ver. 3.14.0)
398 using R (ver. 3.4.2) and RStudio (ver. 1.0.153). Prior to analysis of differential expression, the
399 raw counts were filtered, setting an expression level threshold of a minimum of one count
400 per million reads (cpm) in five or more libraries, resulting in a list of 33 951 expressed genes.
401 The counts were scaled by applying trimmed means of M-values (TMM) scaling. Exact tests
402 were then performed to find genes that were differentially expressed between FW-kept and
403 24-h SW challenged fish. An ANOVA-like test was performed to find genes that were
404 differentially expressed over T1-T6 FW time-points. The test results were filtered for a false
405 discovery rate (FDR) to be less than 0.01 to identify significantly differentially expressed
406 genes. Clustering analysis was performed using Pearson correlation.

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

409 *qPCR*

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

414 *Hormone Assays*

415 Cortisol ELISA assays were performed by Stockgrand (UK).

416 *Statistical analyses*

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

421 The R package JTK cycle was used to assess rhythmicity of transcripts under LD and constant
422 light or dark conditions [30]. For statistical comparison of gene expression between
423 ohnologue pairs in the circadian experiment, expression was normalized to group mean then
424 best fit sixth-order centered polynomial curves were generated by non-linear regression
425 analysis and shared characteristics tested with extra sum of squares F test (Graphpad Prism
426 8.0).

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577

578

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586 **Author contributions**

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

597

598 **Figure legends**

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

600 A. Phylogenetic tree highlighting WGD events and evolution of anadromy. B. The molecular
601 clock network. C. Circos plot showing all Ss4R clock ohnologues chromosomal position and
602 their partners indicated by the red line. Sequence similarity of the loci in a 1mb window is
603 shown on the circus plot; high similarity >95% (red), medium 95-90% (blue), low ~87%
604 (green). Sequence similarity on a genome wide level relates to approximate redipoidization
605 time of Atlantic salmon chromosomes (see grey box). D. Differential regulation of Ss4R pairs
606 in a panel of 11 different tissues. For each Ss4R pair, in each tissue, an expression divergence
607 index (EDI) index was calculated (EDI= abs(log2[Gene1/Gene2)]. The graph shows a violin
608 plot of the distribution of EDI values across all tissues. The vertical black bar represents the
609 median value. Approximate rediploidization time of the loci each pair is found on is
610 represented by a colour: red – late, blue – mid, green – early.

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

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

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

621 A. Photoperiodic gene expression experiment design. LL – constant light, SP – short
622 photoperiod. Time-points for sampling indicated by arrows. B. Expression heatmap of

623 significantly photoperiodic (FDR<0.01) clock genes in the gill. Significant genes cluster into
624 five distinct expression patterns. Individual profiles are represented. Shaded area shows 95%
625 confidence limit where applicable. C. RNA-seq counts per million profiles for divergently
626 regulated ohnologue pairs.

627 **Figure 4. Stress response implicates glucocorticoid receptor signaling in clock ohnologue**
628 **regulation.** A. Diel and circadian profile of plasma cortisol. Time axis is given in continuous
629 hours since the start of the experiment, therefore 1 to 21 represent zeitgeber time (ZT) and
630 25 onwards is equivalent to circadian time (CT)1 to CT29. Due to the sampling protocol time-
631 points 1 and 5 are replotted from time-points 25 and 29. B. Sea-water stress experiment
632 design. LL – constant light, SP – short photoperiod, SW- sea-water challenge. C. Plasma
633 cortisol concentration in blood plasma in sea-water stress experiment. D. Sea-water stress
634 regulation of clock genes. Significantly regulated transcripts (FDR<0.01) are shown in red. FC
635 – fold change. E. Differential sea-water stress regulation of ohnolgue pairs. Significantly
636 different pairs (Analysis of genes where one or both genes are significantly regulated by
637 seawater (FDR<0.01), then submitted to a two-way ANOVA, with sea-water regulation and
638 interaction, p<0.05) are shown in red. F. Predicted transcription factor promoter binding
639 analysis. Both sea-water induced and not-induced gene cohorts were analysed. 50 motifs
640 were specific to the sea-water induced cohort. The top four motifs in each group are
641 displayed.

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

643 A. Dexamethasone-dependent transcript regulation experimental design. After photoperiod
644 manipulation whole gill arches were removed and treated for 24h with glucocorticoid
645 receptor agonist dexamethasone (DEX), or dimethlysulphoxide (DMSO; control). B.

646 Comparative regulation of Cry1-Ch12/22 taken from *in vivo* sea-water stress experiment and
647 *in vitro* dexamethasone treatment. C. As in B for Nr1d2-Ch2/5 & Nr1d2-Ch14/27. D. As in B
648 for Tef-Ch1/28 & Tef-Ch3/6. Braches indicate phylogenetic relationship between gene sets.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

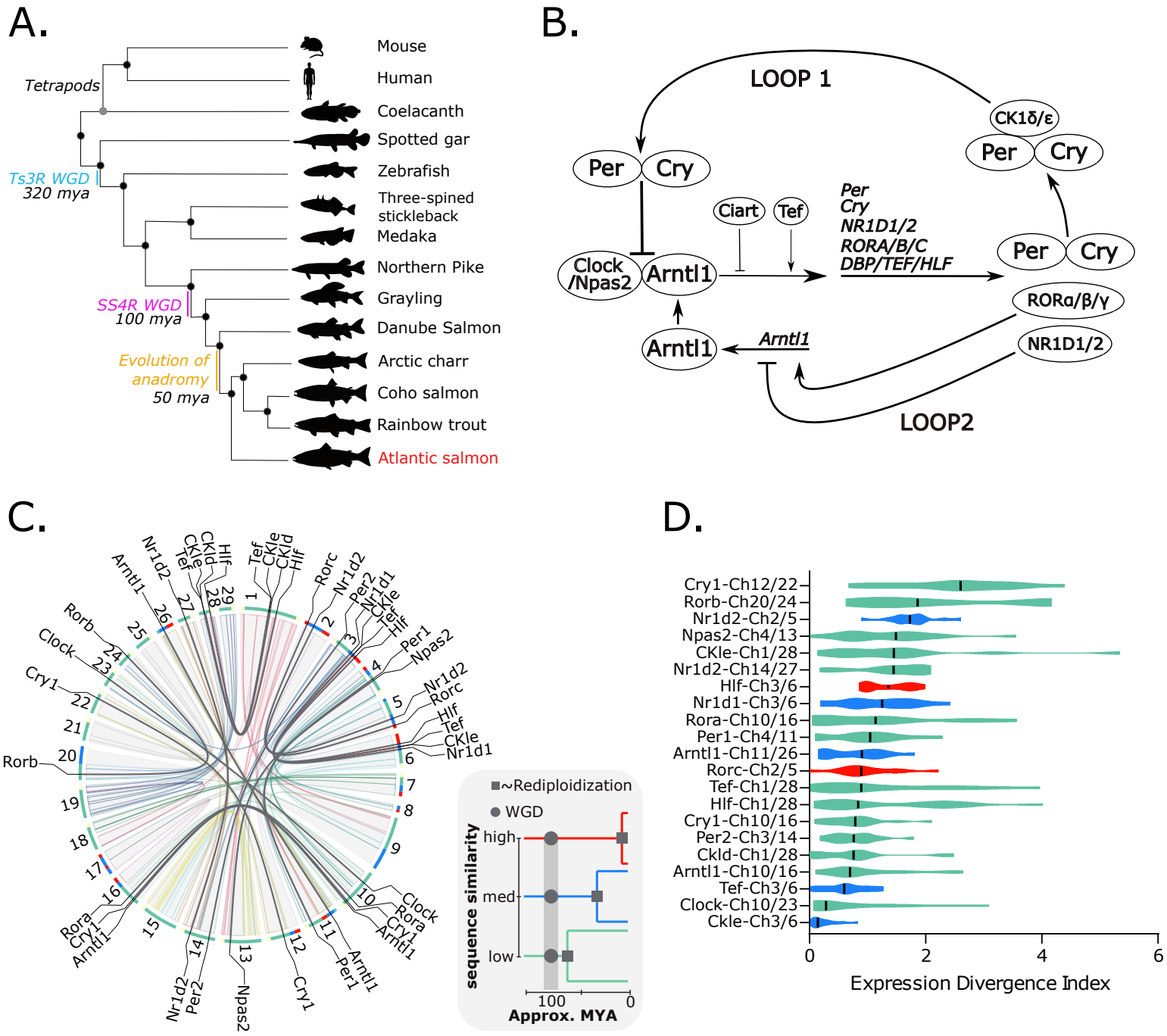

A.
Mouse
Human
Tetrapods
Coelacanth
Spotted gar
Ts3R WGD
320 mya
Zebrafish
Three-spined stickleback
Medaka
Northern Pike
SS4R WGD
100 mya
Grayling
Danube Salmon
Evolution of anadromy
50 mya
Arctic charr
Coho salmon
Rainbow trout
Atlantic salmon
B.
LOOP 1
CK1δ/ε
Per
Cry
Per
Cry
Ciart
Tef
Per
Cry
NR1D1/2
RORA/B/C
DBP/TEF/HLF
Clock /Npas2
Arntl1
Per
Cry
RORα/β/γ
Arntl1
Arntl1
NR1D1/2
LOOP2
C.
Rorb
Clock
Cry1
Nr1d2
Arntl1
Tef
CKle
CKld
Hlf
Rorc
Per2
Per1
Npas2
Nr1d1
Rora
~Rediploidization
WGD
sequence similarity
high
med
low
100
0
Approx. MYA
D.
Cry1-Ch12/22
Rorb-Ch20/24
Nr1d2-Ch2/5
Npas2-Ch4/13
CKle-Ch1/28
Nr1d2-Ch14/27
Hlf-Ch3/6
Nr1d1-Ch3/6
Rora-Ch10/16
Per1-Ch4/11
Arntl1-Ch11/26
Rorc-Ch2/5
Tef-Ch1/28
Hlf-Ch1/28
Cry1-Ch10/16
Per2-Ch3/14
Ckld-Ch1/28
Arntl1-Ch10/16
Tef-Ch3/6
Clock-Ch10/23
Ckle-Ch3/6
0
2
4
6
Expression Divergence Index

Fig. 1

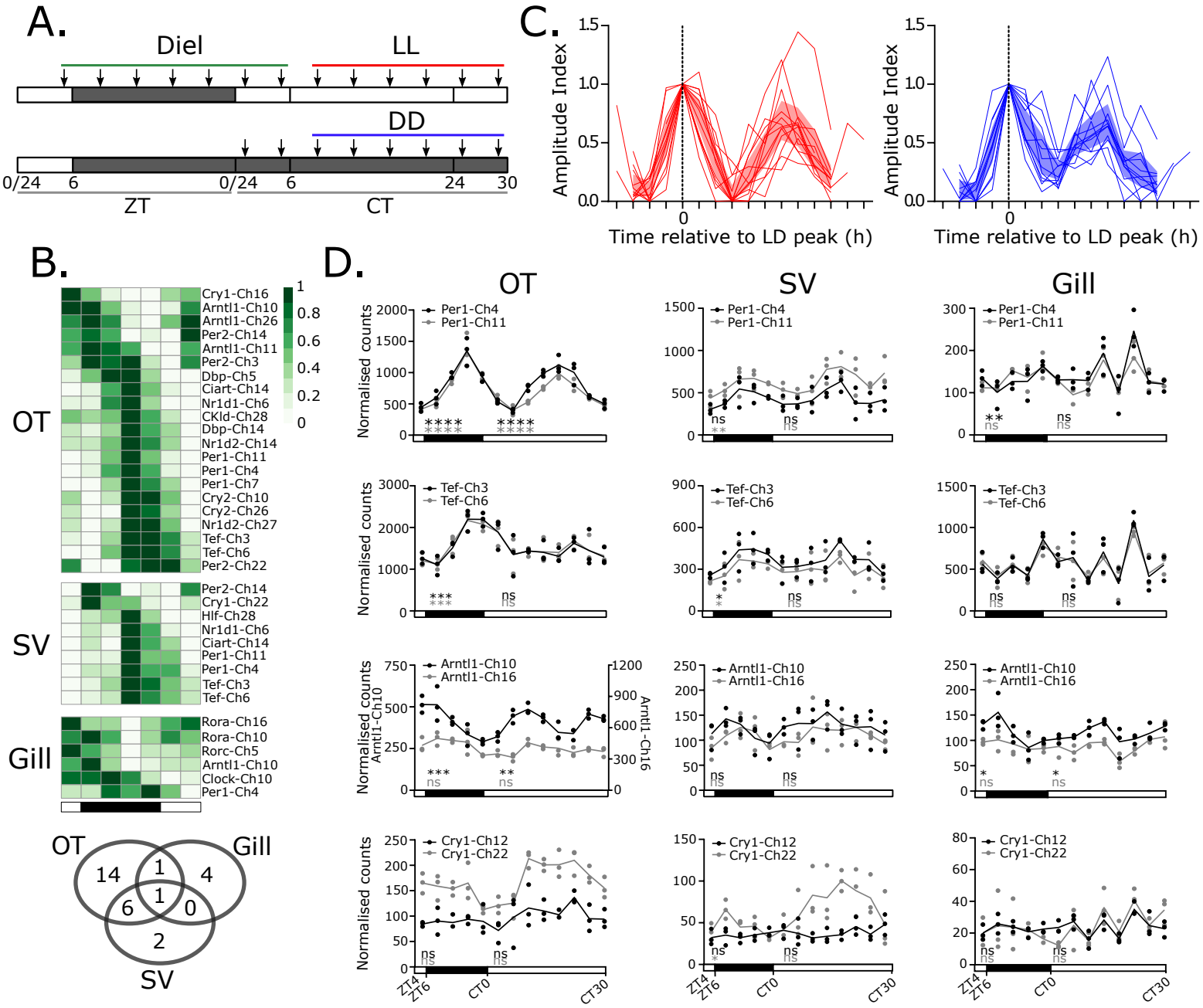

A.
Diel
LL
DD
0/24
6
0/24
6
24
30
ZT
CT
C.
Amplitude Index
Time relative to LD peak (h)
B.
OT
SV
Gill
Cry1-Ch16
Arntl1-Ch10
Arntl1-Ch26
Per2-Ch14
Arntl1-Ch11
Per2-Ch3
Dbp-Ch5
Ciart-Ch14
Nr1d1-Ch6
CKld-Ch28
Dbp-Ch14
Nr1d2-Ch14
Per1-Ch11
Per1-Ch4
Per1-Ch7
Cry2-Ch10
Cry2-Ch26
Nr1d2-Ch27
Tef-Ch3
Tef-Ch6
Per2-Ch22
Per2-Ch14
Cry1-Ch22
Hlf-Ch28
Nr1d1-Ch6
Ciart-Ch14
Per1-Ch11
Per1-Ch4
Tef-Ch3
Tef-Ch6
Rora-Ch16
Rora-Ch10
Rorc-Ch5
Arntl1-Ch10
Clock-Ch10
Per1-Ch4
14
1
4
1
6
0
2
D.
OT
SV
Gill
Normalised counts
Per1-Ch4
Per1-Ch11
Tef-Ch3
Tef-Ch6
Arntl1-Ch10
Arntl1-Ch16
Cry1-Ch12
Cry1-Ch22
ZT4
ZT6
CT0
CT30

Fig. 2

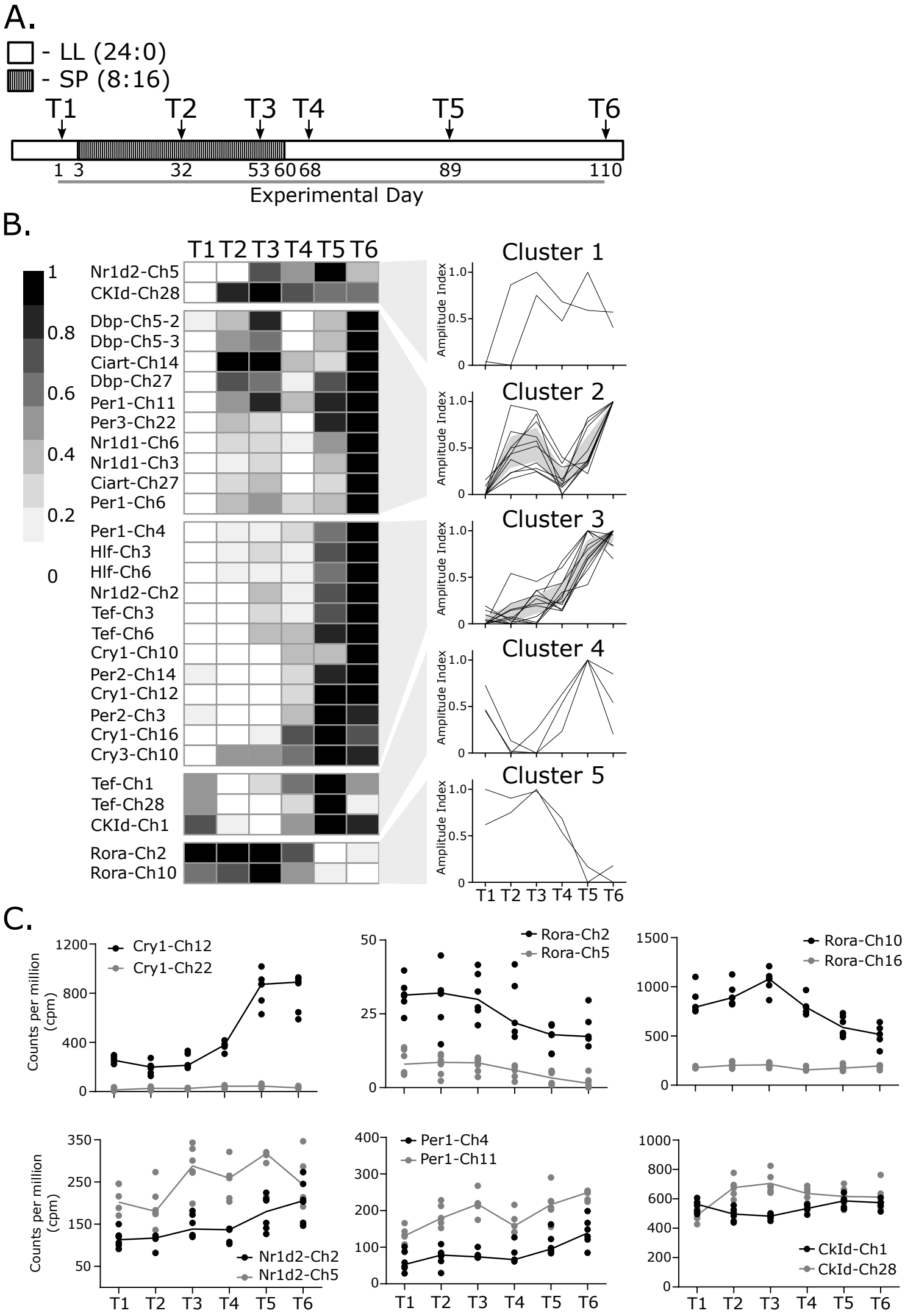

A.
- LL (24:0)
- SP (8:16)
T1
T2
T3
T4
T5
T6
1 3
32
53 60 68
89
110
Experimental Day
B.
T1 T2 T3 T4 T5 T6
1
0.8
0.6
0.4
0.2
0
Nr1d2-Ch5
CKId-Ch28
Dbp-Ch5-2
Dbp-Ch5-3
Ciart-Ch14
Dbp-Ch27
Per1-Ch11
Per3-Ch22
Nr1d1-Ch6
Nr1d1-Ch3
Ciart-Ch27
Per1-Ch6
Per1-Ch4
Hlf-Ch3
Hlf-Ch6
Nr1d2-Ch2
Tef-Ch3
Tef-Ch6
Cry1-Ch10
Per2-Ch14
Cry1-Ch12
Per2-Ch3
Cry1-Ch16
Cry3-Ch10
Tef-Ch1
Tef-Ch28
CKId-Ch1
Rora-Ch2
Rora-Ch10
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Amplitude Index
1.0
0.5
0
T1 T2 T3 T4 T5 T6
C.
Cry1-Ch12
Cry1-Ch22
Counts per million (cpm)
1200
800
400
0
Rora-Ch2
Rora-Ch5
50
25
0
Rora-Ch10
Rora-Ch16
1500
1000
500
0
Nr1d2-Ch2
Nr1d2-Ch5
350
250
150
50
Per1-Ch4
Per1-Ch11
400
300
200
100
0
CkId-Ch1
CkId-Ch28
1000
800
600
400
200
0
T1 T2 T3 T4 T5 T6

Fig. 3

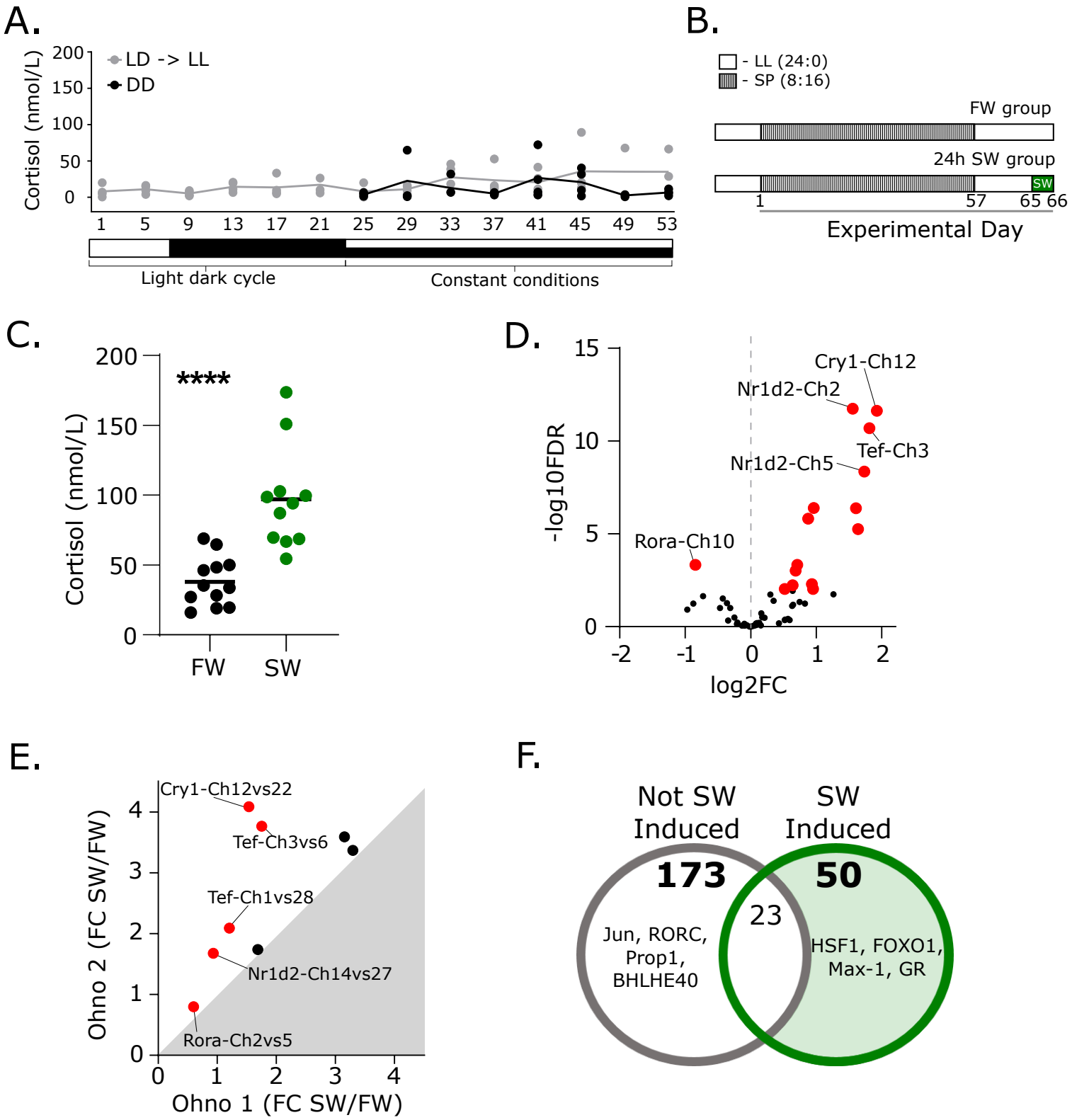

A.
Cortisol (nmol/L)
LD -> LL
DD
Light dark cycle
Constant conditions
B.
- LL (24:0)
- SP (8:16)
FW group
24h SW group
SW
Experimental Day
C.
****
Cortisol (nmol/L)
FW
SW
D.
-log10FDR
log2FC
Cry1-Ch12
Nr1d2-Ch2
Tef-Ch3
Nr1d2-Ch5
Rora-Ch10
E.
Ohno 2 (FC SW/FW)
Ohno 1 (FC SW/FW)
Cry1-Ch12vs22
Tef-Ch3vs6
Tef-Ch1vs28
Nr1d2-Ch14vs27
Rora-Ch2vs5
F.
Not SW Induced
SW Induced
173
50
23
Jun, RORC, Prop1, BHLHE40
HSF1, FOXO1, Max-1, GR

Fig. 4

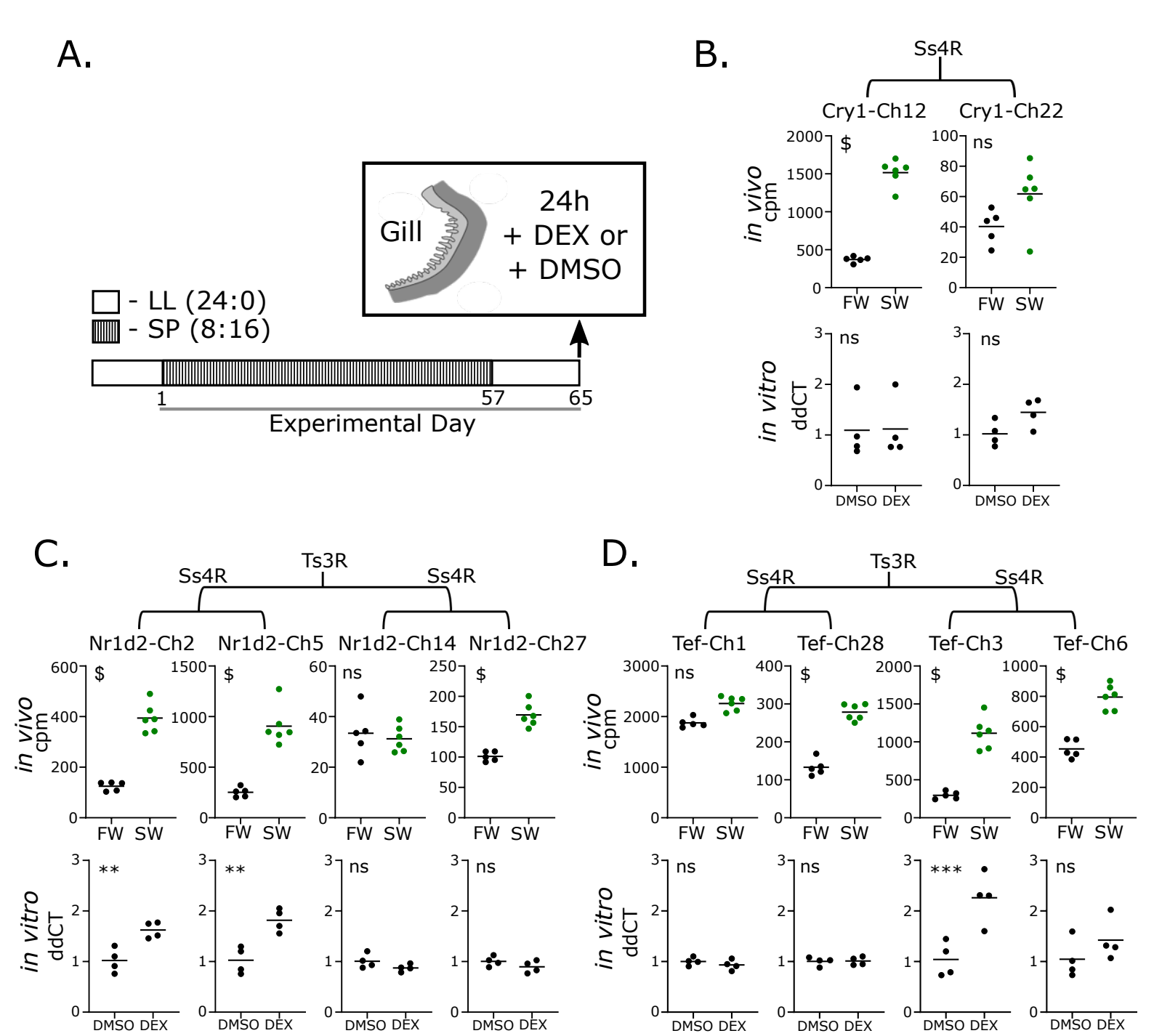
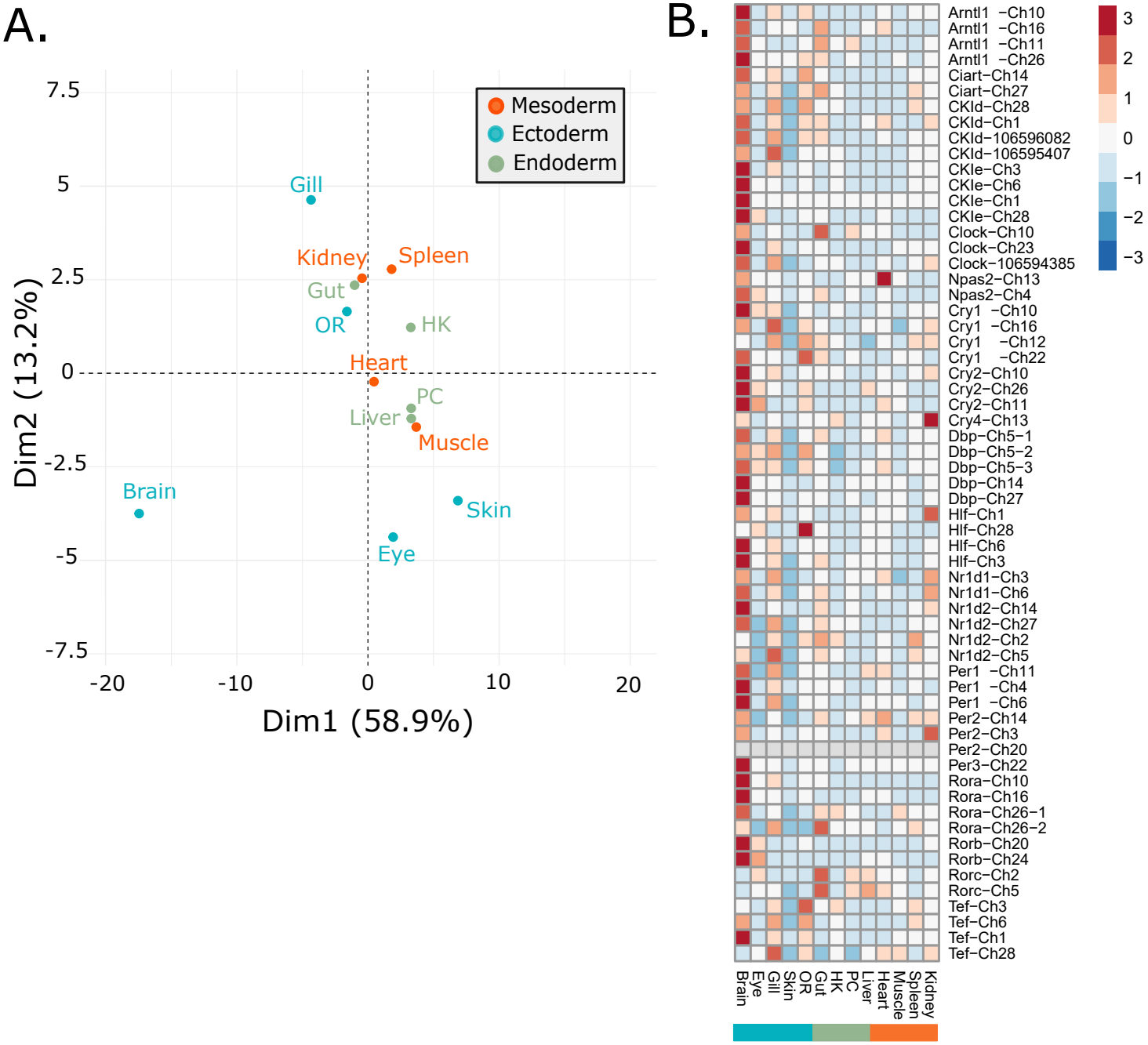
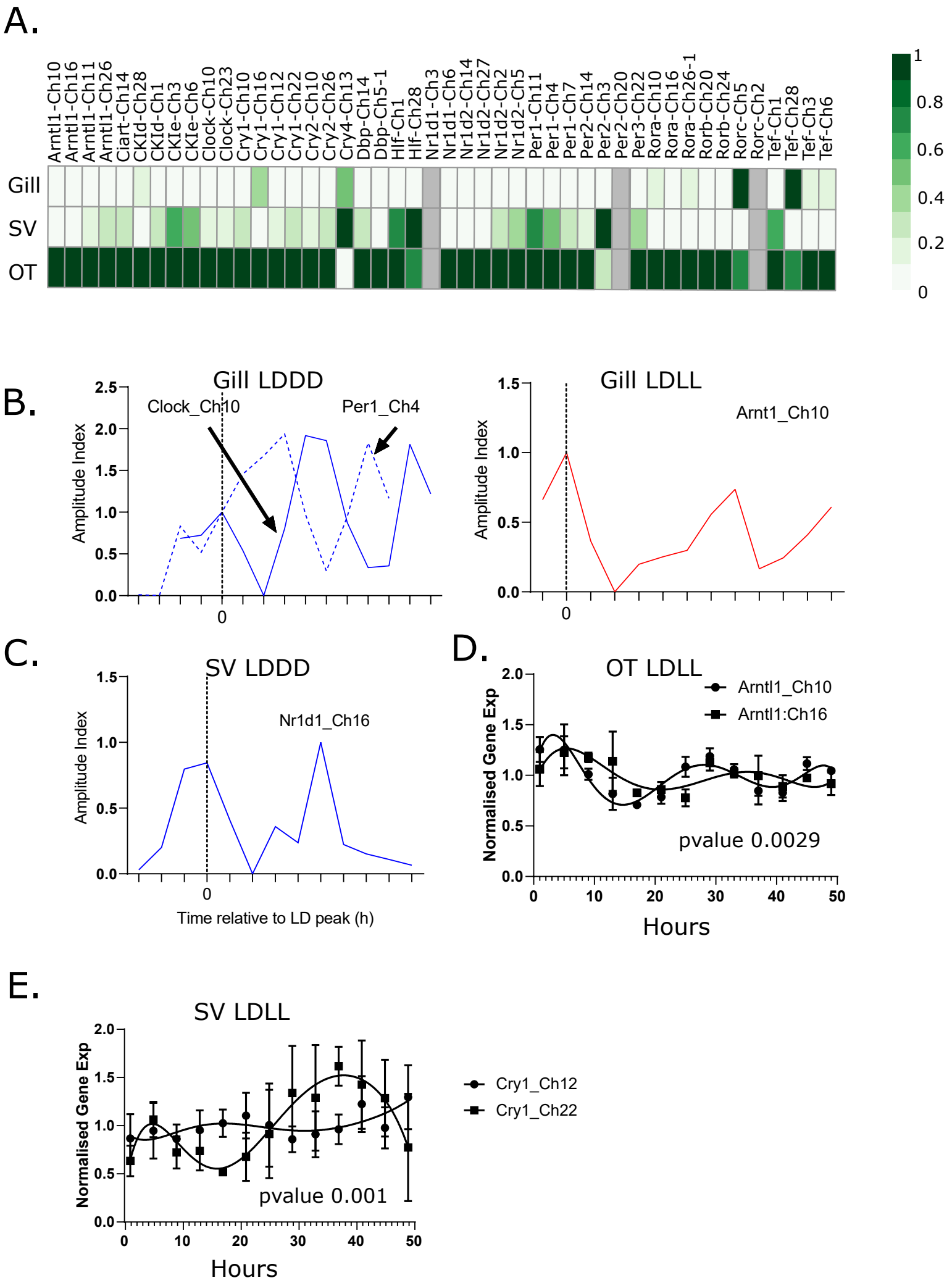

A.
Gill
24h
+ DEX or
+ DMSO
- LL (24:0)
- SP (8:16)
1
57
65
Experimental Day
B.
Ss4R
Cry1-Ch12
Cry1-Ch22
in vivo
cpm
in vitro
ddCT
FW
SW
DMSO
DEX
C.
Ss4R
Ts3R
Ss4R
Nr1d2-Ch2
Nr1d2-Ch5
Nr1d2-Ch14
Nr1d2-Ch27
D.
Ss4R
Ts3R
Ss4R
Tef-Ch1
Tef-Ch28
Tef-Ch3
Tef-Ch6

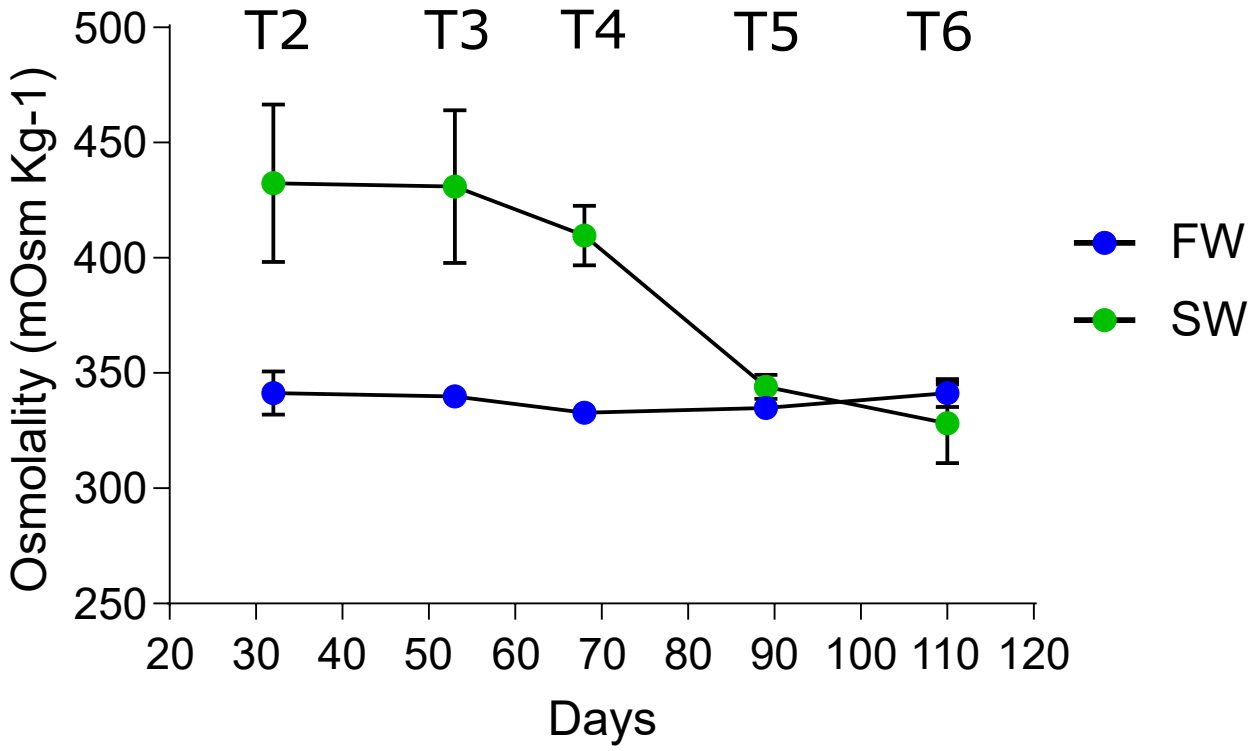
Fig. 5



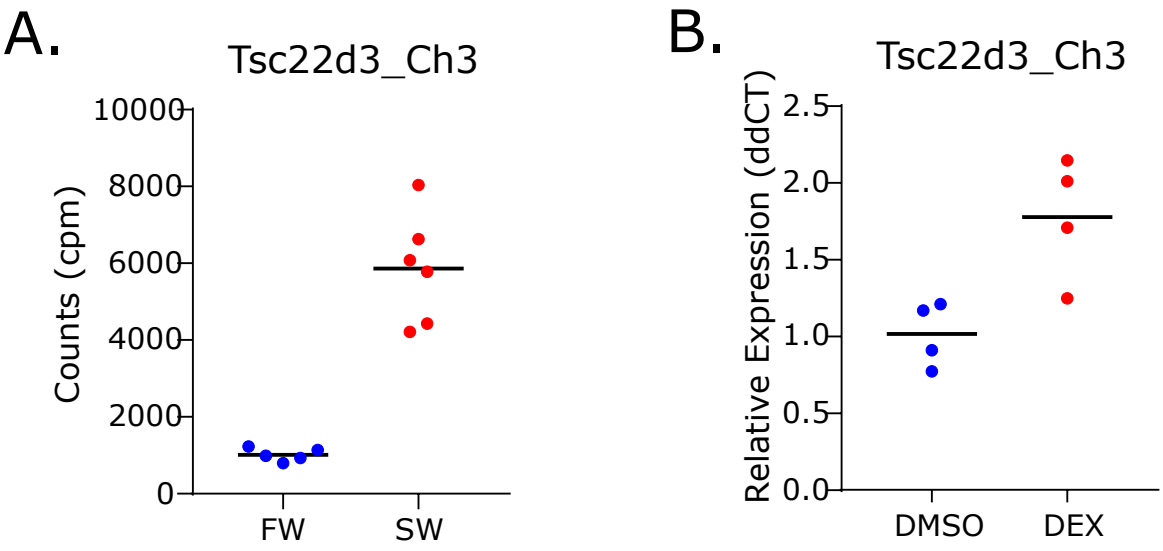

Supplementary Fig. 1




Supplementary Fig. 2




Supplementary Fig. 3




Supplementary Fig. 4