

1 Ontogeny of the circadian system during embryogenesis in rainbow trout
2 (*Oncorhynchus mykiss*) and the effect of prolonged exposure to continuous
3 illumination on daily rhythms of *per1*, *clock*, and *aanat2* expression
4

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17 **Abstract**

18 It is widely held that the development of the circadian system during embryogenesis is
19 important for future survival of an organism. Work in teleosts has been, to date, limited to
20 zebrafish, which provides little insight into the diversity of this system within such a large
21 vertebrate class. In this study, we analyzed the diel expression of *per1*, *clock*, and *aanat2* in
22 unfertilized rainbow trout oocytes and embryos maintained under either a 12:12 light:dark
23 (LD) cycle or continuous illumination (LL) from fertilization. 24-h profiles in expression
24 were measured at fertilization as well as 8, 21 42, and 57 days postfertilization (dpf). Both
25 *per1* and *clock* were expressed in unfertilized oocytes and all embryonic stages, while *aanat2*
26 expression was only measureable from 8 dpf. A reduction in both *per1* and *clock* mean
27 expression level between unfertilized oocytes/0-1dpf embryos and 8-9dpf embryos was
28 suggestive of a transition from maternal RNA to endogenous mRNA expression. While
29 *aanat2* expression was not clearly associated with photic conditions, photoperiod treatment
30 did alter the expression of *per1* and *clock* expression/rhythmicity from as early as 8 dpf
31 (*per1*), which could suggest the presence and functionality of an as yet unidentified
32 “photoreceptor”. As a whole, this work demonstrates that clock systems are present and
33 functional during embryonic development in rainbow trout. Further studies of their
34 expression and regulation will help understand how the environment interacts with
35 embryonic development in the species. (Author correspondence: andrew.davie@stir.ac.uk)

36

37 Keywords: *per1*, *clock*, *aanat2*, rainbow trout, embryo, circadian rhythms

38

39 **Introduction**

40 The clock gene system has been shown to play a multitude of roles across the animal
41 phylum. In essence, this transcription-translation auto-regulatory feedback loop provides a
42 self-sustained timekeeping system, either directly linked to the clock mechanisms themselves or
43 via secondary messengers to maintain a wide range of rhythmic processes from cellular
44 cycling to behavioral rhythmicity in synchrony with the surrounding environment
45 (Pittendrigh, 1993). A basic question about the body clock is when does the system start to
46 cycle, and how does light affect its development. As reviewed by Vallone et al. (2007), the
47 zebrafish has proven to be a useful subject in this respect, exhibiting a clear cascade of
48 rhythmic activity becoming evident as the fish develop. Work by Dekens & Whitmore (2008)
49 has shown that a molecular clock analogous to the zebrafish “peripheral clock” is in fact
50 present and light-entrainable in early embryonic cells prior to the development of any known
51 specialized light-receptor structures, and this clock starts to endogenously cycle within 12 h
52 postfertilization. The same authors proposed that as the embryo develops so the cellular
53 clocks are passed on during differentiation and ultimately mature into the hierarchical
54 circadian system.

55 Traditionally, it has been viewed that a key milestone in the initial development of the
56 circadian system is differentiation of the pineal gland accompanied by the pineal-specific
57 expression of the rate limiting enzyme of melatonin synthesis, namely, serotonin N-acetyl
58 transferase 2 (*aanat2*) (Gothilf et al., 1999). In the Zebrafish, pineal-specific *aanat2*
59 expression is evident within the first 24 h posthatch, while endogenous rhythms in *aanat2*
60 expression can be seen from the second day of embryogenesis (Gothilf et al., 1999).
61 Furthermore, in zebrafish, as with many other teleost species, it appears that the pineal
62 develops photoreceptors before the retina does, and thus, the pineal has been described as the
63 first light sensitive element of the circadian axis to form (Osthholm et al., 1987, 1988).

64 However, it should be noted that irrespective of the timing of photoreceptor development,
65 neural connections from both the retina and pineal to the brain are formed later in the
66 ontogeny, and in actual fact they do so within the same time frame (Ostholm et al. 1988).

67 Clock-gene ontogenetic studies in fish have been performed on zebrafish embryos,
68 which are short lived, taking 24 hours to develop most of their key anatomical structures
69 before hatching and emerging as fully developed larvae at around 72 h (Kimmel et al. 1995).
70 Clearly, the identification and description of developmental landmarks for embryogenesis in
71 the context of biological rhythms is restricted to a short window of only 3 light-dark (LD)
72 cycles before the larvae hatches. On the contrary, rainbow trout (*Oncorhynchus mykiss*)
73 embryos are among the largest of all teleosts and takes up to two months to develop and
74 hatch (temperature dependent) (Ballard, 1973a). This provides a greater range to explore the
75 development of the circadian axis in parallel to other developmental milestones and thus
76 could provide insight into the diversity of the clock-generation system within teleosts. In the
77 current work, we have investigated the ontogeny of diel rhythms in the only two clock genes
78 described in trout to date (*clock* and *per1*) along with *aanat2* expression in rainbow trout
79 embryos and alevins reared under either LD cycles or continuous (LL) lighting conditions.

80

81 **Materials and Methods**

82 *Animals and experimental procedures*

83 Broodstock rainbow trout previously reared under a simulated natural photo-thermal
84 cycle at the Niall Bromage Freshwater Research Facilities (Institute of Aquaculture, Stirling,
85 Scotland, 56°02'35"N/4°00'26"W) were stripped at maturity (2♂:3♀). Eggs were fertilized 4
86 h after sunrise or zeitgeber time 04:00 (ZT 04:00) using a standard dry fertilization technique,
87 with the eggs then being split equally ($n = 1000/\text{treatment}$) and laid down in a monolayer in
88 an egg incubation tray, which was thereafter subjected to either continuous illumination (LL)
89 or an alternating 12 h: 12 h LD photoperiod with lights switching on at ZT 00:00 and off at
90 ZT 12:00. Eggs remained in the trays for the remainder of the experiment and were inspected
91 and any dead eggs were removed every second day by siphoning. At 40 days post fertilization
92 (dpf), which equated to 280 °C-days, eggs were physically “shocked” to remove unfertile
93 eggs (Piper et al., 1982). During incubation, water was continuously aerated, and water
94 temperature averaged 7.1 ± 0.1 °C. At fertilization, 60, 150, 300, and 420 °C-days (8, 21, 42,
95 and 57 dpf respectively), embryos were collected every 4 h over a consecutive 24-h period. In
96 addition, during the first 24-h sample, unfertilized oocytes (submerged in ovarian fluid),
97 maintained at the same constant temperature and LD photoperiod, were sampled at the same
98 times as the fertilized embryos. During sampling, embryos/alevins ($n = 6$) were individually
99 snap frozen over liquid nitrogen vapor and then transferred to -70°C storage for later
100 processing. All procedures were performed in accordance with the Animals (Scientific
101 Procedures) Act, UK, 1986, under the approval of the local ethical review board (Institute of
102 Aquaculture, Ethics board), and in accordance with the ethical standards of the journal
103 (Portaluppi et al., 2010).

104

105 *RNA extraction and cDNA synthesis*

106 Embryos were thawed in 1 ml TRIzol® Reagent (Invitrogen, UK)/100 mg of tissue
107 over ice before homogenization using a IKA Ultra-Turrax disperser and RNA extracted in
108 accordance with guidelines (Invitrogen, UK). RNA pellets were reconstituted in MilliQ
109 water. RNA quality checks were performed with a ND-1000 Nanodrop spectrophotometer
110 (Labtech Int., East Sussex, UK). Furthermore, 1 µg of total RNA was analyzed on a 1%
111 agarose denaturing RNA gel electrophoresis, showing ribosomal RNA of good quality.
112 cDNA was synthesized using 1 µg of DNase-treated (DNA-Free, Ambion, UK) total RNA,
113 25 µM of a 3:1 blend of random hexamers and anchored oligo dT₂₀, 500 µM dNTPs, and 200
114 units of SuperScript™ II RT reverse transcriptase with provided buffer (all from Invitrogen,
115 Paisley, UK) in a final volume of 20 µl. Reactions were incubated for 60 min at 42°C
116 followed by 70°C for 15 min. All samples were then stored at -70°C prior to qPCR analysis.

117

118 *Synthesis of clock, per1, and aanat2 cDNA*

119 Real-time quantitative polymerase chain reaction (qPCR) assays were used to
120 quantify the expression of *clock*, *per1*, and *aanat2*. Accession numbers of the published
121 sequences used to design primers are shown in Table 1. All primers were designed using
122 PrimerSelect Ver. 6.1 program (DNASTAR, www.dnastar.com).

123 Partial cDNA sequences for each target were generated by PCR using 0.5 µM of
124 primers (clkF, clkR; per1F, per1R; aanat2F, aanat2R), one eighth (2.5 µl) of the cDNA
125 synthesis reaction, Klear Taq polymerase with supplied buffer (Kbiosciences, Beverly, UK),
126 and 2.25 mM MgCl₂ in a final volume of 20 µl. The following hotstart PCR strategy was
127 used: 15 min 95°C, 29 cycles 95°C 30 s, X°C 30 s, 72°C 1 min/kb of product size, where
128 annealing temperature X = 58, 60, 60, and 59°C for *clock*, *per1*, and *aanat2*, respectively. All
129 primer pairs generated a single PCR product that was cloned into a 2.1 plasmid (Topo TA,
130 Invitrogen, Paisley, UK) and sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA).

131 The identities of the cloned PCR products were then verified (100% overlapping) using
132 BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

133

134 *Sequence analysis*

135 Sequencing was performed using a Beckman 8800 autosequencer. Lasergene
136 SEQman software (DNASTAR, www.dnastar.com) was used to edit and assemble DNA
137 sequences. ClustalW (Thompson et al., 2000) was used to generate multiple alignments of
138 deduced protein sequences. MEGA version 4 was used (Tamura et al., 2007) to deduce and
139 bootstrap phylogenetic trees using the neighbor joining method (Saitou & Nei, 1987).

140

141 *Quantitative PCR*

142 All cDNA for qPCR was synthesized using SuperscriptTM II reverse transcriptase and
143 supplied buffer components (Invitrogen, Paisley, UK) and an oligo-dT primer (as described
144 above). qPCR primers for *clock*, *per1*, *aanat2*, and the reference genes *β -actin*, and
145 *Elongation factor α* (Table 1) were used at 0.5 μ M, with one-fortieth of the total cDNA
146 synthesis reaction and SYBR-green qPCR mix (ABgene, Epsom, UK) in a total volume of 20
147 μ l. The thermal cycling protocol run in a Techne Quantica thermocycler (Techne, Quantica,
148 Cambridge, UK) consisted of 15 min at 95°C followed by 45 cycles of 95°C for 15 s, X°C for
149 15 s, and 72°C for 30 s followed by a temperature ramp from 70 to 90°C for melt-curve
150 analysis. The annealing temperature (X) was changed as follows: 56°C for *clock*, 66°C for
151 *per1*, 64°C for *aanat2*, 62°C for *β -Actin*, and 61°C for *Elongation factor α* . Melt-curve
152 analysis verified the primer sets for each qPCR assay generated one single product and no
153 primer-dimer artefacts. In addition, a random sample of each qPCR product was than
154 sequenced to confirm its identity and was found to be 100% identical to its relative sequence.
155 Quantification was achieved by a parallel set of reactions containing standards consisting of

156 serial dilution of spectrophotometrically determined, linearized plasmid-containing partial
157 trout cDNA sequences generated as described above. All samples were run in triplicate
158 together with non-template controls, standards, and internal controls to correct expression
159 levels between plates. Results from the fertilization and 60 °C·day samples were normalized
160 with a correction factor generated from the geometric mean of *β-Actin* and *Elongation factor*
161 *α*. While results from 150, 300, and 420 °C·day samples were normalized to *β-Actin* only due
162 to financial limitations.

163

164 *Statistical analyses*

165 Statistical analysis was performed using the InStat Statistical package (V 3.01;
166 GraphPad Software Inc., San Diego, USA). Significant variation in expression within a given
167 24-h period under a specific lighting treatment was tested by one-way analysis of variance
168 (ANOVA). Data were first assessed for normality by the Kolmogorov-Smirnov test and for
169 homogeneity of variances by Bartlett's test, with all data being log transformed to ensure
170 equal variance. Post-hoc comparisons were applied using Tukey's test. A significance of *p*
171 <0.05 was applied to all statistical tests performed. All data are presented as mean ± SEM
172 (standard error of the mean).

173 To model the rhythmic nature of the expression data, a non-linear regression also
174 referred to as cosinor analysis was used to fit, by the method of least squares, the cosine
175 function:

$$176 \quad Y = A + B \times \cos(C \times X - D)$$

177 Where Y is the copy no./μg of totRNA, A is the baseline copy no./μg of totRNA, B is the
178 waveform amplitude (one-half of the peak to trough variation determined by the cosine
179 approximation), C is the frequency multiplier (set to the fix period of 24 h), and D is the
180 acrophase (peak time of the cosine approximation). Cosinor analysis was performed using

181 chronobiological software (“El Temps,” by Prof. Díez-Noguera, University of Barcelona,
182 Spain). Where a significant ($p < 0.05$) 24-h cosine function could be fitted, the expression
183 pattern was described as being rhythmic.

184

185 **Results**

186 *Embryo development*

187 Embryos developed normally: eyeing was complete by 28 dpf (200 °C·days) in both
188 treatments, while hatching began at 48 dpf (340 °C·days) under both treatments and was
189 completed by 51 dpf (362 °C·days) under the LD treatment and by 53 dpf (379 °C·days)
190 under LL conditions.

191

192 *Clock & Per1*

193 Both *clock* and *per1* were actively expressed in rainbow trout oocytes and embryos
194 from fertilization through to hatched alevins. For *per1*, mean expression levels decreased
195 significantly between unfertilized/0-1 dpf and 8-9 dpf, then increased back to original levels
196 at 21-22 dpf, and, thereafter, remained the same or increased further in both photoperiod
197 treatments (Table 2). *Per1* was measureable in unfertilized oocytes and exhibited significant
198 variations in expression over the 24-h period; however, this was not rhythmic, i.e., did not fit
199 a cosinor waveform (Figure 1, Table 3). In the fertilized embryos exposed to LD conditions,
200 there was no significant variation in expression in *per1* expression at 0-1 dpf; however,
201 thereafter there was significant variations in expression levels during each sample window
202 from 8-9 dpf to 57-58dpf (Figure 2). Cosinor analysis revealed rhythmic expression in these
203 conditions with the expression peaking (i.e., acrophase of the rhythm) just before lights-on in
204 all cases (Table 3). Under LL conditions, there was no significant variation in expression over
205 the 24-h period at 0-1dpf and 57-58 dpf (Figure 2). Furthermore, while there were significant
206 variations in expression across the 24-h period at 42-43 dpf, this expression did not fit to a
207 cosinor cycle. Expression was rhythmic at 8-9 dpf and 21-22 dpf; however, the acrophase of
208 the expression was significantly different in both cases, being ZT 20:14 ± 1:12 and ZT 22:55
209 ± 0:44, respectively (Table 3). As with *per1*, *clock* mean expression levels showed a

210 significant reduction at 8-9 dpf compared to unfertilized oocytes/0-1dpf embryos; however,
211 levels thereafter exceeded 0-1 dpf levels for the remainder of the trial (Table 2). Equally,
212 *clock* was measureable in unfertilized eggs, and while this did vary over the subsequent 24-h
213 period, this pattern was not rhythmic (Figure 1, Table 3). In the embryos exposed to LD
214 conditions, *clock* expression showed significant variations in expression levels over the 24-h
215 period at 0-1, 42-43, and 57-58 dpf (Figure 3). Cosinor analysis revealed expression in these
216 conditions was rhythmic and peaked just prior to lights-on, between ZT 23:06 and 00:08
217 (Table 3). Under LL conditions, there was significant variations in diel expression of *clock* at
218 all times, with the exception of 21-22 dpf; however, this expression was not rhythmic
219 (Cosinor analysis $p > 0.05$) (Table 3).

220

221 *aanat2*

222 The expression of *aanat2* was not detectable using the current method in unfertilized
223 oocytes (data not shown), nor in 0-1 dpf embryos (Figure 4). Thereafter, *aanat2* expression
224 was detectable with mean expression levels rising significantly from 8-9 dpf to their highest
225 levels at 21-22 dpf before reducing back to a level comparable to 8-9dpf at the end of the
226 study (Table 2). At 8-9 dpf, embryos exposed to LD conditions displayed significant
227 differences in expression levels over the 24-h period; however, this was not clearly associated
228 with lighting treatment and did not fit to a cosinor cycle (Table 3). At 21-22 dpf, significant
229 elevations in expression were evident within the dark phase of the LD treatment (ZT 14:00 &
230 18:00), while significant increases in expression were evident in LL treatment at ZT 10:00 &
231 14:00. Thereafter, under LD, expression peaked at the end of the dark phase (42-43 dpf) or in
232 the early morning (57-58 dpf), while under the LL conditions no clear patterns in expression
233 were evident. The expression patterns was rhythmic at 21dpf under both LD and LL

234 conditions and at 42 dpf under LD conditions; at all other times, the expression was not
235 rhythmic (cosinor analysis $p > 0.05$).

236

237 **Discussion**

238 The present study reveals that *per1* and *clock* are actively expressed throughout the
239 embryonic development of rainbow trout and that their expression is influenced by light
240 conditions prior to the reported development of known pineal or retinal photoreceptive
241 structures. Furthermore, expression is present in the early phases of development and even
242 within unfertilized eggs, which suggests that clock-gene expression in these early stages may
243 be from maternal origin.

244 To date there has been no diel expression measurement of either *per1* or *clock* in
245 rainbow trout, existing work being restricted to localization studies with limited temporal
246 profiling (Brierley et al., 1999; Mazurais et al., 2000). Since this original work, our
247 understanding of clock genes, and variety of period homologues in teleosts in particular, has
248 increased (Wang, 2008). Thus, an *in silico* analysis of the registered rainbow trout *per1*
249 fragment was performed to confirm its identity. The phylogenetic analysis revealed that it is
250 nested within the *per1b* (previously referred to as *per4*) node of teleost period genes (data not
251 shown), in accordance with the classification of Wang (2008). The translated 411bp partial
252 cDNA fragment possessed between 74 and 84% identity with all registered teleost PER1b
253 protein sequences. The expression pattern of trout *per1*, peaking close to lights-on/sunrise
254 under LD conditions, is a typical pattern of expression for this gene in fish, including
255 Zebrafish (Tamai et al., 2005), Golden rabbitfish (*Siganus guttatus*) (Park et al., 2007),
256 goldfish (*Carassius auratus*) (Velarde et al., 2009), and European seabass (*Dicentrarchus*
257 *labrax*) (Sánchez et al., 2010). In the present study, no significant variations in *per1*
258 expression were measurable in fertilized eggs under LD or LL conditions in the 24-h
259 following fertilization. Thereafter, a rhythm which peaked just prior to lights-on was evident
260 under LD conditions, while no consistent rhythm remained under LL. Within teleosts the
261 entrainment of clocks during early embryonic development has only been studied in

262 zebrafish, with the early work being reviewed by Vallone et al. (2007). Hurd & Cahill (2002)
263 previously demonstrated that while robust locomotor activity rhythms are present in zebrafish
264 from about 5 days post fertilization, these behavioral rhythms are dependent on the
265 entrainment of an endogenous pacemaker mechanism that starts during the second day of
266 embryogenesis and matures by the fourth day. The original hypothesis that a functional,
267 rhythmic, pacemaker was transferred via maternal mRNA (Delaunay, 2000) has been
268 superseded by the work of Dekens & Whitmore (2008). These authors demonstrated that the
269 embryonic clock starts autonomously within 12 h postfertilization and is marked by the
270 increase in *per1b* at the end of the first day of development and followed thereafter by robust
271 cycling peaking just after sunrise under LD conditions. However when zebrafish embryos
272 were maintained under DD conditions following the initial rise in *per1b* at 12 h
273 postfertilization, expression remained constant thereafter. In the present study, when rainbow
274 trout embryos were maintained under LL conditions, rhythmic expression was apparent at
275 both 8-9 dpf and 21-22 dpf; however, thereafter this rhythm was lost. DD conditions were not
276 tested in the present study due to technical limitations. These results are suggestive in the first
277 case of a pacemaker that over time is becoming desynchronized through lack of entrainment
278 leading to arrhythmia. In the Zebrafish PAC2 fibroblast cell line, Vallone et al. (2004)
279 reported that *per1b* expression was suppressed by constant illumination and became
280 arrhythmic within 72 h. The difference in time frames to reach arrhythmia suggests that the
281 *in-vivo* situation in trout is more complex. It is possible that the arrhythmia could be within
282 an individual's pacemaker network, or it could be due to sampling individuals cycling at
283 different endogenous times, or in fact both situations could prevail, but this could not be
284 resolved with the methods of our studies here and, hence, requires further investigation. In
285 addition, our results may also indicate the presence and functionality of a 'photoreceptor'
286 (deep brain photoreceptor?) that can entrain rhythmic expression prior to the development of

287 any such known structure in salmonids (Ostholm, 1987), which demands further
288 investigation.

289 *Clock* mRNA expression in the 24 h postfertilization was rhythmic and peaked just
290 prior to lights-on under LD conditions; however, this rhythm was not evident at 8-9 or 21-22
291 dpf, but then did return again at 42-43 and 57-58 dpf. Under LL conditions, there was no
292 significant rhythm in expression. Reports of *clock* expression in salmonids are limited.
293 Mazurais et al. (2000) was unable to detect any variation in *clock* expression in the brain of
294 juvenile rainbow trout between 2 and 12 h post lights-on using *in-situ* hybridization, while
295 Davie et al. (2009) demonstrated a daylength-dependent expression in Atlantic salmon
296 (*Salmo salar*) parr brains where *clock* was rhythmic only under short-day photoperiods and
297 peaked in the middle of the dark phase. In zebrafish, rhythmic *clock* expression has been
298 described to peak in the late-photophase to mid-scotophase, depending on the tissue studied
299 (Cermakian et al., 2000; Pando et al., 2001; Whitmore et al., 1998); however, during
300 embryogenesis, Dekens & Whitmore (2008) reported that *clock* expression is arrhythmic. It is
301 possible that the current results support the hypothesis of Dekens & Whitmore (2008) that
302 core circadian clock genes are differentially regulated during the embryonic development in
303 teleosts, though more research is needed to confirm this.

304 For both *perl* and *clock*, it is evident that mean expression levels were comparable
305 between unfertilized oocytes and just fertilized embryos. Furthermore, these levels
306 significantly declined from 0-1 to 8-9 dpf before recovering and exceeding initial levels at the
307 later stages of development. Similar transitions in clock-gene expression levels have been
308 reported in zebrafish, in which it has been described as a progression from maternal mRNA
309 that breaks down by the midblastula stage (~4 hpf) before endogenous zygotic expression
310 begins *de novo* and increases as the embryo develops (Dekens & Whitmore, 2008; Ziv &
311 Gothilf, 2006). In rainbow trout, it has been suggested that embryo genomic transcription

312 activation (EGTA) begins from about 4 dpf at 8.5°C (Ignatieva & Rott, 1970); however, the
313 onset of EGTA appears to be transcript specific (Li et al., 2007; Yang, 1999). Thus, in the
314 current study, it is likely that the 8-9 dpf profiles are a reflection of the endogenous
315 embryonic pacemaker expression that then increases as the embryo develops. By 8 dpf, the
316 trout embryos are in the middle of gastrulation (development window = 40-70 °C.days
317 according to Ballard (1973a)), which aligns with the developmental stage when zebrafish
318 endogenous clocks initiate (Dekens & Whitmore, 2008). As such, it appears that the salmonid
319 embryo may prove to be an interesting subject to examine more closely the ontogeny of
320 embryonic clock evolution and specifically the transition from maternal to endogenous clock
321 cycling due to its protracted embryonic development.

322 The expression of *aanat2* in zebrafish embryos has been proposed to act as a marker
323 for pineal photoreceptor development and clock functioning (Gothilf et al., 1999), and it was
324 for this reason it was included in the present work. While *aanat2* expression was not
325 detectable in unfertilized oocytes nor newly fertilized embryos, its expression was
326 measureable from 8-9 dpf onwards, by which time the rainbow trout embryos are in the early
327 stages of gastrulation (Ballard, 1973a). Gothilf et al. (1999) reported *aanat2* expression in
328 zebrafish embryos from 22 h postfertilization localized to the midline of the roof of the
329 diencephalon. Development is clearly faster in this species, and as such zebrafish embryos of
330 this age would be at the 26-somite stage (Kimmel et al., 1995), where the sculpturing of the
331 brain rudiment is already quite advanced. The possibility that expression at this early
332 developmental stage was of maternal RNA origin as proposed in sole (*Solea senegalensis*)
333 (Isorna et al., 2009) has to be rejected, as it was not detectable at the earlier timepoint. Thus,
334 it would be interesting to localize this early *aanat2* expression in rainbow trout to see if it
335 maps closely to regions destined to form the brain as cellular fate is already determined by
336 this stage of gastrulation in trout (Ballard, 1973b). In the subsequent cycles, there appeared

337 no consistent or robust expression pattern. Mean expression levels were at their highest at 21-
338 22 dpf due primarily to extremely high levels of expression at ZT 14:00 and 18:00 under the
339 LD photoperiod and ZT 10:00 and 14:00 under the LL photoperiod. In both cases, these
340 peaks represented a >70 fold increase compared to basal expression levels. Thereafter,
341 expression levels showed a marked decline and dark-specific expression in the LD treatment
342 was not apparent. The lack of a consistent rhythmic pattern in *aanat2* expression in
343 comparison to *per1*, under the LD conditions, could be an indication that *aanat2* expression is
344 not regulated by clock genes in rainbow trout, as previously suggested by Begay et al. (1998).
345 The authors reported that *aanat2* expression, in the rainbow trout pineal at least, is arrhythmic
346 and not responsive to light treatment. The disconnection between clock rhythms and
347 melatonin synthesis in salmonids has been reported on a number of occasions (e.g., Bolliet et
348 al., 1996; Iigo et al., 2007); however, reasons for the disconnection are lacking. In the closely
349 related Chum salmon (*Oncorhynchus keta*), Shi et al. (2004) reported no differences between
350 day and night *aanat2* expression levels in the brain (samples included pineal gland) until after
351 hatching; however, they did observe significant day-night alterations in ocular *aanat2*
352 expression during late embryogenesis and up to 2 days posthatching. It must be
353 acknowledged that the whole embryo approach adopted in the current study negates detection
354 of such tissue-specific expression profiles. However, in general, it appears that *aanat2*
355 expression during embryogenesis in teleosts does not follow a consistent pattern. In zebrafish,
356 robust cyclic expression is measureable from the second day of embryogenesis (Gothilf et al.,
357 1999). In sole, rhythmic expression was not apparent during embryo development, nor during
358 larval metamorphosis. However, it was present during a brief window in larvae following
359 hatching (Isorna et al., 2009), while no significant day night cycling could be measured in
360 turbot embryos (Vuilleumier et al., 2007). Clearly, the functional significance of *aanat2*
361 expression in relation to embryonic development needs to be studied in a species-specific

362 manner. In the case of rainbow trout, it would be important to localize expression and
363 subsequently explore the regulatory mechanisms of *aanat2* expression to dissociate the
364 endogenous, i.e., clock genes (Appelbaum & Gothilf, 2006), versus exogenous, i.e.,
365 photoperiod and temperature (Begay et al., 1998) drivers of this mechanism.

366 Studies performed to date on the development, entrainment, and functional
367 significance of clock systems during embryonic development, in a wide range of species, has
368 suggested that while the system may not be essential for normal embryogenesis, its presence
369 and normal development during this phase is essential for later survival (Vallone et al., 2007).
370 In teleosts, work in this field has focused on zebrafish due to its inherent advantages as a
371 model species; however, the embryonic phase is short due to the rapid development which
372 restricts the opportunity to investigate the clock system. Current results in rainbow trout draw
373 clear parallels with reports in zebrafish (Dekens & Whitmore, 2008), and it is, therefore,
374 suggested that trout embryos could be a productive model to study more closely the ontogeny
375 of clock mechanisms and key processes, like the transition from maternal RNA signalling to
376 endogenous expression. Overall, this work suggests that the traditional view of salmonid
377 embryo development and, in particular, the onset of environmental entrainment should
378 perhaps be re-examined due to advances in our understanding of these mechanisms in other
379 species.

380

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384

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482

483 **Table 1:** Primer name, sequence, predicted amplicon size, and GenBank accession number
 484 for the different genes studied.

485

Name	Sequence	Product size	Accession Number
per1F	5' -GCCAGCCCCACCCAGCAGT- 3'	410 bp	AF 228695
per1R	5' -TCGGCCCGTCAGGAAGGA- 3'		
Qpcr per1F	5' -ACGCCCTCCAGTACGCCCTGAAC- 3'	97 bp	
Qpcr per1R	5' -AGGCTGCAGCCGTGACACTCCTC- 3'		
clkF	5' - GCAACACCCGAAAGATGGACAAGT - 3'	546 bp	AF 266745
clkR	5' -AAGCGGGCCGGAGTGACC- 3'		
Qpcr clkF	5' -AGAGACGCTAAGGCCAGAGTATC- 3'	168 bp	
Qpcr clkR	5' -AAGCCATTTTCGAGTTGAGTTAGG- 3'		
aanat2F	5' -GGAGGGCCCTGCTGGTCTGT - 3'	831 bp	AF 106006
aanat2R	5' -AGGGGGTTCGGGATGCTGTCT- 3'		
Qpcr aanat2F	5' - CCGTCACCACCCCGCTCATAATCA - 3'	101 bp	
Qpcr aanat2R	5' - GTGTGGTCTGGACGGTCAACTGTG - 3'		
actinF	5' -ACCGCGGCCTCCTCTTCTCT- 3'	1040 bp	AB 196465
actinR	5' -GTCCCTCTGGCACCCTAATCACC- 3'		
Qpcr actinF	5' -GCCCTCTTCCAGCCCTCCTTCC- 3'	147 bp	
Qpcr actinR	5' -GCCGGGGTACATGGTGGTTCCT- 3'		
eloAF	5' -TTCAAGTATGCCTGGGTGCTGGAC- 3'	1223 bp	NM_001124339
eloAR	5' -TACCGGCCTTAACAGCAGACTTTG- 3'		
Qpcr eloAF	5' -TCTGGAGACGCTGCTATTGTTG- 3'	182 bp	
Qpcr eloAR	5' -GACTTTGTGACCTTGCCGCTTGAG- 3'		

486

487 **Table 2.** Mean expression levels (copy no./ μg *tot*RNA) for *clock*, *per1*, and *aanat2* during rainbow trout embryogenesis.

	<i>per1</i>		<i>clock</i>		<i>aanat2</i>	
	LD	LL	LD	LL	LD	LL
Unfertilised	7131 \pm 694 ^a	-	3149 \pm 383 ^{bc}	-	-	-
0-1 dpf	7886 \pm 805 ^a	7131 \pm 694 ^b	2832 \pm 356 ^c	2553 \pm 301 ^c	-	-
8-9 dpf	1173 \pm 211 ^b	1175 \pm 190 ^c	115 \pm 24 ^d	119 \pm 18 ^d	1198 \pm 195 ^c	1291 \pm 175 ^d
21-22 dpf	6386 \pm 1421 ^a	7658 \pm 1074 ^b	3973 \pm 506 ^b	4522 \pm 527 ^b	221710 \pm 101708 ^a	194458 \pm 81800 ^a
42-43 dpf	9720 \pm 1591 ^a	10040 \pm 1082 ^a	3633 \pm 402 ^b	3879 \pm 259 ^b	11966 \pm 2911 ^b	10527 \pm 1518 ^b
57-58 dpf	7849 \pm 618 ^a	7892 \pm 655 ^b	24891 \pm 2575 ^a	26315 \pm 2279 ^a	1649 \pm 252 ^c	4558 \pm 1970 ^d

488 *N* = 36 (0-1 dpf) or 42 for all other timepoints; - = data not available; superscript denotes significant differences between timepoints for a given
 489 treatment.

490

491 **Table 3.** Acrophase (circadian peak time) of the daily rhythms of *per1*, *clock*, and *aanat2* expression in whole rainbow trout embryo/alevin
 492 homogenates.

	<i>per 1</i>		<i>clock</i>		<i>aanat2</i>	
	LD	LL	LD	LL	LD	LL
Unfertilized	n.s.	-	n.s.	-	-	-
0 – 1 dpf	n.s.	n.s.	23:06 ± 01:54 h	n.s.	-	-
8 – 9 dpf	23:31 ± 00:59 h	20:14 ± 01:12 h ^a	n.s.	n.s.	n.s.	n.s.
21 – 22 dpf	23:12 ± 02:21 h	22:55 ± 00:44 h ^b	n.s.	n.s.	15:49 ± 02:12 h	13:37 ± 02:36 h
42 – 43 dpf	23:56 ± 02:54 h	n.s.	23:47 ± 02:49 h	n.s.	23:20 ± 03:19 h	n.s.
57 – 58 dpf	21:50 ± 02:48 h	n.s.	00:08 ± 02:26 h	n.s.	n.s.	n.s.

493

494 Acrophases were calculated by non-linear regression fit of a cosine function. Data are expressed as acrophase ± 95% confidence intervals, n.s. =
 495 no significant rhythmic variation in expression over the 24-h period; - = data not available.

496 Superscripts denote significant differences in acrophase between timepoints within a given treatment.

497 **List of Figures**

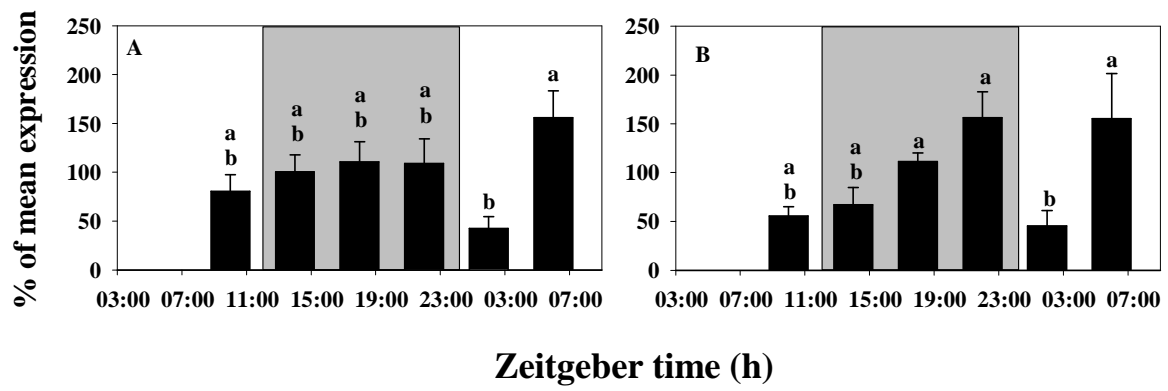
498 **Figure 1:** Expression of *per1* (A) and *clock* (B) mRNA in whole unfertilized oocyte
499 homogenates previously maintained under LD photoperiod conditions. Grey box symbolizes
500 darkness. Data are presented as % of the mean expression levels \pm SEM ($n = 6$ /timepoint).
501 Significant differences between timepoints within a given treatment are shown above the
502 bars.

503 **Figure 2:** Expression of *per1* mRNA in whole embryo/alevin homogenates under LD or LL
504 photoperiod conditions. Grey box symbolizes darkness under the LD conditions. Data are
505 presented as % of the mean expression levels \pm SEM ($n = 6$ /timepoint). Arrow in the 0-1 dpf
506 graphs signifies time of oocyte fertilization. Significant differences between timepoints
507 within a given treatment/developmental stage are shown above the bars. n.s. denotes no
508 significant difference in expression during a given 24-h period. The cosinor waveform is
509 plotted when expression was identified as being rhythmic.

510 **Figure 3.** Expression of *clock* mRNA in whole embryo/alevin homogenates under LD or LL
511 photoperiod conditions. Grey box symbolizes darkness under the LD conditions. Data are
512 presented as % of the mean expression levels \pm SEM ($n = 6$ /timepoint). Arrow in the 0-1 dpf
513 graphs signifies time of oocyte fertilization. Significant differences between timepoints
514 within a given treatment/developmental stage are shown above the bars. n.s. denotes no
515 significant difference in expression during a given 24-h period. The cosinor waveform is
516 plotted when expression was identified as being rhythmic.

517 **Figure 4:** Expression of *aanat2* mRNA in whole embryo/alevin homogenates under LD or
518 LL photoperiod conditions. Grey box symbolizes darkness under the LD conditions. Data are
519 presented as % of the mean expression levels \pm SEM ($n = 6$ /timepoint). Arrow in the 0-1 dpf
520 graphs signifies time of oocyte fertilization. Significant differences between timepoints
521 within a given treatment/developmental stage are shown above the bars. n.s. denotes no

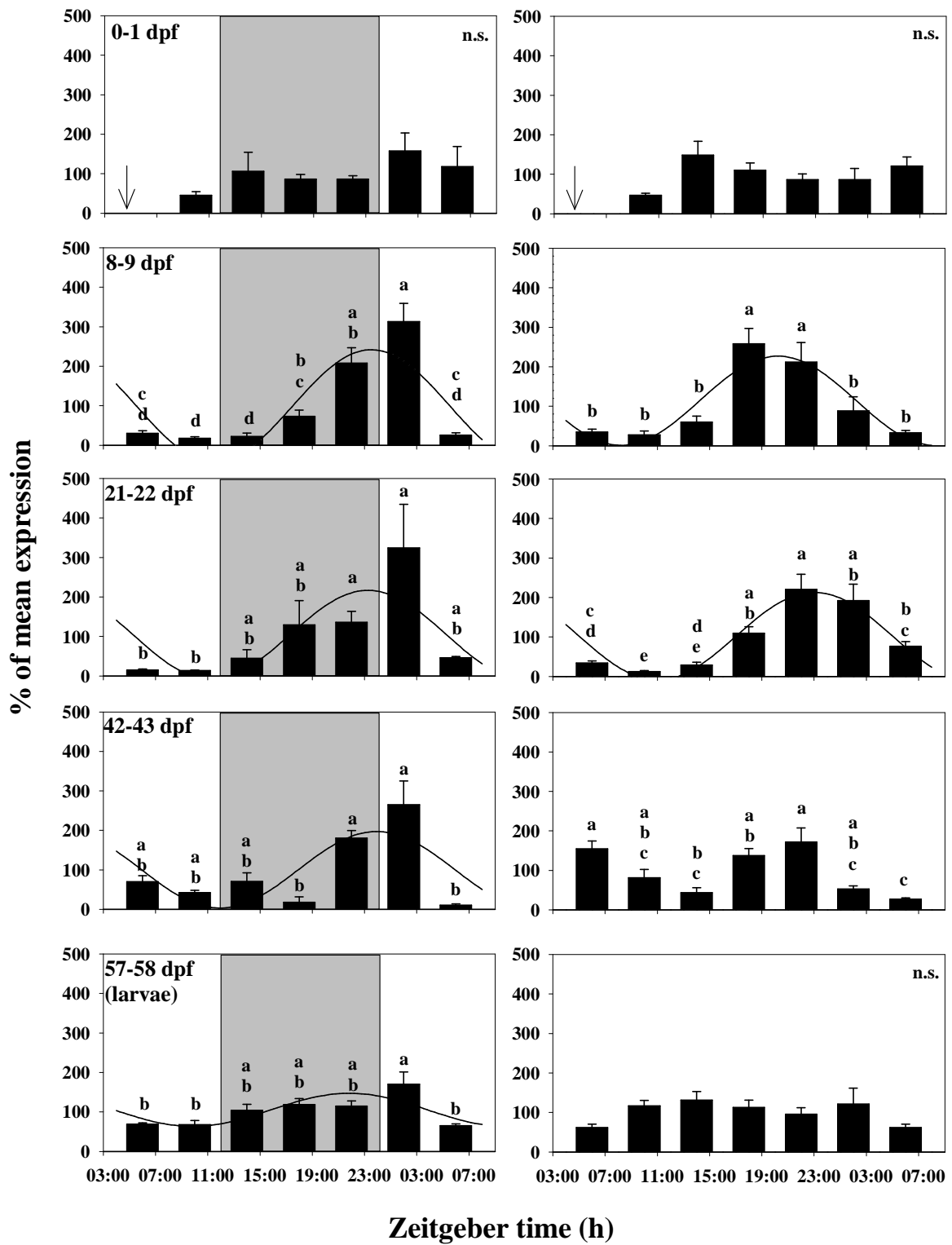
522 significant difference in expression during a given 24-h period. The cosinor waveform is
523 plotted when expression was identified as being rhythmic.
524



525

526 **Figure 1**

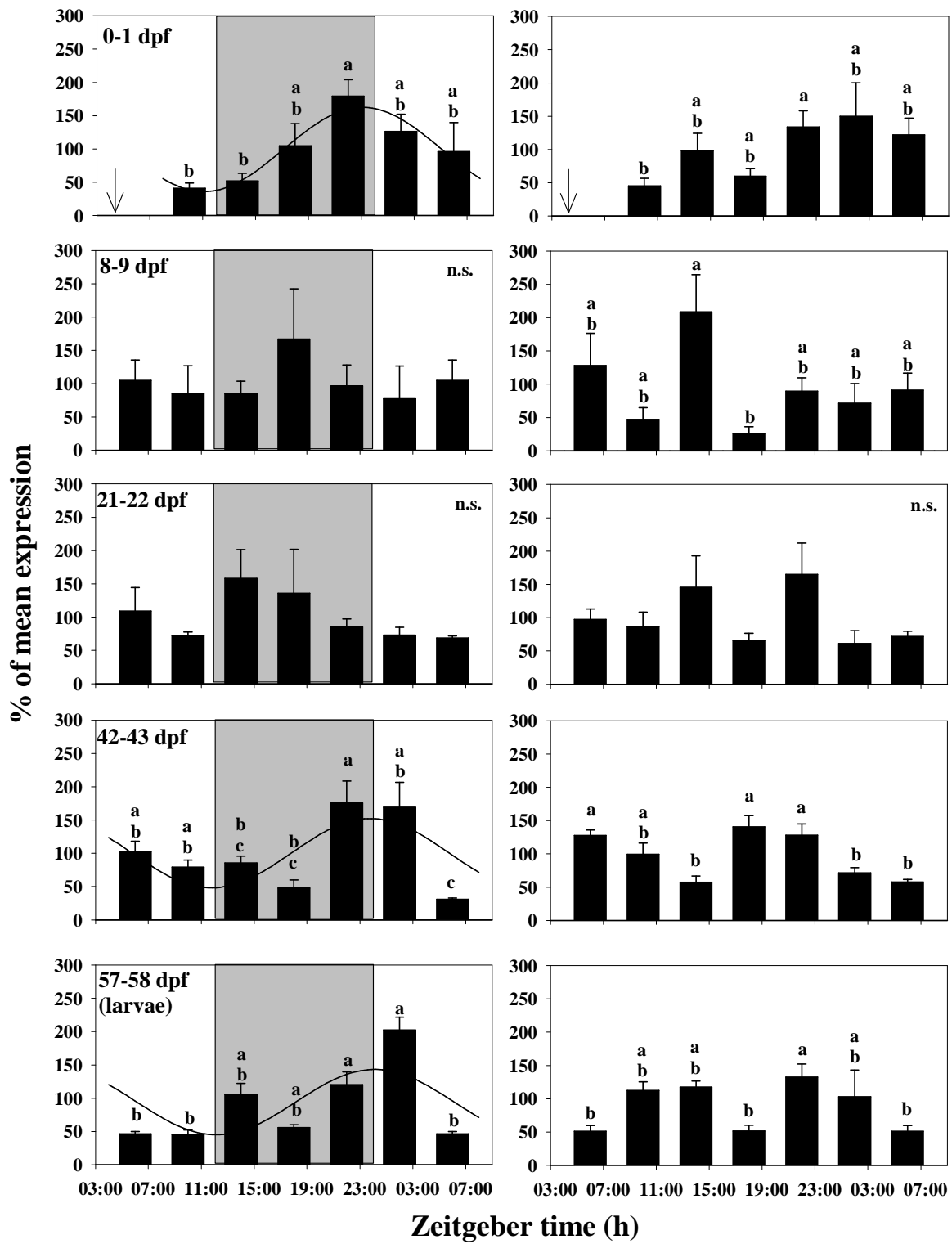
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529 **Figure 2**

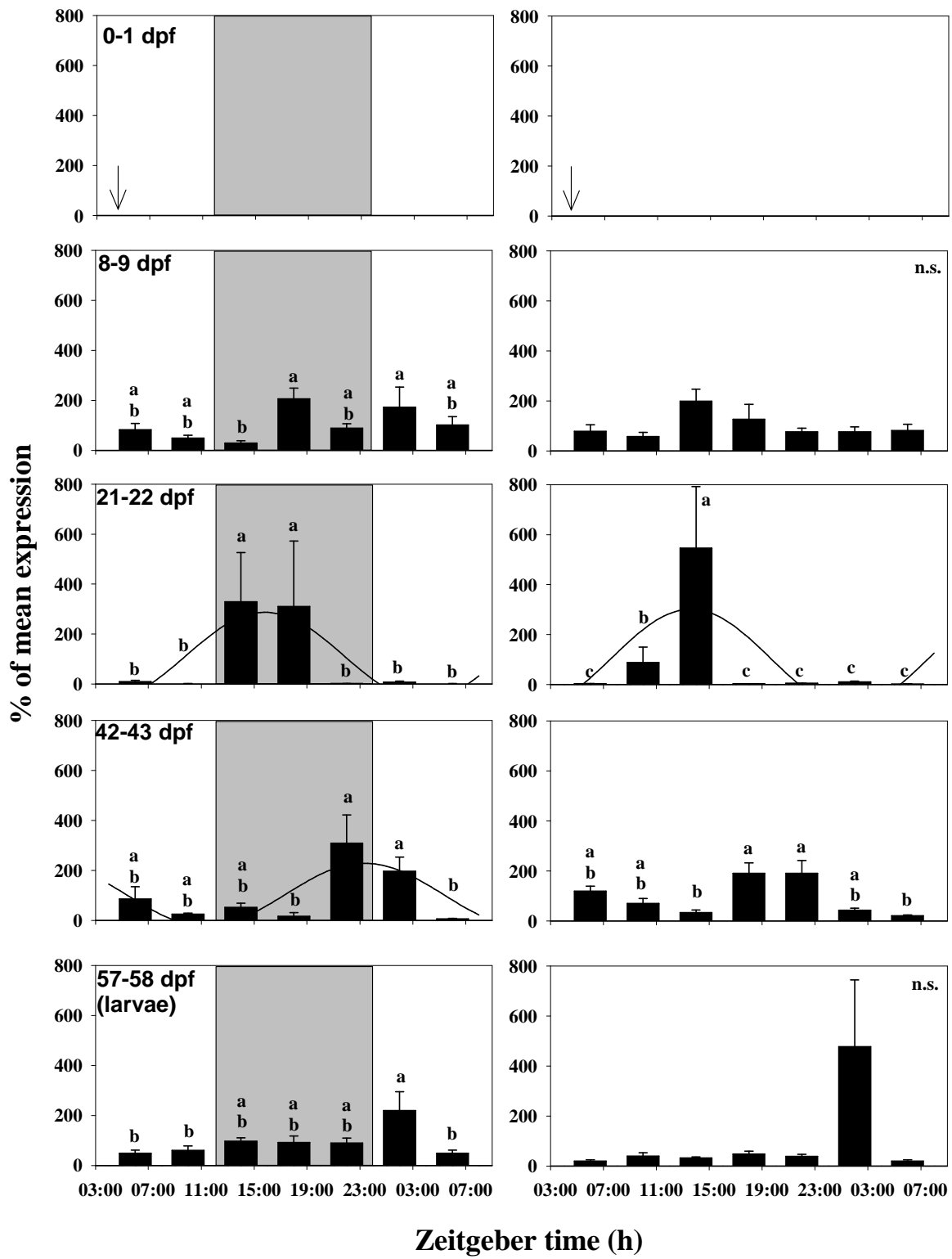
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531

532 **Figure 3**

533



534

535 Figure 4