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

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Abstract

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

Keywords: *per1*, c*lock, aanat2*, rainbow trout, embryo, circadian rhythms

Introduction

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

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

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

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

Materials and Methods

Animals and experimental procedures

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

RNA extraction and cDNA synthesis

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

Synthesis of clock, per1, and aanat2 cDNA

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

Partial cDNA sequences for each target were generated by PCR using 0.5 µM of primers (clkF, clkR; per1F, per1R; aanat2F, aanat2R), one eighth (2.5 µl) of the cDNA synthesis reaction, Klear Taq polymerase with supplied buffer (Kbiosciences, Beverly, UK), and 2.25 mM $MgCl_2$ in a final volume of 20 µl. The following hotstart PCR strategy was used: 15 min 95°C, 29 cycles 95°C 30 s, X°C 30 s, 72°C 1 min/kb of product size, where annealing temperature X = 58, 60, 60, and 59°C for *clock, per1*, and *aanat2*, respectively. All primer pairs generated a single PCR product that was cloned into a 2.1 plasmid (Topo TA, Invitrogen, Paisley, UK) and sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA).

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

Sequence analysis

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

Quantitative PCR

All cDNA for qPCR was synthesized using Superscript™ II reverse transcriptase and supplied buffer components (Invitrogen, Paisley, UK) and an oligo-dT primer (as described above). qPCR primers for *clock*, *per1*, *aanat2*, and the reference genes *β-actin*, and *Elongation factor α* (Table 1) were used at 0.5 µM, with one-fortieth of the total cDNA synthesis reaction and SYBR-green qPCR mix (ABgene, Epsom, UK) in a total volume of 20 µl. The thermal cycling protocol run in a Techne Quantica thermocycler (Techne, Quantica, Cambridge, UK) consisted of 15 min at 95°C followed by 45 cycles of 95°C for 15 s, X°C for 15 s, and 72°C for 30 s followed by a temperature ramp from 70 to 90°C for melt-curve analysis. The annealing temperature (X) was changed as follows: 56°C for *clock*, 66°C for *per1*, 64°C for *aanat2*, 62°C for *β-Actin*, and 61°C for *Elongation factor α*. Melt-curve analysis verified the primer sets for each qPCR assay generated one single product and no primer-dimer artefacts. In addition, a random sample of each qPCR product was than sequenced to confirm its identity and was found to be 100% identical to its relative sequence. 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 $$Y = A + B \times \cos(C \times X - D)$$
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

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

Results

Embryo development

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

Clock & Per1

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

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

aanat2

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

Discussion

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

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

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

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

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

For both *per1* and *clock*, it is evident that mean expression levels were comparable between unfertilized oocytes and just fertilized embryos. Furthermore, these levels significantly declined from 0-1 to 8-9 dpf before recovering and exceeding initial levels at the later stages of development. Similar transitions in clock-gene expression levels have been reported in zebrafish, in which it has been described as a progression from maternal mRNA that breaks down by the midblastula stage (~4 hpf) before endogenous zygotic expression begins *de novo* and increases as the embryo develops (Dekens & Whitmore, 2008; Ziv & Gothilf, 2006). In rainbow trout, it has been suggested that embryo genomic transcription

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

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

no consistent or robust expression pattern. Mean expression levels were at their highest at 21-22 dpf due primarily to extremely high levels of expression at ZT 14:00 and 18:00 under the LD photoperiod and ZT 10:00 and 14:00 under the LL photoperiod. In both cases, these peaks represented a >70 fold increase compared to basal expression levels. Thereafter, expression levels showed a marked decline and dark-specific expression in the LD treatment was not apparent. The lack of a consistent rhythmic pattern in *aanat2* expression in comparison to *per1*, under the LD conditions, could be an indication that *aanat2* expression is not regulated by clock genes in rainbow trout, as previously suggested by Begay et al. (1998). The authors reported that *aanat2* expression, in the rainbow trout pineal at least, is arrhythmic and not responsive to light treatment. The disconnection between clock rhythms and melatonin synthesis in salmonids has been reported on a number of occasions (e.g., Bolliet et al., 1996; Iigo et al., 2007); however, reasons for the disconnection are lacking. In the closely related Chum salmon (*Oncorhynchus keta*), Shi et al. (2004) reported no differences between day and night *aanat2* expression levels in the brain (samples included pineal gland) until after hatching; however, they did observe significant day-night alterations in ocular *aanat2* expression during late embryogenesis and up to 2 days posthatching. It must be acknowledged that the whole embryo approach adopted in the current study negates detection of such tissue-specific expression profiles. However, in general, it appears that *aanat2* expression during embryogenesis in teleosts does not follow a consistent pattern. In zebrafish, robust cyclic expression is measureable from the second day of embryogenesis (Gothilf et al., 1999). In sole, rhythmic expression was not apparent during embryo development, nor during larval metamorphosis. However, it was present during a brief window in larvae following hatching (Isorna et al., 2009), while no significant day night cycling could be measured in turbot embryos (Vuilleumier et al., 2007). Clearly, the functional significance of *aanat2* 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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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’ –GCCCAGCCCCACCCAGCAGT– 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’ –AAGCCATTTCGAGTTGAGTTAGG– 3’		
aanat2F	5’ –GGAGGGCCCTGCTGGTCTGT – 3’	831 bp	AF 106006
aanat2R	5’ –AGGGGGTCGGGATGCTGTCT– 3’		
Qpcr aanat2F	5’ – CCGTCACCACCCCGCTCATAATCA – 3’	101 bp	
Qpcr aanat2R	5’ – GTGTGGTCTGGACGGTCAACTGTG – 3’		
actinF	5’ –ACCGCGGCCTCCTCTTCCTCT– 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

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

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

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

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

	per 1		*clock*		*aanat2*	
	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 ± 0 3:19 h	n.s.
57 – 58 dpf	21:50 ± 02:48 h	n.s.	00:08 ± 02:26 h	n.s.	n.s.	n.s.

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

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

List of Figures

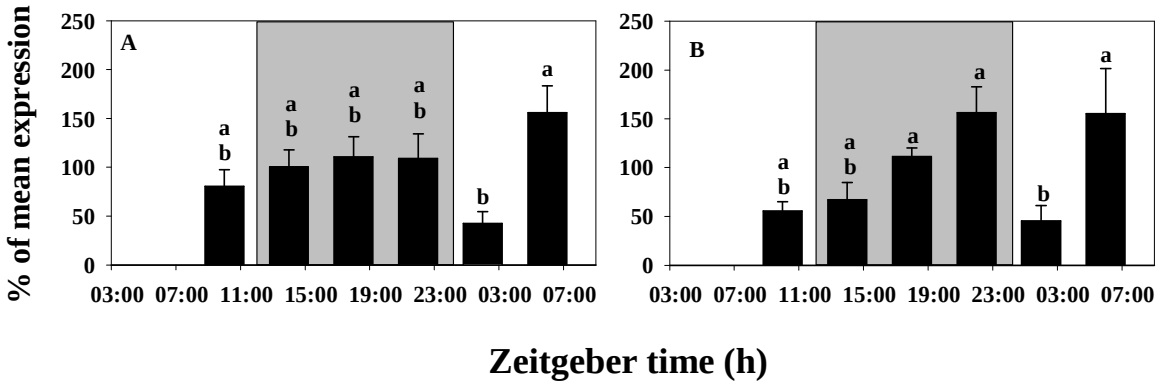
Figure 1: Expression of *per1* (A) and *clock* (B) mRNA in whole unfertilized oocyte homogenates previously maintained under LD photoperiod conditions. Grey box symbolizes darkness. Data are presented as % of the mean expression levels ± SEM (*n* =6/timepoint). Significant differences between timepoints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

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

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

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

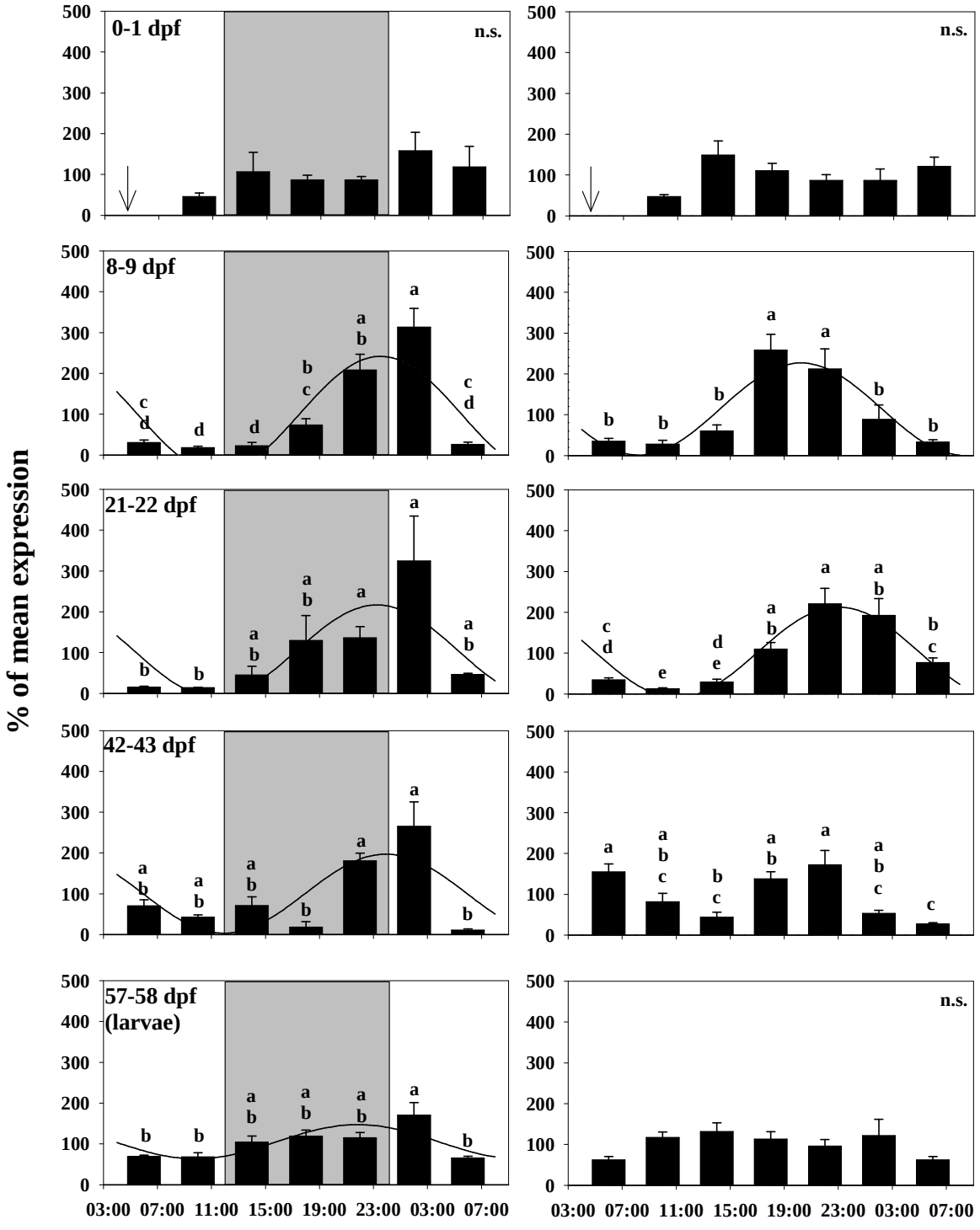
522 significant difference in expression during a given 24-h period. The cosinor waveform is
523 plotted when expression was identified as being rhythmic.
524



525

526 **Figure 1**

527



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529 **Figure 2**

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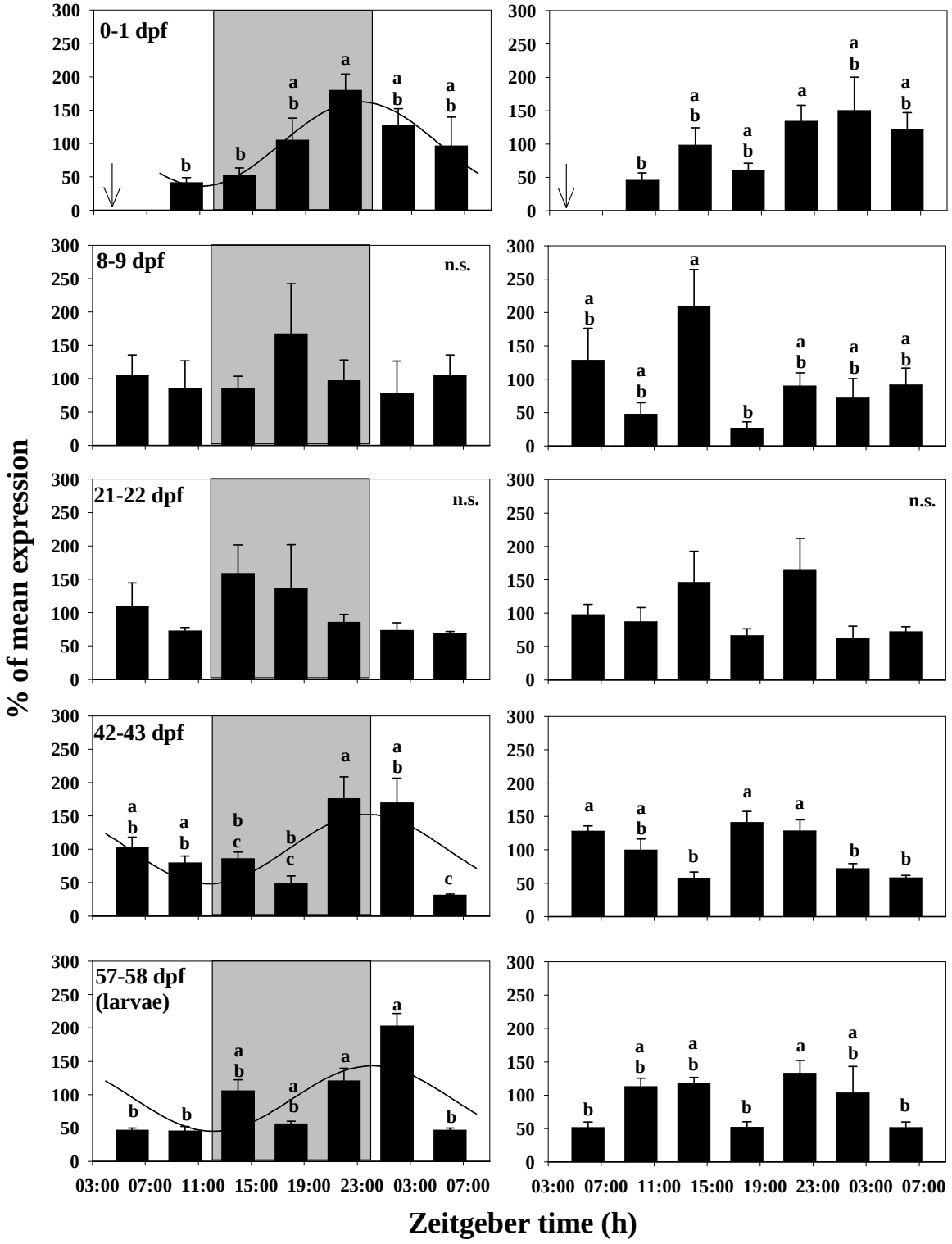

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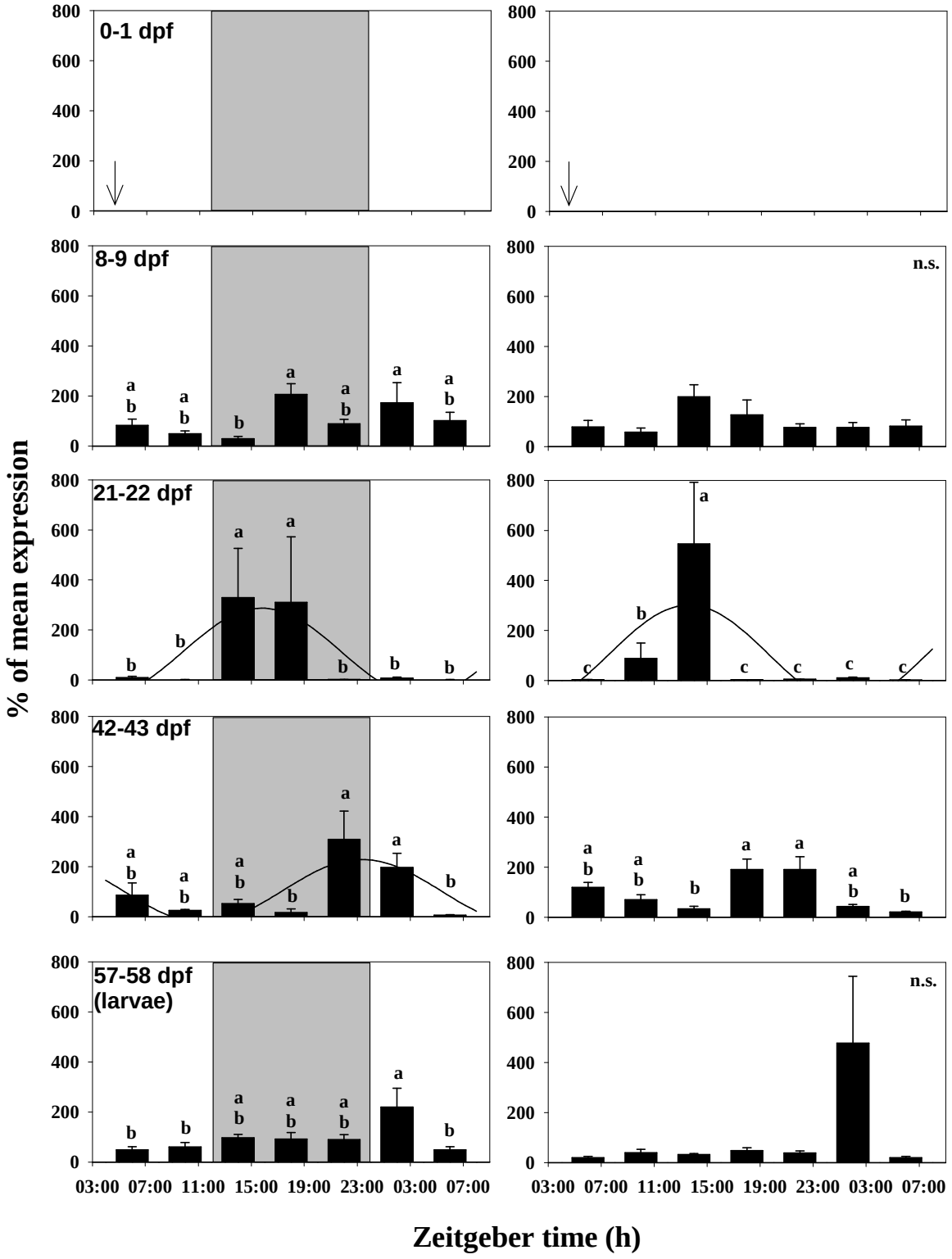
532 **Figure 3**

533



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535 **Figure 4**