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2

3 **Interplay between daily rhythmic serum-mediated**
4 **bacterial killing activity and immune defence**
5 **factors in rainbow trout (*Oncorhynchus mykiss*)**

6

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28

29 **Abbreviations**

30 ALP, alkaline phosphatase; ANTI, anti-protease; CERU, ceruloplasmin; LD, light:dark;

31 LYS, lysozyme; MPO, myeloperoxidase; ZT, *zeitgeber* time

32

ACCEPTED MANUSCRIPT

33 **Abstract**

34 Circadian rhythm is emerging as an important regulator of immune
35 functions. However, there is a paucity of information on the influence of this biological
36 phenomenon in the antimicrobial factors in teleost fish. This study investigated the
37 dynamics and interplay of serum-mediated bacterial killing activity and immune
38 defence factors throughout the light:dark (LD) cycle in rainbow trout (*Oncorhynchus*
39 *mykiss*). The juvenile fish came from two different emergence time fractions (*i.e.*, late
40 and early) that were believed to exhibit behavioural and physiological differences.
41 Serum collected during the day from fish (mean \pm SD: 39.8 \pm 6.3 g) reared under
42 14L:10D photoperiod demonstrated bactericidal activity against *Flavobacterium*
43 *psychrophilum*, *Yersinia ruckeri* and *Aeromonas salmonicida* subsp. *salmonicida* of
44 varying magnitude, but no significant differences between the emergence fractions
45 were observed. A day-night comparison in the same batch of fish revealed time-of-day
46 dependence in the bactericidal activity against *F. psychrophilum* and *Y. ruckeri* amongst
47 emergence fractions. A group of fish (63.3 \pm 4.7 g) from each fraction was entrained to
48 12L:12D photoperiod for 21 days to investigate whether serum bactericidal activity
49 exhibit daily rhythm. Serum-mediated bacterial killing activity against *F. psychrophilum*
50 and *Y. ruckeri* displayed significant daily rhythm in both emergence fractions, where
51 the peak of activity was identified during the light phase. Moreover, several serum
52 defence factors manifested variations during the LD cycle, where anti-protease (ANTI)
53 and myeloperoxidase (MPO) activities exhibited significant daily oscillation. However,
54 there were no remarkable differences in the daily changes of serum factors amongst
55 emergence fractions. Acrophase analysis revealed that the peaks of activity of alkaline

56 phosphatase (only in late fraction), ANTI, lysozyme (only in early fraction) and MPO
57 were identified during the light phase and corresponded with the period when serum-
58 mediated bacterial killing activity was also at its highest. The daily dynamics of
59 bactericidal activity and immune defence factors displayed positive correlation,
60 particularly between MPO and, the two pathogens (*i.e.*, *F. psychrophilum* and *Y.*
61 *ruckeri*). Taken together, the study revealed that serum-mediated bacterial killing
62 activity and immune defence factors remarkably varied during the LD cycle in rainbow
63 trout. In addition, the two emergence fractions displayed nearly comparable
64 immunological profiles.

65

66 **Keywords:** *aquaculture, circadian rhythm, immunity, fish, stress-coping style*

67 1. Introduction

68 The immune system is the classical defence against pathogenic bacteria in
69 fish. In particular, the humoral immunity constitutes a group of molecules that ensures
70 a well-orchestrated action is imposed to a potential threat. Serum has an integral role
71 in humoral immunity as it contains a great number of defence factors such as
72 inhibitors (*e.g.*, transferrins and lectins) and lysins (*e.g.*, lysozyme, C-reactive protein
73 and complement) that are responsible for counteracting the danger associated with
74 pathogenic bacteria [1]. These potent factors are the key players in serum-mediated
75 bacterial killing activity, a vital defence mechanism in a number of fish species [2-7].

76 In recent years, the interaction of immunity and circadian clocks has been
77 the subject of great interest. The circadian cycles established by the endogenous clock
78 enable the organism to anticipate periodic and cyclic changes in their immediate
79 environment (*e.g.*, light-dark cycle) and exerts a pervasive regulatory function to
80 several physiological, behavioural and biochemical processes [8-10]. One of the
81 advantages of this adaptive response is the scheduling of important biological
82 processes to occur at the most crucial times of the day. This fundamental phenomenon
83 is characterised by an oscillatory pattern with a period of approximately 24 h. In
84 mammalian models, it has been shown that humoral (*e.g.*, cytokines, chemokines and
85 cytolytic factors), and cellular immune factors (*e.g.*, T and B cells, dendritic cells) and
86 mechanisms (*e.g.*, phagocytosis, inflammation) exhibit daily rhythmicity [11-13]. These
87 daily changes are essential in the homeostasis, adaptability and protective functions of
88 the immune system. Furthermore, the clock genes that canonically comprise the core
89 molecular machinery of circadian rhythms regulate the immune response to bacterial

90 infection [14], thus, providing a compelling support to the relationship between
91 circadian rhythms and immunity. The physiology and behavior of fish have long been
92 indicated to be under circadian control; however, little is known about the impact of
93 this fundamental process on fish immunity, with only a few reports showing the daily
94 rhythms of humoral and cellular immune effectors [4, 5, 15, 16]. A striking observation
95 has been published recently demonstrating that the ability of tilapia (*Oreochromis*
96 *niloticus*) to mount a humoral immune response to a bacterial endotoxin was gated by
97 the time of the day [5], thus, highlighting the importance of the host immunological
98 rhythm during infection. The daily oscillation of immune defence factors in the serum
99 posits that bactericidal activity is expected to be changing dramatically during the LD
100 cycle as well. To the best of our knowledge, no studies have discussed the daily rhythm
101 of serum-mediated bacterial killing activity in fish.

102 The present study aimed at identifying the dynamic changes in serum-
103 mediated bacterial killing activity against key pathogens during a complete 24 h light-
104 dark (LD) cycle in rainbow trout (*Oncorhynchus mykiss*). Likewise, the daily oscillating
105 patterns of selected humoral defence molecules were explored to provide insight into
106 the underlying factors that may be contributing to the daily changes of serum
107 bactericidal function. The variations in serum-mediated bacterial killing activity and
108 immune defence factors were investigated in a comparative approach by employing
109 rainbow trout originating from two different emergence time (often referred to the
110 time for first feeding) fractions, namely the early- and the late-emerging individuals
111 [17]. In the wild, the emergence time of salmonid is thought to be related to their
112 stress-coping style (SCS): individuals emerging early are more proactive while those

113 emerging late are thought to exhibit a reactive SCS [18, 19]. Proactive SCS is believed
114 to be more resistant to diseases [20, 21], however, too little is known about whether
115 this feature relates to the distinctive immunological robustness amongst emergence
116 fractions, especially those that have already been subjected to domestication.

117

118 **2. Materials and Methods**

119 **2.1. Ethics statement**

120 All fish handling procedures employed in the study were in accordance
121 with national and EU legislation (2010/63/EU) on animal experimentation. The Animal
122 Welfare Committee at DTU Aqua approved the experiment.

123 **2.2. Target pathogens**

124 *Flavobacterium psychrophilum*, *Yersinia ruckeri* and *Aeromonas*
125 *salmonicida* subsp. *salmonicida* (hereafter will be referred to as *A. salmonicida*)
126 isolates were from the private culture collection of the National Veterinary Institute at
127 the Technical University of Denmark (DTU) and had all three been isolated and
128 identified from different disease outbreaks in cultured rainbow trout (*Oncorhynchus*
129 *mykiss*) in 2015. The pathogenicity of the isolates have been determined. The isolates
130 were stored at -80 °C in either for *F. psychrophilum* tryptone yeast extract salts (TYES)
131 broth [22] or for *Y. ruckeri* and *A. salmonicida* veal infusion broth, both with 15 to 20 %
132 glycerol, and were subcultured in agitated cultures at 15 °C (*F. psychrophilum*)/20 °C (*Y.*
133 *ruckeri* and *A. salmonicida*). Strains were taken directly from -80 °C and incubated in
134 one of the described broth types for a minimum of 48 hours before further
135 inoculations were made for the liquid cultures used for the bacterial interaction

136 studies [23]. All bacterial broth culturing was done under agitation. For all bacterial
137 interaction studies the concentration of each pathogen was set to approximately $3 \times$
138 10^3 CFU/ml by diluting the 48 hour bacterial cultures with sterile broth. The estimated
139 CFU/ml were verified by the plate count method by streaking 10-fold dilutions of each
140 culture on either TYES added 1 % agar or Blood Agar.

141

142 **2.3. Fish, husbandry conditions and serum collection during the on-growing** 143 **period**

144 Rainbow trout (*Oncorhynchus mykiss*) eggs were purchased from a local
145 supplier (Piledal Dambrug, Vejle, Denmark) and transported to the hatchery facility of
146 DTU Aqua (Hirtshals, Denmark). These rainbow trout eggs came from a selective
147 breeding programme that has been running for over 20 generations. The eggs were
148 kept in incubation trays with a current of oxygen-saturated water and the temperature
149 was maintained at 10 °C. After hatching, actively swimming larvae were transferred to
150 artificial gravel nests, sheltered by golf balls to simulate a natural gravel condition.
151 These artificial nests functioned as a screening device to fractionate fish based on the
152 time of emergence (see [24] for a complete description of the screening device).
153 Emergence time is referred to the phase when fish start to emerge and swim upwards
154 looking for feed [24]. Egg incubation and fractionation were performed in total
155 darkness. During daily routine monitoring, the room was illuminated for a short period
156 with incandescent bulbs (maximum surface water intensity = ca 32 lux). Two
157 emergence fractions were collected for this study: the early fraction comprising the 20
158 % of the first swim-up fish larvae, and the late fraction that constituted the 20 % of the

159 fish that emerged last. The fish from the two emergence fractions were reared in
160 separate tanks for several months at 12 °C until their use in the experiments. During
161 the on-growing period, fish were reared under 14L:10D photoperiod with lights on at
162 07:00 AM. LED bulbs provided illumination and had a water surface intensity of around
163 320 lux. The hatching, fractionation and husbandry protocols are described in detail in
164 Gesto et al. [17].

165 Eight juveniles (mean \pm SD: 39.8 \pm 6.3 g) were selected from each
166 emergence fraction for the determination of serum-mediated bacterial killing activity
167 (Section 2.5). The fish were anaesthetised (benzocaine solution, 50 mg L⁻¹) and blood
168 was withdrawn from the caudal artery using a syringe fitted with a 21-G needle. The
169 blood was collected at ZT3 (*Zeitgeber* time 3; 3 h after lights on) from fish that were
170 fasted for 24 h. The collected blood was allowed to clot at room temperature for 2 h
171 and at 4 °C overnight. Thereafter, serum was collected by centrifugation at 1500 *g* for
172 10 mins. Aliquoted serum samples were stored at -80 °C until analysis.

173 The influence of time of the day on the bactericidal activity of fish serum
174 was investigated. The procedure for serum collection and preparation was similar to
175 those described above except the time of collection. Serum samples were collected
176 from 8 fish (mean \pm SD: 46.6 \pm 7.5 g) from each emergence fraction during the day
177 (ZT3) and another batch during the night (ZT16; 2 h after lights off). Our preliminary
178 studies revealed that these time-points were suitable to show day-night variations.
179 Sample collection during the night was performed in a room with red illumination (< 3
180 lux) and exposure of an individual anaesthetised fish to the lighting condition was no
181 longer than 3 min. Samples were kept at -80 °C until analysis.

2.4. Entrainment to 12L:12D photoperiod

Fish with an average weight of 63.3 ± 4.7 g (mean \pm SD) in the on-growing holding tanks were transferred to 189-L, cylindrical-conical, thermoplastic tanks in a recirculation system. Each emergence group included 5 tanks, each of which was stocked with 8 fish. A white LED bulb with a maximum water surface intensity of 350 lux on top of each tank provided the illumination. The water temperature was controlled at 15 °C and quality parameters (NO_3^- , NO_2^- , $\text{NH}_3/\text{NH}_4^+$, pH) were monitored every 2 days and kept within safe limits, *i.e.*, $\text{NH}_3\text{-N} < 0.025$ mg/l; $\text{NH}_4\text{-N} < 5$ mg/l; $\text{NO}_2^- \text{-N} < 10$ mg/l; $\text{NO}_3^- \text{-N} < 100$ mg/l; $\text{pH} \approx 7.4$. The photoperiod was set at 12L:12D, with lights on at 07:00 AM (ZT0). The fish were fed at a ration of 1.5 % total biomass per day. The fish were under these conditions for 21 days before sample collection.

Fish were not provided feed for at least 24 h before sample collection. Serum was collected at 6-h intervals (*i.e.*, ZT2a, ZT8, ZT14, ZT18, ZT2b) for a period of 24 h within 2 intersecting daily cycles. To ensure minimal disturbance during sampling, a single tank was dedicated exclusively to a particular sampling point. Blood was withdrawn and serum was collected similarly to the protocol described in section 2.3. The serum aliquots were stored at -80 °C until analysis.

2.5. Serum-mediated bacterial killing activity

The bactericidal activity of serum towards the test pathogens was determined using a co-incubation assay previously optimised for fish serum samples [25] and was modified for spectrophotometric assay [26]. Bacterial activity expressed as cell metabolic activity was measured by the addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 2 mg mL^{-1}) to the mixture of equal volumes of

205 undiluted serum and the target pathogen after a 24-h incubation at 15°C. Bacterial
206 viability was evaluated based on the ability of viable cells to reduce MTT to formazan
207 crystals. The absorbance was measured with a microplate reader (Fluostar Optima) at
208 630 nm and the MTT reduction was thereafter compared with the control group to
209 calculate the percentage of inhibition.

210 **2.6. Soluble immune defence factors in serum**

211 Humoral immune defence factors were characterised by
212 spectrophotometric quantifications in the serum samples collected in Section 2.4.
213 Alkaline phosphatase (ALP) activity was quantified through a kinetic reaction assay
214 using *p*-nitrophenyl phosphate as a substrate [27]. One unit of activity was defined as
215 the amount of enzyme required to release 1 μmol of *p*-nitrophenol product in 1 min.
216 The inhibition of trypsin activity was employed to determine the anti-protease (ANTI)
217 activity in serum [28]. Percentage of inhibition was calculated by comparing it to 100 %
218 control. The level of ceruloplasmin (CERU) was measured enzymatically in a reaction
219 mixture containing *para*-phenylenediamine-sodium azide in acetate buffer, as
220 previously described [29]. The kinetic increase of absorbance was followed for 15 min
221 and 1 unit was defined as an increase of optical density (OD) of 0.001 min^{-1} at 550 nm.
222 Lysozyme (LYS) activity was quantified by a turbidimetric method [30], following a
223 modified protocol for 96-well microplate reaction [31]. A unit of lysozyme activity was
224 defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per
225 minute at 450 nm. Myeloperoxidase (MPO) was measured following previously
226 described protocol [32], with modifications [4], using 3,3',5,5'-tetramethyl benzidine
227 hydrochloride as a reaction substrate. Unit of activity was expressed as OD at 450 nm.

228 All absorbance measurements were conducted in a microplate reader (TECAN GENios,
229 Salzburg, Austria).

230 **2.7. Statistical analyses**

231 All statistical analyses were performed in SigmaStat version 4.0 (Systat
232 Software, London, UK). Student *t*-test for independent samples was used to identify
233 significant differences in the serum-mediated bacterial killing activity between
234 emergence fractions (*i.e.*, early versus late), as well as between collection times (*i.e.*,
235 ZT3 versus ZT16). The level of significance was set at $P < 0.05$. Data on the changes in
236 bactericidal activity and serum defence factors throughout the daily cycle were
237 subjected to a one-way ANOVA after complying the requirements for normal
238 distribution and equal variance. Differences between time points were further
239 delineated by Tukey's multiple comparison test. For data sets that did not follow a
240 Gaussian distribution or did not meet the equal variance requirements, Kruskal-Wallis
241 one-way ANOVA on ranks followed by Dunn's multiple comparison test was
242 alternatively used. The level of significance was set at $P < 0.05$. COSINOR analysis
243 (CRONOBIO, by Prof. Díez-Noguera, University of Barcelona, Spain) was performed to
244 determine the parameters defining the rhythmicity and the significance of daily
245 oscillation. Analysis was performed by fitting a periodic sinusoidal function to the
246 activity values of a parameter across the five ZTs, using the formula: $f(t) = M + A \cos$
247 $(t/\pi/12 - \phi)$, where $f(t)$ is the level of the parameter at given time, mesor (M) is the
248 mean value, A is the sinusoidal amplitude of oscillation, t is time in hours and ϕ is the
249 acrophase. A parameter was considered exhibiting significant daily rhythm when both
250 ANOVA $P < 0.05$ and COSINOR $p < 0.05$ [4].

251 3. Results and Discussion

252 Infections associated with *Flavobacterium psychrophilum*, *Yersinia ruckeri*
253 and *Aeromonas salmonicida* have serious consequences in salmonid aquaculture [33-
254 35]. This study explored the natural mechanism of defence against these pathogens in
255 rainbow trout by exploring their serum-mediated bacterial killing activity. Serum
256 collected at ZT3 from rainbow trout showed potent bactericidal activity against the
257 three pathogens and the magnitude of bactericidal action varied between the target
258 bacteria (**Fig. 1**). The bactericidal activity against a specific pathogen between
259 emergence fractions revealed no significant difference. However, significant
260 differences were identified in the bactericidal activity towards the different pathogens
261 within a fraction. It was further demonstrated that the overall trend of serum
262 bactericidal activity was identical in both fractions. Serum-mediated bacterial killing
263 activity was highest against *Y. ruckeri*: it was around 52 % higher than the activity
264 against *F. psychrophilum* and almost 90 % higher than the activity towards *A.*
265 *salmonicida*. It has been reported earlier that bactericidal activity against *A.*
266 *salmonicida* was relatively low in rainbow trout serum, however, it could be increased
267 by antigenic stimulation [3]. Results from other determinations in the study (**Figs. 2 &**
268 **3**) showed a consistent trend of low bacterial killing activity against *A. salmonicida*. In
269 other animal models, it has been demonstrated that antibacterial activity is highly
270 influenced by the time of the day [14]. In crayfish (*Procambarus clarkia*), a higher
271 survival was observed when the animals were infected with *Aeromonas hydrophila* at
272 CT19 (Circadian time 19) than at CT5 and their ability to significantly lower bacterial
273 load 12 h after infection more effectively when infected at CT19 than at CT5 had been

274 implicated for this difference [36]. Our previous study also demonstrated the
275 differential temporal sensitivity of tilapia to bacterial endotoxin challenge [5]. Thus, we
276 explored whether the time of the day had an impact on the serum-mediated bacterial
277 killing activity in rainbow trout by collecting serum samples for bactericidal assay at
278 ZT3 (day) and at ZT16 (night). Day-night differences in bactericidal activity were
279 exhibited by the early fraction against *F. psychrophilum* and *Y. ruckeri* (**Fig. 2**).
280 Inhibition of *F. psychrophilum* by serum from the early fraction was 60 % higher during
281 the night than during the day. An opposite trend was observed in the activity against *Y.*
282 *ruckeri* for the same emergence group, where bacterial killing activity at ZT3 was
283 almost 58 % higher than at ZT16. Serum samples collected during the night revealed
284 significant differences in bactericidal activity between early and late fractions. At ZT16,
285 serum-mediated bacterial killing activity against *F. psychrophilum* was significantly
286 higher in the early than in the late fraction. Difference between emergence groups was
287 likewise observed against *Y. ruckeri*, where the activity was higher in the late than in
288 the early fraction. These observations indicate temporal gating in serum-mediated
289 bacterial killing activity in rainbow trout, supporting earlier reports that an organism's
290 antibacterial defence is highly influenced by the time of the day [5, 12, 36, 37].
291 Surprisingly, we observed a significant difference in the bacterial killing activity against
292 *Y. ruckeri* between early and late fractions at ZT3 in the second sampling (**Fig. 2**), which
293 we did not observe in the first sample collection (**Fig. 1**). Though we could not
294 affirmatively identify the cause of this difference, we speculate that it may be due to
295 potential size/age-related differences, which have been implicated as a contributory
296 factor in immunological differences in other fish species [37, 38].

297 The light-dark differences observed in the serum-mediated bacterial killing
298 activity against the pathogens led us to hypothesise that this feature may not only be a
299 function of day and night but may perhaps be a phenomenon that exhibits a daily
300 rhythmic pattern. The entrainment results reveal that serum-mediated bacterial killing
301 activity against *F. psychrophilum* and *Y. ruckeri*, but not towards *A. salmonicida*
302 exhibited significant daily rhythm during the LD cycle (**Fig. 3**). The daily rhythmic
303 pattern of bactericidal activity against *F. psychrophilum* between the early and late
304 fractions exhibited an almost identical trend (**Fig. 3A**). The peaks of activity (acrophase)
305 of the two fractions were likewise similar: ZT 9.11 in the early fraction and ZT 10.5 in
306 the late fraction (**Supplementary Table 1**). This result contradicted the earlier
307 observation (**Fig. 2**) that bactericidal activity against *F. psychrophilum* was higher
308 during the night than during the day. This inconsistency could be attributed to the
309 change of photoperiod from 14L:10D to 12L:12D. It could be possible that the equal
310 length of day and night shifted the activity to be more active during the light phase as
311 an adaptive response to a new photoperiod regime. The photoperiodic plasticity of
312 defence mechanisms demonstrated in a number of fish species partly support this
313 conjecture [5, 39, 40]; though our present data could not provide functional relevance
314 associated with this change. There was an obvious difference between early and late
315 fractions in the daily rhythmic pattern of bactericidal activity against *Y. ruckeri* and this
316 was supported by a wide dissimilarity in the acrophases of the two groups (**Fig. 3B**,
317 **Supplementary Table 1**). The acrophase for the early fraction was at ZT 7.05 while for
318 the late fraction was registered 4 hours later, at ZT 11.6. The data reveal that serum-
319 mediated bacterial killing activity against *Y. ruckeri* is possibly dissimilar between the

320 early and late fractions as indicated by their highly variable day-night profile (**Fig. 2**)
321 and daily rhythmic trend (**Fig. 3B**); however, the analysed serum factors did not
322 provide such a strong support (**Fig. 4**). It is yet to be established the biological
323 significance of the difference amongst the fractions, especially on whether the
324 distinction is related to differential susceptibility to *Y. ruckeri* of the different
325 emergence groups. There was a large inter-individual variation in serum-mediated
326 bacterial killing activity against *A. salmonicida*, and no significant rhythm was found
327 (**Fig. 3C**). An *in vivo* time-dependent challenge experiment is a future strategy to
328 explore the underpinnings of the relatively stable bactericidal function against *A.*
329 *salmonicida* in the serum.

330 An array of potent molecules present in serum plays crucial roles in the
331 protective mechanisms against bacterial pathogens [37]. Hence, the participation of
332 immune defence factors was investigated in relation to the observed daily rhythm in
333 serum-mediated bacterial killing activity in the model fish. Five immune defence
334 factors were profiled in the present study, alkaline phosphatase (ALP), anti-protease
335 (ANTI), ceruloplasmin (CERU), lysozyme (LYS) and myeloperoxidase (MPO) (**Fig. 4A-E**).
336 These defence factors are known to be key mediators in the humoral defence
337 mechanisms in rainbow trout [41-43]. Serum ANTI and MPO activities exhibited
338 significant daily rhythms during the LD cycle. ANTI activity was at lowest in the
339 beginning of the dark phase whereas its peak was identified in the early hours of the
340 light phase (**Fig. 4B**). This was substantiated by COSINOR analysis revealing the
341 acrophase at ZT 2.83 for the early fraction and ZT 2.55 for the late fraction
342 (**Supplementary Table 1**). To our knowledge, this is the first report to demonstrate

343 that ANTI activity in fish serum exhibits daily rhythmicity. Protease has been regarded
344 as one of main virulence elements present amongst the extracellular factors in a
345 number of pathogens and a contributor to the pathogenesis of infections [44]. The
346 dynamic changes in ANTI activity may be critical in ensuring that the system targeting
347 potent bacterial proteases is well-regulated by having a peak-rest trend. This
348 partitioning strategy may be more efficient as it allows the system to be at its peak at a
349 certain time and at the same time provides a period to recover and regenerate [4].
350 MPO is produced by immune cells, such as the neutrophils, and plays a significant role
351 in the bactericidal function in fish [5, 45]. In the present study, rhythmicity was
352 observed in the MPO activity with a peak of activity at ZT 10.6 for the early fraction
353 and at ZT 9.57 for the late fraction (**Fig. 4E, Supplementary Table 1**). Serum MPO
354 activity has been demonstrated to exhibit daily rhythm in a number of fish species,
355 including permit (*Trachinotus falcatus*) [4] and in two species of tilapia (*O. niloticus* and
356 *O. mossambicus*) [5, 16]. This poses a possibility that daily rhythmicity of MPO may be
357 conserved within teleost fish and may have an active role in the temporal dynamics of
358 serum humoral immunity in fish. The relatively constant level in the daily activities of
359 ALP, CERU and LYS (**Fig. 4A,C,D**) indicates their involvement in the homeostasis of
360 humoral immunosurveillance throughout the day.

361 There were positive correlations in the daily dynamics of bacterial killing
362 activity and immune defence factors in serum (**Supplementary Table 2**). This was
363 particularly conspicuous between rhythmic bacterial killing activity (*i.e.*, against *F.*
364 *psychrophilum* and *Y. ruckeri*) and rhythmic MPO. We speculate that MPO is one of the
365 key defence molecules involved in the rhythmic bacterial killing activity against *F.*

366 *psychrophilum* and *Y. ruckeri*; nonetheless the present study had limitations drawing a
367 concrete causation. We constructed an acrophase map to show the pictographic
368 relationship of the peaks of activities of bactericidal activity and immune defence
369 factors in serum (**Fig. 5**). The peaks of bacterial killing activity coincided with the
370 acrophases of most of the immune defence factors. Though we could not definitely
371 conclude the direct involvement of these immune molecules in the observed
372 heightened bacterial killing ability at that particular period, this temporal concurrence
373 points to the probable participation of these immune molecules, given their known
374 antimicrobial functions [41-43]. Mechanistic and functional studies should be explored
375 in the future to investigate this implicated relationship.

376 In wild salmonids, there has been a documented correlation between larval
377 emergence time and SCS: early-emerging individuals display characteristics associated
378 with a proactive SCS, including higher boldness, aggression and metabolic rates than
379 late-emerging individuals [18, 19]. Screening strategy based on this stress-coping style
380 is a promising approach in aquaculture, but there is a big lacuna in our understanding
381 of the underlying mechanisms of these differences. The fish used in the present study
382 was from a sister experiment that aimed to understand the physiological differences
383 between the different emergence fractions in rainbow trout [17]. Since earlier
384 evidence suggested that proactive SCS was also more resistant to certain diseases [20,
385 21], we speculated that there might be distinct immunological differences between
386 the two fractions. Employing a comparative approach in the series of experiments,
387 overall results indicated that the early and late fractions exhibited no remarkable
388 immunological differences, at least based on the biomarkers used in the present study.

389 Though there were a few instances that variations existed between early and late
390 fractions (**Fig. 2**), the changes could not affirmatively characterise the immunological
391 distinction amongst the groups as results of other sub-experiments did not offer
392 striking support (**Figs. 3 and 4**). It could be possible that domestication has an influence
393 on the immunological profiles of these two fractions. It was speculated in our previous
394 study that the origin and degree of domestication may be partly responsible in the
395 absence of correlation between emergence time and growth performance, social
396 competitive ability or stress response in this batch of fish [17]. Moreover, a previous
397 study in Atlantic cod (*Gadus morhua*) showing that serum-mediated bacterial killing
398 activity significantly changed following domestication lends support to our implication
399 [2].

400 Taken together, this study showed that serum-mediated bacterial killing
401 activity against *F. psychrophilum* and *Y. ruckeri* exhibited significant daily rhythms
402 during the LD cycle in rainbow trout. However, such daily dynamic changes were not
403 observed in the bactericidal activity against *A. salmonicida*. The daily changes in the
404 levels of key immune defence factors in serum are likely involved in the observed
405 variations in bacterial killing activity. This was supported by the correlation of the daily
406 changes and the concurrence of acrophases of bacterial killing activity and immune
407 defence factors during the LD cycle. The results of the present study add support to
408 the emerging field of chronoimmunology and offer new insights into the interplay of
409 immunity and circadian rhythms in fish.

410

411

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418

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- 554

555 **Figure captions:**

556

557 **Figure 1. Serum-mediated bacterial killing activity against *F. psychrophilum*, *Y.***
558 ***ruckeri* and *A. salmonicida*.** The serum samples were collected at ZT3 from fish reared
559 under 14L:10D photoperiod. Values presented are mean + SE of 8 individual fish. No
560 significant difference in the bactericidal activity against a specific pathogen between
561 the emergence fractions was detected. Bactericidal activity towards the different
562 pathogens within a fraction showed significant differences: different letters indicate
563 significant differences in the early fraction, while different numbers for the late
564 fraction.

565

566 **Figure 2. Day-night variations in the serum-mediated bacterial killing activity against**
567 ***F. psychrophilum*, *Y. ruckeri* and *A. salmonicida*.** The serum samples were collected at
568 ZT3 (day) and ZT16 (night) from fish reared under 14L:10D photocycle. Values
569 presented are mean + SE of 8 individual fish. Different letters indicate significant
570 difference between early and late fractions at ZT3. On the other hand, different
571 numbers indicate significant difference between early and late fractions at ZT16.
572 Asterisk (*) denotes significant difference between ZT3 and ZT16 in either early or late
573 fraction.

574

575 **Figure 3. Daily rhythms in serum-mediated bacterial killing activity against *F.***
576 ***psychrophilum*, *Y. ruckeri* and *A. salmonicida*.** Fish were entrained to 12L:12D
577 photoperiod for 21 days. Thereafter, serum samples were collected at 6-h intervals for

578 a period of 24 h within 2 intersecting LD cycles. Values presented are mean \pm SE of 6
579 individual fish per time-point. Different letters indicate significant difference in the
580 activity of early fractions, whereas different numbers indicate significant difference in
581 the activity of late fractions during the LD cycle. Asterisk (*) denotes that the changes
582 exhibited significant daily rhythm. The broken line (blue: early fraction, orange: late
583 fraction) is the periodic sinusoidal function of the bacterial killing activity in the LD
584 cycle constructed from the rhythmicity parameters revealed by COSINOR. The bar
585 above the graphs show the photoperiod regime: white block represents the light phase
586 while the black counterpart is the dark phase.

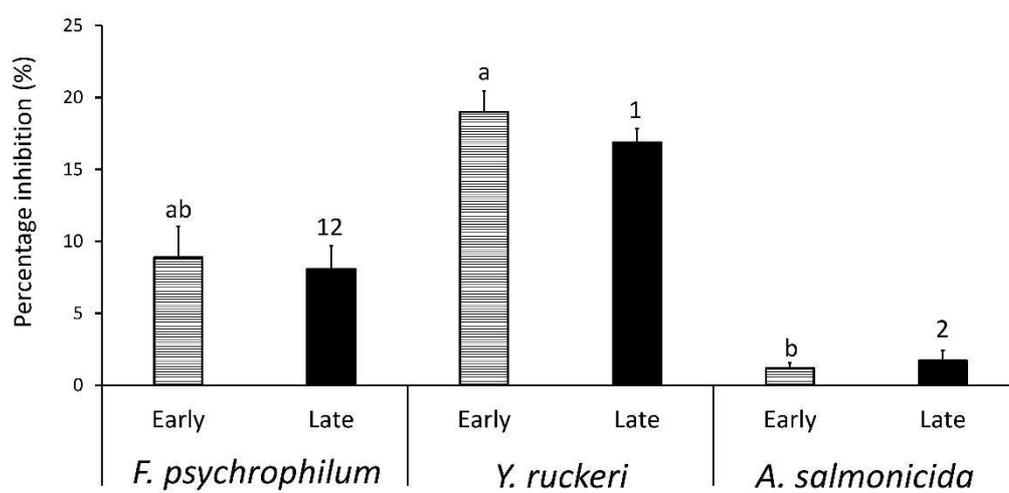
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588 **Figure 4. Daily rhythms in the immune defence factors in serum.** Other details of the
589 graph are given in Figure 3. ALP: alkaline phosphatase, ANTI: anti-protease, CERU:
590 ceruloplasmin, LYS: lysozyme, MPO: myeloperoxidase.

591

592 **Figure 5. Acrophase map.** The acrophase is indicated by symbols and the fiducial limits
593 (set at 95 %) are shown by lateral bars. Symbols with blue fill represent the acrophases
594 from the early fraction while those with orange fill represent the acrophases of the
595 late fraction. The white and black bars above the graph represent the light and dark
596 phase, respectively. Fp = *F. psychrophilum*, Yr = *Y. ruckeri*, As = *A. salmonicida*. Refer to
597 Figure 4 for additional information.

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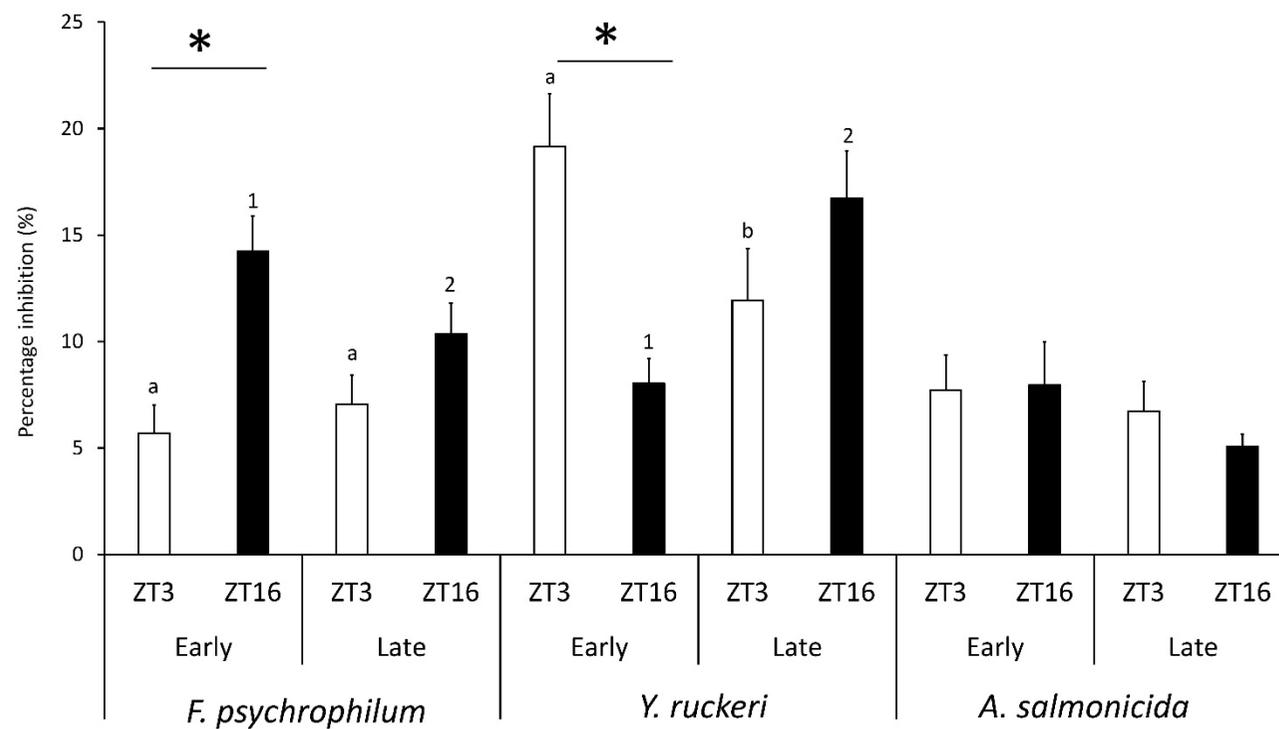


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Figure 1.



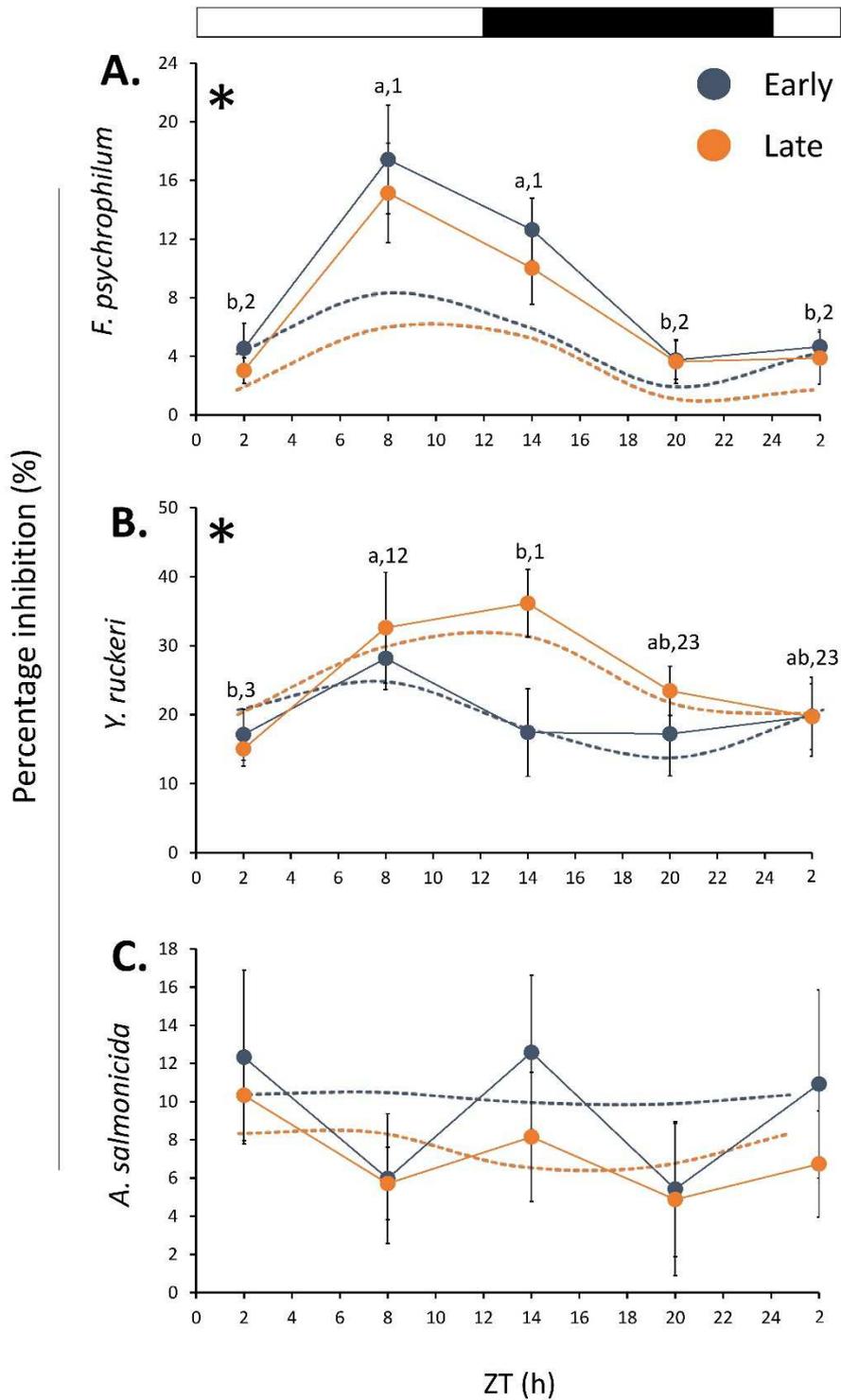
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Figure 2.

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Figure 3.

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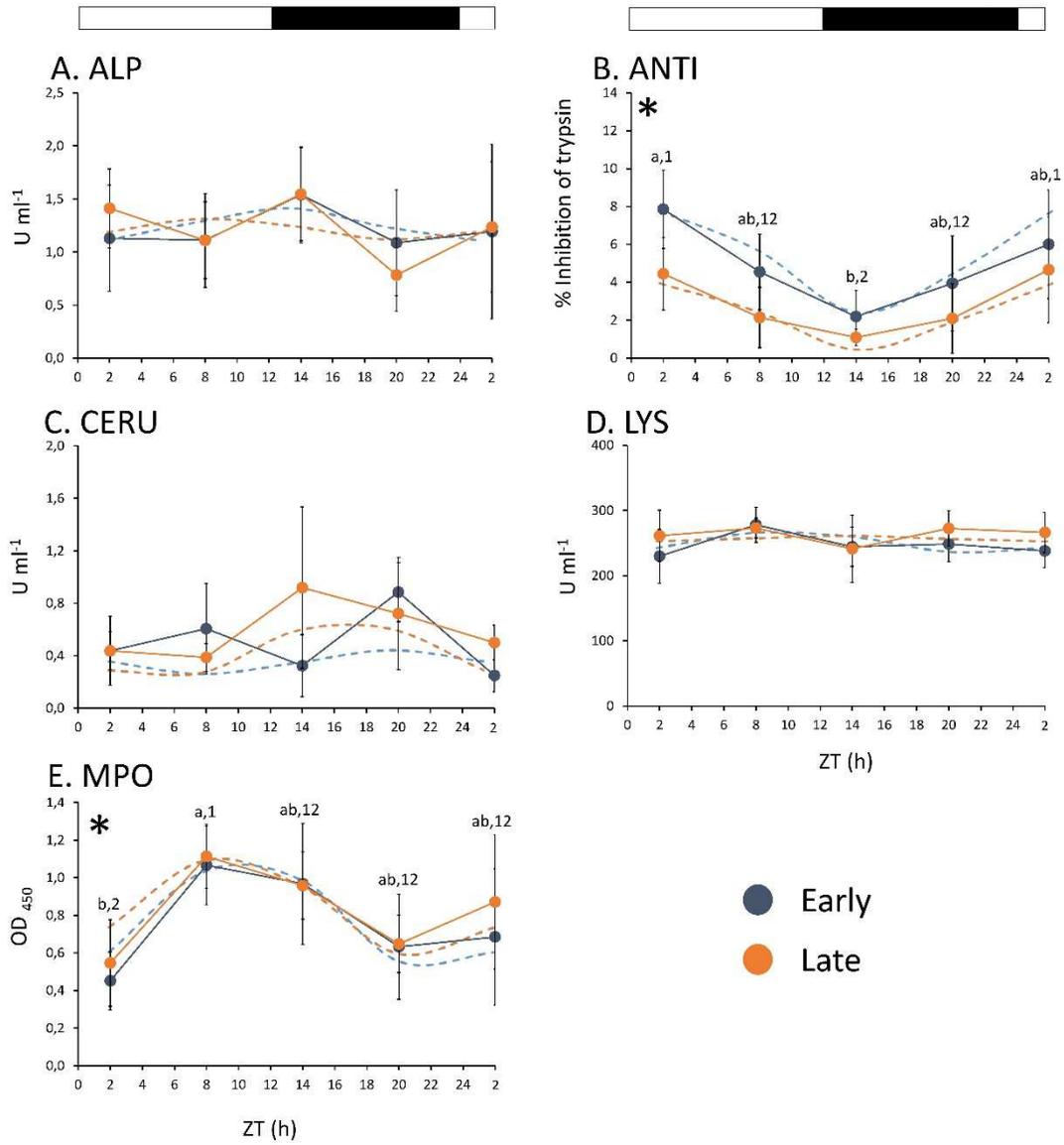
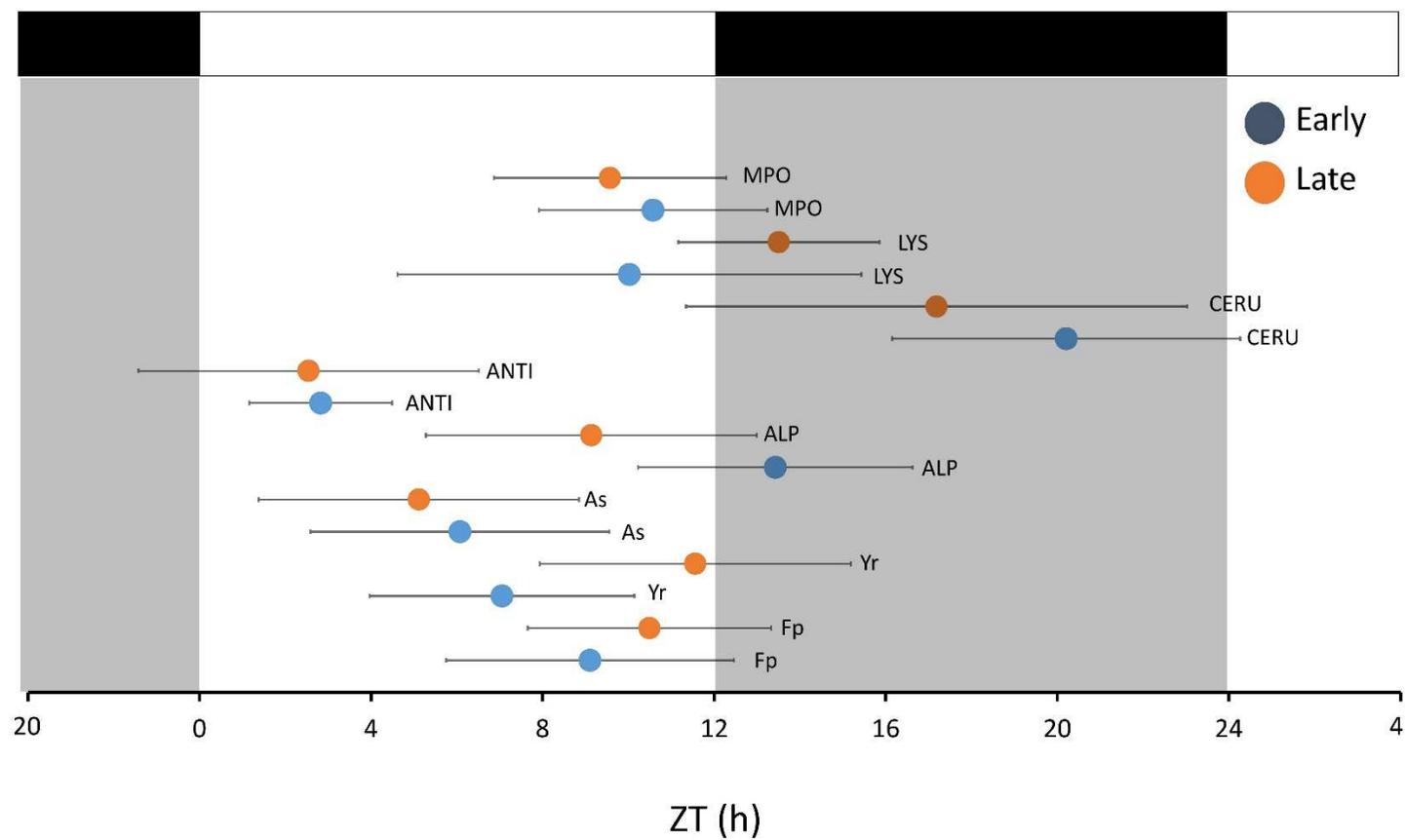
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Figure 4

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Figure 5

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Interplay between daily rhythmic serum-mediated bacterial killing activity and immune defence factors in rainbow trout (*Oncorhynchus mykiss*)

Carlo C. Lazado, Manuel Gesto, Lone Madsen, Alfred Jokumsen

HIGHLIGHTS

- Serum-mediated bacterial killing activity varied dynamically during the LD cycle.
- Bactericidal activity towards *F. psychrophilum* and *Y. ruckeri* displayed daily rhythms.
- The level of immune defence factors exhibited daily oscillatory pattern in serum.
- Activity peaks of immune factors corresponded with the period of highest bactericidal activity.
- Early and late emergence fractions showed nearly comparable immune profiles.