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

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29 **Abbreviations**

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

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

32

Abstract

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

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

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

2.1. Ethics statement

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

2.2. Target pathogens

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

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

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

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

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

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

The influence of time of the day on the bactericidal activity of fish serum was investigated. The procedure for serum collection and preparation was similar to those described above except the time of collection. Serum samples were collected from 8 fish (mean ± SD: 46.6 ± 7.5 g) from each emergence fraction during the day (ZT3) and another batch during the night (ZT16; 2 h after lights off). Our preliminary studies revealed that these time-points were suitable to show day-night variations. Sample collection during the night was performed in a room with red illumination (< 3 lux) and exposure of an individual anesthetised fish to the lighting condition was no 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 ± 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, NH_3/NH^+_4, pH) were monitored every 2 days and kept within safe limits, *i.e.*, NH_3-N < 0.025 mg/l; NH_4-N < 5 mg/l; NO_2^- - N < 10 mg/l; NO_3^- - N < 100 mg/l; pH ≈ 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 3,30,5,50-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: $ƒ(t) = M + A\cos(t/pi/12 - ϕ)$, where ƒ (t) is the level of the parameter at given time, mesor (M) is the
247
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].

3. Results and Discussion

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

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

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

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

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

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

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Figure captions:

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

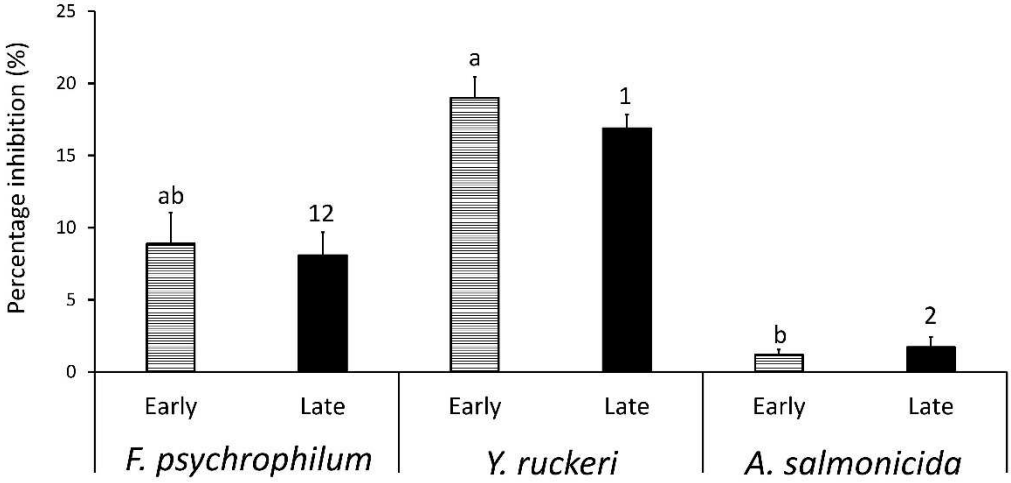
Figure 2. Day-night variations in the serum-mediated bacterial killing activity against *F. psychrophilum*, *Y. ruckeri* and *A. salmonicida*. The serum samples were collected at ZT3 (day) and ZT16 (night) from fish reared under 14L:10D photocycle. Values presented are mean + SE of 8 individual fish.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

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

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

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

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



599

600 Figure 1.

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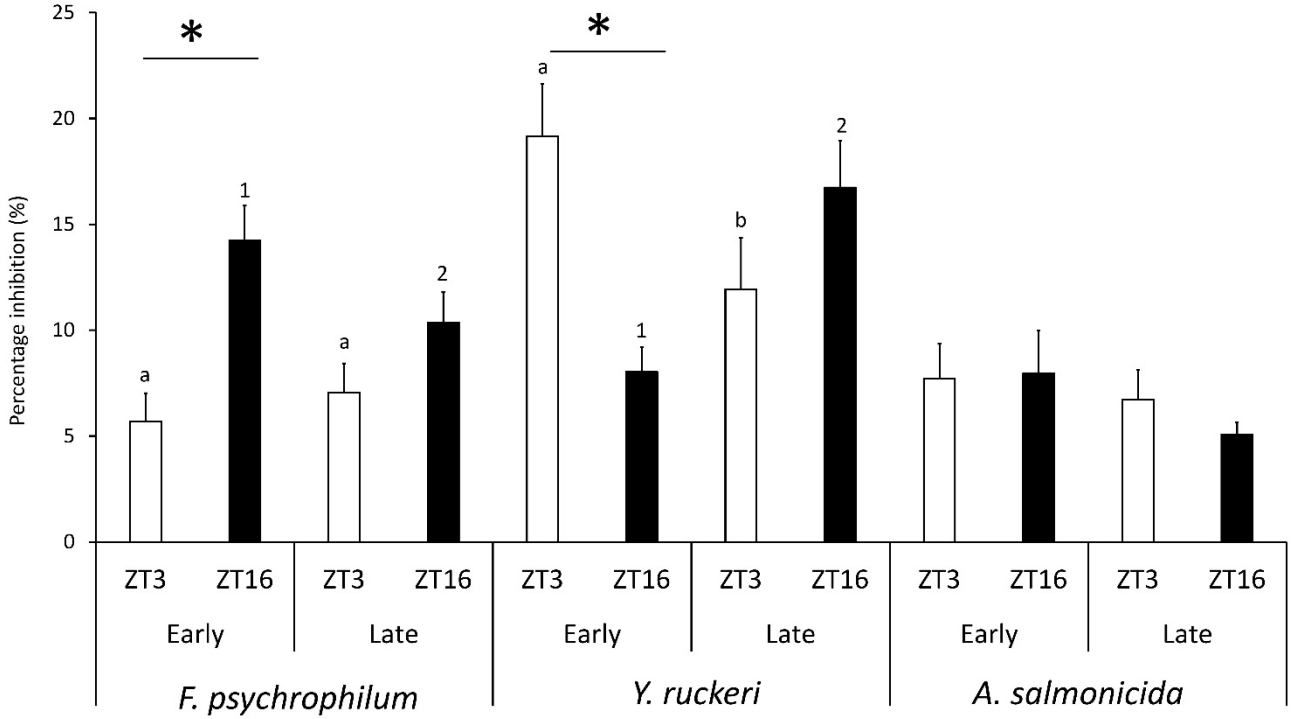
Percentage inhibition (%)
25
20
15
10
5
0
*
*
a
1
a
2
a
1
b
2
ZT3
ZT16
Early
Late
F. psychrophilum
Y. ruckeri
A. salmonicida

Figure 2.

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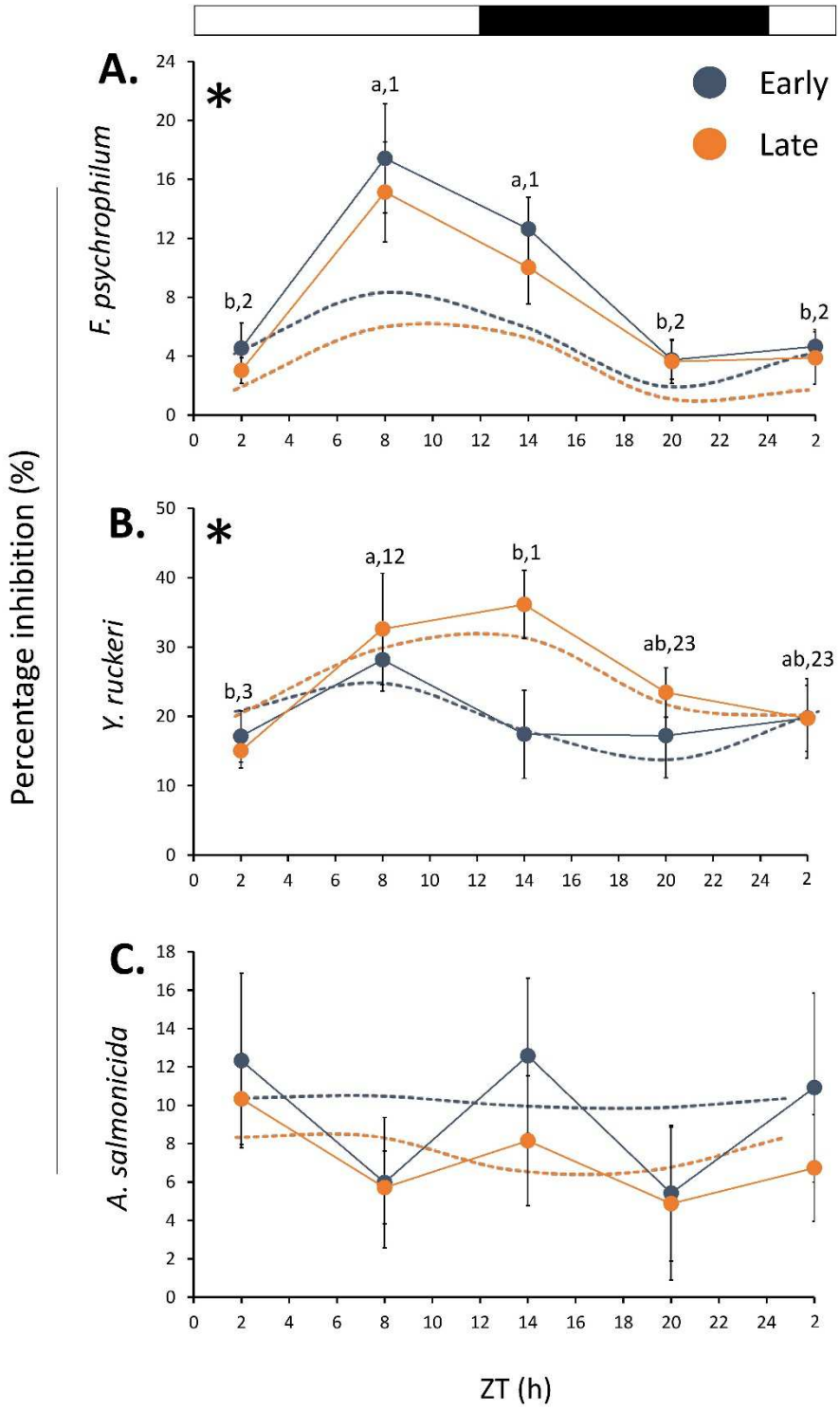

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607 Figure 3.

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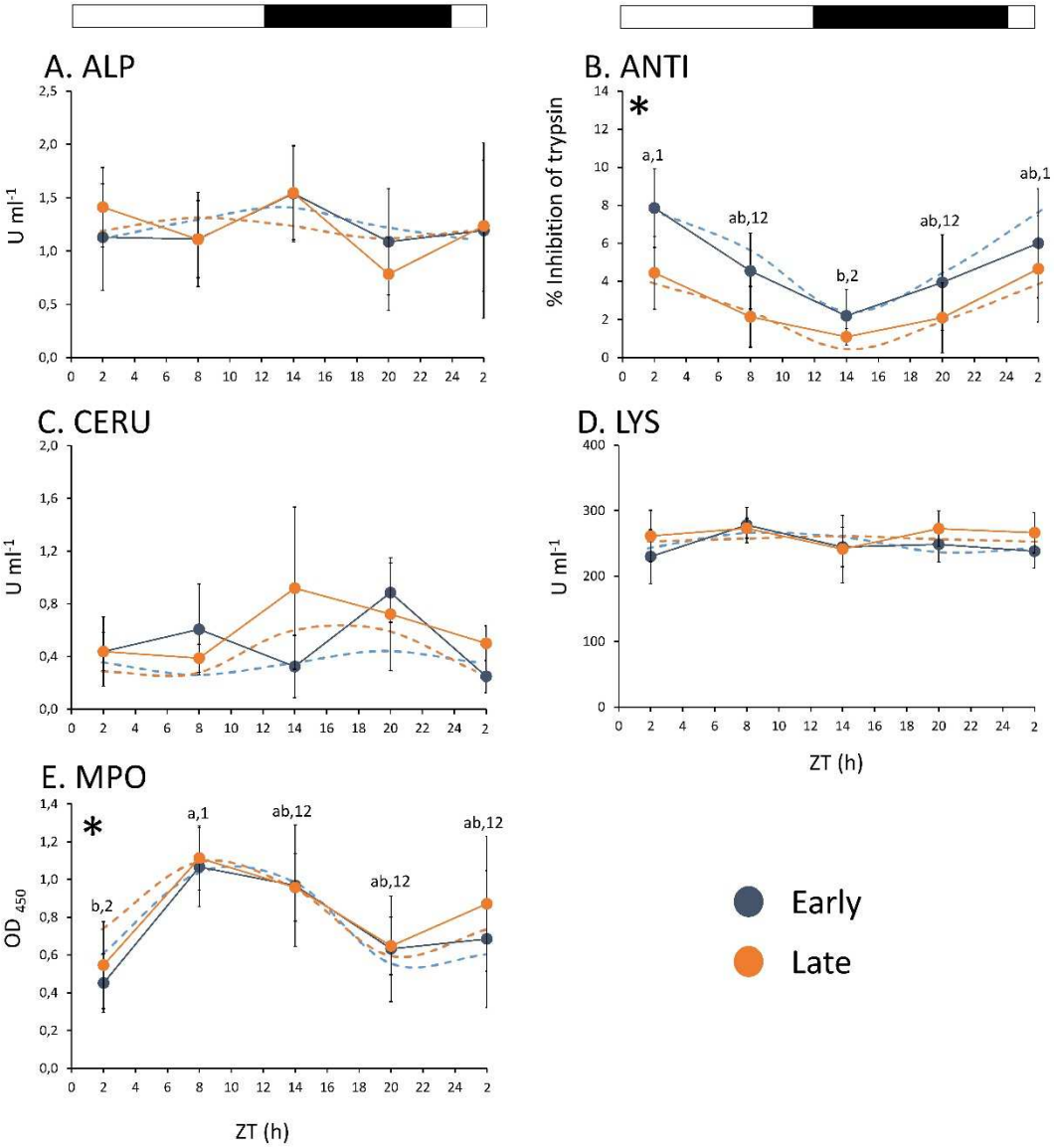

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Figure 4

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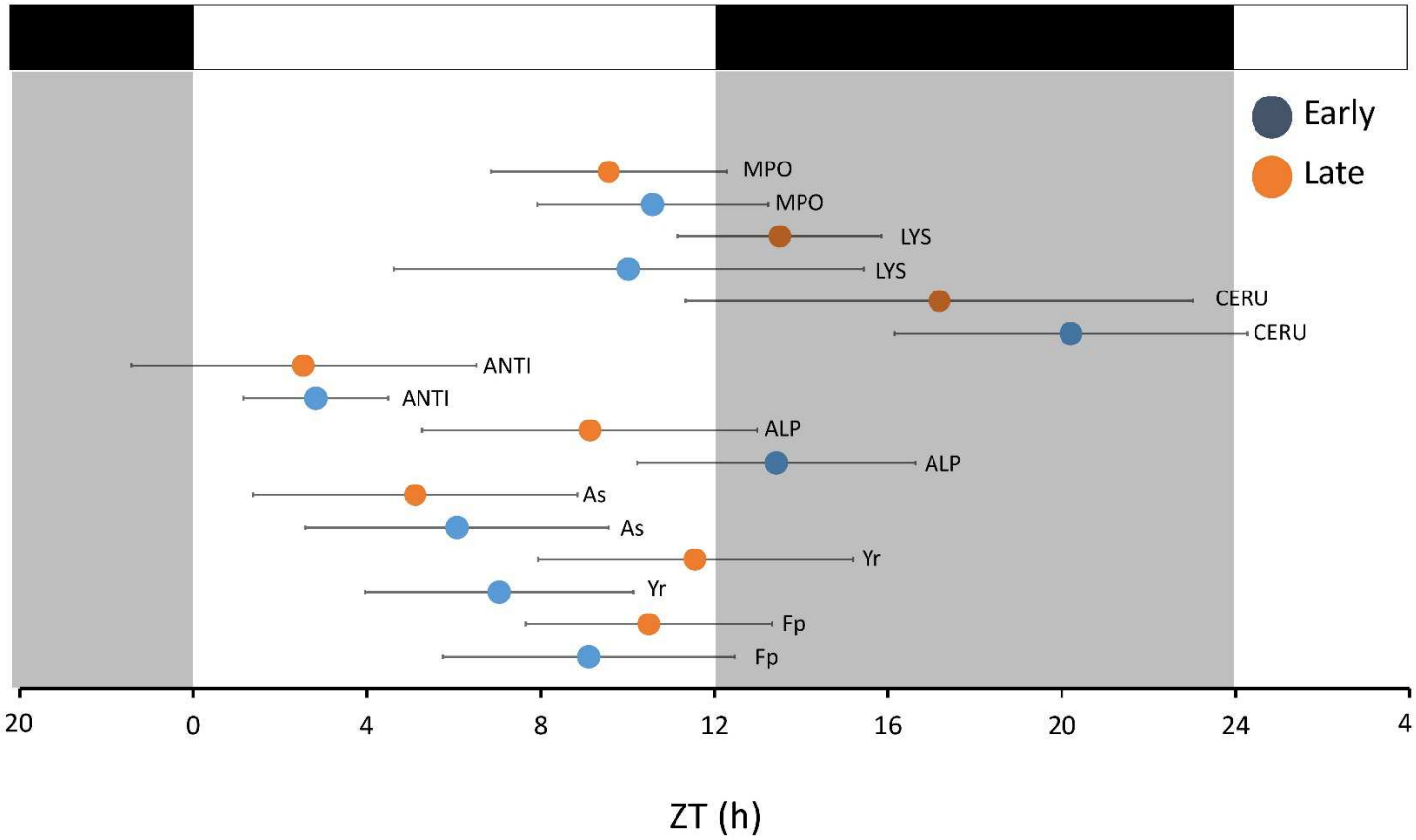

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613 Figure 5

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.