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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; LYS,

31 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

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

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166 The fish were anesthetised (benzocaine solution, 50 mg L^{-1}) and blood was withdrawn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

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

181 **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 undiluted serum and the target pathogen after a 24-h incubation at 15°C. Bacterial

viability was evaluated based on the ability of viable cells to reduce MTT to formazan crystals. The absorbance was measured with a microplate reader (Fluostar Optima) at 630 nm and the MTT reduction was thereafter compared with the control group to calculate the percentage of inhibition.

2.6. Soluble immune defence factors in serum

Humoral immune defence factors were characterised by spectrophotometric quantifications in the serum samples collected in Section 2.4. Alkaline phosphatase (ALP) activity was quantified through a kinetic reaction assay using *p*-nitrophenyl phosphate as a substrate [27]. One unit of activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol product in 1 min. The inhibition of trypsin activity was employed to determine the anti-protease (ANTI) activity in serum [28]. Percentage of inhibition was calculated by comparing it to 100 % control. The level of ceruloplasmin (CERU) was measured enzymatically in a reaction mixture containing *para*-phenylenediamine-sodium azide in acetate buffer, as previously described [29]. The kinetic increase of absorbance was followed for 15 min and 1 unit was defined as an increase of optical density (OD) of 0.001 min^{-1} at 550 nm. Lysozyme (LYS) activity was quantified by a turbidimetric method [30], following a modified protocol for 96-well microplate reaction [31]. A unit of lysozyme activity was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 per minute at 450 nm. Myeloperoxidase (MPO) was measured following previously described protocol [32], with modifications [4], using 3,30,5,50-tetramethyl benzidine hydrochloride as a reaction substrate. Unit of activity was expressed as OD at 450 nm. All absorbance

measurements were conducted in a microplate reader (TECAN GENios, Salzburg, Austria).

2.7. Statistical analyses

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

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

318 indicated by their highly variable day-night profile (**Fig. 2**) and daily rhythmic trend (**Fig.**
319 **3B**); however, the analysed serum factors did not provide such a strong support (**Fig. 4**).
320 It is yet to be established the biological significance of the difference amongst the
321 fractions, especially on whether the distinction is related to differential susceptibility to
322 *Y. ruckeri* of the different emergence groups. There was a large inter-individual variation
323 in serum-mediated bacterial killing activity against *A. salmonicida*, and no significant
324 rhythm was found (**Fig. 3C**). An *in vivo* time-dependent challenge experiment is a future
325 strategy to explore the underpinnings of the relatively stable bactericidal function
326 against *A. salmonicida* in the serum.

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

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

There were positive correlations in the daily dynamics of bacterial killing activity and immune defence factors in serum (**Supplementary Table 2**). This was particularly conspicuous between rhythmic bacterial killing activity (*i.e.*, against *F. psychrophilum* and *Y. ruckeri*) and rhythmic MPO. We speculate that MPO is one of the key defence molecules involved in the rhythmic bacterial killing activity against *F. psychrophilum* and *Y. ruckeri*; nonetheless the present study had limitations drawing a concrete causation. We constructed an acrophase map to show the pictographic

relationship of the peaks of activities of bactericidal activity and immune defence factors in serum (**Fig. 5**). The peaks of bacterial killing activity coincided with the acrophases of most of the immune defence factors. Though we could not definitely conclude the direct involvement of these immune molecules in the observed heightened bacterial killing ability at that particular period, this temporal concurrence points to the probable participation of these immune molecules, given their known antimicrobial functions [41-43]. Mechanistic and functional studies should be explored in the future to investigate this implicated relationship.

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

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

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

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References

[1] A.E. Ellis, Immunity to bacteria in fish, Fish & Shellfish Immunology 9(4) (1999) 291-308.

[2] C.M.A. Caipang, N. Hynes, M.F. Brinchmann, K. Korsnes, V. Kiron, Antimicrobial activity in serum of Atlantic cod *Gadus morhua* L.: comparison between cultured and wild fish, Journal of Fish Biology 73(1) (2008) 115-122.

[3] G.E. Rainger, A.F. Rowley, Antibacterial activity in the serum and mucus of rainbow trout, *Oncorhynchus mykiss*, following immunisation with *Aeromonas salmonicida*, Fish & Shellfish Immunology 3(6) (1993) 475-482.

[4] C.C. Lazado, I. Lund, P.B. Pedersen, H.Q. Nguyen, Humoral and mucosal defense molecules rhythmically oscillate during a light–dark cycle in permit, *Trachinotus falcatus*, Fish & Shellfish Immunology 47(2) (2015) 902-912.

[5] C.C. Lazado, P.V. Skov, P.B. Pedersen, Innate immune defenses exhibit circadian rhythmicity and differential temporal sensitivity to a bacterial endotoxin in Nile tilapia (*Oreochromis niloticus*), Fish & Shellfish Immunology 55 (2016) 613-622.

[6] M. Dong, S.W. Tang, W. Shen, D.W.T. Au, Y.W. Lam, Characterization of the interactions between fish serum proteins and pathogenic bacteria by using label-free quantitative proteomics, The Journal of Immunology 196(1 Supplement) (2016) 63.5-63.5.

[7] J.D. Biller-Takahashi, L.S. Takahashi, F. Pilarski, F.A. Sebastião, E.C. Urbinati, Serum bactericidal activity as indicator of innate immunity in pacu *Piaractus mesopotamicus* (Holmberg, 1887), Arquivo Brasileiro de Medicina Veterinária e Zootecnia 65 (2013) 1745-1751.

[8] G. Vatine, D. Vallone, Y. Gothilf, N.S. Foulkes, It's time to swim! Zebrafish and the circadian clock, FEBS Letters 585(10) (2011) 1485-1494.

[9] M.L. Idda, C. Bertolucci, D. Vallone, Y. Gothilf, F.J. Sánchez-Vázquez, N.S. Foulkes, Circadian clocks, Progress in Brain Research 199 (2012) 41-57.

[10] C.C. Lazado, H.P. Kumaratunga, K. Nagasawa, I. Babiak, A. Giannetto, J.M. Fernandes, Daily rhythmicity of clock gene transcripts in Atlantic cod fast skeletal muscle, PLoS One 9(6) (2014) e99172.

[11] R. Dumbell, O. Matveeva, H. Oster, Circadian clocks, stress, and immunity, Frontiers in Endocrinology 7 (2016) 37.

[12] Anne M. Curtis, Marina M. Bellet, P. Sassone-Corsi, Luke A.J. O'Neill, Circadian clock proteins and immunity, Immunity 40(2) 178-186.

[13] N. Labrecque, N. Cermakian, Circadian clocks in the immune system, Journal of Biological Rhythms 30(4) (2015) 277-290.

[14] L.L. Tsoumtsa, C. Torre, E. Ghigo, Circadian control of antibacterial immunity: findings from animal models, Frontiers in Cellular and Infection Microbiology 6 (2016) 54.

[15] M. Ángeles Esteban, A. Cuesta, A. Rodríguez, J. Meseguer, Effect of photoperiod on the fish innate immune system: a link between fish pineal gland and the immune system, Journal of Pineal Research 41(3) (2006) 261-266.

[16] C. Binuramesh, R.D. Michael, Diel variations in the selected serum immune parameters in *Oreochromis mossambicus*, Fish & Shellfish Immunology 30(3) (2011) 824-829.

[17] M. Gesto, P.V. Skov, A. Jokumsen, Emergence time and skin melanin spot patterns do not correlate with growth performance, social competitive ability or stress response in farmed rainbow trout, Frontiers in Neuroscience 11 (2017) 319.

[18] N. Metcalfe, J. Thorpe, Early predictors of life-history events: the link between first feeding date, dominance and seaward migration in Atlantic salmon, *Salmo salar* L, Journal of Fish Biology 41(sB) (1992) 93-99.

[19] M.Å. Andersson, U.W. Khan, Ø. Øverli, H.M. Gjøen, E. Höglund, Coupling between stress coping style and time of emergence from spawning nests in salmonid fishes: Evidence from selected rainbow trout strains (*Oncorhynchus mykiss*), Physiology & Behavior 116 (2013) 30-34.

469 [20] S.E. Fevolden, R. Nordmo, T. Refstie, K.H. Røed, Disease resistance in Atlantic
470 salmon (*Salmo salar*) selected for high or low responses to stress, Aquaculture 109(3)
471 (1993) 215-224.

472 [21] S.E. Fevolden, T. Refstie, K.H. Røed, Disease resistance in rainbow trout
473 (*Oncorhynchus mykiss*) selected for stress response, Aquaculture 104(1) (1992) 19-29.

474 [22] R.A. Holt, J.S. Rohovec, J.L. Fryer, Bacterial coldwater disease, in: V. Englis, R.
475 Roberts, N. Bromage (Eds.), Bacterial diseases of fish, Blackwell Scientific Publications,
476 Oxford, 1993.

477 [23] L. Madsen, I. Dalsgaard, Reproducible methods for experimental infection with
478 *Flavobacterium psychrophilum* in rainbow trout *Oncorhynchus mykiss*, Diseases of
479 Aquatic Organisms 36(3) (1999) 169-176.

480 [24] J. Vaz-Serrano, M. Ruiz-Gomez, H. Gjøen, P.V. Skov, F. Huntingford, Ø. Øverli, E.
481 Höglund, Consistent boldness behaviour in early emerging fry of domesticated Atlantic
482 salmon *(Salmo salar*): decoupling of behavioural and physiological traits of the proactive
483 stress coping style, Physiology & Behavior 103(3) (2011) 359-364.

484 [25] C.M.A. Caipang, M.F. Brinchmann, V. Kiron, Short-term overcrowding of Atlantic
485 cod, *Gadus morhua*: Effects on serum-mediated antibacterial activity and transcription
486 of glucose transport and antioxidant defense related genes, Comparative Biochemistry
487 and Physiology Part A: Molecular & Integrative Physiology 151(4) (2008) 560-565.

488 [26] C.M.A. Caipang, I. Berg, M.F. Brinchmann, V. Kiron, Short-term crowding stress in
489 Atlantic cod, *Gadus morhua* L. modulates the humoral immune response, Aquaculture
490 295(1) (2009) 110-115.

491 [27] N.W. Ross, K.J. Firth, A. Wang, J.F. Burka, S.C. Johnson, Changes in hydrolytic
492 enzyme activities of naive Atlantic salmon *Salmo salar* skin mucus due to infection with
493 the salmon louse *Lepeophtheirus salmonis* and cortisol implantation, Diseases of Aquatic
494 Organisms 41(1) (2000) 43-51.

495 [28] A. Hanif, V. Bakopoulos, G.J. Dimitriadis, Maternal transfer of humoral specific and
496 non-specific immune parameters to sea bream (*Sparus aurata*) larvae, Fish & Shellfish
497 Immunology 17(5) (2004) 411-435.

498 [29] M. Dunier, C. Vergnet, A.K. Siwicki, V. Verlhac, Effect of lindane exposure on
499 rainbow trout (*Oncorhynchus mykiss*) immunity. IV. Prevention of nonspecific and
500 specific immunosuppression by dietary vitamin C (ascorbate-2-polyphosphate),
501 Ecotoxicology and Environmental Safety 30(3) (1995) 259-268.

[30] R.M. Parry Jr, R.C. Chandan, K.M. Shahani, A rapid and sensitive assay of muramidase, Proceedings of the Society for Experimental Biology and Medicine. 119 (1965) 384-386.

[31] A. Sitjà-Bobadilla, O. Palenzuela, P. Alvarez-Pellitero, Immune response of turbot, *Psetta maxima* (L.) (Pisces: Teleostei), to formalin-killed scuticociliates (Ciliophora) and adjuvanted formulations, Fish & Shellfish Immunology 24(1) (2008) 1-10.

[32] M.J. Quade, J.A. Roth, A rapid, direct assay to measure degranulation of bovine neutrophil primary granules, Veterinary Immunology and Immunopathology 58(3-4) (1997) 239-248.

[33] A. Nematollahi, A. Decostere, F. Pasmans, F. Haesebrouck, *Flavobacterium psychrophilum* infections in salmonid fish, Journal of Fish Diseases 26(10) (2003) 563-574.

[34] E. Tobback, A. Decostere, K. Hermans, F. Haesebrouck, K. Chiers, *Yersinia ruckeri* infections in salmonid fish, Journal of Fish Diseases 30(5) (2007) 257-268.

[35] B. Austin, D.A. Austin, Bacterial Fish Pathogens: Disease of Farmed and Wild Fish, Springer International Publishing2016.

[36] C. Dong, S. Bai, L. Du, Temperature regulates circadian rhythms of immune responses in red swamp crayfish *Procambarus clarkii*, Fish & Shellfish Immunology 45(2) (2015) 641-647.

[37] P.R. Rauta, B. Nayak, S. Das, Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms, Immunology Letters 148(1) (2012) 23-33.

[38] B. Magnadottir, H. Jonsdottir, S. Helgason, B. Bjornsson, T.O. Jorgensen, L. Pilstrom, Humoral immune parameters in Atlantic cod (*Gadus morhua* L.) II. The effects of size and gender under different environmental conditions, Comp Biochem Physiol B Biochem Mol Biol 122(2) (1999) 181-8.

[39] C. Uribe, H. Folch, R. Enriquez, G. Moran, Innate and adaptive immunity in teleost fish: a review, Veterinarni Medicina 56(10) (2011) 486-503.

[40] T.J. Bowden, K.D. Thompson, A.L. Morgan, R.M.L. Gratacap, S. Nikoskelainen, Seasonal variation and the immune response: A fish perspective, Fish & Shellfish Immunology 22(6) (2007) 695-706.

[41] K.H. Røed, S.E. Fevolden, K.T. Fjalestad, Disease resistance and immune characteristics in rainbow trout (*Oncorhynchus mykiss*) selected for lysozyme activity, Aquaculture 209(1) (2002) 91-101.

[42] H.Y. Yildiz, İ.M. Guzey, M.B. Ergonul, Changes of non-specific immune parameters in rainbow trout, *Oncorhynchus mykiss*, after exposure to antimicrobial agents used in aquaculture, Journal of Applied Aquaculture 21(3) (2009) 139-150.

[43] X. Zuo, P.T.K. Woo, Natural anti-proteases in rainbow trout, *Oncorhynchus mykiss* and brook charr, *Salvelinus fontinalis* and the *in vitro* neutralization of fish α2-macroglobulin by the metalloprotease from the pathogenic haemoflagellate, *Cryptobia salmositica*, Parasitology 114(4) (1997) 375-382.

[44] P. Secades, J.A. Guijarro, Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production, Applied and Environmental Microbiology 65(9) (1999) 3969-3975.

[45] R. Castro, M.C. Piazzon, M. Noya, J.M. Leiro, J. Lamas, Isolation and molecular cloning of a fish myeloperoxidase, Molecular Immunology 45(2) (2008) 428-437.

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.

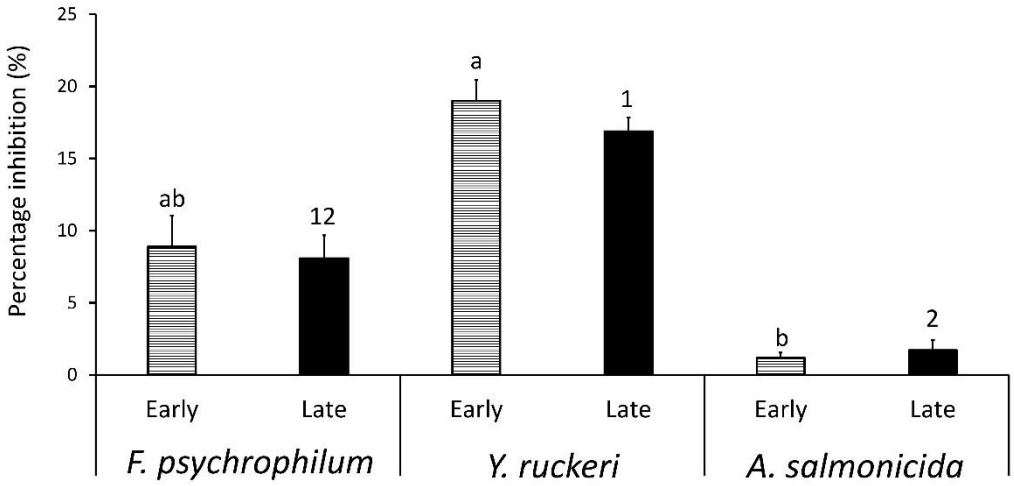
Figure 2. Day-night variations in the serum-mediated bacterial killing activity against *F. psychrophilum*, *Y. ruckeri* and *A. salmonicida*. The serum samples were collected at ZT3 (day) and ZT16 (night) from fish reared under 14L:10D photocycle. Values presented are mean + SE of 8 individual fish. 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.



592

593 Figure 1.

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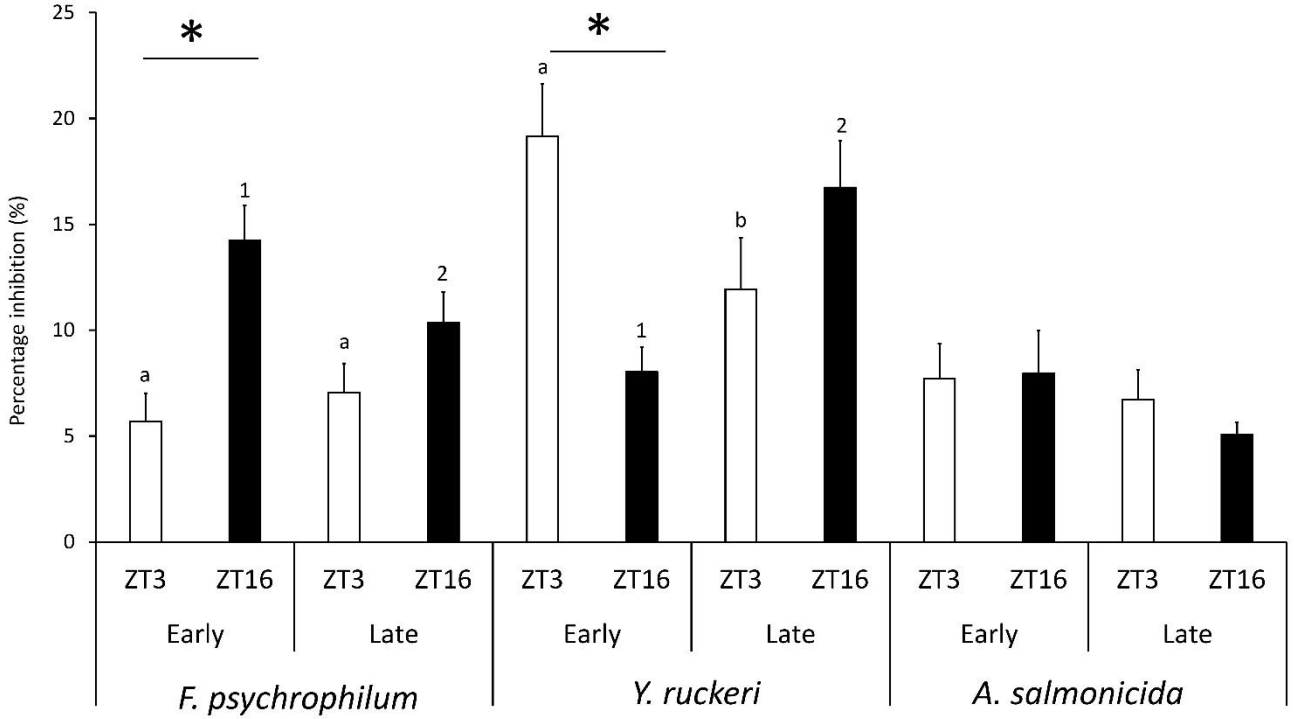

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597 Figure 2.

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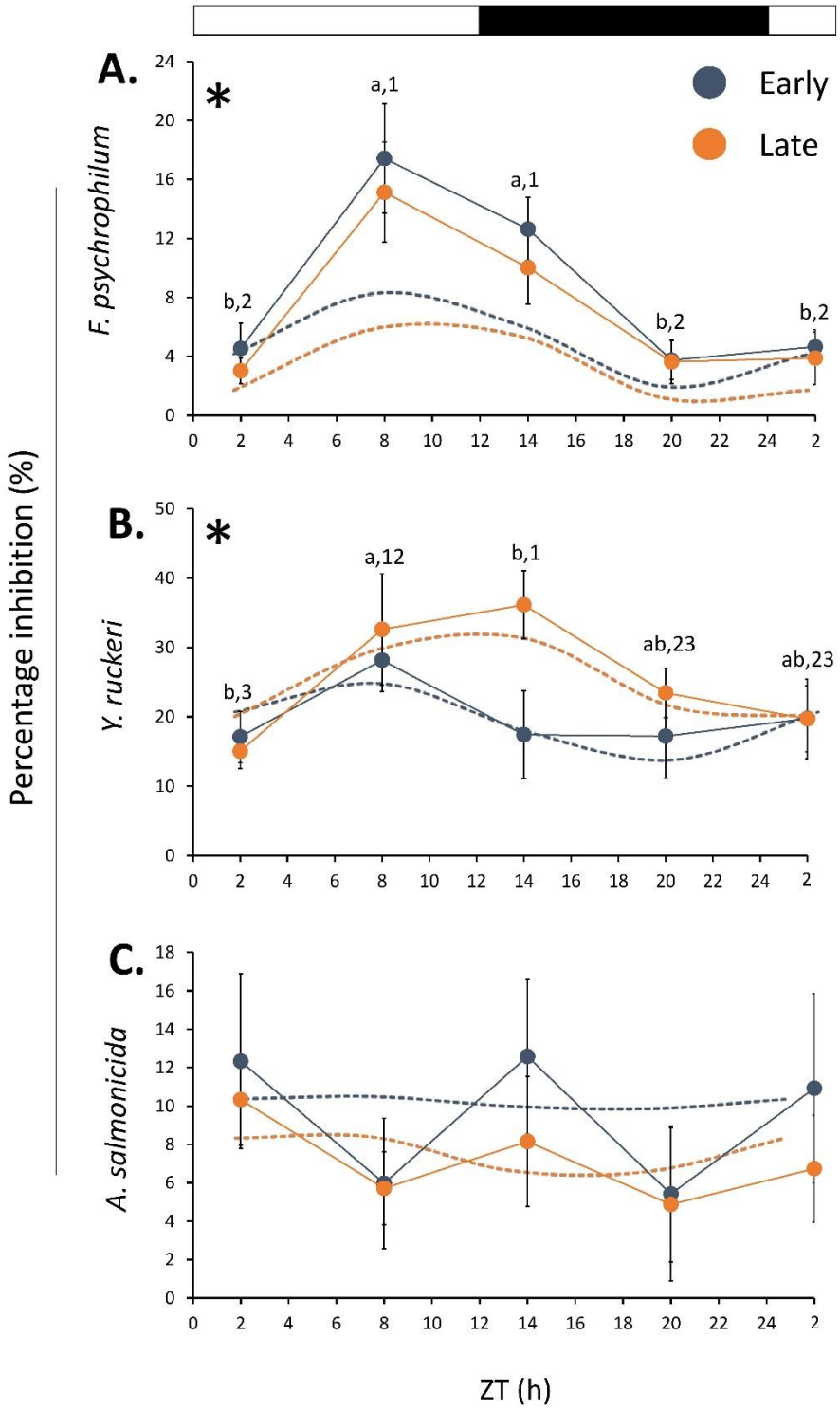

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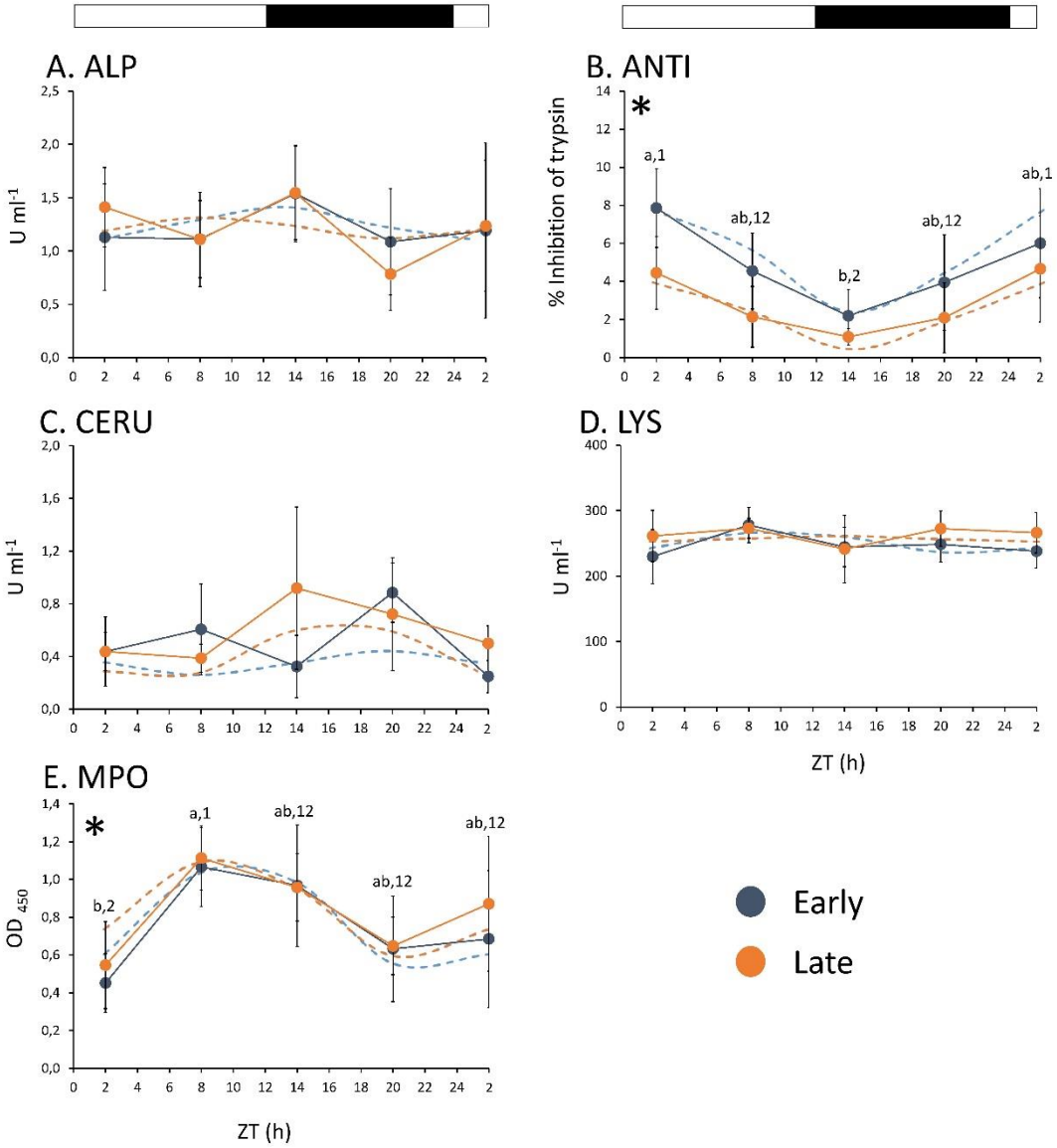

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603 Figure 4

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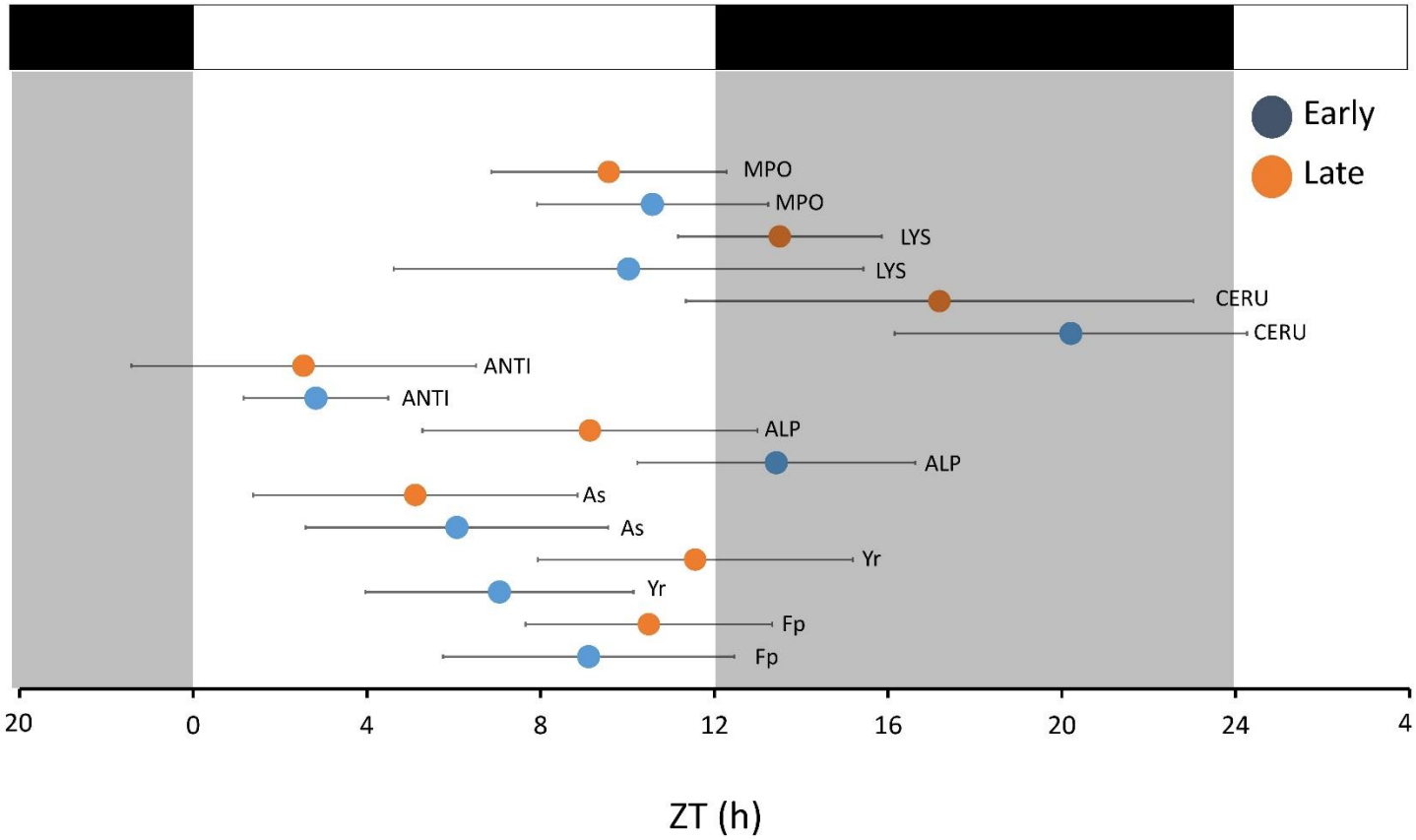

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606 Figure 5