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## Utilising Chronobiology For Sustainable Aquaculture Nutrition & Fish Health

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# UTILISING CHRONOBIOLOGY FOR SUSTAINABLE AQUACULTURE NUTRITION & FISH HEALTH

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PRIFYSGOL BANGOR UNIVERSITY

## I. ABSTRACT

Circadian rhythms are ubiquitous to life and are the most pervasive rhythm in nature; from bacteria to plants and animals, most organisms demonstrate daily physiological, behavioural, and metabolic cycles. Akin to other vertebrates, fish gut microbiota is critical to nutritional provision, metabolic homeostasis, and immune defence and it is evident that metabolism, circadian rhythms of activity and the gut microbiome are inextricably intertwined. As the aquaculture industry turns towards augmentation of fish microbiomes to promote health and productivity, it is suggested that a chronobiological understanding of fish microbiomes and feed treatment times may be crucial for their effectiveness and is necessary to ensure sustainable aquaculture nutrition and fish health. Using experimental feed trials, daily activity assays, and 16S rRNA microbiome profiling, I examined the effect of feed timing on rainbow trout growth, behaviour, and gut microbiome composition and daily rhythms. This study indicates that feed timing has no strong impact on growth, although trout fed early in the light cycle appeared to have a dampened circadian clock. Early fed fish displayed arrhythmic daily activity patterns and fewer gut microbial taxa exhibited rhythmicity in abundance. This dampening of the endogenous circadian clock of the host organism may be detrimental for fish welfare by perturbing the commensal microbiome, potentially instigating resounding effects on the functional pathways of microbiota and consequently fish health.

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# III. DECLARATION

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards. This work has not been accepted, and is not being submitted concurrently, for any award. This work is submitted with full support from the supervisor (Dr Amy Ellison).

# IV. ABBREVIATIONS

| *Symbol or acronym* | *Definition* |
|---|---|
| **GI** | **GASTRO-INTESTINAL** |
| **MF / AF** | **MORNING (EARLY) FEED / AFTERNOON (LATE) FEED** |
| **TRF** | **TIME RESTRICTED FEEDING** |
| **SCN** | **SUPRACHIASMATIC NUCLEUS** |
| **FAA** | **FOOD ANTICIPATORY ACTIVITY** |
| **WHO** | **WORLD HEALTH ORGANISATION** |
| **FAO** | **FOOD & AGRICULTURE ORGANISATION** |
| **bHLH** | **BASIC HELIX-LOOP HELIX** |
| **LD LL DD** | **LIGHT/DARK LIGHT/LIGHT DARK/DARK** |
| **RER** | **RESPIRATORY EXCHANGE RATIO** |
| **ASV** | **AMPLICON SEQUENCE VARIANT** |
| **DAM** | **DROSOPHILA ACTIVITY MONITOR** |
| **PCR** | **POLYMERASE CHAIN REACTION** |
| **SGR** | **SPECIFIC GROWTH RATE** |
| **SL** | **STANDARD LENGTH** |
| **ZT** | **ZEITGERBER** |

# UTILISING CHRONOBIOLOGY FOR SUSTAINABLE AQUACULTURE NUTRITION & FISH HEALTH

CGMG, SCHOOL OF NATURAL SCIENCES (SNS), BANGOR

# 1. INTRODUCTION

## 1.1 Chronobiology and the science of internal biological rhythms

Life on earth must anticipate one, or more, of four periodicities: the seasons, diurnal day/night cycles, the tides, and the lunar phases. These predictable rhythms in the environment have led to rhythmic processes within organisms orchestrated by "biological clocks", allowing the synchronisation of behaviour and physiology to conditions within a periodically changing system (Aschoff, 1984; Foulkes *et al.,* 2016). Earth's four cyclical periodicities result in predictable changes in biotic and abiotic conditions including food availability, temperature, and illumination (Zhdanova & Reebs, 2006). Circadian rhythms are ubiquitous to life and are the most pervasive rhythm in nature; from bacteria to plants and animals, most organisms demonstrate daily physiological, behavioural, and metabolic cycles (Brady, 1987). The vertebrate mammalian circadian system has been well described and is defined by a hierarchy of oscillators, centrally controlled by the suprachiasmatic nucleus (SCN) located in the anterior hypothalamus (Rusak & Zukar, 1979). The SCN is responsible for the coordination of the individual peripheral oscillators which ensure the orchestration of a consistent rhythm at the organismal level (Ko & Takahashi, 2006; Yamazaki *et al.,* 2000; Yoo *et al.,* 2004). The SCN and peripheral oscillators are known to have similar clock mechanisms at the molecular level (Balsalobre *et al.,* 2000; Brown *et al.,* 2005; Van den Driessche, 1989); consisting of a network of transcriptional– translational feedback loops that drive the oscillatory 24-h expression rhythms of core clock mechanisms (Ko & Takahashi, 2006; Lowrey & Takahashi, 2004; Reppert & Weaver, 2002). Core clock mechanisms are defined as protein products of genes that are necessary for the construction and regulation of circadian rhythms throughout cells within the organism (Takahashi, 2004). Within the primary feedback loop there are defined positive elements, which are a division of the basic helix-loop-helix (bHLH), these are known as

BMAL1 and CLOCK. There are also the defined negative arm proteins, comprised of PER, and CRY. Positive arm proteins stimulate the transcriptional activation of negative arm proteins. The negative arms proteins reactivate the positive arm, in a time specific manner, to pause the negative arm's activation prior to degradation and re-initiation, thus establishing the period of the circadian clock. These autoregulatory feedback loops (depicted in Figure 1) complete a full cycle over a 24-h period and constitute the mammalian circadian molecular clock (Ko & Takahashi, 2006). These molecular clocks are autonomous and self-sustaining even within dissociated cultured cells and peripheral tissues, and their discovery has revolutionized understanding of the mammalian circadian system hierarchy (Ko & Takahashi, 2006; Yamazaki *et al.,* 2000; Yoo *et al.,* 2004; Balsalobre *et al.,* 2000; Nagoshi *et al.,* 2004; Welsh *et al.,* 2004; Brown *et al.,* 2005).

Similar to mammalian circadian systems, chronobiological research conducted on zebrafish demonstrated that molecular clock components occur within all tissues in fish (Whitmore *et al.,* 1998, 2000). However, unlike mammals, there is clear indication that independent circadian pacemakers are present within all fish tissues. It is unclear whether fish possess a dominant circadian clock, although there are several separate clocks within the brain and pineal glands, as well as the liver and heart. There are two possibilities, one in which all peripheral oscillators synchronize independently, and the other in which a dominant component orchestrates peripheral clocks. However, contemporary literature suggests that the idea of a central or master clock is no longer a necessary concept in the field of chronobiology. The significance of fish whole-body light sensitivity, however, may be important in the field of fish circadian rhythms, physiology, and aquaculture (Steindal & Whitmore, 2019).

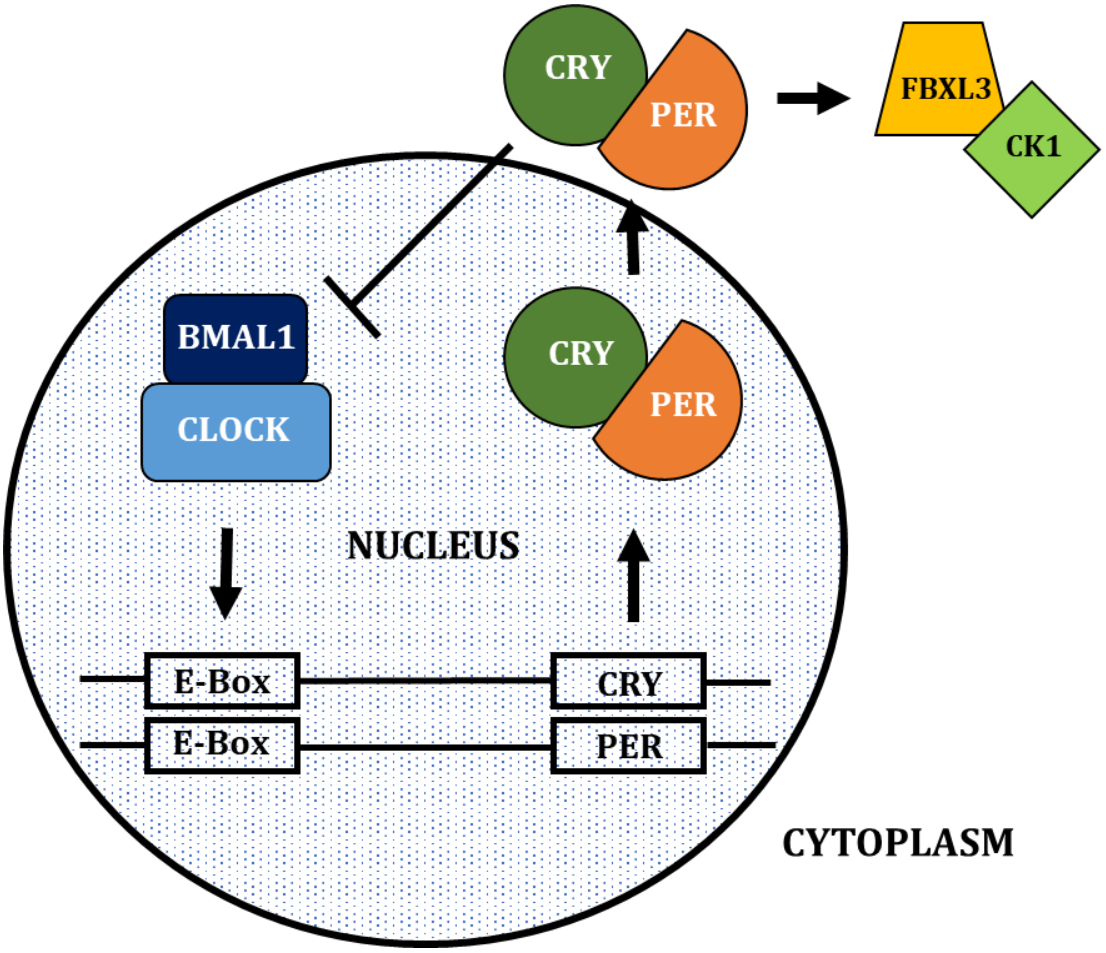


**Figure 1.** The described network of transcriptional-translational feedback loops which constitute the mammalian circadian clock. The core molecular clock consists of a positive arm in which BMAL1 and CLOCK heterodimerize to induce expression of the negative arm comprising of PER and CRY, which then react to inhibit BMAL1 and CLOCK. The negative arm responds to the positive arm protein degradation in a time specific manner, mediated by CK1δ/ε and FBXL3, generating the 24-h circadian period.

### 1.2 The efficacy of aquaculture and the limits of capture fisheries

Sustainable aquaculture expansion continues to be at the forefront of priorities for meeting protein demands of a rising human population (Ahmed & Thompson, 2019) and thus continues to remain the fastest growing food sector (Davies *et al.,* 2019). The global population is expected to rise from 7.5 to 9.1 billion by 2050 and the count of malnourished people continues to increase (FAO, 2021). Therefore, it is necessary that innovative food production solutions are devised to counter this universal problem. Whilst capture fisheries will remain relevant, aquaculture has a crucial role in global food security with overall production increasing 7.5% annually since the 1970s (FAO, 2018). Two of the greatest barriers to intensification of aquaculture in the UK, and indeed

globally, is sustainable, effective fish nutrition (McGhee *et al.,* 2019) and outbreaks of infectious diseases (Rodger, 2019). Currently, disease is a substantial economic liability, at an estimated cost to the aquaculture industry of US $6 billion a year (Gov, 2017), and is a growing concern for farmed fish welfare (Toni *et al.,* 2019). Furthermore, effective use of feeds is increasingly critical as traditional reliance upon fish-derived proteins and oils, sourced from capture fisheries, is progressively more unsustainable (Llagostera *et al.,* 2019). Therefore, it is critical that innovative management strategies are developed that not only promote effective fish feed utilisation and sustainable ingredients, but also provide benefits to fish health and welfare. A greater understanding of fish chronobiology may have significant applications in aquaculture. Yet the question remains; is there a possibility of using chronobiology to augment aquaculture farming systems to tackle the problems facing human nutrition whilst still maintaining fish health and welfare?

Optimisation of feeding regimes as a basis for sustainable and efficient fish farming practices is a promising avenue in aquaculture development (Naylor, 2005). Interestingly, it has been shown that time-restricted feeding (TRF) regimes in mammals can be beneficial to health (Chaix *et al.,* 2019). TRF appears to be critical to metabolic homeostasis via circadian clock regulation of daily oscillations in feeding and fasting rhythms (Figure 2). However, while photoperiod manipulation is a commonly used tool in aquaculture to promote growth and/or control reproduction (Hiu *et al.,* 2019), chronobiological approaches have yet to be applied to feeding and treatment regimens to manage fish health. Chronobiology offers a solution to begin addressing the problems facing contemporary aquaculture practices by improving the efficacy and management of these farming systems (FAO, 2018).

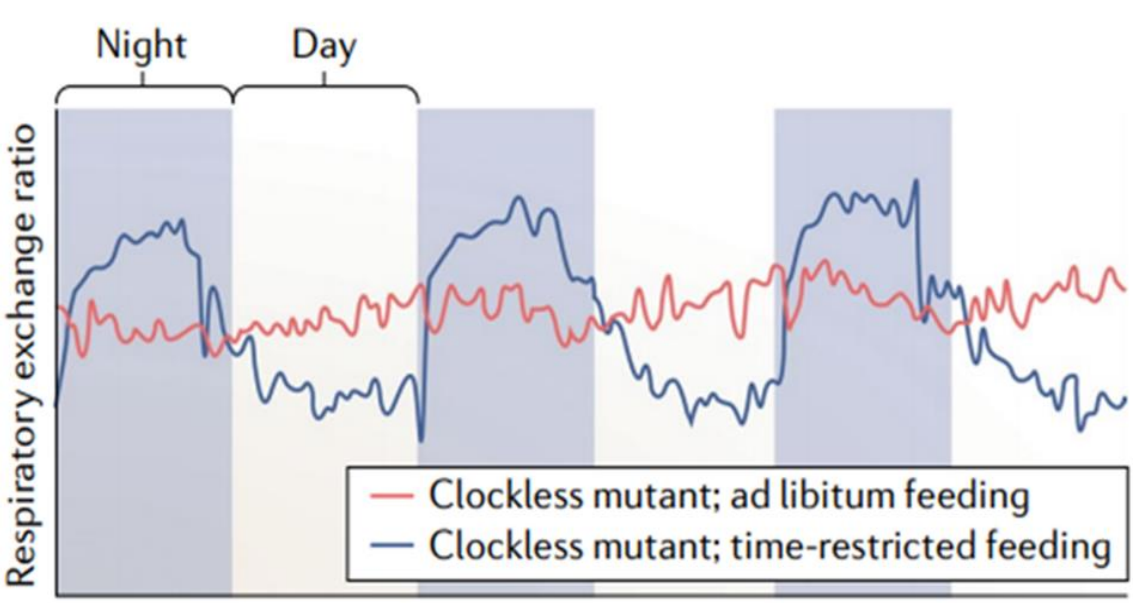


**Figure 2.** A contrast between the mice fed *ad libitum,* and those subject to TRF. The circadian rhythm of the respiratory exchange ratio (RER) is disturbed in clockless mice fed *ad libitum* but is returned to default when subject to TRF (Sourced from Chaix *et al.,* 2018).

### 1.3 The augmentation of circadian feeding and fish activity

Circadian rhythms of activity have been found to synchronize with oscillations of environmental stimuli and light is generally thought to be the predominant factor (Miller, 1978a; Manteifel *et al.,* 1978). It is also evident that fish activity is synchronised with endogenous circadian rhythms. The question remains, are accurate simulations of these biological rhythms replicable within an intensive farming system? Experimental modification of seasonal light cycles and photoperiod manipulation are tools commonly used to augment year-round supply of eggs, whilst also improving the ability to manipulate growth rates (Hansen *et al.,* 1992; Hui *et al.,* 2019). Despite success in exploiting such rhythms, the fundamental physiological mechanisms underlying commercial practices are still misunderstood and research into feed timing, daily activity patterns and their interaction with health/immunity are yet to be fully explored. It has become evident that feeding cycles are crucial to the synchronisation of many physiological, behavioural, and molecular rhythms within animals (Chaix *et al.,* 2018, 2019). Feed timing also appears to be important to food utilization efficiency and thus there is a need to consider feeding rhythms within cultured fish species to improve overall production efficacy (Parker, 1984; Spieler, 1977; Boujard & Leatherland, 1992). However, prior to application of timed feeding regimes within aquaculture, it is necessary

to gain a better understanding of the wider impacts of TRF on fish physiology, behaviour, and health.

### 1.4 Microbiome, Circadian Rhythmicity and Aquaculture

The microbiomes of fish are complex communities comprised of yeasts, viruses, protists, and members of Archaea and Bacteria taxa'. These communities colonise the gills, skin, and gastrointestinal (GI) tract of their host (Merrifield & Rodiles, 2015). An interplay of a multitude of factors determines the composition of the fish microbiome. These include the quality of surrounding water columns and inhabiting microbial communities (Sullam *et al.,* 2012; Wong & Rawls, 2012), host developmental stage, immune status, genetic makeup, and other host specific pressures (Figure 3) (Burns *et al.,* 2016; Ye *et al.,* 2014; Rawls *et al.,* 2006; Hennersdorf *et al.,* 2016). Lastly, dietary intake also influences the composition of microbial communities (Miyake *et al.,* 2015; Scott *et al.,* 2013; Bolnick *et al.,* 2014; Saha *et al.,* 2006; Merrifield & Rodiles, 2015). Akin to other vertebrates, fish gut microbiota is critical to nutritional provision, metabolic homeostasis, and immune defence (Sullam *et al.,* 2012; Gomez & Balcazar, 2008). Research on fish microbiomes is increasingly focused on nutritional management and modification of the microbiota to augment growth and aquaculture productivity, whilst maintaining the welfare and health of the host organism (Figure 4). Despite developments in the field, a comprehensive understanding of the influence of specific gut microbiota on host physiology is still lacking (Egerton *et al.,* 2018). However, there have been associations found between alterations in the activity and composition of the fish microbiome and that of fish physiology and disease susceptibility (Ellison *et al.,* 2021). Excitingly, TRF has been found to positively affect commensal gut microbes in mammals (Ren *et al.,* 2019) and raises the possibility that TRF may be an important tool to manage fish microbiomes for the benefit of aquaculture. It has also been suggested that the overall quality of cultured fish may be influenced by the time of day when the fish are being fed (Steindal & Whitmore, 2019), treated with therapeutic chemicals (Ellison *et al.,* 2021), or being physically disturbed (Sanchez-Vazquez *et al.,* 2019). Therefore, this is increasing interest in how efficacy of production and quality of aquaculture produce might be improved if fish husbandry practices were timed to synchronise with the biological rhythms of fish (Hasan, 2001).

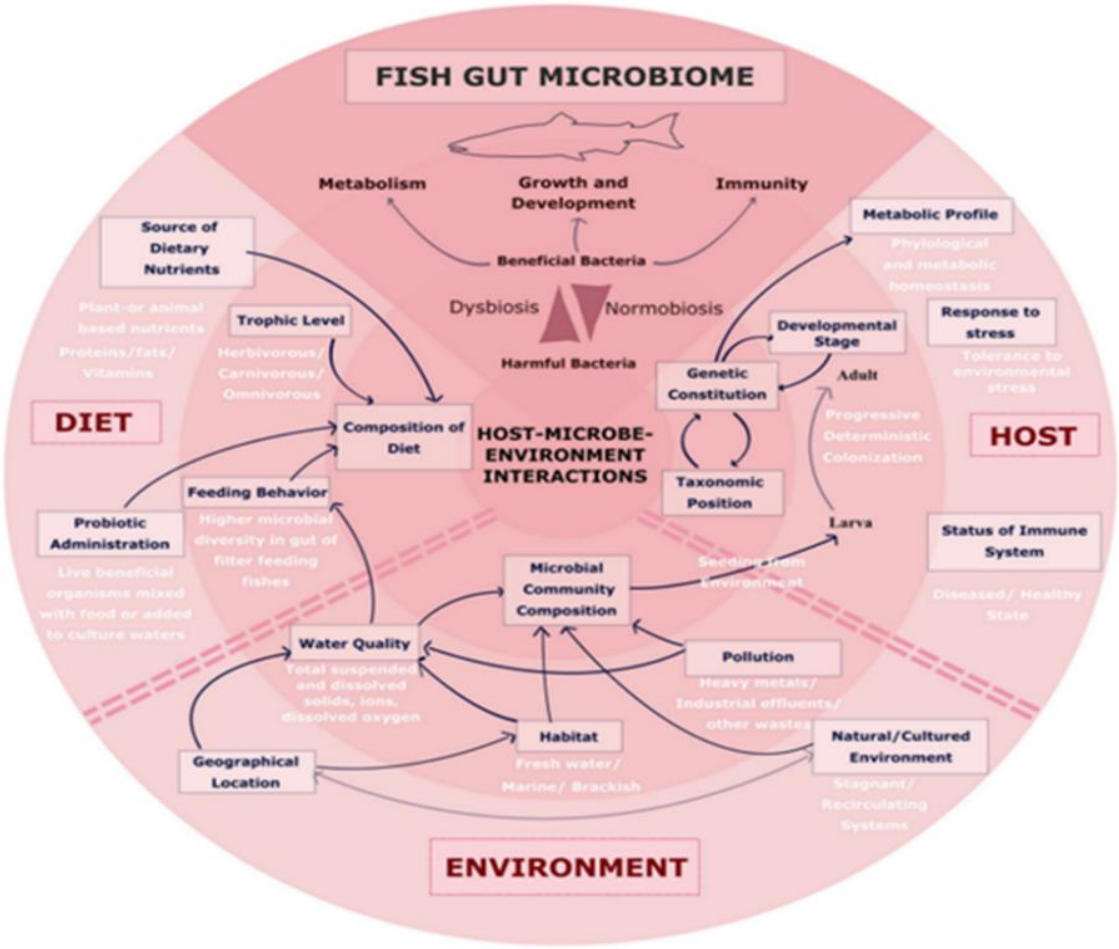


**Figure 3.** The factors influencing the function and diversity of the fish gut microbiome. These may broadly be characterized into host-associated, environmental, or diet-associated factors. All intrinsic and extrinsic factors directly influence either healthy state (normobiosis) or altered microbiota (dysbiosis) both of which effect the development and growth of the fish host (Sourced from Chandni *et al.,* 2018).

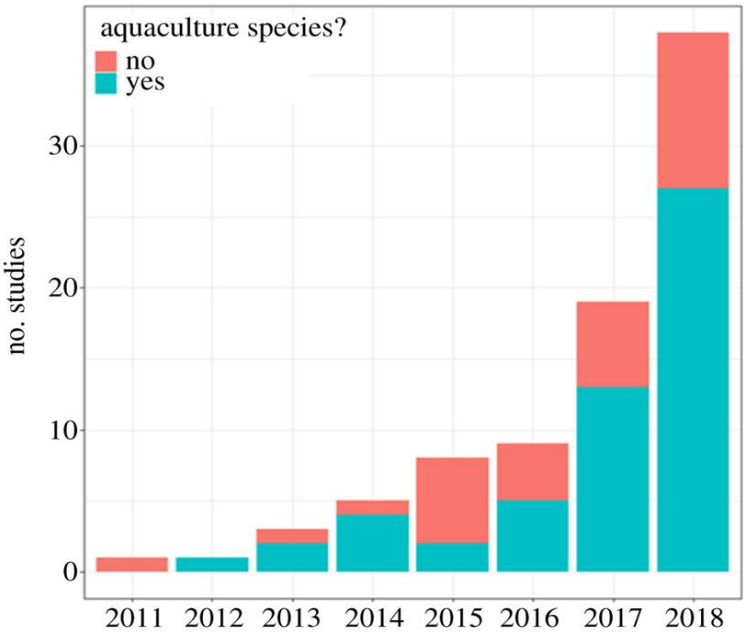


**Figure 4.** Development of studies analysing fish GI microbiomes and whether they are food aquaculture species (aquaculture status sourced from Fishbase (Froese & Pauly, 2000)) (Sourced from Perry *et al.,* 2020).

Recent insights on fish microbiomes, alongside progress in chronobiological research, has led to the question of whether a chronobiological understanding of microbiome dynamics in fish digestive systems can be harnessed to improve aquaculture. In fish and other aquatic organisms, the gut microbiome is necessary for the production of specific amino acids, the digestion of certain diet components (e.g., algal cells), the secretion of compounds that protect against colonisation of the gut by bacterial pathogens, and immune modulation (Figure 5) (Nayak, 2010; Austin, 2006; Xia *et al.,* 2014). This knowledge may be foundational within cultured fish systems in the management of populations, feeding delivery, and growth potential (Ghanbari *et al.,* 2015). The rapid development of aquaculture production has led to an increasing pressure to improve sustainability (Perry *et al.,* 2020). Understanding and augmenting these key microbial-host- environment interactions could contribute significantly towards achieving sustainable aquaculture nutrition whilst maintaining fish health.

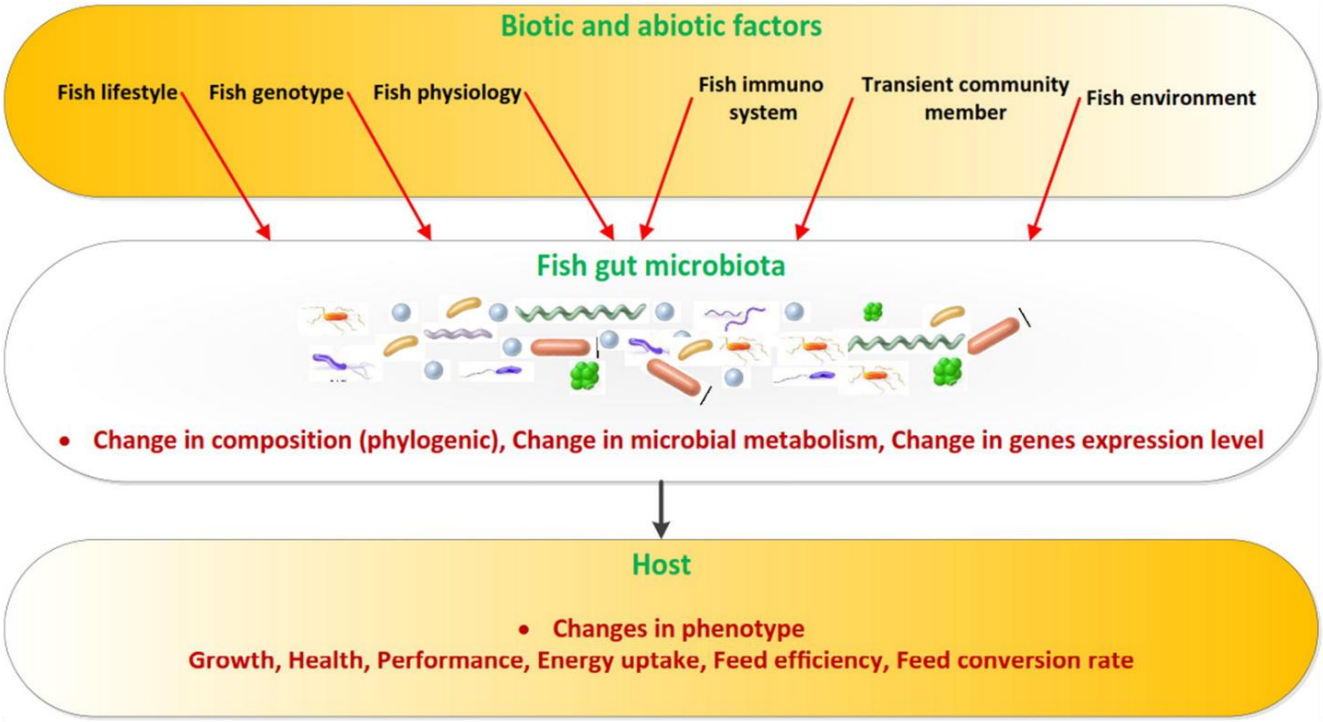


**Figure 5.** The combination of abiotic and biotic factors influencing the function, composition, and metabolic activity of fish gut microbiomes, these factors include genotype, fish pathology (disease status), physiology (including innate and adaptive immune systems), lifestyle (including diet), environment, and presence of transient populations of microorganisms. All these factors influence processes involved in health, energy storage, growth, and performance within fish (Sourced from Ghanbari *et al.,* 2015).

In mammals, gut microbial community composition and metabolism have been found to have daily rhythms, regulated by time of feeding and dietary intake (Chaix & Zarrinpar, 2015; Chaix *et al.,* 2018, 2019). These daily cycles of microbial metabolic activity and community structure can substantially impact host metabolic and immune function (Voigt *et al.,* 2016). Metabolism and circadian rhythms are inextricably intertwined, where peripheral and core circadian clocks synchronise metabolic systems in response to light-dark and sleep-wake cyclic events. In mammals, light exposure and time of feeding are the two principal stimuli that alter the synchronicity of circadian rhythms with cyclical environmental cues of day and night, a process known as entrainment. Environmental circadian perturbation, whereby external timing cues conflict with the internal biological clock, are an acute example following chronic shift work and jet lag in humans. For example, human physiology is adapted to receive food and light coincidently in daytime hours. Feeding at night-time thus elicits conflicting cues, which have been

shown to be associated with a reduced amplitude of clock gene and physiological rhythms which increase the risk of metabolic and cardiovascular health problems (Archer *et al.,* 2014, Dijk et al., 2012; Scheer *et al.,* 2009). Indeed, circadian perturbation is associated with a number of diseases, including metabolic syndrome, type 2 diabetes, and various cancers (Reddy and O'Neill, 2010). Understanding the mammalian cellular clocks response to changes in feeding time is paramount and is likely to translate across disciplines and fields and may well be crucial for the development of chronobiology in aquaculture (Crosby *et al.,* 2019).

Recent studies have observed associations between host circadian clocks and the gut microbiome in mice (Li *et al.,* 2020). Although the gut microbiome is not exposed to light, diurnal host signals induce oscillations of the function (i.e., metabolite production), abundance and composition of the gut microbial communities (Frazier & Chang, 2020; Leone *et al.,* 2015). However, it is unclear whether fish gut microbiomes demonstrate similar circadian rhythmicity or if there is an influence of feed timing on microbiome composition and host-microbe relationships. Using Rainbow Trout (*Oncorhynchus mykiss*) as a model, this study will examine the effects of feed timing on fish growth, behaviour, and gut microbiomes. Collectively, this will provide a new fundamental understanding of how chronobiological augmentation can be utilised to facilitate sustainable aquaculture.

# 2. MATERIALS AND METHODS

## 2.1 Experimental Design and Sample Collection

### 2.1.1. Field of Study and Objectives

Ethics approval was granted by Bangor University approval body, authorising the implementation of this study (See Appendix). Experiments were carried out at the Brambell Aquaria, Bangor University, UK over a period of 16 weeks. Rainbow Trout (*Oncorhynchus mykiss*) were obtained (n=~370) from a commercial hatchery (Northern Trout Ltd.) with an initial mean body weight of 2.36 ± 0.08g and length of 47.0 ± 0.59mm. Fish were acclimated for 4 weeks prior to commencing the experiment. Mortalities were observed during the initial acclimation period but were extremely low during the experimental trials (data not shown). Water temperature was maintained at 12 ± 1°C and

aerated via airlines, maintaining >80% oxygen saturation. Water quality was measured on a weekly basis, pH (7.5 ± 0.2), ammonia (<0.01 mg/L), nitrite (<0.02 mg L−1), and nitrate (<15 mg L−1) were maintained within an appropriate range. A 12 h light/12 h dark photoperiod was maintained throughout the experiment (08:00AM – 20:00PM) using 4W submersible LEDs at the water surface of each tank. 50% tank volume water changes were conducted weekly to minimise accumulation of by-products of organic waste breakdown. At the start of the experiment, batches of 45 fish were randomly distributed into eight 119 L (120x30x38 cm) glass tanks, each provided with aerated re-circulated freshwater at a rate of 1900L $h^{-1}$. Tanks were in two controlled temperature rooms, both of which were light and sound-proof. To avoid disturbance, access to experimental CT rooms was restricted. One treatment group of fish (4 tanks of 45 fish) was subject to early-only feeding (MF) provided at 09:00AM (1hr post zeitgeber; lights on). The second treatment of fish was subject to late feeding at 19:00PM (AF) (1hr pre lights off). Treatment tanks were equally divided between the CT rooms (2 tanks per treatment per room). Fish in both treatments received equal food rations (2-3.5% biomass $day^{-1}$ as recommended by FAO, 2021) by an automatic Oase™ food dispenser programmed to feed at the set treatment time. Commercial Nutripar 0.8mm trout pellets were provided containing 56% Crude protein, 18% Crude fat, 10.2% Ashes, 0.4% Crude fibre, 1.47% Phosphorus, 1.83% Calcium, and 0.97% Sodium.

Growth measures were taken on a weekly basis using a subset of individuals. 40 random individuals per feed treatment were weighed (g) and measured (standard length, SL in cm) for 15 weeks to monitor growth rates. Fulton's condition (*K*) factor, absolute growth rates (AGR), and specific growth rates (SGR) were calculated using the equations described in Table 1. General linear models of length, weight, and condition (*K*) were used to assess variation in growth between feed treatments (Wickham, 2009). Daily locomotory activity patterns were assessed using automated infra-red behavioural arrays, recorded by the Trikinetic DAMSystem3 data acquisition software (Cichewicz & Hirsh, 2018) (Figure 6). A total of 20 fish per treatment were individually monitored in the arrays for a 48h period. Total beam breaks per hour was used as a proxy for activity levels, these counts were then analysed in RStudio using the "Circacompare" package (Parsons *et al.,* 2020) to determine if fish activity had a significant daily rhythm. Analysis was first conducted using "circa single" to assess individual rhythms. The full

Circacompare algorithm was then used to determine overall rhythmicity of treatment groups and statistically compare rhythm mesor, amplitude and phase between groups (Figure 6).

At the end of experimental feeding trials, trout were sampled over a 24h period to encompass a full circadian cycle. Starting at zeitgeber (translation = "time giver") time 0 (ZT 0: 08:00 = lights on in 12:12 LD treatment and first time point of euthanasia), every 4h, ten fish from each treatment were euthanized by use of an overdose of tricaine methanesulfonate (MS222, 500 mg L− 1) followed by destruction of the brain. At timepoints during dark periods, fish were handled and euthanised in dim red light. All sampled fish were weighed (g) and measured (standard length, SL in cm). The distal intestine was dissected using sterilised instruments and immediately stored in 100% ethanol solution at -80 ° C to ensure preservation of the gut microbiome for DNA extraction. All dissections for each timepoint were performed within a 1-hour window.

**Table 1.** Standard aquaculture growth measure equations.

| ***wt = end length/weight, wi = initial length/weight, Ln = natural logarithm, t = time*** | | |
|---|---|---|
| **Fulton's condition factor (*K*) :** | **Absolute growth rates (AGR) :** | **Specific growth rates (SGR) :** |
| $K = W/L^3 \times 100$ | $(w_t - w_i)/t$ | $(Ln(wt) - Ln(wi))/t$ |

### 2.2 DNA Extraction, 16S rRNA gene amplification, & Illumina sequencing and analyses

DNA was extracted from gut tissue samples using QIAamp DNA kits (Qiagen) according to Gill *et al.,* (2016) to maximise lysis of microbiome community and DNA concentrations. PCR amplification of the 16S rRNA V1-2 region was performed via a two-step process for each DNA extract (Vere *et al.,* 2017). A primary tailed amplification of the V1-2 region was performed using modified CS1_27F (5′-AGAGTTTGATCMTGGCTCAG-3') and CS2_338R (5′-TCTGCTGCCTCCCGTAGGAGT-3′) primers with Universal Illumina tails (Vere *et al.,* 2017). The primary amplification was followed by a second round of amplification that introduced indices so that samples could be separated via bioinformatics. The first round 16S amplicons were generated using a PCR reaction containing 2µl of template DNA, 0.5µl CS1_27F, 0.5µl CS2_338R, 12.5µl Q5® High-Fidelity 2X master mix (NEB) and 9.5µl molecular grade microbial free water. The PCR amplification programme consisted of an

initial denaturation set at 98 °C for 2 minutes. followed by 30 four-step cycles of 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 minutes. PCR products were cleaned using Ampure XP beads (Beckman Coulter) according to manufacturer instructions. 1µl of cleaned product from the first PCR was used as template for the second PCR, where a forward and reverse Illumina adaptors and unique i5 or i7 Nextera indexes were added using the universal tail. The PCR reaction included 1µl of template first-round PCR product, 0.5µl i5 indexed primer, 0.5µl i7 indexed primer, 12.5µl Q5® High-Fidelity 2X master mix and 10.5µl molecular grade microbial free water. Cycling conditions included an initial denaturation step of 98 °C for 30 s, followed by 15 four-step cycles of 98 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s, and 10 minutes at 72 °C. Negative controls were included for all PCRs and inspected for a positive band using gel electrophoresis. The same protocol was followed to determine whether the bacterial composition of the dietary feed provided was similar to the microbial composition in the GI tract of the fish.

Qubit® dsDNA Broad Range (BR) Assay Kits were used to determine PCR product concentrations for pooling individual amplicon libraries. Negative controls for extractions and PCR were included for sequencing. All libraries were pooled equimolarly and the final library pool cleaned using Ampure XP beads (Beckman Coulter) according to manufacturer instructions. Libraries were sequenced using a 2 x 250 bp Illumina MiSeq run at the Centre for Environmental Biotechnology (CEB), Bangor University, UK. Paired end demultiplexed Illumina sequencing reads were imported into RStudio (Version 1.4.1717-3, R Core Team, 2021). Sequences were then quality filtered, dereplicated, chimeras identified, and merged using DADA2 (Callahan *et al.,* 2016) with default settings. Classification of Amplicon Sequence Variants (ASVs) were accomplished using Decipher (Murali *et al.,* 2018), trained using sequences representing the bacterial V1-2 rRNA region available from the SILVA database (Pruesse *et al.,* 2007; https://www.arb-silva.de/download/archive/qiime; Silva_132). The classifier was then used to assign taxonomic information to representative sequences of each ASV. Following rarefaction analysis, samples with less than 5000 sequences were excluded from further analyses. RStudio was used to analyse alpha (α-) (Wilcoxon rank sum test) and beta (β-) (pairwise PERMANOVA) diversity measures. ASVs were filtered to exclude those assigned to eukaryotes or eukaryotic organelles and include ones with at least 100 copies in at least two samples. The Bioconductor package, phyloseq (McMuride & Holmes, 2013), was used for sub setting, normalizing, and plotting of the data. The weighted and

196 unweighted UniFrac tests were applied to determine differences in community
197 composition and structure (Lozupone *et al.,* 2007). Differential abundance of ASVs
198 between early and late feed treatments were determined using DESeq (Love *et al.,* 2014),
199 with FDR-corrected p-values less than 0.05 considered significant. Rhythmic analyses
200 (Hutchinson *et al.,* 2015; non-parametric asymmetric waveform-fitting, e.g., empirical
201 JTK cycle -eJTK- analyses) was used to detect and contrast rhythms of bacterial taxa
202 abundance. ASV abundance was also contrasted and correlated against condition (*K*)
203 factor between treatments (only those demonstrating >50% prevalence in all samples)
204 using Spearman's rank correlation coefficient (FDR corrected P values).
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206

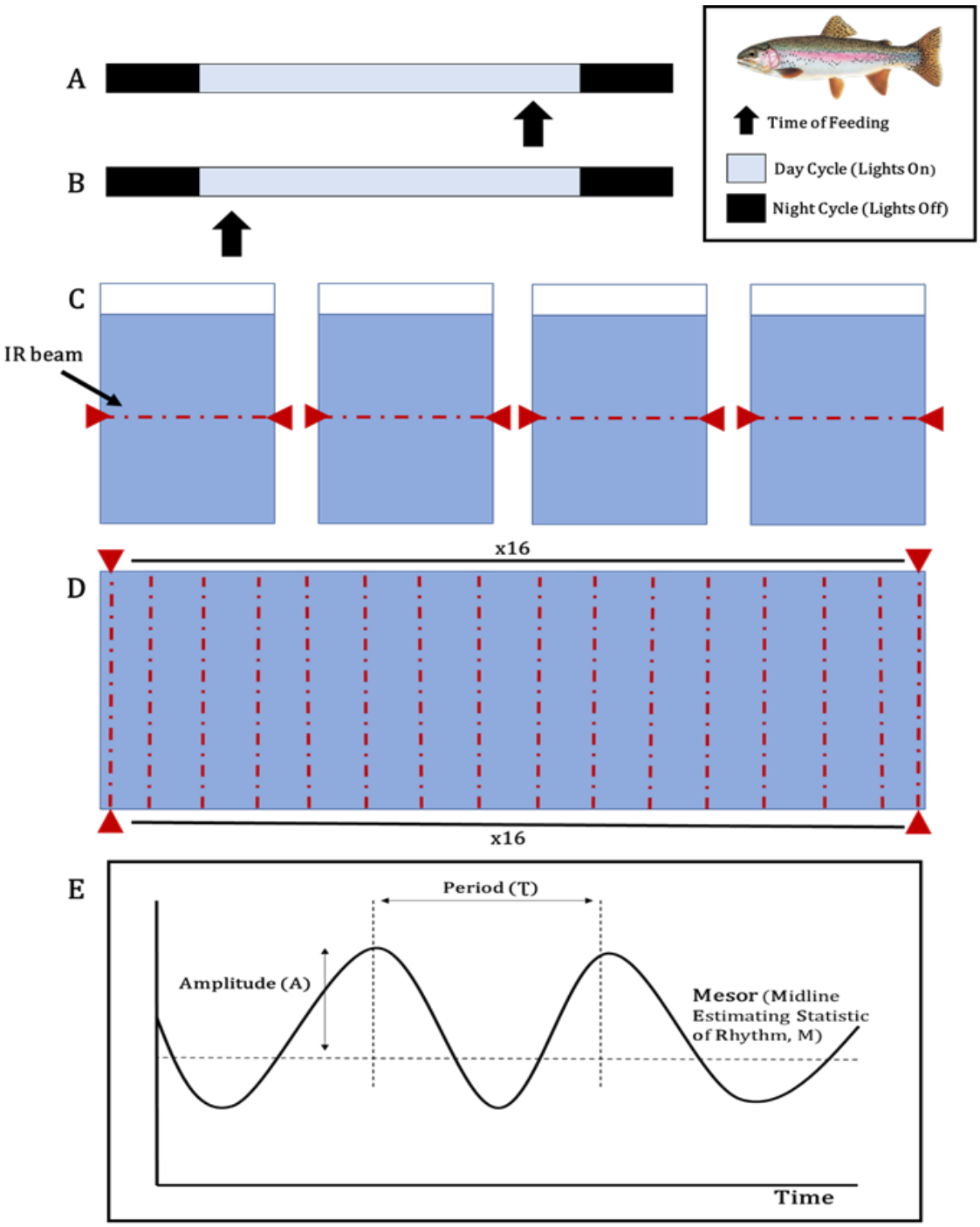



**Figure 6.** Illustrated schematic demonstrating the two daily feeding regimes and the Modified- Drosophila Activity Monitoring System (DAM). (**A**) shows the feed rotation during the late light cycle & (**B**) the feed rotation during the early light cycle. (**C**) shows a lateral view of the four quadruplet tanks. (**D**) shows a top-down view of the DAM system setup. Fish are placed individually in tanks and monitored using DAM monitors. Each monitor has a total of 16 infrared beams (IR). Total beam breaks per hour was used as a proxy for activity levels, data collection is automated via the DAMsystem software, and output files are analysed in R using the CircaCompare package (Parsons *et al.,* 2020). (**E**) depicts rhythm characteristics of a cosinor wave, used in CircaCompare for detecting circadian rhythmicity.

# 3. RESULTS

### 3.1 Dietary treatment has no significant influence on growth

Dietary treatment had no significant impact on the growth of juvenile rainbow trout (*O. mykiss*) over a 15-week trial period (weight: $t_{956}$ = 1.818, df = 1159, *P* = .069, length: $t_{956}$ = -.029, df = 1159, *P* = .977, Figure 7). Standard aquaculture growth measures were calculated (See Table 2) and their specific growth rates (SGR) was greater than 2% per week for fish on both dietary treatments. SGR was not significantly different between treatments ($t_{956}$ = .309, df = 5, *P* = .757). Both treatments had condition factors greater than >1.5 which suggests healthy individuals. However, body condition (*K*) was significantly different between treatments ($t_{956}$ = 5.029, df = 1159, *P* = <.001) with the early treatment (MF) showing an overall greater *K* value compared to the late treatment (AF) (early; 1.92 ± .012, late; 1.85 ± .010) (Supplementary Figure 1). The morning treatment also had a higher representative SGR at the conclusion of the experimental trial (2.61 ± .42%/week, 2.58 ± .27%/week for the early and late respectively).

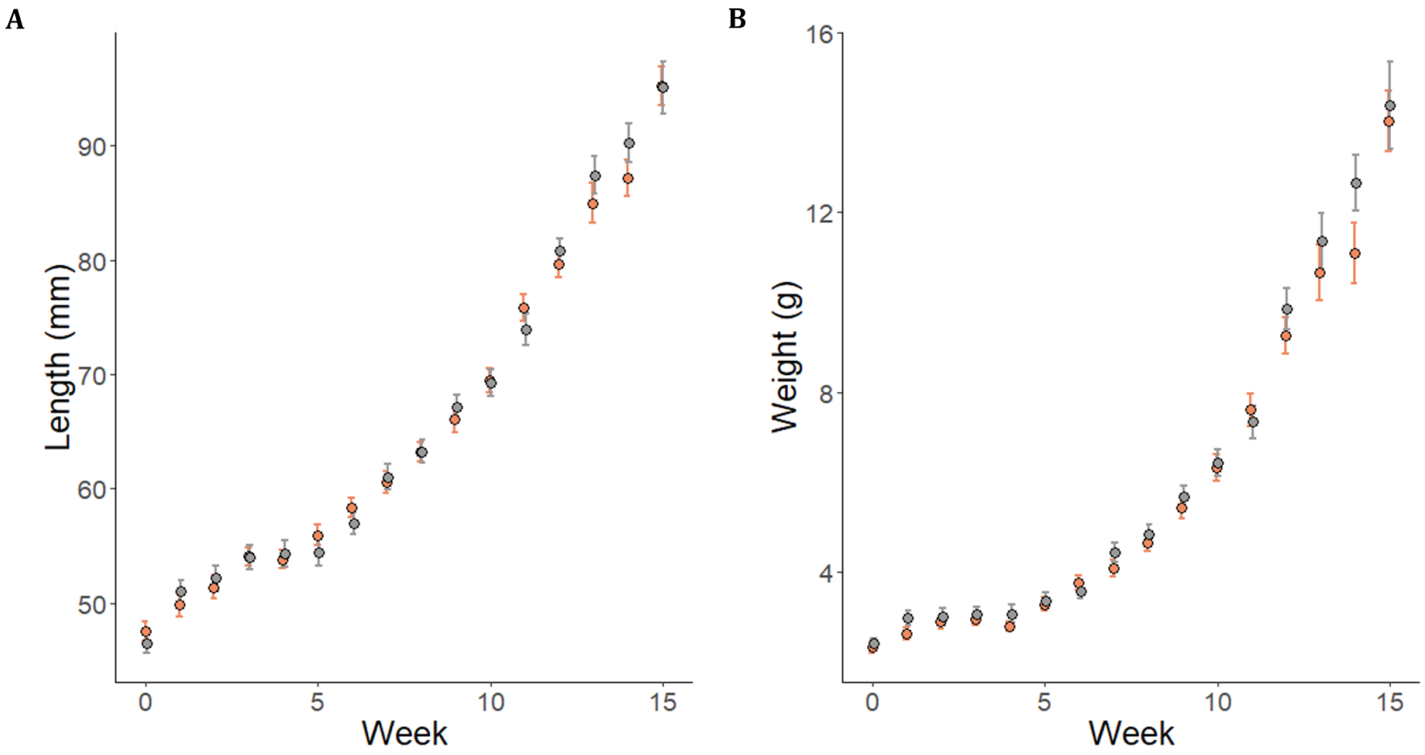



**Figure 7.** Average (**A**) standard length and (**B**) weight of trout (±1 S.E.) over a 15-week growth trial under two feed treatments, early/morning feed (grey) and late/evening feed (orange).

**Table 2.** Standard aquaculture productivity measures for each dietary treatment.

| Absolute growth rate (AGR) | | Specific Growth Rate (SGR) | | Fulton's Condition Factor $\bar{x}$ ($K$) | |
|---|---|---|---|---|---|
| MF | AF | MF | AF | MF | AF |
| 0.79 ± 0.11 g/week | 0.78 ± 0.12 g/week | 2.61 ± .42 %/week | 2.58 ± .27 %/week | 1.92 ± .012 | 1.85 ± .010 |

### 3.2 Circadian rhythmicity is altered by dietary treatment, both at an individual and group level

50% of total individuals (10/20) subject to the early feed treatment displayed circadian rhythmicity in their activity levels (Supplemental Table 1). In contrast, 80% (16/20) of individuals subject to the late feed treatment had significant daily activity rhythms. Significant rhythmicity in activity levels was found at a group level in the late treatment ($P$ = <.001), but activity in the early treatment was overall arrhythmic ($P$ = .155) (Figure 8).

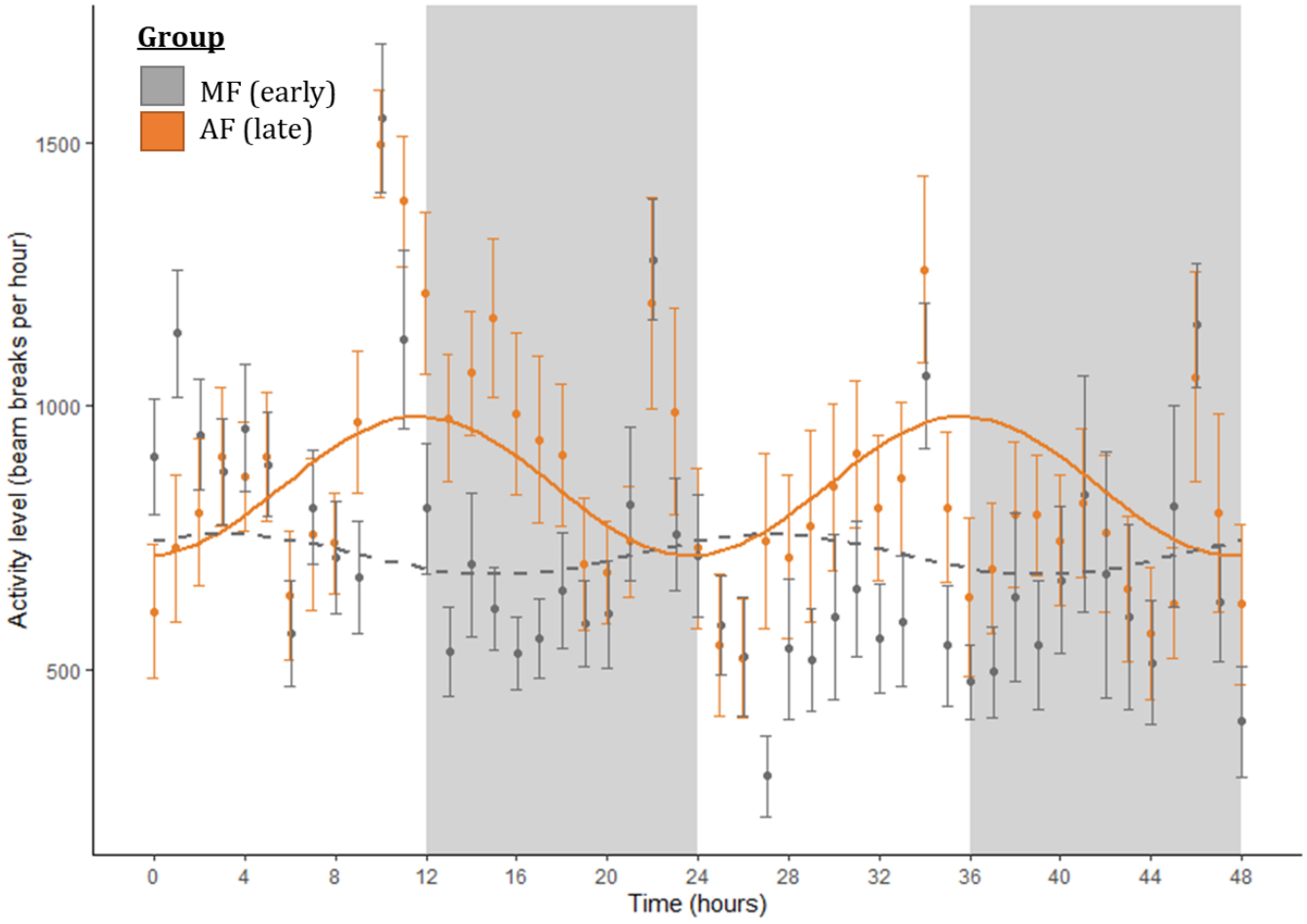



**Figure 8.** Mean Activity (± 1 S.E.) of group level rhythmicity by late feed treatment group (orange), and early feed treatment group (grey). Mean counts of beam breaks per hour was used as a proxy for activity levels. Measurements were taken over a 48h period. Curves denote cosinor waveforms fitted using CircaCompare. Grey shading indicates time periods in darkness. A solid line indicates significant rhythmicity, dashed indicates arrhythmicity.

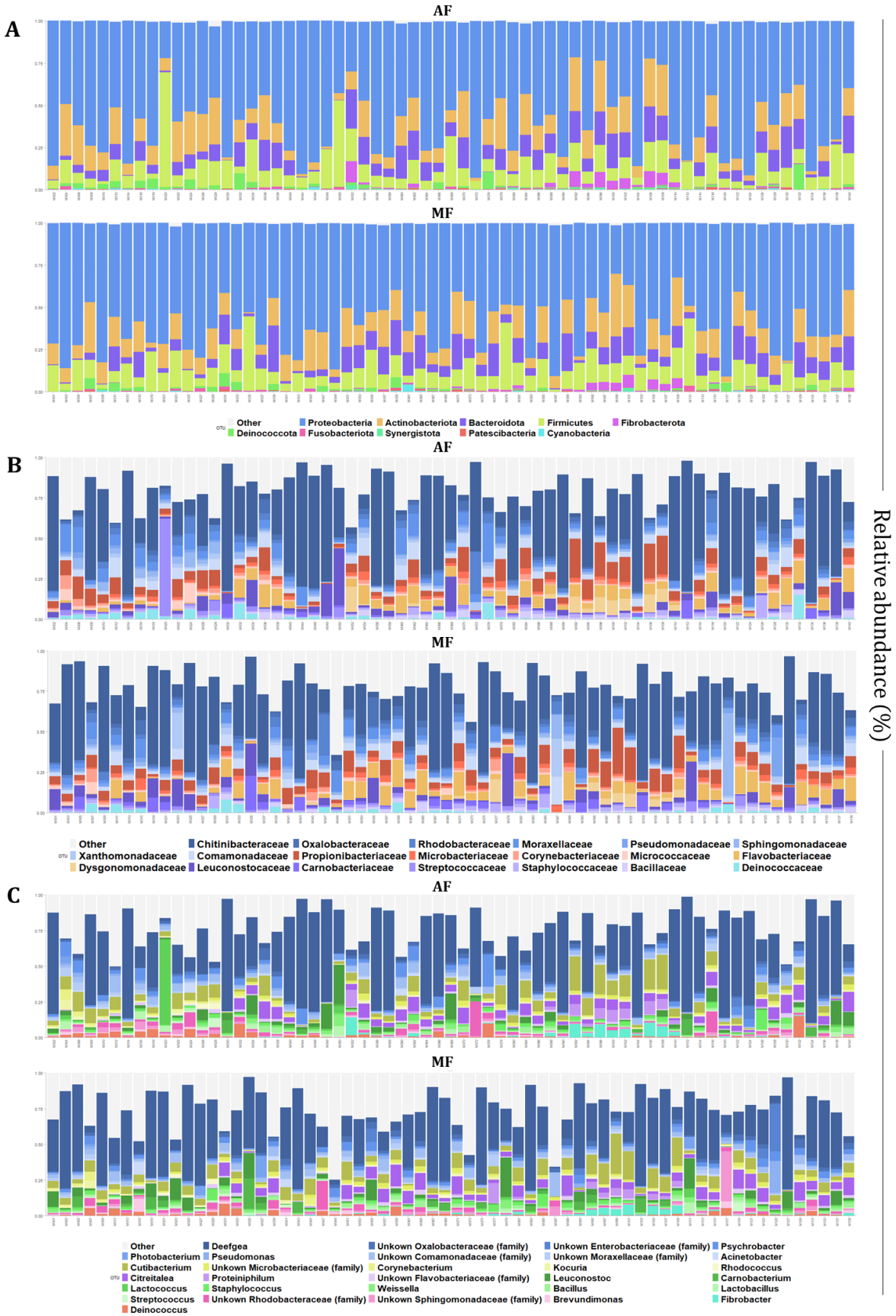
### 3.3 Microbiome composition and abundance was similar between both dietary treatments

The analysis of the 16S rRNA sequencing data revealed an average 53,365 ± 2377 reads per sample (n=131 fish), ranging from 546-122,318 reads. Nine libraries were omitted from the analysis due to small library size (fewer than 5000 reads). After read pre-processing, error correction, chimera removal, and filtering, a total of 2,467 unique amplified sequence variants (ASVs) were found across all samples. Rarefaction curves confirmed a minimum read depth of 5,000 was appropriate to achieve adequate representation of diversity in the trout gut microbiome (Supplementary Figure 2). The profiles of intestinal microbial communities have been presented for each dietary group and individual fish at the phylum, family, and genus taxonomic level (Figure 9). The overall gut microbial community across both treatments was comprised of 23 phyla, 38 classes, 98 orders, 217 families and 576 genera.

Contrasts of α-diversity between treatments showed no significant variation in observed richness for both observed and Shannon (H) diversity metrics (Wilcoxon rank sum test: Observed; $P$ = .80, Shannon; $P$ = .81) (Supplementary Figure 3) (Shannon & Weaver, 1949). Multivariate permutational analysis of β-diversity by ASV indicated that there was no significant segregation of microbiome composition amongst the two dietary treatments (Weighted UniFrac: df = 129, $P$ = .402) (Supplementary Figure 4).

Bacteria associated with the intestine of rainbow trout was substantially different to those prevalent in the composition of the dietary feed provided (Supplementary Figure 5). Irrespective of diet treatment, Proteobacteria (60.6 ± 1.5%), Actinobacteriota (14.1 ± 0.7%), and Firmicutes (12.2 ± 0.8%) comprised the most abundant phyla in the trout gut microbiome (Figure 9); other abundant bacteria phyla included Bacteroidota (9.2 ± 0.6%), and Deinococcota (1.3 ± 0.1%). *Deefgea* (34.14 ± 2.2%), *Cutibacterium* (6.53 ± 0.4%), *Citreitalea* (4.02 ± 0.3%) and *Leuconostoc* (3.35 ± 0.4%) were the most abundant at genus level (Figure 9). Bacterial abundance of *Hymenobacter* was the only ASV significantly different between the two dietary treatments and was greater in abundance in the fish fed late in the light cycle (Wald test: $P$ = <.001). There was no significant variation in bacterial abundance between the two dietary treatments at genus, class, or phylum level. However, Chitinibacteraceae was significantly different at family level (Wald test: $P$ = .04; greater in abundance in the late treatment). Spearman's rank

correlation was computed to assess the relationship between ASV abundance and body condition (*K)* factor. A total of 5 ASVs were significantly correlated (all demonstrating a negligible correlation) regardless of treatment, these included *Citreitalea* (r (62) = .189, *P* = .030), *Polynucleobacter* (r (62) = .184, *P* = .034), *Fluviicola* (r (62) = .195, *P* = .025), *Limnohabitans* (r (62) = .188, *P* = .031), and *Pseudorhodobacter* (r (62) = .198, *P* = .023).




297 **Figure 9.** Proportional abundance of most prevalent (**A**) bacteria *phyla,* (**B**) bacteria
298 *families,* and (**C**) bacteria *genera* represented in amplicon libraries derived from intestinal
299 contents of rainbow trout for both treatment groups.

### 3.3.1 Circadian rhythmicity among trout gut microbiota was more common in the late dietary treatment

164 bacteria genera were rhythmic in relative abundance following eJTK analysis across both dietary treatments (Hutchison *et al.,* 2015). Analysis of circadian rhythmicity prevalence across all bacteria taxa present revealed that 12% of genera present in the early feed treatment (66/554) were significantly rhythmic, whereas 23% (127/548) of total genera present in the late feed treatment were rhythmic in abundance (Figure 10).

28 genera were rhythmic in both early and late treatments. Of which 3/28 (10.7%) had a significantly different mesor, while 1/28 (3.5%) had a significantly altered rhythm phase between treatments. The three genera with significantly different mesor's included *Trichococcus, Fermentimonas,* and unassigned Genera: - *Lachnospiraceae-fissicatena group. Curtobacterium* was the only bacteria genera to display a significantly altered rhythm phase between treatments (Table 3).

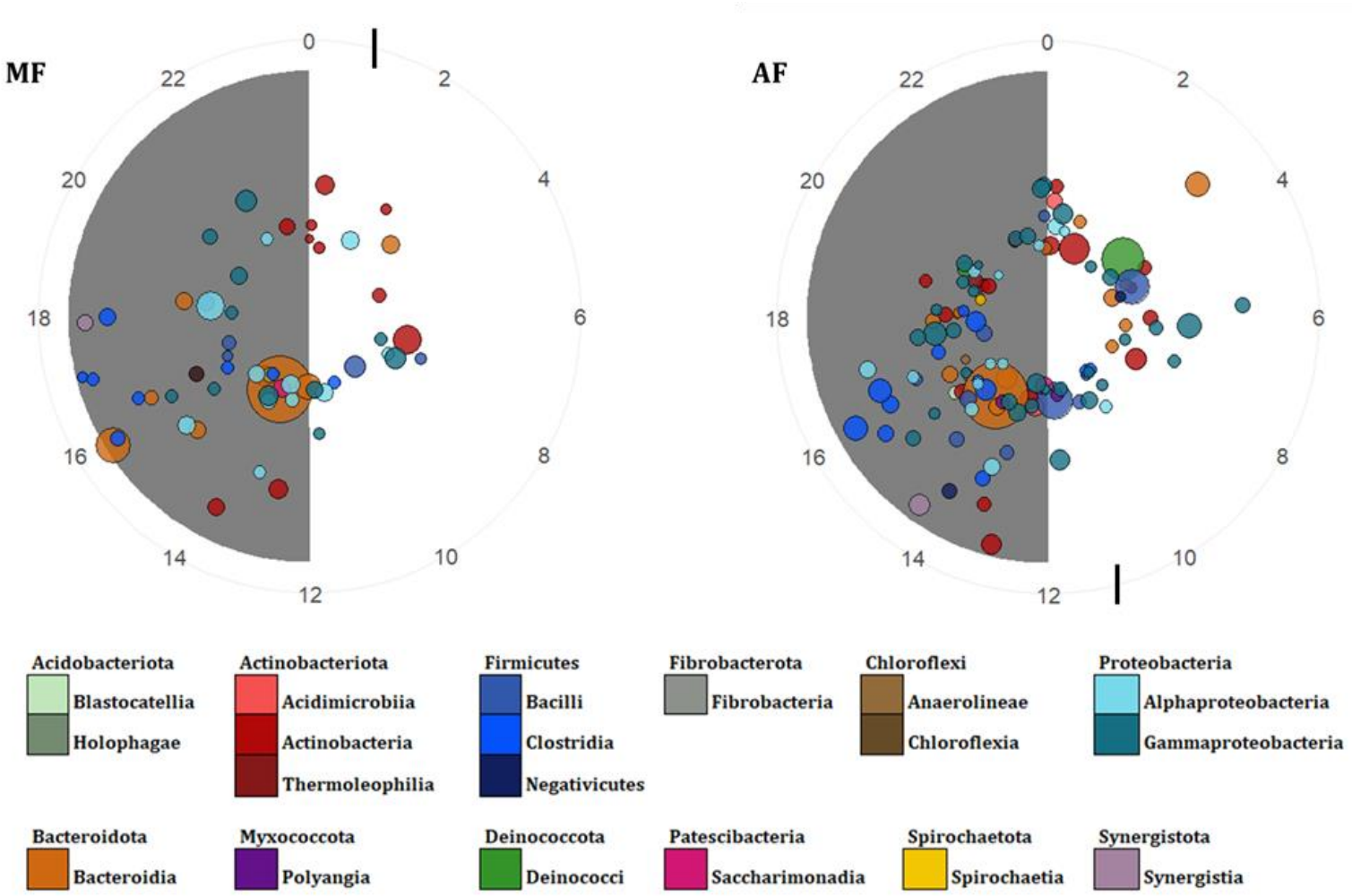



**Figure 10.** Polar plots demonstrating times of peak relative abundance of significantly rhythmic microbiome genera. Each circle represents a genus, colored by class, and scaled by average relative abundance. Radian signifies time of peak and distance from centre indicates significance (more significant/stronger rhythms towards the edge of the plot). The dark band signifies the relative time of feeding for both treatment groups. Time is represented as circadian time and hence, 0:00 hours = lights on (08:00am), 12:00hrs = lights off (20:00pm).

**Table 3.** Summary of shared significant microbiome rhythmic analyses for both treatments (n=28). Rhythm significance determined via eJKT_cycle. Rhythm parameters (mesor, amplitude, relative peak of abundant genera) estimated and contrasted in CircaCompare.

| Family | Genus | Rhythm (FDR P value*) | | Mesor | | | Amplitude | | | Phase (Peak hour) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MF | AF | MF | AF | MF v AF | MF | AF | MF v AF | MF | AF | MF v AF |
| *Acetobacteraceae* | *Unknown. Acetobacteraceae* | 0.000 | 0.004 | 0.0002 | 0.0003 | 0.4492 | 0.0003 | 0.0005 | 0.5028 | 13.229 | 14.695 | 0.525 |
| *Alcaligenaceae* | *Oligella* | 0.003 | 0.000 | 0.0002 | 0.0003 | 0.8306 | 0.0004 | 0.0004 | 0.9644 | 15.568 | 17.932 | 0.238 |
| *Azospirillales Incertae Sedis* | *Stella* | 0.016 | 0.034 | 0.0002 | 0.0001 | 0.3765 | 0.0002 | 0.0002 | 0.8511 | 7.664 | 23.567 | 0.059 |
| *Caldicoprobacteraceae* | *Caldicoprobacter* | 0.010 | 0.000 | 0.0002 | 0.0003 | 0.8054 | 0.0003 | 0.0004 | 0.6295 | 15.908 | 16.934 | 0.559 |
| *Carnobacteriaceae* | *Trichococcus* | 0.043 | 0.004 | 0.0020 | 0.0008 | **0.0265** | 0.0019 | 0.0005 | 0.0732 | 9.162 | 15.010 | 0.179 |
| *Christensenellaceae* | *Christensenella* | 0.000 | 0.000 | 0.0031 | 0.0061 | 0.0603 | 0.0045 | 0.0059 | 0.5289 | 17.124 | 15.875 | 0.439 |
| *Clostridiaceae* | *Clostridium* | 0.000 | 0.000 | 0.0017 | 0.0029 | 0.1567 | 0.0022 | 0.0028 | 0.6027 | 16.680 | 16.029 | 0.710 |
| *Clostridiaceae* | *Clostridium* | 0.000 | 0.000 | 0.0003 | 0.0006 | 0.1185 | 0.0004 | 0.0006 | 0.4774 | 16.948 | 15.663 | 0.482 |
| *Comamonadaceae* | *Methylibium* | 0.028 | 0.005 | 0.0003 | 0.0002 | 0.8795 | 0.0003 | 0.0003 | 0.9457 | 7.151 | 6.398 | 0.807 |
| *Dysgonomonadaceae* | *Proteiniphilum* | 0.000 | 0.000 | 0.0090 | 0.0131 | 0.3132 | 0.0117 | 0.0159 | 0.4876 | 15.813 | 16.459 | 0.682 |
| *Dysgonomonadaceae* | *Fermentimonas* | 0.000 | 0.000 | 0.0010 | 0.0024 | **0.0341** | 0.0013 | 0.0024 | 0.2813 | 16.884 | 15.486 | 0.499 |
| *Fibrobacteraceae* | *Fibrobacter* | 0.000 | 0.000 | 0.0082 | 0.0136 | 0.1470 | 0.0120 | 0.0158 | 0.4960 | 17.473 | 16.282 | 0.396 |
| *Flavobacteriaceae* | *Citreitalea* | 0.025 | 0.012 | 0.0440 | 0.0391 | 0.5189 | 0.0095 | 0.0083 | 0.9084 | 13.500 | 14.292 | 0.867 |
| *Frankiaceae* | *Jatrophihabitans* | 0.004 | 0.002 | 0.0002 | 0.0002 | 0.9655 | 0.0001 | 0.0002 | 0.4827 | 11.662 | 19.109 | 0.094 |
| *Garciellaceae* | *Garciella* | 0.000 | 0.000 | 0.0007 | 0.0013 | 0.0837 | 0.0012 | 0.0017 | 0.3907 | 17.521 | 16.028 | 0.283 |
| *Garciellaceae* | *Unknown. Garciellaceae* | 0.000 | 0.004 | 0.0001 | 0.0003 | 0.1388 | 0.0002 | 0.0003 | 0.3701 | 17.028 | 16.836 | 0.923 |
| *Hymenobacteraceae* | *Hymenobacter* | 0.006 | 0.000 | 0.0011 | 0.0028 | 0.1993 | 0.0013 | 0.0046 | 0.0650 | 3.284 | 3.274 | 0.998 |
| *Lachnospiraceae* | *Unknown. Lachnospiraceae [Eubacterium] fissicatena group* | 0.000 | 0.000 | 0.0010 | 0.0023 | **0.0472** | 0.0015 | 0.0023 | 0.4189 | 17.997 | 16.457 | 0.420 |
| *Lachnospiraceae* | *Unknown. Lachnospiraceae NK3A20 group* | 0.041 | 0.000 | 0.0003 | 0.0004 | 0.3992 | 0.0003 | 0.0003 | 0.7692 | 14.219 | 13.509 | 0.814 |
| *Microbacteriaceae* | *Curtobacterium* | 0.040 | 0.000 | 0.0001 | 0.0003 | 0.2193 | 0.0002 | 0.0004 | 0.1885 | 0.559 | 13.272 | **0.000** |
| *Nocardiaceae* | *Williamsia* | 0.000 | 0.000 | 0.0010 | 0.0015 | 0.3218 | 0.0010 | 0.0011 | 0.8535 | 13.778 | 12.953 | 0.765 |
| *Nocardioidaceae* | *Unknown. Nocardioidaceae* | 0.026 | 0.008 | 0.0001 | 0.0005 | 0.1663 | 0.0001 | 0.0008 | 0.1265 | 0.002 | 6.005 | 0.518 |
| *Nocardiopsaceae* | *Nocardiopsis* | 0.000 | 0.019 | 0.0003 | 0.0002 | 0.6858 | 0.0005 | 0.0003 | 0.6179 | 21.631 | 22.413 | 0.784 |
| *Pseudonocardiaceae* | *Pseudonocardia* | 0.002 | 0.035 | 0.0014 | 0.0010 | 0.7495 | 0.0015 | 0.0018 | 0.8328 | 0.458 | 0.171 | 0.939 |
| *Rhodocyclaceae* | *Azospira* | 0.000 | 0.001 | 0.0023 | 0.0029 | 0.4996 | 0.0012 | 0.0017 | 0.6920 | 9.005 | 6.237 | 0.412 |
| *Ruminococcaceae* | *Anaerotruncus* | 0.000 | 0.000 | 0.0002 | 0.0004 | 0.1643 | 0.0002 | 0.0004 | 0.3246 | 16.341 | 16.282 | 0.984 |
| *Saccharimonadaceae* | *Unknown. Saccharimonadaceae TM7a* | 0.027 | 0.039 | 0.0012 | 0.0009 | 0.4370 | 0.0006 | 0.0005 | 0.8566 | 13.405 | 12.222 | 0.712 |
| *Synergistaceae* | *Unknown. Synergistaceae EBM-39* | 0.000 | 0.000 | 0.0008 | 0.0018 | 0.0800 | 0.0013 | 0.0016 | 0.7147 | 17.903 | 14.352 | 0.121 |

# 4. DISCUSSION

Optimisation of feeding regimes for sustainable and efficient fish farming practices is a promising avenue in aquaculture development. In this study feed timing was hypothesized to influence condition and growth factors, circadian rhythms of activity and the microbial gut composition of rainbow trout (*Oncorhynchus mykiss)*. Additionally, it was hypothesized that alterations of feed times may induce changes in rhythms of relative abundance of gut microbiota. Circadian rhythms of fish activity, gut microbial composition, and the prevalence of rhythmic microbiota were all significantly different between the two dietary treatments. The early feed treatment fish displayed generally arrhythmic behavioural activity and the number of rhythmic gut microbial genera were halved as a result of feeding early in the light cycle. This suggests the circadian clocks of fish are influenced by feed timing. Furthermore, feed timing was also shown to influence the body condition of *O. mykiss.* The dampening of the circadian rhythms of the host and microbiota in the early dietary treatment may be detrimental for fish welfare in aquaculture systems.

Metabolic processes and circadian rhythms are inextricably intertwined, where peripheral and core circadian clocks synchronise metabolic systems in response to light-dark and sleep-wake cyclic events (Adamantidis & Lecea, 2008; Eckel-Maham & Sassone-Corsi, 2013). Over the trial period it was found that body condition between the two treatments was significantly different, with fish fed early in the light cycle having a higher body condition (Table 2). However, final overall size and weight of fish were similar (Figure 7; Table 2). Similar growth between the two feeding regimes is perhaps unsurprising as typical determinants of growth performance (i.e., genetics, stocking density, feeding rate/frequency, water quality, and feed composition) were controlled across both treatments (Imsland *et al.,* 2020; Refstie, 1977; Akalu, 2021). However, increased body condition in fish fed early in the light cycle may suggest altered allocation of nutrition between growth and other processes. Although not studied here, comparisons of food intake and food conversion efficiency between timed feed treatment would be useful to further understand this result.

Circadian rhythms of activity have been found to synchronize with various oscillations of environmental stimuli and light is generally thought to be the predominant factor (Miller,

1978a; Manteifel *et al.,* 1978). In fish, however, feed timing appears to act as a synchronizer or entraining cue (zeitgeber); fish often exhibit food anticipatory activity (FAA) prior to feed deliverance (Sánchez-Vázquez *et al*., 1997). Increased locomotory activity several hours before the deliverance of feed is thought to allow fish to maximize food intake and nutrient utilization (Sánchez-Vázquez *et al.,* 1997; Aranda *et al.,* 2001; Sánchez-Vázquez & Madrid, 2001). This study has demonstrated that feeding time has a strong influence on the circadian rhythm of activity/rest phases in rainbow trout. Early fed fish displayed more arrhythmic behaviour than late fed fish which demonstrated a strong circadian rhythm to their activity levels (Figure 8; Supplemental Table 1). A dampened circadian clock may have resounding effects on metabolic processes (i.e., nutrient utilization) within fish. It has recently been demonstrated that the circadian clock modulates the expression of many digestive enzymes in a cyclic manner as nutrient availability is often predictable on a 24-hour scale (Chaix *et al.,* 2019; Hardin & Panda 2013). The involvement of the circadian clock with the modulation of metabolic processes may explain the association observed between arrhythmic fish fed early in the light cycle and higher body condition factors. From an aquaculture productivity standpoint, the dampened activity resulting from early feeding and the associated increase in body condition may be considered preferable than late feeding for the food production industry. However, fish growth can no longer be the sole consideration for aquaculture success given the increasing issues of fish health and diseases on fish farms.

Currently, disease is a substantial economic burden to the aquaculture industry (Gov, 2017) and is a growing concern for farmed fish welfare (Toni *et al.,* 2019). The influence of feed time on circadian rhythmicity and the association between a dampened circadian clock and increased disease warrants further investigation. It has recently been demonstrated in that circadian disruption via light manipulation results in compromised immune expression in rainbow trout (Ellison *et al.,* 2021). Moreover, emerging work in environmental and genetic mammalian studies indicates that perturbation of the circadian clock is associated with numerous adverse health consequences (i.e., cardiovascular dysfunction, premature death, cancer, metabolic syndrome, immune dysregulation, reproductive problems, learning deficits and mood disorders) (Evans & Davidson, 2013; Maury *et al.,* 2014). Furthermore, studies in humans also suggests that mistimed feeding perturbs the circadian clock and is associated to metabolic syndrome (Arble *et al.,* 2010). Recent studies are linking gut microbial communities to the

physiological function of the host, and dysbiosis of the gut microbiota as a result of circadian disruption is now thought to be critical to metabolic and immune systems (Wang *et al.,* 2020; Durack & Lynch, 2019). In recent years, there is increasing interest in aquaculture for the importance of fish gut microbiota in not only nutrient acquisition/utilisation but also their role in immunity and disease resistance (Perry *et al.,* 2020; Ellison *et al.,* 2021). Here gut microbial communities of trout were profiled under different feed timing regimes.

In agreement with previous studies on rainbow trout, the results showed that Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria were the dominant bacterial phyla present in the gut microbiome of this species. These phyla typically constitute the "core gut microbiota" of rainbow trout regardless of the diet type provided (Wong *et al.,* 2013; Ghanbari *et al.,* 2015; Rimoldi *et al.,* 2018). It has been shown that Proteobacteria, Firmicutes, and Bacteroidetes phyla represent the majority (≥ 90%) of fish intestinal microbiota in most marine and freshwater species (Givens *et al.,* 2014; Ringo *et al.,* 2016). The observation of reoccurring bacterial taxa in the gut microbiota of various fish species suggests that these bacteria are involved in essential host gut functions, such as nutrient absorption, digestion, and immune responses (Rimoldi *et al.,* 2018). In this study, evaluation of overall community structure showed no significant difference in gut microbial composition between feed treatments (Supplementary Figure 4), consistent with the concept of a shared core microbiota. However, some taxa-specific differences were found between the feed regimes. The Chitinibacteraceae family was significantly different in abundance between the two dietary treatments and was increased in the fish fed late in the light cycle. Species of this family have been previously detected in high relative abundance in intestine microbiota of "healthy" *Coreius guichenoti* and *Salmo salar* (Nikouli *et al.,* 2021; Li *et al.,* 2016). However, they are also commonly associated with skin lesions in salmonids (*Salmo trutta* and *Oncorhynchus mykiss,* (Carbajal-González *et al.,* 2011). A *Hymenobacter sp.* was the only ASV significantly different between the two dietary treatments (also greater in abundance in the late feed regime). The significance of the differences and increased abundance of Chitinbacteraceae and *Hymenobacter sp.* in the late treatment may be associated with a strong host circadian rhythm, although it will require further analyses to determine the true causality of this disparity.

For the first time, I demonstrate that fish gastrointestinal microbial communities have substantial daily dynamics (Figure 10; Table 3). A significant proportion of bacterial genera present in rainbow trout intestines exhibit rhythmic changes in relative abundance, similar to recent findings in mammals (Liang *et al.,* 2015; Risely *et al.,* 2021). Although the analysis of circadian rhythms in gut microbiomes is novel, it is now apparent that microbial circadian oscillations are critical to host physiological homeostasis (Frazier & Chang, 2020). Understanding the temporal dynamics of gut microbiomes is essential if we are to identify the mechanisms by which they influence the host organism.

Consistent with previous mammalian studies diurnal oscillations in the relative abundance of Bacteroidetes and Firmicutes were observed (Figure 10) (Liang *et al.,* 2015). Fish fed late in the light cycle displayed consistently higher relative abundances and increased numbers of rhythmic species of Firmicutes, which the majority peaked several hours after the beginning of the dark phase. Recent mice gut microbiome studies demonstrate Firmicutes peaking around the beginning of the light phase and Bacteroidetes peaking several hours after the beginning of the dark phase. However, both phyla present in the trout gut microbiome peaked at the beginning of the dark phase. These contrasting rhythms may be due to the natural differences in mice and trout chronobiology (mice; nocturnal, trout; diurnal/crepuscular). Cyclical fluctuations were also detected in abundance of *Clostridium spp.,* consistent with recent findings in meerkats which demonstrated cyclic oscillations of this genera as an assumed result of temperature-constrained foraging schedules, light-dark cycles, and niche modifications (Risely *et al.,* 2021). It is thought that modulation of microbial circadian rhythms lies largely with host circadian clock genes, however, irregular diets and prolonged exposure to dark cycles disrupts these endogenous rhythms (Perez-Hernandez *et al.,* 2017). Food consumption directly influences the microbial communities present in the GI tract due to the fluctuating accessibility of nutrients available to bacteria present. The ingestion of food is associated with an increase in Firmicutes, whilst fasting is thought to increase the numbers of Bacteroidetes (Liang *et al.,* 2015). In both treatments, there was a greater number of the rhythmic genera with peak abundance in the dark phase than during the light phase. Aside from a disparity in the total number of rhythmic genera (with far greater numbers in the late feed regime), cyclic oscillations of bacteria genera were similarly synchronised across both treatment groups. However, there was an apparent

difference observed in the presence of rhythmicity of a *Deinococci sp.* which was only rhythmic in the late fed fish, peaking several hours into the light cycle.

As discussed previously, activity patterns in the early fed fish were largely arrhythmic, suggesting a dampening of the circadian clock as a result of feed timing. The early treatment also displayed roughly 50% less rhythmic bacterial genera, compared to the late dietary treatment (12 and 23% of total genera present respectively) (Figure 10; Table 3). Recently, trout clock gene expression has been shown to be strongly associated with skin microbiome composition (Ellison *et al.,* 2021). The large disparity between the two dietary treatments in both fish behaviour and microbiome rhythms suggests that feed timing is strongly influencing expression of the molecular clock in trout, although further studies are required to determine the true interaction between gene expression and feed timing. Moreover, feed timing may be influencing the clocks of bacteria directly. Circadian clock mechanisms have been demonstrated in prokaryotes and *Cyanobacteria spp*. are well known to exhibit molecular clock mechanisms (Loza-Correa *et al.,* 2010). More recently, circadian oscillators are being reported for many other bacterial taxa. Some of the bacteria families known to possess their own endogenous clocks include Xanthomonadaceae, Comamonadaceae, Oxalobacteraceae, Pseudomonadaceae, and Flavobacteriaceae, all of which were present in the gut intestines of both dietary treatments (Figure 9). Interestingly, Xanthomonadaceae was only rhythmic in abundance in the late feed treatment. In contrast, Pseudomonadaceae was only rhythmic in fish fed early in the light cycle. Flavobacteriaceae, Oxalobaceraceae and Comamonadaceae were significantly rhythmic in both feed treatments. This disparity and interaction between prokaryote circadian clocks and host physiology as a result of feed timing requires further analysis. Ultimately this will improve understanding of microbial activity relating to functional importance to the host organism.

Despite growing evidence for the links between fish microbiomes and health, still little is known of the functional mechanisms of commensal microbiota in the teleost gut. It is vital that temporal meta-transcriptomic profiling be conducted to decipher the true extent of host-microbe interactions and more specifically their role in nutrition. Furthermore, experimental manipulation of fish microbial communities and fish clock expression may be crucial to understand their relative roles in these daily host-microbiome interactions. Nonetheless, the results presented in this study strongly suggests an association between

the host circadian clock and the temporal patterning of the microbiota found in the gut, as shown by the synchronicity between host activity levels and proportion of rhythmic relative abundance of gut microbiota compared across both treatments.

# 5. CONCLUSION

Akin to other vertebrates, fish gut microbiota is critical to nutritional provision, metabolic homeostasis, and immune defence (Sullam *et al.,* 2012; Gomez & Balcazar, 2008) and it is evident that metabolism, circadian rhythms of activity and the gut microbiome are inextricably intertwined. Although the gut microbiome is not exposed to light, diurnal host signals induce oscillations of the function (i.e., metabolite production), abundance and composition of the gut microbial communities (Frazier & Chang, 2020; Leone *et al.,* 2015). A dampening of the host circadian clock may be detrimental for fish welfare as it perturbs the commensal microbiome, potentially instigating resounding effects on the functional pathways of microbiota, and thus fish health. There is a growing body of knowledge on fish microbiomes, with increasing emphasis on nutritional management, feed timing and modification of the teleost microbiota to augment growth and aquaculture produce, whilst maintaining the welfare and health of the host organism. Despite developments in the field, a comprehensive understanding of the influence of specific gut microbiota on host physiology is still lacking (Egerton *et al.,* 2018). However, there have been associations found between alterations in the activity and composition of the fish microbiome and that of fish physiology and disease susceptibility (Ellison *et al.,* 2021). TRF has been found to positively affect commensal gut microbes in mammals (Ren *et al.,* 2019) and raises the possibility that TRF may be an important tool to manage fish microbiomes for the benefit of aquaculture. This study demonstrates the complex interplay in fish between feed timing, activity patterns, condition factors, and the microbiome. As industry turns towards the augmentation of fish microbiomes to promote health and productivity in aquaculture, we propose that a chronobiological understanding of fish microbiomes and feed treatment times may be crucial for their effectiveness and is necessary to ensure sustainable aquaculture nutrition and fish health.

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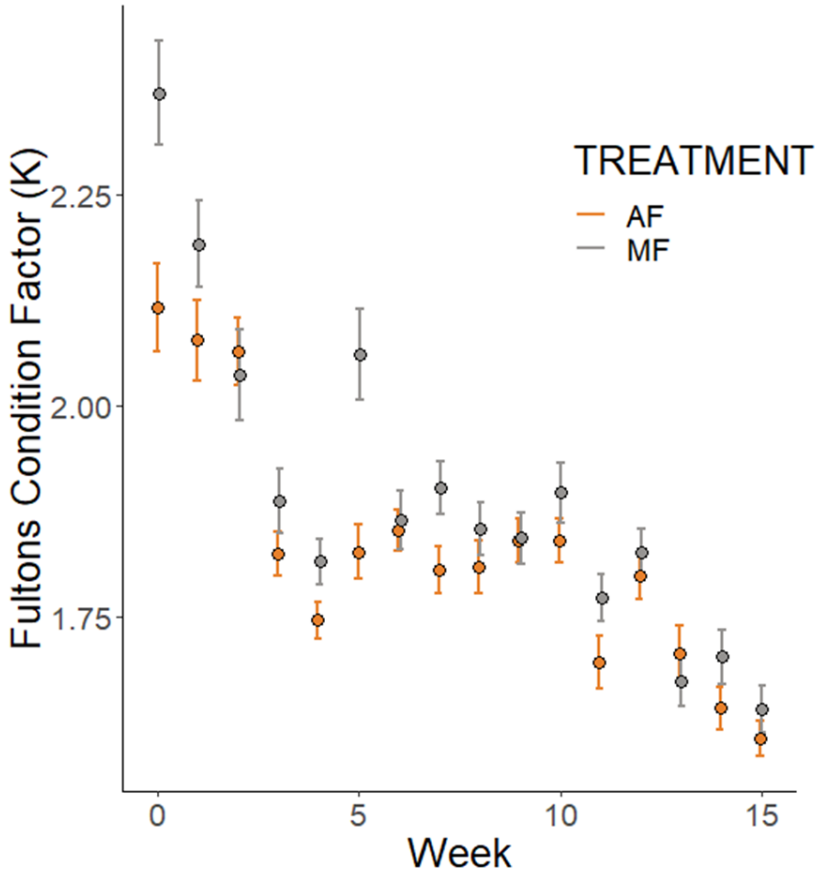
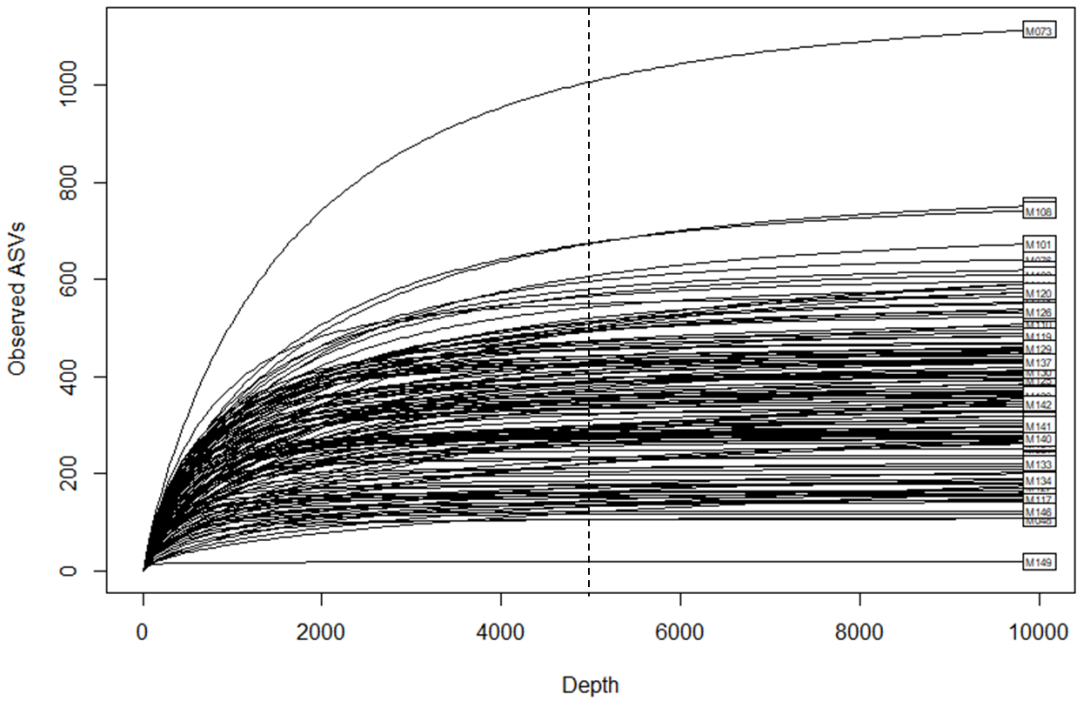
# VI. BIBLIOGRAPHY / APPENDIX

***Supplemental Table 1.*** **Summary of individual fish activity rhythmic analyses. Rhythm parameters (mesor, amplitude, phase) were estimated in CircaCompare.**

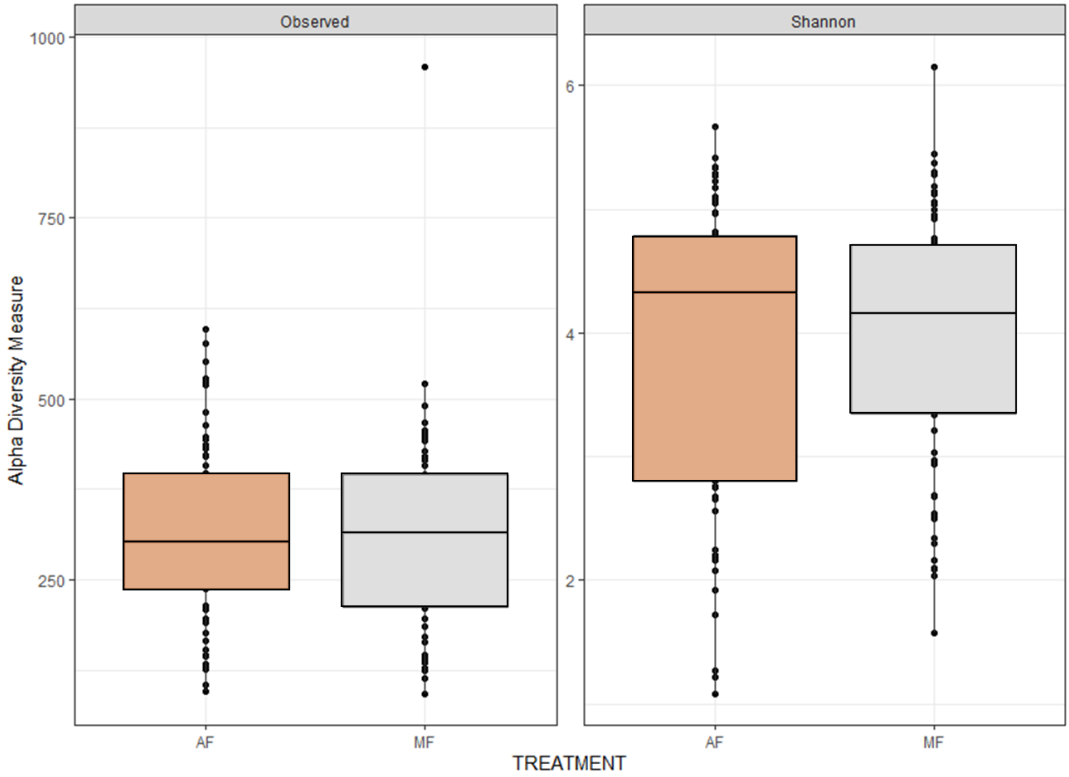
| ID | Rhythm (FDR P value*) | Mesor | Amplitude | Phase (Peak Hour) |
|---|---|---|---|---|
| 001MF | 0.14 | - | - | - |
| 002MF | 0.37 | - | - | - |
| 003MF | 0.08 | - | - | - |
| **004MF** | **<0.001** | **536.35** | **393.41** | **2.50** |
| 005MF | 0.14 | - | - | - |
| **006MF** | **0.00** | **669.19** | **167.65** | **1.78** |
| **007MF** | **0.02** | **585.72** | **183.49** | **8.87** |
| 008MF | 0.05 | - | - | - |
| 009MF | 0.09 | - | - | - |
| **010MF** | **0.00** | **615.09** | **329.50** | **3.77** |
| 011MF | 0.13 | - | - | - |
| **012MF** | **0.02** | **786.06** | **210.06** | **4.91** |
| **013MF** | **0.01** | **512.32** | **256.86** | **1.97** |
| **014MF** | **0.00** | **866.45** | **332.67** | **14.63** |
| **016MF** | **0.00** | **714.13** | **794.41** | **17.71** |
| **017MF** | **<0.001** | **874.41** | **562.07** | **14.25** |
| 018MF | 0.24 | - | - | - |
| 019MF | 0.49 | - | - | - |
| 020MF | 0.13 | - | - | - |
| | | | | |
| **001AF** | **<0.001** | **1120.79** | **590.49** | **2.86** |
| **002AF** | **<0.001** | **728.92** | **716.63** | **14.98** |
| 003AF | 0.13 | - | - | - |
| **004AF** | **<0.001** | **471.63** | **237.52** | **18.45** |
| **005AF** | **0.04** | **924.32** | **246.10** | **14.81** |
| **006AF** | **<0.001** | **900.72** | **429.03** | **16.95** |
| **007AF** | **<0.001** | **453.50** | **400.98** | **13.31** |
| **008AF** | **<0.001** | **779.01** | **478.92** | **13.77** |
| 009AF | 0.13 | - | - | - |
| **010AF** | **0.02** | **872.51** | **297.98** | **8.93** |
| 011AF | 0.25 | - | - | - |
| **012AF** | **0.04** | **907.34** | **261.99** | **11.82** |
| **013AF** | **<0.001** | **732.50** | **317.23** | **5.09** |
| **014AF** | **<0.001** | **776.45** | **325.44** | **6.27** |
| **015AF** | **0.03** | **673.11** | **166.12** | **7.50** |
| **016AF** | **<0.001** | **366.64** | **196.25** | **9.07** |
| **017AF** | **0.01** | **773.04** | **280.61** | **11.39** |
| **018AF** | **<0.001** | **995.82** | **558.81** | **12.70** |
| **019AF** | **<0.001** | **1114.87** | **311.03** | **5.30** |
| 020AF | 0.11 | - | - | - |



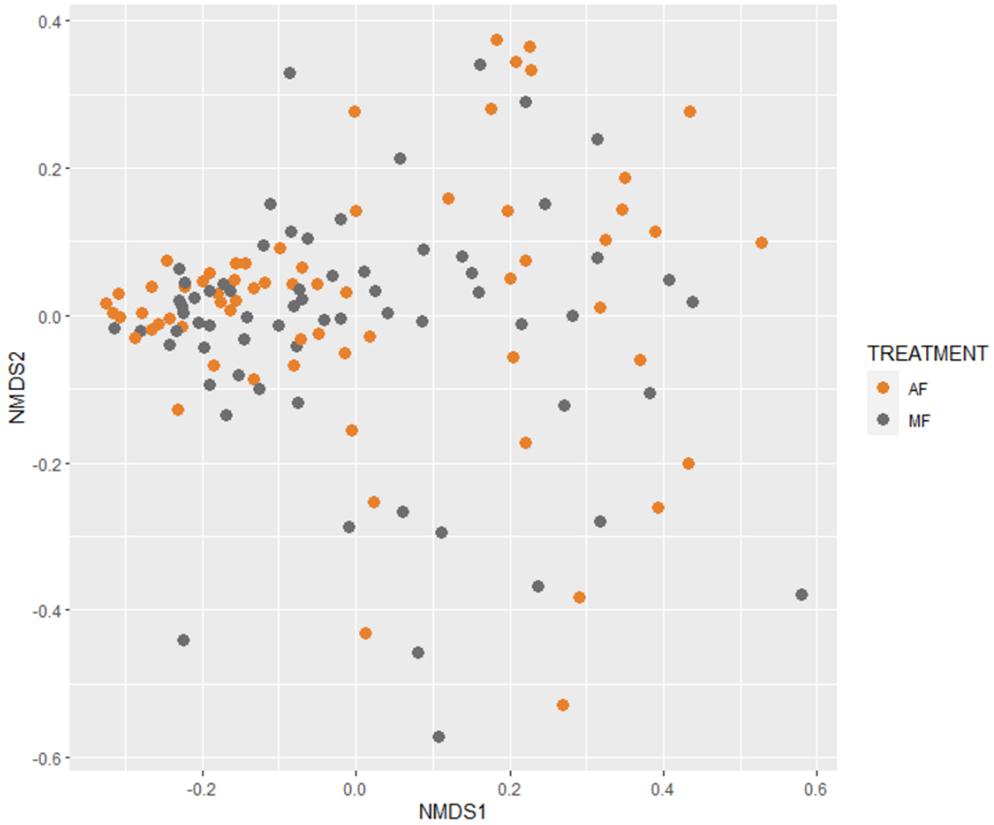

***Supplemental Figure 1.*** Average Condition *(K)* factor (±1 S.E.) over a 15-week growth trial under two feed treatments, early/morning feed (grey) and late/evening feed (orange).



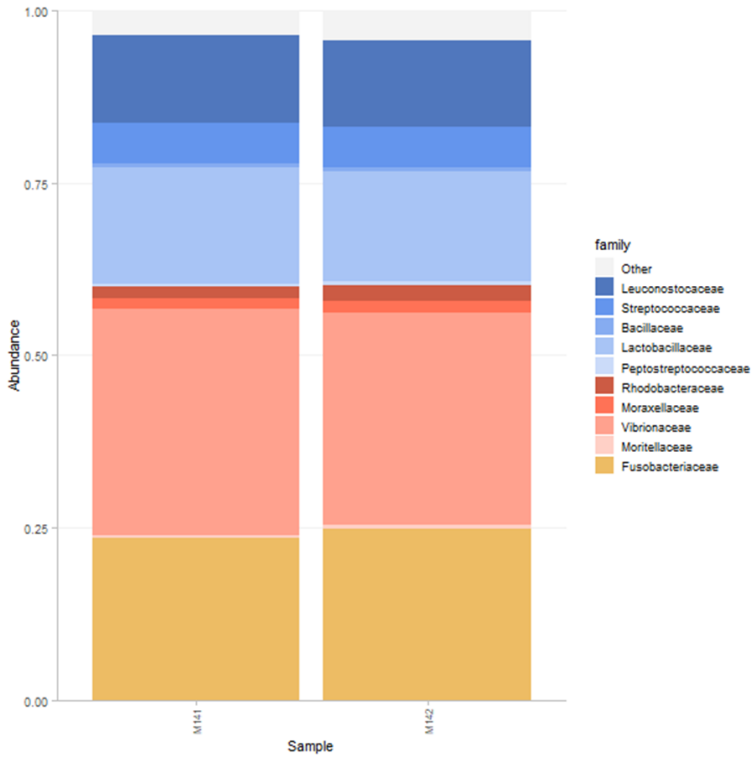

***Supplemental Figure 2.*** Rarefaction plots of detected amplified sequence variants (ASVs) by sampling depth. Dashed line indicates minimum read depth adequate to achieve diversity.




***Supplemental Figure 3.*** Alpha (α-) diversity plots by treatment group.




***Supplemental Figure 4.*** NMDS ordination of microbiome profiles.




***Supplemental Figure 5.*** Proportional abundance of bacteria ***families*** represented in amplicon libraries derived from the feed pellets provided. A total of 10 families are shown.

**X. ETHICS APPROVAL**

PRIFYSGOL BANGOR UNIVERSITY

# COLLEGE OF ENVIRONMENTAL SCIENCE AND ENGINEERING

# RESEARCH PROJECT ETHICAL ISSUES CHECKLIST FOR STAFF AND PHD STUDENTS

*Researchers (staff and PhD) should complete this ethical checklist for all research projects. If you answer 'no' in ALL of sections A B and C below, please keep this form on file as it may need to be referred to when you submit results for publication. If you answer 'yes' in ANY of sections A, B, or C below, further details will be required. Please complete sections 1, 2 or 3 as appropriate and forward the full application with supporting documents to the ethics committee (via Jane Lee).*

To assist with record keeping, please name your checklist files according to the following format: Ethics_surname_year

| **A. Research involving people or biological samples from people?** | **YES** | **NO** |
|---|---|---|
| Does the proposed research involve people or biological samples from people? | | × |
| **B. Research on animals** | **YES** | **NO** |
| Does the proposed research involve live vertebrates or cephalopods?<br>(if working with live invertebrates other than cephalopods please provide a brief description of your work in section 2) | × | |
| **C. Importing material into the UK** | **YES** | **NO** |
| Will the proposed research involve the import of material from outside the UK into the UK | | × |
| **Permission to conduct your research** | **YES** | **NO** |

| | | |
|---|---|---|
| Please confirm you have all required permission to conduct your research (including permission from land owners to access land). Please note it is your responsibility to ensure all permits and permission is obtained. | × | |
| Project title: UTILISING CHRONOBIOLOGY FOR SUSTAINABLE AQUACULTURE NUTRITION & FISH HEALTH | | |
| Proposed start date: 01/10/20 | | |
| Proposed end date: 01/08/21 approximated | | |
| Funding body. Fee Waiver as Bangor University Staff | / | |
| Name of researcher (applicant): Charlie George Max Gregory | / | |
| Email address: osub4a@bangor.ac.uk / charliegeorge.mg@gmail.com | / | |
| **For PhD students only** | | |
| Supervisor's name: | **Dr Amy Ellison** | |
| Supervisor's signature | | |

Please note that it is your responsibility to follow the University's Research Ethics Policy, the General Data Protection Regulation, and any relevant academic or professional guidelines in the conduct of your study. This includes providing appropriate information sheets and consent forms, and ensuring confidentiality in the storage and use of data. It is also your responsibility to ensure that you have all necessary permits to conduct your research. Any significant change to the project over the course of the research should be notified to the secretary of the ethics committee and may require a new application for ethics approval.

---

## Research involving people and human biological samples (please complete if you ticked yes in box A)

| | Research that may need a full review by CESE Ethics Committee | Yes | No |
|---|---|---|---|

| | | | |
|---|---|---|---|
| 1 | Does the research require review by an NHS Research Ethics Committee? If Yes the research should be submitted to the NHS Ethics Committee in the first instance. | | ✕ |
| 2 | Does the research involve children or vulnerable adults, such as those with a learning disability or cognitive impairment, or individuals in a dependent or unequal relationship e.g. your own students? | | ✕ |
| 2a | If you answered Yes to question 2, has the researcher confirmed with Human Resources if a Disclosure and Barring Service (DBS) check is required (replaces CRB check)? | | ✕ |
| 3 | Will the research involve the collection or storage of human tissues (defined as any material containing human cells i.e. including blood, urine and saliva)? | | ✕ |
| 4 | Will the study require the co-operation of a gatekeeper for initial access to the groups or individuals to be recruited? (e.g. students at school, members of self-help group, members of an association?). | | ✕ |
| 5 | Will the research necessarily involve deception or be conducted without participants' full and informed consent at the time the study is carried out (e.g. covert observation of people in non-public places, analysis of social media data)? | | ✕ |
| 6 | Will the study involve discussion of sensitive topics (e.g. illegal behaviour-including resource use which breaks local rules, trade-union membership, political views, health, religion, sexual orientation, ethnic status, genetic data)? | | ✕ |
| 7 | Will the study involve intrusive interventions (e.g. administration of drugs or other substances, vigorous physical exercise)? | | ✕ |
| 8 | Will the study induce psychological stress, anxiety or humiliation or cause more than minimal pain? | | ✕ |
| 9 | Will the research collect data through an online survey?[1] | | ✕ |
| 10 | Will the research involve access to records of personal or confidential information (including genetic, biometric and biological information, concerning identifiable individuals) or collecting information which identifies individuals?[2] | | ✕ |

[1] Such online surveys must be set up in such a way as to ensure they are not unintentionally collecting personal information (IP address etc).

[2] Data which allows individuals to be identified is covered by the General Data Protected Regulation and all that implies. We encourage researchers to consider carefully if they need to collect individually identifying information. If they do then it must be collected, handled and stored in accordance with the act.

**RESEARCH OUTLINE**

If the answer to any of the above questions is yes, then please fill out the box below and submit to the CESE Ethics Committee along with your supporting information (questionnaires or interview protocol, copies of your informant information sheet, consent forms and completed social survey checklist) via Jane Lee (j.lee@bangor.ac.uk).

| |
|---|
| *Outline of proposed research and the research questions:*<br><br>*Identify the target population:*<br><br>*Sampling design (how will target population be sampled):*<br><br>*Define the potential benefits of the research:*<br><br>*Define how data will be stored and what information will be provided to participants about data collection or storage:*<br><br>*Define any potential risks or negative impacts:*<br><br>*Describe how risks will be controlled:* |

| | **Student** | **Supervisor (if PhD research)** |
|---|---|---|
| **Supporting documents** | | |
| A copy of the survey instrument(s) is attached. | | |
| A copy of the participant information sheet and consent forms are attached. | | |
| The research questions are laid out (in accompanying proposal or in RESEARCH OUTLINE above). | | |
| | | |
| **1. Fit to research questions** | | |
| The research questions are precise and answerable. | | |
| The survey questions are necessary and sufficient to answer the research questions. | | |
| The form of data to be collected (scalar, ordinal, categorical or qualitative) has been considered, and will allow appropriate analyses to be conducted. | | |
| | | |
| **2. Target population & sampling** | | |

| | | |
|---|---|---|
| The target population is appropriate and necessary to answer the research questions. | | |
| The proposed sampling method is appropriate. | | |
| The proposed sample size is both achievable AND sufficient to answer the research questions. | | |
| | | |
| **4. Ethics and consent** | | |
| The participant information sheet clearly states: | | |
| the purpose of the research. | | |
| the approximate duration of the survey and what is required of participants. | | |
| whether data will be anonymous/confidential (and who will see the data), how it will be stored and for how long (and any possible reuse e.g. public archiving). | | |
| the participant's rights with respect to withdrawing their consent to hold and process data. | | |
| the name and contact details (usually email) of the researcher. | | |
| The researcher has clear protocols for recording and storing consent. | | |
| | | |
| **5. Piloting & proofing** | | |
| Plans for piloting are appropriate. | | |
| Survey instrument(s) and associated documents checked for spelling, grammar and clarity. | | |
| Survey questions are clear, with appropriate response options (if applicable) and arranged in a logical order. | | |
| Jargon is minimised and any necessary terminology is clearly defined. | | |
| The survey is an appropriate length, and not overly onerous to complete. | | |

## Research on vertebrates and cephalopods (please complete if you ticked yes in box B)

| **Research that may need review by either the CESE Ethics Committee or the University Animal Welfare and Ethical Review Body** | **Yes** | **No** |
|---|---|---|
| 1. Do you intend to perform any actions which fall under the Animals (Scientific Procedures) Act 1986? Please see http://tna.europarchive.org/20100413151426/http://www.archive.official-documents.co.uk/document/hoc/321/321.htm | | ✕ |
| *If yes, please go to question 2. If no, please complete project details section and outline of proposed research and forward to John Latchford (j.latchford@bangor.ac.uk), together with your initial research project ethical issues checklist.* | | |
| 2. Will the research be carried out in the UK? | ✕ | |
| *If yes, please go to question 3. If no, please complete the research outline box below and return to John Latchford (j.latchford@bangor.ac.uk), together with your initial research project ethical issues checklist.* | | |
| 3. Is this research authorised by a current Home Office project licence? | | ✕ |
| *If yes please complete the research outline box below and send the completed form, together with your initial research project ethical issues checklist, to John Latchford (j.latchford@bangor.ac.uk). If no, you must obtain a project licence before starting work. Please see*<br><br>*http://www.homeoffice.gov.uk/publications/science/769901/licences/project-licences/.*<br><br>The completed project licence application must be submitted to and approved by the University Ethical Review Committee (Gwenan Hine, gwenan.hine@bangor.ac.uk) prior to a formal application to the Home Office. *Please also send a copy of this form, together with your initial research project ethical issues checklist, to John Latchford (j.latchford@bangor.ac.uk).* | | |
| 4. Have you got agreement of a Home Office personal licence holder that they will carry out any procedures which fall under the Act? | | ✕ |
| *If yes, please ask the appropriate licence holder to countersign this form and send it to John Latchford (j.latchford@bangor.ac.uk). If no, you will need obtain a personal Home Office licence before starting work. Please see*<br><br>*http://www.homeoffice.gov.uk/publications/science/769901/licences/personal-licences/?view=Standard&pubID=788367. Please also send a copy of this form, together with your initial research project ethical issues checklist, to John Latchford (j.latchford@bangor.ac.uk).* | | |

| **Home Office project licence details (*if you answered 'yes' to question 3*)** |
|---|
| **Home Office personal licence holders (*if you answered 'yes' to question 4*)**<br>I confirm that I will carry out any procedures covered by the Animals (Scientific Procedures) Act in relation to this project. |
| Licence holder's name: |
| Licence holder's signature: |
| Date: |

**RESEARCH OUTLINE**

You must complete this section for all work involving live vertebrates and cephalopods (include a brief description of the work if working with invertebrates other than Cephalopods)

| |
|---|
| *Define the potential benefits of the research*<br>*List species and numbers of animals used and in which country the proposed study will take place*<br>*Describe any risks (including the potential for pain, suffering or lasting harm) to animals used in the study*<br>*Describe how the principles of the 3Rs (Replacement, Reduction, Refinement) have been applied to your study* |

## 3. Importing material into the UK (please complete if you ticked yes in box C)

**RESEARCH OUTLINE**

| |
|---|
| *What material will be imported; what legislation covers the import of this material.* |

N/A

*Outline of proposed research:*

**I. Details of Proposed Research**

**i. Field of Study:** Chronobiological influences of feed timing and gut microbiomes on teleost fish health.

The core purpose of this study is to examine the chronobiological interactive effects of feed timing alongside gut microbiomes on teleost fish health. I will aim to quantify the impact of time-restricted feeding strategies on growth, while also characterising the rhythmicity of fish gut microbiota under varying feeding strategies. Collectively, this will provide a new fundamental understanding of how chronobiology can be utilised to augment sustainable and efficient aquaculture. Alongside this, further insight will be developed on the influence of circadian rhythms within vertebrates and will add to the body of scientific knowledge. One day hoping to allow future fisheries to augment their systems to take full advantage of these endogenous rhythms; providing protein nutrition and calorific value for the ever-growing human population. This study will take place within the Brambell Aquaria, Bangor, utilising both CT1 and CT2 rooms over a period of 6-8 months.

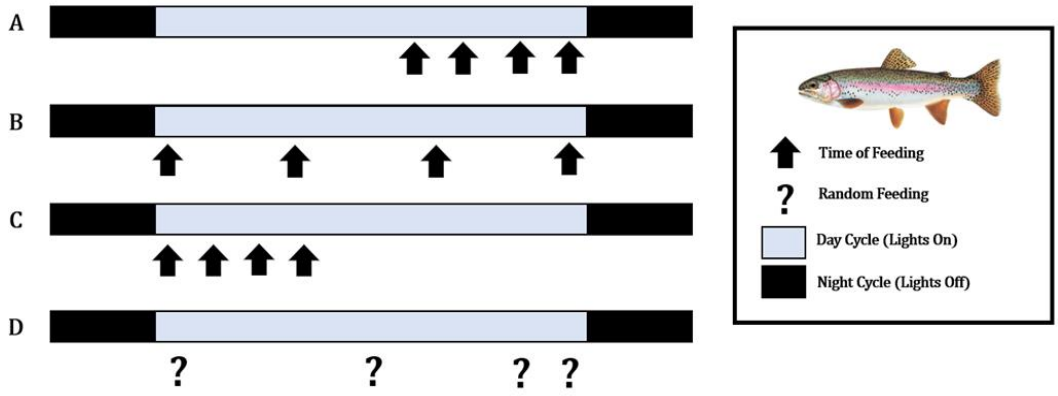



**Figure 1.** Illustrated schematic demonstrating the four daily feeding regimes, (A) 4 times during the late light cycle, (B) 4 evenly spaced feeding times throughout the daily light cycle, (C) 4 times during the early light cycle, and (D) 4 random allocated times interchanged daily.

**ii. Aims and Objectives**

**Aim 1**: Quantify the impact of time-restricted feeding strategies on fish metabolism and immune expression rhythmicity.

**Objective 1.1:** Raise juvenile rainbow trout (*Oncorhynchus mykiss*) on four different time-restricted feeding regimes (Figure 1): 4 random times per day, 4 specific times per day (twice

early and late), 4 early feeds and 4 late feeds. The trout will be delivered feed by Oase™ branded automatic feeders, filled with nutritionally balanced commercial trout feed. Distributed feed will be targeted between 2-6% of body weight and will adjust accordingly with age and size, decreasing as the fish grow as recommended by the FAO. The automatic feeders will be tuned, and the amount of feed delivered per rotation will be adjusted to minimise the amount of uneaten food. The tanks will not have any substrate so uneaten waste food will be noticed immediately upon inspection and will be siphoned out twice daily (if necessary) following the early and late scheduled feed. However, I hope to tune the feeders to provide the adequate amount of food prior to official experimentation as excess, readily available food may skew the results. Growth rates, food conversion efficacy, daily activity levels and survival will be quantified and explored. On a weekly rota a subset of fish will be removed from their tank so that weight and length measurements can be taken. The restraining method used has been proposed by Levin *et al.,* (2011), where removed fish will be restrained in a net, placed on a flat wet sponge and secured with the thumb and forefinger while length measurements are taken. The eyes will be covered with a damp cloth to keep the fish calm. Weight measurements will be taken by placing the fish in a pre-weighed jug of water on a standard metric weight scale and moved back into their tank in as efficient and calm a manner as possible. 400 total fish will be used within the study, regarding stocking density there will be 50 fish between 8x 200L tanks. Each treatment group (n=4) will have a duplicate tank to act as a replicate study. An approximated 30x total fish will be analysed from each treatment group on a weekly basis – 15x per tank for dual replicates - a total of 120. The rainbow trout will be subject to a 12:12 LD light cycle, ranging from 7:30am-7:30pm.

**Objective 1.2:** Over 48hr time-course, sample 6 fish per treatment (n=4) every 4 hours for gut contents for microbiome analysis (See Aim 2). 13-time plots over a 48-hour period will require a total of ~312 approximated fish, rounded to 400 to account for random mortality. (4 feed treatments* 6 sample fish* 13-time plots)

**Aim 2:** Characterise the rhythmicity of fish gut microbiota under different feeding strategies.

**Objective 2.1:** Using 16S rRNA profiling, contrast the diversity and structure of gut microbial communities under the 4 different feeding regimes.

**Objective 2.2:** Using statistical rhythmic analyses identify bacteria taxa exhibiting rhythms in abundance (relative to entire gut community) linked to 1) feed timing, and/or 2) light/day cycles.

**Objective 2.3:** Use co-occurrence network analyses to identify groups of bacteria taxa that potentially directly interact or are indirectly associated during feeding/light cycles, contrasting these interactions/associations between different feeding regimes. Use functional enrichment analyses to identify shifts in bacteria functional pathways (e.g. nutrient acquisition, metabolism) associated with feeding regime and/or time of day.

**II. Risk and Concerns**

**i. Potential for Pain**

The Rainbow Trout used within this study will be subject to irregular feeding times or feeding at uncommon hours, weekly removal for growth and weight measurements and finally the potential to be euthanised for gut microbiome analysis at the end of the study. Regardless of

irregular feeding times it will be ensured they are fed an adequate, nutritionally healthy amount. Regarding the weekly removal for growth and length measurements, a temporary stress period will be induced but previous experience by Dr Amy Ellison has shown that no mortality is shown from this and behaviour returns to normalcy following a brief period. Further methodology drawn from Levin *et al.,* (2011) will also be implemented to minimise stress and ensure an efficient process. The use of only a subset of fish from each tank to obtain statistically robust measures will mean that not all fish are subject to weekly removal and will hope to offer some respite to those having undergone the process prior to remeasurement.

**ii. Principles of the 3 Rs**

Processes will be implemented to mitigate any unnecessary stress or discomfort to the animals, and they will be housed in pristine conditions within an appropriate tank size (~200litre), with weekly 50% water changes, enrichment in the form of artificial plants or tubes to simulate a natural environment. Water will be moved from the standing water setup on the roof of Brambell through the pipe system and left to sit in 3x 80L bins in each room on a weekly basis where chorine and chloramine will both evaporate prior to water changing. External filters rated at 1200 LPH will be used as standard on each tank. Any necessary removal of the trout from their enclosures will be done swiftly and with two nets to avoid chasing them around for any unnecessary extra time – avoiding the fear of predatory behaviour within a small enclosed environment. In regards to destruction, a solution of MS222 and Sodium Bicarbonate will be used at the correct measurements to ensure a painless, stress-less anaesthetic process; after 5-10minutes, upon noting the slowing down and stopping of gill movement, the animal will be removed from the solution and its brain will be destroyed using a sharp, sterile scalpel. The animal waste will be contained within a biological waste bag in a dedicated freezer, ready for authorised removal and destruction. Following the reduction principal of the 3Rs, on a weekly basis a subset of fish will be used for growth and length measurements. This subset will represent and quantify the entirety of the group, providing a model value to avoid subjecting a larger number of fish to removal. The period between sampling and the proportion of fish sampled compared to total sample size will ensure that there is a rotation between sampling for any one individual. Amy Ellison has previously shown the numbers of animals required are the minimum required to obtain statistically robust measures of growth and rhythmic analyses. All tanks will be supplied with $O_2$ through connected airlines/air stones with valves fully open and the outflows of connected external filters rated at 10x LPH of the volume of water will be raised slightly above the water to ensure adequate surface agitation. Following normal procedure within the aquarium these tanks will be tested for ammonia, nitrites and nitrates using a commercial aquarium test kit. All tanks will be cycled prior to introduction of fish using pre-seeded media and test kits will be used to ensure the nitrogen cycle is complete. During the weekly 50% water changes, all uneaten food / fish waste will be siphoned out alongside the water to ensure a clean environment for these organisms.