Description of Scientific Techniques and Concepts Acquired in an International Setting

Taylor Rossiter, Nate Wilson, Miki Tanikawa, Yuma Katada, Kumiko Ito-Miwa, Tomoya Nakayama, Taeko Ohkawa, Takashi Yoshimura

General Introduction/Abstract:

During 9 weeks in the summer of 2024, I had the amazing opportunity to study abroad at Nagoya University in Nagoya Japan. During this study, I worked under Dr. Takashi Yoshimura at the Institute of Transformative Bio-Molecules (ITbM) and the School of Agricultural Sciences learning various scientific techniques and skills. This paper will highlight some of the skills and knowledge that I have acquired during my international study, as well as discuss the cultural experiences that I have had during my stay and how they have shaped me into a global citizen.

Histology:

Objective:

During this experiment, we had two separate learning objectives. The first was to learn how to use the cryostat to obtain tissue sections that we would later use in staining. The second was to learn how to stain those tissue samples using Nissl and hematoxylin and eosin (HE) staining. The tissue samples we used were the fugu brain, gills, eyes, and testes, as well as the mouse brain.

Methods:

All tissue samples were covered in a frozen sectioning compound and were fit to the cryostat stage. The samples were then cut into 20 um portions fixed to glass slides and placed in -80°C until they were used for staining. For both staining methods, all of the samples were baked at 55°C for 15 minutes and washed in running tap water for 4 minutes then rinsed with distilled water. The fugu brain, fugu eyes, and mouse brain were stained using the Nissl staining protocol. The fugu gill and testes were stained using hematoxylin and eosin (HE) staining.

Briefly, the mouse brain Nissl staining was performed using the following protocol. After baking and rinsing, the mouse brain samples were stained at 37°C with 0.1% cresyl violet solution for 10 minutes. The samples were then differentiated into 95% ethanol + 10% acetic acid for 5 minutes, 95% ethanol for 1 minute, and 100% ethanol for 1 minute. After differentiation, the samples were stained with 0.1% cresyl violet solution at 37°C for 10 minutes and differentiated in 100% ethanol for 1 minute. Finally, the samples were dehydrated in 100% ethanol for 15 minutes and were cleared in xylene for 30 minutes.

For the fugu eyes and brain Nissl staining, the samples were stained with 0.1% cresyl violet solution at 37°C for 10 minutes after baking and rinsing. The samples were then differentiated into 95% ethanol + 10% acetic acid for 5 minutes, 95% ethanol for 1 minute, and 100% ethanol for 1 minute. The differentiation was then repeated to remove any excess staining and the samples were then dehydrated in 100% ethanol for 15 minutes and cleared in xylene for 30 minutes.

The fugu gill and testes were stained using hematoxylin and eosin (HE) staining. Briefly, after baking and rinsing the samples were stained in hematoxylin solution for 4 minutes and were rinsed in running tap water for 15 minutes to remove any residual staining. The samples were then counterstained in eosin for 2 minutes and were differentiated in 70% ethanol by dipping the samples quickly 10 times. The samples were then dehydrated in 100% ethanol for 15 minutes and cleared in xylene for 30 minutes.

Results:

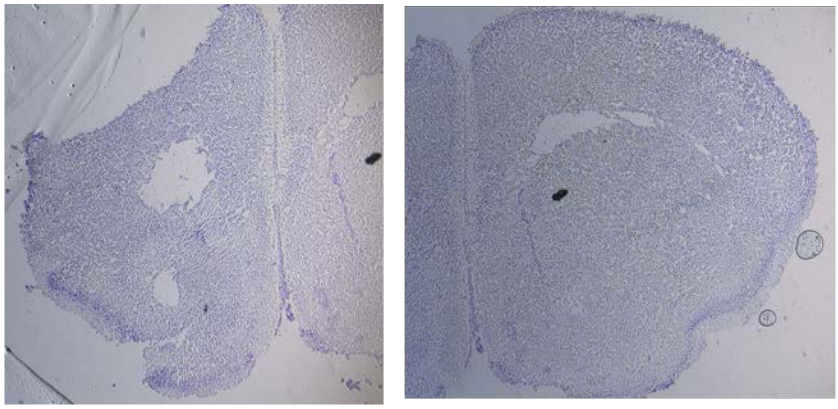

Figure 1: Mouse brain Nissl Stain

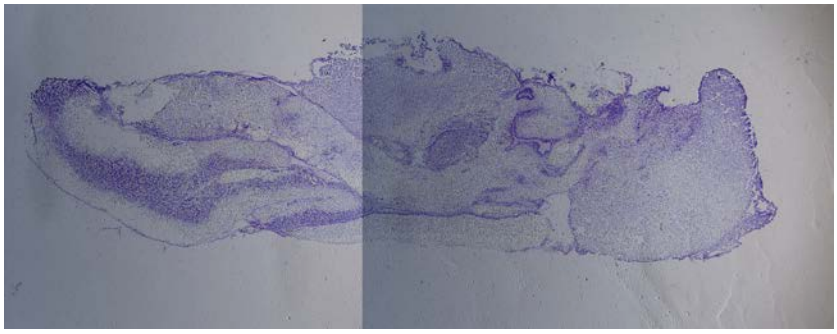

Figure 2: Fugu Brain Nissl Stain

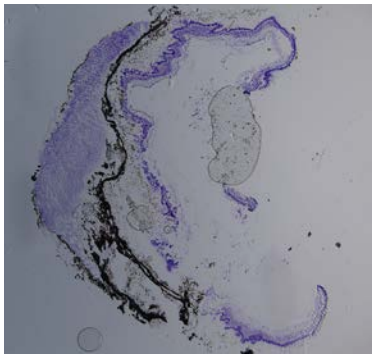

Figure 3: Fugu Eye Nissl Stain

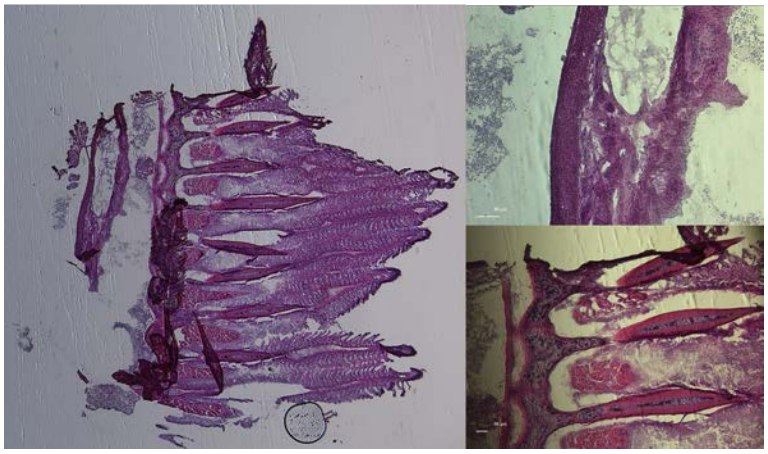

Figure 4: Fugu Gill HE Stain

Discussion:

Tissue samples of mouse brain, fugu brain, testes, gills, and eyes were successfully obtained for staining. Both the Nissl stain and the HE staining were successfully performed. However, many of the samples were damaged or torn when being fixed to the slide during the cryostat stage. In the future, more care should be taken when handling the samples so that they do not get damaged as easily. Additionally, if parafilm samples were used for staining instead of cryostat samples the amount of damage to the tissues may not have been as severe.

Microinjection:

During this experiment, we aimed to learn the techniques necessary to perform microinjections in medaka embryos. To accomplish this, we watched videos on microinjections and then practiced using medaka embryos using tracer dye. During this experiment, we learned how to harvest medaka embryos and we practiced locating the single cell and injecting the embryo with tracer dye. We learned many facts and techniques as well that we need to know in the future to perform a successful microinjection. For example, we learned that placing medaka

embryos on ice slows their development. We also learned that the embryos need to be injected during the single-cell phase of development or else a mosaic will develop.

KaiC SDS-PAGE Project:

Introduction:

Circadian rhythms refer to the internal time clocks that can be observed in various domains of life, from complex homosapiens to simpler, single-celled organisms, like dinoflagellates. Circadian rhythms must be endogenous and entrained, and there must be a temperature compensation of their period. For a rhythm to be endogenous, circadian rhythms must be present even without external factors such as temperature or light meaning they are not directly dependent on them to function. Entraibale refers to the ability of the rhythm to be changed or synchronized to external time cues such as the light-dark cycle. Finally, temperature compensation refers to the idea that the period of circadian rhythms remains constant despite changes in temperature since many of the biological processes that circadian rhythms rely on are temperature-dependent. Period refers to the length of time that it takes for the circadian rhythm to complete a full cycle, the period also determines the timing of specific biological processes that the circadian rhythm depends on. Additionally, the frequency of a circadian rhythm refers to the number of cycles that occur within a day. For example, if the period of the circadian rhythm is 24 hours, their frequency would be 1, since one cycle occurs within 24 hours.

Throughout our stay in Japan, we investigated the circadian rhythm found in cyanobacteria. Previous studies have identified three clock genes in cyanobacteria, *kaiA*, *kaiB,* and *kaiC*, that are essential for the circadian rhythm (Ishiura et al. 1998). The proteins *kaiB* and *kaiC* are expressed by the *kaiBC* protomer, whereas *kaiA* is expressed by the *kaiA* promotor (Ishiura et al. 1998). When overexpressed, *kaiC* was shown to suppress the *kaiBC* promotor; however, when *kaiA* was overexpressed, the *kaiBC* promoter was enhanced demonstrating *kaiC's* importance to the regulation of the cyanobacteria circadian rhythms (Ishiura et al. 1998). *KaiC* has been categorized as a hexamer consisting of two ATPase domains, CI and CII (Pattanayek et al. 2004). The CI domain contains only ATPase activity whereas CII contains both ATPase and phosphatase activity (Pattanayek et al. 2004). *KaiC* has two separate phosphorylation sites, threonine 432, and serine 431 which are sequentially auto-phosphorylated and dephosphorylated leading to four phosphorylation states (Nishiwaki et al. 2007). The first phosphorylation state is the unphosphorylated threonine and unphosphorylated serine *kaiC* (S/T), the second is the phosphorylated threonine and unphosphorylated serine (pT/S) *kaiC*, the third is the phosphorylated threonine and serine (pT/pS), and the final phosphorylation state is the unphosphorylated threonine and a phosphorylated serine (T/pS) state.

The cyanobacteria's phosphorylation rhythm can also be recreated in vitro using all three *kai* proteins (*kaiA, kaiB, and kaiC)* and ATP (Nakajima et al. 2005). The ratio of phosphorylated *kaiC* in the reaction mixture can then be measured over time to graph the circadian rhythm. Previous studies have demonstrated that *kaiC* ATPase activity is responsible for controlling the

period of the phosphorylation rhythm in vitro (Terauchi et al. 2007), however, the relationship between *kaiC's* phosphorylation state and ATPase activity has not been explored. This study aimed to compare the circadian period of 7 *kaiC* mutant proteins (1, 2, 3, 4, S157P, T24S, and 5) and the wild-type *kaiC* to the previously identified *kaiC* ATPase activity using a high-resolution SDS-PAGE to compare the 4 phosphorylation states of *kaiC* and ATPase activity.

Methods:

KaiC Buffer Exchange and Protein Quantification:

To recreate the cyanobacteria circadian rhythm in vitro for analysis, *kaiC* proteins (1, 2, 3, 4, S157P, T24S, 5, and WT) were collected from the -80°C freezer, and protein aggregates were removed by performing a buffer exchange using the Bio-Spin® P-30 Gel Columns with Tris Buffer from BIO-RAD. The reaction buffer used for the exchange contained 20 mM Tris [pH 7.8 at 30°C], 150 mM NaCl, 5 mM $MgCl_2$, and 1 mM ATP. After the exchange, the *kaiC* proteins were quantified using the Bradford method. The dye reagent used was the Quick Start Bradford 1X Dye Reagent from BIO-RAD and the bovine serum albumin (BSA) protein standard had a concentration of 1mg/mL and was obtained from Sigma-Aldrich.

In Vitro Reconstruction of *kaiC* Phosphorylation Rhythm

Once the protein concentrations were confirmed, reaction mixtures were prepared in vitro to mimic the *kaiC* phosphorylation rhythm. To accomplish this 0.04 mg/mL of *kaiA,* 0.04 mg/mL of *kaiB,* and 0.2 mg/mL of *kaiC* (mutant protein or WT) were combined with buffer (20mM Tris, 150mM NaCl), 0.1M ATP, and 0.5M $MgCl_2$ to a final amount of 200uL in a centrifuge tube. One 100uL portion of the *kaiC* reaction mixture was aliquoted into a second centrifuge tube and placed in the block incubator at 30°C at 21:00. 12 hours later, the second tube containing the remaining *kaiC* mixture was placed in the block incubator and 3uL portions were collected from all samples. The 3uL portions were then mixed with a 2xSDS buffer in a 384-well plate. Sampling was repeated at 9:00, 13:00, and 17:00 over a total of four days allowing us to collect a total of 24 samples for each *kaiC* protein in 4-hour increments (96 hours total).

SDS-PAGE

To perform SDS-PAGE on the collected *kaiC* protein samples, high-resolution SDS-PAGE gels were made. A 10% acrylamide separation gel and 4% acrylamide stacking gel were made using an acrylamide/bis ratio of 29.8 to 0.2. When solidified, 20uL of the *kaiC* protein samples were loaded into the gels along with 5uL of the molecular weight marker and ran at 20 mA 300V for 1 hour and 40 mA 300V for 4 hours. When completed, the gels were washed with distilled water and stained with Quick-CBB plus (FUJIFILM) stain overnight. The next day the remaining dye was rinsed off using distilled water and gels were scanned.

Data Analysis

To analyze the *kaiC* SDS-PAGE scans, ImageJ was used to determine the pixel density of each band. Each band on the high-resolution gel corresponds to a different phosphorylation state of the *kaiC* mutant protein. The first band represents a phosphorylated threonine and serine (pT/pS), the second represents a phosphorylated threonine and unphosphorylated serine (pT/S), the third represents an unphosphorylated threonine and a phosphorylated serine (T/pS), and the fourth band represents the unphosphorylated threonine and unphosphorylated serine *kaiC* (S/T) phosphorylation state. The ratio of each phosphorylation state (pT/pS, pT/S, T/pS, and ST) was then determined using Excel along with the overall ratio of phosphorylated *kaiC* (P-*kaiC*). The period (τ) and amplitude (A) of the *kaiC* phosphorylation rhythm after 12 hours of incubation was then estimated by fitting the P-*kaiC* ratio (Y) to a cosine function of time (t) as previously described by (Nakajima et al. 2010), $Y(t)=A\cos(2\pi(t-a)/\tau+b$, using R-studio.

ATPase activity (day-1 KaiC-1) of each *kaiC* protein used in this study was provided to us by our mentor Kumiko Ito-Miwa. The frequency (day-1) of each *kaiC* protein was then calculated using the predicted periods and was compared to the activity of ATPase using Excel software.

Results:

SDS-PAGE

KaiC protein samples (WT, 5, 4, 3, T42S, S157P, 2, 1) were collected every 4 hours for 92 hours and were run using SDS-PAGE. We expected the results to show 4 bands representing the four phosphorylation states of *kaiC.* However, we were only able to achieve two band separations for all of the *kaiC* proteins representing phosphorylated *kaiC* (P-kaiC) and unphosphorylated *kaiC* (kaiC) (Figure 5).

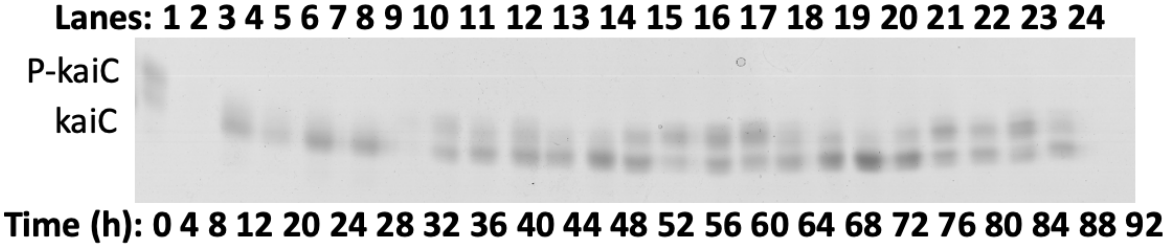

Figure 5: 10% polyacrylamide gel demonstrating the phosphorylation state of mutant *kaiC* protein 3 over 92 hours. The top row represents phosphorylated *kaiC* (P-kaiC) while the bottom row represents unphosphorylated *kaiC* protein (kaiC).

Data Analysis

Through the use of ImageJ and R-studio software, SDS-PAGE gels of sampled *kaiC* proteins were analyzed and the period estimation of the *kaiC* proteins was determined (Figures 6

and 7). Unfortunately, the data did not fit the *kaiC* proteins 5 (Figure 6.B), 4 (Figure 6.C), and 2 (Figure 7.C).

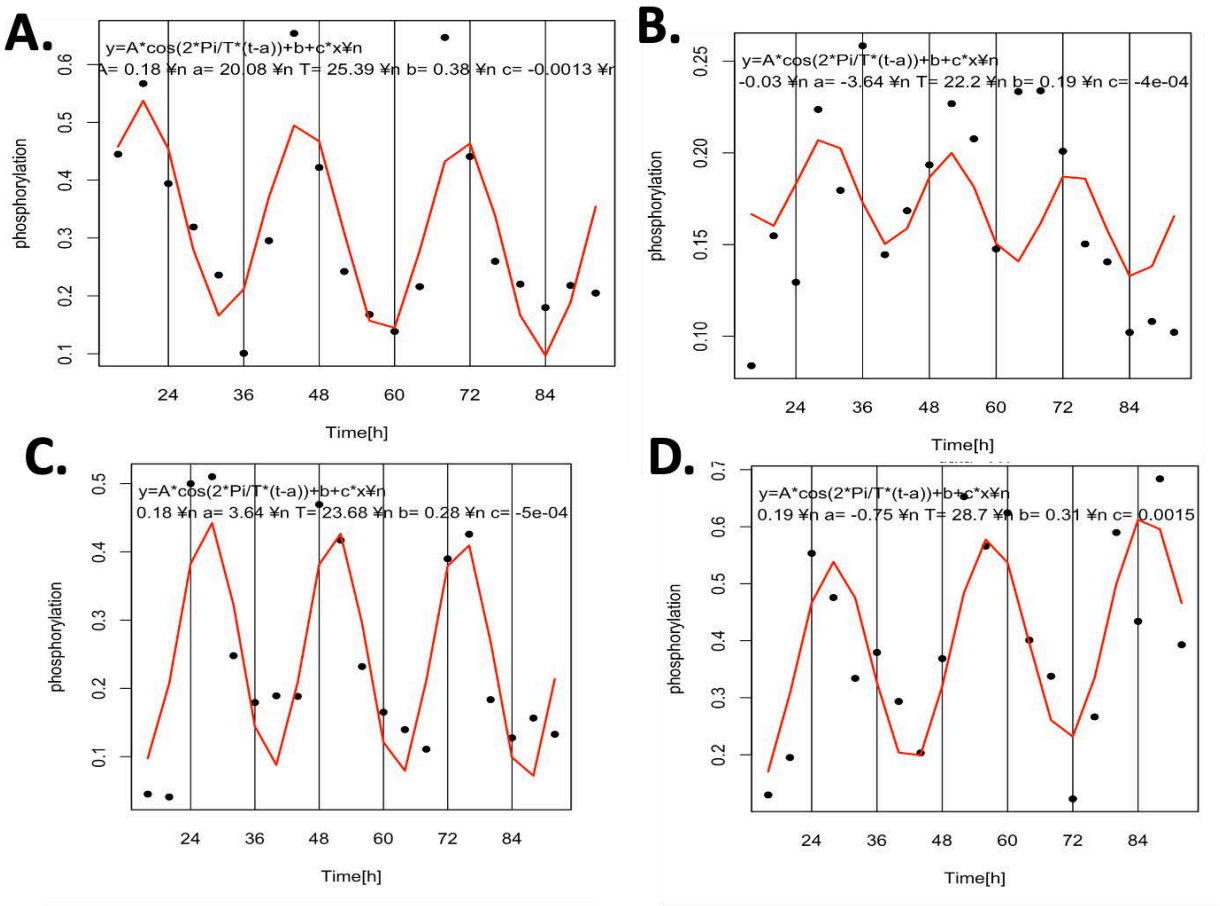

Figure 6: Period estimation of wild-type *kaiC* (A, T=25.39h), and mutant *kaiC* proteins 5 (B, T=22.2h), 4 (C, T=23.68h), and 3 (D, T=28.7h).

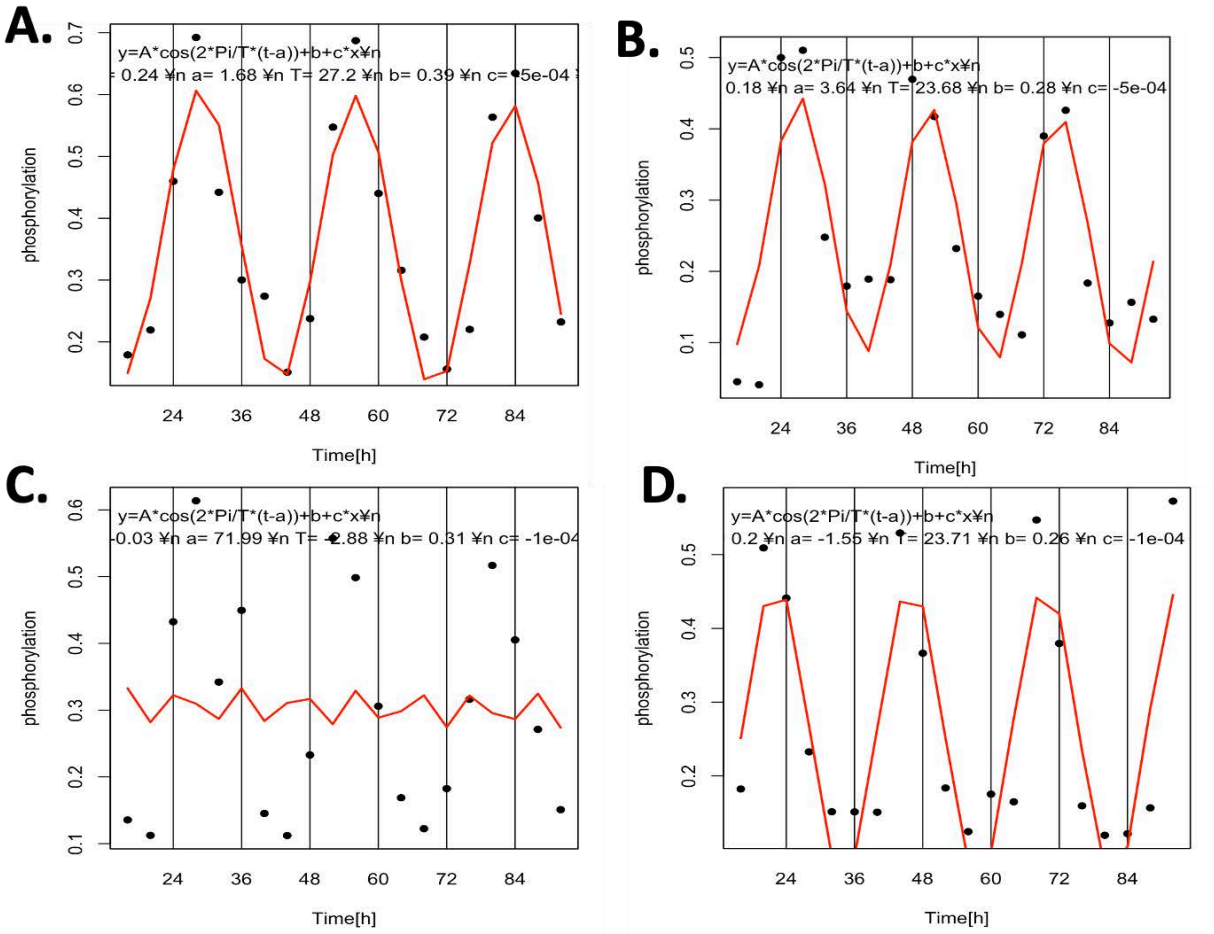

Figure 7: Period estimation of mutant *kaiC* proteins T42S (A, T=27.2h), S157P (B, T=23.68h), 2 (C, T=22.88h), 1 (D, T=23.71h).

ATPase activity (day-1 KaiC-1) for each *kaiC* protein was provided for us and was compared to the frequency of each *kaiC* protein (Figure 8). The frequency of *kaiC* proteins 2, 4, and 1 did not show a correlation with their ATPase activities (Figure 8), and when removed, a trendline was able to be fitted and a relationship between ATPase activity and the *kaiC* frequency was observed (Figure 9).

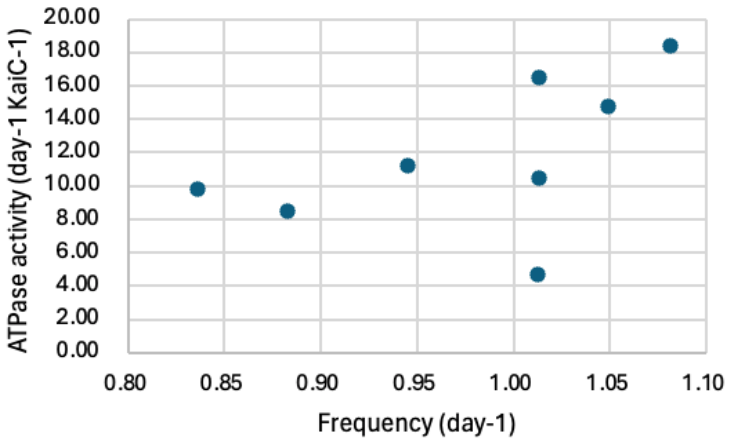

Figure 8: ATPase activity (day-1 KaiC-1) of *kaiC* proteins (WT, 5, 4, 3, T42S, S157P, 2, and 1) versus their frequencies (day-1).

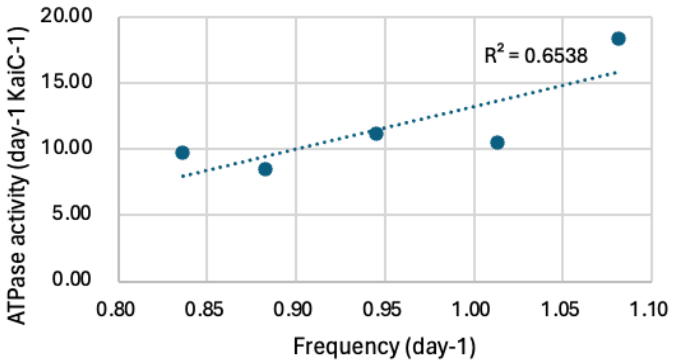

Figure 9: ATPase activity (day-1 KaiC-1) of *kaiC* proteins (WT, 5, 3, T42S, and S157P) versus their frequencies (day-1) (R^2=0.6538).

Discussion:

We were unsuccessful in obtaining high-resolution SDS-PAGE gels showing the four phosphorylation states of the *kaiC* proteins wild-type, 5, 4, 3, T42S, S157P, 2, and 1. We were only able to obtain two bands representing phosphorylated *kaiC* protein and unphosphorylated *kaiC* protein. This may be because we accidentally used expired acrylamide and therefore we were unable to get proper separation of the phosphorylated *kaiC* protein. *KaiC* proteins 5, 4, and 2 did not have a period that would fit the equation. We also believe this is due to the improper separation of the SDS-PAGE gel since many bands were not visible and therefore the data may not be correct. This is also supported by the fact that our period data for the *kaiC* proteins used in this study do not match previously identified periods from other studies. Overall, we do not believe that we successfully recreated the usual phosphorylation rhythm and period of the *kaiC* proteins in vitro and would need to repeat this process to get more accurate results.

For *kaiC* proteins WT, 5, 3, T42S, and S157P we did see a relationship between the ATPase activity of the *kaiC* proteins to their frequencies which is consistent with previous research. However, the relationship that we did determine was not strong (R^2=0.6538), which may also be attributed to the incorrect prediction of the period. To conclude, we believe that future research is necessary to elucidate the true relationship between the phosphorylation state of the *kaiC* proteins and their ATPase activity.

International Experience:

Not only did I gain valuable knowledge during the lab portion of my internship, but I was also able to have valuable experiences that have shaped me into more of a global citizen. During my stay I traveled to many places in Japan including, (but not limited to), Tokyo, Kyoto, Ise, Toba, Osaka, Okasaki, and Nagoya. I was extremely fortunate to have this opportunity since the duration of our stay allowed me to travel frequently and experience the culture in a way that many people do not. Being able to stay in Japan for such a long amount of time really allowed me to get an understanding of the day-to-day life Japanese people have, and after my stay, I truly believe that it is safe to say that the quality of life is much better. The people in general seem much happier and healthier than they do in the United States.

Another thing that stood out to me during my stay in Japan was the attention to detail that everything had. Many stores and restaurants had not only their menus but life-sized models of food displayed outside of their establishments so that people could see the food that they would be receiving and decide if they wanted to eat there. Additionally, when the food was presented to you, it never was in a sloppy or unorganized manner, it was always presented in the same way and never seemed sloppy or just thrown together. Aside from restaurants, I noticed a lot of attention to detail at historical places like temples, shrines, castles, and gardens. Every one of these places was put together very carefully from the smallest flower to the biggest tree was placed there intentionally with a goal. Nothing was overgrown or appeared to not be taken care of and the absolute beauty that many of these places had was outstanding.

During my stay in Japan, I noticed a strong societal norm of working for others instead of yourself and appreciating everyone and everything for what they do no matter how small the task. I have many examples of this but I will list a few that were my favorites. The first thing was the overall attitude of those who were employed in public or customer service. Every cashier, waitress, police officer, or security guard that I met was an extremely polite person whose ultimate goal was to help. There were many times when I was trying to navigate a situation and even through the language barrier they would always do their best to assist me and help me in any way that they could. Additionally, when walking down the street many people would just pick up trash if they saw it and would carry it to the nearest trash can even if it wasn't their trash. These are very admirable actions that I noticed a lot during my stay in Japan and really just became a part of my day-to-day life during my stay. Overall, I strongly believe that I have grown as a person since my experience in Japan and I hope that I will continue to explore and

appreciate other cultures throughout my life to make me a better scientist and more of a global citizen.

Acknowledgements:

I wish to express my gratitude to Dr. Yoshimura and the entire Yoshimura lab for welcoming me and assisting me during my study in Japan. I also would like to thank Dr. Ohkawa and Miwa-san for their support and guidance throughout the *kaiC* SDS-PAGE experiment. I want to express my appreciation to Miki Tanikawa and Yuma Katada for their mentorship throughout my experience and continued friendship and to Dr. Nakayama for teaching us how to perform microinjections in medaka embryos. Finally, I would like to thank Dr. Munakata and Dr. Molina for their support and guidance throughout this program and their commitment to helping us become amazing scientists and global citizens.

Funding:

This work was funded by the National Science Foundation (NSF) Project #1952513.

References:

Ishiura M, Kutsuna S, Aoki S, Iwasaki H, Anderson C, Tanabe A, Golden S, Johnson C, Kondo T. 1998. Expression of a Gene Cluster kaiABC as a Circadian Feedback Process in Cyanobacteria. Science. 281(5382):1519–1523. doi:https://doi.org/10.1126/science.281.5382.1519.

Nakajima M, Imai K, Ito H, Nishiwaki T, Murayama Y, Iwasaki H, Oyama T, Kondo T. 2005. Reconstitution of circadian oscillation of cyanobacterial KaiC phosphorylation in vitro. Science (New York, NY). 308(5720):414–415. doi:https://doi.org/10.1126/science.1108451. [accessed 2021 Mar 22]. https://pubmed.ncbi.nlm.nih.gov/15831759/.

Nakajima M, Ito H, Kondo T. 2010. *In vitro*regulation of circadian phosphorylation rhythm of cyanobacterial clock protein KaiC by KaiA and KaiB. FEBS Letters. 584(5):898–902. doi:https://doi.org/10.1016/j.febslet.2010.01.016.

Nishiwaki T, Satomi Y, Kitayama Y, Terauchi K, Kiyohara R, Takao T, Kondo T. 2007. A sequential program of dual phosphorylation of KaiC as a basis for circadian rhythm in

cyanobacteria. The EMBO Journal. 26(17):4029–4037. doi:https://doi.org/10.1038/sj.emboj.7601832.

Pattanayek R, Williams DR, Pattanayek S, Xu Y, Mori T, Johnson CH, Stewart PL, Egli M. 2006. Analysis of KaiA–KaiC protein interactions in the cyano-bacterial circadian clock using hybrid structural methods. The EMBO Journal. 25(9):2017–2028. doi:https://doi.org/10.1038/sj.emboj.7601086.

Terauchi K, Kitayama Y, Nishiwaki T, Miwa K, Murayama Y, Oyama T, Kondo T. 2007. ATPase activity of KaiC determines the basic timing for circadian clock of cyanobacteria. Proceedings of the National Academy of Sciences. 104(41):16377–16381. doi:https://doi.org/10.1073/pnas.0706292104.