Progress Report on Two Projects: the Study of Seminlunar Rhythms in Grass Puffer and the Study of Critical Temperature in Medaka

Luke Nicholson, Katada Yuma, Michiyo Maruyama, Takashi Yoshimura

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Abstract

The Yoshimura lab at the Institute of Transformative Bio-Molecules (ITbM) and the School of Agricultural Sciences of Nagoya University is primarily interested in determining the molecular mechanisms underlying certain behavioral rhythms. During my stay with the lab, I worked on two distinct projects, the first working to understand the semilunar rhythms of grass puffer and the second working to understand the difference between the seasonal reproductive rhythms of two medaka strains. Both projects are still in progress and as such, no significant conclusions can be made yet.

Study on the Semilunar Rhythms of Grass Puffer

Introduction

It has been observed that there exist biological rhythms related to the lunar phase among many different species: coral spawn near the full moon, honeycomb grouper have a lunar-related spawning cycle, cattle have a lunar-related reproductive cycle, and the lunar cycle results in the variation of duration and onset of sleep among humans. The Yoshimura group is interested in the semilunar spawning rhythms of grass puffer (fugu), which spawn during spring tide. Semilunar rhythms are defined as biological rhythms that are synchronized with the cycle of spring tide

(when high and low tide are at their highest) and neap tide (when high and low tide are at their lowest). It is also important to note that grass puffers demonstrate a behavior coined “tumbling” which describes their characteristic spawning behavior which takes place on the beach. The molecular mechanism for this behavior is unknown and is another point of study for the Yoshimura group.

To begin to uncover the molecular mechanism underlying this semilunar rhythm, Yuma-san acquired samples and identified 125 potential semilunar genes by RNA-seq; he also acquired GSI and histology data for analysis. Of the 125 candidate genes, in order to differentiate between genes that respond directly to stimuli from the moon and genes with endogenous expression variation rhythms, Yuma-san is also rearing grass puffer under constant conditions to identify genes expressing endogenous rhythms by performing RNA-seq analysis.

Experimental Method

Acquiring grass puffer samples

In regards to sampling grass puffer from the wild, we followed a time series sampling protocol, collecting grass puffer according to the lunar phase cycle. We went fishing to collect samples during the new moon, first quarter moon, full moon, and last quarter moon as these time points corresponded with the cycle of spring tide and neap tide. On these excursions, we would examine the spawning behavior (i.e. tumbling or no tumbling), collect seawater to test for any pheromones present, and catch grass puffer using a standard fishing rod with shrimp as the bait. If a particular individual did not look healthy or was too small, we would release the puffer, aiming to only collect healthy adults for the purpose of the study.

Tissue sampling

Once enough samples were collected, roughly 20, we would return to the lab and dissect our samples immediately given that the behavioral phenotype is dynamic and heavily influenced by time/ the lunar cycle. First, semen was collected from male fugu by rubbing the stomach above the gonads and placed in ice for pheromone ELISA measurements. Next, samples were sacrificed in buffered tricaine methane sulfonate (MS222) and then dried and weighed. Plasma was collected and placed in a -80 freezer for hormone measurements and metabolomics. The brain was dissected and the hypothalamus and pituitary were isolated in order to perform ATAC-seq and ChIP-seq (6 fishes/ timepoint); the left and right OE were isolated for qPCR. The head was placed in RNAlater to stabilize RNA for sequencing. The testis was dissected from the body and weighed to determine GSI; a portion of the testis was kept at -80 for pheromone measurements. The whole body and remainder of the head were kept in liquid nitrogen for snRNA-seq (9 fishes/ time point).

Constant condition

In regard to the constant condition, fish were kept under constant LW conditions, long days (14 hours light and 10 hours dark), and warm temperature, with no moon-derived stimuli such as moonlight or tides. Weekly sampling was conducted over a 2-month period. Using these samples, RNA-seq analysis will be performed to extract genes whose expression changes between high and low tides. To maintain samples at the constant condition, each week we prepared fresh seawater to replace the three tanks by using tap water that had sat out for a day – to decrease chlorine concentration – and salt containing other minerals to replicate ocean conditions. A tool that measures specific gravity was used to normalize the salinity. If visible parasites were growing on the fugu samples, we would add 100ppm PFA to the tanks (40 mL 4%

PFA in 20L seawater) to kill the parasites. Finally, we would clean the filters of the tanks and select six individuals for sampling. Following a simplified protocol, we dissected the six individuals and recorded body and gonad weight to determine GSI. The brains were dissected to obtain the hypothalamus, pituitary, and olfactory epithelium; all portions were placed in liquid nitrogen and stored in a -80 freezer to be sequenced at the end of 9 weeks when all samples have been collected.

DanioVision behavioral analysis

I was briefly introduced to the method used for behavioral analysis: larvae are placed in artificial seawater with methylene blue to prevent infection and aid visualization, then placed in separate wells on a 96-well plate, which is then placed in a movement tracking system set to track movement over the course of a week.

Discussion

Observations

As this project is still ongoing, there are no results as of yet, however, there are still observations that can be discussed. I went on four of the fishing expeditions to Minamichita, once at neap tide and the remainder at spring tide. On the first expedition, I caught 1 of the 22 male fugu and the females I caught were released. On the second expedition, it was spring tide, meaning the grass puffer were expected to spawn, however they did not. We hypothesized that this was because of heavy rain the night before and the presence of pollution close to the shore. Nonetheless, there was much more fish than on the first expedition and I personally caught 17 fugu. On the third expedition, we witnessed the tumbling behavior of fugu during spawning.

Samples could be gathered in nets rather than individually caught with a rod. On the final expedition, it was once again spring tide but the typical spawning behavior was not observed, likely for similar reasons to expedition number two. Each time we sampled after the expedition, I was responsible for semen collection as well as dissecting and weighing the gonads to determine GSI measurements.

Future plans

In regards to future plans of the project, the team will continue to sample grass puffers from Minamichita for the remainder of the spawning season and grass puffers from the constant condition for the remainder of the 9 weeks. After this, the team will analyze the different tissues that have been collected from the samples and stored. They will compare all of the appropriate data and analysis (RNA-seq, ATAC-seq, ChIP-seq, GSI, ELISA, and qPCR data) across the gathered time points and between the wild group and control group in order to better study the semilunar rhythms of grass puffer. Finally, Yuma-san has plans to begin behavioral analyses of the larvae as it has been observed that the spawning timing of grass puffer differs between East and West Japan. He hopes to uncover the relationship between the diurnal rhythm and the semilunar clock; the diurnal rhythm with a 24-hour cycle and the tidal rhythm with a 12.4-hour cycle are phase-matched at a 14.8-day cycle and can be the oscillators of the semilunar cycle rhythm. Thus, future plans include examining the differences in diurnal rhythm characteristics between East and West Japan by way of DanioVision behavioral analysis.

Critical Temperature Project

Introduction

Project background

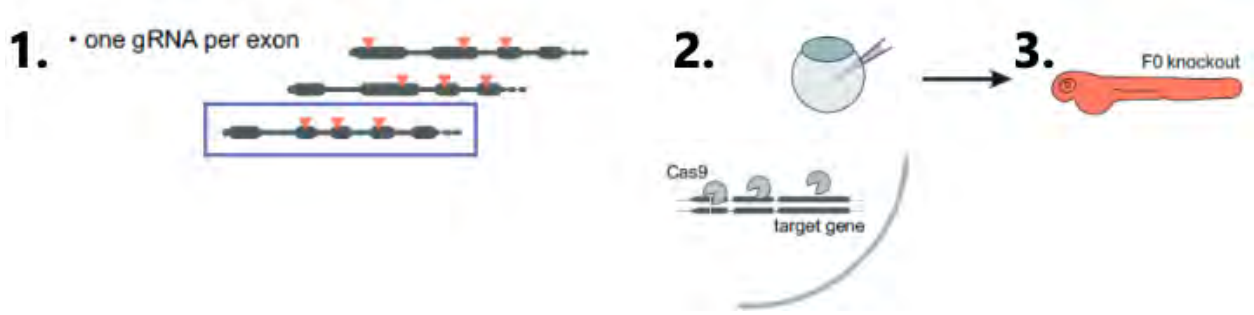
The Yoshimura group has noted that there exist two strains of Medaka, Higashidori (Hi) and Hanamaki (Ha), which exhibit different critical temperatures in regard to seasonal gonadal development. For the sake of this project, critical temperature is defined as the threshold temperature that must be met before male medaka gonads increase in size and allow for reproduction. When this temperature is reached, the gonadosomatic index (GSI, calculated as gonadal weight/ body weight x 100) increases from ~3 to ~10, a significant increase. The mechanism for critical temperature is unknown, thus, these two strains are being studied and genetically manipulated to uncover the genetic and molecular basis for this phenomenon in seasonal spawning patterns.

At the beginning of the project, Maruyama-san chose candidate genes via a screening process that compared the genomes of these two strains of medaka. She reared a (Hi x Ha) F_2 population held at LW conditions (14-hour days, 18°C) and performed QTL (quantitative trait locus) mapping with GSI. Using a multiple imputation method with $p = 0.01$ and $p = 0.05$ genome-wide significance levels (determined by 1000 permutations), potential candidate regions on chromosome 12 were identified with QTL analysis giving a LOD score of 10.54. Next, she narrowed down the number of candidate genes by performing RNA sequencing and qPCR analysis; this process gave candidate genes both in the 95% confidence interval (~4mbp) and just outside this interval (~1mbp). 17 candidate genes were established (potential enhancers, activators, and repressors of critical temperature behavior) and triple CRISPR knockouts will be made for all of them to evaluate behavioral changes.

Triple CRISPR Method

The traditional method of gene editing using CRISPR is to inject a single-cell embryo with a Cas9/gRNA RNP (Cas9/ guide RNA ribonucleoprotein) which binds to a target locus via complementary sequences and induces a double-stranded break. The natural DNA repair mechanism, NHEJ (non-homologous end joining), usually introduces an indel at the target region, either an insertion or deletion, which can result in a non-functioning protein due to a mutated essential residue or frameshift resulting in a truncated protein. However, this method is not very efficient as two generations are required to generate homozygous mutants (this takes about 1 year in medaka). As such, the Yoshimura group adapted the triple CRISPR method from Kroll et al. to increase the efficiency of their genome editing experiments. In brief, this method couples multi-locus targeting with high mutagenesis and is able to consistently convert > 90% of injected zebrafish embryos into biallelic F0 knockouts. Three sgRNAs (single guide RNAs) are designed for one target gene and after being mixed in a 1:1 ratio with Cas9, are injected into single-cell embryos to obtain F0 knockouts (Figure 1). The triple CRISPR method reduces the time of an experiment from months to hours and as such was highly useful for the critical temperature project given that understanding behavioral phenotypes requires large genetic screens.

Figure 1: (Kroll, et al., eLife, 2021)



Experimental Method

Ribonucleoprotein (RNP) solution preparation

To prepare RNP solution for microinjection, three separate mixtures for each sgRNA were made. First, we combined 1 µl sgRNA (100 ng/µl) with 1 µl 1/20 diluted Cas9 protein (500 ng/µl) for each target and placed these three mixtures in the thermal cycler to incubate at 37°C for 10 min. These three mixtures were then mixed to form our RNP solution and stored at 4°C. The original Cas9 protein used was Alt-R s.p. Cas9 Nuclease V3 10 mg/ml (IDT, 1081059). To dilute the Cas9, 0.5µl of the original Cas9 was mixed with 9.5 µl 0f Cas9 working buffer.

Gel preparation

A gel was required to hold embryos in place for microinjection. To prepare this gel, first, we created 1x seawater by dissolving 30 g of "REI SEA MARINE" (IWAKI, 274514) in 1 L of MilliQ; then we diluted this 1x seawater to 0.1x (10 ml 1x sea water + 90 ml MilliQ). Next, we mixed 100 ml of 0.1x sea water with 2 g of purified agar powder (Nacalai tesque, 01056-15) to create a 2% agar gel. The agar was heated up in the microwave and poured into 2 - 3 Petri dishes on ice. After 2 - 3 min, an injection mold was placed on the gel and left to set for more than 20 min. Finally, the mold was removed and the dish was filled with 0.1x seawater and stored at 4°C.

Needle preparation

With a micropipette puller (SUTTER INSTRUMENT, MODEL P-1000), we pulled a 1.0mm OD glass capillary (HARVARD APPARATUS, GC100F-10) into two needles using the following settings: heat 620, pull 35, vel 50, time 250, pressure 500. The tips of these needles were cut with a microforge (NARISHIGE, MF-830), aiming to create 2 µm tips with clean breaks.

Microinjection

To carry out microinjections with single-cell embryos, the first step was to insert a separator into each tank in the evening prior to injection to separate male and female adults. Because social interaction is important in medaka, it was necessary to minimize the separation time. The next day, the separator was removed early in the morning, allowing the couple to mate. After 10 - 15 minutes, the females lay fertilized eggs. Using a net and pipette, the eggs were collected from each female and rinsed. Next, tweezers were used to remove the extraneous filaments clumping the eggs together. Using a stereo dissecting microscope, the embryos were then fixed in the slits of the gel plate and rotated so that the cytoplasmic side faced upwards. Unlike in zebrafish, the RNP solution can only be injected into the small cytosol, not the yolk. The injection needle containing RNP solution was placed in the micromanipulator and was lowered to bring it close to the embryo. Next, the nitrogen gas valve was opened and the pressure microinjector was switched on. Going down the line, the cytoplasm of each embryo was injected with 1 nl of RNP solution. The injection process must occur quickly to avoid embryos progressing to the 2-cell or even 4-cell stage; embryos that have developed beyond the one-cell stage can still be genetically manipulated, however, it becomes more difficult to avoid creating mosaic F0 knockouts. After injecting all the embryos, they were placed in methylene blue to prevent bacterial infection and set to incubate at 26-28 °C.

RNA extraction and qPCR analysis

Although F0 knockouts have not yet been created for every candidate gene and thus the data collection phase of the project has not started yet, I practiced the RNA extraction and qPCR techniques to get a sense of how the results of the microinjection experiments will be analyzed. Practicing with an already studied medaka gene, I first isolated the testes, ovaries, and brains (specifically the hypothalamus and pituitary) from an adult male and female and placed these tissue samples in liquid nitrogen. To extract the RNA from these tissues, first I added 1 ml of QIAzol Lysis Reagent (QIAGEN) to each sample and immediately crushed the tissue in a homogenizer (20,000 rpm, 1 min). 200 µl of chloroform was then added to the homogenized sample, which was then vortexed for 15 s and centrifuged (12,000 x g for 15 min at 4°C). I then collected 600 µl of the supernatant with a cut pipette tip and added it to a gDNA Eliminator Column. These samples were then centrifuged (8,000 x g for 30 s) and the flow-through was collected into 1.5 ml tubes. Next, an equal volume (= 600 µl) of 70% EtOH was added to the sample and vortexed. 700 µl of this mixture was applied to the Rneasy MinElute Spin Column which was centrifuged (8,000 x g for 15 s). The flow-through was discarded and the remainder of the mixture was applied to the column and centrifuged again. Hereafter, we followed the protocol of the RNeasy Plus Micro Kit (QIAGEN), starting from step 5 of the Quick Start Protocol. Having successfully extracted the RNA from the four samples, we then determined the RNA concentration using a NanoDrop. The ratios of RNA/protein and RNA/buffer looked good for all samples, so we proceeded with qPCR.

To perform qPCR, we followed the Takara TB Green *Premix Ex Taq* II Tli RNaseH Plus protocol. To analyze this data, the delta-delta Ct method was used. With the help of excel and Prism, a graph was created to showcase relative expression levels of the target gene in these medaka tissues.

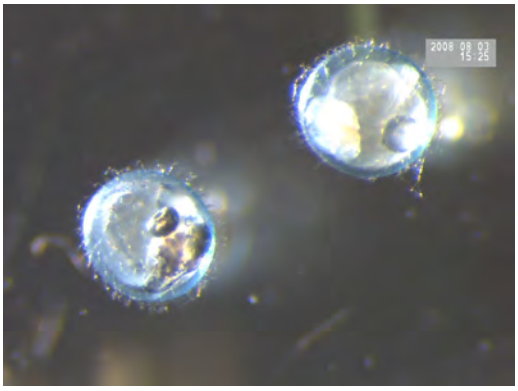
Results

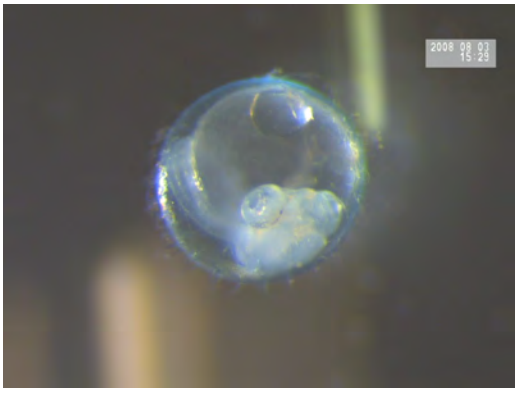
Practice with slc45a

Before performing microinjections with RNP corresponding to the candidate genes, I first practiced making knockouts of the gene slc45a (solute carrier family 45 member gene 2), a pigment coding gene in medaka melanophores. This gene is used to practice the technique as it is easy to recognize the knockout phenotype, transparent eyes. Figure 2 shows imaged medaka embryos several days after performing microinjections.

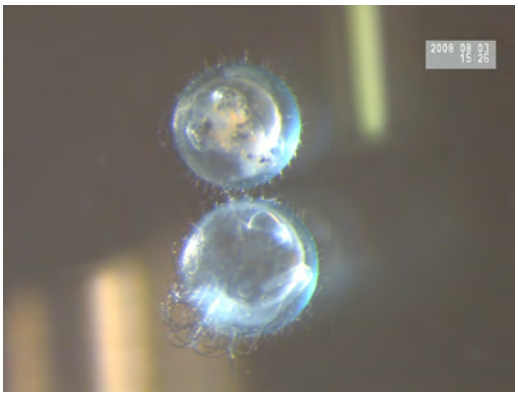
Figure 2:

a) Left: Wild Type Right: Knockout b) Knockout





c) Top: Mosaic Bottom: Unclear



Preliminary results

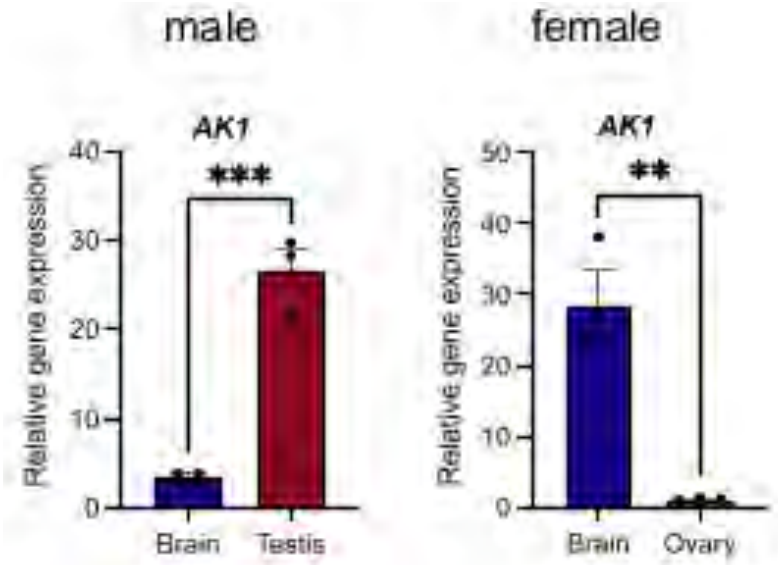
Table 1:

Knockout Gene	# Injected Embryos	Condition (as of 6/14/22)
Candidate 1	186	Hatched (day 12)
Candidate 2	157	Some have hatched (day 10)
Candidate 3	179	Incubating (day 7)

Practice with qPCR analysis

Although not relevant to the critical temperature project, in the name of practice, data was collected on the relative expression levels of a particular medaka gene (Figure 3). The samples from the qPCR were all successfully amplified and had a cycle threshold of around 20. Relative to a particular housekeeping gene, the target gene showed high expression levels in the male testes and female brain whereas it showed low expression levels in the ovaries and male brain. This data was consistent with prior studies, indicating that the qPCR analysis was performed correctly.

Figure 3:



Discussion

Microinjection technique

The technique of microinjection is quite difficult as there are many factors that must be just right to ensure a successful outcome. When I first started practicing the technique by making slc45a knockout medaka, I ran into many difficulties that deserve discussion. First, the timing of injection is very important as the embryos will begin to harden and progress beyond the one-cell stage after about an hour, especially if not placed on ice to slow development. In the event that an embryo enters the two-cell or even four-cell stage, it is still possible to create a successful knockout, however, each cytosol must be injected and if a mistake is made with any, a mosaic phenotype will manifest. Another issue with further developed embryos is the hardness of the egg. The microinjection needles are very fragile and will break more often when the embryo becomes tougher. Not only is it tedious to have to replace the needle after each break, but often times the loaded RNP solution is lost with each broken needle – if too much RNP solution is lost,

more must be made which provides more time for the embryos to further develop, decreasing the efficacy of the experiment.

In regards to collecting freshly fertilized embryos to perform microinjections on, I found that it was necessary to use all 9 medaka breeding pairs to ensure enough healthy eggs could be collected. Sometimes, a particular pair would produce unhealthy embryos characterized by nonuniform appearances and a hardened outer layer, resulting in broken needles and overall difficulty. Other times, unfertilized eggs would be collected, appearing as a yellowish color and much less structurally sound. This would occur if the medaka couple was not given enough time to fertilize the eggs or if the male was not healthy and was having trouble spawning. Three medaka pairs in particular did not produce fertilized eggs on more than one occasion after removing the barrier, potentially indicating that they are unhealthy and should be replaced in future experiments. Finally, sometimes the embryos would die if they were pipetted too vigorously or for other reasons. As such, with so many factors that influenced the collection of viable embryos, it was necessary to use all 9 medaka breeding pairs to account for the potential of unusable eggs. It is also important to note, however, that all 9 barriers were not removed at the same time as this would result in far too many eggs to have to prepare for microinjection; as I learned early on, if I was too ambitious with around 100 eggs to microinject in one sitting, by the time I removed the connecting filaments, plated them on the agar, and rolled them into position with the cytoplasms facing upwards, most of the embryos would have progressed to the four-cell stage and the outer egg would have begun to harden. Thus, to ensure that the efficacy of the technique was optimized, it was imperative that embryos were injected in smaller batches, typically only collecting from 3 medaka pairs at a time.

Preliminary results

I contributed to this project by creating enough triple CRISPR knockouts for three critical temperature candidate genes. Although these knockouts are not ready to be analyzed, we can still make some preliminary assumptions. To truly analyze the phenotype of the triple CRISPR injected embryos, they must reach adulthood to allow for sampling for GSI and qPCR measurements. However, the condition of the embryos noted in Table 1 possibly indicates some promising results. Wild-type embryos typically hatch after 8 days post-fertilization, yet the injected embryos from the candidate gene 1 and 2 groups hatched at days 12 and 10 respectively. This delay in hatching suggests an altered phenotype and is preliminary evidence that the knockout phenotype is being observed.

Future Plan

Prior to joining the project, Maruyama-san had created knockout medaka for 10 of the candidate genes; I was able to create knockouts for 3 more, thus, there are 4 more genes to create knockouts for before the next phase of the project. Once all 17 knockouts have been created and have reached adulthood, the genetically modified phenotype will be assessed. For each gene, two groups of medaka will be reared, one group held at 16 °C and the other at 18 °C in order to observe how the knockouts affect critical temperature. To analyze the knockout phenotype, these adult medaka will be sampled to obtain RNA for qPCR analysis as well as to obtain GSI data.

Conclusion

Both the grass puffer and medaka projects are still in progress and as such, no significant conclusions can be drawn at this time. However, both projects are progressing smoothly and

preliminary results prove to be promising. Both teams should reach the data analysis phase of their experiments by the end of the summer and at this time conclusions will be able to be made.

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