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Zebrafish Feeding and Breeding: Adapting Best Practices for Zebrafish Husbandry

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°C	degree Celsius
L	Liter
RO	Reverse Osmosis
UV	Ultraviolet light
mL	Milliliters

CHAPTER I

INTRODUCTION

Zebrafish (Danio rerio) are a tropical, freshwater fish that serve as a key research model for human health and disease. They are commonly known as Zebrafish because of the deep blue, horizontal stripes along each side of their bodies. The anatomical and genetic similarities shared by humans and zebrafish make establishing a zebrafish laboratory advantageous for both undergraduate research and coursework. Zebrafish are able to be used as a research model for genetic research, which is a quickly growing scientific field.[1]

While Zebrafish naturally inhabit the tropical, freshwater of ponds and rivers in India, over the years their popularity as pets has grown, making them readily available in local pet stores for a very affordable price. While scientific research that utilizes Zebrafish does not typically use fish that are purchased from local pet stores or chain pet retailers, these readily accessible fish can be used to set up a lab and gain experience caring for the fish in a laboratory environment before making the jump to ordering research grade live fish or embryos from a supplier. It is important to establish and standardize the protocols and procedures that will be used in the lab before spending the money on fish from a supplier. It can take several months to have everything set up and running for undergraduate fish research and it is more cost efficient to do this with pet store fish, as morbidity among the fish is a given when sorting out technique.[1]

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Research Models

Zebrafish make excellent vertebrate research models for genetic research for several different reasons. Zebrafish have all the same organ systems that are seen in the human body. This species of fish provides scientists with a research model that has “two eyes, a mouth, brain, spinal cord, intestine, pancreas, liver, bile ducts, kidney, esophagus, heart, ear, nose, muscle, blood, bone, cartilage, and teeth".[1] The only organ systems in the human body that cannot be studied in the Zebrafish model are the respiratory system, as humans have lungs and fish do not, and the integumentary system, as fish do not have skin. In fact, “Many of the genes and critical pathways that are required to grow these features are highly conserved between humans and zebrafish”.[1] Therefore, nearly any type of disease that causes changes in these body parts in humans could also theoretically be studied by using Zebrafish as a model.[1]

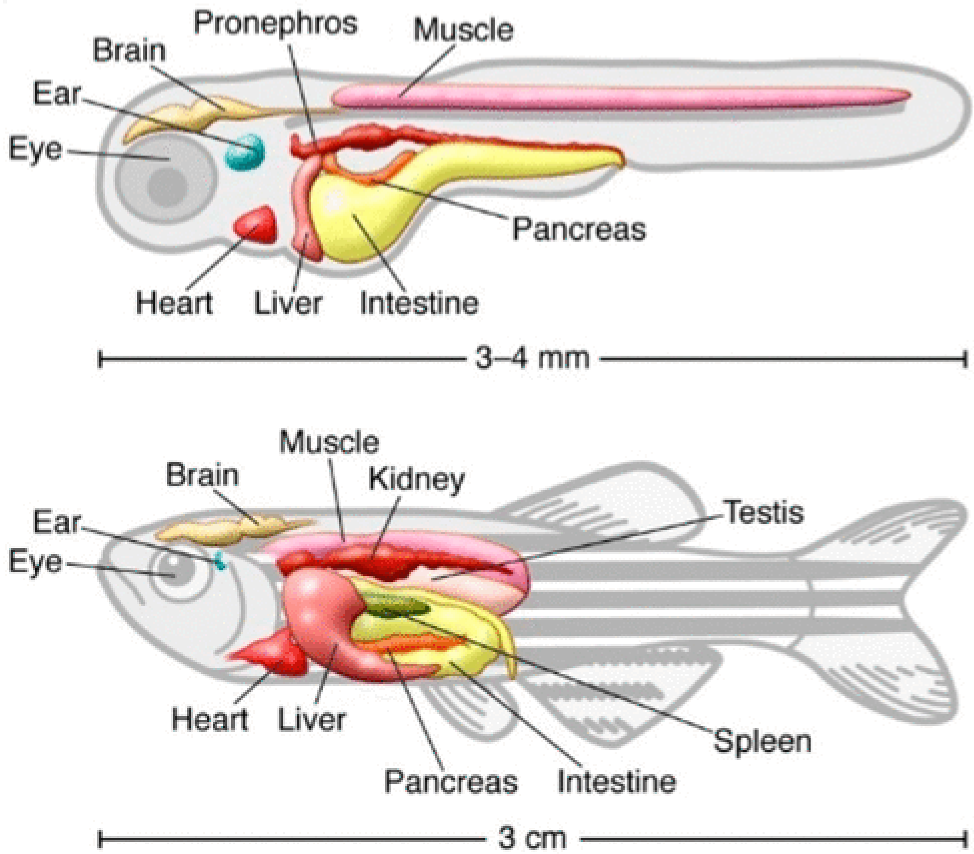

Figure 1.1. Zebrafish organ systems. Top: Larvae. Bottom: Adult[6]

Their anatomical similarities to humans makes Zebrafish an option for what is known as cross-species comparisons. Cross-species comparisons are comparisons that are made "between closely related species that differ in a cognitive or neural character, and comparison between distantly related species that share a cognitive or neural character, can be used to identify adaptations".[2] Other examples of cross-species comparison are mice and rabbits. Rodent models are often more commonly seen as research models than Zebrafish, because the idea of using them as a model is a much newer idea.

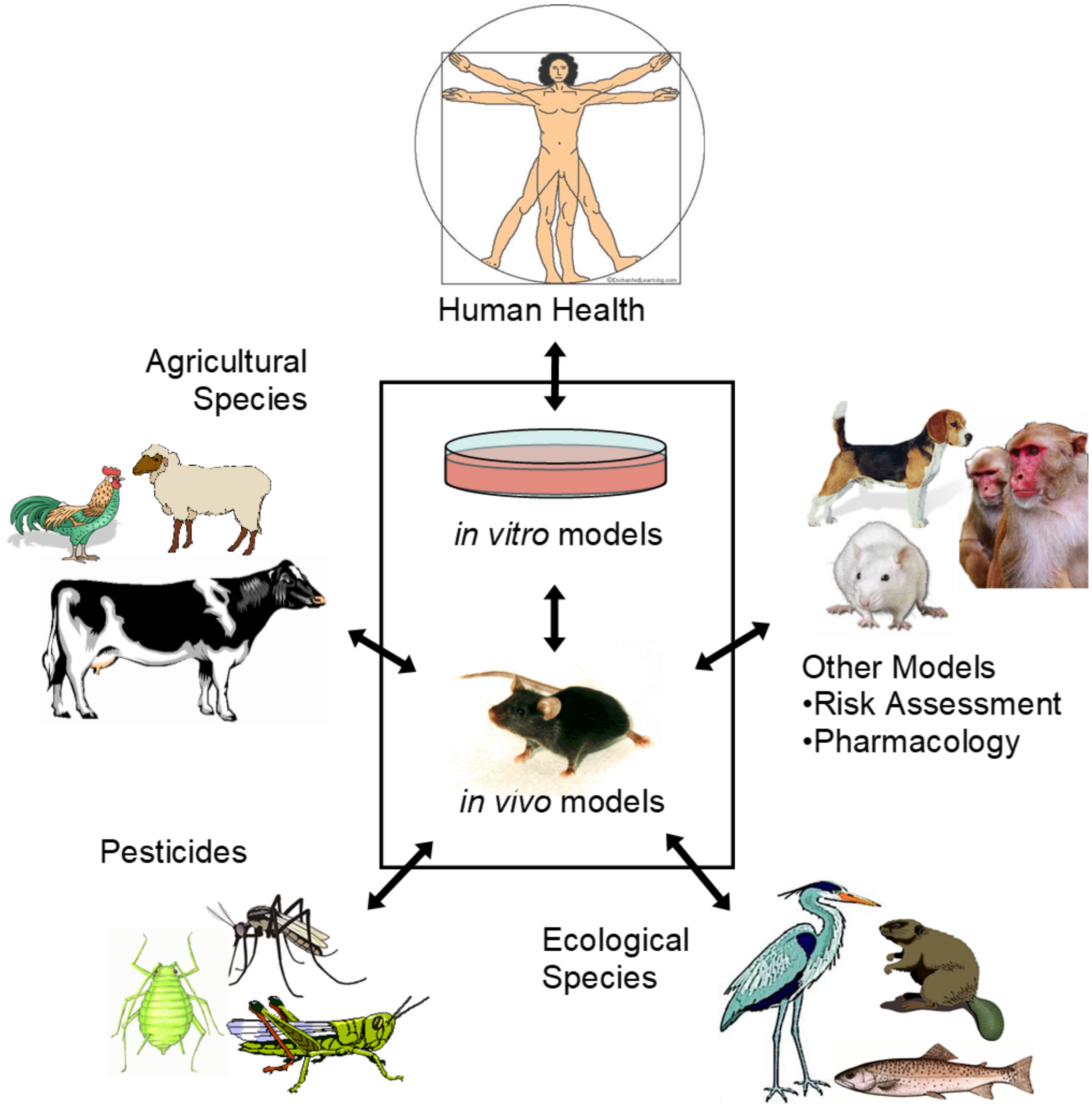

Figure 1.2. Comparative Genomics: Basic and Applied Research[3]

Another reason Zebrafish make great research models is because of their rapid development. It only takes about three months for a Zebrafish to reach sexual maturity, at which point they are considered adults. This is very advantageous for lab work because other research models that are used take much longer than this.[4]

Zebrafish also have a much higher reproductive capacity than most other research models. They breed readily and frequently, about every 10 days. One instance of breeding can result in up to 100 eggs at a time. Mice on the other hand can only have about 3 litters of pups in their lifetime. Each litter will only produce 1 to 10 pups. Because effective and reputable scientific research requires experiments to be repeated several times to prove the validity and accuracy of the results, Zebrafish are a great model for this kind of work in a lab.[1]

Zebrafish are inexpensive to rear, breed, and maintain. They take up much less space in a lab than mice or rabbits, making them a good option for research institutions with limited space for a new lab. They not only tolerate being kept together in large groups, but they actually prefer it. They engage in what is known as shoaling behavior. Because of the location in which they are found out of captivity, they evolved to practice shoaling behaviors in effort to avoid predation. In fact, shoaling has been proven to “reduce stress and aggression among fish held in small groups”.[5] This is beneficial to researchers because they are able to provide the fish an environment that is most similar to the one they thrive in while in the wild and it is cost effective in comparison to other models.

Another advantage to working with Zebrafish is the fact that the embryos develop externally after being laid and fertilized. When working with mice and rabbits, their embryos develop internally, making them more difficult to monitor and work with. External embryo development is beneficial in a lab and research setting because you can actually modify the genetic makeup of lines of Zebrafish by injecting the embryos with DNA or RNA.[1]

The embryos of Zebrafish are completely clear, which allows scientists to see firsthand the different stages of development that the fish go through. When viewing the developing fish under a microscope, you are able to watch its circulatory system develop and begin to see blood circulate through the organism. Being able to watch as the different organ systems develop in your chosen research model is both incredibly fascinating for those studying science and advantageous to your work. Models like mice and rabbits do not provide the researcher with as much clarity in embryology as Zebrafish do.[1]

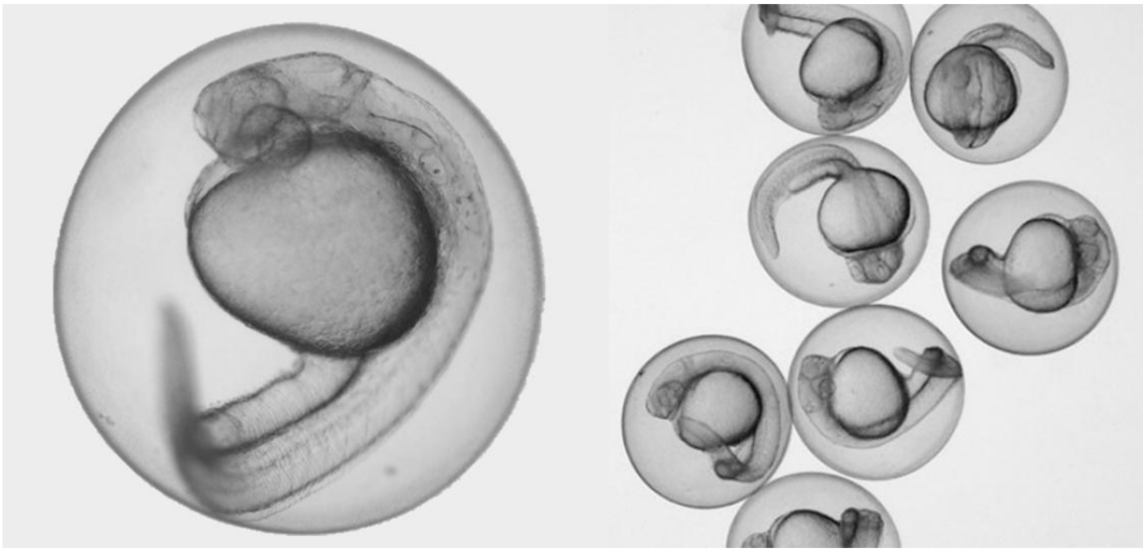

Figure 1.3. Zebrafish Development and Embryology[7]

CHAPTER II

MATERIALS AND CLEANING

Materials

Having the right materials is an integral part of conducting effective research and this was true for the work done in the Zebrafish lab. The first and most important material needed was the research grade fishtank system. The system used in our lab was from "Aquaneering Inc.". Using a research grade tank is incredibly important because without a water system with a successfully functioning nitrogen cycle, fish can not grow to be healthy enough to conduct research on and breed.

The nitrogen cycle is the process of converting the ammonia produced in a fish tank into nitrite and finally nitrate which can be used in assimilation. Ammonia is a natural product that builds up in fish tanks from fish waste, mainly from their gills, as well as their feces and urine, and uneaten food.[8] At safe levels, ammonia is not harmful to fish, but without the nitrogen cycle working effectively, ammonia can accumulate to levels that cause the fish stress and eventually death. This is because "high concentrations of ammonia in the water make it difficult for fish to eliminate ammonia from their bodies", which leads to major damage to their gills and internal organs.[8]

There were several components to our tank system that made it optimal for research. One of the most important parts was the UV light that is used to filter the water. The UV light was to stay on at all times. Just like humans, zebrafish can fall victim to threatening pathogens. "UV filters use ultraviolet light to kill harmful microbes such as algae, bacteria, and parasites.

The job of a UV sterilizer is to prevent pathogens from outbreaking diseases in an aquarium, and keep water from getting cloudy or greenish”.[9]

When using fish for research purposes, it is imperative that the environment they live in is very sanitary, as to not skew the results of the research. Researchers in a lab want to have the freedom to alter other factors, without worrying that an unseen pathogen in the tanks could be the cause for their results.

Another crucial material used in the process of setting up the lab was an accurate thermometer to ensure the water remained not only at a consistent but biologically ideal temperature for our species of fish. Because Zebrafish are a tropical fish, it is very important that the temperature of their water in the tank is as similar to the temperature of natural tropical waters as possible. What we found worked best in our lab for our fish was 26-28°C. The thermometer that was a part of our Aquaneering tank system worked well by providing us accurate readings, so we could keep a close eye on the temperature and ensure the fish were happy and comfortable.

Arguably the most essential material in the lab to the health and happiness of the fish was the food we provided them. Without a balanced source of nutrition, all other measures taken to keep the fish in peak health would be in vain. It seems like it would be a very simple component of the material in the project, but the diet of the fish was carefully selected and scheduled. It was found that the tropical flakes by API were the best source of nutrition for the zebrafish in our care.

These specific flakes are designed with tropical fish, like our Zebrafish, in mind. This meant that the flakes had the optimal amount of protein the fish needed to carry out the biological processes that were necessary for both their overall health and our future research goals. The flakes are also designed to allow for optimal nutrient absorption in the fish.

This brand sells both tropical flakes, like those used in our lab, as well as tropical pellets. We found the flakes were easier for our fish to feed on due to the size of the mouths of Zebrafish. The fish were able to eat the flakes with more ease due to the lighter texture of the flakes, which in turn meant less food waste in the tanks and therefore, less ammonia for the tanks nitrogen cycle to fix.

Another major reason these tropical flakes specifically worked well in a lab setting was due to the fact they are proven to “release up to 30% less ammonia”, which leads to less waste build up in our tank and overall cleaner, clearer water.[10] Overall, when feeding the fish flakes, these were without a doubt the best choice for our specific lab.

Figure 2.1. API Fishcare Tropical Flakes[10]

Brine shrimp were another essential material needed to get our lab protocols established. While feeding fish strictly flakes will satisfy their hunger, brine shrimp can provide them with even more nutrients. Researchers can provide their fish with brine shrimp in multiple ways, such as purchasing live shrimp, but we found it was most practical in our setting to purchase bulk Grade A Brine Shrimp Eggs and hatch the amount we needed in a hatchery.

Figure 2.2. Grade A Brine Shrimp Eggs Used in the Kwekel Zebrafish Lab

The hatchery was another essential material in the process of hatching brine shrimp for our Zebrafish to eat. The hatchery in place in the Kwekel Lab when I began my work there was a simple, plastic container that could hold the 1L of tank water needed to hatch the eggs. The hatcher also had a spout at the bottom to release the shrimp into a smaller container to portion out, as well as a simple aeration system, and stand to hold the hatchery upright. The specific details of the hatching process will be discussed further in Chapter III of this paper, titled "FEEDING".

Figure 2.3. Brine Shrimp Egg Hatchery Used in the Kwekel Zebrafish Lab

A key material in the process of breeding the Zebrafish was artificial plantlife, much like those that can be purchased for household fish at your local pet store. The plants, while plastic, help the fish to feel more like they are in their natural breeding environment: tropical riverbeds. The fish feeling more comfortable gave rise to higher yields of fertilized embryos in the breeding trials run. The specific details of the breeding process will be discussed further in Chapter IV of this paper, titled “BREEDING”.

Figure 2.4. Plastic Plantlife Used for Breeding in the Kwekel Zebrafish Lab

The final material that was essential to everyday work in the lab was our water testing kit. The Kwekel lab uses the API Freshwater Master Test Kit. This kit provides you with everything you need to test pH, high range pH, ammonia levels, nitrate, and nitrite levels. All of these things are important to keep fish healthy enough to breed. The kit also includes glass tubes in which to conduct the tests. Testing the water is a task that must take place every day in a research lab setting, and this kit provides accurate results with ease.[11]

Figure 2.5. API Freshwater Master Test Kit[11]

Cleaning Procedure

Just as the materials being used were essential to conducting scientifically accurate research, so is ensuring the tank system is clean and sanitary for the fish. This helps to ensure that environmental factors are not actually the cause of the results of the research being conducted in the lab. Maintaining a clean and orderly tank is a simple way to protect the fish from pathogens and disease.

The first step in maintaining a clean tank is the type of water used in the tanks. RO water is not only the only water in the tanks, but also the only water that is allowed to come in contact with any piece of the Aquaneering aquarium system. Tap water on something even as simple as the netting we use to move the fish from tank to tank during breeding could expose the fish to environmental factors that could be harmful.

Another important step to keeping a clean tank for the fish is regular cleaning of the individual tanks. Each day in the lab, we would observe which tanks appeared to have debris accumulating in them and clean at least a few each day. Oftentimes it was simply uneaten food at the bottom of tanks, but this is still enough to throw off the ammonia levels and cause harm to the fish. For a deeper clean than what is necessary on a daily basis, tanks can be rinsed with 4% bleach, followed by a rinse of tap water, and finally RO water. Water rinses can be repeated as many times as needed, as to not leave residual bleach on the surface of the tanks.

Daily cleaning of tank pieces first involves moving any fish inhabiting the tank into a different tank. After this you take the smaller tank portion to the sink in the research lab, disassemble the tank pieces, remove the lid portion from the rest of the tank, and empty the water into the sink. Then you are able to use RO water and the designated tank scrubber to scrub and rinse the tank clean. Once all the pieces appear to be clear of debris, you simply reassemble the smaller tank portion, fill the tank back up to capacity with RO water, and place it back on the tank ranks. For clarity, Figure 2.6 below is the stand alone Zebrafish rack used in our lab.

Figure 2.6. Kwekel Lab Aquaneering Zebrafish Rack

Conclusion

As discussed above, the materials used and how clean they are kept is the keystone to conducting effective and scientifically sound research. The most important material choice made in setting up the Kwekel Zebrafish Lab was the tank system. After extensive research, we found that Aquaneering is the leading name in stand alone Zebrafish tank racks for research labs. The Aquaneering rack in our lab cycles water, maintains a constant temperature, and sanitizes the water with a UV light around the clock, making it possible to conduct accurate research without the need to staff the lab at all hours, day and night.

Ensuring that the nitrogen cycle was cycling properly meant greater health of the fish because it meant ammonia levels did not get too high and cause the fish unnecessary stress. We also created a diet of both flake food and brine shrimp for the fish to keep them happy and healthy. When breeding trials were taking place, we saw greater success when artificial plant life was used, so this was deemed an essential material as well.

Cleaning protocols were optimized to use the most efficient methods to keep the aquatic environment clean and sanitary for the Zebrafish. RO water is not only the only water in the tanks, but also the only water that is allowed to come in contact with any piece of the Aquaneering aquarium system. We also created protocols for both a deep clean of the tanks as well as a daily clean. All of these protocols developed for the Kwekel Lab helped to ensure the success and accuracy of future research conducted in the lab.

CHAPTER III

FEEDING

Introduction

Zebrafish feeding protocols utilize two primary food types: dry (flake or pelleted) food and live prey, such as artemia (brine shrimp) or rotifers. Adopting a feeding protocol that incorporates both dry and live feed optimizes considerations of nutrition and cost for maintaining healthy fish. Best practices for feeding were adapted from authoritative sources in the current literature and published standards. Source, amount, regularity, and scheduling were optimized for our new zebrafish laboratory.

Feeding Procedure

The simplest and most efficient food source for Zebrafish used in the Kwekel lab was found to be dry food, specifically API Fishcare Tropical Flakes.[10] The advantages of API Fishcare Tropical Flakes were previously discussed in the “Material” section above. The process by which you give the fish flakes is fairly straightforward. When feeding flakes, a scalpel is used to crush flakes into pieces small enough for the fish to eat. Only ⅛ teaspoon is dispensed into the tanks at a time. This is because the fish can only be fed small amounts that can be eaten within 2-3 minutes at a time. This process is repeated and “pinches” of flakes are dispensed into tanks until fish appear satisfied, which is when they do not eat excitedly anymore.

Not only is the source of food chosen for your research model essential to scientifically sound and reproducible research, but so is the schedule on which they are fed. This is a research component that must be kept very consistent to simulate a feeding schedule in their natural environment. Because of this, the fish are fed daily in the morning and again in the afternoon. Below is the schedule that was set for the alternations of feeding live or dry food.

	S	**M**	**T**	**W**	**T**	**F**	**S**
AM	Dry Flakes	Dry Flakes	Shrimp	Shrimp	Shrimp	Shrimp	Dry Flakes
PM	Dry Flakes	Dry Flakes	Dry Flakes	Dry Flakes	Dry Flakes	Dry Flakes	Dry Flakes

Table 3.1. Kwekel Lab Feeding Schedule

This was found to be the schedule for feeding that led to the happiest and healthiest fish. As mentioned in Chapter II, the process of hatching live brine shrimp for the Zebrafish is very labor intensive. This is why they only have shrimp Tuesday through Friday. With no one working full days in the lab over the weekend, brine shrimp on Saturday, Sunday, or Monday is not realistic. However, when researchers are spending a full day in the lab on M-F, brine shrimp eggs can easily be placed in the hatchery.

Hatching shrimp is a process that takes roughly 24 hours overall. The preparation in the hatchery is essential to a successful hatch. You first fill the hatching device with water from the tank system to the one liter fill line. This is clearly marked on the plastic for easy set up, as shown in Figure 2.3 "Brine Shrimp Egg Hatchery Used in the Kwekel Zebrafish Lab". After adding the system water, you mix in 1 ⅔ tablespoon of Instant Ocean, an aquarium salt solution.

After the elements for the hatch are completely mixed, you are now ready to add the shrimp eggs by sprinkling in a level ½ teaspoon of refrigerated shrimp eggs. If previously frozen, they will need to have thawed in the fridge for 1-2 days before hatching.

With the solution created, the final step is to ensure proper aeration with tubing and device covering and allow it to sit for a minimum of 24 hours. Between hours 24 and 30 after beginning aeration, turn off air source and remove both the covering and tubing from the hatchery. You now allow the contents in water to settle for at least 5 minutes. This is very important so that the waste in the hatchery can settle below the live shrimp. After settling, you can drain off the bottom layer of water. This should be roughly 25-35 mLs and contains the opened shells of hatched shrimp eggs. This is shown in Figure 3.1 below.

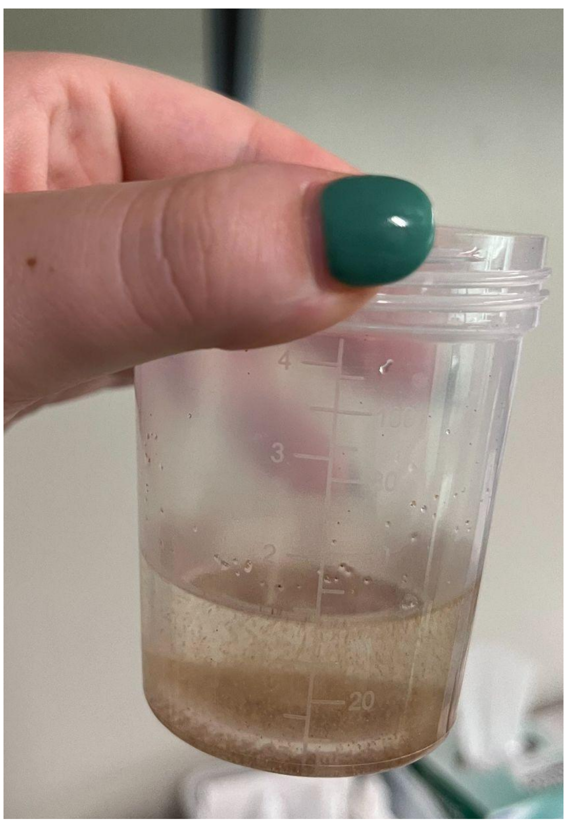

Figure 3.1. Opened Egg Shells in Breeding

Now you will be able to access the live shrimp for feeding. To achieve this, you simply drain into a separate container the next 20-40 mL of water. This contains the hatched brine shrimp that will be best for feeding the fish. This is shown in Figure 3.2 below.

Figure 3.2. Live Brine Shrimp

The next important step is to pipette up a small amount for the fish to eat. We found 500 microliters of hatched shrimp to be the perfect amount for the speed at which our fish ate. Simply pipette this up at a time of feeding and begin feeding to fish by dispensing into tanks. Now all you have to do is add in shrimp periodically. It is important to ensure fish have enough time to eat what has already been dispensed until they appear satisfied. Do not add in the shrimp too quickly, or they will go uneaten. It is also important to feed the shrimp to the fish within 2-3 hours of hatching. After this, the shrimp will begin to die, as they have no food source.

Finally, it is important to clean up your supplies. We found the best way to do this was by rinsing both containers' contents down the sink with generous amounts of water. It is also important to rinse all containers and leave them to dry for next feeding. It usually only takes an hour or two to dry, and then the hatchery can be reassembled with new unhatched eggs for the next day's feeding.

It is essential to store the unhatched eggs as directed on their original packaging. This means you will need portion out and thaw 3-4 weeks worth of shrimp eggs at time to refrigerate. This is shown in Figure 3.3. The rest of the frozen eggs should be kept in their original container, tightly sealed in the freezer and clearly labeled.

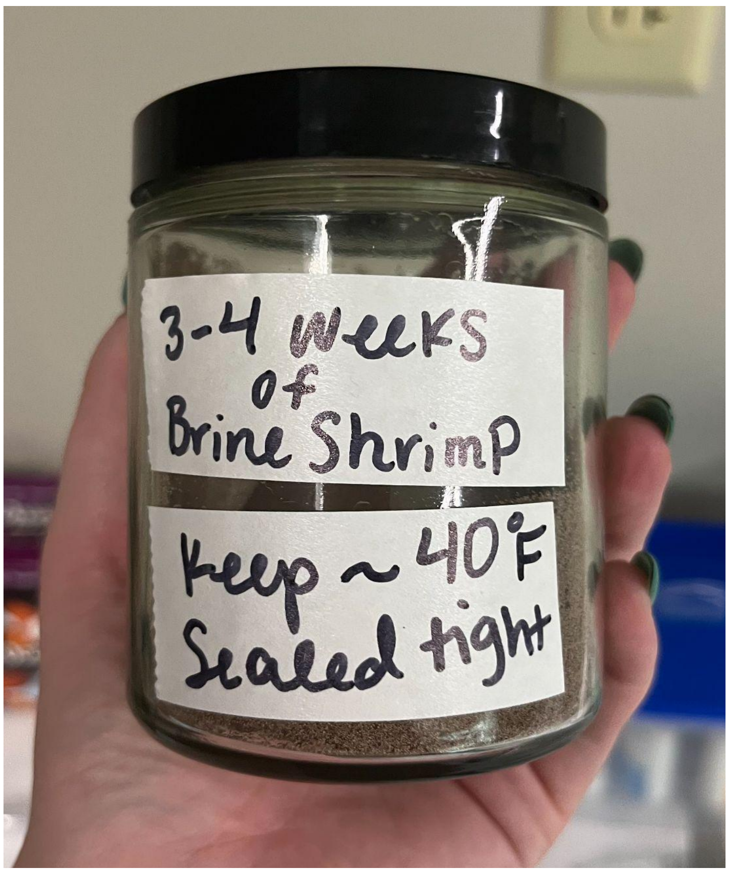

Figure 3.3. Thawed and Portioned Eggs, Labeled Container

Conclusion

As discussed above, Zebrafish feeding protocols utilize two primary food types: dry (flake or pelleted) food and live prey, such as artemia (brine shrimp) or rotifers. Adopting a feeding protocol that incorporates both dry and live feed optimizes considerations of nutrition and cost for maintaining healthy fish. Best practices for feeding were adapted from authoritative sources in the current literature and published standards. The practices were also adapted to best suit the daily work life of our lab. This allowed us to create a set schedule for when the fish are to be fed which type of food source. Source, amount, regularity, and scheduling were optimized for our new zebrafish laboratory and showed overall success.

CHAPTER IV

BREEDING

Introduction

Breeding zebrafish is necessary to maintain stock during ongoing experiments involving necropsy as well as studying developmental changes in zebrafish. Breeding protocols utilize a collection of special apparatus and environmental cues that produce the appropriate behavioral conditions for spawning. Best practices for zebrafish breeding were tested and adapted to optimize egg and embryo production. Optimizing and adapting these protocols for our new laboratory are necessary for establishing a successful zebrafish laboratory with standard operating procedures in supporting future research.

Breeding Procedure

The process of breeding begins long before the fish are allowed to interact in the breeding tank. The afternoon before breeding is to take place, you place females and males in a divided tank with stagnant water. This allows them to become aware of one another, while still separated. At this point, you can add in plastic plant life if you like. This helps the fish feel more like they are in a natural breeding environment. Later in the evening, you are to ensure fish in breeding tanks are covered by a dark covering. This signals to the fish that it is night, since the lab environment has no windows. After covering the fish, you leave the lights to the animal room off overnight.

About 10 hours later, you return to the lab and remove the covering from the tanks and turn on the lights to the room. This tells the fish it is now day time. One perk to the artificial lighting in the room is that you can run breeding trials at any time of day, so long as you give the fish proper light and dark time windows.

Immediately after turning the lights on, remove the divider and tilt the grated tank insert to simulate the tilt of a tropical riverbed. Next all you need to do is leave the breeding tank for 2-3 hours. This is enough time to allow the full breeding process to be completed. After breeding is complete, place all fish into a new tank on the rack.

To collect the eggs, pour out about 80% of the water in the breeding tank. Next you use a plastic pipette to pull the eggs off the bottom of the tank and place them into a well plate. This is shown in Figure 4.1 below.

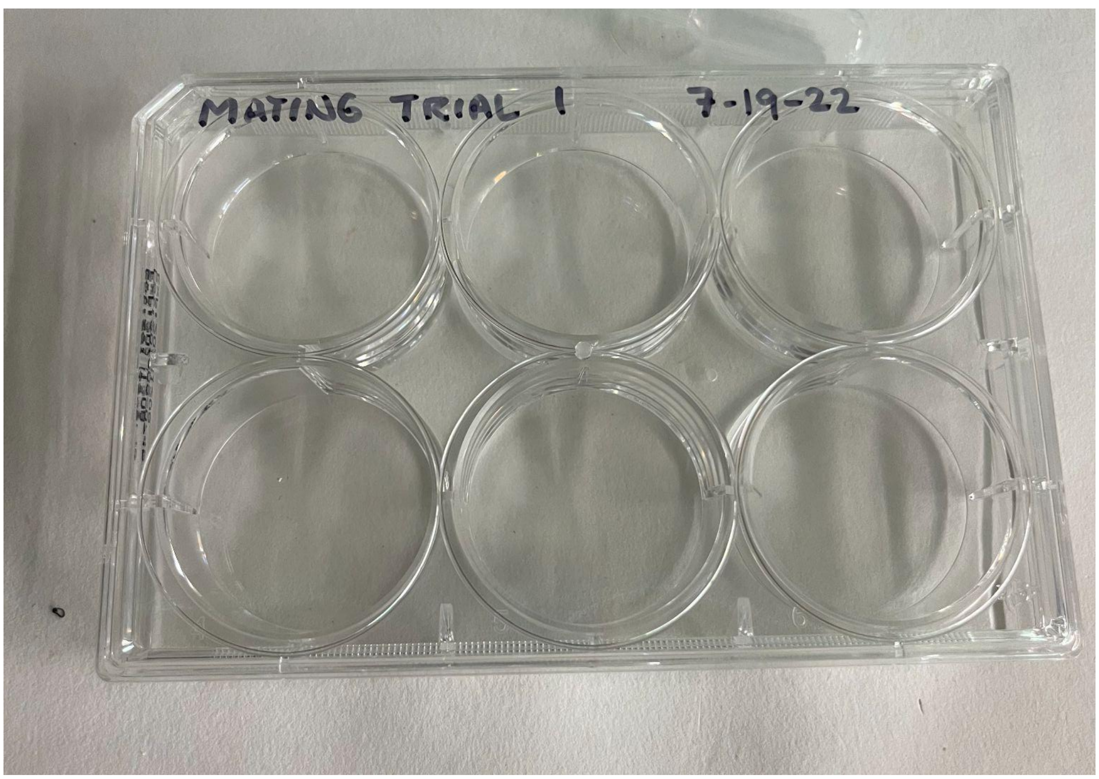

Figure 4.1. Mating Trial 1 in Well Plate

Conclusion

As discussed above, the breeding of zebrafish in a lab is necessary to maintain stock during ongoing experiments involving necropsy as well as studying developmental changes in zebrafish. Breeding protocols utilize a collection of special apparatus and environmental cues that produce the appropriate behavioral conditions for spawning. Best practices for zebrafish breeding were tested and adapted to optimize egg and embryo production. Optimizing and adapting these protocols for our new laboratory are necessary for establishing a successful zebrafish laboratory with standard operating procedures in supporting future research.

CHAPTER V

CONCLUSION AND FUTURE OUTLOOK

Zebrafish (Danio rerio) are a tropical, freshwater fish that serve as a key research model for human health and disease. The anatomical and genetic similarities shared by humans and zebrafish make establishing a zebrafish laboratory advantageous for both undergraduate research and coursework. Zebrafish are able to be used as a research model for genetic research, which is a quickly growing scientific field. These genetic and anatomical similarities will allow students and researchers alike to study anatomy and pathology across species like never before.[1]

As mentioned in this paper, Zebrafish are advantageous for lab work due to their accessibility and affordability. While scientific research that utilizes Zebrafish does not typically use fish that are purchased from your local pet store or chain pet retailer, these readily accessible fish can be used to set up a lab and gain experience caring for the fish in a laboratory environment before making the jump to ordering research grade live fish or embryos from a supplier.[1]

Zebrafish labs are truly the future of scientific research. Zebrafish are smaller and easier to care for than larger models like mice, and they can be more closely housed as well, making them a perfect option for smaller labs that would not traditionally have space for a live research model.

Now that basic protocols in the Kwekel lab have been established, we will be able to further our research. With a basis for how to properly care for, feed, and breed the fish, work in the lab can now move onto more breeding, as well as utilizing necropsy to create histological slides of the fish in our lab. These slides will be able to be used in coursework on the undergraduate level that will deepen students' knowledge of tissues and biology as a whole.

By providing an avenue to study histological slides, human health and pathology, embryology, and disease across species, the foundation that this work has set for the Kwekel lab will help grow students' knowledge and interest in biology for years to come.

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