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THE ZEBRAFISH SCIENCE MONITOR

Volume 3 Issue 5 Monday, July 24, 1995 Eugene, Oregon, USA

HOX GENE EXPRESSION IN TELEOST FINS AND THE ORIGIN OF VERTEBRATE DIGITS

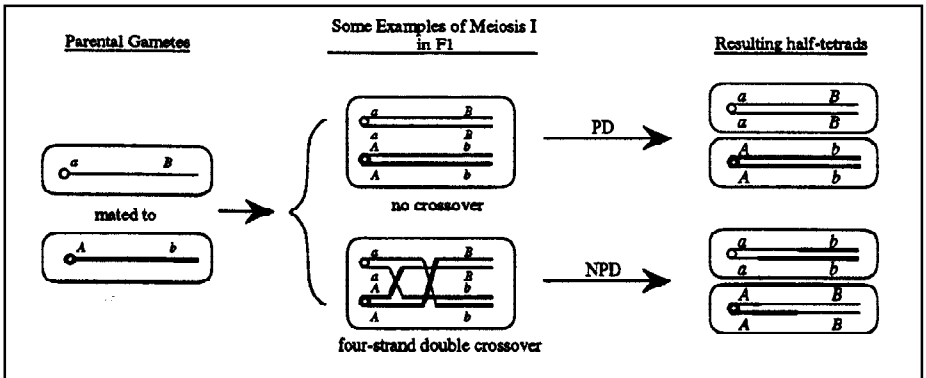
By P. Sordino, F. van der Hoeven, and D. Duboule, Department of Zoology and Animal Biology, University of Geneva, Sciences III, Quai Ernest Ansermet 30, 1211 Geneva 4, SWITZERLAND (*Nature* **375**:678-681, 1995)

Hox genes are essential for growth and patterning of the tetrapod limb skeleton. Mice mutant for the *Hoxd-13* gene have an important delay in morphogenesis owing to reduced proliferation. Based on the appearance of atavisms in such mice, we suggested that modifications of *Hox* gene regulation may have been a source of morphological variation during the evolution of tetrapod limbs. Pectoral and pelvic fins are homologous to fore- and hind-limbs, respectively. To compare the relative importance of *Ho* genes during fin versus limb morphogenesis, we cloned zebrafish (*Danio rerio*) HoxD and HoxA complex genes and analyzed their expression during fin development. The results suggest a scheme for the fin-limb transition in which the distal autopods (digits) are neomorphic structures produced by unequal proliferation of the posterior part of an ancestral appendix.

HALF-TETRAD ANALYSIS IN ZEBRAFISH: MAPPING THE ROS MUTATION AND THE CENTROMERE OF LINKAGE GROUP I

By S.L. Johnson, D. Africa, S. Horne, and J.H. Postlethwait, Institute of Neuroscience, 1254 University of Oregon, Eugene, OR 97403-1254, USA (*Genetics* **139**:1727-1735, 1995)

Analysis of meiotic tetrads is routinely used to determine genetic linkage in various fungi. Here we apply tetrad analysis to the study of genetic linkage in a vertebrate. The half-tetrad genotypes of gynogenetic diploid zebrafish produced by early pressure (EP) treatment were used to investigate the linkage relationships of two recessive pigment pattern mutations, leopard (leo) and rose (ros). The results showed that ros is tightly linked to its centromere and leo maps 31 cM from its centromere. Analysis of half-tetrads segregating for ros and leo in repulsion revealed no homozygous ros individuals among 32 homozygous leo half-tetrads -- i.e., a parental ditype (PD) to nonparental ditype (NPD) ratio of 32:0. This result shows that ros is linked to leo, a mutation previously mapped to Linkage Group I. Investigation of PCR-based DNA polymorphisms on Linkage Group I confirmed the location of ros near the centromere of this linkage group. We propose an efficient, generally useful method to assign new mutations to a linkage group in zebrafish by determining which of 25 polymerase chain reaction (PCR)-based centromere markers shows a significant excess of PD to NPD in half-tetrad fish.



Mutations can be efficiently mapped by half-tetrad analysis in zebrafish. Only no-crossover and four-strand double crossover events between a centromere (*a*) and a mutant locus (*b*) can lead to homozygosity at the mutant locus. Because four-strand double-crossovers will be infrequent, linkage group determination is achieved by surveys of centromere markers in selected homozygous mutant half-tetrads. Markers near the linked centromere will show an excess of parental di-type (PD) half-tetrads over nonparental di-type (NPD) half-tetrads, while markers near unlinked centromeres will show equal numbers of NDP and PD half-tetrads among the homozygous half-tetrads. Adapted from Figure 1. Johnson *et al.* Genetics 139: 1727-1735.

THE ZEBRAFISH SCIENCE MONITOR

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Published at the Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403 USA

KEEPING AND RAISING ZEBRA FISH (*DANIO RERIO*) IN TÜBINGEN

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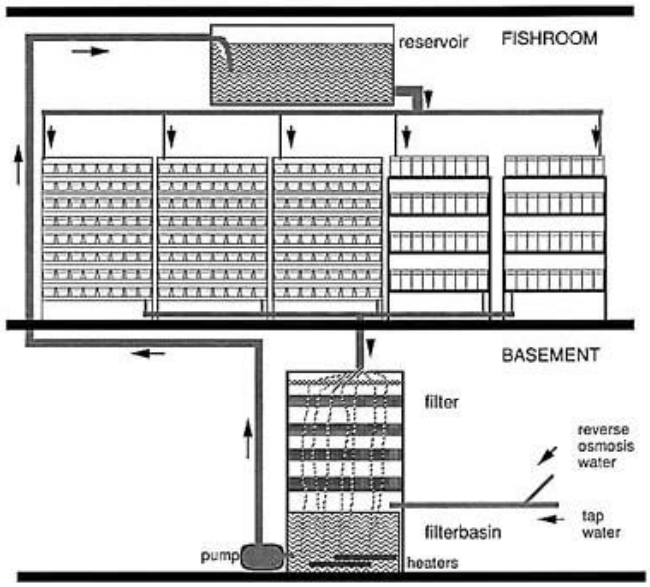
In the Max-Planck-Institut für Entwicklungsbiologie in Tübingen, we are running a large fish facility with about 7000 individual fish containers. Before planning the fish house, we tested many protocols, aquarium types, and procedures over a period of several years. The house HAS now run for three years, during which a very large number of embryonic mutants were identified and are being kept. Although we use a large facility (located in a separate building) that operates on the principles we describe here, we would like to emphasize that small versions of this system, where everything is located in a single room, have been built as well and run successfully in several other laboratories. The principles of the system have been described briefly (Mullins et al., 1994).

AQUARIA: In our lab, mainly concerned with mutant screens, we need a large number of rather small fish containers. These aquaria are interconnected and the water from one room with many (200-1500) aquaria is collected and recycled through one common filter per room. From the filter basin it is pumped up into a large reservoir located above the racks of aquaria in the fish room (Figure 1). From there it is distributed by gravity to the aquaria. The flow rate can be adjusted for each row of aquaria, which gives normally an exchange of 3 tank volumes per hour. In our fish house, the filters are in the basement, while in a second, small facility they lie underneath one of the racks of tanks, in the same room with the aquaria. The two different types of aquaria we use are described below (Figure 2).

Figure 1



A. Overflow system (Figure 2a): Half-sized mouse cages (2.5 l) or other plastic containers are used with a hole with a grid (or a slit) that functions as an overflow. The water is supplied to these containers (7-12 on a shelf) via an overlying pipe with a series of outlets (silicone tubes with cut off eppendorf pipette tips, to ensure even water flow) through which the water squirts. The outlets are spaced such that each container can be supplied with running water (Figure 2a). The water comes from a reservoir at the top of the room, runs into each container, and exits through the overflow and onto the rimmed shelf. Three different mesh sizes (0.3 mm, 1 mm, 2 mm), depending on the age of the fish, are used to keep fish from escaping through the overflow. At one end of the shelf is a pipe

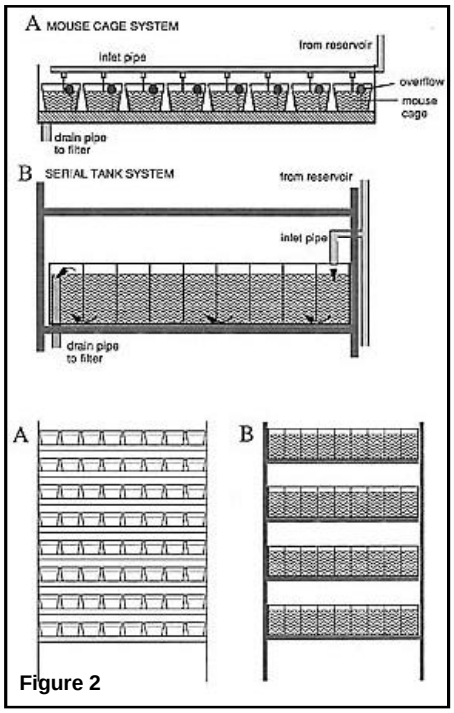


Figure 2

extending below the shelf, where the water is collected, drained into the filter, and recycled as described above. The system holds all sizes of containers, and is therefore very versatile. One can also take out the containers and replace them in different order, or carry them away for setting up matings. We use half- or full-sized mouse cages, rat cages and small 1 liter containers for single fish and pairs of fish in this system.

Recently we have begun to use a hybrid 'serial overflow' (SO) system for keeping small numbers of fish at higher density. In the SO system, mouse cages or rat cages are further subdivided by partitionings as in the serial tank system, with slits on the bottom to allow the dirt to be carried away. SO systems stay cleaner, and seem to work well for smaller numbers of fish. This type of vessels also allows more efficient use of space; for instance, when males and females of the same genotype need to be separated, they can still reside in the same mouse cage (and they even get to see one another).

B. Serial tank system (Figure 2b): Aquaria of the dimension of about 1.20 m length, 60 cm wide and 22 cm high are subdivided by glass partitionings that leave a 1.5 mm slit at the bottom, to give a series of 8-10 interconnected compartments of about 12 liters each. Water flows into the aquarium at one end, runs through the slits, and carries the dirt with it. The water is collected at the other end by an overflow (with a cover grid to keep fish from escaping) which determines the water level. From there, water is drained into a big filter unit; clean water is then pumped up into a reservoir from which it gets distributed again, just by gravity.

Fish densities: Maximum densities are about 10 adult fish per liter of water in the serial tank system. We can raise fish to adulthood (3 month) at densities of up to 40 fish per liter in the overflow system. Usually, we transfer them into tanks after about 2 months when they are big enough not to pass through the slits anymore. The fish seem to do better at higher densities, probably because the tanks are cleaner because the water is kept in constant movement and the dirt is carried out via the overflows and the slits. Fish tend to be aggressive at low densities, so we add some plastic grass to give them a chance to hide.

Illumination: We use a 14h light-10h dark cycle. For convenience, we have lamps above every row of serial tanks; however, a sufficiently bright illumination of the room would probably suffice. The overflow systems do not have their own illumination.

Biological filters: We recycle the water through biological filters of appropriate sizes (1000 liters of water per m^2 of foamed plastic of 10-30 ppi). The water runs by gravity through the filter and then into basins from which it is pumped up into reservoirs that are located above the aquaria in the fish rooms. There are two pumps per filter that run on alternate 12 h cycles; in case one breaks down, the other takes over automatically. The filters provide a large surface area for aerobic bacteria which degrade ammonium compounds to nitrite and finally nitrate. While the former compounds are very toxic to the fish, the nitrate is not. Since the surface area of the filters must be large, we use mats of foamed plastic, through which the water is dribbling into the basin, arranged in a series of shelves with increasing density of foam. The top layer is disposable synthetic filter material which collects the coarse debris; it is checked twice a week for clogging and replaced if necessary. The filter mats are cleaned twice a year in a washing machine. The production of nitrate leads to lower pH. The pH in the fish water is between 6.5 and 7.5, although higher and lower pH may work also. We measure the pH, nitrate and nitrite (using Merck sticks) in the filter basins twice a week. Normally 5% of the water is exchanged with fresh water daily; this is done using a timer. If the pH drops and/or the nitrate increase is rapid (caused by intense feeding), more fresh water is added. In addition, there is a device in the filter basin (a float like in toilets) that causes automatic refilling with fresh water in case the water level drops, because of a leak or after removal of water from the system in fish rooms (eg, for setting up crosses).

Water: Tübingen tap water is hard (approx. 500µS), very rich in $CaCO_3$, and has a pH of 8 or higher. The composition of the tap water is as follows:

Cations	mg/l	Anions	mg/l
Calcium	73.3	Hydrogencarbonate	195.0
Magnesium	13.2	Chloride	17.0
Sodium	11.5	Sulfate	72.0
Potassium	2.2	Nitrate	9.7
Iron	<0.01	Nitrite	<0.01
Manganese	<0.01	Phosphate	0.09
Ammonium	<0.01		

We use a mixture of tap water and reverse osmosis water, at a conductivity of about 350 µS at pH 7.5. This seems to be a good compromise between sufficiently low conductivity to stimulate egg laying and a high enough carbonate buffering capacity at an acceptable pH. All water is filtered through

charcoal before going into the systems. **Temperature:** The temperature of the water is adjusted to 26 C with a number of heaters placed into the filter basin. The temperature in the fish rooms is slightly higher (27 C) to prevent condensation of water on the walls of the rooms. Higher temperatures are uncomfortable for the researchers, they might also reduce the life span of the fish. As we have several heaters for each basin, none of which is sufficient to heat up above tolerable values, there is no danger of overheating. Temperatures dropping to room temperature by failure of the heaters is not dangerous for the fish.

Snails: We also have snails (Florida fresh water snails, *Planorbella spec.*) in our tanks. They clean the walls of algae and eat the surplus food, which has a very positive effect on the water quality. Adult snails are sometimes killed, and baby snails can be eaten by the adult fish, so it is necessary to regularly resupply snails. Snails multiply in the aquaria in which fish are raised. Usually some have to be removed at regular intervals. Even though snails introduce a small amount of extra work, they are very helpful; a tank without snails is easily spotted because the fish are hidden behind growing algae.

Problems with the aquaria: Clogging of the outlets, grids and slits. Snails or little pieces of dirt sometimes get into the outlets and may clog them. We check daily to ensure that the water is clean and running in all aquaria because fish kept at high density may die overnight if the water is not running. The containers in the overflow system stay clean only if there are enough fish to sufficiently stir the water such that the dirt is carried out of the overflow. With only a few adult fish and careless feeding, they may get very dirty and may need to be cleaned regularly (about once every 4 weeks). Running the water at higher rates helps, but there is a limit because the reservoirs may run dry, and the incident of clogging is higher with higher flow rates. In the serial tanks, dirt collects in compartments with no or few fish. The last compartment, from which the dirt has to exit via the overflow should therefore hold many fish (or be cleaned regularly).

***Problems with the water*:** Tübingen tap water is too hard for good egg laying, so it has to be diluted with desalted water. On the other hand, due to its high carbonate content, it has good buffering capacity and usually the pH is stable. If the filters do not work or if their capacity is too low, the pH may rise to values above 8, preventing growth of the nitrifying bacteria in the filter. With too much feeding, this may lead to bacterial growth (turbidity) of the water. We had this problem at the beginning. Frequent water changes and adjusting the pH by mixing the tap water with reverse osmosis water at a ratio of about 1:1 will reduce this problem. In the beginning, we did not use mixed water and our charcoal filter had too low a capacity, which caused frequent and erratic death of baby fish. Strangely enough, the adult fish survived these difficult times remarkably well and even laid eggs.

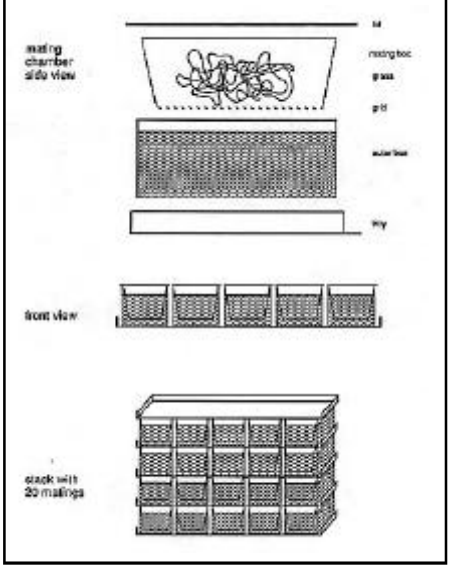
Diseases: As many tanks share a common filter, there is the problem that diseases introduced into one tank may spread very quickly through the entire system. The intensive fish work carried out in our fish house (about 2,000 crosses set up weekly with fish from different aquaria and rooms, up to 200 containers of baby fish started weekly for raising) makes it very difficult to keep individual systems or rooms isolated. It would be orders of magnitude more work, and much more costly. Unlike in the case of mice and humans, fish germs do not spread via the air, so the possibilities of infection are restricted to food, water, or fish introduced from the outside. We do not use food from natural fresh water ponds. The tap water is chlorinated and goes through charcoal. We do not introduce fish from the outside without putting them into quarantine first and treating them with medicines against parasitic infections. We collect eggs from them in the quarantine room, treat the eggs with Clorox (see *The Zebrafish Book* for a protocol), and transfer the babies into the fish facilities. Although we did not start our facility with a disease free population of fish, we did not have any problems with diseases for about four years. We had noticed an occasional skinny fish before, but since less than about 1/1000 of the fish were affected at any time, there was no reason for concern. In the past summer, we started observing significant numbers of sick fish, they got skinny, stopped eating and died eventually. We found that almost all were infected with nematodes (capillaria) and a few in addition with fish tuberculosis (Tb). We do not know where these diseases came from, and whether they were the primary cause of the sickness, but we assume that they were present in our population all along. The problem was more severe in the rooms where many containers held only a few old fish which had not been kept very clean. This probably led to a spreading of the infection, because the nematodes propagate via eggs passed through fish feces, that are taken up by other fish if left around. We did a round of serious cleaning of the tanks, filters and mouse cages, treated the fish with Levamisol, twice within two weeks (which was supposed to be effective against the worms, but not the Tb). The fish were mostly o.k. after this, although we did not get rid of all the worms.

Now we try to remove all infected fish as soon as we detect them, and further, we try to keep the fish containers rather clean to avoid spreading the diseases via the feces of infected fish. An effective treatment to get rid of the worms was published here recently by the Driever lab, which we intend to use to solve this problem. We have also had a serious spread of fish tuberculosis, Tb. Because there seems to be no effective treatment against Tb, we are currently starting individual rooms from sterilized eggs (see *The Zebrafish Book* for a protocol) after disinfecting, hoping that this will solve the problem. It will of course be ideal if the facility can be maintained disease-free in this way. According to our fish veterinarian, both diseases often occur latently in fish populations without harming them, but they can become a problem, when the fish are subjected to some form of stress. During the time we had disease problems, the fish were most certainly subjected to a significant amount of stress, as we kept setting them up frequently for crosses during our screen. Overall, therefore, our impression is that a good genetic background achieved by frequent outcrossing, healthy food, and clean conditions suppresses the level of these infections to a tolerable level.

Food: Adult fish are fed with live, frozen or dry food. We use fresh *Drosophila* larvae, frozen *Bosmina* (a small freshwater crab which can be bought here), green flakes, red flakes, and brine shrimp nauplia, for fish older than 6 weeks. Food, including dry food, is suspended in water in squirt bottles, and squirted through small holes in the lids of the tanks. Larval fish and fish for ready for mating are fed 3 times, adults twice, and adults that are not being used once daily. Baby fish are fed with salt water rotifers or paramecia during the first week of feeding, and later with brine shrimp *nauplia*.

Comments: We believe that a variety of different food is good for fish health. It is clear that dry food alone is inadequate to keep fish in good laying condition. Our fish like *Drosophila* larvae more than anything else, but growing them in large scale for fish food is no trivial task. We do not know for certain, but we suspect that the fly larvae supply the fish with essential vitamins or fatty acids they otherwise do not get in large enough quantities. Alternatively, we feed frozen *Bosmina* that are harvested seasonally from fish-free ponds at sugar factories; but the supply of *Bosmina* is limited. *Artemia nauplia* hatched from dry eggs are liked as well, but they are very small. Furthermore, if unhatched artemia eggs are present in the food, the fish may get seriously constipated. We cannot get live adult *artemia* here. Baby food always was and is the biggest problem. We tried dry baby food several times but found it quite unsatisfactory. *Paramecia* grown according to the protocol of the Driever lab are fine, but sometimes the cultures become contaminated (this may be a problem particular to laboratories also working with flies), together with being expensive and work intensive. For a long time we had a supply of frozen dormant rotifers, which are harvested seasonally from the ponds in sugar factories mentioned above. They hatch within 24h in fresh water and are concentrated by very low speed centrifugation. The supply, however, is limited, and presently we do not have them. A good alternative are salt water rotifers, although this requires raising the babies for some time in salt water to allow the rotifers to survive. *Drosophila* larvae and rotifers have the added advantage that they can be stored at 4 C for two (*Drosophila* larvae) to 7 days (rotifers) before feeding, so they can be prepared in advance. A protocol for growing salt water rotifers is given below.

Figure 3



Raising fish: For egg collection, we place males and females into mating chambers (Figure 3) the preceding afternoon: A plastic box with the bottom replaced by a mesh of stainless steel (2 mm), inserted snugly into an outer box such that there is a space between the mesh net and the bottom of the outer box. We use one liter boxes for single pair matings. Plastic green grass is placed into the inner box. Five such mating chambers are held on one plastic tray, covered with a lid or with another tray (with mating boxes) (Figure 3). The water is fish water from the reservoirs. Depending on the condition of the fish, an average of 50% of single pair matings lay eggs the morning after being set up, and another 100% on the following morning. For cleanliness of the eggs, the fish are not fed on the day they are set up. They may stay in the boxes without food and water changes for two days. The shortest interval for setting up fish for mating is about one week for females, and

three days for males, if the fish are to be used frequently.

The eggs when laid fall through the steel net and are collected from the outer box with a plastic tea sieve and placed into E3 saline (5mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 10^{-5}% Methylene blue). We collect the good eggs and place them in petri dishes within 12 h after laying, up to 100 per 94 mm dish, or 40 per 60 mm dish in E3 medium. The water is changed once on the third day of development.

Swimming larvae (day 5) are placed into fish water (from the reservoir) at a density of 70 fish per mouse cage in about 1 liter. When salt water rotifers (which is what we usually feed now) are fed during the first week, the water is supplemented with sea salt to a concentration of 3g/l such that the rotifers survive for at least several hours. We put the babies directly into mouse cages and place the cages onto the shelves in the overflow system without connecting them to the running water. With clean paramecia or rotifers, the water does not need to be changed in the first week, otherwise it has to be replaced every other day or so. Babies are fed twice a day, such that there is always some food around. The overflow is provided with a 0.3 mm mesh sieve to prevent the babies from being flushed out. At day 14, when the baby fish are big enough to eat brine shrimp, we start feeding brine shrimp *nauplia*, and one snail is added per pot. We feed initially small, but increasing amounts of *artemia* two to three times a day, and let the water dribble through. On day 21, the fine mesh sieve is replaced with a 1 mm sieve and the flow rate is increased to slow running, and another three weeks later they are switched to the 2 mm sieve. The fish can get adult food when they are 6 weeks old or so, and can be put into tanks shortly thereafter. They may already give eggs when they are two months old, but the fecundity is optimal after 4 months or so.

Problems: For babies, the right amount of food is crucial for their survival and vigor. Heavy overfeeding with rotifers may lead to high nitrite levels, underfeeding causes unequal growth rates among the population. Overfeeding with *artemia* is dangerous, as rotting *artemia* spoil the water. A good rule of thumb is that the *artemia* should be mostly eaten after about 20 minutes, at which point the babies will have nice red bellies. If properly done, all 70 fish placed into a container at day five will grow up in these containers until adulthood. If space allows, baby fish can be raised at lower densities, which makes them grow even faster.

Maintenance: for 6 rooms, 5 filters with a total of about 7,000 containers holding between 1 and 100 fish (about 300,000 fish), we have 5 full time people for cleaning the rooms and the plastic boxes, checking and cleaning the tanks, preparing the food, and feeding the adult fish. In addition, the scientific staff, the technicians and seasonal students participate in the tank checks once daily, the growing of baby food and *artemia*, and feeding of the individual fish and the baby fish. Control of outlets, grids, and overflows or slits from each container is done twice a day, this takes about twice two hours for one person for all 7,000 containers, plus two hours daily cleaning of slits and overflows. Every half year or so each system is cleaned thoroughly, the pipes are flushed, and the filter mats are cleaned. The coarse filters are checked daily. 5% of the water is exchanged daily, and the water measurements are made twice a week.

Safety: The systems, aquaria, shelves, filters, plumbing etc. were built by an experienced firm from high quality material (glass, valves, pipes) using a special technique for gluing the glass, rendering the aquaria extremely stable for a long time. For the aquaria, we have glass where ever possible, because it is easy to keep clean and transparent. The firm is not particularly cheap, on the other hand the great safety, stability and ease in maintenance is worth it, we believe, and, once installed, it is a very good investment. So far we have had no catastrophe such as breaking of a tank or a pipe coming loose overnight. The design allows untrained people to grasp very quickly how it works and easily take care of things. This is, we feel, of very great importance. The system is designed so the containers cannot run dry, nor can it be overheated. The worst possible catastrophe would be if both pumps of one filter unit would break down simultaneously. This would cause suffocation of the highly populated tanks, but the individual aquaria would not run dry. During the three years, we had a couple of pump breakdowns, but never both pumps in one room. There are alarm systems for pumps, temperature, water level in the reservoirs above the tanks, water level in the millipore water reservoir, and the ventilation of the rooms. These alarms are important for us because so many people use our facility. For smaller facilities, we believe that regular inspections and checks would be quite sufficient; an alarm system for the water level in the reservoirs above the tanks would probably provide adequate security.

Salt water rotifers. The following brief description is a modification of a protocol that was kindly provided to us by Drs. P. Dhert and P. Sorgeloos, University of Ghent, Belgium. A more detailed protocol is available upon request. We grow *Brachionus spec*. rotifers in 50 liter

round plastic tanks with 15 g/l seasalt in deionized water at about 25 C, with slow-bubbling aeration. Rotifers are inoculated at a density of 100-150 R/ml and are fed on instant Culture Selco food (CS, Inve Aquaculture, Baasrode, Belgium). Depending on the density of the culture, we feed increasing amounts of CS: 6 g at set up, and 6, 7, 8.5, 10, 11, 12 g on subsequent days. After 7 days, the culture has more than 500 R/ml and is harvested through a fine net, and the rotifers are resuspended in 14 liters of seawater (15 g/l sea salt), of which about a quarter is used to reinoculate a new culture. The remainder can be stored in the refrigerator for up to a week and is used for feeding the babies (2x12 ml per mouse cage per day).

PRODUCTION OF ANDROGENETIC HAPLOIDS AND DIPLOIDS

By G.E. Corley-Smith, C.J. Lim, and B.P. Brandhorst, Institute of Molecular Biology & Biochemistry, Simon Fraser University, Burnaby, BC, V5A 1S6, CANADA

We have visual and molecular marker evidence that we have produced haploid and diploid androgenetic zebrafish. Androgenotes inherit all of their chromosomes from their father and none from their mother.

Androgenesis is a technique that can be useful in considering a variety of genetic phenomena, including: rates of meiotic recombination in spermatogenesis, detection and mapping of male specific DNA markers or linkage groups (if any exist), basis of sex determination in zebrafish, and the role of imprinting of essential paternal genes, if any.

Androgenetic haploids are produced by irradiating eggs to destroy the maternal genome, followed by fertilization. By inhibition of either karyokinesis or cytokinesis of the first mitotic division, diploid androgenotes can be produced. For irradiation of eggs we use a cabinet x-ray source that is commercially manufactured for detection of fractures in airplane and other critical parts, the Torrex 150D x-ray inspection system (Faxitron X-Ray Corp., Buffalo Grove, IL, USA; phone (708) 465-9729). The initial cost is approximately $17,000 US. When switched off, the source is extremely safe. Radioisotope sources are also useful for irradiating eggs. Disadvantages of using a radioisotope source for irradiation include: set up expenses, regulations and precautions necessary for isotope use, and the requirement of a room dedicated for irradiation purposes. X-ray dosimetry was performed with a MDH1515 dosimeter using a MDH model 10X5-180 ion chamber (paddle chamber). This was calibrated with a known ^{137}Cs source (NBS ^{137}Cs source #47455).

We heat shock zygotes to inhibit the first mitotic division. After fertilization, eggs are held at 28.5±0.5 C. Eggs are heat shocked 13 minutes after fertilization for 2 minutes at 41.4±0.05 C. Temperatures were measured with a calibrated thermometer (Fisher Scientific, Cat. No. 15041A) with an uncertainty in temperature certified not to exceed 0.03 C.

In a recent experiment, we collected eggs from one female (from a line which originated from zebrafish bought from pet stores in Vancouver Island, B.C. and assumed not to be closely related to Eugene's AB line) and the milt from one male (*AB line from Charline Walker, which has been screened to reduce recessive lethals). The eggs were held in ovarian fluid at room temperature for 50 minutes, the time required for irradiation of eggs. The milt was collected just prior to being used for fertilization and was held in sperm extender. 204 eggs were irradiated with 10,000 R of x-rays. These eggs and 76 eggs which were not irradiated (control group) were then fertilized. 72% of the control group were observed developing normally at 24 hr. All of these subsequently hatched and appeared to be diploids. Of the 204 eggs that were irradiated and subsequently fertilized, 49 were not heat shocked (treatment to produce haploid androgenotes) and 155 were heat shocked to produce diploid androgenotes.

In the group whose eggs were irradiated (to destroy the maternal genome) but not heat shocked, 5 embryos developed, all of which exhibited the haploid syndrome (shortened body, small melanocytes). These are putative androgenetic haploids. In the group whose eggs were irradiated and then heat shocked to inhibit the first mitotic division, 2 normal looking diploids developed (putative diploid androgenotes).

The haploid syndrome can be seen at 24 hours as a shortened body phenotype (Figure 1). At 48 hours, the shortened body is easily noticeable and the difference in size of melanocyte starts to become noticeable (Figure 1) and is pronounced by 96 hours (not shown). The development of putative androgenetic diploid embryos was initially slightly retarded (Figure 1). However, by the end of the first month, these fish achieve approximately the same size as the diploid control fish.

The lack of any embryos that appeared to be diploid in our irradiated and non-heat shocked group suggests that the irradiation dosage was sufficient to destroy the maternal

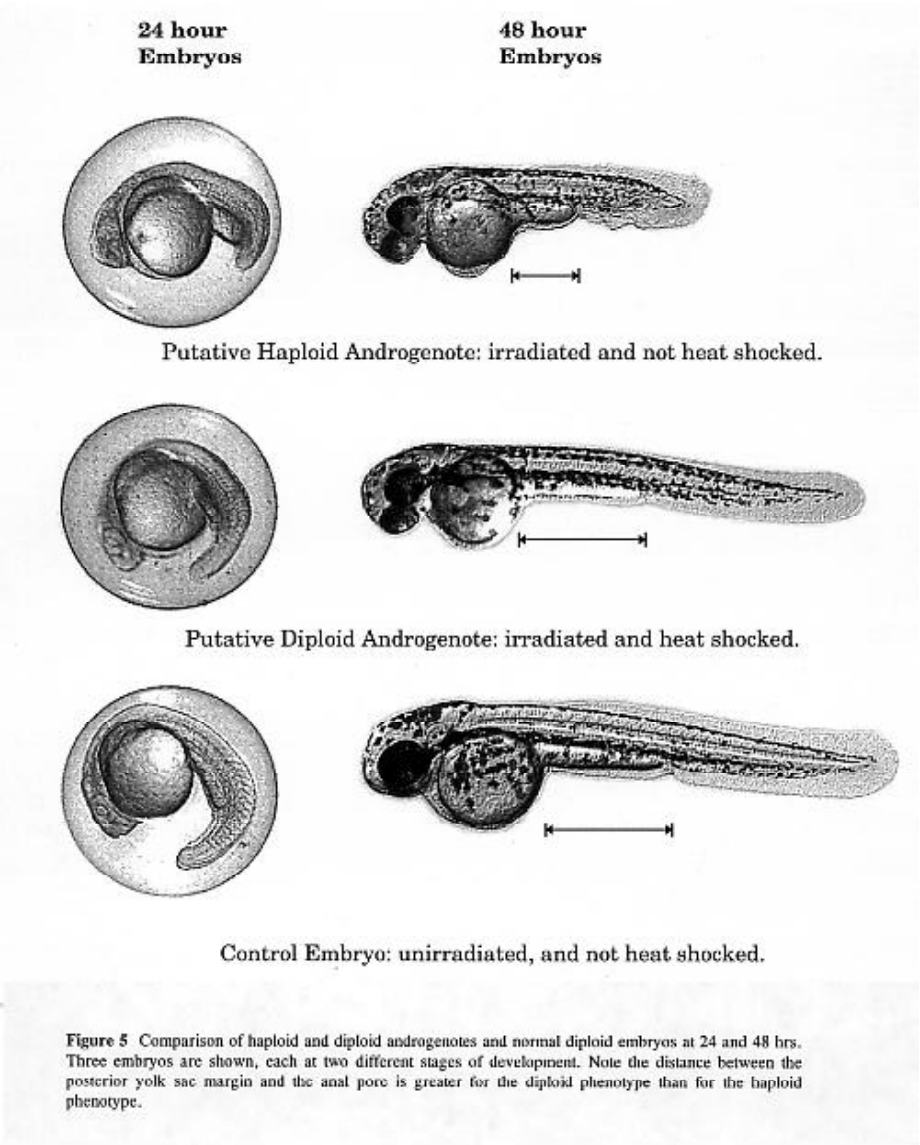


Figure 5 Comparison of haploid and diploid androgenotes and normal diploid embryos at 24 and 48 hrs. Three embryos are shown, each at two different stages of development. Note the distance between the posterior yolk sac margin and the anal pore is greater for the diploid phenotype than for the haploid phenotype.

genome, or somehow impeded its transfer to the offspring. Thus, we believe the surviving diploid embryos in the irradiated and heat shocked group, are indeed androgenetic rather than resulting from a failure to eliminate the maternal genome. Putative haploid androgenotes often hatch, but seldom feed. They usually die during the first few weeks of life. We have apparently healthy, active putative diploid androgenotes which are over a month old.

The percentage of haploid and diploid androgenotes produced relative to our control group was 14% and 2%, respectively. We believe we can increase these production rates with further refinement of the technique. Updates to the technique will be posted on our WWW zebrafish server.

http://darwin.mbb.sfu.ca/imbb/brandhorst/zfish.html

Genetic analysis was performed using polymorphic DNA markers that were fluorescently labeled during PCR and detected on an ABI 373 Automated Sequencer, thus enabling precise sizing and clear identification of markers. None of the maternal specific markers we have identified to date, some of which are homozygous, are inherited by either the haploid or diploid putative androgenotes we have produced. This does not preclude the possibility of some leakage of maternal DNA to the putative androgenotes, but it is compelling evidence that we have produced zebrafish which are lacking a substantial proportion of the maternal genome. We have confidence in our makers as all DNA markers identified in the parents, many of which are not polymorphic, were observed in at least some of the normal diploid progeny. Markers were considered homozygous if they were observed in all 12 normal diploid progeny analyzed.

The sex ratios we will observe in the progeny of our androgenetic zebrafish, may be informative about the mode of sex determination in zebrafish, which is presently not understood. The survival of diploid zebrafish androgenotes suggests a lack of male specific imprinting of essential genes as has been observed in mammalian species.

DELAYED *IN VITRO* FERTILIZATION OF ZEBRAFISH EGGS USING COHO SALMON (*ONCHORHYNCHUS KISUTCH*) OVARIAN FLUID

By G.E. Corley-Smith, C.J. Lim, and B.P. Brandhorst, Institute of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, B.C., V5A 1S6, CANADA

Many experiments that involve *in vitro* fertilization can be facilitated by being able to delay fertilization of zebrafish eggs for periods up to a few

hours after they are extruded from the female. For example, when injecting 1-4 cell embryos, the injections could be done in batches that were fertilized at progressively later times. Delayed fertilization is also required if eggs must be manipulated prior to fertilization; e.g. for microinjection, nuclear transplantation, or irradiation to produce haploid or diploid androgenotes.

We have held zebrafish eggs for over 6 hours after extrusion from the female; when subsequently fertilized, they developed into apparently normal zebrafish. Recently we held 76 zebrafish eggs for 50 minutes between the time the eggs were extruded from the female and when milt was added to fertilize the eggs. In this group, 72% were fertilized and developed as normal looking embryos which hatched. The remaining 28%, observed at 24 hours, showed no embryonic development and appeared unfertilized. The technique does not appear to lead to an increased incidence of abnormal development or decreased fertility of adults. Fish derived from eggs that were held for times exceeding 1.5 hours, have matured and been used for breeding in our lab.

The technique uses the natural body fluid that surrounds mature coho salmon eggs, ovarian fluid. We have successfully used coho salmon and rainbow trout (*O. mykiss*) ovarian fluid. Our batches of coho ovarian fluid collected in 1991 are more effective than our batches of rainbow trout ovarian fluid. However, Pat Gibbs (Gibbs *et al.*, 1994) has used rainbow trout ovarian fluid successfully. We suspect that ovarian fluid from any fish species that holds eggs in a mature state for long periods is likely to be effective. Thus, ovarian fluid from any salmonid species is likely to work, although we expect the most effective ovarian fluid to be from species that undergo prolonged upstream migrations to their spawning streams and consequently probably hold their eggs longer.

When coho ovarian fluid containing zebrafish eggs is diluted with water, the chorions of the eggs rise, and cytoplasmic streaming that leads to formation of the blastodisks is initiated. Although the blastodisk forms, no cell divisions take place. Thus, egg activation without insemination is initiated, and the egg can no longer be fertilized. Very little dilution of coho ovarian fluid is required for initiation of activation. Increasing the proportion of water added, up to a plateau, increases the rate of chorion elevation.

Quality of Ovarian Fluid

Care should be exercised in collecting ovarian fluid. For salmon, if a few eggs are ruptured in a batch of eggs, the subsequent fertilization rate of the non-ruptured eggs is substantially reduced. Contamination of salmon eggs and ovarian fluid with blood reduces their subsequent fertilization rate. Furthermore, dilution of ovarian fluid with water leads to activation of eggs. Thus, we believe the ovarian fluid must be collected in such a manner as to reduce contamination by broken salmon eggs, blood and water.

The female fish from which ovarian fluid is collected should be mature and ready to deliver eggs but should not be overly ripe with broken eggs and watery ovarian fluid. Ovarian fluid from non-ripe females may be useable, but the volume of ovarian fluid will be less.

We normally collect ovarian fluid at a salmon hatchery. We separate some ovarian fluid from the salmon eggs and turn the salmon eggs over to the hatchery staff for fertilization, which is not impaired. Approximately 50 mls of ovarian fluid can be collected per coho salmon. One good batch of 50 mls is sufficient for holding the eggs from over 1,000 zebrafish females.

As batches of ovarian fluid (ovarian fluid from one fish) vary in their effectiveness for holding zebrafish eggs, we test individual batches of ovarian fluid. When a batch is identified that allows for holding zebrafish eggs for 1.5 hours with high subsequent fertilization rates, we then aliquot this batch into 1.5 ml screw cap microcentrifuge tubes and freeze at -20 C or -80 C. Coho salmon ovarian fluid is robust and can undergo repeated freezing and thawing and can be left for several days at room temperature and still function. However, for optimal fertilization rates we suggest more careful storage.

Collection of Ovarian Fluid

Coho salmon die shortly after spawning; thus, eggs are normally collected at hatcheries immediately following lethal cranial trauma. The euthanized female coho salmon is immediately hung by its tail and all the gills on both sides of the had are slit to drain blood. Pushing a wad of paper towels under the operculum will help soak up blood and impede coagulation. After 5 minutes, dry the fish with a towel so that no water can drip into eggs and ovarian fluid when they are collected. Have an assistant hold the fish by its head and tail, with its belly downward over a clean dry bowl, and slit the fish from anus to from of the body cavity. Eggs and ovarian fluid will fall into the bowl. Release eggs from skein (ovarian connective tissue) and remove pieces of skein from bowl containing free eggs and ovarian fluid. Remove 75% of ovarian fluid to 50 ml conical plastic tube. A non-abrasive kitchen strainer or colander, stainless steel or

plastic, is useful to separate eggs from ovarian fluid. Handle eggs gently at all times to prevent breakage. Give the salmonid eggs to hatchery staff and put the ovarian fluid on ice. Back at the lab, centrifuge the tubes of ovarian fluid at 5500 g for 5 minutes at 4 C to sediment cellular debris. Remove the supernatant and freeze it.

Use of Ovarian Fluid for *In Vitro* Fertilization

This technique is similar to normal *in vitro* fertilization as outlined by Charline Walker and George Streisinger in Eugene's *The Zebrafish Book; A Guide for the Laboratory Use of Zebrafish (*Brachydanio rerio). We collect eggs with silanized capillary tubes (Kimax-51, Kimble Products Art. No. 34502, ID 0.8-1.1mm, length 100mm) that we pre-wet with ovarian fluid. When eggs are collected, they are placed into 100µl of salmonid ovarian fluid in a small petri dish. The actual volume of ovarian fluid is adjusted to the number of eggs. All eggs must be covered with ovarian fluid. We get better subsequent fertilization rates when we hold the eggs at room temperature (18-22 C) than when we hold them chilled at approximately 4 C. Immediately before fertilization, we remove most of the ovarian fluid from the zebrafish eggs with a Pipetman, centrifuge it at 5500g for 5 min at 4 C to sediment cellular debris, and then remove the supernatant which can be frozen for reuse. Following removal of ovarian fluid, eggs are fertilized as normally done for *in vitro* fertilization.

A step by step protocol for using coho ovarian fluid for delayed *in vitro* fertilization is expected to be included in an update to *The Zebrafish Book* which is scheduled to be issued later this year. This protocol will also be available on the Eugene WWW zebrafish server <http://zfish.uoregon.edu> and our zebrafish server <http://darwin.mbb.sfu.ca/imbb/brandhorst/zfish.html>.

Acknowledgements

We gratefully acknowledge Pat Gibbs and Charline Walker. This technique was refined based on friendly advice received from both. Also, they were both instrumental in infecting us with their enthusiasm for zebrafish research, and teaching us the basics of running a zebrafish research facility.

ELECTRONIC ZEBRAFISH

By M. Westerfield, Institute of Neuroscience, 1254 University of Oregon, Eugene, OR 97403-1254, USA

At the zebrafish Cold Spring Harbor meeting last year, a committee was selected to investigate establishment of an on-line database for zebrafish information. In November, the NSF announced a special program for biological databases and we applied.

The application was funded and design and implementation of the database system will begin in September. The prototype server will be set up in Eugene within a year and a second mirror site will be available in Boston the following year. We also hope to establish a site in Europe.

The database is intended to contain extensive information about zebrafish stocks, mutants, gene expression patterns, developmental and adult anatomy, and much more. A simple WWW interface will be available for submitting data and obtaining information over the Internet. More details of the proposed system can be obtained at the Eugene WWW site: <http://zfish.uoregon.edu>.

Comments, questions, or suggestions can be directed by email to: MONTE@UONEURO.UOREGON.EDU or DRIEVER@HELIX.MGH.HARVARD.EDU.

A CONSERVED FAMILY OF *ELAV*-LIKE GENES IN VERTEBRATES

(*Proc. Natl. Acad. Sci.* **92**:4557-4561, 1995)

By P.J. Good, Department of Biochemistry and Molecular Biology, LSU Medical Center, 1501 Kings Highway, Shreveport, LA 71130-3932

A large family of genes encodes proteins with RNA recognition motifs that are presumed to bind RNA and to function in posttranscriptional regulation. Neural-specific members of this family include *elav*, a gene required for correct differentiation and maintenance of neurons in *Drosophila melanogaster* and a related gene, *HuD*, which is expressed in human neuronal cells. I have identified genes related to *elav* and HuD in *Xenopus laevis*, zebrafish and mouse that define a family of four highly related vertebrate *elav*-like genes (*elrA*, *elrB*, *elrC* and *elrD*) in fish, frogs, and mammals. In addition to protein sequence conservation, a segment of the 3' untranslated sequence of *elrD* also is conserved, implying a functional role in *elrD* expression. In adult frogs, *elrC* and *elrD* are exclusively expressed in the brain, while *elrB* is expressed in brain, testis and ovary. During *Xenopus* development, *elrC* and *elrD* RNAs are detected by late gastrula and late neurula stages, respectively, while a nervous system-specific *elrB* RNA species is expressed by early tadpole stage. Additional *elrB* transcripts are detected in the ovary and early embryo, demonstrating a maternal supply of mRNA and possibly of protein. These expression patterns suggest a role for different *elav*-like genes in early development and neuronal differentiation. Surprisingly, *elrA* is expressed in all adult tissues tested and at all times during development. Thus, the widely-expressed *elrA* is expected to have a related function in all cells.

CULTURING *Tetrahymena* AS AN ALTERNATIVE BABY FOOD TO PARAMECIA

By M. Gerson and D. Stainier, Department of Biochemistry and Biophysics, UCSF School of Medicine, SanFrancisco, CA 94143-0554, USA

Encouraged by the early report of Speksnijder and Bijmolt, we took advantage of local expertise (i.e. Liz Blackburn's lab) to optimize *Tetrahymena* culture conditions. *Tetrahymena* are pear-shaped, ciliate protozoa, about 30-100 micrometers in size. Although they are not parasites, *Tetrahymena* can be associated with sick or weakened fish, found on skin lesions where they feed upon bacteria and fungi.

As a baby food, *Tetrahymena* offer the advantage of being easier to culture and maintain than paramecia since the media is much less complicated to prepare, and cultures rarely crash. *Tetrahymena* can be used as an exclusive food source for babies until they start eating brine, or can be used in conjunction with paramecia or rotifers. The protocol we use is adapted from one given to us by Cathy Strahl (Blackburn lab) and has provided us with a reliable supply of *Tetrahymena* for the past 6 months.

Media Stock Solution (1 liter of 10X PPYS)

Component	Amount	Source
proteose peptone	200.0 g	Fisher #DF0120-17-6
yeast extract	20.0 g	Fisher #DF0127-17-9
sequestrene (NaFe form)	0.3 g	Ciba-Geigy

1. Combine proteose peptone, yeast extract, and sequestrene in a large flask. Adjust to 1000 ml with warm ddH20 and stir until dissolved.

2. Spin at 7K gsA rotor for 20 min.

3. Pour off supernatant into 200 ml aliquots. (Note: a small amount of solid material in the media will not affect the culture.)

4. Store at -20 C.

Before use:

1. Thaw one aliquot of media and adjust to 1000 ml with ddH20. Aliquot into smaller volumes if desired. Autoclave 20 min.

2. Add P/S/F (10 ml for a 1000 ml culture; P/S/F not required for small cultures). P/S/F = penicillin/streptomycin/fungizone (Gibco/BRL #15240-013, 100 ml bottles). This is 100x for use in PPYS. Can be used at 5x to cure a mold infection in *Tetrahymena* cultures.

Note: medium: 2X for growth +P/S/F 1X for stock tube (strain storage) (no P/S/F)

Tetrahymena Culture

Stock tubes

Tetrahymena can be obtained through Ward's Biology and Lab Supplies, Connecticut Valley Biological, and other biological supply companies. The strain we use is *Tetrahymena Thermophila*.

1. Maintain *Tetrahymena* in 10 ml stock tubes of 1X PPYS (no P/S/F).

2. About 2x monthly take 0.2-0.5 ml from top of stock tube (healthiest cells) and transfer to ~5 ml 1X PPYS. Store at room temperature loosely capped for aeration.

Growth for Harvest

1. Inoculate 0.5-1 ml stock into 50 ml.

2. Grow at 30 C O/N shaking at about 100 rpm (if no shaker is available, aerate/bubble at room temperature) for about 24 h.

3. To inoculate a 1 liter culture, prepare a 50 ml culture ahead of time (about 15-25 ml is enough to inoculate a 1 liter culture). Incubate as for 50 ml culture.

Harvest

1. Prepare egg H_2O (0.3 g Instant Ocean or similar salts/ 1000 ml ddH_2O)

2. - For small cultures, spin cells in 50 ml conical tubes, 5-10 minutes, 20 C at 3000 rpm clinical centrifuge

- For large cultures, spin cells in 500 ml tubes, 10 minutes, 20 C at 5000 rpm gsA rotor.

3. Resuspend pellet in egg H_2O and spin again

4. Resuspend pellet in original volume in egg H_2O

Note that cells swim after spinning, so supernatant must be poured off immediately.

Storage and Use

We dilute 1 liter of *Tetrahymena* into 2 liters of egg H_2O since they seem to keep better when less concentrated. Storage at room temperature also prolongs their life. (Live *Tetrahymena* swim in the water column, while the dead ones fall to the bottom). We generally add 10 ml of *Tetrahymena* per "100 ml" of babies.

ZEBRAFISH DEVELOPMENT IN SPACE

By E.M. Goolish, NASA Ames Research Center, P.O. Box 138, Moffett Field, CA 94035; (415) 604-1961/(415) 604-1701, ED_GOOLISH@QMGATE.ARC.NASA.GOV

Are you studying the developmental biology of zebrafish, or some other aquatic model?

What are the effects of gravity on the particular aspect of their developmental biology which you are studying?

Nasa is developing facilities to maintain aquatic organisms, including zebrafish, on the space shuttle and/or space station to conduct research on the effects of microgravity on the developmental biology of vertebrates.

It is anticipated that short-term (hours to days) thru long-term, multi-generational (>6 months) studies will be possible.

This ambitious effort, a part of the gravitational biology facility project, is soliciting input from members of the scientific community which may be users of the facility once it is operational. Suggestions on the kinds of experiments which would make best use of the facility will be used to define what the system will look like and what capabilities it will have.

* What stages of zebrafish development are most important to you?
* By what mechanisms do you think gravity affects development?
* Can you obtain information from multi-generational studies?
* How do your samples need to be fixed?
* Would other species be better to address your experiemntal objectives?

If you are interested in giving your opinions on these and other issues, please respond to the e-mail address below and include your name, mail address, and telephone/fax numbers; or call me at (415) 604-1961.

I will send you more information about the gravitational biology facility project (gbf), and a short form for outlining particular experiments which you would like to see done in space on the gbf. These reference experiemnts will be used to help design the aquatic habitat.

If you use medaka as a model system, we would like to hear from you too!

We are defining developmental biology very broadly, so if you have creative thoughts on how the aquatic habitat could be used to study other aspects of gravitational biology among aquatic organisms, such as orientation/vestibular or cardiovascular function, please respond with those ideas as well.

ZEBRAFISH WEB SERVER (SOUTH CAROLINA) CHANGES

By R. Vogt, Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA (VOGT@ZEBRA.SC.EDU)

The Zebrafish Web Server in South Carolina has a new name. The University changed the domain name from “scarolina” to “sc”. While this change is supposed to be transitional (2 years), please note the change where appropriate. The new address for this server is:

http://zebra.sc.edu

Also, I relocated the server from a PC to a Sparc Station. File name extensions have similarly changed from “htm” to “html”. If any of you have pointers set to a specific file, please note this change.

Also, please note that I archive many points of methods from this NewsGroup on the server under “Zebrafish Resources” in “Submitted Methodology” (http://zebra.sc.edu/methods/miscmeth.html).

CHROMOSOME COMPLEMENT, C-, AG-NOR AND REPLICATION BANDING IN THE ZEBRAFISH *DANIO RERIO*

in press, *Chromosome Research*

By R.R. Daga[1], G. Thode[1] and A. Amores[2,] [1]Departamento de Genetica, Facultad de Ciencias, Universidad de Malaga, 29071-Malaga, SPAIN; [2]Institute of Neuroscience, University of Oregon, Eugene, OR 97403-1254, USA

The chromosome complement of *Danio rerio* was investigated by Giemsa staining, C-banding (for constitutive heterochromatin), Ag-NOR (silver staining of Nucleolus Organizer Regions), and replication banding (BrdU incorporation in late replicating regions). Zebrafish chromosomes were obtained by the *in vivo* technique from adult fish. The diploid number of this species is 2n=50 and the NF=70 (NF= number of chromosome arms). Chromosomes were ordered approximately according to length. Given the similarity in size and morphology of most zebrafish chromosomes, only three chromosome pairs were unequivocally distinguished after Giemsa staining: the two metacentric pairs (6 and 11) and pair 1, the largest of the complement. Constitutive heterochromatin as demonstrated by C-banding was located at centromeric position of all chomosome pairs. After silver staining, Nucleolus Organizer Regions (NOR) appeared in telomeric position of the long arms of chromosomes 1, 2 and 8. Replication banding pattern allowed the identification of each chromosome pair.

FISHSCOPE

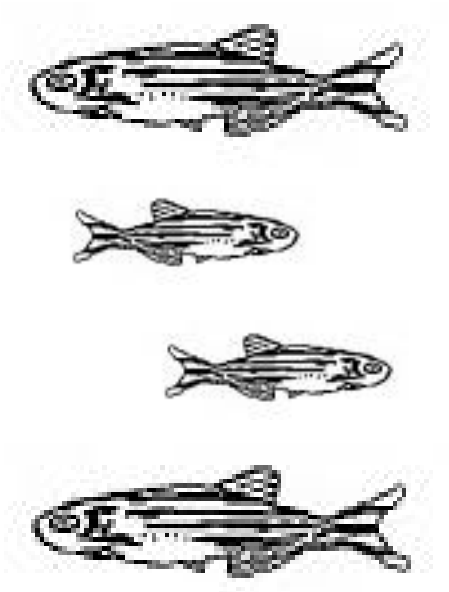
By M.S. Cooper, University of Washington, Seattle, WA 98195-1800, USA (MSCOOPER@U.WASHINGTON.EDU)

We would like to announce a new WWW archive site called FishScope. FishScope contains a set of MPEG and QuickTime time-lapse movies dealing with zebrafish development. These recordings illustrate the cellular mechanics involved in zebrafish gastrulation and neurulation. These time-lapse recordings were made using a scanning laser confocal microscope. FishScope also contains a still image gallery of confocal images of zebrafish embryos stained with fluorescent vital dyes.

The address of FishScope is: http://weber.u.washington.edu/~fishscop

Additional time-lapse recordings of zebrafish and medaka embryos will be added in the near future.

We welcome any feedback and comments about this site.



ZEBRAFISH YAC LIBRARY?

By D.A. Powers, Director, Hopkins Marine Station, Stanford University, Pacific Grove, California 93950

I have contacted a company who may be willing to make a zebrafish YAC library, but they want to obtain some feeling for the level of interest in the zebrafish community. While a number of my colleagues have encouraged me to pursue the creation of YAC library, I have not done an extensive survey of the zebrafish scientific community. If you are interested in having a zebrafish YAC library available for your current or future research, I would greatly appreciate it if you would send me a brief note or e-mail message stating that you are interested in it. This same company is also interested in making cDNA and genomic libraries from puffer fish DNA. If you are interested in any of these libraries, I would be happy to forward the company's name and address to you once they have built the libraries.

You may send your response to:

Dennis A. Powers, Director
Hopkins Marine Station
Stanford University
Pacific Grove, California 93950
(408) 655-6214
FAX: (408) 373-1254
FH.DAP@FORSYTHE.STANFORD.EDU

While they will probably proceed with the puffer fish libraries within a few weeks, they haven't decided about the YAC library and I would greatly appreciate your support in convincing them that the zebrafish community is really interested. If there is any other information that you would like me to pass on to this company, I would be happy to relate whatever you send me.

Address and Publication updates are not included in this version