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The Effects of Temperature on the Dispersion and Reaggregation Stages of Development
in the Annual Killifish, *Austrofundulus limnaeus*

by
Timothy Grant Cleaver

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

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Abstract

The dispersion and reaggregation stages have been described as a unique feature of embryonic development in annual killifish such as *Austrofundulus limnaeus*, a killifish that inhabits ephemeral ponds in the Maracaibo basin of Venezuela. These stages have previously been described as an atypical developmental progression in which blastomeres completely disperse on the surface of the yolk and then reaggregate into a mass of cells to form the embryo. Temperature affects the onset as well as the duration of this stage in related annual fishes. We have undertaken this study to show in detail the behavior of blastomere cells and their distributions as a function of developmental temperature. Embryos incubated at either 25 or 30°C were fixed and stained with Hoescht dye to allow visualization and quantification of cell number during the dispersion and reaggregation phases of development. The location of every cell nucleus on the embryo was assessed through photomicroscopy using inverted epifluorescent microscopy. This analysis revealed that the rate of cell division during the process of dispersion/reaggregation has a typical sensitivity to temperature with Q_{10} values of about 2-3. There is no indication that the pattern of blastomere movement and distribution is different in embryos incubated at 25°C versus 30°C. The primary developmental difference was observed as a temporary plateau in blastomere counts at 25°C followed by great variation of blastomere numbers in subsequent timepoints compared to the degree of variation observed in embryos incubated at 30°C. This trend fits the model that embryos developing at 25°C enter into a brief diapause-like event at the dispersion stage from which they emerge at a variable rate. Of great interest, at both temperatures examined, the majority of the dispersed

blastomeres do not reaggregate and contribute to the formation of the primary embryonic axis. Prior studies have overemphasized blastomere reaggregation in *A. limnaeus* due to the limitations of the sampling methods used as well as overdependence upon a statistical test, the coefficient of dispersion. Thus, the present study sheds light on these early mischaracterizations of *A. limnaeus* development.

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Introduction

For the annual killifish, *Austrofundulus limnaeus*, survival of harsh environmental conditions is mediated by several developmental adaptations that diverge markedly from developmental patterns observed in most teleost fishes. One such adaptation that has been previously described in the lifecycle of *A. limnaeus* is the dispersion and reaggregation stage of embryonic development. During this stage, blastomeres disperse completely over the yolk surface to become randomly distributed at the completion of epiboly, the stage at which the yolk is fully encompassed by embryonic cells, and prior to formation of an embryonic axis (Wourms 1972b). The dispersion and reaggregation stages have also been described as the first of three possible diapause events that may occur during the embryonic development of *A. limnaeus* (Wourms 1972a,c). Recent observations in our laboratory illustrate that increasing the incubation temperature of embryos of *A. limnaeus* from 25°C to 30°C dramatically increases the rate of development and induces the embryos to bypass diapause II and follow a different developmental trajectory (Podrabsky et al., 2010). These embryos were termed “escape” embryos by Wourms because they escape dormancy in diapause II (Wourms, 1972c). The stages of development that are most affected by temperature remain unknown. The focus of this thesis is to investigate the effect of temperature on the rate and nature of the cell movements associated with the dispersion and reaggregation stages of development in *A. limnaeus*.

The Life History of Annual Killifish

Annual killifishes inhabit temporary ponds that fill seasonally but do not persist

throughout the dry season (Myers 1952). The annual killifish *A. limnaeus* inhabits such ponds in the northern regions of South America, primarily in the Maracaibo basin of Venezuela (Podrabsky et al. 1998). The embryos of *A. limnaeus*, which are deposited in sediments during spawning, persist throughout the dry season by utilizing two distinct strategies to resist desiccation and to conserve energy. The egg envelope of the embryo consists of protein fibrils that provide the embryos with some resistance to desiccation (Podrabsky et al. 2001). In order to survive encased in sediment throughout the dry season, the embryos enter into various states of diapause in which metabolism is highly depressed (Podrabsky & Hand 1999b). Diapause is a state of dormancy associated with a decrease in metabolism and a temporary suspension of development or growth. Diapause will occur at genetically prescribed stages and may be either obligate, requiring no external stimulus, or triggered by environmental cues that often precede or indicate unfavorable conditions (Harvey 1962, Tauber & Tauber 1976). Importantly, dormancy in diapause may persist even when conditions favorable for development and growth (Harvey 1962, Tauber & Tauber 1976). Many organisms that are adapted for surviving predictable patterns of harsh conditions, such as seasonal drought, undergo diapause during set stages in development (Tauber & Tauber 1976).

Of the three possible diapause stages that occur in annual killifish (Wourms, 1972c), only the second and third stages of diapause are routinely observed in the normal development of our laboratory strain of *A. limnaeus*. Diapause II occurs midway through development just prior to the initiation of organogenesis, and is also associated with over a 90% reduction in metabolic rate, and a remarkable depression in the rate of protein synthesis. (Podrabsky & Hand 2000). Diapause III occurs as the embryos complete their

development and it is broken when the embryos receive environmental cues that hatching conditions are favorable.

The main characteristic associated with the stage of development during which diapause I may occur is a unique process of dispersion and reaggregation of the deep blastomeres prior to embryogenesis (Wourms 1972b). While metabolic depression is not associated with this stage in *A. limnaeus* as it is during diapause II and diapause III (Podrabsky & Hand 1999, Hand & Podrabsky 2000), developmental delay does occur to various degrees in other species of annual killifish (Markofsky & Matias 1977, Wourms 1972a). The entrance into a prolonged dispersion stage is facultative and as of yet a study on the bioenergetics of embryos in a state of prolonged dispersion has yet to be undertaken.

Temperature has been shown to affect the onset as well as the duration of diapause I and diapause II in an East African species of annual killifish, *Nothobranchius guentheri* (Markofsky & Matias 1977). Despite being classified in a separate family, *N. guentheri* share a strikingly similar developmental pattern to *A. limnaeus* that includes the dispersion/reaggregation phase of development and embryonic diapause. (Murphy et al. 1999). When *N. guentheri* were incubated at elevated temperatures the embryos did not enter diapause II or diapause III (Markofsky & Matias 1977). Embryos that were spawned and maintained at lower temperatures remained in diapause I longer than those that were spawned at elevated temperatures and subsequently maintained in lower temperatures. However, maintaining embryos at higher temperature after spawning negated the prolongation of diapause I that was associated with lower spawning temperatures (Markofsky & Matias 1977). Thus, temperature appears to have an acute

affect on the progression through the dispersion and reaggregation stages of development, suggesting that this stage of development may be very sensitive to changes in environmental temperature.

Cell Movements During Early Development in Teleost Fish

During the early stages of development of a typical teleost, such as *Fundulus heteroclitis* or *Danio rerio*, a zygote will undergo at least nine cell divisions to form a mound of 512 blastomeres at the animal pole of a large yolk cell (Web & Miller 2006). During subsequent cleavage stages the blastomeres differentiate into three layers: the deep cells of the blastoderm, the enveloping cell layer, and the periblast or yolk syncytial layer (Trinkaus & Lyntz 1967, Web & Miller 2006). Two of these cell lines, the enveloping layer and the yolk syncytial layer, will divide and expand to eventually encompass the entire surface of the yolk (Solnica-Krezel 1995). The yolk syncytial layer (YSL), a multinucleated mass of cytoplasm that is continuous with the surface of the yolk cell, begins to extend from its point of origin at the animal pole through the fission of peripheral yolk syncytial nuclei to eventually encompass the entire inner surface of the yolk cell (Web & Miller 2006). As the YSL spreads, the cells of the enveloping layer flatten and divide and eventually encompass the entire yolk as a monolayer, which results in the formation of a narrow space between the enveloping layer and the yolk syncytial layer (Web & Miller 2006). This encompassing process is referred to as epiboly. The third population of cells, the deep blastomeres, exist between the yolk syncytial and enveloping cell layers. At the onset of epiboly, the deep cells begin to move about in seemingly random directions by extending lobopodia (Trinkaus & Lyntz 1967, Web &

Miller 2006). The deep blastomere cells begin as a centralized mass at the animal pole of the embryo. In a typical teleost, soon after the onset of epiboly the blastomeres undergo localized scattering and rearrangement (Trinkaus 1973). Observations in zebrafish, *D. rerio*, show extensive rearrangement through intercalating movements between the tiers of blastomeres during this stage (Warga & Kimmel 1990). The blastomeres soon take on a coordinated directionality in their movements and converge to form the embryonic shield (Trinkaus & Lyntz 1967, Trinkaus 1973), which is a thickening of cells that defines the dorsal side of the embryo. Interestingly, at this stage the cells that comprise the margin of the blastoderm, which has expanded to cover roughly 50% of the yolk mass (50% epiboly) begin to involute, or fold under themselves as the cell layer continues to expand through the process of epiboly; This folded margin of cells is called the germ ring. Cells that undergo involution move towards the anterior (the animal pole) through a process called convergence and extension. Cells that involute are known to form mesoderm and endoderm, while the upper layer of cells forms ectoderm (Warga & Kimmel 1990, Solnica-Krezel 1995). The process of involution continues as the margin of the blastoderm moves toward the vegetal pole until the entire yolk mass is encompassed (Web & Miller 2006). When the expanding margin of the blastoderm converges at 100% epiboly, the cell movements associated with involution and migration of the deep blastomeres has already resulted in the formation of the embryo, with all of the major axes of the body clearly defined (Web & Miller 2006).

A significant divergence from this developmental pattern occurs in the development of all annual killifishes including *A. limnaeus*. The first striking difference occurs at 50% epiboly. Instead of commencing the process of involution, the deep cells

begin to assume amoeboid characteristics and disperse over the surface of the yolk between the expanding yolk syncytial layer and enveloping cell layer (Lesseps et al. 1975, Carter 1991). At the completion of epiboly there is a somewhat evenly distributed arrangement of potentially undifferentiated blastomeres surrounding the yolk (Lesseps et al. 1975). Soon after the completion of epiboly, this spatial arrangement will become random (Wourms 1972b, Carter 1991). The length of time spent in this stage of development is variable and may be dependent upon whether environmental conditions induce diapause I (Wourms 1972b). Blastomeres continue to migrate and undergo cell division during this stage regardless of how long the stage persists. After a period of time, which is dependent upon species, environmental conditions, and subject to interindividual variation (Markofsky & Matias 1977), the blastomere distribution pattern changes from even, to random, and finally to aggregated (Lesseps et al 1975). Cell movements of blastomeres during the dispersion and reaggregation stages of annual killifish are mediated by the extension of lobopodia in an amoeboid like manner (Wourms 1972b). The direction of movement and pattern of distribution of these cells at the dispersed phase is mediated by cell-cell contact inhibition (Lesseps et al 1979). Carter and Wourms observed that blastomere movements are oriented toward a site of reaggregation in *Cynolebias sp.* following the dispersion stage (Carter & Wourms 1991). The cells migrate toward the site of aggregation, typically on the lower hemisphere of the embryo, to form the embryonic shield and then the embryonic axis. After this stage the emergence of body features follows a typical teleost pattern of embryonic development (Carter & Wourms 1991).

It is important to note that the volume of the zygote compared to the volume of

the yolk in *A. limnaeus* is much smaller than that observed in zebrafish (personal observation) and therefore the density of blastomeres as they spread over the yolk is reduced. Ordinarily, the gastrulation period ends at the completion of epiboly, however in the annual killifish it is unknown whether the primary germ layers have differentiated at this stage and there are no morphological structures present that would indicate that gastrulation has occurred.

An evolutionary advantage that may be gained through the annual killifish pattern of dispersion and reaggregation of blastomeres is the delay of a developmental phase that is sensitive to adverse environmental conditions until conditions are favorable (Wourms 1972b). This assertion is supported by the fact that the embryos of *N. guentheri* are far more apt to survive after exposure to temperature extremes if the exposure took place during diapause I or diapause II (Matias & Markofsky 1978). It is interesting that the dispersion stage of development is unique to all annual killifishes within the Order Cyprinodontiformes. All non-annual killifishes of this group develop in a more typical pattern similar to that described above for zebrafish and *Fundulus* (Wourms 1972b). The fact that this occurrence is unique to the annual killifishes could be indicative of a mechanism that is vital for embryo survival in ephemeral aquatic environments.

Preliminary observations within our laboratory have indicated that an elevation of temperature during early development has a profound effect on the rate at which embryos of *A. limnaeus* develop (unpublished data). This has prompted us to consider several possibilities for the effects of temperature on the early development of *A. limnaeus*.

These considerations have led us to form the following hypotheses:

H₀-A— Temperature has no effect on the pattern or distribution of blastomeres during the

dispersion and reaggregation stages of development in embryos of *A. limnaeus*.

H₀-B– The dispersion and reaggregation stages of development have a typical sensitivity to temperature as evidenced by Q_{10} values for the rate of cell division around 2.

H₁ – Elevated temperature causes an atypically high Q_{10} value at some or all of the stages of early *A. limnaeus* development.

H₂ – Elevated temperature causes variation in the spatial distribution of blastomeres during dispersion and reaggregation, perhaps truncating or skipping the dispersion stage altogether.

Materials and Methods

Killifish husbandry and spawning

Adult fish were raised and spawned in 20 l glass tanks and fed a diet primarily of Hikari frozen bloodworms (midge larvae of the Family Chironomidae) with occasional live red earthworms, *Eisenia fetida*. Forty-two mated pairs were allowed to spawn into trays containing a spawning medium of 500 μ m glass beads (Thomas Scientific) according to previously established methods (Podrabsky 1999).. Embryos were separated from the spawning medium by sifting the medium through a screen and were then transferred to plastic Petri dishes with a transfer pipette. Unfertilized embryos were distinguished visually and removed.

Embryos were incubated in the dark at either 25°C or 30°C in plastic Petri dishes filled with embryo medium designed to mimic the osmotic conditions of their native habitat (Podrabsky 1999). To prevent bacterial growth, a final concentration of 0.0001% of methylene blue chloride, was included in the medium during the first 4 days post-fertilization (dpf). A final concentration of 10 μ g/l of gentamycin sulfate (Sigma) was included in the medium after the embryos had reached 4 dpf. The medium was changed daily at which time any dead embryos were removed.

Sampling

The embryos were collected on a total of four separate spawning dates. All embryos from each spawning date were pooled and sampled randomly for the time-course experiments. The embryos that were used to study development at 25°C were

placed at 25°C immediately after the embryos had been separated from the spawning medium. The embryos used to study development at 30°C were incubated at 25°C for eight hours after separating them from the spawning medium and were then placed at 30°C. This was done to decrease the high mortality that occurs if they are incubated at 30°C immediately after spawning. The embryos were sampled starting at 32 hr post-fertilization and then every 24 hr. Additional samples were taken of embryos incubated at 30°C. Following the completion of epiboly, these embryos were sampled every 12 hr. Fifteen to 25 embryos were sampled and fixed at each time point at each temperature for the photomicrograph studies. This provided enough surplus embryos to replace those damaged during the dechoriation or staining procedures. Three groups of 20 embryos each from a single spawning event were frozen for each sampling time point at each temperature for the quantification of DNA.

Embryo fixation, dehydration, and storage

Embryo development was halted by fixation with BT Fix solution (4% paraformaldehyde, 0.15 M CaCl₂, 4% sucrose in 0.1 M NaPO₄, pH 7.4; Westerfield 2000). The chorion of each embryo was pierced with an insect pin while in BT Fix to allow a more rapid infusion of the fixative. Care was taken to not pierce the enveloping layer or yolk in the process. Embryos were transferred to 2 ml microcentrifuge tubes and fixed at 4°C for 48 hours. Following fixation, the embryos were removed from the tubes and the chorion of each embryo was removed with fine tip forceps. Embryos that were damaged during the process were discarded. Intact embryos were placed back in the microcentrifuge tubes with a transfer pipette and dehydrated by a series of 5 min washes

in 50%, 70%, 95%, and 100% methanol. Embryos were washed twice with 100% methanol and then stored in 100% methanol at -20° C.

Hoechst staining

Embryonic membranes were permeabilized by incubating the embryos in phosphate buffered saline (PBS) with 0.5% Tween-20 for 24 hr at room temperature. All wash steps were performed at room temperature without shaking unless otherwise noted. Following permeabilization, the embryos were washed twice in PBS for 5 min each. Hoechst 33258 was used as a nuclear stain. Embryos were incubated for 5 min at room temperature with 10 µg/ml Hoechst in PBS. Staining was followed by a 5 min wash in PBS. The embryos were then soaked in SlowFade Gold antifade (Invitrogen) for at least 45 minutes prior to being cubed in agarose.

Agarose cubes

The embryos were placed in the center of small agarose cubes to provide a stable support scaffold that enabled a series of photographs from six vantage points, one for each face of the cube. This procedure allowed for a comprehensive view of the entire surface area of each embryo. The cubes were molded from a plastic 384 well plate that had the bottom removed with a milling machine. A glass slide served as a removable bottom so that the wells would hold molten agarose. A mixture of 3 parts antifade and one part 6% low melting agarose was brought to 60°C on a heat block and then pipetted into each well. Each well was filled completely and then had one third of the hot agarose removed to create a meniscus in each well. Once the agarose had set, the meniscus

helped to center the embryo as it was placed in the well with forceps. The wells were then topped off with more agarose and allowed once more to set. The false bottom was then removed and the agarose pushed up out of the mold from the bottom. A razor blade was used to cut the agarose into cubes, each with an embryo centered in its interior.

Photomicrography

The embryos were photographed on a Leica DMIRB inverted compound microscope equipped with epifluorescence. The fluorescent light source was set at half of full intensity with the aperture diaphragm adjustment knob to reduce photobleaching. Each cubed embryo was photographed on a large coverslip. From each of the six sides of the cube, a single photograph at 40X magnification and a stack of 11 focal planes (taken at every 20 microns) at 100X magnification were taken with epifluorescence to observe the Hoechst staining. A stack of bright field Nomarski images was also taken on the last side of the embryo to be photographed. Bright field images were not obtained for every side due to the danger of increased photobleaching. The exposure times of the photographs were manually increased in small increments while photographing each embryo to compensate for photobleaching of the Hoechst stain.

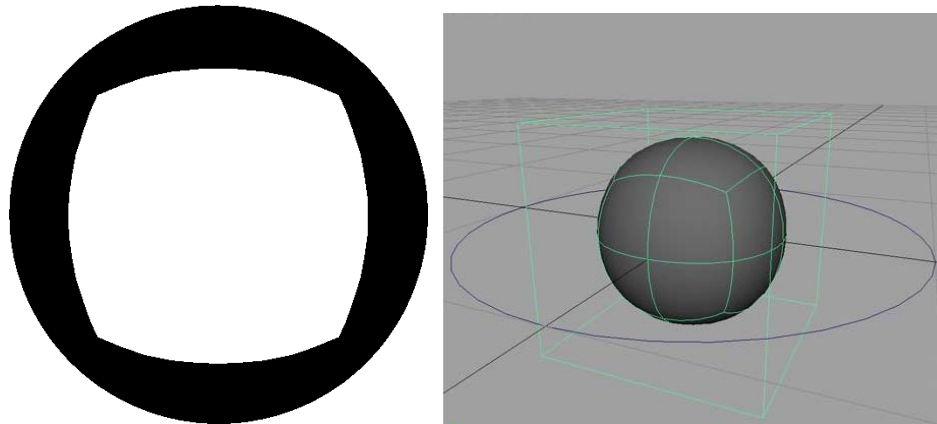
Image processing

Images were digitally processed using ImagePro Plus (Media Cybernetics, Bethesda, MD). The image series taken of each embryo that spanned eleven depths of field were combined into composite images using the Extend Depth of Field function. Two composite images were created: one using the maximum local contrast parameter

and the other using the maximum intensity parameter. The maximum intensity composite pictures proved to be of greater utility for nuclei counting. However the two images could be merged using the Image Merge function to create a more realistic representation of the embryos' nuclei for viewing purposes.

An area of interest that represents one sixth of the surface area of a cubed embryo of average size was visually centered on each embryo (refer to Figure 1). Applying this area of interest to each side of the embryo provides a complete study of an embryo with no overlap, assuming that the embryo is perfectly round and is of exactly average size. The images were then cropped, converted to grayscale, color inverted, and flattened using the Flatten Image function. The gamma was adjusted to 46 from 50 and the contrast to 61 from the original value of 50.

Figure 1: Shape of a two dimensional area of interest imposed upon photographs of spherical embryos taken from six angles within an agarose cube



An area of interest in white superimposed upon a black circular shape representing an embryo (left) and the three dimensional shapes from which the area of interest is derived (right).

Nuclei counting

The composite images from each of the six angles were printed with a laser printer and each image was bisected twice to create four quadrates per image and thus 24 quadrates per embryo. Nuclei that are associated with blastomere cells are small and uniform in size. Larger nuclei are associated with either the enveloping layer cells or the yolk syncytial layer. Distinguishing and totaling the two populations of larger nuclei was not feasible due to their visual similarity so the two populations were counted together for a single total. Blastomere nuclei, which were easily distinguished, were then counted separately.

Statistical analysis

The statistical method used to determine the relative state of dispersion of blastomeres on the surface of the yolk and thus a measure of the early developmental stages was a comparison of the standard error of the variance to mean ratio of the blastomere counts across the 24 quadrates of each embryo. This ratio is called the coefficient of dispersion and is described as $\Sigma(\bar{X} - X)^2 / (n - 1)$ where X is the number of blastomeres in a quadrate, \bar{X} is the mean number of blastomeres in a quadrate, and n is the number of quadrates (24 quadrates). Analysis of variance (ANOVA) was used for all other statistical comparisons with Tukey's and Student Newman-Keuls post hoc tests used to compare individual means.

The effect of temperature on the rate of cell division

Developmental progression was evaluated by comparing the rate of increase in cell number and DNA content during development at 25 and 30°C. A Q_{10} value was calculated by taking the time required to reach a series of developmental benchmarks at 25°C and 30°C for each batch of embryos observed. These series of Q_{10} values were then averaged individually to provide a Q_{10} value for blastomere proliferation and a Q_{10} value for DNA content increase.

DNA quantification

Three groups of 20 embryos each were sampled from a single batch of embryos for each time point at each temperature. Embryo medium was wicked from the embryos by placing them on a nylon mesh that was in contact with a Kimwipe. The mass of the

embryos was then determined prior to freezing them at -20°C where they were stored until use. Embryos were homogenized for 30 sec in 4 volumes (by mass) of TNE buffer (100mM Tris; 2.0M NaCl; 10mM EDTA; pH 7.4) with a hand held homogenizer using a 7mm generator attachment (IKA, UltraTurax). DNA content of the embryo homogenate was quantified fluorometrically. Ten microliters of homogenate were added to a glass cuvette containing 2 ml of room temperature Hoechst 33258 fluorescent dye assay medium. The contents were mixed thoroughly with a micropipette and 3 intensity readings were acquired for each sample. The DNA content of all samples was quantified on the same day using a single batch of assay medium. A standard curve defining the relationship between fluorescence signal and DNA content was generated using a serial dilution of a solution of calf thymus DNA (Sigma) of known concentration.

Results

Developmental progression

The developmental progression of the embryos through the dispersion and reaggregation stages of development was assessed by DNA quantification, a count of blastomere cells present on the surface of the yolk, and the state of dispersion of the blastomeres on the surface of the yolk. We have greater confidence in the accuracy of the methodologies we used to quantify cell number until the cellular aggregate is formed, which occurs around 200 hours post-fertilization at 25°C. The counts that we report for cell number beyond 200 hours post-fertilization are likely to be somewhat lower than the actual number due to nuclei overlap at the aggregate.

Blastomere counts

The total number of blastomeres present on the surface of the yolk of embryos incubated at 25°C increases to around 2500 by 106 hours post-fertilization (hpf) as shown in Figure 2. An additional increase in the number of cells present is not significant until 178 hpf at which the count more than doubles. The time from 106 to 178 hpf could be described as a developmental plateau. Statistical analysis supports a significant increase in the counts taken at 178 hpf from the counts taken 24 hours prior (ANOVA, $p < .003$). A similar pattern is present for embryos incubated at 30°C except the rate is accelerated and there is much less of a plateau at 2500 blastomeres. The number of nuclei increases significantly from the first sample taken at 58 hpf by the fourth sample taken at 94 hpf (ANOVA, $p < 0.019$) and continues to increase significantly again by the next sample

taken at 106 hpf.

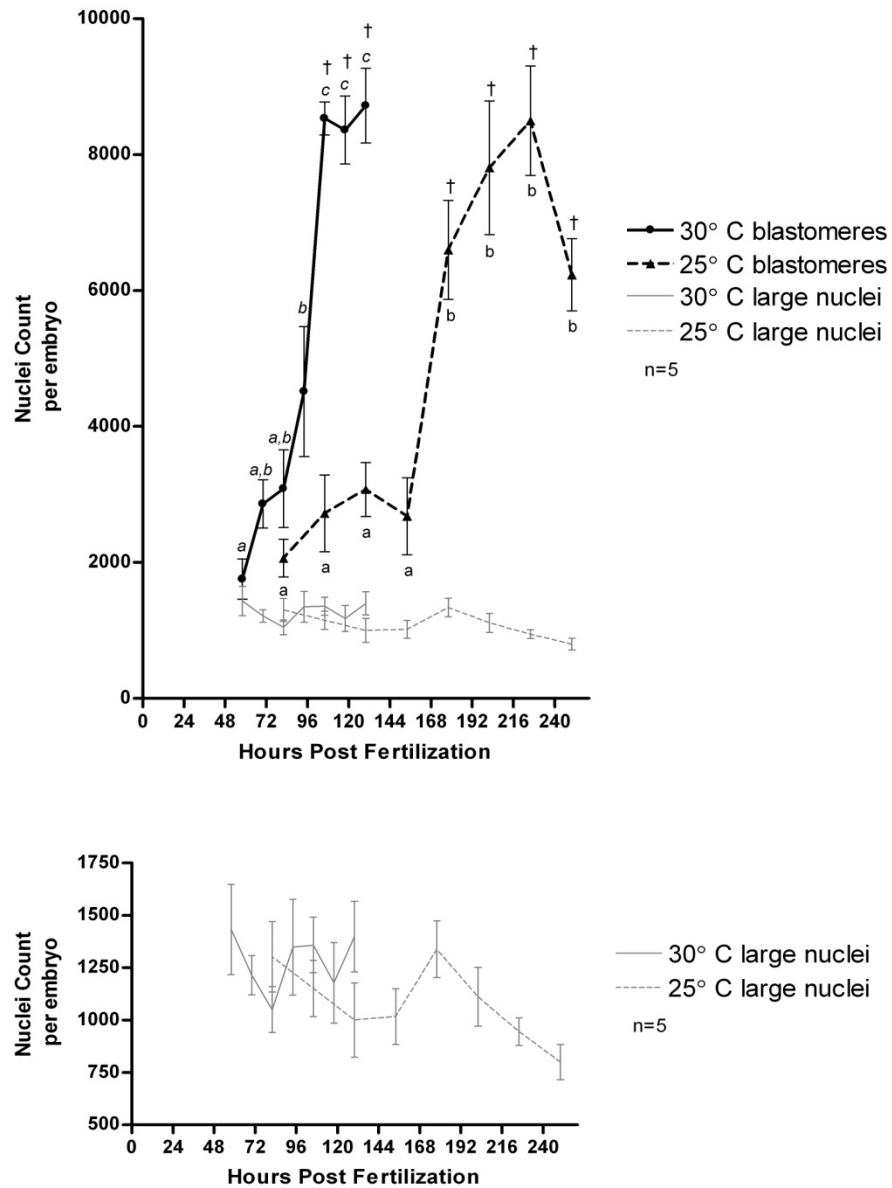
Note that our confidence in accuracy of blastomere counts is diminished after an aggregate has formed on the embryo due to the high density of cells present and the possibility that some nuclei are obscured by neighboring nuclei. By 154 hpf for the embryos incubated at 25°C an obvious aggregate had formed in one embryo of five, but not consisting of a density too high to easily count. However, by 178 hpf there were two embryos with aggregates present, a small portion of each that were so dense as to present a challenge in determining with confidence the precise number of nuclei in highly dense regions of the aggregate. We feel that the possibility for an under representation of the total nuclei count at these timepoints. Highly accurate counts of cell numbers in time point of 178 hpf or later are not possible using these methods. For the embryos incubated at 30°C, aggregates became challenging to count by 106 hpf.

Coefficient of dispersion

The coefficient of dispersion, or the variance to mean ratio, for the spatial distribution of blastomeres on the surface of the yolk divided into 24 quadrates is presented in Figure 3. The coefficient of dispersion can be used to distinguish when a spatial arrangement does not fit a random distribution pattern, whether it is evenly distributed or aggregated. A significant divergence from randomness can be tested using the formula $2 \sqrt{2n / (n-1)^2}$ where n is the number of quadrates. For 24 quadrates, the limits for randomness are $1 \pm .602$. A coefficient of dispersion less than 0.398 would be considered evenly distributed and a coefficient of dispersion greater than 1.602 would indicate aggregation. The coefficient of dispersion increases in the 25°C and 30°C

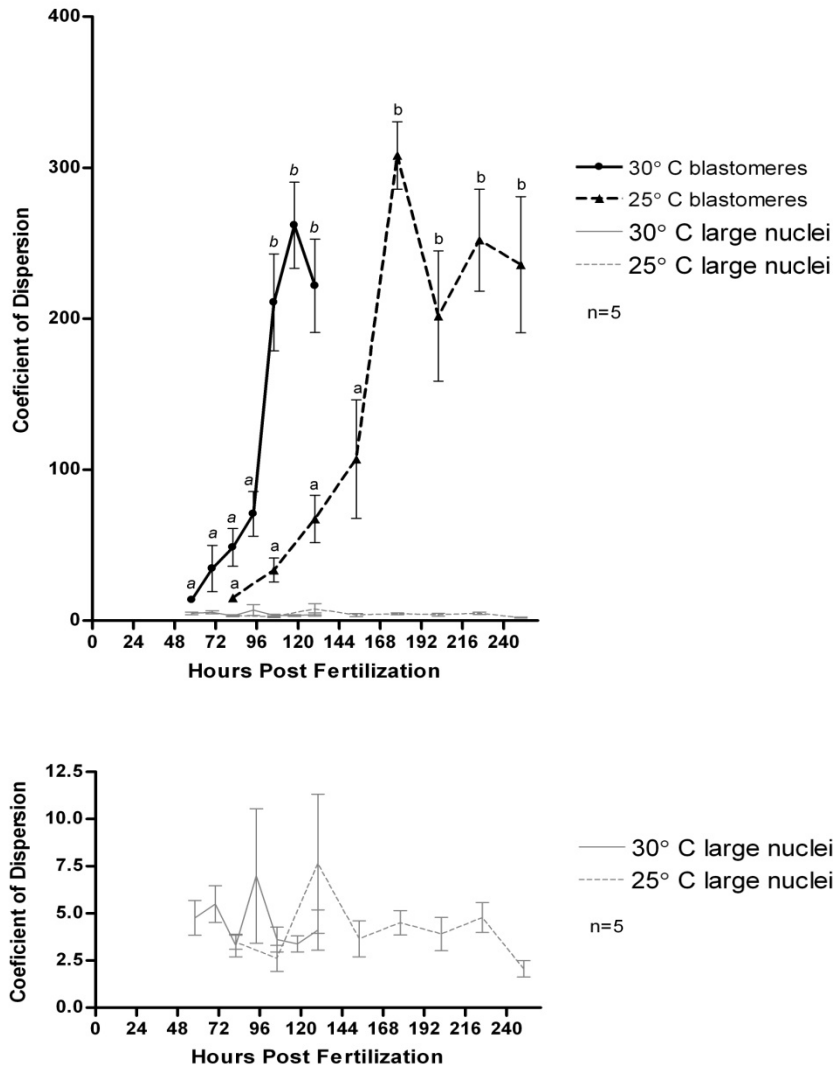
embryos in a manner similar to the blastomere count data in Figure 2. All of the data in Figure 3 exceed the upper limit for a random distribution so the distribution for every time point studied can be considered aggregated. Note that increases in the coefficient of dispersion are necessarily linked to increases in the sample number. At 25° C there is a significant increase in the degree of aggregation at 178 hpf (ANOVA, $p < 0.001$), while a significant increase is observed at 106 hpf in embryos incubated at 30° C (ANOVA, $p < 0.002$).

Figure 2: Nuclei counts at 25 ° and 30 ° C from fertilization to early embryogenesis



Blastomere counts show the mean of five embryos per sampling with error bars depicting the standard error of the mean. ANOVA statistical analysis with Student Neuman-Keuls post-hoc groupings shown with letters a, b, and c. Timepoints that do not share a letter are statistically different ($p < 0.05$). The stages that contain some aggregate areas and may contain more blastomeres than we reported due to the difficulty posed by nuclei overlap are denoted with a †. Larger nuclei belong to yolk syncytial layer and enveloping layer cells and are combined into a single count and shown at a larger (above) and smaller (below) scale.

Figure 3: Coefficient of dispersion for nuclei on the yolk surface at 25 ° and 30° C from fertilization to early embryogenesis



The coefficient of dispersion increases very dramatically over time for embryos incubated at 25 and 30°C (attributed more to increases in blastomere counts than increases in the state of aggregation.) The coefficient of dispersion shown is the mean of five embryos per sampling with error bars depicting the standard error of the mean. ANOVA statistical analysis with Student Neuman-Keuls post-hoc groupings shown with letters a and b. Timepoints that do not share a letter are statistically different ($p < 0.05$). The coefficient of dispersion for the large nuclei is comparatively small as would be expected for a more evenly distributed population of cells that is not increasing in size.

Percentages of blastomeres relative to the animal and vegetal poles of the embryos

The animal pole on the surface of each embryo was defined in this study as the combination of four adjacent, intersecting quadrates whose combined blastomere count exceeded that of any other combination of four adjacent, intersecting quadrates on that embryo. The combined area totals one sixth of the surface area of the yolk and is roughly square in shape. This method provides a method for identifying the most densely populated four adjacent quadrates of the embryo in a manner that is independent of human subjectivity as the original orientation of the embryos is random with regard to camera angle and the aggregated areas.

Figure 4 depicts the percentage of blastomeres that were located within the area defined as the animal pole, the percentage of blastomeres found within equivalent area opposite to the animal pole or the vegetal pole, and the percentage of blastomeres that were located outside of both the animal and vegetal poles for embryos incubated at 25°C. Figure 5 depicts the same information for embryos incubated at 30°C. The relative number of blastomeres present within the animal pole of the embryos incubated at 25°C increases significantly from 26.5% at 82 hpf to 42.6% at 154 hpf (ANOVA, $p < 0.022$). Similar to the data from the embryos incubated at 30°C, the increase is gradual and does not continue beyond this point but rather decreases slightly. It is possible that the decrease may be due to counting inaccuracy in the aggregated areas. The blastomere percentages between poles vary between 57.9% and 37.0% with the only significant differences being between 82 hpf at 57.9% and 178 hpf at 37.0% (ANOVA, $p < 0.014$). The numbers of blastomeres within the vegetal poles vary between 9.9% and 18.6% and does not change significantly or follow a trend during the course of this experiment.

The number of blastomeres present within the animal pole of the embryos incubated at 30°C increases significantly from 26.8% at 58 hpf to 41.0% at 106 hpf (ANOVA, $p < 0.004$). Note that the number of blastomeres in later counts within the animal pole possibly underrepresented due to overlapping blastomeres within aggregated regions. The blastomere percentages between poles vary between 57.6% and 49.0%. The overall trend is for the numbers of blastomeres between the poles to decrease slightly during the time course of the study. The numbers of blastomeres within the vegetal poles vary between 8.0% and 17.0% and do not change significantly or follow a trend during the course of development.

Examples of the photomicrographs used in this study show the blastomere nuclei and other features observed in early *A. limnaeus* development (Figures 6, 7, and 8). The nuclei shown in Figure 7 of the yolk syncytial layer and enveloping layer are not the focus of this study. The nuclei of the blastomeres are distinctly compact in comparison to the nuclei of the yolk syncytial layer and enveloping layer. The embryo in Figure 8 contains an aggregate that has regions of nuclei too dense to count with a high degree of confidence.

Quantification of total DNA content within the embryos

The DNA content of embryos incubated at 25 and 30°C are presented in Figure 9. The DNA content of the embryos incubated at 25°C increases gradually during development. The initial sample reflects a base level of non-genomic DNA, likely mitochondrial DNA found within the yolk, which totals to represent roughly half of the total DNA of the embryos sampled at 250 hpf. The increase per day on any day of

development is never statistically significant from the prior day although the overall increase is significant. The amount of variation in the samples is dramatically higher on and after 106 hpf. The degree of variation is less extensive in the embryos incubated at 30° C and there is a greater increase in total DNA content per unit of time in the embryos incubated at 30°C.

Figure 4: Percentages of blastomeres found within the animal pole (1/6th of yolk surface), vegetal pole (1/6th of yolk surface), and between poles in an embryo incubated at 25° C

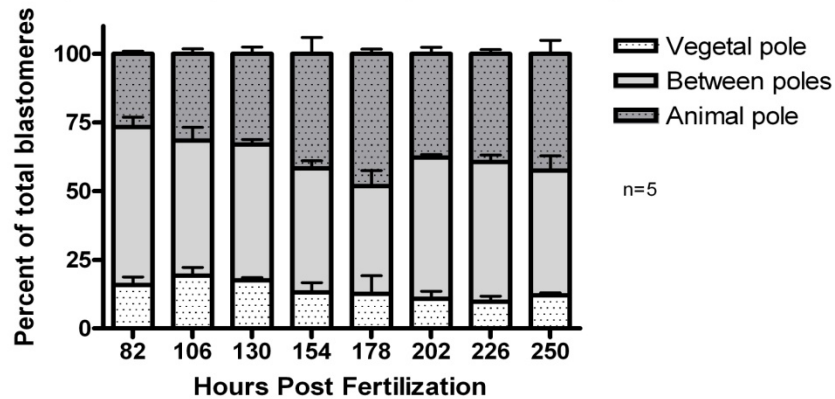
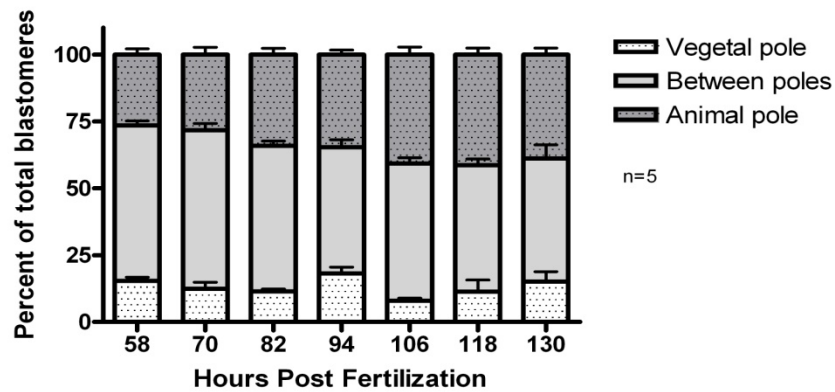
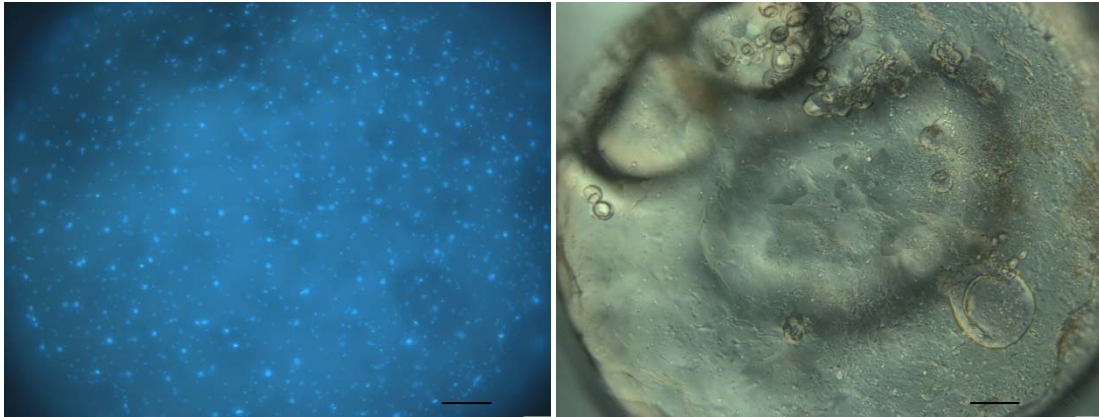


Figure 5: Percentages of blastomeres found within the animal pole (1/6th of yolk surface), vegetal pole (1/6th of yolk surface), and between poles in an embryo incubated at 30° C



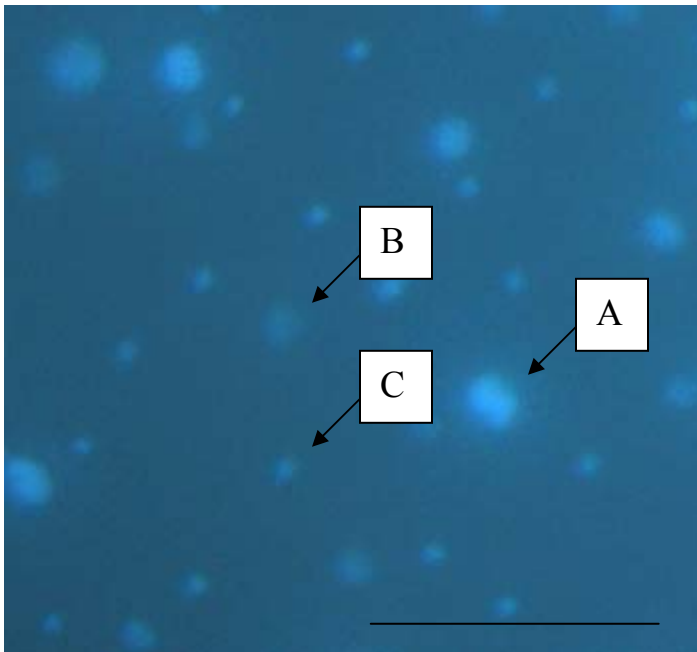
Figures 4 and 5: Percentages of blastomeres found within the animal pole (1/6th of yolk surface), vegetal pole (1/6th of yolk surface), and between poles. The actual percentages of blastomeres found relative to the most populated region of the yolk are shown with error bars depicting the standard error of the mean. The surface area of each embryo was divided into 24 equal quadrates and the 4 adjacent quadrates that contained the greatest number of blastomeres were defined as the animal pole. The opposing 4 quadrates were defined as the vegetal pole. A noticeable increase in the percentage of blastomeres found within the animal pole as development progresses to embryogenesis is clearly observed. However, contrary to the impression given by the coefficient of dispersion, the majority of cells remain outside of the quadrates containing the aggregate at all stages quantified.

Figure 6: Photomicrographs of 2.4 day post fertilization embryo incubated at 30°C



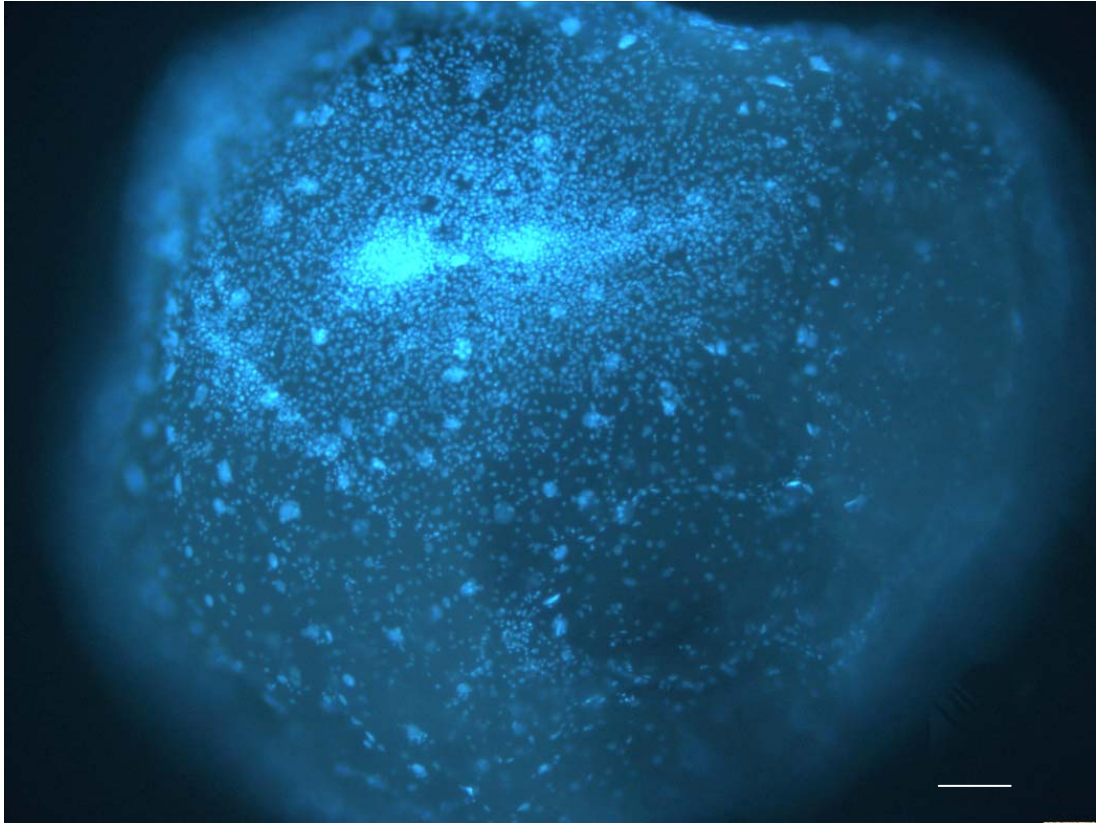
Above left: Composite image taken under epifluorescence after treatment with Hoechst 33258 to stain nuclei. Above right: Composite image of the same embryo taken with bright field microscopy. Images taken at 100x magnification. Scale bar 100 microns.

Figure 7: Nuclei of three cell types observed at the surface of the yolk.



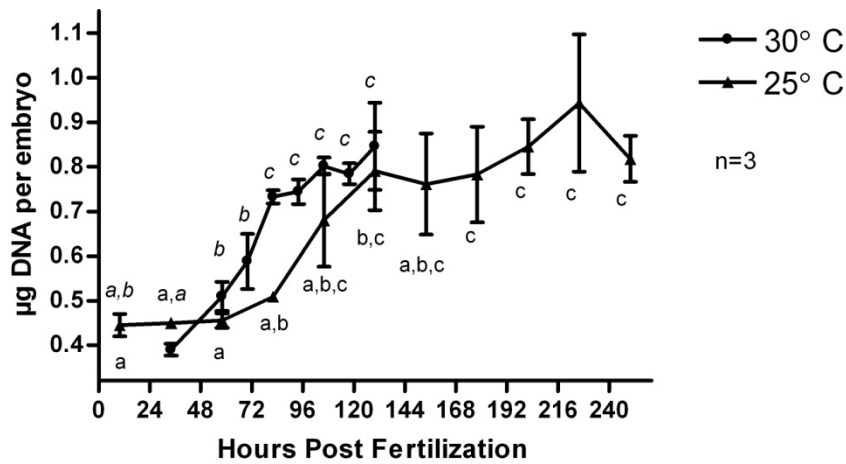
A: Yolk syncytial nuclei. B: Enveloping layer nuclei (presumably.) C: Nuclei of blastomeres, the interest of this study. Scale bar 50 microns.

Figure 8: Photomicrograph of an aggregate forming in the surface of a 5.4 day post-fertilization embryo that had been incubated at 30°C.



Composite image taken under epifluorescence after treatment with Hoechst 33258 to stain nuclei. An aggregate of blastomere cells is in view. Image was taken at 100x magnification. Scale bar 100 microns.

Figure 9: DNA content at 25° and 30° C from fertilization to early embryogenesis



DNA content per embryo increases more rapidly for embryos incubated at 30°C than for those incubated at 25°C. 20 embryos were homogenized and measured simultaneously for total DNA content. The experiment was repeated with the embryos from 3 individual spawning dates. Error bars depict the standard error of the mean. ANOVA analysis with Student Newman-Keuls post-hoc groupings shown with letters a, b, and c. Time points that do not share a letter are statistically different ($p < 0.05$).

Calculation of Q_{10} using DNA content and cell counts

The Q_{10} for *A. limnaeus* during early development was calculated using both increases in DNA content as well and increases in blastomere numbers (Table 1.) The resulting Q_{10} values apply specifically to the developmental process being measured and the two resulting values should not be considered conflicting.

Table 1

Q₁₀ for DNA quantification data and blastomere counts in *A. limnaeus*.

| | | | | | | | | | |
|--------------------|------|------|------|------|------|------|------|--------------------|---------------------|
| | | | | | | | | <u>Mean</u> | <u>S.E.M</u> |
| µg DNA per embryo: | 0.24 | 0.28 | 0.32 | 0.38 | 0.42 | | | | |
| Q ₁₀ : | 1.70 | 1.81 | 1.98 | 2.89 | 2.02 | | | 2.08 | <i>0.24</i> |
| | | | | | | | | | |
| | | | | | | | | <u>Mean</u> | <u>S.E.M</u> |
| Blastomere count: | 2000 | 3000 | 4000 | 5000 | 6000 | 7000 | 8000 | | |
| Q ₁₀ : | 1.85 | 2.34 | 3.28 | 3.06 | 3.08 | 3.35 | 4.09 | 3.01 | <i>0.36</i> |

Q₁₀ measured for the rate of blastomere proliferation as well as increases in DNA content are shown for a series of benchmarks for blastomere count and DNA content. The mean and standard error of the mean are shown in bold and bold italics respectively.

Discussion

The effect of temperature on the rate of development compared to closely related fish

A straightforward assessment of the effect of temperature on the development of *A. limnaeus* is found by evaluating overall change in developmental rate. A method used for comparison of complex biological or biochemical processes across a range of temperature is to calculate the temperature coefficient, Q_{10} (Hochachka & Somero 2002). The effect of temperature on the developmental rate of *A. limnaeus* was estimated by comparing the DNA increases and blastomere count increases between embryos incubated at 25°C and 30°C. The Q_{10} values for both processes average not less than 2 and only slightly greater than 3 (Table 1). We had theorized that if temperature had a very large effect, on the regulation of developmental delay during the dispersion/reaggregation stages of development then the resulting Q_{10} might be extremely high. However, the Q_{10} values reported here are well within the expected range for most biological processes, and are similar to those reported for development in other species of teleosts (Barrionuevo & Burggren 1999). Embryonic metabolic rates, measured in O_2 consumption have a Q_{10} ranging from 4-5 in zebrafish, *Danio rerio*, and vary somewhat throughout subsequent stages of development (Barrionuevo & Burggren 1999). However, the Q_{10} data for heart rate are relatively flat at 1.5-2.5 for all developmental stages of *Danio rerio* (Barrionuevo and Burggren 1999). In trout larvae, *Oncorhynchus mykiss*, Q_{10} values for the interval between 5-15°C was around 2.4 (Mirkovic 1998). A Q_{10} estimate of a more closely related species can be found by comparing developmental rates from fertilization to hatching of the annual fish *Cynopoecilus melanotaenia*. This

species of fish hatches after 14.5 weeks when incubated at 20°C and after 10.1 weeks when incubated at 25°C (Arenzon et al. 2002) which results in a Q_{10} of 2.88. Taken together, these data indicate that the dispersion/reaggregation stages of development in *A. limnaeus* display a typical sensitivity to temperature. Further, the Q_{10} data for *A. limnaeus* do not suggest that 25°C and 30°C are beyond a physiologically relevant temperature range. If an organism is subjected to a temperature that is outside of its thermal window then adverse effects may ensue and Q_{10} values would be rendered invalid (Hochachka & Somero 2002). For instance, when the temperature to which an organism is being exposed are in excess of its tolerated range, the rate of development may no longer increase with temperature and may eventually decrease due to temperature induced damage on the system, yielding a negative Q_{10} (Hochachka & Somero 2002).

A plateau in the 25°C blastomere counts that is not present in 30°C counts

The possibility existed for a large number of development differences between embryos incubated at 25°C and 30°C. Considering the overall increased rate at which embryos raised at 30°C develop in the lab (an overall Q_{10} for development of approximately 5), we hypothesized that the movement and proliferation of blastomeres during the dispersion and reaggregation stages may have been drastically affected by temperature. Specifically, we reasoned that dispersion of the blastomeres would not occur to the same extent or even at all in embryos developing at elevated temperatures, perhaps because there may not be enough time for a complete dispersion event in an embryo that develops at such a rapid rate. We can conclude from our data that the overall increased rate of development observed is not due to a highly temperature sensitive

dispersion and reaggregation stage of development, and that the dispersion phase of development is not skipped at elevated temperatures. The distribution of blastomeres on the yolk appears to be very similar under the two experimental conditions.

It can be seen in Figure 2 that there is a definite plateau in the blastomere count between 106 hpf and 154 hpf in the embryos incubated at 25°C. The plateau is followed by a dramatic increase in the blastomere count at 178 hpf. This plateau is not nearly as prominent in the DNA quantification data in presented in Figure 9. It would be expected that the pattern in cell counts would mirror the DNA quantification data since there is a direct relationship between increases in nuclei number and increases in genomic DNA content. However, the DNA measurements increase steadily, unlike the nuclei counts that increase at distinct time points. Although this scenario would be unexpected, one could speculate that there may be apoptosis just prior to this stage of development and that the remaining DNA material could not be degraded and cleared sufficiently to no longer be observable with our staining methods. A more plausible explanation for this discrepancy would be an arrest in the G₂ phase of the cell cycle during the time period that corresponds to the plateau in cell counts. The possibility of the synchronization of cell cycle at G₂ during a prolonged dispersion stage would be intriguing event in the development of *A. limnaeus*. The advantage of this strategy is not immediately clear. However, such an event would perfectly fit our observation of blastomere counts doubling over the course of 24 hours while DNA content was observed to increase gradually and steadily during the same time course. Additional experiments, especially a detailed analysis of the cell cycle, will likely help to clarify this interesting discrepancy between DNA content and cell number.

The plateau pattern that is seen at 25°C is not echoed in the embryos incubated at 30°C. Instead, there is a steady increase in blastomeres followed by a slightly more dramatic increase at 106 hpf. If this plateau in the development of *A. limnaeus* at 25°C is a real event and not an artifact of the sampling method, then a possible explanation for the phenomenon could be that a temperature induced diapause, however slight, may be occurring. Diapause is ordinarily not observed at this stage of development unless the embryos are subjected to environmental stress (Worms 1972a, c). The embryos observed in this study behaved at 25°C similarly in that their development was delayed slightly compared to embryos incubated at 30°C. If the recovery from this delay was somewhat asynchronous, which would be expected following a diapause event (Wourms 1972c), then the higher degree of variation that is observed after the plateau is supportive of this explanation. Note that the embryos incubated at 30°C do not have the same degree of variation at corresponding time points (Figure 2, Figure 9). This observation is consistent with the hypothesis that any developmental delay that is occurring at 25°C is occurring for variable lengths of time between embryos. Measurements of development taken after such an event would necessarily have a higher degree of variation.

The most logical confirmation for a temperature induced delay or even diapause at this stage of development would be a repeat of the study at low temperatures. An extension of the plateau at that stage, while taking into account the effect on overall developmental rate, would support this idea. If this line of reasoning is followed, a low enough temperature would induce a true diapause in which development is temporarily arrested. In the event of diapause, the synchrony of the development of the embryos would likely be lost as they tend to remain in diapause for variable durations (Wourms

1972c).

The majority of dispersed blastomeres do not reaggregate in A. limnaeus development

The methods employed in this study offer some distinct advantages over those used in prior studies of the dispersion and reaggregation stages of development in annual fishes. Never before has research been able to present a complete snapshot of every cell present on the surface of the yolk at a single point in time. All other methods used have taken observations of somewhat small regions of a yolk surface and have estimated the densities and behaviors of the remaining cell populations. For instance, a mere 3% of the yolk surface area was sampled by Wourms (1972b) in the first comprehensive characterization of dispersion and reaggregation in *A. limnaeus*. We have confirmed that the estimates of prior studies (Wourms 1972b) have projected cell count and densities with reasonable accuracy. However, an apparent disadvantage of merely calculating estimates based on sub samples of the embryo surface area is that a visual representation of the entire embryo at any given point in time could never be referenced, and thus the miscalculation that lead to an exaggeration of degree to which blastomeres reaggregate was never noticed. This study has made it clear that the general depiction of a reaggregating population of blastomeres in *A. limnaeus* has been somewhat misrepresented.

The works by Wourms (1972a, b, c) and Carter (1991) on the dispersion and reaggregation stages of annual fish have focused the depiction of the reaggregation stage on the migration of dispersed blastomeres toward the site of reaggregation. The results of this study illustrate that the majority of blastomeres that are present in an embryo at the

initiation of embryogenesis do not migrate to the site of reaggregation (Figure 4, 5). The eventual fate of these cells cannot be determined from this study. However it is clear that they are not participants in the initial formation of the embryo.

The existence of blastomeres on the surface of the yolk during and after aggregation and embryogenesis has been described once before. In Table 3 of his second 1972 paper, Wourms reports that the cell densities in non-aggregating areas increase from 8.7 on day two to 41.4 on day nine for a 25,000 μ^2 area (Wourms 1972b). This is the only mention of these cells and they are not discussed further. Instead, Wourms emphasizes the degree of aggregation that is seemingly occurring by focusing on the coefficient of dispersion at each stage of development. An overemphasis by Wourms on the coefficient of dispersion has contributed to a general misconception of the actual extent to which blastomeres reaggregate in *A. limnaeus*. The limits for determining divergence from a random distribution are dependent upon the number of quadrates sampled within the area of study. Wourms used this method successfully to determine the point at which the population of cells on the yolk surface had deviated from a randomly distributed arrangement. The confidence limits for his methods were based on 10 quadrates sampled and he was thus able to determine that there was aggregation occurring by 4 days post-fertilization. The wide confidence limit made it impossible to determine whether the cells were evenly distributed, although subsequent studies have demonstrated that blastomeres are evenly distributed at the completion of epiboly (Lesseps et al. 1975). Wourms errs by repeatedly focusing on how rapidly the coefficient of dispersion increases even after the limits for non-randomness had been achieved. In fact, the coefficient of dispersion does become very large at an accelerating rate.

Unfortunately, the rapid increase in the coefficient of dispersion is used to imply that the blastomeres are becoming highly aggregated and he then shifted the focus to cell migration rates. In fact, very little cell migration would be required to achieve the observed coefficients of dispersion. The coefficient of dispersion will double with each doubling of a population that remains stationary and does not migrate between quadrates at all. The rapidly increasing coefficient of dispersion that Wourms emphasized at the expense of minimalizing the population of cells outside of the aggregate was due largely to similarly increasing numbers of cells observed in each quadrate, a product of cell division and not cell migration. In fact, the increase in coefficient of dispersion from 3.325 to 12.460 over the span of day 5 and day 6 could have been achieved with almost no cell movement. If all cells in all quadrates from day 5 had divided at equal rates and had remained stationary then a coefficient of dispersion of 11.407 would have resulted on day 6.

A second assertion by Wourms (1972b) and again by Carter and Wourms (1991) which leads to misconceptions concerning the extent to which blastomeres join the aggregate is that the development of the fishes of the Genus *Cynolebias* follows the same general pattern as that of *Austrofundulus*. The only major distinctions between the development of the two species that are noted in these articles are the number and size of the blastomere population at the completion of epiboly. At the completion of epiboly there are approximately 200 blastomeres in *Cynolebias* compared to around 2000 blastomeres in *Austrofundulus*. The blastomeres of *Cynolebias* are much larger and, being fewer in number, are much easier to observe. There are two possibilities for the lack of reported data on extra-aggregate blastomeres in *Cynolebias* development. The

first and most likely possibility is that they do not occur to any appreciable extent. The second possibility is that they do occur and were not observed. The latter is unlikely due to the ease with which blastomeres of *Cynolebias* can be viewed (Carter & Wourms 1991). From these photographs, the impression is given that if there were more blastomeres present outside of the aggregate at the time of embryogenesis than in the aggregate itself that they would surely be noticed and reported.

Carter and Wourms (1991) painstakingly measured and recorded the blastomere directionalities and velocities during the dispersion and reaggregation stages of *Cynolebias* development. During the later stages of *Cynolebias* reaggregation the blastomeres move at a mean velocity of $1.31 \pm 0.31 \mu\text{m}/\text{min}$ in the direction of the aggregate with very few moving in a direction away from the aggregate (Carter and Wourms 1991). It is easily interpreted from these data that the majority of *Cynolebias* blastomeres join the aggregate. If even the most distant blastomere traveled with this directionality and speed it would easily reach the aggregate in less than two days.

The conclusion based on our data and that reported by Carter and Wourms (1991) on *Cynolebias* is that the assertion that these two species develop in the same manner for this stage of development cannot be accurate. The majority of dispersed blastomeres never reach the aggregate in time to participate in embryogenesis during *A. limnaeus* development. That this has been overlooked until now can be easily explained. The small size of the blastomeres of *A. limnaeus* at the time of reaggregation makes them virtually invisible at lower magnifications using light microscopy. The original observations by Wourms (1972b) were made under 630X magnification that provided a field of view of only 0.3% of the total surface of the yolk at one time. Our methods,

which utilize epifluorescence to clearly illuminate each nuclei as well as image processing of many photomicrographs to form a composite image from multiple depths of field, yield a visual representation of the blastomere locations for nearly an entire hemisphere at a time. Prior technologies have detected the presence of this population of extra-aggregate blastomeres. However, the degree to which they exist, outnumbering the blastomeres that form the aggregate, has not been appreciated prior to our observations.

Implications of the extra-aggregate blastomeres

The percentages of blastomeres that are present on the yolk in regions outside of the area of aggregation remain surprisingly steady from epiboly to embryogenesis (Figure 4, Figure 5). If complete reaggregation were to occur, the percentage of blastomeres during the same stages would be from ~83% at epiboly to 0% at embryogenesis. Instead, we observe 73% (blastomeres are not evenly distributed at the time of the first measurement) just after the completion of epiboly to ~60% at embryogenesis. The extent to which the blastomeres remain outside of an aggregate exceed these percentages considering that there are many extra-aggregate blastomeres present in the quadrates containing the aggregates. The blastomere counts in these regions were totaled along with the counts within the aggregates because of the difficulty in determining a definable edge to an aggregating mass of cells.

The implications of a developmental pattern in which the majority of blastomeres do not participate in embryogenesis are unclear. There are no apparent differences in the distribution of cells at equivalent developmental stages in embryos developing at 25°C or 30°C. Wourms hypothesized that the dispersion stage allows the embryos to avoid the

effects of harmful environmental agents during a sensitive stage in development by postponing embryogenesis until more favorable conditions are present (Wourms 1972b). This is a possibility that remains regardless of how many blastomeres participate in embryogenesis. The questions that arise are which blastomeres join the aggregate and what becomes of those that do not. We do know from the observations of prior researchers that the blastomeres of *A. limnaeus* are motile. What is now unclear is whether they all remain motile throughout the entire dispersion and reaggregation stages or if motility ceases for some or all of the blastomeres. The distribution of cells that we observe could be maintained with either highly motile or almost entirely sessile cells. Cell divisions could account for the presence of the aggregate once an initial imbalance of cells has been established and virtually no movement of blastomeres would be required. The aggregate could also be established through the coordination of a highly motile population of cells. Random directional movements could maintain the relatively stable densities observed elsewhere on the yolk. These possibilities cannot be assessed by examining our data and require further studies on living embryos.

A more important mystery is the eventual fate of the blastomeres that failed to merge with the aggregate. This population of cells increased throughout the stages of dispersion and reaggregation. We have no data to indicate that these cells ceased to divide after the embryo had formed. The question that begs to be answered is what, if anything, do these cells ultimately become. In vertebrates such as mice and humans, the yolk sac is the site at which hematopoietic progenitor cells originate (Palis & Yoder 2001). However, it would be unlikely to expect the same to occur in *A. limnaeus* since extraembryonic hematopoiesis does not occur in teleosts (Detrich et al. 1995). In fact,

hematopoiesis occurs in a dorsal region of the tail bud in most bony fishes (Detrich et al. 1995, Kozlowski et al. 1997). The eventual fate of the extraembryonic cells in *A. limnaeus* remains a mystery. It would seem improbable that such a large population of cells would undergo apoptosis or be otherwise discarded. It would be far more reasonable to expect that these cells would ultimately differentiate into a cell type that is eventually absorbed into the abdominal wall as yolk supplies are exhausted. These ultimate cell fates are of special developmental significance considering their position in a classical development context in which cell and tissue types are derived from a primary germ layer that is established during gastrulation.

Summary

The overall change in the rate of development from fertilization to embryogenesis in *A. limnaeus* embryos that have been incubated at 25°C and at 30°C would not be considered unusual when compared the effects of temperature on developmental rate in other teleost fishes. There is no indication that the pattern of blastomere movement and distribution is different between these two experimental groups and thus the hypothesis that there is an incomplete dispersion stage in embryos incubated at 30°C can be rejected. The primary developmental difference observed was the temporary plateau in development at 25°C that corresponds with the dispersed stage and is followed by increased variation for developmental progression in subsequent stages. This was not observed in embryos incubated at 30°C and this trend is consistent with the hypothesis that embryos developing at 25°C enter into a brief diapause like event at the dispersion stage from which they emerge at a variable rate.

Prior studies have overemphasized blastomere reaggregation in *A. limnaeus* due to the limitations of sampling methods. It is clear from data presented in this study that the majority of blastomeres present on the yolk surface do not contribute to the aggregated mass at embryogenesis. Whether it is cell divisions or the movements of the blastomeres toward a site of aggregation that make the greater contribution to the eventual cell aggregate that forms the embryo cannot be extrapolated from our data alone.

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