TIMING AND 3-DIMENSIONAL ANALYSIS OF MUSCOLOSKELETAL DEVELOPMENT

C-RUI 9510228

PROGRESS REPORT - YEAR 3
(September 15, 1997—September 15, 1998)
NSF Report for 1997-98

During the year we continue processing specimens for mathematical analyses of the shape of selected chondrocranial cartilages as well as for analysis of developmental patterns of muscle formation.

The following students were supported under this grant throughout this period of the grant: Mr. Matthew Smatenick (Senior), Mr. Jon-Eric Burgess (junior), Mr. Joseph Oppong (sophomore), Mr. James Tripp (freshman), Mr. Stephen Spear (freshman), Mr. Michael Franchella (junior), Mr. Charles Swart (graduate student), and Mr. Will Turner (graduate student).

Below is a report of the progress made during the third year summarized under three major sections: 1) Analyses of Shape, 2) Patterns of Muscle Development, and 3) Presentations and Publications.

We requested a No Cost Extension of this grant to August 31, 1999. Copy of request is attached at the end of this report.

Analyses of Shape

A. Mathematical Analyses

Following the Imaging Workshop held in September of 1997, the Dr. Kerckhove and his students pursued ideas presented to us by Stephen Pizer, Kenan Professor in the Department of Computer Science at the University of North Carolina - Chapel Hill.

We are still primarily interested in producing a representation for the shapes of chondrocranial cartilages based on the medial axis transform. In order to overcome the noisy image boundaries that have resulted from the sectioning and realignment process by which tadpole specimens have been treated, we pursue three separate approaches.

Approach 1.— During the Fall of 1997, we worked to develop a code that will produce a stick figure model for the shape of the a given cartilge, e.g., we used the muscular process, a laterally projecting cartilage in the chondrocrania of most tapdoles, as a model. These models are based on a sampling of the inner radius of the figure at a rate proportional to that inner radius. The procedure is described in Pizer, et. al., 1998. Once developed, this program worked well in only a few cases; the variability in the radius data caused most of the resulting stick figures to have boundaries with several points of self-intersection. While it did not help us to overcome our registration problems, this work should prove useful in the analysis and description of images of the muscular process taken from whole-mounted specimens. Furthermore, a protocol for obtaining such images was developed during the summer of 1998.

Approach 2.— The spring semester of 1998 was dedicated to implement a second idea from Dr. Pizer's research group. Here, the medial locus of a figure is extracted from the image by computing a medialness value at each point. Points along a ridge of ``maximal medialness'' are then declared to constitute the medial axis of the figure. The process of computing medialness values involves a convolution integral, which has the effect of smoothing noisy boundaries. Algorithms for extracting the medial locus are

described in the manuscript cited above. A key concept is the ``optimal scale'' at which medialness is to be measured.  The collection of optimal scales forms a surface in ``scale space''. Students worked in developing an algorithm for locating ridges of medialness on the optimal scale surface by using a pullback metric. This constitutes a mathematical advance, since ridges can be located in 2 dimensions directly rather than being projected from scale space; although, it remains to be seen whether this clearer mathematical formulation of the problem will result in a faster algorithm for ridge extraction. A student, James Tripp, is currently working to write up his results in the form of a technical report.

Approach 3.— During the summer of 1998, we worked to apply a shape metric based on distances between circle-preserving transformations of the plane to images of the projected muscular process that were captured by the students. This metric, described further in the next paragraph, can be used to quantify local shape difference along corresponding branches of the medial locus and (by localizing shape difference) provides a useful supplement to traditional shape indices. In the Mathematica programs we've written (manuscript in preparation), the user must assist in partitioning the medial locus for the figure into separate branches and in identifying corresponding branches for comparison. To date, students have processed 8 specimens in this way and their results were presented at the Toronto MathFest (see below). In 1998-99, we plan to expand the number of specimens treated in this way to obtain a more comprehensive collection of data relating muscular process shape to current hypotheses of evolutionary relationships among species.

B. Three-Dimensional Reconstructions from serial sections

One of the goals of this project is to standardize a protocol for measuring chondrocrania shape using computers to reconstruct a 3D image from serial sections. We wanted the protocol to be (1) simple and robust enough to be performed for undergraduates with relatively little training, (2) to use computers and software that are inexpensive and widely available, and (3) provide comparative data accurate enough to be useful in constructing or interpreting phylogenies.

We have partially met these goals. We have a standard protocol that our undergraduates use for generating serial sections and reconstructing 3D images using Macintosh PowerPC computers. Briefly, tadpoles are fixed in one of several related formaldehyde based fixatives, dehydrated, embedded in paraffin wax, and serial sectioned at 10 μm. All serial sections are oriented transverse to the neural axis and begin at the anterior tip of the head. Sections are collected in sequence as they are cut. To reduce the possibility that sections will be lost during staining the slides are silanized. Sections are then stained in a modified Milligan's Trichrome stain, which stains cartilage blue-green and muscle deep red.

Images of stained sections are captured using a stereomicroscope and single chip RGB camera. Section images are aligned by hand and captured in sequence as grayscale TIFF files. These original TIFF files are then copied and the originals are archived. The duplicates are used for creating reconstructions.

Chondrocrania are reconstructed by outlining with a mouse all of the cartilage elements in a particular section. Though tedious, manual tracing is superior to automated procedures because automated segmentation and edge detection algorithms are not as good as the human eye. After outlining, the outline pixels are set to black and the rest of the image pixels are set to white leaving an image that is saved as a binary file. Binary files take less storage space yet contain enough information for our use, since we are interested only in surfaces.

The sequence of binary outlines is used to create the reconstructions, either by projection (NIH Image) through a stack of such images or by interpolation of a surface (SurfDriver). An example of each is shown in Fig. 1.

Fig.1 Reconstruction of the muscularis process from the right palatoquadrate of *Ptychohyla salvadorensis*. Both reconstructions were created from the same set of 169 serial sections. Sections were made transverse to the anterior/posterior axis. The muscularis process is about 2 mm tall at this stage of development. The view is from inside of the chondrocranium from posterior to anterior, with dorsal facing up. The ventral margin of the muscularis process, seen at the lower left edge of the reconstruction, is normally continuous with the rest of the palatoquadrate but was cut off during image capture, leaving a ragged edge. A. Reconstruction by projection of all 169 section using NIH Image. The individual section outlines are close enough together that the image appears nearly solid. B. Reconstruction using SURFdriver showing a view similar to that seen in A, but with a surface interpolated over the section outlines. The surface reconstruction was digitally smoothened to reduce rough edges. QuickTimemovies showing these two views in animated rotation can be found at http://www.science.richmond.edu/~biology/rdesa.www/grant.html.

Limitations of 3D reconstruction from serial sections.—While attractive in concept, there are four practical problems that limit our ability to create accurate reconstructions from serial sections.
Specimen distortion before sectioning. Tadpole tissues are mostly water and shrink during dehydration and embedding, even after thorough formaldehyde fixation. The amount of shrinkage varies but is usually about 10 to 15%.
Specimen distortion during and after sectioning. Sections are compressed as they pass over the blade during sectioning. Thus unlike shrinkage, compression occurs in one direction so that specimens are flattened in the direction of sectioning. We found that

3

the amount of compression can be as much as 50% depending on the rigidity of the wax.

Specimen alignment. Individual sections must be aligned in the reconstruction in the same position they occupied before sectioning, without rotation or translation. Most workers recommend registering sections using external registration or fiducial markers co-embedded with the specimen. We tried a number of different markers but for our tissues none proved as reliable as using the brain as an internal registration marker. All of the tadpole heads were sectioned transverse to the central nervous system, which appears in all but the anterior-most sections, is bilaterally symmetrical, and changes in outline minimally from section to section.

Variation in section thickness and missing sections. Specimen dimensions in the x and y axis can be measured directly on the image, but measurement along the section axis depends on summing section thicknesses. Variation in section thickness or skipping sections thus can alter the depth of the reconstruction. We measured the accuracy and precision of our microtome by reconstructing an object of known dimensions and found section thickness to be accurate to within 5%. Accounting for skipped or missing sections relies on the record keeping ability of the person who does the sectioning. The position of a skipped or missing section is occupied by a blank "placeholder" in the reconstruction.

A more complete description of our protocol is being prepared for publication and will be submitted by December 1998.

Patterns of Muscle Development

A. Myogenic pattern in *Hymenochirus boettgeri*

Heterochronies are known to exist in amphibian myogenesis, particularly in the formation of axial muscles. A common pattern of muscle begins in the myotome with the appearance of mononucleated myotomal myoblasts, which then fuse to form elongated, multinucleated muscle cells. The muscle fiber then synthesizes myofibrils, which is followed by first twitch. Developing muscle in *Xenopus laevis* displays a remarkably early expression of myosin and actin fibers and becomes functional prior to becoming multinucleated. First twitch in *X. laevis* can be observed at about 24 hr post-fertilization, preceding the first heartbeat by about a day and the multinucleation of the myotome by about three days. The similarity in myogenic pattern between *X. laevis* and *B. variegata* may suggest that early myogenesis is an ancestral myogenic condition for anurans since these taxa represent basal lineages within Anura (Ford and Cannatella, 1993). During the past year, we studied another pipid species, *Hymenochirus boettgeri*, to determine whether the pattern of myogenesis seen in *X. laevis* is unique to that species, or represents a pattern specific to the pipid lineage.

A total of 86 specimens of *Hymenochirus boettger*i were examined and staged according to the Nieuwkoop and Faber normal table of development for *X. laevis* (NF stages, Nieuwkoop and Faber, 1956). The specimens ranged from NF 24–50. Nieuwkoop and Faber's normal table of development could not be used to stage *H. boettgeri* specimens between stages 43-45. Changes in intestinal structure are used to distinguish among these stages, but intestinal development in *H. boettgeri* differs significantly from that in *X. laevis.* The next reliable stage marker common to both species is the first appearance of hind limb buds, which identifies stage 46/47.

Determinations of first twitch (18 specimens), first heartbeat (12 specimens), and immunohistochemical staining (33 specimens) were made by using a Nikon dissecting

microscope. Observations of axial myotome multinucleation (23 specimens) were made with a Nikon Optiphot microscope.

To observe muscle cell nuclei, fixed specimens were embedded in glycol methacrylate. Axial muscles were sectioned longitudinally at 2 μm using glass knives, transferred to a slide, and stained for 10-15 sec with 0.1% toluidine blue in 1% sodium tetraborate.

Whole-mount immunohistochemical staining was performed on *Hymenochirus boettgeri* specimens from stages 24–32 to identify the initial presence of muscle protein. For comparison, immunohistochemical staining was also performed on *X. laevis* specimens from stages 17–25 using monoclonal antibody 12/101, a muscle-specific antibody that recognizes an antigen in amphibian skeletal muscle. Antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. The primary antibody was visualized using the Vectastain Universal Kit (biotin-avidin complex) and diaminobenzidine (DAB). Stained embryos were cleared with benzyl alcohol:benyzl benzoate (1:2).

Immunohistochemical staining of the *Hymenochirus boettgeri* specimens detected the initial presence of muscle protein in axial muscle at NF stage 25, approximately 24 hr post-fertilization. At this stage, only the most anterior axial myotome was visible. Muscle protein was not detected at NF stage 24, as indicated by the absence of DAB staining in the dorso-medial part of the embryo. In contrast, muscle protein was first detected in *X. laevis* at NF stage 20.

First twitch of axial muscle, stimulated by poking live specimens with a metal probe, was observed no later than NF stage 27/28, approximately 5 hr after the earliest detected presence of muscle protein. Spontaneous mid-body flexing was observed by stage 30/31. For comparison, *X. laevis* shows stimulated twitching at stage 22/23, and spontaneous flexing at stage 25.

Observation of methacrylate sections revealed the presence of mononucleated myotome cells from stage 24 to stage 42. Because of the difficulties of staging *H. boettgeri* between NF stages 43-45, it was not possible to determine whether myoblasts became multinucleated during these stages. However, the myotome fibers clearly were multinucleated by stage 46/47, which is distinguished by the first appearance of hind limb buds. First heartbeat was not observed in *H. boettgeri* until stage 36/37. It occurs at stage 33 in *X. laevis.*

It is possible that patterns of myogenesis are lineage specific within anurans. If so, then other pipids should display myogenic patterns similar to *X. laevis.* We have found that three landmarks of skeletal muscle myogenesis—muscle protein synthesis, first twitch, and multinucleation— indeed occur in the same sequence in *H. boettgeri* and *X. laevis.* Because these myogenic landmarks are relatively easy to assess, additional phylogenetic comparisons will be possible if live, early stages of additional species can be obtained.

We found that although the sequence of these myogenic events is the same in both pipid species studied, the timing of these events relative to other developmental markers is delayed in *H. boettgeri* compared with their timing in *X. laevis.* The timing of these events is summarized in Fig 2. The earliest stage at which muscle-specific antigens can be detected is NF stage 20 in *X. laevis* and stage 25 in *H. boettgeri.* Antigen expression in *H. boettgeri* is later in absolute time as well as relative developmental age since both species reach NF stage 25 at the same time—about 24 hr post-fertilization at 22° C. First stimulated twitch is also delayed in *H. boettgeri* (stage 27/28, about 29 hr) compared with *X. laevis* (stage 22, about 22 hr). First spontaneous twitch is correspondingly later, occurring as late as stage 31 in *H. boettgeri* versus stage 25 in *X. laevis.* Although relatively late, first twitch still precedes first heartbeat in *H. boettgeri,* which occurs at approximately stage 36 compared with stage 32 in <u>X.</u> <u>laevis.</u> The time and stage of multinucleation were more difficult to compare because the morphological

5

characters used to stage *X. laevis* at stage 43-45 are not present in *H. boettgeri*. Nevertheless, it is safe to conclude that time of multinucleation is not delayed in *H. boettgeri*. <u>Xenopus</u> laevis becomes multinucleated at stage 46, when hind limb buds first appear. Axial muscle is also multinucleated in *H. boettgeri* at stage 46. Thus, the early events of both skeletal and cardiac myogenesis seem to be heterochronically delayed as measured by both absolute time and developmental stage in *H. boettgeri* compared with *X. laevis*, but multinucleated fibers appear at about the same developmental stage.

Muscle development is somewhat delayed in *H. boettgeri* compared with *X. laevis*, but both species have unusually early myogenesis compared with other. One explanation proposed for early muscle function in *X. laevis* is that it is an adaptation for fast development to a free-swimming tadpole. Alternatively, the pattern may arise from historical constraints in the pipid lineage. The work we did in the past year shows that myogenesis in *H. boettgeri*, though occurring slightly later than in *X. laevis*, still occurs much earlier in development than in other anurans studied consistent with an evolutionary conservation of myogenic timing. This work has been submitted and accepted for publication in the Journal of Herpetology (see below).

Fig. 2. Five myogenic events were examined in Xenopus laevis and Hymenochirus boettgeri: 1) the initial presence of a muscle protein in axial muscle (   ), 2) first stimulated twitch of axial muscle (■), 3) first spontaneous twitch of axial muscle (✿), 4) first heartbeat (●), and 5) multinucleation (✿). These developmental events occur between Nieuwkoop and Faber (1956) stages 20—46 (NF stages). The time between stages is not linear. Early muscle development in H. boettgeri appears to be delayed at early stages. However, axial muscle multinucleation occurs at stage 46 in both species.

B. <u>Myogenic pattern in other anurans.</u>

Examining additional pipids, and other genera with faster or slower developmental rates, as well as direct and indirect developing anurans, will be necessary for a more complete understanding of the constraints on myogenic patterns. Therefore, we have continued our comparisons by examining myogenic patterns in five additional species: (1) *Xenopus tropicalis*, (2)*Eleutherodactylus coqui*, (3) *Rana sylvatica* and*Rana utricularia*, and (4)*Agalychnis calydrias*.

Xenopus tropicalis is another pipid species closely related to *Xenopus laevis* (Cannatella and de Sá, 1993), however, *X. tropicalis* is a diploid species instead of the most common polyploid condition exhibit by most *Xenopus* species. Our preliminary results indicate that, as we predicted, the timing and pattern of myogenic markers in *Xenopus tropicalis* is very similar to that seen in *X. laevis.* That is, the first appearance of 12/101 antigen, first twitch, and time of multinucleation are all more like that seen in *X. laevis* than in *H. boettgeri*.

6

Eleutherodactylus coqui is a direct developing frog (=lacks free-swimming larvae). We found that *E. coqui* has a myogenic pattern quite different from the pipids in that muscle specific protein synthesis and first twitch occur relatively later developmentally, but multinucleation occurs earlier developmentally. Overall, the pattern of myogenesis in *E. coqui* more closely resembles that seen in amniote vertebrates such as birds and mammals. These results were presented in preliminary form last year (see below).

Rana sylvatica and *Rana utricularia*. Analysis of these two "slow" and indirect developers is in progress.

Agalychnis calydra, the Red-Eyed tree frog, is a semi-direct developer that develops slowly inside an egg capsule until a relatively later tadpole stage but hatches before limbs develop. These specimens, obtained with the generous assistance of the National Aquarium in Baltimore, have been fixed at various stages and are being processed now.

C. Are there phylogenetic trends in patterns of somitogenesis?

It will be important to extend the comparison to events preceding myogenesis, including mesoderm formation and somitogenesis. Minsuk and Keller (1996) compared the cellular mechanics of mesoderm formation in *H. boettgeri* and *X. laevis* and found major differences in the origin and migration of axial and paraxial mesoderm, which includes the precursors to skeletal myoblasts. In contrast, we have found that the later sequence of myogenesis is largely the same in the two species. These observations have led us to compare patterns of cellular rearrangements during the intermediate steps of somitogenesis. It is known that in *X. laevis* the myotomal precursors are initially oriented perpendicular to the neural tube, along the medial-lateral axis. They then rotate as a block to lie parallel to the anterior-posterior axis (Malacinski et al., 1989). This morphogenetic movement has not been seen in other amphibians studied (*Rana dalmatina, Bufo bufo, Pelobates fuscus, Bombina variegata, Ambystoma mexicanum, Cynops pyrrhogaster*). It may be a morphogenetic pattern unique to pipids. To answer this question, we are examining the pattern of somitogenesis in *X. tropicalis* and *H. boettgeri*. Specimens of the appropriate stages have been fixed and will be embedded in methacrylate for frontal sectioning. They will be stained with a modified Fuelgen/PAS stain to enhance our view of the cell borders. Since somitogenesis proceeds in an anterior-posterior sequence, we expect to see all the stages of myotome cell rotation (or other movement) in the same specimen.

Literature Cited

Cannatella, D. C., and R. O. de Sá. 1993. *Xenopus laevis* as a model organism. Systematic Biology, 42:467-507.

Ford, L., and D. C. Cannatella. 1993. The major clades of frogs. Herpetological Monographs, 7:94-117.

Malacinsky, G. M., A. W. Neff, , G. Radice, and H.-M. Chung. 1989. Amphibian somite development: contrasts of morphogenetic and molecular differentiation patterns between the laboratory archetype species *Xenopus* (anuran) and Axolotl (urodele). Zoological Science 6: 1-14.

Minsuk, S. B. and R. E. Keller 1996. Dorsal mesoderm has a dual origin and
forms by a novel mechanism in *Hymenochirus*, a relative of *Xenopus*.
Devel. Biol. 174: 92-103

Neiuwkoop, P. D. and J. Faber. 1956. Normal Table of *Xenopus laevis* (Daudin).
North-Holland, Amsterdam. 252 pp.

Pizer, S. M., Eberly, D., Morse, B. S., and Fritsch, D. S. 1998. Zoom-invariant figural
shape: the mathematics of cores," Computer Vision and Image Understanding,
Vol. 69, pp.55-71.

Presentations and Publications

During the third year we presented several papers at meetings. In addition some manuscript were published and other were submitted for publication.
* indicates student author

A. Presentations:

• J. Tripp*. 1998. Stable Medialness Measures in the Presence of Boundary Noise. University of Richmond 13th Annual Undergraduate Research Symposium.

• J. Tripp*. Height Ridges and Medial Loci for Image Analysis. Math and Computer Science Departmental Colloquium, University of Richmond. September, 1998.

• Oppong*, J. M. Jr.. Quantification of Shape Difference using SL(2,R). Math and Computer Science Departmental Colloquium, University of Richmond. September, 1998.

• M. Smetanick 1998. Patterns of Musculoskeletal Development in Direct and Indirect Developing Anurans. University of Richmond 13th Annual Undergraduate Research Symposium.

• J. Tripp*. Height Ridges and Medial Loci for Image Analysis. Pi Mu Epsilon, Mathematical American Association. MathFest '98, Toronto Canada, July 1998.

• Oppong*, J. M. Jr.. Quantification of Shape Difference using SL(2,R). Pi Mu Epsilon, Mathematical American Association. MathFest '98, Toronto Canada, July 1998.

• S. F. Spear* and R. O. de Sá. Chondrocranial and internal oral anatomy of *Hyla chrysoseli*s (Anura: Hylidae). Combined meetings American Society Ichthyologists and Herpetologist, Herpetological League, and Society for the Study of Amphibians and Reptiles. Guelph, Canada. July 1998.

• M. Smetanick*, R. O. de Sá, and G. P. Radice. The timing and pattern of myogenesis in *Hymenochirus boettgeri*. Combined meetings American Society

Ichthyologists and Herpetologist, Herpetological League, and Society for the Study of Amphibians and Reptiles. Guelph, Canada. July 1998.

• C. Swart* and R. O. de Sá. Development of the suprarostral plate in pipoid frogs. Combined meetings American Society Ichthyologists and Herpetologist, Herpetological League, and Society for the Study of Amphibians and Reptiles. Guelph, Canada. July 1998.

• W. H. Turner* and R. O. de Sá. Chondrocranial morphology and skeletogenesis in three species of *Physalaemus* (Anura: Leptodactylidae). Combined meetings American Society Ichthyologists and Herpetologist, Herpetological League, and Society for the Study of Amphibians and Reptiles. Guelph, Canada. July 1998.

• R. O. de Sá. The monophyly of anuras in relation to the development of the tadpoles' rostral region. Fith Symposium, Herpetological Association of Africa, Stellembosch, South Africa, September 1998.

• Radice, G. P. 1998. Bringing research in amphibian development into the classroom (and back out again). National Assembly of Project Kaleidoscope's Faculty for the 21st century, Arlington Heights, Illinois.

B. Publications:

• R. O. de Sá and Shannon Hill*. 1998. Chondrocranial anatomy and skeletogenesis in *Dendrobates auratus* (Anura: Dendrobatidae). Journal of Herpetology 32(2):205-210.

• Swart, C*. and R. O. de Sá. 1998. The chondrocranium of the morphology Mexican Burrowing Toad *Rhinophrynus dorsalis* (Anura: Rhinophrynidae). In press: Journal of Herpetology (March 1999).

• E.O. Lavilla and R. O. de Sá. 1998. Estructura del condrocraneo y esqueleto visceral de larvas de *Pseudis minuta* (Anura: Pseudidae). In press: Alytes (1998)

• P. Larson* and R. O. de Sá. 1998. Phylogenetic analysis of the genus *Leptodactylus* (Anura: Leptodactylidae) using chondrocranial morphology. In press: Journal of Morphology (1998)

• M. Smetanick*, R. O. de Sá, and G. Radice. 1998. The timing and pattern of myogenesis in *Hymenochirus boettgeri*. In press: Journal of Herpetology.

• A. T. d'Heursel* and R. O. de Sá. 1998. Comparing the tadpoles of *Hyla geographica* and *Hyla semilineata* (Anura: Hylidae). Accepted in: Journal of Herpetology.

• Kerckhove, M. 1998. A Shape Metric for Blum Ribbons", submitted to: Journal of Mathematical Imaging and Vision.

• R. O. de Sá and C. Swart*. 1998. Development of the suprarostral plate of pipoid frogs. Submitted to: Journal of Morphology.