FINAL SCIENCE REPORT - LMS

Principal Investigators

Debra J. Wolgemuth, Ph.D.
Columbia University College of Physicians and Surgeons

Carey R. Phillips, Ph.D.
Bowdoin College

(1) Objectives:

The goal of these experiments was to determine the effect of microgravity on the early development of the fish medaka. There were two objectives for this flight series. The primary objective was to assess the effects of microgravity on different stages of development and to ascertain whether the relevant developmental questions can be addressed at the gross morphological level or if the issues involve more subtle questions about regulation at the molecular and cellular levels. The secondary objective was the assessment of the utility of flight hardware with the capabilities to perform embryological studies.

We have been able to take advantage of the flight testing phase of the STL-B hardware to also study the effects of microgravity on the early development of the fish, Medaka. Our initial studies involved monitoring the early Medaka development and raising flight embryos for breeding. Images of the developing embryos were collected either via video which was either taken by the astronauts or broadcast to Earth. Sample video images were digitized and stored on a hard drive resident within the on-board STL-B unit. Embryos were fixed at specific intervals, returned to Earth and are being analyzed for the timing and location of molecular events associated with controlling the morphological pattern for the onset of adult structures.

(2) Background:

Initial embryological studies on the effects of microgravity of any organism will involve similar questions and thus require, at least in the past, common technical capabilities. The first obvious question is whether the organism will survive through all stages of development and reproduce, either while in microgravity or after having been exposed to microgravity through a significant period of its development. The second level of questions involve the timing to and between different developmental stages and examination for subtle perturbations in the developmental physiology. Experiments addressing timing issues require continuous observation, a high degree of temperature

control, and the ability to fix embryos at the desired developmental stages. Temperature control is a particularly important problem in space research because the lack of gravity diminishes convection. The lack of convection causes uneven heating of the experimental environment which can lead to false interpretation of the data. The third level of experimentation often involves the ability to change media or add chemical compounds to the developing embryos at defined stages. Lastly, the ability to fix embryos at precise times to perform various molecular analyses becomes highly desirable. The STL-B has been engineered to provide all of these capabilities for small aquatic embryos.

Previous experiments using frog embryos in space (Black, S., Larkin, K., Jacqmotte, N., Wassersug, R., Pronych, S., and Souza, K. 1996. Advances in Space Research 17:209-217) indicate that morphologically normal tadpoles will develop in microgravity, even though some of the early developmental stages show subtle differences from Earth-grown embryos. It appeared that amphibian embryos have the ability to regulate for some variations in developmental pattern when subjected to a microgravity environment. This is an especially curious finding since these same developmental variations have not been observed on Earth. Therefore, our second and third flight experiments focused on gathering more precise information on the developmental morphology of the embryos in microgravity and ground-based studies involved the analysis of molecular events associated with pattern formation. Embryos were fixed during early gastrula through late neurula and are currently being assayed for a variety of gene expression patterns.

There are a number of reasons for the selection of the fish medaka for these experiments (Wolgemuth et al., 1997). The medaka represents an excellent model for vertebrate development. There are a number of laboratories around the world creating libraries of developmental mutants. There are an increasing number of molecular probes being generated, including studies from Dr. Wolgemuth's group (G. Herrada, D.A. Crotty, X.Wang, and D.J. Wolgemuth, in preparation), which will greatly enhance the analysis of regulation events during early development at the molecular and cellular levels. The embryos of medaka are clear and therefore amenable to digital video recordings of the development of internal as well as external organs. Of great importance for the particular studies was the fact that the developmental rate of the embryos can be experimentally controlled through the use of temperature. Finally, it is important to note that the medaka was also selected for a series of studies by Japanese Space Science investigators, thereby increasing the body of information which will ultimately be available to the scientific community.

(3) Methods of Data Acquisition and Analysis:

a) Overview: These studies were undertaken in collaboration with Dr. William Weismann and colleagues at WRAIR. The development of flight certified scientific hardware necessary to address the initial questions being asked by embryologists on the

effects of microgravity on embryonic development has been reported elsewhere (Wiesmann, W.P., L.A., Pranger, E.S., Delaplaine, and T. Cannon. 1994. AIAA Conference on Space Life Sciences. September 1994.). In short, initial embryological studies require gross morphological visual observations over the entire developmental time course, the ability to control the environment of the embryos in terms of oxygen, media flow, and temperature, and the ability to fix embryos at appropriate times for subsequent sectioning and more detailed analysis.

Thirty-six medaka embryos were flown on a modified STL-B hardware system. Embryos were collected within one half hour after fertilization, cleaned of chorionic hairs, transferred to both flight and ground control STL-B growth chambers. The embryos were held at 11.5-12.0°C until one half hour after reaching microgravity, at which time the temperature was raised to 17.5-18.5°C. Embryos in all chambers were monitored by video which was either broadcast to Earth, stored on videotape, or stored as digitized images. The six chambers of the STL-B were fixed with Bouins fixative at pre-programmed times and the embryos returned to Earth for detailed morphologcial and molecular analysis. Digital images and real-time video sequences were taken of the flight, synchronous ground and non-synchronous ground embryos under identical conditions except for the flight environment (acceleration into orbit and microgravity). Embryos were intended to be fixed at the following intervals: Orbit plus 24, 48, 72, 96, 142 and 166 hours. A hardware malfunction led to the fixation pre-flight of chamber #4 (72 hrs.) and the Orbit plus 142 and 166 hour embryos not being fixed until the shuttle landed back on Earth. This was later traced by the STL-B team to intermittent contact on the wiring to the pump board assembly and the flowpath pumps. The wiring was most likely compromised during the exchange prior to NASA turnover. The pumps were checked and functioning properly at the time of turnover. The remaining fixative was injected to the flowpaths during ground processing. The embryos had not reached the hatching phase at this time. Media and gas exchange to the two flowpaths was affected by the intermittent wiring. The effects of the intermittent nature of pumping on the embryos was investigated in the first of three post-flight tests conducted at Bowdoin College (C.R. Phillips).

All of the embryos, both flight and control, have been embedded and sectioned. No gross morphological abnormalities were observed between the flight and control embryos or between flight or control animals and comparably staged untreated embryos. Analysis of the video and digital data is still under examination. However, some conclusions can be drawn, the most important being that overt development was normal.

b) Selection of Fixation Protocols: Our preliminary histological evaluations from STS 70 suggested that 4% paraformaldehyde in the spaceflight condition yielded adequate but not optimal fixation for histological observations. While 4% paraformaldehyde is the fixative of choice for use in fixing tissues for subsequent *in situ* hybridixation analysis, it does not appear to be the best fixative for morphological integrity of medaka embryos, particularly when fixed through the chorion. Since Bouin's fixative had been used by one of us (C.R. Phillips) in an extensive series of studies and was found to yield both optimal

morphological preservation and adequate detection of at least certain cellular proteins by immunohistochemistry, this was the fixative of choice for STS 78.

c) **Video Analysis of Medaka Development in Control and Flight Environments:** Medaka fish embryos are optically clear, allowing direct observation of embryonic development by video-microscopy. Such instrumentation has been developed by our colleagues from Walter Reed as part of the STL-B hardware. The STL-B hardware has flown experiments on the shuttle on three separate occasions. The first, STS 59, was considered a hardware flight test and was not supported by NASA. Medaka embryos were flown on this mission and all systems checked out in terms of biocompatibility. The second and third flights, STS 70 and 78, provided the opportunity for a series of video observations and fixation of embryos for subsequent histological examinations on the effects microgravity on the development of the medaka at various stages.

The first video sequence served as a reference for fish development. There is normally a rotation of the animal pole upwards, relative to gravity, and active cytoplasmic rearrangement towards the animal pole following fertilization. Cytoplasmic components are localized to the animal pole as mitosis begins to partition the egg into cells. It is during the early stages of cell division shown here that the dorsal/ventral and right/left axes are determined. All of the major body organs are spatially determined during gastrulation.

The primary on-orbit activities conducted by the crew was to provide the on-orbit reference to the system at 7 hours after launch and to re-program the embryo positions to accommodate any shifting resulting from launch and orbit. This reprogramming optimized the digital image storage and the on-board recordings by centering embryos in the viewfield. Once the embryos were correctly positioned and the video cables set up, it became possible to receive an earlier than planned, additional downlink. This downlink verified embryo viability and positioning. The first planned downlink was operated by Commander Tom Hendricks, who provided us with a much longer than scheduled downlink. During this downlink, each of the six embryo chambers were reviewed and observed. This proved to provide a good baseline for system operation, performance, and status of the embryos. Subsequent downlinks and on-board recordings were conducted automatically by ground commmanding of the shuttle systems. In many cases the downlinks were longer than originally scheduled, and on-board recordings were more frequent than scheduled. The landing preparation reference was provided following our final scheduled downlink and the termination of the last samples.

Following the mission, ground and flight digital images were decompressed and recorded onto compact disks. Copies of these disks have been provided to one of us (C.R. Phillips) for analysis.

d) Studies of Complete Life Cycle of Medaka Embryos Which Were Allowed to Hatch Upon Return to the Earth Environment: Although not formally part of LMS, the following observations from STS 70 are relevant to the overall scientific mission. Two of the culture chambers containing embryos in the experiments flown in STS 70 were returned to earth unfixed. These embryos and one chamber of control embryos were held at KCS for 9 days at 18°C. They were then shipped to Columbia University in a container to maintain the 18°C temperature. Upon receipt on 7/25/95, they were placed in a controlled temperature environment of 21-25°C, average temperature was 23°C. The lighting regimen in the culture incubator was 12 hours light: 12 hours dark from 7/25/95 to 8/17/95. After 8/25/95, the light/dark cycle was changed 16 hours light: 8 hours dark. The average hatching time, in days post arrival at Columbia University, was 30 days for the flight animals (two groups of six embryos each) and 40 days for the control (one group of 6 embryos). Both series are considerably longer than the typical hatching time for medaka of 10-14 days total at 25°C. This delayed hatching in embryos in the culture chamber is potentially interesting and should be investigated further; however, the numbers in the present study were too small to be evaluated statistically.

Upon hatching, the fry were allowed to develop into adulthood. Over a 4 month-period, 3 control animals and 5 experimental animals survived and began to reproduce. The two females and one male of the control group produced 8 eggs that were recovered but then stopped reproducing, no doubt due to the small number of animals in the tank. In contrast, the space flight animals consistently yielded batches of fertilized embryos from 11-10-95 through 7-16-96. This result demonstrated that animals exposed to microgravity during embryogenesis could reproduce. A sample of the embryos yielded from the flight animals were then removed to a separate culture environment and allowed to develop to adulthood. Upon reaching sexual maturity, these animals began to mate as well. Thus, the progeny of the space flight animals are also fertile.

4) Flight Results Compared with Ground Results:

a) Use of Reduced Temperatures in Studies of Early Embryogenesis in the Microgravity Environment: One of the reasons for selecting the fish medaka as an ideal model for studying vertebrate development in space is its ability to tolerate reduced temperatures during early embryogenesis. This has permitted us to slow down early embryogenesis until the embryos were exposed to microgravity. A series of temperature shift trials have been run to determine the appropriate temperatures in which to hold medaka embryos so that a minimal amount of development has occurred prior to arrival in microgravity. The length of time the embryos are held in a slow developing state depends on the flight hardward turnover time established for each particular flight. In STS-78, temperatures were held at 12°C during loading of the embryos and in the STL-B hardware unit until orbit was achieved. Once in orbit, the system raised the temperature in the chambers to 17.5°C, which allowed development to proceed at a faster rate. The

temperature was monitored throughout the flight and held to within 1°C. An example of the temperature tracking is shown in Fig. 1.

One important consequence of the previous flight experiments was the preliminary observation that flight embryos might develop at slightly different rates than do the ground controls. Analysis of developmental rate differences in fish embryos will be highly dependent on controlling and monitoring the temperature during both the flight and control samples. As noted above, the STL-B can maintain temperatures with a one degree centigrade accuracy. Therefore, in order to study the effects that microgravity might have on rates of development, it is important to understand the effects of small temperature changes, up or down, on the developmental rates between specific stages of embryonic development. We have done extensive studies on the effects of temperatures at 14° C, 15° C, and 16° C, concentrating on the period of development encompassing gastrulation. Interestingly, embryos raised at 14° C appear to develop faster during the initiation of movements at gastrulation than do embryos raised at either 15° or 16° C. (Fig. 2). By mid-gastrulation, the developmental rates are equivalent between embryos raised at these three temperatures. Time lapse video analysis is currently being done to determine the cause and mechanisms involved in these differences during early gastrulation.

Comparison of both developmental heterogeneity within a clutch of embryos and developmental rates between ground control and flight embryos is highly sensitive to temperature fluctuations. The STL-B was designed to minimize the temperature differentials within the growth chamber. This was accomplished by minimizing the volume and replacing the media with new media at the appropriate temperature. The volume of each of the six chambers was 70 microliters and the media was changed 10 times per hour. Ground-based tests indicate that this rate of flow provides adequate oxygenated media and waste removal for normal development.

b) Analysis of Gene Expression in Medaka Embryos: We have begun to analyze the expression of the medaka *Hoxa-4* gene as a marker of embryonic development for analyzing the effect of microgravity on pattern formation and embryonic segmentation. To this end, we are currently determining the expression pattern of medaka *Hoxa-4* during embryogenesis, under normal conditions. Our Northern blot analysis of total RNA isolated from embryos pooled at various stages of development revealed the expression of a major transcript of ~1.7 kb, first detected at stage 21, when the medaka embryos have six to eight somites. Our next experiments will extend this analysis to sections of embryos at various stages of development by *in situ* hybridization on histologically sectioned embryos. This will be critical for studies on the expression of specific genes in flight embryos, as multiple genes could be assayed in the same embryo. We have concomitantly successfully obtained whole mount *in situ* hybridizations with medaka embryos, but feel it is critical to develop the use of sectioned material to maximize data return. An example of whole mount *in situ* hybridization of the homeobox gene *Hoxa-4* is shown in Fig. 3.

c) Effects of Retinoic Acid on Developing Medaka Embryos: Retinoic acid had been shown to be an important regulator of vertebrate development, in both the fish and mouse. In concurrent studies in our lab, we have shown that some Hox genes in the mouse, particularly *Hoxa-4* (Alan Packer and D. J. Wolgemuth, unpublished observations), are regulated at least in part by the administration of all-trans retinoic to pregnant females. Although no retinoic acid binding element has yet been identified in the upstream region of medaka *Hoxa-4*, we wanted to know if this mode of regulation of the expression of this gene in mouse could be conserved through other species. Medaka embryos at various stages prior or at the begining of *Hoxa-4* expression (see above) were treated with all-trans retinoic acid for two hours in the dark. Embryos treated with 1uM did not show any specific phenotype, although none of them were allowed to develop further than stage 27. Embryos treated with 10 uM retinoic acid died at stage 20-21, with a typical curly tail, proving that the normal pattern of body development was strongly affected at that concentration. Whole-mount analysis of treated embryos showed that, at both concentrations, *Hoxa-4* expression in the neural tube was shifted more anteriorly, and that the amplitude of the shift could be affected by the concentration of retinoic acid. Interestingly, the shift observed at stage 25 exhibited two bands of expression, more anterior than the normal limit of *Hoxa-4* expression and very similar to what we have observed in the mouse (A. Packer and D.J. Wolgemuth, unpublished observations).

(5) Conclusions Including Significance and Future Plans:

Earlier flight experiments produced embryos showing truncation of anterior structures. However, analysis of the temperature profile indicated that a temperature spike to near 32°C occurred during the early stages of the flight. We have grown embryos at 32°C in 1 g, both for long durations and for short bursts of time during gastrulation and have not seen the kinds of anterior truncations observed in these early flight experiments. However, we can not rule out that the combination of stresses due to higher temperature and microgravity, in combination might lead to the observed anterior truncations. The temperature problems for the STL-B were corrected and a second flight experiment (STS 70) where the temperature control responded correctly produced normal embryos which subsequently were bred and produced live young. Therefore, the possibility of the combined stress of microgravity and temperature has not been addressed.

Sections of flight and control embryos would ultimately provide detailed information about developmental morphology and molecular events controlling development. However, fish embryos are small and difficult to orient when embedding. Therefore, sectioned embryos tend to lay at various orientations, making it difficult to compare subtle differences in morphology between embryos. We have been working on a method of digitally reconstructing sectioned embryos to make comparisons easier. Digital sections can be used to make 3-D reconstructions or they can be used to make movies of simultaneous "flights" through the embryo. Movies of both flight and ground control embryos can be compared simultaneously as a means of detecting more subtle

morphological differences. Eventually, three dimensional reconstructions of each organ within the embryos will be made and animated through all of the developmental stages which have been fixed in flight and sectioned here on earth. An additional observation that merits future study is the extended hatching time that was observed in both control and experimental medaka embryos flown on STS 70.

(6) Bibliographic Citations of Articles/Presentations Resulting from the Flight (to date)

Wolgemuth, D.J., G. Herrada, S. Kiss, T. Cannon, C. Forsstrom, L.A. Pranger, W.P. Weismann, L. Pearce, B. Whalon, C.R. Phillips. (1997). Vertebrate Development in the Environment of Space: Models, Mechanisms, and Use of the Medaka. **The ASGSB Bulletin**, in press.

(7) Non-technical Summary

In the flight experiment on LMS, June 20, 1996, thirty-six medaka embryos were flown on a modified STL-B hardware system. Digital images and real-time video sequences were taken of the flight, synchronous ground and non-synchronous ground embryos under identical conditions except for the flight environment (acceleration into orbit and microgravity). In addition, ground-based studies have been performed on the use of reduced temperatures to study early embryogenesis, on developing alternative fixation protocols, on gene expression in medaka embryos, and on the effects of retinoic acid on embryogenesis in developing medaka.

FIGURE LEGENDS

Figure 1. The embryo temperatures from the flight unit on STS-78 were kept at 12°C (lower boundary) once loaded into the STL-B. Approximately 9 hours after orbit, the temperature was gradually raised to 17.5°C, where it remained for the duration of the mission. Data points on this graph are instantaneous, not averaged, values which were recorded every 5 minutes.

Figure 2. Effects of reduced temperatures on rates of medaka development.

(2a). Ten embryos each from one clutch were incubated at temperatures of 14.5, 15.5, and 16.5°C. At varying intervals, the embryos were staged according to Kirchen and West (1976). The embryos were monitored from the beginning of gastrulation at approximately 11 hours of development through 72 hours of development at the three temperatures depicted above. The development of embryos at 25°C is shown from a second clutch of embryos. All embryos within a clutch developed synchronously at a given temperature.

(2b). Embryos at the lower temperature appear to develop faster during early to mid-gastrula stages, as shown in Figure 2a. A second clutch of embryos were incubated at 14.5, 15.5, and 16.5°C during the early to late stages of gastrulation. Embryos from this clutch also exhibited increased rates of development at 14.5°C than embryos grown at 15.5 or 16.5°C.

Figure 3. Expression of the medaka homeobox-containing gene Hoxa-4 at specific developmental stages. Embryos were cultured, harvested at specified stages, and subjected to whole mount *in situ* hybridization (D.A. Crotty, G. Herrada, X. Wang, and D.J. Wolgemuth, in preparation). The stages of embryonic development are assigned according to the criteria described by T. Iwamatsu (Zool. Sci., 11: 825-839, 1994).

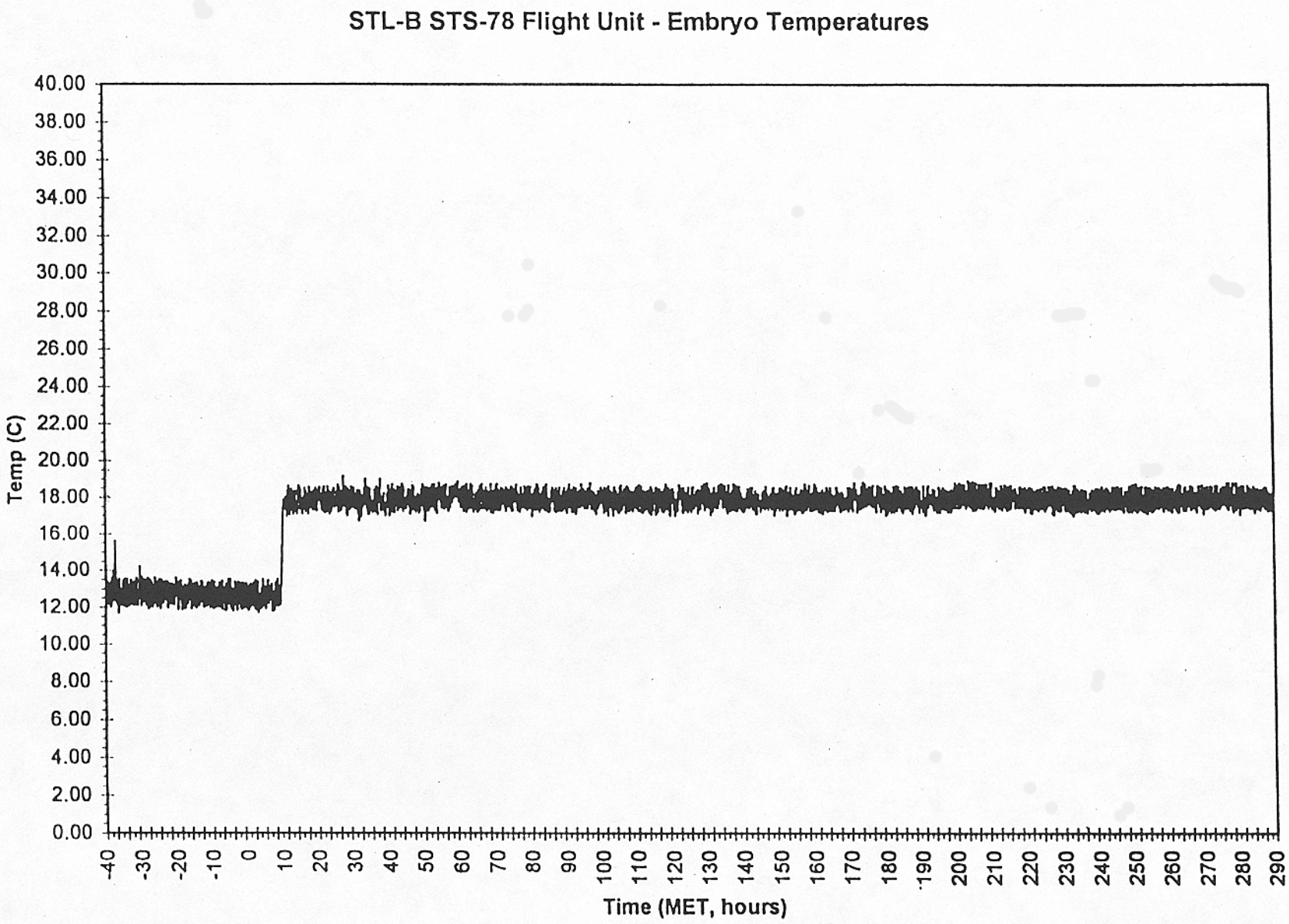
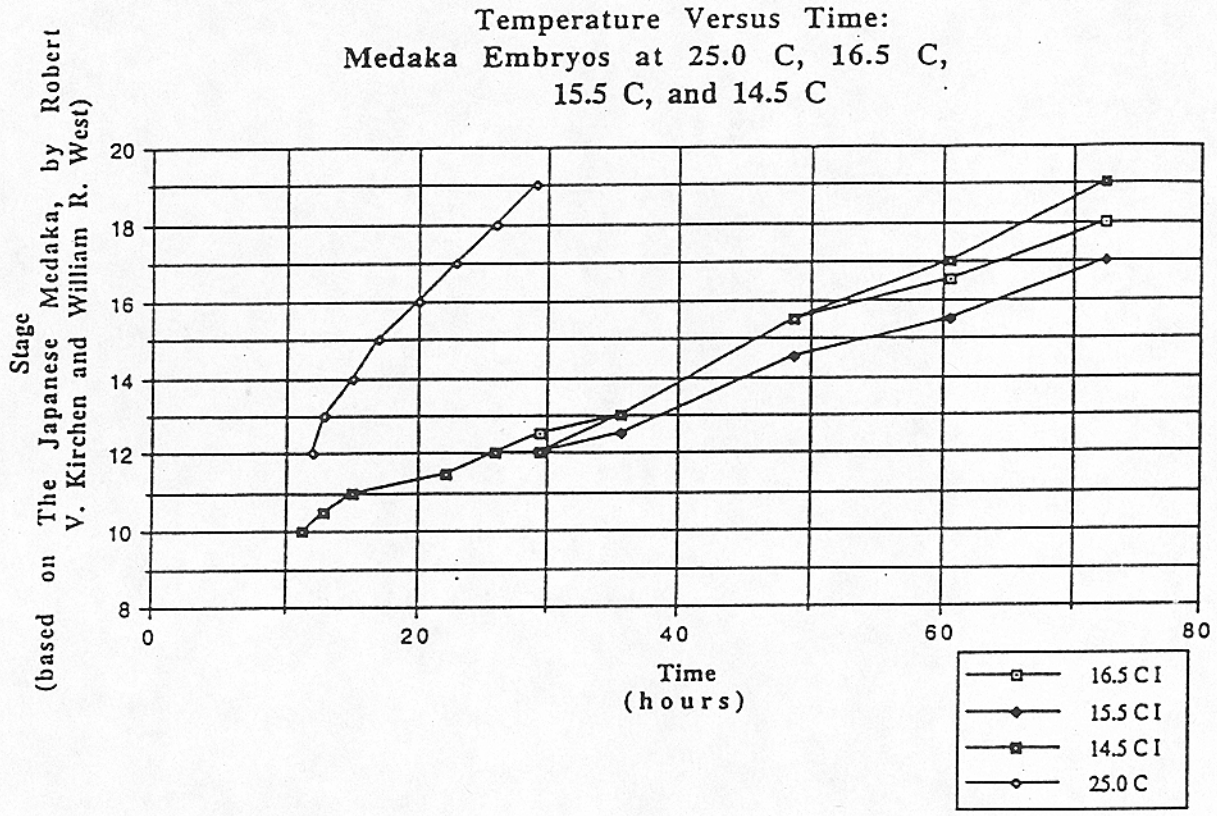

STL-B STS-78 Flight Unit - Embryo Temperatures
Temp (C)
40.00
38.00
36.00
34.00
32.00
30.00
28.00
26.00
24.00
22.00
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
-40 -30 -20 -10 0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260 270 280 290
Time (MET, hours)

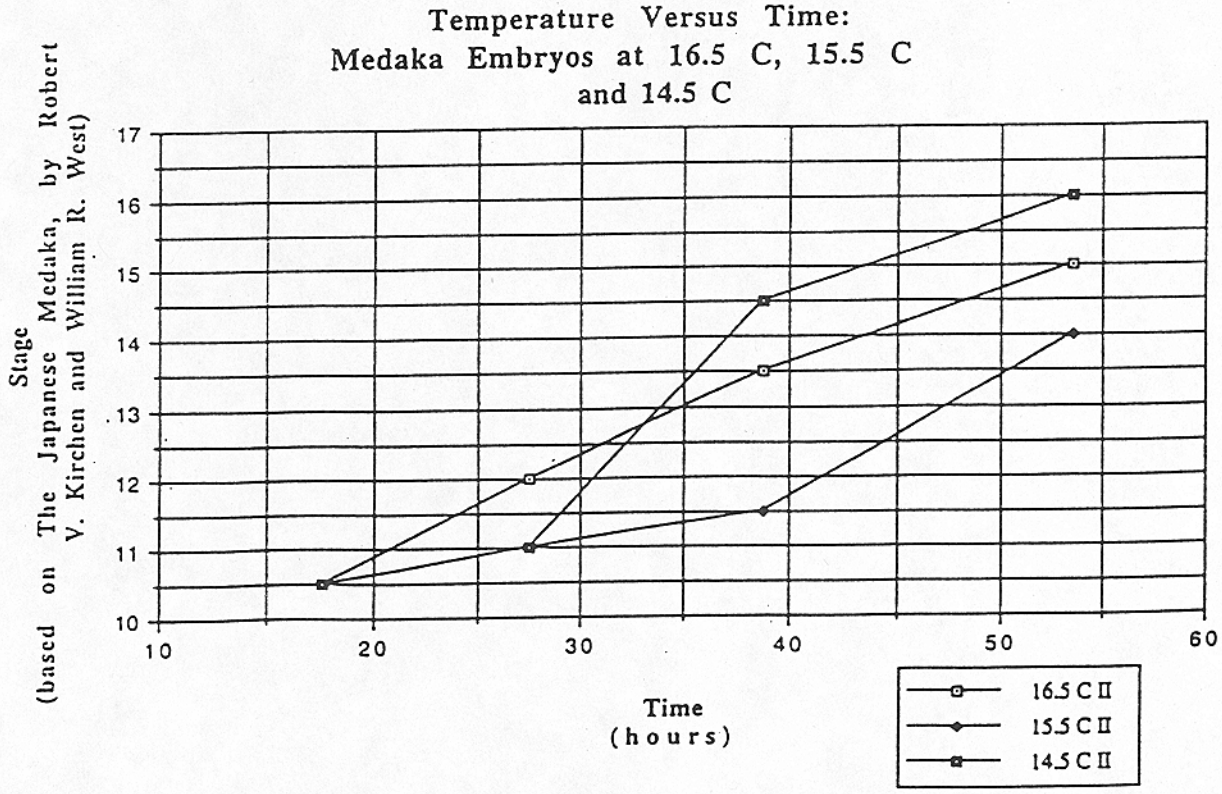
Figure 1.

Figure 2.

[A]


Temperature Versus Time:
Medaka Embryos at 25.0 C, 16.5 C,
15.5 C, and 14.5 C
Stage
(based on The Japanese Medaka, by Robert V. Kirchen and William R. West)
20
18
16
14
12
10
8
0
20
40
60
80
Time
(hours)
16.5 C I
15.5 C I
14.5 C I
25.0 C

[B]


Temperature Versus Time:
Medaka Embryos at 16.5 C, 15.5 C
and 14.5 C
Stage
(based on The Japanese Medaka, by Robert V. Kirchen and William R. West)
17
16
15
14
13
12
11
10
10
20
30
40
50
60
Time
(hours)
16.5 C II
15.5 C II
14.5 C II

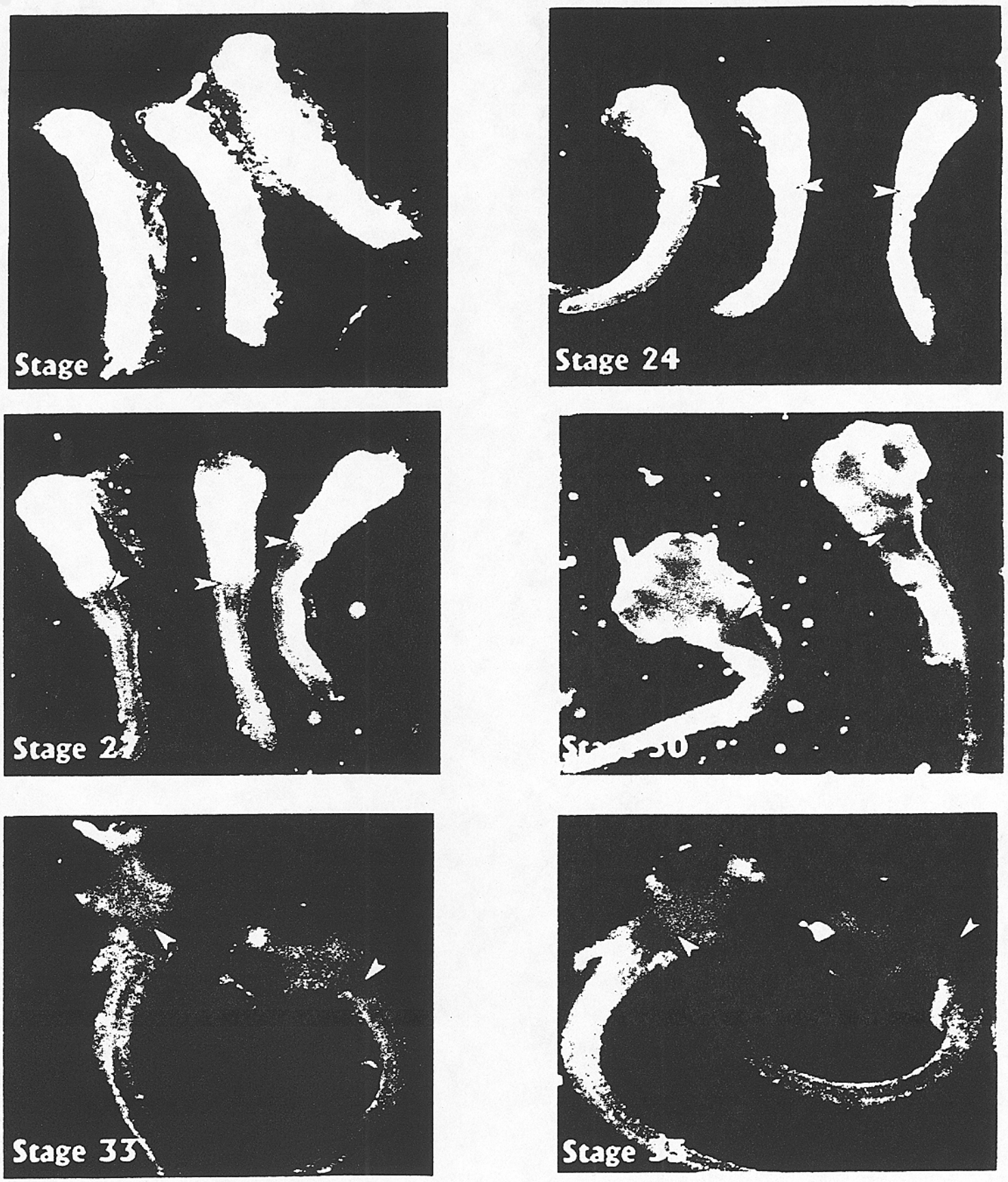


Figure 3.

Expression of Hoxa-4 in different stages of Medaka Embryonic Development

(arrowheads show anterior boundary)