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FINAL REPORT

NASA CONTRACT NAS 9-14632

"SUPPORT OF ASTP/KOSMOS FUNDULUS EMBRYO DEVELOPMENT EXPERIMENT"

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INTRODUCTION: This final report covers the work done in the performance of NASA Contract NAS 9-14632. The major portion of this report will cover the work and data which resulted from the KOSMOS Biosatellite 782 (K-104) flight. The earlier work, that resulting from the ASTP flight, was reported earlier, and has been published by NASA; "Apollo-Soyuz Test Project; Summary Science Report" NASA SP-412; pgs. 281-306. The major refinement in the KOSMOS project lay in the incorporation of a one-gravity onboard control treatment through the use of an onboard centrifuge.

EXPERIMENT BACKGROUND: The experimental organism chosen for these studies is Fundulus heteroclitus (Walbaum), a small shallow-water minnow common to the Atlantic coast of North America. The strain employed produces an egg having a transparent chorion thus allowing observation of development from first cleavage through hatching. The chorion is quite tough and the egg may be manipulated extensively without undue stress on the developing embryo. Because the biology and developmental relationships of the Fundulus are relatively well known (Armstrong & Child, 1965; Oppenheimer, 1938), and because of its physical attributes which allowed aseptic culture, the organism was used as a test organism at the NASA Lyndon B. Johnson Space Center (JSC) during the lunar quarantine. Subsequently the methodology (Boyd & Simmonds, 1974) for continous laboratory production of fertile eggs was developed in this laboratory and the accumulation of a data base for flight experimentation was initiated.

The initial Fundulus experiment was flown on the Skylab 3 mission. The experimental design was quite preliminary in form. The flight package consisted of a plastic bag containing 2 juvenile fish in a compartment with 250 milliliters of synthetic seawater (21 parts per thousand dissolved solids) and 50 fertilized eggs (5 days past fertilization) in a separate compartment with 50 milliliters of seawater. Initially, the juveniles exhibited obvious disorientation reactions (swimming rapidly in loops and circles), but over a period of several days in orbit, they gradually adapted to the weightless environment and to dependence on visual cues for orientation. In this state of adaptation, the locker door surface to which the plastic aquarium was attached served as "down". Adaptation was not complete, however, and occasional disoriented swimming activity occurred. Nearly all the 50 eggs hatched in space, and because of a delay in hatching of the flight fish, several returned alive to Earth. However, a series of unfortunate events resulted in the death of these animals shortly after return. The space-hatched Fundulus fry exhibited no observable tendency toward disoriented swimming acitvity, and their apparent dependence on visual

orientation cues both onboard the Skylab spacecraft and on the recovery ship suggested the possible absence of vestibular input. Preservation of the returned hatchlings was insufficient to prevent deterioration and did not permit definite conclusions regarding the condition of the vestibular system.

The second experiment in the series was the MA-161 experiment flown on the 9-day Apollo Soyuz Test Project mission to confirm and extend the observations of the Skylab experiment (Scheld et al, 1975, 1976). The experiment package consisted of two parts: a series of staged embryos in five individual compartments of a polyethylene bag and a series of preconditioned juvenile fish in a similar bag. Embryos at 32, 66, 128, 216, and 336 hours after fertilization at the time of launch were chosen to represent key stages of development; development occurred at a constant temperature of 295 K (22°C). Juvenile fish were reared from hatching for 21 days in specific visual environments. Experiment packages were mounted on the docking module wall and photographed periodically during the mission to record the swimming activity of the fish and the condition of the eggs. At splashdown and at selected times thereafter, juveniles and hatchlings were tested to assess normalcy of vestibular function and samples were fixed for microscopic examination to assess normalcy of anatomical development.

Juvenile fish in a null-gravity environment exhibited looping swimming activity similar to that observed during the Skylab 3 mission. Hatchlings from the 336 hour egg stage also were observed to loop. At splashdown, both juveniles and hatchlings exhibited a typical diving response suggesting relatively normal vestibular function. The juveniles exhibited swimming patterns suggestive of abnormal swim bladders. Rotating drum tests confirmed that no radical changes in vestibular function had occurred, and no significant differences were found in subsequent light orientation tests. Tests of geotactic response in fish after 6 months or more of development suggested a tendency for the 32 hr flight fish to spend a significantly greater portion of their time in the upper portion of the test apparatus. Other treatment groups exhibited no statistically significant tendency, but in all groups tested there was evidence that the flight fish might be more sensitive to environmental factors. High resolution analyses of oriented locomotor activity (Kleerekoper, et al, 1973) are planned for the remaining experimental animals at maturity.

Extensive light and electron microscopic examination of flight and control materials have revealed no significant differences in the embryological development of the central nervous system, peripheral vestibular apparatus, the eye or the cardiovascular system, of any of the animals examined thus far. The otoliths of flight juveniles were compared by scanning electron microscopy with otoliths from control animals maintained on Earth. The medial surfaces of the sagittae are characteristically grooved and sculptured; the lateral surfaces are smooth with concentric "growth rings" and "crystalline" projections in the groove in larger fish. None of the scanning electron microscopic examinations revealed recognizable differences in size, shape, or surface structure between flight and control juveniles. Histological and ultrastructural analyses of all age categories revealed that the peripheral vestibular system developed fully with complete expression

of the maculae of the sacculus and utriculus. The statoliths of the sacculus and utriculus followed normal sequence, and the lagenal statolith initiated development between posthatching days 4 and 6 in all groups. Except for a decrease in the extent and content of the vitreous cavity, ocular development was judged to be normal. Ultrastructural analyses of the developing statoliths and sensory maculae have revealed no differences between matched groups of animals. Both sustentacular and hair cells followed normal developmental parameters. Central projections and vestibular ganglia contained normal cellular complement and projection configuration. Cartilage development and calcification was identical in flight and control categories. Preliminary analysis of the specimens treated with tritiated thymidine at selected postsplashdown periods has also failed to reveal any significant alterations in the patterns of cellular proliferation within the vestibular, ocular, or central nervous systems of the oldest stages flown. Cells of the ependyma and cortical neuroepithelium, the planum semilunatum of the maculae, and the ora serrata of retina were heavily and consistently labeled in all groups.

Since the results of the animals flown on KOSMOS 782 flight have not yet been published (in Press), we will describe the experimental design, implementation and initial analysis in some detail in the following paragraphs.

The main experiment package of the Kosmos K-104 experiment was based upon the design of the ASTP experiment and modified only to the extent necessary to conform to the new requirements and constraints of the Soviet spacecraft, launch/recovery procedures, and flight hardware. Each experimental unit consisted of a polyethylene bag containing 50 embryos of a given age and 23 ml sterile filtered 21‰ Instant Ocean. An experimental treatment contained 2 units each of five different embryo age groups as follows:

Unit	Calculated Launch Age	Average State of Development
1	32 hr	Mid-to-late gastrula
2	42 hr	Tubular profile of membranous labyrinth apparent. No evidence of statolith precursors
3	66 hr	Appearance of statolith precursors
4	88 hr	Initiation of ventral elongation of medial sensory patch. Initial elaboration of semi-circular canals
5	128 hr	Statoliths formed but without definitive margins

Age was based upon development time at 295 K (22 C). Each treatment group thus contained 100 embryos of each of the 5 different nominal development stages for a total of 500 eggs.

Experimental treatments were as follows:

K-1: Flight treatment, null-gravity (stationary mounted). Five hundred eggs as indicated above were fertilized, allowed to develop and then packaged, chilled, transported and flown as indicated in Table 1.

K-2: Flight treatment, one-gravity onboard control (centrifuge mounted). Eggs were treated identically with K-1.

K-3: Ground Control 1. Eggs were prepared and transported with K-1 and 2. At the time indicated in Table 1 the experiment package was mounted on a 5 rpm tube rotator and subjected to slow rotation (tumbling) in order to simulate the uniform dispersion of eggs in null-gravity.

K-4: Ground Control II. Eggs were prepared and transproted with K-3. The flight package was allowed to remain in a stationary position. Ten eggs were removed from each development group and fixed at time of launch to provide a reference point for guaging the developmental stage at time of null-gravity exposure.

K-5: Ground Control U.S. Eggs were prepared identically with K-1 through 4, transport was simulated and the flight package was mounted as for K-3.

K-6: Dish control. Eggs were fertilized as for K-1 through 5, but were allowed to continue development until hatching under standard environmental conditions in petri dishes.

DATA COLLECTION:

The primary form of data yielded by the experiment was in the form of fixed material for light and electron microscopic analysis. Fixation was in the cold (ice bath) in Kalt-Tandler (Keefe, 1973) fixative. Samples were fixed at recovery and at intervals up to 30 days following recovery. No further sampling of flight or control fish is planned until behavioral testing has been completed and the mature fish have produced eggs for the initiation of a second generation. The primary point of interest in all examinations is the vestibular and other sensory regions.

Postflight observation and testing were carried out to detect possible differences in orientation behavior attributable to the null-gravity exposure during development. The tests employed, light orientation, rotating striped drum and geotaxis test have been described elsewhere (Scheld, et al, 1975; Scheld, et al, 1976) as they were employed for ASTP postflight testing. These same tests were used with only minor modification.

EXPERIMENT EXECUTION:

The basic procedure for preparation of fertilized eggs followed that of the ASTP experiment (Scheld, et al, 1975). Specific timing for this experiment is indicated in Table 1. Eggs fertilized at the appropriate times prior to launch were allowed to develop to known stages of development and then sealed in the plastic packets and chilled to 10°C to retard further development during transport to the launch site. For planning purposes it was assumed that elapsed time of chilled transport would be 72 hours and time from rewarming and loading of flight package to launch would be 8 hours. Progress of development at 10°C for 72 hours has been calculated to be equivalent to 12 hours at standard temperature of 22°C. Fertilization of eggs was thus scheduled to produce embryos that at the time of chilling would be 20 hours younger than the expected launch ages.

Slow chilling of the eggs was effected by placing the sealed plastic bags in plastic beakers containing 1000 ml of water and allowing these to chill to 10°C in a 6°C incubator. The chilled bags were then quickly transferred to the equilibrated 10 degree transporter and packed for shipment.

Transport and launch of the flight package proceeded without incident. In Moscow at approximately 36 hours before launch, control eggs were removed to a separate transporter and held in Moscow for treatment, while flight eggs were taken to the launch site. As indicated in Table 1, eggs were removed from the transporters a few hours prior to launch, loaded into the experiment hardware and then placed onboard the spacecraft.

At recovery flight treated packets were removed from the flight containers, marked and then placed in the 10°C transporter for shipment to the Moscow laboratory. Ground controls were not subjected to this chilling treatment because access to these materials was delayed by airline scheduling, until nearly 1 1/2 days after recovery and chilling of the flight materials had occurred. This deviation was considered to be justified in light of baseline laboratory tests which have indicated that chilling has no harmful effect and because a chilling treatment of control eggs would have caused a considerable delay and complication of the recovery procedure.

An adjunct experiment carried out by Soviet investigators considered the effects of clinostatting upon development. Eggs were fertilized and prepared by the standard procedures to be chilled and shipped 10 days prior to launch. Development, at the time of chilling for shipment, had proceeded for 12 hours, 36 hours and 66 hours respectively in three separate sets of 300 eggs each. Upon arrival in the Soviet Union, eggs from the three age groups were randomly divided into treatment and control groups for clinostat exposure. Periodically during the course of development, samples were removed and fixed for microscopic examination. At approximately 36 hours prior to launch subgroups of the treatments were chilled, transported, and launched with the eggs of the main experiment.

Table 1

Environmental Conditions Encountered by Kosmos Experimental Embryos

Treatment Number	22°C Prechill Incubation Time for Embryos					Temperature/Treatment Profile			
	128 hr	88 hr	66 hr	44 hr	32 hr	Chill Cycle	Transport Cycle 10°C	Warm-up	Flight	Recovery
K-1	108	68	46	24	12	1.75 hrs. (18°C-10°C)	65 hrs (drop to 8.6° for 4-5 hrs)	8 hrs 21°C	19½ day 21° 0-G	52 hrs 10°C
K-2	"	"	"	"	"	"	"	"	" Centrifuge	"
K-3	"	"	"	"	"	"	81 hrs (Temperature stable)	4 hrs 20°C	19½ days 21°C Rotated Moscow	14 hrs 21°C
K-4	"	"	"	"	"	"	"	"	" Stationary Moscow	"
K-5	"	"	"	"	"	"	90 hrs	3 hrs 22°C	20 days 22°C USA Rotated	24 hrs 10°C
K-6	Continuous development at 22°C until hatching									

Recovery Activities

The experiment was carried out according to the timing indicated in Table 1 and in the "Experiment Execution" section of this report. Upon opening of the experiment packages severe retardation and high anomaly rates were apparent in all treatments except the Houston Standard Dish Control. The originally planned sampling and testing schedules were modified to allow for the slower hatching rates, and reduced numbers of samples. All eggs were examined under a binocular microscope and counts of normal (Table 2), abnormal (with types of abnormality indicated) and dead were recorded. Following microscopic examination all eggs were shaken for 20 minutes on a rotary shaker and then examined for hatching 20 minutes or longer after the end of the shaker period. Except for the time in transit form the USSR, this routine was followed until all normal eggs had hatched.

Significant numbers of hatchlings did not emerge until the third and fourth days postflight. All morphologically normal hatchlings exhibited a typical fright-diving response and there were no apparent differences among the treatments with respect to diving response. No changes in orientation were noted in response to undirectional light through the sides or bottoms of the containers. Beginning on the third day post-recovery motion picture recordings were made of normal and disturbed swimming activity, geotactic response and orientation in a rotating striped drum.

Samples of hatchlings and eggs were fixed for microscopic examination on the fourth, seventh and thirtieth days, postflight. Major sampling was from the population of anomalous hatchlings. Only representative samples were taken from the apparently normal population. All samples were post-fixed with osmium and embedded in Epon plastic for sectioning.

Behavioral Testing

It has become obvious from all observation made thus far on Fundulus from flight experiments, that alteration in orientation behavior, if they exist, will be distinguishable only by quantitative analysis. Further, the behavior in young fish is too erratic to allow resolution of subtle differences.

It is now a certainty that development in weightlessness from the earliest time possible under the present experimental constraints, or the additional tenure in space for 8-14 days past time of theoretical full development has no radical effect upon vestibular function. Thus, in the absence of any major quantitative change, the policy adopted is to allow fish to grow to maturity before expending a major effort on behavioral analysis.

Preliminary investigations (Scheld, et al, 1976) of geotactic response in maturing (6-8 month) old ASTP hatchlings have shown a statistically significant difference in tendency of 32 hr hatchlings, only, to swim predominantly in the upper half of the test apparatus. There was also some indication that flight fish in general were more sensitive to their environment. These results are quite preliminary and both ASTP and Kosmos fish are being raised to maturity at which time they will be large enough to register on the photocell detectors of a more sensitive apparatus for quantitating and analyzing patterns of oriented locomotor activity.

Table 2

Normal Hatchlings Record from the Kosmos Mission*

Treatment	Age				
	32 hrs.	44 hrs.	66 hrs.	88 hrs.	128 hrs
K-1 (flight stationary)	6	4	1	14	38
K-2 (flight centrifuge)	4	10	6	11	27
K-3 (ground rotated)	1	1	0	0	1
K-4 (ground stationary)	0	2	0	0	25
K-5 (USA rotated)	0	0	0	0	0
K-6 (USA dish)	99	97	100	97	98

*Not corrected for hatchlings from any eggs given to Soviet investigators. All treatments contained 100 eggs except K-4 which contained 90.

The significance of such subtle changes in behavior is difficult to judge at this point. If high resolution analysis of locomotor behavior in the ASTP fish and the more limited sample of Kosmos fish supports the conclusion from the preliminary geotaxis experiments, then there is good reason to explore the possible consequences much more carefully in fish and ultimately in mammalian systems.

Histological Examination

Although most of the returned experimental and control fish exhibited developmental anomalies it was considered that this material was well worth using for studies of the vestibular development because of the generally lower sensitivity of the vestibular system to teratogenic agents (Solberg, 1938; Oppenheimer, personal communication). The mode of operation for study of this material has been to first examine a large number of specimens from the ASTP experiment and then to follow this with comparative specimens from the Kosmos material. Further, examination began with the oldest and theoretically most probably normal egg groups and progressed downward to the youngest, most sensitive groups.

Studies of the KOSMOS material to date has been carried out at the light and electron microscopic levels on embryos exposed to null-gravity as early as 32 hours post fertilization equivalence (counting transporter time). No significant micro-anatomical differences, traceable to spaceflight effects, have been observed in otolithic structural development or in development of the sensory tissues of the visual and vestibular systems in any of the age groups.

Cause of Anomalous Development

Postflight testing of procedures and materials from the experiment indicates that the probable cause of the high incidence of anomalous development lies in the tape used to label the plastic bags. Tests have shown high anomalies even in unlabelled bags immersed in water containing labelled bags. Analysis of organic volatiles from the tape by gas chromatography/mass spectrometry has resolved only one major component, carbon disulfide, that has known toxic properties in the concentration range potentially available in the experimental packages. All other detectable volatile components were present in much lower concentration and none have known toxic properties. Tests with carbon disulfide have demonstrated the capability for causing death or delay and anomalous development in Fundulus embryos.

CONCLUDING REMARKS

The experiment series to which the present experiment belongs was begun to explore the possible deleterious effects of exposure to weightlessness upon development and growth of biological systems. The results obtained thus far from these experiments are very encouraging in that we can say with considerable confidence that development of Fundulus beyond the gastrula stage is not affected in any major way by weightlessness. At this point it seems somewhat doubtful that we will discover very many minor effects, and it is tempting to speculate that weightlessness may be largely beneficial.

Most discussions of effects of weightlessness upon development appear to assume the weightless state to be a stressful condition. Practical experience with Fundulus development would suggest the opposite. There was indication of generally better health in flight animals of all three experiments of this series. With the possible exceptions of those aspects of development in which gravity is required as a cue for establishment of polarity or as a reference stimulus for sensory development, it is probable that the weightless state provides generally superior conditions for embryo development. This may not be true for situations in which maternal-fetal interaction is part of the process, but even there, the source of stress would be through the parents interaction with the environment while stress on the embryo and stress on the parent resulting directly from pregnancy should be reduced.

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