Hypergravity Induced Gene Enrichment by Subtractive Hybridization

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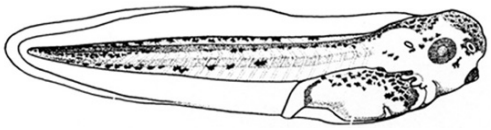
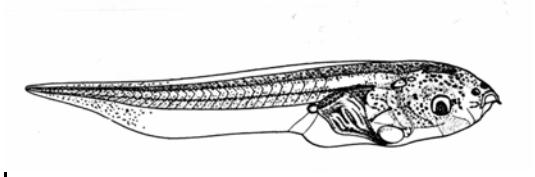
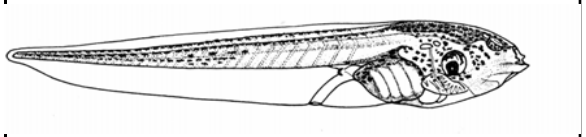
Hypergravity Research

- My lab is interested in how exposure to altered gravity may affect the development of the inner ear and the nervous system
- We are specifically interested in how chronic acceleration will affect the vestibular system during development of the inner ear
- We did a 3 yr study using long arm centrifuges at NASA Ames to provide the hypergravity stimulus
- We have analyzed control and hypergravity treated specimens for changes in gross morphology and cellular structure

Why *Xenopus*?

- Previous studies have demonstrated *Xenopus laevis* adapts well to hypergravity
- Development is well characterized (Kay et. al, 1991)
- *Xenopus* larvae have developed successfully in microgravity (Black *et. al*, 1996)

Black, S., Larkin, K., Jacqmotte, N., Wassersug, R.,Pronych, S., and Souza, K. (1996) Regulative development of *Xenopus laevis* in microgravity. *Adv. Space Res*. 17: 209-217.
Kay, B. and Peng, B., Eds. (1991) *Methods in Cell Biology: Xenopus laevis: Practical Uses in Cell Biology Acdemic press Inc.* San Diego CA.

	Stage	Length (mm)	Features
	28	3.8-4.0	Length and facial features determine stage > 1 day
	41	6.7 - 7.5	Cement “hang” stage ~3 days
	47	12 - 15	Vestibular system has developed ~5 days
	50	20 – 27	Auditory system begins to develop ~15 days

My research objective

- Isolate genes selectively induced by gravity
  - whole larvae
- Precedents
- Approach my goal using **subtractive hybridization**

What is subtractive hybridization?

Method to enrich differentially expressed genes of two populations in a library

cell type, growth phase, diseased states…

What can be done?

screen to find novel genes associated with a condition

NASA AMES 2001 Overview

- **24 ft centrifuge was used to expose stage 28, 41, 46 and 50 *X laevis* to a gravitational force of 2.0g or 3.0g together with OC and 1.0g controls**
- **Centrifuge was stopped every 2 days for animal care, observation and collection**

  Animals were staged according to Nieuwkoop and Faber (1966)

  Animals at important developmental stages were collected for histology, RNA and confocal imaging analysis

Nieuwkoop P.D and Faber, J. (1966) Normal table of *Xenopus laevis* (Daudin). North-Holland Publishing Company, Amsterdam

NASA AMES 2002 Overview

- **48 ft centrifuge was used to expose stage 28, 41, 45, 47 and 50 *X. laevis* to a gravitational force of 4.2g**
- **Centrifuge was stopped every 2-3 days for a maximum of 90 min for animal care, observation and collection**

  As in year 2, animals were collected for gene expression and morphological analysis

Preliminary Results of NASA AMES

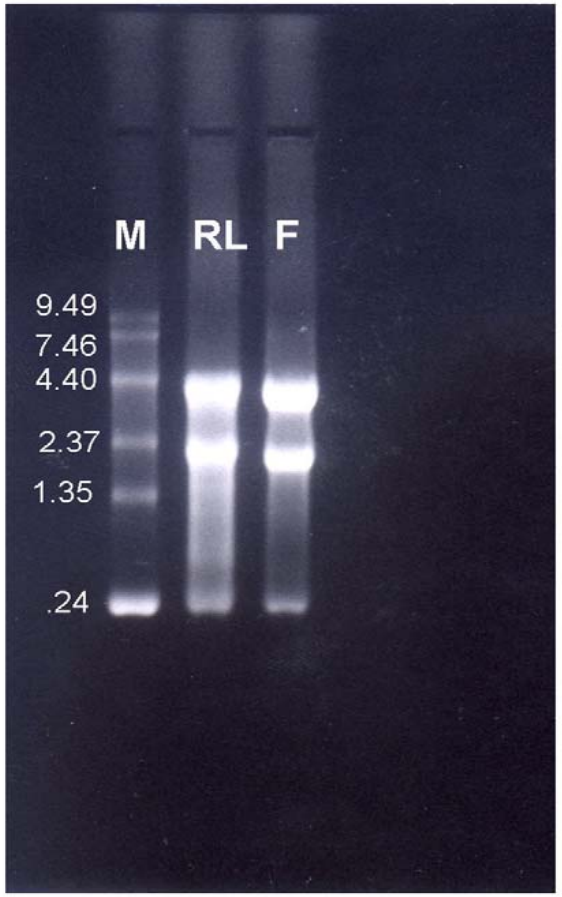
- ***Xenopus*** **exposed to gravitational forces of up to 4.2g developed similarly to those of 1.0g**
- **Survival rates of Xenopus at all conditions were similar**
- **At 4.2g, 10-30% of the specimens at stages 28 and 50 showed looping and disoriented behavior after 5-10 days of centrifugation**

Approach

- **cDNA library methods established (Serrano et. al, 2001)**
- **PCR based approaches when starting tissue is small (2µg RNA)**

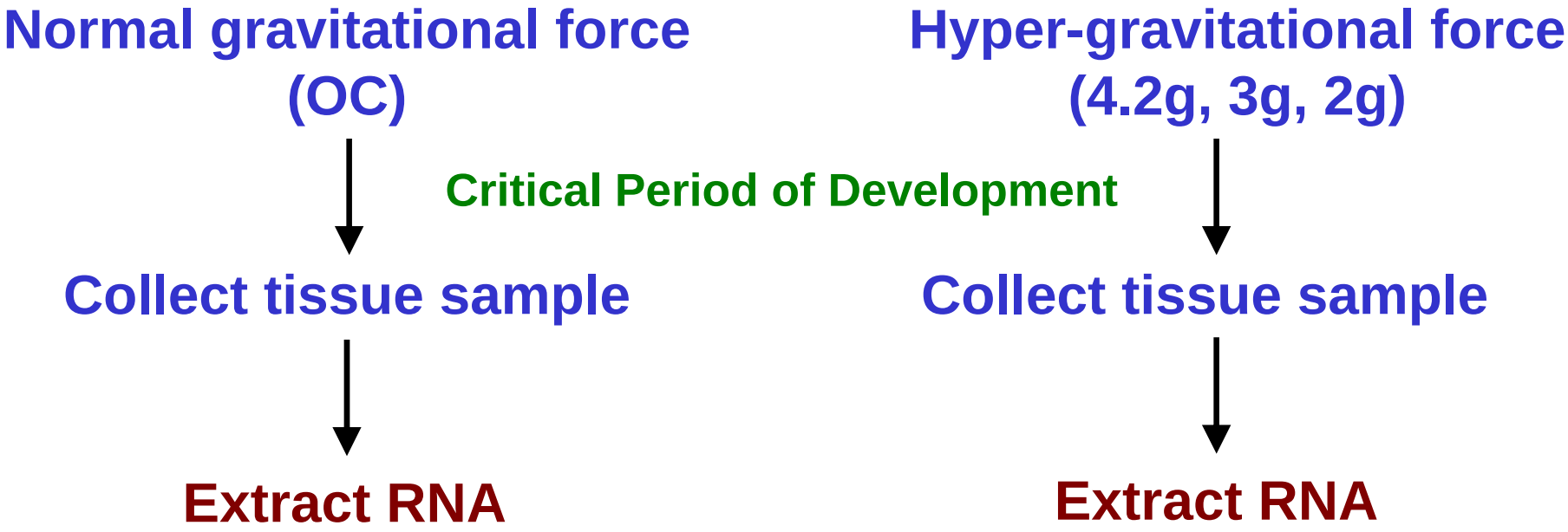
  276 ng RNA/ear

- **Prepare RNA and then hybridization and construct cDNA library**



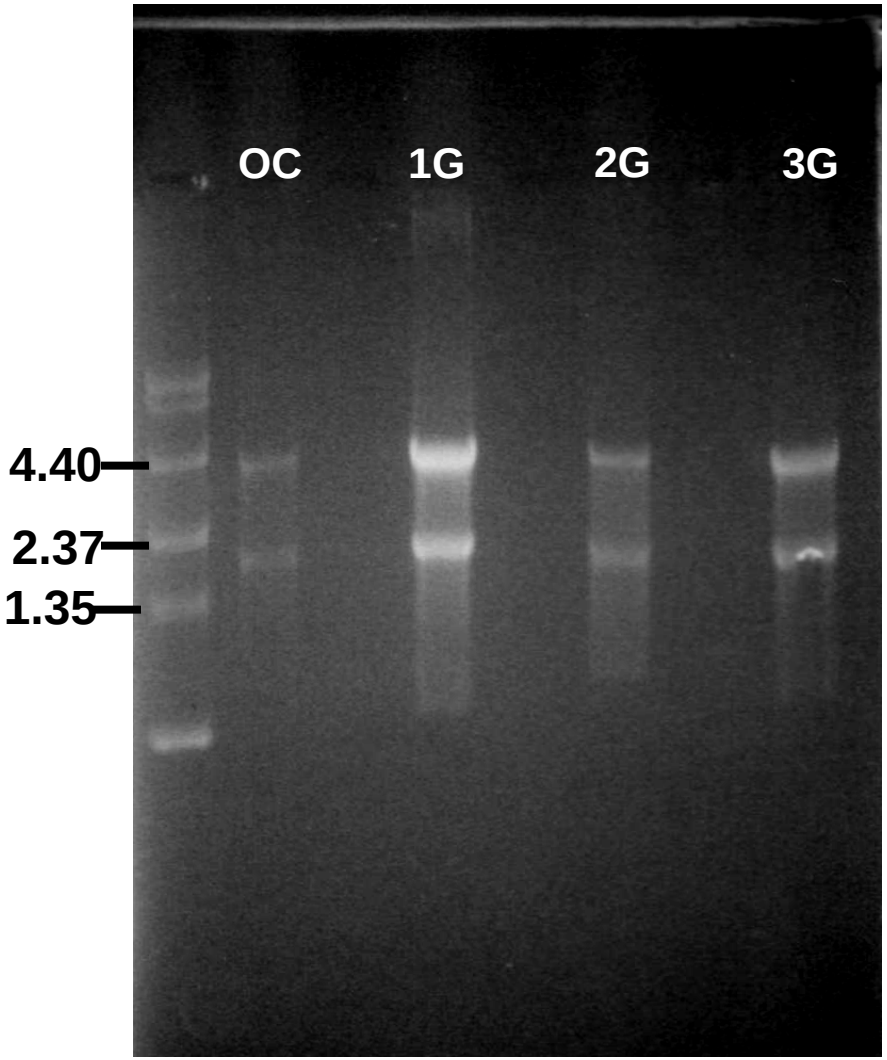
The gel compares 5 µg of RNA from NASA samples (RL) with 5 µg of total RNA isolated from "fresh" larval animals (F) at NMSU. M: Gibco - BRL RNA ladder

Serrano, E.E., Trujillo-Provencio, C., Sultemeier, D, Bullock, W. M., and Quick QA (2001) Identification of genes expressed in the *Xenopus* inner ear. *Cellular and Molecular Biology* 47*:* 1229-1239


Normal gravitational force
(OC)
Hyper-gravitational force
(4.2g, 3g, 2g)
Critical Period of Development
Collect tissue sample
Collect tissue sample
Extract RNA
Extract RNA

Condition	Start (St.)	End (St.)	Days	Samples
1.0G	28	42/43	2	24 Whole
2.0G	28	42	2	25 Whole
3.0G	28	42/43	2	25 Whole
2.0G	28	46/47	8	9 Whole
3.0G	28	47	8	10 Whole
1.0G	28	47/48	11	14 Whole
2.0G	28	47/48	11	10 Whole
OC	28	47/48	11	4 Whole

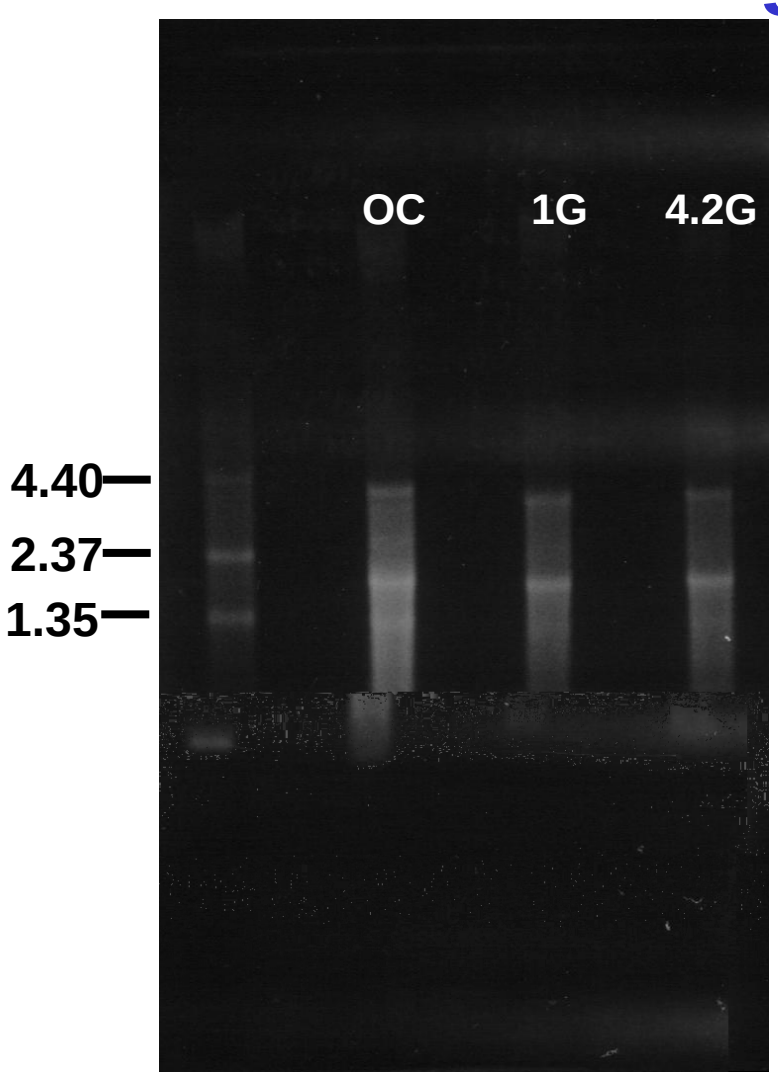
Year 2 Total RNA Has High Integrity



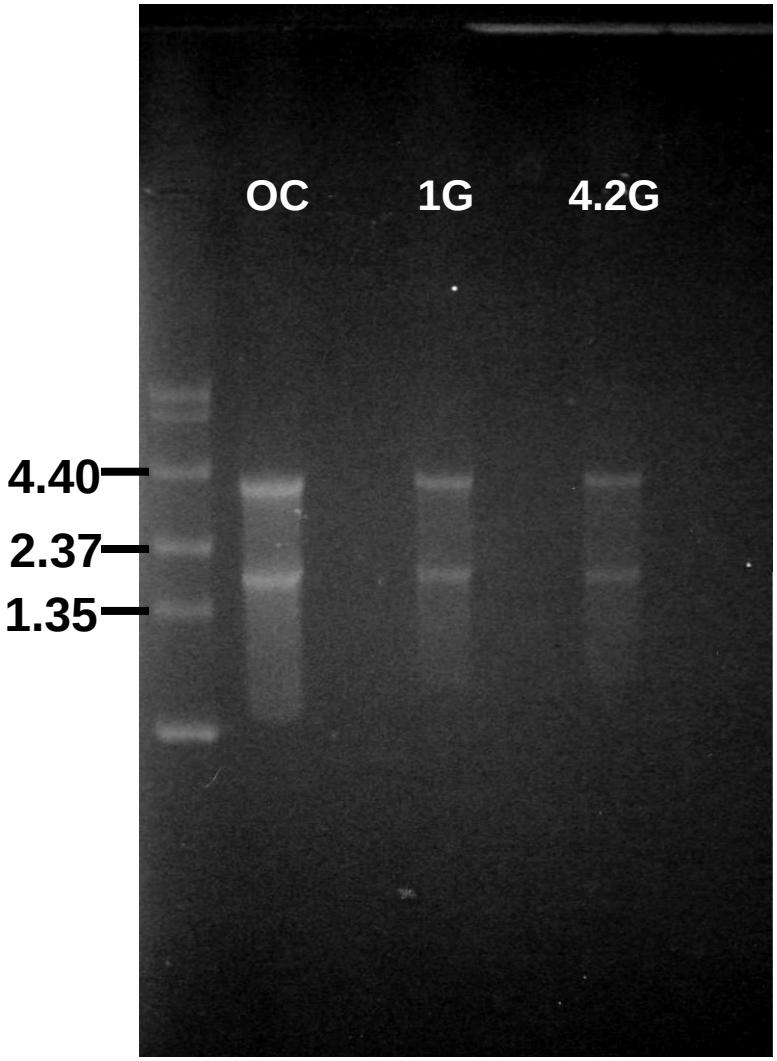
NASA 2001 Total RNA Extraction

Condition	Start (St.)	Start (mm)	End (St.)	End (mm)	Hours	Samples
4.2G	50	22.6	50	24.2	364.3	8 Ears
1.0G	50	22.6	50	25.5	364.3	8 Ears
OC	50	22.6	50	22.6	364.3	8 Ears
4.2G	26	3.3	47	13.0	357	4 Whole
OC	26	3.3	47	12.7	357	11 Whole
4.2G	28	4.2	47	12.3	171.8	6 Whole
1.0G	28	4.2	47	12.9	171.8	4 Whole
4.2G	42	NA	47	12.7	143.3	5 Whole
1.0G	42	NA	47	12.8	143.3	5 Whole
OC	42	NA	47	12.5	143.3	5 Whole

Year 3 Total RNA Samples are Slightly Degradaded



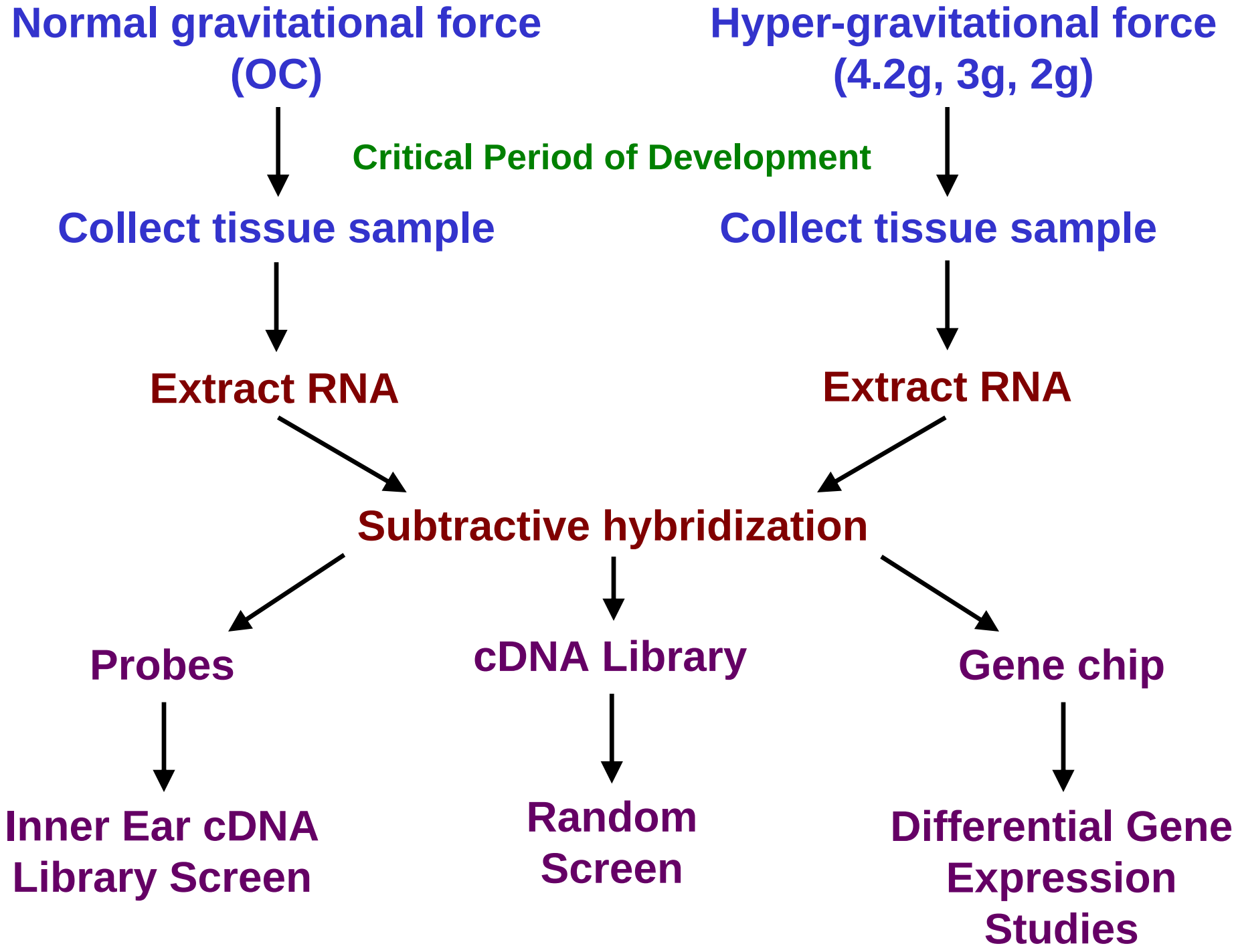
NASA 2002 St. 28 Total RNA Extraction



NASA 2002 St. 42 Total RNA Extraction

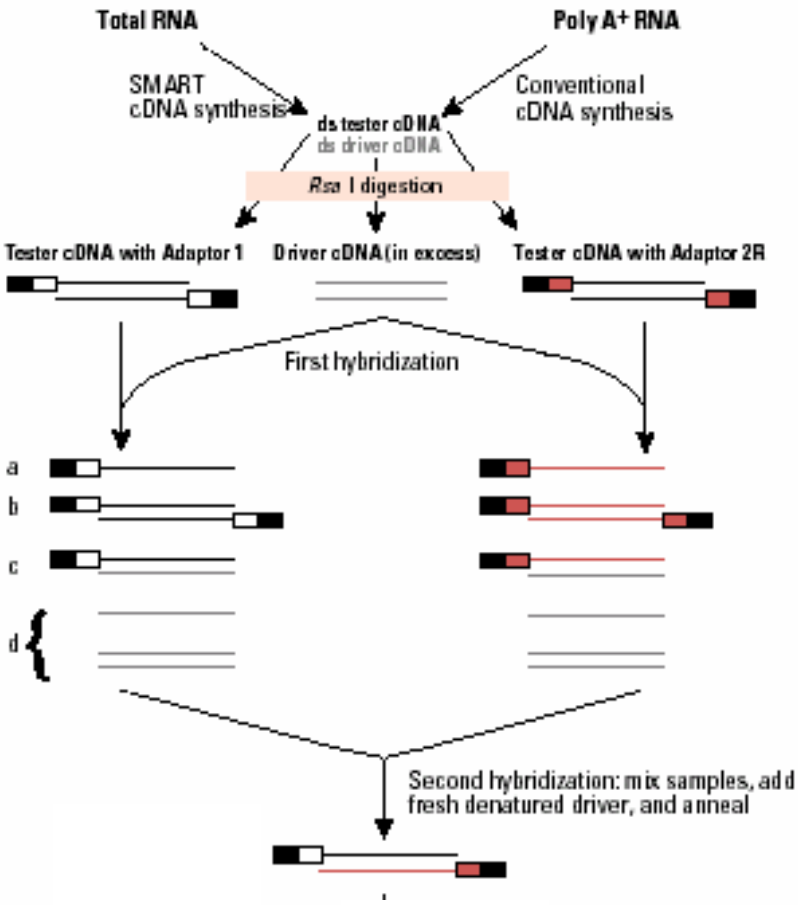
Preliminary Results: Year 2 and 3 RNA Isolation

- Year 2
  - RNA isolations have been successful
  - Visual inspection reveals RNA integrity is good
- Year 3
  - All RNA samples have been isolated
  - RNA appears to be degraded in St. 28 samples
    - St. 28 library construction is questionable
    - Paraffin embedded sample RNA extractions
  - Ear RNA isolations did not yield enough RNA to be visualized
  - Proceed with whole animal cDNA library construction


Normal gravitational force (OC)
Hyper-gravitational force (4.2g, 3g, 2g)
Critical Period of Development
Collect tissue sample
Collect tissue sample
Extract RNA
Extract RNA
Subtractive hybridization
Probes
cDNA Library
Gene chip
Inner Ear cDNA Library Screen
Random Screen
Differential Gene Expression Studies

cDNA Hybridization

Condition	Start (St.)	Start (mm)	End (St.)	End (mm)	Hours	Samples
4.2G	26	3.3	47	13.0	357	4 Whole
OC	26	3.3	47	12.7	357	11 Whole
4.2G	42	NA	47	12.7	143.3	5 Whole
1.0G	42	NA	47	12.8	143.3	5 Whole
OC	42	NA	47	12.5	143.3	5 Whole



Prepare cDNA by either SMART or conventional synthesis.

Separately digest tester and driver ds cDNA to obtain shorter, blunt-ended fragments.

Divide tester cDNA into 2 portions and ligate each to a different adaptor. Driver cDNA has no adaptors.

Hybridization kinetics lead to equalization and enrichment of differentially expressed sequences among ss tester molecules.

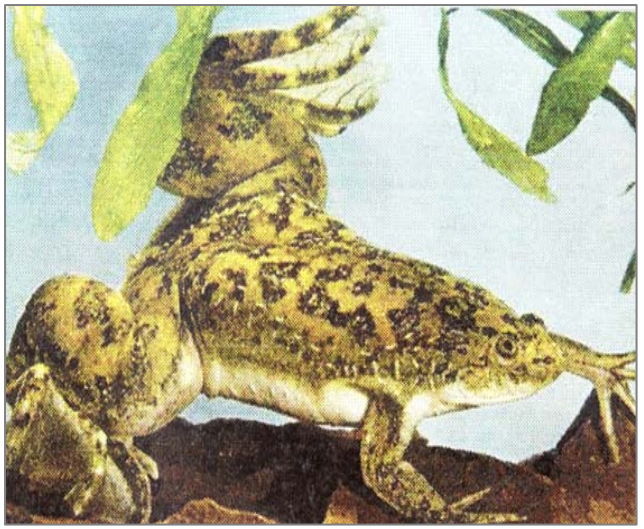
Generate templates for PCR amplification from differentially expressed sequences.

Conclusion

- Preliminary results show *X. laevis* development is affected little, if at all, by chronic acceleration
- RNA samples have been isolated from year 2 and year 3
- Two year 3 samples (St. 26 and St. 42) have been hybridized
- *X. laevis* and *X. tropicalis* inner ear libraries will be screened using DIG-labeled probes for differentially expressed inner ear genes
- Lambda phage cDNA library will be synthesized for subtracted cDNA
- *X. laevis* hypergravity subtractive cDNA libraries can be used for construction of gene chips for microarray analysis in future hypergravity studies

Acknowledgements

- Dr. Elba E. Serrano
- Members of the Serrano Laboratory
- Members of the NASA AMES Research Facility
- Funding provided by NMSGC Graduate Fellowship (2002/2003)
- Supported by a NASA MURED FAR award to EES



MOVIE!!!!