The Application of Biotechnology in Larvi- and Aquaculture

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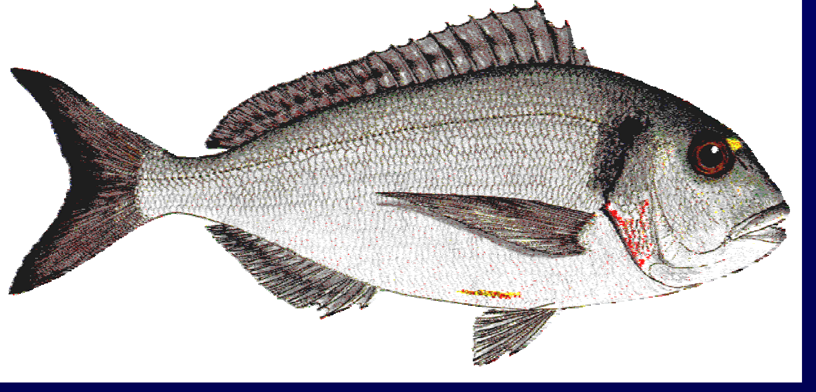
Aquaculture: Meeting the Challenge

- Currently a 30 million ton, $45 billion industry, aquaculture must increase production 3-4 times by 2025
- Become more efficient and cost-effective
- Overcome biological obstacles
- Strong input from modern biology and biotechnology



Principle Platforms of Biotechnology

- Chromosome-set manipulation
- Fermentation
- Cloning
- Protein expression and production
- Gene transfer and transgenics
- Genetic immunization
- DNA fingerprints and genetic markers
- Genomics / bioinformatics
- Post / functional genomics

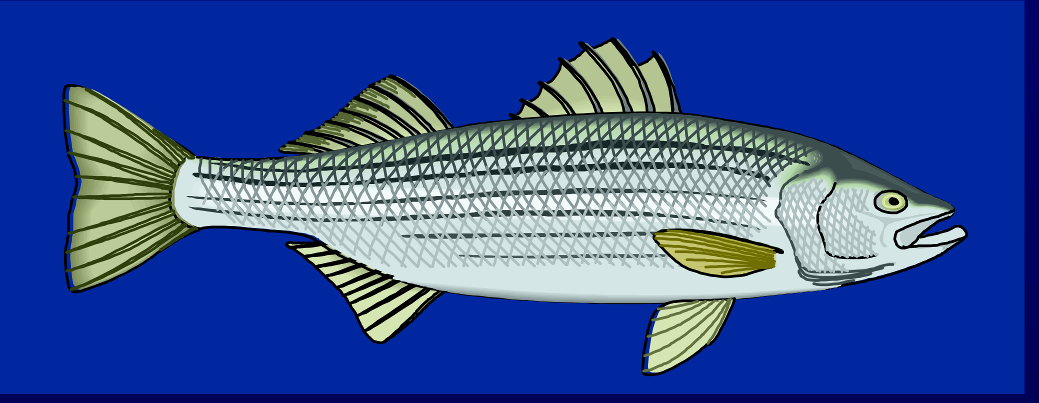
"Bottlenecks" in Commercial Aquaculture

- Reproduction
- Early (larval) development
- Growth
- Nutrition
- Disease / health management
- Interactions with the environment
- "Biosecurity"

Reproduction: The Issues

- No ovulation and spawning
- Unpredictable ovulation and spawning
- Reproduction / growth interactions
- Sterility

Gilthead seabream

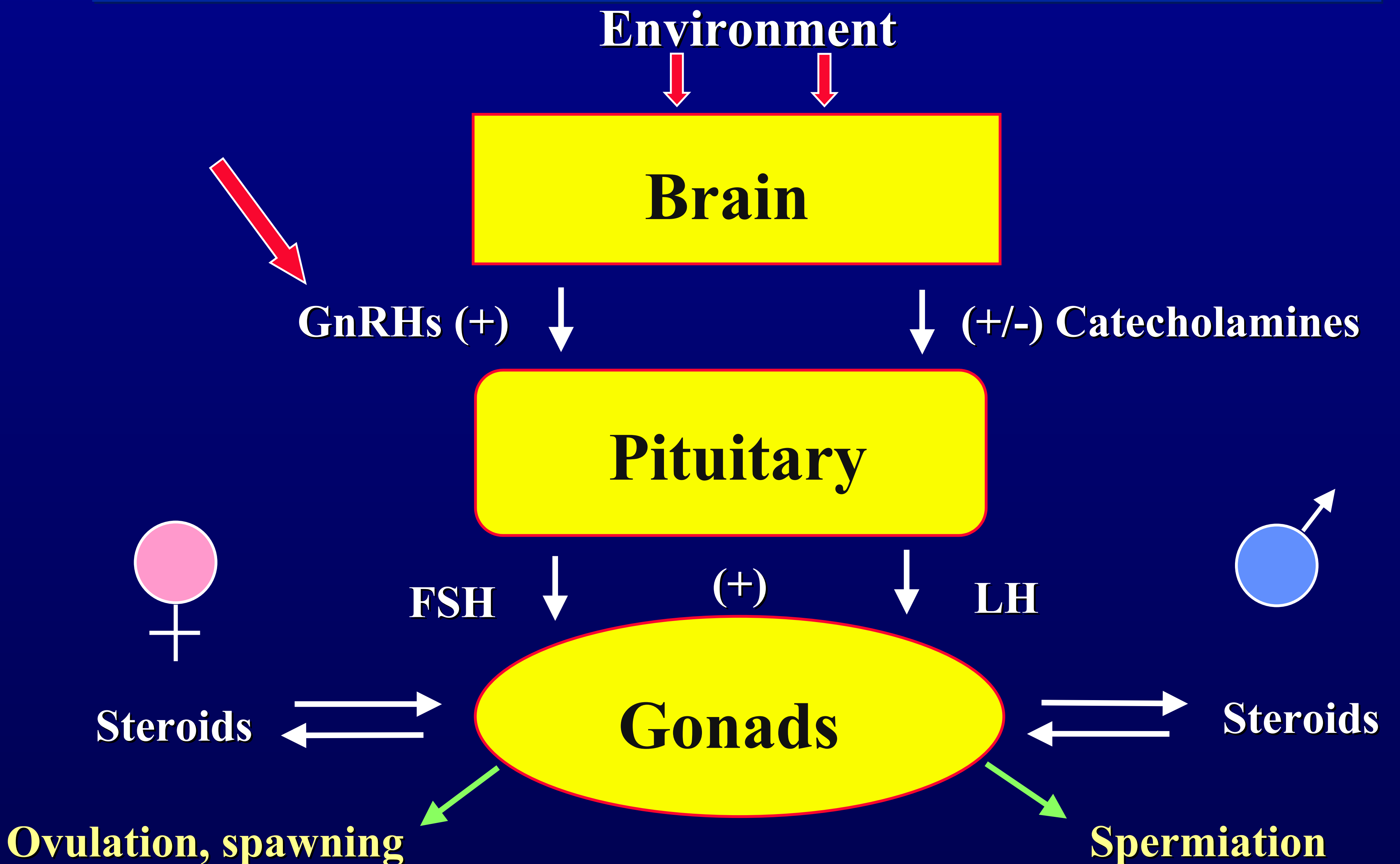


Striped bass

Captive versus….

Wild fish

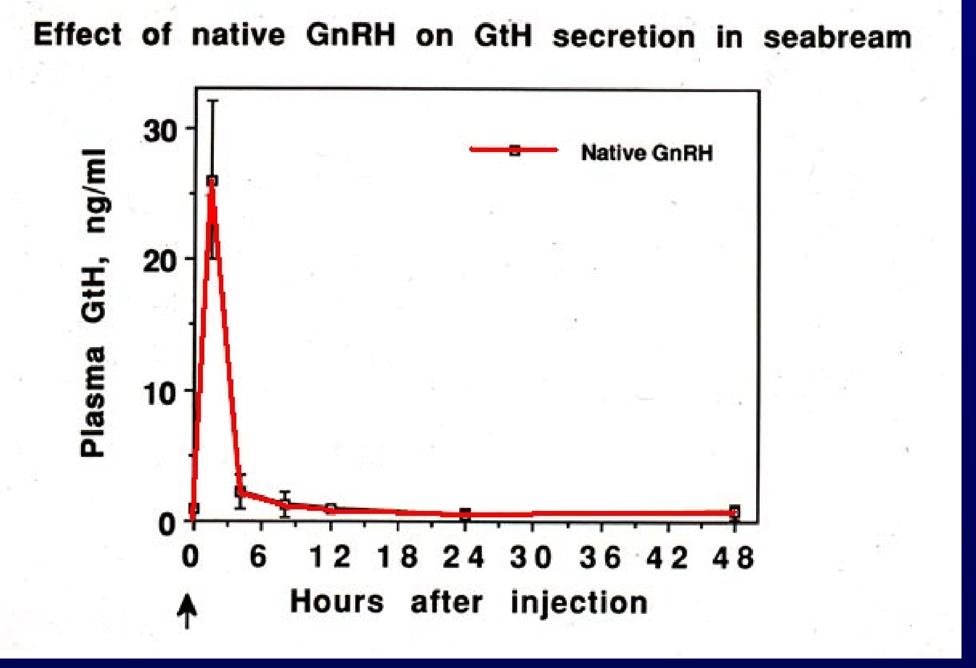
Regulation of Fish Reproduction



Fish Gonadotropin-Releasing Hormone (GnRH)

1 2 3 4 5 6 7 8 9 10

pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-GlyNH2

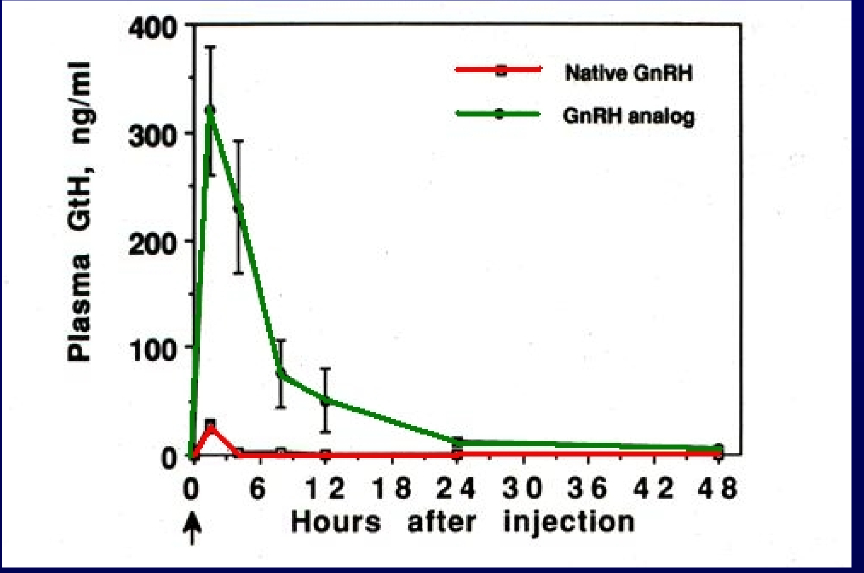


Peptide Engineering: GnRH and GnRH Analogs

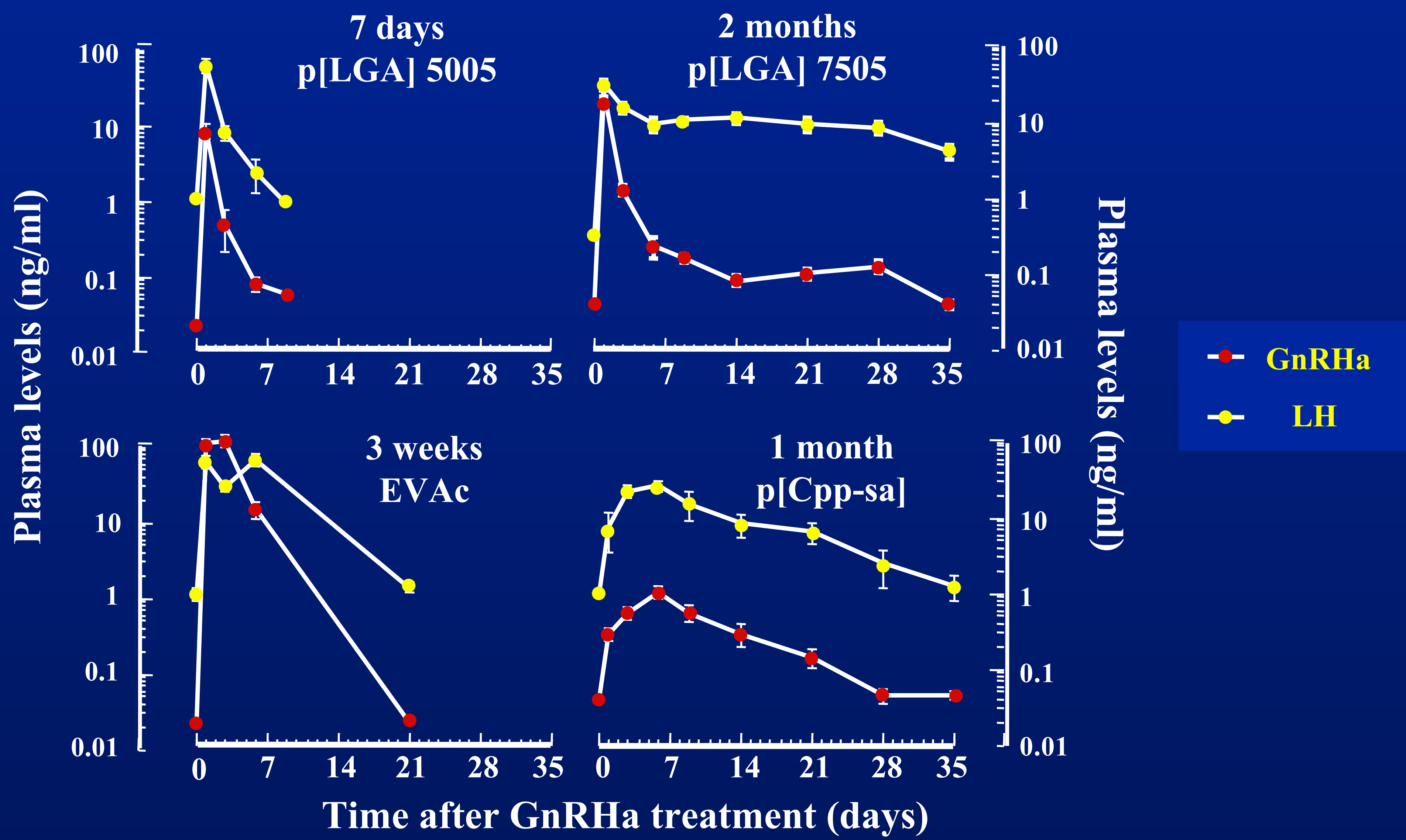
1 2 3 4 5 6 7 8 9 10

pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-GlyNH2

pGlu-His-Trp-Ser-Tyr-DAla-Trp-Leu-Pro-NEt



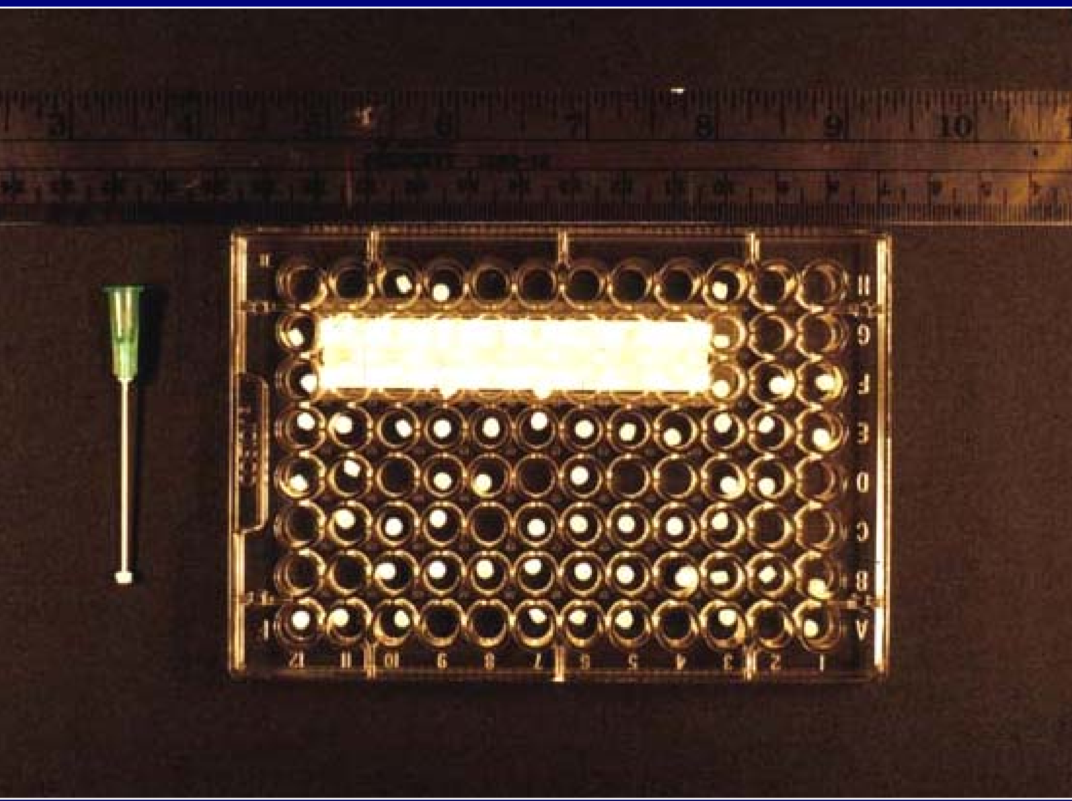
GnRHa and LH Levels After Treatment With Different GnRHa Sustained-Release Delivery Systems



Application of GnRHa Sustained Release Technology to Induce Ovulation, Spawning and Sperm Production

- Gilthead seabream
- Red seabream
- Red porgy (Pagrus)
- Dentex
- European seabass
- Striped bass
- White bass
- American shad
- Gray mullet
- Grouper
- Turbot
- Plaice
- Flounder
- Sturgeon
- Snook
- Pompano
- Atlantic salmon
- Coho salmon
- Sockeye salmon
- Chinook salmon
- Rainbow trout
- Brown trout
- High hats

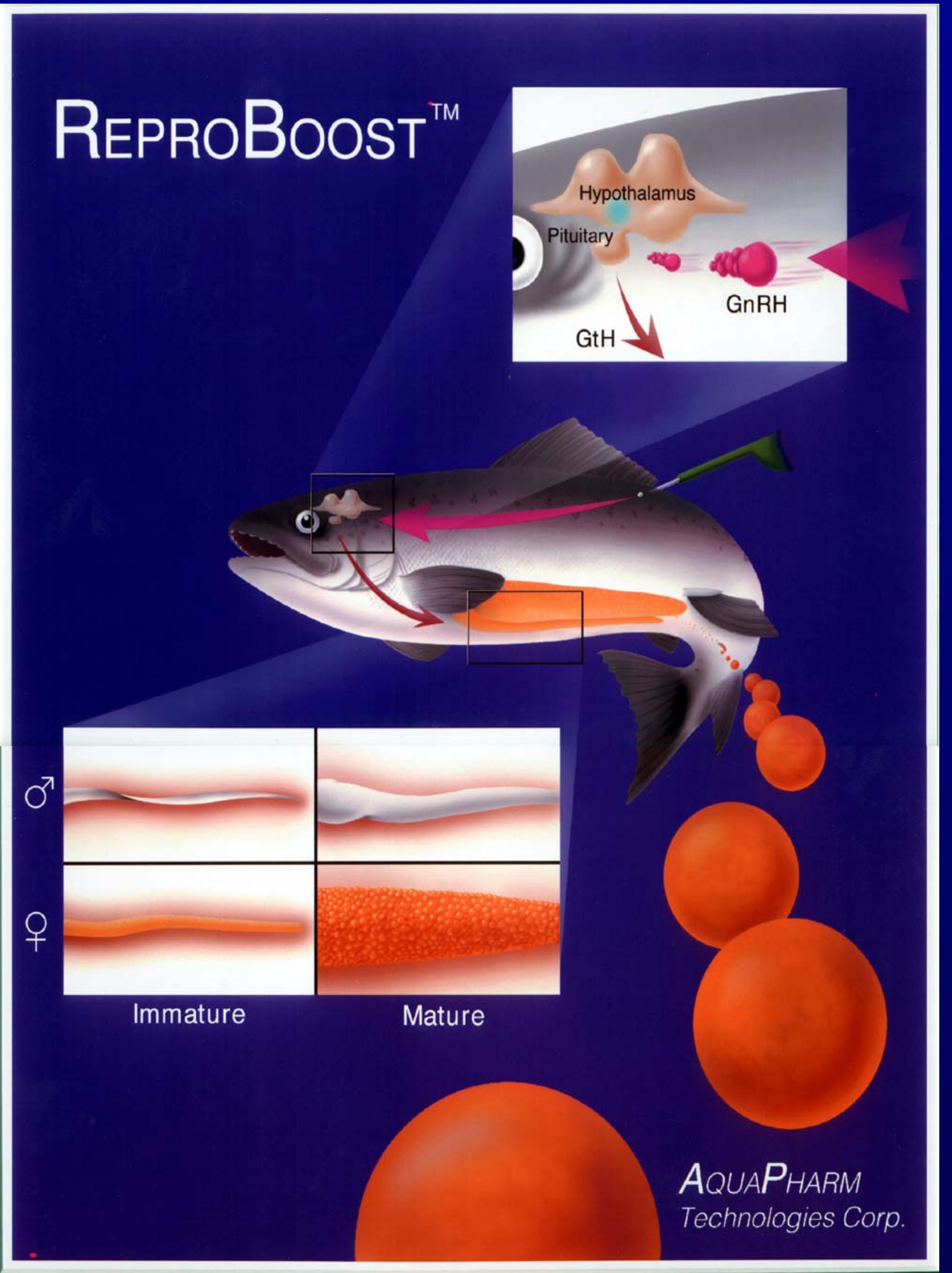
GnRHa-based delivery systems



Implants

Microspheres

GnRH implants are commercially available



Emerging Technologies for the Manipulation of Spawning

Understand the nature of the captivity-induced alterations in the GnRH system and correct them

??

Inducer (environmental, hormonal)

?

Promoter	Reporter: GnRH

?

GnRH mRNA

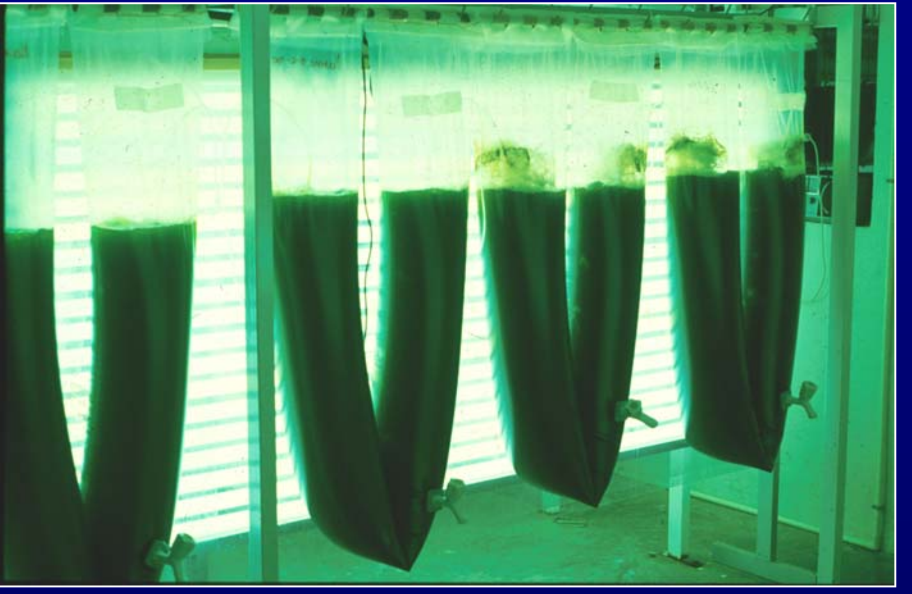
?

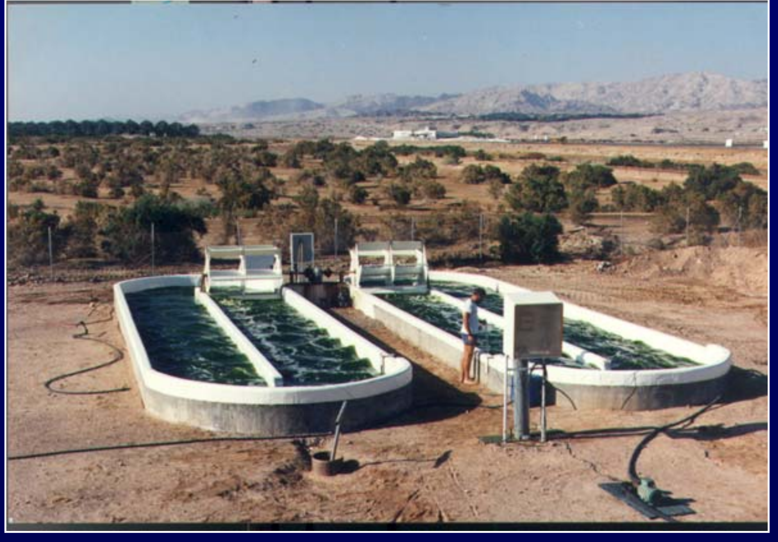
FOM, Ovulation & Spawning

Early Development:
The Issues

- Low survival rates
- Dependency on live food
- Very specific nutritional requirements

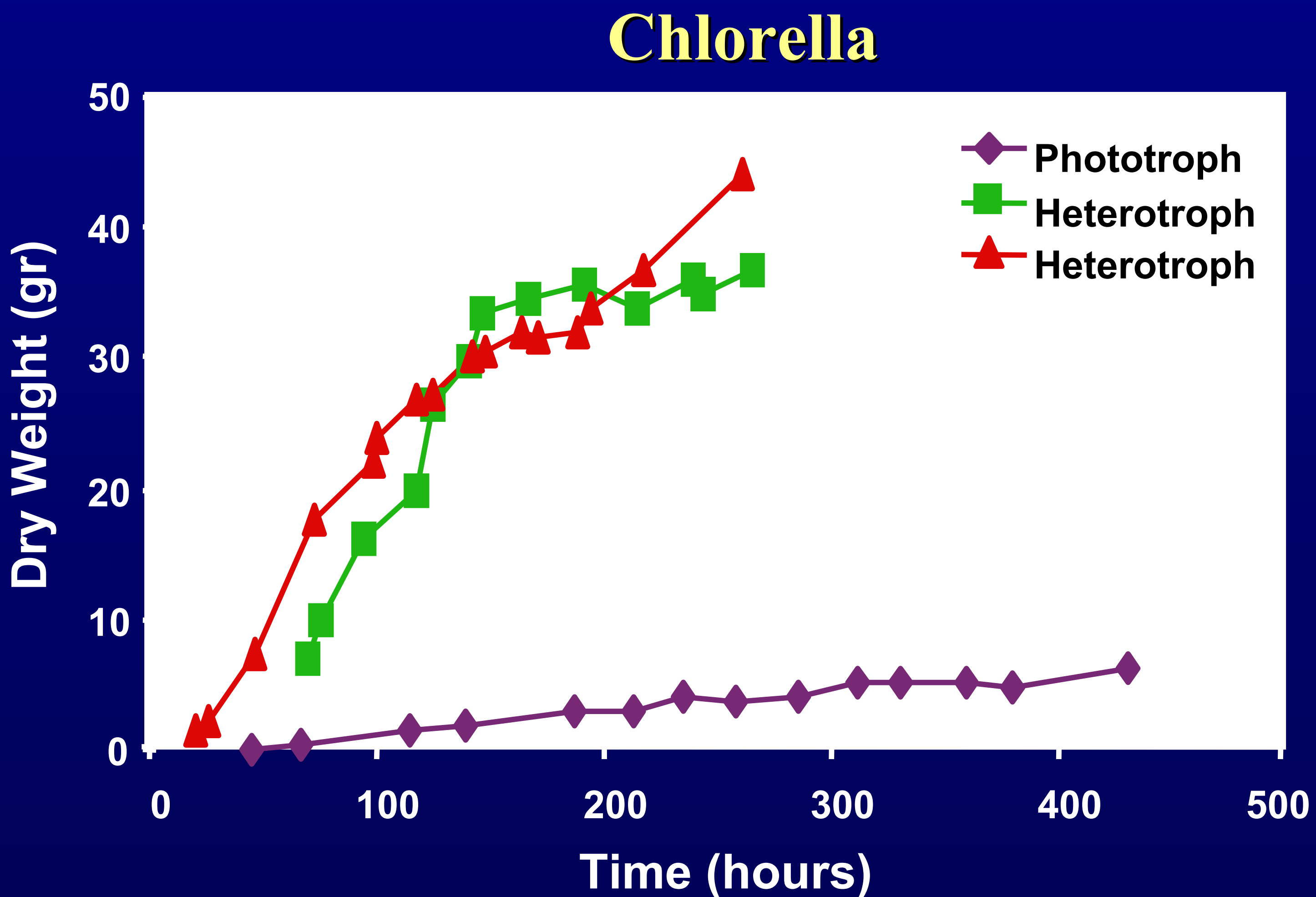
"Food Chain" in Larviculture





O. Zmora et al., NCM

Heterotrophic Mass Culture of Algae

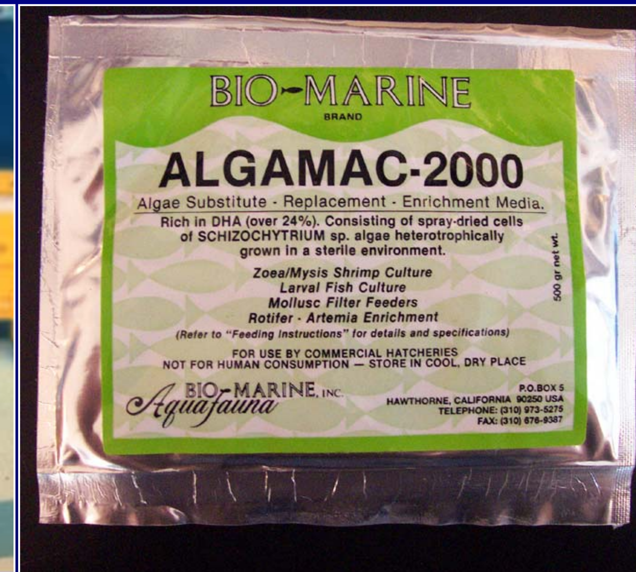


Algae Species Grown Heterotrophically

Cyclotella
Ankistrodesmus
Chlamydomonas
Chlorella
Chlorococcum
Crypthecodinium (DHA)
Amphora

Dunaliella (pigments)
Euglena
Nannochloropsis
Nitzschia (EPA)
Ochromonas
Tetraselmis
Schizochytrium

Lipid and DHA/EPA Ratios in Artemia
A Comparison of Various Enrichment Diets

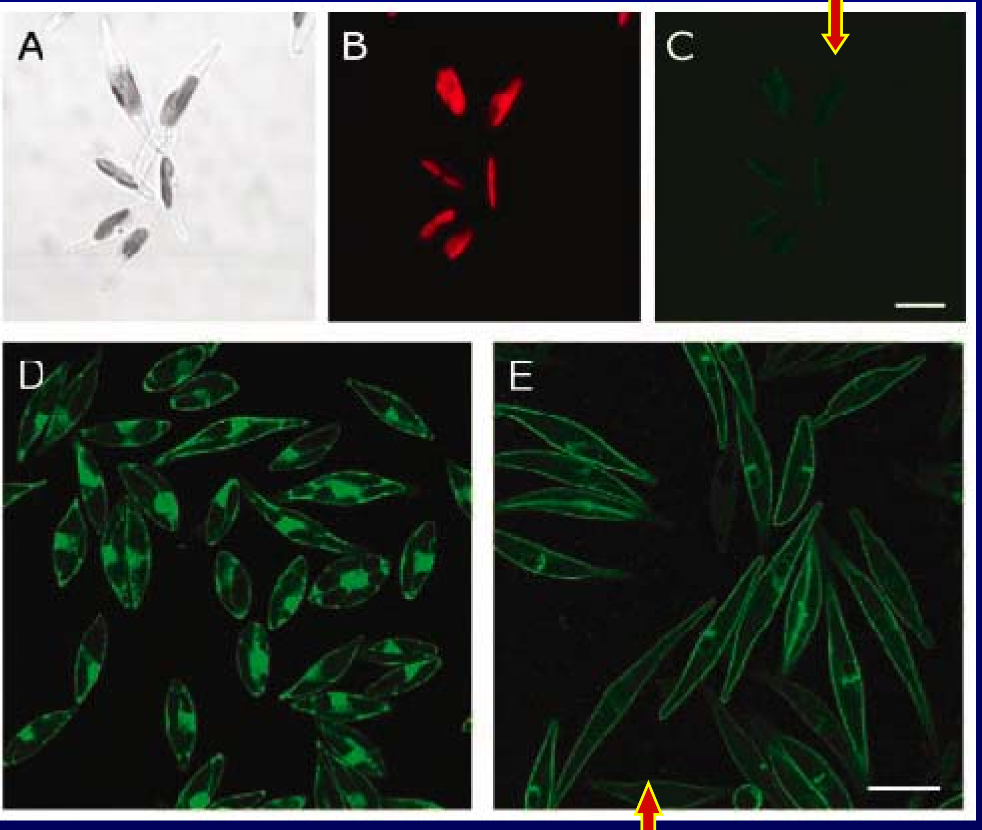


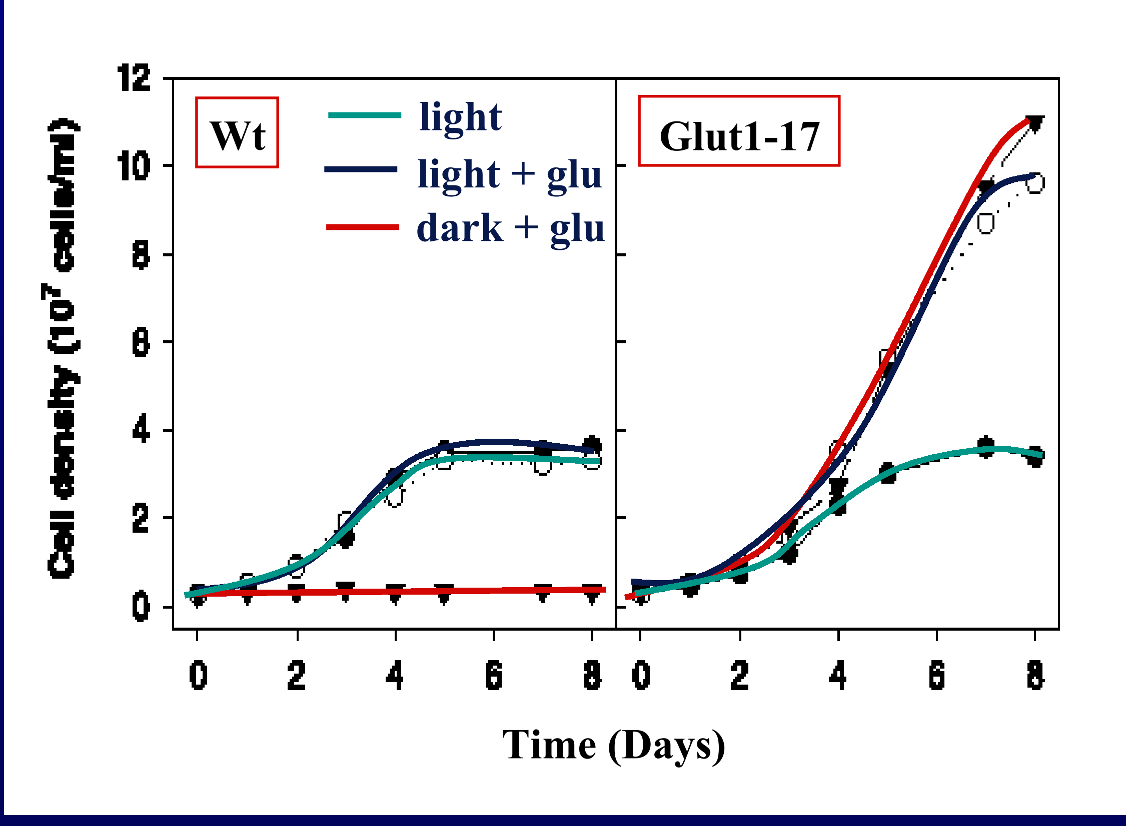
Diet	Lipid (%)	DHA/EPA Ratio
Microdiet	15.08 ± 1.47	0.78 ± 1.47
DHA Selco	20.58 ± 0.28	1.4 ± 0.1
Algamac 2000	17.70 ± 3.80	2.3 ± 0.1
Aquagrow	26.36 ± 2.58	3.75 ± 0.17

Harel, Place & Behrens, 2000

Genetic Engineering of Heterotrophic Microalgea

Control





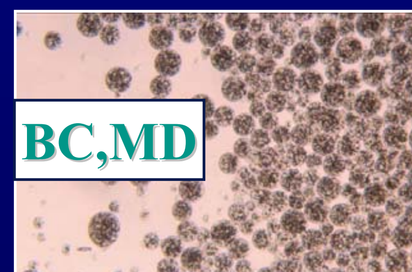
Glut1-GFP transformed

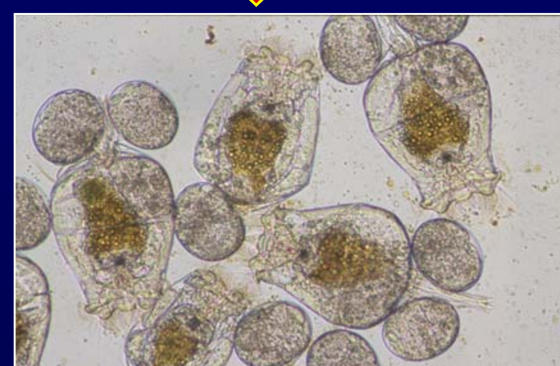
Zaslavskaia et al., Science, June 2001

Delivery of Beneficial Compounds to Larvae
(The Ghent Group!)

Recombinant
(algae, bacteria, yeast)

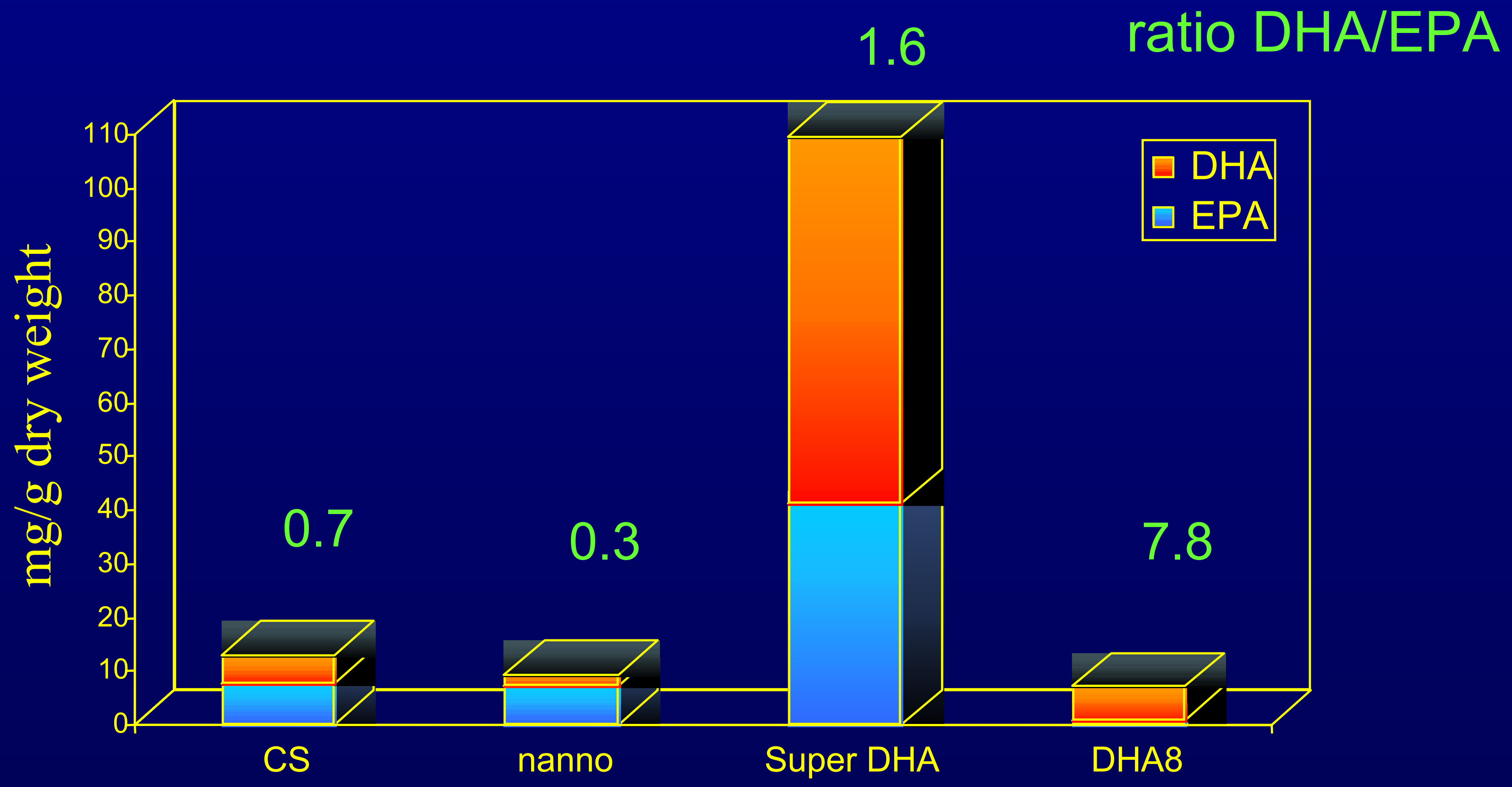
- Nutrients
- Therapeutics
- Vaccines
- Hormones
- Enzymes
- Pigments
- DNA





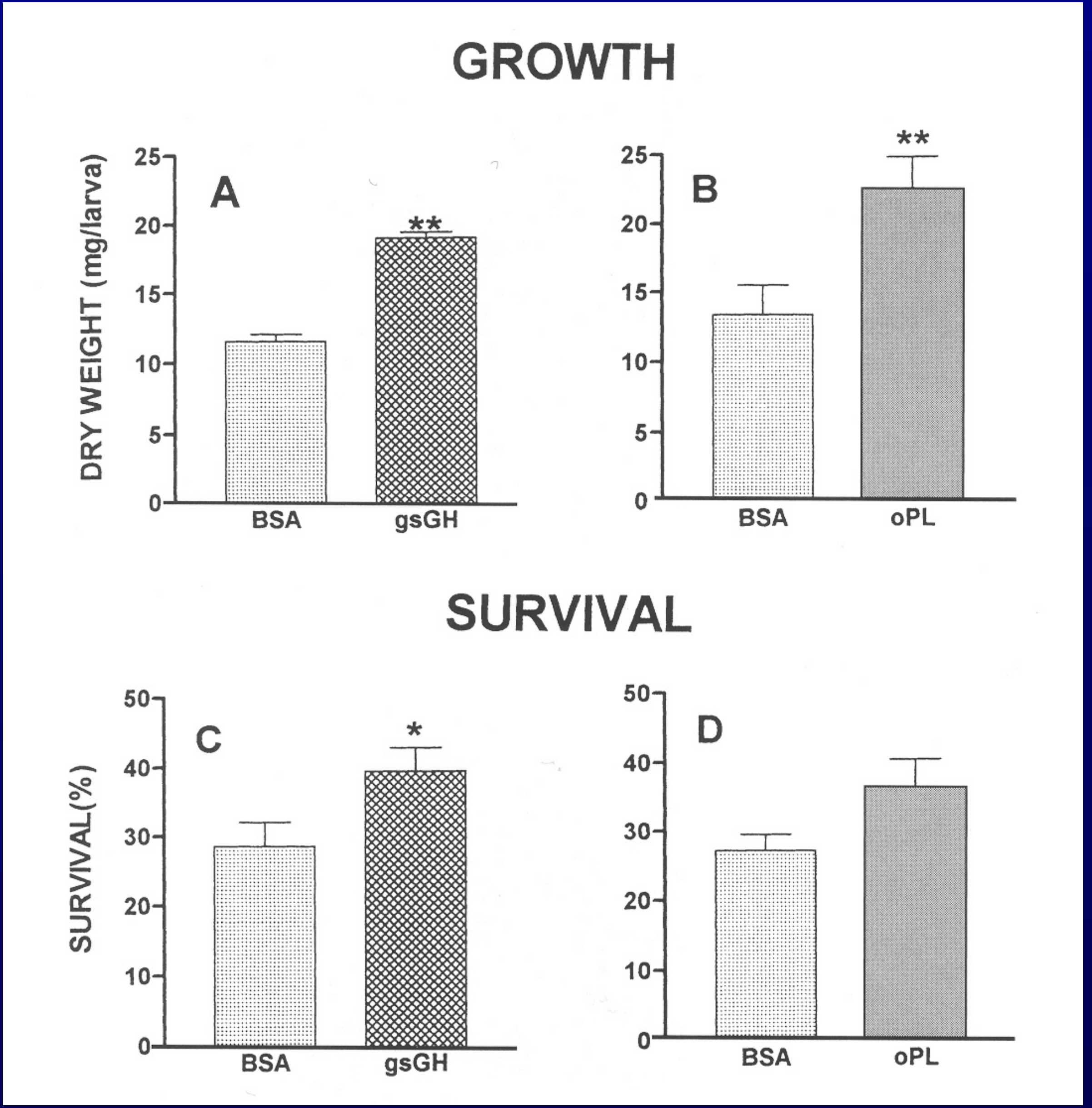
- Improved
- Genetically modified

Fatty acid concentration in enriched rotifers



P. Sorgeloos, Ghent University

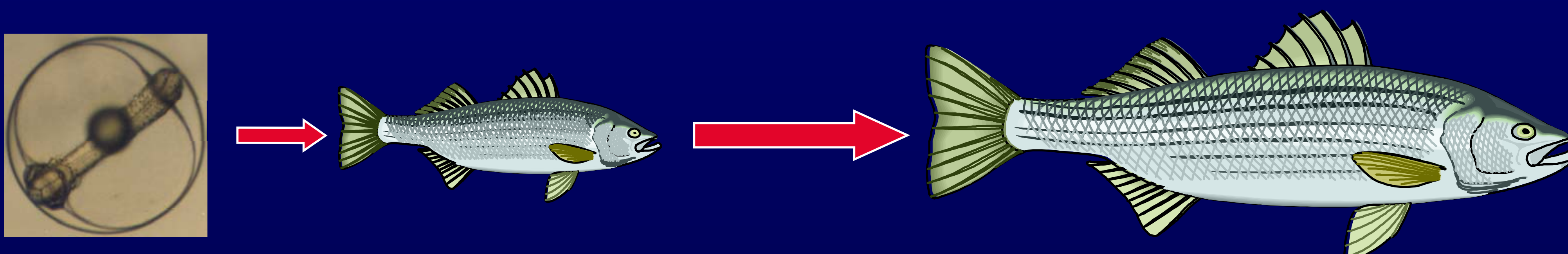
Recombinant Growth Hormone: Enhancing Larval Growth and Survival in Seabream



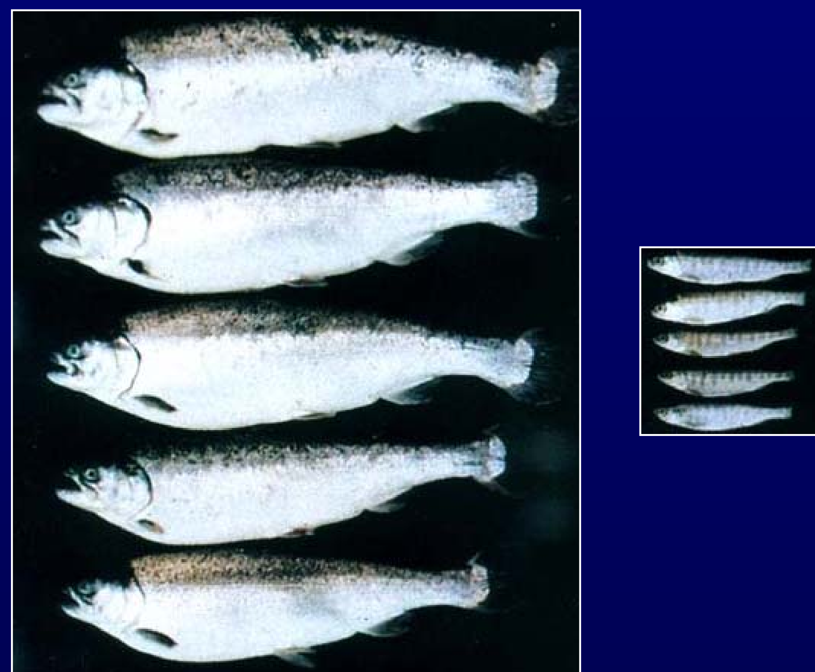
Ben-Atia et al., 1999

Growth:
The Issue

- Long time to market size

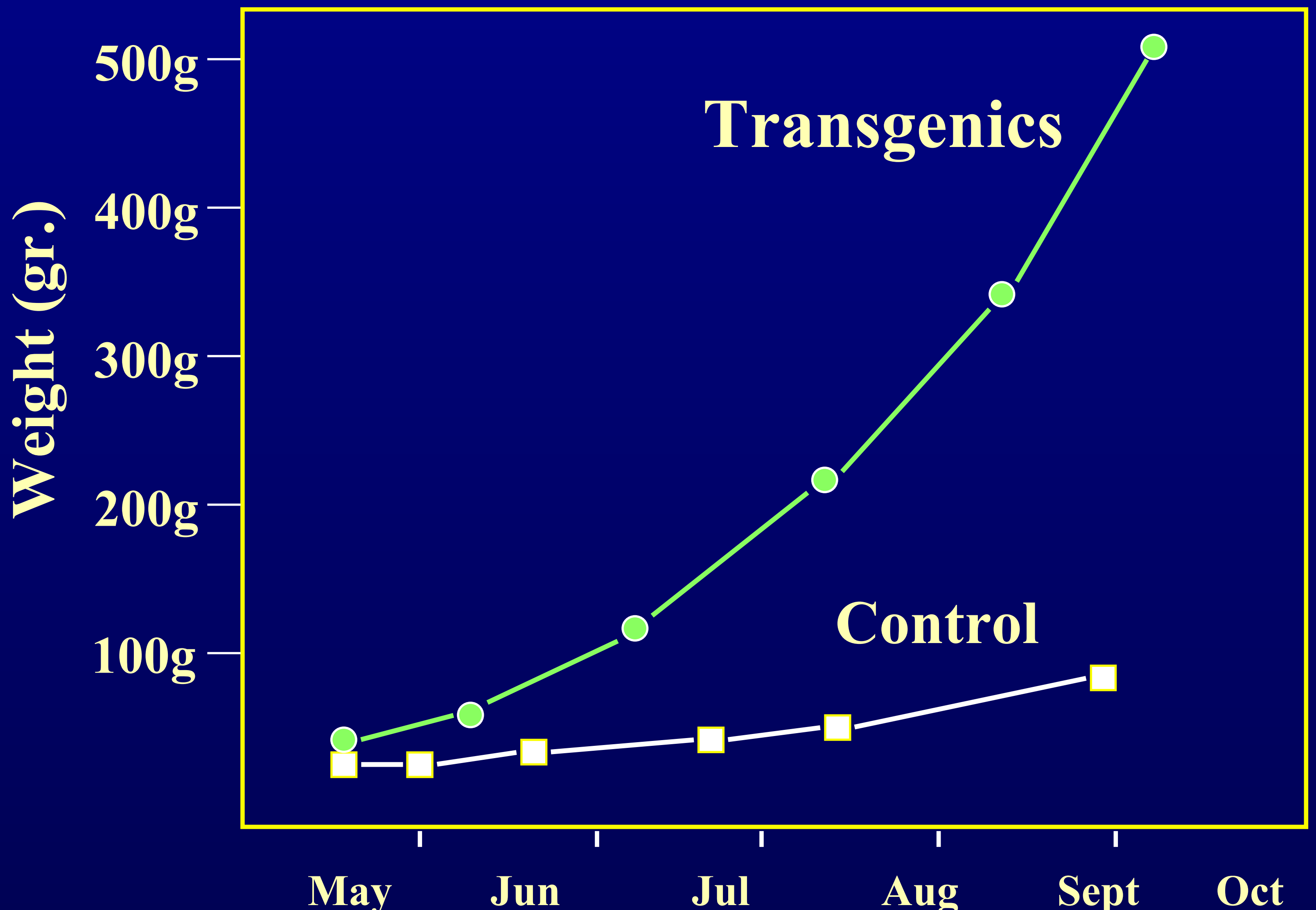


“Extraordinary Salmon Growth”



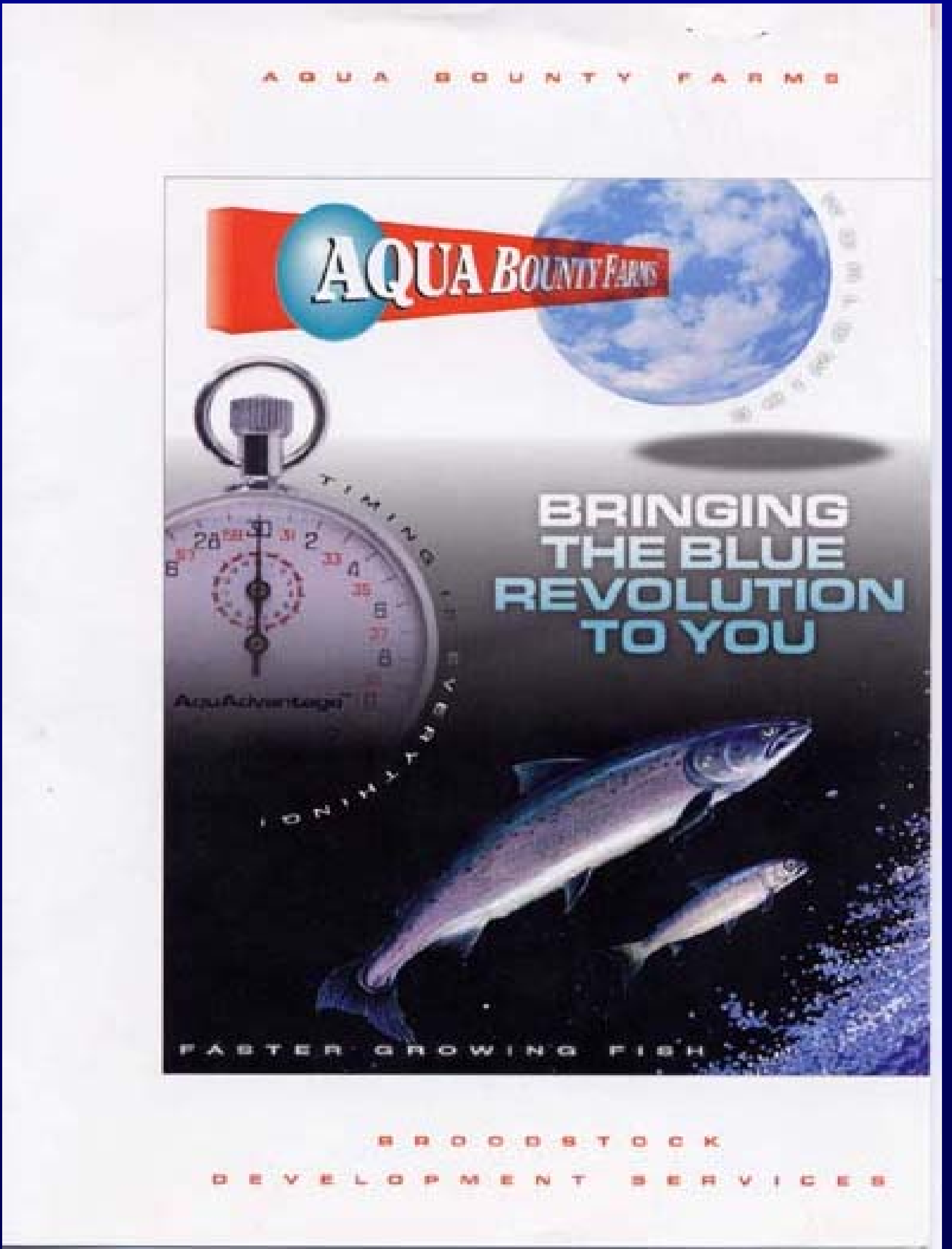
Devlin et al., 1994
(coho salmon)

Growth Rate in Transgenic Atlantic Salmon



Hew, C.L. and Fletcher, G., 1997

Transgenic, Fast Growing Salmon Are Commercially Available



Transgenic Fish: Public Concerns

SCIENCE

Make Way for Frankenfish!

What happens to these ordinary salmon if the genetically modified lunkers ever get loose?

By FREDERIC GOLDEN

WHETHER SERVED AS RAW SUSHI, grilled steak or in thin smoked slices, most of the salmon you eat these days is not the sleek sport fish that has been a favorite of anglers since Izaak Walton but rather a chunky, sluggish creature raised in captivity. Indeed, salmon caught in the wild accounts for less than half of all salmon sold in the U.S.

Now gene splicers have cooked up a replacement that sounds like a fish tale: a veritable superfish, one that can grow at least twice as fast, resist disease and outmate competitors. If approved, it could provide protein to millions of people at a time when fish stocks are perilously low. But as you might expect, some critics are carping. They consider the supersalmon a biological time bomb that could destroy the remaining natural salmon populations and wreak other environmental havoc. To them, the supersalmon is nothing less than a "Frankenfish."

Unlike other genetically modified foods—so-called Frankenfoods—the supersalmon was born almost accidentally. About 20 years ago, a fish researcher in Newfoundland found that even though his saltwater tank had frozen, the flounder in it survived. Adapted to icy Canadian waters, the fish turned out to have a gene, known in other polar fishes, that produces an antifreeze protein. While trying to splice this gene into salmon so it too could be grown in colder waters, scientists made a second accidental discovery: they found that while the gene didn't keep the salmon from freezing, a portion of it, when stitched onto a salmon's growth-hormone gene, greatly speeded development—up to five or six times as fast as in the early months and about twice as fast overall. Patenting their discovery, the scientists started a company in Waltham, Mass., called A/F Protein (A/F stands for antifreeze).

The company has 10,000 to 20,000 Atlantic supersalmon swimming in endless circles in 136 tanks at three locations in Canada's Maritime provinces. The hope is that these fish will soon be producing eggs for commercial aquaculture not just in Canada but in New Zealand, Chile and the U.S. as well. By turning to the supersalmon, says Elliot Entis, A/F's president, fish farmers could double production without doubling costs because the fish converts food into body mass so much more efficiently than ordinary salmon. That, he says, would mean "more fish for more people at a lower price."

But this so-called blue revolution may not reach U.S. shores for a while. Although gene scientists in the U.S. have been tinkering with a variety of marine creatures—not only salmon and trout but also carp, catfish, tilapia and shrimp—these efforts are drawing criticism similar to that directed at genetically modified foods. Opponents, who complain about the fertilizers and other pollutants released into coastal waters by the fish farms, are especially concerned about the potential impact on the gene pool. They note that domesticated fish regularly escape from their pens into the wild and breed with native stocks, upsetting the balance of nature.

No one knows what ripple effects might occur if the new supersalmon escaped into the wild. One of the few studies done by U.S. researchers found a lower survival rate for eggs produced by transgenic fish. Still other studies show that despite their name, so-called superfish have diminished muscle strength and swimming performance. Says Canadian fish geneticist Robert Devlin: "Science, at the moment, is unable to give us a reliable assessment of risk."

Entis and others reply that whatever the risk, it could be lowered to almost zero by raising the fish in closed tanks rather than in storm-exposed pens. Still another tactic under consideration is shocking the fertilized eggs so they create fish that cannot reproduce—a marine equivalent of the self-destructing terminator gene that [illegible] once considered putting in patented plant seeds.

Fearing a consumer backlash, New Zealand King Salmon, a major producer of Chinook salmon—the largest Pacific salmon—announced last week that it was suspending its gene-modification experiments. Entis, by contrast, believes he can win acceptance of his supersalmon through public education. "We have to show we have nothing to hide," he says.

But don't count on getting supersalmon lox on your Sunday-morning bagel anytime soon. The Food and Drug Administration must first approve introduction of the fish into the U.S., something that probably won't happen before 2001. —Reported by Dick Thompson/Washington

Bigger the Better?

These salmon are siblings, yet one grew spectacularly, thanks to a gene transplant

15.8 in. (40 cm)

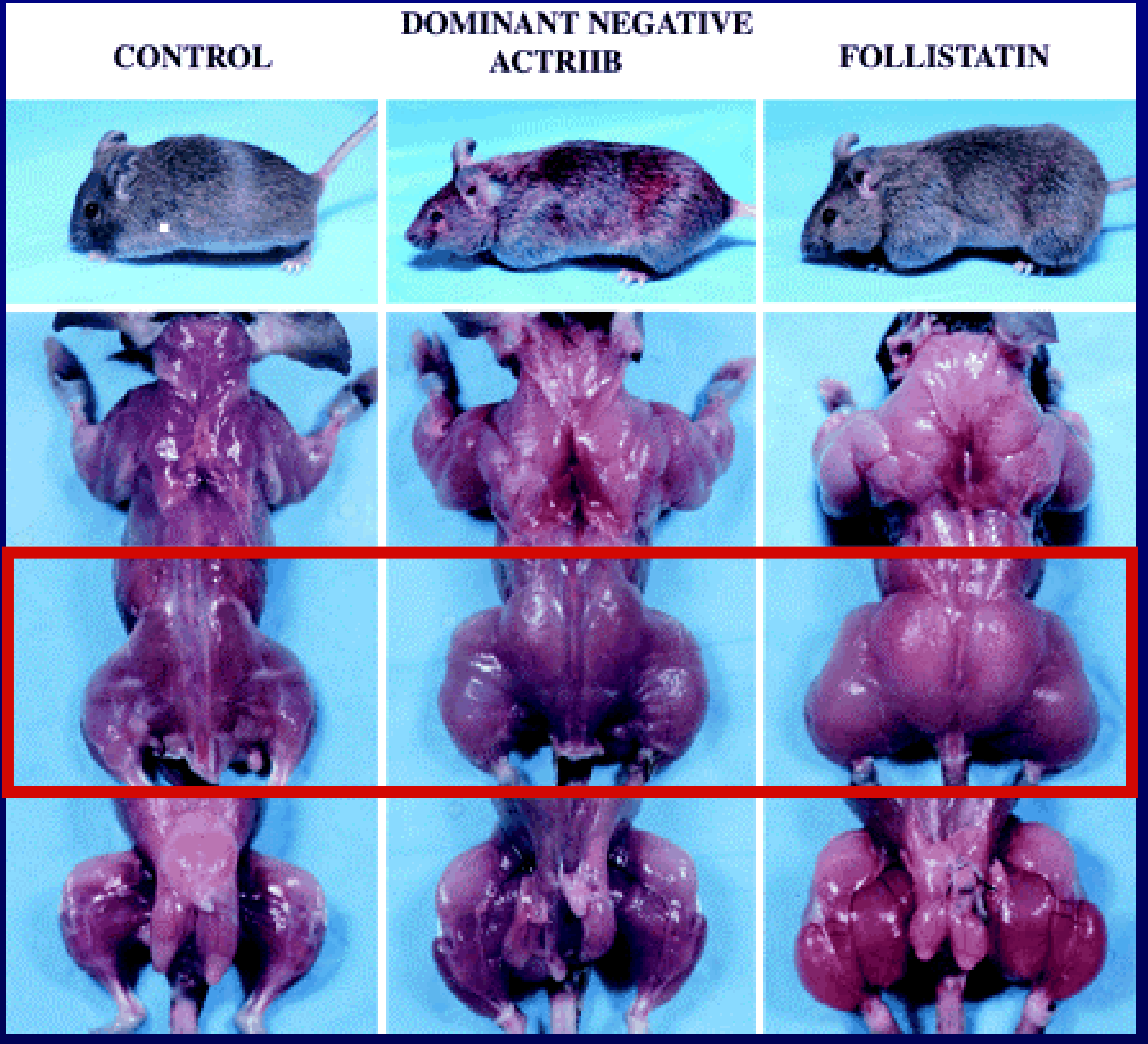
4.8 in. (12 cm)

Time Magazine, March '00

Transgenic Fish: Addressing Public Concerns

- Use “all fish” constructs
- Avoid viral promoters
- Make the transgenic fish sterile
- Contain GM fish in recirculated aquaculture systems

Enhancing Muscle Growth by Regulating Myostatin (GF) Activity



Lee and McPherron; PNAS, August 2001

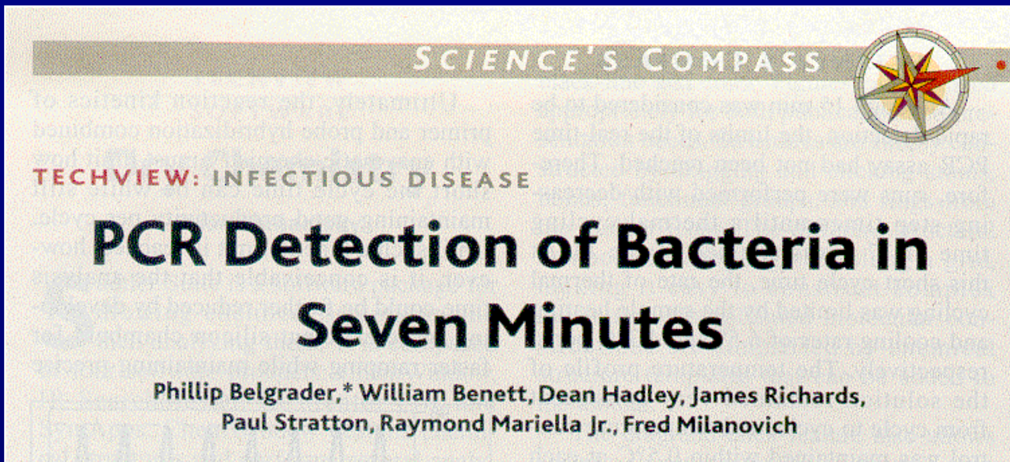
Disease / Health Management: The Issues

- Intensification of aquaculture is accompanied by increased susceptibility to pathogens
- Losses due to disease are in the billion $ range annually

Animal Health: The Challenges

- Early diagnostics (histology, immune, molecular)
- Efficient vaccination (microbes, expressed antigens, DNA)

“Real-time” Quantitative RT-PCR: TaqMan



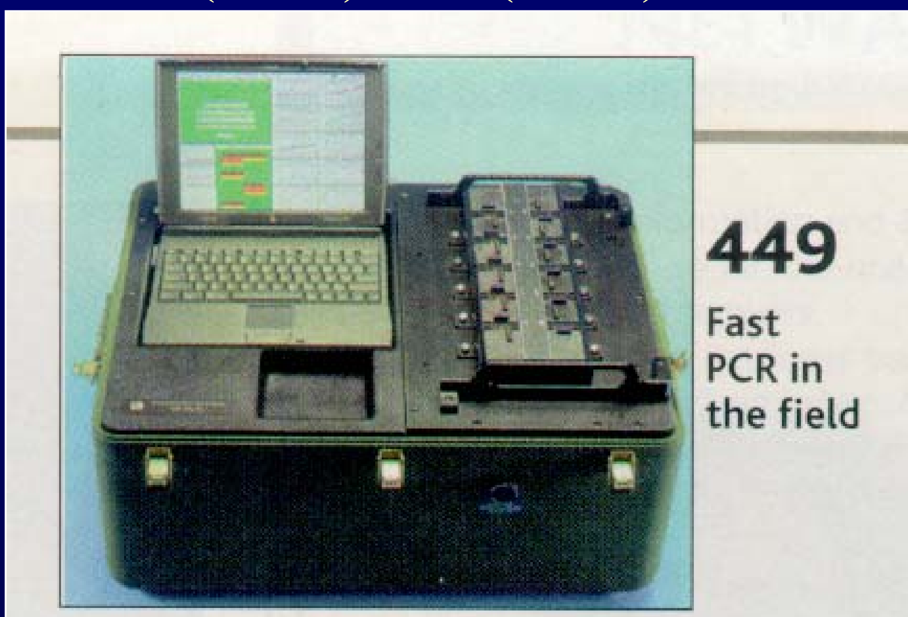
SCIENCE'S COMPASS

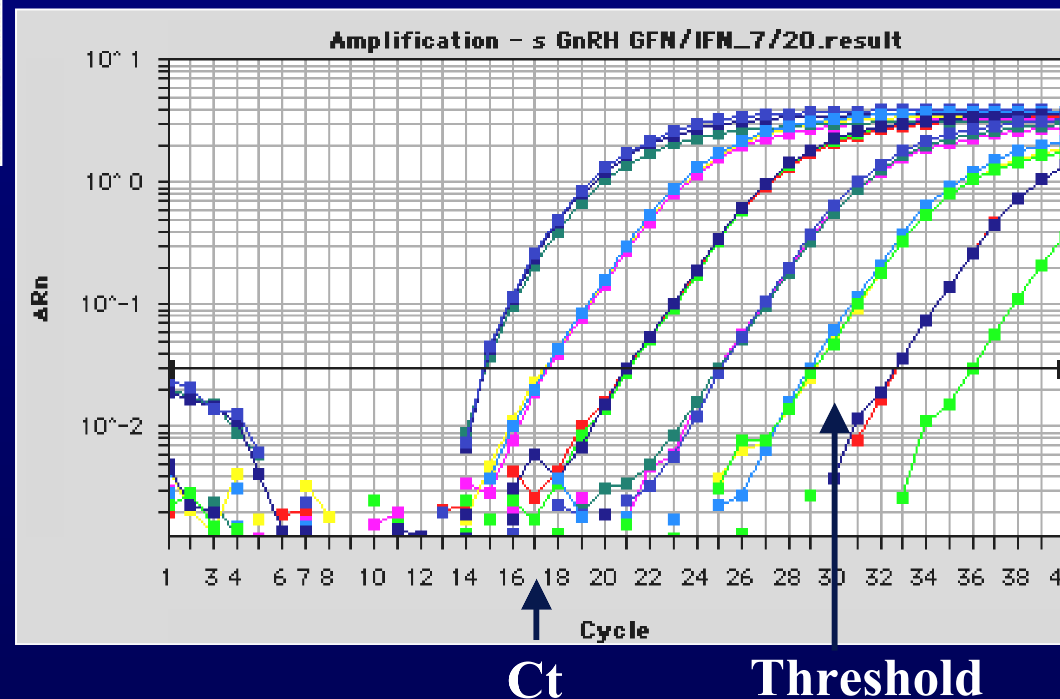
TECHVIEW: INFECTIOUS DISEASE

PCR Detection of Bacteria in Seven Minutes

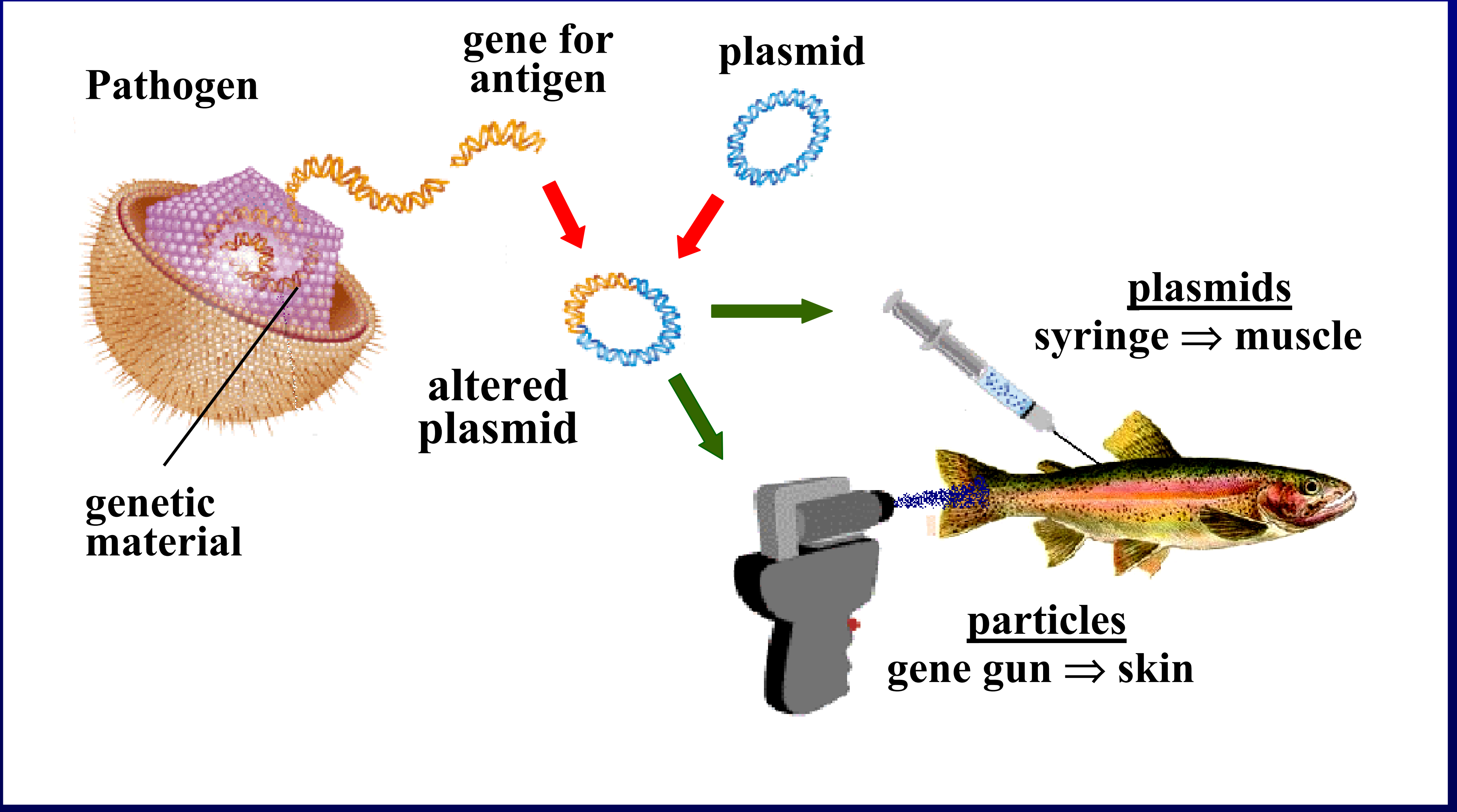
Phillip Belgrader,* William Benett, Dean Hadley, James Richards, Paul Stratton, Raymond Mariella Jr., Fred Milanovich

Science (1999) 284 (5413): 449-450

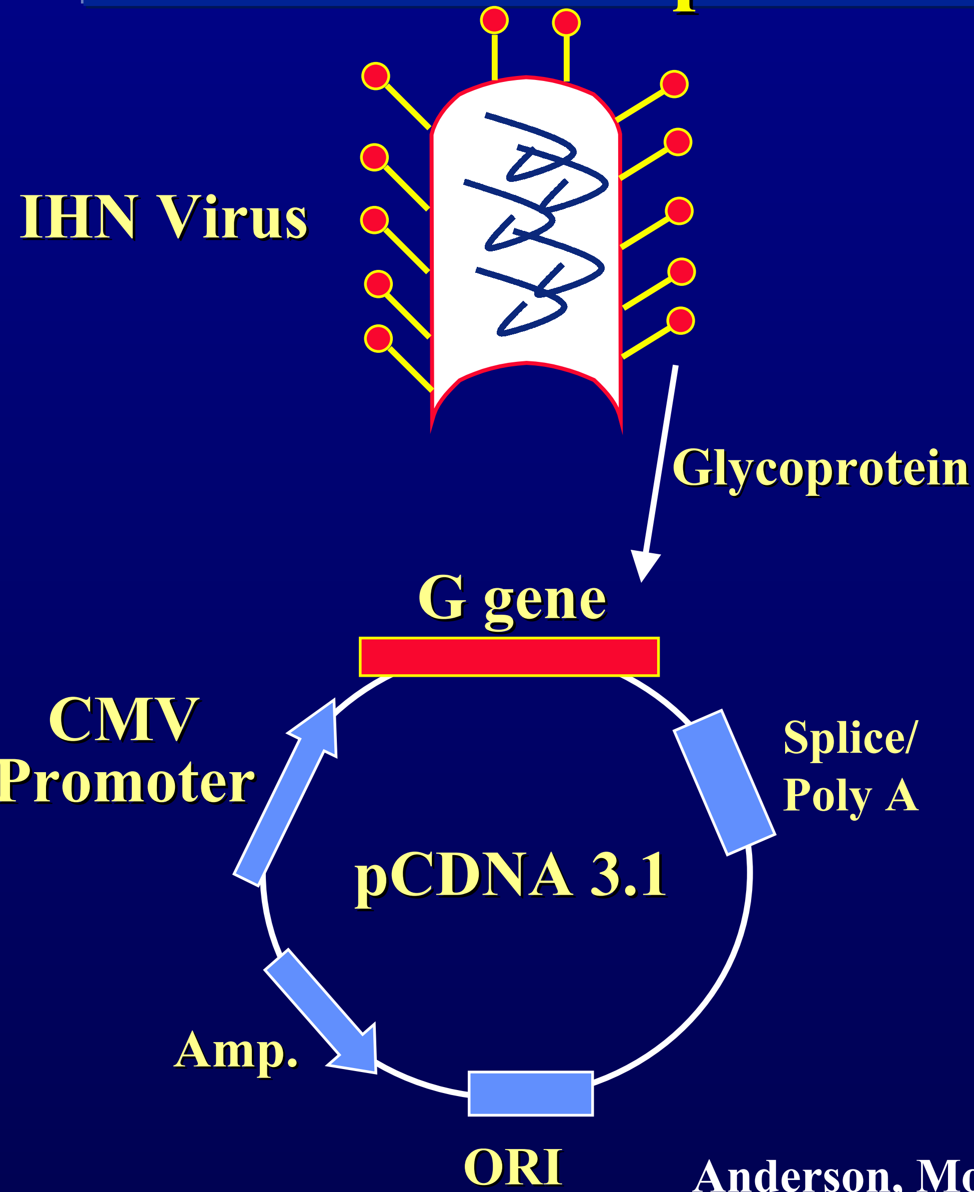


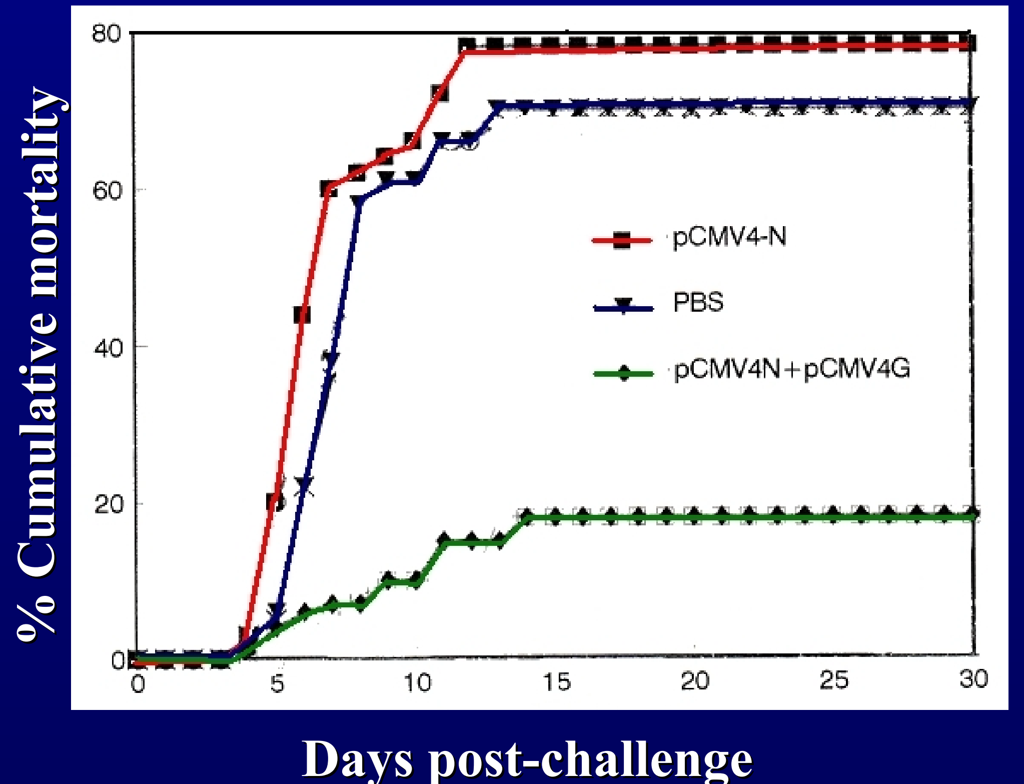


Genetic / DNA Vaccination



Genetic Immunization Against Infectious Hematopoietic Necrosis (IHN) Virus





Anderson, Mourich, Fahrenkrug, LaPatra, Shepherd & Leong, 1996

Fish Vaccination - Labor Intensive

Interactions with the Environment: The Issues

POLICY FORUM: ECOLOGY

Nature's Subsidies to Shrimp and Salmon Farming

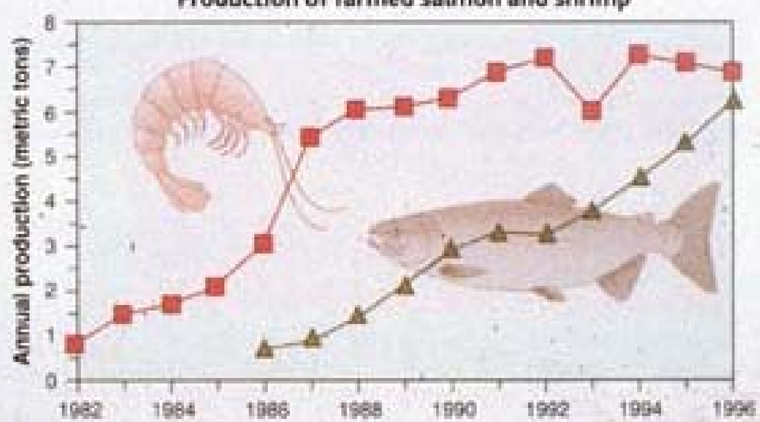
Rosamond L. Naylor, Rebecca J. Goldburg, Harold Mooney, Malcolm Beveridge, Jason Clay, Carl Folke, Nils Kautsky, Jane Lubchenco, Jurgenne Primavera, Meryl Williams

Seafood production is undergoing a dramatic transition. While many fisheries stocks worldwide have declined precipitously, fish farming (or aquaculture) has boomed. Global aquaculture production more than doubled in weight and value between 1986 and 1996, and it currently accounts for over one-quarter of all fish consumed by humans (*1*).

Here we focus on the environmental impact of two of the most lucrative and widely traded aquaculture products: shrimp and salmon. Globally, these crops make up only 5% of farmed fish by weight but almost one-fifth by value (*2*). Both shrimp and salmon farming have expanded and intensified rapidly as a result of technological changes in production and strong demand

adds to the world's food supply. For herbivorous species, this assumption is generally valid (*4*). For species raised as carnivores, however, the opposite may be true. Farmed species such as shrimp and salmon are fed nutrient-rich diets containing large amounts of fishmeal and fish oil extracted

Production of farmed salmon and shrimp



by a factor of 7. The explosive growth of shrimp farming has been supported by national governments, private investors, and international development agencies motivated to generate foreign exchange, private profits, and employment. Farmed shrimp is produced mainly in developing countries for markets in industrialized nations, at a global value exceeding $6 billion annually (*2, 3*).

In shrimp aquaculture, young shrimp, primarily tiger shrimp (*Penaeus monodon*) and Pacific white shrimp (*P. vannamei*), are reared to marketable size in ponds of varied stocking densities. Higher stocking densities are typically supported by increased pumping and aeration of water and greater input of commercial feed and chemicals. Shrimp feed contains about 30% fishmeal and 3% fish oil, and intensive shrimp farming actually results in a net loss of fish protein (*5*).

The rapid growth of shrimp aquaculture has masked the industry's erratic production on a regional scale. The record-breaking 1986 shrimp crop in Taiwan was followed by a spectacular collapse in yields the next year. This boom-and-bust pattern has been repeated in China, Thailand, and In-

Naylor et al.; Science, 2000

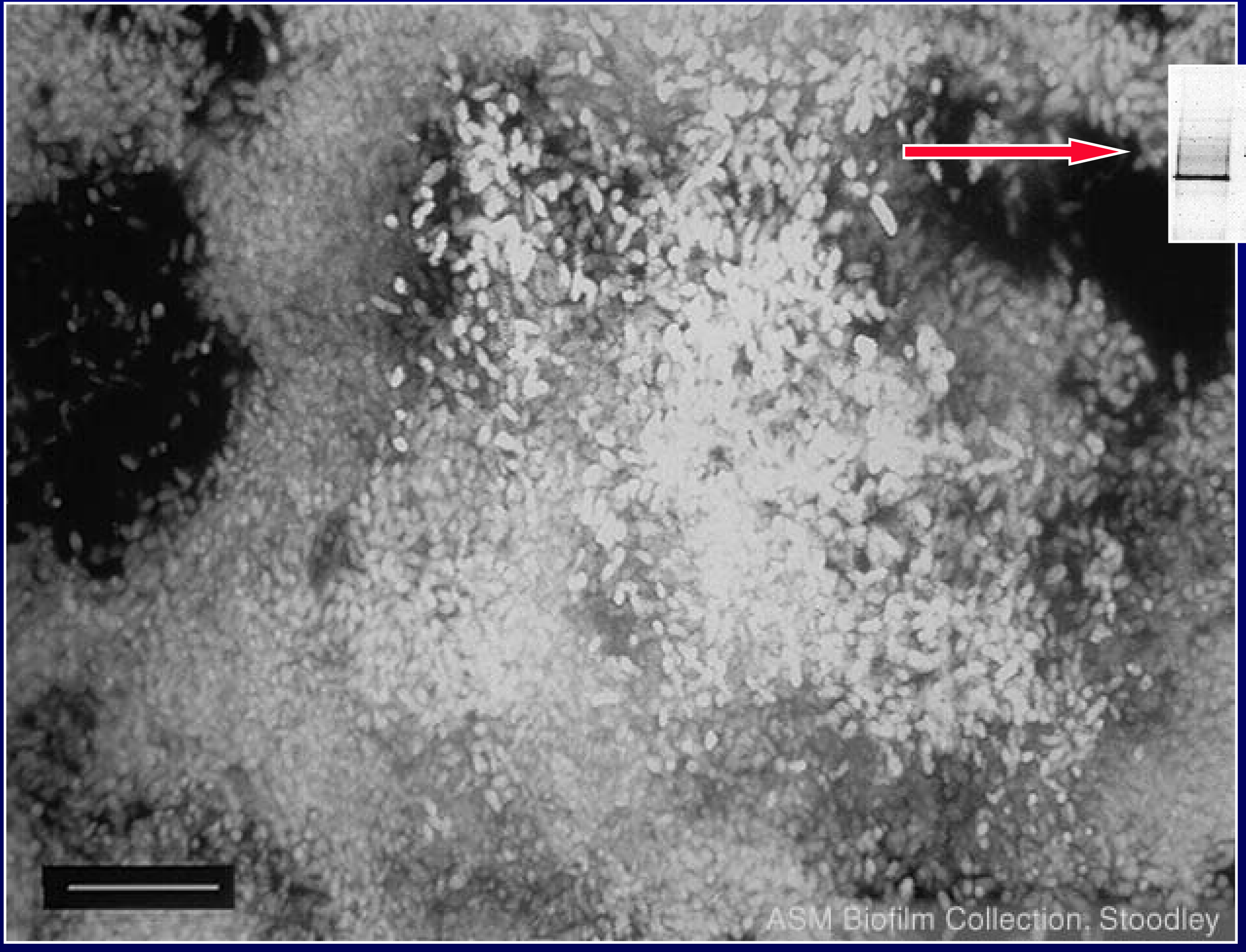
Interactions with the Environment: The Issues

- Effect of aquaculture on the environment
  - Chemical pollution (nutrients, antibiotics)
  - Biological pollution (escapees, GMOs, disease)
- Effect of the environment on aquaculture
  - Pollutants
  - Algal blooms
  - Pathogens
  - Suboptimal conditions

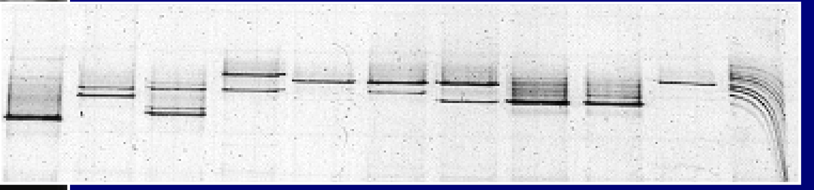
Recirculated, Fully Contained Marine Aquaculture

- No pollution, disease-free, clean, flexible, generic, **GMOs** and non-native species
- Applicable for rural and urban locations

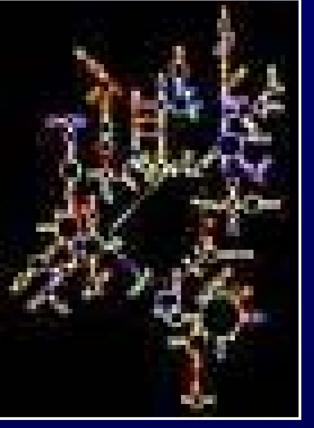
Characterization and Improvement of Microbial Communities in Biofilters



DGGE fingerprinting



16S rRNA analysis



Gene Transfer, Transgenics: Potential Applications

- **Improve performances**

  Reproduction; early development; growth; digestion; disease resistance; aggression

- **Develop new feeds**

  Plant-stuff with adequate proteins and lipids

- **Tailor fish to the market**

  Taste; flesh color; fat content; body form

- **Basic research**

  Reproduction, development, growth, immunology

Post / Functional Genomics: From Genes to Functions

- **DNA microarrays / gene chips**

  Measure differential gene expression of the entire genome on 1 chip

- **PROTEins encoded by the genOME**

  Full array of all proteins present in an organism, a tissue or a cell

- **Protein microarrays / chips**

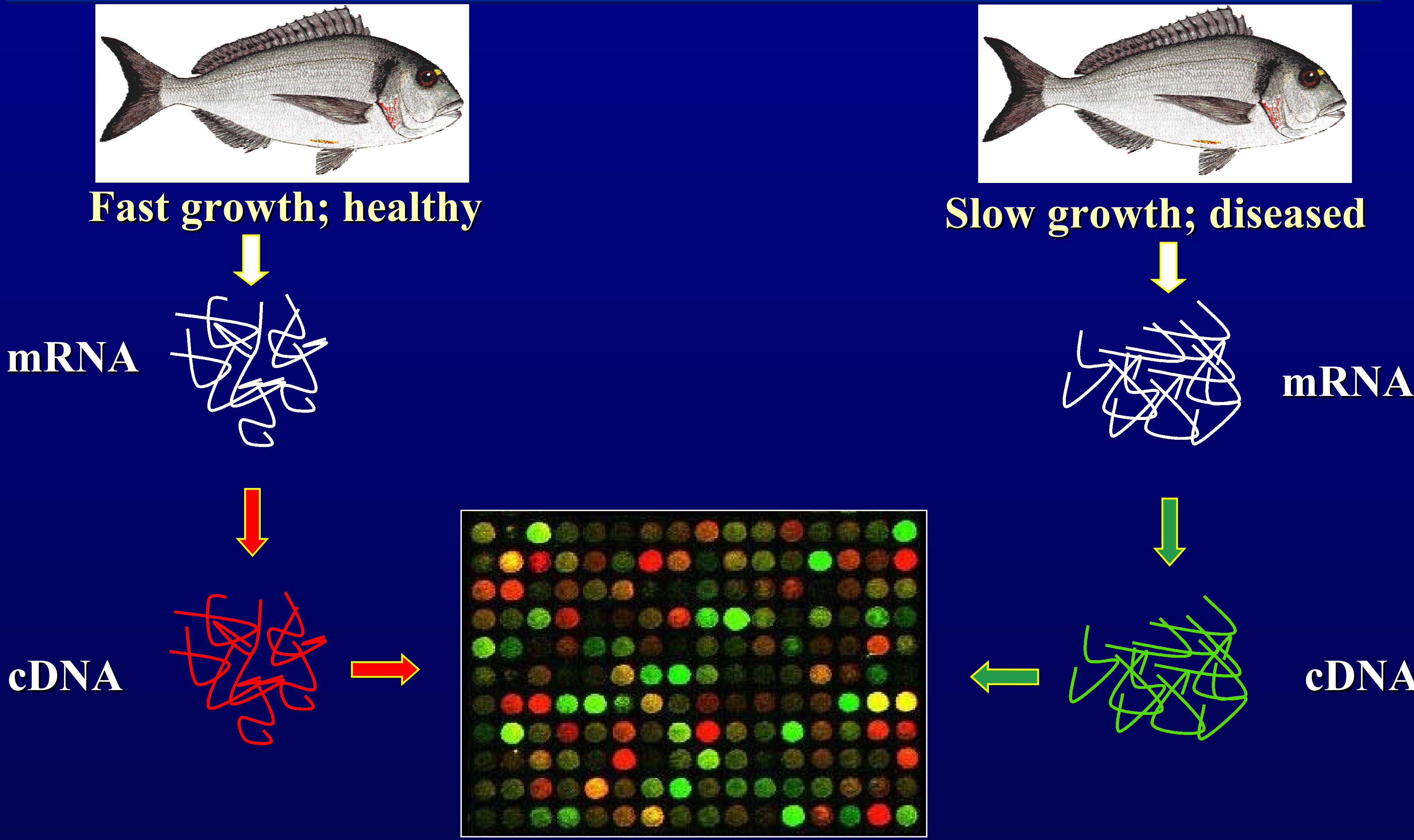
  Measure differential abundance of thousands of proteins on one chip (with specific antibodies)

- **Tissue microarrays / chips**

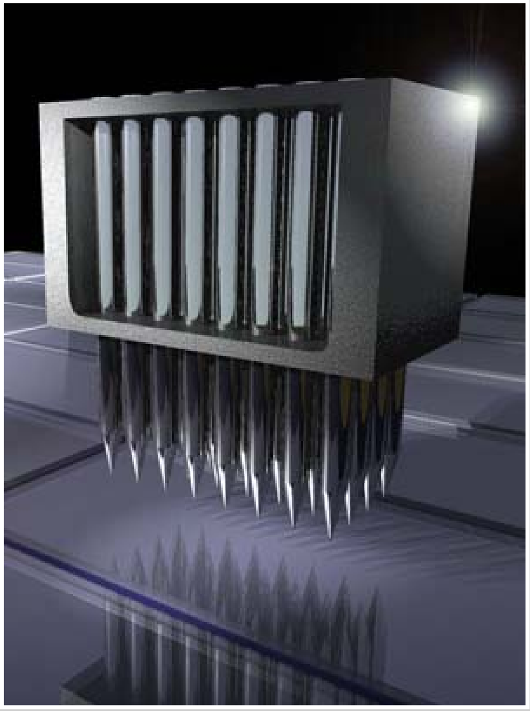
  Monitor gene and protein expression patterns in hundreds of tissues on one chip

- **Protein activity chip…. (still a dream…)**

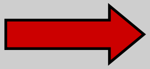
DNA Microarrays / Gene Chips

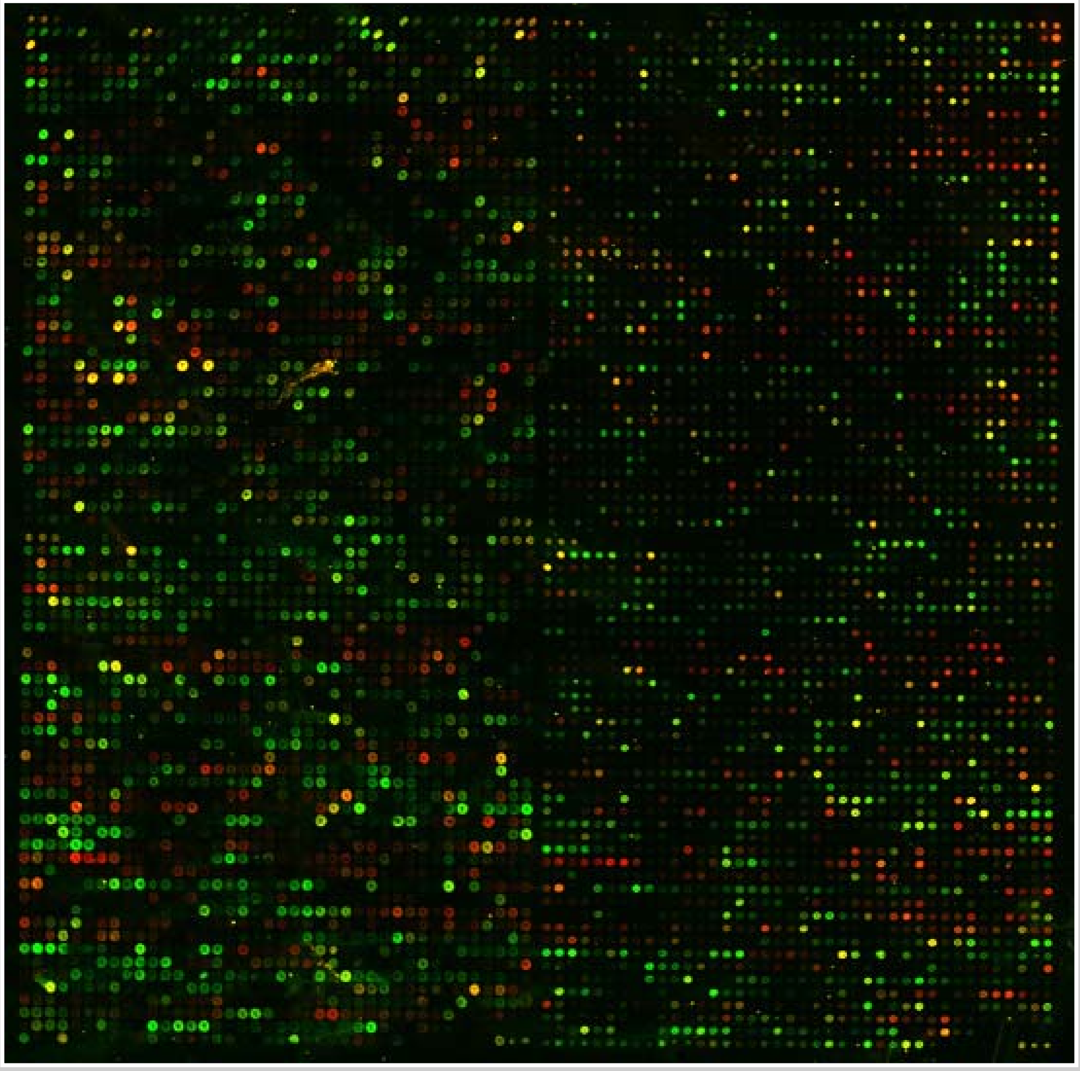


Functional Genomics: Gene Chips



Robotic ‘arrayer’



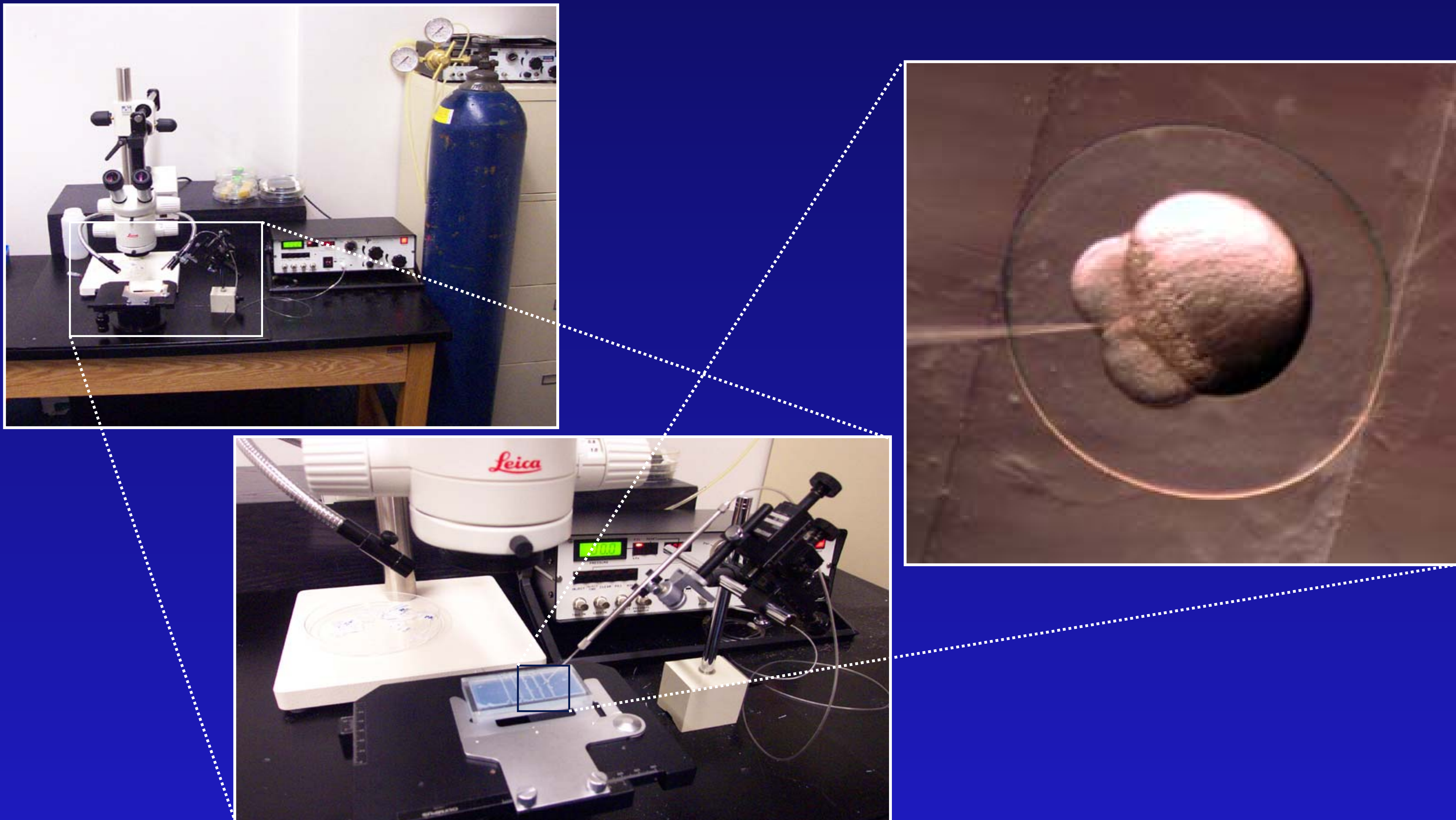


The entire yeast genome on a chip!; Dr. Patrick Brown, Stanford U.

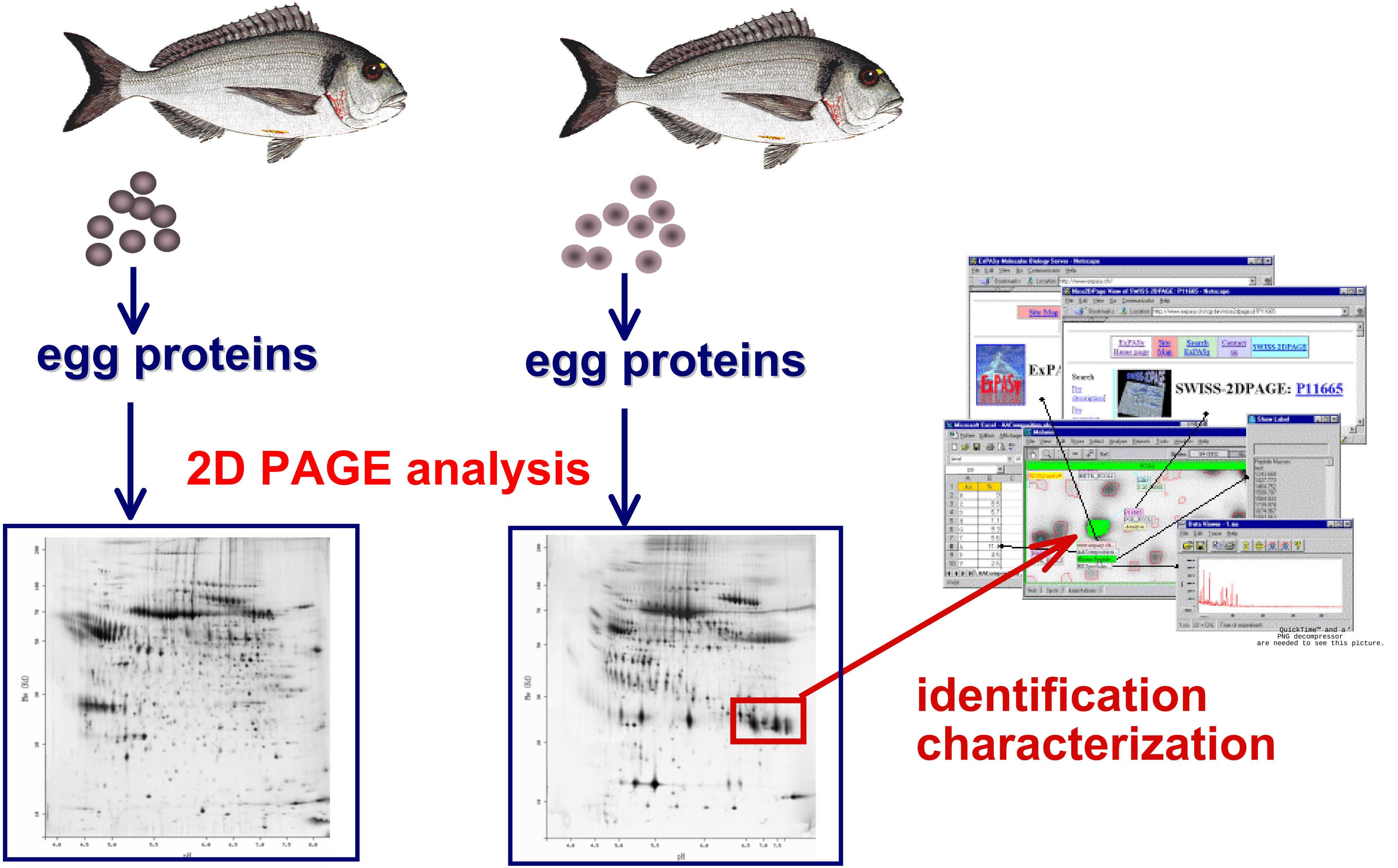
Acknowledgements:

- Drs. J. Trant, J. Du, A. Place, G. Vasta, M. Harel, H. Schreier, V. Frenkel- COMB, Baltimore, MD
- Dr. Amos Tandler, Odi Zmora, NCM, Eilat
- Dr. Patrick Sorgeloos, University of Ghent
- Dr. Choy Hew- National U. of Singapore
- Dr. Scott Lapatra- Clear Springs Foods Inc., Idaho
- Dr. Paul Behrens, Martek Biosciences, MD

Microinjecting DNA into zebrafish embryos



Proteome Analysis



Genetic Selection: Integrating Molecular Genetics

- **Identify beneficial traits:**

  Growth rate; food conversion; flesh quality; fecundity; age at maturity; disease resistance

- **Identify and map the relevant gene(s):**

  Single genes; multiple genes (QTLs)

  (Danzmann et al., 1999; Rodriguez et al., 1999; Davis and Hetzel, 2000)

- **Link gene markers to gene(s) of interest**
- **Perform marker-assisted selection**

Gene Linkage Mapping in Crustaceans and Finfish

• Zebrafish	Kelly et al., 2000
• Salmon	Lie et al., 1997
• Catfish	Liu, 1999
• Tilapia	Kocher et al., 1999
• Kuruma shrimp	Moore et al., 1999
• Tiger shrimp	Moore et al., 1999

Gene Transfer, Transgenics: Future Directions

- **Better delivery of transgenes**

  Vectors; sperm; lipofection; gene guns

- **Better integration of transgene**

  Transposable elements (Ivics, Z., Izsvak, Z. & Hackett, P.B., 1999); NLS (Collas, P., Husebye, H. & Alestrom, P., 1996)

- **Knock out /knock in technology**

  Embryonic stem cells (Hong, S., Chen, S. & Schartl, M., 2000)

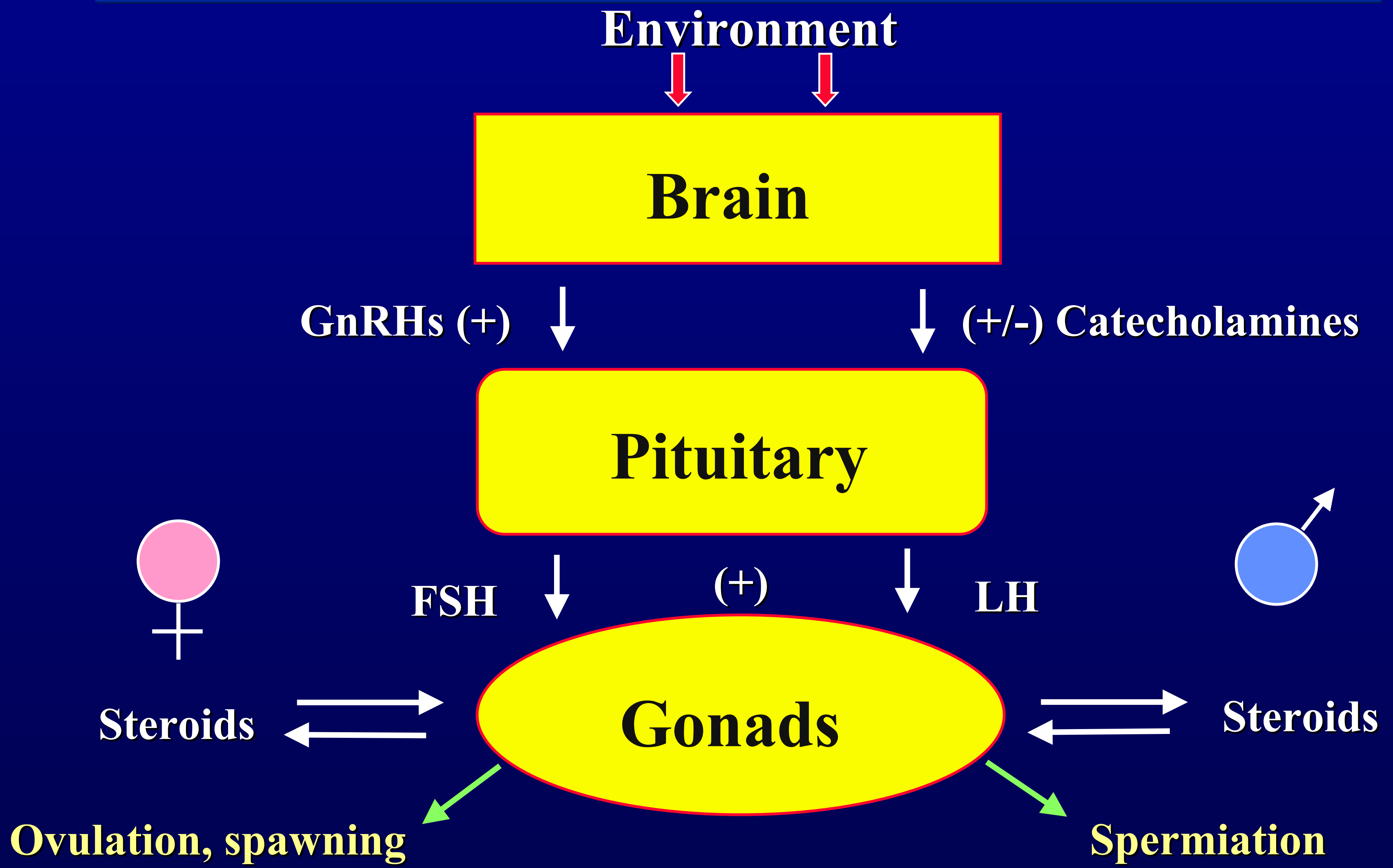
- **Spatial and temporal-specific expression**

  Promoters; constructs

Expression of Recombinant Substances: Potential Applications

- Hormones (GH, Prol, GtHs, GFs,…)
- Protective antigens (viral coat proteins,...)
- Enhancers of immune system (glucans,...)
- Antibacterial compounds (peptides, lysozymes)
- Enzymes (phytase, cellulase, desaturase,...)
- Others (antifreeze proteins,…)

Regulation of Fish Reproduction



Genetic Engineering for the Control of Reproduction

Inducer (water, feed)

Inducible Promoter	Anti-GnRH or GnRH

Anti-GnRH mRNA → Sterility

GnRH → Spawning

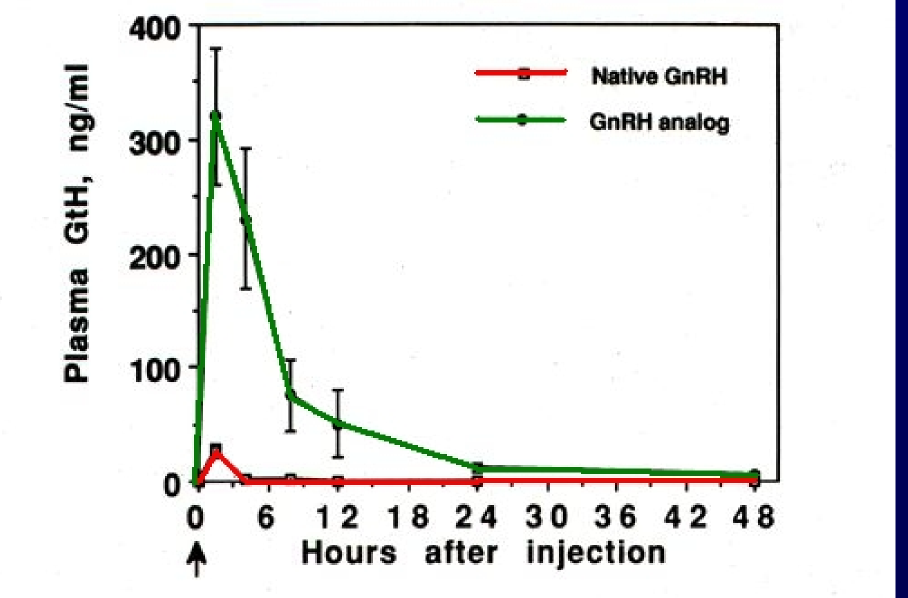
Uzbekova, Alestrom, Hanley & Breton, 2000

Peptide Engineering: GnRH and GnRH Analogs

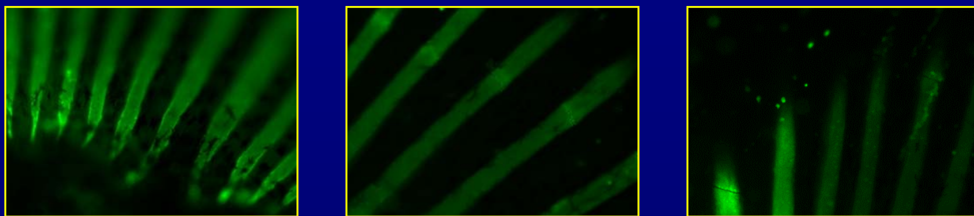
1 2 3 4 5 6 7 8 9 10

pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-GlyNH2

pGlu-His-Trp-Ser-Tyr-DAla-Trp-Leu-Pro-NEt

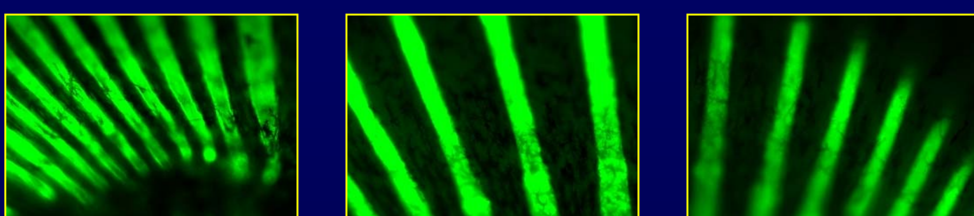


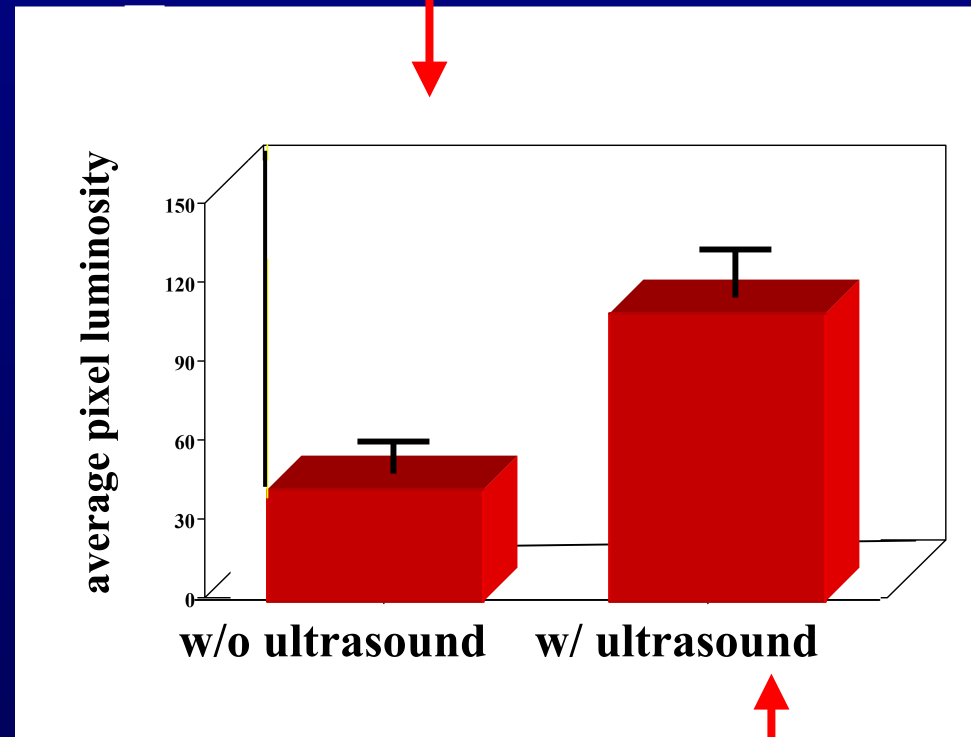
Ultrasound Enhanced Uptake of Calcein



Calcein w/o ultrasound

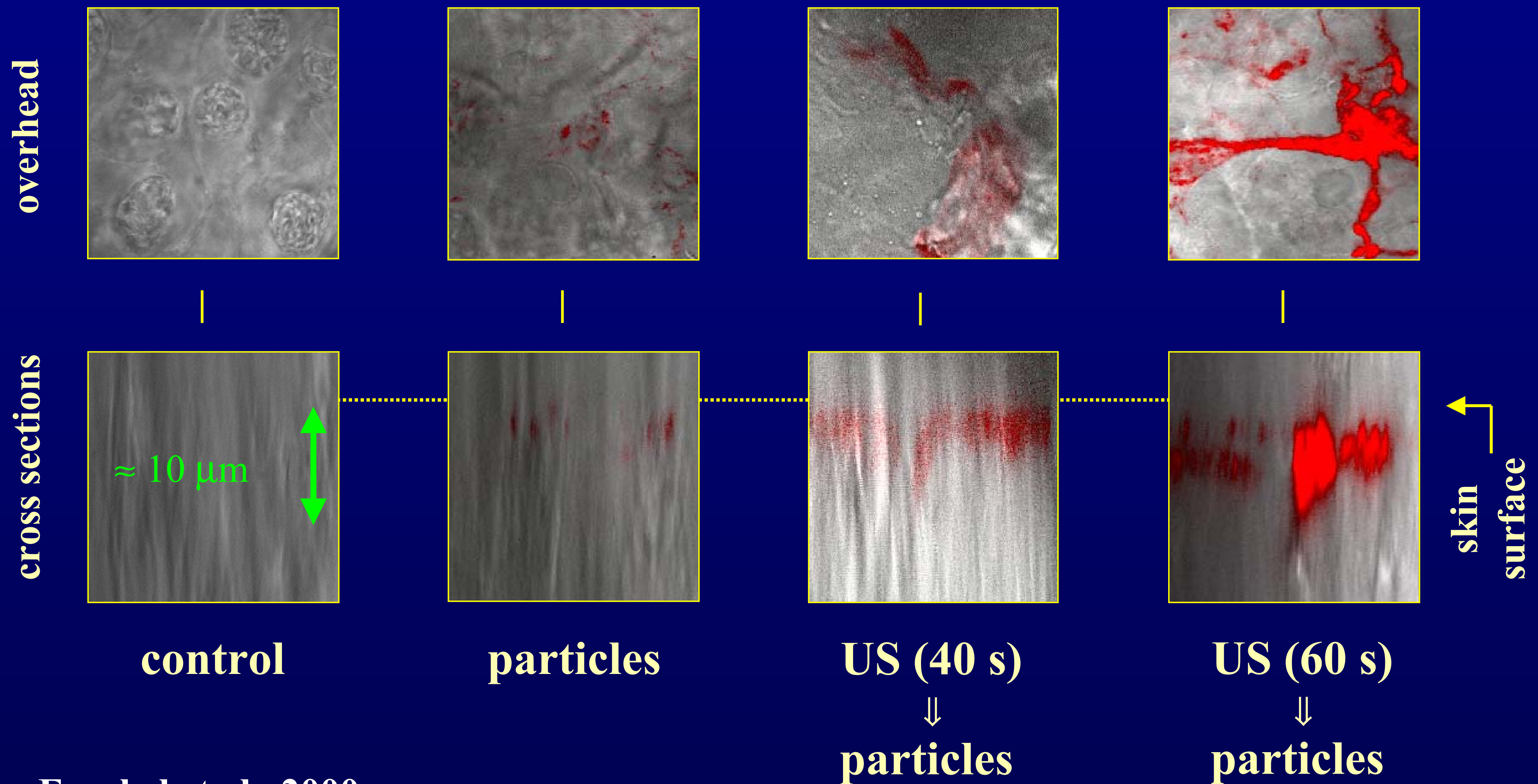
Calcein w/ 30 second ultrasound





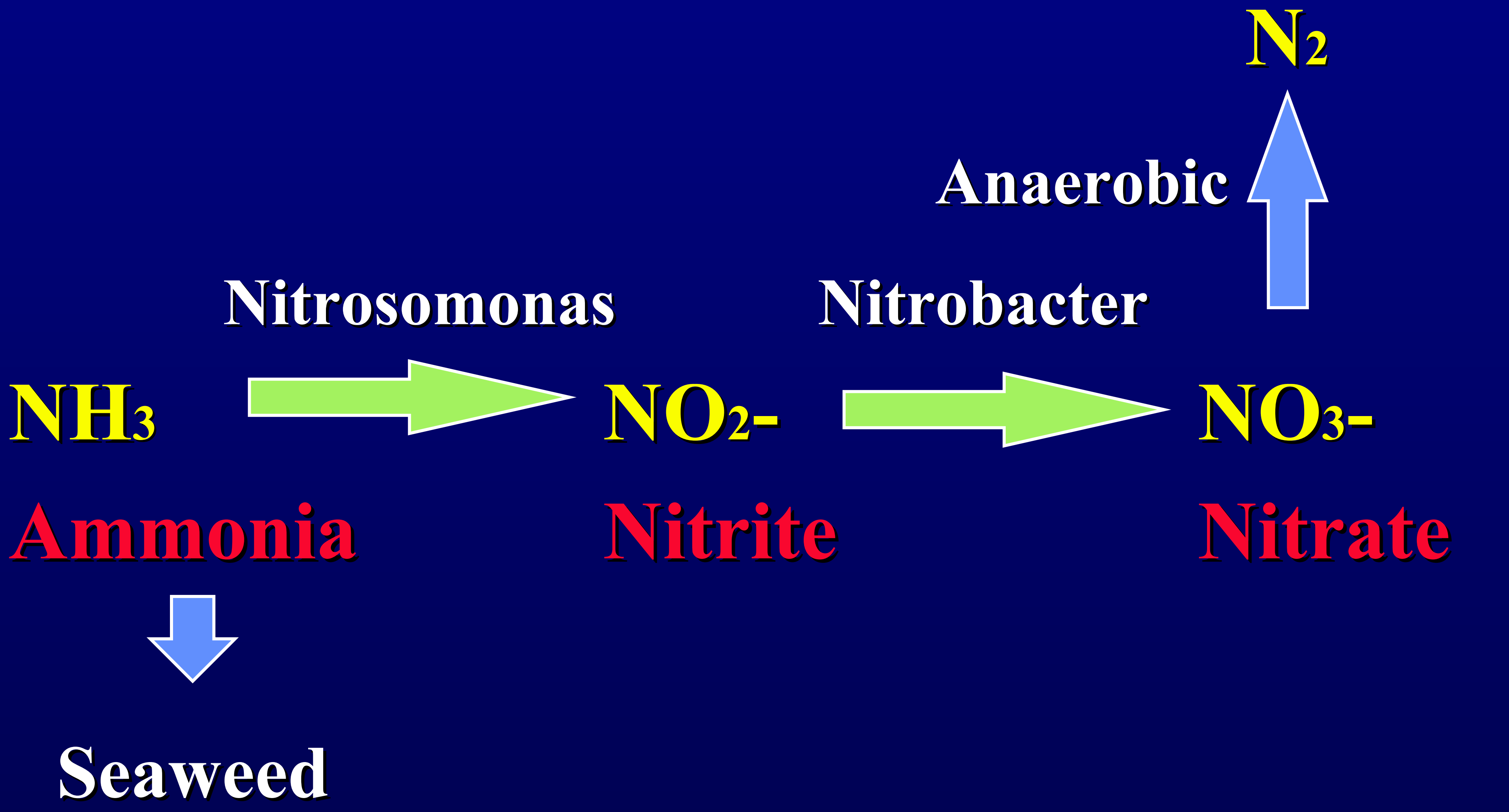
Frenkel et al., 2000

Ultrasound-mediated delivery of biopolymer/DNA nanospheres

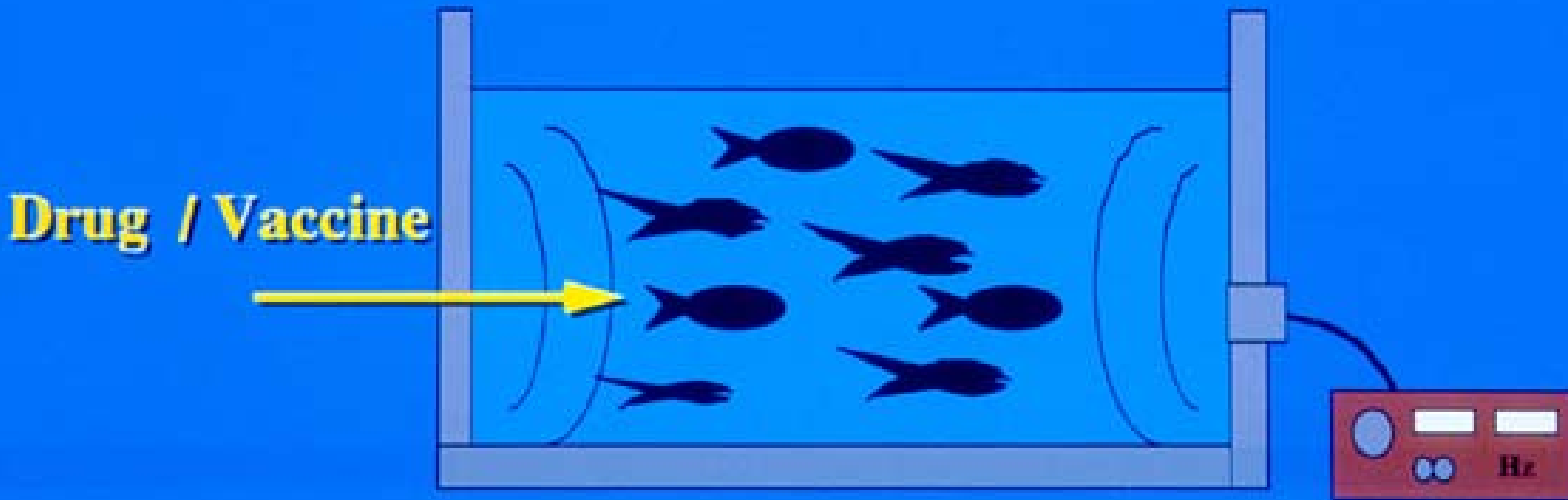


Frenkel et al., 2000

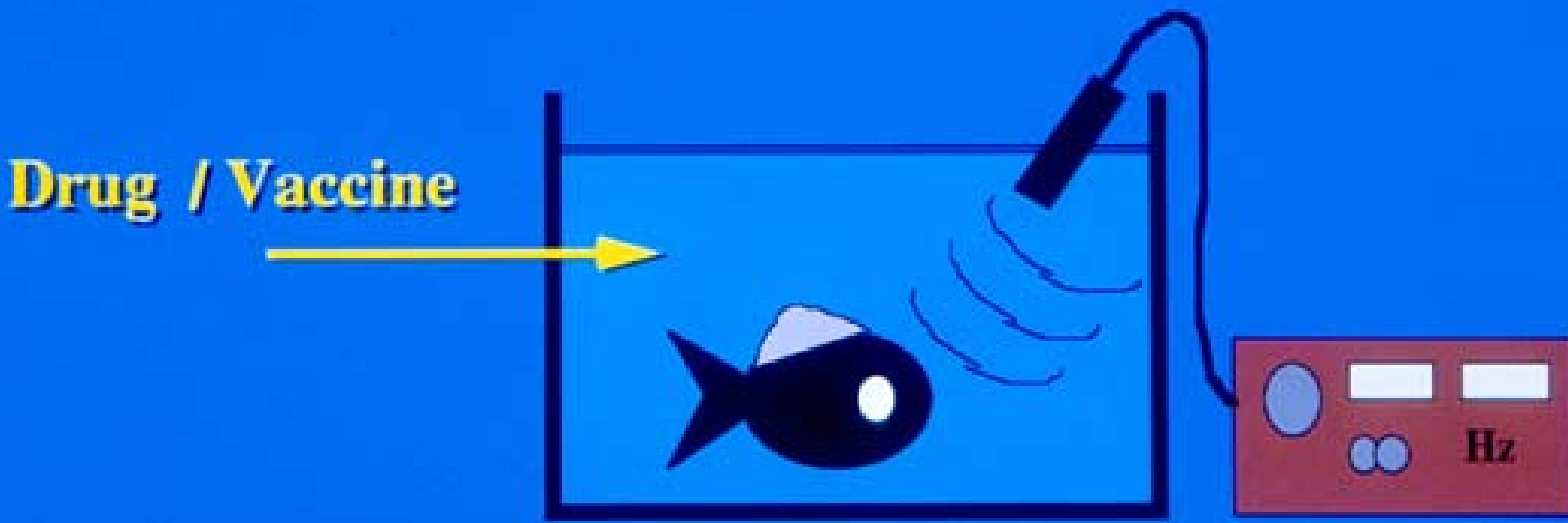
Biological Filtration



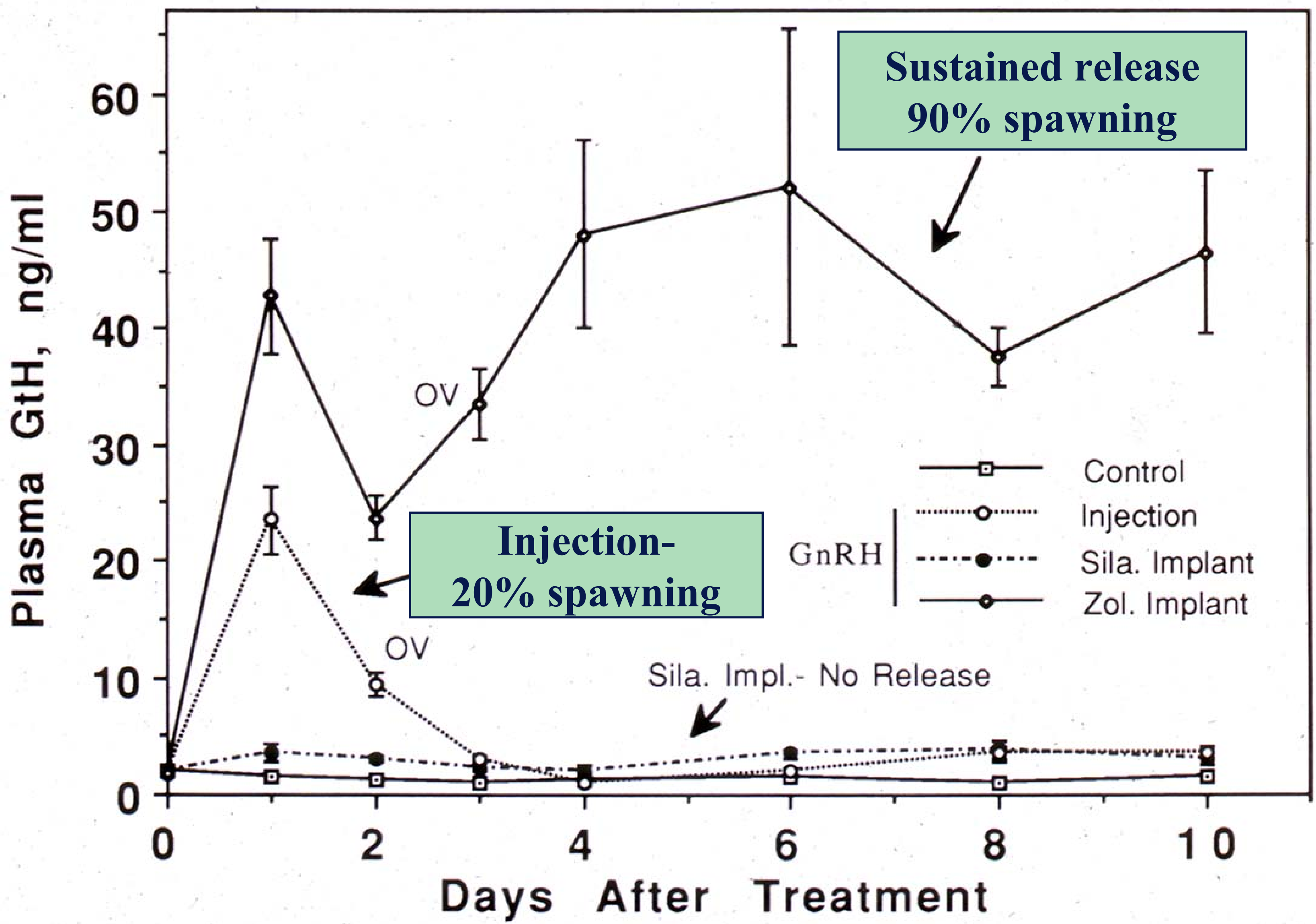
Ultrasound-commercial application



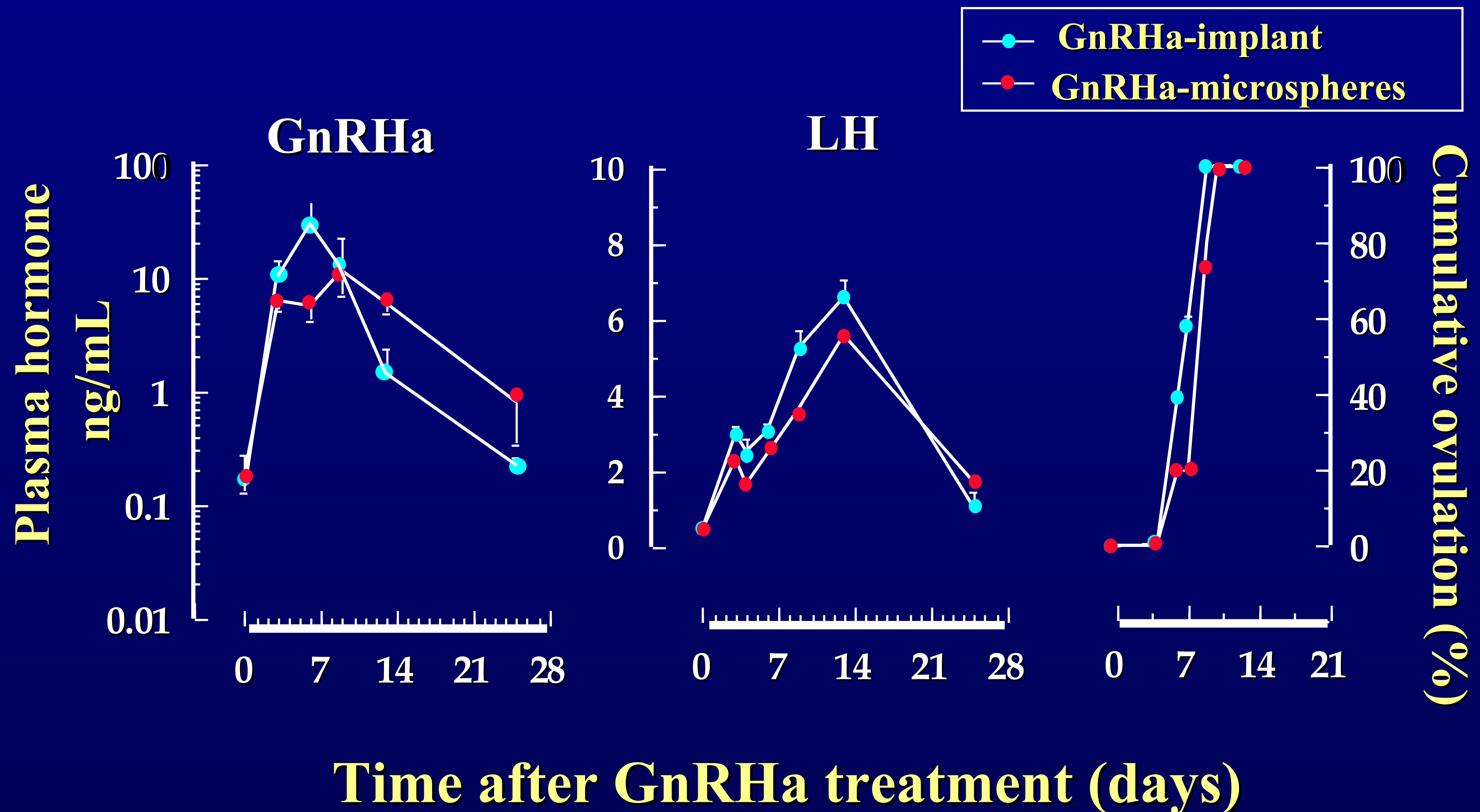
Ultrasound-experimental



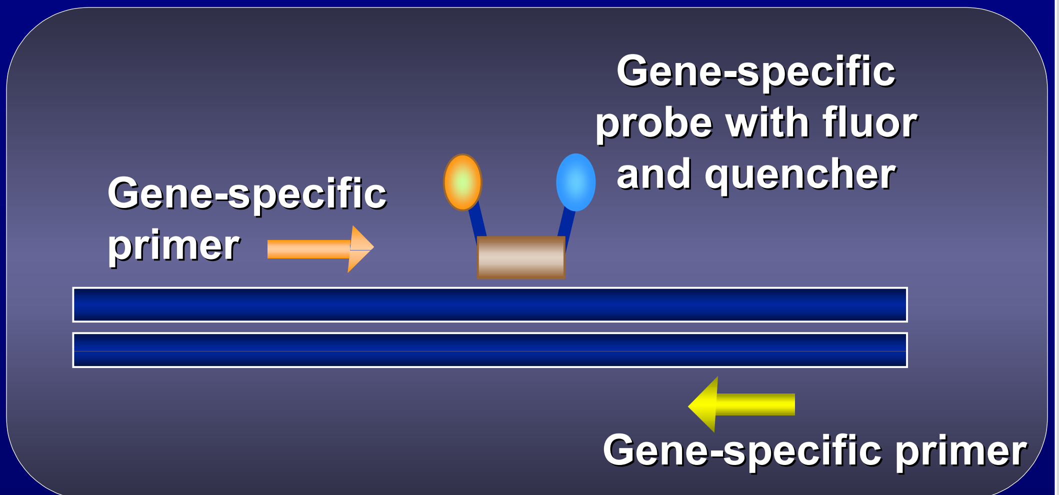
Modes of GnRHa administration in gilthead seabream

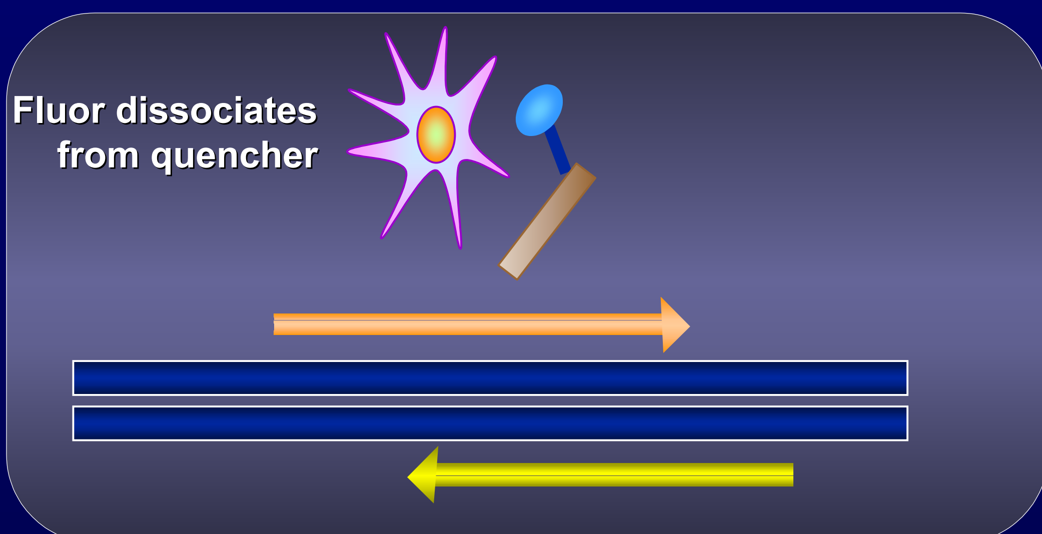


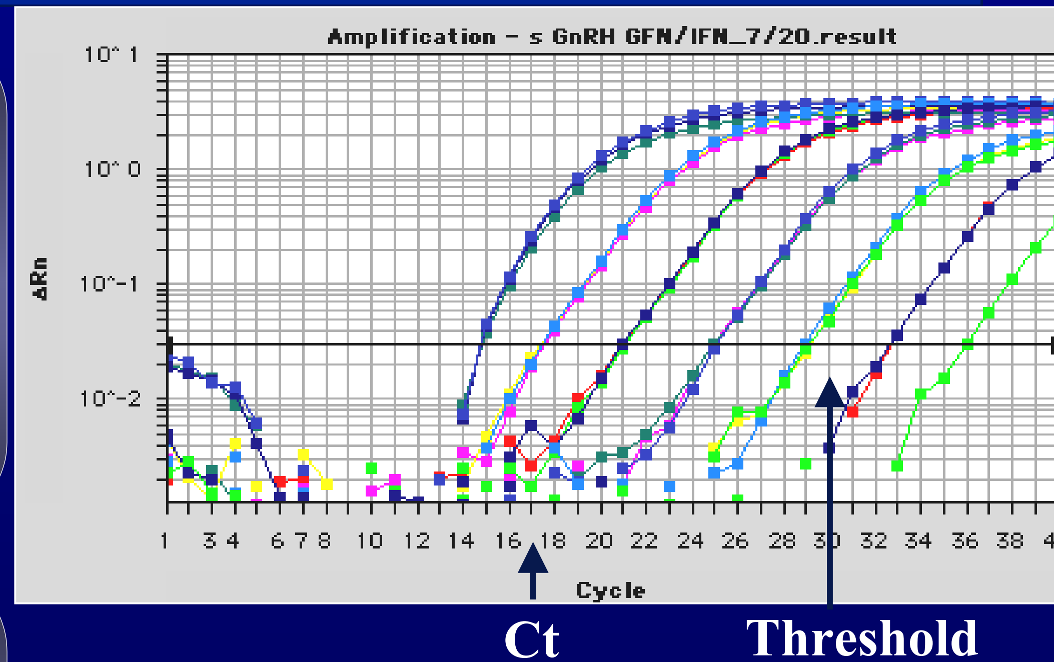
Plasma hormone levels and ovulation in striped bass females after treatment with GnRHa-delivery systems



Monitoring Larval Development Through Gene Expression: Real Time RT-PCR







Nutrition:
The Issues

- Food cost accounts for 50% of growout expenses
- Aquaculture industry spends $ 6 billion/year on feed
- Projections for 2010 are $ 10 billion
- Heavy dependence on fish meal (1/3 of all fisheries; 6 mmt/year)

Source: USDA; FAO

The Immediate Challenge: Replace Fish Meal with Plant Meal

- "Production of fatty acid components of high value drying oils in transgenic soybean embryos"

  Cahoon et al.; PNAS, 1999

- Develop "algal meal"