# Use of Morphometrics and Biochemical Assays to Study the Development of Larval Tautog.

Final Report on CMER NOAA/NMFS RESEARCH TOPICS - 1998:

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Topic 19. Effect of Dietary Fatty Acid and Amino Acid Composition on the Growth Rate and Body Composition of Larval Tautog (Tautoga Onitis) and on the Reproductive Success of Adult Tautog
(Contact: Dean Perry and Laurel Ramseyer, Milford Laboratory)

## Introduction

"For tautog aqua culture to advance, the effect of diet quality on egg production, larval survival and larval growth must be quantified" (Perry and Ramseyer, 1997). This request for analytical assistance required a special laboratory that is familiar with dealing with both biochemical analyses and the concepts of morphological growth and development of embryos. Our laboratory accepted the challenge and developed the analytical skills to answer the questions posed.

The dietary question focused on determining what algal feeding schedule was most efficient in providing the maximum levels of polyunsaturated fatty acids (PUFAs) ingested by the protozoan culture (rotifers) that are to be fed to larval tautogs.

The growth rate aspects were approached with a morphometrics approach which was aimed at developing methods to analyze embryonic, larval and young tautog development to allow comparison of different feeding protocols of growth rates. Talking with investigators at the Milford Laboratory it became clear that the objective was to establish when it was possible to stop feeding larvae with rotifers, which are expensive to rear, and replace them with commercial larval fish food.

## Materials and Methods

HPLC of PUFAs was carried out using the analytical equipment of the Food Science Laboratory at UMass Amherst. Lipid was extracted by chloroform methanol extraction, which produces total protein and total lipid fractions which are then measured gravimetricly. The lipid is then further processed to produce a FA fraction. The FA fraction is further analyzed for PUFAs via HPLC.

Samples of algae were obtained from the NOAA Milford Laboratory algal growth facility on a visit by JG Kunkel and Joe Zydlewski to Milford. Several 250 ml centrifuge bottles of algae of Plymouth and T-iso strains were centrifuged to obtain extractable amounts of algal cells. These cells were frozen and brought back to Amherst for analysis. Cultures of rotifers at Milford laboratorywere fed algal strains or yeast and harvested at different times after feeding and subsequently analysed.

Samples of tautog embryo and larval tissue were obtained from the Milford Laboratory tautog rearing program. Samples of three large food items: European Green

crab *Carcinus maenas*, Blue Mussel *Mytilus edulis* and Rock crab *Cancer irroratus* were obtained from the Milford Laboratory.

Morphometric research was carried out in the lab of the investigator JG Kunkel in the Biology Department at UMass Amherst. Embryonic, larval and young Tautog material was not available in a regular fashion due to rearing limitations at Milford and as a result we adjusted our protocols to use other sources of developing fish as models for developing the morphometric approaches we proposed. Tilapia larvae were recruited from the Bioshelters Corp. Zebrafish larvae were obtained from the UMass Amherst zebrafish rearing facility of Rolf Karlstrom. Salmon digital images were recruited from the Contes Anadromous Fish Laboratory research program of Ben Lecher. The Salmon digital set of images is indexed by a Microsoft Access Database.

Embryos and larvae studied at stages that are motile were anaesthetized with ms-222 to immobilize them during imaging.

Morphometric analysis was carried out using several hardware and software components. Image capture of larvae was obtained using a Parco fixed magnification Stereo microscope with a photomicrograph module. Several digital cameras have been utilized for different aspects of this research. A Kodak DC290 digital camera provides high resolution 1752x1200 pixel single images but with a slow turnaround time. A Sony videocamera provides 800x600 images with rapid turnaround. The single images are the result of multi-image average which does provide super resolution under optimal lighting conditions. A digitizing board installed in a Dell 600 Mhz PC was used to grab images from the video camera.

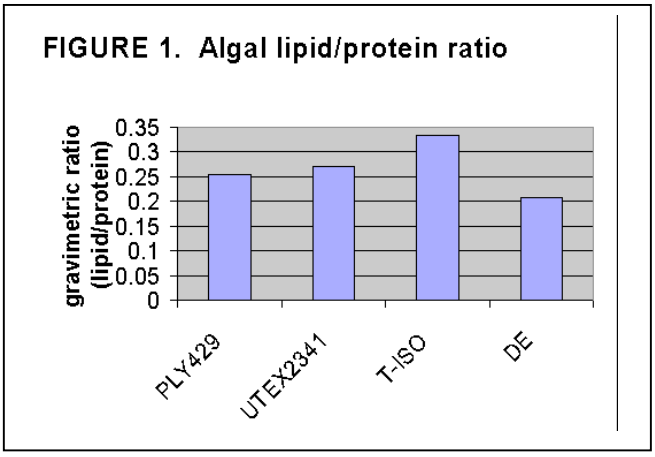
Analytical software used included the tpsSuite of PC compatible software of James Rohlf (Rohlf, 1997A-D). The digitizing software tpsDig was used to obtain the digital coordinates. Alternately ImageJ, an update of NIH Image, is available for all computer platforms and can be used for determining pixel coordinates. Both digitizing softwares have capabilities of calibrating the digital data using a known standard.

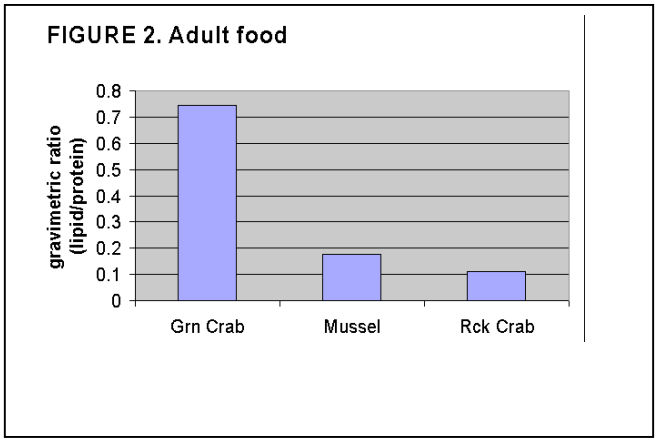
Fourier based analysis for pattern recognition is a potential approach to recognizing individual fish by their patterned markings. Two pieces of software were evaluated for this purpose. MatLab version 5.0 was obtained in trial version. R, Copyright 2003, from The R Development Core Team,Version 1.6.2 was installed and evaluated for its Fourier analysis package capabilities. R is a freely distributed under the terms of the GNU GENERAL PUBLIC LICENSE Version 2, June 1991. The R package was found to be more useful due to its extensive user base which includes researchers who have produced a basic Fourier analysis package which includes FFT functions as well as a convolution function which can be applied to both 1-dimensional signal data as well as 2-Dimensional image data. In addition R has a scripted programming language that looks and feels like Fortran programming as well as allowing system calls to the operating system from which any system accessible computations can be made via computationally intensive subroutines written in C or any other library language. The Comprehensive R Archive Network (CRAN) is a collection of web sites that carry identical material, consisting of the R distribution(s), the contributed extensions, documentation for R, and binaries.

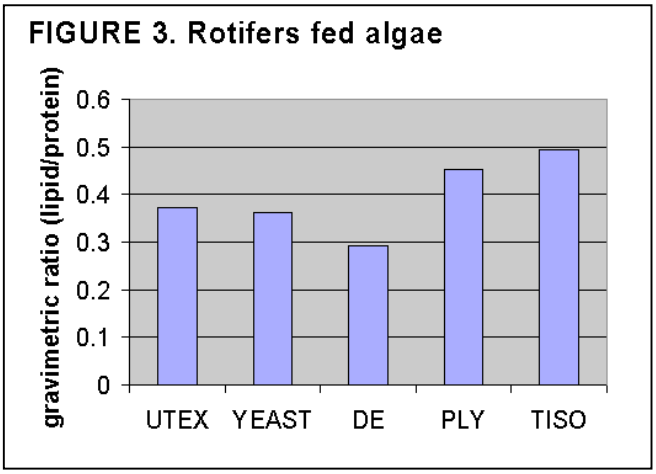
# Results

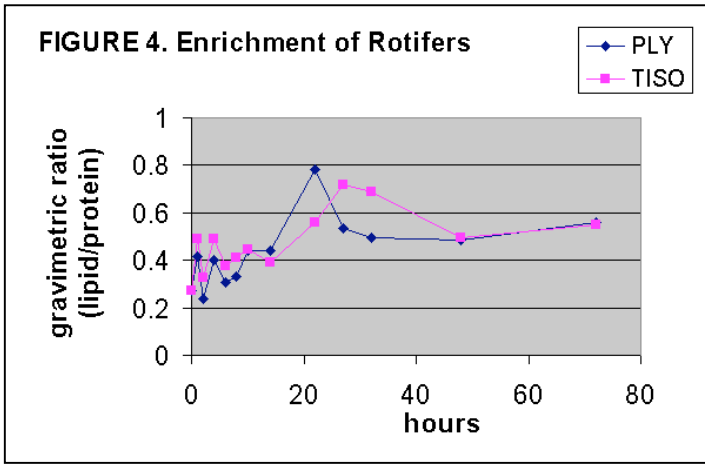
I. Lipid protein ratios

Crude lipid/protein ratios were determined on Tautog aquaculture food resources by determining lipid and residual protein content gravimetricly after chloroform/MeOH lipid extraction. The resultant data indicate that the alga strain T-iso has the greatest lipid content among the algae, Fig. 1. Green crabs are the best lipid source among the adult food items tested, Fig. 2. T-iso followed by Ply are the best source of lipids for overnight enrichment of rotifers, Fig. 3. In the time series of rotifer enrichment it appears that 24 hours of enrichment achieves the highest lipid/protein ratios in the rotifers, approaching 0.7-0.8:1 by 24 hours after which the ratio declines, Fig 4.




II. PUFA content in algae

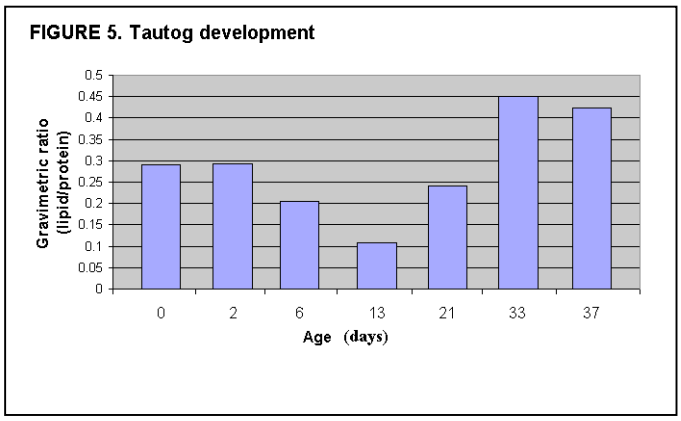
Algal strains were harvested from growing cultures at NMF, Milford lab, and were analysed for their total lipid relative to protein content (Fig 1) and then the PUFAs were estimated as a percentage of total FA in their C16-C22 content (Fig 6) and their n-3, n-4 and n-6 content (Fig 7). It is clear that the Plymouth 429 strain of algae has the highest n-3/n-6 PUFFA ratio by a substantial margin. Given that a high n-3/n-6 PUFA ratio is a prime reason for feeding rotifers on algae it is perhaps an important alga to use in the preloading process.

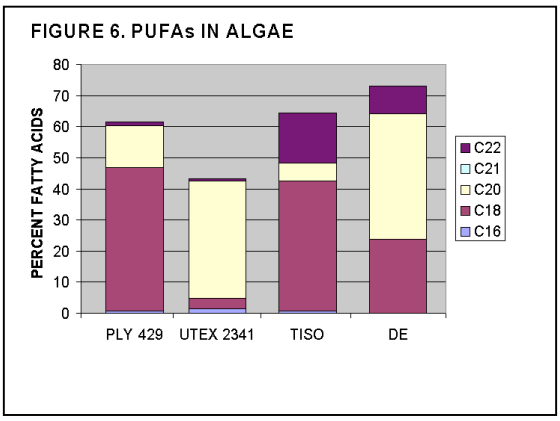
III. PUFA content in adult food items

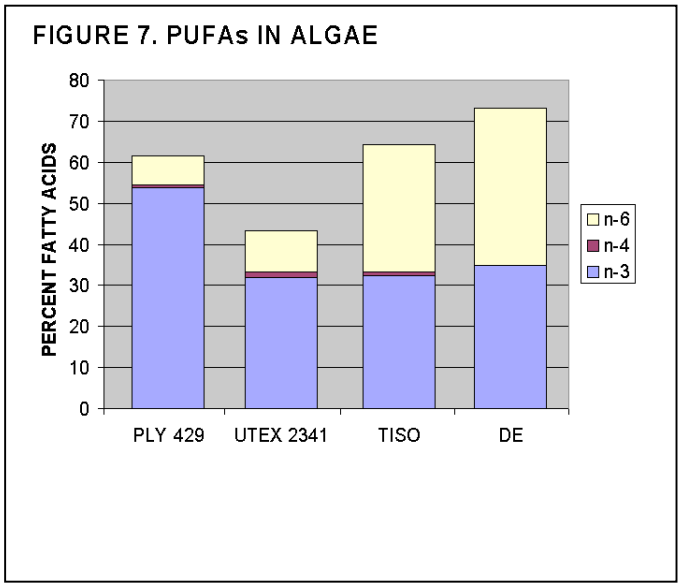
Adult food items were provided by NMF, Milford lab, and were analyzed for their FA content (Fig 2) and then the PUFAs were estimated as a percentage of total FA in their C16-C22 content Fig 8, and their n-3, n-4 and n-6 content (Fig 9).

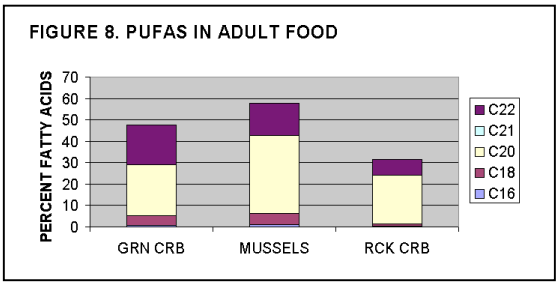
IV. PUFA content in rotifers enriched with algal strains overnight

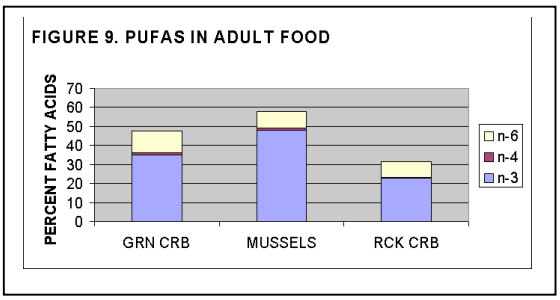
Rotifer cultures enriched with algae overnight (18 hours) were provided by NOAA, Milford lab, and were analyzed for their FA content relative to total protein (Fig 3). It is clear that the two strains T-iso and Ply 429 produce the largest loading of lipid to protein in the gravimetric assay. The PUFAs were further estimated as a percentage of total FA. In

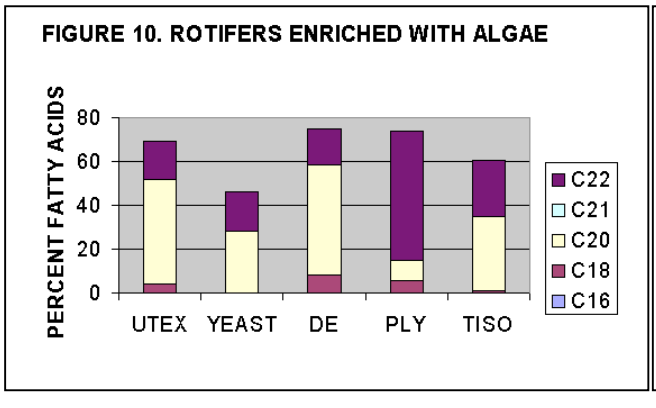


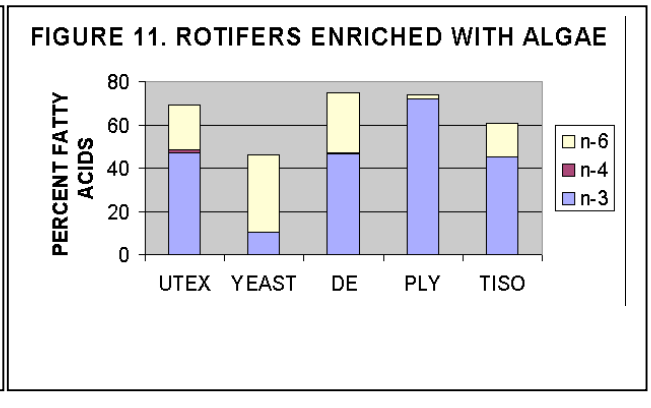



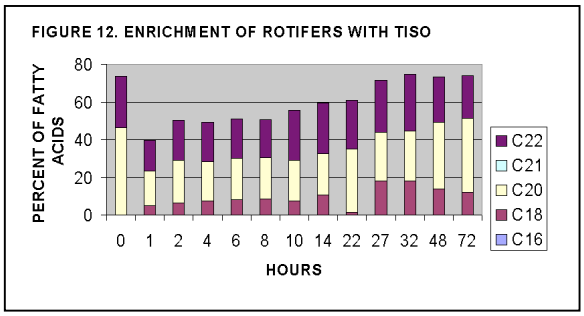



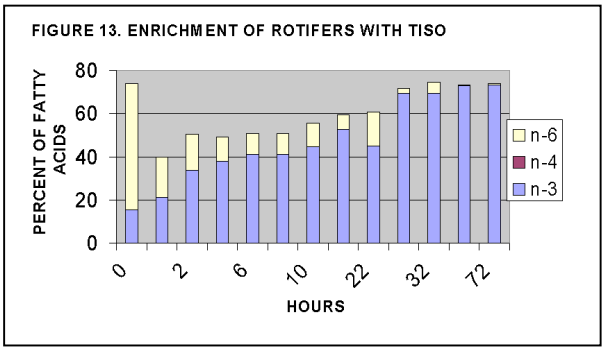



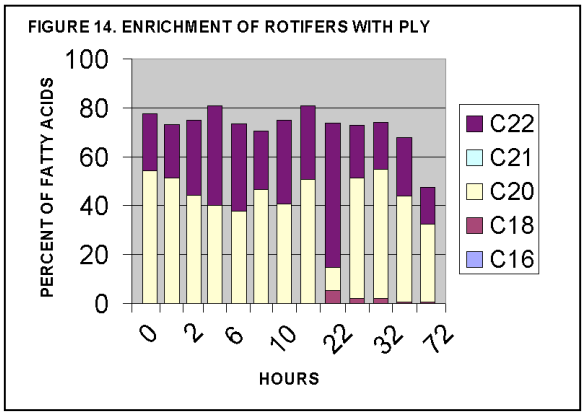



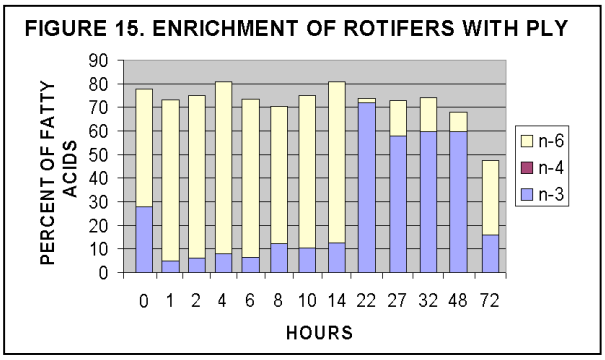



this first enrichment experiment the rotifers were enriched overnight (18 hours) with one of five different foods and concentrated using a sieve plus centrifugation of the sieve contents. This was a one-time-point analysis that resulted in one estimate of lipid (Fig 3) for each algal strain, Figs. 10 and 11. The analysis of the PUFAs of 18 hour enrichment rotifers shows the substantial improvement of all algal feeds over the basal yeast feeding of rotifers. Within the algal feeds, the Plymouth 429 strain provides the highest n-3/n-6 PUFA ratio by 18 hours. A second experiment concentrated on T-iso and Ply in a time course of enrichment, Fig 4. This time course was done in parallel with the two algal strains and the initial 8 hours of sampling was done at NMF, Milford Lab and the cultures transported to UMass Amherst and subsequent sampling continued there. The two time courses are illustrated in Figs. 12-15. The time courses suggest that maximal rotifer enrichment would be achieved at 20-24 hours based on lipid/protein ratios (Fig. 4) and C18-C22 enrichment (Fig. 12, 14) and n-3,4,6 content (Fig. 13, 15). In general it seems that Plymouth 429 is the overall best alga to use as feed when the 18 and 22 hour data of gravimetric and PUFA analysis are considered.



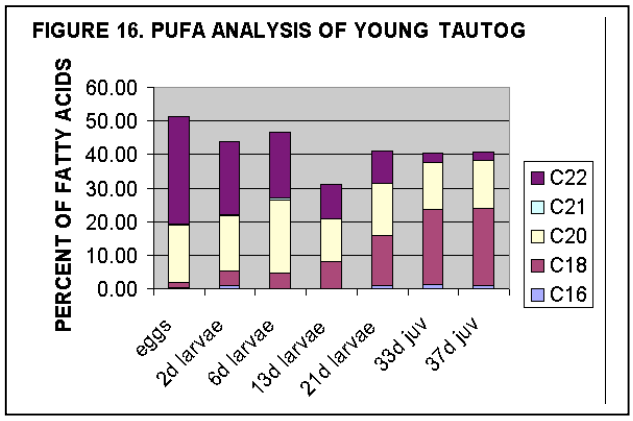



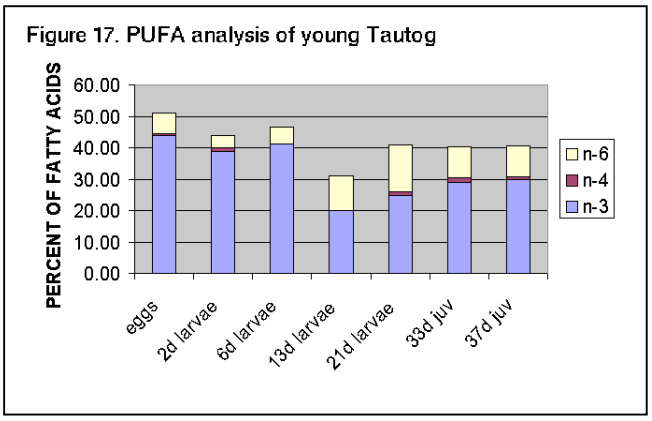

V. Lipid content during tautog development in aquaculture

Tautog eggs (0 days), larvae (2 - 21days after hatching) and juveniles (33 and 37 days after hatching) sampled from the aquaculture facility at NMF, Milford and submitted to total lipid analysis (Fig 5). The larvae at 21 days have resumed lipid accumulation, presumably from eating rotifers.

VI. PUFA transfer to larvae and juveniles

The 21day larvae and 33 and 37 day juveniles of aquaculture reared tautogs (and perhaps 13 day larvae) demonstrate increased evidence of PUFA content of their FA fraction as a result of feeding by various algal strain enriched rotifers, Fig. 16, 17. Unfortunately, these tautog larvae were not in an experiment designed to establish the preferable algal strain fed




for enrichment. They were all fed algal enriched rotifers of unspecified strain on a similar schedule. It is not clear if they would have a higher n-3/n-6 PUFA ratio if they had been fed on rotifers that had been primed with Plymouth 429 algae. The fact that early egg and 2d and 6d larvae have higher n-3/n-6 PUFA ratios may stem from the feeding of the maternal adult with large food items of high n-3/n-6 PUFA ratio. The early high n-3/n-6 PUFA ratios are presumably maternally inherited.

VII. Conclusions on PUFAs

The n-3/n-6 ratio of PUFAs has been extensively discussed in its role in health related issues (FSA, 2000). Since the tautog larvae and juveniles reared at the Milford Labs were not on a specific diet regimen of one type of algal enriched rotifer, it is not possible to suggest which enriched rotifers would have resulted in better growth of the tautogs or when they could be switched to dry fish food. However it was clear from analysis of the algae and the algal enriched rotifers that there were clear differences in the PUFA content of different algal strains and rotifers enriched by them, and that the appropriate timing of enrichment could produce rotifers of substantially higher n-3/n-6 PUFA ratio content. Rotifers are routinely reared on yeast and then enriched by feeding with algae just prior to being fed to the tautog larvae. It is clear from our PUFA analyses that yeast has a low n-3/n-6 PUFA ratio. It takes at least 22-24 hours of enriching with an algal feed before the rotifers acquire a high n-3/n-6 PUFA ratio (Fig 13, 15).

With respect to dietary effects on egg production we are able to report the n-3/n-6 PUFA ratios of the three large item foods: European Green crab *Carcinus maenas*, Blue Mussel *Mytilus edulis* and Rock crab *Cancer irroratus*. Each of these food items has a high n-3/n-6 PUFA ratio which is expected from marine organisms. The Blue Mussel tissue has the highest n-3/n-6 PUFA ratio (fig 9).

VIII. Morphometrics Analysis

A varied and flexible morphometric analysis was pursued. None of the projects are mature enough for firm biological conclusions but several methodological insights are worth reporting. These insights will be transmitted associated with the subprojects with which they were associated.

A. Morphometric Development of Tautogs

The ad hoc rearing protocols of the Tautog rearing program at Milford Labs could not provide large enough samples of larvae at close enough intervals in an experimental design contrasting nutritional factors. For a thorough morphometric approach to be developed we needed abundant material that could be divided into experimental treatment groups exposed to different nutritional sources. We instead decided to adopt organisms from fish rearing programs which were already established. We decide to use conveniently reared organisms to develop the technology that might be applied to Tautogs in the future.

B. Morphometric Development of Tilapia and Zebrafish

Tilapia and zebrafish were available in larger numbers and could be separated into treatment groups that provide a suitable statistical basis for establishing protocols of morphometric analysis. Zebrafish developmental series are well studied and a time line of images of embryonic development is available from the ZFIN database. The development has been studied at several temperatures and the timing of a developmental stage at any temperature within the normal temperature range 25-32 C can be computed using a linear equation. This is an immense benefit, which allows experiments to be designed for any acceptable temperature.

Patricia Squitiero and I have been studying zebrafish development and have developed several protocols that are beneficial to a morphometric approach intent on detecting differences between experimental treatments. One of the most effective protocols to record structure has been to use stereo pairs to record structure. This approach allows more detail to be discriminated by eye and could allow better determination of the location of 2-dimensional landmarks to be estimated given the subjective impression of better resolution of landmark location. The effectiveness of this latter protocol will need to be established by comparative studies focused specifically on this aspect of improved resolution. Such experiments will be established using undergraduate students to follow individual zebrafish embryos and larvae through their developmental path using stereo images to follow normal development. The stereo pairs will then be used as single images and as pairs to establish the trajectory of developing landmarks. By taking multiple stereo pairs at close time intervals on several embryos we will be able to establish an estimate of the error about the shape trajectory over time. The landmarks obtained from stereo pairs

Fig 18. Red/green stereo pair of two zebrafish embros. Using red green glasses allows improved resolution to be seen.

are predicted to provide less variability about their morphometric trajectories. This hypothesis will be tested using analysis of dispersion of the trajectory data. I have recently translated my package that measures analysis of dispersion from J to the more convenient statistical environment R. This will facilitate our use of this analysis of dispersion package.

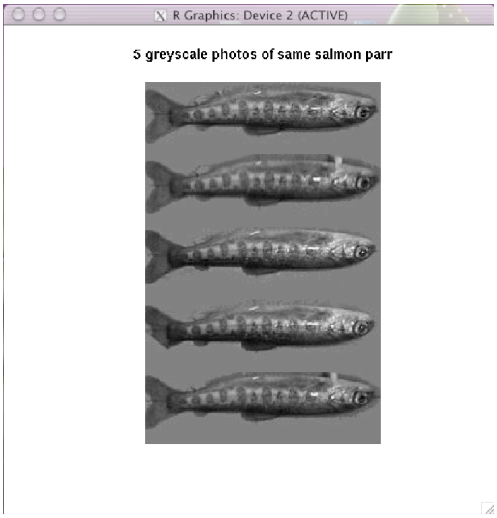
The R package has been installed on the newly installed Macintosh Os-X work stations at the Biological Computer Research Center and a course in its use is proposed for fall 2003 semester. Since the R package is available for free for all computer platforms we are confident that we will be able to develop a culture of R use in the biology sector of students that will allow us to obtain students interested in helping us in our stereo pair approach to landmark analysis.

C. Fourier Analysis of Parr to Smolt transformation in Salmon.

The parr to smolt transformation in atlantic salmon is a critical phase change that may present itself as an appropriate and economically important enough issue that applying Fourier analysis may be a feasible and logical methodology. If the proportion of parr that will smolt in a given season could be predicted early enough in the season, the size and value of the future migrating Salmon population could be available to sport and commercially interested parties.

The principle is this. The bar pattern on the side of a parr fingerlings may be a unique fingerprint for that fish (Fig 19). Fourier analysis is able to encode 2-dimensional patterns and these patterns can be compared by convolution of the FFTs of a model pattern (Fig 19 B) with an FFT of an unknown fish or fishes (Fig 19A) that one wishes to check for similarity. A convolution produces a 2-dimensional density distribution that would be tallest for a model with the identical fish (Fig 20). Non-identical fish should provide less strong convolution density distributions. It is certain that growth and slight or big differences in lighting may have a substantial effects on the shape and strength of an identity reaction and research alone will determine if this technique has sufficient power to be useful in recognizing a parr after a release and subsequent recapture. It may be that a special photographic arena may need to be designed to improve the reproducibility of lighting conditions and to eliminate glare distortions. If reidentifications are possible in release recapture situations, the morphometrics comparing the size and shape of the

A.




B.


Figure 19. Multiple photos (A) of same parr salmon which should give an identity reaction with the model form (B).

advanced parr to the earlier parr could be sufficient to predict if the parr will smolt this season. Use of the R programming environment is likely to be useful in developing this protocol of comparing models (photos of newly recaptured parr) with an archived library of prior captured and released parr. An additional feature of properly convolving parr models with archived pictures will be the scaling of the later parr model with a presumably smaller dimensioned younger fish prior to doing a convolution.

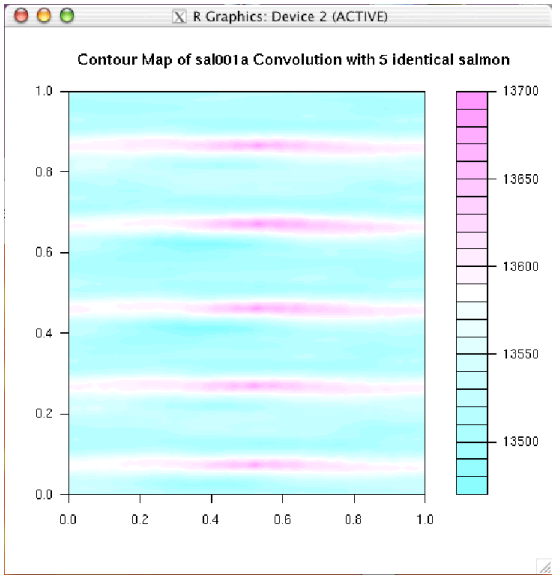



Figure 19. The model parr of Fig 18B is convoluted with the multi-image file of Fig 18A. The higher magenta patterns indicate the density distributions of identity reactions to the five photos of the identical fish.

Given the patterning evident in older Tautog it is possible that the Fourier comparison protocols developed during this approach to mark recapture study of salmon could be used for other fish exhibiting patterns such as the Tautog.

IX. Morphometric conclusions.

It is clear that we have not met the specific objectives of the original Tautog Research Request (Topic 19. 1998). However the specific applications to the tautog were prevented by unanticipated lack of abundant expendable embryos for our morphometric experimentation. None-the-less we have made good use of the available resources that the NOAA/CMER funds provided and we expect that our developed morphometric protocols would be applicable to following tautog development if an effort in that direction is desired. The protocols for these methods will be published at our laboratory protocol and ancillary data site, URL: http://www.bio.umass.edu/biology/kunkel/pub/.

The use of the statistical programming and calculation environment, R, is being encouraged by a users group, the Biometry Project, centered on the UMass campus which maintains an interactive WikiWiki website for cooperation and communication at URL: https://bcrc.bio.umass.edu/phpwiki/index.php/BiometryProject. My implementation of Analysis of Dispersion using R will be reported there as well as examples of its application to sample data sets.

X. Active Personnel during entire project.

1. Joe Kunkel, PI. Morphometric analysis and protein purification and analysis.
2. Joe Zydlewski, Postdoctoral Associate. Lipid analysis and immunologic assay.
3. Ray Moniz, Undergraduate. Senior undergraduate student assisting Joe Zydlewski in HPLC.
4. Rahul Sharma, Undergraduate. Graduate technician assisting Joe Kunkel in immunology.
5. Jeff Xu. Graduate student working on image analysis.

6. Patricia Squitiero. Undergraduate student working on zebrafish morphological and physiological development.

7. Arne Christiensen. Graduate student working partly on zebrafish biochemical development which used morphological states as reference points for comparing biochemical developmental observations.

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