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AGE DETERMINATION OF *HOMARUS AMERICANUS* USING AN INDEX OF LIPOFUSCIN ABUNDANCE

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ABSTRACT

A lipofuscin abundance index is used for age determinations (Sheehy, M.R.J., 1989) of *Homarus americanus*. Lipofuscins are identified on histological preparations of the brain by fluorescence microscopy and histochemical staining. Lipofuscin abundance is quantified by semi-automated measurements of its yellow auto-fluorescence under U.V. excitation. The fluorescence is measured either by digital image analysis of the preparations or by analogue photometry of fluorescence intensity. The between-age-groups variability is much greater than the between-individuals variability, allowing precise age discriminations.

A preliminary calibration on preparations from cultured lobsters of known age (Courtesy Bodega Bay, U.C. Davis team) is conducted. Lipofuscin provides a precise age index (R^2=.95). No significant differences are found between lipofuscin accumulations in individuals of a same age group. A 100% discrimination of individuals into correct age groups could be achieved. Over the range of ages studied, a positive exponential or an allometric model adequately represent lipofuscin accumulation vs age. The Lipofuscin/age relationship should not be calibrated on lobsters reared at constant temperature for age determinations of wild lobsters because the lipofuscin contents can differ considerably.

Calibrations are attempted on wild lobsters using age determinations based on modal analysis of young individuals and life

cycle information. The heterogeneity of lipofuscin abundance reveals that ages in small size modal groups may already be quite variable. The life history and genetic characteristics of each individual might also affect lipofuscin metabolism. The waters of the Gulf of St Lawrence are strongly stratified in summer (-1ºC to 22ºC) and provide a very heterogeneous habitat. Individuals life histories may widely differ.

Notwithstanding the difficulties encountered for calibration, our best working hypothesis suggested an age of 5 years for a 63 mm carapace length (minimal legal size) males and 8 years for one female of the same size.

INTRODUCTION

After the recent decline of the cod fishery, the lobster fishery now provides the most important catch in value within the Gulf of St Lawrence. The forecasting of lobster recruitment to the fishery several years in advance is of growing importance for management as well as economic planning. Unfortunately, the estimation of the abundance of un-recruited age groups is hindered by the present difficulty to estimate the age of lobsters.

For age determinations one must rely exclusively on sizes. The molt and growth schedules may vary considerably between individuals and between locations. Only the very first modes in the size-frequency distributions of the youngest benthic stages may be attributed to age groups. Large modal sizes are artefacts created by a combination of harvesting regulations, and molt groups. Lobsters having molted the same number of times may belong to very different age groups. This is partially due to the fact that lobsters may molt less than once a year as they grow older. Females for instance will alternate molting and spawning in such a way that they will normally skip a molt while incubating eggs.

Attempts to age lobster by other means than size have not been fully successful. For instance, the number of antennular segments or the number of ommatidia have proved to be better correlated with size than with age (Henocque, 1987; Shelton,1991).

We attempt to adapt a technique developed by Sheehy (1989) for age determinations of a crayfish, *Cherax cuspidatus*. The accumulation of lipofuscins in tissues during aging is found in a wide range of animals, from arthropods to mammals. The biochemistry of the accumulation in the human eye has been described (Eldred and Lasky,

1993). It appears that lipofuscin accumulation is actually the consequence of an incomplete destruction of metabolites during the process of rejuvenation of the cells. The rejuvenation results from a destruction (lysis) and rebuilding of cellular structures, but the process of lysis would become incomplete with senescence. Tissues in which the cells are thought to have stopped dividing (postmitotic tissues) should contain an amount of lipofuscin which is a function of age. By calibrating the lipofuscin/age relationship and fitting an adequate model, it should be possible to read age as a function of amount of lipofuscin deposited.

MATERIAL AND METHODS

Biological material

Thirty four lobsters from early benthic to commercial sizes were collected from the wild in Malpeque Bay (Prince Edward Island) by trawling (Conan *et al.*, 1994). The lobsters were selected from modal groups ranging in size from 30 to 80 mm. The individuals were measured, weighed and sexed. Caution was taken not to chose lobsters with missing legs or signs of regeneration that might have hindered growth. The sampling had been designed to be simultaneously used for surveying and mapping the abundance of all size categories of lobsters (Conan *et al.*, this meeting). It is felt that the lobsters selected well represent average characteristics of the Malpeque Bay stock.

Eighteen lobsters of known age were obtained from an aquaculture facility (Courtesy Bodega Bay laboratory, University of California Davis). Three sets of six individuals were provided each from three hatching events which had occurred on 11 August 1991, 19 February 1992, and 6 August 1992. At the time of the tissue sampling these lobsters were respectively 42, 37 and 31 month old.

After trials on the heart and other tissues, the olfactory lobe of the brain was selected as an expected post-mitotic tissue for measurement of lipofuscin abundance. Prior to dissection the lobsters were anesthetized at cold temperature. The brains of the animals from the wild were dissected in vivo. The cephalothoraxes of the animals from the aquaculture facility were fixed in toto immediately after sacrifice. The fixations were made for 24 hours in 10% formalin in seawater. The lobsters from the aquaculture facility were shipped and remained in the fixative medium for 3 days.

Lipofuscin abundance index

Lipofuscin deposits were identified and quantified on histological serial histological sections of the olfactory lobe by selective histochemical staining (Pearse, 1985) and by U.V. induced auto-fluorescence. We had initially considered extraction and titration by standard biochemical methods, but we were deterred from this procedure by the fact that "lipofuscins" a complex mixture of products which, so far, cannot be easily detected in solution. Lipofuscin granules conversely have a definite structure and can be identified in histological preparations by auto-fluorescence or staining.

The lipofuscin deposits were located by comparing sections stained by reactants specific to different biochemical characteristics of lipofuscin, and by observation under U.V. light within a range of wavelengths specific for lipofuscin auto-fluorescence. Histological preparations were made from 6µm serial sections in paraffin wax using standard mounting procedures. Lipofuscin-specific staining was achieved using either Sudan black periodic acid-Schiff, or haematoxylin-eosin (Pearse, 1985). Unstained preparations were observed under a fluorescence microscope, using 330 to 380 nm UV excitation light.

The abundance of lipofuscins in the deposits within a section was quantified by measuring the auto-fluorescence they produce either by image analysis of a digitized colour picture (IADCP) of the section, or by direct analogue measurement of the light emitted (DAMLE) by a specific area of the section. Initial trials for directly digitizing the image of the slide under microscope, using a digital camera, and feeding the data directly into a computer did not provide high enough a resolution in colours and a sufficient pixel definition. A higher definition, both in colour resolution and in definition was obtained by taking a colour photographic and digitizing it using a standard page scanner. In DAMLE, the amount of light emitted by the lipofuscin was measured by a photometer mounted on a Leitz fluorescence microscope. The power of resolution of the photometer was enhanced by a monochromator and a photomultiplier. The position and size of the field measured was adjusted manually. We proceeded by measuring the light emitted both by the preparation in toto, and by 3 conspicuous lipofuscin granules.

In the IADCP, the scanned digital data was processed using the software "Adobe Photoshop". Colours were scanned into three components red, green, and blue (RGB) for display on three colour plane monitors. The intensities of the red, green, and blue

components for each pixel were classified into frequency histograms which were analyzed separately. Since each picture was a different photographic frame with its own scale of colours, each picture needed to be calibrated separately. For calibration, the range of intensities in red, green, and blue is measured on histograms taken from fields on the digitized photograph which appear to contain lipofuscin exclusively. The modal sub-components characteristic of lipofuscins were then identified and estimated on the digitized *in toto* photograph. In this process the frequencies of the intensities within the range identified as characteristic of lipofuscin were simply summed. After initial trials, for simplicity, the analyses were conducted only on the green component which contained most of the information on the characteristic yellow fluorescence of lipofuscin. The index of lipofuscin is represented by the number of fluorescent pixels or by the ratio of the number of fluorescent pixels representing the organ.

In the DAMLE, the measures of light emitted we automatically processed by the MPV-STAT software provided by Leitz. Direct measurements of light intensity emitted within the selected range of wavelengths either by the lobe *in toto* or by the selected field, were provided as output.

RESULTS

Location and aspect of lipofuscin concentrations.

Lipofuscin appeared to be randomly distributed within the olfactory lobe in the form of structured granules of sizes ranging from 2 to 6 µ. The granules fluoresced in bright yellow (560 to 620 nm) under 330 to 380 nm UV light. The granules are conspicuously revealed by Sudan Black, periodic acid-Schiff reaction, and Haematoxylin-eosin.

The distribution of lipofuscins along a sequence series of serial sections is presented in figures 1 and 2. The data is provided both as counts of fluorescent pixels (1) and as the ratios of fluorescent pixels to the total number of pixels within the section of the lobe (2). Figure 1 shows a quadratic relationship between counts of fluorescent pixels and their serial (Spearman rank correlation P=.47) location which was satisfactorily corrected by taking the ratio index (Fig 2). Subsequently all further statistics were calculated for ratio indices.

A histogram of the frequencies of counts or ratios of fluorescent pixels on a complete series of 74 serial sections of an olfactory

lobe (figure 3) showed a symetrical distribution which was approximated by a normal curve. A Student test shows that a random selection of 12 sections would provide an estimate of mean ratio within +/- 10% of the true mean in 95% of cases. For practical purposes only 12 sections were fully analyzed for each lobster in further work.

Relationship between lipofuscin abundance measured by IADCP and age in reared lobsters

The best fit was obtained for an exponential or allometric models (R^2 = .95) rather than a simple linear (figure 4, 5, and table 1). There were no significant difference (P>.05) between lipofuscin abundance indices of different individuals within the same age group (ANOVA and Kruskall-Wallis). The average lipofuscin abundance indices for individuals to different age groups did not overlap. The between-age-group difference was significant (P<.0001) when tested by ANOVA.

Within each age group there was little correlation between the lipofuscin abundance and the weight of the individuals (Fig. 6, R^2 < .25). It was the age independently from the weight of the individual which appeared to determine its lipofuscin content.

Relationship between lipofuscin abundance measured by IADCP and age of wild lobsters

The lipofuscin contents of lobsters taken from the wild in Malpeque Bay were much lower than those of reared lobsters of comparable size. However, the wild individuals were thought to be much older than the reared lobsters, based on previous tagging and modal analyses of size frequency distributions. The lipofuscin content vs age relationship of wild individuals could not be calibrated from data on reared individuals.

Alternatively age were estimated from modal analysis and previous knowledge on the molting seasons and molting frequencies of lobsters in Malpeque Bay (Moriyasu, 1984). Figure 7 and table 2 illustrate the relationship between lipofuscin abundance and age as defined from the size of the individuals studied and the position of the first 6 modes in the size frequency distributions. There was a definite relationship between lipofuscin content and presumed age, and a discrimination between presumed ages was achievable for most individuals on the basis of their lipofuscin content However, there were significant differences between the lipofuscin contents of individuals within any of the modal groups at the 5% level, when

tested by ANOVA. The mean lipofuscin contents of the different modal groups did not differ significantly when compared by ANOVA (Table 3).

Measurements of lipofuscin abundance by DAMLE and age

The DAMLE results were comparable to the IADCP results, but the discrimination between age groups was not as good, and there were significant differences ($P < .05$) between individuals within the same groups. Measurements on individual granules did not provide a better resolution than measurements on the lobe *in toto*. The DAMLE was a faster but less selective procedure than the IADCP. Photometric measures were possibly corrupted by light emissions from sources other than lipofuscin.

DISCUSSION

Our experimental results can be explained in the context of the current interpretations of lipofuscin metabolism. Within an age group, the lobsters of known age obtained from a rearing experiment all came from the same brood, and their indices of lipofuscin accumulation did not differ significantly. This may have resulted from the fact that they all were genetically similar, that they had been reared under similar conditions, but also that they had precisely the same age. Conversely lobsters taken from the wild and attributed to the same age group had significantly different indices of lipofuscin accumulation. Their actual birth dates must have been spread over a whole spawning season of about a month since they most likely originated from distinct broods. They had experienced different life conditions in an environment which is highly seasonally and spatially variable, ranging in temperatures from -1 to 20 ºC, and in salinity from 20 to 33 per mil.

The animals obtained from a rearing experiment provide simplified information which is useful for testing models of lipofuscin accumulation vs age. The relationship appeared to be curvilinear and monotonic with a slope increasing with age (over the age interval studied). An exponential or an allometric model provided a good fit. This is similar to observations on *Cherax* for the early part of the lifespan only (sheehy, et al., 1994).

Although the inadequacy of using reared animals to calibrate the age/lipofuscin abundance relationship is understandable, it is not fully explained. We have no information on the influence of genetics or on environmental factors on the process of lipofuscin

accumulation in lobsters. The variability of lipofuscin contents in animals taken from the wild and attributed to the same age group increases considerably with presumed age. This may reveals an artefact due to imprecise age determination by modal analysis starting from the smallest modal sizes and increasing to the largest. The number of molts and the time interval between molts is known to diverge between individuals mostly after onset of maturity which is reached at a size varying considerably between individuals.

Although very provisional, the age/lipofuscin content model we produced can be tentatively used for estimating the age of lobsters captured in Malpeque Bay. We recommend however, cautious interpretations since we do not have information on the year to year variability in lipofuscin accumulations, or on environmental effects..

The most efficient way of calibrating the age/lipofuscin content for lobster in their natural environment would involve in tagging and recapture of early benthic stages of the lobster. It would then be possible to analyze the between-brood, the between-location and between-year variability. The experiment should be run over a minimum of ten years to allow for a comprehensive coverage of the presumed life span of commercially harvested lobsters. This may seem a tedious task, but in the present context of total lack of precise information on the age structure of natural lobster populations, and of the commercial catch, the investment would be highly profitable.

ACKNOLEDGMENTS

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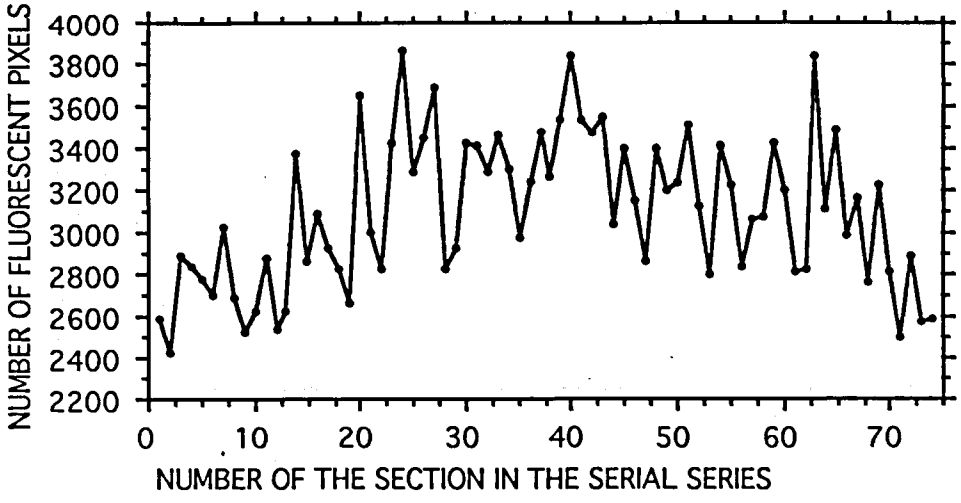


Figure 1- Lipofuscin deposits along 74 histological 6 µm serial sections of the olfactive lobe of lobster. The deposits appear smaller towards the extremities due to the lobular shape of the organ.

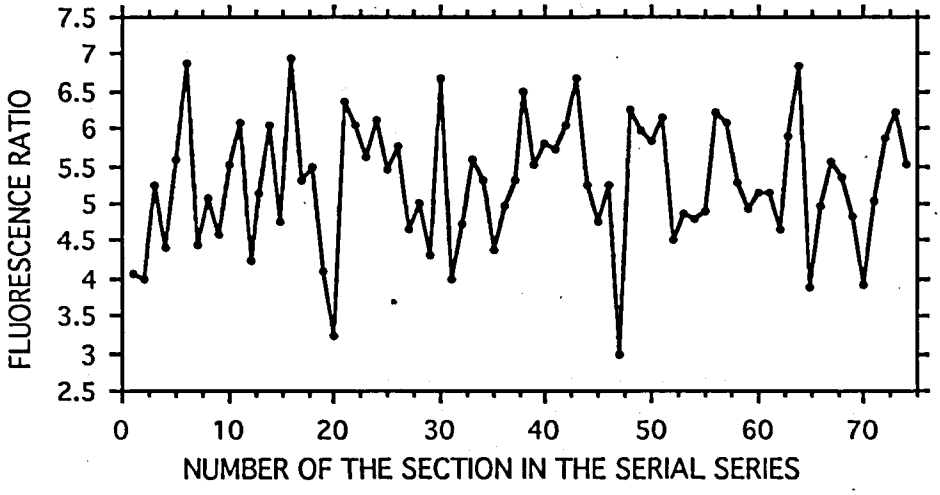


Figure 2- Lipofuscin deposits along 74 histological 6 µm serial sections of the olfactive lobe of lobster. The fluorescence ratio is the ratio of the number of fluorescent pixels to the total number of pixels representing the organ. This type of index provides similar results independently of the position of the section.

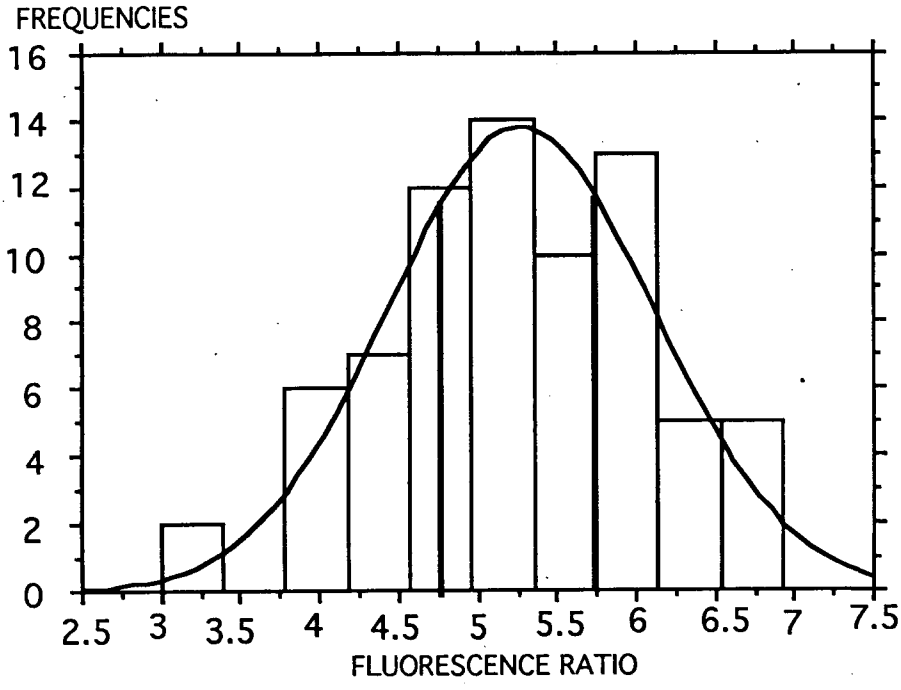
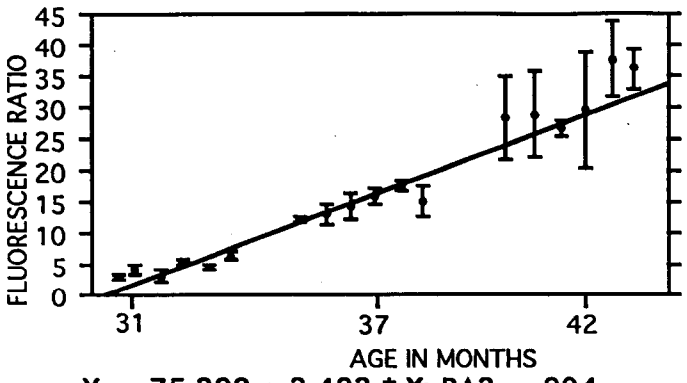
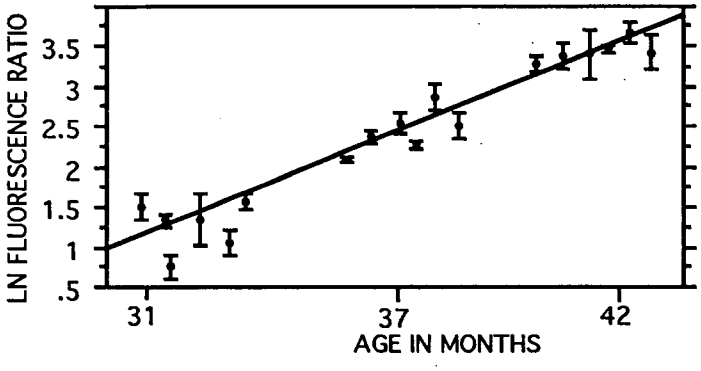


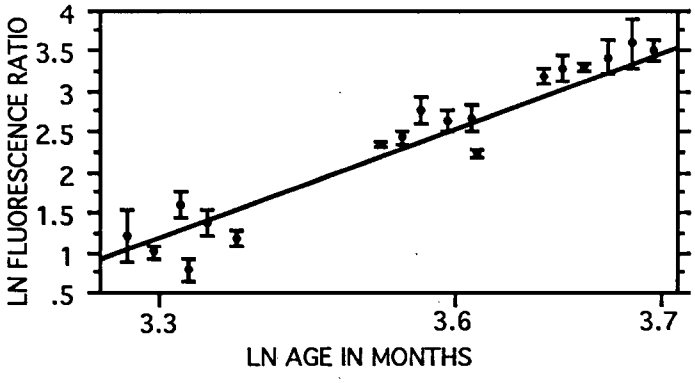
Figure 3- Frequency distribution of the fluorescence ratios in 74 serial sections of the olfacory lobe of a lobster. The distribution can be approximated by a normal curve.Twelve random samples would provide an estimate within +/- 10% of the mean in 95% of cases.



Y = -75.388 + 2.483 * X; R^2 = .894



Y = -5.21 + 0.207 * X; R^2 = 0.951



Y = -24.516 + 7.481 * X; R^2 = .955

Figure 4-Reared lobsters. Linear, exponential and allometric models for fluorescence ratio vs age data. All lobsters within a group had identical ages although the points were shifted in abscissa for clarity of the display.

Table 1- Fit of models to Fluorescence ratio vs age relationship for reared lobsters. Residual variance for untransformed variables.

LINEAR MODEL	16.7
EXPONENTIAL MODEL	9.0
ALLOMETRIC MODEL	9.1

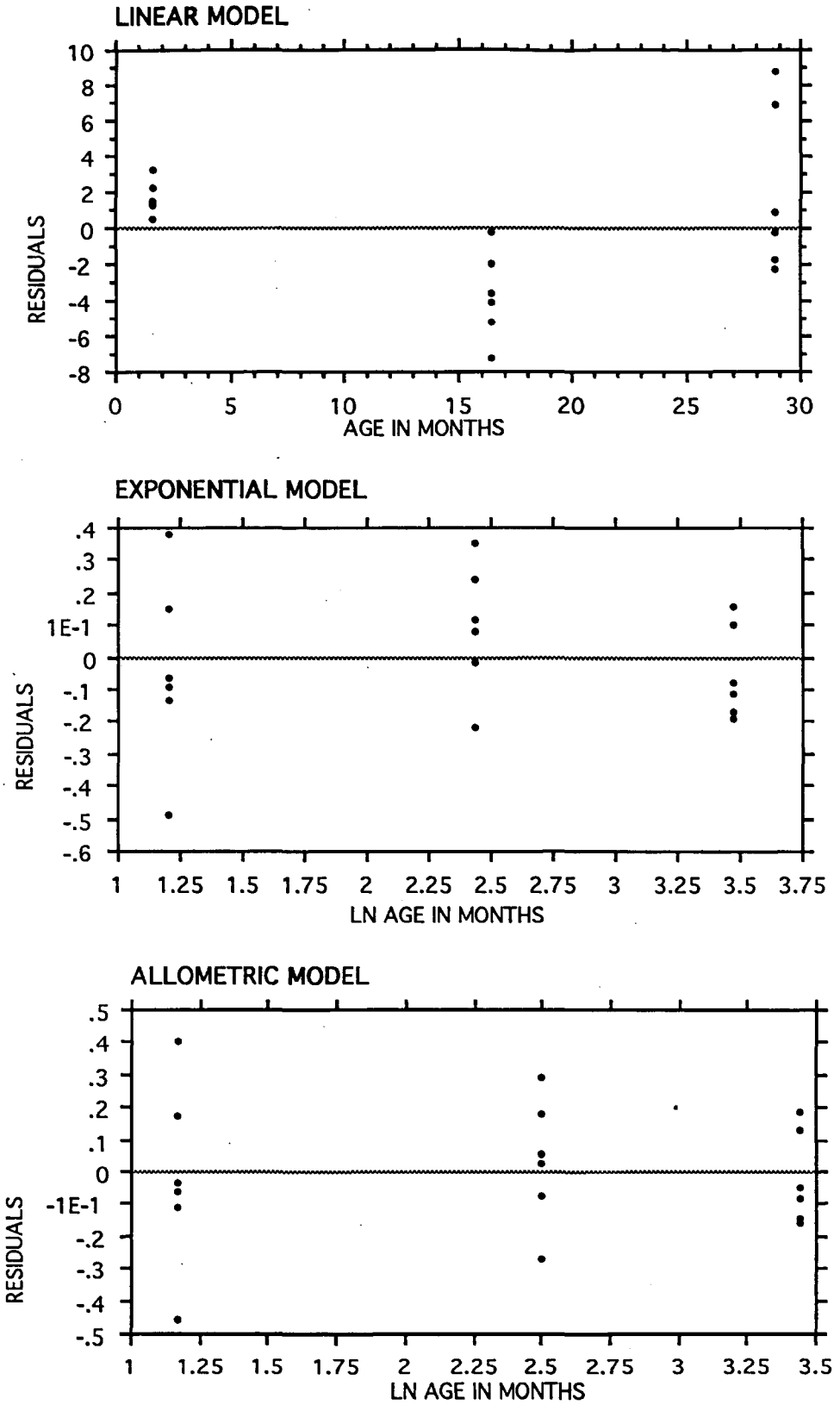
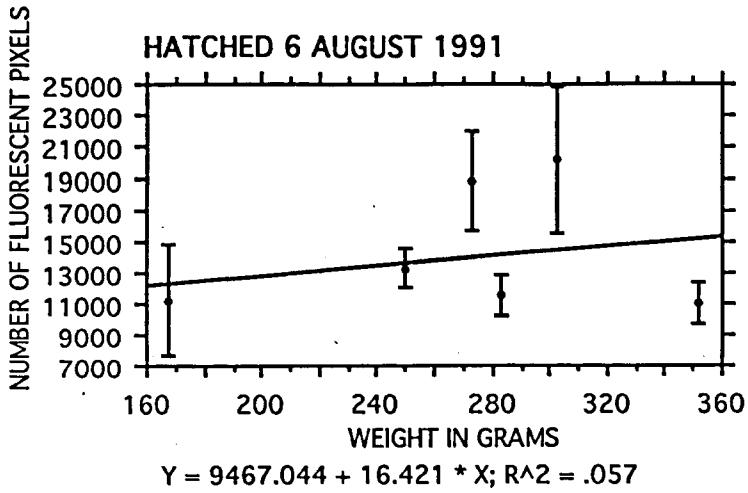
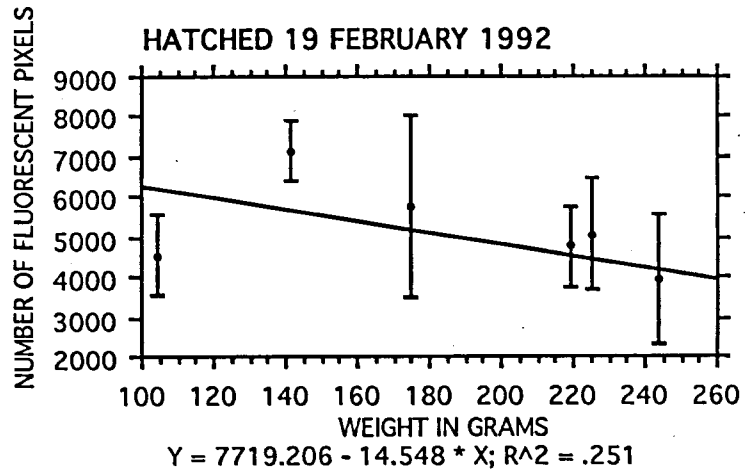


Figure 5-Residual plots for the linear, exponential and allometric models of fluorescence ratio vs age for untransformed variables. The allometric model provides the most balanced residuals and the exponential model is the next best. The linear model is not satisfactory.





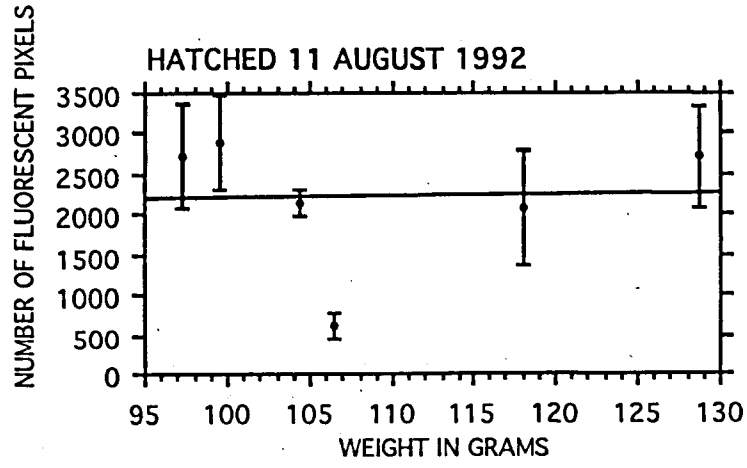
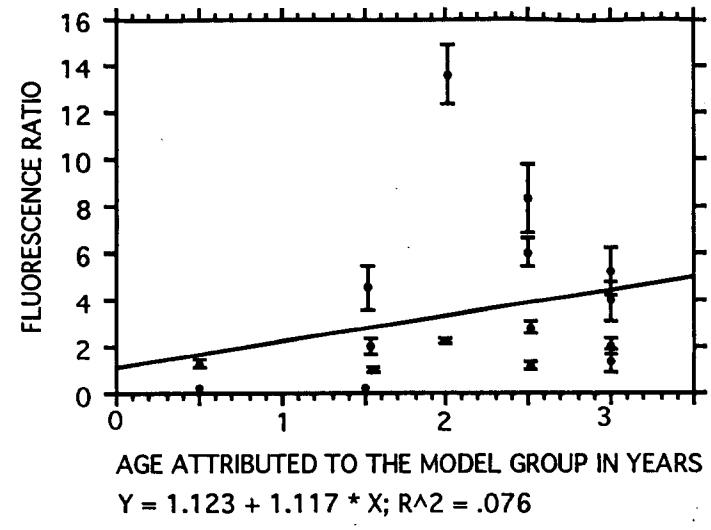
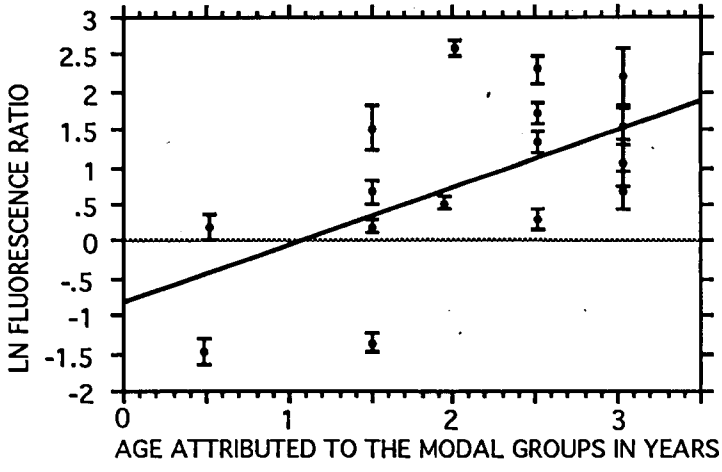


Figure 6-There was no apparent relationship between fluorescence ratio and weight of lobsters within a same age group. It appeared to be the age and not the weight of the lobsters which determined the fluorescence ratio. If the number of fluorescent pixels is independet from weight, the fluorescence ratio should be more independent from the weight of the individuals.





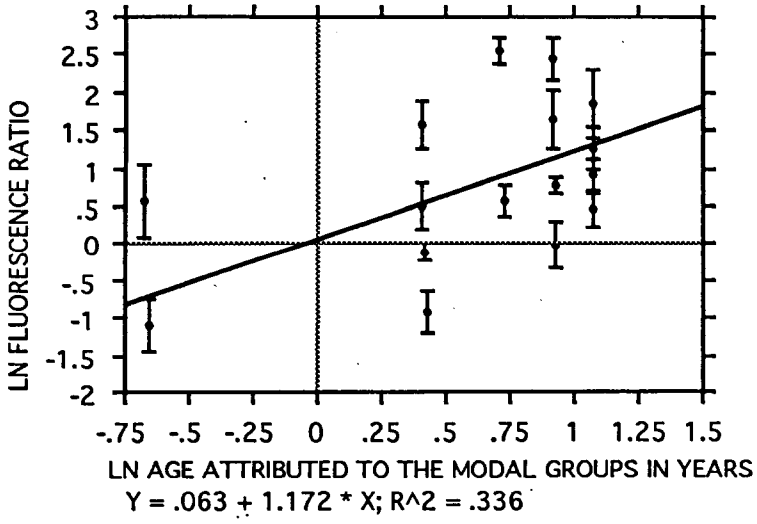


Figure 7-Lobsters from the wild. Linear, exponential and allometric models for fluorescence ratio vs presumed age.

Table 2- Fitting models to Fluorescence ratio vs age of wild lobsters. Residual variance for untransformed variables.

LINEAR MODEL	11.36
EXPONENTIAL MODEL	12.99
ALLOMETRIC MODEL	12.34

Table 3- Analysis of Variance in fluorescence ratio between individuals within the first 6 modal groups presumed to be age groups. The differences are not significant. Regressions on a linear exponential or allometric model do show that a correlation exists, however.

	SIGNIFICANCE
FLUORESCENCE RATIO	.29
LN FLUORESCENCE RATIO	.17