TRIAL OF THE USE OF LIPOFUSCIN AGE-PIGMENT FOR AGE DETERMINATION OF WESTERN ROCK LOBSTER (*Panulirus cygnus*)

FRDC Project # 93/090
15/8/93 - 14/2/95

FINAL REPORT

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in collaboration with the
Western Australian Fisheries Department

and

Funded by the
FISHERIES RESEARCH AND DEVELOPMENT CORPORATION

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Non-technical summary:

1. The western rock lobster fishery represents one of Australia's most valuable marine resources. To ensure, year by year, that the fishery is harvested in both a maximal, and yet sustainable way, there is a need for continuous and intensive research determining if and how the lobster population is changing (its population dynamics) in response to fishing and other factors.

2. The ongoing monitoring of the population dynamics of the rock lobster for management purposes has necessitated the acquisition of data on age composition. Present methods for age determination of the lobster and other crustaceans have a number of limitations. Body size is not a very reliable indicator of age. Thus, it is important to seek alternative approaches to age determination of the western rock lobster.

3. One such alternative approach is the lipofuscin ageing method. Lipofuscin is a relatively insoluble fluorescent lipid compound which naturally accumulates with age as a metabolic byproduct in the nervous tissue of a wide range of organisms. Early attempts to biochemically measure lipofuscin in crustacean tissues have encountered considerable methodological difficulties but the measurement technique has been refined by the utilization of fluorescence microscopic and computerized image analysis techniques. This procedure has yielded some very promising results. In 7 years of research by the principal investigator using freshwater crayfish, lipofuscin was found, over a range of conditions, to be a consistently better predictor of age than body size. This suggests considerable potential for lipofuscin as a tool for both strategic and routine age determination in fisheries research but further validation of the technique is required.

4. The overall objective of the lipofuscin age pigment studies on western rock lobster is to produce an accurate alternative ageing and validation tool which ultimately assists in the management of the fishery. The specific objective of this project was to determine whether lipofuscin age pigment in the brain of the rock lobster could provide a more reliable measure of age (year class) than the traditional carapace length measurement.

5. Lipofuscin concentrations were determined for a sample of 198 lobster from the inshore reefs at Seven Mile Beach, Western Australia. Modal analysis of a lipofuscin concentration-frequency histogram was used to provide an indication of population age structure. The results of this procedure were compared with those from the conventional size-based methodology.

6. In the sampled group, several extra year-classes were apparent from the lipofuscin data in addition to those indicated by a conventional size-frequency histogram from the same group. Modal separation for older year-classes appeared superior when using lipofuscin concentration. Size groups appeared to consist of a wide range of ages suggesting the possibility that groups in the size-frequency histogram were artefacts of the sampling regime.

7. Analysis of the progression of the lipofuscin concentration groups indicated a remarkably constant lipofuscin accumulation rate of about 0.34% by volume per year, with detectable accumulations commencing after the first post-settlement year. The mathematical model generated thus provided a preliminary basis for age estimation of lobster using lipofuscin concentration.

8) Lipofuscin measurements suggested that the sample was composed primarily of individuals of 3 yr to 8 or 9 yr of age. This confirmed the previously-determined range of ages known for this area from tagging studies, although this was not apparent from size-frequency data.

9) The relationship between carapace length and lipofuscin concentration suggested a faster growth rate for males than females, as expected. The growth curve for juvenile lobster was very similar to that published in the previous literature. In future it should be possible to construct complete growth curves for wild *P. cygnus* at various locations by using the relationships between lipofuscin concentration and carapace length. The variability of size-at-age, as determined by lipofuscin analysis was also consistent with earlier studies.

10) Project results strongly supported the proposition that a lipofuscin ageing method would be useful for age determination in this fishery and others and that such a technique would provide greater accuracy than size-based methods presently in use. A supplementary study is now required to validate the present results. This study will involve determination of lipofuscin accumulation rate from known age juvenile lobster reared in the laboratory at ambient sea temperatures. Preparations for this study are now underway.

11) The present approach seems to discriminate juvenile year classes in *P. cygnus* very well and it would be extremely informative to conduct a further study, identical to the present one but examining individuals above legal size, This would indicate the complete extent to which year classes can be resolved in lipofuscin frequency histograms from the *P. cygnus* population.

12) Studies seeking a more rapid and less expensive lipofuscin quantification protocol are also proceeding.

Background to the R & D Project:

The Western Australian rock lobster fishery is the most valuable single species fishery in Australia worth some 200 million annually in export dollars. The resource is heavily harvested with a high unit value and the primary policy objective of the authority managing the fishery is to ensure that fishing effort exerted on the resource is contained at sustainable levels. This produces a need for continuous and intensive research primarily directed toward assisting the management authority, through revision of the fishery data, population modelling and refinement of catch forecasting. The western rock lobster fishery is considered to be one of the best researched and managed fisheries in the world (Australian Government, 1991). The ongoing modelling of the population dynamics of the rock lobster for management purposes has necessitated the acquisition of data on age composition of the lobster stocks. Presently there are no entirely adequate techniques for ageing the lobster. An alternative approach, using lipofuscin age pigment, has shown considerable promise in several years of laboratory trials (see below). The present project represents the first large-scale field trial of the method in a crustacean fishery. It follows an earlier pilot study (FRDC 92/148) in which the presence of age pigment in the lobster was demonstrated and a preliminary age relationship was established.

Project objective:

"To determine whether lipofuscin age pigment in the brain of the rock lobster can provide a more reliable measure of age (year-class) than the traditional carapace length measurement."

Introductory technical information concerning the research need:

Previously, age estimation of lobster has been achieved using the conventional method of modal analysis of size (carapace length) - frequency histograms from instantaneous samples of the population. Such modes are interpreted as year classes. They are assumed to result initially from seasonally intensified periods of reproduction and recruitment, with modes in larger size classes ideally reflecting progressive, cohesive, growth of such recruits over successive years. As is often the case, modal analysis of western rock lobster length-frequency data has been made difficult or impossible by growth rate variability between individuals and the tendency for growth rate to decline at maturity. Both of these phenomena tend to cause modes to merge with each other, particularly in older age groups, so that age classes cannot be distinguished. For *Panulirus cygnus*, the maximum longevity of which exceeds 25 years, only the 2+ or 3+ year classes can usually be separated in this way, older groups becoming progressively harder to distinguish. Mathematical approaches to the separation of age group modes have been attempted but have given conflicting results except where modes were already obvious (Chittleborough, 1976). Tag-recapture and laboratory rearing studies have provided supplementary information for the construction of growth (size at age) models but the wide divergence in growth rate of lab-reared individuals (Phillips *et al.*, 1983) can mean that back-calculated ages from such models are quite inaccurate (Sheehy, 1992). As emphasized by Crossland *et al.* (1988), the "establishment of a precise age criterion has wide and important implications for research and for the management of the western rock lobster fishery, and, by extension, for lobster fisheries elsewhere" Thus it is important to investigate alternative approaches to age determination of this species.

One such alternative approach is the putative lipofuscin ageing method. Lipofuscin is an autofluorescent lipoprotein which accumulates with age in the nervous tissue of a wide range of organisms and is thought to represent a relatively insoluble metabolic by-product. The first attempted use of lipofuscin for age determination of a crustacean was that of Ettershank

(1983) on Antarctic krill. Following its inception, the proposed lipofuscin ageing method for aquatic organisms proved controversial. Unfortunately, a substantial effort during the 80's by researchers interested mainly in fisheries applications, both here and overseas, has proved fruitless because of the use of an invalid biochemical quantification procedure inherited from mammalian and insect gerontology (Sheehy, in press). However, since 1989, an alternative histological approach to the quantification of lipofuscin has produced some very promising findings. Using a decapod, the freshwater crayfish, *Cherax*, as a model aquatic organism, nervous tissue lipofuscin was found to be a consistently better predictor of age than body size, over a range of constant laboratory temperatures and under variable field conditions (Sheehy, 1990, 1992; Sheehy *et al.*, 1994, 1995). Lipofuscin accumulation is independent of growth. Based on an analysis of individual variation in relation to rate of increment, it was predicted that year-class separation in frequency histograms would be better separated when using lipofuscin concentration as the age index rather than carapace length and that lipofuscin might therefore offer a whole new dimension for age determination. The present study represents a test of that prediction.

Detailed Description of Research Methodology

[For justification of methodology see technical introduction above]

Experimental animals:

One hundred and ninety-eight *Panulirus cygnus* collected in mid-October 1993 from the gazetted research area on the inshore coastal reef off Seven Mile Beach, Dongara, Western Australia were used for this study. These individuals had not yet migrated into deeper water and were not reproductive. A stratified sample (uneven sampling effort) provided lobster from a range of sizes and ages. Sampling was undertaken over 2 days at 3 different locations: 100m from shore in water approximately 2 m deep, 200m offshore in water approximately 4m deep and 1km offshore in water approximately 20m deep. Collapsible 'parlour and bedroom' pots with small mesh were used at the two shallower locations and standard cane pots were used at the deeper site. Sampling was conducted so as to obtain a total of about 200 individuals with approximately equal representation in each of at least two, and preferably more, 10 mm size classes. The rationale for this protocol was that a sample size of about 200 was considered a minimum requirement for adequate modal analysis of frequency histograms and in order to test modal separation a sample containing at least two, but preferably more, year-classes was required. Average growth of juveniles/year was known to be around 10 mm carapace length. The sample used for analysis contained 104 females and 94 males, with 7 individuals between 30.0 and 30.9 mm carapace length, 43 between 40.0 and 49.9 mm, 50 between 50.0 and 59.9 mm, 50 between 60.0 and 69.9 mm and 48 between 70.0 and 79.9 mm. There were several 'whites' in the latter group.

Dissection and fixation of brain tissue:

Each lobster was first anaesthetized by placing in icy sea-water. Its carapace length, weight, sex and any other pertinent details were recorded. Bone cutters were then placed in the mouth of the lobster and a 1cm long incision made laterally from each side of the mouth to sever the circumoesophageal commissures. To facilitate handling and fixative penetration, the horns, antennae, antennules and eye stalks were severed as close as possible to their bases, ensuring clean cuts so as not to stretch nerves and damage the brain. Bone cutters were then inserted into the hole where the right antenna was removed and a cut made through the carapace so as to snip out only the 'face' of the lobster (the brain lies immediately inside the carapace between the bases of the eye stalks). The incision passed from the base of the antenna to the area just

behind the horns, then down through the other antenna base and then across and around the flat forward projecting plates under the clear cuticular window at the base of the eye stalks. As the circular cut was completed, care was taken not to tear the face away from the rest of the body as this could again rip nerves and damage the brain. Once the face was loose, a pair of fine scissors was inserted into the underlying tissue and the face was gently snipped away so that about a 2 cm depth of tissue including the brain was retained underneath. Any traces of digestive gland and gut contents were rinsed from the dissected tissues in clean sea-water. The sample was placed in a leak-proof 500 ml container of chilled (4°C) fixative and left chilled, but not frozen, for at least 2 days. Fixative consisted of 10% v:v formalin in clean sea-water with 16% w:v sucrose (as an osmotic adjuster). After fixation the head was thoroughly rinsed free of fixative solution and the brain dissected out under sea-water by careful removal of overlying yellowish fatty tissue. All projecting nerves were trimmed at their bases except for the right circumoesophageal connective which was left relatively long so as to facilitate orientation of the brain for future sectioning. The isolated brain was then longitudinally halved with a very sharp scalpel using a slicing rather than a pressing action. The right half with the long commissure was used for further processing.

Histological processing:
Brains were processed for standard wax embedding and sectioning by passage through the following solutions: phosphate buffer rinse (3 hr); ethanol, 70% (3 hr), 85% (3 hr), 95% (3 hr), 100% (2 hr); ethanol:xylene 1:1 (1 hr); xylene (2 x 30 min); embedding wax at 60°C (30 min); embedding wax under vacuum at 60°C (15 min). For the present large sample, brass rods were used to carry stacks of Lynx disposable tissue baskets. Three rods each carrying 22 baskets, each basket containing 3 specimens, were sufficient to carry and process the whole batch at once. The rods were placed in a tall measuring cylinder and processing chemicals exchanged in the cylinder when required. Right brains halves were embedded by placing the sliced midline surface of the brain half against the bottom floor of the embedding pan, using the remaining commissure for manipulation via fine tweezers. Serial 6-µm longitudinal sections were cut through the tissue blocks. All sections containing the right olfactory cell mass were mounted sequentially on slides.

The general techniques for lipofuscin diagnosis and quantification have been fully described in the principal investigators previous publications (Sheehy, 1989, 1990; Sheehy and Wickins, 1994). Brain sections were de-waxed in 3 x 2 min changes of xylene and mounted under a coverslip in DePeX medium. Autofluorescence of sections was observed under a Zeis-Jenaval epifluorescence microscope with a 100x Planapochromat oil immersion objective while exposed to 450 nm blue excitation light. So as to encompass the full range of variation in concentration of lipofuscin, 10 sections were selected at evenly spaced intervals through the olfactory cell mass. The fluorescent emission from the sections above the cut-off filter wavelength of 515 nm was photographed on 400 ASA Kodak colour print film

Image analysis:
A Dapple 2GS Image Analyzer with Imageplus software was used for evaluation of pigment quantities in the micrographs. Micrograph images were video-captured in standardized lighting conditions after maximizing the contrast of the video signal. By adjusting grey-scale cut-off levels, the instrument was able to discriminate brightly fluorescing lipofuscin granules from darker background tissue and produce binary images of this. Binary images were manually edited to remove noise and include any granules which had not been automatically discriminated. The perimeter of the tissue in each section was traced using a mouse and the

tissue area determined from this. Background tissue area in the sections was strictly defined as the cross-sectional area of the neurone cell bodies and lipofuscin granules only, not fibres or membranous material. Software calculated fractional area of lipofuscin granules in each olfactory cell mass section. This value, averaged over the 10 micrographs, gave an estimate of the average lipofuscin volume fraction in the right olfactory cell mass of each lobster.

Analytical treatment of data:
Both arithmetic and geometric means were employed for determining average lipofuscin concentrations from the 10 replicate images for each lobster. Geometric means were calculated as antilog $\frac{1}{n} \Sigma \log (L+ c)$, where L was the lipofuscin concentration in each section and c was a small constant (0.04) to compensate for zero concentration values. Means were also weighted accorded to the cross-sectional area of tissue present in the images with means from lesser areas having lower weights.

An analysis of covariance (ANCOVA) of the relationship between carapace length and lipofuscin concentration was conducted to examine any sexual differences in this relationship which might indicate differing growth rates.

From the carapace length data, a standard size-frequency histogram was constructed. The size of class intervals in frequency histograms can have a marked effect on histogram appearance and the ability to detect year-class modes. Selection of a class interval of 3 mm for the present carapace length-frequency histogram was based on Chittleborough's (1976) practice of applying a sliding average across 3 x 1 mm intervals to smooth distributions. The results obtained by him from such histograms were validated using tag-recapture studies and were therefore considered reliable. A lipofuscin concentration-frequency histogram was also constructed. For valid comparison with the carapace length-based histogram, class interval was chosen so as the bulk of the data, excluding a few outlying individuals at the upper extreme, lay within the same number of intervals as present in the size-frequency histogram. This class interval was 0.1% VF.

In order to elucidate modes (putative year-classes) in frequency histograms, Battacharya's (1967) method for separation of gaussian components was used through the ELEFAN 1.11 program (ICLARM, Makati, The Philippines). Goodness of fit of the expected frequency distribution with the actual distribution was checked with χ^2 tests. Modal separation indices (Sparre, 1989) were also calculated using this program.

Individuals were designated to a particular putative year-class based on the position in which they occurred in either the OLCM lipofuscin concentration- or carapace length-frequency histogram. All modes overlapped to some extent, so the expected proportion of individuals from each of the adjacent modes in each class interval had to be determined. At each interval, the appropriate proportion of individuals with lower carapace lengths or OLCM lipofuscin concentrations were arbitrarily designated to the left mode of the overlapping pair and the remaining individuals with larger carapace lengths or OLCM lipofuscin concentrations were designated to the right mode. The distribution of OLCM lipofuscin concentrations within individuals from the same size mode was examined to give an indication of the temporal integrity of each size class.

Intermodal distances in the OLCM lipofuscin concentration-frequency histogram were used to determine the OLCM lipofuscin accumulation rate in the field. Using OLCM lipofuscin concentration-based year-class estimations a linear regression of OLCM lipofuscin concentration on age was obtained.

Results:

The raw data set is presented in Appendix 1. Figure 1 shows the relationship between carapace length and OLCM lipofuscin concentration for the present sample (filled circles) superimposed over the growth curve for *P. cygnus* as described by Phillips *et al.* (1992). The relative position of the lipofuscin concentration and age axes was determined using the modal-analysis based calibration described below. Note that the present data closely overlie the expected trend. Hollow circles represent data from the pilot study in which individuals came from a different sampling location and were, in part, selected for their large body size. Note also that the upper size range of lobsters collected in the present sample corresponded closely to the legal carapace length limit of 76mm. Analysis of covariance of carapace length vs lipofuscin concentration regressions for males and females indicated that the slopes of the equations were similar but the intercepts were significantly different ($p<0.05$). For a given lipofuscin concentration, males tended to have a greater carapace length than females. Assuming similar lipofuscin accumulation rates for males and females, this indicated that females were slower growing. The figure also gives an indication of the degree of variation in size-at-age.

Figure 2A shows the carapace length-frequency distribution for the whole lobster sample, with superimposed gaussian components as determined by the Battacharya method (see Appendix 2 for results summary). Three distinct modes were apparent at mean carapace lengths of 45.6 mm, 59.5 mm and 73.1 mm. These modes were subsequently designated as modes '3', '4' and '5', respectively (see discussion) There may also be a small mode at 35.0 mm ('2'). Figure 2B shows the OLCM lipofuscin concentration-frequency histogram for this sample. OLCM lipofuscin concentrations ranged from 0.131% to 3.459% VF. The Battacharya method discriminated 6 normal components within the data. Modes were at 0.331, 0.680, 0.990, 1.333, 1.714 and 2.024% VF. Expected frequencies within carapace length- and OLCM lipofuscin concentration-frequency histograms based on the mixture of normal distributions indicated in Figure 2 did not differ significantly ($P>0.05$) from the observed distributions according to χ^2 tests (see Appendix 2). Figure 2C shows the lipofuscin concentration-frequency distribution utilizing geometric rather than arithmetic means. Note the compression of data and slightly better resolution of modes at the upper end of the distribution. The arithmetic mean-based concentration-frequency histogram has, for the present, been used in further analyses and discussion.

In Fig 3A-D, the distribution of OLCM lipofuscin concentrations of individuals falling into each of the 4 modes in the carapace length-frequency distribution is shown. The first OLCM lipofuscin mode was primarily composed of individuals from the 45.6 mm modal size class, which was considered to represent individuals of approximately 3 years of age (see discussion). This was taken as a reference age for the following year-class designations for higher modes in the OLCM lipofuscin concentration-frequency histogram.

It can be seen that each size group contained individuals of a wide range of OLCM lipofuscin concentrations. Most of the few individuals from the smallest apparent size group (c.l. 35.0

mm, '2') occurred in the first OLCM lipofuscin mode along with a majority of individuals from the 45. 6mm c.l. size class (3 yr). A substantial proportion of individuals from this second size group (c.l. 45.6 mm, '3') fell also into the OLCM lipofuscin mode designated as 4 yr, with a decreasing proportion of individuals up to the sixth mode (8 yr). The third size group (59.5 mm, '4') contained individuals primarily in the 4-6 yr OLCM lipofuscin modes, but with some individuals as young as 3 yr and with a decreasing proportions of individuals at higher ages. The 4th size group (73.1 mm, '5') contained individuals mainly in the 5yr and higher OLCM lipofuscin modes. The highest OLCM lipofuscin concentrations were encountered in some individuals in this group indicating individuals of 8 years or older. On the other hand this size group also appeared to contain a few individuals as young as 3 yrs of age.

Although each size-based mode contained a considerable mixture of age groups, the first three modes in the OLCM lipofuscin concentration-frequency histograms were identifiably composed of individuals primarily from the corresponding three major modes in the carapace length-frequency histogram. A comparison of modal separation indices was therefore undertaken (Table 1). It can be seen from this table that individuals in the '2' and '3' size groups were apparently not separable using OLCM lipofuscin concentration which was quite low in these individuals. Separation of modes designated as 3-5 yrs was better for carapace length than OLCM lipofuscin but the carapace length modes were apparently not pure age groups. The separation of the OLCM lipofuscin concentration modes was well above the critical value of 2. Carapace length apparently could not discriminate individuals above 5 yrs of age whereas there was good separation of modes for individuals from 6-8 yrs of age based on OLCM lipofuscin concentration.

Modal averages from the OLCM lipofuscin concentration-frequency histogram formed a straight line when plotted against their designated year-class (Figure 4). This line had a slope of about 0.34% VF per year and an intercept indicating that resolvable OLCM lipofuscin accumulation begins at an age of about 1.9 yr.

Discussion:

The high level of scatter in the relationship between carapace length and OLCM lipofuscin concentration (and predicted age) found in this study (Fig 1.) was consistent with earlier results for this species and others (see Final Report FRDC 92/148). Based on the expected relatively high correlation of OLCM lipofuscin concentration with chronological age, this scatter was indicative of the known wide variation in size-at-age for *P. cygnus* (Phillips *et al.*, 1992). Chittleborough (1976) found that yearly growth rates in tagged juveniles of similar size at Seven Mile Beach varied by an order of magnitude in some cases.

The good fit, over the sample range, of the relationship between lipofuscin concentration (physiological age) and carapace length, with the conventionally derived growth curve for *P. cygnus* (Phillips *et al.*, 1992) was as expected, and consistent with previous results for *Penaeus monodon* (Sheehy *et al.* 1995a). These results strongly suggested that this relationship would be very useful for the determination of complete growth curves where calibration data from only a limited part of the lifespan is available using conventional techniques. Of great significance, the recent extensive survey of OLCM lipofuscin in tagged known-age European lobster from three landing sites (England, Scotland, Wales), showed that lipofuscin accumulation rate in the field was unaffected by either sex or geographical location (Belchier, pers. comm.).

The higher elevation of the relationship between carapace length and lipofuscin concentration for males than for females as demonstrated by analysis of covariance indicated that for a given lipofuscin concentration (age), males tended to have a greater carapace length than females. Assuming there were no sexual differences in OLCM lipofuscin accumulation rate, as concluded from previous studies on other species (Sheehy 1989, 1990, 1992, Sheehy *et al.*, 1994, 1995b, Belchier, pers. comm.), the present results show that females were slightly slower growing. This is consistent with the known growth characteristics for this species (Chittleborough, 1976). Since this growth rate difference was small however, and sample sizes were not sufficiently large for separate treatment (France *et al.* 1991), data for both sexes were pooled for size-frequency analyses.

The size-frequency histogram for the sample showed a small mode at 35.0 mm and 3 more distinct modes at 45.6 mm, 59.5 mm and 73.1 mm carapace length. These modes fell very close to the average values determined by Chittleborough (1976) for 2,3,4 and 5 year old individuals respectively, at Seven Mile Beach, from modal analysis and validated with tagging studies. These data therefore formed a suitable reference for calibration and comparison of OLCM lipofuscin as an age determinant. As indicated in Figure 1. the sample was limited to individuals less than the legal size of 76 mm carapace length.

When plotted in a similar format the OLCM lipofuscin concentration-frequency histogram showed several additional modes. There was only a limited correspondence between individuals comprising the 3 largest modes in each histogram. Each mode in the size-frequency histogram apparently consisted of individuals from a fairly wide range of OLCM lipofuscin concentrations. An interpretation of this result was that the size-frequency modes actually did not represent very pure age classes and that individuals aggregated on the reefs in groups of similar size rather than similar age. This interpretation was consistent with the findings of Chittleborough (1976) who presented evidence that the size of individuals in the wild was related to the depth at which they lived rather than their age, with larger individuals occurring in deeper water. Not only do larger individuals move to deeper water, but they grow faster there, where density dependent resource restrictions were less (Chittleborough and Phillips, 1975).

If these size-frequency modes did not strictly represent the outcome of seasonal recruitment pulses then other explanations for their occurrence must be sought. The sample regime involved collection of individuals from three different depths and there was a known size gradient on the inshore reefs with depth. This may partly explain the predominantly tri-modal appearance of the size distribution. It is apparent that the sample is artificially sheered off at the top end by the legal size limit. It could be reasonably expected therefore, that the sample might contain smaller individuals from age classes older than 5 years. This appears to be exactly what the lipofuscin concentration-frequency distribution is showing. At this point the evidence suggests that the trimodal appearance of the size-frequency histogram, with lack of differentiation of year classes at the top end, represents an artefact of the sampling regime and/or offshore migration of larger individuals and/or increasing variation in size at age.

Although the variance in mean OLCM lipofuscin determinations was very high in some older individuals due to the clustered nature of the pigment in older brains, this problem was overcome with the use of geometric and weighted means. The lipofuscin data clearly showed the existence of individuals from older cohorts than shown by the size-based data. Resolvable

modes were present for individuals up to 8 years of age. From tagging studies, Chittleborough and Phillips (1975) reported that nursery reefs contained a few individuals up to 7 years of age, although this could not be determined from size-frequency data. The present study confirmed and possibly slightly extended this range. This result, showing better separation of older year-classes, was exactly as postulated from previous studies on *Cherax.* comparing size and OLCM lipofuscin as age indices (Sheehy, 1992; Sheehy *et al.*, 1994). Thus, the present approach seems to discriminate juvenile year classes in *P. cygnus* very well and it would be extremely informative to conduct a supplementary study, identical to the present one but examining individuals above legal size, This would indicate the complete extent to which year classes can be resolved in lipofuscin frequency histograms from the *P. cygnus* population.

There is presently some uncertainty as to whether the lowest lipofuscin mode represents a three year old year-class or a two year old one. The present interpretation that this mode is comprised of three year olds was based on the fact that the average carapace length of individuals in the mode was similar to that for Chittleborough's (1976) three year olds. If this interpretation is correct then OLCM lipofuscin might not be useful for separating the youngest age groups, 1-3, because of its low concentration early in development. On the other hand it is possible that the lowest lipofuscin concentration mode predominantly represents very large two year olds disproportionately captured by the sampling gear. If this is the case then all age designations on the lipofuscin histogram would reduce by 1 year and the predicted age at which lipofuscin begins to accumulate would correspond to settlement age rather than 1.9 years. We intend now to conduct a study on known age lobster reared in the laboratory at ambient temperatures to resolve this question and verify our predicted lipofuscin accumulation rate.

Since a stratified sampling procedure was used for sampling, with differing gear and effort applied to obtain a range of sizes, a precise interpretation of the relative proportions of individuals in each modal group in terms of survivorship is not appropriate. However, the apparent predominance of individuals 3-7 years of age in the sample, as indicated by OLCM lipofuscin concentration, was broadly consistent with the findings of Chittleborough (1970).

Rate of OLCM lipofuscin accumulation (modal progression) appeared remarkably constant over the progression sampled in this survey, averaging about 0.34% VF per year. The OLCM lipofuscin accumulation curve for *P. cygnus* did not show the slight curvature with advancing age apparent for laboratory reared *C. quadricarinatus* (Sheehy, 1992; Sheehy *et al.*, 1994). This curvature was not apparent for field-reared *C. quadricarinatus* (Sheehy *et al.*, 1994) and as speculated in that paper, the present result again raised the question as to whether this non-linearity was a laboratory artefact. Alternatively, very old rock lobster were not sampled in the present study so any slowing of accumulation rate in old age may not have been detectable. Based on the observed variance in OLCM lipofuscin concentration in putative year-classes, in relation to the predicted yearly lipofuscin accumulation rate (Fig. 4), the 95% confidence intervals for age predictions based on OLCM lipofuscin concentrations are likely to be narrow indeed i.e. $< \pm 1$year.

In light of the prediction model established in the present work:

$$A = \frac{L + 0.641}{0.342}$$

where A was chronological age (yr) and L was OLCM lipofuscin concentration (% volume fraction, VF), and provided this relationship holds for locations other than Seven Mile Beach, the ages of lobster sampled in the pilot project (FRDC 92/148) can be more reliably approximated. For example, the largest individual sampled was a male with a carapace length of 153.4 mm, a whole wet weight of 2750g and an average OLCM lipofuscin concentration of 4.87% VF. Based on the latter variable this lobster's chronological age was in the order of 16 years.

In summary, the objective of this project which was to compare year-class resolution in frequency histograms based on size or lipofuscin concentration has been achieved with what appears to be a very positive outcome.

Implications and recommendations

The results of this project potentially have somewhat profound implications for research and management of the western rock lobster, other crustacean fisheries and also for more general population dynamics research on crustaceans and other groups. Inability to determine age accurately through conventional methods has proved a significant obstacle previously. Laboratory based research preceding this study predicted that lipofuscin concentration might provide a substantially better means for determining year class in crustaceans than the presently used methods (Sheehy *et al.*, 1994). That prediction seems to have held true in the present field trial on the *P. cygnus* fishery.

In the next phase of developing this method into a tool for strategic and routine fisheries research use we need to:

(i) Validate the present results which indicate a yearly lipofuscin accumulation rate in *P. cygnus* of 0.34% using known age lab-reared animals.

(ii) Establish a more rapid and less expensive quantification protocol applicable to routine use.

In order to achieve objective (i) we are currently preparing a sample of known-age (laboratory reared) lobster (2-5 yr) for analysis.

In order to achieve objective (ii) the principal investigator is examining alternative lipofuscin quantification procedures. Recent attempts to extract and biochemically assay lipofuscin from crustacean brain have not proved successful due to the low solubility and exceedingly small quantities present (Sheehy, in press). As part of the principal investigators present contract on lipofuscin-based age determination of the European lobster, a study will be made of further alternative approaches, which have already demonstrated promise in early trials. If successful, this technology should be readily transferable to *P. cygnus* and other crustaceans.

For future consideration it would be extremely informative to conduct a supplementary study, identical to the present one, but examining individuals above legal size. This would indicate the complete extent to which year classes can be resolved in lipofuscin concentration-frequency histograms from the *P. cygnus* population. Can the whole lifespan be resolved in this way? Furthermore, the present results strongly suggest that the relationship between carapace length and lipofuscin concentration would be very useful for the determination of complete growth curves for *P. cygnus* from a range of locations e.g. the Abrohlos Islands.

Intellectual property
Not applicable at this stage

Technical summary of all information developed as part of the research.
i) Lipofuscin concentration in the olfactory lobe of *P. cygnus* appears to be a superior index for accurate year-class determination over the currently available size-based method.
ii) The application of weighted geometric means to the analysis for the first time has improved year-class resolution in the lipofuscin concentration-frequency histogram.
iii) The relationship between carapace length and lipofuscin concentration appears to have great potential for the determination of complete growth (size-at-age) curves for *P. cygnus.*

Acknowledgements:
The assistance of J. Penn, R. Brown, N. Caputi, C. Chubb, N. Hall, H. Jones, N. Sumner, M. Rossbach and C. Skepper of the W.A. Fisheries Department; D. Wright of CSIRO Fisheries W.A.; and Glen Hopper and Juliet McGrath at the University of Queensland, Zoology Department is gratefully acknowledged.

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Table 1. *Panulirus cygnus.* Comparison of modal separation indices (S.I.) for carapace length-frequency (Fig 2A) and OLCM lipofuscin concentration-frequency (Fig 2C) histograms from the sample of lobster from Seven Mile Beach, Western Australia, in October 1993.

Modes (year classes)	Carapace length S.I.	Lipofuscin concentration S.I.
2-3	2.77	0
3-4	3.58	2.91
4-5	3.34	2.78
5-6	0	2.56
6-7	0	3.2
7-8	0	3.93

Figure 1. *Panulirus cygnus.* The relationship between carapace length and OLCM lipofuscin concentration for the present sample (filled circles) superimposed over the growth curve for *P. cygnus* as described by Phillips *et al.* (1992). Hollow circles represent data from the previous pilot study in which individuals came from a different sampling location and were in part selected for their large body size. Horizontal line represents 76mm carapace length minimum legal size limit.

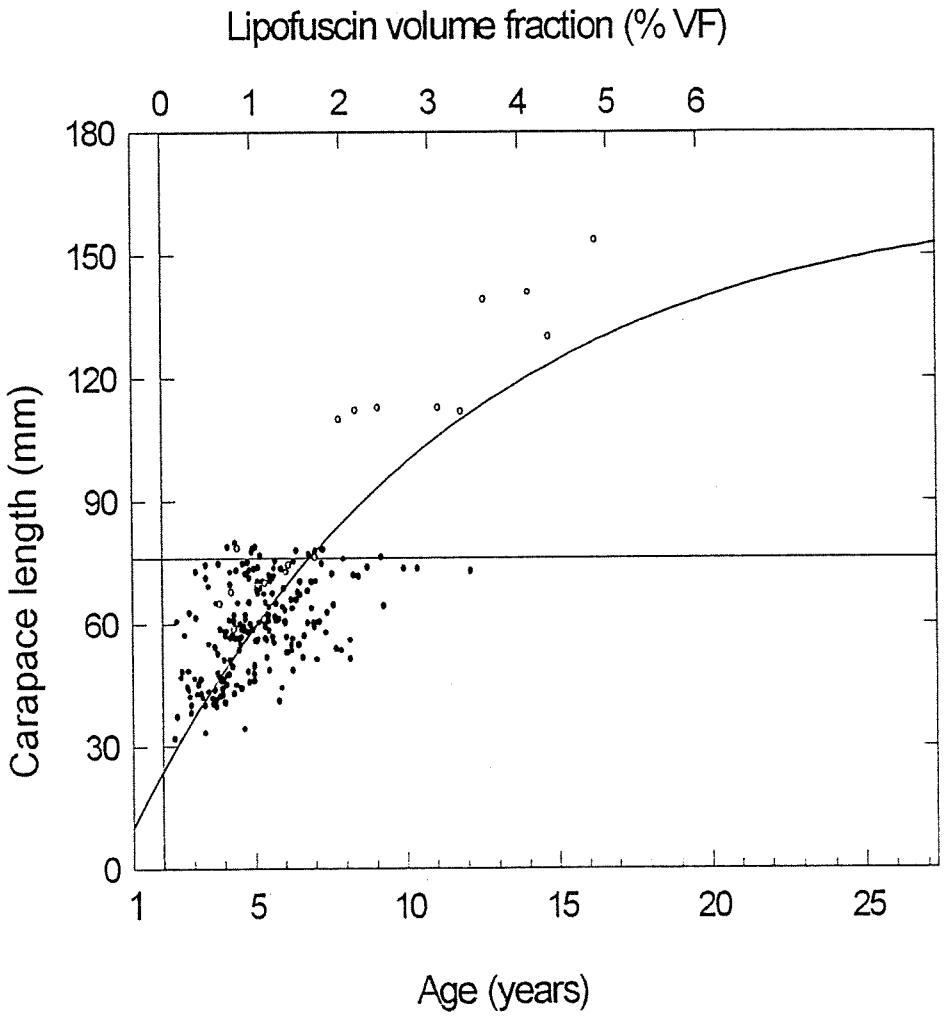


Figure 2. *Panulirus cygnus.* Frequency histograms and component normal distributions fitted with ELEFAN 1.11 using the Battacharya method for A) carapace length and B) OLCM lipofuscin concentration (arithmetic means). Designations '2'-'5' are based on Chittleborough's (1976) year-class determinations.

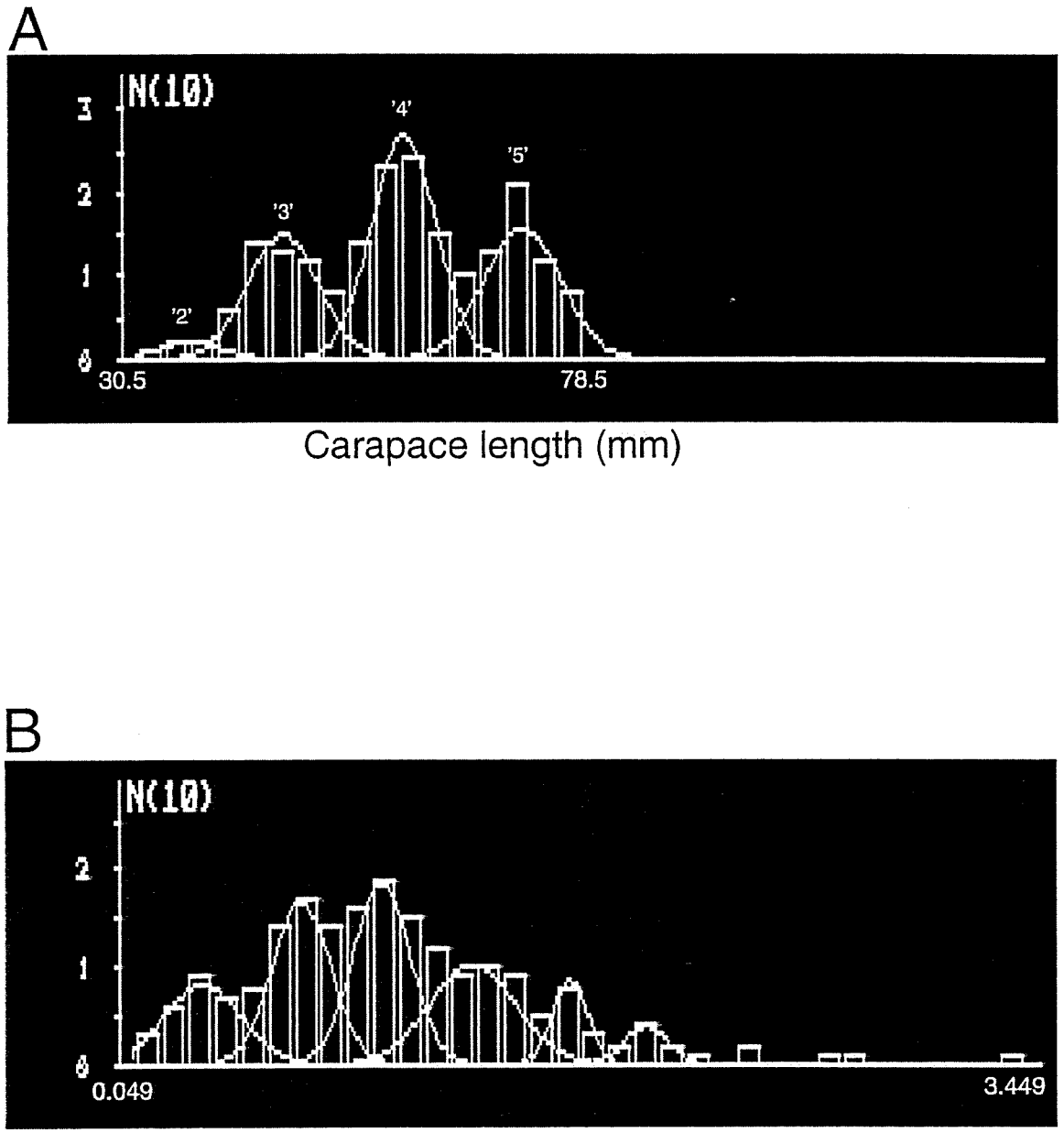


Figure 2C. *Panulirus cygnus.* Frequency histogram for OLCM lipofuscin concentration recalculated using weighted geometric means.

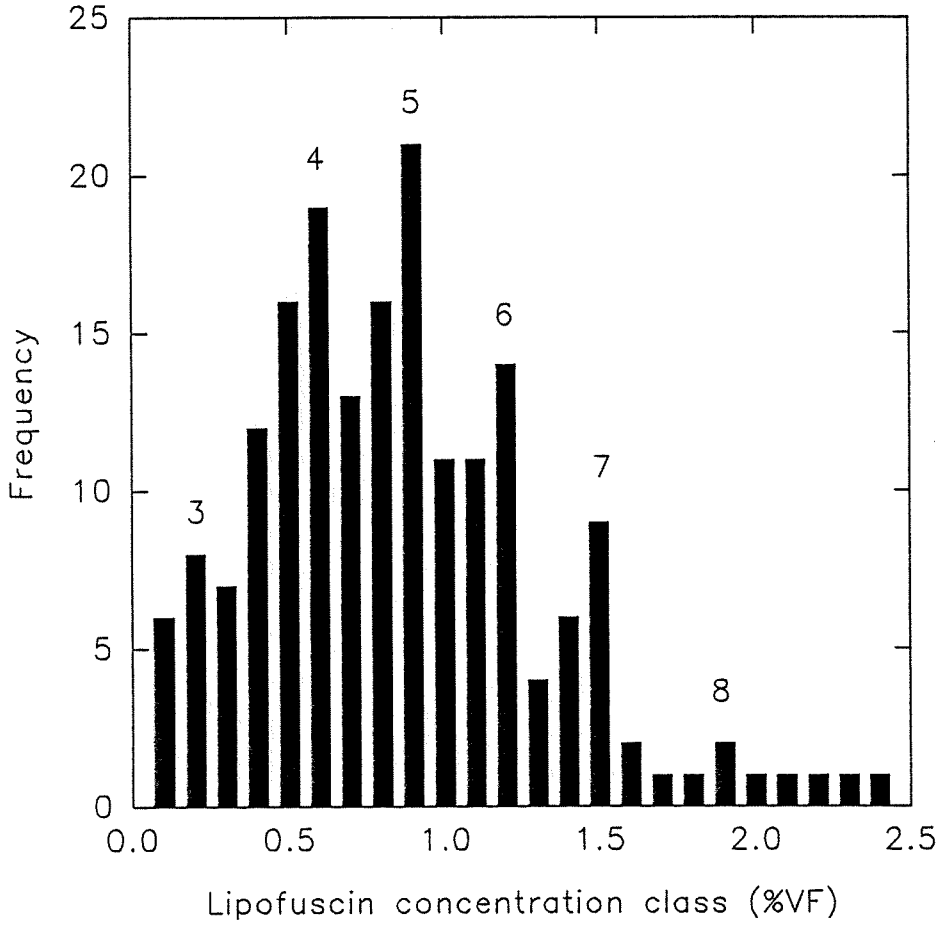


Figure 3. *Panulirus cygnus.* OLCM lipofuscin concentration-frequency histograms (arithmetic means) indicating the location of individuals falling into each of the four modes visible in the carapace length-frequency histogram shown in Figure 2A. Year-class designations on the second OLCM lipofuscin histogram are calibrated based on the fact that the first mode was primarily composed of individuals classified as 3 yr olds by Chittleborough (1976), from size-frequency analysis, tagging and laboratory rearing studies.

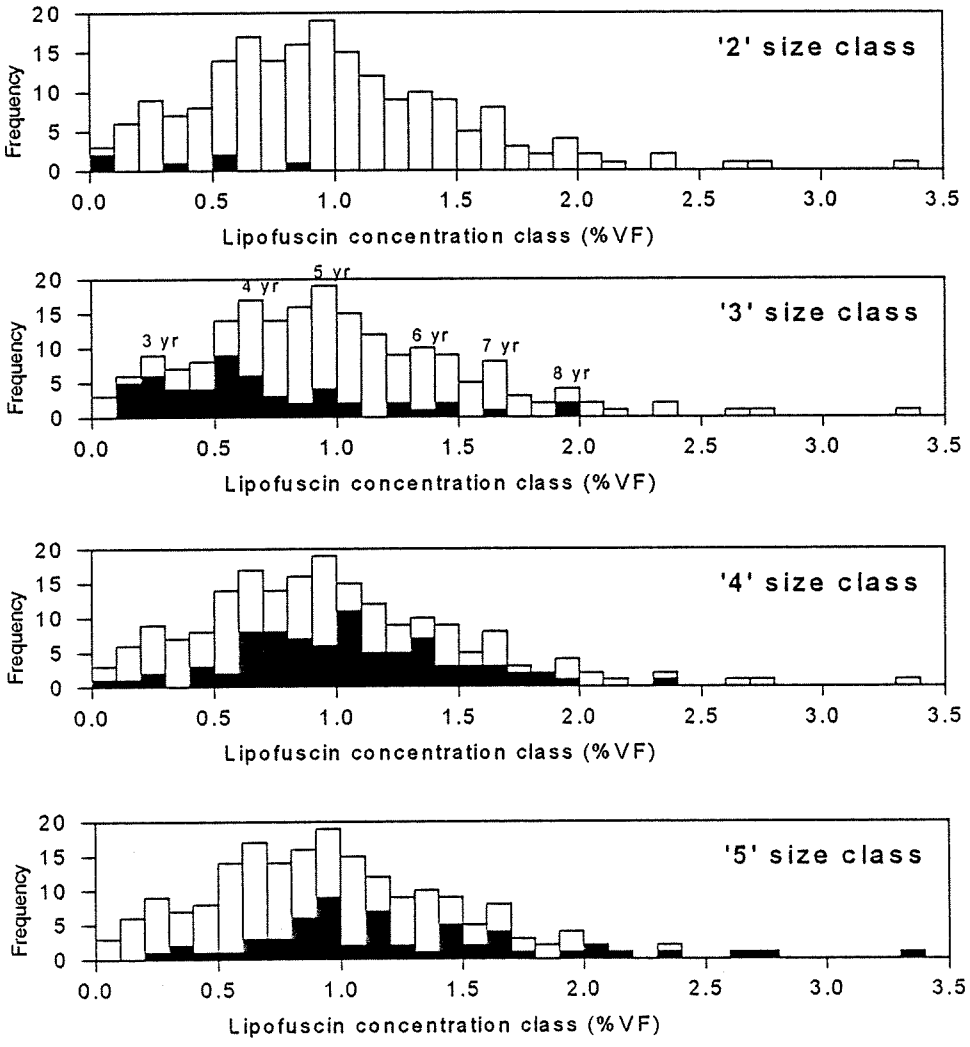
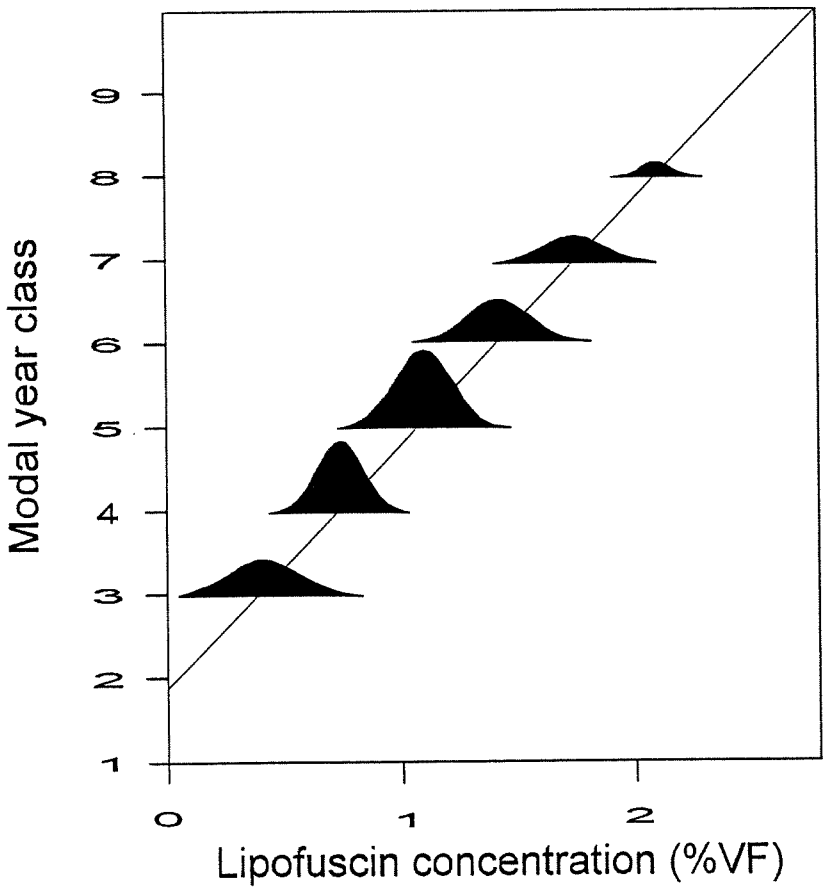


Figure 4. *Panulirus cygnus.* Progression of OLCM lipofuscin concentration modes between year-classes based on the frequency histogram depicted in Fig 2B with a regression line fitted through mean lipofuscin concentrations in each group.



Appendix 1. *Panulirus cygnus.* Raw data set for FRDC Project #93/090

ID #	Carapace length (mm)	Body weight (g)	Sex	Arithmetic mean OLCM lipofuscin concentration (%VF)	Variance of lipofuscin estimate	Modal position (carapace length)	Modal position (lipofuscin concentration)
300	64.9	241.4	F	0.6	0.1	4	4
301	40.5	72.2	F	0.7	0.17	3	4
302	71.3	334.8	F	2.2	1.51	5	8
303	67.3	299.8	M	1.24	0.87	5	5
304	62	214.7	F	1.2	0.35	4	5
305	61.3	214.3	M	0.38	0.19	4	3
306	65.7	261.7	M	1.5	0.59	4	6
307	71.8	348.9	F	2.14	0.6	5	8
308	63.7	233.3	M	1.46	2.82	4	6
309	56.8	167.2	M	0.79	0.07	4	4
310	56.1	178	F	1.07	0.58	4	5
311	58	191.9	F	0.71	0.06	4	4
312	53.3	140	F	1.44	0.83	3	6
313	51.6	125.5	F	1.57	0.72	3	7
314	70.2	356.16	F	1.06	0.31	5	5
315	63.1	236	M	1.38	0.71	4	6
316	65.7	275	F	1.46	0.07	4	6
317	58.8	199.3	M	0.81	0.27	4	4
318	65.8	274.8	F	1.45	0.86	4	6
320	64.7	279.3	F	1.29	0.28	4	6
321	63.7	230.6	M	1.67	0.46	4	7
322	54.6	144.9	M	1.54	0.79	4	6
323	68	265.8	M	1.63	2.9	4	6
324	60.5	223.7	F	1.14	0.36	4	5
326	45.9	89.8	M	0.65	0.81	3	4
327	46.4	88.4	M	0.69	0.19	3	4
328	60.6	210.6	M	0.74	0.11	4	4
329	65.1	248.2	F	1.15	0.26	4	5
330	60.7	233.1	F	1.28	0.1	4	6
331	64	255.3	F	1.2	0.67	4	6
332	43.9	91.11	F	0.89	0.22	3	5
333	58.2	172	M	1.2	0.68	4	6

334	61.1	204.9	M	0.93	0.25	4	5
335	42.4	70.6	M	0.68	0.38	3	4
336	51	135.9	F	0.68	0.29	3	4
337	31.8	33.3	F	0.13	0.01	2	3
338	72.5	329	M	0.76	0.26	5	4
339	43.9	82.68	M	0.67	0.34	3	4
340	55.5	151.4	F	1.06	0.2	4	5
341	56.1	172.9	M	1.46	0.78	4	6
342	60	202.7	F	1.71	1.19	4	7
343	48.1	113.4	M	0.96	0.2	3	5
344	42	69.8	F	0.65	0.14	3	4
345	46.3	95.4	M	0.44	0.17	3	3
346	73.3	400.4	F	2.72	0.99	5	8+
347	67.1	269.8	M	1.14	1.13	5	5
348	78.7	446.9	M	0.73	0.15	5	4
349	53.3	157.8	F	2.01	0.54	3	7
350	62.5	221.2	F	1.85	0.74	4	7
351	73.2	363.8	F	1.33	0.46	5	6
352	58.5	192.1	F	1.01	0.55	4	5
353	67.1	314.6	F	1.07	0.41	5	5
354	61.5	229	M	1.17	0.36	4	5
355	47.8	104	M	0.61	0.22	3	3
356	56.4	182.2	M	1.15	0.33	4	5
357	42.7	75.5	F	0.8	0.19	3	4
358	73.5	370.2	F	2.3	0.76	5	8
359	53.5	147.1	M	0.86	0.14	3	4
360	40.8	61.1	M	1.32	0.85	3	6
361	64.9	276.7	M	0.84	0.2	5	4
362	71.9	332.3	M	1.9	1.36	5	7
363	56.2	170.7	F	0.82	0.21	4	4
364	57.7	189.8	F	1.84	1.21	4	7
365	53.7	150.3	F	1.94	1.15	4	7
366	46	96.1	M	1.03	0.22	3	5
367	59.1	170.3	F	1.71	0.94	4	6
368	41.6	78.2	F	0.44	0.07	3	3
369	37.1	51.23	M	0.17	0.02	2	3
370	59.1	191.08	M	0.92	0.35	4	5

371	40	65.9	M	0.32	0.02	3	3
372	49.3	122	M	0.78	0.09	3	4
373	44.8	92.6	F	0.83	0.6	3	4
374	58.3	167.8	M	0.88	0.41	4	5
375	60.5	223.3	F	1.19	0.16	4	5
376	41.9	73.41	F	0.31	0.1	3	3
377	57.1	180.8	F	0.25	0.06	4	3
378	34.1	38.3	F	0.91	0.21	2	4
379	44.1	86	F	1.33	0.26	3	6
380	73.6	362.5	M	1.06	0.47	5	5
381	45	98.2	M	0.4	0.13	3	3
382	56.2	172.7	M	0.76	0.18	4	4
383	56.9	179.9	F	1.59	1.1	4	7
384	69	298.1	M	0.51	0.05	5	3
385	77.4	444.3	M	1	0.25	5	5
386	61.1	210.8	M	1.31	0.9	4	6
387	72.7	349.1	M	0.37	0.13	5	3
388	61.6	243	F	1.27	0.76	4	6
389	59.8	205.8	F	0.87	0.15	4	5
390	54.7	150.1	F	0.87	0.35	4	5
391	64	255.3	F	1.36	0.22	4	6
392	51.2	121	M	2.1	1.92	3	8
393	64.5	277.2	F	1.91	0.37	4	7
394	46.9	102.2	F	0.64	0.13	3	4
395	54.1	156.4	M	0.59	0.1	4	4
396	60.1	218.4	M	1.15	0.11	4	5
397	70.3	354.8	F	1.21	0.7	5	5
398	60.2	199.7	M	1.08	0.21	4	5
399	39.5	61	M	0.61	0.4	2	3
400	67	282.1	F	1.55	0.67	5	6
401	77.9	402.9	M	1.8	0.49	5	7
402	70.3	306	M	1.56	0.36	5	7
403	48.3	109.8	M	0.29	0.02	3	3
404	55.7	171.2	F	1.04	0.41	4	5
405	65.2	265.4	F	0.97	0.29	4	5
406	55.8	171	F	2.09	4.51	4	8
407	52.4	151.8	F	0.62	0.29	4	4

408	46.4	100	F	0.37	0.06	3	3
409	43.2	76.9	M	0.51	0.08	3	3
410	57.9	189.8	M	0.95	0.74	4	5
411	50.9	145.2	F	0.76	0.07	3	4
412	39.9	57.8	F	0.48	0.12	3	3
413	41.2	76	F	0.61	0.07	2	3
414	68.5	318.4	F	1.36	1.15	5	6
415	70.3	377.2	F	1.72	1.05	5	7
416	58	192.7	F	0.92	0.69	4	5
417	55.9	177.5	M	1.16	0.35	4	5
418	33.1	34.8	M	0.48	0.05	2	3
419	48.5	114.7	M	1.19	0.65	3	5
420	60.4	203.7	M	1.76	0.11	4	7
421	56.4	178.4	F	0.86	0.66	4	4
422	73.3	379.4	F	2.86	27.87	5	8+
423	59.7	202.6	M	0.99	1.95	4	5
424	44.9	92.8	F	0.72	0.15	3	4
425	62	232.6	F	0.93	0.11	4	5
426	54.9	175.8	M	0.52	0.1	4	3
427	62.5	234.2	M	0.3	0.09	4	3
428	56.8	185.7	M	0.7	0.24	4	4
429	58.5	186	M	0.65	0.21	4	4
430	52.9	146.5	F	1.4	1.22	4	6
431	60.1	224.9	F	1.64	0.73	4	7
432	47.5	104.6	M	0.74	0.22	3	4
433	76.6	444.7	F	1.65	1.33	5	7
434	68.6	327.7	M	1.1	0.77	5	5
435	56.6	178.9	M	0.88	0.26	4	5
436	43.5	88.03	M	0.29	0.03	3	3
437	44.4	86.8	F	0.27	0.04	3	3
438	51.6	142.3	F	1.17	0.22	3	5
439	62	232.9	F	0.81	0.31	4	4
440	60.5	210.5	M	0.17	0.04	4	3
441	74.7	364.9	M	0.94	0.48	5	5
442	56.5	173.5	M	1.24	0.47	4	5
443	71	344.1	M	1.24	0.57	5	5
444	60.1	210.2	M	1.38	0.6	4	6

445	64.2	260.6	F	2.49	0.97	4	8+
446	60.4	208	F	0.8	0.41	4	5
447	54.6	154.5	M	1.45	0.56	4	6
448	73.4	361.3	F	1.25	1.04	5	6
449	68.2	337.2	F	1.06	0.68	4	5
450	55.2	162.2	F	1.26	0.4	4	6
451	51.1	126.4	F	1.73	1.26	3	7
452	67.7	283.5	M	1.52	0.57	5	6
453	58	198.6	F	0.71	0.19	4	4
454	48.2	110.2	M	0.22	0.04	3	3
455	59	200.4	M	1.15	0.66	4	5
456	69.6	304.8	M	0.76	0.51	5	4
457	60.3	192.5	M	1.38	1.23	4	6
458	49.4	123.6	F	1.03	0.44	3	5
459	47.3	103.5	M	0.73	0.2	3	4
460	45.7	90.6	F	0.97	0.28	3	5
461	56.5	176.3	F	1.4	0.65	4	6
462	47.5	103.2	F	1.03	0.37	3	5
463	42.7	78	F	0.44	0.1	3	3
464	41.6	77.9	F	0.57	0.21	3	3
465	49.6	118.8	F	1.03	0.34	3	5
466	60.5	211.5	F	0.79	0.33	4	4
467	46.7	95.7	F	0.21	0.04	3	3
468	37.9	59.7	M	0.31	0.1	3	3
469	48.5	111.01	F	1.47	0.2	3	6
470	43.7	77.4	F	0.59	0.16	3	4
471	40.3	68.4	F	0.58	0.14	3	3
472	42.6	73.37	M	0.39	0.07	3	3
473	74.5	412	M	0.91	0.48	5	4
474	74.8	411.3	M	0.96	0.29	5	5
475	78.2	449	M	1.01	0.39	5	5
476	72.6	349.6	M	3.46	24.34	5	8+
477	70.3	336.7	F	1.12	0.23	5	5
478	71.1	325.3	M	0.97	0.44	5	5
479	71.8	373	F	1.2	0.16	5	5
480	71.5	353.7	M	1.25	0.34	5	5
481	77.6	471.1	F	1.51	0.36	5	6

482	74.6	391.9	F	1.8	0.63	5	7
483	71	350.8	M	0.48	0.11	5	3
484	76	384.6	F	2.46	1.1	5	8+
485	77.6	415.3	M	1.71	3.23	5	7
486	73	349.1	F	0.97	0.19	5	5
487	72.1	392.5	F	0.92	0.19	5	5
488	75	393.5	M	1.47	0.45	5	6
489	74.4	403.2	F	0.63	0.03	5	4
490	74.3	420.6	F	0.49	0.21	5	3
491	73	353	M	0.83	0.3	5	4
492	78.5	457.9	F	1.04	0.39	5	5
493	75.2	436.9	F	1.26	0.15	5	6
494	70.1	339.9	M	1.68	0.65	5	7
495	75.6	369.8	M	2.03	3.26	5	8
496	73.2	366.8	M	1.02	0.26	5	5
497	79.7	450.2	M	0.81	0.33	5	4
498	76.5	439.7	F	1.09	0.25	5	5
499	70	345.4	F	1.55	0.77	5	6

Appendix 2. Summary of modal analyses of OLCM lipofuscin concentration-frequency and carapace length-frequency histograms using the Battacharya method and ELEFAN 1.11.

BHATTACHARYA'S METHOD FOR SEPARATING FREQUENCY DISTRIBUTIONS
Summary results for SAMPLE No. 1 , File : CARA

GROUP No.	MEAN	STANDARD DEV. (s.d.)	POPULATION (N)	SEPARATION INDEX (S.I.)
1	35.00	3.603	6.280	-
2	45.61	4.065	50.770	2.766
3	59.47	3.669	82.030	3.584
4	73.07	4.395	57.440	3.373

S.I. should be >= 2 for groups to be meaningfully separated

ML (mm)	OBSERVED FREQUENCY	GROUP No. 1	GROUP No. 2	GROUP No. 3	GROUP No. 4	EXPECTED FREQUENCY
30.50	1.00	0.86	0.01	0.00	0.00	0.87
33.50	2.00	1.84	0.14	0.00	0.00	1.98
36.50	2.00	1.97	1.03	0.00	0.00	3.00
39.50	6.00	1.06	4.32	0.00	0.00	5.37
42.50	14.00	0.28	10.52	0.00	0.00	10.80
45.50	13.00	0.04	14.87	0.01	0.00	14.92
48.50	12.00	0.00	12.20	0.24	0.00	12.44
51.50	8.00	0.00	5.80	2.11	0.00	7.91
54.50	14.00	0.00	1.60	9.55	0.00	11.15
57.50	23.00	0.00	0.26	22.11	0.02	22.39
60.50	24.00	0.00	0.02	26.23	0.22	26.47
63.50	15.00	0.00	0.00	15.95	1.26	17.21
66.50	10.00	0.00	0.00	4.97	4.62	9.59
69.50	13.00	0.00	0.00	0.79	10.62	11.42
72.50	21.00	0.00	0.00	0.06	15.34	15.40
75.50	12.00	0.00	0.00	0.00	13.90	13.90
78.50	8.00	0.00	0.00	0.00	7.91	7.91
81.50	0.00	0.00	0.00	0.00	2.82	2.82
84.50	0.00	0.00	0.00	0.00	0.63	0.63
87.50	0.00	0.00	0.00	0.00	0.09	0.09
90.50	0.00	0.00	0.00	0.00	0.01	0.01
93.50	0.00	0.00	0.00	0.00	0.00	0.00
96.50	0.00	0.00	0.00	0.00	0.00	0.00
99.50	0.00	0.00	0.00	0.00	0.00	0.00
102.50	0.00	0.00	0.00	0.00	0.00	0.00
105.50	0.00	0.00	0.00	0.00	0.00	0.00
108.50	0.00	0.00	0.00	0.00	0.00	0.00
111.50	0.00	0.00	0.00	0.00	0.00	0.00
114.50	0.00	0.00	0.00	0.00	0.00	0.00
117.50	0.00	0.00	0.00	0.00	0.00	0.00
120.50	0.00	0.00	0.00	0.00	0.00	0.00
123.50	0.00	0.00	0.00	0.00	0.00	0.00
126.50	0.00	0.00	0.00	0.00	0.00	0.00
129.50	0.00	0.00	0.00	0.00	0.00	0.00

ML (mm)	COMPONENTS OF CHI-SQR.
30.50	--
33.50	--
36.50	0.334
39.50	0.077
42.50	0.946
45.50	0.248
48.50	0.015
51.50	0.001
54.50	0.730

81.50						
84.50	0.00	0.00	0.00	0.00	0.63	0.63
87.50	0.00	0.00	0.00	0.00	0.09	0.09
90.50	0.00	0.00	0.00	0.00	0.01	0.01
93.50	0.00	0.00	0.00	0.00	0.00	0.00
96.50	0.00	0.00	0.00	0.00	0.00	0.00
99.50	0.00	0.00	0.00	0.00	0.00	0.00
102.50	0.00	0.00	0.00	0.00	0.00	0.00
105.50	0.00	0.00	0.00	0.00	0.00	0.00
108.50	0.00	0.00	0.00	0.00	0.00	0.00
111.50	0.00	0.00	0.00	0.00	0.00	0.00
114.50	0.00	0.00	0.00	0.00	0.00	0.00
117.50	0.00	0.00	0.00	0.00	0.00	0.00
120.50	0.00	0.00	0.00	0.00	0.00	0.00
123.50	0.00	0.00	0.00	0.00	0.00	0.00
126.50	0.00	0.00	0.00	0.00	0.00	0.00
129.50	0.00	0.00	0.00	0.00	0.00	0.00

ML (mm)	COMPONENTS OF CHI-SQR.
30.50	--
33.50	--
36.50	0.334
39.50	0.073
42.50	0.946
45.50	0.248
48.50	0.015
51.50	0.001
54.50	0.730
57.50	0.017
60.50	0.230
63.50	0.284
66.50	0.018
69.50	0.220
72.50	2.033
75.50	0.261
78.50	0.001
81.50	--
84.50	--
87.50	--
90.50	--
93.50	--
96.50	--
99.50	--
102.50	--
105.50	--
108.50	--
111.50	--
114.50	--
117.50	--
120.50	--
123.50	--
126.50	--
129.50	--

Chi-square value = 5.41 ; degrees of freedom = 6 ;(note that chi-square val
was cumulated after classes with expected frequency < 5 were combined
with adjacent classes, see dashed lines). At 95% level of confidence, the
expected distribution is not significantly different from the observed
distribution. Frequencies=0.000 under the GROUP headings can be anyth
>= 0.0001; they were truncated for clarity.
WARNING : The chi-square value is meaningless
not consist of actual frequ
unreliable.

BHATTACHARYA'S METHOD FOR SEPARATING FREQUENCY DISTRIBUTIONS
Summary results for SAMPLE No. 1 , File : LIPO

LIPOFUSCIN FREQUENCY CONCENTRATION

GROUP No.	MEAN %VF x10	STANDARD DEV. (s.d.)	POPULATION (N)	SEPARATION INDEX (S.I.)
1	3.10	1.455	30.040	-
2	6.83	1.114	46.630	2.906
3	9.89	1.094	50.920	2.775
4	13.38	1.633	42.080	2.556
5	17.07	0.679	14.500	3.196
6	20.03	0.826	8.070	3.927

S.I. should be >= 2 for groups to be meaningfully separated

%VF x10	OBSERVED FREQUENCY	GROUP No. 1	GROUP No. 2	GROUP No. 3	GROUP No. 4	GROUP No. 5
1.00	3.00	2.63	0.00	0.00	0.00	0.00
2.00	6.00	5.87	0.00	0.00	0.00	0.00
3.00	9.00	8.16	0.03	0.00	0.00	0.00
4.00	7.00	7.07	0.52	0.00	0.00	0.00
5.00	8.00	3.83	3.71	0.00	0.00	0.00
6.00	14.00	1.29	11.78	0.02	0.00	0.00
7.00	17.00	0.27	16.67	0.44	0.00	0.00
8.00	14.00	0.04	10.54	3.52	0.04	0.00
9.00	16.00	0.00	2.97	12.29	0.24	0.00
10.00	19.00	0.00	0.37	18.57	1.06	0.00
11.00	15.00	0.00	0.02	12.17	3.25	0.00
12.00	12.00	0.00	0.00	3.46	6.82	0.00
13.00	9.00	0.00	0.00	0.43	9.85	0.00
14.00	10.00	0.00	0.00	0.02	9.77	0.00
15.00	9.00	0.00	0.00	0.00	6.66	0.05
16.00	5.00	0.00	0.00	0.00	3.12	1.91
17.00	8.00	0.00	0.00	0.00	1.01	8.25
18.00	3.00	0.00	0.00	0.00	0.22	4.06
19.00	2.00	0.00	0.00	0.00	0.03	0.23
20.00	4.00	0.00	0.00	0.00	0.00	0.00
21.00	2.00	0.00	0.00	0.00	0.00	0.00
22.00	1.00	0.00	0.00	0.00	0.00	0.00
23.00	0.00	0.00	0.00	0.00	0.00	0.00
24.00	2.00	0.00	0.00	0.00	0.00	0.00
25.00	0.00	0.00	0.00	0.00	0.00	0.00
26.00	0.00	0.00	0.00	0.00	0.00	0.00
27.00	1.00	0.00	0.00	0.00	0.00	0.00
28.00	1.00	0.00	0.00	0.00	0.00	0.00
29.00	0.00	0.00	0.00	0.00	0.00	0.00
30.00	0.00	0.00	0.00	0.00	0.00	0.00
31.00	0.00	0.00	0.00	0.00	0.00	0.00
32.00	0.00	0.00	0.00	0.00	0.00	0.00
33.00	0.00	0.00	0.00	0.00	0.00	0.00
34.00	1.00	0.00	0.00	0.00	0.00	0.00

%VF x10	GROUP No. 6	EXPECTED CONTR. TO CHI-SQR.	COMPONENTS CHI-SQR.
1.00	0.00	2.63	--
2.00	0.00	5.87	0.003
3.00	0.00	8.19	0.080
4.00	0.00	7.60	0.047
5.00	0.00	7.54	0.039
6.00	0.00	13.09	0.063
7.00	0.00	17.39	0.009
8.00	0.00	14.13	0.001

17.00	8.00	0.00	0.00	0.00	1.01	8.25
18.00	3.00	0.00	0.00	0.00	0.22	4.06
19.00	2.00	0.00	0.00	0.00	0.03	0.23
20.00	4.00	0.00	0.00	0.00	0.00	0.00
21.00	2.00	0.00	0.00	0.00	0.00	0.00
22.00	1.00	0.00	0.00	0.00	0.00	0.00
23.00	0.00	0.00	0.00	0.00	0.00	0.00
24.00	2.00	0.00	0.00	0.00	0.00	0.00
25.00	0.00	0.00	0.00	0.00	0.00	0.00
26.00	0.00	0.00	0.00	0.00	0.00	0.00
27.00	1.00	0.00	0.00	0.00	0.00	0.00
28.00	1.00	0.00	0.00	0.00	0.00	0.00
29.00	0.00	0.00	0.00	0.00	0.00	0.00
30.00	0.00	0.00	0.00	0.00	0.00	0.00
31.00	0.00	0.00	0.00	0.00	0.00	0.00
32.00	0.00	0.00	0.00	0.00	0.00	0.00
33.00	0.00	0.00	0.00	0.00	0.00	0.00
34.00	1.00	0.00	0.00	0.00	0.00	0.00

SVF10

GROUP No. 6	EXPECTED	COMPONENTS CONTR. TO CHI-SQR.
1.00	0.00	2.63 --
2.00	0.00	5.87 0.003
3.00	0.00	8.19 0.080
4.00	0.00	7.60 0.047
5.00	0.00	7.54 0.028
6.00	0.00	13.09 0.063
7.00	0.00	17.39 0.009
8.00	0.00	14.13 0.001
9.00	0.00	15.50 0.016
10.00	0.00	20.01 0.051
11.00	0.00	15.44 0.012
12.00	0.00	10.28 0.289
13.00	0.00	10.27 0.158
14.00	0.00	9.79 0.004
15.00	0.00	6.71 0.779
16.00	0.00	5.03 0.000
17.00	0.00	9.26 0.171
18.00	0.00	4.42 0.457
19.00	0.00	1.80 --
20.00	0.00	3.86 0.005
21.00	0.00	2.23 --
22.00	0.00	0.30 --
23.00	0.00	0.01 --
24.00	0.00	0.00 --
25.00	0.00	0.00 --
26.00	0.00	0.00 --
27.00	0.00	0.00 --
28.00	0.00	0.00 --
29.00	0.00	0.00 --
30.00	0.00	0.00 --
31.00	0.00	0.00 --
32.00	0.00	0.00 --
33.00	0.00	0.00 --
34.00	0.00	0.00 --

Chi-square value = 2.173 ; degrees of freedom = 5 ;(note that chi-square val was cumulated after classes with expected frequency < 5 were combined with adjacent classes, see dashed lines). At 95% level of confidence, the expected distribution is not significantly different from the observed distribution. Frequencies=0.000 under the GROUP headings can be anything >= 0.0001; they were truncated for clarity.

WARNING : The chi-square value is meaningless if the sample analyzed did not consist of actual frequencies. Also, tests with df <10 are unreliable.