Transfer of Lipofuscin technology to the Central Ageing Facility

FINAL REPORT

S. G. Robertson K. Krusic-Golub

and A. K. Morison,

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Project No. 96/154

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NON-TECHNICAL SUMMARY

96/154 Transfer of Lipofuscin technology to the Central Ageing Facility

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Objectives:

1. To provide the Central Ageing Facility with the equipment necessary to undertake age determination of crustaceans using the morphological lipofuscin technique.
2. To test the success of the technology transfer against results previously obtained by the developer of the technique, Dr Sheehy of Queensland University.
3. To undertake a preliminary trial of the technique on southern rock lobsters.

Non Technical Summary

Lipofuscin is a metabolic by-product that accumulates in tissue of the nervous system, particularly the brain, and its occurrence has been shown to be widespread in crustaceans. Because it accumulates in the nervous tissue over time, the concentration of lipofuscin can be related to chronological age. A technique determining the age of crustacea using the ratio of the area of lipofuscin granules to neurons in the olfactory lobe cell mass (OLCM) in the brain of crustaceans has been developed by Dr M.R.J. Sheehy. The method was developed using a suite of crustaceans (Sheehy 1994), then transferred to Western Australian rock lobster (FRDC 93/090), and is currently being used for age determination of the European lobster (*Hommarus gammarus*).

The aim of this project was to transfer lipofuscin technology to the Central Ageing Facility (CAF) at the Marine and Freshwater Resources Institute (MAFRI).

Initially, a staff member from the CAF studied the technique with Dr Sheehy at the University of Queensland to become familiar with various aspects of the technique and thus assist with implementation of the method at the CAF. Image analysis and fluorescence microscopy, which are key components for the lipofuscin technology, are already in use by the CAF. Additional micrometry equipment including filter blocks, an immersion lens with a

high numerical aperture to enable photomicroscopy, and a photomicroscopy unit with low light capabilities, were purchased.

Samples and data from three species of crustacea were used in this study, *Hommarus gammarus* (European Lobster), *Cherax quadricarinatus* (Redclaw), and *Jasus edwardsii* (Southern Rock Lobster). The CAF's image analysis system was customised and tested using images of samples from *H. gammarus* supplied by Dr Sheehy. This allowed the system to be calibrated against previously estimated lipofuscin concentrations. Image analysis macros were trialed until no significant differences existed between the results obtained by Dr Sheehy and the results obtained by the CAF. The images supplied by Dr Sheehy were also used to assess inter and intra variability of lipofuscin fractions between and within images.

Dissection, fixation and histological preparations of samples was tested using *C. quadricarinatus.* The NATA accredited Geelong Pathology Unit performed the histological components of this project. Additional trials were undertaken with the six *J. edwardsii*. The olfactory lobe was examined for the accumulation of lipofuscin, and the relationship between lipofuscin volume fraction and carapace length were investigated. The area of the brain which contained the highest concentrations of lipofuscin, was the olfactory lobe in both the redclaw and southern rock lobster. Lipofuscin concentrations were highest in the larger and presumably older individuals in both species.

The transfer of the morphological lipofuscin technique was completed with all objectives being achieved. Following the success of this project the CAF is in a position to develop protocols to apply the method on a larger scale to provide age estimates for the southern rock lobster and other commercially important crustaceans. It is proposed that work in this area be conducted with continued consultation with Dr Matt Sheehy.

KEYWORDS: Lipofuscin, age determination, crustacea

FINAL REPORT

Background

The major problem with assessing rock lobster stocks has been the inability to determine ages. In most cases growth has been inferred from tagging studies. However, there are statistical problems associated with applying these data to age structured models. Conventional methods of ageing using hard parts are not applicable to rock lobsters because the exoskeleton is discarded at each moult.

Recently, attention has been focused on lipofuscin, a fluorescent pigment used for estimating the age of vertebrates (Hill 1988) and invertebrates, particularly crustaceans (Nicol et al. 1991, Sheehy 1989). Lipofuscins are a metabolic by-product that is stored in tissue of the nervous system, particularly the brain, and its occurrence has been shown to be taxonomically widespread in crustaceans (Sheehy 1990a). As lipofuscin is a nervous by-product that accumulates in the nervous tissue over time, the quantification of the amount of lipofuscin can be related to metabolic and chronological age (Sheehy, 1995). This approach can be a superior predictor of age compared with body-size parameters frequently used for this purpose (Sheehy 1990b, Sheehy et al. 1994). The method requires the identification of the regions of the nervous system where lipofuscins accumulate most rapidly, and then quantifying the amount of lipofuscin using fluorescence microscopy and image analysis. The morphological technique relies on the serological law that states that the volume fraction is equal to the surface area fraction. To quantify the volume of lipofuscin in the nervous tissue, the surface area of the lipofuscin can therefore be measured.

Lipofuscin was examined as a means of ageing western rock lobsters (*Panulirus cygnus*) (Crossland et al. 1988). However, Dr. Matt Sheehy, Zoology Department, University of Queensland, has since modified and refined the use of the lipofuscin as a means for estimating the age of crustaceans. Previous errors in the methodology have been rectified and a trial using the western rock lobster produced promising results (Sheehy, pers. comm.). Lipofuscin has also been recorded from the European clawed lobster (Sheehy and Wickins 1994) and further work on this species is currently being undertaken.

The Central Ageing Facility (CAF) has developed state-of-the-art technology to determine the age of fish for stock assessment purposes, and has won acclaim from international visitors for its system. Both image analysis and fluorescence microscopy, which are key components for the lipofuscin technology, are already in use in the CAF for other purposes relating to fish ageing. The current system could be readily adapted for lipofuscin analysis. With a minimum of additional training and extra equipment, the CAF would be in a position to offer lipofuscin analysis as an additional tool for ageing studies on crustaceans or other animals.

The proposed project would enable the transfer of the lipofuscin technique, developed by Dr Sheehy over a ten-year period, to the CAF. As an initial step, the CAF has sent a staff member to visit and train with Dr Sheehy in Queensland to learn details of the method and the equipment requirements. It is proposed that equipment be purchased to allow the same

methods to be employed by the CAF. Tests of the success of the transfer would ensure that results are comparable and follow the established methods. Once the technology has been successfully transferred, initial trials can be run for the southern rock lobster, *Jasus edwardsii*. This will allow the development of a research proposal next year to fully test the validity of the method for this species.

Need

Estimation of critical population parameters for growth and mortality of rock lobsters are difficult because of the lack of a reliable method of age estimation. Stock assessment methods that are applicable are also limited by the inability to monitor age composition of the catch.

The Victorian fishery for southern rock lobsters is showing a long-term decline in catch rates, an indicator of declining stock size and possible growth over-fishing. Without accurate knowledge of the age structure of the population, it is not possible to accurately estimate the relative importance of changes in recruitment, mortality and growth in producing the observed trend.

Once a validated and reliable method to age rock lobsters has been established, age data would be available for the stock assessment process, allowing for age structured models to be applied to the assessment of rock lobster stocks.

Presently, basic biological information such as age at legal size, and variations of age at minimum legal size is not known. The application of the lipofuscin technique could, in the long term, make these data available to management.

Objectives

Objective 1 To provide the Central Ageing Facility with the equipment necessary to undertake age determination of crustaceans using the morphological lipofuscin technique.

Objective 2 To test the success of the technology transfer against results previously obtained by the developer of the technique, Dr Sheehy of Queensland University.

Objective 3 To undertake a preliminary trial of the technique on southern rock lobsters.

Methods and Results

Overview

Lipofuscin is an autofluorescent compound: to observe lipofuscin it is necessary to stimulate the fluorescence using ultraviolet light. The colour of the emitted light after excitation using particular wavelengths by filtering was previously demonstrated by Sheehy (1995). The lipofuscin volume fraction can be quantified by measuring the surface area of the lipofuscin as a ratio to the neurons using image analysis techniques. Errors in the measurement of the surface area of the lipofuscin granules are exacerbated by the space between the tissue, caused by "teasing" in the microtomy preparation of the olfactory lobe. This is adjusted by removing the "negative space" in the image analysis component of the procedure.

As the wavelengths required to stimulate fluorescence of the lipofuscin are specific, specialised microscopy equipment is needed. The low light levels produced by the background fluorescence of the nervous tissue require specialist lenses with high numerical

apertures and photomicrography equipment. It is these equipment requirements that were purchased to enable the transfer of the technique to the Central Ageing Facility.

Equipment

The CAF acquired the necessary equipment for the transfer of the morphological lipofuscin technique. These included a 100x fluorescence oil immersion lens for the Leica Laborlux compound microscope. In addition, a photomicrography system, the MPS 60 Photoautomat (automatic camera) was used for all photomicrography. A 12.5 x intermediate objective was used between the camera and the oil immersion lens. This gave a final magnification for photomicrography of 1250x. To excite the lipofuscin in the sections it was also necessary to purchase a filter block (I3) for the detection of the autofluorescence exhibited by the lipofuscin granules at the described wavelengths. The I3 filter block has a fluorescent emission cut off above 550 nanometres (nm) and an excitation wavelength of 450 nm (Birk, 1984), in accord with the techniques and equipment developed by Dr Sheehy. A low-light video camera was not purchased due to insufficient fluorescence levels for live data acquisition.

Samples

The samples used for this pilot study came from three species of crustacea. These were *Homarus gammrus*, *Cherax quadricarinatus* and *Jasus edwardsii*. Eight images of *H. gammarus* were supplied electronically via email by Dr Sheehy. A commercial aquaculturalist in Maroochydore supplied a sample of *C. quadricarinatus*. A sample of six *J. edwardsii* were purchased live from the Apollo Bay Fishermen's Co-operative Pty. Ltd.

The *H. gammarus* image samples were supplied to test image-processing techniques developed by the CAF, to automatically determine the amount of lipofuscin. These images were from one individual with a range of lipofuscin concentrations from 0.856 to 1.538 % lipofuscin (Figure 1 and Figure 2). The results were derived by Dr Sheehy using confocal laser microscopy and COMOS software. It was originally proposed that tests to determine the compliance with Dr Sheehy's results would be based on samples of the western rock lobster (*Panulirus cygnus*), however, these samples were not available as Dr Sheehy was investigating the lipofuscin concentrations on *H. gammarus* in the UK.

The *Cherax quadricarinatus* (redclaw) sent to the CAF included a range of size classes (from 30 mm to 140 mm carapace length). Individual animals were subsampled from the specimens supplied to encompass the range of length classes within the sample. These were held in aquaria until pre-processing. Holding aquaria were established at the Marine and Freshwater Resources Institute. Aquaria were monitored for physical chemistry (pH, dissolved oxygen, salinity and temperature); data on these parameters are presented in Appendix 3.

The samples of *J. edwardsii* selected for the study consisted of two small, two medium and two large individuals. These samples were pre-processed on receipt at MAFRI. The carapace lengths of these samples are presented in Table 1.

Sample Preparation

Dissection protocols established by Sheehy (1995) were used in this pilot study. The individuals were chilled to morbidity using a water/ice slurry. Animals were then removed and biological information was recorded. This included the carapace length and weight.

The same pre-processing protocols were used for both the *J. edwardsii* and *C. quadricarinatus* samples. In the case of *C. quadricarinatus*, the declawed weight was also recorded (Table 1).

For *C. quadricarinatus*, dissection scissors were placed in the mouth and an incision was made laterally through the carapace to sever the circumoesophagael commissures. The anterio-dorsal section of the carapace was then removed (Figure 3). The circumoesophagael commissures were cut close to the gut, which leaves the commissures at a maximum length to facilitate ease of handling during the histological embedding process. To aid with fixation, excess tissue was removed from inside the section of carapace and the antennae, eyestalks, and rostrum was also removed. The section of carapace containing the brain was then fixed in 4% neutral formalin solution for a minimum period of two weeks before histological processing. Due to the thickness of the carapace of *J. edwardsii*, it was necessary to use a high-speed electric saw, blade dimensions 22mm x 0.65 mm, (Arlec Supertool ET571, 10,000 RPM) to make the incision from the mouth through the carapace. The same protocols used for *C. quadricarinatus* were followed once the anterio-dorsal section of the carapace was removed from the specimen.

After the samples had been fixed for a two-week period, the brains were dissected from the anterio-dorsal section of the carapace. The brain lies directly between the eyestalk bases and behind the rostrum (Figure 3, Figure 4). All connecting nerves were carefully cut using surgical scissors while the brain was positioned using the circumoesophagael commissures. The dissection were performed with the aid of a dissection microscope at 6.4x and reflected light. After dissection from the carapace, the brain was placed into 4% neutral formalin solution until histological preparation.

Histology

The histology for this pilot study was performed by the NATA registered Geelong Pathology Service. Samples were supplied to the Geelong Pathology Service after being fixed in 4% formalin for a further two-week period. The brain samples were dehydrated using standard histological practices (Sheehy 1995) before embedding in wax for sectioning. The samples were serially sectioned at 6 µm. This ensured the area including the posterior olfactory lobe (area of interest, as determined by Dr Sheehy) was obtained. This equated to between 70 and 120 sections per animal. The sections were fixed to slides, rehydrated using standard histological techniques, and mounted on a series of numbered slides corresponding to the particular sample. A coverslip was mounted on each slide using D.P.X. (dissolved perspex in xylene). The histology for both *C. quadricarinatus* and *J. edwardsii* followed the same protocols.

A sub-sample of the *C. quadricarinatus* sections was stained with Hemoxylin and Eosin stain. This was performed to familiarise staff with the morphology of the sectioned nervous tissue and identify the olfactory lobe position within the cell mass. This allowed staff to later identify the olfactory lobe on unstained samples used in fluorescent photomicroscopy. Further, samples of *J. edwardsii* were stained using Sudan Black B (Figure 5 and Figure 6). This is a lipophilic stain used for the detection of lipids (including lipofuscin) in tissue samples. This has previously been used for validating the morphological lipofuscin technique (Sheehy 1995). Both staining protocols followed NATA standards and protocols and were performed by the Geelong Pathology Service.

Photomicrography and image processing

The olfactory lobe was identified in each section under 400x magnification. The magnification was increased to 1000x and switched to epifluorescence using the I3 filter block. Random areas within the olfactory lobe containing visible lipofuscin granules were photomicrographed. The photomicrographs were taken using the MPS-60 Photoautomat with ISO 400 Kodak gold film. The exposure time for the photomicrographs was standardised to 2 minutes 30 seconds. Due to low levels of background autofluorescence using the I3 filter block, the samples were photomicrographed again using the D block (excitation filter 460nm). The D filter block provided higher background autofluorescence of the nervous tissue than the I3 filter block with the lipofuscin granules still visible (Figure 7 and Figure 8). The number of photomicrographs per specimen was variable, between 0 and 10 for *C. quadricarinatus* and 0 and 26 for *J. edwardsii*. This variability was a function of the histological preparation on the area of the nervous tissue. Numbers of micrographs per sample are given in Table 2. Species information, slide number, position of the section on the slide and the number of frames from each of the olfactory lobe were printed on the bottom of each photomicrograph by the MPS 60 photoautomat for identification during image processing.

All photomicrographs were digitised to colour bitmap format (8 bit RGB) using a Hewlett and Packard Deskjet IIc using Deskscan II V2.1a software. All images were saved and stored on a writable compact disk for further processing.

The images were loaded to Optimas and converted to 8 bit grey images (256 levels of grey) for image processing. The images were pseudo coloured using the image RGB (Red, Green, and Blue) output lookup tables. The process of manipulating the lookup tables enhanced the image, highlighting the lipofuscin in a cyan colour and increased the contrast between the edge of the neurons and areas of negative space (areas of space between the neurons "teased" apart by the microtomy process). The red output lookup table was set so that values between 150 to 255 were zero. The green output lookup table was set so that values between 200 and 255 were set to 255 and values between 0 and 30 were set to 0. The blue lookup table was set so that values between 100 to 255 were set at 255 and values between 0 and 30 were set to zero (Figure 9). These values were applied to each image for processing. The original image and the processed image from a *J. edwardsii* sample are shown in Figure 10 and Figure 11.

The region of interest (ROI) selected for processing, excluded the image boundaries. The detection of the lipofuscin granules was accomplished by setting the image threshold to below the value of brightness values to which the lipofuscin fluoresced. The negative space was determined in the same manner, by selecting a value at which the negative space was automatically measured. This value was then used as the lower limit threshold. This effectively divided the image into three regions, negative space, background tissue and lipofuscin granules. These predetermined values, one for the upper boundary, and one for the lower boundary, were used as constants for each subsequent image. Threshold values were determined for each species. The lower boundary used for both species was a grey value (GV) of 25, while the upper threshold was set to a GV of 80 for the *C. quadricarinatus* and a GV of 90 for the *J. edwardsii*. The lower GV was determined by the amount of background fluorescence. As the background fluorescence levels increased as a function of the species, it was necessary to increase the lower GV to define the edge of the

neurons. The images photomicrographed using the I3 filter block were not processed using this technique, as the background fluorescence did not illuminate the nervous tissue to a level that could be photomicrographed for *C. quadricarinatus*.

Comparison of known lipofuscin concentrations

Sample images of *H. gammarus* supplied by Dr Sheehy were obtained using confocal laser microscopy and COMOS software (Sheehy, pers. comm.). The images are from one individual. This allowed two aspects of the transfer of the technique to the CAF to be explored. These were i) the application of the CAF custom macros was tested on samples of 'known' lipofuscin concentrations as determined by Dr Sheehy, and ii) the level of intra-sample lipofuscin concentration variance using CAF software was compared with the variance from COMOS software analysis. The negative space within these images was assumed to be negligible (Sheehy, pers. comm.) In response to this fact, the lower limit GV was set to zero. The morphological lipofuscin granules can be seen in Figure 12. The size ranges of the granules counted from this image are displayed in Table 3.

Data automatically extracted from the image was written to MS Excel via Dynamic Data Exchange (DDE). Information about the sample being processed (header information), along with areas of the foreground (lipofuscin) and background (negative space), and the area of the ROI in square microns were written to Excel after each image was processed. All objects extracted from the image were assigned a consecutive integer index. Objects in the foreground were assigned positive indices while background objects were assigned negative indices. Summary photomicroscopy data extraction details are presented in Table 4. for *C. quadricarinatus* and Table 5 for *J. edwardsii*.

The Optimas macro written for the processing of the images and quantification of positive and negative space along with the lipofuscin concentrations is presented in Appendix 4.

The *H. gammarus* images supplied by Dr. Sheehy were used to compare the lipofuscin volume fractions obtained through the use of COMOS confocal laser microscopy and the image analysis images written by the CAF. From the eight images supplied by Dr Sheehy, the lipofuscin volumes combined arithmetic mean and standard error were 1.083% (0.092%) using COMOS / laser microscopy. The mean and standard error obtained using the custom Optimas macros were 1.158% (0.131%) respectively. These results were not significantly different as tested using a paired Student's T test ($t=-1.09$, $df=7$, $p=0.31$). The lipofuscin volume fractions calculated using the two techniques can be seen in Table 6 (laser confocal microscope / COMOS software), Table 7 (Central Ageing Facility Optimas software) and Figure 13 (direct comparison between methods).

Lipofuscin fractions in *C. quadricarinatus* and *J. edwardsii*

The results from the *C. quadricarinatus* samples are shown in Table 4. The arithmetic means are calculated from each image processed. The cumulative mean and standard errors were calculated from the combined means for each sample. The lipofuscin volume fraction increased in relation to carapace length can be seen in Figure 14. The age details of these individuals were not available for comparison.

The results from *J. edwardsii* images are shown in Table 5 using the same processing methodologies as were used for the *C. quadricarinatus* images. Of the six samples of *J. edwardsii* only four were processed. The samples were accounted for by a) missing the

olfactory lobe in the microtomy in one sample and b) contamination of the series of slides by a foreign stain that caused high levels of fluorescence from all tissue on the slide.

The mean and standard error (SE) lipofuscin volume fractions in the *J. edwardsii* samples ranged from 0.23% (0.03) to 1.03% (0.17) for individuals with carapace lengths 139.89 mm to 163 mm respectively. These data appear correlated to carapace length (Figure 15).

Discussion

The distribution of the lipofuscin granules in the *J. edwardsii* appears to be generally more contagious than the more uniform distribution of lipofuscin granules in *C. quadricarinatus*. This can be seen in Figure 7 and Figure 8. The neurons appear to be of similar size in the olfactory lobe cell mass (OLCM) of both *J. edwardsii* and *C. quadricarinatus*. The neurons in the in the *J. edwardsii* samples are more angular in appearance and tend to be more easily "teased apart" by the microtomy procedure. This led to a significantly higher area of average negative space for *J. edwardsii* (717 µm, n=49) than for *C. quadricarinatus* (332 µm, n=64, t=-6.31 df=102, P<0.05). The background fluorescence is also higher for the *J. edwardsii* images. This is accounted for in the image processing by increasing the foreground threshold level during classification of the lipofuscin granules.

Lipofuscin granules appear to be distributed throughout the OLCM of *J. edwardsii* and can be quantified as a ratio to the neurons through the application of image analysis macros written by the CAF. The accumulation of the lipofuscin appeared to be correlated with carapace length.

The pilot study suggests that lipofuscin increases with carapace length and presumably chronological age in the OLCM of *J. edwardsii*. The variance in the lipofuscin volume fraction is high between individuals of given length classes. The validation of lipofuscin volume fractions as a function of chronological age was beyond the scope of this pilot study due to the small sample sizes.

Conclusion

The project for the transfer of the lipofuscin technique has been successful in the delivery of the three objectives of the initial proposal.

The CAF has demonstrated the ability to process images in accord with the methodologies developed by Dr Sheehy. These are evidenced by the volume fractions of lipofuscin calculations obtained from images supplied by Dr Sheehy. The comparison between Dr Sheehy's results and the results from the CAF were not significantly different, although the variance using the CAF written Optimas macros was slightly higher than those obtained using COMOS imaging software.

The presence of lipofuscin in the OLCM tissue from *J. edwardsii* samples was demonstrated. Images displaying lipofuscin granules can be seen in Figure 7 and Figure 8. The Central Ageing Facility now has the equipment and the technical ability to examine the relationship between chronological age and lipofuscin volume fractions in populations of the southern rock lobster *Jasus edwardsii* and other crustacea for the purpose of providing data for stock assessment.

Benefits

The major beneficiary of this research is the southern rock lobster fishery, particularly in Victoria, but also in Tasmania and South Australia. However, now that the techniques has been transferred, the CAF will be able to provide age estimates for other Australian rock lobster fisheries, and other Australian crustacean fisheries (following appropriate validation studies).

Potential benefits will be through higher average catch rates in the medium to long term because of more accurate stock assessment and a reduction in the risk of stock collapse.

Further Developments

The processes and the protocols for morphological lipofuscin measurements have now been established at the CAF. This will allow the development of a validated ageing project in the future with the aims being providing management and scientists with age structure data on the southern rock lobster stocks.

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List of Tables

Table 1. Summary of biological data for *C. quadricarinatus* and *J. edwardsii*. De-clawed weight was recorded for *C. quadricarinatus* only.

Species	Specimen	Length (mm)	weight (nc)	weight(c)	Prep date
C.quadricarinatus	1	104.99	189		19/05/97
C.quadricarinatus	2	94.69	160	237	19/05/97
C.quadricarinatus	3	109.46	237	358	19/05/97
C.quadricarinatus	4	93.59	114	128	19/05/97
C.quadricarinatus	5	79.50	82	110	19/05/97
C.quadricarinatus	6	101.89	179	229	19/05/97
C.quadricarinatus	7	87.51	115	143	19/05/97
C.quadricarinatus	8	86.03	113	145	19/05/97
C.quadricarinatus	9	69.30	66	81	19/05/97
C.quadricarinatus	10	67.06	49	65	19/05/97
C.quadricarinatus	11	75.13	76	96	19/05/97
C.quadricarinatus	12	71.44	60	71	19/05/97
C.quadricarinatus	13	63.86	47	58	19/05/97
C.quadricarinatus	14	104.21	215	332	20/05/97
C.quadricarinatus	15	62.32	44	62	20/05/97
C.quadricarinatus	16	91.76	138		20/05/97
C.quadricarinatus	17	75.85	76	91	20/05/97
C.quadricarinatus	18	69.57	67	80	20/05/97
C.quadricarinatus	19	77.41	76	89	20/05/97
C.quadricarinatus	20	82.76	91	107	20/05/97
C.quadricarinatus	21	61.33	44	59	20/05/97
C.quadricarinatus	22	64.26	48	65	20/05/97
C.quadricarinatus	23	67.60	62	73	20/05/97
C.quadricarinatus	24	65.81	51	69	20/05/97
C.quadricarinatus	25	69.60	51	60	20/05/97
J.edwardsii	1	119.97	696		25/8/97
J.edwardsii	2	139.89	1160		25/8/97
J.edwardsii	3	117.29	803		25/8/97
J.edwardsii	4	136.86	1157		25/8/97
J.edwardsii	5	163.00	2121		25/8/97
J.edwardsii	6	169.00	2294		25/8/97

Table 2. Number of photomicrographs taken per specimen.

		Block Type		
Species	Specimen	D	I3	N
C. quadricarinatus	1	0	1	1
C. quadricarinatus	2	12	0	12
C. quadricarinatus	3	0	5	5
C. quadricarinatus	4	0	10	10
C. quadricarinatus	5	2	0	2
C. quadricarinatus	6	0	1	1
C. quadricarinatus	12	2	5	7
C. quadricarinatus	14	16	9	25
C. quadricarinatus	15	11	0	11
C. quadricarinatus	16	15	0	15
C. quadricarinatus	17	12	13	25
J. edwardsii	1	0	12	12
J. edwardsii	2	12	26	38
J. edwardsii	3	12	11	23
J. edwardsii	4	16	12	28
J. edwardsii	6	12	12	24

Table 3. Lipofuscin granule areas extracted from image using foreground image thresholding. Areas of granules in square microns.

Granule	Area	Granule	Area	Granule	Area
L1	2.13	L17	1.28	L33	5.01
L2	0.61	L18	0.51	L34	0.64
L3	1.85	L19	0.36	L35	0.93
L4	2.24	L20	5.40	L36	11.60
L5	0.79	L21	2.07	L37	1.51
L6	2.26	L22	1.60	L38	6.46
L7	2.60	L23	4.93	L39	1.07
L8	2.56	L24	1.34	L40	4.82
L9	0.85	L25	3.20	L41	0.92
L10	1.19	L26	4.84	L42	5.76
L11	0.49	L27	1.19	L43	0.28
L12	1.09	L28	0.28	L44	2.07
L13	0.49	L29	0.45	L45	0.77
L14	2.11	L30	1.05	L46	0.23
L15	7.15	L31	1.32		
L16	0.45	L32	1.43		

Table 4. Lipofuscin volume fraction means and standard errors from *C. quadricarinatus* images processed using the CAF Optimas macros.

Specimen	Image	Image Area	Negative Space	Adjusted Image Area	Lipofuscin	% Lipofuscin	Std. Err/Mean
	C0204302.bmp	6444.2	26.26	6417.94	119.72	1.87	
	C0204303.bmp	6390.4	35.49	6354.91	132.33	2.08	
	C0204304.bmp	6445.1	187.31	6257.79	93.54	1.49	
	C0204305.bmp	6444.8	12.33	6432.47	86.38	1.34	
2	C0204306.bmp	6431.7	133.40	6298.30	45.42	0.72	
	C0204307.bmp	6472.2	90.38	6381.82	70.80	1.11	
	C0204308.bmp	6458.7	148.72	6309.98	67.42	1.07	
	C0204309.bmp	6539.8	57.09	6482.71	172.24	2.66	
	C0204311.bmp	6512.8	125.22	6387.58	57.07	0.89	**0.20**
	C0204312.bmp	6431.7	103.39	6328.31	44.82	0.71	**1.39**
	C0504321.bmp	6620.9	333.29	6287.61	40.51	0.64	
5	C0504322.bmp	6404.7	217.72	6186.98	42.14	0.68	
	C0504323.bmp	6620.9	297.74	6323.16	45.64	0.72	**0.13**
	C0504324.bmp	6566.8	125.16	6441.64	77.55	1.20	**0.81**
	C1405211.bmp	6647.9	483.55	6164.35	159.39	2.59	
	C1405212.bmp	6593.9	340.71	6253.19	180.56	2.89	
	C1405213.bmp	6620.9	509.28	6111.62	164.12	2.69	
	C1405214.bmp	6620.9	635.37	5985.53	102.09	1.71	
	C1405301.bmp	6431.7	440.85	5990.85	88.35	1.47	
	C1405302.bmp	6485.8	216.28	6269.52	162.34	2.59	
	C1405303.bmp	6296.6	231.71	6064.89	108.83	1.79	
14	C1405304.bmp	6377.7	460.99	5916.71	198.73	3.36	
	C1405306.bmp	6620.9	409.31	6211.59	135.88	2.19	
	C1405307.bmp	6431.7	312.75	6118.95	129.25	2.11	
	C1405308.bmp	6485.8	551.54	5934.26	223.98	3.77	
	C1405309.bmp	6539.8	171.54	6368.26	142.96	2.24	
	C1405310.bmp	6539.8	507.43	6032.37	187.08	3.10	
	C1405311.bmp	6539.8	654.05	5885.75	271.11	4.61	**0.21**
	C1405312.bmp	6647.9	1206.32	5441.58	129.22	2.37	**2.63**
	C1505218.bmp	6701.9	271.96	6429.94	55.06	0.86	
	C1505301.bmp	6377.7	79.83	6297.87	74.95	1.19	
	C1505303.bmp	6458.7	138.90	6319.80	132.86	2.10	
	C1505304.bmp	6377.7	2.17	6375.53	40.39	0.63	
	C1505305.bmp	6566.8	16.08	6550.72	55.32	0.84	
15	C1505306.bmp	6620.9	92.27	6528.63	84.80	1.30	
	C1505307.bmp	6485.8	2.96	6482.84	34.27	0.53	
	C1505308.bmp	6593.9	35.15	6558.75	21.41	0.33	
	C1505309.bmp	6593.9	141.02	6452.88	34.33	0.53	
	C1505319.bmp	6620.9	81.27	6539.63	75.60	1.16	**0.15**
	C1505320.bmp	6647.9	258.56	6389.34	32.52	0.51	**0.91**
	C1603315.bmp	6485.8	916.95	5568.85	169.68	3.05	
	C1603316.bmp	6674.9	1109.14	5565.76	298.98	5.37	
	C1603317.bmp	6620.9	513.11	6107.79	115.88	1.90	
	C1604302.bmp	6431.7	760.93	5670.77	235.60	4.15	
	C1604303.bmp	6458.7	484.96	5973.74	292.27	4.89	
	C1604305.bmp	6431.7	741.24	5690.46	270.94	4.76	
16	C1604406.bmp	6431.7	817.63	5614.07	275.15	4.90	
	C1604407.bmp	6539.8	636.08	5903.72	149.17	2.53	
	C1604408.bmp	5999.3	180.51	5818.79	251.09	4.32	
	C1605209.bmp	6620.9	695.66	5925.24	219.60	3.71	
	C1605310.bmp	6837.1	702.79	6134.31	125.53	2.05	
	C1605311.bmp	6431.7	439.10	5992.60	128.38	2.14	**0.35**
	C1605312.bmp	6323.6	750.69	5572.91	139.66	2.51	**3.56**
	C1703201.bmp	5944.1	62.31	5881.79	40.84	0.69	
	C1703202.bmp	6431.7	45.11	6386.59	100.31	1.57	
	C1703301.bmp	6593.9	14.33	6579.57	117.52	1.79	
	C1703302.bmp	6729	7.39	6721.61	66.23	0.99	
	C1703304.bmp	6539.8	2.82	6536.98	110.63	1.69	
17	C1703305.bmp	6485.8	71.79	6414.01	61.81	0.96	
	C1703306.bmp	6674.9	47.73	6627.17	53.15	0.80	
	C1703307.bmp	6404.7	845.03	5559.67	102.68	1.85	
	C1703308.bmp	6647.9	99.61	6548.29	99.61	1.52	
	C1703309.bmp	6566.8	1142.49	5424.31	124.15	2.29	**0.18**
	C1703409.bmp	6566.8	62.37	6504.43	160.56	2.47	**1.51**

Table 5. Lipofuscin volume fraction means and standard errors from *J. edwardsii* images processed using the CAF Optimas macros.

Specimen	Image	Image Area	Negative Space	Adjusted Image Area	Lipofuscin	% Lipofuscin	Std. Err / Mean
	J0205301.bmp	6269.6	210.01	6059.59	28.04	0.46	
	J0205302.bmp	6350.6	198.42	6152.18	13.31	0.22	
	J0205303.bmp	6296.6	198.65	6097.95	8.83	0.14	
	J0205304.bmp	6431.7	181.64	6250.06	24.43	0.39	
	J0205305.bmp	6337.4	362.68	5974.72	17.09	0.29	
2	J0205306.bmp	6431.7	599.60	5832.10	13.34	0.23	
	J0205308.bmp	6404.7	474.89	5929.81	15.91	0.27	
	J0205309.bmp	6458.7	1030.97	5427.73	7.93	0.15	
	J0205310.bmp	6539.8	596.84	5942.96	5.53	0.09	
	J0205311.bmp	6431.7	645.27	5786.43	12.95	0.22	
	J0205312.bmp	6377.7	364.12	6013.58	8.07	0.13	**0.03**
	J0205407.bmp	6458.7	393.40	6065.30	6.85	0.11	**0.23**
	J0305301.bmp	6310.4	538.34	5772.06	23.92	0.41	
	J0305302.bmp	6431.7	432.64	5999.06	8.38	0.14	
	J0305305.bmp	6459.8	769.00	5690.80	24.49	0.43	
	J0305306.bmp	6323.6	883.24	5440.36	28.77	0.53	
3	J0305307.bmp	6257.6	846.15	5411.45	34.87	0.64	
	J0305308.bmp	6377.7	290.38	6087.32	37.77	0.62	
	J0305310.bmp	6539.8	1081.33	5458.47	40.03	0.73	
	J0305311.bmp	6647.9	1492.24	5155.66	24.51	0.48	**0.07**
	J0305312.bmp	6296.6	684.09	5612.51	4.63	0.08	**0.45**
	J0405311.bmp	6080.4	1095.77	4984.63	137.80	2.76	
	J0405312.bmp	6350.6	752.81	5597.79	19.01	0.34	
	J0405316.bmp	6269.6	1085.67	5183.93	46.18	0.89	
	J0407201.bmp	6296.6	716.14	5580.46	36.84	0.66	
	J0407202.bmp	6242.5	394.39	5848.11	19.75	0.34	
	J0407203.bmp	6296.6	834.58	5462.02	107.70	1.97	
	J0407204.bmp	6296.6	1271.76	5024.84	78.76	1.57	
4	J0407205.bmp	6269.6	742.51	5527.09	60.37	1.09	
	J0407206.bmp	5566.9	904.32	4662.58	83.19	1.78	
	J0407207.bmp	6080.4	1010.32	5070.08	65.08	1.28	
	J0407208.bmp	6162.7	854.87	5307.83	11.88	0.22	
	J0407209.bmp	6350.6	845.25	5505.35	77.80	1.41	
	J0407210.bmp	6215.5	936.53	5278.97	63.72	1.21	
	J0407213.bmp	6134.4	965.44	5168.96	36.28	0.70	
	J0407214.bmp	6107.4	553.12	5554.28	28.38	0.51	
	J0407215.bmp	6107.4	491.45	5615.95	26.21	0.47	**0.17**
	J0407216.bmp	6485.8	468.21	6017.59	12.92	0.21	**1.03**
	J0605301.bmp	6404.7	206.49	6198.21	41.66	0.67	
	J0605302.bmp	6539.8	648.27	5891.53	37.74	0.64	
	J0605303.bmp	6404.7	768.46	5636.24	122.88	2.18	
	J0605304.bmp	6485.8	1213.31	5272.49	31.20	0.59	
6	J0605305.bmp	6323.6	1229.14	5094.46	24.80	0.49	
	J0605306.bmp	6404.9	1030.21	5374.69	31.34	0.58	
	J0605307.bmp	6485.8	1032.55	5453.25	97.21	1.78	
	J0605308.bmp	6674.9	597.63	6077.27	5.59	0.09	
	J0605309.bmp	6485.8	610.13	5875.67	5.92	0.10	
	J0605310.bmp	6512.8	1132.72	5380.08	85.13	1.58	**0.21**
	J0605311.bmp	6647.9	479.10	6168.80	36.98	0.60	**0.85**

Table 6. Dr.M.R.J. Sheehy's processed results for the images supplied to the CAF using confocal laser microscopy and COMOS® software

	Image Area	Lipofuscin	% Lipofuscin	Std. Err/Mean
01.tif	11703	100.210	0.856	
03.tif	11703	134.020	1.145	
04.tif	11703	100.540	0.859	
05.tif	9715	90.360	0.930	
06.tif	11680	110.030	0.942	
07.tif	11703	115.660	0.988	
08.tif	11665	179.380	1.538	**0.092**
09.tif	11703	164.770	1.408	**1.083**

Table 7. Central Ageing Facility's processed results for images supplied by Dr Sheehy using custom Optimas macro.

	Image Area	Negative Space	Adj. Image Area	Lipofuscin	% Lipofuscin	Std. Err/Mean
01.tif	11703	102.56	11600.43	71.30	0.62	
03.tif	11703	56.72	11646.28	152.44	1.31	
04.tif	11703	87.36	11615.63	93.06	0.80	
05.tif	9715	223.22	9491.78	89.15	0.94	
06.tif	11680	195.11	11484.88	121.56	1.06	
07.tif	11703	6.01	11696.98	161.96	1.36	
08.tif	11665	36.81	11628.17	203.94	1.75	**0.131**
09.tif	11703	124.47	11578.53	162.10	1.40	**1.158**

List of figures

lipofuscin granules thresholded to foreground.

Figure 13. Comparison between calculated lipofuscin concentrations from images supplied by Dr.M.R.J. Sheehy (COMOS® software) and CAF's custom Optimas macros.

Figure 14. Lipofuscin volume fraction (%) against carapace length (mm) for *C. quadricarinatus.* N = 6

Figure 15. Lipofuscin volume fraction (%) against carapace length (mm) for *J. edwardsii*. N = 4

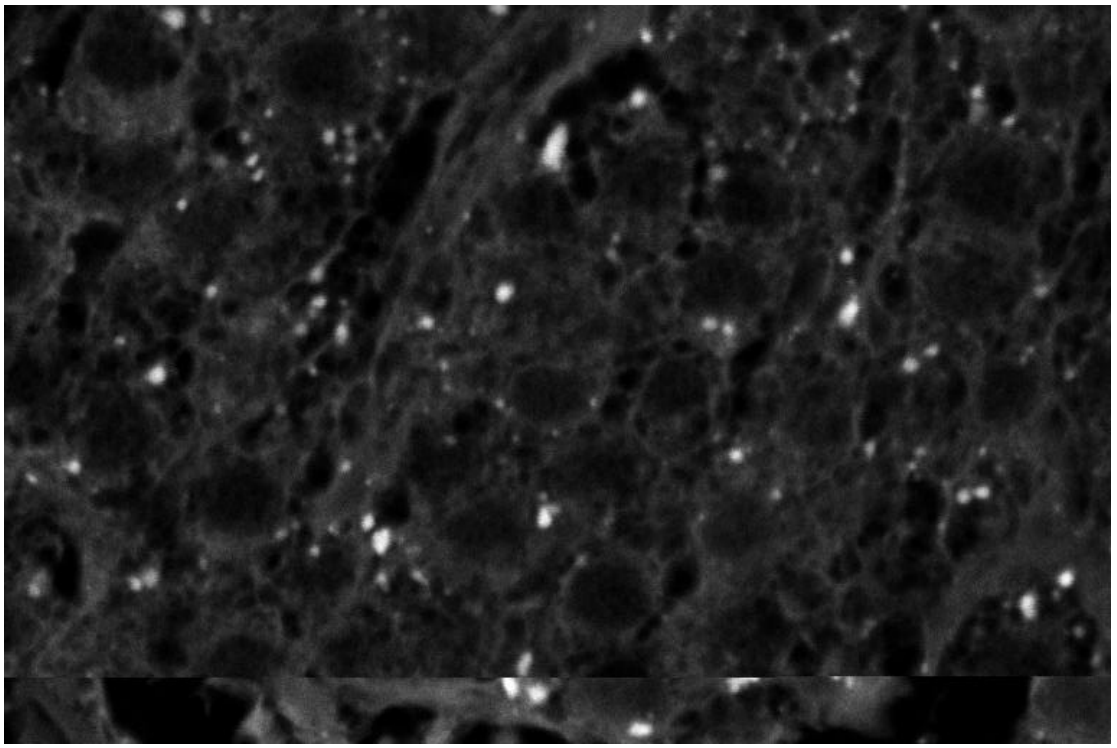


Figure 1. Calibration images from Dr.Sheehy of scanning laser confocal micrographs of *Hommarus gammarus*. Area of image 11703µm. Lipofuscin fraction volume 100.54 µm

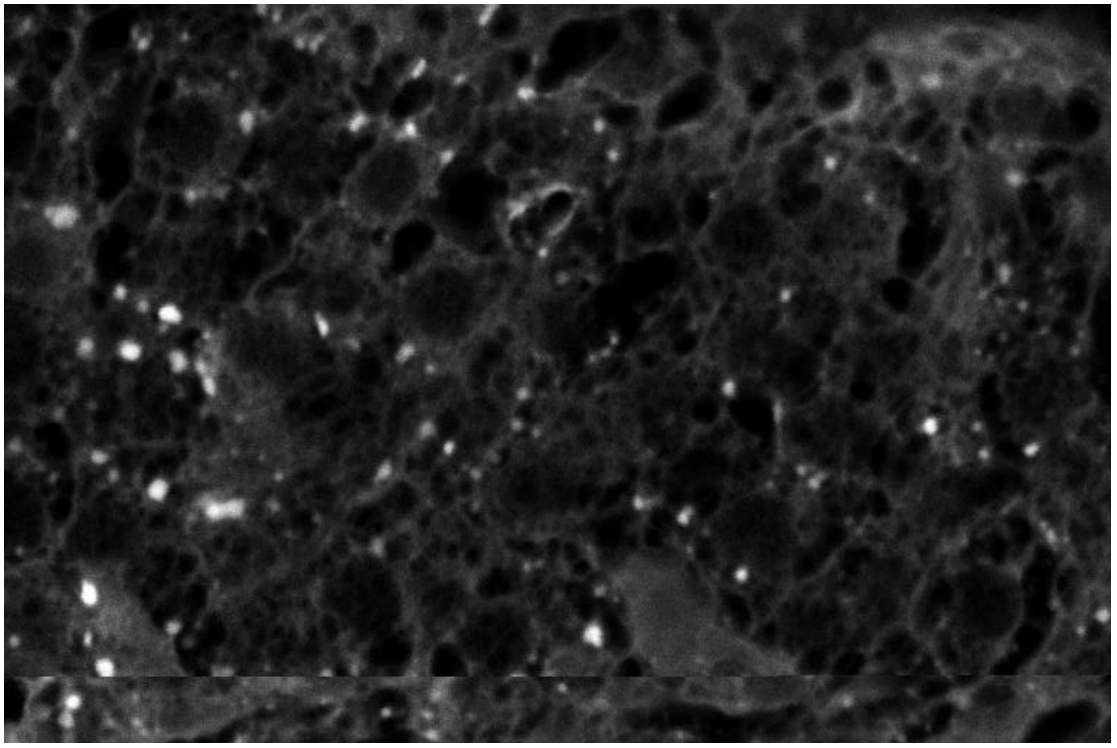


Figure 2. Calibration images from Dr.Sheehy of scanning laser confocal micrographs of *Hommarus gammarus*. Area of image 11680µm. Lipofuscin fraction volume 110.03 µm

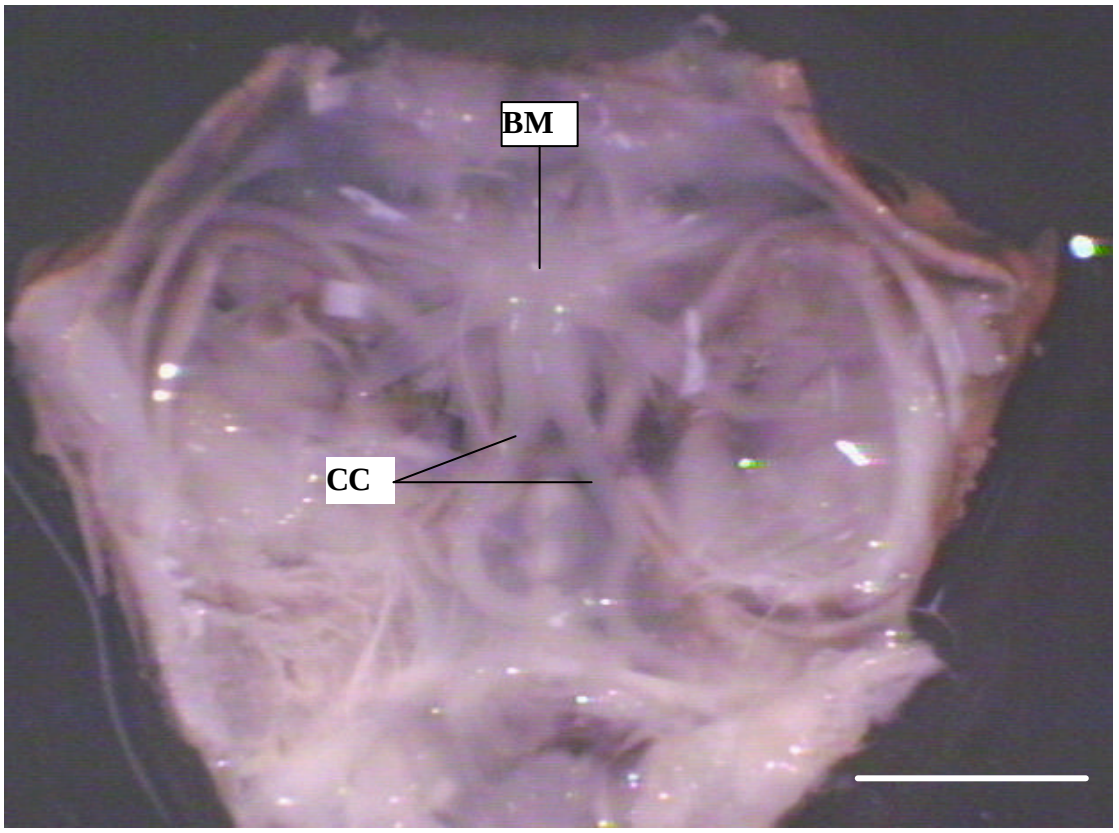


Figure 3. Transverse section of the anterio-dorsal section of the carapace. The brain mass (BM) and the circumoesophageal commissures (CC) are marked.
Scale bar = 5 mm

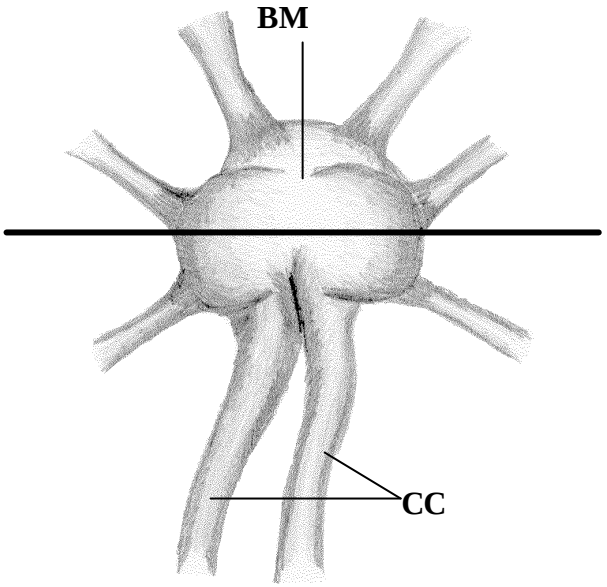


Figure 4. Diagram of central brain mass (BM) of *C. quadricarinatus* indicating the position of the circumoesophageal commissures (CC) used for histological manipulation. The plane of sectioning is indicated (broken line).

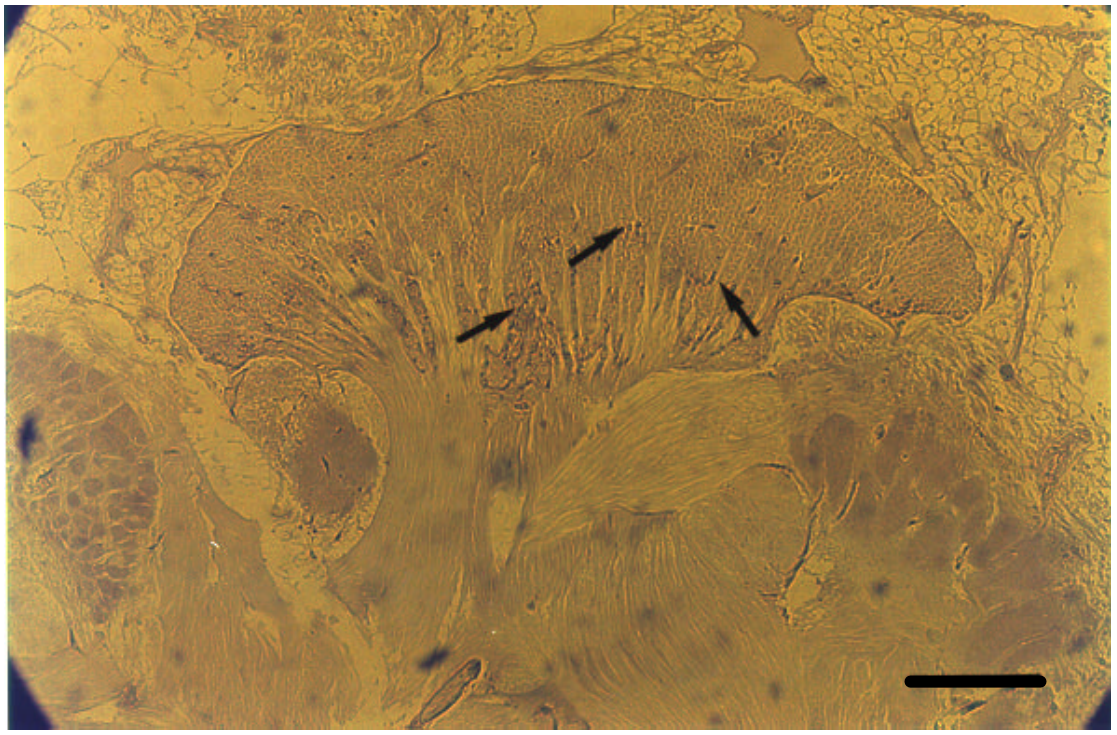


Figure 5. Olfactory lobe (mushroom shaped) of *J. edwardsii* stained with sudan black under transmitted light showing the lipofuscin granules (black) arrowed. Carapace length 169mm. Magnification 125x. Scale bar = 100 µm

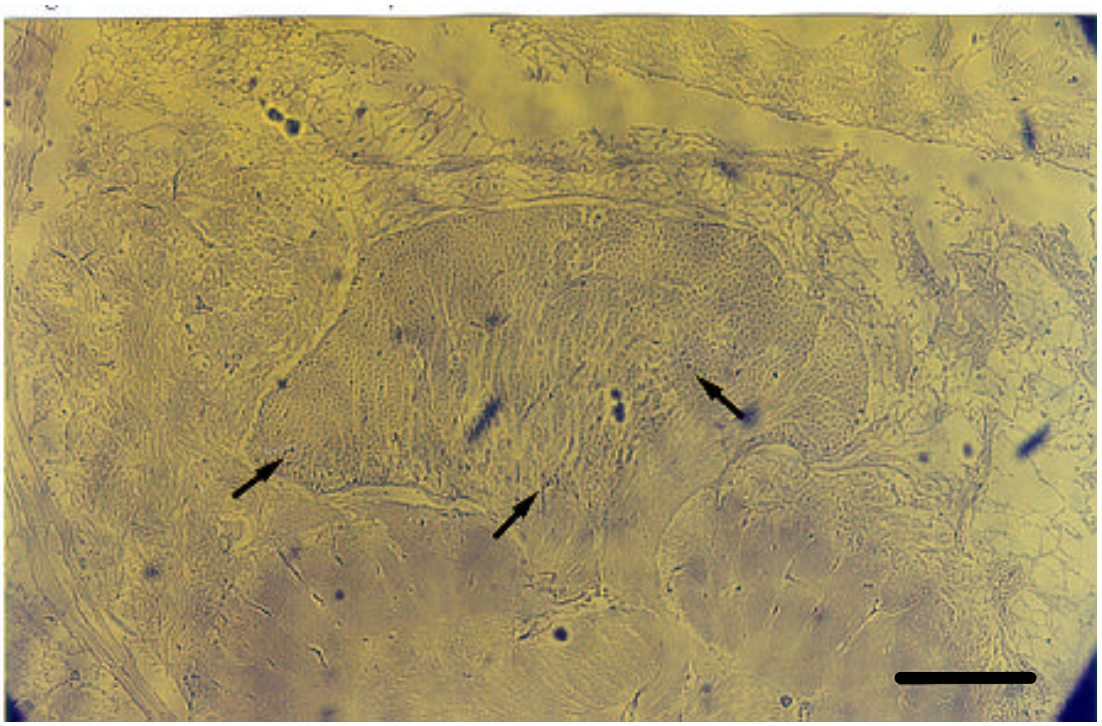


Figure 6. Olfactory lobe (mushroom shaped) of *J. edwardsii* stained with sudan black under transmitted light showing the lipofuscin granules (black) arrowed. Carapace length 136.8mm. Magnification 125x. Scale bar = 100 µm

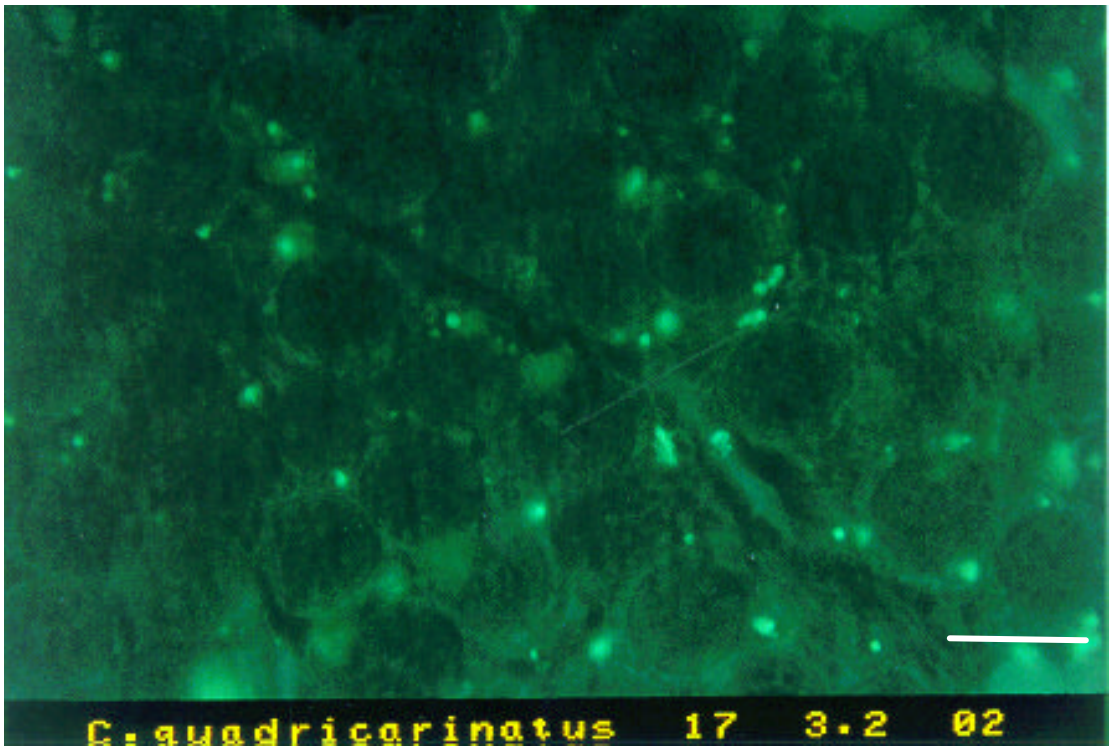


Figure 7. Flourescent lipofuscin granules (light green) against the background flourescence (green) of the neurons and axons from the olfactory lobe of *C. quadricarinatus*. Carapace length 75.85mm Magnification 1250x. Scale bar = 10 µm

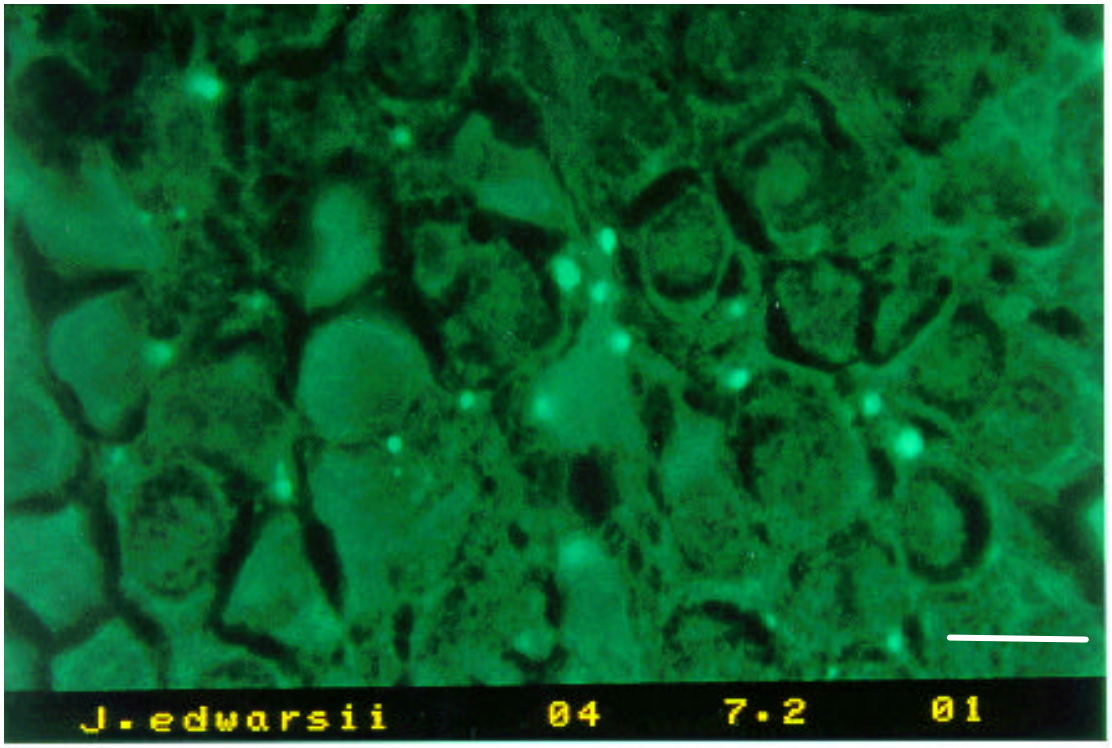


Figure 8. Fluorescent lipofuscin granules (light green) against the background flourescence (green) of the neurons and axons from the olfactory lobe of *J. edwardsii*. Carapace length 136.86mm Magnification 1250x. Scale bar = 10 µm

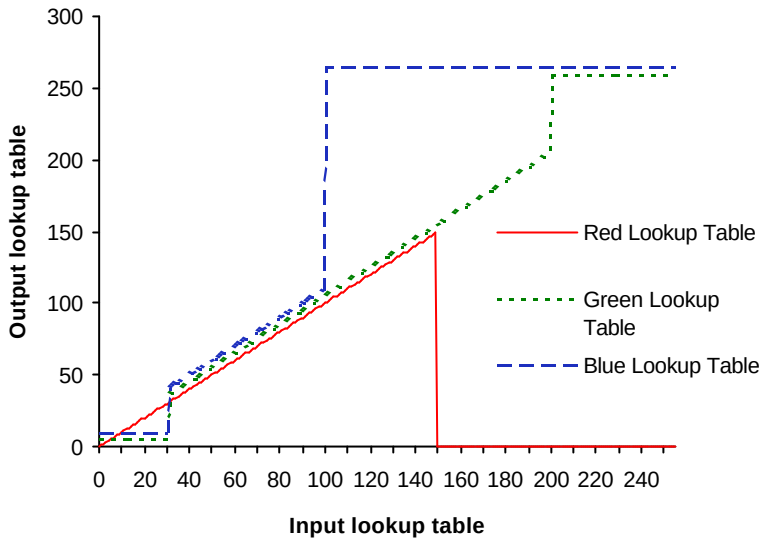


Figure 9. Values of Lookup Tables used to enhance the lipofuscin granules and neurons in image analysis.

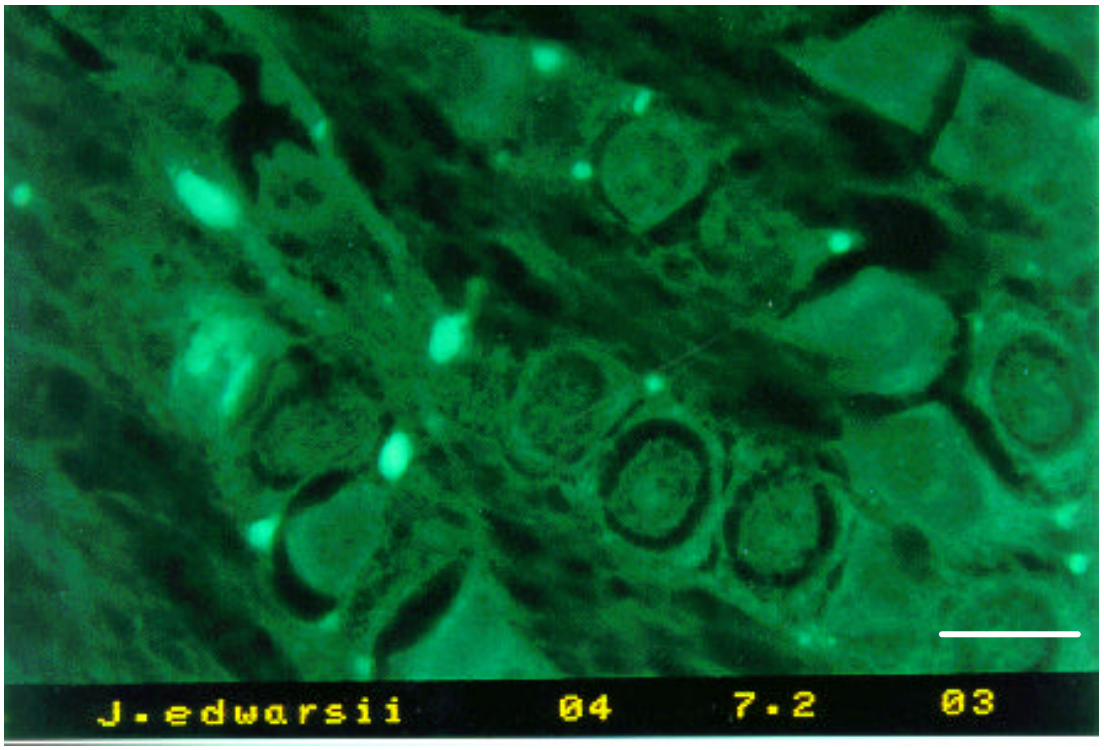


Figure 10. Fluorescent lipofuscin (light green) against the background fluorescence of the neurons (green) for *J. edwardsii*. Magnification 1250x Scale bar = 10 µm

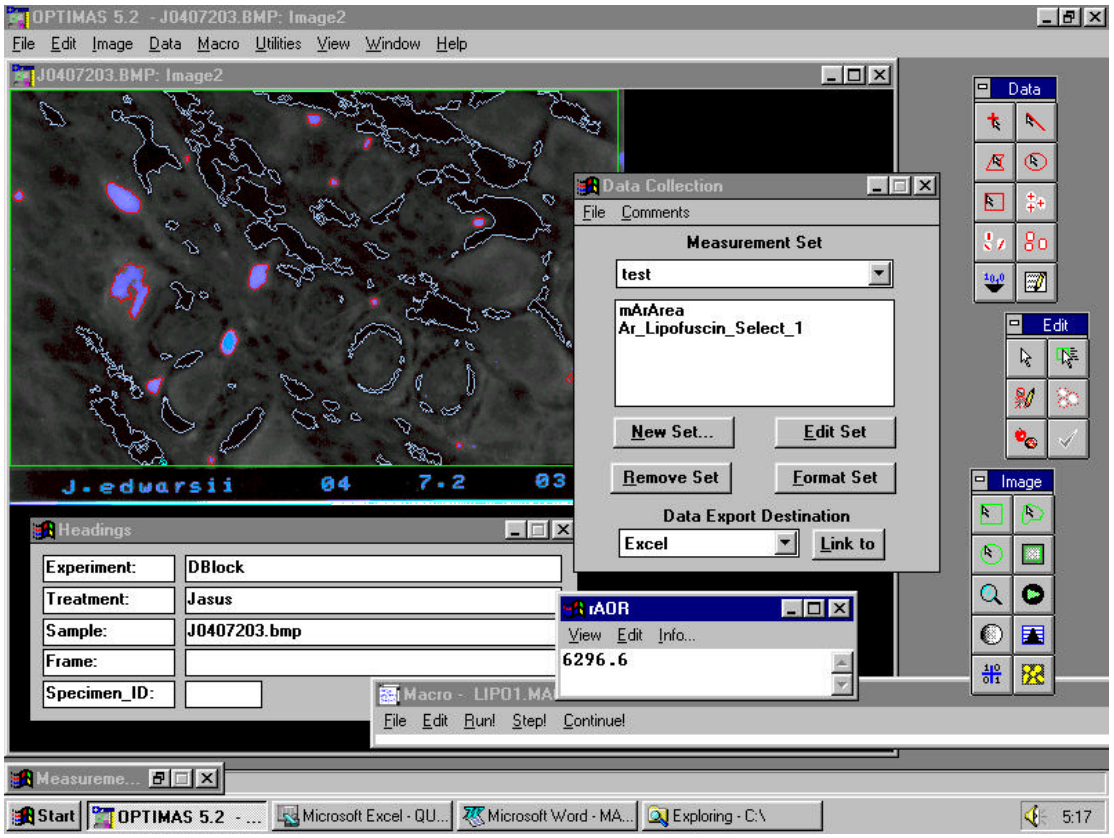


Figure 11. The same image as Figure 10 after manipulation of lookup tables and conversion to 8 bit grey. The fluorescent lipofuscin and space are automatically outlined in order to calculate respective areas.

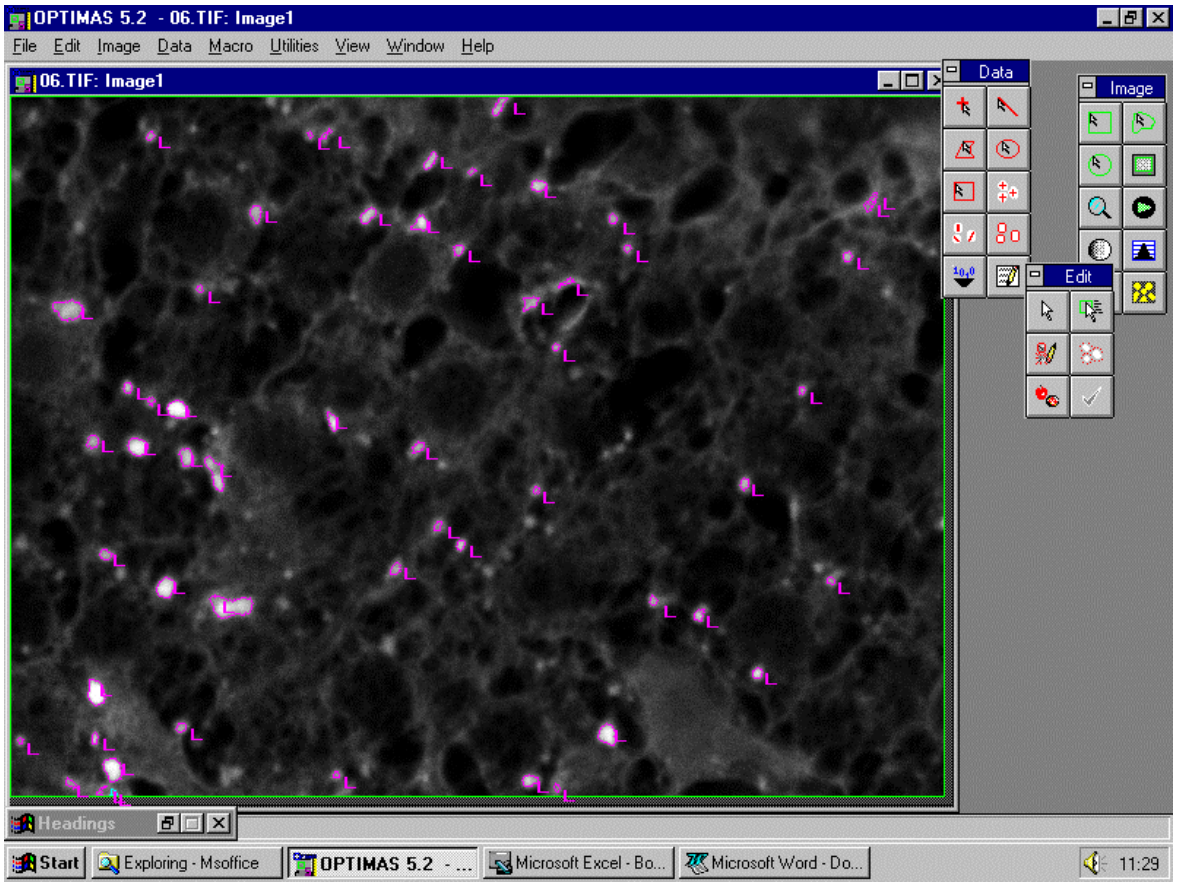


Figure 12. Image of *H. gammarus* provided by Dr Sheehy and processed at the CAF showing lipofuscin granules thresholded to foreground.

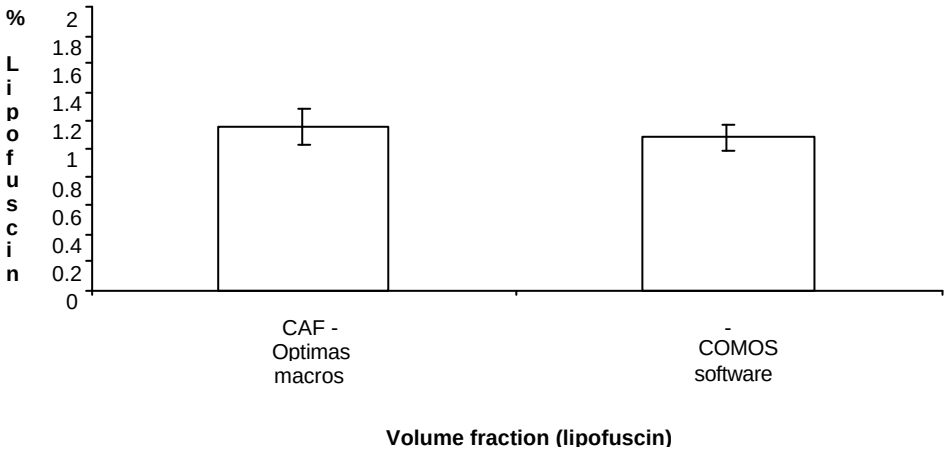


Figure 13. Comparison between calculated lipofuscin concentrations from images supplied by Dr. M.R.J. Sheehy (COMOS® software) and CAF's custom Optimas macros.

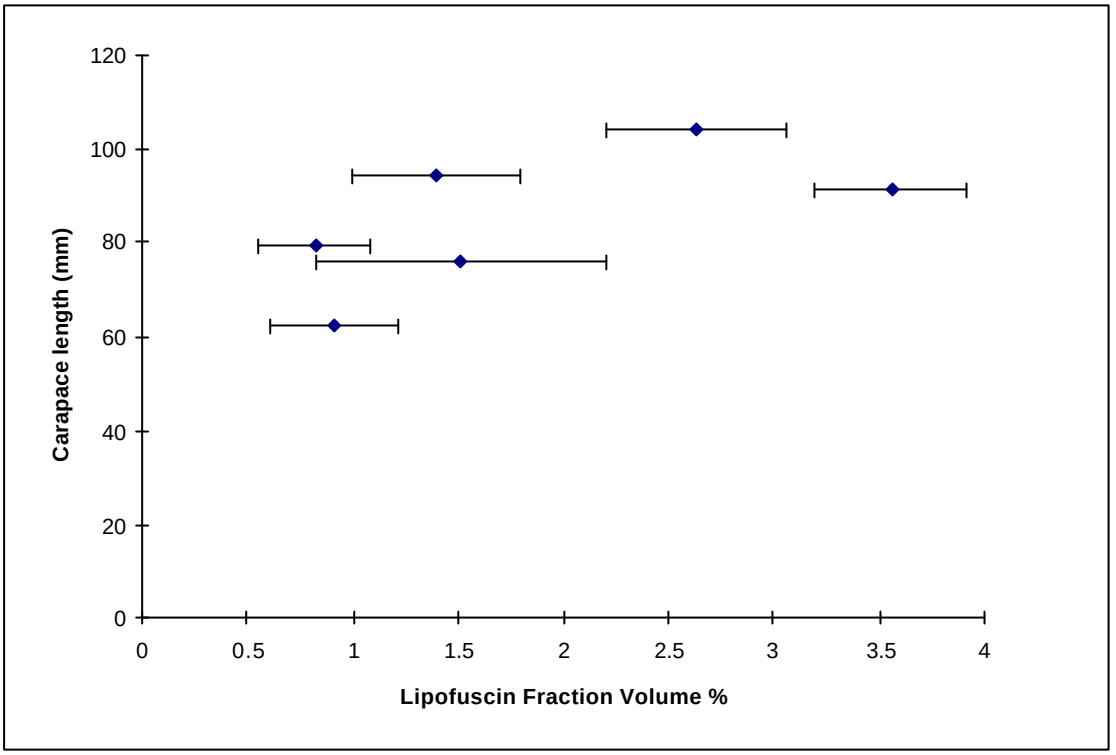


Figure 14. Lipofuscin Fraction Volume (%) against carapace length (mm) for *C. quadricarinatus*. +/-SE

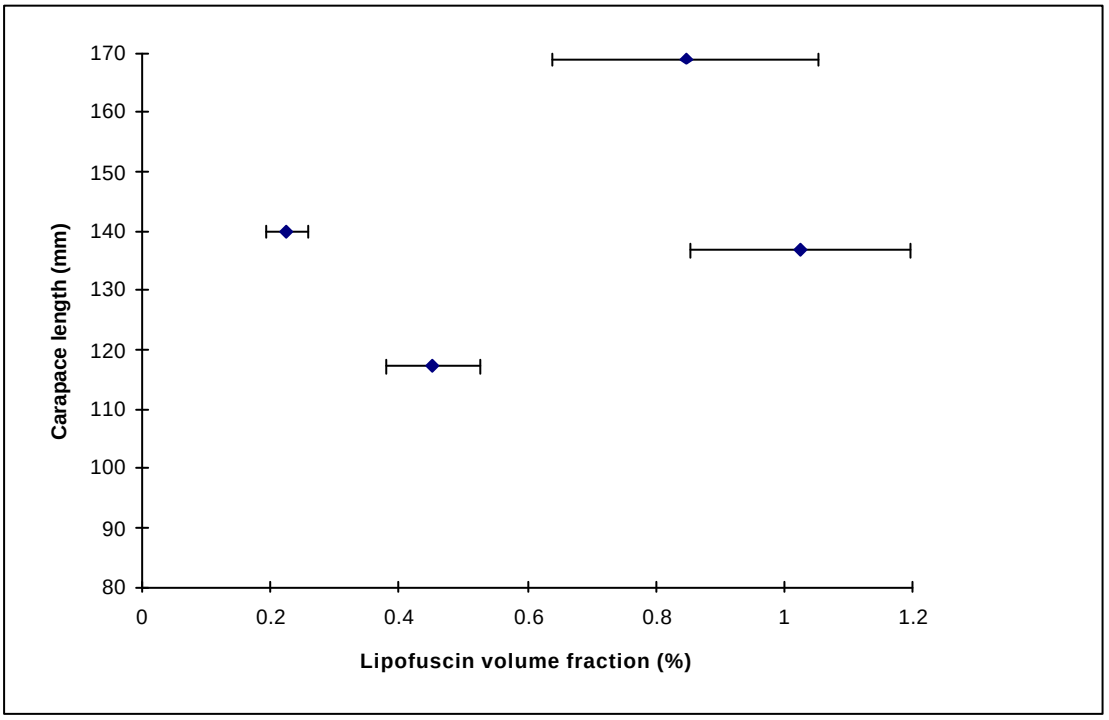


Figure 15. Lipofuscin volume fraction (%) against carapace length (mm) for *J.edwardsii* +/-SE

Appendix 1

Intellectual Property and Valuable Information

No intellectual property of commercial importance has been developed from this report. The proportion of intellectual property for this project is 71% (Fisheries Research and Development Corporation) and 29% (Marine and Freshwater Resources Institute).

Appendix 2

Staff

Alexander Morison	Project co-ordinator
Simon Robertson	Image analysis and report preparation
Kyne Krusic-Golub	Report preparation and photomicroscopy

Appendix 3

Aquaria - Physical chemistry (Table)

The aquaria environment was monitored for dissolved oxygen pH, and temperature on a daily basis during the week. No measurements were taken during the weekends. The results are presented in Table below and Figure overleaf. *C. quadricarinatus* were fed commercial fish pellets *ad libertum.*

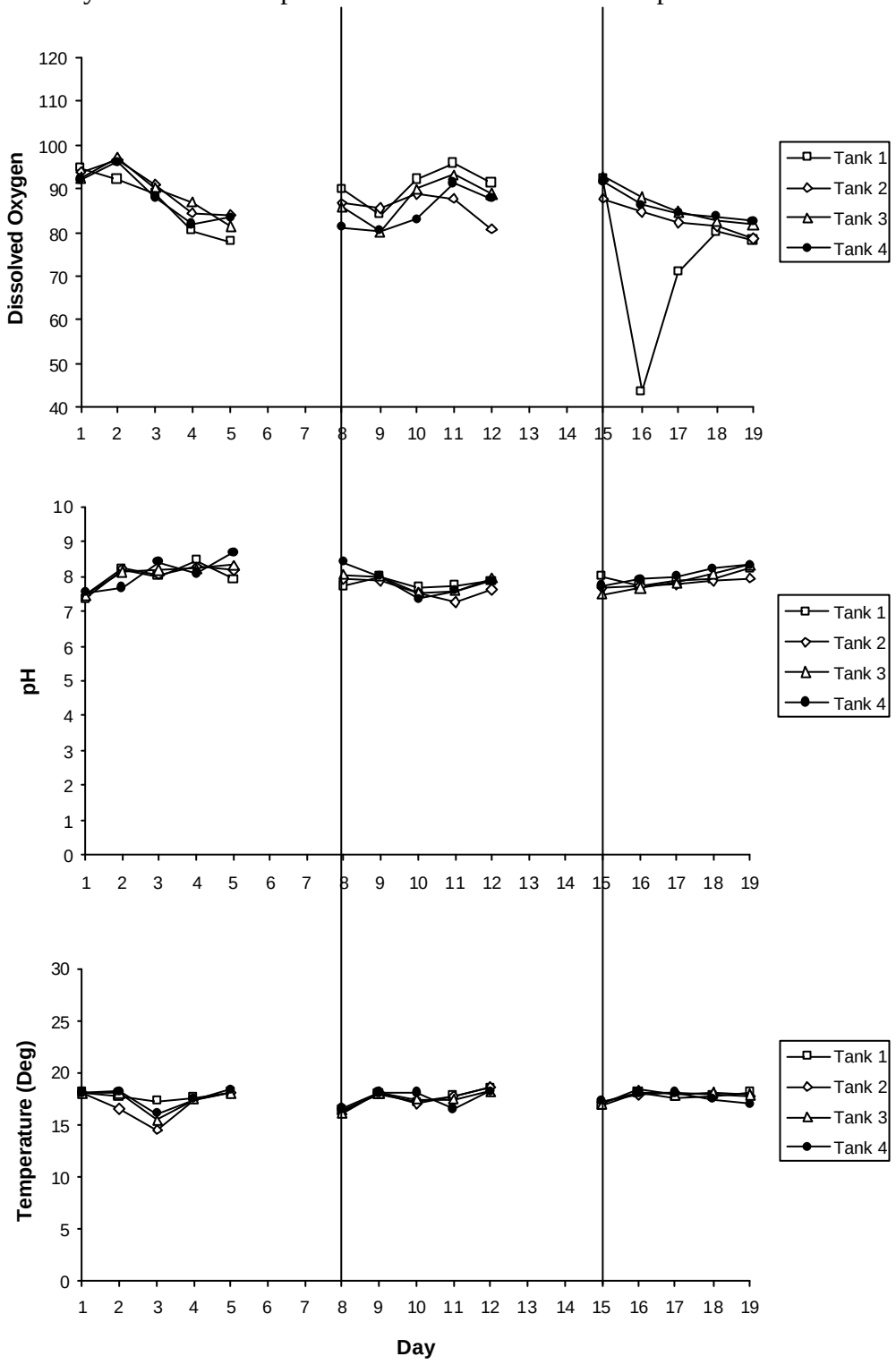
Daily dissolved oxygen, pH, temperature and salinity values for aquaria holding *C. quadricarinatus* under laboratory conditions for a period of three weeks.

	DO				pH				Temp (deg)				Salinity (ppt)			
Day	Tank No				Tank No				Tank No				Tank No			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1	95	94	93	92	7.4	7.5	7.5	7.6	18	18	18	18	1	1	1	1
2	92	97	97	96	8.2	8.2	8.1	7.7	18	17	18	18	1	1	1	1
3	89	91	90	88	8	8.1	8.2	8.4	17	15	16	16	1	1	1	1
4	81	85	87	82	8.5	8.3	8.2	8.1	18	18	17	18	1	1	1	1
5	78	84	82	83	7.9	8.2	8.3	8.7	18	18	18	18	1	1	1	1
6	Weekend															
7																
8	90	87	86	81	7.7	7.9	8	8.4	16	16	16	17	1	1	1	1
9	84	86	80	81	8	7.9	8	8	18	18	18	18	1	1	1	1
10	92	89	90	83	7.7	7.6	7.6	7.3	17	17	17	18	1	1	1	1
11	96	88	93	92	7.7	7.3	7.6	7.6	18	18	18	17	1	1	1	1
12	91	81	89	88	7.9	7.6	7.9	7.9	19	19	18	18	1	1	1	1
13	Weekend															
14																
15	92	88	93	92	8	7.7	7.5	7.7	17	17	17	17	1	1	1	1
16	44	85	88	87	7.7	7.7	7.7	7.9	18	18	18	18	1	1	1	1
17	71	82	85	85	7.9	7.8	7.8	8	18	18	18	18	1	1	1	1
18	80	82	83	84	7.9	7.9	8.1	8.2	18	18	18	17	1	1	1	1
19	78	79	82	83	8.2	8	8.3	8.3	18	18	18	17	1	1	1	1
Mean	84	86	88	86	7.9	7.8	7.9	8	18	17	18	18	1	1	1	1

Appendix 3 - Continued

Aquaria - Physical chemistry (Figure)

Daily dissolved oxygen, pH and temperature for aquaria holding *C. quadricarinatus* under laboratory conditions for a period of 3 weeks. Vertical line represents a 70% water change.



Appendix 4

Lipofuscin volume fraction macro

```
// Gets the volume of the lipofuscin using lipo.thr threshold file
// Central Ageing Facility 1997

// ********************Set for species*****************************

UpperLevel=80;
LowerLevel=25;

/*  used in macro development  */
integer j;
j=0;

/* Begin */
EscapeControl(2); /* restore on exit */
BeginOrEndUpdateBlock( TRUE);
/*  Set array for lookup tables
Upramp = (0..255);
RedLUT[0..255] = Upramp;
RedLUT[150..255] = 0;
GreenLUT[0..255] = Upramp;
GreenLUT[200..255] = 255; // highlight gray values from 200-255 in CYAN
GreenLut[0..30]=00;
BlueLUT[0..255] = Upramp;
BlueLUT[100..255] = 255;
Bluelut[0..30]=0;
delete (Upramp);
BeginOrEndUpdateBlock( FALSE);

// ************Set threshold levels**********************************
//  Create areas and display
                CreateEmptyClass( "Space", 2, 'A');
                CreateEmptyClass( "Lipofuscin", 2, 'B');
                ThresholdsGray = 0:LowerLevel :: UpperLevel:255;
                CreateArea(,,TRUE);
                UpdateAllClassData();

// Display message
                MacroMessage( "Out of ",TotalTally," areas found, ",
                Ar_Space_Tally_0, " were space, while ",
                Ar_Lipofuscin_Tally_1, " was lipofuscin ganules." );

// Get area of roi in calibrated units
```

```
// Extract Roi Array
                rRoi=ROI;
// Get X and Y's from Roi[Array]
                rX1=Roi[0];
                rY1=Roi[3];
                rX2=Roi[1];
                rY2=Roi[2];
/* Used to check size of calculated roi
                MacroMessage(totext(rX1));
                MacroMessage(totext(rY1));
                MacroMessage(totext(rX2));
                MacroMessage(totext(rY2));
*/
// Get X and Y's of horizontal and verticals
                rX=rX2-rX1;
                rY=rY2-rY1;
// Calculate AreaOfRoi
                rAOR=rX*rY;
                viewbox(rAOR);
// display area of roi
                SetExport(mArArea,1,True);
                hAreas=CreateArea(,,TRUE);
                MultipleExtract(True);
                rSpace=ClassifyScreenObjects(Space,hAreas);
```