# FACTORS AFFECTING ACCUMULATION OF LIPOFUSCIN AGE PIGMENT IN ARTHROPOD NEURAL TISSUE AND ITS USE AS AN ECOLOGICAL TOOL FOR AGE DETERMINATION.

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

Duane Barros Fonseca B.Sc., M.Sc.

Department of Biology

University of Leicester

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

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Duane Barros Fonseca

# General Abstract

Factors affecting the accumulation of arthropod neurolipofuscin
by Duane Barros Fonseca

Lipofuscin pigment deposition is a widespread manifestation of cellular ageing and also a useful age indicator in ecological research. This project aimed to explore factors that modulate neurolipofuscin accumulation, using two experimental arthropod species, the locust, *Locusta migratoria,* and the freshwater crayfish, *Pacifastacus leniusculus*. Neurolipofuscin was quantified using validated confocal microscopy methods.

The pattern of age-related accumulation of neurolipofuscin in *L. migratoria* was found to differ from that previously reported for crustaceans, with little accumulation prior to maturity and an exponential increase with high variability thereafter. While this pattern of accumulation may not be very useful for age determination, the finding that neurolipofuscin concentration was highly correlated with population survivorship suggested that lipofuscin formation or its precursors may be deleterious and that insect ageing may be equally well explained by stochastic as well as evolutionary theories of ageing.

It was shown, for the first time in any crustacean, that unilateral eyestalk ablation reduces neurolipofuscin accumulation rate in the contra-lateral eyestalk of *P. leniusculus*. It is hypothesized that this represents either reduced lipofuscinogenesis due to neurohormonal effects on oxidative catabolism or increased lipofuscin degradation by accelerated proteolysis following CNS damage. This finding means that longitudinal measurements of lipofuscin, i.e., in the same individual, by eyestalk biopsy, cannot be used to assess natural lipofuscin accumulation rates in individuals of unknown age.

Neurolipofuscin accumulation rate in ablated signal crayfish was found to be strongly inversely correlated with physiological age, with old individuals generally losing lipofuscin after ablation. Although this pattern is likely to be an artefact of ablation, it is the first quantitative evidence of in vivo reversibility of lipofuscin accumulation for any species and has important gerontological implications.

Annual cohorts were detected by modal analysis of a neurolipofuscin concentration histogram for a pond population of *P. leniusculus* for the first time. Growth curves fitted to the length-at-age data obtained from this analysis were compared with results of three conventional methods for growth curve estimation: size-frequency analysis, anniversary tag-recapture and laboratory rearing. While there was generally good agreement between the methods for the early part of the growth curve, this analysis highlighted problems with extrapolation of growth rates from laboratory rearing to the field and the inability of conventional size-frequency analysis to detect older age groups. Neurolipofuscin methodology is the only approach that can give age-length data for older individuals in the wild population and measurements of longevity.

Overall, the study has provided significant new information in the field of lipofuscin research and for lipofuscin based age determination methodology, and has suggested various avenues for future research.

This thesis is dedicated to Constância.

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# Chapter 1

# General Introduction

## 1.1 Significance of lipofuscin

Two dominant themes in current biological research are the mechanisms of ageing and the sustainable development of living resources. Something that draws these two apparently disparate fields together, lipofuscin, the so-called age pigment, is the topic of this thesis

With the average age of first world human populations steadily increasing, biogerontological research into the causes of, and ways of mitigating the devastating consequences of ageing is intense at this time. At the heart of the ageing process, at a cellular level, is the formation and accumulation of lipofuscin. This is considered to be the most well known and phylogenetically widespread morphological hallmark of normal ageing (Porta, 2002a), and it is also symptomatic of a number of age-related diseases. Lipofuscin may be either a proximate cause or an effect of the ageing process, but which of these it is, remains controversial at this time.

With respect to sustainable development, upwardly spiralling human population numbers and the often inadequately fettered pursuit of food, wealth and recreation have placed many of the world's renewable living resources and their habitats under extreme exploitation pressure. With respect to world fisheries, which are a principal area of interest underlying this thesis, about 48% are considered to be fully exploited, 17% overexploited and 9% depleted (Anon., 1999). The United Nations Convention on the Law of the Sea (Anon., 1982) and the Code of Conduct for Responsible Fisheries (Anon., 1995) require that wild marine fisheries are sustainable. Sustainability produces long-term human material and non-material well-being.

Sustainability means exploitation that 'meets the needs of the present without compromising the ability of future generations to meet their own needs' (Brundtland, 1987). Sustainability is achieved through effective resource management. Such management requires development of a comprehensive resource 'knowledge system', one biological input to which is the traditional stock assessment. Stock assessments require information on three processes that regulate the population dynamics of any species, reproduction, growth and mortality. The rates of each of these processes may themselves be age dependent and quantification of them is usually by way of mathematical indices known as population parameters. These include the so-called generalized growth parameters and mortality coefficients, which are in turn preferably derived from data on numbers-at-age and size-at-age in the population in question. Thus, accurate chronological age determination ultimately forms the basis for the sustainable exploitation of animal populations. In regard to the topic of this thesis, research in the last two decades has demonstrated the value of physiological age indices, such as the lipofuscin and pteridine pigments, for chronological age determination in some problematic animal groups (Mail *et al.*, 1983; Sheehy, 1990).

Age pigments have proved to be a useful tool for studying the biological ageing process, for age determination, and for understanding the population dynamics of crustaceans, particularly with regard to the assessment of commercially important stocks. However, further expansion of, and refinements to, the application of the ageing method are desirable and much remains to be discovered in regard to the endogenous and external factors that may affect lipofuscin accumulation. As the title indicates, the broad

objective of this thesis was to contribute to biogerontological and ecological knowledge, by further examining factors that affect accumulation of lipofuscin in arthropod neural tissue and its use as an ecological tool for age determination.

The purpose of this General Introduction is not to provide a comprehensive review of all of the recent literature on lipofuscin, which is vast, particularly in the medical arena. Rather, it aims to provide a minimum of necessary background information, to highlight several current central questions in lipofuscin and lipofuscin-based age determination research and to guide the reader through the rationale for, and development of, the research contained in each of the 4 experimental chapters of the thesis. Each of these chapters is designed to be self-contained, with its Abstract, Introduction, Methods, Results and Discussion, in order to facilitate rapid publication in scientific journals. This format necessarily leads to some repetition, but attempts have been made to minimize this wherever possible. Finally the General Discussion attempts to evaluate any limitations of the work and highlight the significance and contribution to knowledge of the findings.

## 1.2 Lipofuscin as a tool for crustacean age determination

Age determination of animals is often carried out by the examination of permanent skeletal structures that bear growth rings. This is analogous to dendrochronology in trees. Such rings form in the otoliths, scales, fin rays, dentary and opercular bones of fish, the shells and statoliths of molluscs and, exceptionally for crustaceans, in the shells of cirripedes (barnacles). The rings may reflect periodic reductions in growth rate that occur each winter, or during

reproductive periods. If the periodicity of the rings can be validated as annual (or daily), this method can provide a reliable measure of chronological age.

However, there are situations in which this type of age determination is not possible. For example, in many tropical fishes, clear growth rings in hard parts are not present, either because of the lack of marked seasonality in temperature or because reproduction occurs continuously throughout the year (Longhurst and Pauly, 1987). In crustaceans, the method normally cannot be used because growth occurs via periodic moulting, during which all hard parts are shed. To circumvent this limitation some alternatives exist. Rearing experiments using captive individuals can be used to obtain precise chronological age information; however, the results often cannot be extrapolated for animals in the wild because of the abiotic and biotic environmental differences. Field mark and recapture programmes, using either known age-juveniles or a known time span between release and recapture, may suffice but are normally restricted to large commercial fisheries and can be expensive. Modal progression analysis (MPA) using length-frequency histograms is the most popular alternative because it is widely applicable, technically simple and often the cheapest option. However, the main limitation is the unreliable assumption that size is coupled with age (Hilborn and Walters, 1992).

In the 1980s, a new approach was proposed by Ettershank (1983), who attempted to use 'extractable' lipofuscin for age determination of the Antarctic krill, *Euphausia superba*. Lipofuscin is a "waste" product resulting from cellular metabolism that accumulates in secondary lysosomes (Terman and Brunk,

1998). As the name suggests, lipofuscin in tissue sections is brownish in colour under bright field and its ultrastructure is characteristically irregular with electron-dense, lamellated and vacuolar components. It has affinity for fat stains such as Sudan Black (Porta *et al.*, 2002). However, the main property of lipofuscin, as far its identification is concerned, is its autofluorescence in unstained tissue sections. In crustacean neural tissue, using an excitation wavelength of 514 nm (green) and a barrier filter of 550 nm, *in situ* lipofuscin is identified by its emission maximum at 590 nm (yellow/orange) (Belchier, 1996).

Lipofuscin is found in many tissues, but the most conspicuous accumulations are observed in post-mitotic cells such as those of the brain and heart (Terman and Brunk, 1998). The theory is that in post-mitotic tissues, lipofuscin, which has been considered undegradable, is not "diluted" by cellular division, resulting in an age-related accumulation. It is this accumulation that forms the basis of attempts to use it for age determination.

## 1.3 Lipofuscin formation and biochemistry

Lipofuscin formation is not completely understood. The most detailed information on the mechanism of lipofuscin formation is available for human retinal pigment epithelium (RPE) cells (Liu *et al.*, 2000; Ben-Shabat *et al.*, 2002; Katz and Robison, 2002). Lipofuscin formation in RPE cells is closely related with the visual cycle, i.e., the recycling of visual pigments (rhodopsin). In this process, light absorbed by the retina isomerises rhodopsin (*viz.* 11-*cis* retinal group) to an aldehyde (all-*trans* retinal), which is reduced to vitamin A (all-*trans* retinol) and, subsequently, in dark conditions, is oxidized and

isomerised back to 11-*cis* retinal (Stryer, 1995). Any all-*trans* retinal that is formed but accidentally escapes reduction to vitamin A, can react with the amino groups of proteins and other biomolecules to form amine-retinal Schiff bases. After further reactions, these Schiff bases are transformed to a phosphatidylpyridinium bisretinoid (A2-PE) which is the precursor of the main fluorophore of lipofuscin in RPE cells, *viz*, a pyridinium bisretinoid (A2E). All these processes occur outside RPE cells, *viz.* in photoreceptor outer segments. When outer segments are phagocytosed by RPE cells (a function of RPE cells is the periodic phagocytosis of the outer segment membrane) A2-PE is transformed into A2E. This transformation probably occurs inside lysosomes. It is important to recognize that the process is specific to lipofuscin formation in the vertebrate photoreceptor and is unlikely to explain lipofuscin formation in other tissues or the invertebrate eye, where a different rhodopsin-metarhodopsin cycling exists.

The biochemical basis of lipofuscinogenesis in the lysosomes of other tissues remains a controversial issue. Brunk *et al.* (1992) proposed a mechanism involving oxidative stress reactions on phagocytosed material. Reactive oxygen species (e.g., $H_2O_2$) diffusing into secondary lysosomes would initiate peroxidation processes and intermolecular cross-linking that would lead to lipofuscin formation. The basis for the lipoperoxidation theory of lipofuscinogenesis is found in the original *in vitro* studies of Chio *et al.* (1969). The assumptions underlying this research have now been seriously called into question (Jolly *et al.*, 2002; Porta, 2002a, b). One uncertainty is the distinct fluorescent colour of the by-products of lipid peroxidation (*viz.*, blue) and conspicuously different golden yellow colour of *in situ* lipofuscin (Eldred *et al.*,

1982). Other experimental evidence (Porta *et al.*, 1980, 1981, 1982; Katz *et al.*, 1993) has demonstrated no correlation between the accumulation of lipofuscin and lipoperoxidative processes.

Available evidence on the biochemistry of lipofuscin indicates that its composition is quite variable. Jolly *et al.* (2002) showed that proteins were the main component of lipofuscin in equine thyroid glands, but a high individual variability in lipofuscin composition was also observed. Porta *et al.* (2002) reported that proteins (30-70%) and lipids (20-50%) are the main components of lipofuscin, while a small amount of carbohydrates (4-7%) and traces of metals (Al, Cu, Fe, and Zn) are observed.

*In situ* fluorescence, once the main and irrefutable trait of lipofuscin, has also been called into question recently. Palmer *et al.* (2002), studying storage bodies from sheep with the pathological condition, neuronal ceroid lipofuscinosis, suggested that the putative fluorescence might actually be a type of light scatter resulting from the packing of proteins rather than a true light emission.

## 1.4 Lipofuscin, ageing, and stochastic processes

Lipofuscin accumulation reflects physiological age rather than chronological age. Several studies have related lipofuscin accumulation to metabolic rate. Sohal (1981a) performed an experiment on houseflies in which the metabolic rate (oxygen consumption) was reduced by restricting physical activity. Rate of *in situ* lipofuscin accumulation was also reduced. Low activity flies also had significantly longer life spans. Sheehy *et al.* (1995a) showed that freshwater

crayfish raised at the highest temperatures had the highest lipofuscin accumulation rates and the shortest lifespans, suggesting that they aged more rapidly due to higher metabolic rates. In homeotherms, there is not a straightforward inverse correlation between metabolic rate and life span (e.g., birds live up to 4-fold longer than mammals of similar size, even though the former have a higher metabolic rate; Perez-Campo *et al.*, 1998). In invertebrates an inverse correlation has been observed to be true most of time (Sohal, 1985; Sohal *et al.*, 2002; Van Voorhies 2001, 2002), although some contrary evidence has recently been presented (Promislow and Haselkorn, 2002). A source of confusion regarding this issue is that the metabolic potential (i.e., total consumption of oxygen during life) often does not change in animals subject to different treatments (Yan and Sohal, 2000), suggesting that there is no change in metabolic rates. However, this compensation in oxygen consumption does not preclude changes in some biological functions (e.g., rates of enzymes synthesis and degradation, and balance between different metabolic pathways) that can occur at different temperatures and activity levels (Sohal, 1985).

The "Free Radical Theory of Ageing" (Harman, 1956) is currently the most accepted stochastic theory of ageing (Finkel and Holbrook, 2000). Briefly, it proposes that reactive oxygen species (e.g., the superoxide radical) leaking from the electron transport chain (i.e., cellular respiration) promote cellular damage such as peroxidation of unsaturated fatty acids, oxidation and cross-linking of proteins, and DNA damage. Since the main source of production of reactive oxygen species is non-enzymatically controlled (i.e., complex III in the mitochondrion electron transport chain), the formation of reactive oxygen

species is usually correlated with metabolic rate. Studies demonstrating the effect of the oxidative stress on the accumulation of lipofuscin are Sohal and Brunk (1990), Fleming *et al.* (1992) Ishii *et al.* (1998) and Sitte *et al.* (2001). However, as noted earlier, some previous studies on lipid peroxidation have not supported this theory of lipofuscin formation (Porta *et al.*, 1980, 1981, 1982).

## 1.5 Lipofuscin and evolutionary theories of ageing

To date, no study has specifically discussed the accumulation of lipofuscin in relation to the evolutionary theories of ageing. Because ageing occurs after maturation and reproduction, natural selection would appear to have limited opportunities to influence senescence processes (Kirkwood and Austad, 2000). In fact, ageing puzzled evolutionists because it is a deleterious trait that has been maintained by natural selection (Charlesworth, 1994). To explain ageing from an evolutionary point of view three main theories were proposed: (i) the 'mutation accumulation theory' (Medawar, 1952), which is based on the small advantage gained in selective removal of deleterious mutations after reproduction is complete; (ii) the 'pleiotropy theory of ageing' which states that a mutation that increases early life fecundity (Darwinian fitness) would be favoured by selection, even at the cost of deleterious effects late in life (Williams, 1957); and (iii) 'disposable soma theory' which explains the trade-off between reproduction and survival on the grounds that an optimal energy investment in reproduction diverts resources from cell maintenance and repair, resulting in ageing and culminating in death (Kirkwood, 1977). The latter theory offers opportunities to explore the

relationship between stochastic processes that may underlie the accumulation of lipofuscin and the evolution of ageing and longevity.

## 1.6 Central issues in lipofuscin research

Several current questions and controversies were defined by the recent Sixth International Symposium on Lipofuscin and Ceroid Pigments (Porta, 2002c). These include nomenclature, biochemistry of formation, degradability/turnover, deleteriousness to cell function and quantification.

### 1.6.1 Nomenclature

Inconsistent use of terminology has created misunderstanding within the field. For example, ceroid pigments, although they present superficially similar *in situ* fluorescence and granular morphology to lipofuscins they are, nevertheless, distinct from them. Ceroids are observed in pathological conditions such as inherited neurodegenerative disorders (neuronal ceroid lipofuscinoses, NCL, collectively known as Batten's disease) (Porta *et al.*, 2002). This accumulation results from autosomal recessive mutations leading to primary lysosomal storage diseases which cause impairment of protein catabolism (Joly *et al.*, 2002; Mole, 1999). Therefore, often-used terms such as 'lipofuscin/ceroid pigments' only create confusion (Nagy and Porta, 2002). Katz and Robison (2002) have recently attempted to clarify the definition of lipofuscin as pigments that (i) consist of intracellular secondary lysosome deposits, (ii) have yellow autofluorescent emission when excited by near ultraviolet or blue light, and (iii) accumulate during normal senescence.

### 1.6.2 Biochemistry

Although much information is available on the biochemistry of formation of lipofuscin in mammalian RPE cells, little is known for other tissues and animal groups. Based on what has been learned from RPE cells, a key issue is to clarify the role of oxidation and free radical damage on the formation of lipofuscin. Free radical damage is likely to be concentrated inside lysosomes; however, a detailed biochemical description of this process, based on analytical data, has never been presented. Lipofuscin has largely defied biochemical diagnosis because: it is biochemically extremely heterogeneous both between tissues and species, it is not very soluble, and it is exceedingly difficult to obtain in pure form in sufficient quantities for thorough analysis.

### 1.6.3 Degradability/turnover

The traditionally accepted view of lipofuscin is that it accumulates with age because it is an undegradable, heavily cross-linked, waste product. This notion is consistent with the (now questionable) lipoperoxidation theory of lipofuscinogenesis. The idea that lipofuscin might be turned over in cells, with age-related accumulation simply resulting from an imbalance between rate of formation and rate of loss, was first proposed following a study by Ivy *et al.* (1984) in which the inhibition of proteolysis in lysosomes led to accumulation of "lipofuscin-like" pigments whose nature was not clearly defined. Thereafter, some studies (e.g., Ivy, 1992) claimed that such lipofuscin-like substances were indistinguishable from natural lipofuscin. However, whether these pigments are in fact the same as true lipofuscin remains a source of

controversy (Porta *et al.*, 1995; Boulton and Ellis, 1999; Terman and Brunk, 1998, 1999).

### 1.6.4 Deleteriousness

Whether the accumulation of lipofuscin granules in cells inflicts a detrimental effect is an open question because it has been exceedingly difficult to conclusively demonstrate negative consequences directly resulting from lipofuscin accumulation. Recently, Terman (2001) produced the "Garbage Theory of Ageing" which proposes that the accumulation of lipofuscin physically and chemically impairs cellular functions and increases the likelihood of cell death. *In vitro* studies on human RPE cells have shown that lipofuscin causes the impairment of lysosomal function and loss of membrane integrity (Holz *et al.*, 1999; Schutt *et al.*, 2000; Davies *et al.*, 2001). In human fibroblasts exposed to oxidative stress, lipofuscin-loaded cells have increased sensitivity to that stress (Terman *et al.*, 1999a) and reduced renewal of proteins resulting in worn-out/damaged organelles (Terman *et al.*, 1999b; Sitte *et al.*, 2001). However, Porta *et al.* (2002) claim that substantial natural *in vivo* accumulations of lipofuscin in some parts of the brain from an early age do not appear to result in any deleterious effects. Ceroid pigments do apparently lead to damage, however, and in some of the works cited above (e.g., Terman *et al.*, 1999 a, b) the pigment under investigation would have been more appropriately referred to as ceroid.

### 1.6.5 Quantification

A further controversy in lipofuscin studies has been the issue of lipofuscin quantification by solvent extraction and spectrofluorimetry. The use of this

method by numerous workers, mainly in the 1980s, but still occasionally today, is based on the assumption that soluble lipid peroxidation products are a precursor of lipofuscin. As noted earlier, this assumption is currently being questioned. It has been shown that results obtained by extraction methods are spurious (Sheehy, 1996) because extracted "lipofuscin" (better referred to as "extractable fluorescent substances" (Sohal, 1987)) does not bear any quantitative relationship with *in situ* lipofuscin quantified by microscope-based methods. Sheehy (1996) demonstrated that the intensities of extractable fluorescent substances were sometimes positively correlated with tissue mass used in the assay or the body mass of the individual involved, which could produce a misleading age-related trend when mass is broadly related to age.

## 1.7 Development of the lipofuscin ageing method

Since the establishment of reliable microscope-based quantification methods for lipofuscin (Sheehy, 1989, 1990a), including the introduction of confocal microscopy (Belchier *et al.* 1994), information has been gathering in the field. Several studies on the potential of lipofuscin as a crustacean age determinant were able to demonstrate very significant linear or curvilinear relationships between brain lipofuscin (now termed neurolipofuscin) concentration in crayfish and chronological age (Sheehy, 1990b, 1992; Belchier *et al.*, 1998). Further developments intending to quantify the effect of temperature (Sheehy *et al.*, 1994) showed a significant positive relationship between environmental temperature and neurolipofuscin accumulation rate in crayfish. Similar studies on laboratory-reared lobsters, *Homarus gammarus*, were conducted

(O'Donovan and Tully, 1996; Tully *et al.* 2000) and reached similar conclusions.

Sheehy *et al.* (1998), Bluhm and Brey (2001), and Bluhm *et al.* (2001) successfully used neurolipofuscin to resolve age groups in wild populations, which opened up new possibilities for use of the method where there was no captive or tagged known-age calibration material. Recently, effects of temperature on neurolipofuscin accumulation rate were also modelled in wild *H. gammarus* populations resulting in an equation to correct neurolipofuscin accumulation rate for spatial and temporal variations in the average sea temperature experience of individual animals (Sheehy and Bannister, 2002).

The only other modulator of neurolipofuscin accumulation rate in crustaceans that has been reported to date is dietary anti-oxidant (*viz.*, vitamin C and E) level (Castro *et al.*, 2002). It was found that shrimps subjected to a higher dietary antioxidant concentration exhibited a slight but significant reduction in neurolipofuscin accumulation rate, suggesting that oxidative processes are involved in neurolipofuscin formation.

Unique insights gained by neurolipofuscin-based age-structure analysis have begun to emerge recently for U.K. lobster stocks (Sheehy *et al.*, 1999; Sheehy 2001; Sheehy and Bannister, 2002). Because neurolipofuscin accumulation rate is known to be inversely proportional to age at maturity and longevity, the significant differences in neurolipofuscin accumulation rates between sites, but the lack of differences in growth rates that were found in known-age microtag recaptured calibration material, suggested that there may be major geographical variations in life history traits relevant to stock assessment that

are not detected by conventional size-based methods (Sheehy and Bannister, 2002)

Estimates of extreme longevity for some individuals off the Yorkshire coast suggested the presence of offshore refugia and confirmed previous low natural mortality rate estimates (Sheehy *et al.*, 1999). Slower than expected average growth rates revealed by neurolipofuscin studies have implications for established stock assessments; fishing mortality may be lower and the advantages for yield of raising minimum landing size may be greater than previously estimated. Recruitment to legal size was found to be extremely protracted with at least six year-classes, and probably many more, recruiting to the fishery each year (Sheehy *et al.*, 1999). The minimum size limit therefore provides partial protection for a substantial age range of lobsters off Yorkshire even in more heavily fished inshore waters. However, it would also propagate size-selective fishing impact. Slower growing, small maturing individuals have many more opportunities to reproduce before entering the fishery. The effect of fluctuations in year-class strength on the fishery will be damped, by protracted recruitment producing stable size-compositions but hiding serious year-class failures. This explains how major declines in some U.K. stocks could have occurred without being reflected in altered size distributions. Any benefits of restocking would not be fully realized for many years. Protracted recruitment complicates efforts to predict lobster landings from historical data on larval or early-benthic phase abundance. It also has very important implications for the perception of the spawner-recruit relationship (SRR) because it generates artefactual curvature and scatter (Sheehy, 2001). Therefore, the traditional assumption that the asymptotic

SRR in lobsters reflects density dependent compensatory mortality and resilience to exploitation may be incorrect. This would affect principle arguments against U.K. restocking and reinstatement of the ban against capture of berried females; spawner biomass may have been unknowingly depleted. It would explain the often-observed contradiction between assumption of density dependence and field observations of low population densities. It would also explain how significant increases in clawed lobster landings could sometimes occur despite hypothesised recruitment bottlenecks (Sheehy, 2001)

Age structure analysis has shown that year class strength in *H. gammarus* is climatically modulated (sea temperature and onshore wind strength) (Sheehy and Bannister, 2002). This helps explain the poor recovery rate of depleted cold-water Norwegian and Scottish populations, where settlement success will be poorer. The effect of climate is an important consideration for current discussion on stock enhancement by protection of spawning stock or by release of hatchery-reared juveniles. For northern populations, the environment may impose greater limitations. It suggests that predicted climate change would impact U.K. lobster stocks. The finding also represents a major advance toward development of recruitment forecasting in the absence of quantitative larval sampling. Finally, it is evidence against damping by a 'recruitment bottleneck' (Sheehy and Bannister, 2002).

Overall the results of the neurolipofuscin-based age determination studies on *H. gammarus* confirm serious deficiencies in the size-based stock assessment methods that have had to be used in the past (Sheehy and

Shelton, 2001). Variable, environmentally regulated settlement and minimal correlation between age and size make traditional steady-state, pseudo-cohort and size-based stock assessment models largely redundant. Many of these problems could be overcome by age-structured analyses. Acquisition of year-on-year sets of age, growth, and mortality data for each coastal district by undertaking large numbers of lobster age determinations routinely would form the most reliable approach possible for assessing U.K. lobster stocks in future.

## 1.8 Rationale for the research in this thesis

The first experimental chapter of this thesis, **Chapter 2**, reports the results of a study on neurolipofuscin accumulation in the brain in relationship to survivorship in the African migratory locust, *Locusta migratoria*. The rationale for this work was threefold:

(i) There was insufficient information on morphological lipofuscin accumulation in neural tissues of insects. Although lipofuscin occurrence had been reported in insects (e.g., Sohal and Donato, 1979; Sohal, 1981b; Ettershank *et al.*, 1983), such studies either used unreliable extraction methods or used a very limited number of age groups covering a small proportion of the life span.

(ii) Finding a new broadly applicable indicator of age in insects would open the doorway to new approaches for study of population dynamics in this economically important group.

(iii) Studies on the evolutionary/genetic basis for lifespan and mortality rate lack suitable indices of fitness and physiological age. Neurolipofuscin, as an index of physiological ageing, had not yet been used in this context, although insects were frequently used as models in studies of the mechanism and evolution of ageing.

*Hypothesis: Lipofuscin accumulates in the brain of L. migratoria in a similar pattern to that observed for various crustaceans and this accumulation is related to mortality rate.*

**Chapter 3** reports the results of an investigation into the effects of unilateral eyestalk ablation on the neurolipofuscin accumulation rate in the remaining eyestalk in the signal crayfish, *Pacifastacus leniusculus.* The rationale was that a current problem for use of neurolipofuscin-based ageing on populations in the wild is the need to have a known-age calibration. If neurolipofuscin measurements could be made on one eyestalk and then, after a known time interval, on the other, to determine the neurolipofuscin accumulation rate, this would eliminate the need for known-age individuals. For this approach to be successful, however, a question to be answered would be whether the removal of one eyestalk would cause a change in the rate of neurolipofuscin accumulation in the contralateral eyestalk.

*Hypothesis: In P. leniusculus, unilateral eyestalk ablation does not affect the rate of neurolipofuscin accumulation in the remaining eyestalk.*

**Chapter 4** reports the results of a tag-recapture study in which unilaterally eyestalk-ablated *P. leniusculus* of a wide range of physiological ages (but

unknown chronological ages) were released into the field for 1 year, and then recaptured and neurolipofuscin concentration in the remaining eyestalk measured. The purpose of this study was to attempt to address the question of whether neurolipofuscin accumulation rate is age dependent and declines in old age. Some previous studies showed a strong linear relationship between age pigment concentration and chronological age (e.g., Belchier *et al.*, 1998). Others showed a curvilinear relationship, with age pigment accumulation rate declining at older ages (e.g., Sheehy, 1992). Three explanations have been proposed for the latter observation (Sheehy, 1992; Sheehy *et al.*, 1994, 1995). Firstly, it could be a normal correlate of slowing metabolism in an ageing brain or, secondly, an artefact of age-specific mortality in experiments which have, by necessity, sampled laboratory populations at different ages rather than the same individuals over time. Finally, it could be an artefact of abnormal physiological depression due to prolonged rearing under unnatural laboratory conditions. The last possibility is supported by observations that when grown under field conditions, *Cherax quadricarinatus* did not exhibit the same declining neurolipofuscin accumulation rate with advancing age that laboratory populations reared over the same period did (Sheehy *et al.*, 1994). Using eyestalk ablation techniques, it may be possible to resolve this question by measuring neurolipofuscin accumulation rates in individuals of a range of ages growing under natural conditions.

*Hypothesis: Neurolipofuscin accumulation rate declines with age, even in individuals growing under natural conditions.*

**Chapter 5** reports the results of a study that attempted to detect annual cohorts in a field population of *P. leniusculus* by neurolipofuscin concentration frequency analysis, and then compare the length-at-age data and growth curve so obtained with more traditional methods of growth curve estimation including size-frequency, tag-recapture and laboratory rearing. The rationale was that *P. leniusculus* is an important aquaculture and invasive species in Europe. The identification of annual cohorts by the neurolipofuscin concentration frequency method would enable neurolipofuscin concentration to be calibrated against chronological age without the need for known-age tagged or laboratory reared individuals, opening up new possibilities for investigating the population dynamics of this species. Also, the experiment, if successful, would provide corroboration for previous simulations of the neurolipofuscin technique on tropical freshwater crayfish (Sheehy *et al.*, 1994) and field trials on western rock lobsters (Sheehy *et al.*, 1998).

*Hypothesis: Neurolipofuscin can be used to detect annual cohorts in populations of signal crayfish, P. leniusculus by neurolipofuscin concentration-frequency analysis.*

# Chapter 2

## Neurolipofuscin accumulation after the onset of sexual activity mirrors survival pattern in locusts: Are evolutionary or stochastic theories of insect ageing applicable?

## 2.1 Abstract

Recent studies have explained insect lifespan by focusing on evolutionary theories of ageing to the exclusion of simple stochastic processes. Here attempts are made to further evaluate the applicability and compatibility of the two classes of theories of ageing for understanding insect senescence. To do this, parallel observations of manifestations of senescence at both a population and a cellular level are made in the African migratory locust, *Locusta migratoria* (Orthoptera: Acrididae). Life history traits, such as mean age at maturity and longevity, the form of the survival curve, and sexual differences in terms of natural selection are interpreted based on what is known about locust biology and population ecology. Simultaneously, the time-dependent deposition of yellow autofluorescent lipofuscin granules, the most consistent and phylogenetically constant morphological change of eukaryotic ageing, is used as a measure of the progression of *in vivo* cellular senescence in the locust brain. Interpretation of these cytological results emphasizes stochastic theory. It is found that ageing and longevity in the locust are often equally well explained by stochastic or evolutionary theories and the results highlight the fact that the two classes of theories of ageing are not mutually exclusive. Both make an important contribution to understanding, but neither, yet, has all of the answers.

## 2.2 Introduction

Evolutionary explanations of ageing, such as 'mutation accumulation' (Medawar 1952; Williams, 1957) or 'disposable soma' theory (Kirkwood, 1977; Kirkwood and Holliday, 1979) are sometimes treated as being diametrically opposed to stochastic explanations, such as 'free radical' (Harman, 1956, 1972) or 'glycosylation' theories (Cerami, 1985), as noted by Holliday (1995). For example, the observation that the queens of advanced eusocial insects such as ants, bees and termites, which live in heavily guarded nests and experience low extrinsic mortality rates, live 100 times longer than individuals of solitary species, has been interpreted as strong evidence in favour of evolutionary theory (i.e., low rate of ageing is selected for when there is low extrinsic mortality rate) and against purely mechanistic explanations, i.e., molecular damage by trivial or random causes (Keller and Genoud, 1997). Other observations suggest, however, that it would be prudent not to dismiss the role of stochastic processes when evaluating the impact of the evolution of eusociality on insect lifespan. For example, egg-laying queens are often flightless, physically confined within the brood chamber of the nest, and may have a significantly lower metabolic rate than foraging individuals of the same age (Fahrenholz *et al.,* 1992). Experimental restriction of flight with resultant reduction in metabolic rate and mitochondrial oxidative damage has been found to dramatically increase life span in flies (Sohal, 1981b; Yan and Sohal, 2000).

There may indeed be experimental observations that current formulations of neither class of theories of ageing can easily address. Alternatively, evolutionary and stochastic theories may both validly explain ageing, but at different levels of biological organisation levels (Arking, 1998). The purpose of the present chapter is to

further explore and discuss the applicability and compatibility of the two classes of theories of ageing for understanding insect senescence, using a convenient and novel example, that of the African migratory locust, *Locusta migratoria* (Orthoptera: Acrididae). Manifestations of ageing at both population and cellular levels were observed. At population level, life history traits, such as mean age at maturity and lifespan, the form of the survival curve and sexual differences in these traits, are interpreted in terms of natural selection and evolutionary theory of ageing, based on what is known about locust biology and population ecology. At a cellular level, changes in the central nervous system are considered likely to play a pivotal role in death by senescence. The time-dependent *in vivo* deposition of lipofuscin pigment, the most consistent and phylogenetically constant morphological change of ageing (Porta, 2002a), was used as a biomarker of brain senescence. It has been established that lipofuscin consists of secondary lysosomes containing autophagocytosed but incompletely degraded damaged cellular structures including mitochondria (Sohal and Wolfe, 1986; Porta, 1991; Terman and Brunk, 1998). Lipofuscinogenesis is proportional to rate of oxidative metabolism (Sohal, 1981a). Intra- and interspecific comparative studies have shown significant inverse correlations between lipofuscin accumulation rate and lifespan (e.g. Sheehy *et al.,* 1995b, Sheehy, 2002b) and it has been proposed that the particular molecular events that may underlie lipofuscin formation are the primary events in senescence, i.e., damage associated with free radicals and glycosylation (Harman, 1956, 1972; Tappel, 1973; Cerami, 1985; Yin, 1992; Terman, 2001; Jolly *et al.,* 2002), although the role of lipoperoxidation remains unproven (Porta, 2002a, b). Therefore, the interpretation of the cytological results for locusts emphasizes stochastic theories of ageing.

It is important to note here that, while a large number of previous studies has reported putative age-associated increases in lipofuscin concentration in insects, the vast majority of these results (reviewed in Sheehy and Roberts, 1991) must now be discounted due to application of an invalid quantification method involving organic solvent extraction and spectrofluorimetry of soluble fluorophores (see Sheehy, 1996; 2002a; Porta, 2002a; Palmer *et al.,* 2002). In the remainder, confined to *Drosophila* and *Musca domestica*, data are very limited, being either qualitative (e.g., Hermann *et al.,* 1971; Miquel, 1971; Sohal and Allison, 1971; Sohal and Sharma, 1972; Miquel *et al.,* 1974; 1976; Sohal, 1973, or quantitative but tabulating only 2-4 age groups spanning less than half of the observed life span (Sohal and Donato, 1979; Sohal, 1981a; Sohal *et al.,* 1984). An additional novelty of the present study is that it is the first detailed quantitative description for an insect, of the pattern of *in situ* lipofuscin accumulation with age, over most of the observed maximum lifespan, using reliable quantification methodology.

## 2.3 Material and Methods

Populations of gregarious phase *L. migratoria* were established in two 40x40x40 cm cages from the egg pods of laboratory stock, at an initial density of approximately 70 hatchlings per cage. Cultures were maintained under a controlled light and temperature regime (12h L at 32° C: 12h D at 25° C). Locusts were fed daily with wheat seedlings and bran and sand-filled containers were provided for oviposition.

Locust development and onset of maturation, copulatory activity, oviposition and adult mortality were monitored (twice a day by visual inspection) in the first population until there were no survivors. In the second population, 6 individuals per age group (3 males and 3 females) were sampled at 3, 6, 10, 17 and 18 weeks after

hatching (total N = 30). The supra-oesophageal ganglia (brains) of these individuals were isolated and fixed in 10% formalin for subsequent neurolipofuscin analysis.

Neurolipofuscin was identified in 6-μm wax histological sections of the locust brain by its diagnostic yellow autofluorescence, irregular granular appearance, particle size composition (mainly from 1 to 5 μm in diameter), insolubility and age-related accumulation. Although deposits were widespread in the brain, they were most conspicuous in the pars intercerebralis (Fig. 2.1). Here, neurolipofuscin was quantified using confocal laser scanning microscopy and image analysis of histological sections as described in Sheehy (2002a) and the references cited therein (see Appendix). Briefly, tissue sections were excited at 514 nm and autofluorescent emission was detected at wavelengths >550 nm. Confocal images of the pars intercerebralis were acquired in a standard way: only sections containing both the central body and the pars intercerebralis were used, and the area within the pars intercerebralis that contained the densest aggregations of neurolipofuscin granules was captured in all available sections (n = 10 - 17) through the region of interest. Images were collected so as to minimize inclusion of indeterminate particles, particularly on section margins, and of non-lipofuscin containing areas such as fibre tracts, holes and at tissue edges. Badly damaged sections or samples were discarded. Images were Kalman-averaged during acquisition and subsequently processed to globally or locally contrast-enhance and smooth areas of interest. Brightly fluorescing neurolipofuscin granules were then discriminated from the less intense background by manual grey-scale thresholding. To avoid subjective bias, images were analyzed 'blindly' of specimen age or other identifier. The cross-sectional area fraction of neurolipofuscin granules within the usable tissue area of each image was calculated as a weighted geometric average (Sheehy *et al.*, 1998) of

replicate image measurements (see Appendix). Following convention, mean area fractions are reported as percentage volumes.

After confirming the independence of factors, age and sex, Kruskal-Wallis (Scheirer-Ray-Hare Extension) two-way ANOVA (Sokal and Rohlf, 1995, p. 446) was used to assess the effect of age and sex on neurolipofuscin concentration. The strength of association between locust survivorship and neurolipofuscin concentration was assessed with Spearman rank correlation.

## 2.4 Results

### 2.4.1 Maturation, reproduction and mortality

The first adult locusts appeared in culture during the fourth week after hatching, having passed through 5 nymphal stages. In monoculture, juvenile mortality was observed to be primarily due to cannibalism at moulting. There was no cannibalism amongst adults and from 4 to 9 weeks after hatching there was no adult mortality (Fig. 2.2). The first copulations were observed after 5 weeks in culture and the first ovipositions after 7 weeks. Shortly afterwards, at 9 weeks, survival began to decline sharply and this mortality was attributed exclusively to senescence. Maximum observed life spans for females and males were 14 and 23 weeks, respectively.

### 2.4.2 Neurolipofuscin accumulation

Two-way Kruskal-Wallis ANOVA confirmed a very highly significant association between age and neurolipofuscin concentration in the pars intercerebralis ($H = 20.77$, $\chi^2$ critical = 9.49, df = 4, $P = 0.0003$) (Fig. 2.2). Age specific individual variation in neurolipofuscin concentration increased markedly with age. No significant sexual

difference in neurolipofuscin concentration at age was resolved (H = 0.06, $\chi^2$ critical = 3.84, df = 1, $P$ = 0.81) and there was no significant interaction between the factors, age and sex (H = 0.27, $\chi^2$ critical = 9.49, df = 4, $P$ = 0.99).

During the early adult period, up to 10 weeks of age, when there was no adult mortality, neurolipofuscin concentration was negligible. Thereafter, it increased exponentially in close association with the decline in adult survivorship. The maximum neurolipofuscin accumulation rate achieved, between 17 and 18 weeks of age, was 0.34 % vol. per week. A highly significant inverse correlation between neurolipofuscin concentration and overall locust survival was observed ($r_s$ = -0.97, $P$ = 0.008).

## 2.5 Discussion

### 2.5.1 Short lifespan

The locust's natural lifespan of just a few weeks is short compared with that of many other metazoans (Comfort, 1964; Lamb, 1977). The habitat requirements for locusts are vegetation for food and shelter and bare ground for oviposition. As a consequence, they tend to be most abundant in areas with vegetation mosaics that are characterized by their environmental instability, such as periodic drought, which results in very high mortality (Farrow, 1975; Chapman, 1976; Joern and Gains, 1990). Under these harsh somewhat unpredictable seasonal environments, adult locusts are selected for early maturation (Joern and Gains, 1990). Individual fitness is enhanced by early laying and production of the maximum possible number of eggs. These eggs develop rapidly enough to reach the 'reproductive window' of the year and reduce the period of exposure to extrinsic sources of mortality (Joern and Gains,

1990). Assuming a finite pool of energetic resources, such that a trade-off is required between allocation to reproduction and allocation to somatic maintenance, the consequence of high fecundity from an early age in locusts is predicted to be a short lifespan (Stearns, 1976; Kirkwood, 1977; Calow, 1983; Reznick, 1985; Joern and Gains, 1990).

Alternatively, the occurrence of some potentially life-shortening stochastic processes can be identified for locusts. A neurolipofuscin accumulation rate of 0.34% in only one week (from 17 to 18 weeks) in the CNS of the locust is about 200 times faster than that reported for a very long-lived (70+ years) arthropod, the European lobster (Sheehy, 2002b). This suggests that locusts, like other short lived animals, have much less efficient defence, disposal or repair mechanisms for cellular damage (Holliday, 1995), and/or that they are exposed to much higher rates of such damage than long-lived animals. The latter possibility is consistent with two aspects of locust biology. First, locusts are indigenous to tropical latitudes and they actively seek and absorb heat through behavioural adaptations and dark colouration (Dempster, 1963, Uvarov, 1966). As well as accelerating metabolic rate, thermophilic physiological preferences would be expected to increase vulnerability to cell damage by protein denaturation and oxidative stress (Sohal and Weindruch, 1996; Beckman and Ames, 1998). The association between environmental temperature and levels of cellular damage is supported by inter- and intra-specific evidence of positive correlations between neurolipofuscin accumulation rate and temperature in arthropods (Sheehy, 2002b). Second, flying insects such as the locust are selected for higher metabolic rates, even when resting, compared with flightless species (Reinhold, 1999), and thus possibly there is a higher mitochondrial free radical production. This fact may

have contributed to the high neurolipofuscin accumulation rates and short lifespans observed even in the caged locusts.

### 2.5.2 Rapid post-maturation decline in survivorship

Locusts are opportunists. They are able to massively amplify reproductive output under favourable environmental conditions giving rise to the well-known swarms of 'biblical proportion' that may contain around $10^9$ individuals, at densities of 150 $m^{-2}$, weighing $1.5x10^6$ kg (Chapman, 1976; Joern and Gaines, 1990). In the experimental cultures, a rapid reduction in survivorship was observed shortly after the first copulations and ovipositions. This seems to be a perfect example of the 'disposable soma'; a life history strategy which trades-off apparently energetically very expensive reproduction against subsequent survival. An optimal energy investment in reproduction would divert resources from other vital homeostatic processes resulting in ageing and culminating in death (Kirkwood, 1977). Such processes could include, for example, the removal of the oxidized and/or cross-linked proteins of lipofuscin by proteolytic enzymes (Ivy *et al.,* 1984). The activity of such enzymes has been shown to decrease *in vitro* in senescing human fibroblasts (e.g. Sitte *et al.,* 2000). Likewise, the cost of reproduction produces increased susceptibility to oxidative stress (reduced resistance to Paraquat exposure) in *Drosophila* (Harshman and Haberer, 2000; Salmon *et al.,* 2001).

Partridge (1986) reviewed the range of potential costs that may be associated with reproduction in male and female insects. Experimental data have not always upheld expectation. For example, number of eggs per clutch and clutch frequency are not correlated with lifespan in some locusts (Dean, 1981; Joern and Gaines, 1990). Likewise, the production of sperm is unlikely to be a significant cost associated with

decreasing survivorship in male locusts after first mating because most of the spermatogenesis is completed before the adult instar (Uvarov, 1966). Evidence from comparative studies of parthenogenic and normal females of *Schistocerca gregaria* and *Melanoplus sanguinipes* suggests that the primary determinant of lifespan may be associated with sexual activity itself rather than egg production (Hamilton, 1955; Dean, 1981).

Rather than offering a contradictory alternative to evolutionary theories of ageing, stochastic theory may help explain more precisely what some of the costs of reproduction may be. In this regard, findings of this investigation showing the pattern of neurolipofuscin accumulation throughout life in locusts, stands apart from other comparable studies in insects. Previous studies, which measured trends in solvent extractable fluorescence, that is now considered to have been contamination by unrelated pteridine pigments rather than lipofuscin (Sheehy and Roberts, 1991), invariably found linear or near linear accumulation patterns throughout juvenile and/or adult development that bore no obvious association with reproductive onset. In contrast, the present results are the first to suggest a quantitative inverse correlation between cellular waste product accumulation and survivorship, with a delay in the appearance of significant neurolipofuscin deposits and mortalities until after the first reproductive activity. The form of the neurolipofuscin accumulation curve is unlikely to be due to selective survival of a more viable type of animal carrying more age pigment. Previous studies have shown that neurolipofuscin accumulation rate, as an index of physiological age, is inversely proportional to lifespan, both intra- and interspecifically (Sheehy *et al.*, 1995a; Sheehy, 2002b). Individuals with relatively greater lipofuscin loads would, therefore, be expected to die sooner rather than later. The form of neurolipofuscin accumulation in locusts is

important because it suggests that reduction in lifespan following reproduction is due, at least in part, to ageing, rather than to risk of death by some trivial cause (e.g., increased mortality risk during reproduction due to exposure to predators), a fundamental assumption underlying several key evolutionary studies, for which proof has been lacking (Partridge, 1986). Whether lipofuscin itself is toxic or otherwise deleterious to the cell, remains an open question (Katz, 2002).

What stochastic factors associated with reproduction could intensify the production of cellular damage? One strong, but largely unexplored, possibility is that such damage is a direct consequence of mate searching, aggressive competition for mates and/or intersexual conflict. Such behaviour in locusts involves kicking, jumping, flight and stridulation (Uvarov, 1966; Steedman, 1990). Ragland and Sohal (1973) and Sohal and Donato (1979) found that physical activity level in competing male houseflies could be manipulated by altering the sex ratio present in culture and that increased activity was correlated with increased metabolic rates, shorter lifespans and greater neurolipofuscin accumulation rates. These early studies have never been elaborated on.

Another interpretation of the rapid decline in survivorship and acceleration of neurolipofuscin accumulation at maturity in locusts is that it reflects the end of juvenile development and mitotic activity. Mitosis appears to be critical for the clearance of intracellular detritus including lipofuscin (Terman, 2001)

It is noteworthy that the patterns of survivorship and neurolipofuscin accumulation, relative to maturation, in some longer-lived aquatic arthropods are different to those of locusts. In freshwater crayfish and marine lobsters, changes in survivorship and neurolipofuscin accumulation rate are not obviously associated with the onset of

reproduction (Sheehy, 1990b; Belchier *et al.,* 1998). Unlike locusts, such species exhibit indeterminate growth and strong positive correlation between adult body size and reproductive performance that should select for improved survivorship after first reproduction (Williams, 1957; Sheehy *et al.,* 1999). Also, one could speculate that the relationship between reproductive activity and oxidative stress may be different in the aquatic environment, but to date there is no experimental evidence demonstrating it.

### 2.5.3 Sexual differences in survival

Male *L. migratoria* survive longer than females in the absence of extrinsic sources of mortality and the lower feeding rate of adult males compared to that of females has been suggested as tangible evidence of lower male reproductive costs (Strong, 1967). In the wild, the shorter lifespan of females can produce swarms with a significant higher abundance of males (Steedman, 1990). However, in various other locust species, females live longer than males (Uvarov, 1966). It is difficult, therefore, to apply the traditional evolutionary explanation that reproductive costs for males may be less because sperm are individually less costly to produce than eggs, which is likely to be an oversimplification (Partridge, 1986). Interestingly, despite closely mirroring the overall population survivorship pattern, no significant difference was found in neurolipofuscin accumulation rate between the sexes in *L. migratoria.* This appears to be generally true for Crustacea also (see references in Sheehy, 2002b). This suggests that sex-dependent lifespan determinants other than ageing (or at least brain ageing) may be operative. For example, in some Diptera non-senescent increases in female mortality rate following reproduction appear to be due to toxicity of male seminal fluid (Wolfner *et al.,* 1997) or to internal injury from the armoured male genitalia (Blanckenhorn *et al.,* 2002).

### 2.5.4 Late life mortality kinetics

Possibly due to the limited sample size used, the present locust survivorship curves showed no indication of levelling off that would suggest the type of late-life declining mortality rates that have been observed in medflies (Carey *et al.,* 1992) and various other organisms including man (Vaupel *et al.,* 1998). However, in view of the controversy surrounding this phenomenon, the notable paucity of available hypotheses to explain it (Partridge and Mangel, 1999), and the general theme of the present paper, some comments and proposals on this topic seem appropriate here.

According to evolutionary theory of ageing, specifically 'mutation accumulation' theory (Medawar, 1952; Williams, 1957) mortality rate should increase after reproduction because there is no selection against accumulating deleterious mutations that are only expressed after reproduction (Vaupel *et al.,* 1998). It has been suggested (Vaupel *et al.,* 1998; Partridge and Mangel, 1999; Luckinbill and Foley, 2000) that individual variability in frailty or rate of ageing is one possible explanation for the reduction of mortality rates. Selective mortality of the fastest ageing individuals leaves a subpopulation of old more slowly ageing individuals exhibiting a mortality rate slower than the original population average. There is much evidence from studies on neurolipofuscin accumulation in arthropods (Sheehy, 2002b), including the present results, which clearly demonstrates population heterogeneity in the rate of cellular ageing that is not simply attributable to measurement error. Donato *et al.* (1979a, b) first proposed selective mortality of fast-ageing individuals to explain late life declines in the trajectory of age-associated biological parameters such as age pigment accumulation in flies.

An alternative explanation for late life declines in population mortality rates, which arises from earlier observations of declining neurolipofuscin accumulation rates in old crustaceans, is that individual rate of ageing slows in old age (Sheehy, 1992; Sheehy *et al.,* 1995). There is mechanistic evidence to support this view. Kern and Wegener (1984) showed that brain metabolism in flies declines with senescence. Only in young brains is respiration coupled to ATP synthesis. This is accompanied by morphological change to the mitochondria including disarranged cristae and accumulation of lipofuscin-like residual material. De Grey (1997) has proposed that mitochondria with reduced respiratory function due to mutation or deletion affecting the respiratory chain suffer less frequent lysosomal degradation, because they inflict free radical damage more slowly on their own membranes. Once such a mutation occurs in the mitochondrion of a post-mitotic cell, the mitochondria carrying it will selectively populate the cell thereby destroying the cell's respiratory function. The accumulation of cells that have undergone this transition results in ageing at the organismal level.

It is possible that feedback inhibition of oxidative damage of the type proposed by De Grey (1997) may not only be an explanation for ageing in general, but be responsible for a decline in the rate of ageing of individuals of advanced age. Previously observed declines in population mortality and neurolipofuscin accumulation rates in older arthropods could be manifestations of this. Under this hypothesis, rate of ageing in the oldest of the old would slow at an ever-declining rate until some minimum threshold for viability was reached. Rather than 'catastrophe' (i.e. positive feed-back whereby errors exponentially generate more errors), this process would be 'slow death by a thousand cuts'. Such a scenario creates, in theory, an unexpected paradox whereby, for the very oldest individuals in a population, the instantaneous

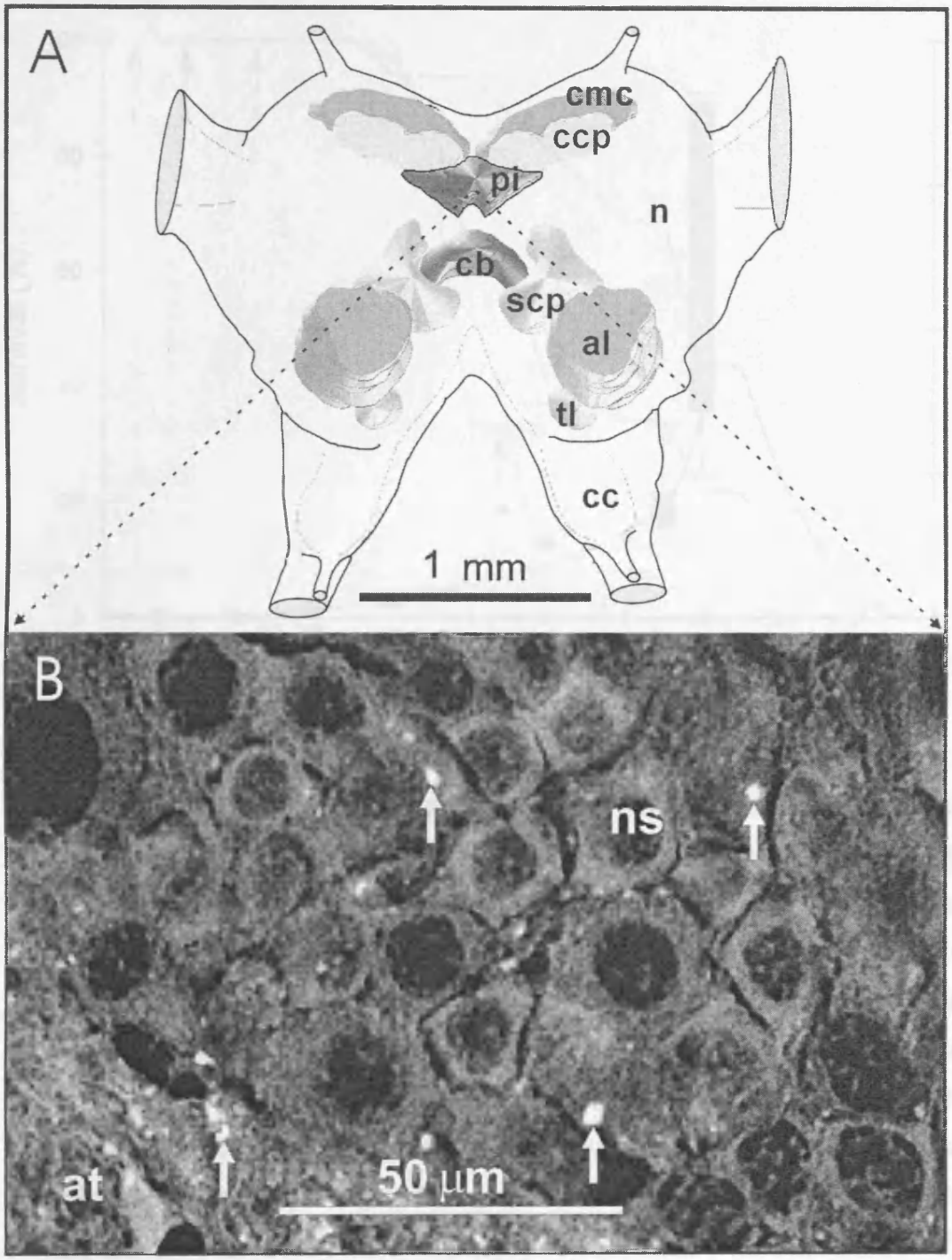
expectation of life increases with increasing age. If rate of ageing does decline at advanced age, it is difficult to see how available formulations of evolutionary theory of ageing could, on their own, explain this phenomenon.

## 2.6 Conclusion

Work on invertebrates continues to provide informative insights into the ageing process (Yeoman and Faragher, 2001). This study has demonstrated that observations on ageing and longevity in a chosen insect species are often equally well explained by both evolutionary and stochastic theories and it has highlighted the fact that the two classes of theories of ageing are not mutually exclusive. Because genes (which can be late acting) and their products, as well as interaction with the environment, determine a life history, it is logical that the features of that life history should be explicable in terms of both adaptive and stochastic processes. There seems little basis for rejection of one or other set of theories, both of which make an important contribution to the understanding of insect ageing, but neither of which yet has all of the answers.

Legend to figure 2.1 overleaf.

**Fig. 2.1**. (A) Diagrammatic representation of the organisation of the locust brain (supra-oesophageal ganglion), in dorsal view, with partial 3D serial section reconstruction of relevant internal structures: al, antennal lobe; cb, central body; cc, circumoesophageal commissure; ccp, calyx of corpora pedunculata; cmc, mass of cells filling the calyx; n, neuropile; pi, pars intercerebralis; scp, stalk of corpora pedunculata; tl, tritocerebral lobe. Nomenclature from Satija (1958) and Bullock and Horridge (1965). (B) Autofluorescent neurolipofuscin granules (some arrowed) in the pars intercerebralis (unstained 6-μm wax section); at, axon tracts; ns, neurosomata.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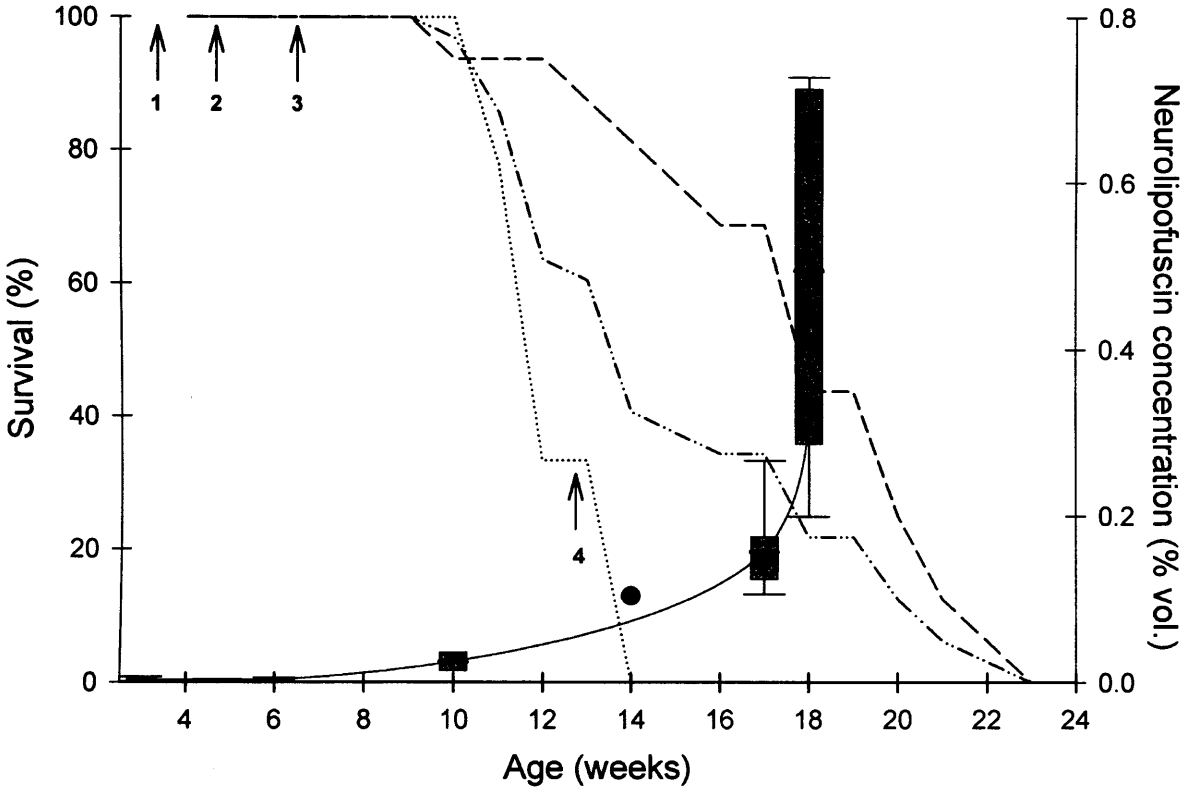



**Fig. 2.2.** Relationship between age, survivorship, reproduction and neurolipofuscin accumulation in the locust; 1, first adults appear; 2, first copulations; 3, first ovipositions; 4, end of ovipositions; dotted line, female survivorship; dashed line, male survivorship; dotted-dashed line, total survivorship; solid line and boxes, neurolipofuscin accumulation pattern; thick centre line on box, age-specific mean; thin centre line on box, age-specific median; grey-shaded area, 25$^{th}$ - 75$^{th}$ percentile; error bars, 5$^{th}$ and 95$^{th}$ percentiles. ●, represents the only sample available for a 14 wk old locust (male). Note that these two data sets came from different populations (cultures), but they were plotted together to facilitate the interpretation of the results.

# Chapter 3

## Unilateral eyestalk ablation reduces neurolipofuscin accumulation rate in the contra-lateral eyestalk of a crustacean, *Pacifastacus leniusculus*

## 3.1 Abstract

A principal requirement for use of the lipofuscin method as an ecological and fisheries research tool for crustacean age determination is a calibration, i.e., a quantitative relationship between tissue lipofuscin concentration and chronological age that is relevant to the natural population under investigation. Current approaches, involving known-age individuals or analysis of cohorts in neurolipofuscin concentration frequency distributions of the wild population, have advantages and disadvantages. A possible alternative involves initial biopsy of lipofuscin-loaded tissue from an eyestalk followed, after a time, by sampling of the second eyestalk, providing two successive lipofuscin measurements from the same individual (of unknown absolute age) and, thus, the neurolipofuscin accumulation rate in the intervening period. This approach was tested using known-age individuals of a convenient decapod model, the signal crayfish, *Pacifastacus leniusculus*. By comparison with untreated controls, no significant effect on body size or survival was detected a year after unilateral ablation. However, a 61% reduction in average neurolipofuscin accumulation rate in the remaining eyestalk occurred over the same period. It is hypothesized that this represents either reduced lipofuscinogenesis due to neurohormonal effects on oxidative catabolism or accelerated degradation due to damage-induced persuasive effects on cysteine protease activity in the central nervous system. In view of the current results it is unlikely that this approach can be employed as a means of calibrating neurolipofuscin accumulation rate.

## 3.2 Introduction

Lipofuscins, or age pigments, are yellow-autofluorescing lysosomal storage bodies that accumulate in non- or slowly dividing tissues during normal senescence (Katz and Robison, 2002). This age-related accumulation has been proposed (Ettershank, 1983) and applied (e.g. Sheehy and Bannister, 2002) as an ecological and fisheries research tool for age determination of crustaceans. Crustaceans cannot be aged reliably by traditional annulus or size-based methods due to periodic shedding of the integument and growth variability. Because lipofuscin deposition is a function of metabolic rate, its concentration reflects physiological age rather than chronological age (e.g. Sohal, 1981a; Sheehy *et al.*, 1994; Beckman and Ames, 1998). In order to employ it for chronological age determination in ectotherms, it is first necessary to calibrate lipofuscin concentration to the passage of calendar time under appropriate environmental conditions.

Three approaches for obtaining such calibrations have been proposed: i) captive rearing of individuals of known chronological age under comparable thermal conditions to those at the field location of interest (e.g. Sheehy *et al.*, 1994; Sheehy *et al.*, 1995b; Belchier *et al.*, 1998), ii) analysis of putative age cohorts in frequency histograms of *in situ* lipofuscin concentration in individuals from the wild population (e.g., Sheehy *et al.*, 1998; Bluhm and Brey, 2001; Bluhm *et al.*, 2001), and iii) recapture of tagged, known chronological age individuals that were released into the wild population as early juveniles (Sheehy *et al.*, 1996, 1999; Sheehy and Bannister, 2002). Each of these approaches has advantages and disadvantages.

Captive rearing at controlled temperatures can provide a very convenient way to obtain a lipofuscin vs. age relationship that is relevant to the wild population. However, it may be difficult to determine or reproduce the precise thermal regime occurring in nature, especially for migratory species or those living under heterogeneous thermal conditions. It will also be impossible to hold some medium to long-lived species in the laboratory in sufficient numbers over the entire lifespan, while other species cannot be maintained in the laboratory at all. There are also legitimate concerns about the effects of artificial diets and restriction of physical activity in captive individuals, as calorific intake, dietary antioxidant concentration and physical activity level are principal determinants of rate of physiological ageing and lipofuscin accumulation (e.g. Sohal, 1981b; Castro *et al.*, 2002). Studies comparing lipofuscin accumulation rates in artificially reared and wild-caught individuals from similar thermal regimes, but which have not controlled for dietary or physical activity differences, have generally not borne out these concerns (Sheehy *et al.*, 1994; Sheehy *et al.*, 1995b; Sheehy *et al.*, 1998). However, where the relevance of results from experimental laboratory treatments to wild populations remains unclear, it is prudent to use such laboratory calibrations as a guide only and in combination with other approaches.

Modal analysis of lipofuscin concentration frequency histograms is analogous to the conventional approach that employs body size data. Its principal advantages are that it is carried out on individuals that have aged under natural environmental conditions and modal progression should, therefore, give a reliable indication of average annual lipofuscin accumulation rate in the field. Because neurolipofuscin concentration-at-age is typically less variable

than size-at-age, modes in such histograms are likely to be better resolved than those based on body size for the same sample (Sheehy *et al.*, 1994). Disadvantages are that when lipofuscin accumulation rate is slow or very sensitive to interannual variations in temperature, or many age groups are present relative to sample size, resolution of discrete annual modes may be difficult or impossible. For example, these limitations apply to long-lived European lobster in U.K. waters (Sheehy and Shelton, 2001). The approach cannot directly give the accumulation pattern over the whole lifespan due to relative scarcity of older individuals in wild populations. Also, establishing the appropriate bin widths for the histogram may require some independent knowledge of the approximate annual lipofuscin accumulation rate, such as data obtained from another calibration approach or by comparative analysis of data for other species (Sheehy, 2002b). Modal analysis of lipofuscin concentration distributions should only be used where modes are obvious and well separated.

Measurement of lipofuscin concentration in tag-recaptured known-age animals released into the wild at an early age, prior to the onset of neurolipofuscin accumulation, provides the most robust way of obtaining a calibration under natural conditions. Microtagging does not appear to affect neurolipofuscin accumulation rate (Sheehy and Bannister, 2002). Disadvantages are that for many species, it may be logistically difficult or impossible to obtain a sufficient number of permanently tagged known-age early juveniles to ensure an adequate number of recaptures in commercial catches years later after losses due to natural mortality and dispersal. Recaptures may also be heavily biased toward age classes newly recruiting to

the fishery, with little chance of obtaining lipofuscin concentration-at-age data over the whole or even most of the natural lifespan.

A fourth possibility has been proposed (Sheehy *et al.*, 1996), but has not yet been tested. Measurement of certain age specific biological parameters, such as lipofuscin accumulation in the brain, normally involves the death of the individuals involved. However, the presence of neurolipofuscin in the paired eyestalks of decapod crustaceans creates the possibility of obtaining two successive eyestalk ganglion samples from the same individual, from which lipofuscin accumulation rate in the intervening period can be determined. Potential advantages of such an approach would be that it could be conducted by tag-release-recapture under field conditions without the need for large numbers of juveniles of known absolute chronological age. It could be used on individuals covering much of the size range present in the field to obtain a lipofuscin accumulation trajectory in a manner analogous to the conventional Ford-Walford plot for moult increment data. For this approach to be feasible, the effect of the initial eyestalk ablation on subsequent mortality and lipofuscin accumulation rate in the contralateral eye needs to be assessed. This chapter reports results of the first such assessment, using the signal crayfish, *Pacifastacus leniusculus*, as a convenient decapod model.

## 3.3 Materials and Methods

Two groups, each of 15 known-age *P. leniusculus* were reared from hatching and individually isolated to avoid cannibalism, in 30 l laboratory aquaria on a 12L: 12D room lighting cycle. Ambient water temperature averaged 15.8 °C over the duration of the study (range: 10.6 °C - 18.8 °C). Air stones fitted to an

under-gravel filter system provided oxygenation. Crayfish were fed *ad libitum* with dry fish food pellets (30% protein), frozen peas (6% protein) and cuttlefish bone as a source of calcium. The first (control) group of crayfish was reared for 2.3 yr, at which time all surviving individuals were sacrificed and both left and right eyestalks removed and fixed in 10% formalin for subsequent neurolipofuscin analysis. In the second (treatment) group, crayfish were reared until they had attained a suitable size for unilateral ablation (1.3 yr of age). Individuals were then immersed for up to 10 minutes in an anaesthetizing solution of 0.25% clove oil (McRae *et al.*, 1999) until tail-flick movements had ceased. Following ablation of the right eyestalk, crayfish were left to recover in fresh, vigorously aerated water, and then returned to aquaria for ongrowing. After a further 1 yr, ablated animals were sacrificed and the remaining left eyestalks removed and fixed for neurolipofuscin analysis. In the treatment group, the size range of females and males, at start of the experiment (1.3 yr), varied from 31 mm to 41 CL (5 ♀) and from 30 mm to 37 mm CL (10 ♂).

Lipofuscin was quantified using the histological, confocal microscopic and image analysis methods described in Sheehy (2002a), Sheehy *et al.* (1996) and the references cited therein. Confocal images (e.g., Fig. 3.1) of all (n = 15 - 20) sections of the region of the medulla terminalis cell cluster A containing fibre tract connections to the hemiellipsoid body were collected for each eyestalk. A geometric weighted average of the area fraction of lipofuscin in each image was used to quantify the lipofuscin concentration, in each eyestalk. By stereological convention this was expressed as a % vol.

## 3.4 Results

After 2.3 yr there were 10 survivors in the control group and 12 in the treatment group. This difference in survival was not statistically significant (Z test comparison of proportions with Yates' correction, $P = 0.68$). The average neurolipofuscin concentrations in 2.3 yr old left and right eyestalks from control individuals and left eyestalks from previously ablated individuals were 1.67 % vol., 1.76% vol. and 1.27% vol., respectively. There was no significant bilateral difference in mean eyestalk neurolipofuscin concentration in the control individuals (ANOVA, $P > 0.05$, Table 3.1, Fig. 3.2). However, the mean neurolipofuscin concentration of the remaining eyestalk of the ablated individuals was significantly different to and lower than that in the control eyestalks (ANOVA, $P = 0.0061$, Table 3.1, Fig. 3.2). Assuming a linear neurolipofuscin accumulation pattern from soon after hatching, as found by Belchier *et al.* (1998) for this species, average eyestalk neurolipofuscin accumulation rate in the unablated individuals was estimated to be 0.75 % vol./yr with an average neurolipofuscin concentration of 0.98 % vol. at 1.3 yr of age (ablation). The difference between this concentration and that observed in the remaining eyestalk of the ablated individuals after 2.3 yr (1.27% vol.) indicated an average neurolipofuscin accumulation rate of 0.29 % vol.$\cdot$yr$^{-1}$ following ablation, a 61% reduction from that observed in the control individuals. There was no significant sexual difference in neurolipofuscin concentration in the 2.3 yr old crayfish after taking into account the effect of ablation (two-way ANOVA, sex, $P = 0.66$; group, $P = 0.006$; interaction, $P = 0.89$). No significant differences in total carapace length due to either ablation or sex were found (number and mean CL: control group – 4 ♂, 46.5 mm; 6 ♀,

41.5 mm; ablated group – 8 ♂, 43.6 mm; 4 ♀, 45.5 mm. Two-way ANOVA: sex, $P = 0.38$; group, $P = 0.75$; interaction, $P = 0.06$).

## 3.5 Discussion

### 3.5.1 What do the results indicate about post-ablative lipofuscin accumulation?

There are several possible explanations for the reduced neurolipofuscin concentrations in the contralateral eyestalks of ablated crayfish observed in this study. Firstly, it is uncertain whether the effect represents an acute change in rate of neurolipofuscin accumulation immediately following ablation, followed by a return to normal, or whether a chronic or permanent effect on ablated individuals occurred. Secondly, the results may reflect either reduced neurolipofuscinogenesis or, alternatively, increased degradation or loss of the pigment. Consideration of the functional consequences of unilateral eyestalk ablation is useful for interpreting the findings.

### 3.5.2 Functional consequences of unilateral ablation

#### 3.5.2.1 Central nervous system (CNS) damage

There are many neural interconnections between the left and right sides of the crustacean CNS (Blaustein *et al.*, 1988; Sullivan and Beltz, 2001) and Hansen and Schmidt (2001), for example, have demonstrated that unilateral ablation of one antennule can impair development of the olfactory system bilaterally.

Studies on vertebrates have shown that following physical damage to the CNS there is upregulation of cysteine protease activity for dendritic

remodelling, which can reach far beyond the immediate site of injury (e.g. Banik *et al.* 1997; Ray *et al.*, 1999). These proteases include lysosomal acid cathepsins (Yashon *et al.*, 1975), which have also been implicated in the degradation of lipofuscin. For example, cathepsin B, is a component of neurolipofuscin granules (Jung *et al.*, 1999) and Ivy *et al.* (1984) found that application of a lysosomal cysteine protease inhibitor (leupeptin) accelerated the accumulation of lipofuscin-like material in the brain. One possible explanation for the present results for ablated crayfish is, therefore, a damage-induced general upregulation of proteolytic degradation of neurolipofuscin in the remaining eyestalk ganglia.

#### 3.5.2.2 Visual impairment

A second consequence of unilateral eyestalk ablation is partial loss of vision. Vision may be used in predator avoidance, agonistic encounters and detection of moving prey but does not appear to be of great importance compared with chemo- and mechanoreception in benthic decapods, which inhabit a characteristically low-light environment. In American lobsters, *Homarus americanus*, eyestalk loss occurs, either genetically or accidentally, in nature (Waddy *et al.*, 1995) and several of the above behaviours are barely affected by blindfolding (Atema and Voigt, 1995; Kaplan *et al.*, 1983; Snyder *et al.*, 1992). Chapman *et al.* (2000) showed that total and permanent blinding of Norway lobsters, *Nephrops norvegicus*, did not significantly affect their long-term survival, growth or reproduction. Therefore, direct effects from partial vision loss on known modulators of physiological ageing and lipofuscin

deposition, such as feeding rate (caloric intake) and movement (physical activity) (Sohal, 1981b) seem unlikely.

#### 3.5.2.3 Neurohormone imbalance

Thirdly, the eyestalks of decapods are the primary production and storage sites of several neurohormones including crustacean hyperglycaemic hormones (CHHs), moult inhibiting hormone (MIH) and mandibular organ inhibiting hormone (MOIH), which play major metabolic regulatory roles. These include control of blood glucose level (Chang and O'Connor, 1983; Chang *et al.*, 1993; Liu *et al.*, 1997), which is likely to be relevant for lipofuscin formation and/or turnover, as discussed below. There is evidence that lost hormone production in unilaterally ablated animals is not fully compensated for by increased output from the remaining eyestalk (Borst *et al.*, 1992; Jo *et al.*, 1999; Tsukimura and Borst, 1992). The main reported effect of bilateral ablation of eyestalks is the acceleration of moult cycle, due to the loss of MIH.

##### 3.5.2.3.1 Relationship between neurohormone and blood glucose levels

In crustaceans, CHH has an action analogous to glucagon in mammals. It regulates glucostasis, the maintenance of a continuous blood glucose supply, by modulating the conversion of glycogen stores in the hepatopancreas and abdominal muscle (Keller *et al.*, 1985; Santos and Keller, 1993a; Sedlmeier, 1982, 1985) (insulin-like peptides appear not to be involved (Sanders, 1983)). This function is particularly important for the oxidative metabolism of neural tissue, which does not have significant energy reserves of its own (Ames, 2000; Verri *et al.*, 2001). Because CHH is also a general stress hormone

(Chang *et al.*, 1999; Santos and Keller, 1993b), redistinction between immediate handling or trauma-related stress responses and longer term effects from hormonal imbalance due to ablation have not always been apparent in previously reported experimental results. However, there is evidence that reduced CHH levels in the haemolymph can lead to subsequent hypoglycaemia in crustaceans (Chang and O'Connor, 1983; Keller *et al.*, 1985; Sarojini *et al.*, 1995). For instance, McWhinnie and Saller (1960) found that normal blood glucose levels were reduced by 35% at 14 days after ablation.

Therefore, eyestalk ablation in crustaceans would appear to mimic the effect of dietary caloric restriction by reducing blood glucose levels, and the anti-ageing effect of caloric restriction is well established across many animal groups including crustaceans (Sohal and Weindruch, 1996). Several mechanistic explanations for this phenomenon have been proposed (reviewed by Masoro 2001) and those relevant to lipofuscin formation or turnover are as follows.

#### 3.5.2.3.2 Relationship between blood glucose, oxidative metabolism and lipofuscinogenesis.

The first possibility is that lowered blood glucose levels produce a short to long-term hypometabolic state in the organism. This appears to be the case in at least some of the mammals studied (Masoro, 2001). While the precise biochemical pathways responsible for lipofuscin generation are likely to be extremely complex and remain largely unknown, a positive correlation between rate of lipofuscin accumulation and the general rate of oxidative

metabolism is well established (Fleming *et al.*, 1992; Nakano and Goho, 1992; Nakano *et al.*, 1995; Sheehy *et al.*, 1995a; Sohal, 1981b; Sohal and Brunk, 1989; Tully *et al.*, 2000). More specifically, rate of damaging free radical production on the electron transport chain is significantly influenced by the availability of mitochondrial energy substrates, i.e. blood glucose (Dröge, 2002). However, whether lipofuscinogenesis actually involves the 2-3% of the oxygen consumed by aerobic cells that is diverted to the formation of reactive oxygen species (Sohal and Weindruch, 1996), as proposed under the widely quoted lipoperoxidative theory of lipofuscinogenesis (see Donato and Sohal, 1981), remains unproven (see Porta, 2002).

### 3.5.3 Relationship between blood glucose, proteolytic activity and lipofuscin turnover.

An alternative mechanism by which reduced blood glucose levels could affect lipofuscin accumulation rate may be by attenuating age-associated declines in cellular repair processes such as the proteolysis of damaged cellular components (Ward, 1988; Masoro, 2001) that seem to be responsible for lipofuscin degradation. Increased proteolytic activity has been associated with calorific restriction in rats (Lee *et al.*, 1999) and the results of Nan *et al.* (1995) and Chen and Chia (1995) for shrimps suggest that eyestalk ablation may produce a shift to protein-dominated metabolism.

## 3.6 Conclusion

This experiment has demonstrated that unilateral eyestalk ablation has an apparent anti-ageing effect on the crayfish CNS, leading to a significant

reduction in the accumulation rate of neurolipofuscin in the contra-lateral eyestalk of signal crayfish. Various explanations for this effect have been proposed including either reduced lipofuscinogenesis or an increased rate of lipofuscin degradation due to far reaching effects of eyestalk ablation on oxidative metabolism and/or proteolytic activity in the CNS. These explanations, although plausible, must remain hypothetical until further research is conducted. With respect to the principal objective of the study, it is recommended that the proposed ablation technique not be used for calibration of lipofuscin-based age determinations.

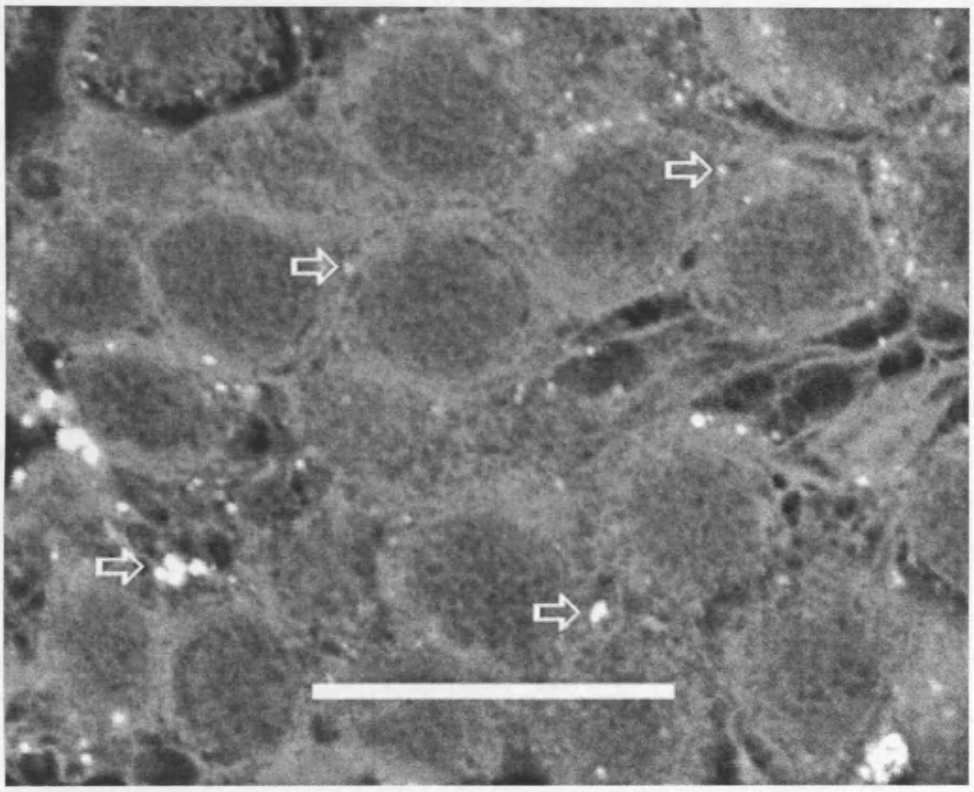


**Fig. 3.1.** Autofluorescent neurolipofuscin granules (some arrowed) in the cytoplasm of globuli neurones and glia in cell cluster A of the medulla terminalis of the eyestalk of the signal crayfish, *Pacifastacus leniusculus* (unstained 6-μm wax section). Scale bar = 50 μm.

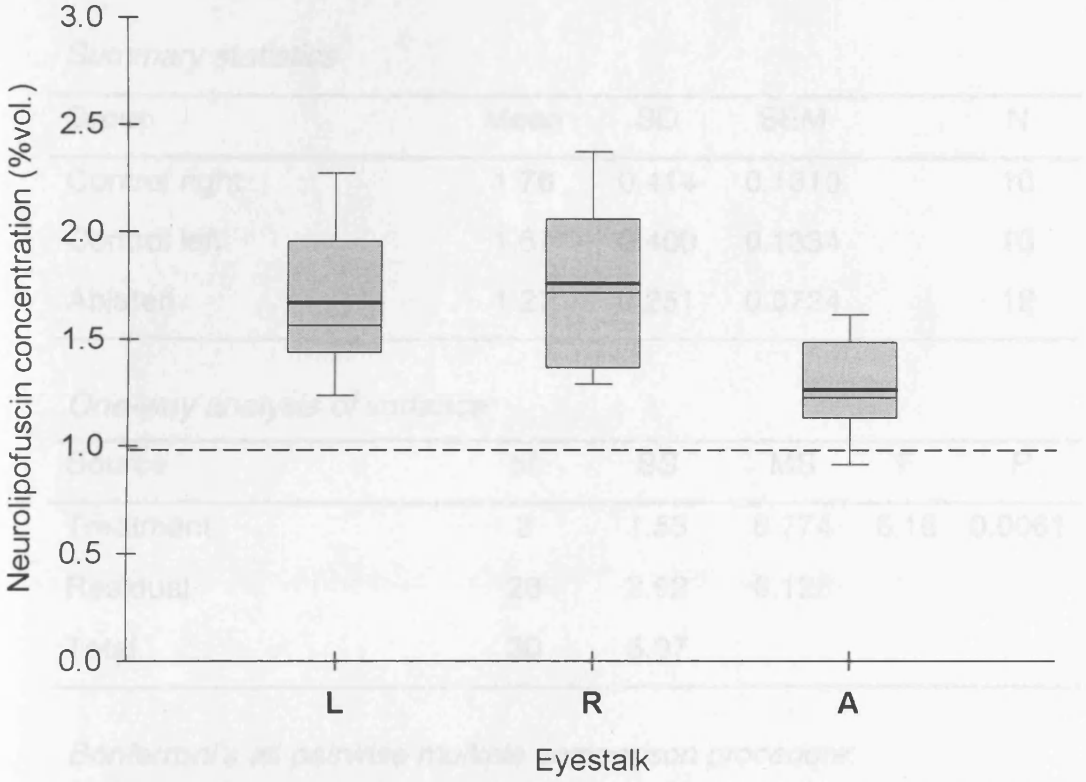



**Fig. 3.2.** Neurolipofuscin concentration in the eyestalks of 2.3 yr old signal crayfish. R, unablated control group, right eyestalk; L, unablated control group, left eyestalk; A, ablated at 1.3 yr group, remaining eyestalk 1 yr later. Thick centre line on boxes, mean; thin centre line on box, median; grey-shaded area, 25$^{th}$ - 75$^{th}$ percentile; error bars, 5$^{th}$ and 95$^{th}$ percentiles. Horizontal dashed line, mean neurolipofuscin concentration at the time of ablation.

**Table 3.1.** Results summary for ANOVA, testing for effects of unilateral eyestalk ablation at age 1.3 yr on neurolipofuscin accumulation rate in the remaining eyestalk over the following year. df, degrees of freedom; F, F-statistic; MS, mean square; N, number of samples; *P*, probability; SD, standard deviation; SEM, standard error of the mean; SS, sum of squares; t, t statistic.

*Summary statistics*

| Group | Mean | SD | SEM | N |
|---|---|---|---|---|
| Control right | 1.76 | 0.414 | 0.1310 | 10 |
| Control left | 1.67 | 0.400 | 0.1334 | 10 |
| Ablated | 1.27 | 0.251 | 0.0724 | 12 |

*One-way analysis of variance:*

| Source | df | SS | MS | F | *P* |
|---|---|---|---|---|---|
| Treatment | 2 | 1.55 | 0.774 | 6.16 | **0.0061** |
| Residual | 28 | 3.52 | 0.126 | | |
| Total | 30 | 5.07 | | | |

*Bonferroni's all pairwise multiple comparison procedure*:

| Comparison | Diff. of means | t | *P*<0.05 |
|---|---|---|---|
| Control right vs control left | 0.0897 | 0.551 | No |
| Control right vs ablated | 0.4955 | 3.265 | **Yes** |
| Control left vs ablated | 0.4058 | 2.597 | **Yes** |

**Note:** Kolmogorov Smirnov normality test with Lilliefors' correction: $P = 0.13$ (passed); Levene median equal variance test: $P = 0.37$ (passed); Statistically significant effects are shown in bold.

# Chapter 4

# Quantitative evidence of *in vivo* reversibility of natural lipofuscin accumulation

## 4.1 Abstract

Whether or not deposits of the so-called age pigment, lipofuscin, in important organs such as the brain, heart and eye, can be reversed once they have formed is one of the central questions in current age pigment research since, at least in some cases, the presence of large amounts of lipofuscin in cells may be deleterious and contribute to age-related disease. Due to popular dogma that lipofuscin accumulates with age because it is undegradable, the idea of lipofuscin turnover in cells has received little attention and, due to various methodological difficulties, the few previous studies in this area have yielded inconclusive results. Considerable research has now been conducted on neurolipofuscin in decapod crustaceans and this group offers a rare opportunity for longitudinal studies of lipofuscin accumulation because two successive neurolipofuscin determinations can be obtained from the same individual by eyestalk biopsy. The purpose of the present study was to assess lipofuscin accumulation rate in individuals of a wide range of physiological ages to determine if this rate is constant, as often assumed, or changes throughout life. Using validated lipofuscin quantification procedures, it was found, for the first time, that, once formed, lipofuscin can be lost from tissue in an *in vivo* system during the normal ageing process, and in the absence of pharmacological intervention. This is contrary to the popular dogma that lipofuscin accumulates because it is undegradable and/or not turned over. In neurons, the mechanism of loss probably involves exocytosis and blood transport. If ways can be found, such as antioxidant treatment, which sufficiently reduce or eliminate the reactions that cause lipofuscinogenesis, the results introduce the possibility that any existing deposits of this most universal manifestation of cellular ageing can be reversed.

## 4.2 Introduction

The accumulation of lipofuscins, so-called age pigments, in the secondary lysosomes of post-mitotic or slowly dividing tissues is a phylogenetically widespread hallmark of normal ageing (Porta, 2002a). With the exception of that in the human retinal pigment epithelium (RPE), the biochemistry of lipofuscinogenesis has largely defied characterization due to its probable complexity and the difficulty involved in isolating and dissolving the pigment in sufficient quantities. From a very large amount of mainly circumstantial evidence, it has been proposed that the particular molecular events that underlie lipofuscin formation are also the primary events in senescence, i.e., damage induced by free radicals and glycosylation (e.g. Harman, 1956; 1972; Tappel, 1973; Cerami, 1985; Yin, 1992; Terman, 2001; Jolly *et al.*, 2002). It has also been proposed that the presence of quantities of lipofuscin in cells may be deleterious in itself (Terman, 2001). Both of these propositions, although plausible, are not definitively proven (Porta, 2002b; Katz, 2002).

Experimental interventions that can slow the rate of lipofuscin accumulation, such as administration of dietary antioxidants, restriction of physical activity or caloric intake and lowering environmental temperature in poikilotherms, are well known (e.g. Sohal, 1981b). What is not clear, however, is whether lipofuscin, once formed, can be lost or removed from cells. Because of the possibility of a causal link between lipofuscin accumulation, normal ageing and age-related disease, this question is of considerable interest. The traditional view has been that lipofuscins are formed via conjugated Schiff base crosslinkage of primary amine-bearing biomolecules by bifunctional aldehydes produced as a result of free-radical damage to lipid membranes, and they are undegradable once formed (Tappel, 1973). This would explain both their

characteristic autofluorescence and time dependent accumulation. Several lines of experimental evidence, including analysis of fluorophores and lipoperoxidation modalities suggest, however, that this popular theory may not be correct (Porta, 2002a, b).

Alternatively, it is possible that lipofuscin in cells is continually turned-over and the characteristic age-related increase in its concentration results from an imbalance between the rate of formation and the rate of degradation or elimination of the pigment, with more being produced than is removed. Due to popular acceptance of lipoperoxidation theory and the non-degradability of lipofuscin, the idea that lipofuscin is turned over in cells has not received much attention and, as reviewed by Katz (2002), difficulties in interpreting the few previous experiments on this question do not permit a definitive conclusion to be drawn. Previous problems include (1) the use of invalid lipofuscin quantification techniques involving organic solvent extraction of fluorophores, (2) conflicting results on the effect of compounds such as centrophenoxine on lipofuscin accumulation and (3) the possibility that lipofuscin-like material that is rapidly deposited after protein malnutrition and treatment with the protease inhibitor, leupeptin, differs fundamentally from normal age pigment. Such material may be an 'immature', readily degradable form that becomes undegradable after prolonged lysosomal processing (Terman and Brunk, 1999). Lipofuscin formation and processing in *in vitro* cell cultures may not be the same as that *in vivo* (Katz *et al.*, 1999; Rubin, 2002). The pattern of lipofuscin accumulation may also differ between laboratory and wild populations (Sheehy, 2002b).

In order to avoid the previous problems, experiments on lipofuscin turnover need to (1) employ reliable lipofuscin quantification methodology, (2) avoid experimentally

accelerated deposition of residual material of uncertain relevance to true lipofuscin and (3) employ *in vivo*, rather than *in vitro*, methods. Previous studies on lipofuscin turnover have been confined to mammals. However, the study of invertebrates continues to provide informative insights on ageing; there are many similarities between senescence processes in the invertebrate and vertebrate central nervous system (CNS), (see for example Yeoman and Faragher, 2001). Many invertebrates age relatively rapidly and some experimental treatments or manipulations can be performed that are not practicable or feasible for vertebrates or humans. Over the past 15 years, Sheehy and co-workers have established decapod crustaceans as model organisms for the study of neurolipofuscin accumulation. Lipofuscin in the decapod CNS is like that described for other invertebrates and vertebrates in its morphology, fluorescence spectral properties, histochemistry, ultrastructure and age-related accumulation (Sheehy, 1989; Sheehy, 1990b; Sheehy and Wickins, 1994; Belchier, 1996; Sheehy *et al.*, 1996; Medina, 2000; Sheehy, 2002a; Bluhm *et al.*, 2002). The experimental system used in this chapter is based on measurement of *in situ* neurolipofuscin granules in the central nervous system using a fully validated morphometric quantitation procedure (Sheehy, 2002a). It also has the advantage of providing longitudinal information; two successive neurolipofuscin determinations can be obtained from the same individual by eyestalk biopsy (Fonseca *et al.*, in press).

In a recent study (Chapter 3) on freshwater crayfish, *Pacifastacus leniusculus*, of a single known chronological age (1.3 yr), it was found that unilateral removal of an eyestalk, including its internal optic ganglia, produced a significant anti-ageing effect in parts of the remaining CNS. Ablation reduced the average neurolipofuscin accumulation rate in the contralateral eyestalk over the subsequent year to 39% of

normal (Fonseca *et al.*, in press). Since this experimental system involves manipulation to reduce (rather than accelerate) neurolipofuscin accumulation during normal ageing, in an *in vivo* context, using proven neurolipofuscin quantification techniques, it fulfils all of the three criteria for valid study of lipofuscin turnover noted above. Possible causes for the observed reduction in neurolipofuscin accumulation rate following eyestalk removal in crayfish include induction of a hypometabolic state (reduced lipofuscinogenesis) by hormonal imbalance or a general upregulation of cysteine protease activity (increased lipofuscin degradation) induced by physical damage to the CNS (Fonseca *et al.*, in press). Since the efficiencies of systems that may be relevant to lipofuscin formation and processing, such as metabolic rate, the concentration of protective antioxidants, damage recycling by auto- and hetero-phagocytosis/proteolysis and waste disposal by exocytosis, are known to vary with age (e.g. Kern and Wegener, 1984; Berra *et al.*, 2002; Grune *et al.*, 2001), the purpose of the present study was to determine the degree of physiological age-dependence of the anti-ageing effect of eyestalk ablation in crayfish.

## 4.3 Material and Methods

### 4.3.1 Experimental treatment

Two hundred and seventy-nine (129 ♂ and 150 ♀) signal crayfish, *P. leniusculus*, of a wide range of sizes (27 mm to 76 mm total carapace length) and physiological ages (as indicated by their neurolipofuscin concentrations, see chapter 5) were obtained from an established experimental pond population (Ullesthorpe Garden Centre, Ullesthorpe, Leicestershire, U.K.; lat. 52° 29'.09N, long. 001° 16'.05W; mean water temperature, 10.3 °C). Each individual was immersed for up to 15 minutes in

an anaesthetizing solution of 0.25% clove oil (McRae *et al.*, 1999) until motionless. The right eyestalk was then excised with fine scissors and fixed in a saline solution containing 4% formaldehyde for subsequent neurolipofuscin analysis. An inert plastic individually numbered tag was inserted into the abdominal musculature. For post-operative recovery, crayfish were placed in tanks of fresh, vigorously aerated water for at least 24 hours and then returned to the experimental pond for ongrowing. After a further 1 yr, a total of 29 surviving experimental crayfish was recaptured, sacrificed and neurolipofuscin measurements were obtained from the remaining eyestalk. The approach used is analogous to 'anniversary' tag-recapture and the Ford-Walford plot commonly used in fisheries research for length data except that, here, neurolipofuscin concentrations at release and recapture are used.

### 4.3.2 Neurolipofuscin quantification

Neurolipofuscin was quantified using the histological, confocal microscopic and image analysis methods described in Sheehy *et al.* (1996), Sheehy (2002a) and the references cited therein. Briefly, tissue sections were excited at 514 nm and autofluorescent emission was detected at wavelengths >550 nm. Sections of the region of the medulla terminalis cell cluster A containing fibre tract connections to the hemiellipsoid body were retained for each eyestalk. The area containing the densest aggregations of neurolipofuscin granules was captured at high magnification in all available sections (n = 10 - 20) through the region of interest. Images were collected so as to minimize inclusion of indeterminate particles, particularly on section margins, and of non-lipofuscin containing areas such as fibre tracts, holes and at tissue edges. Images were Kalman-averaged during acquisition and subsequently processed to globally or locally contrast-enhance and smooth areas of interest.

Brightly fluorescing neurolipofuscin granules were then discriminated from the less intense background by manual grey-scale thresholding. To avoid subjective bias, images were analysed 'blindly' of specimen identity. A geometric weighted average (Sheehy *et al.*, 1998) of the area fraction of neurolipofuscin in each section was used to quantify the age pigment's concentration, in each eyestalk. By stereological convention, this is expressed as a percentage volume.

## 4.4 Results

Out of 279 crayfish released, 43 crayfish (39 ♀ and 4 ♂) were recaptured one year later. Due to missing tags in recaptured crayfish and to poor histological condition of samples obtained either at mark or recapture, the data set was reduced to 29 samples for neurolipofuscin analysis (26 ♀ and 3 ♂). Neurolipofuscin concentrations in the right eyestalks of the experimental *P. leniusculus* at the time of ablation ranged from 0.21% vol. to 2.27% vol. (average 0.98% vol.). There was a strong positive linear association between these concentrations and those of the corresponding left eyestalks, 1 yr after ablation ($r = 0.90$; $P < 0.0001$; intercept, $a = 0.51$ and slope, $b = 0.54$) (Fig. 4.1A). However, there was a strong inverse linear association between right eyestalk neurolipofuscin concentration at the time of ablation and the apparent neurolipofuscin accumulation rate in the remaining eyestalk over the following year ($r = 0.87$, $P < 0.0001$, $a = 0.51$, $b = -0.46$) (Fig. 4.1B). These rates ranged from 0.68% vol.$\cdot yr^{-1}$ to -0.66% vol.$\cdot yr^{-1}$ (average 0.06% vol.$\cdot yr^{-1}$). On average, the higher the neurolipofuscin content of the individual at the time of ablation, the lower the apparent accumulation rate in the remaining eyestalk. At initial neurolipofuscin concentrations below 1.1% vol. at the time of ablation, a net accumulation of pigment typically continued over the following year. Conversely, at initial neurolipofuscin

concentrations above 1.1% vol., a net loss of pigment typically occurred over the following year. Previous results for laboratory reared and ablated *P. leniusculus* (Fonseca *et al.*, in press) of a single known chronological age did not differ significantly from those of the present study ($P > 0.05$) (Fig. 4.1A and 4.1B).

## 4.5 Discussion

### 4.5.1 Do the results definitively demonstrate that neurolipofuscin accumulation is reversible?

As noted in the introduction, the experimental configuration used in this study circumvented inherent problems in previous studies due to flawed lipofuscin quantification methodology and the questionable relevance of *in vitro* studies and of experimental induction of lipofuscin-like material by protease inhibition. In order to properly interpret the results of the study, other issues require consideration as follows:

#### 4.5.1.1 Individuality of rate of ageing and selective mortality

Patterns of age-related change predicted from population averages may differ significantly from individual patterns of change. For example, Donato *et al.* (1979a, b) demonstrated that variation in the trajectory of the mean lipofuscin accumulation rate of a hypothetical population composed entirely of individuals exhibiting constant lipofuscin accumulation rates could be generated by selective mortality of faster ageing individuals and subpopulations. The longitudinal design of the present study, whereby lipofuscin accumulation rates were estimated from sequential samples from the same individuals, eliminated the possibility of this type of artefact.

#### 4.5.1.2 Bilateral variation in eyestalk neurolipofuscin concentrations

The use of sequential eyestalk sampling to determine the neurolipofuscin accumulation rate of the individual, and the resulting interpretation of Fig. 4.1B as indicating loss of neurolipofuscin from the eyestalk neural tissue, is underpinned by two assumptions. The first is that left and right eyestalks have similar neurolipofuscin concentrations at any particular instant in time and second, where any bilateral variation occurs, that neither left or right sides are biased toward having higher or lower neurolipofuscin concentrations at any age. The validity of these assumptions has been confirmed by previous studies. Belchier *et al.* (1998) found that in known-age *P. leniusculus* of a similar range of neurolipofuscin concentrations to those of the present study, chronological age explained 92.4% of the observed variation in lipofuscin concentration with only a small remaining variance component (7.6%) being attributable to a combination of bilateral variation and measurement error. Within the bilateral variance component, there was no bias toward left or right; Fonseca *et al.* (in press) found that the mean neurolipofuscin concentrations in the left and right eyestalks of 2.3 yr old *P. leniusculus* did not differ significantly ($t = 0.55$, $df = 17$, $P > 0.05$). In European lobster, *Homarus gammarus*, of a wide range of ages, the relationship between contemporary left and right neurolipofuscin concentrations was found to be highly significant ($r = 0.93$, $P < 0.0001$) with a slope ($b = 0.98$) not differing significantly from unity ($P > 0.05$) (Fig. 4.2) (Sheehy, 2002a). There is also no possibility of the introduction of lateral bias by the lipofuscin measurement procedure. The selection of eye samples for neurolipofuscin analysis was based solely on the availability of recaptured crayfish, without any prior knowledge of either the ablated or the recaptured eyes' neurolipofuscin concentrations and the neurolipofuscin analyses themselves were performed blindly

of specimen identity. It must be concluded therefore that the trends apparent in Fig. 4.1 represent a real physiological age-dependent decline in lipofuscin accumulation rate in the individual following eyestalk ablation, with lipofuscin loss at advanced age.

### 4.5.2 Could lipofuscin loss occur during normal ageing in crayfish?

While the present results demonstrate that neurolipofuscin can be lost from the CNS of individual ablated crayfish, they do not indicate whether age-dependent changes in neurolipofuscin accumulation rates or neurolipofuscin losses also occur in unablated crayfish. It is known from previous ablation studies on crayfish of a single known-age that unilateral ablation abnormally reduces lipofuscin accumulation rate in the remaining eyestalk (Fonseca *et al.*, in press). The present results indicate that, due to the age dependent decline in neurolipofuscin accumulation rate, there is, on average, no further neurolipofuscin accumulation in the remaining eyestalk of an ablated crayfish after a concentration of 1.1% vol. is reached. If, however, neurolipofuscin accumulation rate does naturally decline with advancing age, this could explain previous observations of curvilinear lipofuscin accumulation patterns in crayfish populations (Sheehy, 1992; Sheehy *et al.*, 1994, 1995a), otherwise attributed to age-specific selective mortality or metabolic depression after prolonged laboratory culture. The possibility of, potential mechanisms for, and significance of reduced rates of physiological ageing in the very oldest individuals of a population, have been discussed in Chapter 2.

An additional line of evidence for neurolipofuscin loss in the crustacean CNS is the recent work of Tully *et al.* (2000) on *H. gammarus*. In this study, known-age juvenile lobsters were reared in the laboratory under simulated seasonally oscillating water

temperatures. The pattern of neurolipofuscin accumulation with age in these lobsters showed clear seasonal oscillation with the largest increments in simulated summers. However, in some cases, the average neurolipofuscin concentration of lobsters sampled in simulated winter was lower than that of lobsters sampled in the preceding summer, implying a loss of neurolipofuscin in cool temperatures. Though indicative, this result is not definitive because longitudinal lipofuscin measurements were not involved and, as the authors note, the age-specific population averages obtained were associated with relatively large confidence intervals.

### 4.5.3 Mechanism of neurolipofuscin loss

It is difficult to draw definitive conclusions about the dynamics of lipofuscin granules from static micrographs. However, ultrastructural studies of neurolipofuscin in the Norway lobster, *Nephrops norvegicus* (Blackman, 1996) indicate that while some lipofuscin occurs in the neurosomata and neurites, the majority is extraneuronal and located in the glial cells (Fig. 4.3A). Within glia, large aggregations of irregular granules were found, suggesting both the coalescence of smaller individual granules and the clustering of larger ones. A number of instances of intra-neuronal lipofuscin granules in close proximity to the plasma membrane were found, some apparently in the process of exocytosis from the neurone and into the glial cell. The fact that there are concentrations of neurolipofuscin in the glial cells suggests that there might be a transfer from neurons to glial cells. The mechanism for this is obscure. However, in at least some cases there were distinctive vesicles immediately between the granule and the glial cell membranes (Fig 4.3B) or discontinuities in the membranes adjacent to the granules (Fig. 4.3C, D), suggesting that there may be some breakdown process occurring. These observations appear similar to those of Bluhm *et al.* (2002)

on two Antarctic caridean shrimps, *Notocrangon antarctica* and *Chorismus antarcticus*. In the brains of these species, mature lipofuscin granules "extended in many cases into the plasma membrane or even into the neighbouring cells". In a vertebrate, Srebro (1966), suggested lipofuscin turnover in the brains of frogs based on observations of apparent transfer of lipofuscin from neurons to glial cells.

As noted by Bluhm *et al.* (2002), observations of apparent exocytosis of lipofuscin in the CNS contradict recent assertions that lipofuscin cannot be exocytosed (Terman, 2001; Terman and Brunk, 1999). The bases for these assertions are results from *in vitro* cell culture, whereas the investigations on intact neural tissue highlight the key role of heterocellular interaction within an undisturbed environmental context for effective lipofuscin disposal. Phagocytes could play a comparable role to glia for collecting lipofuscin granules in other tissues, for example in the retinal pigment epithelium. Observations of exocytosis of lipofuscin from neurones have an important bearing on the question of the deleteriousness of lipofuscin itself. It is difficult to see how a mechanism for ejection of lipofuscin from neurones could have evolved unless the presence of the granules was detrimental to those cells. The formation and export of lipofuscin seems likely, therefore, to be an adaptation to reduce cellular impairment.

While it is likely that there is a mechanism for disposal of lipofuscin from crustacean neurones via exocytosis and uptake by glia, it is not clear by what mechanism the loss of neurolipofuscin from the eyestalks (apparent in Fig. 4.1) could occur. That would require its removal from the glial cells. Several previous reports in the literature, however, shed light on this question. For example, Cavanagh *et al.* (1993) observed lipofuscin-like dense bodies in glial processes near to blood vessels in rat

brain. Monteiro (1991) discussed the possibility of transit of lipofuscin-like dense bodies from neuroglia towards endothelia in the cortex of rats. Brizzee *et al.* (1974) found lipofuscin around microglial blood vessels in the brains of macaques. In brains of monkeys, El-Ghazzawi and Malaty (1975) reported strong evidence of lipofuscin removal from nerve cells to the capillary endothelium. Singh and Mukherjee (1972) observed aggregations of lipofuscin granules near to blood vessels in brains of birds. In the spinal ganglia of rays, Glees *et al.* (1986) found evidence of removal of lipofuscin from nerve cells to capillary wall. Previous studies on the CNS of vertebrates suggest, therefore, that the mechanism for lipofuscin loss may involve blood transport to another site, perhaps for degradation or excretion. Note that while the present study, and those cited above, provide strong evidence of lipofuscin mobility within, and loss from, particular tissues, they provide no evidence on the degradability of lipofuscin granules themselves.

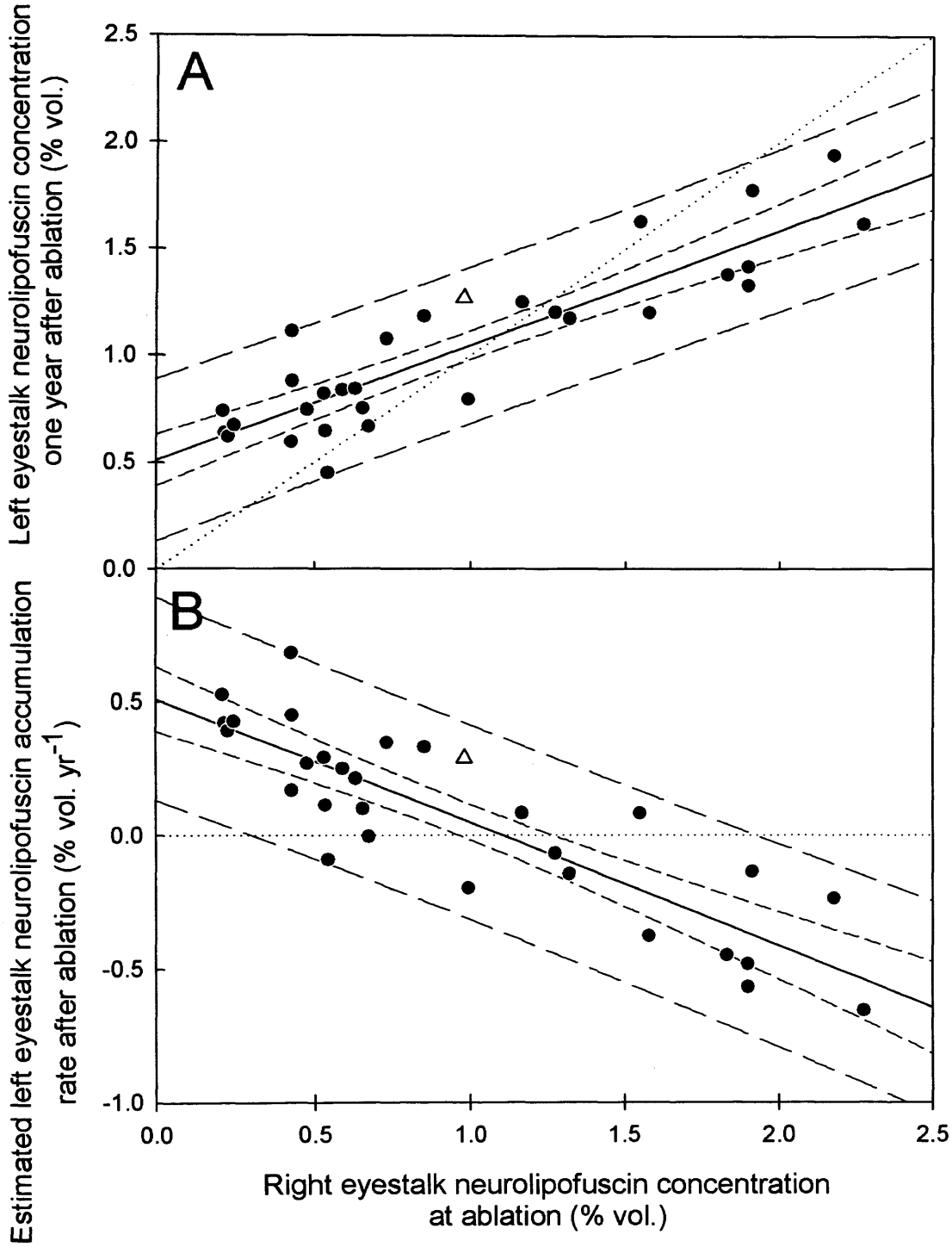
## 4.6 Conclusions

Using validated lipofuscin quantification procedures and longitudinal sampling methods, this study is the first to quantitatively demonstrate that, once formed, during the normal ageing process, lipofuscin can be lost from tissue in an *in vivo* system and in the absence of pharmacological intervention. This is contrary to the popular dogma that lipofuscin accumulates because it is undegradable and/or not turned over. In neurons, the mechanism probably involves exocytosis and blood transport. Whether similar turnover occurs in other tissues remains to be tested. If mechanisms can be found, such as antioxidant treatment, which sufficiently reduce or eliminate the reactions that cause lipofuscinogenesis, our results introduce the

possibility that any existing deposits of this most universal manifestation of cellular ageing can be reversed.

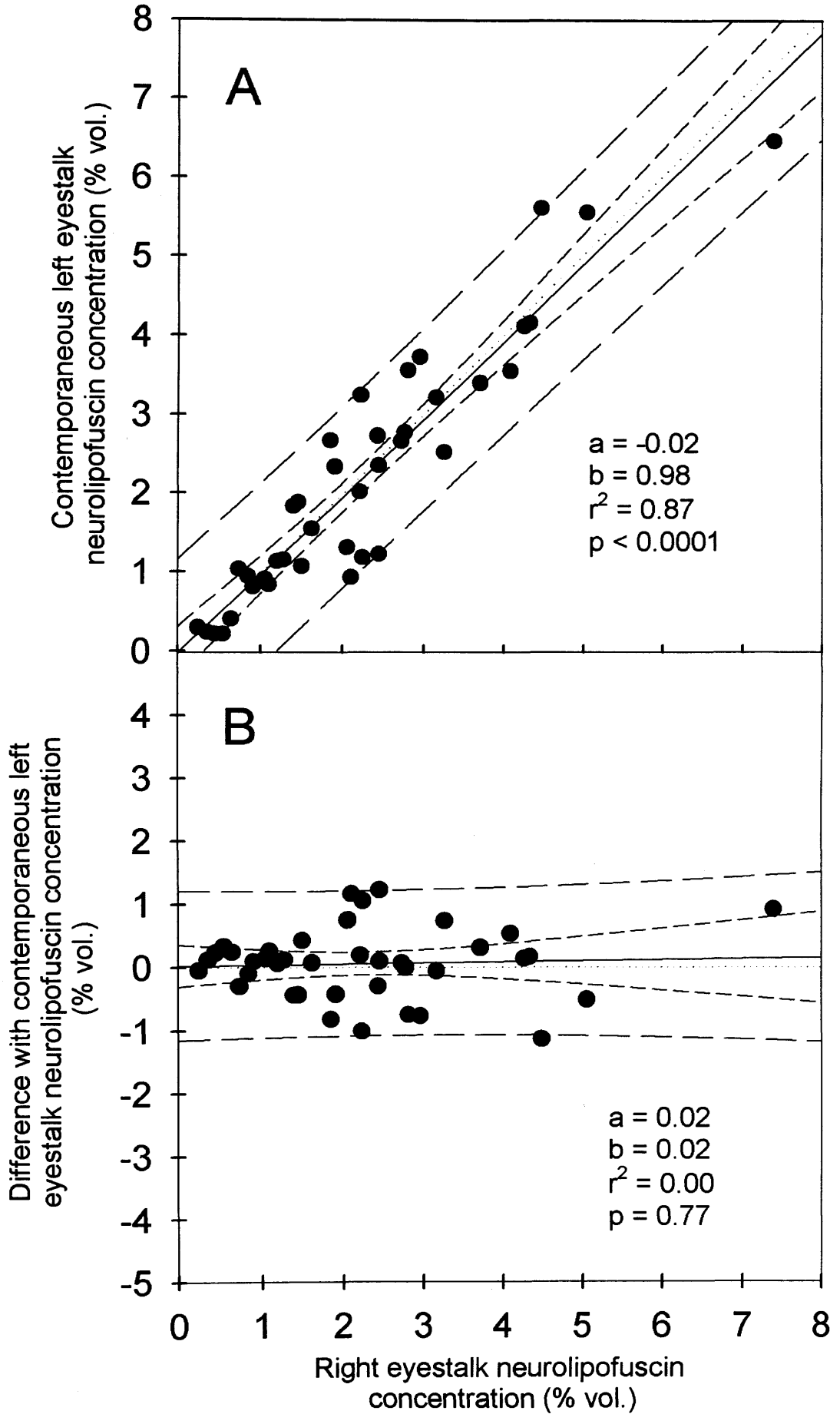
Legend to figure 4.1 overleaf.

**Fig. 4.1.** Relationships between neurolipofuscin concentration in cell cluster A of the right eyestalk terminal medulla of *Pacifastacus leniusculus* at the time of ablation, and (A) neurolipofuscin concentration in the left terminal medulla, one year after ablation, or (B) estimated average neurolipofuscin accumulation rate in the left terminal medulla in the year after ablation. Data from this study, filled circles. Data from Fonseca *et. al.* (in press), hollow triangle. Best-fitting linear regression, 95% confidence limits and prediction limits, solid, short-dashed and long-dashed lines, respectively. Level below which, on average, a net loss of neurolipofuscin from the remaining eyestalk occurred, dotted line.


A
B
Left eyestalk neurolipofuscin concentration one year after ablation (% vol.)
Estimated left eyestalk neurolipofuscin accumulation rate after ablation (% vol. yr-1)
Right eyestalk neurolipofuscin concentration at ablation (% vol.)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
0.0
0.5
1.0
1.5
2.0
2.5

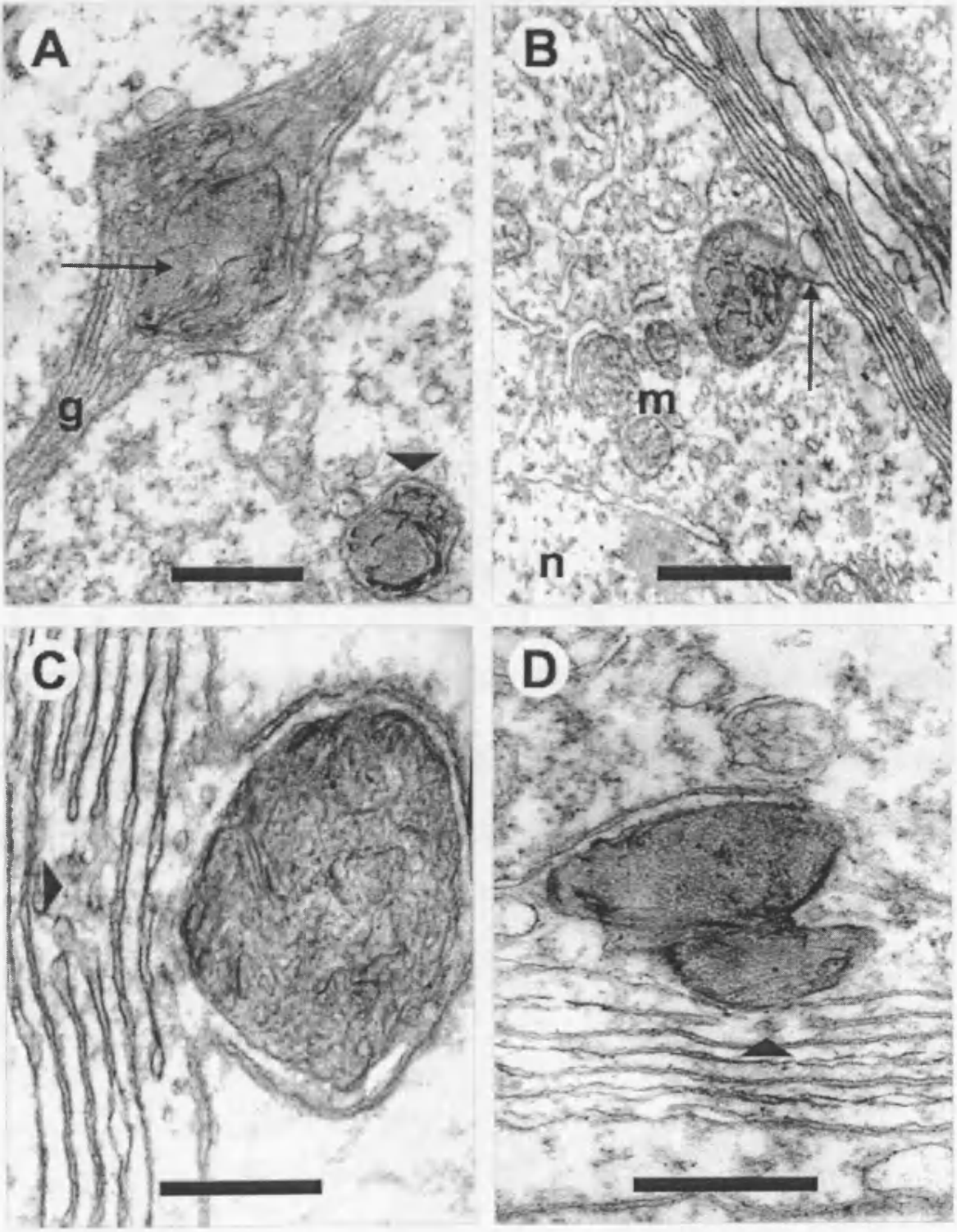
Legend to figure 4.2 overleaf.

**Fig. 4.2.** (A) Relationship between contemporaneous neurolipofuscin concentrations in the left and right eyestalks of *Homarus gammarus.* (B) Relationship between contemporaneous neurolipofuscin concentrations in the left eyestalk and difference between left and right eyestalks of *Homarus gammarus* (from Sheehy, 2002a, by permission). Best-fitting linear regression, 95% confidence limits and prediction limits, solid, short-dashed and long-dashed lines, respectively. In both graphs, point at which bilateral concentrations are identical, dotted line.


A
Contemporaneous left eyestalk neurolipofuscin concentration (% vol.)
a = -0.02
b = 0.98
r2 = 0.87
p < 0.0001
B
Difference with contemporaneous left eyestalk neurolipofuscin concentration (% vol.)
a = 0.02
b = 0.02
r2 = 0.00
p = 0.77
Right eyestalk neurolipofuscin concentration (% vol.)

Legend to figure 4.3 overleaf.

**Fig. 4.3.** Electron micrographs (from Blackman, 1996, by permission) showing neurolipofuscin in cell cluster A of the terminal medulla of *Nephrops norvegicus.* (A) Neurones are surrounded by multiple glial membranes, **g**. Neurolipofuscin granules are found both within the neurosomata (arrowhead) and the glial cell cytoplasm (arrow), the latter being more common. Both granules shown exhibit a fine granular matrix and irregular membranous inclusions. Scale bar = 2 μm. (B) A neurolipofuscin granule associated with the outer membrane of a nerve cell and apparently in the process of exocytosis. There is a distinctive vesicle (arrow) between the granule and the glial membrane. Also shown are part of a cell nucleus, **n**, and a group of 3 mitochondria, **m**. Scale bar = 2 μm. (C) and (D) Neurolipofuscin granules adjacent to nerve cell membranes and apparently undergoing exocytosis. In both cases, the glial membranes show gaps (arrowheads) adjacent to the neurolipofuscin granules. These gaps may be associated with the mechanism for externalising the granules. The irregularly shaped granule in (D) may have formed by fusion of smaller granules. Scale bars = 0.5 μm and 1 μm, respectively.


A
g
B
m
n
C
D

# Chapter 5

## Comparison of growth trajectory estimates for a decapod crustacean from size-frequency analysis, anniversary tag-recapture, laboratory rearing and neurolipofuscin analysis.

## 5.1 Abstract

The generalized growth curve describes the average growth pattern of individuals in a population. It is fundamental to understanding the population dynamics of any species. This investigation sought to compare three conventional methods for growth curve estimation: size-frequency analysis, anniversary tag-recapture and laboratory rearing with a novel approach employing neurolipofuscin-based age determination, using a single experimental population of signal crayfish, *Pacifastacus leniusculus*. Generally good agreement in average growth rate estimates for younger individuals was found for all methods. However, the study highlighted some known, but often overlooked, problems with the conventional approaches. Results from laboratory rearing were limited to younger age groups and were less representative of growth in the field. Size frequency analysis failed to discriminate older age groups, thereby overestimating growth rate and seriously underestimating longevity. Anniversary tag recapture results were subject to catchability biases and/or low survivorship problems. On the other hand the neurolipofuscin-based approach was uniquely able to resolve otherwise hidden older age groups. It was the only method to successfully detect sexual differences in the growth rate of mature individuals. It is the only method that can produce length-at-age data for old individuals of moderate- to long-lived crustaceans. Thus, it is the only method to directly provide longevity and natural mortality rate estimates. Such estimates form the basis of many further analyses such as production and productivity, lifetime reproductive potential, the spawner-recruit relationship and stock assessments to name but a few.

## 5.2 Introduction

The generalized growth curve describes the average growth pattern of individuals in a population. It is fundamental to understanding the population dynamics of any species. For example, the von Bertalanffy growth function (VBGF), in combination with the size-body mass relationship and the size-frequency distribution gives a mechanism by which productivity can be estimated (Crisp, 1984). Growth curves can also give clues to interpret environmental changes affecting growth rates such as variations in temperature and food availability (Devries and Frie, 1996).

In crustaceans, four traditional methods have been employed to estimate growth functions in the absence of age determination methods comparable to annulus reading in the permanent hard parts of other invertebrates and fish (Hartnoll, 1982). The most accurate method employs size-at-age information derived from marked known-age early juveniles released into the wild for subsequent recapture. This type of tag-recapture study may last for 10 years or more with the most valuable information coming from the oldest individuals. However, tagging can be time consuming and expensive and the method requires a supply of known age juveniles. In fisheries research, recapture normally requires effort levels available only through the commercial harvest and even then returns can be few or non-existent. Large numbers of releases are usually required to get sufficient recaptures. Data coverage for the entire growth curve of long-lived species is not possible.

Size-at-age data for generalized growth modelling can also be obtained by captive rearing of known age individuals. However, this method is generally discounted because laboratory environments are seldom able to mimic the wild due to unnatural photoperiods, temperature cycles, feeding regimes or space limitations. Mesocosms,

ocean pens and outside enclosures provide more natural conditions but still cannot emulate natural feeding regimes and population densities.

The second approach is to synthesize information on moult increments and intermoult period. Provided that moult increment and intermoult period are known for the entire size range, a complete growth curve can be estimated. This information can be obtained by laboratory observation; however, the same limitations regarding representativeness apply as mentioned above. An alternative is to use tag-recapture, under the (questionable) assumption that tagging itself does not affect moulting. Under this approach, size at release is plotted against size increment at recapture and any clusters in the size increments for any particular release size are assumed to reflect 1, 2 or more moults while at liberty (e.g., Hepper, 1967). As well as providing an estimate of size-specific moult frequency in the population, the total size increment divided by the estimated number of moults while at liberty gives mean size-specific moult increment. It may however, be difficult to detect such clustering in tag-recapture based moult increment data. A viable alternative was suggested by (Shelton and Chapman, 1987, 1995) who developed "living-tags". These represent small pieces of living dermal tissue and exoskeleton, which, when implanted into the host, continue to grow and moult in synchrony with the host and accumulate unshed exoskeletal layers, thereby recording the moult history. Due to size-specific variation in gear-selectivity and catchability, and low survival rate of individuals in the fishery, it may once again be difficult to get data over the entire size range. An important problem is the overestimation of average moult frequency in the population due to the tendency of recently moulted individuals to be hungrier and more likely to be caught in baited traps than non-moulting individuals (Cobb, 1995). Radiometric

measurement of carapace age is another approach to estimating the moult frequency of larger, longer-lived species (Le Foll *et al.*, 1989).

The third approach is known as 'anniversary' tag-recapture. The method normally involves analysis of recaptures occurring approx. 1 year after release, i.e., 1 year older (hence 'anniversary' method). A plot of size of fish at release vs. size at recapture (Ford Walford plot) yields a line from which K (curvature parameter, often referred to as growth rate) and $L_\infty$ (asymptotic size), parameters of the von Bertalanffy growth function, can be derived (Sparre and Venema, 1998). Advantages are that the method does not require a supply of known age individuals. The entire growth curve can be estimated from a small amount of recapture data. However the same limitations on the representativeness of recaptures apply as noted above. The result is that growth rate estimates for upper and lower parts of the population size range can be less reliable.

The last method is the analysis of size frequency data. This very widely used method is based on the fact that many species, particularly those in temperate seas, have a confined spawning season, normally during the warmer part of the year. The effect is that the population age distribution is not even but clustered into discrete groups or cohorts of similar birth date but separated by one-year intervals representing the time delay between each annual spawning season. This structure can often be detected by looking at the size frequency distribution of the population, which shows a number of peaks corresponding to annual cohorts. Peak positions give an indication of mean size-at-age from which a growth curve can be derived. An extension of this approach is modal progression analysis, whereby individual peaks are traced in time series of size frequency histograms, again to give estimates of mean size-at-age. Limitations

include the fact that wide growth rate variation within and between age groups and deceleration of growth with age means that older age groups, in particular, are often difficult to distinguish. The technique is useful only for fast-growing fish and assumes no size selective movement of fish into or out of the population.

The lipofuscin method is a more recent approach for obtaining growth curves (Belchier *et al.*, 1998; Bluhm and Brey, 2000; Bluhm *et al.*, 2001; Sheehy *et al.*, 1995; Sheehy *et al.*, 1998; Sheehy *et al.*, 1999). This method involves measurement of the age pigment in selected regions of neural tissue in known-age animals (Belchier *et al.*, 1998; Sheehy *et al.*, 1995b, 1998, 1999) in order to establish a relationship between neurolipofuscin loading and chronological age. Alternatively, successful attempts to obtain calibrations by resolving age groups in populations using modal analysis of neurolipofuscin frequency histograms have been carried out by Sheehy *et al.* (1998), Bluhm and Brey (2001) and Bluhm *et al.* (2001). The estimated age-length data obtained is then used for growth curve fitting.

Comparison of growth curves using neurolipofuscin information has been carried out previously. Belchier *et al.* (1998) utilised a calibration derived from cultured known-age signal crayfish, *Pacifastacus leniusculus,* to age wild crayfish of unknown age. Size-at-age data from both cultured and wild groups produced very similar VBGFs despite slightly different temperature experiences in the laboratory and field. However, Sheehy *et al.* (1998) demonstrated sex differences in growth curves of juvenile *Panulirus cygnus* that were not observed in growth curves estimated by length-based methods. Sheehy *et al.* (1999) showed a slower growth trajectory of young lobsters *Homarus gammarus* when age was estimated by the neurolipofuscin approach. A finding shared by all these studies was that both size-at-known age or

size-at estimated-age was very scattered, i.e., much size variation could not be explained by age or age determination error.

However, the lipofuscin technique for growth estimation is still relatively new and requires ongoing evaluation. The present investigation is the first comprehensive comparison of growth curves obtained by various conventional methods and the lipofuscin method using a single experimental population of signal crayfish *Pacifastacus leniusculus*. Detailed conventional growth data for this species is also available in the literature for comparison and the age-related accumulation of lipofuscin in its neural tissue has already been described (Belchier *et al.*, 1998).

## 5.3 Material and Methods

### 5.3.1 Experimental crayfish

The experimental *P. leniusculus* population used in this study was located in a pond at the Ullesthorpe Garden Centre (Ullesthorpe, Leicestershire, U.K.). *P. leniusculus* was introduced into this pond in 1988 and is the only crayfish species known to occur there. The pond is isolated from other waterways, has a surface area of approximately 500 $m^2$ and a usual maximum depth of 1.5 m. Mean water temperature during the year of the study was 10.3 °C (monthly mean range: 3.8 °C – 16.4 °C). Size frequency analysis, anniversary tag-recapture and neurolipofuscin concentration-frequency analysis were conducted on the pond crayfish to estimate their growth as described below.

In addition, gravid female broodstock were obtained from the pond and held in laboratory tanks until hatching of eggs in order to obtain known-age individuals for ongrowing. Thirty of these hatchlings were maintained in the laboratory for 2.3 yr and

their growth monitored. They were reared individually, to avoid cannibalism, in 30 l laboratory aquaria on a 12L: 12D room lighting cycle at ambient water temperatures averaging 15.8°C (range: 10.6 °C - 18.8 °C). They were fed *ad libitum* with dry fish food pellets and frozen peas, and cuttlefish bone was given as a source of calcium.

### 5.3.2 Growth curve estimation

#### 5.3.2.1 Size-frequency analysis

Pond population sampling for size-frequency analysis was carried out in summer 1999 (from 23/07 to 09/08). Cylindrical 'Swedish traps' (40 mm mesh size) with inverted cones at both ends were used to catch crayfish. A few fine mesh traps (4 mm mesh size) were also utilised to catch smaller animals. Tinned cat food was used as bait. Traps were inspected the day after setting. Trapped crayfish were sexed and measured for rostral carapace length (CL, mm) defined as the distance between the posterior median border of the carapace and the tip of the rostrum.

Carapace length-frequency histograms (CLFH) were constructed using 2 mm bin widths in order to be comparable with previous size-frequency analyses for a nearby population of this species (Great River Ouse, Buckingham) the cohort configurations of which had been independently corroborated by tag-recapture and synthesis of moult increment and frequency data (Guan and Wiles, 1999). Based on the need to maximize sample size and previous findings (Guan and Wiles, 1999) that growth rate did not differ significantly between males and females over the size range predominating in the present CLFH, data for both sexes was pooled. One hundred and eighty-three crayfish were used for this analysis (see section 5.3.2.4). In order to estimate the modal composition of the CLFH, automated peak-fitting software was used (Peak Fit v. 4, SPSS Inc.). As is common practice, the frequency distribution

was first smoothed. A light Fast Fourier Transformation (FFT) (8.1%) was used. This filter level was chosen so as to avoid over-smoothing and information loss while at the same time removing what appeared to be noise spikes in the distribution, in order to produce visually plausible normal components comparable to those reported in Guan and Wiles (1999). Gaussian peaks were then fitted to the smoothed distribution by an automated least squares fitting procedure. This routine first fitted peaks to local maxima. Based on analysis of the residuals deriving from this fit, any additional 'hidden' normal components were then fitted. No baseline was subtracted prior to fitting and peak widths (i.e., standard deviations) were allowed to vary during the fitting. To assess the separation of neighbouring peaks, separation indices (SI) (Sparre and Venema, 1998) were calculated. Putative modes with separation indices <2 are usually considered dubious. The first CL mode was designated age 1+ on the basis that young of the year (0+) individuals are too small to be trapped. Further, because sampling was performed approximately 3 months after the main hatching period for signal crayfish (May) (Lowery, 1988; Holdich *et al.*, 1995), an additional 0.25 yr had to be added to the mean age of each cohort. The VBGF was estimated from these data by minimization of least squares linear fitting of the relationship between the designated age of each cohort and its average CL.

#### 5.3.2.2 Anniversary tag-recapture

Two hundred and ninety-six crayfish captured from the pond were tagged, and CL and sex were recorded. Tagging involved immersion for up to 15 minutes in an anaesthetizing solution of 0.25% clove oil (McRae *et al.*, 1999) until tail-flick movements had ceased. The right eyestalk was then excised with fine scissors and fixed in a saline solution containing 4% formaldehyde for subsequent neurolipofuscin

analysis. Thereafter, a 2 mm long numbered yellow plastic tag (a cut-down tubular fish tag supplied by D. Bova, Marine Laboratory, Aberdeen) was inserted in the abdomen, using a pair of forceps, through a small cut on the ventral surface of the second abdominal segment. After tagging, crayfish were kept in the laboratory for 24 hours for observation of survival. From those, 279 survived (129 ♂, mean CL = 50.1 mm, range from 27 mm to 76 mm CL; 150 ♀, mean CL = 51.9 mm, range from 27 mm to 73 mm CL), which were released back into the pond in July-August 1999.

An intensive trapping recapture programme was carried out after approximately one year. Recaptured individuals were first measured (as above) and then immobilized by immersion for up to 10 minutes in ice water. They were then sacrificed for tag removal and identification. CL at recapture was recorded. This tag-recapture programme is exactly the same reported in Chapter 4.

Fabens' (1965) method was used to estimate the von Bertalanffy growth parameters ($L_\infty$ and K) from the tag-recapture data. In this model, the dependent variable is size increment ($\Delta$CL, mm) and the independent variables are size at marking (CL mark) and time elapsed between mark and recapture ($\Delta$T, yr). Fabens' method was used because it does not assume equal time intervals between release and recapture, unlike the Ford-Walford plot. A non-linear estimation was performed by minimization of least squares.

#### 5.3.2.3 Laboratory rearing

Direct length-at-age data was obtained from the laboratory rearing study for ages 0, 1.3 and 2.3 yr. These data were fitted by VBGF using minimization of least squares.

#### 5.3.2.4 Neurolipofuscin analysis

Sheehy *et al.* (1998), Bluhm and Brey (2001) and Bluhm *et al.* (2001) showed that in comparably configured histograms, better resolution of age groups, particularly older ones, can be achieved by analysing neurolipofuscin concentration frequencies rather than standard size frequencies. This is because individual variation in lipofuscin concentration at age is typically less than that for size, and growth rate tends to decline more sharply with age than does lipofuscin accumulation rate. In the present study, neurolipofuscin concentrations were measured in cell cluster A of the eyestalk terminal medulla of 183 pond-caught crayfish using histological procedures as described in the previous chapters. This sample size resulted from sub-sampling the total catch obtained from 23/07/1999 to 09/08/1999, using a design length-stratified with proportional allocation. This procedure was carried out because quantifying neurolipofuscin in all crayfish (circa 300) would be extremely time demanding. A neurolipofuscin concentration frequency histogram (NCFH) was constructed so as to give a similar number of bins in the range containing the bulk of the data to that in the above mentioned size-frequency histogram. This was achieved by selecting a class interval of 0.05 % vol. neurolipofuscin concentration. The frequency distribution was then subjected to precisely the same FFT level and Gaussian component fitting routine as described above for the size-frequency data. After resolving putative annual age groups in the NCFH, the mean concentration of each age group was then linearly regressed against its assigned age to provide a calibration by which the chronological age of pond-caught crayfish could be estimated.

Size-at-estimated age data was fitted by VBGF using minimization of *least squares*. In order to deal with the uneven spread of these data, particularly the lack of large old

males and the smallest individuals of both sexes, model fitting was performed using the following approach: $L_\infty$ was first obtained using an unconstrained curve fit. The $L_\infty$ so obtained and a $t_o$ of –0.15 (which forced the curve to have a realistic origin of 4 mm CL on hatching) were then constrained to these values in a least squares curve fit to find K alone.

## 5.4 Results

Only 34 crayfish were recaptured from the pond after 1 year (although the total recapture was 43 crayfish, in nine animals the tag was not found) (Fig. 5.1). The average CL of recaptured females was 58.7 mm (range from 51 to 72 mm), while the average CL of recaptured males was 62 mm (range from 58 to 66 mm). As only 4 of all recaptures were males, Fabens' VBGF parameter estimates for females only are provided (Table 5.1).

Similarly configured CL and neurolipofuscin concentration frequency distributions for the same 183 pond caught-individuals are shown together in Fig. 5.2 for comparison. The left panels, a and c, show for CL and neurolipofuscin, respectively, raw frequency distributions with lightly smoothed FFTs superimposed on them. The right panels, b and d show the smoothed frequency distributions with estimated individual Gaussian components and the summed frequency of these estimated modes superimposed. Seven putative annual age groups (arrowed), 1+ – 7+ years were identified in the CLFH and eleven (1+ – 11+ years) in the neurolipofuscin concentration frequency histogram NCFH). In both cases, goodness-of-fit between the smoothed frequency distribution and the sum of its estimated Gaussian components was very highly significant, although neurolipofuscin distribution showed a higher fitting standard error. Overlap of putative age groups was higher in the

CLFH than in the NCFH, particularly for the oldest age groups. Nevertheless, separation indices were higher than 2 for all ages groups, in both frequency histograms.

From the modal configuration of the NCFH described above, the slope of the linear regression of the modal mean neurolipofuscin concentrations on designated modal age (Fig. 5.3) indicated an annual neurolipofuscin accumulation rate of 0.20% (95% confidence limits: 0.18 - 0.21) and an intercept (-0.05 %) which was not significantly different from zero (95% confidence limits: -0.14 – 0.05), i.e., neurolipofuscin concentration was nil at the time of hatching.

All CL-at-age data and generalized growth curve segments generated for the available data ranges by the various approaches employed in the study are shown together in Fig. 5.4 and Fig. 5.5, respectively. Growth parameter estimates are shown in Table 5.1. Results from Guan and Wiles (1999) are also plotted for comparison. For the laboratory-reared crayfish, there were no significant differences in mean CL between sexes (♀, CL = 35.2 mm; ♂, CL = 34.3 mm) at 1.3 yr ($t = 0.49 < t_{0.05(2),6} = 2.45$, $P = 0.64$) or at 2.3 yr of age (♂, CL =: 44.6 mm; ♀, CL = 43.1 mm) ($t = 0.86 < t_{0.05(2),20} = 2.08$, $P = 0.40$). Average growth rate for laboratory-reared crayfish was higher than that predicted by alternative methods for the pond crayfish. Individual variation in CL-at-known age appeared slightly less than that for CL-at-estimated age in the pond reared individuals. For ages less than approximately 3.5 years (50 mm average CL) there was very little difference in the growth trajectory by any of the methods employed or due to sex. Mean age at reaching 50 mm CL differed by a maximum of ± 0.5 yr depending on either the sex of the crayfish or the method employed.

At older ages and sizes, the CL-at-neurolipofuscin estimated age data produced the most complete coverage and indicated that mean male and female growth trajectories diverged. The largest females spanned a much greater range of ages, lived considerably longer and reached asymptotic length much earlier than males. The largest crayfish caught was a male (CL = 76 mm, W = 96.02 g) that had an estimated age of 4.8 yr. The smallest (and estimated youngest) crayfish caught was a 1 yr old female (CL = 27 mm, W = 4.96 g), while the oldest crayfish was an estimated 12.6 yr old female (CL = 72 mm, W = 85 g).

The female growth curve estimated by anniversary tag recapture was extremely similar over the entire size range to that derived for females by neurolipofuscin analysis, as evidenced by comparison of the VBGF parameters in Table 5.1. Sexual differences in the adult growth trajectory were not resolved by modal analysis of CLFHs by Guan and Wiles (1999) probably because no cohorts of females older than 3+ yr were detectable in their CLFHs. The upper growth trajectory suggested by the pooled-sex CLFH in the present study was much steeper than that estimated by any of the other methods.

## 5.5 Discussion

The only length-at-known age data employed in this study were those derived from laboratory rearing and the short growth curve segment so derived is therefore the only one that is known with complete certainty. Consequently, it is possible that the elevated trajectory of this curve compared with all of the other estimates obtained is due to measurement errors in the latter. Secondly, it is important to establish the theoretical difference between the approaches. The length-at-age approach assumes that changes in length are explained by the age of the animal, while mark-recapture

methods, for example, assume changes in length are explicable in terms of the length of the animal at release. The difference between length-at-age and mark-recapture approaches was emphasised by Francis (1988) who suggested that growth parameters estimated by mark-recapture and length-at-age were not comparable, although this interpretation has been considered misleading by James (1991). Thirdly, since the growth results from the pond population that were collected by various methods tend to corroborate each other, it may simply be that, individual rearing (with *ad libitum* feeding) and/or the greater average water temperature experienced by laboratory-held crayfish (15.8°C) compared to those in the Ullesthorpe pond (10.3°C) expedited their growth.

The generally good agreement between the growth curves for juvenile pond crayfish suggests (but does not prove) the reliability of each of the methods employed for estimating growth in young age groups. Size-frequency analysis works well when annual growth is fastest, i.e., in the young, because modal separation is maximal and the young age groups' modes are therefore easiest to discern reliably. Anniversary tag recapture also tends to work best for younger individuals because in the fisheries context, size-specific variation in gear selectivity and catchability, and low survival probability can mean that large old individuals are normally harder to obtain, release and recapture in sufficient numbers to adequately define the upper end of the growth curve. The present anniversary tag-recapture experiment produced a fairly typical recapture rate of 10% of tags but, as it involved an unexploited population, was unusual in that reasonably good coverage of the very largest female sizes was obtained. The largest female sampled in the present study (73 mm CL) represented 98% of the estimated asymptotic length.

One of the assumptions of mark-recapture experiments is that tagging does not affect the growth rate. The tags that were implanted in this study were small and were unlikely to have affected moulting or caused significant internal damage. Moreover, one might expect that unilateral eyestalk ablation would affect the growth rate (e.g., reducing moult frequency). However, data obtained in the laboratory (Chapter 3) did not demonstrate a significant effect of unilateral ablation on the growth rate of crayfish (see also Meade and Watts, 2001). The close correspondence between the tag-recapture based female growth curve and those derived from other methods in this study also supports this assumption. It seems that no size-related mortality operated in females in the wild. For example, the proportion of females recaptured which had, at the time of release, CL ≤ 52 mm (i.e., mean of released females) (53%) is very similar to the overall proportion of females below mean CL at the time of release (56%). However, catchability biases and/or low survivorship problems of another type were, nevertheless, encountered. Specifically, it remains unclear why almost all tag recaptures were female.

Regarding size-frequency analysis of the pond crayfish sample, very familiar difficulties were experienced in selecting the appropriate bin width to avoid excessive 'noise' on the one hand and signal loss on the other hand. Several bin widths were assessed and the data for all 297 trapped crayfish were compared with those for the subset of 183 individuals for which a neurolipofuscin determination was also made. In the end, the most objective approach was considered to be adoption of the same histogram configuration as Guan and Wiles (1999). That study used a much larger sample size and modal age designations were independently corroborated by other methods. In order to compensate for increased noise associated with the lower sample sizes used in comparison with Guan and Wiles (1999), light Fourier

smoothing of the frequency distribution was found to be extremely useful. Again, potential subjectivity in the selection of smoothing level was minimized by using Guan and Wiles data as a reference to assess the likely modal configuration in the Ullesthorpe data. Application of a running average across bins is an alternative size-frequency smoothing approach that is sometimes used in the literature. The subsequent process of locating normal components (putative annual cohorts) within the distribution was objectified by application of a totally automated peak fitting routine (Peak Fit v. 4).

No matter how good the software package, it will be impossible to distinguish annual cohorts in size frequency distributions if there is, on average, little or no growth between years. The fact that the size-frequency data of Guan and Wiles (1999) contains no female modes older than 4+ yr and that the growth curve constructed by modal analysis of the pooled CLFH in the present study has a relatively low curvature parameter value, K, and an unusually high estimated asymptote, $L_\infty$ (Table 5.1), strongly suggests that some older age groups amongst the largest sizes of crayfish have been missed in the CLFH due to slow growth and modal overlap. Indeed, this is an acknowledged common problem with modal analysis of size frequency data and appears to be confirmed by examination of the length-at-neurolipofuscin estimated age data for females in Fig 5.4. This shows that females up to about 12 years of age (probably some of the initial implants to the pond in 1988) are likely to be present in the sample but are indistinguishable by size from individuals less than half that age. This conclusion is also supported by the female growth curve generated by anniversary tag-recapture. It should be emphasised that size variation at age is a normal phenomenon, particularly in crustaceans, and that the common tendency of plotting mean length at age is misleading because it suggests that length is a

measure of age (Hilborn and Walters, 1992). The use of a growth curve fitting mean length-at-age data (an approach used in length-based analysis), causes relevant information about size variability at age to be lost. Biased parameter estimation may result (Isaac, 1990; Rosenberg and Beddington, 1987).

The theoretical basis for use of neurolipofuscin concentration histograms to detect age groups is precisely the same as that for standard size frequency analysis. Seasonal synchronous spawning followed by annual growth (or lipofuscin accumulation) will lead to clusters of ages in the population which can be detected as modes in frequency histograms. The advantage of using neurolipofuscin is that it normally gives better-resolved modes than size data because there is less individual variation and reduction in increment with advancing age. The clarity of putative annual cohorts in the present neurolipofuscin data for *P. leniusculus* was not as good as that previously reported for the western rock lobster, *Panulirus cygnus* (Sheehy *et al.*, 1998), the Antarctic shrimp, *Notocrangon antarcticus*, (Bluhm and Brey, 2001) or the Antarctic amphipod, *Waldeckia obesa* (Bluhm *et al.*, 2001), however it was better than that reported for the European lobster, *Homarus gammarus* (Sheehy and Shelton, 2001). The reason for such differences is not known, although in the case of very long-lived species such as *H. gammarus*, neurolipofuscin accumulation is likely to be too slow relative to individual variation at an annual level for separate modes to be discriminated. Following protocols previously employed for *P. cygnus*, the possibility for subjectivity when configuring the neurolipofuscin concentration frequency histogram and smoothing the data was eliminated by 1) using precisely the same individuals, 2) using precisely the same number of bins to carry the main bulk of the observations, and 3) applying precisely the same FFT smoothing level as applied to the size frequency data. When applied to *P. leniusculus* this approach

yielded several additional older age groups, particularly of females, that were not present in the CLFH. The unique ability of the neurolipofuscin-based technique to extract hidden older age groups has been noted previously for other crustaceans (Bluhm and Brey, 2001; Bluhm *et al.*, 2001; Sheehy *et al.*, 1998). In the present study, it was the only method to successfully detect average growth differences in mature male and female *P. leniusculus.* It is the only method that can produce length-at-age data for old individuals of moderate- to long-lived crustaceans. Thus, it is the only method to directly provide longevity and natural mortality rate estimates. For such species, objective methods for determining the precision of such neurolipofuscin-based age estimates are now available (Sheehy and Bannister, 2002).

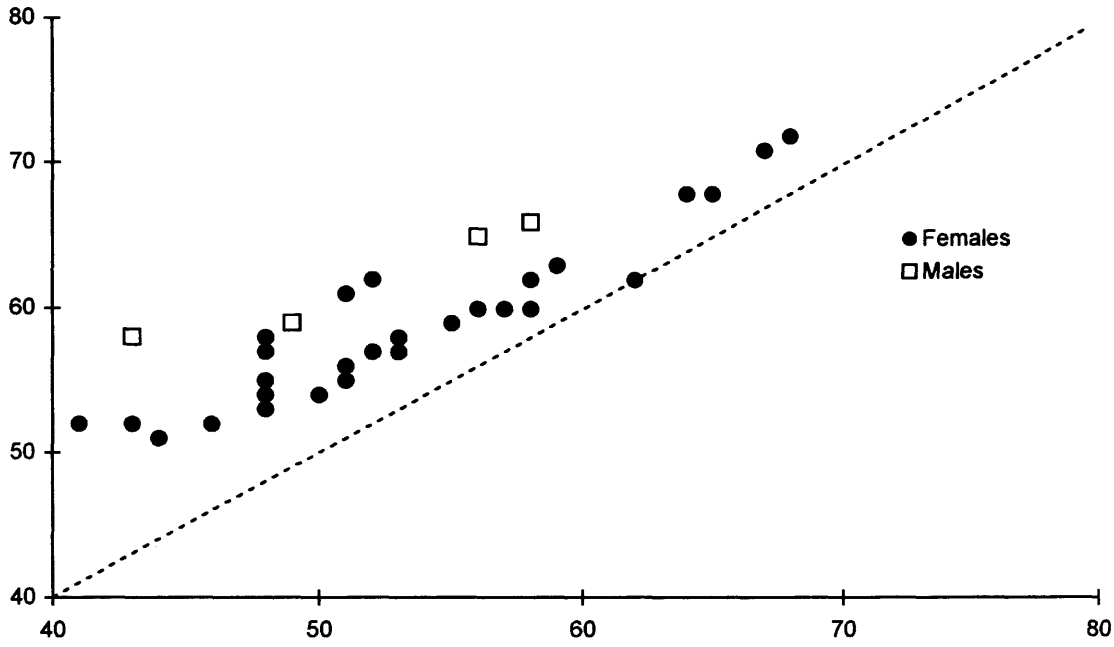
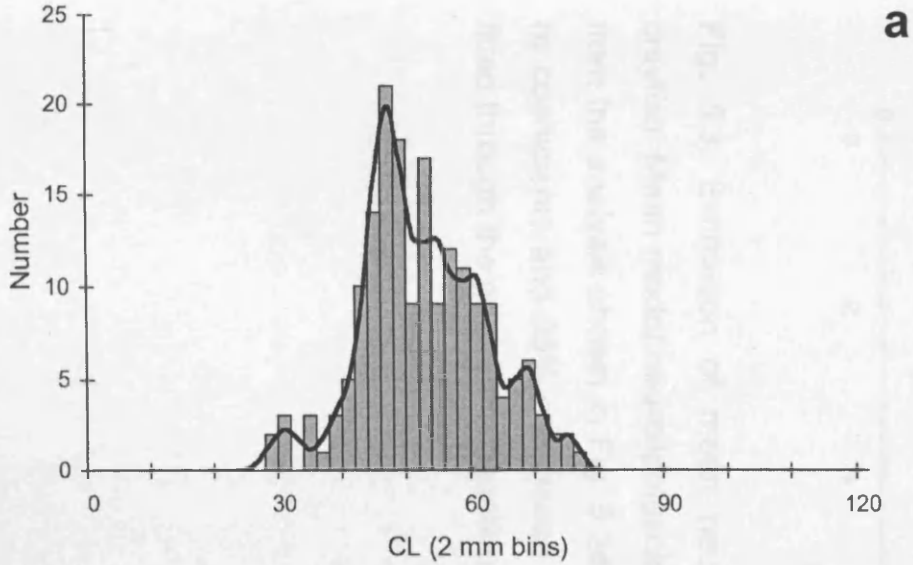
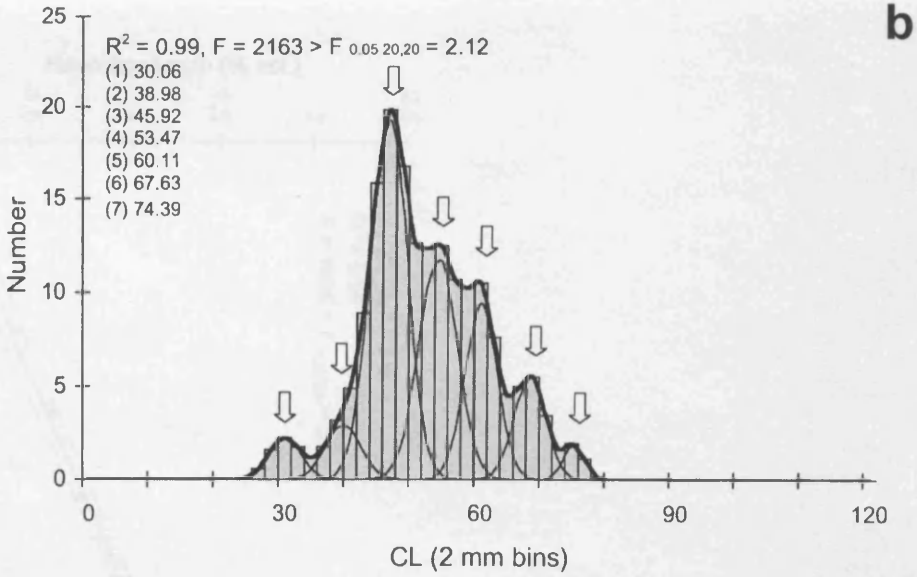


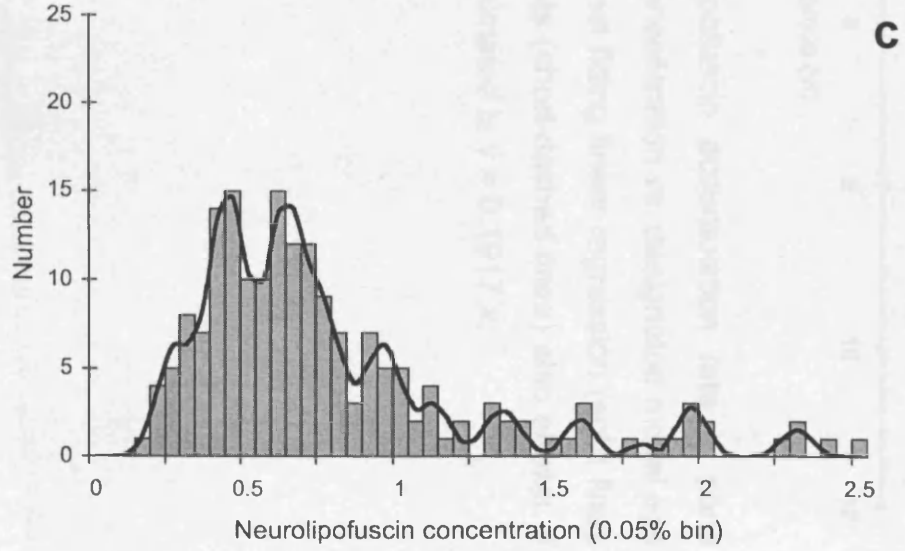

**Fig. 5.1.** Results of tag-recapture study for *Pacifastacus leniusculus* from Ullesthorpe pond. Plot of CL at release vs. CL at recapture, approximately 1 year later. □, males; ●, females; dashed line, nil growth threshold.

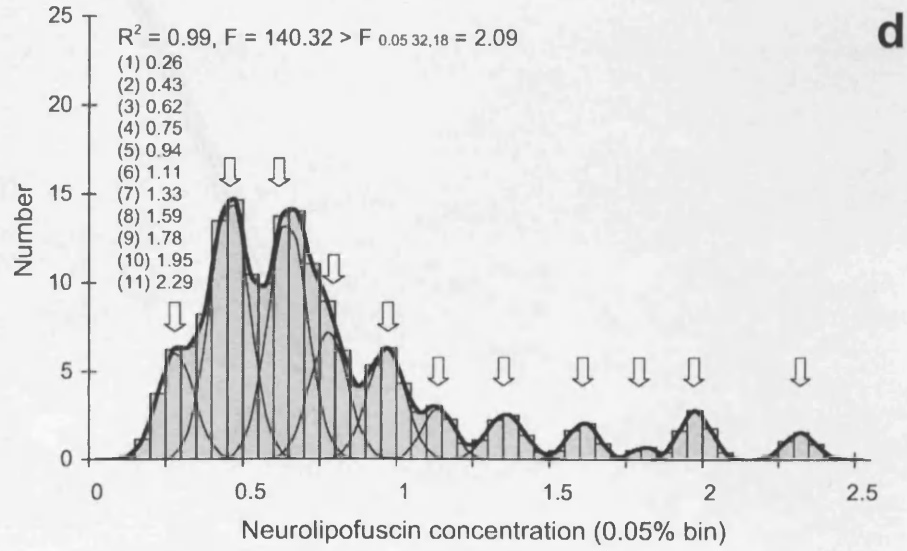
Legend to figure 5.2 overleaf.

**Fig. 5.2.** Results of carapace length (CLFH) and neurolipofuscin (NCFH) frequency histograms analysis for *P. leniusculus* from Ullesthorpe pond. All panels involve the same 183 individuals. Panels a and c show for CL and neurolipofuscin, respectively, raw frequency distributions with lightly smoothed FFTs superimposed on them (solid line). Panels, b and d show for CL and neurolipofuscin, respectively, the smoothed frequency distributions with estimated individual Gaussian components (thin lines) and the summed frequency of these estimated modes superimposed (thick lines). Putative annual age groups are arrowed. In panels b and d the modal means and goodness-of-fit statistics are also shown.


a
Number
CL (2 mm bins)


b
$R^2$ = 0.99, F = 2163 > $F_{0.05\ 20,20}$ = 2.12
(1) 30.06
(2) 38.98
(3) 45.92
(4) 53.47
(5) 60.11
(6) 67.63
(7) 74.39
Number
CL (2 mm bins)


c
Number
Neurolipofuscin concentration (0.05% bin)


d
$R^2$ = 0.99, F = 140.32 > $F_{0.05\ 32,18}$ = 2.09
(1) 0.26
(2) 0.43
(3) 0.62
(4) 0.75
(5) 0.94
(6) 1.11
(7) 1.33
(8) 1.59
(9) 1.78
(10) 1.95
(11) 2.29
Number
Neurolipofuscin concentration (0.05% bin)

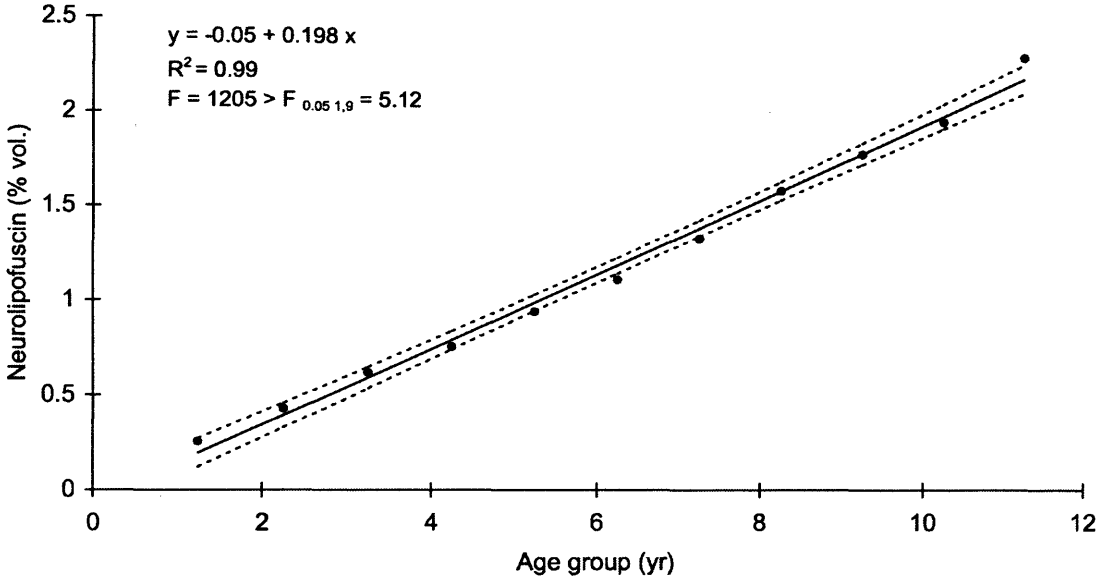
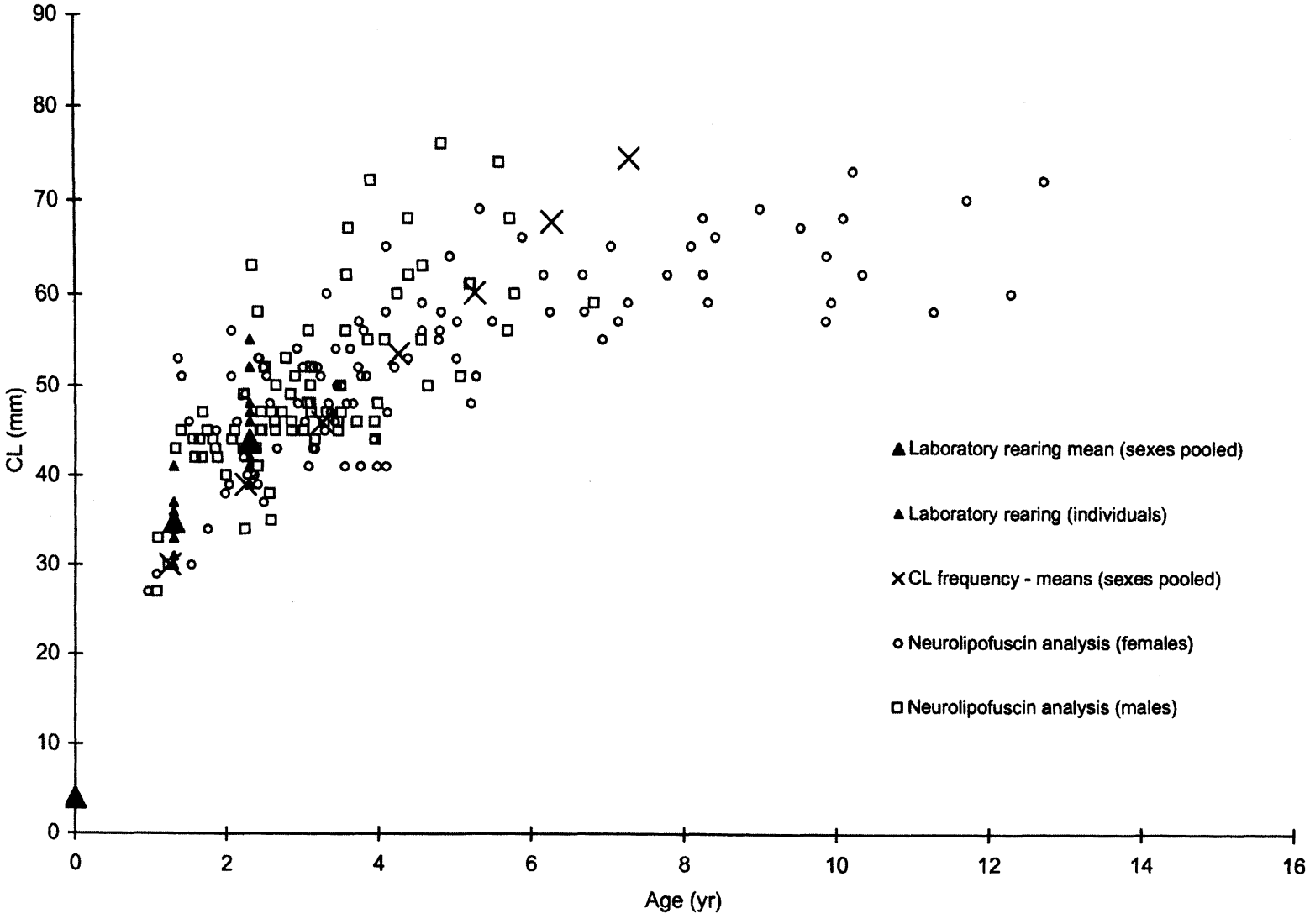



**Fig. 5.3.** Estimation of mean neurolipofuscin accumulation rate in pond crayfish. Mean modal neurolipofuscin concentration vs. designated modal age from the analysis shown in Fig. 5.2d. Best fitting linear regression (solid line), its coefficients and 95% confidence limits (short-dashed lines) also shown. If fitted through the origin, the equation estimated is y = 0.1917 x.

Legend to figure 5.4 overleaf.

**Fig. 5.4.** Plot of length-at-age or mean length-at-age for known-age laboratory reared crayfish and for pond crayfish whose age was estimated by modal analysis of carapace length frequency histograms or neurolipofuscin concentration frequency histograms.


CL (mm)
Age (yr)
0
10
20
30
40
50
60
70
80
90
0
2
4
6
8
10
12
14
16
Laboratory rearing mean (sexes pooled)
Laboratory rearing (individuals)
CL frequency - means (sexes pooled)
Neurolipofuscin analysis (females)
Neurolipofuscin analysis (males)

Legend to figure 5.5 overleaf.

**Fig. 5.5.** Growth curve estimates derived from the age length data shown in Fig. 5.4 or from anniversary mark-recapture of pond crayfish. Also shown are growth curves from Guan and Wiles (1999), estimated by modal analysis of carapace length frequency histograms.

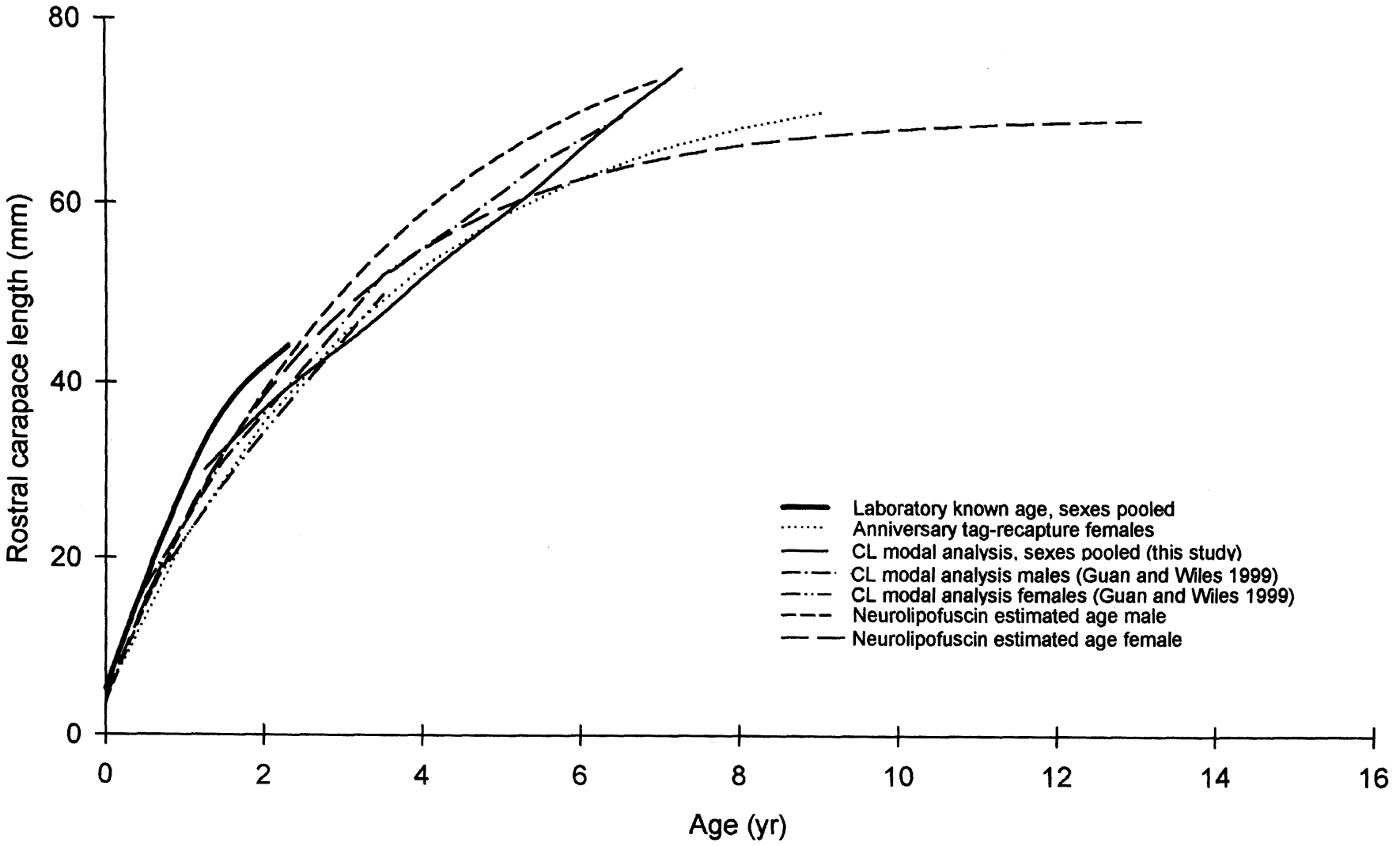

Rostral carapace length (mm)
80
60
40
20
0
0
2
4
6
8
10
12
14
16
Age (yr)
Laboratory known age, sexes pooled
Anniversary tag-recapture females
CL modal analysis, sexes pooled (this study)
CL modal analysis males (Guan and Wiles 1999)
CL modal analysis females (Guan and Wiles 1999)
Neurolipofuscin estimated age male
Neurolipofuscin estimated age female

**Table 5.1.** Parameters of the von Bertalanffy growth model estimated from the various methods employed. Note that the value of $t_0$ was fixed during fittings (see fitting details in material and methods).

| Method | | Parameters | | |
|---|---|---|---|---|
| | Sex | $L_\infty$ (mm) | K ($yr^{-1}$) | $t_0$ (yr) |
| Laboratory known age | Pooled | 54.73 | 0.67 | -0.15 |
| CL modal analysis | Pooled | 85.18 | 0.24 | -0.15 |
| Anniversary tag-recapture | Females | 74.18 | 0.30 | -0.15 |
| Neurolipofuscin estimated age | Females | 69.25 | 0.38 | -0.15 |
| | Males | 83.70 | 0.29 | -0.15 |

# Chapter 6

# General Discussion

The first objective of the study on *L. migratoria* (Chapter 2) was to identify lipofuscin in the brain; the second was to assess its relationship with chronological age. The results confirmed that lipofuscin, as identified by its characteristic fluorescence, micron-size irregular granular appearance, association with neurosomata, resistance to solvent extraction and age-related accumulation, occurred in the brain of the locust. As well as being the most comprehensive coverage of the lifespan for any insect, using reliable lipofuscin quantification methodology, this is the first reported occurrence of age-pigment in any member of the Orthoptera. As for all of the Crustacea examined previously, no significant sexual differences in lipofuscin accumulation rate were found in locusts. However, the exponentially increasing pattern of lipofuscin accumulation with age differed quite markedly from the linear or slightly curvilinear ones reported previously for crustaceans (e.g. Sheehy, 1990b; Sheehy *et al.*, 1994; Belchier *et al.*, 1998). There also appeared to be a very high degree of individual variation in the oldest age group. On the face of it, this suggests that lipofuscin would not provide a very precise index of age for population dynamics studies in *L. migratoria.*

However, the experiment was subject to a number of limitations that need to be addressed in an experimental replication. Firstly, because it was difficult to judge *a priori* how long male and female locusts would survive under the environmental conditions employed, it was difficult to decide the appropriate numbers of males and females to collect within each age group and the appropriate sampling interval, so as to give adequate, but manageable sample sizes for neurolipofuscin analysis. A minimal number of individuals (3 males, 3 females) was opted for in each age group. Ideally, samples at least double this size are required. In addition, due to fixation

problems, most of the samples for a whole age group (14 weeks) were lost. A replicate study is underway in Leicester that will address these problems.

With respect to the third objective of the study, which was to examine the relationship between lipofuscin accumulation and survivorship, the results, if they can be replicated, would have some implications for the study of ageing.

Firstly, the apparently close correlation between numbers of locusts surviving after the onset of reproduction and the average lipofuscin concentration in their brains suggests, as noted earlier, that post-reproductive mortality in this species is due to senescence and not just increased risk of accidental death. This has been a major uncertainty in some of the previous key studies on the evolution of ageing that employed insect models (Partridge, 1986). Secondly, although correlation does not prove causality, the results at least suggest the possibility that lipofuscin formation itself is detrimental to the brain and may be a cause of death. In an interspecific context, a significant inverse correlation between neurolipofuscin accumulation rate and maximum lifespan has also been observed in the decapods studied so far (Sheehy, 2002b). A negative association between lipofuscin accumulation and survival has been observed in transgenic strains of fruit fly, *Drosophila melanogaster,* producing high levels of reactive oxygen species. In these, premature death during eclosion was associated with large amounts of lipofuscin in the Malpighian tubules (Reveillaud *et al.*, 1991). Mutant nematodes, (*Caenorhabditis elegans*, *mev-1*), exhibiting hypersensitivity to oxygen concentration, accumulated lipofuscin at a relatively higher rate and had reduced lifespan (Hosokawa *et al.*, 1994). Some *in vitro* studies suggest that lipofuscin accumulation impairs several cellular functions. For instance, studies on cultured human retinal pigment epithelial

(RPE) cells have shown that lipofuscin causes the impairment of the lysosomal function and loss of membrane integrity (Holz *et al.*, 1999; Shutt *el al.*, 2000). *In vitro* studies of human fibroblasts exposed to oxidative stress, showed that ceroid/lipofuscin-loaded cells have increased sensitivity to oxidative stress (Terman *et al.*, 1999a), and reduced renewal of proteins, which results in worn-out/damaged organelles (Terman *et al.*, 1999b; Sitte *et al.*, 2001). However, evidence derived from *in vitro* studies on lipofuscin must be interpreted with caution (Rubin, 2002). Deleterious effects from lipofuscin have never been convincingly demonstrated (Porta, 2002a; Porta *et al.*, 2002; Palmer *et al.*, 2002). The finding of strong associations between lipofuscin accumulation and survivorship in *L. migratoria* in an *in vivo* context may be a step in the right direction toward resolving this issue, but clearly there are factors modulating the naturally differing lifespans of male and females locusts other than neurolipofuscin accumulation in the brain, which shows no sexual differentiation.

However, there are also some difficulties associated with the interpretation of the apparent correlation of neurolipofuscin and survivorship in *L. migratoria.* This correlation was based on the pooled survivorship data for both sexes. Examination of Fig. 2.2, indicates that males and females have different survivorship patterns, that all females were dead by week 14 and that prior to that age there was very little neurolipofuscin accumulation in females. This suggests that there was no sharp rise in neurolipofuscin accumulation rate after maturation, that lipofuscin concentration was not very strongly inversely correlated with female survivorship and that this association existed only for males. This apparent inconsistency is, in fact, an artefact of the experimental design. By sampling individuals for neurolipofuscin measurement from one cage and monitoring survivorship in another adjacent cage, for the

purposes of the experiment, two separate subpopulations of locust were inadvertently created. For reasons that remain unclear, females lived longer, on average, in the cage that was sampled for lipofuscin than in the cage monitored for survivorship. Lipofuscin does in fact accumulate rapidly in old female locusts after reproduction; the 17- and 18-week neurolipofuscin data both contain females. This problem can be resolved by ensuring that both neurolipofuscin measurements and survivorship monitoring are conducted on the 'one' experimental population and that the sexes are treated separately with respect to correlation between neurolipofuscin concentration and survivorship. The follow-up study underway in Leicester will resolve these points.

The initial rationale for the work reported on eyestalk ablation (Chapter 3) was the need for alternative ways to obtain the lipofuscin accumulation rates needed for crustacean age determinations in the absence of known-age reference samples, which are often difficult to obtain under suitable environmental conditions. The possibility of longitudinal sampling (i.e. sequential measurements in the same individual) of neurolipofuscin concentration by successive sampling from each of the two eyestalks suggested an entirely new way in which such rates might be obtained, provided the removal of the first eyestalk did not affect lipofuscin accumulation rate. Unfortunately, however, this hypothesis must now be rejected. Eyestalk ablation was found to significantly affect rate of neurolipofuscin accumulation in the remaining eye. The technique cannot be used for reliable calibrations in lipofuscin-based age determinations. On the other hand, this is one of the more important findings of the thesis because of its complete novelty and because of what it may tell us about the mechanisms of lipofuscin formation.

The result was perhaps not too surprising in light of knowledge available at the commencement of the study on the major role that the eyestalk plays in hormonal regulation, metabolism, growth and reproduction in decapods (Chang and O'Connor, 1983; Fingerman, 1995; Keller *et al.*, 1985; Skinner, 1985). On the other hand, rather than the observed decrease in lipofuscin accumulation rate, one might have expected an increase, perhaps associated with increased stress following ablation of the first eyestalk.

The proposition that the fall in neurolipofuscin accumulation rate may be due to trauma-induced enhancement of protease activity for neuronal remodeling, with a resultant increase in lipofuscin degradation is also entirely novel. This is plausible, however, based on various lines of available evidence about the effect of trauma on protease activity for dendritic remodelling in mammals and on the likely role of proteases in neurolipofuscin turnover, as cited in Chapter 3. Whether this hypothesis or a more conventional 'metabolic depression' explanation is correct, remains to be tested.

The results of this chapter are important because they open up a new approach for exploring the factors that modulate lipofuscin formation and/or degradation, which could in turn yield insights into mechanisms of ageing itself. While no other evidence of anti-ageing effect arising from neural injury was found in the literature, the role of the nervous system in controlling invertebrate ageing and longevity is documented (Boulianne, 2001; Mattson *et al.*, 2002; Wolkow, 2002). For instance, Parkes *et al.* (1998, 1999) demonstrated that the over-expression of superoxide dismutase (SOD) in motor neurons of *Drosophila* extended lifespan, while over expression in muscles did not. Klichko *et al.* (1999) observed that brains of *Drosophila* have lower levels of

cytosolic SOD than other tissues, suggesting that neural tissues are more susceptible to oxidative damage. Apfeld and Kenyon (1999) demonstrated that the life span of the nematode *Caenorhabditis elegans* is extended when worms have reduced sensory perception caused by defective sensory cilia. Apparently, sensory perception in nematodes is linked to a lifespan-regulating transduction pathway (DAuer Formation, DAF-2), in which *daf-2* encodes an insulin receptor homologue. Lowered levels of this insulin receptor increase the lifespan, possibly because of an increased resistance to oxidative stress (Guarente and Kenyon, 2000). According to Wolkow *et al.* (2000), a longer lifespan was observed only in worms with DAF-2 pathway signalling affected neurons, while no lifespan extension was observed when this signalling was affected in muscle or intestinal cells.

Although plausible, the 'accelerated proteolysis' explanation for the inhibiting effect of eyestalk ablation on neurolipofuscin accumulation in the remaining eyestalk assumed that lipofuscin could, in fact, be turned over and was not undegradable. The latter assumption had been a controversial issue in the literature. Previous studies on this topic had yielded inconclusive results, as noted in Chapter 4, due to (1) use of inappropriate lipofuscin quantification techniques, (2) irreproducible results, (3) the possibility that lipofuscin-like material that is rapidly deposited after protein malnutrition and protease inhibition differs fundamentally from normal age pigment and (4) the possibility that lipofuscin formation and processing *in vitro* cell cultures may not be the same as that *in vivo* (Katz *et al.*, 1999; Rubin, 2002). However, although originally conducted for an entirely different reason, the experiment described in Chapter 4 was to resolve these problems and provide major insight on the question of reversibility of lipofuscin accumulation.

The original purpose of the Chapter 4 was to determine whether apparently declining neurolipofuscin accumulation rates that had been observed previously in freshwater crayfish (Sheehy *et al.*, 1994) were an artefact of prolonged laboratory rearing under unnatural conditions. Clearly, it is important to know the form of lipofuscin accumulation for the oldest age groups in the population, as age prediction of these individuals is likely to involve extrapolation beyond the range of any known-age reference material. Although it has been concluded that the effect of any curvilinearity in the lipofuscin concentration at age relationship should only be significant at lipofuscin concentrations exceeding 90% of the maximum observed value, i.e., for very old age groups (Sheehy and Bannister, 2002) further data on this issue is needed. The question may also be relevant to understanding the reason for the declining mortality rates that have been recently observed in the oldest individuals of many animal populations including man, as cited in Chapter 2. Do they result from a reduction in the rate of ageing in the oldest of the old?

Because there was no prospect of rearing known age individuals of the experimental model, *P. leniusculus,* in the field for the time necessary to reach old age, i.e., more than 10 years, the approach that was adopted was analogous to the anniversary tag recapture/Ford-Walford method commonly used in fisheries research. However, instead of comparing size at release with size at recapture after one year at liberty for as wide an initial size range as possible, the neurolipofuscin concentration at release (in the ablated eyestalk) was compared with the neurolipofuscin concentration at the time of recapture (in the remaining eyestalk) for as wide an initial physiological age range (lipofuscin concentration range) as possible. It was hoped that this would yield a complete set of longitudinal data showing annual neurolipofuscin accumulation rates in individuals of the full spectrum of ages present

in the field. This would indicate, not only the mean lipofuscin accumulation rate of individuals at the field site, which could then be used as a calibration for age determinations, but also indicate any age-dependent variation in neurolipofuscin accumulation rate. In order for this approach to yield insights into natural neurolipofuscin patterns it first had to be confirmed that ablation itself did not cause any abnormal effects. As reported for Chapter 3, this initial test failed. The outcome of the experiment reported in Chapter 4 was, that it confirmed that neurolipofuscin accumulation rate declines with age in *P. leniusculus* but, it was impossible to say whether or not this phenomenon was part of the normal ageing process.

Despite this disappointing result with respect to the initial rationale and objective of the study, the result is nevertheless probably the most important outcome of the thesis in that it is the first conclusive and quantitative evidence of the *in vivo* reversibility of lipofuscin accumulation in any species. This possibility is important, not only for lipofuscin-based crustacean age determination work, but also in the field of gerontology.

With respect to crustacean age determination, Tully *et al.* (2000), in their recent study of the effects of simulated seasonally oscillating water temperatures on lipofuscin accumulation rate in the brain of *H. gammarus,* found that it was highest in summer and lowest in winter. However, in some cases, the average neurolipofuscin concentration of lobsters sampled in simulated winter was lower than that of lobsters sampled in the preceding summer, implying a loss of neurolipofuscin in cool temperatures. The authors were not able to put forward a mechanism for such loss and they suggested instead that it was simply a statistical artefact due to large age-specific individual variation. One candidate for future evaluation of this possibility in a

wild population is the penaeid, *Farfantepenaeus paulensis*, from southern Brazil. This species follows the pattern of some coastal penaeids in which the adults leave estuarine regions at the end of autumn and migrate to warmer waters (Dall *et al.*, 1990). However, adults of *F. paulensis* sometimes fail to leave the estuarine region in autumn, remaining buried in the muddy bottom during winter, and eventually leaving in spring (D'Incao, 1990, 1991).

The results of Chapter 4 of this thesis now confirm that lipofuscin loss can occur from crustacean neural tissue. This loss probably involves exocytosis, possibly followed by blood transport to another location for degradation or excretion (Blackman, 1996). It remains unclear whether part or all of this loss was due to ablation or whether it occurs naturally. If it can be proved that neurolipofuscin loss also operates in very cool environments, this would have implications for age determination models that incorporate correction for spatial and temporal variations in water temperature (e.g. Sheehy and Bannister, 2002). These models have assumed that when the temperature drops below the minimum threshold for lipofuscin accumulation, lipofuscin concentration remains static rather than begins to fall.

In gerontology, any demonstration of a mechanism that can reverse manifestations of ageing is of obvious interest. The knowledge that lipofuscin can be exported from cells may provide both insight into the mechanism of its formation and impetus to find pharmacological or other practicable means to reduce it. The possibility of an ageing reversal effect by low temperatures in lobsters is extremely interesting. Hypothermia in humans has a well-known protective effect against brain damage by hypoxia but possible anti-ageing effects would require longer periods of exposure to the cold than are feasible for survival (cryopreservation of the corpse is of course the

ultimate anti-ageing treatment). The best and only evidence we have of the inhibitory effect of the cold on ageing in living mammals comes from the work of Papafrangos and Lyman (1982). These authors found that in Turkish hamsters, lifespan was proportional to, and lipofuscin accumulation rate inversely proportional to, the amount of time spent in hibernation. However, no evidence of lipofuscin loss was presented. These arguments assume that the presence of lipofuscin is undesirable, but this may be a spurious assumption. On the contrary, lipofuscin formation may be beneficial by serving to compartmentalize toxic substances in a protective wrapping and then export them from the cell.

Finally, some very interesting ideas have been proposed recently on the idea of lipofuscin degradability. De Grey and Archer (2001) in their paper entitled 'Why don't graveyards fluoresce? Anti-aging applications of the bacterial degradation of lysosomal aggregates' produce preliminary findings that some Gram-positive bacteria found in the soil (Nocardioforms) are able to digest lipofuscin!

While microtagging and release of early juveniles into the wild, with as long a period at liberty as possible before recapture, is the most accurate method for collecting the lipofuscin concentration-at-age calibration needed for lipofuscin-based age determination, this approach may be impractical in many cases and very time consuming and expensive in others. Recapture rates are typically low and therefore large numbers of releases are required. Also, it may not be possible to obtain known age juveniles for many species, which cannot be successfully hatched and reared to tagging size in sufficient numbers in the laboratory. The purpose of Chapter 5 was to test, using *P. leniusculus*, a method for obtaining the calibration directly from the wild population without the need for tagging. This method was the analysis of

neurolipofuscin concentration frequency histograms from the field population and the identification of annual cohort modes. This approach had been originally attempted by Ettershank (1983) for krill but with inconclusive results, due to use of a flawed measurement method, then theoretically validated by simulation for freshwater crayfish (Sheehy *et al.*, 1994) and finally successfully applied to a natural population of rock lobster (Sheehy *et al.*, 1998). At the time of commencing the work on this thesis, the latter study was the only successful application of the technique to a natural population and the proposed study on *P. leniusculus* was considered an important test for previous simulations and the lone field results. However, work on Antarctic shrimp and amphipods (Bluhm and Brey, 2001; Bluhm *et al.*, 2001), published while the *P. leniusculus* study was in progress, has now provided much needed corroboration for the approach.

The result of Chapter 5 supports the hypothesis that neurolipofuscin can be used to detect annual cohorts in populations of *P. leniusculus*. It provides further corroboration for the applicability of the method, at least for crustaceans of short to moderate lifespan. Furthermore, the study represents probably the most comprehensive simultaneous comparison ever reported, on a single crustacean population, of traditional and novel approaches for obtaining the basic length-at-age or length increment-at-length data needed to estimate generalized growth. As such, it provides a rare perspective for evaluation of the various approaches. The purpose of the study was to test the advantages of the neurolipofuscin method; it was not to compare various modelling approaches that have been developed for optimal fitting of growth data. These purely mathematical approaches are abundant in the fisheries literature (e.g., Sainsbury, 1980; Wang *et al.*, 1995; Smith and Botsford, 1998; Wang, 1998) and frequently suffer from weakness in the basic size data. For

crustaceans, they have invariably suffered from absence of the basic age data that is available to finfish modelling.

There was generally a surprising degree of similarity between the results of the different methods for estimating early growth, given the widespread analysis of the biases and other problems inherent in the traditional methods, which occurs in the scientific literature (e.g. Sheehy *et al.*, 1999). The most serious problem to surface in the comparative analysis on *P. leniusculus*, apart from the well known problems inherent in extrapolating data obtained by captive rearing to the wild, was underestimation of the number of older age groups present in the population when using traditional length frequency analysis. Age underestimation is the commonest type of error that occurs in age determination work (Campana, 2001). Length-frequency analysis is an extremely popular method because it is widely applicable, simple, quick, inexpensive and can be performed in the field. Yet it is the approach most prone to error. Age estimation error in the form of underestimation of longevity has led to serious scientific errors in stock assessments and multimillion pound losses (Beamish and McFarlane, 1983).

The anniversary tag-recapture approach, when applied to *P. leniusculus,* yielded a growth curve very similar to that obtained by fitting a VBGF to the length-at-lipofuscin estimated age. This tag-recapture approach is also widely used in fisheries research and stock assessment because it does not require a supply of known age individuals and the entire growth curve can be estimated from a small amount of data. The criticism most often levelled at it is that it is very difficult to get data for the largest, oldest individual that may exist in the population (Sheehy *et al.*, 1999). This is because recaptures are normally fishery dependent, and in a fishery, there is often

strong gear selection for a particular size group and the chance of survival much beyond recruiting to legal size is low. The upper part of the growth curve may therefore be unreliable. This problem was not experienced in the *P. leniusculus* study. Good recapture coverage of the upper size range was achieved. While, favourable for achieving a reliable growth curve, this highlights a limitation of the pond study. It was conducted on an unexploited population and therefore many of the biases that are inherent in fishery dependent sampling will not have been present.

An advantage of lipofuscin-based age determination is that it provides age-at-length data for individuals (albeit with error) whereas the conventional size-based methodology, e.g. anniversary tag-recapture, generates the coefficients of the VBGF, in other words, a mean growth relationship. Length-at-age data for individuals allow more flexibility in assessing different growth models for crustaceans. For instance, Sheehy *et al.* (1999) found that an inverse logistic model described better the growth of clawed lobsters than traditional growth models such as the VBGF. Preliminary trials showed that both the Gompertz and logistic models were suitable for the present *P. leniusculus* data.

## 6.1 Conclusions

Although, many questions on the factors affecting lipofuscin accumulation in arthropod neural tissue and its use as an ecological tool for age determination remain, or have now arisen, this thesis has made significant contributions to knowledge in the research field. The main novel findings are as follows:

- Lipofuscin occurs and can be quantified in the brains of locusts using the well-established methodology employed for crustaceans.
- Neurolipofuscin accumulates in locusts following a pattern distinct to that observed in crustaceans.
- There is evidence suggesting that neurolipofuscin accumulation in locusts in inversely correlated with survival.
- Mechanistic and evolutionary theories of ageing should be integrated to explain the ageing process.
- Neurolipofuscin accumulation rate in signal crayfish is reduced by eyestalk ablation.
- It is hypothesized that this reduction results from either reduced lipofuscinogenesis due to neuro-hormonal effects, oxidative catabolism or accelerated lipofuscin turnover by trauma induced protease upregulation.
- Lipofuscin can be lost from neural tissue.
- Neurolipofuscin frequency distributions provide a better resolution of age groups than length frequency distributions in signal crayfish.
- Length-based methods underestimate maximum longevity of signal crayfish, leading to biases in growth trajectory estimates.
- The more detailed length-at-age data obtained by the neurolipofuscin approach permits individual growth variability to be assessed (after accounting for age determination error), which is important for testing generalized growth models other than the VBGF.

The study has also produced many avenues for future research. Replication of the experiment conducted in Chapter 2 using larger sample sizes is already underway. The mechanism of reduction of lipofuscin accumulation rate following eyestalk ablation is a key question arising from Chapter 3, as is the question of whether and how lipofuscin is exported from nerve cells. With regard to Chapter 5 and the use of lipofuscin for crustacean age determination, there is a planet full of heavily exploited crustacean fisheries, the management and sustainable development of which would greatly benefit from improved demographic information.

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# Appendix

## Histological procedure

Animals taken to the laboratory after sampling were "decapitated" and "heads" were fixed in 10% formol saline. Removal of tissue by dissection was performed as soon as possible to avoid fluorescence artefacts associated with prolonged formalin fixation (Sheehy, pers. com.). Tissues used included the supra-oesophageal ganglion (locusts) and eyestalk ganglionic complex (crayfish).

After dissection, tissues were dehydrated and embedded according to the following protocol:

| | |
|---|---|
| Phosphate buffered saline (pH 7.4) | 1 h. |
| Alcohol (IMS) 70% | 1 h. |
| Alcohol (IMS) 85% | 1 h. |
| Alcohol (IMS) 95% | 1 h. |
| Alcohol (IMS) 100% | 1 h. |
| Alcohol (IMS) 100% : Xylene 100% (1:1) | 1 h. |
| 1$^{st}$ Xylene 100% | 30 min. |
| 2$^{nd}$ Xylene 100% | 30 min. |
| Infiltration in 48 °C melting point wax at 60 °C | 20 min. |
| Transfer to 2$^{nd}$ wax and vacuum at 60 °C | 20 min. |

Tissues were finally embedded in moulds filled with melted wax (60 °C). The supra-oesophageal ganglion of the locust was orientated to permit horizontal sectioning, while the eyestalk ganglionic mass of the crayfish was set for transverse sectioning

Blocks were allowed to harden overnight. Sectioning was performed using a Historange microtome set at 6 μm section thickness. Serial sections ("ribbons") were collected and floated in a water bath (40 °C) to allow stretching. Gelatine was added to the water bath so that, when mounted, the sections adhered firmly to the microscope slides. After floating onto a standard microscope slide (normally 3 ribbons of 11 sections per slide) sections were allowed to dry overnight. Mounting was performed after three dewaxing baths in xylene 100% (two minutes each). Coverslips were mounted using DPX mountant. No staining was utilised.

## Region used to quantify lipofuscin

The supra-oesophageal ganglion of *Locusta migratoria* has three main regions (protocerebrum, deuterocerebrum and tritocerebrum). The protocerebrum, which is anterior, has a conspicuous cell mass (pars intercerebralis), the "central body" neuropile, and the neuropiles of corpora pedunculata (Satija, 1958; Bullock and Horridge, 1965). Lipofuscin was quantified in the pars interecebralis.

Eyestalks of *Pacifastacus leniusculus* have four distinct ganglia (Blaustein *et al.*, 1988). The most proximal is the medulla terminalis. Adjacent to one of the

neuropiles of medulla terminalis is the hemiellipsoid body, which is associated with 'cell mass cluster A'. Lipofuscin was quantified in this cell mass.

To identify different anatomical regions, sections of the supra-oesophageal ganglion of locusts and crayfish eyestalks were stained utilising either haematoxylin-eosin or Mallory's triple stain (Humason, 1979). Once the appropriate region had been identified, unstained sections were used for lipofuscin measurements.

Stained sections of the supra-oesophageal ganglion of locusts were used for a three-dimensional reconstruction. For this purpose, 'MacStereology' (version 2.8) software was utilised. A complete set of serial sections of the supra-oesophageal ganglion was digitized, imported into MacStereology, then stacked in appropriate orientation and with appropriate spacing. Individual features were 'linked' throughout the whole set of serial sections in which they occurred in order to create external and internal structural surfaces in the reconstruction.

## Image collection

Unstained sections were observed using a Zeiss inverted epifluoresence microscope with a green excitation filter (514 nm). Images of the appropriate region (pars intercerebralis in supra-oesophageal ganglia of *L. migratoria* and cluster A cell mass in the medulla terminalis of the eyestalks of *P. leniusculus*) were captured using a confocal laser scanning system (Bio-Rad Lasersharp MRC) fitted to the microscope. For each section, the region with the most conspicuous lipofuscin accumulation was selected and an image was

acquired. To acquire images the following settings were used: objective 63x; zoom 1.6x; Kalman collection filter; five image scans. After collection, each image was saved, as a Bio-Rad PIC file, on a magneto-optical disk.

Images were acquired in a standard way so that estimates were always obtained from the same location. In *L. migratoria* only sections containing both the central body and the pars intercerebralis were used, whereas in *P. leniusculus* only images containing the axon tract connection to the hemiellipsoid body were used.

## Image analysis

Images were analysed using COMOS software, version 7 (Bio-Rad). Lipofuscin granules appear brighter against the darker cellular background and the proportion of the image occupied by lipofuscin was obtained for each image by measuring the ratio between the area of lipofuscin granules and the total area of the background cells. Before analysis, images were processed, globally and locally, by smoothing and contrast-enhancing. Thereafter, image analysis was performed by thresholding each image using a grey scale from black to white (0-255). The threshold corresponding to the lowest lipofuscin intensity was determined by visual inspection and all pixels above this threshold were selected to estimate the ratio.

## Calculation of the mean lipofuscin value

Lipofuscin concentration was expressed as volume fraction (% vol.). A geometric weighted average (Sheehy *et al.*, 1998) defined by:

$$\%\text{vol.} = 10^{\left\{\frac{\sum[\log(\frac{Lx100}{A}+c)xA]}{\sum A}\right\}} - c$$

was used to estimate the volume of lipofuscin per sample (% vol.), where L is the total cross-sectional area of lipofuscin in an image ($\mu m^2$), A is the cross-sectional area of suitable tissue in an image ($\mu m^2$) and c is a constant = 0.04. The reason for the use of area weighting is that different sections may differ in background area, and, such differences could introduce bias in the estimate of mean lipofuscin concentration. Also, occasionally, very large granules appear, usually in isolation. The use of a geometric average removes the effect of these outliers to give a better estimate of central tendency.