

Estimating age and growth in long-lived temperate freshwater crayfish using lipofuscin

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SUMMARY

1. In ecological studies on freshwater crayfish, determination of basic population parameters is often complicated by the lack of a suitable age estimation method.
2. Previously, lipofuscin age pigment in the olfactory lobe cell masses (OLCM) of short-lived tropical crayfish has been used for accurate age determination. Here we present the first test of this method on a longer-lived, temperate species, the signal crayfish, *Pacifastacus leniusculus*.
3. Confocal fluorescence microscopy and image analysis of histological sections were used to quantify OLCM lipofuscin in a reference sample of Swedish *P. leniusculus* from several known year-classes, reared under naturally variable temperature conditions. Lipofuscin concentration was linearly associated with age ($r^2 = 92.4\%$) and produced much more accurate age estimates than conventional body size-based procedures.
4. A model derived from the crayfish of known-age was used to estimate the ages of wild *P. leniusculus* from an English stream. The relationship between lipofuscin-estimated age and carapace length suggested relatively slow growth in this wild population, consistent with a high population density and severe competition. The analysis also extended the known longevity of *P. leniusculus* to approximately 16 years.
5. The lipofuscin method for determining age and growth may be widely applicable to freshwater crayfish, with probable further potential both within and outside the Crustacea.

Introduction

Information on growth is fundamental for understanding the population dynamics of freshwater crayfish. A modern molecular method employs cellular RNA concentration as a physiological index of growth rate. This technique is suitable for detecting fine changes in relative instantaneous growth rates of individual crayfish over short periods of time (days) in response to changes in feeding regimes (Edsman, Järvi & Niejahr, 1994), but is less useful for long-term generalized growth patterns. It is difficult to measure growth of crayfish in the wild because they cannot be precisely aged (Lowery, 1988). The problem is widespread in the Crustacea; hard parts bearing seasonally-induced

growth rings are normally not retained through the moult. Conventional mark-recapture techniques require large sample sizes (Lowery, 1988) and the application of size-frequency histograms to the analysis of crayfish population age structures has a number of limitations (Laurent, 1988; Sokol, 1988; France, Holmes & Lynch, 1991).

A recent alternative approach to age determination based on lipofuscin, a widely occurring neuronal age pigment, has produced positive results for the Australian tropical red-claw crayfish, *Cherax quadricarinatus* (von Martens) (Sheehy, 1989; 1990a,b, 1992; Sheehy, Greenwood & Fielder, 1994). For this species,

the quantity of lipofuscin in the individual's olfactory lobe cell masses (OLCM) is a better predictor of age than body size, the variable typically used for age estimation. However, *C. quadricarinatus* is quite short-lived (approximately 3–5 years) and studies on species with different life history patterns and from different climates are needed to test the ageing method more widely. Here we assess the performance of OLCM lipofuscin concentration for determining age and defining generalized growth in a much longer-lived, temperate species, the well-known northern hemisphere signal crayfish, *Pacifastacus leniusculus* (Dana) is indigenous to north-western North America but was introduced to Europe in the 1960s to replace depleted European native crayfish populations. It is now widespread in Europe where it forms the basis of significant fisheries in several countries. It is ideal for aquaculture but can displace native species such as *Astacus astacus* (L.) and *Austropotamobius pallipes* (Lereboullet) (Lowery & Holdich, 1988). Thus, the ecology of *P. leniusculus* is currently of enhanced scientific interest.

For this study a sample of *P. leniusculus*, exceptionally from seven known year-classes, was available to examine the relationship between lipofuscin deposition and age.

Materials and methods

Crayfish

Two groups of *P. leniusculus* were used for this study. The reference group of fifty-nine crayfish of mixed sex was reared for up to 6.5 years from hatching in tanks at the Institute of Freshwater Research, Drottningholm, southern Sweden. Water supply to the tanks was pumped from nearby Lake Mälaren (59°20'N, 17°52'E) through an open circulation system. Water temperature in the tanks closely approximated that in the lake (annual mean 9.1 °C, monthly mean 1.1–18.3 °C). The crayfish were reared at a relatively high density and fed a varied diet of corn, green peas, commercial fish and crayfish feed, fresh fish pieces, shrimps and alder leaves. The age composition of the sample was as follows: 0.5 years, $n = 10$; 1.5 years, $n = 9$; 2.5 years, $n = 10$; 3.5 years, $n = 11$; 4.5 years, $n = 10$; 5.5 years, $n = 4$; 6.5 years, $n = 5$. Ages were known to within a few days.

The second group of eleven wild crayfish of mixed

sex, a range of sizes and unknown ages was selected from catches from an established population in Gaddesby Brook, Leicestershire, England (52°40'N 0°50'W, annual mean temperature 10.5 °C, monthly mean 2.5–17.5 °C). This breeding population arose from aquaculture escapees around 1985.

Lipofuscin measurement

Crayfish olfactory lobes were prepared for lipofuscin measurement essentially as described in Sheehy (1989; 1990c) and Sheehy & Wickins (1994). Lipofuscin in the left olfactory lobe cell mass of each brain was detected and measured using a Bio-Rad Lasersharp MRC 600 confocal laser scanning system fitted to a Zeiss Axiovert 10 inverted epifluorescence microscope and employing COMOS version 7 software (Belchier, Shelton & Chapman, 1994; Sheehy *et al.*, 1996). Sections were excited at 488 or 514 nm and autofluorescence was detected through a 63× oil immersion objective. Approximately every third section through the OLCM was used for lipofuscin measurement. This produced a total replicate number for each individual of twelve to twenty-seven, depending on brain size. Each section was positioned in the image frame so as to maximise the amount of lipofuscin present and minimise the amount of background tissue not containing globuli neurone somata. The neutral density filter, confocal aperture and electronic gain of the instrument were adjusted when necessary to optimize video image clarity. Kalman averaging of three sequential image scans and a scan window zoom factor of 1.0 were used for acquiring all images. Digital images were recorded on optical disk. Brightly autofluorescing lipofuscin granules were readily discriminated from the darker background of neurone somata using manual greyscale thresholding. The outline of the cell-mass background in the image was traced manually. Areas not containing characteristic globuli neurone somata were excluded. The total cross-sectional areas of both lipofuscin and the background cells were calculated by the software. The area fraction of lipofuscin in each image, as a percentage, was calculated by dividing the cross-sectional area of lipofuscin granules by the total background area of neurone somata, and multiplying by 100. The geometric average area fraction over all sections examined for each individual was calculated. In line with stereological convention, the average was recorded as, and equivalent to, the

Table 1 Regression models defining the relationships between *A*, age (years) and *L*, OLCM lipofuscin concentration (%VF); *C*, carapace length (mm) and *W*, body mass (g) in experimental *Pacifastacus leniusculus*; prediction equations for back-calculation of age from these dependent variables and transforms used prior to statistical analyses

Predictor variables	Descriptive models	Age prediction models	Parameter values	<i>r</i> ² (%)	Linear transforms	Normal/homoscedastic transforms
Lipofuscin concentration (% VF)	$L = a + bA$	$A = \frac{L-a}{b}$	$a = -0.00823$ $b = 0.237$	92.4	—	$A^{1/2}, L^{1/2}$ $A^{1/4}, L^{1/4}$
Carapace length (mm)	$C = c_{\infty}[1 - e^{-k(A-t_0)}]$	$A = t_0 + \frac{\ln(\frac{C}{c_{\infty}} + 1)}{-k}$	$c_{\infty} = 69.8$ $k = 0.233$ $t_0 = -0.105$	84.1	$f(A) = 1 - e^{-k(A-t_0)}$ $f(C) = -\ln(-\frac{C}{c_{\infty}} + 1)$	$[f(A)]^{1/2}, C^{1/2}$ $[f(A)]^{1/4}, C^{1/4}$
Body mass (g)	$W = w_{\infty}[1 - e^{-k(A-t_0)}]^3$	$A = t_0 + \frac{\ln(\frac{W}{w_{\infty}} + 1)}{-k}$	$w_{\infty} = 77.6$ $k = 0.316$ $t_0 = 0$	60.5	$f(A) = (1 - e^{-k(A-t_0)})^3$ $f(W) = -\ln(-[\frac{W}{w_{\infty}}]^{1/3} + 1)$	$[f(A)]^{1/4}, W^{1/4}$

volume fraction in per cent (%VF) of lipofuscin in the region of the OLCM under examination. In this procedure, (i) the use of the highest objective magnification, (ii) quantification of the region containing the highest lipofuscin concentration in each section, (iii) use of a high replicate number of sections and (iv) application of a geometric mean, are considered important for accurate lipofuscin measurement.

Statistical analysis

For the known-age Swedish *P. leniusculus*, standard and cubic von Bertalanffy (negative exponential) models were used to define relationships between age and rostral carapace length or wet body mass. Parameter values for these non-linear models were optimised for the data using standard iterative least-squares estimation methods. The relationship between age and OLCM lipofuscin concentration was well described by a linear model. Wherever necessary and possible, data were transformed (Table 1) so as to fulfil assumptions for application of parametric statistics. Possible effects of age and/or sex on the average OLCM lipofuscin concentration, carapace length or mass of each group of experimental crayfish were assessed using one-way and two-way parametric anova. Normality and homogeneity of variance of groups were tested using the Kolmogorov Smirnov test (with Lilliefier’s correction) and the Levene Median test, respectively. Where test groups did not fulfil

assumptions of normality and homogeneity of variance, additional nonparametric one-way Kruskal–Wallis analyses of variance on the medians of the ranked data were performed.

Best-fitting equations describing the relationships between the independent variable, age, and the three dependent variables, OLCM lipofuscin concentration, carapace length and body mass, were appropriately reorganized for back-calculation of age (Table 1). In order to assess and compare the accuracy of the predictor variables, three mathematical indices were calculated: (i) mean age prediction errors (MAPEs), (ii) 95% confidence limits for age predictions and (iii) age resolution factors (Sheehy *et al.*, 1994). Age prediction error for each known-age crayfish was calculated as the absolute difference between its predicted age and its true age, as a percentage of the latter. These individual values were then used to calculate MAPEs for each age-group and for each of the three predictor variables. Ninety-five per cent confidence intervals for age estimates were calculated on the transformed data according to the regression method described by Sokal & Rohlf (1981; p. 498) for inverse predictions and then back-transformed for further analysis and graphical presentation. Confidence limits for lipofuscin-based age estimates on the wild crayfish were approximated by interpolation or extrapolation of those obtained for the known-age crayfish data. Resolution factors represent lipofuscin-, carapace length- or body mass-predicted ages

divided by their 95% confidence intervals and then averaged for each age group. The higher the resolution factor, the finer the degree of age discrimination provided by the predictor variable.

Results

Analyses of variance did not reveal any significant differences in median OLCM lipofuscin concentrations ($P = 0.46$), carapace lengths ($P = 0.77$) or body masses ($P = 0.95$) between males and females in the reference sample of Swedish *P. leniusculus*. On the other hand, highly statistically significant ($P < 0.001$) differences in the mean or median OLCM lipofuscin concentrations, carapace lengths and body masses were found between the different age groups (Fig. 1, Table 1).

Accumulation of lipofuscin in the OLCM of *P. leniusculus* was linear and strongly associated with age over the seven year-classes ($r^2 = 92.4\%$). Stepwise multiple regression of the transformed variables showed that age was the primary predictor of OLCM lipofuscin concentration ($P < 0.001$) and that neither carapace length or body mass explained a significant part of the remaining variance in this relationship. The relationships of carapace length and body mass with age were well defined by standard and cubic von Bertalanffy functions, respectively, although more variable ($r^2 = 84.1\%$ and 60.5% , respectively) than for OLCM lipofuscin concentration.

Mean age prediction errors confirmed that OLCM lipofuscin concentration was a more accurate age predictor than carapace length for all seven age groups of the reference sample of Swedish *P. leniusculus* and better than body mass in all but the youngest (0.5 year) age-group (Fig. 2a). The MAPE for all fifty-nine specimens based on lipofuscin (13.5%) was significantly different to ($P = 0.005$), and lower than that for carapace length (27.3%). Age estimation error was highest for mass-based age estimates. Confidence intervals for age predictions based on OLCM lipofuscin concentration were relatively narrow compared with those based on either carapace length or body mass over the entire age range studied, due to the relatively high variation in size-at-age (Figs 1 and 2b). When using the latter predictors for older individuals, confidence in age estimates declined markedly as the asymptotic size or mass was approached. Estimated upper 95% confidence limits for carapace length- and body mass-based age estimates became infinitely large for pre-

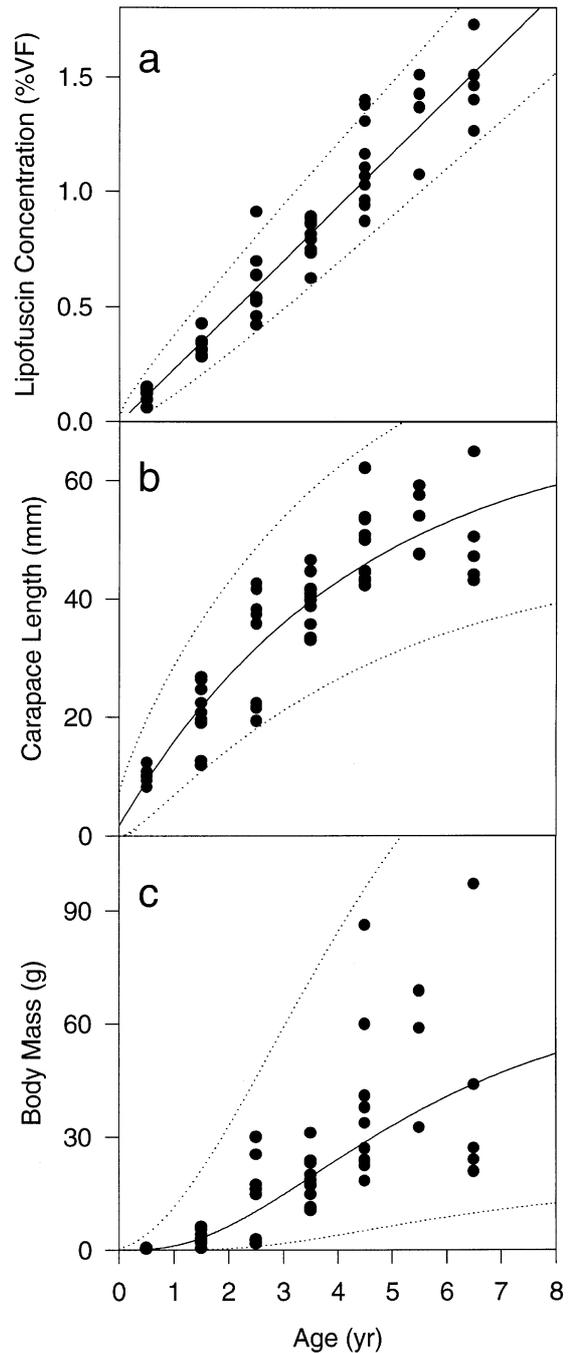


Fig. 1 Relationships between age and (a) OLCM lipofuscin concentration (b) rostral carapace length and (c) wet body mass in cultured Swedish *Pacifastacus leniusculus*. Solid lines represent best-fitting linear and nonlinear regressions (Table 1) and dotted lines represent 95% confidence limits for age predictions.

dicted ages above about 4.8 and 3.3 years, respectively (Fig. 1b and c). At younger ages, the ability of size and mass to resolve age-groups was still comparatively

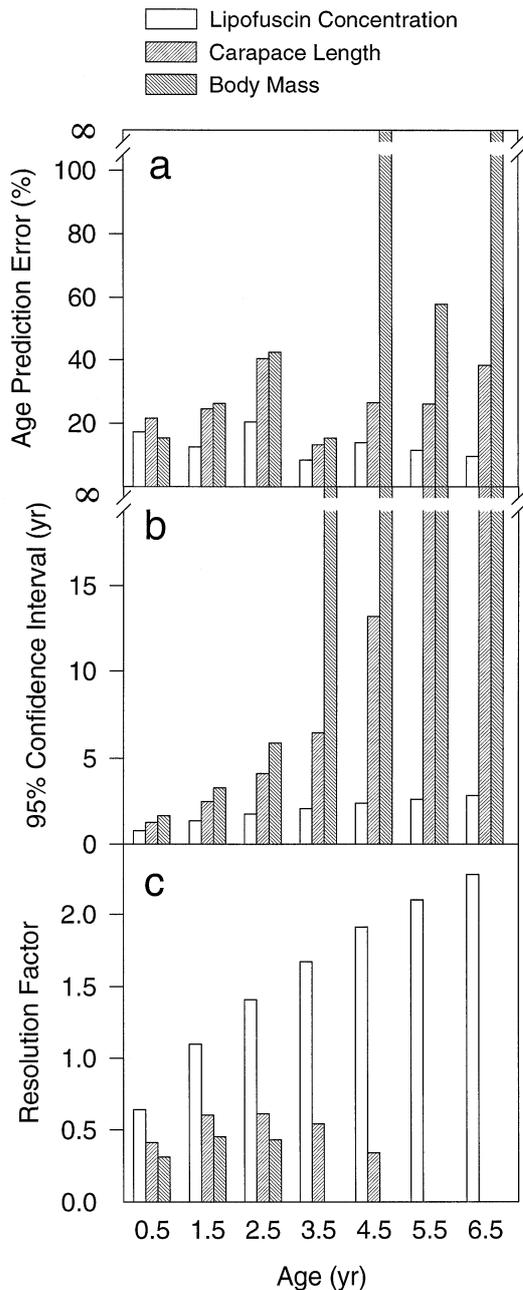


Fig. 2 (a) Mean age prediction errors, (b) 95% confidence intervals for age predictions and (c) age prediction resolution factors for different age groups of Swedish *Pacifastacus leniusculus* based on the relationships in Fig. 1 and Table 1.

poor. In contrast, the relative resolving power of OLCM lipofuscin concentration as an age predictor increased with advancing age (Fig. 2c).

The best-fitting relationship between carapace length and lipofuscin-estimated age for the reference sample of Swedish *P. leniusculus* ($r^2 = 78.6\%$) (Fig. 3)

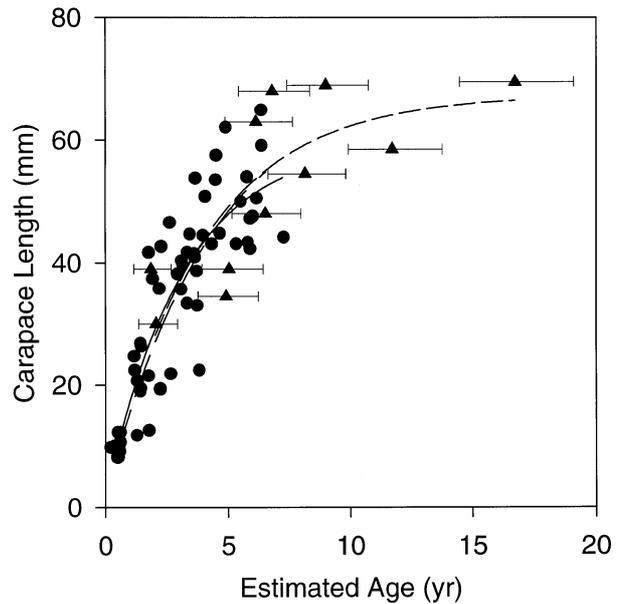


Fig. 3 Relationships between OLCM lipofuscin concentration-estimated age and carapace length in *Pacifastacus leniusculus*: Swedish cultured crayfish of known age (●), English wild crayfish of unknown age (▲) with horizontal error bars representing 95% confidence intervals for age estimates. Also shown are best-fitting regressions for carapace length vs. OLCM lipofuscin concentration-estimated age in Swedish crayfish (—) and English crayfish (---), as well as for carapace length vs. actual age in Swedish crayfish, from Fig. 1(b) (— — —).

was almost identical to that between carapace length and actual age for these crayfish ($r^2 = 84.1\%$) (Table 1, Fig. 3). The best-fitting relationship between carapace length and lipofuscin-estimated age for the wild English *P. leniusculus* also closely overlaid and extended from those of the Swedish reference sample, indicating that the crayfish from the two locations had very similar growth patterns. The largest individual in the English stream sample, a female with a carapace length of 69.5 mm, had a predicted age of 16.7 years (estimated 95% confidence interval 14.5–19.1 years). This individual was close to the predicted average maximum size for this population and differed little in size from individuals half its age.

Discussion

Many aspects of our data are similar to those for *Cherax quadricarinatus* growing in naturally variable water temperatures (Sheehy *et al.*, 1994). Values of r^2 for the relationships between age and OLCM lipofuscin concentration in both cases were 92–93%. Olfactory

lobe cell mass lipofuscin accumulated primarily as a function of age and was not influenced by size or sex. Average OLCM lipofuscin accumulation rate in the shorter-lived tropical species (2.0% VF/year) was about 10 times faster than in the longer-lived, temperate species (0.2% VF/year). This finding fits previous observations on the inverse relationship between species longevity and rate of physiological ageing (Sheehy, Greenwood & Fielder, 1995; Sheehy *et al.*, 1996). The average age prediction error obtained for the *P. leniusculus* in this study using OLCM lipofuscin concentration (13.5%) was very similar to that achieved for *C. quadricarinatus* (16.7%). In both studies this was about double the accuracy that was achieved using carapace length-based prediction (27.3% and 32.5%, respectively). In absolute terms, at a predicted age of 2 years for example, the 95% confidence limits for a lipofuscin-based estimate were -0.33 and $+0.76$ years in the case of *C. quadricarinatus* whereas they were wider at -0.75 and $+0.89$ years for *P. leniusculus*. These results confirm expectations that the absolute resolving power of a lipofuscin-based age index would decline for longer-lived species, but not its relative resolving power (i.e. as a proportion of the lifespan) (Sheehy, 1992). Irrespective of lifespan, the accuracy and resolution of age estimates based on lipofuscin are consistently better than those using body size.

For *C. quadricarinatus* (Sheehy *et al.*, 1994), there was evidence of seasonal variation in OLCM lipofuscin accumulation rate, with higher rates in summer than winter. This possibility could not be assessed in the present study because all age groups were collected at the same time of year. For longer-lived, slower-ageing species such as *P. leniusculus*, however, such short-term variations probably will not be distinguishable from individual variation within age groups.

With reference to freshwater crayfish and in response to criticism of the technique in the literature, France *et al.* (1991) re-appraised the long-established and very widely used method of modal analysis of size–frequency histograms for determining age and growth. They concluded that it could be useful provided that four tenets were strictly adhered to: (i) only crayfish collected from areas of rock substratum should be used and (ii) trapping data should be excluded (as both trapping and substratum variability introduce size biases into the data); (iii) species should have a longevity not exceeding about 4 years since, due to variability in growth, modes for year-classes older

than about 3+ are notoriously difficult to separate and (iv) sample sizes should be greater than about 200 to compensate for random variability in the data. Unfortunately, for various reasons, these criteria are difficult to achieve. On the other hand, lipofuscin-based age and growth estimation does not have any of these constraints.

Since lipofuscin concentration is a measure of relative physiological age, the main requirement for estimating chronological age using this technique is a calibration of the lipofuscin accumulation rate for the species under consideration. This can be obtained (as in this study) from individuals reared in the laboratory under a natural temperature regime or, preferably, from field mark–recaptures (Sheehy *et al.*, 1994, 1996; Belchier, 1996). For seasonal spawners, an independent approach is lipofuscin concentration–frequency analysis of the wild population (Sheehy *et al.*, 1994; Sheehy *et al.* in press). A number of factors could theoretically affect lipofuscin accumulation, although sex, brood origin, diet, oxygen supply (Sheehy, 1990c) and growth (Sheehy *et al.*, 1994, 1995, 1996; de Kerros *et al.*, 1995; Wahle *et al.*, 1996) seem to have no influence. Environmental temperature does, however, have a significant effect (Sheehy, 1990c; Sheehy *et al.*, 1994, 1995) and therefore the same calibration should not be indiscriminately applied to populations from very different thermal regimes. There are still insufficient data comparing lipofuscin accumulation in wild populations from different thermal ranges.

Olfactory lobe cell mass lipofuscin should prove useful for estimating generalised growth. In our study, the growth curve estimated from the reference sample of Swedish *P. leniusculus* using lipofuscin-estimated ages was virtually identical to that fitting the actual size-at-age data. Variation in body size at a given OLCM lipofuscin concentration provides a good indication of the range of sizes-at-age, particularly where the within-age-group variation in OLCM lipofuscin concentration can be accounted for. The use of the laboratory-derived calibration for lipofuscin-based age estimation of wild *P. leniusculus* from Gaddesby Brook is considered to be valid due to the similar environmental temperature regimes in the two places. This produced a best-fitting generalized growth curve for the English *P. leniusculus* which closely overlaid that for the Swedish signal crayfish, indicating that growth of the crayfish in the two locations was extremely similar. The predicted generalised growth

curve for these populations of *P. leniusculus* lies in the lower part of the range reported in the literature for various countries (Hogger, 1986). Initially this appears counterintuitive since the growth rate of *P. leniusculus* in England has been, in some cases, the highest on record. However, these high growth rates have been achieved by introductions into waters not previously carrying crayfish stocks. They are not sustained as population densities increase, due to competition for food and space (Hogger, 1986; Lowery & Holdich, 1988). The predicted relatively slow growth of *P. leniusculus* in Gaddesby Brook is consistent with observations that there is a high population density and many males have missing or regenerating chelae, indicating intense competition (Holdich *et al.*, 1996; Harris & Young, 1996). No statistically significant sexual differences in growth patterns for our *P. leniusculus* were detected. Although differences have been reported in the literature (Guan & Wiles, 1996), they are small compared to the wide variation in size-at-age.

The use of calibrated lipofuscin age indices will provide more realistic longevity estimates for wild individuals than has been possible before, because they are independent of body size and unlimited by artificial rearing conditions or project duration. If the slight decline in OLCM lipofuscin accumulation rates in old individuals that has been observed previously (Sheehy, 1992; Sheehy *et al.*, 1994, 1995) eventually proves to be more than an artefact of prolonged laboratory rearing, then estimated longevity will be minima only. In our sample of *P. leniusculus* from Gaddesby Brook, all individuals but one had lipofuscin-estimated ages less than 12 years. The greatest longevity for *P. leniusculus* reported in the literature is 12 years (Momot, 1984). This also happens to be the period of time over which *P. leniusculus* have existed in Gaddesby Brook from their introduction in 1985 to our sample collection in 1996. Even bearing in mind our 95% confidence limits for the estimate, the oldest individual in the sample, a female of predicted age 16.7 y, is likely to be one of the original colonists, introduced as an adult. This estimate extends the reported longevity of this species.

The findings of this study, in combination with previous results for other species, are evidence for the wide applicability of a lipofuscin-based method for determining age and general patterns of growth in freshwater crayfish. The problem of age estimation in

ecological research and living resource management is a far-reaching one. Given the apparent ubiquity of cellular lipofuscin deposition in animals, the usefulness of lipofuscin-based ageing methods may well extend beyond the Crustacea.

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