

COMMENT

Questioning the use of biochemical extraction to measure lipofuscin for age determination of crabs: Comment on Ju et al. (1999, 2001)

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ABSTRACT: In recent years, there has been considerable progress in the application of the cellular ageing biomarker, lipofuscin (LF), to age determination in crustacean fisheries in cases where no other reliable ageing methods exist. Two general approaches are available for LF measurement, the first involving fluorescence microscopy and image analysis of histological sections, and the second employing spectrofluorimetry of crude tissue extracts. However, for many years, controversy raged, mainly within the gerontological arena as to the validity of the second approach. Marine and fisheries ecologists may not be as aware of the problem. For example, Ju et al. (1999, Mar Ecol Prog Ser 185:171–179; 2001, Mar Ecol Prog Ser 224:197–205 and elsewhere) report application of the LF ageing method to demographic analysis of the economically important blue crab *Callinectes sapidus* using biochemical quantification procedures. This is an important issue because useful conclusions about valuable bioresources cannot be drawn where analysis techniques are flawed. In the present paper I discuss the main concerns regarding past attempts to quantify LF biochemically for age determination of crabs: (1) extracted fluorescence remains unidentified; (2) no correlation between extracted fluorescence and *in situ* LF concentration has ever been demonstrated; (3) there is no evidence that normalization to cellular protein produces a reliable LF assay; (4) dependence of fluorescence intensity on extracted tissue mass can be misleading; and (5) high throughput biochemical measurement does not compensate for absence of specificity. Despite the inherent challenges associated with devising a reliable biochemical quantification method for neurolipofuscin, there remains little doubt that such a development will be a significant advance both in marine- and fisheries ecology and in the wider arena of gerontology.

KEY WORDS: Age determination · Biochemical extraction · Crabs · Error · Histology · Lipofuscin quantification · Protein normalization · Specificity

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Introduction

Ju et al. (1999, 2001) report application of the lipofuscin (LF) ageing method to the economically important blue crab *Callinectes sapidus*. They employ spectrofluorimetry of whole chloroform-methanol extracts of crab brains and eyes to measure LF. These papers and others have stimulated recent interest among fisheries biologists seeking to improve upon existing size-based stock assessments for crabs and other crustaceans by

acquisition of age-structured population data. While the application to fisheries assessments of autofluorescent LF in neural tissue (neurolipofuscin) using validated microscope-based quantification procedures has made good progress in recent years (e.g. Kodama et al. 2005, 2006), the purpose of this brief critique is to highlight serious reservations concerning whole-extract spectrofluorimetry as a means of LF measurement. Criticism of this technique, while extensive, has circulated primarily in the gerontological literature

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over the last 2 decades (Eldred 1987, Sohal 1987, Sheehy & Ettershank 1988, Sheehy & Roberts 1991, Sheehy 1996, 2002, Palmer 2002, Brunk & Terman 2002), culminating in Porta's description of the method as 'totally inadequate' (Porta 2002, p. 62) and highly counterproductive to the field (Porta et al. 2002). It may be helpful, therefore, to bring this issue to the attention of marine and fisheries biologists, particularly those new to the field, in the present forum. It is an important issue because there is risk of harm to valuable biore-sources if assessment and management are based on false premise. In the following sections, I summarize the main concerns as they pertain to past work on crabs.

Extracted fluorescence remains unidentified

The fluorescent material under investigation in *Callinectes sapidus* has not been biochemically characterized. Ju et al. (1999) and Ju & Harvey (2002) follow previous studies in assuming that extracted fluorescence arises from native lipid-peroxidation components or precursors of LF. However, as clarified by Porta (2002), such assumptions are ultimately based on unwarranted extrapolation of the influential early *in vitro* studies of Chio et al. (1969) and Chio & Tappel (1969) to diverse animal tissues in studies such as Fletcher et al. (1973), upon which Ettershank's pioneering, but methodologically questionable attempts to develop LF as an ecological tool were subsequently based (Ettershank 1983, Ettershank et al. 1983, Sheehy & Ettershank 1988, Sheehy & Roberts 1991). Recent molecular analysis suggests that lipid peroxidation products are indeed a component of LF (Schutt et al. 2003, Szweda et al. 2003) but they are unlikely to be the source of extractable auto-fluorescence. Dissolution of isolated neurolipofuscin granules results in a complete loss of fluorescence (Palmer et al. 2002). The latter authors have proposed that neurolipofuscin granule 'autofluorescence' is actually a type of light scatter due to the orderly packing of protein molecules into subcellular organelles. On the other hand, a few fluorophores from LF in the human retinal pigment epithelium (RPE) have been successfully extracted, purified and biochemically characterized (Eldred & Lasky 1993, Parish et al. 1998, Fishkin et al. 2005) but these turn out to be novel molecules and not lipid peroxidation products. They emit at orange wavelengths like *in situ* LF, but unlike the violet fluorescence of *C. sapidus* extracts. Ju et al. (1999) explain this colour discrepancy in terms of a concentration-dependent shift in emission wavelength—a theory originally proposed by Yin & Brunk (1991), again based on *in vitro* experiments. However, Brunk & Terman (2002, p. 613) have recently clarified that this discrepancy 'can

also arise from differential extractability of fluorophores with varying spectral characteristics, suggesting that LF quantification based on extracts is most certainly misleading and should be avoided'. It has long been known that animal tissues contain a diverse array of autofluorescing compounds, many of which are unrelated to age or LF, and which excite and emit at similar wavelengths to those claimed by Ju et al. (1999) for LF. These include derivatives of the amino acid tryptophan, simple proteins, carotenoid-, vitamin A-, vitamin B6- and folic acid-derivatives, and pyridine nucleotides to name but a few (Udenfriend 1962, 1969, Sheehy & Ettershank 1988). An additional source of confusion and misdiagnosis in past 'soluble lipofuscin' studies is that an unrelated class of chloroform-methanol-soluble autofluorescent pigments, the pteridines, can, like *in situ* LF, accumulate with age. However, age-dependent pteridines appear to be restricted to certain insects and appear to represent a form of nitrogenous waste disposal by storage excretion in order to conserve water in a terrestrial environment (Sheehy & Roberts 1991). Due to the difficulty of isolating LF in sufficient quantities, and its heterogeneity and poor solubility, sophisticated techniques are required to purify, biochemically diagnose and reliably assay mammalian RPE LF fluorophores (Eldred & Lasky 1993, Reinboth et al. 1997, Parish et al. 1998, Fishkin et al. 2005). No such techniques have been developed for neurolipofuscin or invertebrate LF in any tissue. This suggests a high probability of contamination and very poor specificity for LF in the measurement procedure of Ju et al. (1999). Under such conditions, and without quantum yields for crab LF fluorophores, the conversion of extract autofluorescence intensities to $\mu\text{g-LF}/\text{mg-protein}$ units (Ju et al. 1999, p. 173, 2003, p. 317) does not seem appropriate.

No correlation between extracted fluorescence and *in situ* lipofuscin

Contaminating insect pteridines aside, no correlation has ever been found between the amounts or concentrations of lipid-soluble fluorescent materials and the amounts of *in situ*, visually verified and morphometrically measured LF (Porta 2002) as demonstrated by studies on a range of species including crustaceans (Donato & Sohal 1978, Bieri et al. 1980, Beatty et al. 1982, Sheehy 1996, 2002). As a result, Sheehy (1996, p. 430) 'strongly recommended that researchers, particularly on aquatic species, avoid using the original biochemical assay procedure for LF and its more recent modifications'. No comparison of extracted fluorescence and *in situ* LF concentrations has been presented for *Callinectes sapidus*.

No evidence that normalization to cellular protein produces a reliable lipofuscin assay

Ju et al. (1999, 2003) assert that fluorescence normalization to cellular protein produces a sensitive measure of LF in *Callinectes sapidus* because it eliminates problems with the accurate measurement of the mass of small pieces of tissue. However, they do not present any evidence to support either element of this assertion. If the problem with the extraction approach to LF quantification was simply inaccurate measurement of small tissue masses, then the strong correlations ($r = 0.9$) reported between extracted autofluorescence intensity and the microgram and milligram masses of individual dried *Daphnia carinata* (Sheehy & Ettershank 1988) and fresh crayfish brains (Sheehy 1996), respectively, should not have been possible. Furthermore, Crossland et al. (1988) considered protein concentration as an alternative to fresh weight for normalizing fluorescence extracted from lobster eyes, but rejected this approach because it resulted in greater variability. Ju et al. (1999) do not address these earlier findings.

Dependence of fluorescence intensity on extracted tissue mass can be misleading

Not surprisingly, indeterminate extractable autofluorescence intensity or mass-normalized intensity is often a direct linear or curvilinear function of the size or amount of the extracted tissue, organ or animal (Crossland et al. 1988, Sheehy & Ettershank 1988, Sheehy 1996). This can create a misleading impression of direct age-relatedness of the soluble fluorescence if organ or animal size is broadly correlated with age (Sheehy 1996), as can be the case for crabs (Sheehy & Prior 2008, this volume). This pitfall will be further obscured if the strong dependence of fluorescence on tissue sample mass is masked by the random variability that is reportedly introduced by protein normalization (Crossland et al. 1988). In such circumstances, why use extractable fluorescence intensity as a proxy for age when size is much easier to measure? Indeed, Crossland et al. (1988, p. 18) concluded that fluorescence extracted from lobster eyes was 'no more effective than the traditional measure of chronological age, carapace length.' No data on the relationship between raw fluorescence and eye size or extracted tissue mass have been presented for *Callinectes sapidus*. The question of whether a trivial relationship between

indeterminate extracted fluorescence intensity (or normalized fluorescence intensity) and tissue sample mass, body size, or instar in *C. sapidus* has been misconstrued as age-dependent LF accumulation can be easily resolved by comparison of extracted fluorescence intensities from the eyes of individuals of the same known age but very different sizes, and from individuals of the same size but very different known ages, all grown under identical environmental conditions. Such individual growth variability is common in lab-reared decapods. While true LF can correctly identify chronological ages in such circumstances (Sheehy 1990), it is expected that extractable autofluorescence can not. No such comparison has yet been reported for *C. sapidus*.

High throughput measurement does not compensate for absence of specificity

In response to previous expressions of concern regarding their methodology (Sheehy 2000), Harvey et al.¹ argue simply that solvent extraction is faster than histology and image analysis. This may be true, but what value is rapid measurement if it is not measuring the compound of interest? It would be convenient if the solution to the problem of quantification of LF by spectrofluorimetry of solvent extracts was only as simple as the mere removal of supposed error associated with the measurement of the mass of the tissue being extracted. On the contrary, accurate biochemical measurement of this highly complex, poorly soluble, spatially and temporally variable target material that is usually present in vanishingly small quantities within a complex mix of contaminants has proved problematic to gerontologists for decades. Despite this challenge, there remains little doubt that future development of a rapid, specific, and precise biochemical assay for neurolipofuscin, probably based on criteria other than soluble lipid autofluorescence, will be an important development in marine and fisheries ecology, and in the wider arena of gerontology.

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