Table

Table 1. Determination of directed network (digraph) complexity or connectivity measured as Connectance ratio (C_V - Conn %).

Source Node*	Edge/Link (interaction)	Target Node*	Biomarker for interaction value	Interaction value (edge/link weight)	
				Control	*Fasted*
LF	LF-AUT	AUT	*LMS*	1	1.146128
LF	LF-NL	NL	*LMS*	1	1.146128
NL	NL-AUT	AUT	*Lipid*	1	1.173186
LOX	LOX-NL	NL	*MDA*	1	1.222716
LOX	LOX-AUT	AUT	*MDA*	1	1.222716
LOX	LOX-LF	RB	*MDA*	1	1.222716
AUT	AUT-RB	RB	*Lf*	1	1.161368
AUT	AUT-AUT	AUT	*Lf*	1	1.161368
E				**8**	**9.456328**
||E||				**8**	**9**
C_V **%**				***32***	***36***

Interaction attributes for the links (edges/arcs) in the directed cellular physiological network are based on the standardised mean biomarker values as a proportion of the control value. **E** is the sum (**Σ)** of the links using the weight for each edge/arc (i.e., **Σ** interaction values). Connectance % -- $C_V = (||E|| / V^2) \times 100$, where ***V*** is the number of nodes in a directed network or digraph, ***||E||*** is the nearest integer function of ***E*** (Bonachev, 2003; Davis, 1997; Moore, 2010). Weight values are all $\log_{10}$ transformed. Mann-Whitney U-test on Control v Fasting – $\mathbf{P \leq 0.01}$, n = 8. *LMS – lysosomal membrane stability; Lipid – cytosolic and lysosomal neutral lipid (triglyceride); MDA – malondialdehyde; Lf – lipofuscin*. *See Figure 2 for node accronyms.

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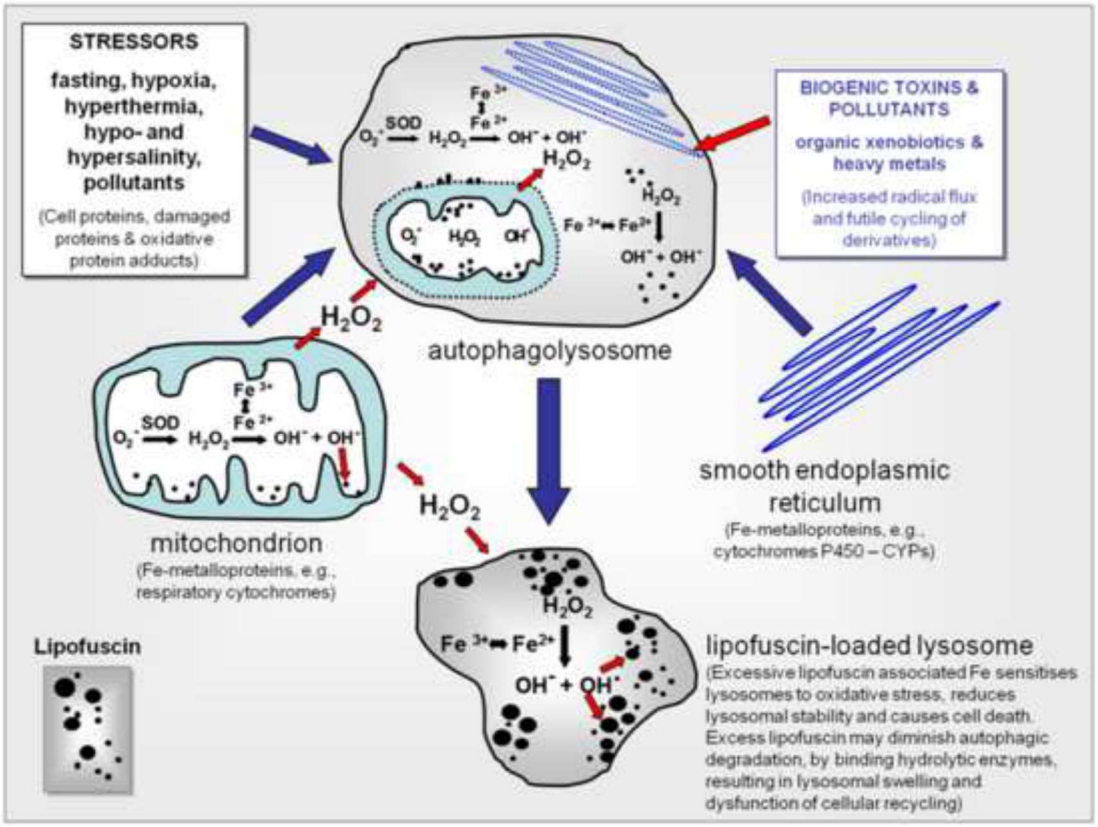

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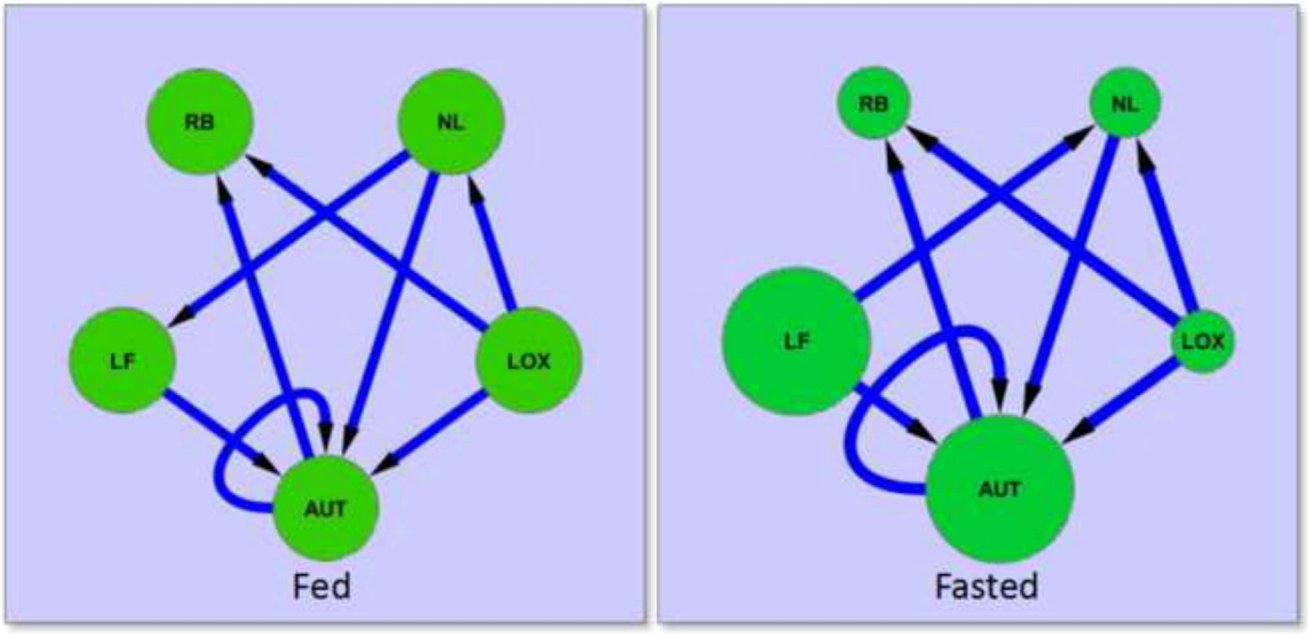

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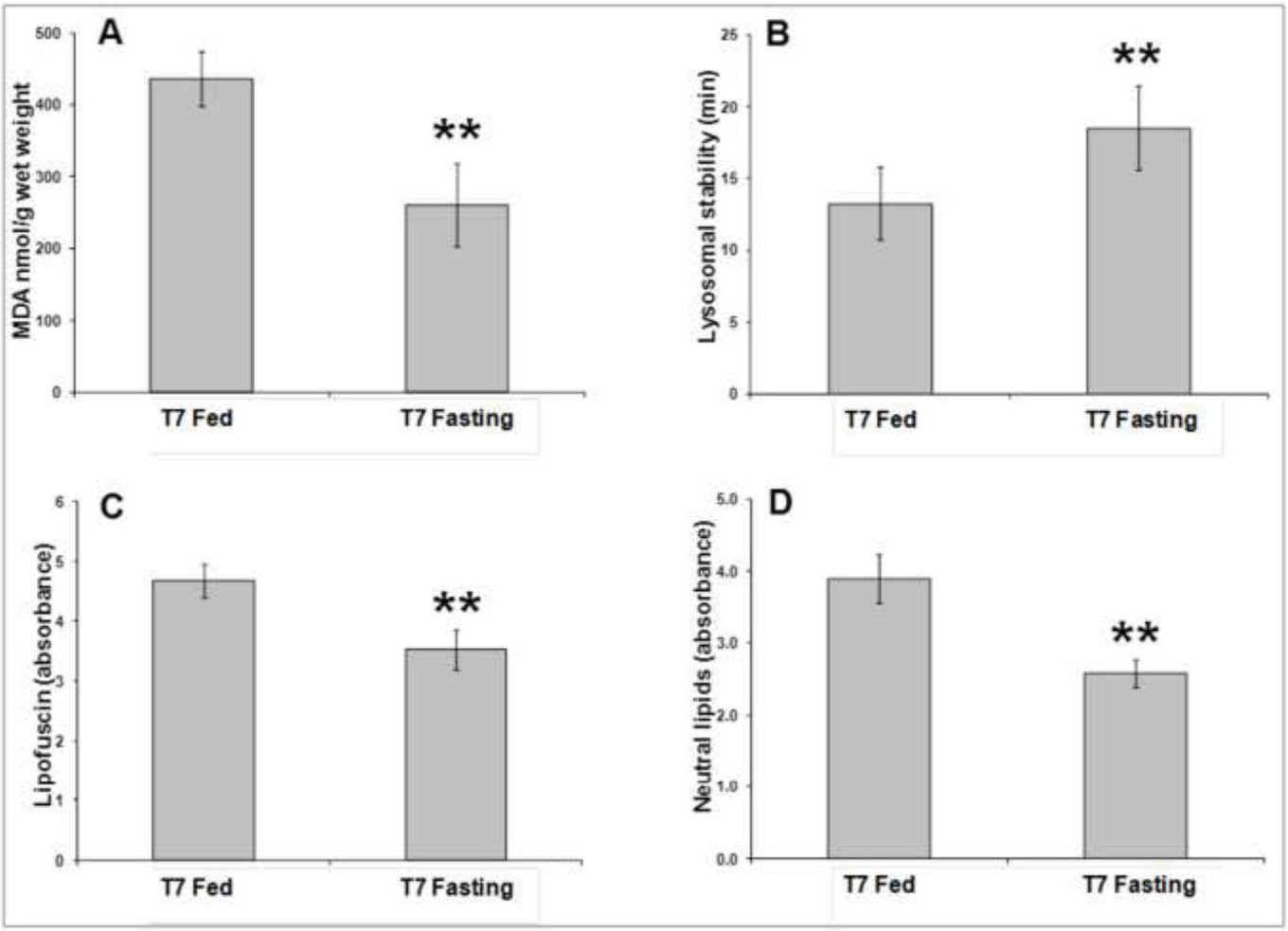

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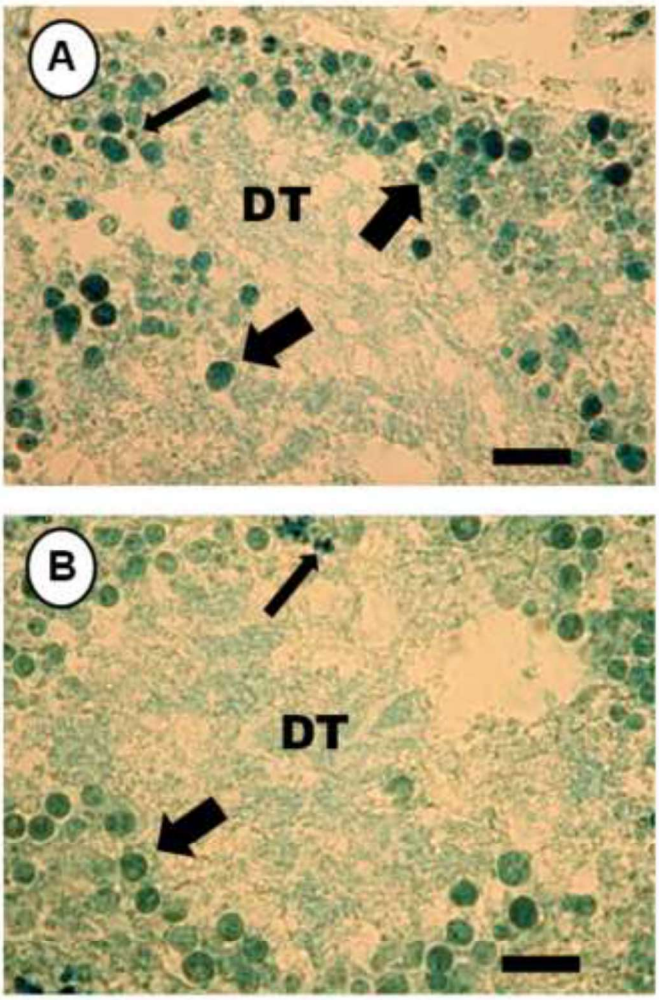

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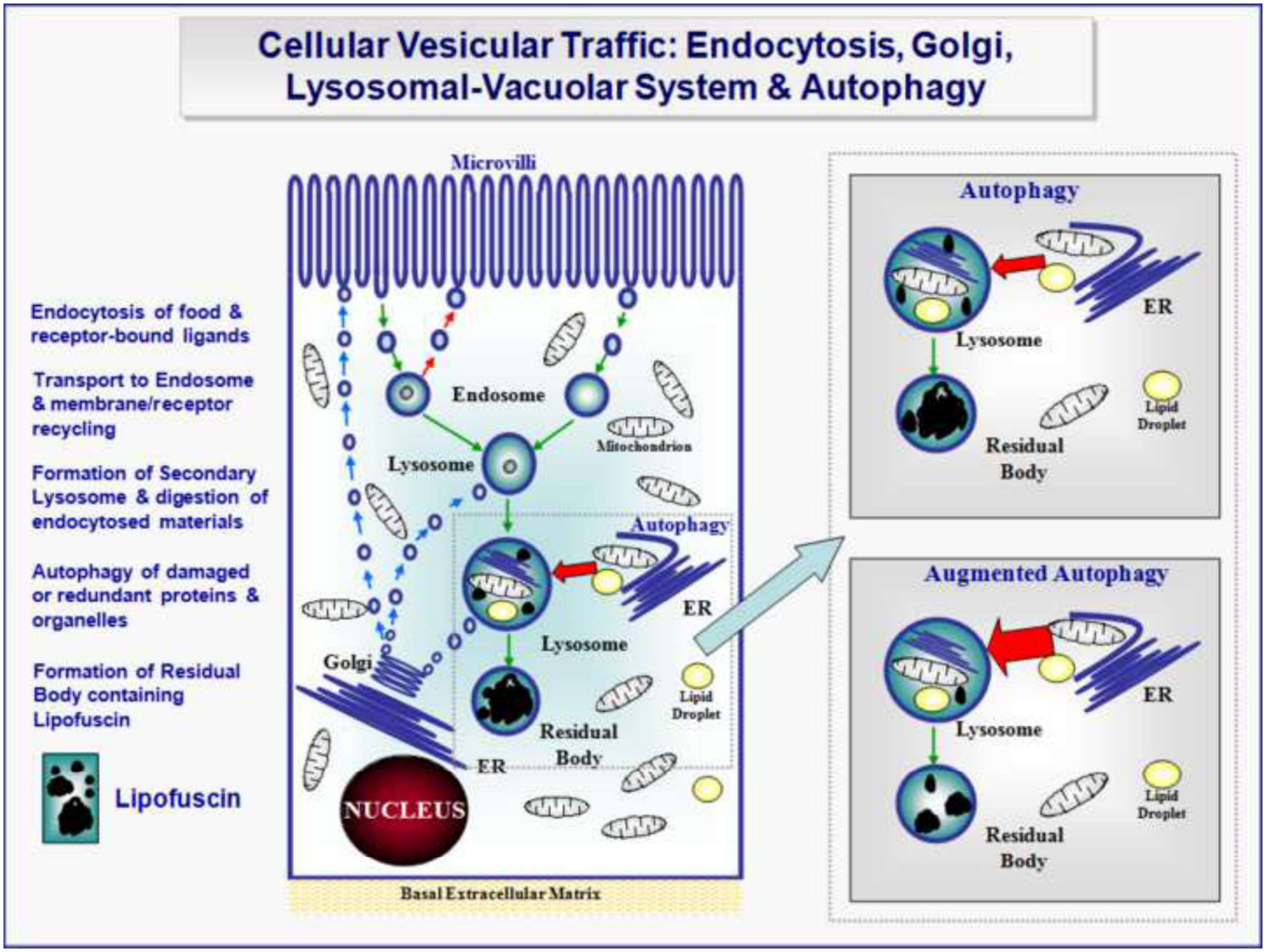

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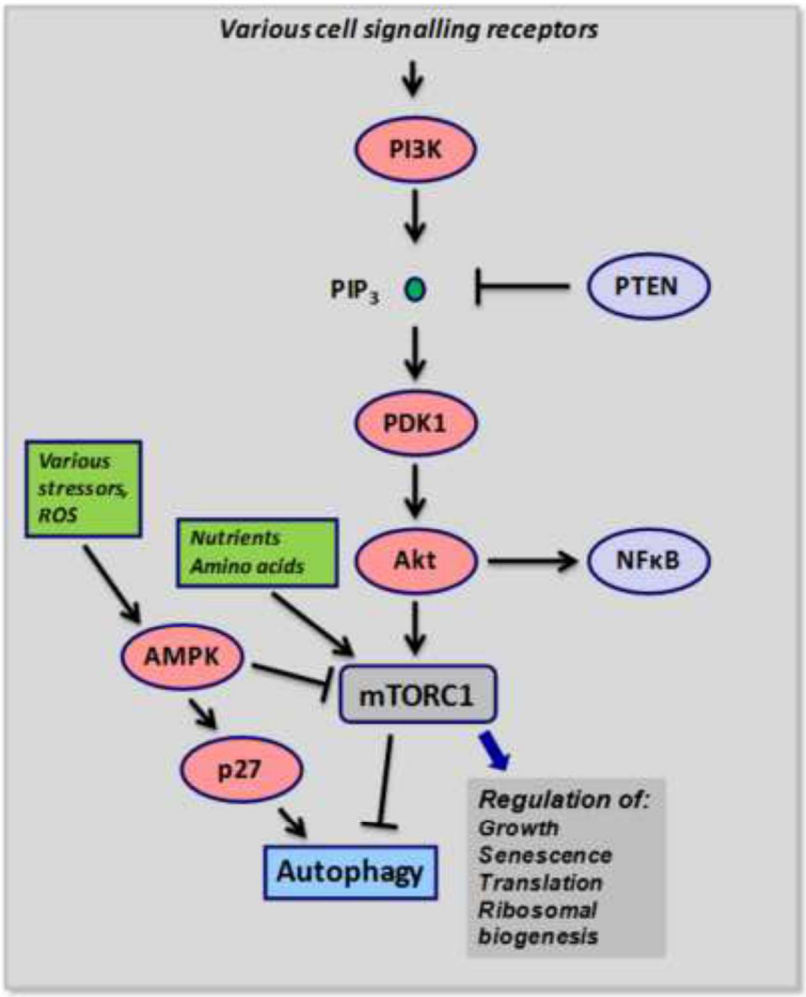

Figure Legends

Fig. 1. Conceptual model for the role of reactive oxygen species (ROS) and lipofuscin in lysosomal autophagy and oxyradical-mediated cell injury. This model draws on one proposed by Brunk and Terman (2002) and adapted by Moore et al. (2006a). Fe^{2+} - ferrous cation; Fe^{3+} - ferric cation; $O_2^{\bullet}$ - superoxide; OH^- - hydroxyl anion; $OH^{\bullet}$ - hydroxyl radical; SOD – superoxide dismutase.

Fig. 2. Topology of cellular physiological networks (directed - *digraphs*) for fed and fasted treatments, constructed using ***Cytoscape 2.8*** (Shannon et al., 2003). LF – lysosomal function; AUT – autophagic function; NL – lysosomal and cytoplasmic lipid (triglyceride); LOX – lipid peroxidation (malondialdehyde); RB – lipofuscin generation in late secondary and tertiary lysosomes. Node size indicates the attributes for the physiological process based on the relevant biomarker values (see Table 1). The networks are constructed as attribute circle networks based on degree (i.e., number of links / node). Biomarkers related to node values: LF (LMS – lysosomal membrane stability); AUT (LMS - lysosomal membrane stability used as a proxy as no direct measurement of autophagy was made); NL (Lipid – cytosolic and lysosomal neutral lipid - triglyceride); LOX (MDA – malondialdehyde); RB (Lf – lipofuscin).

Fig. 3. Effects of fasting on **A** - lipid peroxidation (MDA), **B** - lysosomal stability (minutes – based on latency of β-glucuronidase) and accumulation of **C** - lipofuscin and **D** - lipid (absorbance in arbitrary units) in hepatopancreatic cells. Bar graphs show the relative effects in fasting (day 7) and control (day 7) animals. Mean value ± 95% confidence limits, n = 10 (Asterisk - $P \leq 0.01$, Kruskall-Wallis test between Fed day 7 control – T7 Fed and Fasting day 7 – T7 Fasting treatments).

Fig. 4. Micrographs of snail hepatopancreas showing a digestive tubule (DT) reacted with the Schmorl test for lysosomal lipofuscin (dark staining) in control - T7 Fed (A), and reduced reaction product for lipofuscin in fasting - T7 Fasting (B) hepatopancreatic digestive cells. Many of the secondary lysosomes are enlarged in the fasting treatment compared with the fed controls. Bold arrows - lipofuscin in late secondary lysosomes; small arrows - lipofuscin in tertiary lysosomes. Scale bar = 10 μm.

Fig. 5. Diagramtic representation of the normal autophagic turnover of old or damaged proteins and organelles (e.g., mitochondria, endoplasmic reticulum – ER) which results in the gradual accumulation of lipofuscin and other aggregates. Stress-induced augmented autophagy reduces the accumulation of lipofuscin and aggregates by recycling organelles and protein more rapidly.

Fig. 6. Simplified diagram of the multiple cell signalling pathways involving mTOR (see Laplante & Sabatini, 2009, 2012, for a more extensive chart of mTOR related cell signalling). Overactivity of mTORC1 is believed to trigger inflammatory processes which can result in pathological injury and processes leading to many cancers and degenerative diseases. PI3K -phosphatidylinositol-3 kinase; PIP_3 - phosphatidylinositol 3,4,5 trisphosphate; Akt - serine/threonine kinase Akt or protein kinase B (PKB); mTORC1 - mammalian target of rapamycin complex 1; NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells; PTEN - phosphatase and tensin homolog; AMPK - 5' adenosine monophosphate-activated protein kinase; p27 - cyclin-dependent kinase inhibitor; ROS – reactive oxygen species. Activation □; inhibition □

Supplementary MATLAB .fig files

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Anti-oxidative cellular protection effect of fasting-induced autophagy as a mechanism for hormesis

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Abstract

The aim of this investigation was to test the hypothesis that fasting-induced augmented lysosomal autophagic turnover of cellular proteins and organelles will reduce potentially harmful lipofuscin (age-pigment) formation in cells by more effectively removing oxidatively damaged proteins. An animal model (marine snail - common periwinkle, *Littorina littorea*) was used to experimentally test this hypothesis. Snails were deprived of algal food for 7 days to induce an augmented autophagic response in their hepatopancreatic digestive cells (hepatocyte analogues). This treatment resulted in a 25% reduction in the cellular content of lipofuscin in the digestive cells of the fasting animals in comparison with snails fed *ad libitum* on green alga (*Ulva lactuca*). Similar findings have previously been observed in the digestive cells of marine mussels subjected to copper-induced oxidative stress. Additional measurements showed that fasting significantly increased cellular health based on lysosomal membrane stability, and reduced lipid peroxidation and lysosomal/cellular triglyceride. These findings support the hypothesis that fasting-induced augmented autophagic turnover of cellular proteins has an anti-oxidative cytoprotective effect by more effectively removing damaged proteins, resulting in a reduction in the formation of potentially harmful proteinaceous aggregates such as lipofuscin. The inference from this study is that autophagy is important in mediating hormesis. An increase was demonstrated in physiological complexity with fasting, using graph theory in a directed cell physiology network (digraph) model to integrate the various biomarkers. This was commensurate with increased health status, and supportive of the hormesis hypothesis. The potential role of enhanced autophagic lysosomal removal of damaged proteins in the evolutionary acquisition of stress tolerance in intertidal molluscs is discussed and parallels are drawn with the growing evidence for the involvement of autophagy in hormesis and anti-ageing processes.

Key words: *anti-ageing, age-pigment, autophagy, caloric-restriction, cell network model, cytoprotection, hormesis, lipid peroxidation, lipofuscin, lysosome, lysosomal membrane stability, mollusc, mTOR, protein aggregates, reactive oxygen species, stress tolerance*

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Introduction

Normal metabolic generation of reactive oxygen species (ROS), including oxy-radicals, can cause oxidative attack on the protein machinery and organelles of the cell (Livingstone, 2001; Regoli, 2000). Increased removal of damaged cellular constituents by autophagy will conserve cell function; and also reduce the amount of age-pigment (lipofuscin) produced (Cuervo, 2004; Moore et al., 2006a, b, c, 2007). Consequently, an effective capability to up-regulate the autophagic process will be advantageous to organisms exposed to environmental influences such as many environmental toxins and pollutants which can contribute to increased generation of ROS (Moore, 2008; Moore et al., 2006c). Lipofuscin accumulates in lysosomes as a result of peroxidation of autophagocytosed proteins associated with protein aggregates and oxidatively damaged organelles; and was previously considered to be just cellular junk (Fig.1; Brunk & Terman, 2002). However, recent evidence indicates that lipofuscin binds iron, which generates ROS, probably resulting in exacerbation of oxidative damage and sequestration of proteases, thereby, inhibiting lysosomal degradation (Brunk & Terman, 2002; Grune et al., 2004). This in turn may lead to “incomplete or failed autophagy” with autophagic accumulation of essentially undegradable damaged organelles, proteins, phospholipids and lipids that will produce more lipofuscin (Brunk & Terman, 2002; Cuervo, 2004; Grune et al., 2004, Lüllmann-Rauch, 1979; Moore et al., 2006a, b, c, 2007).

Molluscan species such as bivalve mussels and marine snails provide useful models for studying autophagic function, as autophagy can be readily induced by starvation, salinity change, hyperthermia and hypoxia in the cells of the hepatopancreas or digestive gland, which is the liver analogue in molluscs (Bayne et al., 1978; Lowe et al., 2006; Moore, 2008; Moore & Halton, 1973, 1977; Moore et al., 1986, 2007; Owen, 1970). These species have been extensively investigated, particularly with respect to the harmful effects of pollutant chemicals such as toxic metals and polycyclic aromatic hydrocarbons (Moore et al., 1985). Previous studies using bivalve molluscs have indicated that fasting-induced autophagy has a cytoprotective effect against oxidative stress (Moore, 2004; Moore et al., 2006b, 2007); and Moore and Stebbing (1976) demonstrated that autophagy was involved in hormesis induced by very low concentrations of copper, cadmium and mercury in a colonial hydroid. Hormesis is a biphasic dose response to an environmental agent characterized by

low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect (Mattson, 2008).

This investigation was designed to test the hypothesis that augmented autophagic turnover of oxidatively damaged proteins reduces lipofuscin (age-pigment) formation in hepatopancreatic digestive cells of the marine snail or periwinkle *Littorina littorea*. Snails were subjected to fasting (nutritional deprivation) for a period of seven days in order to induce autophagy (Moore & Halton, 1973; Moore et al., 1986), and the relative content of intralysosomal lipofuscin was then determined cytochemically in comparison to fed control snails. Additional parameters measured included lysosomal membrane stability, cytoplasmic and lysosomal neutral lipid (triglyceride) and lipid peroxidation.

Modelling of whole biological systems from cells to organs is gaining momentum in cell biology and disease studies. This pathway is essential for the derivation of explanatory frameworks that will facilitate the development of a predictive capacity for estimating outcomes or risk associated with particular disease processes and therapeutic or stressful treatments (Moore & Noble, 2004). In this context, a parallel modelling exercise used a modified version of the generic cell network model described by Moore (2010) in order to accommodate the available biomarker data. The original generic model was developed from extensive published data in the environmental toxicology and biomedical literature, and the large-scale organisation of metabolic networks (Cuervo, 2004; Di Giulio & Hinton, 2008; Jeong et al., 2000; Klionsky & Emr, 2000; Zhang & Zhang, 2009). This cellular interaction network was constructed around the essential processes of feeding, excretion and energy metabolism (Moore, 2010). Protein synthesis and degradation, including lysosomal autophagy, are also incorporated in the model as are the major protective systems (Cuervo, 2004; Di Giulio & Hinton, 2008; Livingstone et al., 2000; Moore, 2008). In order to determine whether complexity can be used as an indicator of health, the hypothesis that pathology involves a loss of biological complexity has been tested using the above mentioned generic physiological interaction network.

System complexity and network topology was evaluated using network connectedness (connectance C_V%), as well as node size, node degree, interaction weighting and network diameter. Previous research has shown that the complexity of the whole system increases when sub-systems, such as detoxication and anti-oxidant protective processes, augmented autophagy, protein degradation and

induction of stress proteins, are up-regulated and start to interact significantly as part of a response to low-level stress, (i.e., biphasic or hormetic response; Moore, 2010). However, with increasing severity of stress, cell injury and higher-level functional impairment lead to physiological dysfunction and breakdown of the whole interaction network with consequent loss of complexity (Moore , 2010). The type of network model used in this investigation (i.e., network and graph theory) will provide a mathematical formalism that can facilitate the system-level interpretation of health and dysfunction in living cells (Moore, 2010).

Mathematical models provide the conceptual and mathematical formalism to integrate molecular, cellular and whole animal processes (Allen & McVeigh, 2004; Allen & Moore, 2004; Moore & Noble, 2004). This will help to target "the knowledge gaps", and contribute to the development of a "theoretical ecotoxicology". Such tools will prove invaluable for the future safeguarding of the aquatic environment and the development of legislation for integrated ecosystem management.

Materials and Methods

Animals and husbandry

Snails (*L. littorea*) of shell length 20-25 mm were collected from the intertidal shore at Port Quin harbour (North Cornwall) in July 2008.

The experimental animals were held in a re-circulating seawater system for 2 days at 15±1°C and allowed to graze freely on sea lettuce (the green alga *Ulva lactuca*) also collected from Port Quin. Water quality parameters were monitored during the course of the experiment (dissolved oxygen, ammonia, nitrate, nitrite, pH and salinity (34.7 $\pm$ 0.5 psu).

All experimental animals which climbed above the water level in their tanks were regularly detached and replaced on the bottom of the tank to try to ensure similar migratory behaviour and energy expenditure in both fed and fasting treatments.

Experimental treatments

After 2 days the snails were divided into two treatment groups (algal fed *ad libitum* on the green alga *U. lactuca* and fasting) with 2 replicates of 10 snails for each

158 experimental treatment at 15±1°C and allowed to graze freely. The snails were
159 exposed to a natural regime of daylight and darkness (July - natural daylight
160 conditions). The treatments were maintained for a period of 7 days; and samples
161 taken at the start of the experimental treatments (0 days and after 7 days) with equal
162 numbers of animals (2 x 5) being sampled from both replicates.
163
164 ***Sample preparation***
165 Sails were sacrificed by removal of the shell and excision of the visceral mass. The
166 visceral mass containing the liver analogue or hepatopancreas was tranversely
167 sectioned to provide a tissue sample approximately 5 x 5 x 5 mm in volume. Tissue
168 samples for cytochemistry were subsequently frozen in liquid nitrogen onto
169 aluminium chucks for tissue sectioning with a cryostatic microtome (Bright Ltd.) as
170 described in Moore et al. (2004). The remaining animals (2 x 5 from each replicate)
171 were used for biochemical analysis of malondialdehyde (MDA).
172
173 ***Tissue sectioning and cytochemistry***
174 Duplicate sections for each animal were cut at a thickness of 10 µm and transferred
175 to clean glass microscope slides. Frozen tissue sections were reacted cytochemically
176 for lysosomal stability (using latency of β-glucuronidase), lipofuscin (Schmorl
177 reaction), triglyceride or neutral lipid (oil red-O) (Bayliss High, 1984; Moore, 1976,
178 1988; Moore et al., 2004, 2008).
179
180 Cytochemical reaction products were measured in the tissue sections (5 fields at x
181 400 magnification in each duplicate section) for lipofuscin and neutral lipid
182 (triglyceride) respectively using a graded series of photomicrographs with reaction
183 intensities previously determined by microdensitometry (Moore et al., 2006c).
184
185 ***Lipid peroxidation – malondialdehyde (MDA)***
186 Lipid peroxidation was measured in hepatopancreatic tissue as concentration of MDA
187 as described in Shaw et al. (2004). Whole digestive glands were washed with ice
188 cold 0.9% NaCl and homogenised with 20 mM TRIS-HCl, pH 7.4 (1:10 w:v) at 4°C.
189 The homogenate was centrifuged at 3,000 *g* at 4°C for 20 minutes. Tissue MDA
190 levels were derivatised in a 1 ml reaction mixture containing a final concentration of
191 6.7 mM 1-methyl-2-phenylindole, (dissolved in acetonitrile), 150 µl 37% hydrochloric
192 acid and 200 µl sample or standard (10 mM 1,1,3,3-tetramethoxypropane, in 20 mM
193 TRIS-HCl, pH 7.4). The tubes were vortexed and incubated at 45°C for 40 minutes.

Samples were cooled on ice, centrifuged at 15,000 *g* for 10 minutes and read at 586 nm. Results were expressed as MDA nmol/g wet weight.

Network modelling of biomarker data

Model description

The generic cell model described by Moore (2010) has been developed from extensive published data in the environmental toxicology and biomedical literature, and the large-scale organisation of metabolic networks (Cuervo, 2004; Di Giulio & Hinton, 2008; Jeong et al., 2000; Klionsky & Emr, 2000). The generic cellular interaction network was constructed around the essential processes of feeding, excretion and energy metabolism. Protein synthesis and degradation, including lysosomal autophagy, are also incorporated in the model as are the major protective systems (Cuervo, 2004; Di Giulio & Hinton, 2008; Livingstone et al., 2000; Moore, 2008). A subset of the generic model was used in this investigation in order to accommodate the available data (Fig. 2). The cellular physiological networks were constructed using ***Cytoscape 2.8*** (Shannon et al., 2003).

Analysis of cell system complexity

Whole system complexity in the directed cellular physiological network was evaluated using connectedness (Bonchev, 2003). Topological complexity was measured as connectedness or connectance (*Conn %*) is the ratio between the number of links ***E*** in the interaction network and the number of links in the complete graph having the same number of nodes or vertices (***V***) (Bonchev, 2003). Connectedness relates the number of nodes (vertices) ***V*** and links or edges (**arcs** in a directed link) ***E*** where the connectance ratio, C_V, of a directed graph (digraph) with ***V*** nodes or vertices is then:

$$C_V = [(1 / max (C_V)]||E|| \times 100$$

which reduces to:

$$C_V = (||E|| / V^2) \times 100$$

for typical digraphs that allow every node to connect to every other node, where $||E||$ is the nearest integer function of ***E*** (Davis, 1997). This method uses the sum of the **edge weights** rather than the **edge** count and allows for self-loops or arcs, as with the autophagy process (Fig. 2).

Biomarker data were used to attribute proportional weight values (illustrated as edge width; Fig. 2) to the interactions (edges) between cellular physiological processes

(nodes) as shown in Table 1; and to the nodes, as node size (Fig. 2). The various biomarker mean values were standardised to a proportion of Control (Fed 7 days) values. These values (***x***) were used for biomarkers that normally decrease with pathology (e.g., lysosomal membrane stability), while biomarkers that normally increase with pathology (e.g., neutral lipid, lipid peroxidation & lipofuscin) were further transformed to (x^{-1}). These values were normalised using $log_{10}(10.x)$ transformation and then inputted as the weight values for the network interactions (edges/links). The standardised biomarker values were used to set node size for comparisons of network topology (see Fig. 2).

The cell physiology networks generated (Fig. 2) were also tested for generic network structure by analysing the relationship between nodes and links according to network theory (Jeong et al., 2000; Zhang & Zhang,2009). Node degree was determined from the number of edges (arcs) associted with a specific node (summation of in-arcs and out-arcs), and network diameters were calculated according to the equation:

$$D \approx l_n V / l_n k$$

where ***D*** is the network diameter; ***V*** is the number of nodes; and ***k*** is the mean number of edges per node.

Statistical analysis

Multiple range and Kruskall-Wallis tests were applied to the treatment groups replicate data using Statgraphics Plus 5.0. The proportional edge (interaction) values in the Control and Fasting treatments were tested using the non-parametric Mann-Whitney U test (2-tailed). Node size values were tested using the non-parametric Z-test (2 population proportions categorical test).

Results

Fasting for 7 days resulted in a significant increase in lysosomal membrane stability and significant reductions in lipid peroxidation (MDA), lysosomal content of lipofuscin and cytoplasmic/lysosomal neutral lipid in the hepatopancreatic digestive cells compared with the fed animals (pooled replicates, $P \leq 0.01$, n = 10, Kruskall – Wallis test, Figs. 3 & 4).

Structurally the digestive tubules of the hepatopancreas were similar in appearance in both treatments with secondary and tertiary lysosomes being present in similar numbers in the digestive cells, although there was evidence of enlargement of secondary lysosomes in the hepatopancreatic digestive cells, indicative of autophagic response, in the fasting treatment (Fig. 4). Lipofuscin was primarily localised in late seconday lysosomes and tertiary lysosomes (residual bodies) in the digestive glands from snails in both treatment groups (Fig. 4). Snails sampled at the start of the experiment had a similar lipofuscin content in their digestive cells to those of the fed controls (92.3% ± 14.3; 95% CL as percentage of the day 7 fed control).

Inputting the biomarker data into the directed cellular interaction network (digraph) model (Fig. 2) allowed the determination of the **system complexity**. Complexity values as connectance ratio for the two experimental treatments are shown in Table 1, with a considerable significant increase in connectivity in the fasted condition compared with the fed controls (**$P \leq 0.01$**, ***Mann-Whitney U test***, n = 7, 2-tailed test).

The fed and fasted network topologies differ in node size (Fig. 2; ***Z- test***, score is -2.0702, **$P < 0.05$**, n = 5, 2-tailed test), although network diameters remained the same for both treatments. The determination of node degree indicated that autophagy was the most highly connected node with 5 degrees (i.e., summation of 1 out-arc, 3 in-arcs and 1 loop), making it an important physiological hub (Fig. 2). The network diameters (***$D \approx I_n N / I_n k$***) were small (***$D \approx 1.56$***), which is consistent with biological networks and remained unchanged by the fasting treatment (Zhang & Zhang, 2009).

Discussion

A reduction in lysosomal lipofuscin was observed following a period of experimental fasting. A similar reduction in lipofuscin has been shown in the digestive cells of fasting marine mussels exposed to ionic copper (Moore et al., 2007). The inference here is that augmented autophagy is reducing the lipofuscin content of these hepatopancreatic cells; and previous studies with these snails have shown that fasting induces autophagy (Moore et al., 1986).

Concurrent reductions in lipid peroxidation (MDA) and cellular/lysosomal lipid content were also observed, along with increased lysosomal membrane stability indicating that the hepatopancreatic digestive cells were measurably healthier in the fasting

snails (Moore et al., 2006a, b). Fasting animals may have had lower energy requirements, which could possibly contribute to a reduction in ROS generation, particularly in their mitochondria; although all animals had similar migratory activity imposed to minimize differences in energy expenditure (see Materials & Methods). However, in many molluscs the mitochondria are probably not a major source of ROS generation in hepatopancreatic cells: the lysosomal compartment is the main source as disccussed later (Fig. 1; Winston et al., 1991). Furthermore, the fasting animals will not be in a serious starvation situation within the time period of the experiment (7 days), since the hepatopancreatic digestive cells of gastropod molluscs are rich in reserves of glycogen and lipid (Moore and Halton, 1973, 1977). These factors considered together will hopefully have minimized this issue as an interpretational problem, however, further experimentation to determine the contribution of mitochondrial *versus* lysosomal generation of ROS would undoubtedly be helpful in further clarifying this.

Autophagy is often considered to be primarily a survival strategy in multicellular organisms, which either is initiated by stressors (e.g., restricted nutrients, hyperthermia, hypoxia, salinity increase and toxic chemical contaminants; Cuervo, 2004; Klionsky & Emr, 2000; Levine, 2005; Levine & Kroemer, 2008; Moore & Halton, 1973, 1977; Moore et al., 1986, 2006a, b, c). However, recent evidence indicates that autophagy is much more than just a survival process and is, in fact, intimately involved in cell physiology (Fig. 5; Cuervo, 2004; Eskelinen et al., 2009; Lockshin & Zakeri, 2004; Mizushima et al., 2008; Moore, 1988, 2004; Moore et al., 1980; 2006a).

Cells use autophagy and the ubiquitin–proteasome system as their primary protein degradation pathways (Cuervo, 2004; Klionsky et al., 2007; Kraft et al., 2010; Lamb et al., 2013). While the ubiquitin–proteasome system is involved in the rapid degradation of proteins, autophagy pathways can selectively remove protein aggregates and damaged or excess organelles. Although autophagy has long been viewed as a relatively random cytoplasmic degradation system, the involvement of ubiquitin as a specificity factor for selective autophagy is rapidly emerging (Kraft et al., 2010). Indeed, recent evidence also suggests strong interactions (crosstalk) between proteasome-mediated degradation and selective autophagy (Kraft et al., 2010).

Consequently, the autophagic processes have been increasingly shown to have cytoprotective functions against ageing and many diseases including cancers,

neurodegenerative diseases (Cuervo, 2004; Ferrari et al., 2011; Hippert et al., 2006; Mizushima et al., 2008; Ohsumi, 2014; Rubinsztein et al., 2011; Salminen & Kaarniranta, 2009; Selvakumaran et al., 2013; Trocoli & Djavaheri-Mergny, 2011; Zhang et al., 2012). Autophagic lysosomal digestion can be triggered by many environmental stressors including caloric restriction (CR), hypoxia, ROS, exercise, many toxins and phytochemicals, and sunlight and vitamin D mediated via the vitamin D receptor - VDR (Chatterjee etal., 2014; Delmas et al., 2011; Ferrari et al., 2011; Mestre & Columbo, 2013; Moore et al., 2008; Wu & Sun, 2011; Zhang et al., 2012).

Augmented autophagy is controlled by switching off the mTOR (mechanistic target of rapamycin; part of mTORC1 – mTOR complex 1) kinase: mTOR signalling is involved in many aspects of cell growth-regulation and has also been implicated in some cancers (Fig. 6; Asnaghi et al., 2004; Lamming et al., 2013; Laplante & Sabatini, 2009, 2012; Levine, 2005; Proud, 2002). mTOR kinase is also coupled with a nutrient sensing pathway; and is switched off by lack of nutrients (see review by Proud, 2004). This kinase is evolutionarily conserved in eukaryotes and has been variously described in yeast, nematodes, molluscs, insects, crustaceans and mammals (Cammalleri et al., 2003; Beaumont et al., 2001; Levine, 2005; Klionsky & Emr, 2000). The mTOR signalling system is classically switched off by nutrient deprivation (i.e., amino acids), with resultant up-regulation of autophagy in mammals, which has been described in mussels and marine and terrestrial snails (Fig. 6; Bayne et al., 1978, 1979; Bergamini et al., 2003; Cuervo, 2004; Moore & Halton, 1973, 1977; Moore et al., 1979; Moore et al., 1985, 1986; Proud et al., 2002). Autophagy, when triggered by inhibition of mTOR and other mTOR-independent pathways (e.g., SIRT 1 - NAD-dependent deacetylase sirtuin-1 and VDR – vitamin D receptor), is probably an important component of hormetic responses, particularly in anti-ageing processes (Blagosklonny, 2011, Kim et al., 2012; Martins et al., 2011; Moore & Stebbing, 1976; Rubinsztein et al., 2011; Salminen et al., 2012; Wu & Sun, 2011). Calorie restriction (CR) is now well established as having beneficial effects in a wide range of organisms by increasing lifespan and reducing the risk from age-related cancers, cardiovascular and neurodegenerative diseases (Mattson & Wan, 2005; Fontana et al., 2010).

Molluscan hepatopancreatic lysosomes are also a major site for generation of reactive oxygen species (ROS), including oxyradicals, as demonstrated by Winston et al. (1991) in isolated digestive cells. Within the lysosomes of normal unstressed

hepatopancreatic digestive cells, ROS are probably generated by transition metal ions, such as iron and copper, which accumulate in lysosomes from exogenous sources, such as algal and microbial food, and also by autophagic degradation of endogenous metallo-proteins (Fig. 1; Brunk & Terman, 2002; Moore et al., 2006a, 2007). Although molluscan digestive cell lysosomes spontaneously generate oxyradicals such as superoxide, they also contain a superoxide dismutase, which may protect the lysosomal membrane from excessive oxidative damage (Livingstone et al., 1992; Winston et al., 1991). Exposure of mussels to copper and some PAHs also results in increased ROS; as well as does re-immersion in seawater following a period of anoxia as described above (Livingstone, 2001; Moore, 2008; Moore et al., 2008; Regoli, 2000). Re-immersion following anoxia is probably analogous to reperfusion injury (Robin et al., 2007). Copper exposure also increases the concentration of protein carbonyls and lipofuscin (Kirchin et al., 1992; Moore et al., 2007).

Periwinkles are robust animals that frequently live in fluctuating environments such as estuaries where they are subjected to variable nutritional, temperature and salinity regimes, as well as repeated air exposure and re-immersion in seawater. Consequently, this essentially stressful fluctuating environment will tend to trigger repeated autophagic events, which by effectively removing inappropriately altered proteins and damaged or redundant cellular constituents will result in more efficient "cellular house-keeping" and help to minimise the formation of harmful lipofuscin and other aggregates (Fig. 4; Bergamini et al., 2003; Brunk & Terman, 2002; Cuervo, 2004; Hawkins & Day, 1996; Hipkiss, 2006; Kirchin et al., 1992; Moore, 1988, 2004; Moore et al., 2006a, b, c and unpublished data).

This more efficient cellular functionality may underpin the ability of intertidal molluscs such as periwinkles and mussels to survive, and often thrive, in environments that are subject to man-made stresses such as chemical pollution (Moore, 2004; Moore et al., 2006a, b, c).

Changes in lysosomes have been used as biomarkers of ageing (aging) in a wide range of organisms including nematodes, fruit flies, molluscs and mammals (see review by Cuervo & Dice, 2000; Hole et al., 1992, 1993). In general there is a trend for decreasing proteolytic capability with increased age that has been linked with a gradual decline in the efficiency of the autophagic process (Cuervo & Dice, 2000). However, Bergamini et al. (2003) have proposed that repeated triggering of the

autophagic system by nutrient deprivation or caloric restriction will prevent the decline in proteolytic capacity and, hence, contribute to increased lifespan probably through the maintenance of more efficient “cellular housekeeping”. This may parallel the situation of intertidal animals like periwinkles and mussels that live in an environment where autophagy is repeatedly switched on and off as discussed above, thus maintaining an effective capacity for the removal of altered proteins, membranes and organelles that are damaged by ROS and hypoxia-induced methylglyoxal (Fig. 5; Cuervo, 2004; Hawkins & Day, 1996; Hipkiss, 2006; Kiffen et al., 2004; Moore, 2004; Moore et al., 2006a, b, c, 2007; Regoli, 2000). Further investigation of the role of lysosomal autophagy in conferring resistance to stress is required but the possibility raises provocative questions about the possible role of ongoing and fluctuating low levels of stress in the evolution of stress tolerance (Moore, 2008; Moore et al., 2006a, b, c, 2007).

There are also parallels between repeated stimulation of autophagy by mild environmental stressors (Moore (2008) and the growing anti-ageing evidence for fasting and caloric restriction (CR) induced autophagic removal of damaged or old intracellular proteins and organelles (see Fig. 5; Cuervo, 2004, 2008; Cuervo & Dice, 2000; Madeo et al., 2010; Rubinsztein et al., 2011).

Many readily detectable harmful pathological reactions occur at the cell and tissue level as a result of environmental insult (Moore, 2002a, b; Lowe et al., 2006). Some of these can be used as “early warning distress signals” that further cellular and tissue damage will occur unless the causative factors are removed. A key objective for ecotoxicology is to develop prognostic biomarkers and generic simulation models for responses to environmental change in whole systems, that are based on current and developing knowledge of genomic, proteomic, metabolomic, cellular and higher level biological processes. Access to such tools will be essential in the future for environmental managers and regulators; where they will be used in integrated environmental evaluation strategies for risk assessment and prediction in order to effectively manage resource sustainability (Moore, 2002b).

However, one of the major difficulties in predicting impact and risk is our current ability, or rather the lack of it, to link harmful biological effects of environmental stressors in individual animals with their ecological consequences. This problem has resulted in a “knowledge-gap” for those seeking to develop effective policies for sustainable use of resources and environmental protection. The key issues are

complex and interfacial and require a cross-disciplinary approach. These include the effects of the physico-chemical environment on the speciation/binding and uptake of pollutant chemicals; and inherent inter-individual and inter-species differences in vulnerability to toxicity, in particular, the toxicity of complex mixtures. It is also essential to be able to link the impact of pollutants on whole biological systems (biocomplexity), from cells through the higher order interactive levels of organisation to functional ecosystems (using ensemble averages), leading eventually to "ecosystem health" (Allen & McVeigh, 2004; Allen & Moore, 2004).

Consequently, the immense complexity of the interactive functional level of biology dictates that we think in terms of integrated models that address this problem in a way that our limited mental computing capability cannot (Noble, 2002a, b, c). In essence, can mathematical modelling of biological processes shed light on cell injury, pathology, disease and ecosystem damage? Fortunately, models can help to make a coherent whole from disparate data sets; and are also useful conceptual indicators for the design of experiments that rigorously test current paradigms (Allen & Moore, 2004; Lauffenburger & Linderman, 1993; Moore, 2002a, b; Moore & Allen, 2002). In fact, ecotoxicologists' collective neglect of modelling has become a serious impediment to progress. However, the recent work of Noble (2002a, b) in developing numerical physiological models, and that of Düchting et al. (1996) with a tumour model, has opened a new avenue for the future in many areas of biomedicine and toxicology (Hunter et al., 2002a, b; Noble, 2002b). In traffic management and chemical engineering, physics and epidemiology, for example, it is well understood that complex systems can be accurately understood only by constructing quantitative mathematical models (Maddox, 1998). However, ecotoxicologists are still largely working in the dark in this respect; and will remain so, until realistic models have been built for the process describing how the specificity of the whole system response matches that of the external signal or potentially harmful perturbation it receives (Allen & McVeigh, 2004; Allen & Moore, 2004; Moore, 2002a; Moore & Noble, 2004).

Complexity of a cellular biological system can be used as an indicator of homeostasis (Lewis et al., 1992; Moore, 2010; Sedivy, 1999). Consequently, inputting the biomarker data from this experiment into a directed cell physiology network model showed that there was a statistically significant increase in system complexity indicating increased homeostasis and health status (Table 1). Network topology was

also significantly different in terms of node size (Fig. 2). These results support the hypothesis that hormesis is occurring in the fasted animals and this is in line with the predictions for the effects of mild stress on the cellular physiology described by Moore (2010). The network models also demonstrate that autophagy is an important hub in the cellular physiology of the system being tested, which lends support to the overall hypothesis (Fig. 2). The network approach demonstrates that cell injury and pathology can be defined as a loss in system complexity, while an increase can indicate hormesis (Lewis et al., 1992; Moore, 2010; Sedivy, 1999). Consequently, cellular networks can be used to integrate information from biomarker data; and to direct the selection of biomarkers and design of experiments, in order to develop suites of tests that will demonstrate which links are active or inactive, and to what degree, thus providing mathematical formalism for an objective evaluation of health status for potential use in risk assessment (Moore, 2002b, Moore et al., 2004). Cellular interaction networks also have considerable potential for integrating multi-biomarker data for evaluation of whole system "health status" (Moore, 2010).

Acknowledgements: This study was supported in part by funding from a Research Fellowship awarded by the Department of Science and Innovative Technology (DSIT), University of Eastern Piedmont, Alessandria, Italy; and support from a Plymouth Marine Laboratory Fellowship (PML, Plymouth, UK) to Prof. M N Moore. The authors are not aware of any conflicts of interest.

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