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Polyunsaturated fats, membrane lipids and animal longevity

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Polyunsaturated fats, membrane lipids and animal longevity

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Fatty acids are essential for life because they are essential components of cellular membranes. Lower animals can synthesise all four classes of fatty acids from non-lipid sources, but both omega-6 and omega-3 cannot be synthesised *de novo* by 'higher' animals and are therefore essential components of their diet. The relationship between normal variation in diet fatty acid composition and membrane fatty acid composition is little investigated. Studies in the rat show that, with respect to the general classes of fatty acids (saturated, monounsaturated and polyunsaturated) membrane fatty acid composition is homeostatically regulated despite diet variation. This is not the case for fatty acid composition of storage lipids, which responds to diet variation. Polyunsaturated fatty acids are important determinants of physical and chemical properties of membranes. They are the substrates for lipid peroxidation and it is possible to calculate a peroxidation index (PI) for a particular membrane composition. Membrane PI appears to be homeostatically regulated with respect to diet PI. Membrane fatty acid composition varies among species and membrane PI is inversely correlated to longevity in mammals, birds, bivalve molluscs, honeybees and the nematode *Caenorhabditis elegans*.

Introduction

Our approach generally determines our perspective and so it is with fats. It is common to use an anthropo-centric approach rather than an evolution-centric approach when teaching lipid biochemistry. Normally, such teaching starts with triglycerides and energy storage before considering the more complex subject of phospholipids and membrane structure. Yet from an evolutionary perspective, the opposite is the case, phospholipids precede triglycerides. Most basic biochemistry was evolved by bacteria, long before eukaryotes (including the multicellular animals) appeared on the scene, and in many ways most of our biochemistry is still microbial in nature. Because bacteria do not synthesise triglyceride molecules, and thus do not use them as an energy store, the evolution of lipogenesis (synthesis of fatty acids) was thus not likely selected as a process to store energy. Much more likely, the selective pressure for the evolution of lipogenesis, and many other aspects of lipid metabolism was to make membranes from non-lipid sources. The evolution of energy storage in the form of triglyceride molecules is best viewed as a later evolutionary modification (by eukaryotes) of the fundamental processes evolved (by prokaryotes) to make membrane lipids (i.e. phospholipids). An indication of this is the fact that triglyceride molecules are synthesized by modification of a phospholipid (i.e. phosphatidic acid).

Fatty acids are essential for life because they are essential components of membranes, not because they are a source of energy. Theodoseus Dobzhansky famously said “Nothing in biology makes sense except in the light of evolution”, and so it is with respect to lipid metabolism. From an evolutionary perspective, much of lipid metabolism is more important because of its implications for membrane function rather the storage of metabolic energy. This is our perspective in this contribution. The role of lipids in membrane function is important not only because their physical nature influences the activity of membrane proteins but also because they are chemically modified as part of many processes. These processes include the use of membrane lipid products for chemical signaling and as we will assert later, there is increasing evidence that they are also intimately involved in determining the longevity of different animals.

All living organisms have lipid membranes, and just as DNA has been described as an "eternal molecule", so lipid membranes could be considered an "eternal structure" as such membranes are the products of pre-existing membranes. The early evolution of lipid biosynthesis and the importance of lipid membranes is discussed by Lombard et al. (2012). The core components of the membranes of bacteria and eukaryotes are phospholipid molecules each consisting of a pair of fatty acids attached to a hydrophilic headgroup.

Bacteria and plants are capable of making all their membrane lipids '*de novo'* from non-lipid sources and thus fats are not essential nutrients for them. Animals can synthesise both saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) from non-lipid sources and these fatty acids are thus not essential components of the diet, but many animal species are incapable of '*de novo*' synthesis of both omega-6 and omega-3 polyunsaturated fatty acids (i.e. n-6PUFA and n-3PUFA) and therefore must obtain them preformed in their diet. These molecules are thus sometimes also called "essential fats".

Essential fats: the nutritional requirement for polyunsaturated fats

The process of lipogenesis, catalysed by the multienzyme fatty acid synthase complex, produces palmitic acid, a 16-carbon saturated fatty acid (16:0). Elongase enzymes are responsible for the production of longer chain (normally 18-, 20-, or 22-carbons) fatty acids from palmitic acid. A series of different desaturase enzymes are further responsible for introducing double bonds into specific locations of fatty acid chains. A schematic describing the metabolic transformations involved in the synthesis of various fatty acids is presented in Figure 1. While relatively simple animals, such as *Caenorhabditis elegans* possess a complete range of desaturase and elongase enzymes (e.g. see Shmookler Reis et al. 2011) and thus do not have any dietary requirements for essential fats, this is not the case for all animals. For example, "higher" animals, such as rats and humans (and likely all vertebrates, including fish) are unable to synthesise n-6 PUFA or n-3 PUFA from the 18-carbon MUFA (oleic acid, 18:1n-9) because they have neither a Δ12-desaturase nor a

Δ15-desaturase. This is the reason these two types of PUFA are essential dietary components for these animals. Furthermore, they are also unable to interconvert n-6 PUFA and n-3 PUFA and therefore these are separate and independently essential dietary requirements.

Plants are capable of *de novo* synthesis of both n-6 PUFA and n-3 PUFA and generally plant-based foods are the ultimate source of these important fatty acids in the food chain. Plant-based foods generally provide only 18-carbon PUFA, and almost no 20-carbon or 22-carbon PUFA, with α-linolenic acid (18:3n-3) found in the photosynthetic parts of plants (i.e. their "leaves") while linoleic acid (18:2n-6) is found in seeds and flowers. Considering only PUFA, as a generalization, it can be said that "leaf-based" foods contain predominantly n-3 PUFA, specifically 18:3n-3, while foods based on "seeds" (including grains and nuts) contain predominantly n-6 PUFA, specifically 18:2n-6 (see Hulbert and Abbott, 2012).

Generally, animal-based foods (e.g. meats, dairy, or fish) contain a mixture of both n-6 PUFA and n-3 PUFA and unlike plant-based foods, these include significant amounts of 20-carbon and 22-carbon PUFA. The balance between n-3 PUFA and n-6 PUFA in animal-based foods varies and can be influenced by the type of plant-food the animal is fed (e.g. grass-fed meat and dairy food has more n-3 PUFA than grain-fed meat and dairy). The PUFA balance of some food types is illustrated in Hulbert and Abbott (2012). The shift over the last half-century in the PUFA balance of the modern human food chain has been to dramatically increase n-6 PUFA at the expense of n-3 PUFA and has had a number of detrimental health consequences, but that is a story for another time.

Initially, dietary fats were considered necessary only as a source of energy and fat-soluble vitamins. The discovery that a fat-free diet was detrimental for the growth and survival of rats (Burr and Burr 1929) followed by the discovery, a short time later, that the essential factor in fats required by rats was not a vitamin but was instead the fatty acid 18:2n-6 (Burr and Burr 1930) resulted in a reconsideration. However, it took sometime after this discovery for it to be accepted that 18:2n-6 was also an essential nutrient for humans. This was largely because diet experiments on humans

were not carried out for a long enough time. Comparative physiologists are well aware that time is relative in species that differ in body size. An 8-week experiment in rats when translated to a human experiment would be either ~1 year (relative to metabolic rate differences) or 4-5 years (relative to differences in maximum lifespan). Over time it was discovered that both 18:2n-6 and 18:3n-3 were independently essential dietary requirements in humans (see Holman 1992; 1998).

Considering the general public awareness of fish oils as a source of omega-3 fats, it is ironic that fish were the first vertebrates to be shown to have an essential requirement for omega-3 fats in their diets. During the aquaculture boom of 1960s, the use of corn and corn oil as a substitute for the normal food of trout resulted in the lethal condition known as "transport shock". It was discovered that this condition was due a deficiency of n-3 PUFA. While corn oil was able to provide plenty of 18:2n-6 to the trout, it was unable to provide adequate n-3 PUFA which was essential to avoid the lethal shock syndrome, as well as heart myopathy and fin erosion (Castell et al. 1972). It was a decade later that the first example of human ill-health due to n-3 PUFA deficiency was reported (see Holman et al. 1982; Allport 2006). Quantitative determination of the dietary requirements for these two classes of PUFA is of course limited to very few species but is generally regarded to be quite small. For humans, the dietary requirements for n-6 PUFA and n-3 PUFA are estimated to be respectively ~2% and ~1.5% of energy intake (ISSFAL).

We know very little about the "essentiality" of these fats for other species but it is generally assumed that both n-6 PUFA and n-3 PUFA are essential components of the diet for most "higher" animals. Because, by definition, animals eat other organisms (or their products), they will generally consume the small amounts of fats required even if their diet does not macroscopically appear to contain fat. This is because their diet includes the cell membranes of their food. For example, even lucerne (a dry apparently fat-free food) contains significant amounts of 18:3n-3 (Else and Hulbert unpublished measurements). Even animals that use nectar as a primary energy source, will also consume either pollen and/or insects, and while this is often described as being a necessary nitrogen source (or more precisely a source of protein), it is also a source of other nutrients such as the

essential PUFA. Pollen is a very good source of both essential n-6 PUFA and essential n-3 PUFA, which together can constitute nearly 60% of total fatty acids (Manning 2001; Haddad et al. 2007). The role of the gut microflora in *de novo* synthesis of essential fatty acids and their provision to the host animal is a little investigated area.

As mentioned above, *C. elegans* has a full complement of elongase and desaturase enzymes and thus PUFA are not essential in their diet. The situation for insects is more complex. Although most insects appear unable to synthesise n-6 PUFA, species from four orders (Orthoptera, Homoptera, Isoptera and Neuroptera) have been shown to be capable of producing *de novo* 18:2n-6 (but not 18:3n-3) from ^{14}C-labelled acetate (Cripps et al. 1986; de Renobales et al. 1987). Thus n-6 PUFA are not essential dietary requirements for these insects, but n-3 PUFA may so be.

There are other cases of animals having a deficiency in desaturase enzymes that have dietary consequences. For example, most mammals are able to synthesise arachidonic acid (20:4n-6) from linoleic acid (18:2n-6) obtained in the diet but this is not the case for cats, which lack seem to lack both Δ6- and Δ5-desaturase activity (Rivers et al. 1975). This means that cats (and presumably other felids) are obligate carnivores, because plant foods do not provide 20:4n-6 and they cannot synthesise them, they must eat other animals to obtain the necessary 20:4n-6 for incorporation into their membrane lipids.

Of course, whether a component of an animal's diet is an essential diet requirement depends on two criteria. The first is that the animal is incapable of *de novo* synthesis of the component. The second is that the component is essential for the normal function of the animal. In the case of mammals, both these criteria are met for classifying n-6 PUFA and n-3 PUFA as essential fats. This is because (i) they lack Δ12- and Δ15-desaturases, and (ii) n-6 PUFA and n-3 PUFA are necessary for normal function in that they are the precursors of important chemical messengers based on 20-carbon PUFA (e.g. biologically-active eicosanoids and endocannabinoids) and some other PUFA (e.g. 22:6n-3) are necessary for normal neuronal function in mammals.

However, both criteria are not necessarily met for all animal species. As mentioned above some insects are incapable of *de novo* synthesis of 18:2 n-6 (while others are capable). Furthermore, as well as being unable to synthesise 18-carbon PUFA some insects are incapable of producing 20-carbon PUFA from any 18-carbon PUFA they may obtain in their food. Of the eight desaturase genes that have been identified in the *Drosophila melanogaster* genome seven are Δ9-desaturases capable of producing MUFA from SFA but there are no Δ6- or Δ5-desaturases which are necessary to produce 20:4n-6 (Fang et al. 2009). Similarly, the blowfly *Calliphora stygia* appears unable to convert dietary 18-carbon PUFA to 20-carbon PUFA (Kelly et al. 2013). A detailed study of the lipidome of adult *Drosophila melanogaster* found no 20-carbon PUFA in either phospholipids or triglycerides (Hammad et al. 2011) supporting the inability of *Drosophila* to produce such PUFA. Insects unable to synthesise such PUFA may still incorporate them into their membrane lipids if they obtain them preformed in their food. This is the case with *Calliphora* which being carrion-feeders have a larval diet consisting of meat or fish (see Kelly et al. 2013). However, because they are found in membrane lipids of an insect does not necessarily mean they are essential for normal function. For example, mosquitoes require 20- and 22-carbon PUFA for survival but this is not necessarily the case for other Dipteran insects (Canavoso et al. 2001). Eicosanoid production and its importance in insect biology has been reviewed by Stanley (2006). Endocannabinoid production may not be part of the normal function of insects as cannabinoid receptors are absent in insects (McPartland et al. 2001). The role of dietary PUFA and whether they are essential in the biology of insects will likely be complex because lipids and their derivatives have many other roles in insects (e.g. pheromones, waxes) that they do not have in mammals. Similarly, we have much to learn about the importance of dietary fats and the distribution of desaturase enzymes in other invertebrate animals. Pereira et al. (2003) have reviewed desaturase enzymes in lower eukaryotes.

In general, there is no special requirement for specific fatty acids as a source of energy in that SFA, MUFA and PUFA are all capable of providing metabolic energy (via β-oxidation) and the amount of energy per fatty acid molecule depends on chain length much more than the degree of

unsaturation. The essential nature of particular fats will depend largely on the specific role in membranes lipids; either whether they are the sources of significant lipid signaling molecules derived from specific membrane lipids or whether they are necessary to provide specific physical properties to a membrane.

Possibly the most polyunsaturated fatty acid naturally occurring in membranes, namely docosahexaenoic acid (22:6n-3), should be considered in this second category. In vertebrates, this highly polyunsaturated fatty acid is present in substantial amounts in both the retina and brain and is important for normal vision and neuronal function. Its functional importance has been most studied in retinal membranes where it has been shown to be largely responsible for the optimal physical membrane environment for light-activated rhodopsin to initiate the visual signal (Litman and Mitchell 1996). An indication of its importance in providing this optimal membrane bilayer environment is that the G-protein signaling cascade initiated by activated-rhodopsin is 50% slower when 22:5n-6 replaces 22:6n-3 in the retinal membrane bilayer (Mitchell et al. 2003). This highly polyunsaturated fatty acid is notably present in fish where (together with 20:5n-3) it is considered the active ingredient of fish oils. It is obviously not essential for neuronal function in all animals as, for example, insects such as honeybees have highly functional neuronal systems yet it is not present in phospholipids of the head in honeybees (Haddad et al. 2007). It is not present in phospholipids of blowflies fed fish as larvae or provided fish oil or krill oil as adults (Kelly et al. 2013).

Although invertebrates such as *C. elegans* and *Drosophila* are incapable of the synthesis of 22:6n-3, it can be made from dietary 18:3n-3 by mammals. This synthesis however is unlike that of most other PUFA (see Figure 1), in that it involves first the production of 24:6n-3 which is consequently transferred to the peroxisome where a single cycle of β-oxidation removes a 2-carbon unit and produces 22:6n-3 (and hydrogen peroxide). A similar sequence is also the pathway for synthesis of 22:5n-6 in mammals. The complex intracellular process for synthesis of these two highly polyunsaturated fats was elucidated by Sprecher and colleagues (Sprecher et al. 1999) after the observation that individuals with Zwellwegger's Syndrome, a peroxisomal disorder, were

unable to synthesise 22:6n-3. Of course, 22:6n-3 can also be obtained preformed in food, and fish are often a common source. In fish, some of the 22:6n-3 will synthesized by the fish itself (from 18:3n-3) and some will be obtained preformed from the food chain. Marine protists and dinoflagellates are good sources of 22:6n-3 (Ward and Singh 2005) as are some marine algae (e.g. Sayanova et al. 2011). Although mammals use the peroxisomal pathway to synthesise 22:6n-3, many organisms at the base of the marine food chain use other metabolic pathways. For example, desaturases in animals generally consume O_2 during the process of desaturation and are thus aerobic enzymes, however some marine bacteria synthesise 22:6n-3 by an anaerobic pathway and have been suggested to also inhabit the gastrointestinal tract of some fish and molluscs (see Valentine and Valentine 2010). To our knowledge, the relative contribution of these various sources to 22:6n-3 content of fish and other seafood is unknown.

Dietary fats and membrane fatty acid composition: conformers or regulators?

Dietary fatty acids are not all similarly handled once digested and absorbed. This was demonstrated when individual ^{14}C-labelled fatty acids were added to the diet of rats that were then maintained in metabolism cages for the following 24h (Leyton et al. 1987a; 1987b). Expired CO_2 was continuously collected for ^{14}C measurement and at the end of the 24h period, rats were killed and the ^{14}C content in liver lipids measured. The results of this study are presented in Figure 2, and as can be seen, fatty acids differed in their partitioning between oxidation for provision of metabolic energy and their incorporation into either liver phospholipids or liver triglycerides. There is an inverse relationship in $^{14}CO_2$ excretion and incorporation of ^{14}C-labeled fatty acid into liver lipids. In attempting to understand the results of studies like this, it would be common to consider first the relative oxidation of different fatty acids and then consider their relative degree of incorporation into liver lipids. In line with the perspective we have outlined in the Introduction, we would suggest that these results may be more easily understood by first considering the large differences in the relative incorporation of different fatty acids into membrane lipids (specifically liver phospholipids

in this study). Compared to the incorporation of the different ^{14}C-labelled fatty acids into liver phospholipids, their incorporation into liver triglycerides was small and relatively constant. The 24h incorporation of ^{14}C-labelled fatty acids into liver triglycerides varied from 0.4% to 0.8% of the oral dose. In contrast the 24h incorporation into liver phospholipids varied from 0.5% to 18% of the oral dose, with the 18:1n-9 being the fatty acid least incorporated and 20:4n-6 being the fatty acid most incorporated into liver phospholipids. There was much greater incorporation of labeled fatty acid into liver phospholipids than into liver triglycerides, the only exception being 18:1n-9. Our interpretation is that the low exhalation of $^{14}CO_2$ for particular fatty acids is a reflection that once ingested they are rapidly sequestered to the membrane lipid pool. The differences between their incorporation into phospholipids would appear to be related to differences in the relative importance of the individual fatty acid for the remodeling of membrane lipids.

It is little appreciated that membrane lipids continually undergo very rapid cycles of deacylation and reacylation (i.e. fatty acids are being continually removed and replaced at both the sn-1 and sn-2 carbons of the glycerol backbone of the lipid). This process is described as 'membrane remodeling' and is the fundamental reason why membranes can respond very quickly to environmental change. It is also likely very important in the control of membrane fatty acid composition. It is a very old process in evolutionary terms. It is intimately involved in the temperature induced changes in membrane fatty acid composition that were described as 'homeoviscous adaptation', first in bacteria (Sinensky 1974) and later realized to be a common process in many other organisms, being best studied in fish (e.g. Hazel 1995). When *E. coli* are subjected to low temperature they replace 16:0 in the membrane lipids with 18:1n-7 acyl chains via the process of membrane lipid remodeling and this change is very rapid, being evident within 30s of the lowering of temperature (Rock et al. 1996).

Membrane lipid composition is determined by the combination of both the *de novo* synthesis of phospholipids and their remodeling by deacylation/reacylation cycles. Pulse labeling experiments on rat liver cells maintained in cell culture have shown that *de novo* synthesis produces primarily

four molecular species of both phosphatidylcholine (PC) and phosphatidylethanolamine (PE), namely 16:0-18:2, 16:0-18:1, 16:0-22:6, and 18:1-18:2 (Schmid et al. 1995). The other molecular species of PC and PE are produced by deacylation/reacylation of the four *de novo* synthesised molecular species of phospholipid (Schmid et al. 1995). For example, the major membrane lipids of liver cells include the 18:0-20:4 molecular species of both PC and PE and these are produced by remodeling (note that *de novo* synthesis did not include any phospholipids containing 18:0 or 20:4n-6), with placement of 18:0 in the *sn-1* position and 20:4n-6 in the *sn-2* position. Once produced by this deacylation/reacylation process, these 18:0-20:4 membrane lipids appear to not be further remodeled. It is of note that the study of Leyton et al (1987a; 1987b) showed 20:4 and 18:0 to be the ^{14}C-labelled fatty acids showing the greatest incorporation into liver phospholipids (see Figure 2).

Remodeling of membrane lipids is important in the rapid removal of damaged fatty acids. Oxidative stress can result in peroxidative damage to PUFA in membrane lipids (to be discussed in more detail later) and this damage can interfere with membrane structure and function. When isolated liver cells were subjected to oxidative stress it was observed that levels of both PC and PE and their fatty acid composition were maintained during oxidative stress but that the level of cellular triglyceride decreased and furthermore the fatty acid composition of triglycerides changed with a decrease in their PUFA content (GironCalle et al. 1997). At first perusal these results may be interpreted to indicate that lipid peroxidation is occurring in triglycerides but not in phospholipids. However, pulse-labelling experiments showed the situation was more complex. Lipid peroxidation was occurring to PUFA in the PC and PE but these damaged PUFA were being rapidly replaced with undamaged PUFA by the deacylation/reacylation cycles of the remodeling process. Furthermore, the triglyceride molecules were providing the undamaged PUFA in this process. Additionally, there was no increase in the rate of deacylation/reacylation during the oxidative stress (GironCalle et al. 1997) and thus the normal rate of deacylation/reacylation of membrane lipids is rapid enough to cope with an increased rate of PUFA damage during oxidative stress. It is likely

that the rapidity of this process is also capable of maintaining membrane fatty acid composition relatively constant in situations where membrane 20-carbon PUFA (such as 20:4n-6) are being consumed for processes such as eicosanoid production. The enzymes involved in remodeling of membrane lipids (e.g. phospholipases, acyltransferases) will be important components of the regulation of membrane composition.

Thus, as well as being considered as energy stores, triglycerides should also be considered as important stores of PUFA for membrane remodeling during the processes of deacylation / reacylation of membrane lipids. In this respect, it is of interest that trout have been measured to have a very high turnover of triglyceride during rest. This triglyceride turnover rate during rest exceeds several times the energy requirements for sustained locomotion and furthermore does not increase during exercise. It was suggested that the high turnover of triglycerides may be needed by these ectotherms, primarily for the rapid restructuring of membrane lipids especially during temperature change (Magnoni et al. 2008).

Because PUFA are essential, their absence in the diet of mammals (i.e. an essential fatty acid-deficient diet) will result in a modified membrane fatty acid composition. Such a diet results in membrane lipids with a much greater MUFA content and also the presence of significant amounts of 20:3n-9 (known as mead acid), an unusual PUFA that is able to be made *de novo* by mammals because its synthesis does not require Δ12- or Δ15-desaturases (Holman 1992 and see Figure 1).

There have been many studies that show membrane fatty acid composition is influenced by fatty acids in the diet. Most only examine two diets (e.g. control and experimental) and many are only concerned with a particular fatty acid (often not a part of the normal diet of the animal being investigated) and the diet examined is either devoid of, or has the particular fatty acid in substantial excess. Surprisingly, there are relatively few studies that attempt to examine the normal situation. In order to quantify the normal relationship between diet composition and membrane composition in the rat, we recently used twelve iso-caloric moderate-fat (25% energy) diets that were identical, except for their fatty acid profile (which varied widely), to determine the relationship between diet

and membrane fatty acid composition in an out-bred (i.e. genetically diverse) strain of rat. The diets were fed to the rats for 8 weeks (equivalent to ~one year in humans in relative metabolic rate terms) and the fatty acid composition of phospholipids (= membrane lipids) and triglycerides (= storage lipids) from a range of tissues measured (Abbott et al. 2012). We used the "conformer - regulator" perspective, commonly used in comparative physiology, to examine the relationship and some of the results are presented in Figure 3. As can be seen from this figure, the % SFA, % MUFA and % PUFA content of membrane lipids remains relatively constant for skeletal and cardiac muscle, liver and brain of the rat despite considerable variation in their relative content in the diet. These results show that, although there was variation in content of individual fatty acids, membrane lipids are homeostatically regulated with respect the broad categories of fatty acids (SFA, MUFA and PUFA). This was not the situation for fatty acid composition of triglycerides (of both adipose tissue and plasma), which showed much greater conformity to diet composition. Such homeostasis is not restricted to total membrane lipids as it was also observed when a more limited data set from the literature was earlier examined for the membrane fatty acid composition of liver plasmalemma, cardiac sarcolemma, cerebral synaptosome and cerebral myelin membranes of the rat, as well as for individual phospholipids classes of rat liver plasma membranes (Hulbert et al. 2005). In the twelve diet study (Abbott et al. 2012), although total PUFA content of membrane lipids was homeostatically regulated in the rat tissues, the balance between n-3 PUFA and n-6 PUFA in membrane lipids was strongly influenced by their balance in the diet (especially when n-3 PUFA was <10% of total PUFA) and this likely has significant implications with respect to health (Abbott et al. 2012). This is of interest, in view of the fact that Lands et al. (1982) showed that the acyltransferase enzymes, responsible for catalyzing the incorporation of PUFA into the sn-2 position of membrane phospholipids during membrane remodeling, have a very high preference for PUFA but do not discriminate well between n-3 PUFA and n-6 PUFA.

Membrane polyunsaturates: advantages and disadvantages

The fatty acid chains in membrane lipids have the dominant influence on the physical properties on the membrane bilayer they constitute. For example, membrane bilayers consisting solely of 18:0-18:0 PC have a phase transition temperature of 55^{o}C (Silvius 1982) which means that it is only at temperatures >55^{o}C that the membrane will be 'fluid' (more precisely described as liquid-crystalline) and thus able to function normally. While this may be satisfactory for an early prokaryote inhabiting an extremely hot environment it will not enable normal function at temperatures commonly experienced in today's world. Changing the phospholipid head group would little help this prokaryote to function in the normal ambient temperature range as, for instance 18:0-18:0 PE has a transition temperature of 74^{o}C, while for 18:0-18:0 PS phosphatidylserine (PS) it is 68^{o}C, for 18:0-18:0 phosphatidylglycerol (PG) it is 55^{o}C (Silvius 1982). However, replacing the fatty acids with either shorter chains or more unsaturated fatty acid chains would be a great help as it will significantly lower the transition temperature. For example, for 16:0-16:0 PC the transition temperature is 41^{o}C, while for 18:0-18:1 PC it is 6^{o}C, and for 16:0-18:1 PC it is -2^{o}C, while for 16:1-16:1 PC it is -36^{o}C (Silvius 1982). When the bacteria *E. coli* is grown at 37^{o}C, the lipids making up their membranes are primarily SFA-MUFA molecular species, followed in relative abundance by SFA-SFA molecular species and then MUFA-MUFA molecular species (Ishinaga et al. 1979). They have no PUFA in their phospholipids. When grown at the lower temperature of 17^{o}C they decrease the relative abundance of SFA-SFA while increasing abundance of both SFA-MUFA and MUFA-MUFA molecular species of membrane lipids (Ishinaga et al. 1979). Thus PUFA are not necessary for homeoviscous adaptation in *E coli* and the incorporation of only additional MUFA into membrane lipids is adequate to maintain normal membrane 'fluidity' and function at the lower temperature. As mentioned previously such temperature-induced changes in membrane fatty acid composition are very rapid.

Although it is often stated that the role of PUFA in membrane lipids is to maintain membrane 'fluidity', it is obvious that they are doing something extra and more than just providing a "liquid-crystalline" membrane environment. Both Lee (1991) and Hazel (1995) have discussed

the inadequacy of the simplistic concept of 'fluidity' (or its inverse, 'viscosity') for describing the influence of highly polyunsaturated fatty acids on membrane function. As Hazel (1995) pointed out for the fish 'homeoviscous' response to low temperature, change in the MUFA content of membrane lipids was adequate to maintain membrane 'fluidity', the enhanced incorporation of 22:6n-3 into membrane lipids was doing something extra. Similarly, in the study of 22:6n-3 in retinal lipids cited above (Mitchell et al. 2003) both 22:5n-6 and 22:6n-3 will provide a highly 'fluid' membrane, yet there is a much greater amplification of the visual signal in a membrane bilayer with 22:6n-3 than one with 22:5n-6. We do not have adequate terminology to describe the effect of 22:6n-3 on membrane function. It appears to 'speed up' many membrane processes, likely by causing greater molecular movement in the lateral plane of the membrane bilayer and thus, for example, facilitating the interaction of membrane bound proteins (e.g. G-protein cascades). The physical properties that PUFA impart to membrane bilayers is described by Feller et al. (2002) while a specific comparison of the various conformations that 22:6n-3 and SFA adopt in membrane bilayers is found in Feller (2008). The extreme molecular motion of 22:6n-3 in a membrane lipid is beautifully demonstrated in an animation created and made available by Scott E Feller of Wabash College (see "500 ps dynamics of a stearoyl docosahexaenoyl PC lipid molecule" on the bottom of the 'lipids animation' page of the website http://www.lipid.wabash.edu/). From this animation, which is a molecular dynamics simulation of 0.5 nanoseconds (i.e. 1/100 millionth of the duration of an action potential), it is manifest that 22:6n-3 will impart considerable lateral motion and energy to other molecules in a membrane bilayer.

The fatty acid composition of membrane lipids (especially highly polyunsaturated fatty acids such as 22:6n-3) have been postulated to be important in determining the activity of membrane-associated processes, and in turn the cellular metabolic activity of different animals. The differences in basal metabolic rate of vertebrate species, notably the ectotherm-endotherm difference and body-size differences in BMR, have been proposed to be due to a combination of tissue size differences and cell metabolic activity. The reader is referred to other sources for more

detailed discussion of this proposal (Hulbert and Else 2000; Hulbert 2007). Of particular importance in experimentally demonstrating the role of membrane lipids in influencing the activity of membrane proteins have been species cross-over studies on Na^+,K^+-ATPase molecular activity (Else and Wu, 1999; Wu et al., 2004).

Apart from the different physical properties that PUFA, MUFA and SFA impart to membrane bilayers, there are also significant differences in their chemical reactivities, especially with respect to attack by free radicals. These differences likely have implications for ageing and longevities of animals (Pamplona et al. 2002; Hulbert 2005). A 'C=C' double bond weakens the bond energy of the C-H bonds present on the neighbouring C atom (the allylic hydrogens), and this is especially true if there are double-bonded carbons on either side of a C-H bond. In most PUFA, there is a methylene group (-CH_2-) between each pair of carbon-carbon double bonds (i.e. –CH=CH-CH_2-CH=CH-) and thus the hydrogen atoms attached to these methylene carbon atoms (the *bis*-allylic hydrogens) are attached with the lowest bond energies of any of the hydrogen atoms in the fatty acid chains. Thus compared to other hydrogen atoms in the fatty acid chain, a *bis*-allylic hydrogen (and its associated electron) is relatively easily removed by radicals with enough energy, to create a PUFA chain that, in the process of being attacked by the radical, itself becomes a carbon-centred radical (see Halliwell and Gutteridge 2007 pp 239-241). The carbon-centred lipid radicals, thus created, can combine with an O_2 molecule in the membrane to become a lipid peroxide radical which, in turn, can attack other *bis*-allylic hydrogens in other PUFA chains and thus initiate an auto-catalytic chain of lipid peroxidation. Neither SFA nor MUFA have such *bis*-allylic hydrogens and consequently are much less prone to lipid peroxidation. The more polyunsaturated the fatty acid, the more *bis*-allylic hydrogens it posseses and the more prone it is to lipid peroxidation. The *in vitro* rate of oxygen consumption during peroxidation has been measured for methyl esters of individual fatty acids and demonstrated for example that 22:6n-3 undergoes peroxidative damage at a rate eight-times that of 18:2n-6 and 320-times that of 18:1n-9 (Holman 1954). The relative rate of peroxidation of different acyl chains has been used to convert the fatty

acid composition of a particular membrane to a Peroxidation Index (PI) for that particular membrane (see Hulbert et al. 2007 for details). The calculation of PI for a particular membrane fatty acid composition is an exercise to indicate the theoretical susceptibility of that particular membrane lipid composition to lipid peroxidation.

From the twelve-diet study (Abbott et al. 2012) carried out to determine the normal relationship between diet composition and membrane composition in the rat, that was described above, it is possible to calculate the PI for each diet as well as for tissue phospholipids. These have been plotted against each other in Figure 4. This diet PI - membrane PI relationship showed an even greater "homeostatic regulator" response than the diet – membrane relationships for SFA, MUFA, and PUFA presented in Figure 3. For a membrane composition completely "conforming" to diet composition, the slope of the relationship equals "1", while for a perfect "homeostatic regulator" (i.e. membrane composition uninfluenced by diet composition) the slope equals "0". While the SFA, MUFA and PUFA 'diet-membrane' relationships (see Figure 3) had average slopes of 0.01, 0.06, and 0.07 respectively for the four tissues examined, the average slope of the PI 'diet-membrane' relationship was -0.03 (see figure 4A and 4B). Thus, at least in the rat, fed a moderate fat diet, although it is possible to change the relative abundance of individual fatty acids in membrane lipids by altering diet fatty acid profile, it is not possible to alter the PI value of membrane lipids. It is not possible to alter the calculated susceptibility of the membrane to peroxidative damage by alteration of the diet fat profile. Such homeostasis is likely not restricted to mammals, as also plotted in Figure 4 (see Fig 4C) are the results of a similar attempt to alter the membrane composition of an insect by diet fat manipulation. In this experiment, larvae of the blowfly *Calliphora stygia* were fed (for their entire larval life) one of six diets that differed only in fatty acid composition. The fatty acid composition of phospholipids of both the larvae and adults derived from those larvae was measured and although there were diet-related differences in individual membrane fatty acids measured, there was essentially no effect of diet on the PI of the respective membrane lipids (Kelly et al. 2013). Thus in two experiments designed to modify diet

fatty acid composition in a reasonably normal physiological manner (i.e. without extreme changes in presence/absence of different fats), it has not been possible to alter, in both the rat and blowfly, the calculated peroxidation susceptibility of their membrane lipids.

Membrane polyunsaturates: longevity of mammals and birds

After membrane fatty acid composition was shown to vary with body size of mammals (Couture and Hulbert 1995), it was also shown that the PI of membrane lipids was inversely correlated with maximum lifespan of mammals (Pamplona et al. 1998). The relationship between membrane fatty acid composition and maximum lifespan was labeled the "membrane pacemaker" theory of ageing (Hulbert 2005) and is a modification of the "oxidative stress" theory of ageing. It emphasizes the importance of lipid peroxidation and its reactive products in the determination of longevity and has been reviewed in detail elsewhere (Hulbert et al. 2007; Pamplona 2008). It was initially created as a hypothesis to provide a mechanistic explanation for the huge variation in maximum lifespan of different mammal species. Such variation in species maximum longevity is many times the variation in lifespan that has been achieved within species, either by experimental manipulation or genetic mutation. As such, the "membrane pacemaker" theory is thus based on correlation and only proposes, rather than proves, a possible "cause and effect" relationship. The relationship between skeletal muscle PI and maximum lifespan for a variety of species is presented in Figure 5.

Any experimental method to investigate the mechanisms of ageing has to extend lifespan rather than shorten it. This is because methods that shorten lifespan (e.g. feeding a poison) may have nothing to do the normal mechanisms of ageing but methods that extend lifespan presumably modify the normal processes of ageing. The most common physiological manipulation used to extend longevity in mammals is calorie-restriction (CR). It is thus of interest that this experimental treatment was shown to modify membrane fatty acid composition of rat liver such that the susceptibility of membrane lipids to peroxidative damage was significantly decreased (Laganiere and Yu 1987; Lambert et al. 2004). This finding has been verified for other tissues (e.g. Cefalu et al.

2000; Lee et al. 1999), as well as for different phospholipids classes (Jeon et al. 2001; Cha and Jones 2000). Furthermore, in mice it has been shown that membrane fatty acid changes are an early effect of CR, being manifest after one month of CR (Faulks et al. 2006; Chen et al. 2012). The finding that CR changes membrane composition supports the proposition that the correlations observed between membrane composition and maximum lifespan of mammal species may represent a causal connection.

Although maximum lifespan of mammals is correlated with body mass (e.g. Hulbert et al 2007) there are some mammal species that are exceptionally long-living for their size. These include humans, echidnas, and naked mole rats which have recorded maximum lifespans that are approximately four-times the value predicted from their body mass. From their body size, the predicted maximum lifespan of a 70kg human is ~26y, of a 3kg echidna it is ~13y, and for a 30g naked mole rat it is ~4.5y (using equations from Hulbert et al. 2007). Yet the recorded maximum lifespan for these species are respectively 122y, 50y and 31y (all from the 'AnAge' database). Membrane fatty acid composition of skeletal muscle and liver mitochondria has been measured for all three species and all have a membrane composition with a PI one would expect for their maximum longevity (see Hulbert et al. 2007; 2008). The data points for these three long-living mammal species are highlighted in Figure 5.

Bats are another group of mammals that have some exceptionally long-living species but little information is available concerning membrane fatty acid composition for this group nor for the importance of torpor in extended longevity of bats. A recent report (McGuire et al. 2013) of the fatty acid composition of skeletal muscle of the 25-30g hoary bat (*Lasiurus cinereus*), which has a maximum lifespan of 14y compared to a predicted 4.5y, suggests that their extended longevity is not associated with membrane lipids with a low peroxidation susceptibity. Although it has not been measured in bats, torpor has been shown to extend lifespan of hamsters (Lyman et al. 1981) but the effect was quite small and thus the effects of torpor would not appear to be great enough to explain a 3-fold extended longevity of the hoary bat.

Within some mammal species, longevity has been shown to differ and this is likely related to the genetics of the particular mammal. For example, some wild-derived strains of mice (*Mus musculus*) have been shown to have an extended longevity compared to a genetically-diverse population of mice derived from an intercross lab strain of mice (Miller et al. 2002) and have also been shown to have lower PI values for their skeletal muscle membrane lipids (Hulbert et al. 2006). This is of interest because these different mice stocks were maintained in the same environment and fed the same diet for their entire lives. This finding thus supports a genetic basis for determining differences in membrane fatty acid composition, either via different enzyme isoforms (such as acyltransferases) or possibly by genetically-determined differences in hormone concentrations. Such longevity differences are not restricted to wild-derived strains of mice. The *Ames dwarf* mouse strain has a recessive mutation in the *Prop1* gene and homozygous mice are dwarf and also exhibit extended lifespan compared to heterozygotes, which are normal in size. These long-living dwarf mice have low plasma levels of a number of hormones including thyroid hormones (Bartke and Brown-Borg 2004). Low thyroid hormone levels have been shown to alter membrane fatty acid composition in rats and mice (Hulbert 2000) and thus it is of interest that dwarf homozygous *Ames* mice have membrane fatty acid composition with a lower PI than their heterozygous littermates (Valencak and Ruf 2013).

In humans, longevity has also been shown to be in part genetically determined (heritability of ~0.25; Herskind et al. 1996). It is of interest that a study of the children of nonagenarians (who thus have a high probability of a long life) and a matched control group of humans showed them to have red blood cells phospholipids with a significantly lower PI than the controls (Puca et al. 2008). Of course, it is not possible to definitively attribute this to genetic differences between the two groups since there may be also be environmental (especially dietary) differences between the two populations.

A recent study, using a phylogenomic approach to identify genetic targets of natural selection for long lifespan in mammals (Jobson et al. 2010), yielded some interesting results with

respect to the proposition that membrane lipid composition is important in the determination of maximum longevity. These authors compared ~5.7 million codon sites across 25 mammal species and showed that genes involving lipid composition and (collagen-associated) vitamin C binding have undergone increased selective pressure in long-lived mammals, whereas genes associated with DNA replication/repair have not. They also found that many of the candidate genes previously associated with ageing, from genetic mutation studies, have played no detectable role in the evolution of longevity in mammals.

Birds, as for mammals, show an allometric relationship whereby large birds tend to live longer than small birds, but birds differ from mammals such that, on average, birds have a maximum lifespan about twice as long as similar-sized mammals (Hulbert et al. 2007). Similar to the observation that membrane fatty acid composition of mammals varies with body size (Hulbert et al. 2002a) so does it also vary for the skeletal muscle (Hulbert et al. 2002b) and liver mitochondria of birds that vary in body size (Brand et al. 2003). In both birds and mammals, the percent of total unsaturated FA in membrane lipids does not vary with body size but the percent of PUFA decreases and the percent of MUFA increases with body size. However, membrane fatty acid composition is not identical in the two groups. For example, the skeletal muscle phospholipids of birds and mammals have similar percent total unsaturated FA (birds are 90% of mammal value), but birds have much less PUFA (birds are 75% of mammals) and much more MUFA (birds are 160% of mammals) than mammals (Hulbert et al. 2002a; 2002b). This means that birds will have membranes lipids much less susceptible to peroxidative damage (i.e. a lower PI value) than similar-sized mammals. Interestingly, when the PI of bird skeletal muscle phospholipids is plotted against their maximum lifespan, they appear to fall on the same relationship as mammals (see Figure 5).

While, on average, birds have maximum longevities twice that of similar-sized mammals, some individual bird-mammal comparisons show much greater differences. The most studied bird-mammal comparison is the pigeon-rat comparison. While the species are approximately the same body size, there is a seven-fold difference in their maximum longevities (from AnAge database).

This large longevity-difference has been used by many investigators to examine various aspects of the "oxidative stress" theory of ageing; namely antioxidant levels, mitochondrial ROS production, level of oxidative damage. One of the most often-cited bird-mammal differences, that bird mitochondria produce less ROS than mammalian mitochondria, is based on the pigeon-rat comparison. More precisely, it is based largely on findings from the comparison of the *in vitro* production of hydrogen peroxide by heart mitochondria from pigeons and rats, provided succinate as substrate and expressed on a "per mg mitochondrial protein" basis (e.g. Barja et al. 1994; Lambert et al. 2007). Although pigeons and rats are similar in body size, their hearts are very different. Pigeons have large-slow hearts, while rats have small-fast hearts. For example, the resting heart rate of pigeons is 178 beats/min (Grubb 1982), while it is 418 beats/min for rats (Lin and Horvath 1972) and the mass of the pigeon heart is approximately five times that of the rat (Montgomery et al. 2011). It is questionable whether heart mitochondria are the best source of mitochondria for such comparisons. Most of the previous pigeon-rat comparisons have studied only parts of the oxidative stress theory and have sometimes provided contradictory results. A recent study has revisited the pigeon-rat comparison and examined seven antioxidant systems in up to four tissues, concentration of four products of oxidative damage in up to four tissues, *in vitro* ROS production by mitochondria from three tissues, and the PI of membrane lipids from seven tissues. This comprehensive comparison found the only substantial and consistent difference between pigeons and rats related to membrane fatty acid composition (Montgomery et al. 2011).

As in mammals, there is considerable variation among similar-sized birds in their maximum longevity. For example, petrels and albatrosses (*Procellariiformes*) and parrots (*Psittaciformes*) are relatively long-living birds while fowl (*Galliformes*) are relatively short-living birds. Membrane lipids from the heart of petrels have significantly more MUFA and less PUFA than those of fowl with the consequence that the PI of cardiac phospholipids is lower in petrels than in fowl (Buttemer et al. 2008). The same group that carried out the extensive pigeon-rat comparison cited above also recently reported the results from a similarly comprehensive parrot-fowl comparison. They

compared three parrot species with two quail species, that exhibited an average five-fold longevity difference between the two groups, but found no consistent difference in the *in vitro* ROS production by both isolated mitochondria from three tissues and by intact erythrocytes between the long-living parrots and short-living quail (Montgomery et al. 2012a). Similarly, no consistent pattern of antioxidant defenses and oxidative damage could explain the longevity difference (Montgomery et al. 2012b). They also measured the fatty acid composition of phospholipids from a number of tissues from both parrots and quail and although there were some small differences between parrots and quail these were not consistent between tissues and furthermore they found no consistent difference in the PI of membrane lipids of long-living parrots and short-living quail (Montgomery et al. 2012c). The PI values from skeletal muscle (both pectoral and leg muscle) are plotted against the maximum lifespan of these five bird species in Figure 5. An interesting point from Figure 5 is that the muscle PI values for the parrots is what one would expect for birds with their respective maximum longevities, and furthermore within the three parrot species there is an inverse relationship between PI and maximum longevity. The two quail species have much shorter lifespans than one might predict from their muscle PI values. Indeed, this study showed that as far as most of the oxidative stress theory parameters measured the short-living quail were similar to the long-living parrots, and leaves the obvious question: why don't quail live as long as parrots?

This section examining the relationship between membrane composition and the maximum longevity of mammals and birds has been largely based on the correlation between species. There has been some consideration of the association between membrane composition and longevity within species both with respect to genetic differences and also experimental treatment such as calorie-restriction. We believe a strong circumstantial case for a possible "cause and effect" association between membrane composition and longevity has emerged but the obvious experimental test for such an association would be to alter membrane fatty acid composition and examine whether there are the expected changes in longevity. Dietary manipulation is an obvious possible means to try to change membrane composition but from the consideration of the limited

influence of diet on membrane composition described in the earlier section, especially from the data for the rat (see Figure 4), which shows that variation in diet PI had no effect on membrane PI for a number of tissues, there is the possibility dietary manipulation may not be successful in altering membrane composition appropriately. Valencak and Ruf (2011) report such an experiment where they fed 8-week-old mice three different diets for the rest of their lives and recorded their individual lifespans, as well as measuring the fatty acid composition of liver and heart phospholipids. One group (n=14) were fed a normal rat chow, while the other two groups were fed this rat chow with either added sunflower oil (n=12) or added salmon oil (n=14). They found no difference in longevity between the groups but did find some membrane fatty acid differences. The relative SFA, MUFA and total PUFA content of the tissue phospholipids was relatively little changed by the diets, with the main fatty acid changes being, understandably, in the balance between the n-3 PUFA and n-6 PUFA. There were significant effects on the PI of the liver and heart phospholipids and although there were no differences in longevity between the different diet groups, they did find a correlation between heart MUFA content and lifespan when data for all three diets were combined.

Membrane polyunsaturates: longevity of invertebrates

There is also evidence of an association between membrane polyunsaturates and longevity in invertebrates. A study of bivalve molluscs that compared the longest-living metazoan species, the mud clam *Arctica islandica* (maximum reported longevity = 507y) to four other shorter-living bivalve species (although two of these species had maximum reported longevities of 106y and 92y) found a negative relationship between the PI of gill mitochondrial membranes and maximum longevity (Munro and Blier 2012). They also observed a similar relationship when the fatty acid composition of 'gill cell debris' phospholipids was measured, which shows that the relationship was not restricted to mitochondrial membranes. Both relationships are plotted in Figure 6A. The membrane fatty acid compositions measured for the molluscan gill mitochondria had higher PI values than would be predicted for liver mitochondria from mammals of the same maximum

longevity, and the authors suggest that this may be associated with other unusual fatty acids in their membranes, some of which (plasmalogens) may be important membrane antioxidants. This will be discussed in more detail in a later section.

Another invertebrate where difference in membrane fatty acid composition has been associated with longevity differences is the honeybee, *Apis mellifera* (Haddad et al. 2007). Female honeybees can become either "workers" or "queens" depending on what they are fed during their larval life (Winston 1987). Although they are genetically identical, queen honeybees can live for years (maximum reported lifespan is 8y) while workers only live for weeks, normally dying during a foraging trip, but even if they are prevented from foraging, workers only live a maximum of a few months (see AnAge datase). During their adult life there is another difference. Within the first few days of adult life, workers begin eating pollen and honey, while queens are fed mouth-to-mouth (with "royal jelly") by worker bees during their entire adult life, never being allowed to consume pollen (Winston 1987). This is of interest because pollen has a high PUFA content (Manning 2001, Haddad et al. 2007). Worker larvae, freshly-emerged (<12h) worker bees, and both young queen bees and old queen bees all have phospholipids with a low PUFA content and a high MUFA content and consequently a low PI value. During the first week of adult life, worker bees commence eating pollen and after this period worker bees have phospholipids with an elevated PUFA content and reduced MUFA content and consequently an increased PI value (Haddad et al. 2007). For many years it has been thought that there is something in "royal jelly" responsible for the extended longevity of queen bees, however another interpretation is that being fed mouth-to-mouth during their entire adult life is a way of keeping queen bees away from PUFA and this is responsible for their long-life, i.e. it is what is <u>not</u> in "royal jelly" that is important for the long-life of the queen bee. The relationship between PI and longevity of female honeybees is presented in Figure 6B.

A significant invertebrate where a connection between membrane fatty acid composition and longevity has been documented is the nematode *Caenorhabditis elegans*. This species was the first to have its genome sequenced and has become the prime model organism for the genetic

investigation of longevity. In 1983, the first long-living mutant of this nematode, *age-1*, was discovered (Klass 1983), but it was after the discovery of the long-living *daf-2* mutant (Kenyon et al. 1993) that research in this area dramatically increased. There have now been a very large number of genes identified by researchers that modify ageing in *C. elegans,* yet the biochemical mechanisms responsible for this influence have been elusive. Recently, fat metabolism has emerged as likely having a significant role, largely because a number of lipid metabolism genes have been implicated in *C. elegans* longevity (Ackerman and Gems 2012). In many of the reports the emphasis has been on storage lipids with relatively scant attention paid to membrane fatty acid composition. We would suggest that this is another example of where the approach used to teach lipid biochemistry has influenced the perspective used to interpret results (see Introduction). While much interpretation initially centred on lipid stores (or adiposity) and the provision of energy, it has become evident that long-living mutants differ in their adiposity and there is no consistent relationship between fat stores and extended longevity (Hou and Taubert 2012). It is likely that triglyceride deposits in *C. elegans* are not just energy stores but also function as fatty acid stores for the maintenance of membrane composition by the remodeling processes described earlier for rat liver cells. It is emerging that it is fatty acid composition of lipids, rather than the amount of lipids, that is important in the determination of longevity in *C. elegans*.

Many of the studies reporting fatty acid composition of lipids from *C. elegans* do not differentiate between triglycerides and phospholipids yet phospholipids are the dominant cellular lipids being 2-3 times more abundant than triglycerides and furthermore phospholipids and triglycerides differ in their fatty acid composition in wild type *C. elegans*. For example, while there are 28-62% more SFA in phospholipids than in triglycerides, there is 44-106% more MUFA and 566-707% more PUFA in the phospholipids than in triglycerides (calculated from data in Hillyard and German 2009). Similarly, Brock et al. (2007) report that the PUFA represent 46% of total fatty acids in phospholipids but only 12% in triglycerides of wild type *C.elegans*. It is in the membranes of *C. elegans* that the vast majority of PUFA are found. Likely all of these membrane PUFA are

synthesised by *C. elegans*, in that *E. coli* are their normal food source in the laboratory and *E coli* do not have PUFA in their lipids. The dominant fatty acid in *E. coli* is 16:0 (e.g. Ishinaga et al. 1979) and although *C. elegans* are capable of lipogenesis and thus the *de novo* synthesis of 16:0, most of the 16:0 in lipids of *C. elegans* is directly absorbed from digestion of the *E. coli* in their diet (Perez and van Gilst 2008). Unlike mammals, *C. elegans* has a full range of elongase and desaturase enzymes and are thus capable of the synthesis of all their MUFA and PUFA from 16:0, and thus they have no essential fatty acid dietary requirements (for metabolic chart see Shmookler Reis et al. 2011). They do not synthesise 22:6n-3 and this highly polyunsaturated fat is not normally found in their membrane lipids. Generally, the most polyunsaturated fatty they have in their membrane lipids is 20:5n-3. They have three Δ9 desaturase enzymes responsible for producing MUFA from SFA and there is considerable redundancy among these enzymes in that there is essentially no difference in fatty acid composition of the lipids of normally fed *C. elegans* strains where these enzymes are individually genetically ablated. However, ablation of all three Δ9 desaturase genes is lethal as it prevents the production of both MUFA and PUFA (Brock et al. 2006). This is likely indicative of the homeostatic regulation of membrane fatty acid composition.

A study of particular interest is that of Shmookler Reis et al. (2011). These authors report the fatty acid composition of lipids of six mutant strains of *C. elegans* (including *age-1* and *daf-2*) that span a ten-fold range in longevities among a uniform genetic background. They found strong correlations between the fatty acid composition (especially parameters related to the highly polyunsaturated PUFA) of these *C. elegans* strains and their longevity. Although they measured total lipids because of the dominant contribution of phospholipids to total lipids (see above), it is likely that these correlations reflect a relationship between membrane fatty acid composition and longevity in this species. The relationship between PI and relative longevity of these *C. elegans* strains is presented in Figure 6C. The slope of the relationship is similar to that previously observed in mammals and birds (Figure 5) as well as for the more limited data sets for bivalve molluscs (Figure 6A) and honeybees (Figure 6B).

As discussed at the end of the previous section on mammals and birds, an obvious test of the membrane pacemaker theory of ageing is to experimentally alter membrane fatty acid composition of a species and determine if there are predictable changes in maximum longevity. Experiments attempting to alter the membrane fatty acid composition of the adult blowfly *Calliphora stygia* by raising them on larval diets that differ dramatically in fatty acid composition have been carried out and while the relative content of individual fatty acids changed, there were compensatory changes in other fatty acids that resulted in no change in the PI of either larval or adult phospholipids (see Figure 4). Not surprisingly, there were no differences in the maximum longevity of these blowfly fed the different diets (Kelly et al. 2013).

As with other species, it appears diet manipulation in *C. elegans* can produce changes in the relative abundance of individual fatty acids in phospholipids that are countered by compensatory changes in other fatty acids. For example, Hillyard & German (2009) found there was no effect on maximum longevity of *C. elegans* when 20:5n-3 and 22:6n-3 was added to their normal *E. coli*-based food. Although there was an increased incorporation of both fatty acids in the phospholipids (20:5n-3 content increased from 28% to 32% of total fatty acids while 22:6n-3 increased from 0% to 11%), there were compensatory changes in the incorporation of other PUFA into phospholipids such that the total PUFA content of phospholipids was 56% and 54% respectively in 20:5n-3 and 22:6n-3 supplemented nematodes compared to 54% in non-supplemented worms (Hillyard & German 2009).

A distinct experimental advantage of *C. elegans* is that it is possible to use a different mechanism to potentially influence membrane composition. By feeding the worms *E. coli* engineered to produce specific RNAi it is possible to selectively inhibit the production of specific enzymes. This was done in the Shmookler Reis et al. (2011) study where some of the desaturase and elongase enzymes were knocked-down by RNAi and both the effect on longevity and the resistance to oxidative stress (hydrogen peroxide exposure) was measured. Knock-down of the fat-4 gene (which codes for the Δ5 desaturase enzyme responsible for the conversion of 20:3n-6 ->

20:4n-6 and 20:4n-3 -> 20:5n-3) produced the greatest resistance to hydrogen peroxide exposure and also the greatest experimental longevity extension. Although they did not measure whether membrane fatty acid composition was altered in these nematodes, other researchers have compared the fatty acid composition of total lipids in fat-4 mutant (i.e. complete knock-out of the fat-4 gene) with wild-type *C. elegans* (Watts and Browse 2002) and report the complete absence of 20:4n-6 and 20:5n-3 but elevated 20:3n-6 and 20:4n-3 levels in the lipids of the fat-4 mutant compared to wild type. Interestingly, there was negligible difference in total PUFA (38% vs 40%) but a decrease in the PI value we have calculated for lipids of the fat-4 mutant compared to wild-type (123 vs 173). Future work using *C. elegans* should elucidate whether membrane composition and the effects on lipid peroxidation processes are important in ageing and the determination of lifespan.

Other membrane lipids: possible roles in longevity?

As described earlier, in most PUFA there is a single methylene group ($-CH_2-$) between each pair of double bonds and it is the H atoms attached to this methylene carbon (the *bis*-allylic hydrogens) that are removed in the first step of free radical initiated lipid peroxidation. Although uncommon, there are some PUFA where the pairs of double bonds in the fatty acid are separated by a number of $-CH_2-$ groups, rather than a single methylene group. These are called "non-methylene-interrupted" (NMI) fatty acids and because of their distinctive structure they provide the physical properties of PUFA without the disadvantage of being very susceptible to free radical attack (because they lack *bis*-allylic hydrogens) and the consequent initiation of lipid peroxidation. They are peroxidation-resistant PUFA and occur in significant amounts in the membrane lipids of marine bivalves (Kraffe et al. 2004) but not in endotherms or insects. Interestingly, NMI fatty acids were observed by Munro and Blier (2012) to constitute 15-18% of total FA, and 37-42% of total PUFA in the membrane lipids in the exceptionally long-living clam *Arctica islandica* (maximum longevity = 507y) but only 5-6% of total FA, and 12-13% of total PUFA in the membrane lipids of the much shorter-living bivalve *Mya arenaria* (maximum longevity = 28y). The three bivalve species in this

study with intermediate longevities had intermediate levels of NMI fats in their membrane lipids (Munro and Blier 2012).

Another feature of the membrane lipid composition of marine bivalves is that they have significant amounts of plasmalogens (Kraffe et al. 2004). These membrane lipids differ from regular phospholipids in that the carbon chain in the *sn-1* position is linked to the glycerol backbone by a vinyl ether linkage instead of the regular ester linkage. In the bivalves studied by Munro and Blier (2012), plasmalogens were a significant component of membrane lipids and were significantly lower in mitochondria of the shortest-living species compared to the other four species. There was however no significant trend with longevity of the molluscs. High plasmalogen levels in membrane lipids have been associated with extended longevity in mammals. Naked mole rats (*Heterocephalus glaber*) have a maximum longevity (~30y) that is about 7-8 times that of mice (*Mus musculus*; ~4y) and the plasmalogen levels in the tissues of naked mole rats, especially the phosphatidylcholine plasmalogens, are several times the levels found in mice (Mitchell et al. 2007). Plasmalogens are of considerable interest because it has been suggested that as membrane lipids they may be significant endogenous membrane antioxidants. The vinyl ether linkage includes a double bond that is attacked especially rapidly by reactive species, so "sparing" other susceptible membrane lipids. The products of this attack decompose rapidly into molecules that, unlike products of peroxidative attack on PUFA chains in membranes, do not propagate lipid peroxidation (Halliwell and Gutteridge 2007). In this way, plasmalogens are membrane lipids that are ROS scavengers and can thus stop the autocatalytic process of lipid peroxidation.

Conclusion

From an evolutionary perspective, lipid metabolism is best understood by first considering the importance of fatty acids in membrane lipids rather than as primarily a form of energy storage. The lack of Δ12- and Δ15-desaturase enzymes in 'higher' animals means that, unlike SFA and MUFA, both omega-6 and omega-3 PUFA cannot be synthesised *de novo* by these 'higher' animals

and consequently are essential components of their diet. The amount of SFA, MUFA and total PUFA in a normal diet has little influence on their relative abundance in membrane lipids, such that membrane fatty acid composition is homeostatically regulated with respect to these broad categories of fatty acids. This is not the case for storage fats, which are strongly influenced by diet. The processes of continual deacylation/reacylation of membrane lipids is likely important in the regulation of membrane fatty acid composition and the characteristics of the enzymes responsible are likely the path whereby genes determine the fatty acid composition of membranes. Some of the enzymes responsible for this process of continual membrane remodeling, the acyltransferases, do not differentiate well between omega-6 and omega-3 PUFA. Unlike, SFA and MUFA, all PUFA are capable of being peroxidised and the more polyunsaturated the fatty acid the more prone it is to lipid peroxidation. Knowing its fatty acid composition, it is possible to calculate a theoretical susceptibility of a membrane to peroxidative damage; its peroxidation index (PI). For a number of species, PI is also homeostatically maintained in that, it is little influenced by the PI of diet fat. Membrane composition differs between animal species and the PI of membrane lipids is inversely related to the maximum lifespan. Such a correlation has been documented in endothermic vertebrates and also in a more limited number of investigations of invertebrate species. An interesting finding is the emerging role of membrane fatty acid composition in the longevity of one of the most investigated animal models, the nematode *Caenorhabditis elegans*. Gene knockdown studies in this nematode do support a cause-effect relationship, which could profitably be emulated in other genetically malleable species.

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Figure legends

Figure 1. An outline of the metabolic pathways involved in the synthesis of fatty acids. Individual fatty acids are identified by "number of C atoms: number of double bonds (and the position of the most terminal double bond)". The most common fatty acids in mammals are in black text while those in grey text are least common. SFA, MUFA and PUFA respectively refers to saturated, monounsaturated and polyunsaturated fatty acids. Enzymes involved are either identified by name or letters. E refers to elongase enzyme, while Δ12-D, Δ15-D, Δ9-D, Δ6-D, and Δ5-D refer to specific desaturase enzymes. Black solid arrows represents reactions occurring in the cytoplasm or endoplasmic reticulum, black dashed arrows indicate partial β-oxidation occurring in peroxisomes, while blue dashed arrows denote the desaturase enzymes lacking in higher animals.

Figure 2. The percent of oral dose of ^{14}C-labeled fatty acids that, over 24h, were either excreted as CO_2 (left hand figure) or incorporated into liver lipids (right hand figure) in the rat. Data are from Leyton et al. (1987a; 1987b).

Figure 3. Relationship between diet fatty acid composition and the fatty acid composition of membrane lipids (phospholipids; a,b,d,e) of muscle, brain, heart, liver and storage lipids (triglycerides; c,f) of adipose tissue and plasma in the rat. Data are from Abbott et al., (2012). Groups of rats (n=6 per group) were fed, for 8 weeks, one of 12 isocaloric medium fat (25% energy) diets that differed only in their fatty acid profile. Dashed line represents line of perfect conformity between diet fat composition and membrane fat or storage fat composition.

Figure 4. Relationship between diet peroxidation index and membrane lipid peroxidation index of (a, b) rat tissues and (c) membrane lipid peroxidation index of larvae and thorax of adult blowfly *Calliphora stygia*. Data for rat tissues (graphs a & b) are from Abbott et al. (2012) while the data for the blowfly are from Usher et al. (2013).

Figure 5. Relationship between maximum lifespan and peroxidation index of skeletal muscle phospholipids for mammals and birds. Data points are for those described in figure 3A of Hulbert (2010) with the additional data points for parrots and quail from Montgomery et al. (2012c). Equation is relationship determined from all data points.

Figure 6. Relationship between maximum longevity and peroxidation index for three invertebrates. a) relationship for phospholipids of gill mitochondria and gill cell debris from five species of bivalve molluscs that differ in maximum longevity (data from Munro, Blier 2012); b) relationship for phospholipid peroxidation index and maximum longevity difference between worker and queen honeybees (data from Haddad et al. 2007); c) relationship between total lipid peroxidation index and relative lifespan of strains of *C. elegans* that differ in longevity (data from Shmookler Reis et al. 2011).

figure 1

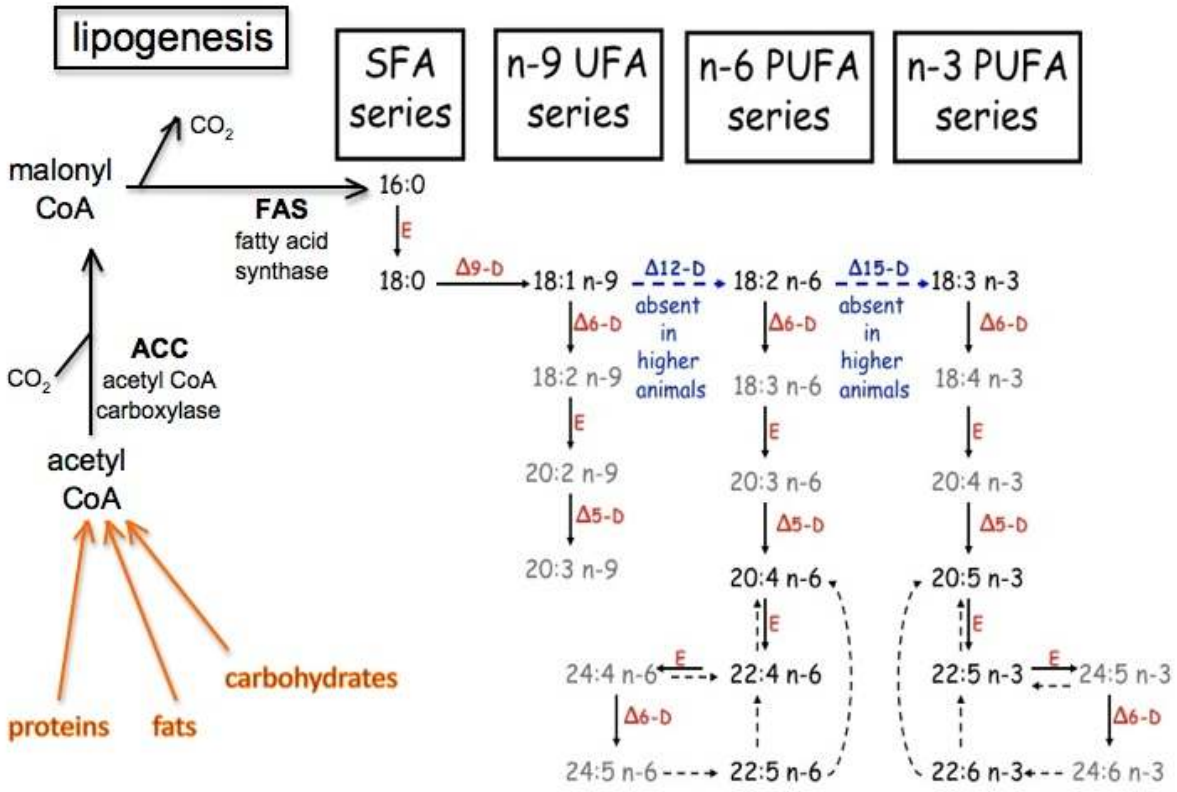

figure 2

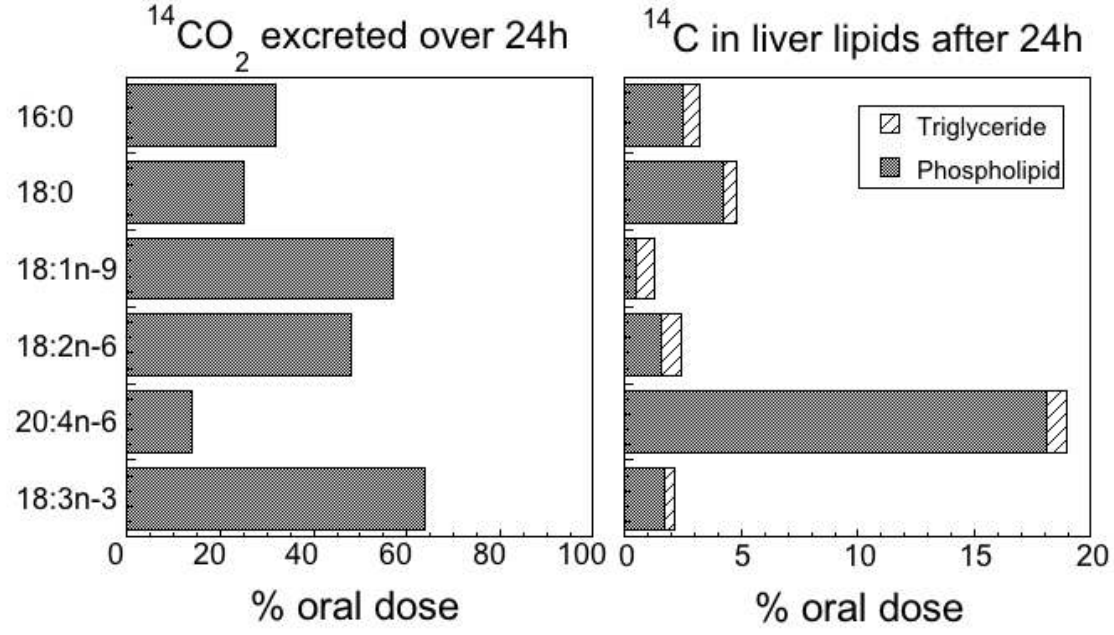

figure 3

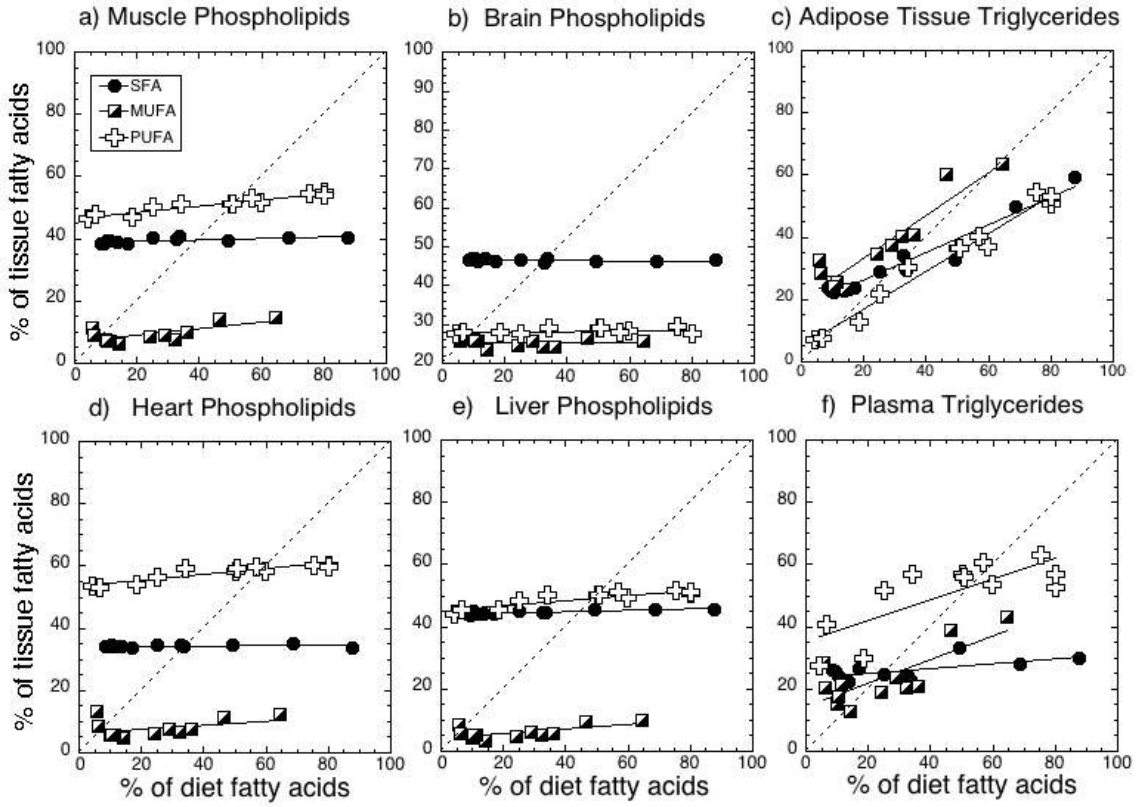

figure 4

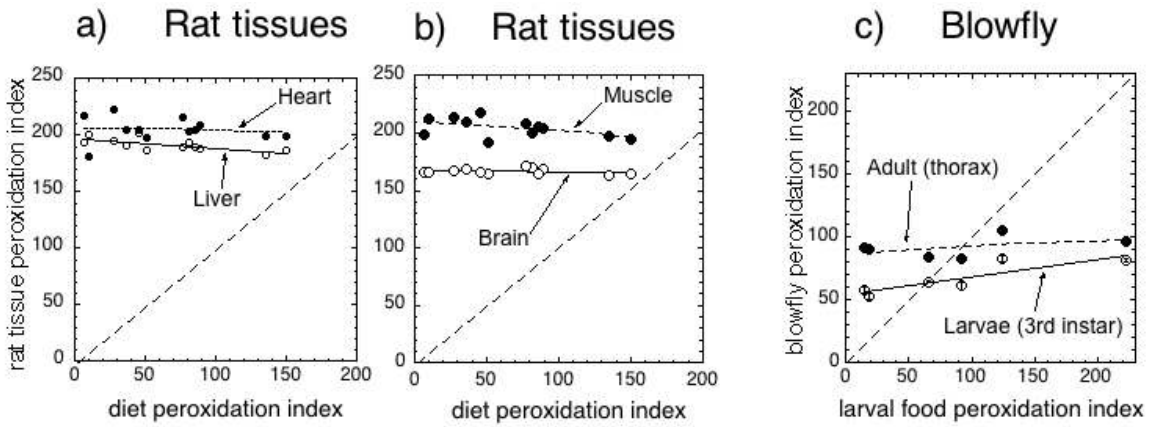

figure 5

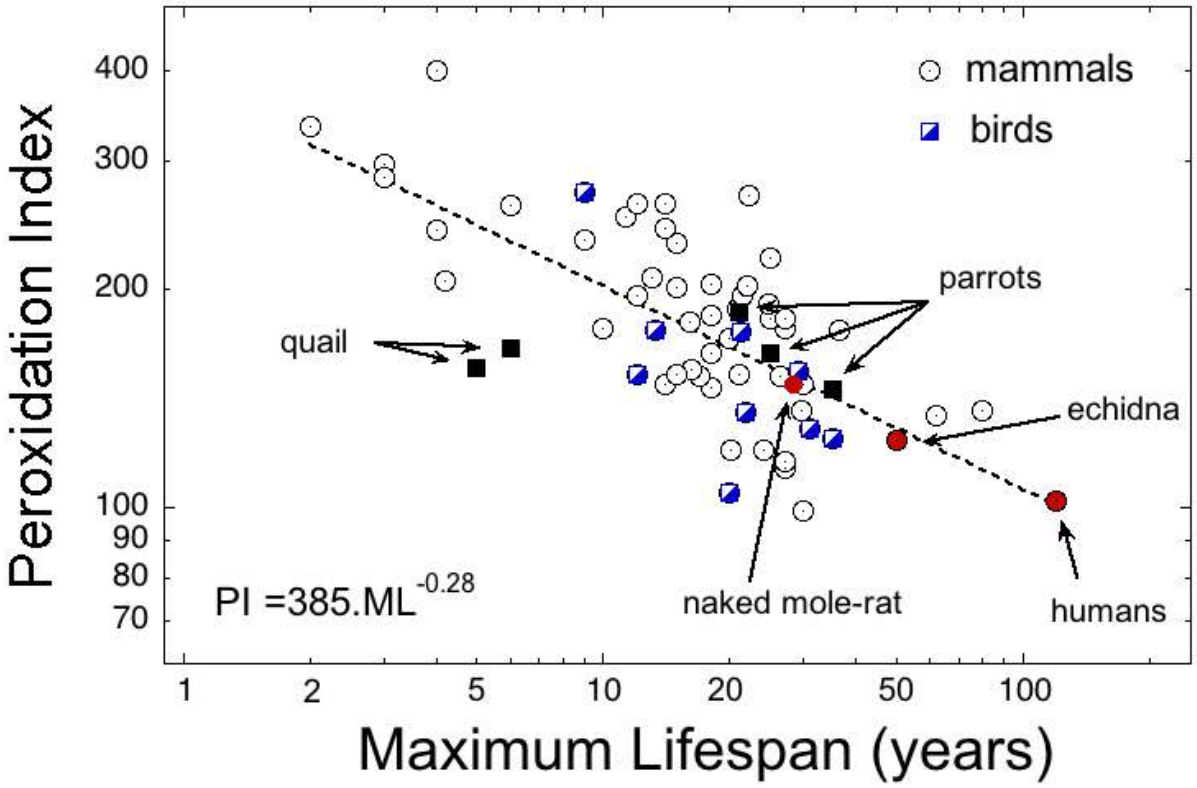

figure 6