The possible role of membrane lipids in the exceptionally long life of the short-beaked echidna, *Tachyglossus aculeatus*.

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ABSTRACT

The short-beaked echidna (*Tachyglossus aculeatus*) is an exceptionally long-living mammal having a maximal lifespan of ~50 years. This is about four times that predicted from its body mass and, consequently, its longevity quotient is ~4. This longevity quotient is similar to two other exceptionally long-living mammalian species; the naked mole-rat (*Heterocephalus glaber*) and *Homo sapiens*. In recent times, the types of fats that make up cellular membrane have been implicated in the determination of a species' maximum lifespan. This modification of the oxidative stress theory of aging, which has been called the membrane pacemaker theory of aging, derives from the fact that polyunsaturated fats are very susceptible to lipid peroxidation whereas monounsaturated fats are resistant to peroxidation. As a test of the theory we measured the fatty acid composition of membrane lipids isolated from tissues of echidnas. We found that, as in the other long-living mammals, echidna membranes are more monounsaturated and less polyunsaturated than would be predicted from their body size and that the peroxidation index of their membrane lipids is what their maximum longevity would predict.

Key words: Aging, maximum life span, membrane fatty acids, lipid peroxidation.

Introduction

The short-beaked echidna, *Tachyglossus aculeatus*, is a fascinating animal for a number of reasons. As well as its evolutionary history, physiology and ecology, it is of interest because of its exceptional longevity. Mammals differ in their maximum life span, which is characteristic for each species, and maximum life span has been related to the body size of the species with large species, in general, living longer than smaller species. The equation $MLSP = 10.2 \times M^{0.22}$ relates maximum lifespan (MLSP, years) to body mass (M, kg) and predicts that a mammal weighing 3-4 kg should live for a maximum of only 13-14 years (Hulbert *et al* 2007). Yet, the maximum longevity recorded for an echidna in captivity is 50 years (Philadelphia Zoo; 1903-1953) and a free-living individual has been observed over 45 years (see Augee *et al* 2006). This means that the echidna have a maximum life span that is almost four times that expected for its body size.

There are a few other mammal species that also exhibit exceptional longevity, all unrelated to echidnas. The naked mole-rat (*Heterocephalus glaber*) is an African subterranean rodent that as an adult is about the same size as the common house mouse (*Mus musculus*), yet while mice live for a maximum of 3-4 years, the naked mole-rat has a recorded maximum lifespan of ~28 years (Buffenstein 2005). Similarly, many bat species live several times longer than predicted by their body mass (Austad and Fischer 1991). Our own species, *Homo sapiens*, should have a maximum lifespan of only 26 years according to the relationship for mammals in general (see equation above), yet there are reliable records of humans living for more than 120 years (Carey and Judge 2000). In comparison, the largest land mammal, the elephant, has a maximum life span of only 80 years (Carey and Judge 2000). Thus, the echidna belongs to a relatively small group of mammals that exhibit exceptional longevity. The data for these three exceptionally long-living species compared to mammals in general are illustrated in Figure 1.

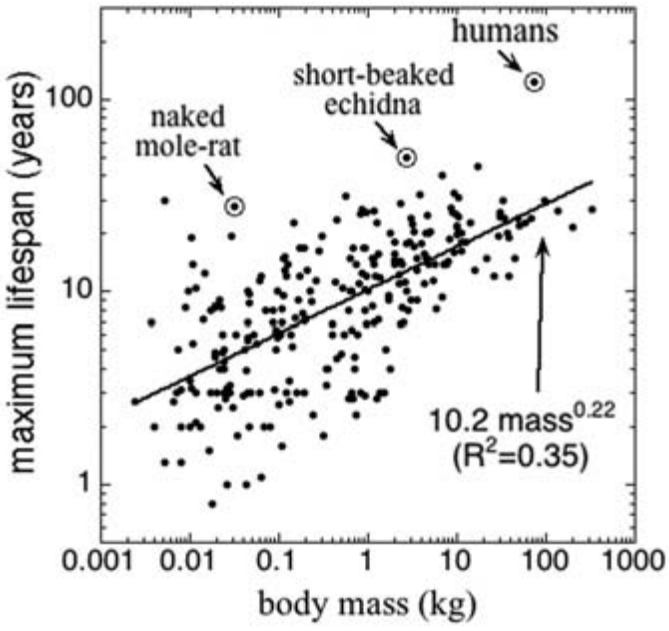

Figure 1. The relationship between the maximum lifespan and body mass of mammalian species (taken from Hulbert et al 2007) with the data points for three exceptionally long-living mammals (humans, short-beaked echidnas and naked mole-rats) highlighted.

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What particular mechanisms and properties determine the characteristic maximum longevity of each species is currently unknown. Early in the last century, it was proposed that maximum lifespan was determined by the 'speed of life', i.e. the metabolic rate of a species (Rubner 1908). This was later called the "rate of living" theory of aging and, while it has much popular appeal, expressed in phrases such as "live fast - die young", there is considerable scientific evidence that longevity is not determined solely by metabolic rate. This evidence includes the following observations; 1) voluntary activity (in humans and rats) does not shorten lifespan, 2) there is no inverse correlation within a population (mice and fruitflies) between metabolic rate and lifespan of individuals, 3) birds have higher metabolic rates than similar-sized mammals yet do not have shorter longevity but have longevities that average more than twice that of similar-sized mammals, 4) although calorie restriction extends longevity in a wide range of animals it does not reduce the mass-specific metabolic rate (for details see Hulbert *et al* 2007). A recent analysis found only 26% of the variation in maximum life span among mammal species is associated with variation in basal metabolic rates (Hulbert *et al* 2007). Although the metabolic intensity of a mammal species plays some role in determination of its longevity there are obviously other properties or processes involved as well. In the case of the echidna, one such mechanism may be hibernation. For example, Lyman et al (1981) showed that hamsters allowed to hibernate and drop their body temperature to 5°C had a significantly longer lifespan (mean longevity = 914 days) than hamsters kept at 22°C for their whole life-time (mean longevity = 812 days). Furthermore they also found that within the hibernating hamsters, good hibernators lived longer than poor hibernators. Consequently it is likely that part of the explanation of the exceptional longevity of echidnas might be due to its hibernating behaviours. However, in view of the fact that hibernation increased the longevity of hamsters by an average 13%, it seems unlikely that hibernation can completely explain the exceptional longevity of the short-beaked echidna.

Recently, it has been found that the fatty acid composition of membrane lipids varies in a systematic manner among species and it has been proposed that this may be part of the explanation of the different longevities of different species. This proposal has been called the "membrane pacemaker" theory of aging and is described in detail elsewhere (Hulbert 2005; Hulbert *et al* 2007). The "membrane pacemaker" is a modification of the "oxidative stress" theory of aging and emphasizes lipid peroxidation as a key process during aging. Of the three types of fatty acids; saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA), only the PUFA are capable of being damaged by peroxidation and both SFA and MUFA are unable to be peroxidised (Holman 1954). Consequently membrane lipids with a high PUFA content are very susceptible to peroxidative damage while those with a high MUFA content are very resistant to damage from peroxidation. Furthermore the products of lipid peroxidation are very potent "reactive molecules" that can do further damage to other important cellular molecules such as proteins and nucleic acids (including DNA).

Membrane fatty acid composition has been shown to be correlated with body size in mammals, with small (short-living) mammals having more polyunsaturated membrane lipids than larger (long-living) species (Couture and Hulbert 1995; Hulbert *et al* 2002). Furthermore, it has been shown recently that the exceptionally long-living naked mole-rat has membrane lipids with a lower level of peroxidation-prone PUFA and a higher content of peroxidation-resistant MUFA than the similar-sized but much shorter-living house mouse (Hulbert *et al* 2006a). Similarly, for our size, we *Homo sapiens* have a comparatively peroxidation-resistant membrane lipid composition (Hulbert *et al* 2007). The study reported here was undertaken to examine whether the fatty acid composition of membrane lipids from the long-living echidna differed similarly from that predicted for a mammal of its body size.

Materials and Methods

Samples of echidna skeletal muscle and liver were obtained from a variety of sources. Small pieces of abdominal wall muscle were taken from two anaesthetized echidnas during removal of telemeters from their peritoneal cavity in the environs of Brisbane, Queensland. Samples of abdominal muscle and leg muscle (hind limb) were also taken from two frozen echidna carcasses at the University of Wollongong. Two frozen samples of leg muscle and four frozen liver samples were also obtained from the University of Queensland. For three of the liver samples there was enough tissue for mitochondria to be prepared by methods described previously for fresh liver (Brand *et al* 1991). The phospholipid fatty acid composition of mitochondria isolated from frozen rat liver is the same as that of mitochondria isolated from fresh rat liver (Hulbert, unpublished results). Tissues were obtained from studies approved by the respective animal ethics committees of the University of Wollongong and the University of Queensland.

Total lipids were extracted by hand using glass/glass homogenizers and ultrapure grade chloroform/methanol (2/1, vol/vol) containing butylated hydroxytoluene (0.01% wt/vol) as an antioxidant. Total lipid extracts prepared at the University of Queensland were dried and sealed in glass vials under a nitrogen atmosphere. These were transported to the University of Wollongong for further analysis. Phospholipids were separated from neutral lipids by solid phase extraction on silica Sep-Pak columns (Waters, Midford MA, USA). Phospholipids were transmethylated, and fatty acid methyl esters were separated by gas-liquid chromatography on a Shimadzu GC-17A gas chromatograph (Shimadzu Corp., Kyoto, Japan) with a fused silica capillary column. Individual fatty acids were identified by comparing each peak's retention time to those of external standards, and expressed as the mol % of total fatty acids.

The peroxidation index is a measure of the calculated susceptibility of the phospholipid fatty acids to peroxidative damage and is calculated as PI = (0.025 x % monoenoics) + (1 x % dienoics) + (2 x % trienoics) + (4 x % tetraenoics) + (6 x % pentaenoics) + (8 x % hexaenoics) (Holman 1954).

Results

Previous studies reporting the fatty acid composition of phospholipids from skeletal muscle and liver of mammals ranging in size from mice to cattle (Hulbert *et al* 2002) and of phospholipids from liver mitochondria of mammals ranging from mice to horses (Porter *et al* 1996) have led to published equations which allow calculation of the phospholipids fatty acid composition expected for a 3kg mammal. Figure 2 presents the composition measured for the echidna tissues relative to the expected values calculated from these previous studies. As can be seen, skeletal muscle phospholipids from the echidna have expected levels of total unsaturated fatty acids but higher-than-predicted MUFA levels and lower-than-predicted PUFA levels. As can also be seen, echidna phospholipids from both whole liver and liver mitochondria have lower-than-predicted total unsaturates, higher-than-predicted MUFA and lower-than-predicted PUFA levels.

Because echidna phospholipids are relatively rich in peroxidation-resistant MUFA and relatively poor in peroxidation-prone PUFA, we would expect that the cell membranes of echidnas would be relatively resistant to peroxidation damage from the free radicals produced by normal metabolism. When the fatty acid composition of membrane phospholipids is combined with the peroxidation-susceptibility of individual fatty acids, it is possible to calculate a peroxidation index (PI) for the membrane. This is a single number which encapsulates the susceptibility of the membrane to peroxidative damage, the higher the value of PI the more susceptible is the membrane to peroxidative damage the lower the PI value, the more resistant is the membrane to peroxidation. The calculated PI values for skeletal muscle phospholipids and liver mitochondria phospholipids are respectively 121 and 79. The expected PI values for a 3kg mammal are respectively 202 and 138 (Hulbert Beard and Grigg, 2008). Thus we can calculate that the PI values for echidna phospholipids measured in the current study are 57-60% of that predicted for a 3kg mammal. However, although the PI values are lower than expected for their body size, they are what one would expect for a mammal of their maximum longevity. This is illustrated in Figure 3, where the relationship between maximum life span and PI values for skeletal muscle phospholipids and liver mitochondrial phospholipids are plotted for a number of mammal and bird species. The echidna data points conform well to the relationship for mammals and birds in general.

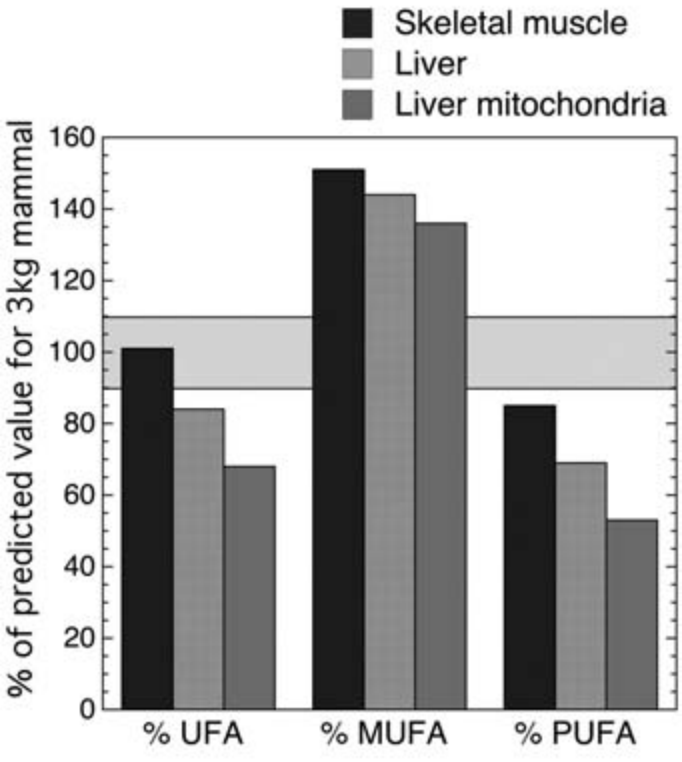

Figure 2. The fatty acid composition of phospholipids from short-beaked echidnas compared to that predicted for a mammal of the same body size (3kg). The predictions for muscle and liver phospholipids have been calculated from the equations of Hulbert et al. (2002) while those for liver mitochondrial phospholipids are from the equations of Porter, Hulbert and Brand (1996). The hatched area represents 90%-110% of predicted values. UFA = unsaturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Discussion

The present finding that the membrane lipids of echidnas are not very polyunsaturated but highly monounsaturated for a mammal of its size is similar to recent measurements of the membrane lipids from the naked mole-rat, another exceptionally long-living mammal (Hulbert *et al* 2006a). It is also similar to measurements reported for the exceptionally long-living primate; *Homo sapiens* (Pamplona et al. 1996; Hulbert 2005). This lower-than-expected level of PUFA and higher-than-expected level of MUFA in membrane lipids means that the membranes of these long-living mammals are resistant to lipid peroxidation and this is consistent with the membrane pacemaker modification of the oxidative stress theory of aging.

There are other examples that support a connection between longevity and the fatty acid composition of membranes. Although birds are in general longer-living than similar-sized mammals, they have membrane lipids with a lower PI than similar-sized mammals and the relationship between maximum longevity and PI does not differ between mammals and birds (see Figure 3). Similarly, there are wild-derived strains of *Mus musculus* that have an extended longevity compared to lab mice when kept under identical conditions (Miller *et al* 2002) and these mice also have membrane lipids with lower PI than the lab mice (Hulbert *et al* 2006b). Queen honey bees live much longer than worker honey bees and they too have membrane lipids which are more monounsaturated than those of worker bees (Haddad *et al* 2007). Within humans, longevity is partly inherited (Herskind et al 1996) and it has been shown recently that the children of nonagenarians have red blood cell membranes with a significantly reduced PI compared to controls (Puca *et al* 2008). Calorie restriction, the only physiological treatment shown to extend longevity in a broad range of animals, has been shown to cause a decrease in the PI of membranes in rats (Laganiere and Yu 1987) and mice (Faulks *et al* 2006).

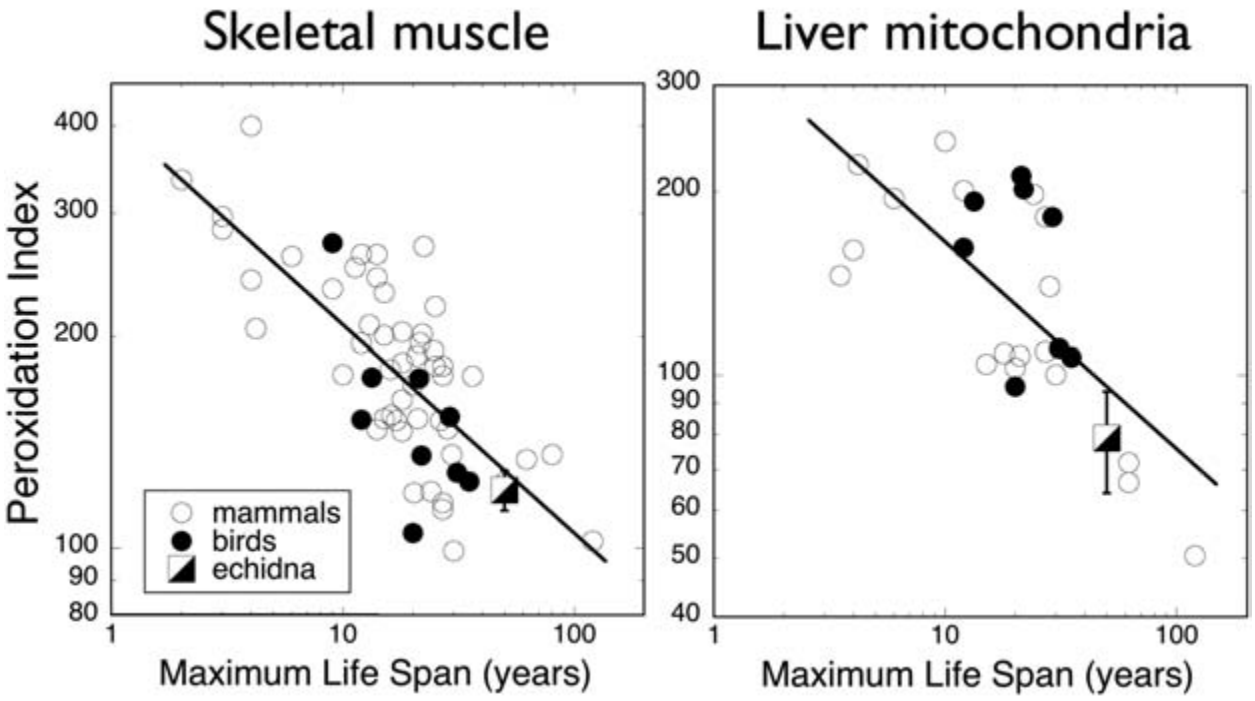

Figure 3. The relationships between the maximum life span of mammals and the peroxidation index of (i) skeletal muscle phospholipids (left-hand graph) and (ii) liver mitochondrial phospholipids (right-hand graph). The mammal data (unfilled circles) for skeletal muscle are the combined data points from Hulbert (2005) and Valencak and Ruf (2007). The mammal data for liver mitochondria are the combined data points from Hulbert (2005) and Pamplona *et al* (1998). The bird data (filled circles) are from Hulbert (2005). The data points for echidnas are from the present study with the error bars representing ± 1 S.E.M.

The reason that membrane PUFA influence longevity is because the products of oxidative damage to membrane PUFA are themselves powerful reactive oxygen species that in turn damage other cellular molecules oxidatively. This provides a positive feedback loop in the processes normally considered to constitute the pathways of oxidative damage. The greater the PUFA content of membrane lipids the greater the intensity of this feedback loop. The biochemistry of this is discussed in more detail elsewhere (see Hulbert *et al* 2007). In Figure 4 we provide a schematic that describes the membrane pacemaker perspective. Although not discussed here it has also been shown that variation between species in metabolic rate is also associated with membrane composition. Those mammals with higher mass-specific metabolic rates also have more polyunsaturated membranes. This too is discussed in more detail elsewhere (see Hulbert and Else 2000).

For illustrative purposes we have chosen in Figure 4 to present a comparison between two similar-sized mammals that differ in maximum longevity and for which we have membrane composition data. These are the echidna and the rabbit (*Oryctolagus cuniculus*). The fatty acid data for the rabbits are for tissues from feral rabbits killed in the wild (from Hulbert *et al* 2002). Whereas echidnas have a documented maximum lifespan of 50 years, surprisingly, the maximum longevity of rabbits is not certain. The AnAge database (http://genomics.senescence.info/) states for rabbits: "There are many anecdotes concerning the longevity of rabbits. It has been estimated that both in the wild and in captivity they rarely live more than 9 years. Although a rabbit in Australia called 'Flopsy' reportedly lived 18.8 years in captivity after being caught in the wild, but this record cannot be confirmed. Record longevity in zoos and parks is only 7.9 years belonging to one female at Frankfurt Zoo. Further studies are necessary to better estimate the maximum longevity of these animals." Certainly rabbits are much shorter-living than echidnas. As can be seen from Figure 4, associated with their shorter maximum lifespan, the membrane lipids of rabbits are both more polyunsaturated and less monounsaturated than those of echidnas.

A final comment should be made concerning the connection between the membrane composition and the fatty acid composition of the diet. Evidence is emerging that the fatty acid composition of membrane lipids is regulated and although the fatty acid composition of storage lipids (triglycerides) reflects the fatty acid composition of the diet, the fatty acid composition of membrane lipids (phospholipids) is not a reflection of diet but is controlled by enzymes that are regulated genetically. For example, the mice strains that differed in longevity showed significant differences in membrane fatty acid composition even though they were fed identical diets (Hulbert *et al* 2006b). Studies on laboratory rats also show that the MUFA content of membrane lipids is relatively independent of the MUFA content of the diet (Hulbert *et al* 2005). However, while echidna membrane lipids have a high MUFA content, their natural diet is also high in MUFA. In the wild, echidnas are termite/ant eaters and the lipids that

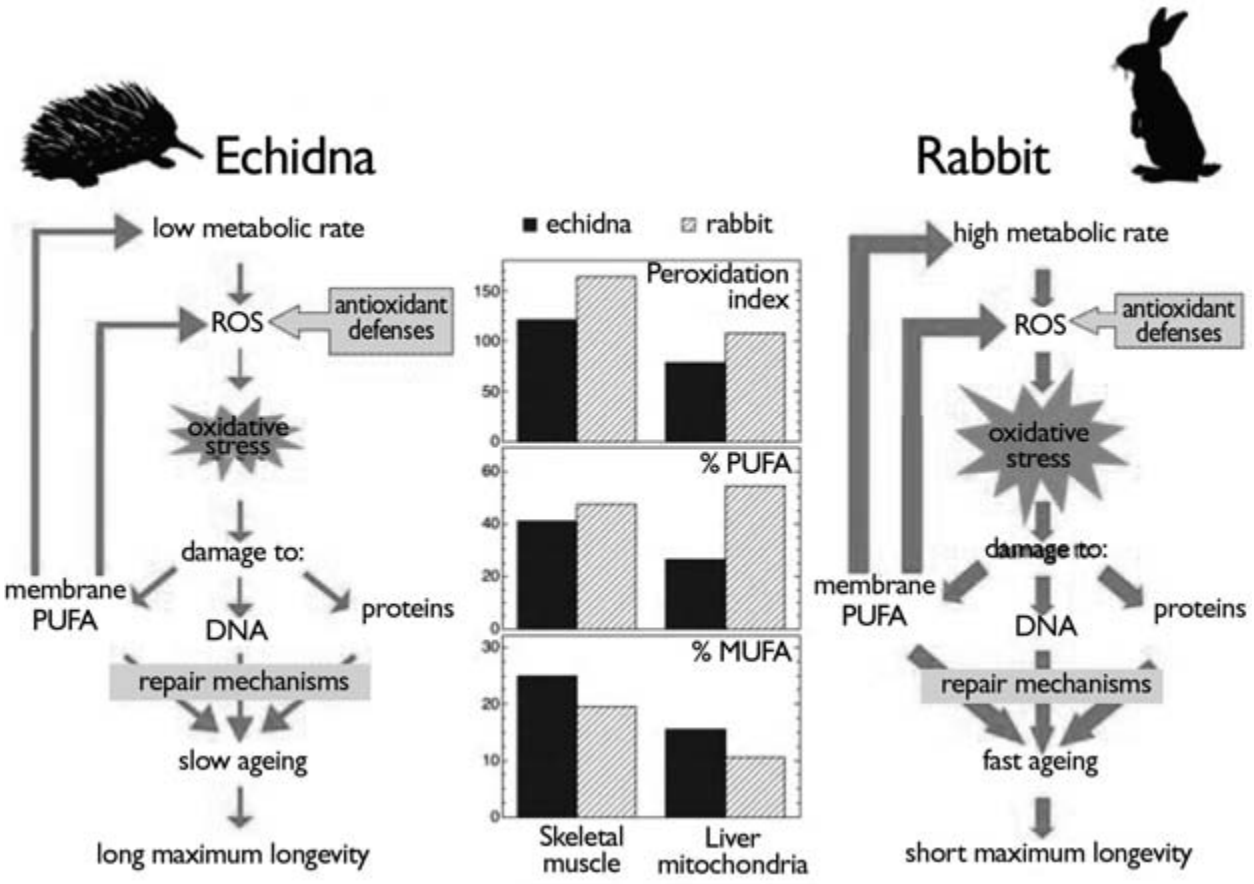

Figure 4. A comparison of the rabbit and the short-beaked echidna: two similar-sized mammals with differing maximum longevities. The middle figure compares the data for the two species. The schematic diagrams on each side outline the membrane-pacemaker theory of aging for each species, with the thicker arrows in the rabbit representing a greater intensity of the process in the rabbit compared to the echidna.

make up these insects are highly monounsaturated. Indeed measurement of the fatty acid composition of adipose tissue (primarily triglycerides) of echidnas shows them to be essentially a reflection of the fatty acid composition of lipids of ant larvae (Falkenstein *et al* 2001). While the fatty acid composition of membrane lipids reported here are for echidnas from the wild, and thus on their natural diet, the maximum longevity value is from a captive echidna at the Philadelphia Zoo that "was on a diet of half a pint of whole milk and a raw egg daily" (see p125 of Augee *et al* 2006). Such a captive diet would be low in MUFA and high in PUFA. The question as to whether the maximum longevity of the wild echidnas (on a high MUFA diet) might actually exceed 50 years could be answered by comparing the life spans of echidnas kept on different diets or, more directly, by radiotracking known age individuals in the wild until their demise, hoping to get data from one or more which survives to die from 'natural causes'. However, at this time in our lives, the current authors will have to pass on such experiments and leave that to the next generation of zoologists!

Rabbit-Echidna comparison

	Rabbit	Echidna
mass	3kg	3kg
MLSP	10 - 18 yrs	50 yrs
MUSCLE		
UFA	67.3%	65.9%
MUFA	19.6%	25.0%
PUFA	47.7%	40.9%
PI	164	121
LIVER MITO:		
UFA	65.0%	41.9%
MUFA	10.6%	15.5%
PUFA	54.4%	26.5%
PI	108	79

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