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DNA MMR systems, microsatellite instability and antioxidant activity variations in two species of wild bats: *Myotis velifer* and *Desmodus rotundus*, as possible factors associated with longevity

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Abstract The accumulation of oxidative damage to biomolecules, such as DNA, is known to induce alterations in the cell's mechanisms and structure that might lead to the aging process. DNA mismatch repair system (MMR) corrects base mismatches generated during DNA replication that have escaped the proofreading process. In addition, antioxidant enzymes can reduce reactive oxygen species effects in order to protect cells from oxidizing damage. In order to determine the importance of these associated factors during the aging process, in this study, levels of MMR proteins MSH2 and MLH1, as well as microsatellite markers, were compared in liver, lung, and brain of juvenile, adult, and old, both female and male, individuals from two species of wild bats: the short-lived *Myotis velifer* and the longer lived *Desmodus rotundus*. Catalase, glutathione peroxidase, and superoxide dismutase were also analyzed to determine if the antioxidant protection correlates negatively with DNA damage. Antioxidant activities were higher in the longer lived *D. rotundus* than in *M. velifer*. Furthermore, old *M. velifer* but not old *D. rotundus* bats had reduced MMR levels and increased microsatellite instability. Therefore, although our results correlate the reduced MMR efficiency, the deficient antioxidant activity, and the increase in DNA damage with the aging process, this is not always true for all living organisms.

Keywords Aging · Antioxidant activity · Chiroptera · DNA damage · Microsatellite instability · Mismatch repair

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Introduction

Harman's theory of aging proposes that the accumulation of oxidative damage to biomolecules induces alterations in the cell's mechanisms and structure that might lead to

the aging process (Harman 2003; Barouki 2006). Increased DNA damage and decreased DNA damage repair stand out as important features in this process (Bokov et al. 2004; Gorbunova et al. 2007). Hence, antioxidant enzymes, which reduce the effects of reactive oxygen species (ROS), are expected to protect cells from oxidizing damage to biomolecules such as DNA (Wei et al. 1996; Xu et al. 2011). Catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) are considered to be the most important representatives of cellular antioxidant defense and have, therefore, been widely studied (Scandalios 2002; Martin et al. 2006).

DNA mismatch repair system (MMR) is an evolutionarily conserved process that corrects base mismatches generated during DNA replication that have escaped the proofreading process (Kunkel and Erie 2005). Along with the base excision repair (BER) and the nucleotide excision repair (NER) systems, which correct changes in DNA sequence (Bernstein et al. 2002; Moustacchi 2000; Nakano et al. 2005), MMR accomplishes its role through the interaction of protein heterodimers that bind to the damaged DNA sequence. The MutSH family of proteins, which was originally found in *Escherichia coli,* recognizes errors in the genome sequence after replication and is known to be integrated in eukaryotic organisms by MSH2, MSH3, and MSH6 (Jiricny 2006; Kültz 2005; Mullins et al. 2005). MSH2 binds to MSH6 in the presence of base mispairing and other mismatches forming the MutSα heterodimer (MSH2–MSH6) (Peltomäki 2001; Santuci-Darmanin and Paquis-Flucklinger 2003). Then, the MutSα heterodimer binds to the damaged region and recruits the MutLH family proteins, MLH1 and PMS2, to form the MutLα heterodimer. Finally, the DNA-MMR complexes initiate the signaling process in order to replace the DNA lesion through the action of DNA polymerase activity (Phung et al. 1999; Saribasak et al. 2008).

Since MSH2 is the key component of the MMR system, when its expression decreases or its interactions fail to initiate the DNA repair signal, the malfunctioning of cells can derive in injury and disease (Denver et al. 2005). Recent evidence suggests that the activity of the MMR system decreases when cells are near or at the senescent stage (Young et al. 2003); furthermore, MSH2 deficiency increases the formation of microsatellite regions. Microsatellites are frame-shift mutations consisting of repeated units from one to six nucleotides which, when inserted into the DNA, can expand it, changing the original sequence and altering the normal genetic expression of key cellular components and pathways (Alazzouzi et al. 2005; Lin and Wilson 2009).

Increased frequency of microsatellite regions, related to MSH2 deficiency, has been reported to promote degenerative conditions in the cell (Chang et al. 2008); however, studies correlating the alterations in MMR systems with aging or senescence are few. Experimental models like *Caenorhabditis elegans Msh2* knock-out have significantly decreased life expectancy (Estes et al. 2004). It has also been corroborated that damaging stimuli, such as oxidative stress due to increased ROS production, can induce an increase in genetic variability in strains of *Trypanosoma cruzi* with deficiencies in their MMR pathways (Campos et al. 2011; Machado-Silva et al. 2008). Our group has previously shown that MSH2 levels are reduced with age in CD1 breeding female mice due to increased methylation in its gene promoter region (Conde-Pérezprina et al. 2008).

As a different model, bats (class: Mammalia, order: Chiroptera) offer particular and unique characteristics for aging studies. Their longevity is three times that of the rest of their small terrestrial mammalian counterparts (up to 25 years of age), and their metabolism is highly active because of their flight capacity, a characteristic similar to birds (Neuweiler 2000). We studied two chiropteran species, *Myotis velifer* (J. A. Allen, 1890) and *Desmodus rotundus* (E. Geoffroy, 1810).

M. velifer, the cave myotis, is an insectivorous bat that has an average lifespan of 8 to 12 years (11.3 years in the field) based on mark-recapture studies and an average adult weight of 10.1 g (Wilkinson and South 2002). As its common name states, *M. velifer* is a cave inhabitant, where several thousands of individuals can congregate during spring and summer, while at early fall, they migrate to higher altitudes to mate and hibernate (Fitch et al. 1981). Since this bat can undergo hibernation, it is considered a heterothermic organism (Jürgens and Prothero 1987). On the other hand, the vampire bat (*D. rotundus)* has an average lifespan of 12 to 20 years (Lord et al. 1976) and an average adult weight of 32.2 g (Wilkinson and South 2002). Vampire bats feed on the blood of stock animals (Balmori 1998; Brunet-Rossini and Austad 2004). These bats are homeothermic and do not undergo hibernation (Balmori 1998; Brunet-Rossini and Austad 2004); however, like *M. velifer*, *D. rotundus* lives in communal roosts using tree holes, caves, or other structures as refuges (Greenhall et al. 1983; Wilkinson 1985).

The objective of this study was to compare the MMR systems, the levels of DNA damage, and antioxidant enzyme activities in two species of wild bats, one short lived, *M. velifer*, and one longer lived, *D. rotundus*, in order to determine the contribution of these factors to the aging process. Our hypothesis is that the long-lived *D. rotundus* will show increased levels of antioxidant activity compared to *M. velifer*. DNA MMR proteins MSH2 and MLH1 were analyzed in liver, lung, and brain of juvenile, adult, and old bats, both female and male. To determine a possible association between MMR deficiency and DNA damage, microsatellite sequences, specific for each species, were quantified. Finally, activities of CAT, SOD, and GPx were analyzed to determine if the antioxidant protection correlates negatively with DNA damage.

Organisms of both species showed variations of MSH2 and MLH1 levels according to age. In *M. velifer*, the reduced levels of MMR proteins in old organisms correlate with an increase in microsatellite instability (MSI), determined as an accumulation of microsatellite markers MS3DO2 and MS3EO2, but not for DESMOO and DESM01 markers in *D. rotundus*, which are reduced since adult age correlating with reduced levels of MMR proteins at this stage. Antioxidant enzyme activities tended to decrease when bats achieved old age, except for SOD activity which was higher in older animals of both species. Furthermore, the longer lived species had higher levels of antioxidant enzyme activities than the short-lived species, suggesting that antioxidant response of those animals might be related to their success to achieve long life.

Materials and methodology

Chemicals

All chemicals and reagents were of the highest analytical grade and were mostly purchased from SIGMA (St. Louis, MO). The reagents obtained from other sources are detailed throughout the text.

Animals

Bat collection

Captures were performed in two different refuges located in the central part of Mexico, mostly in the Mexican state of Puebla. Most juvenile and adult *M. velifer* and all the *D. rotundus* were captured inside the Chicomostoc Cave (19°57'54"N, 97°36'09"W, 1,420 masl), in Nauzontla, Puebla (refuge 1). In order to capture both species of bats, a harp trap was placed 10 m at the interior of the cave principal entrance during the evening, before dark, and the bats were captured at the moment when they came out to feed. It has been suggested that animals caught in this way are exposed to lower levels of stress since they are well hydrated and rested when they leave their roosts. Other *M. velifer* were captured from a roost in a tunnel (refuge 2) located in the state border between Puebla and Tlaxcala (19°37'14"N, 98°02'02"W; 3,220 masl). The tunnel is 57.5 km in straight line from Chicomostoc cave in Nauzontla, but it is 1,800 m higher. Inside the tunnel, the bats were collected with hoop nets during the evening, while they were dormant.

Organism handling after their capture

The captured bats were individually transported in canvas bags to a room located near the cave (Apulco, Puebla). In the room, the animals were identified, and the biological material for the study was collected. The identification of the species was done according to Medellín et al. (1997). The bats were kept no more than 10 h before euthanasia, which was performed according to the rules and stipulations of the Mexican official ethics standard 062-ZOO-1999 and to the guidelines approved by the American Society of Mammalogists (Gannon et al. 2007). Tissues that were not used for this work were used by other researchers from our group for their experiments.

Even though formal necropsies were not undertaken, all the bats were thoroughly examined, and none presented evidence of trauma or disease, such as open wounds or infections caused by bacteria and fungus. The only notable feature was the occurrence of common ectoparasites such as Spinturnicid mites (Acari) and Streblid flies (Diptera). During the tissue extraction, the normal anatomic organization of the principal systems and the maintenance of the morphological structure were checked as an indication of the bats health state.

Age class identification

Groups were categorized by age and sex, based on determinations previously established for these

species (Kunz and Anthony 1982; Morris 1972) as follows:

1. Skeletal criteria.
   (a) Ossification of the cartilaginous epiphyseal growth plates of the third metacarpal–phalangeal joint (Kunz 1990). This feature was evaluated firstly in the field, at the moment of the capture by transilumination, and corroborated secondly at the lab, by microscopy of the third finger after transparentation, as well as bone (Alizarin S red) and cartilage (Alcian blue) staining (Springer and Johnson 2000).
   (b) Dental deterioration. The bat skulls were cleaned using *Dermestes spp.* (carrion beetle larvae that only eat the soft material and leave the bone), and the jaws were observed at the microscope to determine the degree of dental deterioration. For *D. rotundus*, the classification was done according to Núñez and de Viana (1997) and Davis et al. (2010). For *M. velifer*, to our knowledge, there are no reports on dental categorization; hence, a classification scheme was created based on dental development using Carleton et al. (1982), starting from the absence of wear to an advanced degree of wear. Canines, premolars, and molars were analyzed.
2. Somatic development criteria.
   (a) Dermatological features. Patagium and pelage texture, lubrication, coloration, and flexibility were used following Martin et al. (2001).
   (b) Allometric features. Body weight was obtained with an electric portable balance (Ohaus) (± 0.1 g). Forearm length was determined with a Vernier caliper (± 0.1 mm).
3. Reproductive criteria.
   (a) Males. Position and size of both testicles and epididymis were analyzed.
   (b) Females. Mammary gland development and pregnancy were considered.
4. Particular information of each species lifestyle, like birth season, time of sexual maturity, etc, was also taken into consideration when collecting animals; individuals 3 months old and younger were not captured for this study.

Study groups

Sixty animals were collected in total, 15 females and 15 males of each species, and divided into three groups: juvenile bats, adult bats, and old bats. Each age group included five bats of each species.

Myotis velifer

M. velifer is a monoestrous, seasonal species. Sexual maturity is achieved in females during the first year of age and in males during the second year of age (Krutzsch 2009). Most of the juvenile bats that were obtained from the Chicomostoc cave (September–April) were sexually immature specimens, since birth occurs in May. Juvenile cave bats were estimated to have been either 4 months old (born during the year) or 14–15 months (born the year previous to collection). Adult bats were captured during August–September, before their annual migration to higher elevations. The old bats were captured during November–December. Old bats were selected from individuals that are part of an ongoing demographic study that began in 1983, when individual bats were captured and marked with numbered plastic rings. Animals known to be older than 6 years were selected.

(a) *Juvenile* (> 4 months<1 year and 3 months): slender individuals (8.5–9.0-g body weight, < 44-mm forearm length), pristine skin; incompletely fused epiphyses, teeth without wear. The testis and epididymis were undetectable in the interfemoral membrane due to their small size. The female had inconspicuous mammary glands, and the nipples were barely perceptible.
(b) *Adult* (> 1 year and 4 months<4 years): epiphyses closed by ossification, mild dental wear. The males could be distinguished by their larger size (9.0–11.0-g body weight, 44–46-mm forearm length). The testicles were scrotal. According to Krutzsch (2009), testicular diameter in adults ranges from 3 to 9 mm, depending on the spermatogenesis stage. These males were captured in August–September when the spermatogenesis

was finishing; therefore, the testis was small, while the epididymis was clearly distended. In the females, the mammary glands were middle size, and the skin around the nipples was without pelage, as occurs in this species at the end of lactation. No gestating females were used in this study.

(c) *Old* (> 6 years); robust specimens (> 11.0-g body weight, 46–49-mm forearm length), closed epiphyses, thickened metacarpus, maximal grade of ossification and dental deterioration. Males and females showed skin wear (e.g., dry patagium) and rough pelage. No traces of sexual activity were found. Scrotal testicles in males and small nipples in females.

Desmodus rotundus

Even though *D. rotundus* is a continuously breeding species, the vampire bat has two main seasons of reproduction, with the majority of juveniles produced in fall. The first episode of reproduction is in April–May and the second in October–November. Postnatal growth stops at 5 months of age (Greenhall et al. 1983). Based on their reproductive patterns, animals were collected in November–December to capture juvenile bats born the previous spring at roughly 8 months of age and to capture females that showed no evidence of recent lactation. Females that might have had their offspring during October–November (based on the mammary gland size and shape) were not collected or used in this study.

(a) *Juvenile* (> 3 months < 1 year and 3 months): slim individuals (24.5–29.0-g body weight, 56–59-mm forearm length), pristine skin; incompletely fused epiphyses, teeth without corrosion. In the male, the testis was located in intra-abdominal position. It has been reported that the female vampire bats have their first birth at 15 months of age (Wilkinson 1985), so female with inconspicuous mammary glands were selected.

(b) *Adult* (> 2 year < 6 years): silky and shiny pelage; epiphyses ossified, mild dental wear. The adult males could be distinguished by their larger size (29.0–35.0-g body weight, 60–63-mm forearm length). Vampire bats are a polygamous species that live in "*harems*." (Park 1991). Hence, adult males are aggressive and show notorious marks and wounds inflicted by other bats while defending their harems. Male's testicles were scrotal, and according to Wilkinson (1985), testicular diameter is 5 mm during 12–14 months of age. The alpha males could be distinguished by their corpulence and aggressiveness. Females that had nursed showed bite marks on their nipples, since lactating vampire bats stick their teeth in their mother's nipple while she carries them.

(c) *Old* (> 10 years); full-bodied specimens (> 35.0-g body weight, > 63-mm forearm length), closed epiphyses, thickened metacarpus, maximal grade of ossification and dental deterioration. Males and females showed skin wear, including dry patagium, gray pelage, and some old healed wounds. No traces of sexual activity were found. Scrotal testicles in males and worn nipples in females.

Protein extraction and analysis

Proteins were extracted from liver, lung, and brain from the experimental animals using a T-PER Extraction Kit (No 78510, Thermo Fischer Scientific) with protease inhibitor Complete (Santa Cruz Biotechnology, Inc). These organs were chosen because of their lower cellular turnover exchange rate and their particular susceptibility to oxidative stress: The liver and the brain are highly dependent on appropriate oxygen consumption in order to maintain their energetic metabolism, which is one of their main features, whereas the lungs are continuously exposed to high pO_2 (López-Torres et al. 1993). Consequently, these three tissues generate large amounts of free radicals, and any impediment in ROS removal should generate oxidative damage in these tissues.

Total protein concentration was determined spectrophotometrically at 595 nm using a commercial Bradford reagent (Bio-Rad, Hercules, CA, USA) (Bradford 1976).

MSH2 and MLH1 were quantified by Western Blot using specific antibodies (Santa Cruz Biotechnology, Inc.). Total protein levels were previously quantified in each sample (Laemmli 1970). Proteins were separated on 12 % SDS–PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen), and probed with anti-MSH2 and anti-MLH1 (Santa Cruz

Biotechnology, Santa Cruz, CA, USA). Membranes were washed three times with TBS–Tween and incubated with anti-mouse IgG secondary antibody (Pierce Biotechnology, Rockford, IL, USA) for 1 h. After three consecutive washes, the blots were developed using a commercial chemiluminescence reagent (SuperSignal West Pico Chemiluminescent Substrate, Thermo Fischer Scientific). The proportion of these proteins was quantified by densitometric analysis, using a Kodak IMAGEN GEL DOC using Kodak software (v. 3.1). Since there are no specific antibodies for bats, polyclonal antibodies were used. In order to determine if the antibodies recognized proteins obtained from the Chiroptera order, all the antibodies were standardized and compared with samples from mouse, rat, and a human cell line (data not shown).

DNA extraction and microsatellite determination

DNA was isolated from the same tissues using the chaotropic NaI method (Wang et al. 1994), as modified by Matos et al. (2001). The DNA concentration was determined spectrophotometrically at 260 nm. Its purity was assessed by ensuring that the A260/A280 ratio was >1.75, and its integrity was confirmed by agarose gel electrophoresis.

Genomic DNA from the obtained samples was used in the microsatellite PCR assay. Primers were used for this assay according to information from microsatellite sequences specifically determined by Trujillo and Amelon (2009) for *Myotis*; for *D. rotundus*, new primers were designed by modifying the information published by Piaggio et al. (2008) (GenBank: EF591570.1). The primers used for *M. velifer* were marker MS3DO2: 5'-CTAAGACCCTTTCCAGCTCTCA-3' (forward) and 5'-GATACCATCACTCTTTCCCCTG-3' (reverse), (amplification product 229 pb). For the marker MS3EO2: 5'-GCCAATAAGAGCCCAGACATAC-3' (forward) and 5'-GGGGATTAGGGATAGGTTAGCA-3' (reverse), (amplification product 409 pb). For *D. rotundus*, the primer designed for microsatellite was named DESMOO: 5'-AGGTGGGTACTGC CCAGGATTATT-3' (forward) and 5'-AGGAGC CCAGCATCTGACTTCCTT-3' (reverse), (amplification product 200 pb). For microsatellite DESMO1: 5'-TTAGCAAGGAGCCAGCAGAGCATA-3' (forward) and 5'-TCGTTCGGAACTTCTACCGCATCA-3' (reverse), (amplification product 300 pb). In each reaction, 2 μL (200 ng) of DNA per sample was used, as well as 5 μL PCR 10× Buffer (166-mM $(NH_4)_2SO_4$, 670-mM Tris–HCl pH 8.8, 67-mM $MgCl_2$, 100-mM β-mercaptoethanol), 1.0-mM deoxynucleotide triphosphate (dNTPs), 20 mM of each primer, and 1.25 U of GoTaq DNA polymerase (Promega, Madison, WI, USA), the total volume was 25 μL. PCR amplifications were performed as follows: 94°C for 5 min (hot start), denaturalization 94°C for 30 s, annealing 55°C for 30 s, elongation 72°C for 30 s, for 35 cycles, with a final step at 72°C for 4 min. Since amplification was done out of total genomic DNA, to ensure that the target microsatellite sequences were being amplified, PCR products were sequenced using ABI Prism BigDye Terminators, version 3.1, on an ABI3100 automated sequencer according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA) as described by Villarreal-Molina et al. (2007) (data not shown). The products were separated in 0.8 % agarose gels and stained with ethidium bromide. The proportion of product was quantified by densitometric analysis, using a Kodak IMAGEN GEL DOC using Kodak software (v. 3.1).

Antioxidant enzyme activity

Antioxidant enzyme activity was analyzed spectrophotometrically in liver, lung, and brain. Each sample (100 mg), processed with an OMNI TH Homogenizer, was used for enzymatic assays. CAT activity was quantified using the protocol established by Aebi (1984) which evaluates H_2O_2 decrease at 240 nm. One unit of catalase activity (UCAT) was considered as the amount of enzyme necessary to catalyze 1 μmol of H_2O_2 per minute. GPx activity was analyzed spectrophotometrically at 340 nm through a protocol described by Ahmad and Pardini (1998). One unit of GPx activity indicates how much enzyme is required to neutralize H_2O_2 using NADPH (Flohé and Günzler 1984). SOD activity was evaluated through the xanthine/xanthine oxidase system, based on protocols by Paoletti et al. (1986); Stirpe and Della Corte (1969). The superoxide anion formed through this system reacts with nitro blue tetrazolium (NBT) which generates formazan dye that can be measured spectrophotometrically at 560 nm. One unit of enzymatic activity

in this assay is considered as the amount of SOD needed to inhibit 50 % of the superoxide reaction with NBT.

Data analysis

The data are reported as means ± SD. One-way ANOVA tests, followed by the Tukey–Kramer variance analysis, were used to compare between each group by sex and age. A 0.05 level of probability was used as a minimum criterion of significance in all analysis.

Results

MMR protein level analysis

MSH2 and MLH1 levels were determined in liver, lung, and brain of *M. velifer* (Fig. 1) and *D. rotundus* (Fig. 2). All the data were normalized against the protein content obtained from the juvenile animals, which was designated one. The representative blots and the densitometry presented in Fig. 2 show a decrease in both MSH2 and MHL1 protein content in the old animals when compared with the adult and juvenile animals. Specifically, MSH2 levels in liver and lung (Fig. 1a, b) from the old females were approximately 25 % lower than those in the adult females; in the old males, MSH2 levels were only 10 % lower as compared with adult males ($p< 0.05$). In brain (Fig. 1c), MSH2 was 19 % lower in the adult female bats when compared with the juvenile females, while the male adults presented a 24 % decrease against the juvenile ones ($p< 0.05$). MLH1 levels in *M. velifer* were 11 % lower in the female adults than in the juvenile ones; MLH1 levels continued decreasing to an approximate 15 % in the old animals (Fig. 1d) compared to the younger ones. In males, the difference between adult and juvenile animals varied between tissues and was more evident in brain samples (Fig. 1f), where the decrease was close to 25 % ($p< 0.05$). The average protein content found for MSH2 in liver differed by sex in the old groups; females decreased their levels 10 % more ($p<0.05$) than the males. Another difference between genders was observed in the adult group brains, where there was a higher decrease in females' MLH1 levels versus males (12 %) ($p<0.05$).

In *D. rotundus*, MSH2 and MLH1 levels were significantly lower in the adult group when compared to the juvenile ones (Fig. 2a–f) ($p<0.05$). In particular, MSH2 levels in liver (Fig. 2a) were drastically reduced in the female adults compared with the juvenile females (approx. 43 %, $p<0.05$); while in lung tissue, the decrease was 27 % (Fig. 2b, $p<0.05$), and in the brain, the reduction was only 14 % (Fig. 2c, $p<0.05$). In adult males, MLH1 protein levels are approximately 20 % lower in each of the three tissues (Fig. 2d–f). Females showed greater age-related reductions in MSH2 than males. Liver tissue has the greatest difference (23 %) between adult and juvenile female bats ($p< 0.05$). Males and females showed significant differences in MLH1 levels in the brain but not other tissues (13 % less in females, $p<0.05$) (Fig. 2f).

DNA microsatellite assessment through PCR analysis

Figure 3a–f shows the results obtained for the microsatellite sequences for *M. velifer* (MS3DO2 and MS3EO2). In all the cases, an increase in microsatellite incidence was observed in the tissues obtained from old bats. In old females, liver and lung showed an increase of 40 % and 18 %, respectively, of the MS3DO2 marker when compared with the adult female bats ($p<0.05$) (Fig. 3a, b). The MS3EO2 marker was higher in the lungs of old bats compared with the adult and juvenile female bats ($p<0.05$) (Fig. 3d, e). Brain tissue obtained from old female bats showed an increase of 26 % and 29 %, respectively, for each marker, MS3DO2 and MS3EO2 ($p<0.05$), in comparison with the adult female group (Fig. 3c, f). In old males, MS3EO2 was 45 % higher in liver and 22 % higher in lung compared with the same tissues of adult bats ($p< 0.05$); MS3DO2 levels in liver from old bats were 13 % higher than the levels observed in adult bats ($p<0.05$). In brain tissue, both markers in old males were also significantly higher (45 % in MS3DO2 and 28 % in MS3EO2) compared with the adults ($p<0.05$). We also observed sex differences in MS3DO2 levels. Old females had 21 % more MS3DO2 in liver tissue compared to old males in old groups, while in brain samples, old

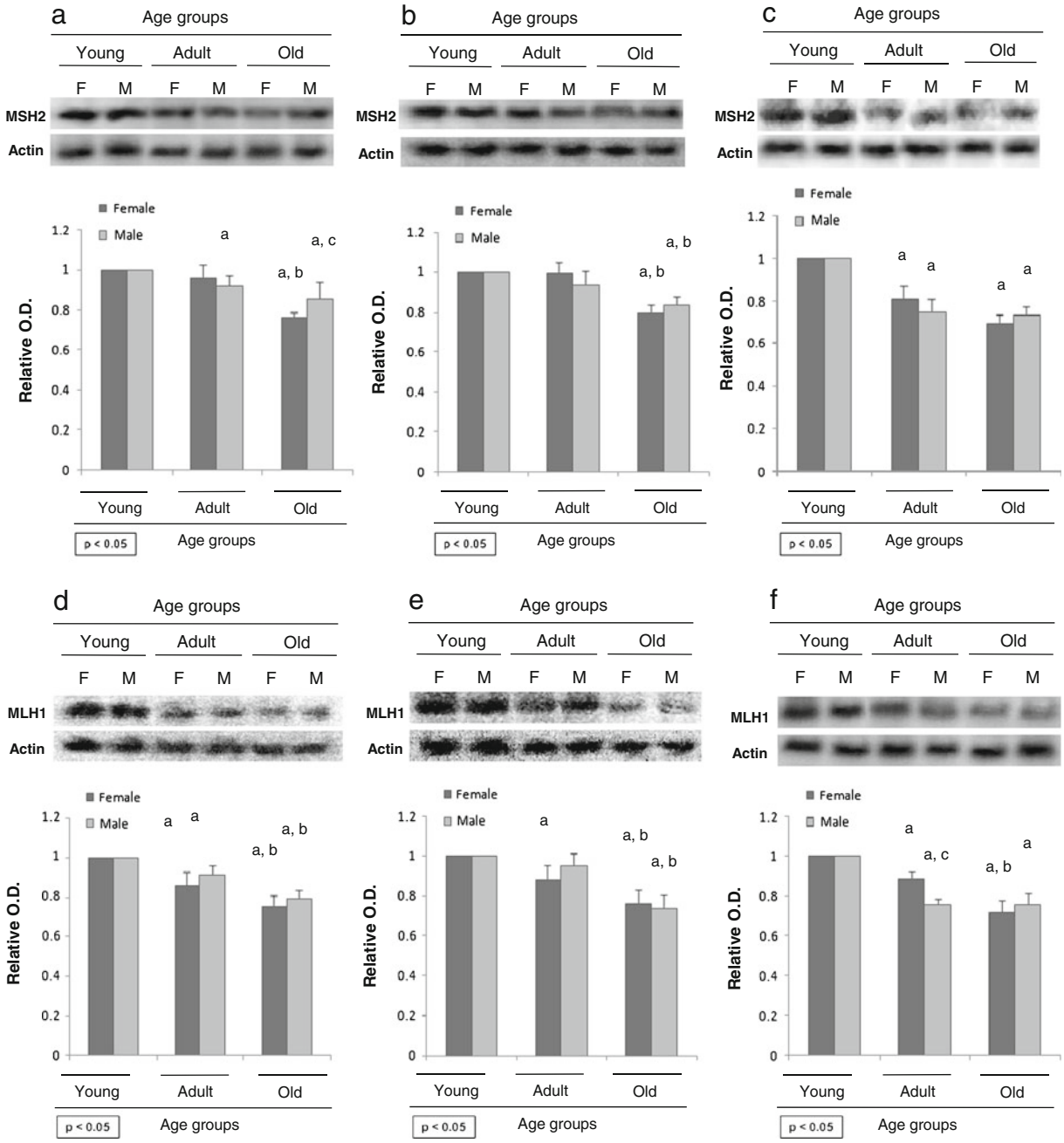

Fig. 1 Levels of MMR proteins in *Myotis velifer*. The figure shows representative blot and densitometry performed for MSH2 levels in **a** liver, **b** lung, and **c** brain as well as MLH1 levels from **d** liver, **e** lung, and **f** brain. MMR protein levels were compared between age and gender groups. Relative optic density was normalized against juvenile animals as control. Each point represents the mean ± S.D. of five determinations performed in independent donor animals. Statistical significance with respect to control (juvenile group) (*a*) or adult (*b*) $p<0.05$ was considered. Letter *c* stands for statistical significance between genders ($p<0.05$). MSH2 protein weight= 100 kDa; MLH1 protein weight= 85 kDa; actin protein weight= 42 kDa

males had 33 % more MS3DO2 compared to old females. In the case of MS3EO2, sex differences were found only in lung tissue, 26 % greater in old female bats compared to old male bats.

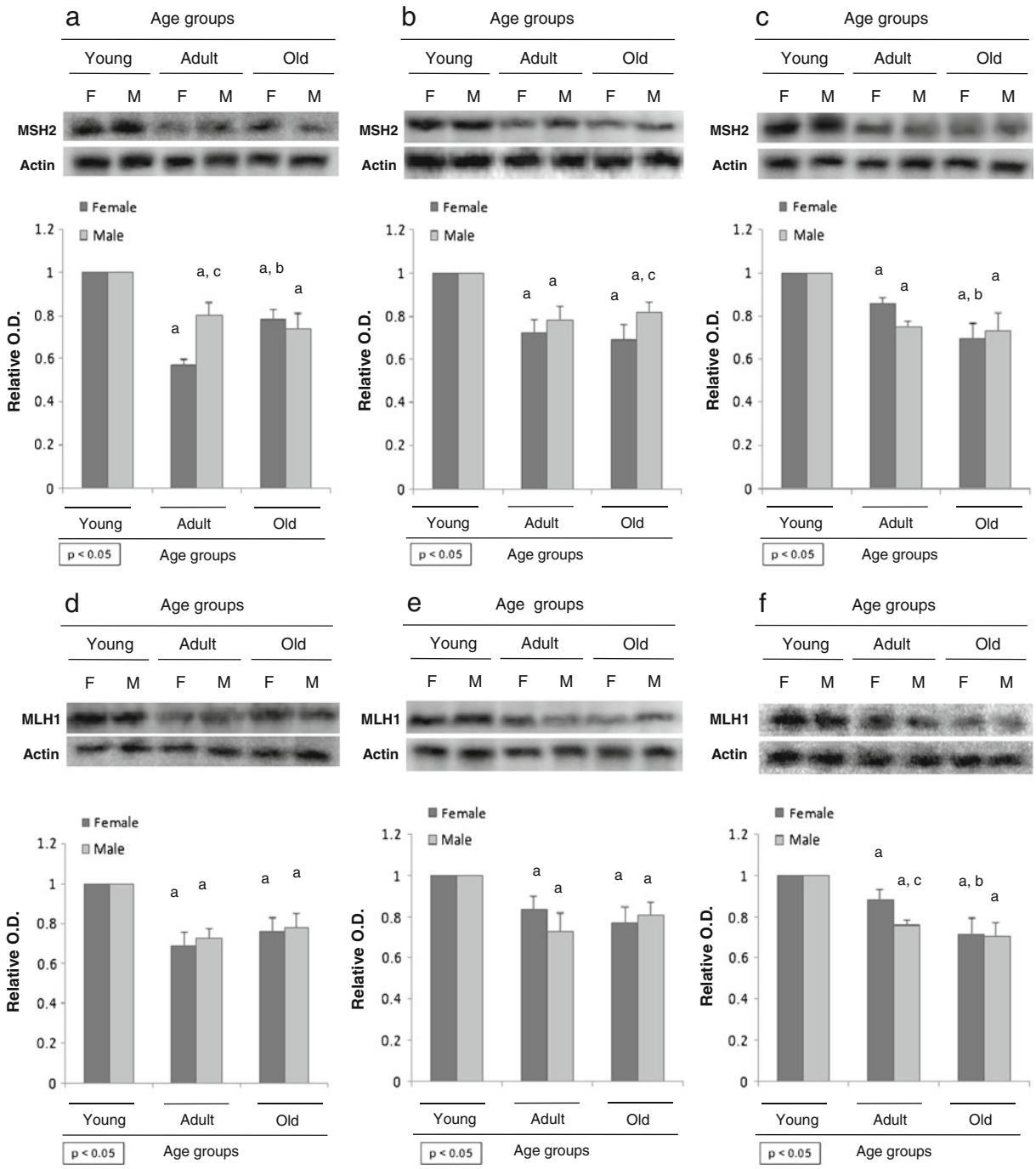

Fig. 2 Levels of MMR proteins in *Desmodus rotundus*. The figure shows representative blot and densitometry performed for MSH2 levels in **a** liver, **b** lung, and **c** brain as well as MLH1 levels from **d** liver, **e** lung, and **f** brain. MMR protein levels were compared between age and gender groups. Relative optic density was normalized against juvenile animals as control. Each point represents the mean ± S.D. of five determinations performed in independent donor animals. Statistical significance with respect to control (juvenile group) (*a*) or adult (*b*) $p<0.05$ was considered. Letter *c* stands for statistical significance between genders ($p<0.05$). MSH2 protein weight= 100 kDa; MLH1 protein weight= 85 kDa; actin protein weight= 42 kDa

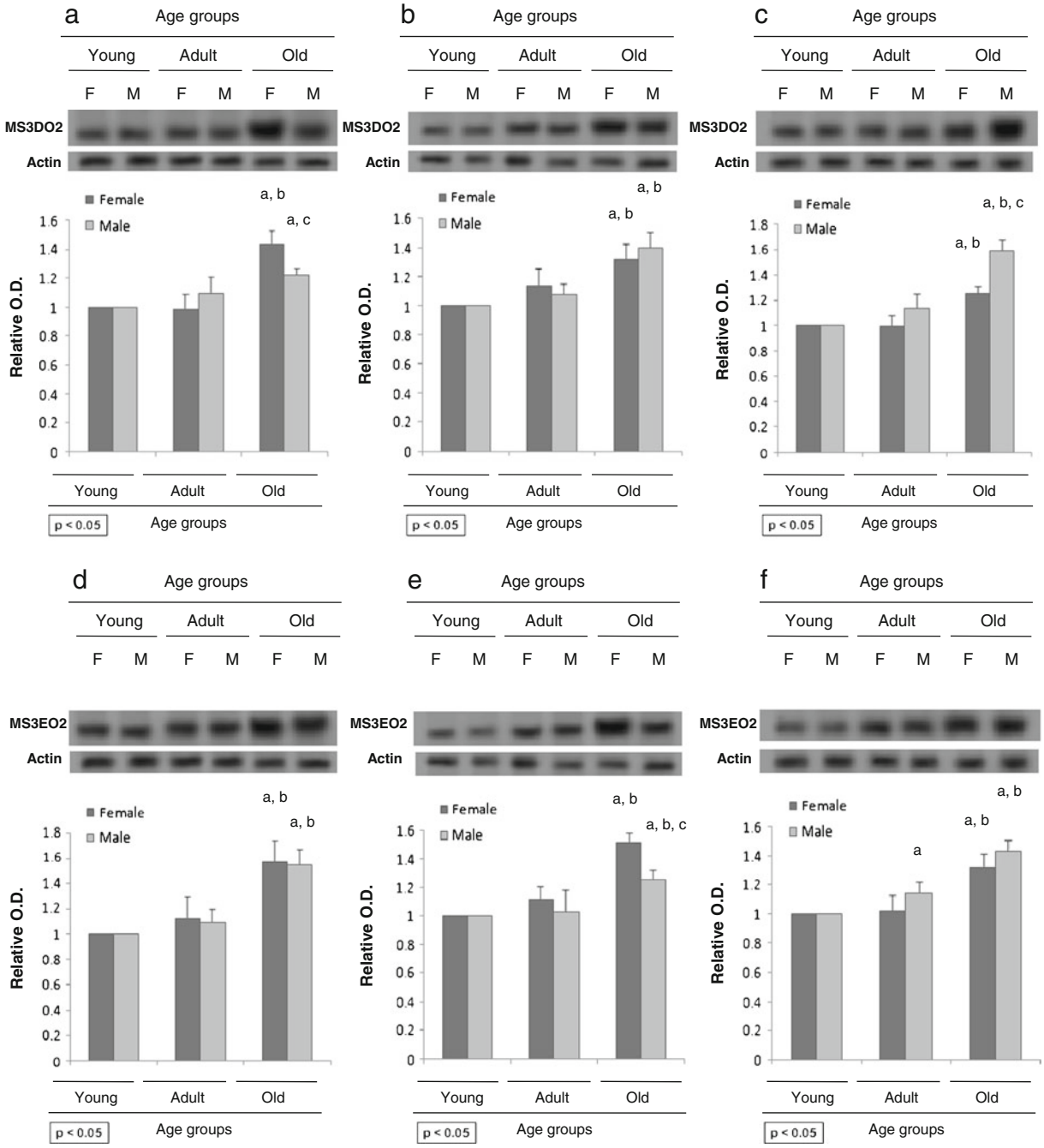

Fig. 3 Microsatellite markers in *Myotis velifer*. The figure shows representative images and densitometry performed for microsatellite MS3DO2 levels in **a** liver, **b** lung, and **c** brain as well as MS3EO2 levels from **d** liver, **e** lung, and **f** brain. Microsatellite levels were compared between age and gender groups. Relative optic density was normalized against juvenile animals as control. Each point represents the mean ± S.D. of five determinations performed in independent donor animals. Statistical significance with respect to control (juvenile group) (*a*) or adult (*b*) $p<0.05$ was considered. Letter *c*) stands for statistical significance between genders ($p<0.05$). MS3DO2 weight= 229 pb; MS3EO2 weight= 409 pb; actin weight= 150 pb

In *D. rotundus*, the microsatellite markers DESMOO and DESMO1 were lower in the adult group compared with the juvenile and old groups (Fig. 4a–f) ($p<0.05$), but in general, both microsatellite markers increased in

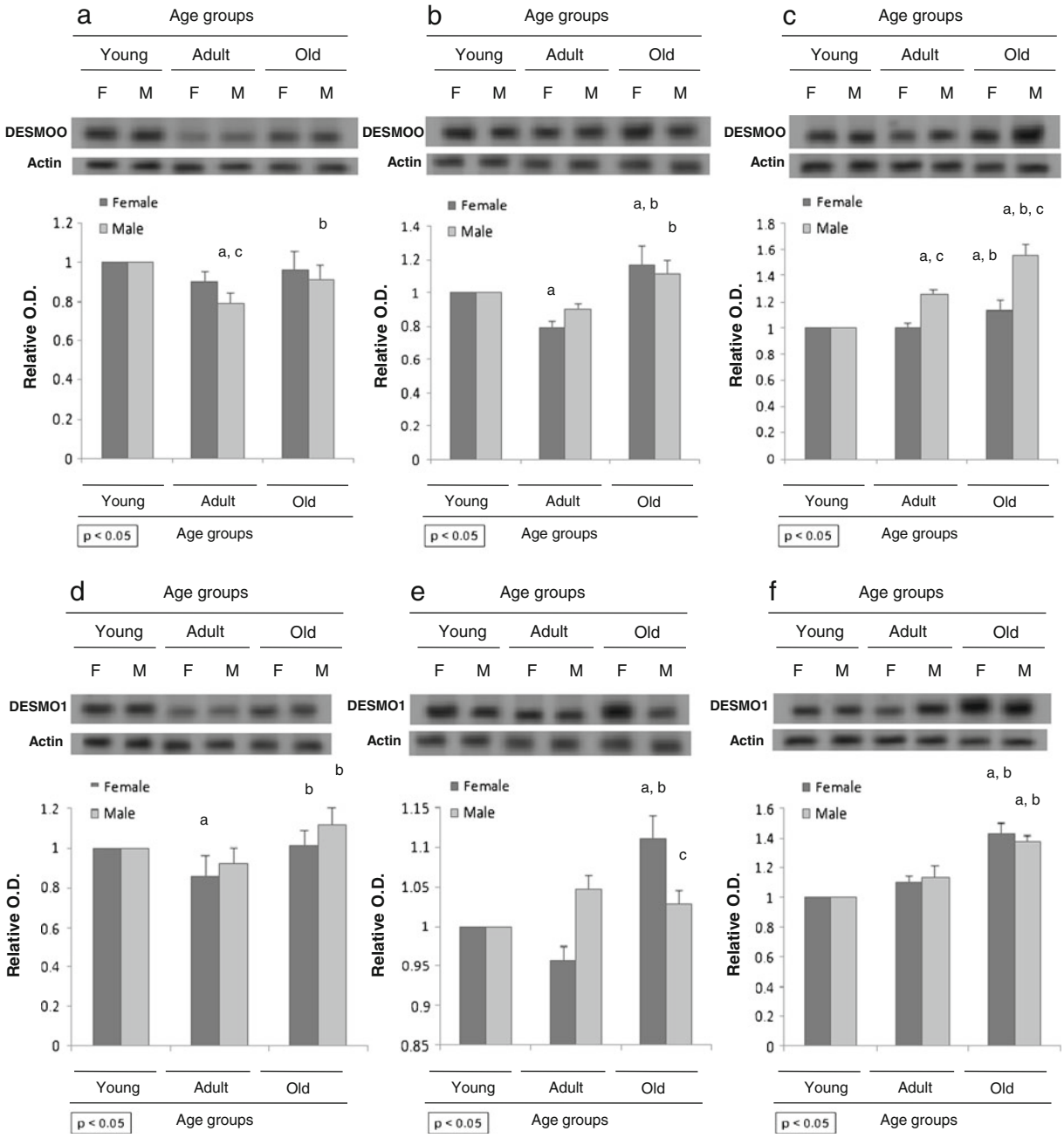

Fig. 4 Microsatellite markers in *Desmodus rotundus*. The figure shows representative images and densitometry performed for microsatellite DESMOO levels in **a** liver, **b** lung, and **c** brain as well as DESMO1 levels from **d** liver, **e** lung, and **f** brain. Microsatellite levels were compared between age and gender groups. Relative optic density was normalized against juvenile animals as control. Each point represents the mean ± S.D. of five determinations performed in independent donor animals. Statistical significance with respect to control (juvenile group) (*a*) or adult (*b*) $p<0.05$ was considered. Letter *c* stands for statistical significance between genders ($p<0.05$). DESMOO weight= 200 pb; DESMO1 weight= 300 pb; actin weight= 150 pb

the old groups, and this was especially evident in the brain (Fig. 4c, f) ($p<0.05$). For females, the DESMOO marker decreased in liver and lung (Fig. 4a, b) ($p<0.05$) in the adult group in comparison with the juvenile group (10 % and 21 %, respectively) ($p<0.05$). In males, DESMOO increased 20 % in liver and 10 % in lung,

Table 1 Catalase enzymatic activity measured in *M. velifer* and *D. rotundus*

CAT activity (mmol/g)							
Myotis velifer				*Desmodus rotundus*			
	Liver	Lung	Brain		Liver	Lung	Brain
Females				Females			
Young	10.34±0.28	7.43±0.09	0.950±0.21	Young	13.39±0.22	7.11±0.24	2.46±0.33
Adult	9.47±0.12	5.77±0.33	0.76±0.41	Adult	11.42±0.30	10.74±0.42	1.89±0.52
Old	4±0.19*,**	3.95±0.16*	0.27±0.14*,**	Old	9.79±0.25	5.62±0.57	0.78±0.43*
Males				Males			
Young	9.51±0.22	8.8±0.21	3.11±0.31	Young	12.92±0.31	8.42±0.15	3.21±0.49
Adult	6.72±0.14	7.67±0.45	2.53±0.88	Adult	11.35±0.41	9.26±0.17	4.17±0.25
Old	3.33±1.19*,**	2.78±0.17*,**	0.97±0.33*	Old	8.43±0.38	7.69±0.38	1.43±0.37*

* Statistically different from young groups; ** statistically different from adult groups

while in the brain, the increase was of 13 % ($p<0.05$) when comparing juvenile vs. adult bats.

Antioxidant enzymatic assay analysis

The antioxidant enzymatic activities are shown in Tables 1, 2, and 3. Activities of CAT and GPx for both species were lower in old than in juvenile and adult bats, particularly in brain ($p<0.05$).

In *M. velifer*, liver tissue had the highest CAT activity in both juvenile females and males ($p<0.05$); CAT activity was significantly lower in old animals (60 % and 66 %, respectively) ($p<0.05$) (Table 1). GPx activity was lower in old bats compared with juveniles (47 % and 52 %, respectively) (Table 2). In *D. rotundus*, both CAT and GPx activities were higher than the activities of those enzymes in *M. velifer* ($p<0.05$). In particular, GPx activity was highest in *D. rotundus* juvenile females' liver tissue; activity declined by 43 % in old females and by 53 % in old males ($p<0.05$). With the exception of CAT activity in the brain and GPx activity in lung of males, antioxidant enzyme activity in tissues from old *D. rotundus* was similar to that in tissues from adult *M. velifer*.

Table 2 Glutathione peroxidase enzymatic activity measured in *M. velifer* and *D. rotundus*

GPx activity (mmol/g)							
Myotis velifer				*Desmodus rotundus*			
	Liver	Lung	Brain		Liver	Lung	Brain
Females				Females			
Young	21.71±0.62	17.36±0.44	13.87±0.52	Young	41.73±0.85	30.90±0.75	13.54±0.98
Adult	16.13±0.22	14.23±0.17	8.12±0.78	Adult	36.12±0.80	22.41±0.49*	7.31±0.67*
Old	10±0.74*	5.82±0.64*,**	3.61±0.54*,**	Old	18.21±0.64*,**	11.72±0.79*,**	1.45±0.35*,**
Males				Males			
Young	27±0.82	15.84±0.91	8.76±0.81	Young	36.19±0.84	13.27±0.75	6.31±1.01
Adult	19.38±0.24*	12.30±1.05	5.13±0.44	Adult	27.74±0.61*	12.26±0.96	5.68±0.49
Old	14.21±0.98*	7.42±0.56*,**	2.39±0.92*	Old	18.99±0.38*,**	5.24±0.13*,**	2.07±0.62

* Statistically different from young groups; ** statistically different from adult groups

Table 3 Superoxide dismutase enzymatic activity measured in *M. velifer* and *D. rotundus*

SOD activity							
Myotis velifer				*Desmodus rotundus*			
	Liver	Lung	Brain		Liver	Lung	Brain
Females				Females			
Young	1,640.84±35.9	568.21±30.65	240.73±49.08	Young	2,144.07±24.8	732.89±19.20	240.73±49.08
Adult	1,540.24±57.13	464.87±25.21	348.17±41.21	Adult	1,998.75±24.98	807.71±42.66	348.17±41.21
Old	1,935±35.77	591.31±27.10	407.12±32.99	Old	2,311±46.09	994.74±33.74	407.12±32.99
Males				Males			
Young	1,471±25.77	573.84±32.44	336.88±36.30	Young	2,009±13.21	628.17±10.31	210.74±21.01
Adult	1,530.42±21.41	512.80±31.61	292.31±47.11	Adult	1,792.33±31.02	632.40±38.11	311.02±4.77
Old	1,766.21±17.09	688.39±28.03	422.28±47.05	Old	2,206.55±45.19	819.22±7.29	475.24±23.67

* Statistically different from young groups; ** statistically different from adult groups

Discussion

Microsatellite instability (MSI) results from accumulated mutations that are believed to occur in an organism (Narine et al. 2007). It has been previously suggested that DNA oxidative damage might induce MSI upon replication of the damaged DNA (Jackson and Loeb 2000). Therefore, it is important to understand the relationship between efficiency of antioxidant mechanisms, metabolic rate, DNA damage, and longevity. However, data supporting the relationship between DNA oxidative damage and MSI formation are scarce and inconclusive. ROS have been considered a DNA damage-inducing factor that has an impact on genetic fidelity and cell homeostasis. It has been demonstrated that MSI increases in the presence of an MMR deficiency scenario, particularly in the absence of a functional MSH2 expression; in models such as genetically modified *E. coli* strains with mismatch repair deficiency, a frame-shift frequency due to MSI increased by '300-fold was observed (Jackson et al. 1998). Modified mouse strains that combine MLH1/ATM (ataxia–telangiectasia) deficiencies, along with superoxide dismutase 1 (SOD1) dysfunction, present an increase in oxidative stress and radiation sensitivity, along with a dramatic increase in aggressive lymphomas; however, these animals did not show any difference on their lifespan (Ziv et al. 2005).

In this study, MSH2 and MLH1 levels were lower in the old groups of *M. velifer* compared with the juvenile and adult groups. On the other hand, *D. rotundus* had different MMR protein levels than those found in *M. velifer* in the adult and old age groups. The fact that vampire bats (the long-lived species in this study) had reduced levels of MMR could indicate that another process or signal might modify expression of these proteins due to a reduction in errors in the DNA sequence, like BER or NER efficiency. At the same time, an increase (> 40 %) of the microsatellite markers MS3DO2 and MS3EO2 in *M. velifer* was observed in the old groups, while the levels of microsatellite markers DESMOO and DESMO1 for *D. rotundus* were not as high as the ones observed in cave bats, being lower in the adult groups compared with the juvenile controls (10 % to 20 %).

Old *M. velifer* had greater levels of microsatellite instability, which correlates with reduced levels of MMR proteins; similarly, *D. rotundus* had reduced levels of microsatellite markers and also reduced levels of MMR proteins. Since *M. velifer* is a short-lived species compared with *D. rotundus*, these factors could suggest associations between DNA damage and repair failures that might affect the longevity in bats.

MSH2 and MLH1 expression during aging have not been studied in bats, but experiments conducted with genetically modified MSH2 *knock-out C. elegans* showed that with each generation of worms, fertility and survival rate decreased due to an increase in mutations attributed to the lack of MMR efficiency resulting from reduced MSH2 expression (Estes et al. 2004). Using T cells from young and old human donors to model the process of T cell clonal expansion

in vitro, it was determined that levels of oxidative DNA damage and microsatellite instability were increased in an age-associated manner along with decreasing DNA repair capacity. Results obtained from another study using T cell clones from extremely healthy very old donors indicated that DNA repair was better maintained and oxidative DNA damage remained in the same degree (Pawelec et al. 2004). In humans, the cumulative risk for colorectal cancer increases in patients of 70 years of age or more, compared to cumulative risk in patients 30 and 50 years of age. This increase was not only correlated with an increase in MSH2 and MLH1 mutations in the 70 year old patients, but also with the increased rate of mutations in the MLH1 gene male patients compared with female patients in all three age-dependent groups. Female patients also showed increased mutation in the MSH2 gene compared with male patients (Choi et al. 2009). Besides inherited mutations that could affect functionality, this information might account for a sex difference in susceptibility to aging and degenerative diseases in organisms as they grow older, resulting from different defects in DNA repair signaling.

According to Lyman (1970), bats sustain very high oxygen consumption when active, a characteristic that contrasts with the drastic drops in oxygen consumption when they become torpid. Records of individuals surviving more than 30 years in the wild now exist for five species of bats. Of these, three species belong to the genus *Myotis*, which also have the capacity for hibernation. Wilkinson and South (2002) have suggested that bat lifespan significantly increases with hibernation, body mass, and occasional cave use, but decreases with reproductive rate, while the influence of diet remains unclear. It has also been shown that *M. velifer* has increased antioxidant activities during hibernation; cognate data for *D. rotunda* are lacking (Salmon et al. 2009).

However, there might be additional causes that could influence bat longevity. *M. velifer* is prone to live in caves, a condition that coupled with its capacity to fly, enables these bats to better avoid and escape predators (Fitch et al. 1981). Hibernation also plays a significant role in bat longevity due to reduced extrinsic mortality: Hibernating bats can cope with periods of food unavailability, while the cave provides a protected environment, thus reducing the risk of starvation and predation. Therefore, it has been suggested that hibernating bats possess greater longevities than tropical species (Wilkinson and South 2002). *M. velifer* populations inhabiting the central part of Mexico do not hibernate, but pass through intermittent phases of profound torpor during the hibernation period. Conversely, tropical bats, like *D. rotundus*, are also long-lived; hence, hibernation might not be the only factor to increase longevity in all bats species. In this regard, *D. rotundus* is not a recurrent cave dweller like *M. velifer* and does not hibernate, and although it is able to fly, this vampire bat sprints in order to reach for its prey (such as cattle); therefore, it is more exposed to different types of predators. Nevertheless, it has been estimated that bats that sometimes roost in caves live longer than bats that either never or always roost in caves (Wilkinson and South 2002). These features are interesting since, in theory, the least exposed species to environmental factors, like cave bats, should have a higher survival rate and longer lifespan. On the other hand, according to the evolutionary theory of aging, senescence is attributed to the declining strength of natural selection at successive ages after sexual maturity. This means that the genetic accumulation of late-acting deleterious alleles and/or selection for alleles with pleiotropic effects might modify the longevity of these species (Austad and Fischer 1991). The vampire bat, although it is more exposed to environmental hazards and predators, also lives in tropical areas, which allows conditions in humidity and temperature that promote genetic traits and food availability that could improve life quality and, therefore, its longevity. Besides, since *Desmodus* is not a cave dweller like *Myotis*, it is not exposed to other type of toxic factors like the by-products generated by its life cycle. Cave bats generate large concentrations of ammonia primarily by the degradation of urea and excrements that might generate physiological stress in species like *M. velifer*, which *D. rotundus* does not experience (McFarlane et al. 1995). Nitrogen degradation metabolism is known to generate high levels of nitrosative and oxidative stress (Oliveira et al. 2010).

CAT and GPx activities were lower in the old female *M. velifer* bats, compared with adult and juvenile animals of the same species (Tables 1 and 2). Also, the males showed a linear decrease in antioxidant enzyme activities with age. These observations are similar to those made previously on different bat species like *M. nigricans* (Wilhelm et al. 2007), indicating a mechanism whereby these long-lived

organisms can compensate for their high metabolic rate with different antioxidant responses. In *D. rotundus*, decreased antioxidant activities were observed in old bats; however, antioxidant enzyme activities in the old vampire bats were equivalent to those recorded in the samples from adult cave bats. The higher antioxidant enzyme activity might give vampire bats an advantage in terms of antioxidant protection, which, in turn, could improve its longevity, given that this species does not undergo torpor. SOD activity increased in the old bats, showing a considerable difference compared with CAT and GPx activity (Table 3). This could indicate that old bats increase their protection against oxidative stress. It could also suggest that the oxidative damage in these organisms is higher and that the activity measured is indicative of the tissue exposure during their longer lifespans.

Another important feature to consider is the different diets of the two species, since the differences found in antioxidant profiles might reflect food differences between species (blood versus insects). It has been reported that vampire bats present high levels of oxidative damage due to the high intra-cellular iron content in blood (Ferreira-Cravo et al. 2007), yet in our study, this high level of oxidative damage did not translate into species differences in DNA instability, since DNA seems to be better protected against damage in *Desmodus.* This difference in DNA instability might be related to the antioxidant profile observed in the tissues studied. In addition, differences in antioxidant levels among tissues were also observed. These tissues might be exposed to different levels of oxidative stress. For instance, the lungs are constantly exposed to higher oxygen tension than most of the tissues; therefore, they might have special antioxidant needs. The liver and the brain are known to be very energy-demanding tissues, with elevated numbers of mitochondria, and therefore also highly susceptible to oxidative stress, particularly the brain.

In order to determine if there is a correlation between MSH2 or MLH1 levels with the MSI and if this kind of DNA damage correlates with the enzymatic activities, a mathematical regression was performed. Figure 5 summarizes the results obtained for this analysis for *M. velifer* only using MSH2 (Fig. 5a–c) and CAT (Fig. 5d–f); however, it is important to remark that this regression was also performed with MLH1 and the other antioxidant enzymes (data not shown). Figure 6 shows the same analysis performed for *D. rotundus*. In Fig. 5a–c, it can be seen that for *M. velifer*, a decrease in MSH2 correlates with an increase of MSI, and the same can be observed in Fig. 5d–f with the decrease in antioxidant activities. This condition is more evident when old bats are compared with adult and juvenile bats. However, in the case of *D. rotundus* (Fig. 6), the tendency differs considerably. Again, according to the evolutionary theory of aging, cellular constituents can eventually fail due to errors and damage accumulation. In contrast, considering the "rate of living" theory, error accumulation would be related to physiological processes as reflected by mass specific metabolic rates. In *M. velifer*, these could explain why the microsatellite instability is higher than *D. rotundus* and could be associated with the high metabolic rates found for other bat species (Austad and Fischer 1991).

Another important feature to take into consideration when comparing the maximum longevity among species is the relationship between body mass and longevity. In this study, it is of particular interest because *D. rotundus*, which lives longer than *M. velifer*, is also larger. Based on a regression of body mass and maximum longevity, the cave *Myotis* might live 2.7 times longer than expected, whereas the vampire bat 4.8 times, thus coinciding with our results.

Morphological and molecular phylogenetic analyses have revealed that the Vespertilionidae family, to which *Myotis velifer* belongs, originated before Phyllostomidae, to which *Desmodus rotundus* belongs; however, the genus *Desmodus* is older (appeared 26 million years ago) than *Myotis* (diverged 20 million years ago) (Teeling et al. 2005). There are three species of vampire bats known (*D. rotundus*, *Diphylla ecaudata,* and *Diaemus youngi*) that belong to the Desmodontinae subfamily (Jones et al. 2009). The only reported data about longevity are related to *D. rotundus*; hence, no comparisons within the species in the subfamily can be made.

On the other hand, the genus *Myotis*, with about 100 members (Simmons 2005), is distributed throughout the world, except in the polar region, and represents one of the most diverse and successful radiations among mammals. There are lifespan data reported for 22 species of *Myotis* (Wilkinson and South 2002), with different geographic dispersion: 9 Paleartic species (Eurasia) 25.4±6.3 years average lifespan, 12 Neartic (North America) 17.6±6.9 years average lifespan, and just 1 Neotropical species (Center-South America) 7.0-years average lifespan. Phylogenetic analyses indicate that the Paleartic species are the ancient ones, while the Neotropical is the more recent

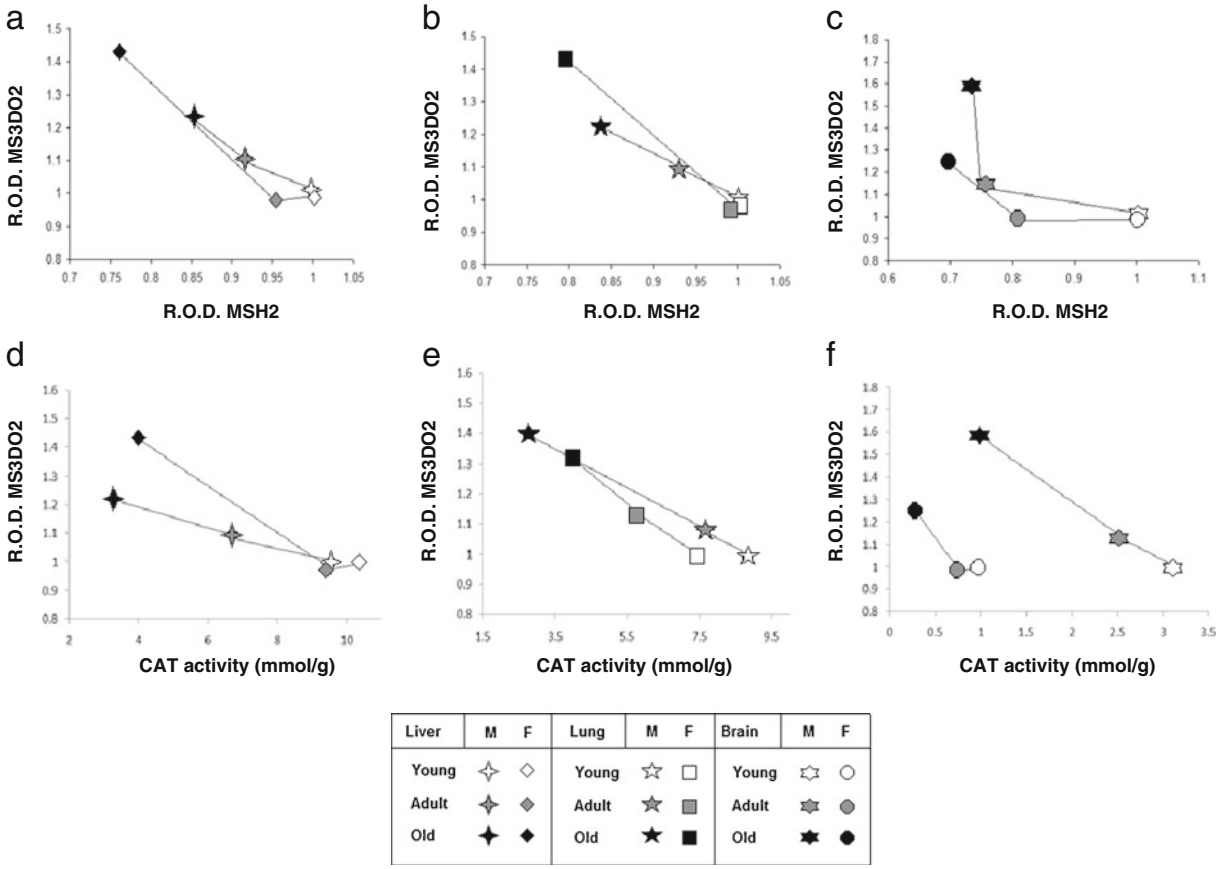

Fig. 5 *Myotis velifer* comparison between MMR levels and MSI. The figures shows comparisons between MSI levels against MMR proteins levels found in **a** liver, **b** lung, and **c** brain as well as comparing MSI levels against CAT activity levels from **d** liver, **e** lung, and **f** brain in female (*F*) and male (*M*) bats of *Myotis velifer*

species (Stadelmann et al. 2007). It is notable that *Myotis* longevity tends to diminish according to its geographic affinity, from the old temperate region (Paleartic) to the recent (Neartic) and, in turn, to the tropical region. This suggests a possible relationship between longevity reduction and *Myotis* dispersion to temperate and tropical environments, as well as to the decrease in hibernation duration, or maybe it is just that there are longer and better data of bat longevity from the temperate regions. Interestingly, most of the species that inhabit cold or temperate zones (Paleartic and Neotropical) hibernate, but they just breed one offspring per year (monoestrous, low reproductive rate), while the one Neotropical species (*M. nigricans*), which does not hibernate, is polyestrous, with a higher reproductive rate of three offsprings per year. Remarkably, these females have a smaller body weight compared to other *Myotis* (4 vs. 10.1 g *M. velifer*).

Since organisms that hibernate, and probably those who enter into torpor, have higher longevity rates, it is intriguing that *D. rotundus* does not adjust to this pattern. Indeed, *D. rotundus*' lifestyle has some features that does not fit in the expected longer lifespan model, such as a gestational period of 7 months, feeding from blood (which contains elevated levels of iron), and having considerable levels of stress due to its need to protect its reproductive niche from satellite males. However, this species lives almost two times more than *M. velifer*. In fact, the linear regression of Fig. 5 for *M. velifer* completely shows what is expected, a decrease in repair and antioxidant enzymes with age and an increase in DNA damage; however, Fig. 6 for *D. rotundus* presents a very different behavior, which is difficult to explain. In an attempt to understand this phenomenon, we have to take into consideration that since *D. rotundus* cannot enter

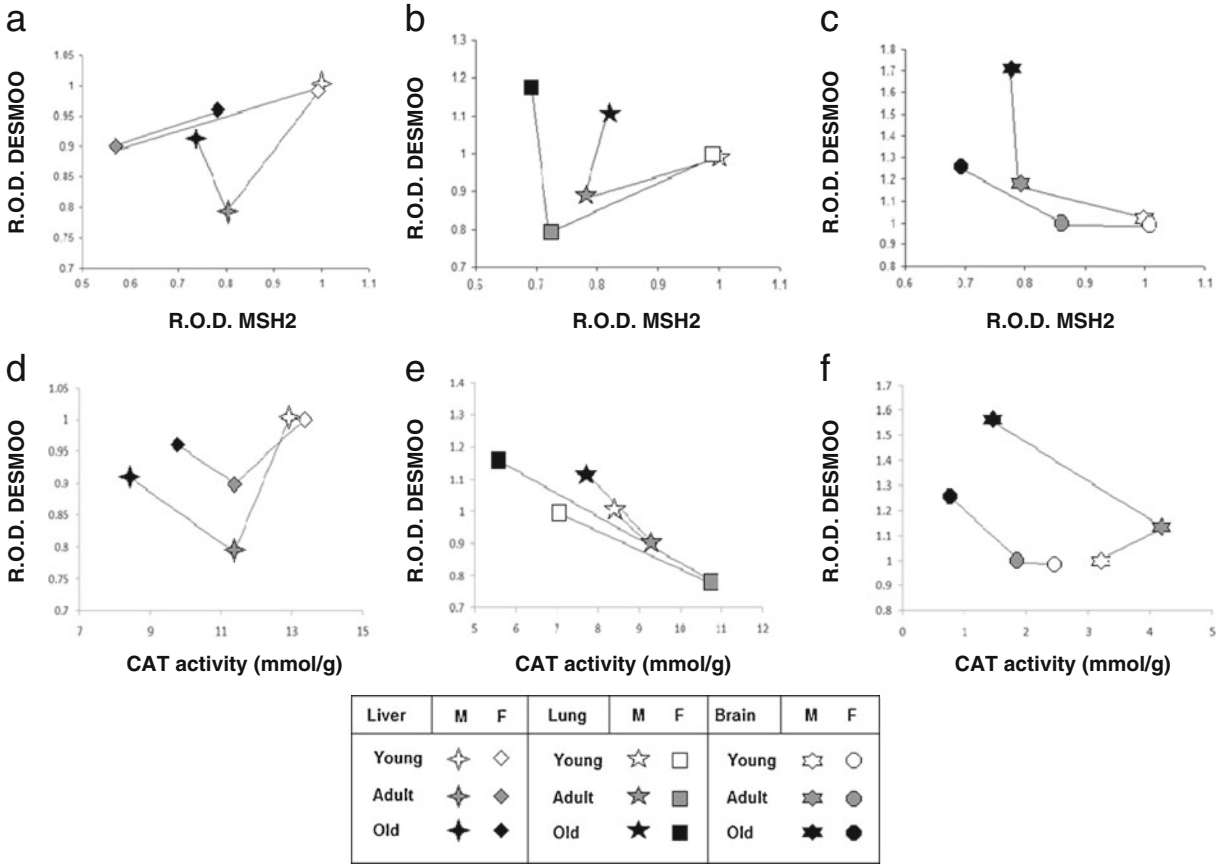

Fig. 6 *Desmodus rotundus* comparison between MMR levels and microsatellite instability. The figures shows comparisons between MSI levels against MMR proteins levels found in **a** liver, **b** lung, and **c** brain as well as comparing MSI levels against CAT activity levels from **d** liver, **e** lung, and **f** brain in female (*F*) and male (*M*) bats of *Desmodus rotundus*

into torpor, metabolic energy consumption is higher compared with *M. velifer*. This could partially explain the higher CAT and GPX activities, as a mechanism to counteract the endogenous generation of ROS. SOD activity was also higher in old bats from both species, which might account for a higher superoxide production. However, this alone cannot explain why it differs with the notion that an increased metabolism increases oxygen consumption and damage. Due to the fact that *D. rotundus* is a homeothermic organism that does not enter torpor, it forces the scenario of having increased antioxidant activities compared with organisms like *M. velifer* that can hibernate; these might induce an adaptive effect on the cells to a low dose of oxidative stress, which might activate the expression of cytoprotective and antioxidant proteins, allowing pro-oxidants to emerge as important hormetic agents (Luna-López et al. 2010). The hormetic effect might explain, at least partially, the different pattern of response in *D. rotundus*, which suggest an adaptive response to all of these factors helping to maintain a healthy and prolonged lifespan. However, more experiments have to be done to completely understand this phenomenon.

Final regards

Throughout this paper, we have established that the levels of MMR proteins MSH2 and MLH1 have differences in tissues from Chiroptera species *Myotis velifer* and *Desmodus rotundus* according to age, and the same can be said for the quantification of microsatellite instability which also varies with age.

Therefore, the information obtained from the old groups indicates that these are the ones with the significant variations pointing to associated factors that might lead the aging process. However, more studies are needed since these factors are usually linked with tumorigenic processes rather than the aging condition. Finally, antioxidant capabilities through enzymatic activities can give insights about the cell homeostasis in these species, but hardly explain alone why the behavior in *D. rotundus* contradicts the hypothesis presented here. The low expression of MMR proteins, reduction in microsatellite markers, and higher antioxidant activities, along with a prolonged lifespan, suggest that there might be another factor in *D. rotundus* that could be modifying its behavior compared with the one seen in *M. velifer*. In this respect, more studies are needed, specifically studies that could show a broader spectrum of damage through levels of oxidation in DNA in the form of adducts, as well as protein and lipid oxidation as markers of oxidative stress.

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