

## RESEARCH ARTICLE

# Muscle myonuclear domain, but not oxidative stress, decreases with age in a long-lived seabird with high activity costs

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## ABSTRACT

In birds, many physiological parameters appear to remain constant with increasing age, showing no deterioration until 'catastrophic' mortality sets in. Given their high whole-organism metabolic rate and the importance of flight in foraging and predator avoidance, flight muscle deterioration and accumulated oxidative stress and tissue deterioration may be an important contributor to physiological senescence in wild birds. As a by-product of aerobic respiration, reactive oxygen species are produced and can cause structural damage within cells. The anti-oxidant system deters oxidative damage to macromolecules. We examined oxidative stress and muscle ultrastructure in thick-billed murre aged 8 to 37 years ( $N=50$ ) in pectoralis muscle biopsies. When considered in general linear models with body mass, body size and sex, no oxidative stress parameter varied with age. In contrast, there was a decrease in myonuclear domain similar to that seen in human muscle aging. We conclude that for wild birds with very high flight activity levels, muscle ultrastructural changes may be an important contributor to demographic senescence. Such gradual, linear declines in muscle morphology may eventually contribute to 'catastrophic' failure in foraging or predator avoidance abilities, leading to demographic senescence.

**KEY WORDS:** Thick-billed murre, *Uria lomvia*, Muscle ultrastructure, Aging

## INTRODUCTION

Physiological aging is progressive and irreversible in nature and manifests ubiquitously across the animal kingdom (Partridge and Gems, 2002; Nussey et al., 2008). Failure of numerous control systems and accumulation of somatic damage to cellular machinery are common denominators in most aging hypotheses (Partridge and Gems, 2002; Bize et al., 2014). Birds are interesting study models of aging because, when compared with similar-sized mammals, they demonstrate higher metabolic rates, yet longer lifespans, providing one of many counter-examples to simplistic formulations of the 'rate of living' theory (Hulbert et al., 2007; Jimenez, 2018). Some examples of physiological aging include progressive telomere shortening with age, reduced whole-animal metabolic rate, increased oxidative damage and decreased immune system function in older animals, among others, and have been shown to manifest in wild populations. For example, in aging tree swallows

(*Tachycineta bicolor*), telomeres are shorter and immune function decreases with age (Vleck et al., 2011). However, the exact mechanism responsible for decreases in tissue-level function and increases in somatic damage in wild animals has yet to be pinpointed. Accumulation of damage seems to lead to increases in pathology and mortality; thus, understanding physiological processes responsible for damage accumulation or cellular malfunction is at the root of understanding the length of an organism's life (Partridge and Gems, 2002).

Wild animals demonstrate age-related declines in probability of survival, fecundity and reproduction, with some species surviving well after reproductive senescence (Nussey et al., 2008). Physiological aging presumably underlies demographic senescence, and can provide insight into the causes of demographic senescence (Nussey et al., 2008; Soulsbury and Halsey, 2018). Because a larger proportion of long-lived than short-lived animals die from senescence, long-lived animals, such as seabirds, are interesting model systems to examine physiological senescence (Ricklefs, 2010). Long-lived seabirds demonstrate variation with respect to their whole-organismal physiology as they age. For example, resting metabolic rate decreases with age in thick-billed murre (*Uria lomvia*), while daily energy expenditure in response to a challenge (handicapping) increases with age (Elliott et al., 2014a, 2015), perhaps implying better energy management in older birds. However, other physiological traits, such as stress responses and declining immunity, show little change with age in long-lived birds, suggesting that physiological senescence is delayed in these birds beyond the age at which birds start to show reproductive senescence (Lecomte et al., 2010; Elliott et al., 2014b, 2015). Some long-lived seabirds show no evidence of demographic senescence if the last year of life is removed from analyses, suggesting that seabirds may suffer 'catastrophic' mortality whereby physiological condition is maintained until there is a catastrophic failure of a physiological system leading to immediate death (Ricklefs, 2010). Loss of skeletal muscle function owing to oxidative damage could be an example of such a system. For example, muscle function could have little impact on mortality or reproduction until it reaches a critical point from which death from starvation or predation soon follows.

One commonly measured physiological process that may determine aging rates is oxidative stress. Oxidative stress results from damage caused by byproducts of mitochondrial respiration, in the form of reactive oxygen species (ROS). At high levels, ROS can overwhelm the antioxidant system, causing damage to DNA, proteins and lipids, impairing cellular function, increasing repair mechanism function and, if all else fails, causing cell death (Skrup and McWilliams, 2016). The antioxidant system within cells includes enzymatic antioxidants, such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), which catalyze the conversion of ROS to less biologically insulting molecules. Other antioxidant molecules such as vitamin E and C act as chain-breaking antioxidants, which can scavenge ROS, remove

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them once they are formed and further halt propagation of peroxidation (Skrup and McWilliams, 2016). Membrane lipids can be highly prone to unabated oxidative damage as a result of increases in ROS production. During lipid peroxidation (LPO), an errant electron propagates across membranes, altering lipid composition and decreasing cellular function (Hulbert et al., 2007). The ‘oxidative stress’ theory of aging states that aging is not a genetically programmed phenomenon, but happens because of the deleterious damage owing to unabated ROS production on the genetic machinery. This theory links the balance between the accumulation of cellular damage and pro-oxidant concentration over time, thus giving rise to the process of aging. However, the generalization of the oxidative stress theory to all organisms is still being disputed (Sohal and Allen, 1990; Hindle et al., 2010; Speakman et al., 2015). The oxidative stress theory of aging has been extensively examined in wild animals (e.g. Costantini and Bonadonna, 2010; Lecomte et al., 2010; Devevey et al., 2010; Alonso-Álvarez et al., 2010; Herborn et al., 2016), but almost exclusively in blood because of its simplicity for sampling. Given that red blood cells (RBCs) are regularly replaced and so oxidative damage may be repaired by replacing the cell, and they do not have high densities of mitochondria (and ROS production), RBCs are not ideal models to examine oxidative stress; more active tissues, such as muscle, may be a better matrix to examine how oxidative stress varies with age.

Reduced muscle performance and athleticism with age is commonly observed in humans and laboratory mammals, but such populations typically die from other causes (Ricklefs, 2010). In the wild, such declines could lead directly to starvation or predation, indicating a potential mechanism for actuarial senescence in wild animals (Hindle et al., 2009, 2010). Such mechanisms have seldom been examined in wild animal populations; for example, an uncommonly measured parameter of aging is the ultrastructure of skeletal muscle (Hindle et al., 2009, 2010). Birds are able to phenotypically adjust their muscle mass during increases in workloads, such as migration (e.g. Swanson, 1991; O’Connor, 1995; Cooper, 2002), or for increased thermogenic requirements (Milbergue et al., 2018); however, how muscle fiber ultrastructure changes during aging in long-lived seabirds has only been documented in a single species, the black-legged kittiwake (Brown et al., 2019). In humans and laboratory rodents, normal muscle aging patterns show a decrease in muscle fiber diameters with age through sarcopenia (Young et al., 1985; Lexell et al., 1988; Frontera et al., 2000), an age-related impairment of muscle function that includes deterioration of quantity and quality of muscle, decreases in myofibrillar protein expression, mismatch in rates of protein synthesis and degradation, and a decrease in force generating capacity (Cristea et al., 2010). Muscle fiber diameter is optimally regulated, as described by the ‘optimal fiber hypothesis’, the concept that muscle fibers will have a diameter that minimizes metabolic cost while also allowing for efficient diffusion of O<sub>2</sub> and ATP (Johnston et al., 2006). Muscle fiber diameter in birds is usually 10–100 µm across muscle phenotypes (Kinsey et al., 2011). Larger fibers may induce diffusion constraints of aerobic metabolism (Kinsey et al., 2011). Smaller fibers would increase the basal cost of the tissue because of increases in membrane surface area over which Na<sup>+</sup>/K<sup>+</sup>-ATPase are pumped to maintain membrane potential (Jimenez et al., 2013). Furthermore, muscle fiber size has been linked to whole-animal metabolism (Jimenez et al., 2013). Fiber diameter changes may be closely tied to other ultrastructural changes within muscle tissue itself, such as myonuclear domain (MND) and capillaries per fiber area (CFA). MND is defined as the

amount of cytoplasm within a muscle fiber that each nucleus is responsible for servicing (Qaisar and Larsson, 2014). MND is an important physiological trait in aging muscle because muscle atrophy is often accompanied by a loss of nuclei in the muscle fiber (Deschenes, 2004). Capillary density is also a key trait to measure in aging muscle of wild animals as capillaries provide oxygen and nutrients to power muscles, a necessity during foraging. Capillary density usually follows the aerobic potential around the muscle fiber, meaning that slow oxidative fibers have higher densities of capillaries compared with fast glycolytic (anaerobic) ones (Torrella et al., 1998). However, capillary density more closely matches fiber size than aerobic potential of the fiber (Bosutti et al., 2015).

Thick-billed murres have the most expensive flight costs of any homeotherm measured to date, and also dive deeper and longer than equivalent-sized penguins, potentially leading to oxidative damage from prolonged hypoxia (Elliott et al., 2015). Thus, murres have a long lifespan despite high metabolic rates. Because of their costly flight, murres may be particularly sensitive to wear and tear on the muscles; a murre living to 35 years would have roughly 1.5 billion wingbeats during its lifetime (assuming 8 Hz during flight and 2 Hz during diving), compared with 0.15 billion steps for both a typical North American human (*Homo sapiens*) living to 80 years or a typical cheetah (*Acinonyx jubatus*) living to 20 years (Hudson et al., 2012). Moreover, the longer lifespan of birds and bats compared with mammals has been attributed to their ability to fly away from predators or fly towards food (Munshi-South and Wilkinson, 2010), and even a small loss of flight performance could be fatal. We examined linkages between muscle oxidative stress, muscle fiber structure and age in thick-billed murres to better understand physiological aging in a long-lived seabird. We analyzed the pectoralis major muscle, a muscle that seems to be homogeneously composed of fast-twitch oxidative-glycolytic fibers, similar to other seabirds (Caldow and Furness, 1993). The pectoralis muscle is responsible for the down stroke in wing beats during underwater and above-water flight (Torrella et al., 1998). We looked at age-related changes in muscle oxidative stress, including enzymatic antioxidant activities of CAT, SOD and GPx, total antioxidant capacity in the form of scavenging capacity against peroxy and hydroxyl radicals, and LPO damage. We also measured muscle fiber diameter, MND and CFA. In particular, we predicted a decline in antioxidant capacity and an increase in lipid peroxidation, and that fiber diameter, MND and CFA would decrease similarly to as in humans and laboratory rodents.

## MATERIALS AND METHODS

### Tissue collection (include age range of colony)

We studied thick-billed murres [*Uria lomvia* (Linnaeus 1758)] at the Coats Island west subcolony (62.95°N, 82.00°W; Nunavut, Canada) during 17 to 22 July 2018. Intensive banding of chicks since 1981 means that many birds are of known age; our oldest bird was 37 years old (i.e. banded in 1981). Birds were captured using a noose pole during the last 10 days of incubation, when birds are most strongly tied to the egg, to minimize disturbance. Upon capture, birds were weighed with a Pesola scale (to the nearest ±5 g), and morphometrics were taken with a caliper (tarsus, culmen; ±0.1 mm) or wing ruler (wing chord; ±1 mm). While one person held the bird, a second person used water to open up a small area of skin in the right breast 1.5 cm posterior and 1.5 cm to the right of the anterior end of the keel. Some down feathers were removed to expose bare skin. The bird was then injected with lidocaine (0.05 ml; 25 mg total) and ketoprofen (0.1 ml; 0.1 mg total) in three

injections of the area. A scalpel was then used to open up a slit in the skin, and a 6 mm biopsy punch was used to remove a muscle biopsy, with tweezers and scissors used to remove the punch itself. We then stitched the skin closed, applied Vetbond and released the bird. The punch was cut in half, with half placed in a vial and put in a nitrogen shipper at  $-80^{\circ}\text{C}$  (for oxidative stress analysis) and half placed in 4% paraformaldehyde (for muscle ultrastructure analysis). Birds recaptured at a later date as part of other projects showed little scarring where the stitches were applied. All birds were observed to fly away without altered ability. Oxidative stress has been shown to be reduced once an animal enters into its reproductive phase (Blount et al., 2016), which may make it more challenging to detect any aging-related patterns (Urvik et al., 2016). We reduced variation owing to reproductive stage by choosing birds that were all at the same stage (late incubation). Reproductive success in our population increases rapidly up to approximately age 15 years and declines after age 25 years (Elliott et al., 2014b), and so we used the following age categories: young,  $<15$  years; middle, 15–25 years; and old,  $\geq 26$  years. According to the longevity database, AnAge, maximum lifespan (MLSP) for this species is 29 years, whereas the oldest bird we sampled was 37 years old. Thus, our sample collection includes most of the age range of this species.

#### Homogenates for oxygen radical absorbance capacity assay, and CAT, SOD and GPx activity

To estimate antioxidant capacity, and CAT, SOD and GPx activity, we homogenized approximately 0.1 g of muscle tissue using a Fisher Scientific homogenizer (model 125) in a 1:9 (mass:volume) 20 mmol  $\text{l}^{-1}$  HEPES, 1 mmol  $\text{l}^{-1}$  EGTA and 90 mmol  $\text{l}^{-1}$  mannitol buffer solution. We then centrifuged homogenates for 20 min at 17,000 rpm using an Eppendorf centrifuge (5417C) at  $4^{\circ}\text{C}$ . The supernatant was extracted and stored at  $-80^{\circ}\text{C}$  until further testing. All samples were run within 1 week of homogenizing. We used commercial kits (Cayman Chemical Company, Ann Arbor, MI, USA) to measure the activity of CAT (Cayman Chemical 707002), SOD (Cayman Chemical 706002) and GPx (Cayman Chemical 703102) and followed the company's protocols for each enzyme. For all oxidative stress measurements, we collected  $N=41$  individuals, ranging from 8 to 37 years old.

#### Oxygen radical absorbance capacity assay

We estimated antioxidant capacities against peroxy and hydroxyl radicals, two of the more damaging forms of ROS, using a microplate-based version of the competitive oxygen radical absorbance capacity (ORAC) assay (Cao and Prior, 1999; Prior and Cao, 1999). In this assay, when *in vitro* production of radicals exceeds the antioxidant capacity of the tissue, these ROS modify the algal pigment phycoerythrin (545/575 nm), found at a final concentration of  $1.67 \times 10^{-5}$   $\mu\text{mol l}^{-1}$ , and decrease its fluorescence. This assay was temperature controlled ( $30^{\circ}\text{C}$ ) and the decrease in phycoerythrin was monitored using a microplate reader (Infinite M200 Pro, Tecan, Austria). Peroxyl radicals were generated by 320 mmol  $\text{l}^{-1}$  2,2'-azobis(2-amidinopropane) dihydrochloride, and hydroxyl radicals were generated in separate plates by adding 0.25  $\mu\text{l}$  per well of 10 mmol  $\text{l}^{-1}$   $\text{CuSO}_4$  and 0.667 mol  $\text{l}^{-1}$  ascorbate mixture. ORAC values for peroxy and hydroxyl radicals were determined by integrating the area under the fluorescence decay curve. Tissue from each individual was measured in duplicate for both peroxy and hydroxyl radical absorbance capacity; replicates were averaged prior to statistical analysis. This method has been used and validated for tissue homogenates before (Cao and Prior, 1999; Prior and Cao, 1999).

#### LPO damage assay

We estimated LPO damage using a microplate-based version of the ferrous oxidation of xylenol orange (FOX) assay (Gay and Gebicki, 2003; Hermes-Lima et al., 1995; Wolff, 1994). Tissues were weighed, and approximately 0.1 g of tissue was placed in a 1:9 (mass:volume) solution of methanol containing 4 mmol  $\text{l}^{-1}$  2,6-di-tert-butyl-4-methylphenol (BHT). We homogenized each tissue using a Fisher Scientific homogenizer (model 125). Homogenates were centrifuged at 5000 rpm in an Eppendorf centrifuge (5417C) at  $4^{\circ}\text{C}$  for 5 min. Supernatants were immediately incubated in a 90% methanol solution containing 36 mmol  $\text{l}^{-1}$  sulfuric acid, 0.25 mmol  $\text{l}^{-1}$  ammonium iron sulfate and 0.1 mmol  $\text{l}^{-1}$  xylenol orange for 30 min before plating in duplicate wells in a 96-well plate. Lipid hydroperoxide concentration was monitored using a microplate reader (Infinite M200 Pro, Tecan) at an absorbance of 595 nm. Standard curves were generated using cumene hydroperoxide as a positive control.

#### Muscle sectioning and staining

After fixing pectoralis muscle in 4% paraformaldehyde, we placed muscle sections in 30% sucrose overnight to cryoprotect samples. Each biopsy was divided in two pieces: one oriented in cross-section (for fiber diameter and fiber cross-sectional area measurements), and the other oriented longitudinally (to measure longitudinal nuclei size for MND determination). Tissues were mounted in optimal cutting-temperature compound (Scigen Scientific) and allowed to equilibrate to  $-20^{\circ}\text{C}$  in a Microm HM 505 N cryostat before sectioning. Sections were cut at 30  $\mu\text{m}$ , picked up on plus slides (Thermo Fisher Scientific), air-dried at room temperature and then stained with a 250 mg  $\text{ml}^{-1}$  solution of wheat germ agglutinin (WGA; Molecular Probes, Inc.) with Alexa Fluor 488, 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Inc.) and *Griffonia simplicifolia* lectin 694 (GSL; Molecular Probes, Inc.) for 30 min, then rinsed in avian Ringer's solution for 60 min. WGA can non-specifically bind to tissue; thus, longer rinse times are necessary. WGA is a lectin that binds to glycoproteins on the basement membrane of the fiber sarcolemma and effectively outlines the fiber periphery to allow measurements of fiber size (Wright, 1984; Jimenez et al., 2013). DAPI binds to nuclei and GSL-II binds to capillaries. Stained slides were examined with a Zeiss 710 laser filter confocal microscope. Polygons were traced along the fiber periphery using ImageJ (Jimenez et al., 2013). Each stain was analyzed using each stain's individual channel. Polygons were initially drawn in WGA images in ImageJ and could be kept in place as the other two stains were quantified. To manually count capillaries and myonuclei, we used split images of each of our stains, so that we could identify capillaries and myonuclei independently of the other two stains. If there were oblique sections, we did not use that section to quantify any of our parameters. We did not count any fibers that showed any degree of muscle fascicle separation or freeze fracture. Forty-five fibers were randomly chosen and measured for averages for each individual thick-billed murre. For all histological measurements, we collected  $N=50$  individuals, ranging from 8 to 37 years old.

#### Nuclear domain determination

Fiber margins were traced using ImageJ and resultant polygons were used to calculate fiber cross-sectional area, while nuclear cross-sectional area and diameter (from fiber cross-sections) and nuclear lengths (from longitudinal sections) were calculated by outlining DAPI-stained nuclei. Number of nuclei per millimeter of fiber ( $X$ )

was calculated following the method of Schmalbruch and Hellhammer (1977):

$$X = NL/d + l, \quad (1)$$

where  $N$  represents the number of myonuclei per cross-section of fiber,  $L$  is the desired length of the fiber segment (1000 mm),  $d$  is the thickness of the section (30  $\mu\text{m}$ ) and  $l$  is the mean length of a muscle nucleus, measured from longitudinal sections. The volume of cytoplasm per nucleus or MND ( $Y$ ) was calculated by:

$$Y = CL/X, \quad (2)$$

where  $C$  is the cross-sectional area of the muscle fiber measured for each species. Nuclear number volume, which is the number of nuclei per volume of cell, is the inverse of the MND,  $Y$ .

### Capillaries per fiber area

Fiber cross-sectional area and number of capillaries around the fiber were analyzed using ImageJ. Capillary counts were measured by manual count of the number of capillaries touching the fiber. CFA was obtained by dividing the capillary counts by muscle fiber cross-sectional area of each individual fiber (Brown et al., in press; Ross et al., 2017).

### Statistics

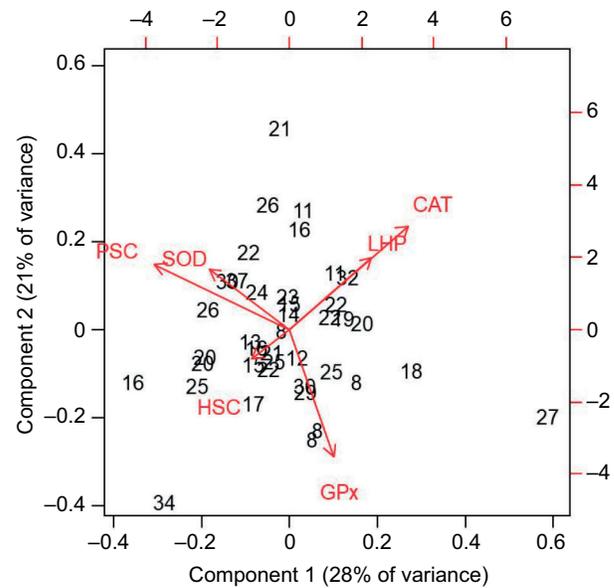
We used R 3.6.0 to complete all statistical analyses. We calculated body condition as the residual of body mass on the first principal component of size (wing chord, tarsus, culmen), as this measure of body condition correlates most closely with body lipids in murre (Jacobs et al., 2012). For oxidative stress, we conducted a principal components analysis to examine relationships among the various parameters; as there were only three muscle ultrastructure parameters, we did not use a principal components analysis for muscle ultrastructure. We then used a general linear model to examine whether each of the oxidative stress or muscle ultrastructure variables were explained by body mass, body size (head-bill length, as this was the only parameter to correlate with body mass), body condition (residual of body mass on body size), sex, age or age<sup>2</sup>. We used a significance level of 0.05.

### RESULTS

Body mass was independent of sex, age, age<sup>2</sup>, wing chord and tarsus (all  $P > 0.25$  in omnibus model), and positively associated with head-bill length ( $t_{47} = 2.52$ ,  $P = 0.02$ ). Body condition was independent of sex, age, age<sup>2</sup>, wing chord and tarsus. Thus, we included body mass and body condition in subsequent analyses.

#### Muscle oxidative stress in aging thick-billed murre

A principal components analysis showed that the first axis (loading most heavily, negatively, on SOD and peroxy scavenging capacity, and positively on CAT) explained 28% of the variance, the second



**Fig. 1. Principal components analysis of oxidative stress parameters in thick-billed murre.** CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase; PSC, peroxy scavenging capacity; HSC, hydroxyl scavenging capacity; LPO, lipid peroxidation. The numbers on the graph refer to the age (years) of each individual.

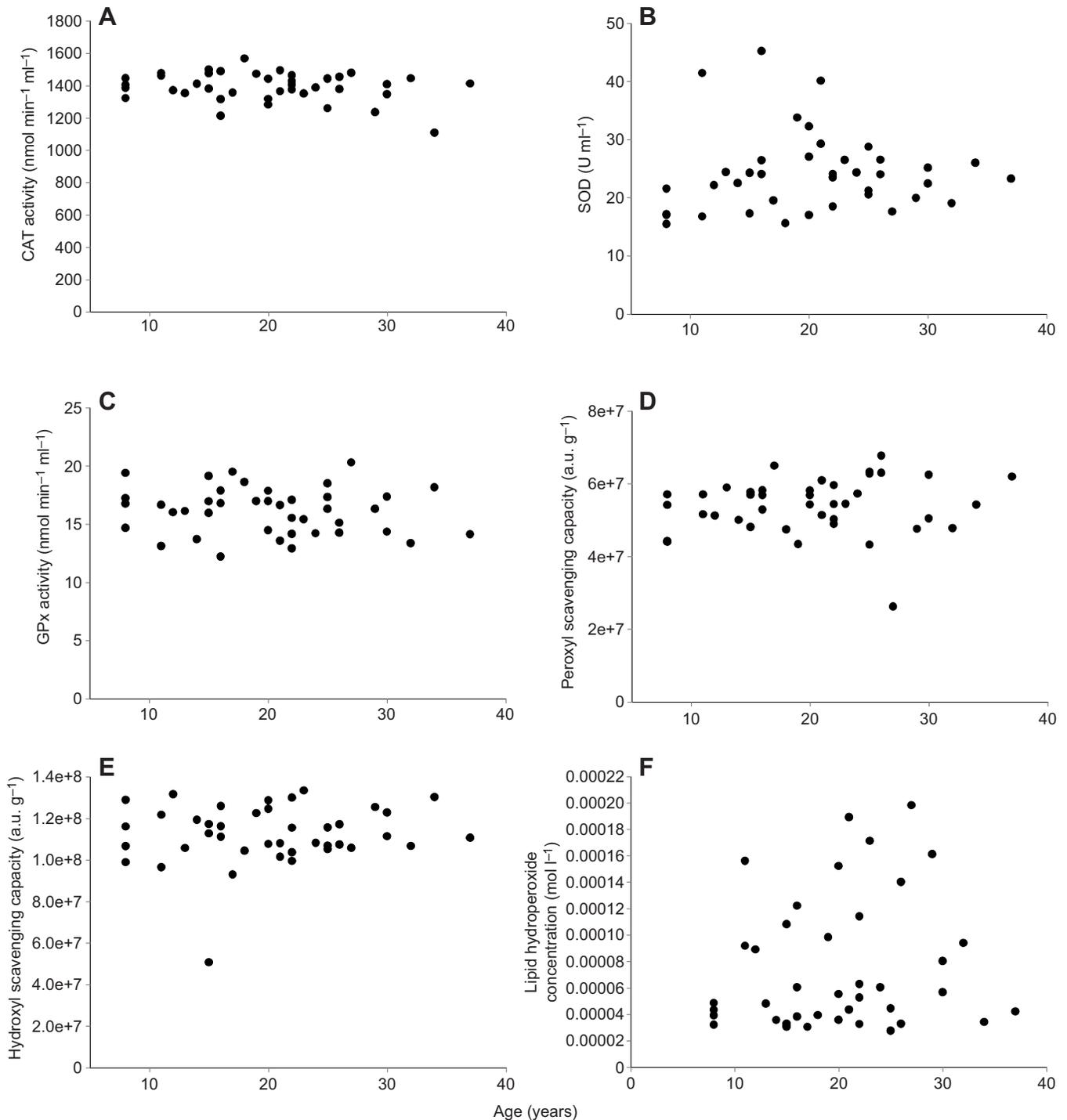
axis (loading most heavily, positively, on CAT and SOD, and negatively on hydroxyl scavenging capacity and lipid hydroperoxide) explained 21% of the variance, the third axis (loading most heavily, negatively, on GPx and, positively, on CAT) explained 17% of the variance and the fourth axis (loading most heavily, negatively, on lipid hydroperoxide and hydroxyl scavenging capacity) explained 16% of the variance, with no other axis explaining more than 11% of the variance (Fig. 1). There was no strong clustering in the first or second component with age (Fig. 1, Table 1). Given the lack of strong correlations or axes among the oxidative stress parameters, we examined each parameter separately. No parameter varied significantly with age (Fig. 2). When considered in a general linear model with body mass, body size and sex, no parameter varied with age (CAT activity,  $P = 0.1309$ ; GPx activity,  $P = 0.272$ ; SOD activity,  $P = 0.9445$ ; peroxy scavenging capacity,  $P = 0.6380$ ; hydroxyl scavenging capacity,  $P = 0.2362$ ; LPO damage,  $P = 0.444$ ). CAT activity increased with body size ( $P = 0.0235$ ) while SOD activity was higher in females ( $P = 0.0334$ ). Hydroxyl scavenging capacity increased with body mass ( $P = 0.0462$ ) and decreased with body size ( $P = 0.033$ ).

#### Muscle histology in aging thick-billed murre

When considered in a general linear model (with body mass, body size and sex), fiber diameter ( $P = 0.8360$ ) and capillaries per

**Table 1. Results from a principal components analysis of the oxidative stress parameters**

	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5	Axis 6	Axis 7
Catalase	0.363	0.408	0.520		0.305	0.568	0.113
Glutathione peroxidase	0.247	0.162	-0.750	0.244	-0.201	0.457	0.204
Superoxide dismutase	-0.561	0.415				0.303	-0.637
Peroxy scavenging capacity	-0.357	-0.197	0.371	0.477	-0.565	0.229	0.312
Hydroxyl scavenging capacity	-0.338	-0.431		-0.590	0.170	0.497	0.267
Lipid peroxidation damage	0.133	0.430		-0.592	-0.615	-0.132	0.211
Standard deviation	1.379	1.190	1.079	1.039	0.858	0.556	0.472
Proportion of variance	0.278	0.207	0.171	0.158	0.108	0.045	0.033



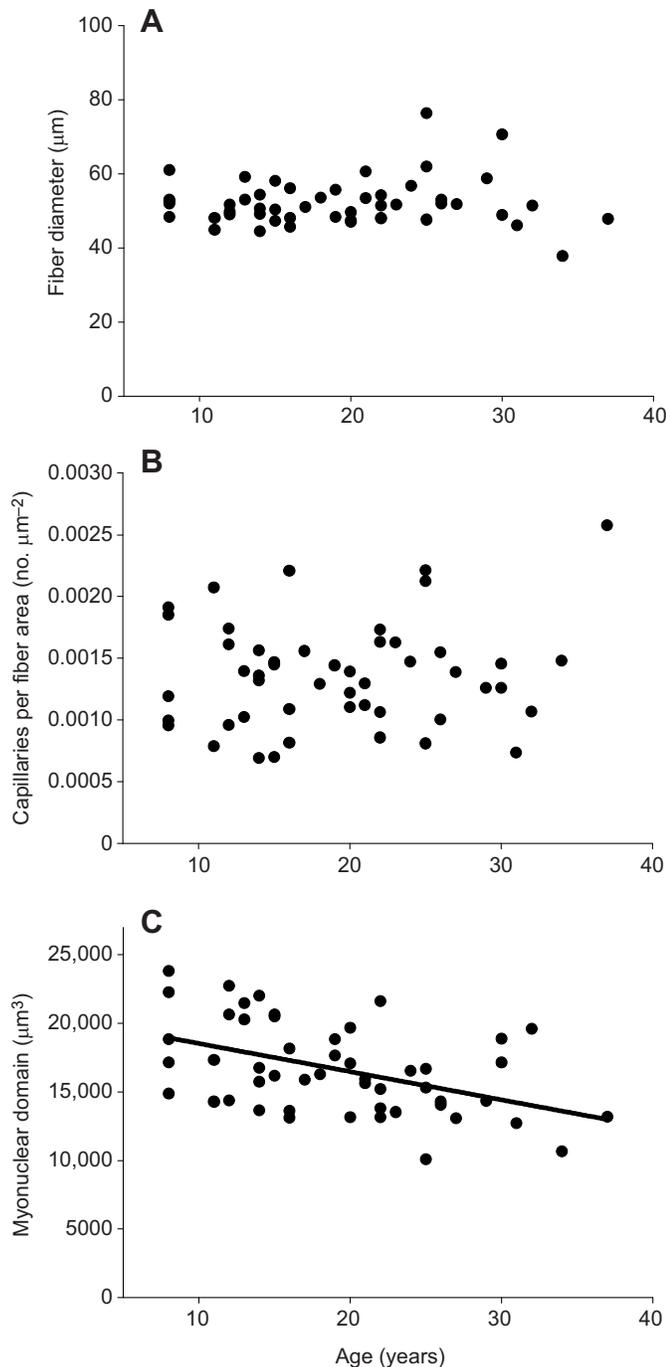
**Fig. 2. Muscle oxidative stress in aging thick-billed murres.** When considered in a general linear model with body mass, body size and sex, no parameter varied with age (CAT,  $P=0.1309$ ; GPx,  $P=0.272$ ; SOD,  $P=0.9445$ ; PSC,  $P=0.6380$ ; HSC,  $P=0.2362$ ; LPO damage,  $P=0.444$ ). For all oxidative stress measurements, we collected  $N=41$  individuals, ranging from 8 to 37 years old.

fiber area ( $P=0.2459$ ) did not vary with age (Fig. 3), though CFA was significantly higher in males compared with females ( $P=0.0103$ ). MND significantly decreased with age ( $P=0.00145$ ; Fig. 3).

## DISCUSSION

As hypothesized and observed widely in humans and laboratory mammals, MND declined with age. In contrast, we found no

relationship with fiber diameter, CFA or any measure of antioxidant capacity or oxidative stress. We conclude that muscle senescence may be important in thick-billed murres. As our dataset is purely cross-sectional, we cannot exclude the possibility that selective disappearance of individuals with high MND may have contributed to our trend. However, as mortality increases non-linearly with age in our population, it seems unlikely that such a trend could lead to a roughly linear decline in MND with age.



**Fig. 3. Muscle histology in aging thick-billed murres.** When considered in a general linear model with body mass, body size and sex, fiber diameter ( $P=0.8360$ ) and capillaries per fiber area ( $P=0.2459$ ) did not vary with age. Myonuclear domain significantly decreased with age ( $P=0.00145$ ). For all histological measurements, we collected  $N=50$  individuals, ranging from 8 to 37 years old.

Thick-billed murres have the highest flight costs of any homeotherm measured, and whole-animal metabolism decreases with increasing age (Elliott et al., 2015). To some, these high flight costs may equate to the fact that murres should have high oxidative stress costs. However, the relationship between metabolism and ROS production is not straightforward; that is, an increase in whole-animal oxygen consumption does not always yield an increase in ROS production and concomitant changes in the oxidative stress

system (Stier et al., 2014). This may be due to the fact that oxygen consumption can be coupled with either ATP production or heat depending on whether the ATP synthase or mitochondrial uncoupling proteins (UCPs) are driving respiration (Ricquier and Bouillaud, 2000; Divakaruni and Brand, 2011). UCPs are a family of proteins embedded in the inner mitochondrial membrane; their function is to allow protons to cross the inner mitochondrial membrane without producing ATP, uncoupling ATP production and potentially leading to significant decreases in ROS production (Cooper-Mullin and McWilliams, 2016). Though we see aging-related changes in whole-animal metabolism, and sustained high metabolic rates in this species, we did not see any age-related changes to the oxidative stress system as thick-billed murres aged. Indeed, perhaps the lower whole-animal metabolism in older individuals contributed to an absence of apparent oxidative stress, especially given that these birds apparently maintained their investment in antioxidant defense.

We know that shorter-lived mammals have higher rates of oxidative stress compared with longer-lived birds of similar sizes (Jimenez et al., 2019). Within birds, long-lived species have lower oxidative stress compared with shorter-lived species (Costantini and Bonadonna, 2010). Others have sampled blood from long-lived seabirds during incubation, measured oxidative stress and found differences across the incubation period only in females (Lecomte et al., 2010). In the albatross, another long-lived bird, blood samples from aging birds (8–47 years old) showed no age-dependent changes in immunity, oxidative stress or antioxidant defenses, though reproductive senescence had happened after 30 years of age, and foraging activity decreased with age (Lecomte et al., 2010), similar to our findings. In the common gull (*Larus canus*), blood oxidative stress measurements during breeding did not change with age; however, there was a positive correlation between glutathione levels and lifespan in female birds (Urvik et al., 2016). However, others have found age-related effects on bird oxidative stress, albeit in various patterns across lifespan. For example, in a study using whole blood, the greater flamingo (*Phoenicopterus ruber roseus*) exhibited decreases in resistance to an oxidative attack in young birds and old birds, whereas middle-aged birds had higher resistance to oxidative stress (Devevey et al., 2010). Similarly, in red-legged partridges (*Alectoris rufa*), RBCs had higher lipid damage and higher glutathione levels in younger and older birds; however, uric acid and total antioxidants were higher in older birds compared with younger birds (Alonso-Álvarez et al., 2010). In contrast, a population of aging European shags (*Phalacrocorax aristotelis*) showed higher oxidative stress in blood in older birds, and younger, adult-sized birds showed significant within-individual variation, whereas older birds showed little variation in oxidative damage (Herborn et al., 2016). We were not able to detect any changes in the oxidative stress system as thick-billed murres aged. Thus, it may be complicated to demonstrate generalizable patterns of aging in the oxidative stress system.

Body size is positively correlated with longevity across bird species. Additionally, long-lived birds increase monounsaturations and decrease polyunsaturations of cell membranes, a trait that may have co-evolved with an increase longevity (Galván et al., 2015). In long-lived seabirds, petrels and albatross, membranes were found to be 40-fold less peroxidizable than polyunsaturated fatty acid (PUFA) membranes, and had significantly more monounsaturated fatty acids than membranes from Galliformes (Buttemer et al., 2008). Decreases in PUFA membranes can also decrease the potential for propagation of ROS, thus decreasing LPO damage. Intrinsic factors that could affect oxidative stress include body mass, where decreased body mass

often leads to longer lifespan (Briga et al., 2019). We found a positive correlation between body mass, CAT activity and hydroxyl scavenging capacity, possibly implying that larger body masses had to expend extra energy increasing their antioxidant system. Our lack of age-related effects on various aspects of the oxidative stress machinery implies that murrens maintain a high level of somatic maintenance into very old age, despite the fact that resting metabolism decreases with age (Bize et al., 2014; Elliott et al., 2015), though others have been able to detect senescence in oxidative stress in very old, long-lived birds, such as wandering albatrosses (Costantini et al. 2014). As daily energy expenditure increases with age during stressful periods (Elliott et al., 2014a), the absence of a trend in oxidative stress may represent a compromise between lower oxidative demands for old birds during rest and higher oxidative demands during activity. It may also be because long-lived birds have such low levels of oxidative stress that aging does not alter this mechanism more (Costantini and Bonadonna, 2010). Another possibility is that, at non-basal levels, such as those during pathology or environmental stress, oxidative stress may show age-related effects not detected at basal levels. Although murrens still must commute long distances to foraging grounds every 12 h during incubation, daily energy expenditure is substantially higher during chick-rearing (Elliott et al., 2014a); thus, oxidative stress may demonstrate differences during that time. At basal levels, using knockouts/transgenic methods, increasing the antioxidant machinery in mice has not changed or altered lifespan. However, when these mice are challenged with a pathology, decreased oxidative stress owing to increases in the antioxidant system retards the pathology (Salmon et al., 2010), implying that oxidative stress differences may have implications for the onset of mortality owing to pathology. Others have found that oxidative stress in a long-lived long-distance migrant, the light-bellied brent goose (*Branta bernicla hrota*), tended to change only when extrinsic factors change, i.e. in migration stages or when air temperature abruptly changes (Bodey et al., 2019).

We did not find a significant correlation between muscle fiber diameter and age, or fiber diameter and body mass. Thus, there seems to be no apparent atrophy with increasing age in thick-billed murrens. In humans, normal muscle aging patterns show a decrease in muscle fiber diameters with age through sarcopenia (Young et al., 1985; Lexell et al., 1988; Frontera et al., 2000), though it is unclear whether age-related decreases in muscle fiber diameter are a general trend in most animals or just the pattern observed in humans and mammals (Young et al., 1985; Lexell et al., 1988; Frontera et al., 2000). Similar non-significant changes in fiber diameter were observed in another long-lived seabird, the black-legged kittiwake (Brown et al., 2019). Additionally, we did not find a correlation between CFA and age. Thus, activities such as foraging or escaping predators, where muscles are critically relied upon and need efficient diffusion of O<sub>2</sub> and ATP, are not impaired in aging murrens, as in the case of aging black-legged kittiwakes (Brown et al., 2019). In three different species of bird, CFA in aerobic and anaerobic fibers closely match energetics and locomotory patterns exhibited by each species (Torrella et al., 1998), thus CFA may be a trait that is maintained across a bird's lifespan.

Studies have shown conflicting results when examining variation in number of myonuclei in age for humans, mice and birds (e.g. Van der Meer et al., 2011). Others highlighted that variations in MND size were ascribed only to physiological or pathological conditions (e.g. Hughes and Schiaffino, 1999). In mammals, a decrease in innervation appears to be causative for sarcopenia and concomitant decreases in MND (Hughes and Schiaffino, 1999). Through

sarcopenia in mammals, muscle atrophy is often related to an apoptotic decrease in the number of nuclei in muscle; however, there have been cases demonstrating exceptions to this 'rule' (Cristea et al., 2010). Satellite cells comprise the seemingly stem-like population of myotube precursors that exist in close association with myotubes. They are recruited into the existing muscle fiber in times of strain or repair (Brooks et al., 2009; Bruusgaard et al., 2010). During aging, studies have shown inconclusive results as to whether satellite cells are reduced or remain constant (e.g. Brooks et al., 2009). Because of this, whether MND changes with age has also been an unresolved question. Some have found that slow oxidative muscle fibers in rats demonstrated a decrease in MND with age, whereas fast-glycolytic muscle fibers did not show a MND with age in rats and humans (Brooks et al., 2009; Cristea et al., 2010). We found a significant decrease in MND with age, similar to findings in humans (Manta et al., 1987; Cristea et al., 2010). However, fiber diameter did not change with age, as predicted by mammalian work. Thus, it may be that chronic exercise, as in the case of birds that forage, may limit the usual sarcopenia seen in mammals with age, as well as loss of function in satellite cells. Functionally, this change can be viewed as a decrease in protein turnover load per nuclei, if all nuclei are still equally producing products; in contrast, it could imply that not all nuclei are producing products at fast enough rates, thus more nuclei have to be recruited into each existing muscle fiber (Brooks et al., 2009). Others have found that filling up muscle fibers with nuclei by exercising may be beneficial in senescence so as to prevent muscle weakness in the elderly (Bruusgaard et al., 2010), which may be the mechanism used by thick-billed murrens to maintain proper muscular function into old age.

In conclusion, thick-billed murrens showed declining MND with age. Similar to how declining whole body metabolic rate may be one mechanism for maintaining a constant level of oxidative damage, declining MND may represent recruitment of nuclei into existing fibers or reduced protein turnover per nuclei, thus leading to maintenance of fiber performance with age – as implied by the constant fiber diameter with age. Thus, reduced physiological (muscle) performance may be an important precursor to actuarial (mortality) senescence, although the mechanism does not appear to be via oxidative damage.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: A.G.J., K.H.E.; Methodology: A.G.J., E.S.O.; Software: E.S.O.; Validation: A.G.J.; Formal analysis: K.H.E.; Investigation: A.G.J., E.S.O.; Resources: A.G.J., K.H.E.; Data curation: E.S.O.; Writing - original draft: A.G.J.; Writing - review & editing: K.H.E.; Visualization: A.G.J., K.H.E.; Supervision: A.G.J., K.H.E.; Project administration: K.H.E.; Funding acquisition: A.G.J.

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