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The naked mole rat (NMR; Heterocephalus glaber) is the longest-living rodent known [maximum lifespan potential (MLSP): >28 yr] and is a unique model of successful aging showing attenuated declines in most physiological function. This study addresses age-related changes in endothelial function and production of reactive oxygen species in NMR arteries and vessels of shorter-living Fischer 344 rats (MLSP: ~3 yr). Rats exhibit a significant age-dependent decline in acetylcholine-induced responses in carotid arteries over a 2 yr age range. In contrast, over a 10 yr age range nitric oxide (NO)-mediated relaxation responses to acetylcholine and to the NO donor SNAP were unaltered in NMRs. Cellular superoxide anion (O2•−) and H2O2 production significantly increased with age in rat arteries, whereas they did not change substantially with age in NMR vessels. Indicators of apoptotic cell death (DNA fragmentation rate, caspase 3/7 activity) were significantly enhanced (10±30%) in arteries of 2 yr-old rats. In contrast, vessels from 12 yr-old NMRs exhibited only a ~50% increase in apoptotic cell death. In the hearts of NMRs (2 to 26 yr old), expression of endothelial NO synthase, antioxidant enzymes (Cu,Zn-SOD, Mn-SOD, catalase, and glutathione peroxidase), the NAD(P)H oxidase subunit gp91phox, and mitochondrial proteins (COX-IV, ATP synthase, and porin, an indicator of mitochondrial mass) did not change significantly with age. Thus long-living NMRs can maintain a youthful vascular function and cellular antioxidant phenotype relatively longer and are better protected against aging-induced oxidative stress than shorter-living rats.

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Maximum life span potential (MLSP) varies more than 40,000-fold across the animal kingdom and even within rodents considerable variability exists (6) (Fig. 1). Comparative studies of species with disparate longevity may provide useful insights into the mechanisms determining successful aging. The naked mole rat (NMR; Rodentia: Bathyergidae; Heterocephalus glaber), living more than 28.3 yr, is the longest-living rodent known (7). These mouse-sized eusocial subterranean animals, like humans, have a longevity quotient of 5 [the ratio of actual maximum lifespan potential to that predicted by body mass using the allometric equations of Austad and Fisher (5)], which is approximately eightfold greater than that of laboratory mice and rats (Fig. 1, A and B).

The NMR seems to be an ideally suited animal model with which to study successful agings, and we have begun to characterize age-related changes in physiological function in different organ systems in NMRs (reviewed recently in Ref. 6). These studies demonstrated that unlike laboratory mice and rats, NMRs show retarded age-related changes in body composition and metabolism and surprisingly do not seem to exhibit typical age-related pathology (e.g., tumors) (6). Recently, we reported that tissues of NMRs, even at a young age, are more resistant to oxidative stress than those of shorter-living rodents (22). The present study was designed to characterize age-related changes in vascular function in NMRs.

Epidemiological data show that cardiovascular disease is responsible for a large percentage of age-specific mortality in humans. Despite the growing incidence of aging-related coronary artery disease, stroke, and diabetic vasculopathy, the factors determining successful vascular aging are still poorly understood. Studies based on laboratory rats and mice reveal that vascular aging in these short-lived species (MLSP ~3–3.5 yr) is characterized by gradual proatherogenic functional alterations, including impaired endothelial nitric oxide (NO) mediation, increased production of reactive oxygen species [ROS, such as superoxide (O2•−) and hydrogen peroxide (H2O2)], and enhanced endothelial apoptotic cell death (11–14, 23, 36). These changes are, at least in part, due to age-related cellular phenotypic alterations, including downregulation of endothelial NO synthase (eNOS) and antioxidant enzymes, increased expression/activity of NAD(P)H oxidases, and dysregulated expression of the electron transport chain components (including COX-IV) resulting in an increased mitochondrial ROS production. To date, such studies have not been undertaken in long-lived NMRs, even though this species may expose mechanisms involved in resisting vascular aging.

In long-living animals there are three putative adaptive mechanisms by which vascular cells can delay/limit oxidative stress-induced damage (Fig. 1, C–F). The first mechanism is change in the rate of age-related decline in NO production and/or age-related increases in ROS generation (Fig. 1C). This would alter the time to reach a critical threshold below which cellular function declines. The second is change in the physiological reserve. In other words, if a young long-living animal...
had high NO production rates and lower ROS generation, even if the rate of decline during aging is the same as that of shorter-living animals, then it would take longer to reach the critical threshold, below which physiological function is severely compromised. Finally, the long-living animal may exhibit increased tolerance for age-related increases in ROS production. This may manifest in tolerance of accrued damage without affecting physiological function or superior antioxi-
dant defenses and/or repair mechanisms thereby minimizing the accrual of damaged tissue.

The present study was designed to assess, primarily over a 10-yr period, age-related changes in endothelial function, $O_2^{-}$- and $H_2O_2$ production, and vascular apoptotic cell death in NMR and compare these age-related changes with those observed over a 1-yr time interval in shorter-lived rats. Specifically, we tested the hypothesis that age-related decline in endothelial NO mediation, increased production of ROS, and/or enhanced apoptotic cell death in successfully aging NMRs is delayed.

METHODS

Animals. All animal-use protocols were approved by the Institutional Animal Care and Use Committee of the City College of New York and the New York Medical College, Valhalla, NY. Young (<2 yr old; n = 8) and ~12-yr-old (n = 6, which is the oldest currently available age-group in this species) NMRs (weighing 25–38 g) were from the well-characterized colonies maintained in Dr. Buffenstein’s laboratory at City College of New York (CCNY) (3, 6, 30, 33, 38). In addition, tissue samples harvested from 26-yr-old NMRs were also used in biochemical aspects of this study. 3-, 14-, 16-, and 24-mo-old Fischer (F344) rats (n = 10 in each group) were purchased from the National Institute of Aging and were maintained under standard laboratory conditions. Rats were maintained on a standard rat chow diet and provided with both food and water ad libitum, whereas mole rats are fed a diet of fresh fruits and vegetables supplemented with a high-protein cereal (Pronutro, Bokomo, South Africa). All animals were disease free with no signs of systemic inflammation and/or neoplastic alterations. Longevity quotients were calculated from the ratio of maximum longevity to the predicted maximum lifespan potential as determined by body mass. The allometric equation of Austad and Fisher (5) for nonflying eutherian mammals was used (Fig. 1; predicted longevity = 10.67 × $M_{100}^{0.189}$, where $M$ is body mass in kg).

Vessel isolation and functional studies. Animals were euthanized by an overdose of pentobarbital, as described previously (13, 14). The carotid arteries and the aorta of each animal were carefully exposed and isolated from the surrounding tissues. The vessels were cleaned from the adventitia by using an operating microscope and microsurgery instruments.

Endothelial function was assessed as previously described (9, 16). In brief, carotid arteries of each animal were cut into ring segments 2 mm in length and mounted on 40-μm stainless steel wires in the myographs chambers (Danish Myo Technology) for measurement of isometric tension. The vessels were superfused with Krebs buffer solution (in mM: 118 NaCl, 4.7 KCl, 1.5 CaCl2, 25 NaHCO3, 1.1 MgSO4, 1.2 KH2PO4, and 5.6 glucose; at 37°C; gassed with 95% air and 5% CO2). After an equilibration period of 1 h during which a optimal passive tension was applied to the rings (as determined from the vascular length-tension relationship), relaxations of precontracted (by $10^{-6}$ mol/l phenylephrine) vessels to acetylcholine (ACh; from $10^{-10}$ to $10^{-4}$ mol/l) and the NO donor $N$-nitro-L-arginine methyl ester (L-NAME 3 $10^{-5}$ mol/l) were obtained. The effects of the NO synthase inhibitor $N^\text{G}$-nitro-L-arginine methyl ester (l-NAME 3 $10^{-5}$ mol/l) on ACh-induced responses of NMR vessels were also tested.

Measurement of vascular superoxide production. Production of $O_2^{-}$ was determined in segments of the same carotid arteries that were previously used for functional studies. Hydroethidine, an oxidative fluorescent dye, was used to localize superoxide production in situ as we previously reported (12, 35, 37). In brief, vessels were incubated with hydroethidine ($10^{-6}$ mol/l; at 37°C for 60 min). The arteries were the washed three times, embedded in OCT medium, and cryosectioned. Vascular sections were imaged using a Zeiss AxiosCam.
Mrm camera mounted on a Zeiss Axiovert 200 fluorescence microscope (Carl Zeiss, Gottingen, Germany). Images were captured at ×20 magnification and analyzed using the Zeiss Axiovision imaging software. Ten to fifteen entire fields per vessel were analyzed with one image per field. The mean fluorescence intensities of ethidium bromide-stained nuclei in the endothelium and medial layer were calculated for each vessel. Thereafter, these intensity values for each animal in the group were averaged. Vessels coincubated with Tiron were used as negative controls.

Measurement of vascular H$_2$O$_2$ production. The cell-permeant oxidative fluorescent indicator dye C-H$_2$DCFDA [5-(and 6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate-acetyl ester, Invitrogen, Carlsbad, CA] was used to assess H$_2$O$_2$ production in isolated aortic segments according to the modified protocols of Miura et al. (26). C-H$_2$DCFDA is a 2’7’-dichlorofluorescein derivative that has longer retention within the cells. In brief, vessel segments were treated with C-H$_2$DCFDA (10$^{-5}$ mol/l; at 37°C for 60 min). Thereafter, the arteries were washed three times. Fluorescent images of the endothelial layer of en face preparations were captured and analyzed using the Axiovision software. Ten to fifteen entire fields from each vessel of every animal were analyzed with one image per field. Fluorescent images of unstained arterial segments from the same animal were obtained to determine the average level of background fluorescence intensity. The background-corrected mean DCF fluorescent intensities of each image from each animal were averaged. These intensity values for each animal in the group were then averaged. Vessels coincubated with PEG-catalase were used as negative controls.

Western blot analysis. To determine whether in NMRs aging alters the expression of major cellular antioxidant systems, NAD(P)H oxidase, and/or mitochondrial proteins, Western blot analysis was performed as described (12, 13). Because of the limited amount of vascular tissue available, we have measured protein expression in homogenates of NMR hearts (age groups: 2, 5, 13, and 26 yr old). Primary antibodies directed against eNOS (no. 610298, Transduction Laboratories), catalase (no. 16731, Abcam, Cambridge, MA), glutathione peroxidase-1 (no. 16798, Abcam), Mn-SOD (no. SOD-110, Stressgen, Ann Arbor, MI), Cu,Zn-SOD (no. SOD-100, Stressgen), and gp91$^{	ext{phox}}$ (no. 07-024, Upstate Biotechnology), porin (A31855, Molecular Probes), cytochrome oxidase subunit IV (COX-IV; no. 4844, Cell Signaling), and complex V (no. A21350, Molecular Probes) were used. Anti-β-actin (no. 6276, Abcam) was used as loading control.

DNA fragmentation assay. Arteries were lysed and cytoplasmic histone-associated DNA fragments, which indicate apoptotic cell death, were quantified by the Cell Death Detection ELISA$^{	ext{Plus}}$ kit (Roche Diagnostics, Indianapolis, IN) as previously described (14), using an Infinite M200 plate reader (Tecan, Research Triangle Park, NC). Results are reported as normalized arbitrary optical density (OD) units.

Caspase activity assay. Arteries were homogenized in lyses buffer, and caspase activities were measured using Caspase-Glo 3/7 assay kit according to the manufacturer’s instruction (Promega, Madison, WI). In 96-well plates, 50-μl sample was mixed gently for 30 s with 50 μl Caspase-Glo 3/7 reagent and incubated for 2 h at room temperature. The lyses buffer with the reagent served as a blank. Luminescence of the samples was measured using an Infinite M200 plate reader (Tecan). Luminescent intensity values were normalized to the sample protein concentration.

Data analysis. Data were normalized to the respective young control mean values and are expressed as means ± SE. Statistical analyses of data were performed by Student’s t-test or by two-way ANOVA followed by the Tukey post hoc test, as appropriate. $P < 0.05$ was considered statistically significant.
function of chronological age, whereas in Fig. 3B, maximal relaxation to ACh is plotted against the age of the animals of each species as a percentage of MLSP. Both mice and rats show a marked decline in induced maximal vessel relaxation with age. Despite more than an order of magnitude difference in age-period monitored compared with that shown for the laboratory rodents, maximal vessel relaxation was unchanged in NMRs.

Cardiac expression of eNOS did not change significantly in NMRs throughout the entire lifespan (2–26 yr; Fig. 4A: original Western blot analyses, B and C: summary data for eNOS expression, as a function of chronological age and the age of the animals as a percentage of MLSP, respectively).

Vascular \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) production. Analysis of nuclear ethidium bromide fluorescence intensities showed that aging was associated with significant increases in endothelial \( \text{O}_2^- \) production in the carotid arteries from F344 rats. In contrast, \( \text{O}_2^- \) production in NMR carotid arteries did not change significantly with age (Fig. 5, A and B).

Vascular apoptotic cell death. Previously, we have demonstrated that DNA fragmentation and caspase 3/7 activity are a good measure of apoptotic cell death in blood vessels (14). In F344 vessels there was a significant increase in DNA fragmentation rate and caspase 3/7 activity after midage (Fig. 8, A and C). In arteries of 12-yr-old NMRs, DNA fragmentation rate was also significantly higher than that observed in young vessels (Fig. 8A). Figure 8, B and D, shows the changes in DNA fragmentation rate and caspase 3/7 activity as a function of the age of the animals as a percentage of MLSP. These data show that the DNA fragmentation rate and caspase 3/7 activity in vessels of 12-yr-old NMRs are similar to those in the arteries of rats at the same relative biological age (Fig. 8, B and D).

Fig. 3. A: maximal relaxation induced by acetylcholine in carotid arteries of NMRs and F344 rats as a function of chronological age (A) and relative age (expressed as a percentage of maximal lifespan potential; B). Responses of carotid arteries of young, middle-aged, and aged C57 mice are also shown for comparison. Data are means ± SE. *P < 0.05 vs. young; #P < 0.05 vs. rat.

Fig. 4. A: original Western blot showing expression of endothelial NO synthase (eNOS) in hearts of NMRs and F344 rats. B and C: line graphs are densitometric data (means ± SE) as a function of chronological age (B) and relative age (expressed as a percentage of maximal lifespan potential; C).
DISCUSSION

This study set out to assess whether successful vascular aging in long-living NMRs was correlated with lower rates of age-related changes in NO and ROS production; an augmented physiological reserve facilitating an extended period before age-related declines compromise function and/or greater tolerance for age-related damage without affecting functionality. There are three salient findings of this study: 1) Age-related declines in endothelial function are delayed in long-lived NMRs compared with shorter-living rats. In addition, only slight increases in endothelial ROS generation are observed over a 10-yr period, whereas age-related increases in vascular apoptotic cell death are attenuated in NMRs. If age-related changes in the latter variable are expressed as a relative proportion of MLSP, both rats and mole rats follow a similar relative time course in cell death response.

We have confirmed in the present study that aging is associated with a substantial decline in NO-mediated dilations in rat and mouse arteries (11, 12, 17) (Fig. 2A and Fig. 3). This is attributed to age-dependent increases in O$_2$•ABOUT as generation and downregulation of eNOS in short-lived laboratory rodents (Fig. 4), which in turn leads to impaired bioavailability of NO (12). In contrast, endothelial NO synthesis (Figs. 2B and 3) and expression of eNOS (Fig. 4) is preserved throughout the long NMR lifespan (2–26 yr). NO is known to exert vasculoprotective and antiatherogenic effects, and its decreased bioavailability with age is likely to contribute to the development of atherosclerotic vascular disease (reviewed in Ref. 11). Recent studies have demonstrated that a genetic lack of eNOS accelerates cardiovascular aging, with a concomitant impairment of cardiac performance (Dr. Pal Pacher, personal communication, 2006) and altered cardiac (31) and vascular gene expression profiles (Csiszár and Ungvari, unpublished data, 2005). Thus one can speculate that the preservation of NO synthesis helps to maintain a youthful vascular phenotype in aged NMRs.

Because relaxation of young and aged NMR carotid arteries in response to an exogenous NO donor is comparable (Fig. 2C), it seems that the sensitivity of smooth muscle cells to NO also does not change over at least a 10-yr period in these mouse-sized rodents. These attenuated changes in NO bioavailability could explain the apparent lack of atherosclerosis in aged NMRs (Buffenstein, unpublished observation, 2005).

There is extensive evidence that age-related decline in NO synthesis in short-lived rodents is, in part, due to an increased oxidative stress and accrued oxidative damage (reviewed in Ref. 11). We, therefore, also compared endothelial ROS production in rat and NMR vessels. O$_2$•ABOUT production did not change significantly between arteries from 2-yr-old and 12-yr-old NMRs, whereas substantial increases in O$_2$•ABOUT production in rat arteries were evident over a far shorter time interval (1.75 yr) when data from blood vessels of 3-mo-old and 24-mo-old rats were compared (Fig. 5, A–C). Measurement of endothelial...
DCF fluorescence showed that H$_2$O$_2$ production also significantly increased with age in rat arteries. Although 12-yr-old NMR arteries also tended to produce somewhat more H$_2$O$_2$ than young NMR vessels, H$_2$O$_2$ generation in aged NMR vessels was significantly less than that in vessels of both middle-aged and old (14- and 24-mo-old) rats (Fig. 5, D and E) but was similar to young rats. These findings support our premise that age-related changes in physiology and morphology are retarded in NMRs (6, 30). In previous studies, we noted that NMRs, unlike other mammals, did not exhibit age-related changes in basal metabolism, gastrointestinal absorption, or body composition (30), and similarly, bone integrity (Buffenstein, Grun-Kramer, and Jepsen unpublished data, 2005) and reproductive function (6) are maintained well into the third decade of life. Recent comparison based on four rodents with disparate maximum longevity [NMRs, mice, guinea pigs, and Damara mole rats (Cryptomys damarensis)] that range between 3 and >28.3 yr revealed that initial cellular

![Fig. 6. Original Western blot analyses showing expression of gp91phox (A), Mn-SOD (B), Cu, Zn-SOD (C), catalase (D), and Gpx-1 (E) in the hearts of 2-, 5-, 13-, and 26-yr-old NMRs. Line graphs are densitometric data (means ± SE) as a function of relative age (expressed as a percentage of maximal lifespan potential).](http://ajpheart.physiology.org/)

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O$_2^-$ and H$_2$O$_2$ production does not show a strong correlation with MLSP (22) and thus cannot be a key determinant of lifespan potential in these rodents.

NAD(P)H oxidase activity in NMRs like that of mice, rat, and humans, contributes significantly to basal vascular O$_2^-$ production (12, 15, 17). In rat and mouse blood vessels, NAD(P)H oxidase increases with age thereby augmenting O$_2^-$ production, decreasing the bioavailability of NO (1, 11, 12, 14, 17, 23, 36), increasing cardiac oxygen demand (1), and promoting vascular inflammation (11). Our findings that in NMR tissues, O$_2^-$ generation (Fig. 5) and expression of the gp91phox subunit of NAD(P)H oxidase (Fig. 6A) did not increase with age raises the possibility that this longer-living species may be protected against harmful factors, such as increased pro-inflammatory cytokine production that activate NAD(P)H oxidase in shorter-living species. We plan to address this question in future studies.

Cellular mitochondrial biogenesis is regulated by a NO-dependent mechanism (28), and there is increasing evidence for an age-dependent dysregulation of mitochondrial protein expression and a decline in mitochondrial mass in various organs of short-lived rodents. The findings that in aged NMRs mitochondrial mass (assessed by porin content) and expression of electron transport chain components (Fig. 7) are maintained support the view that preserved NO bioavailability may contribute to the preservation of a youthful cellular phenotype in longer-living species. Recent data suggest that mitochondria are a major source of vascular H$_2$O$_2$ production (20, 24), which significantly contribute to increased H$_2$O$_2$ levels in aged rat arteries (Csiszár and Ungvari, unpublished observation, 2006). Studies are currently underway to characterize mitochondrial H$_2$O$_2$ generation in NMR tissues.

Oxidative stress-induced cellular apoptotic pathways and concomitant endothelial cell injury have been implicated in the initial phases of coronary artery disease (8). Studies by this and other laboratories revealed that advancing age in rats promotes apoptotic cell death (at least in part due to an impaired bioavailability of anti-apoptotic NO) in various tissues, including peripheral arteries (Fig. 8) (14) and the heart (21, 29, 34). Interspecies differences in this regard between rats and NMRs reveal that retardation of apoptotic cellular pathways may play a pivotal role in the prolonged longevity of NMRs. Age-related increases in vascular apoptotic cell death are delayed in NMRs (Fig. 8, A and C). This difference is most pronounced when 2-yr-old rats and NMRs are compared. Indeed it appears that the time course for age-related changes in apoptotic pathways correlated better with MLSP than with chronological age and follow a similar relative time course in both rats and NMRs when expressed as a function of MLSP (Fig. 8, B and D). It is presently unclear why cells of NMRs are more protected against oxidative insults, remain viable, and resist apoptosis than those of other rodents (Mele and Buffenstein, unpublished data). We have previously shown that vascular cells of young long-lived Damara mole rats and NMRs exhibit a marked resistance to oxidative stress-induced cell death (22). Higher NO bioavailability due to the preserved expression of eNOS (Fig. 4A) and retarded development of oxidative stress during aging (Fig. 5) coupled with a preserved cellular resistance against oxidative stress in aged NMRs (Csiszár and Ungvari, unpublished data, 2006) are likely to protect against an increased rate of apoptosis. Interestingly, NMR dermal-derived fibroblasts show greater resistance in vitro to a wide range of cytotoxic challenges than shorter-living rodents (Mele and Buffenstein, unpublished data; Salmon et al. unpublished data). Similarly skin-derived fibroblasts of other longer-lived species as well as mutant mouse models of extended longevity (such the Ames and Snell dwarf mice) are more resistant than shorter-living rodent species to some but not all oxidative stressors (25, 27, 32). Surprisingly, other mouse models of extended longevity (caloric and methionine-restricted dietary treatments) do not show such resistance to oxidative stress (19). The mechanisms underlying the increased oxidative
stress resistance in long-living animals are, to date, not completely understood.

One would expect that long-lived animals have an efficient antioxidant defense and thus would accrue less oxidative damage. However, recent studies revealed that this is not the case in NMRs (2–4). NMR antioxidant defenses are not superior (3, 6), and indeed activities of most antioxidants are similar to those of mice. NMRs expression of these major antioxidant enzymes, however, is preserved during aging (Fig. 6, B–E; 3). In mice, on the other hand, Mn-SOD activity in the liver and other organs increases with age, whereas the activity of catalase and cGPx declines (3).

Surprisingly, NMR tissues, even from young animals, have high levels of lipid peroxidation, protein carbonyls, and DNA damage, although age-related increases in damage accrual are attenuated (2). Despite higher rates of damage even from an early age than that observed in mice, NMRs continue to thrive for an additional 26 yr, whereas young mice have less than 3 yr of life left. We are currently testing the hypothesis that cells of NMRs are extremely tolerant to oxidative and other types of damage because they possess very effective repair mechanisms. Preliminary data for DNA repair in response to gamma radiation support this premise (Buffenstein, Podlutsky, and Austad, unpublished data, 2006).

Limitations of the study. In this study we used the oldest available NMRs to study age-related vascular alterations. We do not have functional data on senescent NMRs approaching their maximal lifespan. Thus it is yet unknown whether this species will eventually develop cardiovascular pathophysiology that is similar to that found in rats. NMR and rat arteries differ in their size, and it can be a challenge to vascular biologists to determine whether vessels are comparable by function/location or by size when comparing animals that differ substantially in body mass. Because we assessed age-related functional changes in vessels from corresponding anatomical location from both species, it was possible to compare the rate of vascular aging irrespective of body-size dependent morphological differences. Nevertheless, it would be definitely interesting to determine whether NMR vessels from other organs (e.g., coronary arteries) or from the level of the microcirculation also exhibit resistance to aging-related functional deterioration. Future studies should also test the hypothesis that long-lived animals are resistant to cardiovascular diseases, of which their development is facilitated by oxidative stress using established disease models (including streptozotocin-induced diabetes and doxorubicin-induced heart failure).

In conclusion, long-living NMRs can maintain a youthful vascular function longer and are better protected against aging-induced oxidative stress and apoptotic cell death than shorter-living rodents (Fig. 8E). It is likely that the resulting slower rate of cardiovascular aging contributes to the exceptional longevity of this species.
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