Germ cell nests in adult ovaries and an unusually large ovarian reserve in the naked mole-rat

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Abstract

The naked mole-rat (NMR, *Heterocephalus glaber*) is renowned for its eusociality and exceptionally long lifespan (> 30 y) relative to its small body size (35–40 g). A NMR phenomenon that has received far less attention is that females show no decline in fertility or fecundity into their third decade of life. The age of onset of reproductive decline in many mammalian species is closely associated with the number of germ cells remaining at the age of sexual maturity. We quantified ovarian reserve size in NMRs at the youngest age (6 months) when subordinate females can begin to ovulate after removal from the queen's suppression. We then compared the NMR ovarian reserve size to values for 19 other mammalian species that were previously reported. The NMR ovarian reserve at 6 months of age is exceptionally large at $108,588 \pm 69,890$ primordial follicles, which is more than 10-fold larger than in mammals of a comparable size. We also observed germ cell nests in ovaries from 6-month-old NMRs, which is highly unusual since breakdown of germ cell nests and the formation of primordial follicles is generally complete by early postnatal life in other mammals. Additionally, we found germ cell nests in young adult NMRs between 1.25 and 3.75 years of age, in both reproductively activated and suppressed females. The unusually large NMR ovarian reserve provides one mechanism to account for this species' protracted fertility. Whether germ cell nests in adult ovaries contribute to the NMR's long reproductive lifespan remains to be determined.

Reproduction (2021) **161** 89–98

Introduction

Reproductive lifespan in female mammals, both within and among species, is largely dependent on the size of the ovarian reserve of germ cells at the age when individuals become sexually mature (Gosden *et al.* 1983, Gosden & Telfer 1987). Thereafter, the rates of germ cell attrition and reduced oocyte quality, as well as aging-associated decline in hypothalamic-pituitary function, are additional factors that influence the timing of reproductive senescence (reviewed in Gore *et al.* 2015). Events that occur long before pubertal onset can have major impacts on the size of the ovarian reserve at sexual maturity. For example, the number of primordial germ cells (PGCs) that successfully migrate from the embryonic epiblast to the genital ridges during early embryonic development has an effect on the ovarian reserve, as does PGC mitotic activity during migration (Funkuda 1976, Findlay *et al.* 2015, Gomes Fernandes *et al.* 2018). Continued mitotic activity of PGCs and oogonia within the developing ovary similarly contribute

to the maximum number of female germ cells, which is typically achieved during fetal life – embryonic days 13.5–15.5 (E13.5–E15.5) in mice (Myers *et al.* 2014). In humans, during the ninth week post-fertilization, proliferating PGCs begin to differentiate into oogonia, which have higher mitotic activity than PGCs. This activity continues to 20 weeks of gestation (Motta *et al.* 1997, Pereda *et al.* 2006, Mamsen *et al.* 2011). As female germ cells transition from mitotic activity to the initiation of meiosis and formation of germ cell nests, the first major wave of germ cell apoptosis gets underway (Coucounanis *et al.* 1993). This occurs at E13.5–E15.5 in mice and at 10–12 weeks post-fertilization in humans.

A second and more pronounced wave of germ cell loss occurs when nests break down and individual primordial follicles (PFs) form (Pepling 2006, Rodrigues *et al.* 2009). The formation of PFs, which are composed of an oocyte surrounded by flat pre-granulosa cells, is essentially complete by postnatal 5 (P5) in mice. In humans, PF formation begins at 17–20 weeks gestation and continues until term (Motta & Makabe 1986a,b, Motta

et al. 1997), followed by a 39% decline in the number of PFs between birth and puberty (Wallace & Kelsey 2010). In mice, during the interval between P5 and sexual maturity, approximately half of the non-growing PFs are lost due to atresia and when they transition to growing, primary follicles (Tingen *et al.* 2009). This despite the fact that these growing follicles have no chance of being fertilized and contributing to individual fitness. In the absence of a mature gonadotropin milieu, follicle growth is arrested well before the peri-ovulatory stage, which leads to follicle atresia and continuous declines in the size of the ovarian reserve of PFs (reviewed by Forabosco & Sforza 2007, Monget *et al.* 2012, Grive & Freiman 2015, Ge *et al.* 2019).

The size of the ovarian reserve at sexual maturity shows substantial variability both within females of the same species and among different species of mammals. The intra- and inter-species differences in ovarian reserve size are thought to be major contributors to differences in female reproductive longevity (Finch & Kirkwood 2000). Another critical factor that is likely associated with reproductive longevity is adult lifespan because there is no point in having a decade's worth of eggs if a given species' maximum lifespan is measured in months or a few years. Gosden and Telfer (1987) compared ovarian reserve sizes among 19 species of mammals whose maximum adult lifespans (MALS) covered nearly 100 years, and body weights spanned four orders of magnitude (pipistrelle bat to cattle, refer to Table 1). They noted the numbers of PFs in peri-pubertal

females scaled allometrically with both body weight ($2700 \times M^{0.47}$) and maximum adult lifespan ($820 \times L^{1.58}$). The authors concluded that larger mammals generally live longer than smaller species and that an increased ovarian reserve size in larger species could be an important adaptation for longer adult lifespans (Gosden & Telfer 1987).

The naked mole-rat (NMR, *Heterocephalus glaber*) is an exception to the general rule that small terrestrial mammals have relatively short lifespans (Healy *et al.* 2014). In fact, NMRs are the longest-lived rodent with a maximum lifespan of 35 years, which is five-fold greater than predicted allometrically for a 35–40 g rodent (Edrey *et al.* 2011, Ruby *et al.* 2018). In addition to being renowned for their remarkable longevity, female NMRs show no decline in fertility and fecundity into the third decade of life, and they essentially breed until they die (Buffenstein 2008). Further, NMRs are one of only two eusocial species of mammals (Jarvis & Bennett 1993), with just one reproductively active female (the queen) in subterranean colonies that number in the tens to hundreds of animals (Jarvis 1991). Whereas all other females within a colony are reproductively suppressed by the queen's aggressive behaviors, these subordinate females can become *reproductively activated* when removed from the colony and the queen's suppression. Whilst under the queen's suppression, subordinate females maintain a hypogonadotropic-hypooestrogenic-anovulatory state (Margulis *et al.* 1995, Clarke & Faulkes 1997, Holmes *et al.* 2009, Swift-Gallant *et al.* 2015,

Table 1 List of species with their code numbers, orders and numbers of primordial follicles.

Code No.	Species	Order	No. primordial follicles*	% of follicles growing†	Body mass (kg)§	Maximum lifespan (y)§
1	Bandicoot (<i>Isodon macrourus</i>)†	Marsupialia	12,440	4.9	1.600	6.8
2	Common shrew (<i>Sorex araneus</i>)	Insectivora	3560	–	0.009	3.2
3	Pipistrelle bat (<i>Pipistrellus pipistrellus</i>)†	Chiroptera	3268	7.2	0.005	16.6
4	Greater horseshoe bat (<i>Rhinolophus ferrumequinum</i>)†	Chiroptera	7950	–	0.023	30.5
5	House mouse (<i>Mus musculus</i>)	Rodentia	4270	16.5	0.021	4.0
6	Wood mouse (<i>Apodemus sylvaticus</i>)	Rodentia	3170	3.2	0.023	6.3
7	Bank vole (<i>Clethrionomys glareolus</i>)	Rodentia	4380	5.6	0.021	4.9
8	Field vole (<i>Microtus agrestis</i>)	Rodentia	2858	5.0	0.046	4.8
9	Norway rat (<i>Rattus norvegicus</i>)	Rodentia	5180	–	0.300	3.8
10	Guinea pig (<i>Cavia porcellus</i>)	Rodentia	29,200	–	0.728	12.0
11	European rabbit (<i>Oryctolagus cuniculus</i>)	Lagomorpha	75,120	4.0	1.800	9.0
12	Domestic cat (<i>Felis catus</i>)	Carnivora	74,520	2.1	3.900	30.0
13	Domestic dog (<i>Canis familiaris</i>)	Carnivora	150,380	19.8	40.000	24.0
14	Sheep (<i>Ovis aries</i>)	Artiodactyla	105,450	0.7	80.000	22.8
15	Swine (<i>Sus scrofa</i>)	Artiodactyla	420,000	–	130.000	27.0
16	Cattle (<i>Bos taurus</i>)	Artiodactyla	210,000	–	750.000	20.0
17	Common marmoset (<i>Callithrix jacchus</i>)†	Primates	17,220	10.9	0.255	22.8
18	Rhesus monkey (<i>Macaca mulatta</i>)	Primates	100,000	16.1	8.235	40.0
19	Human (<i>Homo sapiens</i>)	Primates	302,000	4.0	62.035	122.5
NMR	Naked mole-rat (<i>Heterocephalus glaber</i>)	Rodentia	108,588	3.0	0.035	31.0

Adapted from Gosden and Telfer (1987), with the naked mole-rat added. Body mass and maximum lifespan values are from the AnAge database. Bold values are indicative of species for which the data listed in the AnAge database did not meet the inclusion criteria set by Healy *et al.* (2014).

*Mean number per pair of ovaries (Gosden & Telfer 1987); †Fraction (%) of total follicles that are in the growing stages; §Body mass and maximum lifespan values from the AnAge database; *Species excluded by Gosden & Telfer (1987) from their PF x MALS graph.

Faykoo-Martinez *et al.* 2018). Thus, NMRs provide the opportunity to investigate mechanisms critical to reproductive longevity with unprecedented internal biological controls. First, they allow dissociation of the relative importance of longevity and body size for determining the ovarian reserve and, second, they allow dissociation of chronological age and reproductive maturation.

While the histological images of NMR ovaries reported by Kayanja and Jarvis (1971) suggested an unusually large number of primordial follicles, to our knowledge, the size of the ovarian reserve in NMRs has not been previously enumerated or reported. The earliest age at which a subordinate female NMR can be reproductively activated is 6 months (Buffenstein *et al.* 2012). As such, we counted the ovarian reserve of PFs in NMRs of this age in order to make comparisons with the 19 mammalian species examined by Gosden and Telfer (1987), who evaluated ovaries from peripubertal females. Additionally, in the process of evaluating ovaries from 6-month-old NMRs, we noted the presence of germ cell nests. Lei and Spradling (2013) defined a germ cell nest as 'germ cells that clump morphologically; the interconnected nature of such cells cannot be determined from morphological observation', and their definition applies to our observations in NMRs. Breakdown of germ cell nests is typically complete long before 6 months of age in other mammalian species including mouse and human (Pepling 2006, Hartshorne *et al.* 2009, Findlay *et al.* 2015), and nests are absent in adult ovaries. Having observed germ cell nests in ovaries from 6-month-old NMRs, we interrogated ovaries from young adult females (1.25–3.75 years of age) to determine whether germ cell nests are present in both reproductively suppressed and reproductively activated NMRs. Herein, we show that long lifespan and protracted female fertility are associated with an exceptionally large ovarian reserve in the NMR relative to its small body size, and that germ cell nests are present in ovaries of young adults, regardless of reproductive status.

Methods

Animals

All experimental procedures followed federal and institutional guidelines and were approved by the University of Toronto Animal Care Committee. Naked mole-rat colonies were housed in polycarbonate cages of three sizes (large: 65 cm L × 45 cm W × 23 cm H; medium: 46 cm L × 24 cm W × 15 cm H; small: 30 cm L × 18 cm W × 13 cm H) connected by tubes (25 cm L × 8 cm D) and lined with corn cob bedding. Reproductive activation was achieved by pair housing an adult female with an adult male from a different colony for 4 weeks in a single, medium polycarbonate cage. Naked mole-rats were kept on a 12 h light:12 h darkness cycle at 28–30°C and fed a diet consisting of sweet potato and wet 19% protein mash *ad libitum* (Teklad global diet; Envigo).

Ovary collection and processing

Immediately following euthanasia by isoflurane, ovaries from five 6-month-old NMRs were removed and immersed in 10% buffered formalin overnight. Until the time of euthanasia, these females had been housed with their natal colony, including their queens, and therefore, they were reproductively suppressed. Ovaries were transferred to 70% ethanol and shipped overnight on ice packs to Cornell University. Ovaries were then washed three times in 70% ethanol, embedded in paraffin and serial sectioned at 6 µm. Every tenth section from one ovary per animal was mounted on glass slides and stained with hematoxylin and eosin (H&E). Imaging and counting methods are described subsequently.

For young adult NMRs, ovary halves from four sets of age-matched subordinate and reproductively activated females were analyzed ($n=4$ each). These females ranged in age from 1.25 to 3.75 years. The maximum age difference between age-matched subordinate and reproductively activated females was 3 months. These young adult females were part of a larger, neurobiology study (Faykoo-Martinez *et al.* 2018), and their ovaries were dissected and processed in a different manner than the 6-month-old NMRs. Ovaries were dissected, cut in half with a scalpel and immersed in 4% paraformaldehyde for 1–2 h and then transferred to PBS-0.1% Triton X-100 and stored at 4°C until shipped. One half of an ovary was dedicated to this study, and upon delivery, ovary halves were washed three times in 70% ethanol, embedded in paraffin and serial sectioned at 6 µm. Every tenth section was mounted on glass slides and H&E stained. Every tenth section from each of eight animals was viewed with a Leica DM 2500 optical microscope and digital images of germ cell nests were obtained with a Leica DFC295 camera (Leica Biosystems). The examination of young adult ovaries for germ cell nests was qualitative rather than quantitative because only approximately one half of each ovary was available for study.

Germ cell counting

For ovaries from 6-month-old NMRs, digital images of every tenth H&E stained section were prepared with an Aperio CSO ScanScope (Leica Biosystems) and downloaded to a tablet (Surface Pro, Microsoft, Redmond, WA). The image processing package Fiji for ImageJ (Schindelin *et al.* 2012) was used to count PFs, germ cells within nests, and growing follicles by ticking off these objects with a stylus. To avoid double counting across sections, only germ cells for which the nucleus was visible were counted. Following the methods of Gosden and Telfer (1987), the total numbers of PFs per animal were estimated by multiplying the average raw counts from one ovary by two, and then by both the sampling frequency (10) and a correction factor as described by Abercrombie (1946). The equation for the correction factor is: (section thickness (6 µm) ÷ (section thickness (6 µm) + average germ cell nucleus diameter (18.1 µm)) = 0.25). We measured oocyte nuclear diameters in 10 representative PFs from each animal using Fiji to calculate the average diameter of 18.1 µm.

To calculate the predicted size of the NMR ovarian reserve based on their body weight, we first used the power function reported by Gosden and Telfer (1987) for 19 species of

mammals. We also calculated the predicted size of the NMR ovarian reserve based on our value for MALS in the NMR and using the Gosden and Telfer power function, which excluded the bandicoot, pipistrelle and greater horseshoe bats, and common marmoset. Because Gosden and Telfer (1987) did not include the raw data that they used to generate their power functions involving body weight and MALS, we searched the AnAge database for the body masses and maximum lifespans (MaxLS) of the same 19 mammalian species, plus the NMR (<https://genomics.senescence.info/species/>; accessed 4 May 2020). We used the AnAge values to produce linear log-log regression lines and power functions ($Y = aX^b$) for the numbers of PFs in the 19 species (JMP, SAS, Cary, NC). We similarly generated power functions after excluding the four species that Gosden and Telfer (1987) excluded from their analysis of PF $\times$ MALS to determine how the predicted numbers of PFs in NMRs compared with the results derived from the AnAge values. We also generated power functions after limiting our analysis to the 10 Gosden and Telfer species that had high-quality AnAge data for body mass and MaxLS, using the parameters for data quality set forth by Healy *et al.* (2014). Their exclusion criteria for MaxLS included those species with less than 10 longevity records, or with low or questionable data quality as defined by the AnAge database (de Magalhães *et al.* 2007, de Magalhães & Costa 2009). Healy *et al.* (2014) included the NMR in their report of lifespan variation in birds and mammals, and we entered the AnAge values for NMR body mass and MaxLS into all of the power functions that we generated using the AnAge database.

Immunofluorescent imaging of germ cell nests

To enhance the visualization of germ cell nests in ovaries from 6-month-old and young adult females, some of the remaining sections underwent immunofluorescence for the germ cell marker DDX4 (a.k.a. mouse vasa homolog (MVH)) and YBOX2 (Y box protein 2, a.k.a. (MSY2), which is present exclusively in diplotene-stage and mature oocytes (Gu *et al.* 1998). Slides were deparaffinized and rehydrated with three washes of Safeclear (Fischer Scientific) for 10 min each, followed by three washes of each concentration in a graded series of ethanol (100, 95, 80, 70%) for 5 min. After twice rinsing the slides for 5 min in distilled water, the slides were incubated in 10 mM sodium citrate (pH 6.0) for 40 min at 95°C. Permeabilization was performed in 0.2% Triton-X 100 in PBS for 1 h. Sections were blocked for 4 h in blocking solution (2.52 mg/mL glycine, 10% goat serum, 3% BSA, 0.2% Tween20) and then incubated overnight at room temperature (RT) in 1:100 dilution of rabbit anti-DDX4 or anti-YBOX2 polyclonal antibody (Abcam 13840; Sigma SAB4502102, respectively) in blocking solution. After two 10-minute washes with 0.1% Tween20 in PBS (PBS-Tw20), the slides were incubated in goat anti-rabbit secondary antibody AlexaFluor®594 and goat anti-rabbit AlexaFluor 488 (Jackson ImmunoResearch) for 2 h at RT. The slides were twice rinsed with PBS-Tw20 for 5 min, counterstained with DAPI and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Sections containing germ cell nests were imaged with a Zeiss Axiolmager M2 microscope and Axiocam 506, and the images were processed using Zen 2 (Carl Zeiss AG).

Results

Six-month-old NMRs had an average ($\pm$ s.d.) of 108,588 ($\pm$ 69,890) PFs (Table 1), and oocytes within nests accounted for 1.4% ($\pm$ 0.4%) of the non-growing germ cells. Growing follicles accounted for 3% ($\pm$ 1.1%) of the total number of follicles, which is the lowest fraction of growing follicles amongst the four Rodentia species for which Gosden and Telfer (1987) enumerated the fractions of growing follicles (Table 1). Based on an average body mass of 35 g, the NMR has an exceptionally large ovarian reserve as compared to other similarly sized mammals (Fig. 1A). In comparison to the seven other species weighing less than 100 g that were evaluated by Gosden and Telfer (1987), the size of the ovarian reserve in NMRs is more than an order of magnitude greater than the species that was closest in body weight to the NMR, that is, the greater horseshoe bat (*Rhinolophus ferrumequinum*, Fig. 1A). Whereas the guinea pig (*Cavia porcellus*, #10 in Fig. 1A) has the largest ovarian reserve (29,200) among the five other rodent species, its body weight is an order of magnitude greater than the NMR.

We calculated the predicted ovarian reserve size for 6-month-old NMRs to be 5,703 PFs based on body mass, using the equation reported by Gosden and Telfer (1987), which is $PF = 27,700 \times M^{0.47}$. We used body mass data from the AnAge database to produce power functions for PFs and body mass, and either included all 19 species (Fig. 2A) or excluded nine species based on the exclusion criteria used by Healy *et al.* (2014) (Table 2). The different power functions yielded a relatively narrow range of results (5617–6420 PFs) for the predicted size of the NMR ovarian reserve, which is 16.9- to 21.4-fold less than our result (108,588).

Owing to its eusociality, the age of onset of adulthood in NMRs can be somewhat arbitrary, since all females within a colony, save for the queen, will be reproductively inactive. Whereas 6 months is the youngest age at which female NMRs can be reproductively activated (Buffenstein *et al.* 2012), not all animals have reached adult body size and the onsets of vaginal patency and ovulatory activity upon removal from the queen's suppression are highly variable between 6 and 12 months of age (Holmes, unpublished observations). Conversely, growth has largely plateaued and onset of reproductive activation is less variable in females at 1 year of age (Swift-Gallant *et al.* 2015, Faykoo-Martinez *et al.* 2018), and therefore, for the purpose of determining MALS we have assigned this age as the onset of adulthood regardless of reproductive state, that is, suppressed or activated. Following Gosden and Telfer (1987), we calculated MALS for the NMR to be 34 years, based on the difference between their maximum longevity (35 years) and the youngest age at which we considered them to be adults (1 year). Upon plotting the values for the NMR on the Gosden and Telfer graph for PF numbers vs MALS (Fig. 1B), we find

Figure 1 (A) Variation with body weight (kg) in the numbers of primordial follicles per species. (B) Variation with the length of the maximum adult lifespan in the numbers of primordial follicles per species. The linear regressions with 95% CIs and the allometric formulas do not include the naked mole-rat data points. Refer to Table 1 for the list of species codes. Adapted with permission from Gosden and Telfer (1987).

the data point for the NMR lies within the 95% CI that was based on results from 15 other mammalian species. We calculated the predicted ovarian reserve size for 6-month-old NMRs to be 215,551 PFs based on MALS using the power function reported by Gosden and Telfer (1987), which is $PF = 820 \times L^{1.58}$. Our estimate of MALS for the NMR (34 years) lies closest to the rhesus monkey (*Macaca mulatta*, #18 in Fig. 1B), which had an ovarian reserve size of 100,000 PFs and a MALS of 35 years.

We used maximum lifespan (MaxLS) values from the AnAge database, and either included all 19 species (Fig. 2B) or excluded certain species based on the exclusion criteria used by Gosden and Telfer (1987) and by Healy et al. (2014), to produce power functions for PFs and MaxLS (Table 3). The different power functions yielded a

Figure 2 (A) Variation with body weight (g) in the numbers of primordial follicles per species. (B) Variation with the length of the maximum lifespan (MaxLS) in the numbers of primordial follicles per species. Values for body weight and MaxLS are from the AnAge database. The linear regressions with 95% CIs and the allometric formulas do not include the naked mole-rat data points. Refer to Table 1 for the list of species codes.

broad range of results for the predicted size of the NMR ovarian reserve (51,991–215,551 PFs). When the lifespan data for the pipistrelle and greater horseshoe bats were excluded, the predicted NMR ovarian reserve size was greater than we measured. Conversely, when bat lifespan data were included, the predicted NMR ovarian reserve size was less than we measured (Table 3).

Primordial follicles accounted for the vast majority of the ovarian reserves of the 6-month-old NMRs, but germ cell nests can be seen scattered throughout the ovaries (Fig. 3). Generally, each nest contained approximately four germ cells, with nine being the maximum number of germ cells in a single nest. For the young adult NMRs, three of four reproductively suppressed and two of four reproductively activated females had germ cell nests

Table 2 Predicted size of the ovarian reserve in the naked mole-rat (NMR) using the power function from [Gosden & Telfer \(1987\)](#) for body mass (BM). The power functions for which values from the AnAge Database were used include the same 19 species reported by Gosden & Telfer (1987), as well as power functions when the same four species were excluded. Additional power functions were based on the 10 species in common between the Gosden & Telfer (1987) and Healy *et al.* (2014) studies. The NMR value for body mass (BM; 35 g) is from the AnAge Database.

Source	BM (kg)	Formula	Predicted	Actual	Fold difference
Gosden & Telfer (1987)	0.035	$27,700 \times M^{0.47}$	5703	108,588	↑ 19.0
AnAge Database	0.035				
19 species		$25,347 \times M^{0.45}$	5617	108,588	↑ 19.3
15 species [†]		$24,789 \times M^{0.47}$	5082	108,588	↑ 21.4
10 species [§]		$29,424 \times M^{0.45}$	6420	108,588	↑ 16.9

*Excluded the same four species as [Gosden and Telfer \(1987\)](#), that is, bandicoot, pipistrelle and greater horseshoe bats, and common marmoset.

§Limited to the 10 species' values from the AnAge database that met the inclusion criteria set by [Healy *et al.* \(2014\)](#).

within their ovaries ([Fig. 4](#)). Germ cell nests were present across the age range examined, that is, in females that were 1.25 and 3.75 years of age at the time of collection. In both 6-month-old and young adult females, the germ cells within nests labeled positive for the germ cell marker DDX4 and the oocyte marker MSY2 by immunofluorescence ([Figs 3 and 4](#)). Follicle growth had advanced further in the activated than in the suppressed females, and only reproductively activated females showed signs that they had ovulated, that is, presence of corpora lutea and/or corpora albicans (not shown).

Discussion

In this study, we have determined that the NMR has an exceptionally large ovarian reserve relative to other mammalian species of similar body weight. This is likely to be one of the means by which female NMRs are able to remain fertile and fecund into their third decade of life. However, it remains to be definitively determined if the large ovarian reserve in NMRs at 6 months of age is due to: (1) enhanced mitotic activity of PGCs and oogonia during pre- and/or postnatal life, and/or (2) reduced germ cell atresia during the mitosis-meiosis transition and during nest breakdown/PF formation. Whereas neo-oogenesis in postnatal and adult mammals is a contentious issue in the field of reproductive biology ([Johnson *et al.* 2004](#), [Begum *et al.* 2008](#), [Tilly *et al.* 2009](#), [Albertini & Gleicher 2015](#),

[Horan & Williams 2017](#), [Wang *et al.* 2017](#)), the NMR's large ovarian reserve and protracted fertility warrant consideration for neo-oogenesis in this species. Indeed, the unusual finding of germ cell nests in NMRs that are years old would be consistent with neo-oogenesis, but not proof of it. From the morphological appearance of the germ cell nests in NMR ovaries, we are unable to determine at this time whether the structures formed relatively recently or at a much earlier age, including prenatally, and failed to breakdown and form PFs. Also, it remains to be determined whether the nests in adult NMR ovaries would subsequently breakdown and form PFs that have the capacity to activate, grow, complete meiosis, ovulate, fertilize and produce viable offspring. Advances in folliculogenesis have made it possible to activate PFs and complete the growth and maturation process *in vitro* ([Eppig & O'Brien 1996](#), [Cortvriendt & Smitz 2002](#)), and therefore, it might be possible to isolate germ cell nests from adult NMR ovaries and manipulate them in culture to determine whether they form PFs and complete all of the steps necessary for fertilization and the production of viable offspring.

[Lei & Spradling \(2013\)](#) defined a germline cyst as 'a cluster of interconnected germ cells generated by mitotic divisions with incomplete cytokinesis', and whether adult NMR ovaries contain germline cysts remains to be determined. In the current study, we have classified clusters of germ cells within NMR ovaries as nests based on their morphology and immunoreactivity

Table 3 Predicted size of the ovarian reserve in the naked mole-rat (NMR) using the power function from [Gosden and Telfer \(1987\)](#) for maximum adult lifespan (MALS; 34 years). Naked mole-rat value for MALS is based on our own determination, as described in the text. Gosden & Telfer (1987) excluded four of 19 species for their MALS formula. The power functions for which values from the AnAge Database were used include the same 19 species reported by Gosden & Telfer (1987), as well as power functions when the same four species were excluded. Additional power functions were based on the 10 species in common between the Gosden & Telfer (1987) and Healy *et al.* (2014) studies. The NMR value for maximum lifespan (MaxLS; 31 years) is from the AnAge Database.

Source	Lifespan, years	Formula	Predicted	Actual	Fold difference
Gosden & Telfer (1987)	34*	$820 \times L^{1.58}$	215,551	108,588	↓ (2.0)
AnAge Database	31 [†]				
19 species		$712 \times L^{1.35}$	72,956	108,588	↑ 1.5
15 species [†]		$592 \times L^{1.56}$	124,622	108,588	↓ (1.1)
10 species [§]		$416 \times L^{1.41}$	51,991	108,588	↑ 2.1

*MALS; [†]MaxLS; [‡]Excluded the same four species as [Gosden & Telfer \(1987\)](#), that is, bandicoot, pipistrelle and greater horseshoe bats, and common marmoset; [§]Limited to the 10 species' values from the AnAge database that met the inclusion criteria set by [Healy *et al.* \(2014\)](#).

Figure 3 Representative H&E photomicrographs (A and C) of an ovary from a 6-month-old NMR. Arrows point to germ cell nests. (B and D) Representative immunofluorescent images (DDX4 red, MSY2 green, DAPI nuclear stain blue) of germ cell nests. Images in (A) and (B) are from adjacent sections from one region of an ovary, and images in (C) and (D) are from adjacent sections from a different region of an ovary. Arrows within the DAPI only panels point to the corresponding nuclei of the oocytes within the nests.

for MSY2, which is present exclusively in diplotene-stage and mature oocytes (Gu *et al.* 1998). As part of a separate study, we attempted to identify interconnected germ cells in embryonic and neonatal NMR ovaries by immunofluorescence for the bridge protein TEX14, but this was unsuccessful because the antibody for TEX14 (Abcam ab41733, Cambridge, MA) did not work in this species. TEX14 is present in intercellular bridges within mouse germline cysts before E14.5 (Greenbaum *et al.* 2009). If we are able to identify intercellular bridges in embryonic NMR ovaries using different anti-TEX14 antibodies or by electron microscopy, we will then expand our search to include adult ovaries.

Whereas the size of the NMR ovarian reserve appears to be exceptionally large relative to its body weight (Fig. 1A), the size of the ovarian reserve is unremarkable relative to its adult longevity (Fig. 1B). However, the size of the NMR ovarian reserve might actually be relatively small in relation to its MALS, if the NMR's protracted fertility is taken into consideration. Peri-pubertal NMRs and rhesus monkeys have similar ovarian reserve sizes and MALS, but unlike female rhesus monkeys, female NMRs show no aging-associated decline in fertility and fecundity (Buffenstein 2008). Conversely, captive rhesus monkeys produce offspring well into their twenties, but thereafter they cease to be reproductively active, which results in a post-reproductive lifespan that could last another decade (Gagliardi *et al.* 2007).

Gosden & Telfer (1987) did not indicate if the values used for MALS were for mammals in their natural environments or in captivity, however, many of the species in their study are domesticated (e.g. cat, dog, sheep, swine, cattle), and therefore, we assumed the MALS values were based on animals that had been maintained in captivity. We made the same assumption for the NMR and set its MALS to 34 years. Gosden and

Telfer (1987) excluded the bandicoot, marmoset, and the pipistrelle and greater horseshoe bats from their PF vs MALS figure, and they stated, 'In most species, there was sufficient reliable data for estimating the maximum adult lifespan by subtracting the characteristic pubertal age from total longevity'. Presumably, reliable MALS data were not available for these four species at the time of publication. Interestingly, we now know that the pipistrelle and greater horseshoe bats are relatively long-lived as compared to rodents and insectivores of similar size (Healy *et al.* 2014), and it is apparent that the bats' PF values lie far to the right of the regression line in Fig. 2B. Gosden & Telfer (1987) speculated that 'these species may depend on parsimonious utilization of follicles in order to prevent premature loss of fecundity'. Whether the NMR has an ovarian reserve that is relatively large or small for its long lifespan appears to depend on which species are included in the allometric analysis, in particular bats (Table 3), and whether lifespans are based on captive or wild data. If it proves to be correct that the NMR has a small ovarian reserve relative to its exceptionally long reproductive lifespan, then this species should also be considered for parsimonious utilization of their germ cells, especially if they do not demonstrate adult neo-oogenesis. Whereas the growing fraction of total follicles was relatively small at 3% in NMRs (Table 1), this does not necessarily support parsimonious utilization of follicles in this species. Gosden & Telfer (1987) noted from their data that the 'fraction of growing follicles are far too variable to be useful indicators of the rate of depletion of the follicle store'. The remarkably large number of non-growing PFs in NMR ovaries is likely a major contributor to the low fraction of growing follicles in this species.

There are several different methods for estimating the size of the ovarian reserves in mammals (Tilly 2003),

Figure 4 Representative H&E photomicrographs of ovaries from (A and B) a 2-year, 10-month-old reproductively suppressed female, and (C and D) a 2-year, 7-month-old reproductively activated female. Arrows point to germ cell nests. (E and F) Representative immunofluorescent images (DDX4 red, MSY2 green, DAPI nuclear stain blue) of a germ cell nest in an ovary from a 3-year, 8-month-old reproductively suppressed female. Arrows within the DAPI only panels point to the corresponding nuclei of the oocytes within the nest.

thus, we attempted to follow the method of [Gosden & Telfer \(1987\)](#) as closely as possible in order to make accurate comparisons among NMRs and the 19 species of mammals that they included in their study. This is not to say that this method is necessarily the optimum way to estimate ovarian reserve size, especially in light of recently developed methods that evaluate whole ovaries rather than ovarian sections of varying thicknesses and sampling frequencies ([Faire et al. 2015](#), [Malki et al. 2015](#), [Rinaldi et al. 2018](#), [McKey et al. 2020](#)). These so-called ‘whole-mount’ immunofluorescent approaches could prove to be more accurate than serial sectioning, though it might be an impractical approach for some species due to ovarian size, consistency, and availability of antibodies with adequate tissue penetration and cross-reactivity. Thus far, we have had limited success with NMR ovaries in this regard. Nevertheless, no matter what formula is used to calculate the ovarian reserve size

based on counts from serially sectioned NMR ovaries, the raw counts for 6-month-old females from this study ($21,805 \pm 14,034$ PFs per ovary) leave no doubt that this species has an abundance of non-growing germ cells for its body size. In addition to using the [Gosden & Telfer \(1987\)](#) formula for estimating ovarian reserve size from serial-section counts, we also calculated the size of the NMR ovarian reserve using another method described by [Bristol-Gould et al. \(2006a\)](#). Their method yielded a result for the NMR ($112,332 \pm 71,904$) that differed from the Gosden and Telfer method by only 3.7%. [Bristol-Gould et al. \(2006b\)](#) estimated the ovarian reserve size in 6-month-old CD1 mice to be 1487 ± 109 PFs per animal, a value that is 75 times less than in NMRs of the same chronological age. And whereas 6-month-old mice will soon begin to show signs of reproductive aging within a matter of months ([Chiang et al. 2010](#), [Hirshfeld-Cytron et al. 2011](#), [Briley et al. 2016](#)), female NMRs will have their entire decades-long reproductive lifespan ahead of them when they reach this age. The one critically important caveat being that almost all female NMRs will never realize their vast reproductive potential because their queen will prevent them from doing so.

In conclusion, we can now add an exceptionally large ovarian reserve and the presence of germ cell nests in adult ovaries to the NMR’s extensive list of remarkable adaptations, which include an unusually long lifespan, tolerance to prolonged anoxia, and near-complete cancer resistance ([Tian et al. 2013](#), [Park et al. 2017](#), [Ruby et al. 2018](#)). Certainly, the large ovarian reserve relative to its small body size is one mechanism that can contribute to protracted fertility in long-lived NMRs, but it remains to be determined whether parsimonious germ cell usage and/or adult neo-oogenesis are also contributing factors. Female reproductive lifespans are comparable in NMRs and humans, which are measured in decades in both species. Therefore, elucidating the means by which NMR females escape aging-associated declines in fertility and fecundity might lead to the development of interventions that could preserve fertility as women age.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was funded by NSERC Discovery Grant RGPIN 2018-04780, NSERC Discovery Accelerator Supplement RGPAS 2018-522465, and an Ontario Early Researcher Award to M M H, and NSERC PGS D Scholarship to M F-M. M A B-E was supported by the Eunice Kennedy Shriver National Institute of

Child Health & Human Development of the National Institutes of Health under Award Number R00HD090289 to M A B-E and P50HD096723 Pilot Project.

Author contribution statement

N J P conceived the study, analyzed data, wrote the initial and final drafts of the manuscript. A M P performed H&E histology, enumerated germ cells, reviewed and edited the manuscript. M F-M collected NMR ovaries, processed and shipped them for histology, reviewed and edited the manuscript. M A B-E performed immunofluorescence histology, microscopy and imaging, prepared figures, reviewed and edited the manuscript. D F A conceived the study, reviewed and edited the manuscript. M M H conceived the study, provided NMR ovaries, reviewed and edited the manuscript.

Acknowledgements

The authors thank the Cornell University Histology/Cytology Core Facility for the preparation of paraffin blocks of naked mole-rat ovaries, and Roger Gosden and Evelyn Telfer for their original work on the numbers of primordial follicles in 19 species of mammals.

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Received 1 June 2020

First decision 30 June 2020

Revised Manuscript received 29 September 2020

Accepted 5 November 2020