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The scent of senescence: sexual signalling and female preference in house mice

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Abstract

Sexual signals are expected to be costly to produce and maintain, thus ensuring that only males in good condition can sustain their expression at high levels. When males reach senescence they lose physiological function and condition, which could constrain their ability to invest in costly sexual signals, decreasing their attractiveness to mates. Furthermore, females may have evolved mating preferences that cause avoidance of senesced males to enhance fertilization success and viability of offspring. Among mammals, the size of antlers and other weapons can decrease with senescence, but changes in olfactory sexual signals have been largely unexplored. We examined changes in olfactory signals with senescence in house mice (*Mus musculus domesticus*), where males excrete volatile and involatile molecules in scent marks that elicit behavioural and priming responses in females. Compared to middle-aged males, the urine of senesced males contained a lower concentration of involatile signalling proteins (major urinary proteins or MUPs), and associated volatiles that bind to these proteins. The reduced intensity of male scent will affect the longevity of scent signals deposited in the environment and, accordingly, females were less attracted to urine from senesced males deposited 12 h previously. Females also discriminated against senesced males encountered behind a mesh barrier. These results reveal that investment in olfactory signalling is reduced during senescence and suggest that senesced males and their scent may be less attractive to females.

Introduction

Males of many species produce and maintain costly sexual signals to advertise their phenotypic quality and attract mates. Costs associated with the expression of such traits help to ensure that signalling remains honest (Grafen, 1990), but also mean that investment in sexual signalling can negatively affect other life history components. As a consequence, males are expected to vary their investment in sexual signalling over their lifetime in a way that will maximize reproductive success (Partridge & Endler, 1987). Investment is predicted to be less than maximal in younger age classes over a diverse range of conditions, but increase with age (Kokko, 1997). However, as males reach late ages they suffer from a progressive loss of function, accompanied by a decrease in fertility and an increase in mortality, which defines senescence (Kirkwood & Austad, 2000). Senesced males may thus be physiologically constrained in their ability to produce costly sexual signals.

Changes in sexual signalling with age, whether as a result of strategic signalling effort or an inability to produce costly traits, are likely to influence male attractiveness. Females also may have evolved to select males on the basis of age to increase fertilization success or to be assured of genetic quality. The ability to survive is a testament to viability, such that females that select older over younger mates may be more likely to gain good genes for viability in their offspring (Trivers, 1972;

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Manning, 1985; Kokko & Lindstrom, 1996). In agreement with this theory, females often do prefer older over younger adult males (Zuk, 1988; Wetton *et al.*, 1995; Kempenaers *et al.*, 1997; Komers *et al.*, 1999). However, mating with males that are senesced may incur fitness costs to females and their offspring that outweigh possible viability benefits. For example, senesced males are often the least fertile (Brooks & Kemp, 2001) and may have accumulated more germ-line mutations through their life-time (Pizzari *et al.*, 2008). Thus, in contrast to the preference for middle-aged over younger adult males, females may purposely select against males that are senesced (Jones *et al.*, 2000; Velando *et al.*, 2008) to increase fertilization success or offspring genetic quality (Pizzari *et al.*, 2008; Dean *et al.*, 2010).

Age-dependent sexual selection has been investigated extensively in relation to morphological weapons such as antlers and horns in mammals, where weapon size increases as males reach prime ages and, in some species, decreases during senescence (Mysterud *et al.*, 2005; Vanpe *et al.*, 2007). Investment in rutting, an important behaviour influencing access to mates, can also show a large decline with male senescence (Nussey *et al.*, 2009). In many other mammals sexually dimorphic odours have important roles in sexual selection; these signals are often left in the environment in the form of scent marks (Blaustein, 1981). Changes in olfactory signalling and subsequent female response have been observed between young adult and middle-aged male meadow voles (Ferkin, 1999, 2010) and laboratory mice (Osada *et al.*, 2003, 2008), but the effects of senescence on olfactory signalling have been little explored. As investment in sexual scent signalling may have metabolic costs (Gosling *et al.*, 2000; Radwan *et al.*, 2006; Zala *et al.*, 2008), senesced males may be unable to maintain signalling at the same high level as younger males.

The molecular basis and temporal dynamics of sexual scent signalling are best understood in the house mouse (*M. musculus domesticus*), where male urine scents have a major influence on female attraction and mate choice (Hurst & Beynon, 2004; Hurst, 2009). Males produce a number of androgen-dependent urinary volatiles, including 2-*sec*-butyl-4,5-dihydrothiazole ('thiazole') and 2,3-dehydro-*exo*-brevicomin ('brevicomin'), that stimulate females to sniff male scents (Jemiolo *et al.*, 1985) and bring females into oestrus (Jemiolo *et al.*, 1986; Novotny *et al.*, 1999). These highly volatile scent components are bound to involatile major urinary proteins (MUPs), which are excreted at very high concentrations in adult male mouse urine (Beynon & Hurst, 2004). This binding of volatiles to MUPs substantially slows their release from scent marks, extending the emission of airborne volatile signals over many hours that would otherwise be lost within a few minutes (Hurst *et al.*, 1998). The MUPs themselves convey information, allowing females to recognize scent owners from the individual-specific pattern of MUPs in scent marks (Cheetham *et al.*, 2007), to avoid inbreeding with close kin sharing the same MUP pattern (Sherborne *et al.*, 2007) and to assess male genome-wide heterozygosity (Thom *et al.*, 2008). Darcin, a male-specific MUP expressed by all adult male wild house mice, acts as a sex pheromone that attracts females to spend time near a male's scent and stimulates females to remember and become attracted to the airborne urinary odour of that particular male (Roberts *et al.*, 2010). Darcin also binds and extends the release of one of the most abundant androgen-dependent volatiles in male urine, 2-*sec*-butyl-4,5-dihydrothiazole (Armstrong *et al.*, 2005). The concentration of MUPs and bound ligands is thus likely to have a major effect on the intensity, duration and decay of male urinary signals.

Here we use mice recently derived from the wild and representative of heterozygous mice in wild populations to test whether senescence reduces the concentration of MUPs and volatile ligands in male mouse urine, and whether this reduces the duration over which volatiles remain in male scent marks and their attractiveness to females. We also test whether senescence influences the attractiveness of males themselves, which is critical in understanding how senescence influences male reproductive success.

Methods

Changes in MUP output with senescence

Subjects

Urine samples were collected from a colony of mice that derived from several populations in the North West of England, UK. These mice had been crossed and outbred in captivity for up to six generations. Stocks recently derived from the wild were used in this experiment rather than domesticated laboratory mice as long term selection in the laboratory can substantially alter the expression of sexually selected signals (Cheetham *et al.*, 2009; Roberts *et al.*, 2010). Further, inbred laboratory animals can have much shorter lifespans compared to animals derived from the wild (Harper, 2008), which may influence the degree and onset of senescence. Although mice are a key model organism in ageing research, surprisingly little is known about the ageing of mice in the wild. Reports of mortality in natural populations of mice vary greatly; for example, the average lifespan of a mouse in an island population can be just 100 days, whereas in the open steppe of Russia 22% of mice were reported to have survived to over 21 months old (Berry & Bronson, 1992). To our knowledge no study has documented senescence in wild populations of mice, but the ages of senesced individuals in this experiment correspond to increased mortality rates among mice from two different lines recently derived from separate wild populations and bred in the laboratory (Miller *et al.*, 2002).

To test whether urinary MUP concentration of individual males decreases with senescence, and to measure the rate of decline among adult males of different ages, we collected urine samples from 11 males once a month over a 6-month period. These males were derived from animals outbred in captivity for one to three generations (i.e. the animals in this study were second to fourth generation) and were either middle-aged adults ($n = 6$, age range 12–17 months) or were in the early stages of senescence ($n = 5$, age range 25–26 months) at the start of the 6-month sample period. Senesced and middle-aged males did not differ significantly in the number of generations since their ancestors were collected from the wild (mean generations ± SE for senesced males: 2.6 ± 0.6; middle-aged males: 3.0 ± 0.0; *t*-test for unequal variance: $t = -0.67$, $P = 0.54$). Males were born in enclosures (122 cm × 60 cm × 76 cm), housed singly at 28 days old (48 cm × 11.5 cm × 12 cm), and given equivalent experience of conspecifics through life (occasional olfactory, visual and acoustic contact with females after they were 6 months old).

Under more natural conditions, selective disappearance of males and variation in signalling effort with differing social experience could mask any correlation between MUP concentration and senescence at a population level. Thus, we also examined whether there was a significant correlation between male age and MUP concentration within a group of colony males of different ages (18–33 months, $n = 33$) that varied in their individual lifetime experiences with conspecifics. These males were a random sample of mice from our laboratory that had been used in behavioural experiments that allowed different experience of conspecifics (although none of the males had mated): some males had been involved in competitive interactions with other males and had experience of females, while others had been largely isolated through life. Social experience was randomly distributed across ages and males were derived from animals outbred in captivity for two to five generations. While there was a correlation between age and the number of generations in captivity among these males ($n = 33$, $r_s = -0.83$, $P < 0.001$), number of generations was not correlated with either urinary protein concentration ($n = 33$, $r_s = 0.25$, $P = 0.17$) or protein concentration corrected for dilution ($n = 33$, $r_s = 0.26$, $P = 0.15$). Furthermore, controlling for the number of generations had no impact on the relationships between age and sexual signalling (see results). The males were born in cages (45 cm × 28 cm × 13 cm) then housed singly at 28 days old (48 cm × 11.5 cm × 12 cm). Cages of all males were lined with Corn cob Absorb 10/14 substrate. Mice had paper-wool bedding material and *ad libitum* access to food (Lab diet 5002, International Product Supplies Limited, London, UK) and water. Mice were maintained on a 12:12 h reversed light cycle with white lights off at 09:00. Samples were collected during the dark phase under dim red lighting.

MUP concentration

Urine was collected by holding males by the scruff of the neck over a clean Eppendorf tube and the bladder massaged gently using the tip of the index finger and thumb to stimulate urination; samples were stored immediately at −20 °C. To establish the proportion of urinary protein that consisted of MUPs, urine samples were run on SDS-PAGE gels as described by Laemmli (1970). For each gel, the same volume of urine was added from each male (diluted 1:40 for gels in Fig. 1, 1:50 for Fig. 2) such that the amount of protein added per lane varied between 0.25–3 μg, depending on each male's urinary protein concentration; the volume of MUP bands was quantified using the densitometry program Total Lab Quant (TotalLab Ltd, Keel House, Garth Heads, Newcastle upon Tyne, UK). Total urinary protein concentration was assessed using the Coomassie plus® protein assay reagent kit from Perbio Science UK Ltd (Unit 9, Atley Way, North Nelson Industrial Estate, Cramlington, Northumberland NE231WA) as described by Cheetham *et al.* (2009). To correct for urinary dilution we measured urinary creatinine (Beynon & Hurst, 2004) also using the method of Cheetham *et al.* (2009). However, as creatinine is a by-product of muscle metabolism and muscles become smaller and weaker with age (McArdle *et al.*, 2002), we only used this correction for urine dilution when creatinine did not vary with age.

Epididymal sperm counts

To test whether there was a relationship between MUP concentration and male reproductive senescence, we measured epididymal sperm counts in 13 males aged 21–32 months with varying experience of conspecifics. These males were a random subsample of the individuals that were used to test for a correlation between male age and urinary MUP concentration. In mice, epididymal sperm counts are lower in senesced males when compared to young adults and can correlate with male fertility in old age (Parkening, 1989; Ten *et al.*, 1997). Males were culled humanely then the left epididymis was dissected and macerated for 1 min with a scalpel blade in 0.1 mL of 1% citrate solution. A further 0.9 mL of citrate was added and the mixture was left to stand for a further minute. After mixing, a small amount of the preparation was added to each chamber of an improved Neubauer haemocytometer, which was left to stand for 15 min in a sealed container on moist cotton wool before sperm were counted manually under a microscope.

Changes in volatiles with senescence

We measured the amount of 2-*sec*-butyl-4,5-dihydrothiazole and 2,3-dehydro-*exo*-brevicomin in urine samples from middle-aged ($n = 5$, 15–20 months old) and senesced males ($n = 6$, 28–29 months old) that each had equivalent lifetime experience of conspecifics. These were the same males that were used to assess changes

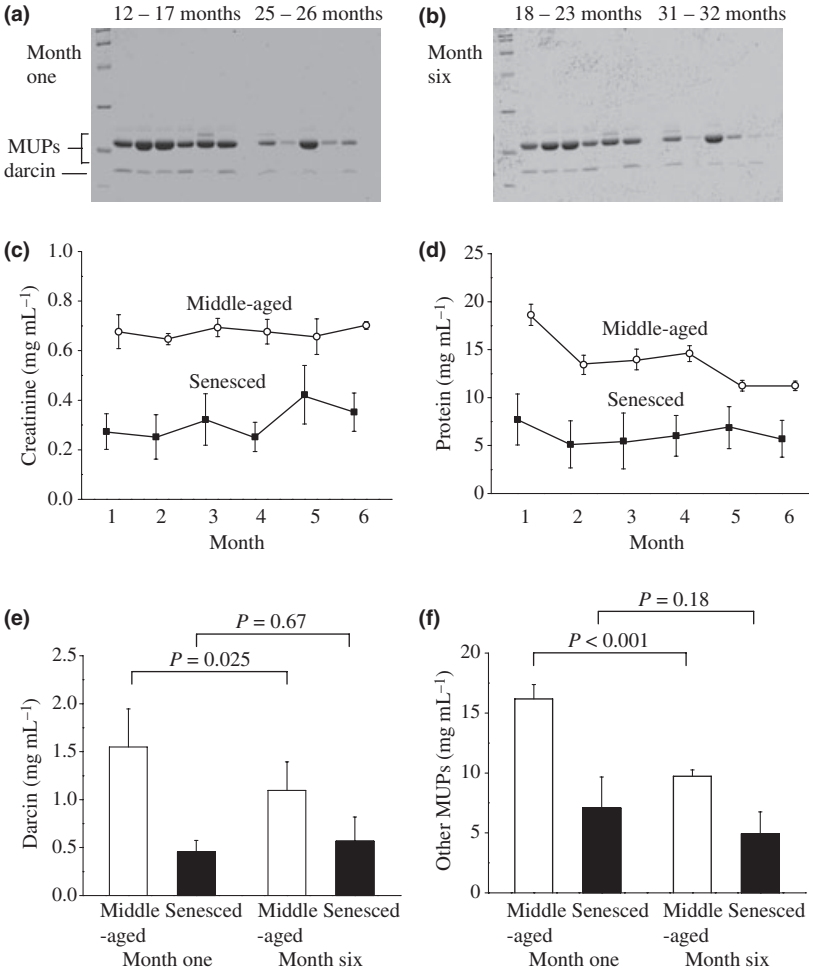

Fig. 1 Decreased urinary MUP concentration in senesced males compared to middle-aged adults. SDS-PAGE confirmed that nearly all urinary protein was MUP in both middle-aged and senesced males sampled at (a) the start and (b) the end of a 6-month period. (c) Senesced males (■) consistently excreted a lower concentration of urinary creatinine than middle-aged males (○) over the 6 month sample period. (d) Urinary protein concentration was greater among middle-aged than senesced males, but this difference became smaller over time as middle-aged males decreased urinary protein concentration. Densitometry of bands on SDS-PAGE confirmed that both (e) darcin and (f) other MUPs were reduced among senesced compared to middle-aged males. *P* values are for separate repeated measures GLMs for males of different age classes.

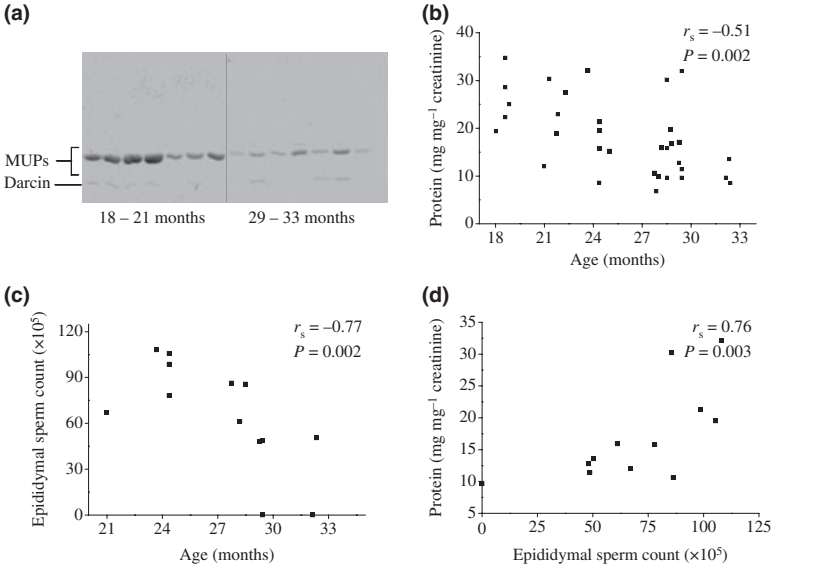

Fig. 2 MUP output gradually decreases with old age, corresponding with reproductive senescence. (a) SDS-PAGE of urine (0.1 μL of urine added from each male) sampled from the upper and lower quartiles of male ages confirmed that most urinary protein was MUP and that MUP output declined among senesced males. (b) Urinary protein (corrected for urine dilution) correlated negatively with male age. (c) The oldest males had the lowest epididymal sperm counts. (d) Epididymal sperm counts correlated strongly with urinary protein output.

in MUP concentration over a 6-month period but urine samples were collected for volatile analysis only once. In 1 mL sealed glass vials, urine samples (50 μL) were added to 50 μL of AnalaR grade hexane (Fisher Scientific, Loughborough, Leics, UK). This hexane contained an internal standard [10 pg μL^{-1} of decaflourobenzophenone (Sigma-Aldrich Ltd, Gillingham, Dorset, UK)] to allow correction for differences in extraction between samples. Vials were vortexed for 10 s and incubated for 10 min at room temperature (18–24 °C). The hexane layer was then removed to an autosampler vial. This extraction process was repeated twice more using the remaining sample then the vial was sealed for GC–MS analysis. Solvent extracts (1 μL of the hexane extract) were analysed using a DB-5MS 20 m × 0.18 mm inner diameter × 0.18 μm film capillary column (J&W Scientific, Folsom, CA, USA) fitted to a Thermo Electron Trace GC fitted with a splitless injector. The detector was a Thermo Electron Polaris ion-trap mass spectrometer. Data acquisition, tabulation and analysis were controlled using Xcalibur software. All mass spectra were obtained by electron ionization at an ionization potential of 70 eV. Analysis of samples was in full scan mode (50–600 *m/z*). The extracted ions at 60 *m/z* and 125 *m/z* were used to monitor thiazole and brevicomin respectively (Robertson *et al.*, 1993), while those at 362 *m/z* were used to monitor the internal standard. The abundance of volatiles and the internal standard were determined using the peak area. We also tested whether urine samples from middle-aged (n = 6, 18–23 months old) and senesced males (n = 5, 31–32 months old) differed in abundance of thiazole after 10 μL of urine was spotted as a scent mark and left for 10 min, 4, 8 and 12 h prior to analysis. Urine from each individual was spotted on eight replicate 20 mm glass fibre discs and placed under a box to minimize external factors influencing volatile loss. At each time point two replicate discs per male were removed to 100 μL of hexane and extracted to measure residual thiazole as described above.

Female preference between males and between urine scents

Subjects

Females were bred and housed in enclosures (116 cm × 58 cm × 80 cm) or cages (45 cm × 28 cm × 13 cm), in groups of two or three after weaning and tested when adult (age range: 9–15 months old); all females had experienced regular olfactory, visual and acoustic contact of unrelated males and females through life. Eight of the females used in the choice test between males had also mated and given birth to a litter, 5–11 months prior to the experiment. Unless otherwise stated the origins of mice and the cage/enclosure conditions were the same as described for males. Experimental procedures were carried out in the dark phase under dim red light. To bring females into oestrus or proestrus during the preference tests, soiled bedding and substrate from an unfamiliar male, unrelated to those used in the experiment, was added to each female's cage or enclosure 3 days prior to each trial (Marsden & Bronson, 1964; Cheetham *et al.*, 2007).

Urine scent preference tests

Fifteen females were given paired choice tests between the airborne odours emanating from urine streaks derived from senesced (n = 17, age range 27–33 months, age mean = 29.3) vs. younger adult males (n = 14, age range 4–19 months old, mean = 11.8 months). Each female was tested twice using urine from different males in each test (females were always tested with urine from males they had not encountered previously), at least 2 weeks apart, once when urine marks were allowed to dry for only 10 min prior to the test, and after urine was allowed to dry for 12 h prior to testing. A unique pair of males was used in each trial, with urine from individual males used in one or two trials of each type. Tests were balanced for both order (half the females received the 10 min urine first) and for the sides on which adult and senesced urine were presented.

Females were housed in pairs in two enclosures (116 cm × 58 cm × 80 cm) that were connected by two gates (40 mm diameter); females could move between enclosures except during experimental tests, when the two females were confined and tested in separate enclosures. Each enclosure contained a food and water hopper and a clear Perspex sheet (30 cm × 39 cm) placed above two small bricks (3 cm high) to create a covered central area. Urine streaks were presented on microscope slides at either end of the enclosure, equidistant (21 cm) from the edge of the cover sheet and end wall. This required females to move into an open area of the enclosure to investigate male scent marks. The microscope slides were presented in mesh holders that prevented direct contact, ensuring that females could only respond to airborne volatiles emanating from the urine streaks. However, as females are only attracted to a male's airborne urinary scent after they have contacted darcin in the male's urine, which stimulates them to learn the airborne scent (Roberts *et al.*, 2010), females were given direct contact with 10 μL of each urine sample, presented simultaneously on separate microscope slides under the central covered area, 1 h prior to the test (aged for the same time as the urine used in the test). Scent marks used in the test were created by streaking 10 μL of urine in a 4 cm line on a microscope slide, which was then left to dry for 10 min or 12 h. Females were tested with urine from males that were unrelated and unfamiliar, using different pairs of males in each test. Immediately before each test, females were confined away from the test arena while the scent stimuli were placed in the mesh holders; one female was then allowed to re-enter each test arena for a 10 min trial. Trials were recorded remotely and analysed by an

experimenter blind to both the location of different aged scents and the amount of time samples had been left to dry. The total amount of time spent in the open area within a 10 cm radius of scent samples was recorded, together with time spent sniffing each scent (nose in contact with the mesh barrier).

Choice test between males

Fourteen females were given a paired choice between middle-aged ($n = 6$, age range 17–22 months) and senesced males ($n = 5$, age range 30–31 months). Each female was presented with a different pair of middle-aged and senesced males, with individual males used in up to three tests. Males were presented behind separate mesh barriers at opposite ends of a three chambered apparatus, created by connection of three cages (45 cm × 28 cm × 13 cm) with plastic tunnels (42 mm diameter); females were able to move freely between cages and allowed olfactory, visual and acoustic contact with the males. Soiled substrate from the home cage of each male (2.5 g) was placed in front of the corresponding barrier to allow females direct contact with the male's scent. Trials were 1 h in duration, recorded remotely and then watched by an experimenter blind to the location of the senesced male. We recorded the amount of time and number of visits made by females to the cage area in front of each male (12.5 cm × 41 cm), together with the frequency of scent marking by females on Benchkote that lined the cage area in front of each male. Marking patterns were visualized using a Bio-Rad Fluor-S MultiImager (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Scent mark numbers were counted automatically using the 'Analyze Particles' tool in IMAGEJ version 1.38 × (http://rsb.info.nih.gov/ij/).

Data analysis

Statistical tests of changes in olfactory signalling with senescence were carried out using the SPSS software package, version 16. Correlations between urinary protein concentration, sperm number and male age were assessed using Spearman's rank tests. When controlling for the number of generations since males were derived from the wild, General Linear Models (GLM) were used and the number of generations included as a covariate. Repeated measures GLMs, applying the Greenhouse–Geisser correction where necessary, assessed changes in urinary protein or creatinine concentration over 6 months. A repeated measures GLM assessed the loss of thiazole from scent marks with time since deposition and male age, while the amount of thiazole and brevicomin in fresh urine from middle-aged and senesced males were compared using Student's t and Mann–Whitney U-tests respectively. To assess female preference for middle-aged over senesced males or their scents, we calculated the bias in response to the middle-aged minus senesced stimulus (excluding any trials where females visited neither stimulus). Linear mixed effects (LME) models, fitted by maximum likelihood using the lme4 package in R, assessed whether the bias was significantly greater than zero. The identities of the middle-aged and senesced stimulus males were added as random effects to control for the use of some males in more than one trial; prior female breeding experience was included in the model for female preference between males but all females were naive in tests of preference between urine stimuli. The bias in time spent sniffing urine was transformed logarithmically to approximate normality for analysis (Kolmogorov–Smirnov and Shapiro–Wilk tests, NS).

Results

Decrease in MUP output with senescence

We first tested whether urinary MUP concentration was lower in senesced males (25–26 to 31–32 months) when compared to middle-aged males (12–17 to 18–23 months), and whether MUP concentration decreased as males aged over a 6-month period. Nearly all urinary protein was MUP, with no increased leakage of other proteins into urine even among the older senesced males (Fig. 1a, b). Males that were senesced had a lower concentration of creatinine in their urine (Fig. 1c; effect of age class: $F_{1,9} = 19.41$, $P = 0.002$; interaction between month and age class: $F_{2.4,45} = 1.50$, $P = 0.24$), possibly due to the loss of muscle mass with age (McArdle *et al.*, 2002) or to an unexpected age-related increase in water intake. Although protein concentration could not therefore be corrected for urine dilution using creatinine, urinary protein concentration was significantly reduced among senesced compared to middle-aged males over the 6 month sample period (Fig. 1d; $F_{1,9} = 12.52$, $P = 0.006$). Notably, the difference in protein concentration between the two age groups decreased over the 6-month period (Fig. 1d; interaction between month and age class: $F_{3.1,45} = 5.18$, $P = 0.005$), reflecting a sharp rate of decline among males that were middle-aged at the start of this period ($F_{5,25} = 16.34$, $P < 0.001$). Males that were already senesced and had a low protein concentration at the start of sampling did not decrease MUP output any further over the next 6 months ($F_{5,20} = 1.46$, $P = 0.29$).

As not all MUPs have the same functions in sexual signalling, we also assessed more specific changes in MUP output using densitometry of MUPs separated by SDS-PAGE, using samples taken in the first and last month of the sample period. The male-specific MUP darcin plays a special role as a male sex pheromone, responsible for attracting females to spend time near male scent marks and stimulating a learned attraction to that male's airborne scent. Changes in darcin concentration followed the same pattern as total protein concentration, with a difference between senesced and middle-aged males in the rate of decline in concentration over time (Fig. 1e;

interaction between month and age class: $F_{1,9} = 5.20$, $P = 0.048$). Over both months there was only a tendency for middle-aged males to excrete a higher concentration of darcin (effect of age class: $F_{1,9} = 3.84$, $P = 0.082$), because there was a difference in the first month ($t_9 = -2.63$, $P = 0.027$) but this was no longer significant by the sixth month ($t_9 = -1.27$, $P = 0.24$). The main MUP band on SDS-PAGE comprises a number of very similar MUP isoforms that provide the individual-specific MUP patterns used for individual and kinship recognition (Mudge *et al.*, 2008). Although not male-specific, males generally express a significantly higher concentration of these MUPs than females. Senesced males had a lowered output of these MUPs compared to middle-aged males (effect of age class: $F_{1,9} = 10.53$, $P = 0.01$). However, as with total urinary protein, middle-aged males showed a much sharper decrease in output of these MUPs over the sample period when compared to middle-aged males (Fig. 1f; interaction between month and age class: $F_{1,9} = 8.50$, $P = 0.017$).

We also tested whether there was a relationship between senescence and MUP concentration across males ranging from middle-aged to senesced (18–33 months) that had more variation in their lifetime experience of conspecifics. Again, the oldest males expressed the lowest concentration of urinary protein ($n = 33$, $r_s = -0.39$, $P = 0.025$), which was almost all MUP (Fig. 2a). In this case, however, age had no effect on urinary creatinine ($n = 33$, $r_s = -0.02$, $P = 0.90$), allowing protein output to be corrected for urine dilution to provide a more accurate measure. This revealed an even stronger negative relationship between male age and urinary protein output (Fig. 2b). We also examined epididymal sperm number in a subset of these males; as expected, the oldest males had the lowest epididymal sperm counts (Fig. 2c). Further, epididymal sperm count correlated strongly with urinary protein output (Fig. 2d), suggesting that males with the lowest MUP output were the least fertile. After controlling for the number of generations since the ancestors of each male were caught in the wild, there was still a strong effect of male age on urinary protein concentration ($F_{1,32} = 7.22$, $P = 0.012$), protein concentration corrected for dilution ($F_{1,32} = 9.32$, $P = 0.005$) and sperm number ($F_{1,12} = 8.89$, $P = 0.014$). A strong relationship also persisted between epididymal sperm count and urinary protein output ($F_{1,12} = 9.49$, $P = 0.012$).

Changes in volatile abundance

Senesced males (sampled at 28–29 months) excreted the signalling volatiles 2-*sec*-butyl-4,5-dihydrothiazole ('thiazole') and 2,3-dehydro-*exo*-brevicomin ('brevicomin') at approximately one third of the concentration observed among middle-aged males (15–20 months) (thiazole: mean ± SE for senesced males: 19.8 ± 6.2; middle-aged males: 63.2 ± 17.2; $t_9 = -2.56$, $P = 0.03$; brevicomin: senesced males: 1.4 ± 0.9; middle-aged males: 4.9 ± 1.3; Mann–Whitney $z = -1.68$, $P = 0.09$; data are expressed in arbitrary units relative to an internal standard). While MUPs are involatile and remain unchanged after deposition, these volatile ligands bind to MUPs but are gradually lost from male scent marks over time. Brevicomin is lost quickly, within about 20 min (Cavaggioni *et al.*, 2006). However, the release of thiazole is extended over a period of approximately 24 h, with an initial rapid loss followed by a much slower release of the thiazole bound to darcin, which has a strong binding affinity for this volatile (Armstrong *et al.*, 2005). We thus assessed the amount of thiazole remaining over a 12 h period after urine was deposited in small scent marks. As expected, the amount of thiazole remaining decreased with time since deposition ($F_{3,27} = 18.1$, $P = 0.002$), with a much more rapid loss over the first 4 h (Fig. 3). The amount of thiazole remaining in the urine of senesced males was lower at each time point ($F_{1,9} = 9.2$, $P = 0.014$), although the extent of this difference varied with time since deposition (interaction between male age class and scent mark age: $F_{3,27} = 10.0$, $P < 0.012$). Notably, the amount of thiazole remaining in senesced male urine after 4 h was similar to that in middle-aged male urine after 8 or 12 h; after 12 h, middle-aged males still retained a low but detectable level of thiazole but almost all had gone from senescent male urine (Fig. 3).

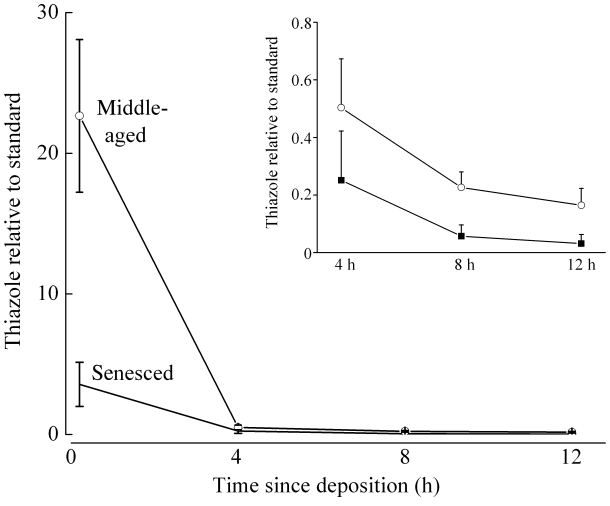

Fig. 3 Abundance of thiazole remaining in urine spots from senesced and middle-aged males left to dry for different time periods. Open circles (○) denote middle-aged males (18–23 months), closed squares (■) denote males that were senesced (31–33 months). The amount of thiazole remaining at scent marks decreased over time and the difference between middle-aged and senesced male urine varied with the length of time that urine spots were left to dry in the environment.

Female attraction to senesced males and their scent

Urine preference test

The effects of senescence on male scent signals are only likely to influence male reproductive success if these alter the male's attractiveness to females, or if their scent signals are simply less detectable. We tested the time that females spent in the vicinity of airborne odours from senesced (27–33 months old) vs. younger adult (4–14 months old) male scent marks (10 μL urine), and the time spent closely investigating the scents. These were presented in separate open areas of a female's home enclosure that normally were visited only rarely. When scent marks were freshly deposited, there was no difference in the time spent near or closely sniffing urine from adult or senesced males (Fig. 4a). However, when scent marks were 12 h old, females spent more time in the vicinity of the younger adult male's scent (Fig. 4b). This was due both to more time spent closely sniffing at the younger male's scent, and to more time in the vicinity not sniffing (Fig. 4b).

Male preference test

To test whether females were less attracted to senesced males themselves (30–31 months old) compared to middle-aged adults (17–22 months old), we assessed female preference when males were presented behind separate barriers that allowed olfactory, visual and acoustic contact over a 1 h test. There was no difference in the frequency of visits (Fig. 5a) or time spent in the vicinity of senesced vs. middle-aged males (Fig. 5b). However, females deposited more scent marks in front of the middle-aged male than in front of the senesced male (Fig. 5c), suggesting a preference for communicating with the middle-aged male. Females were tested with (n = 8) or without (n = 6) prior breeding experience, but this factor had no influence on their bias in scent mark placement (χ^2 = 0.19, d.f. = 1, P = 0.66).

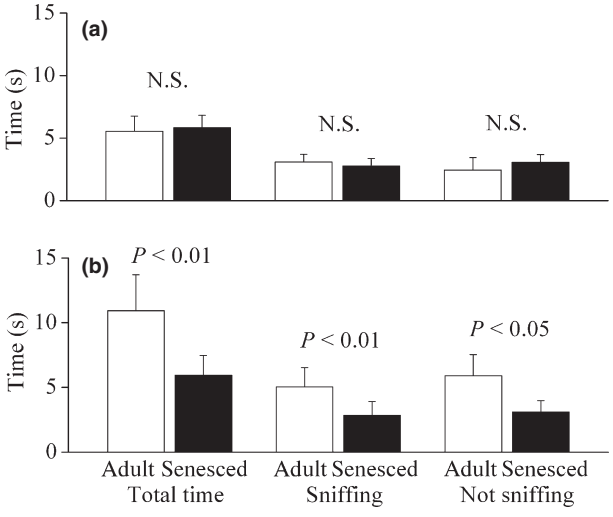

Fig. 4 Female preference between younger adult (4–19 months old) and senesced (27–33 months old) male urinary volatiles. Females were tested with scent marks aged for (a) 10 min or (b) 12 h. There was no difference in response when scent marks were aged for 10 min, but females spent less time in the open area near senecesed male urine when aged for 12 h, due to both less time sniffing the scent stimulus and less time in vicinity not sniffing. *P* values are from LME models assessing whether there was a significant bias against urine from senesced males.

Discussion

Our results demonstrate a reduction in the concentration of each of the male-specific scent signalling molecules examined in the urine of senesced male mice compared to middle-aged adults. This involved both volatile and involatile androgen-dependent sexual signals, each showing an approximately three fold reduction compared to younger middle-aged males. The two androgen-dependent volatiles 2-*sec*-butyl-4,5-dihydrothiazole ('thiazole') and 2,3-dehydro-*exo*-brevicomin ('brevicomin') are important for stimulating female investigatory contact with a male's scent, and have been found to stimulate as much investigation as intact male urine when both male volatiles are presented together (Jemiolo *et al.*, 1985). These two compounds also act synergistically to stimulate oestrus in females (Jemiolo *et al.*, 1985) as well as stimulating male competitive aggression when added to castrated male urine (Novotny *et al.*, 1985). As both of these hydrophobic volatiles are bound by the central cavity of MUPs (Robertson *et al.*, 1993), the reduced concentration of these volatiles in senesced males may be due, at least in part, to the reduced concentration of the MUPs that carry them. This reduction in MUP concentration was evident in the male-specific sex pheromone darcin (Roberts *et al.*, 2010), as well as in the polymorphic MUPs that are expressed by both sexes and encode individual and kinship identity (Hurst *et al.*, 2001; Cheetham *et al.*, 2007; Sherborne *et al.*, 2007).

Although females may encounter fresh urine from males during direct interactions, most urine scent marks are likely to be encountered many minutes or hours after their initial deposition. A consequence of the binding of volatiles to MUPs is that this reduces the rate at which these highly volatile signalling components evaporate, increasing both the time over which airborne volatile signals continue to be emitted from scent marks and the amount of signal that remains within an aged scent mark. This is particularly the case for thiazole, which mostly binds to the male sex pheromone MUP darcin, extending its release over many hours (Armstrong *et al.*, 2005). Urine spots from both middle-aged and senesced males lost the vast majority of thiazole over a 12 h period. However, the greatly reduced initial concentration of thiazole and darcin in fresh senescent male urine meant that this volatile was lost from these scent marks

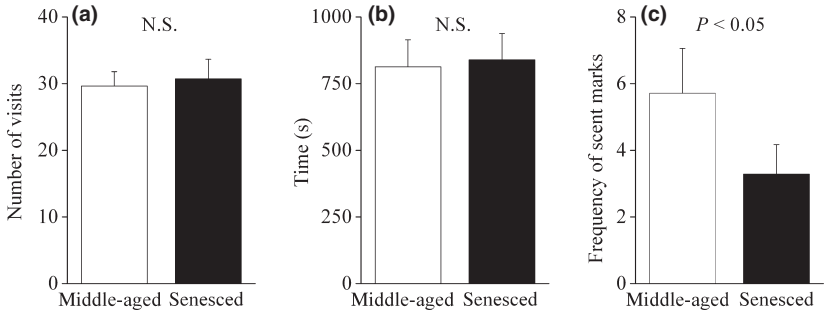

Fig. 5 Female preference between a senesced (30–31 months old) vs. a middle-aged (17–22 months old) male presented behind mesh barriers. There was no difference in (a) the number of visits or (b) the amount of time spent in vicinity of middle-aged or senesced males, but (c) more scent marks were deposited in front of middle-aged males. *P* values are from LME models assessing whether there was a significant bias against senesced males. Prior breeding experience among females had no effect on the lack of bias in number of visits (χ^2 = 1.72, d.f. = 1, P = 0.19) or time in vicinity (χ^2 = 0.10, d.f. = 1, P = 0.75), or on the bias for more scent marks in front of middle-aged males (χ^2 = 0.19, d.f. = 1, P = 0.66).

more quickly. This may thus affect the ability of females to detect a senesced male's ageing scent marks. We found no difference in the time that females spent near freshly deposited urine from senescent compared to younger adult males. In these first few minutes after deposition, there is a large release of volatiles including thiazole (Armstrong *et al.*, 2005; Cavaggioni *et al.*, 2006; current study), providing a high concentration of airborne odours that may be detected by females. However, as scent marks age, initial differences in the concentration of volatile signals between males may be more important because a reduced volatile signal is lost more quickly. Consistent with this, females spent less time near urine from senescent compared to younger adult males when urine marks were 12 h old, when the senescent male urine had lost almost all thiazole. Other volatiles, not measured here, may also be lost more quickly from senesced male scent, and may have contributed to the lower attractiveness of senesced male compared to younger adult male scent. Our results further highlight the importance of examining responses to scents not only when fresh but also when they have aged, as this may better reflect the information that remains in scent deposits when they are encountered under natural conditions.

Females also appeared able to distinguish between middle-aged and senesced males when encountered directly, depositing more scent marks near to the middle-aged males. Female mice are typically stimulated to scent mark more in the vicinity of males (Coquelin, 1992), and more in the vicinity of a preferred male when given a choice between two males (Rich & Hurst, 1999). This suggests that their scent mark signals may be directed towards particular males and perhaps reflect a mate preference. Although females did not spend more time near to middle-aged males compared to senesced males, this may be because they were confined in a small unfamiliar arena where they were unable to interact freely with the males and spent much time exploring both chambers.

A relationship between sexual signal production and senescence may arise due to changes within individuals as they age or as a consequence of selective disappearance or appearance of individuals of certain phenotypes (Van de Pol & Verhulst, 2006). Some differences between males that were middle-aged and senesced could have been generated from selective disappearance of particular individuals, such as when high quality individuals invest heavily in early life sexual signalling but die young (Hunt *et al.*, 2004). Nonetheless, our 6 month longitudinal study of ageing males indicates that a reduction in urinary MUP concentration occurs within individuals as they become senesced. Lower total urinary MUP concentration with senescence was also observed between individuals, both when comparing middle-aged and senesced males and when we tested for a correlation between male age and urinary MUP concentration across a group of males with a variety of social experience. It seems likely that these differences were a result, at least in part, of the decrease in male urinary MUP concentration that occurs with senescence. When comparing middle-aged and senesced males over a 6-month period, we also observed consistently lower creatinine levels in the urine of senesced males; by contrast, there was no evidence of a relationship between age and urinary creatinine levels in the cross-section of males examined. These two studies were purposely very different in their design and thus the discrepancy in results may be a consequence of the cross-sectional nature of the latter study or the variation that was allowed in social experience. Changes in urinary creatinine with age have previously been demonstrated in laboratory rats, with creatinine concentration increasing between the ages of 4 and 18 months but then dropping by the time individuals reach 24 months (Wu *et al.*, 2008). The authors suggest that such age related changes could occur as a result of changes in muscle mass,

nutritional status or glomerular filtration rate. We did not test which of these factors may have led to differences in creatinine output between senesced and middle-aged males, but our results highlight the need to check for differences between males in creatinine before using this marker to correct for urinary dilution.

Decreases in sexual signal production with senescence may occur for various reasons. Investment in olfactory signalling is frequently suggested to be costly (Gosling *et al.*, 2000; Radwan *et al.*, 2006; Zala *et al.*, 2008), so senesced males, which have a loss of physiological function, may be unable to pay the costs of producing these signals at a high level. Alternatively, decreased sexual signal production may be an adaptive life history strategy for males. While males have been predicted to increase investment in sexual signals as they become older under a variety of conditions (Kokko, 1997), a recent state-dependent model of age-related reproductive investment predicts that individuals should partition relatively less to reproduction with age (McNamara *et al.*, 2009). As sexual signalling is a major component of male reproduction, it is possible that reduced olfactory signalling during senescence may reflect an adaptive reduction in male reproductive effort. The most likely mechanistic cause for decreased MUP output is change at the transcriptional level in the liver. MUPs produced by male laboratory rats also decrease with senescence, which is due to decreased transcription in the liver as a result of a decline in androgen sensitivity (Roy *et al.*, 1974). A decline in testosterone levels or in androgen sensitivity could further account for the decrease in thiazole and brevicomin with senescence, as expression of both these volatiles is testosterone dependent (Novotny *et al.*, 1985). In addition, sexual signalling may decline during senescence as a consequence of oxidative stress (Velando *et al.*, 2008), a pathological process particularly associated with ageing (Beckman & Ames, 1998). In species with carotenoid-based visual traits (Metcalfe & Alonso-Alvarez, 2010), there is evidence that sexual signalling varies with an individual's antioxidant status (Bertrand *et al.*, 2006). Oxidative stress could thus be another physiological factor contributing to the reduction in olfactory sexual signalling in senesced male mice, although further research is required to understand whether olfactory sexual signals vary specifically with oxidative stress.

Our results are consistent with the hypothesis that females select against senesced males as mates (Hansen & Price, 1995; Beck & Promislow, 2007; Velando *et al.*, 2008), and are able to detect senescence through scent cues, although the impact on mate choice per se remains to be tested. Females may incur a number of fitness costs from mating with senesced males, one of which may be a decrease in fertilization success due to lower male fertility (Brooks & Kemp, 2001). Here, the oldest males had the lowest epididymal sperm counts, which could suggest that these males were the least fertile. An additional fertilization test would be required to determine this, however, as quantitative differences in sperm characteristics of senesced males do not always influence fertilization success (Gasparini *et al.*, 2010). Epididymal sperm counts correlated strongly with urinary MUP concentration, suggesting that this olfactory sexual trait could reveal information to females regarding male ejaculate quality, as expected under the phenotype-linked fertility hypothesis (Sheldon, 1994). As sperm from senesced males also have more random germline mutations (Velando *et al.*, 2008), offspring from such males can suffer reduced viability (Jones *et al.*, 2000; Pizzari *et al.*, 2008), which is likely to cancel any viability benefits females would gain from mating with these older males. Female selection against senesced males is therefore likely to be an adaptive choice that enhances fertilization and increases offspring reproductive success.

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