RESEARCH ARTICLE

Flight restriction prevents associative learning deficits but not changes in brain protein-adduct formation during honeybee ageing

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SUMMARY

Honeybees (*Apis mellifera*) senesce within 2 weeks after they discontinue nest tasks in favour of foraging. Foraging involves metabolically demanding flight, which in houseflies (*Musca domestica*) and fruit flies (*Drosophila melanogaster*) is associated with markers of ageing such as increased mortality and accumulation of oxidative damage. The role of flight in honeybee ageing is incompletely understood. We assessed relationships between honeybee flight activity and ageing by simulating rain that confined foragers to their colonies most of the day. After 15 days on average, flight-restricted foragers were compared with bees with normal (free) flight: one group that foraged for ~15 days and two additional control groups, for flight duration and chronological age, that foraged for ~5 days. Free flight over 15 days on average resulted in impaired associative learning ability. In contrast, flight-restricted foragers did as well in learning as bees that foraged for 5 days on average. This negative effect of flight activity was not influenced by chronological age or gustatory responsiveness, a measure of the bees' motivation to learn. Contrasting their intact learning ability, flight-restricted bees accrued the most oxidative brain damage as indicated by malondialdehyde protein adduct levels in crude cytosolic fractions. Concentrations of mono- and poly-ubiquitinated brain proteins were equal between the groups, whereas differences in total protein amounts suggested changes in brain protein metabolism connected to forager age, but not flight. We propose that intense flight is causal to brain deficits in aged bees, and that oxidative protein damage is unlikely to be the underlying mechanism.

INTRODUCTION

The tremendous metabolic scope of insect flight makes invertebrates such as flies and bees excellent tools for investigating connections between physical activity, ageing-related processes and lifespan (Sohal et al., 1984; Yan and Sohal, 2000; Magwere et al., 2006; Rueppell et al., 2007; Piazza et al., 2009; Vance et al., 2009). Honeybees exemplify how insect flight activity can vary from intense and continuous during days with access to flowering plants and good weather, to short bursts of activity or complete inactivity during periods with changing or poor weather conditions and variable food availability (Winston, 1987). The bee provides an interesting model in ageing research because of its social biology and the availability of good tools for quantification of individual brain function (Münch et al., 2008). However, it is not entirely clear how physical activity affects honeybee ageing. In flying insects, this relationship has primarily been studied in houseflies (*Musca domestica*) and fruit flies (*Drosophila melanogaster*), and has centred on flight muscle mitochondria as a source of reactive oxygen species formation (Sohal et al., 1984; Magwere et al., 2006). Flight activity increases oxidative stress and mortality in houseflies (Yan and Sohal, 2000) and similarly reduces oxidative stress resilience and survival in fruit flies (Magwere et al., 2006). Oxidative stress, longevity and ageing are also likely linked to flight activity in honeybees (Neukirch, 1982; Seehuus et al., 2006a; Behrends et al.,

2007). Here, current knowledge suggests that cellular defence and repair mechanisms are influenced by workload, behavioural state and chronological age in a complex and tissue-dependent manner (Williams et al., 2008; Elekonich, 2009).

Honeybees live in colonies that are almost entirely female. A highly reproductive queen is responsible for egg laying, and essentially sterile female workers (helpers) perform complex social tasks in a temporal, age-associated sequence. Young adult workers typically conduct within-nest tasks such as brood care, but within the third week of life the behaviour of the worker bees changes and they start taking foraging flights to collect nectar, pollen, propolis and water in the field (Winston, 1987). This behavioural ontogeny is flexible because nest tasks and foraging can be conducted by worker sisters of identical chronological age. A bee can forage for over 10 h daily, covering in-flight distances of up to 21.5 km (Neukirch, 1982; Winston, 1987). During flight, bees attain one of the highest mass-specific metabolic rates measured in any animal (Suarez, 2000). Foragers, which engage in frequent foraging and flight activity, are susceptible to induced oxidative stress (Seehuus et al., 2006b), and functional decline can be detected as forager age increases, in days since the individual's first onset of foraging; mechanical senescence of the wings (Finch, 1990), lowered flight capacity (Vance et al., 2009), reduced frequency of foraging trips (Tofilski, 2000) and poorer scores in tests of associative learning

(Behrends et al., 2007; Scheiner and Amdam, 2009) are all features of aged individuals. The latter result connects forager age (i.e. days of foraging labour) to brain ageing. Yet because the transition from nest tasks to foraging is not only behavioural, but also associated with changes in brain gene and protein expression (Whitfield et al., 2003; Garcia et al., 2009), general metabolism (Crailsheim, 1986; Harrison, 1986; Toth and Robinson, 2005; Wolschin and Amdam, 2007a) and cell-based innate immunity (Amdam et al., 2005), it is unclear whether the connection is conferred by flight activity *per se* or whether it emerges because of other factors inherent to forager physiology.

Here, we asked how the honeybee brain is influenced by flight opportunity and forager age. Four groups of worker honeybees were hosted in triplicate, natural colonies. Two groups had low forager age (on average 5 days since foraging onset), and two groups had high forager age (15 days on average). Of those with low forager age, chronological age was known for one group and varied (i.e. unknown) for the other; both groups had free access to flight. Of those with high forager age, chronological age varied, and one group had free access to flight whereas flight was constrained to a daily burst in the other. The resulting four experimental groups were contrasted to investigate how flight activity and forager age influenced sensory sensitivity, learning performance and protein adduct formation in the brain, while the role of chronological age was monitored.

Our data demonstrate that associative learning ability remains intact in flight-restricted workers, whereas free-flying bees senesce. In contrast, the majority of brain protein changes were related to forager age, independent of flight opportunity. We report on total protein patterns as well as malondialdehyde (MDA) and ubiquitin (Ub) levels in two sample fractions of the brain. MDA protein adducts are secondary damages of lipid peroxidation and endogenous mutagens, whereas Ub protein accumulation indicates age-related deficits in systems for protein degradation and control. Both markers are frequently used in research on ageing (Pryor and Stanley, 1975; Niedernhofer et al., 2003; Martinez-Vicente et al., 2005; Simonsen et al., 2008).

MATERIALS AND METHODS
Chemicals
Protease inhibitor was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Primary antibodies, polyclonal rabbit anti-MDA (MD20A-R1a) and monoclonal anti-mono- and poly-ubiquitinylated conjugates (clone FK2, PW8810) were from Academy Bio-Medical (Houston, TX, USA) and BioMol (Postfach, Lausen, Switzerland), respectively. Carnation oil was from Natur og Helse (Sætre, Norway) and UniPosca colour pens were from Mitsubishi Pencil Company Ltd (Worcester, UK). Cy-conjugated secondary antibodies were all purchased from Jackson Immunochemicals Ltd Europe (Newmarket, Suffolk, UK). Other materials for immuno-blotting and protein quantitation, electrophoresis and gel staining were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents were analytical-grade chemicals purchased from Sigma-Aldrich (Munich, Germany) or Merck (Darmstadt, Germany).

Experimental design
The study was conducted at the Norwegian University of Life Sciences, Aas, Norway. We used nine honeybee (*Apis mellifera* Linnaeus 1758) colonies, each with an initial 8000–10,000 workers and one queen. The nine queens were sisters mated at the same breeding station. These colonies provided triplicate host environments for each of three general test sets of bees: workers of low forager age with free access to flight, workers of high forager age with free access to flight and workers of high forager age with restricted access to flight. Furthermore, the triplicate colonies with workers of low forager age each contained two distinct age-structured groups: one group of known chronological age and one where chronological age was set to vary, resulting in a total of four experimental groups.

The two treatment groups of low forager age, but with different variance in chronological age, were used as an internal control. Ageing can progress largely independent of chronological age in honeybees, and forager age is the better predictor of senescence and survival. Our *a priori* expectation, therefore, was that the two worker groups with low forager age were pre-senescent regardless of age-structure and would produce similar results during testing. In addition, hive manipulations (see below) were adjusted so the cumulative time available for flight was comparable between these two treatment groups of low forager age and the one treatment group of high forager age and restricted access to flight. This design enabled us to study forager age while cumulative flight opportunity was approximately constant (see Fig. 1).

Workers were marked to track group identity (flight restriction, chronological and forager age). For bees of known chronological age, a comb with brood was removed from each of the three designated host colonies and incubated overnight (33°C, 80% relative humidity). The next day, <24 h-old bees were marked with UniPosca water-based pigment ink before combs and bees were reintroduced into their natal colonies. The remaining six colonies received similar (sham) handling. Flight activity was monitored daily for 1–2 h. When marked bees began foraging, identified as returning from flight with distended abdomen (indicating nectar load) or with pollen carried on the hind legs, they were given a new paint mark on the thorax. In parallel, returning foragers were marked *en masse* at all nine hive entrances to produce sampling populations of bees with varying chronological age and known forager age, as described previously (Wolschin et al., 2009). These procedures were repeated during the experimental time window (May–August 2008), resulting in a continuing access to bees from all treatment groups and colonies.

Different colours/combinations of pigment ink were used so that every marked cohort could be traced. The complete experimental design was repeated twice, first as a pilot and then as a full-scale experiment. For the pilot, sample sizes were limited and did not justify independent statistical processing (44 bees in total). Our report (detailed in Results) thus centres on the full-scale experiment. To demonstrate the robustness of this setup and consistency of the results, pilot data for flight opportunity, sensory and behavioural changes are given as supplemental material (supplementary material Fig. S1). To ensure that the status of all nine hives was roughly equal at the start of each experiment brood, honey and pollen frames were either removed or added. The status of the colonies with regard to egg laying, capped brood, pollen and honey reserves was also inspected at regular intervals during the experimental period.

Experimental regulation of flight opportunity
Flight was restricted by time-controlled lawn sprinklers that were directed at the entrances of the colonies to create an artificial cool rain (~15°C), as described previously (Schneider et al., 1986; Huang and Robinson, 1996). In parallel, the triplicate colonies were provided with shade to further discourage foraging during the periods when flight was restricted. Within minutes after sprinklers shut down, flight-restricted colonies initiated foraging and continued
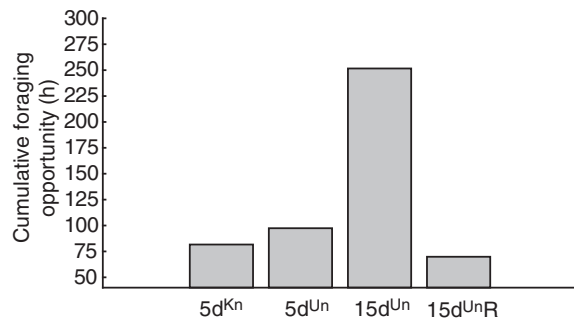
Fig. 1. Cumulative foraging opportunity for honeybee workers during the main experiment. Flight occurred when the sun was up, air temperature was >12°C and precipitation over the last 24 h period was <3 mm. The four treatment groups differed in forager age, foraging opportunity and age structure. X-axis: 5d and 15d give the forager age as the mean number of days since onset of foraging labour, and superscript Kn or Un indicate that chronological age was known or unknown, respectively. The data demonstrate that flight-restriction by rain (R) was successful in controlling foraging opportunity, such that bees with a forager age of ~15 days experienced opportunities similar to bees that foraged for ~5 days.

flight activity until sunset. The success of the lawn sprinklers in limiting flight was validated every day by a 1–2 h inspection during the peak foraging period (09:00–14:00 h).

Within-colony temperature was not controlled during the experiment because previous studies have shown that artificial rain created at the hive entrance has a limited effect on colony temperature and non-foraging related behaviour in foragers (Riessberger and Crailsheim, 1997). Norwegian summers are also cool with fluctuating weather conditions (http://www.vips-landbruk.no/); in 2008, when this study was undertaken, daily mean temperatures ranged from 4 to 22°C during the experimental period (May–August), and the mean daily temperature difference measured was 12°C when comparing daily maximum and minimum temperatures, whereas maximum differences ranged up to 20°C. Thus the role of experimentally induced temperature differences as a factor in this study is restricted compared with natural temperature changes.

From daily inspections of colonies during the pilot setup, we could predict that foraging activity occurred when the following conditions were met: the sun was out, temperatures were >12°C and precipitation was minimal (<3 mm within a 24 h period). The daily inspections also confirmed that the status of colonies exposed to artificial rain in terms of motility, reaction threshold, brood production and other non-flight-related behaviours was not visibly altered during the experimental period. To account for effects of ambient conditions on flight, the duration of flight restriction was adjusted daily based on air temperature measurements made at 15:00 h. Specifically, the artificial rain was turned on each morning at 02:30 h (before dawn) and turned off at 16:00 h (<15°C), 17:30 h (15–20°C) or 19:00 h (>20°C). As a result, flight restriction did not eliminate the daily opportunity for flight, because the restriction was lifted earlier in the afternoon during periods of cool weather to allow a burst of flight activity before temperatures dropped further throughout the evening. Sunrise was at 04:00–05:00 h and sunset was at 22:00–23:00 h during the experimental period.

At the end of the study, we estimated how the flight-restriction scheme had influenced the bees' opportunity for flight throughout the experimental period (Fig. 1). To obtain this estimate, the sprinkler system's data log was compared with weather data for the University campus (59°39′52″N, 10°47′40″E). Weather information was obtained from the Norwegian National Agricultural Meteorological Service (http://www.vips-landbruk.no/).

Sampling of bees

The experimental bees were recognised by their paint marks and collected inside each hive in the morning before foraging flights began (05:30–08:30 h). They were then transported to the laboratory (400 m from the apiary) in well-ventilated containers within 1 h of collection. Upon arrival in the laboratory, bees were either processed for brain protein extraction or tested for individual gustatory responsiveness and associative olfactory learning performance. To minimise effects of hive-to-hive variability, workers from all colonies (and treatment groups) were collected and processed together, numbering 40–50 bees in total each sampling day.

Measurements of gustatory responsiveness

Bees were mounted in holders (see Hammer and Menzel, 1995) before gustatory responsiveness was quantified, as detailed previously (Wang et al., 2010). In brief, operators were blind to the treatment identity of the bees. Sucrose–H_2O solutions were presented one at a time by touching the bees' antennae with a syringe. Solutions were presented in ascending order of concentration (0, 0.1, 0.3, 1.0, 3.0, 10 and 30% sucrose) at a minimum of 2 min intervals. If a bee extended her proboscis (tongue) when presented with the stimulus, a proboscis extension response (PER) was recorded. The PERs were tallied at the end of the assay, giving each bee a gustatory response score (GRS) between 0 (no PER) and 7 (PER to all concentrations). GRS is an established measure of honeybee gustatory responsiveness (Scheiner et al., 2004).

Olfactory conditioning

Bees that responded to at least 30% sucrose in the gustatory responsiveness test were trained by classical conditioning to associate an odour (carnation) with a 30% sucrose reward (Bitterman et al., 1983). Before conditioning, bees were tested for their spontaneous response to the positive conditioning stimulus (CS+; carnation) and the alternative (unrewarded) odour cineole. Each bee was placed in a constant neutral air stream for 5 s before odour was presented. Exposure to odour, which was produced as 5 ml air was pressed through a 20 ml syringe containing a filter paper with 2 μl of scented oil, lasted 5 s. The bee sat in the air stream for another 8 s before she was removed. The inter-stimulus interval was at least 5 min. Bees that responded spontaneously to either odour were not tested further (N=35), whereas the remaining workers (N=243 in total) were subjected to conditioning to the CS+.

During conditioning, each bee underwent the exposure schedule to the air stream and odour outlined above, but 1 μl sucrose reward, the unconditioned stimulus (US), was applied to the antennae 3 s after exposure to CS+ began. From the second conditioning trial, PER expressed before presentation of CS+ was recorded as conditioned PER. In total, six CS+/US pairings were performed for every bee with inter-trial intervals of 10 min on average. After the six conditioning trials, the bees were presented with the cineole and the CS+ (carnation) to determine whether they could discriminate between the two. Each bee was first presented with the cineole without reward, and next with the CS+ without reward. Finally, conditioned PERs were tallied for bees with CS-specific memory, i.e. those that responded to CS+ but not to the alternative odour in the final test (N=220). This summation resulted in integer learning score values ranging from 0 (bees never responded to CS+ during

conditioning) to 5 (bees learned during the first CS+/US pairing, and showed conditioned PER in all following trials).

Preparation of brain protein extracts and protein quantification

For the purpose of western blotting, eight samples per experimental group were selected. To avoid an uneven representation and bias in the downstream analysis, the triplicate host colonies were all represented among the eight samples that underwent analysis. For preparation of protein extracts, individual bee brains were dissected on ice and immediately homogenised in $1\times$ PBS buffer supplemented with $1\,\mathrm{mmol\,l^{-1}}$ Na$_2$EDTA and Complete Protease Inhibitor cocktail for 10 s using a handheld Pellet Pestle Motor (Sigma-Aldrich, Steinheim, Germany) before being centrifuged at $1000\,\boldsymbol{g}$ for 10 min at 4°C. Supernatants were then transferred to new Eppendorf tubes and the pellets were suspended in $50\,\mathrm{mmol\,l^{-1}}$ Tris-HCl, pH 7.4, 2% sodium dodecyl sulphate (SDS), including Complete Protease Inhibitor cocktail, before being sonicated for 20 s using a tip-sonicator at 25 W output. All samples were aliquoted before storage (–80°C). Five μl from each sample was set aside for protein measurement, and the remaining material was used for western blotting. Sample protein concentration was determined by the Bradford protein-dye binding assay with bovine serum albumin (BSA) as standard (Bradford, 1976). These measurements were conducted in duplicate using a Tecan Sunrise plate reader (Grödig, Austria) at 595 nm absorbance.

Detection of MDA and Ub adducts in brain protein fractions

SDS-polyacrylamide electrophoresis (SDS-PAGE) and western blotting for detection of MDA and Ub adducts were conducted in accordance with the methods of Laemmli (Laemmli, 1970) and Towbin et al. (Towbin et al., 1979), respectively, using the Mini-Protean Tetra Cell system (Bio-Rad Laboratories). Every gel was loaded with two samples from all four treatment groups, and the sample locations within the gel of each group were rotated between each gel separation. This processing scheme ensured that technical error, such as blot-to-blot variation and edge effects, would not confound our results. In total, four blots summing to eight biological replicates from each experimental group were run.

For electrophoretic separation of the different brain protein fractions, i.e. the $1000\,\boldsymbol{g}$ supernatant and $1000\,\boldsymbol{g}$ pellet, the two fractions of each individual brain were separately diluted to $2\,\mathrm{mg\,ml^{-1}}$ in $1\times$ Laemmli buffer and heated to 95°C for 4 min before 30 μg protein from each sample was loaded on 10% polyacrylamide gels for electrophoretic separation. Eight different samples and 5 μl molecular weight standard (Precision Plus Protein Standards, Dual Color) were loaded and run in parallel on each gel. To control for differences in the amount of protein loaded per well, Sypro Ruby staining was used as a loading control (Aldrige et al., 2008). Thus, following SDS-PAGE, electro-separated proteins were subsequently blotted onto polyvinylidin fluoride membranes, stained with 10 ml Sypro Ruby Protein Blot solution for detection of the overall protein pattern, and thereafter blocked with 1% BSA in PBS with 0.05% Tween-20 (PBS-T) for 1 h before the blots were stained with antibodies.

To visualise MDA protein adducts, the blots were next incubated with rabbit anti-MDA at 1:200 dilution for 1 h. Membranes were rinsed twice afterward, incubated for 15 min and finally washed for $2\times5\,\mathrm{min}$ with PBS-T before being incubated with secondary goat anti-rabbit Cy5 antibody diluted 1:400 in 1% BSA in PBS-T for 1 h. Finally, the washing procedure with PBS-T was repeated. To visualise mono- and poly-Ub proteins, the same protocol was used but with mouse monoclonal anti-mono- and anti-poly-ubiquitinylated conjugates (clone FK2) at 1:1000 dilution and a goat anti-mouse Cy3 labelled secondary antibody at 1:400 dilution.

Grey scale images of all Sypro-Ruby- and antibody-stained blots were captured using a Typhoon Variable Mode Imager 8600 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equipped with ImageQuant TL v2005 image analysis software (GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). For detection of Sypro Ruby, laser/filter settings were 633/610BP30. For Cy5- and Cy3-conjugated antibodies, combinations of 633 nm exitation/670BP30 emission and 532 nm/580BP30 emission filters were used, respectively. All gels were scanned simultaneously and pixel saturation was avoided during image acquisition by using the in-built quality control system of the ImageQuant TL v2005 image analysis software package. Quantitative data for individual protein bands were later retrieved for analysis using Carestream Molecular Imaging Software v.5.0.2.30 (Carestream Health, Inc., Rochester, NY, USA). All protein bands were manually detected using the Manual ROI option.

Fluorescent stains like Sypro Ruby have a broad linear dynamic range (Nishihara and Champion, 2002) and are frequently used as loading controls and for quantitative protein determination following gel electrophoresis and western blotting, as demonstrated previously (Schulenberg et al., 2005; Aldridge et al., 2008; Lush et al., 2008; David et al., 2010). Hence, in line with such an approach, intensity values of MDA and Ub protein adducts were corrected for background staining and normalised to the total protein load within the 14–200 kDa gel as detected by Sypro Ruby total protein staining following western blotting. However, for a more comprehensive assessment and evaluation, all data (corrected and uncorrected) were analysed and are reported herein.

Statistical analysis

We used a conservative approach to data analysis by including only bees that expressed at least one conditioned PER plus CS-specific memory in the learning assay (scores 1–5, $N=165$). This strategy was employed as bees were tested from spring to fall, and preliminary analyses showed that the occurrence of unsuccessful learning (score=0) was connected to sampling time [it has been previously suggested that the propensity of honeybees to express learning is affected by season (Scheiner et al., 2003)]. The categorical GRS and learning score data did not conform to assumptions of normality, as determined by probability plots of residuals. These scores, therefore, were first analysed by the non-parametric Kruskal–Wallis test. When a significant result was detected, Mann-Whitney U-tests with Bonferroni correction were used for *post hoc* comparisons. Colony could not be included as blocking factor for non-parametric analysis. However, by separate analysis, colony was not found to influence GRS (Kruskal–Wallis test, $H=9.947$, d.f.=8, $P=0.269$, $N=165$) or learning score (Kruskal–Wallis test, $H=7.804$, d.f.=8, $P=0.453$, $N=165$). Thus, it is unlikely that our behavioural data were confounded by colony-associated effects.

The effect of treatment on total protein amounts was estimated by one-way ANOVA. MDA and Ub data were initially analysed with main-effects ANOVA, where treatment group, blot, lane and protein band identity were categorical factors. Colony was not included as a blocking factor because each colony was represented by only two to three bees. Thus, in addition to the cost of degrees of freedom to the colony factor, we already had limited power to control for colony-level influences. The finding that colony did not affect phenotype in our previous and more powerful analysis of 165 individuals further indicated that the collected bees were not

strongly influenced by this factor in general. Probability plots of residuals and Levene's test were used to assess the assumptions of normality and homogeneity of variance. Along with blot and lane location within blot (technical error factors), the band identity came out as a significant variable. As band identity was simply an algebraic number given to each band in ascending order starting from the top of each lane in the gel, its significance only reflected how some protein bands within the same sample category are typically more intense than others. Thus, the effect of band identity was neither dependent on treatment nor on technical factors. To allow possible between-treatment variation in each protein band to count equally toward the analysis, independent of bee *per se*, we therefore used a variance normalisation by calculating a *z*-score for each band. Similar to the approach of Smedal et al. (Smedal et al., 2009), the normalised data were analysed by a main-effects ANOVA with bee coded as a random factor and blot and lane included to control technical error variance. Fisher's least significant difference (LSD) test was used for *post hoc* comparisons. All calculations were performed in Statistica 6.0 (StatSoft, Tulsa, OK, USA).

RESULTS
Opportunity for flight

Summation of local weather data and the schedule for artificial flight-restriction verified that our experiment was successful in producing worker groups with treatment-specific opportunity for flight and foraging (Fig. 1). Cumulatively, bees with high forager age (15 days on average) and free access to flight had accrued approximately three times more hours of exposure to good foraging conditions (sun, air temperature >12°C, minimal precipitation; 252 h in total) in comparison with the three remaining treatment groups. The number of hours accumulated by bees with high foraging age and restricted flight (70 h) was similar to that of the two control groups with low forager age (5 days on average) and free access to flight (82 and 97 h; Fig. 1).

Effect of flight restriction and forager age
Gustatory responsiveness

The median GRS of the workers remained unchanged despite the manipulations of the colonies and bees (Kruskal–Wallis test, $H=2.160$, d.f.=3, $P=0.541$, $N=27$–34; Fig. 2A). This result is in accordance with previous data showing that forager age, in general, does not have a consistent effect on honeybee gustatory responsiveness (Behrends et al., 2007; Scheiner and Amdam, 2009). Gustatory responsiveness can be affected by genotype (Scheiner and Arnold, 2010) and factors in the colony environment such as food availability and pheromones from larvae (Pankiw and Page, 2001; Pankiw et al., 2001). The fact that the median GRS was similar between our treatment groups, therefore, further indicated that the design of the study did not give rise to unintended and potentially confounding differences between the nine experimental colonies.

Associative learning performance

The median learning score, which reflected the ability of workers to associate an odour with a sucrose reward, was significantly affected by the treatment scheme (Kruskal–Wallis test, $H=9.210$, d.f.=3, $P=0.027$, $N=27$–34; Fig. 2B). Specifically, bees with high forager age and free access to flight performed significantly poorer than the other groups (Mann–Whitney *U*-tests, Bonferroni corrected, $P<0.01$). The bees with similar high forager age that were restricted from flight opportunity, moreover, maintained a median learning ability that was indistinguishable from the two control groups with low forager age (Mann–Whitney *U*-tests, $P=0.329$). It has been
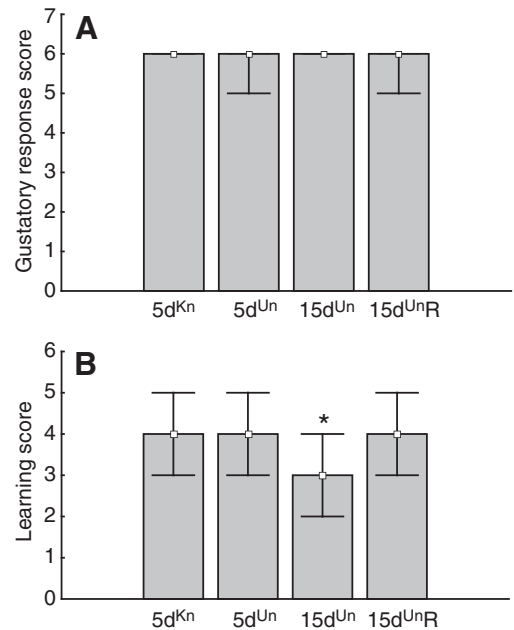

Fig. 2. Gustatory responsiveness and associative learning ability. Medians and 25–75% interquartile ranges are given for our second and main experiment. (A) Gustatory responsiveness measured with an ascending series of sucrose concentration. The sensory responses of individual bees were quantified as a gustatory response score (GRS), where high scores convey high gustatory sensitivity. No differences were detected between groups. (B) Pavlovian (associative) learning ability measured by an olfactory learning paradigm with 30% sucrose as reward. High learning scores signify good performance in the test. High forager age (~15 days) and free access to flight resulted in reduced learning ability. Sample sizes: 5d^Kn=29, 5d^Un=27, 15d^Un=31, 15d^UnR=34. Asterisk indicates significant difference by Bonferroni-corrected Mann–Whitney *U*-tests ($P<0.05$).

shown that associative olfactory and tactile learning performance declines in bees with high forager age (Behrends et al., 2007; Scheiner and Amdam, 2009); however, it was unclear whether this pattern emerged from flight activity or other factors inherent to the physiology of foragers. Our results are consistent with a causal connection between flight activity and brain ageing.

Total protein concentration of brain

With regard to group effects, we found that the total protein amount in the 1000 *g* supernatant fraction of the brain was the same between the treatment groups (one-way ANOVA, $F_{3,28}=2.515$, $P=0.079$, $N=8$; Fig. 3A). For the 1000 *g* pellet fraction, however, the total protein amount was significantly different (one-way ANOVA, $F_{3,28}=10.152$, $P<0.001$, $N=8$; Fig. 3B). *Post hoc* tests determined that the two groups with low forager age had significantly less total protein in this fraction than the two groups with high forager age (Fisher's LSD, $P<0.01$). Because the two groups with low forager age were characterised by different compositions of chronological age (known *vs* variable), and the two groups with high forager activity were characterised by different cumulative flight opportunity, this fraction-specific difference in brain protein concentration is best explained by forager age and not by the chronological age or flight activity of the bees.

Amount of 14–200 kDa proteins in brain

From gel separation and western blotting of the 1000 *g* supernatant fraction, we found that the total amount of protein visualised by
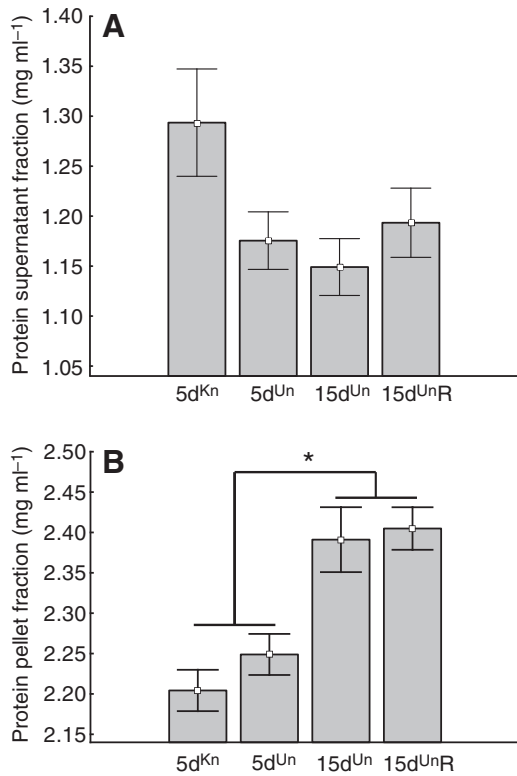
Fig. 3. Total protein amounts (mg ml^{-1}) in extracts of honeybee forager brains. Means ± s.e.m. are shown for (A) the 1000 $\boldsymbol{g}$ supernatant and (B) the 1000 $\boldsymbol{g}$ (insoluble) pellet fraction by Bradford protein-dye binding assay. The total protein concentrations of the pellet fraction increased at high forager age. Asterisk indicates significant difference by Fisher's LSD ($P<0.05$, $N=8$).

Sypro Ruby was significantly different between the worker groups (main-effects ANOVA, $F_{2,19}=3.987$, $P=0.036$, $N=8$; Fig. 4A,C). *Post hoc* tests revealed that this overall statistical difference was associated with forager age, not chronological age or flight opportunity; i.e. the groups with high forager age showed increased amounts of visualised protein in comparison with the groups of low forager age (Fisher's LSD, $P<0.05$). This result suggests that the protein matrix composition of the brain supernatant fraction changes with honeybee forager age. For all samples, the same amount of protein (30 µg) was loaded onto the gels, but the visualised amount in the 14–200 kDa range differed depending on the forager age of the bees. This effect was specific to the supernatant, as an equivalent analysis on the pellet fraction did not detect similar differences ($F_{2,15}=0.391$, $P=0.683$, $N=7$; Fig. 5A,C).

Brain MDA-protein adduct formation

For the 1000 $\boldsymbol{g}$ supernatant fraction, our analysis of variance-normalised MDA-stained protein bands (z-scores) showed that the total amount of MDA-protein adducts was significantly different between the treatment groups (main-effects ANOVA on band intensities, bees coded as a random variable, $F_{2,467}=7.032$, $P<0.001$, $N=8$; Fig. 4B,D). *Post hoc* analysis revealed that, when intensity values were normalised to the total amount of protein within the range of the gel (relative intensity, Fig. 4D), the bees with high forager age and free access to flight had lower MDA-protein adduct levels than the other groups (Fisher's LSD, $P<0.05$). The bees with similar high forager age but restricted flight opportunity had MDA levels indistinguishable from those of the two groups with low forager age (Fisher's LSD, $P>0.05$). This pattern of MDA-protein adduct formation was best explained by flight activity and resembled the treatment-specific patterns we had attained for associative learning (Fig. 2B *vs* Fig. 4D).

To determine how our correction for the total amount of visualised protein affected the data, we repeated the ANOVA for the net intensity values, i.e. for MDA-protein levels that were only corrected for background and variance-normalised for band identity (see Materials and methods). This test also detected a significant treatment effect in the supernatant brain fraction (main effects ANOVA, $F_{2,467}=11.418$, $P<0.0001$; Fig. 4E) but it did not detect patterns that were well-explained by either flight opportunity or forager age. The clearest result was that bees with high forager age and restricted access to flight had the largest amount of MDA-protein adducts in their brains (Fisher's LSD, $P<0.01$), whereas a general trend toward increasing levels with higher forager age was weakly suggested (see Fig. 4E for further patterns and details).

Treatment-specific effects were not detected when the same set of analyses was run for the brain pellet fraction, neither for relative intensities corrected for total amount of visualised protein (main-effects ANOVA on band intensities, bees coded as a random variable, $F_{2,267}=0.209$, $P=0.812$, $N=7$) nor for the net intensity values ($F_{2,267}=1.236$, $P=0.292$; Fig. 5B–E).

Brain Ub protein adduct formation

Our analysis of mono- and poly-Ub proteins did not detect large quantities of this form of protein adducts in any sample (Fig. 6A,B). Although levels were negligible in the insoluble 1000 $\boldsymbol{g}$ pellet fraction, the 1000 $\boldsymbol{g}$ supernatant showed quantifiable amounts. However, ANOVA failed to detect an influence of the treatment scheme for relative intensities (main effects ANOVA on band intensities, bees coded as a random variable, $F_{2,147}=1.000$, $P=0.370$, $N=8$; Fig. 6C) and for net intensities ($F_{2,147}=1.046$, $P=0.354$; Fig. 6D). Collectively, these results suggest that mono- and poly-Ub proteins do not accumulate in the honeybee worker brain during ageing.

DISCUSSION

This study demonstrates that flight restriction has a positive effect on learning performance as foraging honeybees age. More precisely, as determined by the ability foragers have to associate an odour with a sucrose reward, we established that bees with free access to flight declined in learning performance after ~15 days of foraging labour whereas bees that were foragers for the same amount of time, but restricted from flight, retained levels of brain function similar to that of bees with shorter foraging experience. Moreover, when analysing brain levels of total protein and two types of protein adducts, MDA and Ub, only one variable corresponded to the pattern of learning ability: the mean amount of MDA proteins in the soluble supernatant fraction relative to the total protein amount visualised for each sample. For this variable, the detected amount was lowest in bees with high forager age and free access to flight. Our remaining significant protein data were, in general, best explained by forager age – the exception being the net amount of MDA protein in the supernatant fraction, which was highest in the flight-restricted bees.

Our study did not monitor the feeding behaviour of the foragers. Foragers rely on a diet that is primarily composed of carbohydrate, but they also consume pollen-derived protein, fatty acids, lipids, sterols, vitamins and minerals that are fed to them by nest bees *via* trophallaxis (Winston, 1987). All of these substances can affect the brain (Fukui et al., 2002; Fitsanakis et al., 2009). Also, as shown
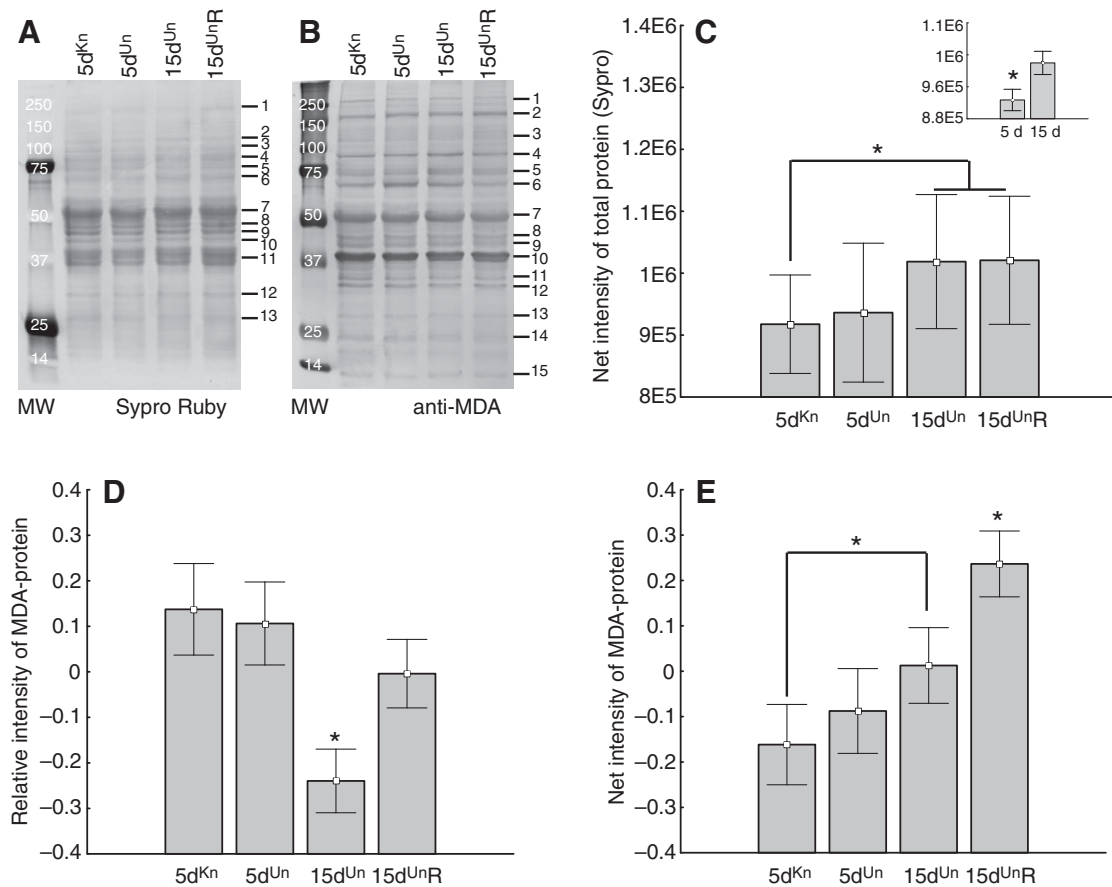
Fig. 4. Total electrophoretically separated proteins and anti-MDA staining after western blots on the soluble brain fraction. Examples of blots are given for (A) Sypro Ruby stained proteins (total soluble protein) and (B) anti-MDA stained proteins. MW, molecular weight. (C) Net intensity of total proteins within the 14–200 kDa range, as determined by Sypro Ruby staining. Inset: simplified comparison of bees of low (~5 days) vs high forager age (~15 days), with the largest quantities detected at high forager age. (D) Relative intensity of MDA-protein staining normalised to the total amount of visualised proteins and (E) net intensity of MDA-protein staining, demonstrating that the pattern of MDA detection is complex. Data in C–E are means ± s.e.m., N=8. Asterisks indicate significant differences by Fisher's LSD (P<0.05).

in *D. melanogaster*, dietary protein and carbohydrate can have opposing effects on insulin-like signalling cascades that influence a plethora of processes including metabolism, growth, longevity, learning and memory (Zhao and Alkon, 2001; Buch et al., 2008). However, although we do not provide a direct measure of feeding behaviour here, gustatory responsiveness was equal between the treatment groups. Variation in honeybee gustatory responsiveness has been linked to many factors including satiation and food-related behaviour (Ben-Shahar and Robinson, 2001; Pankiw and Page, 2001). Therefore, we suggest that feeding patterns and related social processes were largely similar in this study, and were thus not likely to influence the results.

Our data on associative learning performance answer the question of whether rates of functional brain ageing in honeybee foragers are best explained by physiology or flight activity. Flight activity *per se* is not required for foragers to maintain their caste-specific physiology (Toth and Robinson, 2005). Accordingly, if physiology were the driver of senescence, learning deficits would be equally present at high forager age regardless of the flight-restriction scheme. This was not what we observed. Instead, our results establish the connection to flight activity and suggest that foragers can retain brain function for an extended period, i.e. beyond 15 days of foraging labour, when natural foraging opportunities of colonies are few. In honeybee workers it is already established that

unfavourable environmental conditions may favour sustenance over senescence through adaptive responses and prolong life. More precisely, in winter, honeybee workers survive as *diutinus* or 'winter bees', which have elevated levels of lipids and protein and live for up to 280 days with negligible signs of senescence (Koehler, 1921; Maurizio, 1954; Sekiguchi and Sakagami, 1966; Fluri et al., 1977; Shehata et al., 1981; Fluri et al., 1982; Amdam and Omholt, 2002; Seehus et al., 2006a). Accordingly, in a wider context, our findings relating to the effects of artificial rain and limited foraging opportunities may therefore indicate that similar adaptive responses can also be utilised by honeybee colonies during summer in response to environmental cues to delay functional decline in workers. This is in line with previous studies that highlight ageing and functional decline in bees as a plastic and mouldable process; old foragers that revert to nursing activities partly regain physiological and cognitive functions and have a protein profile that is a hybrid between the proteome of nest bees and foragers (Amdam et al., 2005; Seehuus et al., 2006a; Wolschin and Amdam, 2007b; Baker et al., 2010). Alternatively, although our data on MDA-protein adduct levels point to the contrary, old foragers that undertake fewer foraging flights may also have more energy to invest in repair and maintenance – perhaps explaining why they are superior in learning to same-aged foragers that have been foraging more actively. The prediction that environmental factors such as rain and prevention
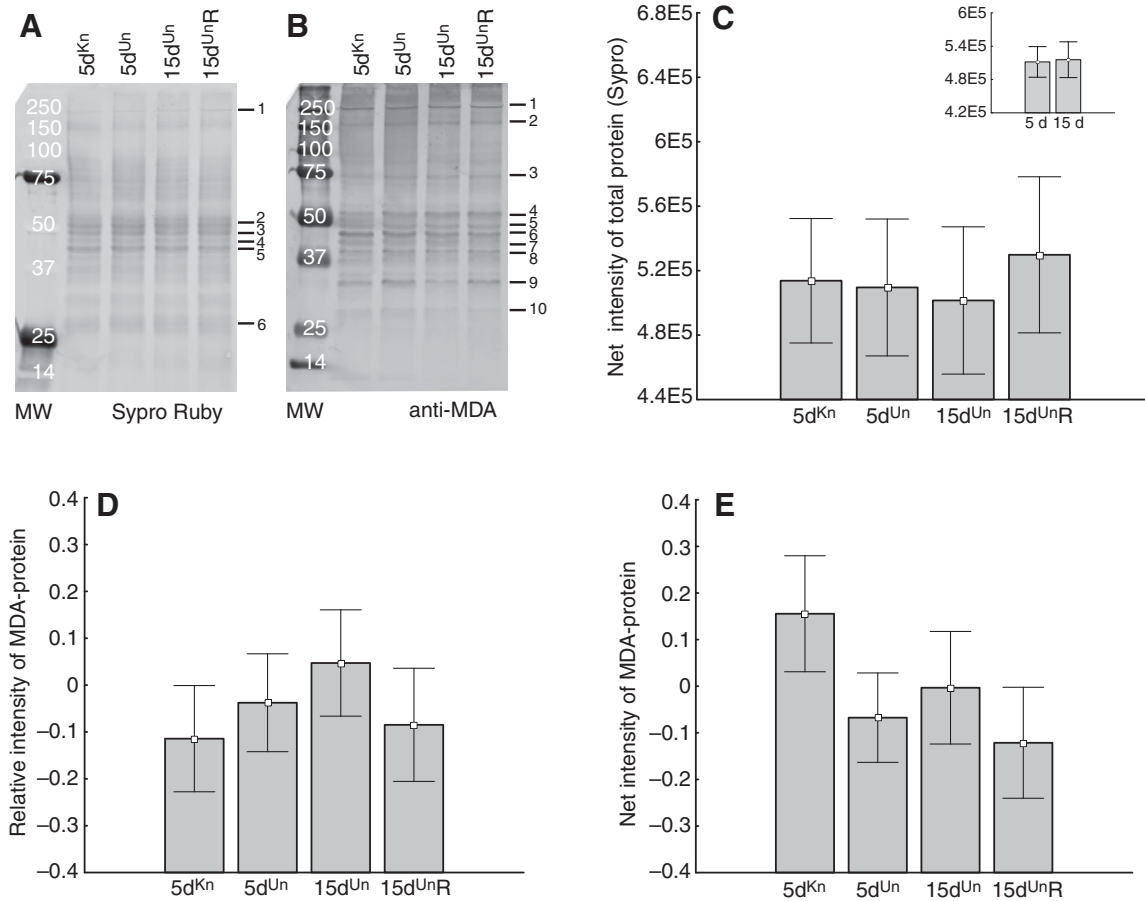
Fig. 5. Total electrophoretically separated proteins and anti-MDA staining after western blots on the insoluble (pellet) brain fraction. Examples of blots are given for (A) Sypro Ruby stained proteins (total protein separated from the pellet) and (B) anti-MDA staining. (C) Net intensity of total proteins within the 14–200 kDa range, as determined by Sypro Ruby staining. Inset: simplified comparison of bees of low forager age (~5 days) vs high forager age (~15 days), with no differences detected between groups. Differences were also not identified for (D) the relative intensity of MDA-protein staining normalised to the total amount of visualised proteins and (E) the net intensity of MDA-protein staining. Data in C–E are means ± s.e.m.; N=7.

from foraging favour sustenance in old worker bees, including underlying biochemical changes, importance of social parameters as well as putative consequences for colony fitness, can now be tested with new experiments.

In our study, high forager age conferred a significant increase in total amounts of insoluble (pellet) brain proteins, as measured by the Bradford protein-dye binding assay, in addition to higher amounts of soluble (supernatant) proteins detected in the 14–200 kDa range. These results suggest that the honeybee brain's protein metabolism and/or its localisation of intracellular proteins changes at high forager age (Alaux et al., 2009). In this context, accumulation of mono- and poly-Ub protein adducts could point to age-dependent deficits in metabolic turnover processes that involve the lysosome and proteasome complex (Martinez-Vicente et al., 2005; Simonsen et al., 2008). However, our experiment did not reveal significant changes in the level of Ub protein adducts. The increase in total insoluble brain proteins, as well as higher soluble amounts in the 14–200 kDa range, therefore, is unlikely to indicate that bees with high forager age have deficient systems for brain protein degradation/turnover. Perhaps the results rather reflect on previously established patterns of maturational brain plasticity, including brain growth, or other maturational changes in the brain metabolism of the foragers (Fahrbach et al., 1995; Fahrbach et al., 2003; Alaux et al., 2009).

Oxidative damage to intracellular macromolecules such as formation of MDA-protein adducts is considered a marker of ageing (Stadtman and Levine, 2000; Levine and Stadtman, 2001) and, accrued over a lifetime, it causes age-related functional impairment and death (Harman, 1956). The central nervous system (CNS), and especially the brain, is a sensitive target for reactive oxygen species (Mattson and Magnus, 2006; Sayre et al., 2008). During vertebrate and invertebrate ageing, CNS defence and repair mechanisms are overwhelmed by oxidative insults, leading to impaired memory, learning and sensomotoric function (reviewed by Mattson and Magnus, 2006). Physical activity may slow CNS ageing through hormesis, a process that stimulates beneficial cellular stress and repair processes involving cytokines and growth factors (Mattson et al., 2004; Cotman et al., 2007; Radak et al., 2008; van Praag, 2009). Immobility or overtraining, however, can be harmful, and during short bursts of intense activity, compensatory mechanisms may not activate and oxidative damage is high (Radak et al., 2001; Nybo and Secher, 2004; Rosa et al., 2007). In flying insects, like other organisms, ageing is classically associated with augmented oxidative stress and manifestations thereof (Sohal, 1981; Sohal et al., 1981; Agarwal and Sohal, 1994; Yan and Sohal, 2000; Magwere et al., 2006; Toroser et al., 2007; Simonsen et al., 2008), including oxidative damage in the brain (Sohal and Donato, 1979). However, the non-additive effects of exercise can make it difficult to
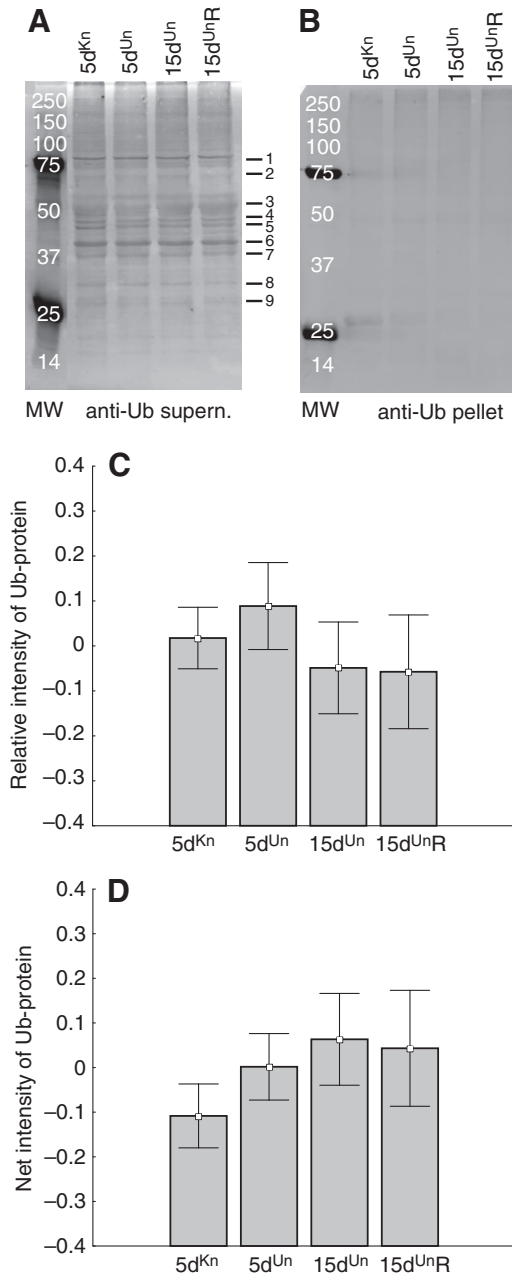
Fig. 6. Levels of mono- or poly-ubiquitinated (Ub) proteins. Examples of blots are given for (A) the soluble supernatant fraction and (B) the insoluble pellet fraction. The staining patterns of the pellet samples were not quantified due to low intensities. (C) Relative Ub-staining intensity in the supernatant fraction normalised to the total amount of visualised proteins. (D) Net Ub-staining intensity. No significant changes were detected for Ub. Data in C,D are means ± s.e.m.; N=8.

generalise about the beneficial effects of physical activity on the ageing brain.

The data presented herein shows that flight restriction in old forager bees is accompanied by a paradoxical increase in secondary protein damages resulting from lipid peroxidation. Moreover, surprisingly and seemingly contradictory to the oxidative stress theory of ageing (Harman, 1956), the learning deficits of the bees with ~15 days of free access to flight were not accompanied by a similar increase in the MDA-protein adduct levels of the brain. This result was independent

of whether the data on relative or net MDA amounts were considered. For the former measure, i.e. corrected by the visualised amount of electrophoretically separated proteins, the senesced group had the lowest MDA levels. For the latter measure, the flight-restricted group showed significantly higher MDA levels. Differences were only detected in the $1000g$ supernatant fraction of the brain, which contains intact organelles such as mitochondria in addition to water-soluble cytosolic proteins. Thus, we suggest that the complex pattern of MDA detection in this study points to changes in the biochemistry of the honeybee brain, as supported by the diverging amounts of separated 14–200 kDa proteins in the same samples.

Our results on MDA-protein adducts in old forager brains is surprising relative to previous findings of increased concentrations of thorax fluorescent pigment and brain carbonylated proteins in honeybee foragers compared with flight-inactive nest bees (Young and Tappel, 1978; Seehuus et al., 2006a), which suggested that flight activity in bees, like in other insects, can be associated with oxidative damage, including lipid-peroxidation-induced changes to proteins. However, lack of correlation between oxidative stress and functional senescence has been demonstrated previously in *D. melanogaster* (Cook-Wiens and Grotewiel, 2002) and rats (Ogonovszky et al., 2005), and although much data link oxidative stress to lifespan, the relationship between oxidative stress and age-related functional decline remains somewhat ambiguous. Based on this study alone, it cannot be firmly excluded that MDA-protein adduct levels were not influenced by factors other than rain and access to flight. For instance, we did not control for any non-foraging behavior or social factors such as the activity level of individual bees and their location in the hive (Riessberger and Crailsheim, 1997; Stabentheiner et al., 2010; Amdam, 2010). Still, in light of apparent discrepancies from the oxidative stress theory of ageing in previous studies with *D. melanogaster* (Cook-Wiens and Grotewiel, 2002) and rats (Ogonovszky et al., 2005), it is nonetheless plausible that long foraging experience in honeybee workers induces learning impairment through mechanisms that do not directly involve oxidative stress or that an acclimatisation response to foraging activity can enhance protection toward lipid peroxidation and resulting levels of protein damage.

Interestingly, it was recently suggested that fatty acid (FA) composition is a key driver of species differences in longevity (Hulbert et al., 2007). Caste differences in the level and ratio of monounsaturated (MUFAs) to polyunsaturated FAs (PUFAs) were also demonstrated in honeybees, and were postulated to explain the up to 30-fold longevity difference between workers and queens (Haddad et al., 2007). FAs differ in their susceptibility to oxidative stress: although PUFAs are prone to oxidation, MUFAs and saturated FAs are highly resistant. Thus, the lower the degree of FA saturation, the more susceptible tissues are to lipid peroxidation and secondary detrimental effects (Haddad et al., 2007). The brain is rich in fat, and FAs can influence learning and memory performances (Yehuda et al., 2005). This effect is seen in rats fed a diet depleted in n-3 PUFA, which decreases the level of docosahexanoic acid (a long-chain n-3 PUFA) in the brain as well as the animals' performance in olfactory discrimination tasks (Greiner et al., 2001). Docosahexanoic acid depletion alone also affects the velocity by which rats learn to discriminate odours (Hichami et al., 2007). Thus, although this is not the only plausible explanation (Rikans and Hornbrook, 1997; Das, 2003), we propose that changes in tissue FA composition may account for why functional brain aging is not associated with high MDA levels in bees. Specifically, we hypothesise that flight activity increases the FA saturation level of the honeybee brain. This lowers the tissue's peroxidation index and

limits oxidative damage, but could also impair olfactory learning performance by altering CNS properties.

This study presents new information on how the honeybee worker brain is influenced by flight opportunity and forager age and demonstrates the utility of the honeybee as a model with which to study the relationship between ageing and physical exercise. In people, connections between physical exercise, workload, health and late-life cognition are complex and often influenced or obscured by genetic and environmental factors (Herrmann and Guadagno, 1997; Harris et al., 2007; Fors et al., 2009). Model organisms such as the honeybee can offer better access to underlying cause and effect relationships. Related questions to those posed in this study were asked in insects before (Sohal and Donato, 1979; Yan and Sohal, 2000; Magwere et al., 2006), but the bee offers opportunities for individual-level analyses that are not equally available to *M. domestica* and *D. melanogaster*. Honeybee flight activity can be manipulated in the animal's natural environment, and individual brain function can be tested in the laboratory and field with established paradigms. Furthermore, the relatively large size of the bee enables molecular analysis of tissues without extensive pooling of the biological material. These properties, in addition to the rich social biology of the honeybee model, provide a resource for future studies of how physical activity affects the aging brain.

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CNS	central nervous system
CS	conditioned stimulus
FA	fatty acid
GRS	gustatory response score
MDA	malondialdehyde
MUFA	monounsaturated fatty acid
PBS	phosphate buffered saline
PBS-T	PBS with 0.05% Tween-20
PER	proboscis extension response
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene fluoride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ub	ubiquitin
US	unconditioned stimulus

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