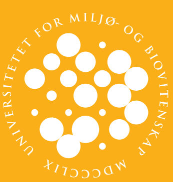
UNIVERSITETET FOR MILJØ- OG BIOVITENSKAP



Abstract

Growing old is often related to a decline in physiological function, performance, survival or reproduction.(Kirkwood 2002) But studies among animals have showed that an increased chronological age doesn't automatically leads to these physical changes.(Rueppell, Christine et al. 2007) Honeybees (*Apis mellifera)* has showed a remarkable plasticity of aging with queens having an average lifespan of 1–2 years, while workers have an average lifespan of 140 days in the winter and 15–38 days in the summer(Munch and Amdam 2010).

Regarded as a marker of cellular aging, lipofuscin, a fluorescent non-degradable material, will be expected to accumulate in the cells of aging animals(Gray and Woulfe 2005; Double, Dedov et al. 2008).

By obtaning bees of the same age, and manipulating them into different social roles in the hive, this study could observe how the accumulation of lipofuscin can be a process controlled by other factors then just increased chronological age. Age-matched bees from with different social roles in the hive were sampled, and using a Leica SP5 Confocal laser scanning microscope, it was possible to create images that enabled the quantification of lipofuscin within the tissue samples from the bees.

The results showed a rapid accumulation pattern of lipofuscin among bees that had been engaged in foraging activities for more than 13 days, with the bees that was engaged in nursing activities showing no such accumulation. This illustrates the strong effect of these social factors among honey bees, and with studies on functional senescence that show significant decline patterns for older forager groups after about 2 weeks of foraging(Behrends, Scheiner et al. 2007; Williams, Roberts et al. 2008), this study suggests that the accumulation of lipofuscin is more related to the onset of senescence, then just being a harmless wear-and-tear product purely related to the chronologically age of an animal.

Sammendrag

Økt alder er ofte forbundet med en nedgang i fysisk form, overlevelsesevne og repoduksjonsevne. (Kirkwood 2002) Men studier av dyr har vist at økt kronologisk alder ikke nødvendigvis fører til disse fysiske forandringene. (Rueppell, Christine et al. 2007) Honningbier (*Apis mellifera)* har vist fleksible aldrings monster, hvor dronningen kan ha en levealder på 1 til 2 år, mens arbeiderene har en gjennomsnittlig levealder på 140 dager om vinteren og 15 til 38 dager om sommeren(Munch and Amdam 2010). Det ikke-nedbrytbare autofluoriserende stoffet lipofuscin, blir ofte referert til som en aldersmarkør, og vil hos dyr som blir eldre forventes å øke i mengde.(Gray and Woulfe 2005; Double, Dedov et al. 2008). Ved bruk av bier med samme alder, og ved å manipulere disse til å innta forskjellige sosiale roller i en bikube, tok denne studien for seg hvordan oppsamling av lipofuscin i cellene kan bli styrt av andre prosesser enn bare økt kronologisk alder. Prøver fra bier som hadde hatt forskjellige roller i kuben, men som hadde samme alder, be innhentet og ved bruk av Leica SP5 Confocal laser scanning microscope kunne det skapes bilder hvor akkumuleringen av lipofuscin kunne studeres.
Resultatene viste et raskt akkumuleringsmønster blant bier som hadde hatt en rolle hvor de jobber primært utenfor kuben (samlebier) i mer enn 13 dager. Mens bier som jobbet inne i kuben viste ingen slike tegn til akkumulering.
Dette illustrerer den sterke effekten de sosiale rollene i en bikube kan ha. Og sammen med studier hvor man har sett en nedgang i fysisk form blant bier som har vært samlebier i to uker(Behrends, Scheiner et al. 2007; Williams, Roberts et al. 2008), indikerer dette at akkumuleringen av lipofuscin henger mer sammen med prosesser i kroppen som dog ofte inntrer når man blir eldre, enn bare med den kronologiske alderen til et dyr.

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1. Introduction

Senescence is often referred to as the process of an age related decline in physiological function, performance, survival or reproduction(Holliday 1997; Kirkwood 2002)
But the onset of senescence, and when an individual is considered old varies greatly among animals, and is a regulated process that is not a homogeneous development within a population. Among honey bees, *Apis mellifera,* the queens have an average lifespan of 1–2 years, while workers have an average lifespan of 140 days in the winter and 15–38 days in the summer(Munch and Amdam 2010). The lifespan and the biological differences between these two castes are determined by social and dietary factors that the individual bees are exposed to during their development (Corona, Hughes et al. 2005).
The biological processes that lead to senescence and the differences of when individuals develop these symptoms are a long lasting problem are major research topics in gerontology, molecular and evolutionary biology (Kirkwood 2002; Remolina, Hafez et al. 2007)
The reasons for aging and senescence are many, and include the accumulation of genetic damage or mutations in genes, chromosomes, and mitochondria, cross-linking of collagen and elastin, damage by reactive reactive oxygen species (ROS), abnormal modifications of proteins and accumulation of insoluble aggregates, a decline in the immune functions and autoimmunity, hormone imbalance and a decline in homeostasis, and the deposition of lipofuscin and advanced glycation endproducts (Terman and Brunk 2004; Holliday 2006; Rajawat, Hilioti et al. 2009). All these factors can either by themselves or in combination with each other lead to a dysfunction or complete failure of the major organ systems. When this happens in the heart, the brain, or other crucial organs, death can be imminent, or it can lead to more moderate loss of fitness by the organism. The onset of senescence therefore has multiple causes, and the progression of senescence have shown to be variable not only in individuals among the same species, but also in the organs and tissue within an organism, and in the cell types within the tissue (Rattan 2006).

1.1 The honey bee (Apis mellifera)

The social society of the honey bee is a well known model for system theory, behavioral ecology, molecular biology, neurobiology, and aging (Page and Erber 2002; Keller and Jemielity 2006; Wolschin, Munch et al. 2009)

Eusocial insects are characterized by society were the individuals belong to different castes. The two castes in a honey bee population are the queen caste that is reproducing and the functionally sterile non-reproductive worker caste. The latter will gather and process food, care for the young, build and maintaine the nest, and defend it. Notably, these two castes, with highly differentiated phenotypes, will develop among sibling (Amdam and Seehuus 2006; Honeybee Genome Sequencing 2006).

The worker caste can be divided into two different sub-castes; the long-lived winter bee, and the shorter lived summer sub-castes that include nurse bees and the forager bees.

The summer bee will have a normal life span of about 25 to 35 days, whereas the winter bee can live up to 6 to 8 months.(Amdam and Omholt 2002).

The nurse bee will perform tasks within the hive, such as brood care or ‘‘nursing”, and hive maintenance. The winter bee also develops from young nurse bees during periods when brood rearing is ceasing.Foragers will normally develop from older nurse bees, and will be performing tasks outside the hive, collecting pollen, nectar, water and plant resins (propolis).

Bees that change from nursing to foraging, will not just change their social role, but will also undergo physically changes.(Seehuus, Norberg et al. 2006; Rueppell, Bachelier et al. 2007)

When the worker is in its nurse stage, it will use a set of paired head-glands called the hypopharyngeal glands to produce a protein rich jelly that is used for brood rearing(Kubo, Sasaki et al. 1996), while the foragers will secrete alpha-glucosidase, which converts nectar into honey. The foragers will also secrete low levels of digestive endopeptidases, and thereby not be able to obtain nutrients from pollen on their own. The forager bee will therefore be depending on nurse bees to feed it. Furthermore, in the forager bee, the hypopharyngeal glands as well as the abdominal fatbody and the circulating hemocytes will undergo an atrophic process during transition from the nurse to the forager stage (Amdam, Aase et al. 2005). The hypopharyngeal glands consists of a long slender main channel with alveolar clusters of glandular secretory cells. There are approximately 550 of these alveolar units associated with each of the channels. These alveolar clusters, known as acini, consist of 8-12 glandular cells, which are each connected to a duct cell. These duct cells will form a bundle, and connect the secretory cells with the main channel. In the junction between the duct cells and the glandular cells, there is an end apparatus which is a specialised structure that allows secretions to leave the glandular cells. This secretion will be transported further by the duct cell into the main channel. There are also several tracheoles present between the secretory cells within each acinus, to provide for oxygen and carbon dioxide exchange. The two main channels open up into the suboral plate of the hypopharynx, so that the secretion ends up being released trough the mouth of the bee (Silva de Moraes and Bowen 2000; Deseyn and Billen 2005).

The secretion by the HP glands is believed to not only differ between the nurse bees and the foragers, but also between nurse bees of different age. Younger nurse bees will secrete royal jelly, which is fed to the queen and larvae. While older bees will secrete a enzyme rich secretion that are mixed with pollen to provide the amino acid needed by the foragers (Silva de Moraes and Bowen 2000). The activity of the HP gland has also shown to be related to the feeding of larvae. Studies have shown that nurse bees with larvae to feed will have a higher activity in their HP glands, and can maintain this activity for three days after the removal of larvae, while the presence of eggs and pupae did not have the same effect. (Holliday 1997)

A nurse bee's shift from nursing to foraging duties is motivated by social signals. These signals will create a negative regulatory feedback loop between the *vitellogenin* gene and the juvenile hormone. This hormone is suppressed by the activity of the *vitellogenin* gene, while the bee is a nurse. Vitellogenin is produced in the fat body, and the reduced signalling from it will lead to an increase of the juvenile hormone. This causes an endocrine signal feed that further inhibits the synthesis of vitellogenin protein, which in turn also affects the hypopharyngeal glands.(Amdam and Seehuus 2006)

The transformation from nurse to forager is an age-dependent role-change that occurs among honey bees, where nurse bees change to foraging at an age of about 14 days. However, this transition can occur earlier (precocious foraging) or much later, when nurse bees continue with nest tasks for more than 30 days (over-aged nurse bees). This is a flexible system, that also largely depends on colony demands.(Kubo, Sasaki et al. 1996)

Several studies have been performed where there have been created single cohort colonies (SCC). This involves hives where all the bees have the same chronological age.(Powell, Wang et al. 2005; Behrends, Scheiner et al. 2007; Remolina, Hafez et al. 2007; Dukas 2008) This manipulation of the bee society forces some of the bees to start foraging at an earlier age than what they would normally do, because of the lack of older nurse bees that would normally transform into foraging. By the creation of SCC's and the use of this social manipulation, it has been possible to obtain foragers and nurse bees of similar age, thus creating the opportunity to study aging symptoms among bees of similar chronological age, but with different social roles. Several factor concerning aging and physical changes in bees have been studied, including; olfactory learning performance, body mass, the production of reactive oxygen species and oxidative carbonylation, which all showed a greater dependency towards social role than to chronological age(Suter. M, Reme. C et al. 2000; Seehuus, Krekling et al. 2006; Rueppell, Bachelier et al. 2007; Williams, Roberts et al. 2008)

There have also been reversion studies, were the foragers have been separated from the nurse bees. In hives consisting of only foragers, the lack of nurse bees will force some of the forager to

revert back to nursing (Amdam, Aase et al. 2005). The bees' transition from nursing to foraging, as former studies have shown, increase the amount of juvenile hormone and reduces the bees immunity by extensive hemocyte death. When the bees reverted back to nursing, a drop in the endogenous juvenile hormone titer and an increase in the hemolymph vitellogenin level were observed. The proliferation of new cells also participated in restoring the bees' immunity in this study.

1.2 Lipofuscin

Cellular degradation processes, will normally provide a continuous turnover of cellular components, but deficiencies in this processes, or an increased production of damaged or poorly functional structures, can lead to a build up of this structures within a cell (Terman and Brunk 2004). This structures includes altered macromolecules, damaged mitochondria and other organelles.(Brunk and Terman 2002) And as an organism becomes older, the amount of such structures in a cell will often increase.

Another feature of this build up of such material, is the accumulation of a fluorescent, non-degradable material within intracellular granules, called lipofuscin(Szweda, Camouse et al. 2003; Terman and Brunk 2004; Jung, Bader et al. 2007). As the cells have no way of degrading this material, the concentration of lipofuscin within a cell can only be diluted, by the division and/or growth of a cell.(Gray and Woulfe 2005). Originating from a variety of intracellular structures, the lipofuscin will often accumulate at the primary site of waste disposal, the lysosome .(Brunk and Terman 2002) The lipofuscin is a chemically and morphologically polymorphous material, and has in many cases been considered a harmless wear-and-tear product in many different organisms(Terman and Brunk 2004) Lipofuscin has been described as age dependent fluorescent intracellular granules, and as an hallmark of aging, it has an accumulation rate that inversely correlates with longevity(Szweda, Camouse et al. 2003; Terman and Brunk 2004) The accumulation of lipofuscin is also being used to estimate the age of certain organism, as the build-up of lipofuscin in many cases are considered to be associated with the chronological age of the organism. With a nearly linear accumulation of lipofuscin with increasing age in the nervous systems among some creatures in the wild,(Gray and Woulfe 2005) this has proven in some cases to be a more accurate method of age determination than a body size-based method (Belchier, Edsman et al. 1998)

The term lipofuscin represents granules enclosed by a single membrane (Szweda, Camouse et al. 2003) with a mixture of different chemical substances that may vary between cell types.(Szweda,

Camouse et al. 2003; Terman and Brunk 2004; Jung, Bader et al. 2007) With a lack of a defined substance with a specified chemical composition(Yamada, Kumazawa et al. 2001), the properties of lipofuscin are variable within certain limits.(Terman and Brunk 1998)
It mainly consists of lipid degradation residues(19–51%) and oxidatively modified proteins(30–58%), in which the latter shows a variable content of amino acids, while the lipid components mostly consists of triglycerides, free fatty acids, cholesterol and phospholipids.(Terman and Brunk 2004; Jung, Bader et al. 2007)
As a marker of cellular aging, the build up of lipofuscin would be expected to increase as an organism gets older. (Gray and Woulfe 2005; Double, Dedov et al. 2008) But as several studies have shown, the aging plasticity among bees has great impact on several physiological changes in bees.(Amdam, Aase et al. 2005; Seehuus, Krekling et al. 2006; Dukas 2008)
In this study we aim to test if the accumulation of lipofuscin in the hypopharyngeal glands in bees is solely a direct result of an increasing chronological age, or can be explained as a function of the bee's social role in the colony.

2 Methods

2.1 Marking of newly emerged bees from brood combs

To obtain worker bees of the same chronological age, frames of sealed brood that was about to emerge were collected from several different hives. These frames were placed in ventilated boxes and placed in an incubator at 34 ºC and with 60-70 % relative humidity. When the bees emerged, they were brushed of the frames into a trey and marked individually on their dorsal thorax with a bullet tip pen (Uni POSCA, Mitsubishi Pencil Co. Ltd.), with a specific color representing that date. This color would later allow to identify the bees belonging to a specific single age cohort (SC), by the day of emergence. The bees were also checked for mites, *Varroa destructor,* to avoid infesting the hives. Bees that were infected with these mites were excluded from the study.
Marked bees were placed into new combs containing honey, and stored in the incubator. This marking were performed over a period of 30 hour, after which the combs were placed in a hive outside.

In each hive there were added 3000 unmarked young nurse bees, in addition to the 3000-4000 marked bees. The unmarked bees were nurse bees obtained from other hives, and would typically be older than the marked bees. These unmarked bees were added to increase the total size of the hive, making it more robust and relieve some of the pressure on the youngest SC bees to begin foraging.
In each hive a mated queen was also confined in a cage sealed with edible candy. This would enable the worker to slowly eat its way through the candy thus releasing the queen. This would also make the workers used to the presence of the queen, and not rejecting her.
The three hives were placed at the outskirts of the campus area at UMB, on a single line, approximately half a meter apart of each other.

2.2 Marking forager bees

The hives were observed daily for foraging activity of SC bees. To not risk marking nurse bees as foragers, marking would not be performed when orientation flight was observed. When orientation flight was no longer observed, marking would resume. Parts of the entrances were blocked with rubber foam so that foragers returning to the hive with nectar or pollen only had a five centimeters opening to enter the hive. This made it possible to catch the bees with forceps. When returning from their first foraging flight, the bees were caught and would receive an additional paint mark.
Over time, foragers will vanish and the number of foragers will decline if nurse bees do not transform into foragers. The group of foragers from which the samples were obtained, could not be reinforced by new foragers, and had to be large enough to ensure that there were bees to sample from on the last sample day (17 days of foraging). The rate in which nurse bees differentiate into foragers may vary, (Amdam and Seehuus 2006), so continuous observation and marking were performed until the number of newly observed forager on one day exceeded 500. Bees were collected every fifth day, from inside the hive. Bees with only one marking representing the their chronological age, which inserted their heads into cells feeding and cleaning the larvae, and didn’t fly up when touched by a forcep, were considered nurse bee. Bees that had two markings on them were considered forager. The bees were collected using forceps and put in a ventilated box.

From each sample day, a minimum of 8 and a maximum of 14 bees from both groups were obtained, resulting in a total of 16 to 28 bees collected on each sample day.

2.3 Sample preservation

The boxes that contained the bees were placed in a refrigerator for <15 min (until the bees showed no activity). This will work as an anaesthetic on the bees (Correa-Fernandez and Cruz-Landim 2010) .Then the boxes were placed on ice for another 15 min.

The head of the bee were cut of using a scissor (the headless body of the bee was placed in a trey, since it could still become active, and thereby sting). Then the heads were placed in a tube with 10 ml 4% paraformaldehyd in phosphate-buffered saline (Ph 7,2). All work with paraformaldehyde was performed at a ventilated station. Each tube would contain no more than six heads, and the tubes would be placed on a shaker to make sure the heads were always covered in paraformaldehyde, and then stored in a refrigerator over night. After one night, the paraformaldehyde was replaced with PBS. The tubes were then placed back on the shaker in the refrigerator for 25 min. This was repeated two more times, so that the heads were rinsed 3 x 25 min in PBS. Then the PBS was replaced with 30% sucrose, and stored in a refrigerator over night. For long term storage, the heads would after one night in 30% sucrose be placed in Eppendorf tubes and covered with Tissue-Tek O.C.T. (Miles Laboratories, Elkhart, IN, USA). Then the tubes were placed in a –80°C freezer. Upon use, the frozen heads were thawed in a refrigerator, and placed in PBS to rinse of remaining Tissue-Tek.

By using a forcep, it was possible to grab the tongue (proboscis) of the bee and holding the head in place.

The head of the bee was opened by first making an incision above the antennas from the right eye to the left eye. Then another incision was made along the left eye. A third incision was made parallel to the first one, but behind the ocelli. And a last incision along the right eye down towards the first incision. When removing the cuticle, it was important to make sure that any parts of the brain or glands weren’t attached to the cuticle. On the other hand, a thin white layer would often be covering the inside of the cuticle.

Fig 1: The head of a bee. The ocelli can be seen on the top of the head.

The glands were extracted using forceps, and placed in PBS. The solution was gently stirred, so not to destroy the glands, before the glands were placed in 50% sucrose on a microscope slide, and covered with a cover glass. The edges on the cover glass were sealed with nail polish. The microscope slides with the samples were then placed on a tray covered with tinfoil, to avoid exposure to light, and stored in a refrigerator.

2.4 Microscopy

For scanning the gland samples, I used a Leica SP5 Confocal laser scanning microscope. Different settings on the laser or the detector may affect the detected size of a lipofuscin granule in a scanned image. It was therefore essential to establish fixed settings for laser power and detector sensitivity. These settings had to be able to cover all of the samples, ranging from specimen with small amounts of lipofuscin and little signal, to specimen with high amount of lipofuscin. The depth and the number of sections of the scan also had to be fixed. Pictures were taken from defined regions and depth levels, with the main duct clearly visible. Also, to reduce any bias caused by potential day-to day technical variations, a balanced number of samples from all test groups were scanned every session.

When the samples were scanned for lipofuscin it was used a sequential scan. Lipofuscin was detected using laser lines and detectors set to λ=514nm and 561nm, and 570-650nm accordingly. This scan will also reveal autofluorescent trachea and other non-granular structures. By including a second channel with a shorter wavelength, the lipofuscin was not detected on this scan. Comparing signals from both channels allowed to distinguish the lipofuscin from other unspecific background signals.

Both channels used the same setting for x,y and z. The logical size of x and y was 1024µm, while the physical size was 154.58µm. I used 11 z steps with a total physical size of 10,07µm. All

specimens were scanned at similar depth levels with the main duct clearly visible. This was done to avoid that any uneven lipofuscin accumulation in different areas would bias the measurements. The first channel included a laser at 405nm at 30%, and a photon detector (PMT 1) ranging from 410nm-450nm. This detector had a gain at 791. The second channel had two lasers λ= 514nm and 561nm, both at 30%. It had a detector (PMT 3) ranging from 570nm-650nm, with a gain at 701.I used a 40ximersion lens, and a zoom at 2, 5.

For scans that later were used to assess acini sizes, the settings for the laser power and detector sensitivity were also fixed. Three lasers were used; 405nm at 30%, 514nm at 40%, and 561nm at 40%. And with two detectors active; PMT 1 (410nm-450nm) with a gain of 790,8, and PMT 3 (570nm-650nm) with a gain of 695,7. Each z-step size was fixed at 4,99 but the amount of z steps were adjusted for each time, so that the scan would cover the maximum diameter of the acini. The number of z steps ranged from 4 to 14. I used a 20x lens, a zoom of 1 and a pinhole size of 60,7

2.5 Image analysis

Image processing was performed using ImageJ. (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/). From the scanning procedure, there were produced 11 images x six frames from each 40x scans, and a varied number of images x six frames from the 20x scans. Images belonging to the same frame from each scan were added on top of each other to create one hyperstack/image (2D maximum projection). A gaussian filter of 5 were applied to all pictures to minimize noise. Two similar sized regions of interest were analysed per image.

From the LF granule situated most to the left in each half of the picture, a straight line was drawn to the nearest LF granule to the right. The line from one granule to the next would always be going from the left to the right, thus preventing that the selection of LF granules being dominated by clusters of densely packed granules, with the line going in circles. The line was always made edge to edge, and the shortest nearest-neighbour distance was chosen. Only distances inside the acini were measured. If the line crossed the central duct or the area between the acini, the distance would be discarded.

The lipofuscin granules that were included in the distance measurement were also used in the size measurement, starting from the right, and following the distance line to the left. The size of each lipofuscin granule was outlined and the program would automatically calculate the area of the granule. After all images were analysed, the identity of the individual were revealed, and the data were sorted accordingly. This procedure were also used to measure the size of the acini. The

data-values from each individual were then presented by their 1. 2. and 3. quartile value. These values would then be used in the statistical analyses.

3 Results

This result sections consists of three parts. The first two parts address parameters (lipofuscin granule size and density) that describe lipofuscin accumulation in foragers and nurse bees of different age groups. The third part tests for possible differences in acini size among foragers and nurse bees.
For all three parameters data is given for age-matched foragers (F) and nurse bees (N). This means that each group of forager had a nurse group with the same chronological age. Each test group consisted of five individuals n=5. The groups were categorised by the number of foraging activity days according to the forager group.

3.1 Lipofuscin granule size

First I asked if lipofuscin accumulates in the hypopharyngeal glands over time by examining the lipofuscin granule size. I also addressed the question of a possible accumulation could be related to the chronological age or the worker role of the bee (forager/nurse)

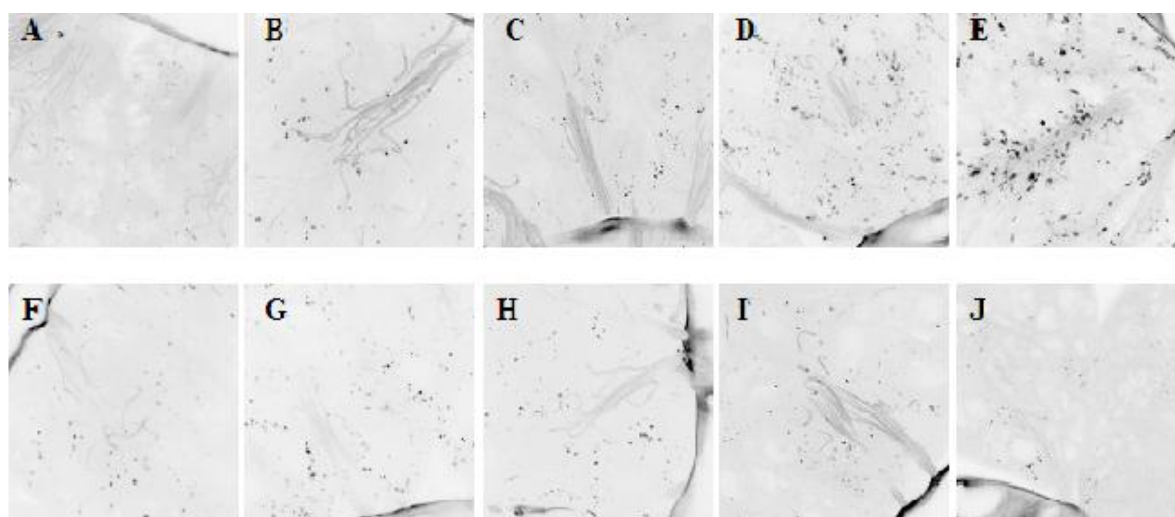

Fig 2: Image A-E represents lipofuscin accumulation in foragers from each age groups (day1- day17) as dark spots
Image F-J represents lipofuscin accumulation in nurse from each age group (day1-day17) as dark spots

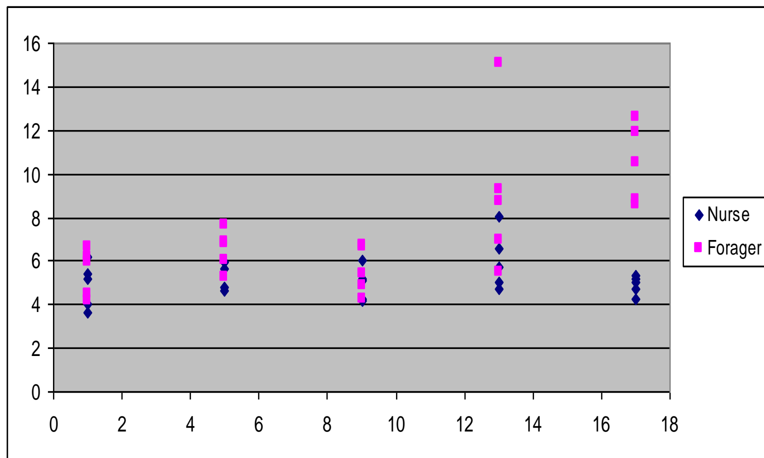

Fig 3: represents the median value of the size of the lipofuscin granules The y values are given in μm^2, and the x represents days of foraging

Figur 2 A-J shows representative microscopic images of lipofuscin accumulation in nurse and foragers. A difference between the young forager (A) and the old forager (E) can be detected, but no such difference is visible between the young nurse bee (F) and the old nurse bee (J). This indicates that there is a change in the lipofuscin accumulation among the foragers, and this does not occur in nurse bees.

Figure 3 gives the lipofuscin size when median values per individual were considered. This shows some overlap in lipofuscin granule size in the first three age groups, but a greater differentiation among the older groups.

Statistics:

An effect of age was detected in foragers by contrasting the youngest and oldest group (Fday1 vs. Fday17; $P_{F1 vs\ F17}<0.01$). This is supported by linear regression analyses (giving a result of n=25, R square= 0.43926988, and a P<0.01) In nurses no such age effect was detected as revealed by comparing the youngest and oldest nurse group (Nday1 vs. Nday17; $P_{N1\text{vs.N}17}$=0,9939)This, as well, is supported by linear regression analyses (with the result of n=25, R square= 0.00962506 , and a P= 0.6408)

Then I tested for possible differences between age-matched pairs of nurses and foragers on lipofuscin granule size. I only detected significant differences between nurses and foragers for days 13 ($P_{F13\text{vs. N}13}<0.01$) and day 17 ($P_{F17\text{vs.N}17}<0.01$). In all, the data suggests that age related lipofuscin accumulation results in significant differences between foragers and nurse bees but only after about 13 days. In contrast younger foragers and nurse bees do not differ with respect to lipofuscin accumulation.

3.2 Lipofuscin granule density

Next I asked if the density of the lipofuscin granules, by measuring the distance between them, would increase or decrease over time. And is there a difference between age-matched nurse and foragers bees in liofuscin density.

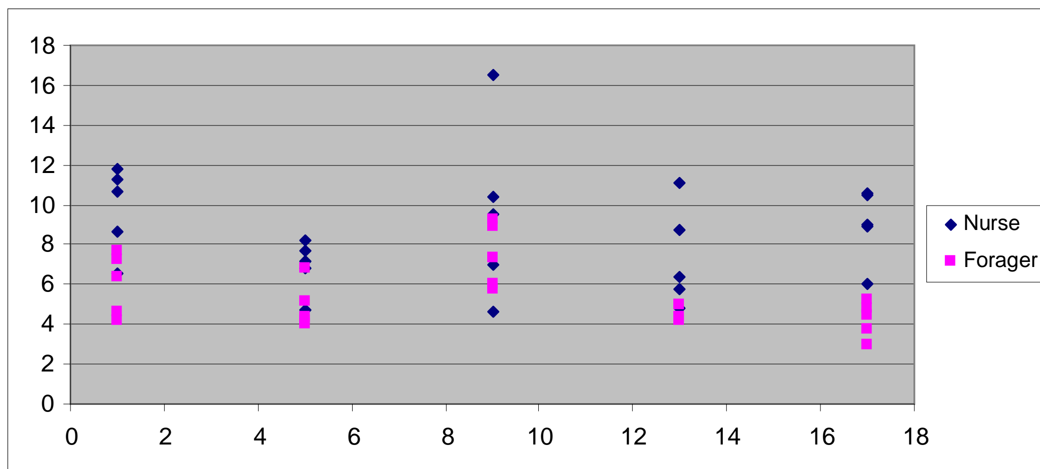

Fig 4 represents the median value of distance between the lipofuscin granules. The y values are given in µm, and the x represents days of foraging

Fig 2 A-E shows the accumulation of lipofuscin and the density of these. But a clear difference in the distance between each granules is not easily represented here.

Figure 4 gives the median distance between the lipofuscin granules of each individual. This shows some difference between age-matched nurse and foragers, but little variation between foragers belonging to different age groups, and nurse bees of different age groups.

Statistics:

A two-factorial ANOVA with the median value of the distance between lipofuscin granules as a dependent variable, and sub cast (forager/nurse) and age difference as independent factors, showed significant effects for caste, but not for age difference and the interaction between both factors

(F_{caste}=27,36, P<0.01; $F_{\Delta age}$=3,19, P=0.0228; $F_{caste\ x\ \Delta age}$=0,75, P=0,5587)

Also, using linear regression analysis, no effect of time were detected among the foragers n=25, R square= 0.11901977, and a P=0,0912, or nurse bees n=25, R square= 0.00375796, and a P= 0.7709.

Post-hoc test, however, showed that there was a significant difference between foragers and nurse on day 1 ($P_{F1\text{vs. N1}}P<0.01$), day 13($P_{F13\text{vs. N13}}P<0.01$), and day 17($P_{F17\text{vs. N17}}P<0.01$). This data indicates that the distance between the lipofuscin granules does not change significantly within each worker type over time. But instead there is a difference already present from day one.

3.3 Acini size

If there is a constant amount of lipofuscin separated into any given number of granules within a cell, any fusion of these granules could form a bigger granule. But the total amount of lipofuscin will not necessarily have increased.
A decreasing size of the acini, could also lead to a concentration of the non degradable lipofuscin that are already present.
To further asses the accumulation of lipofuscin, I therefore also asked if there was a change in the acini size over time.

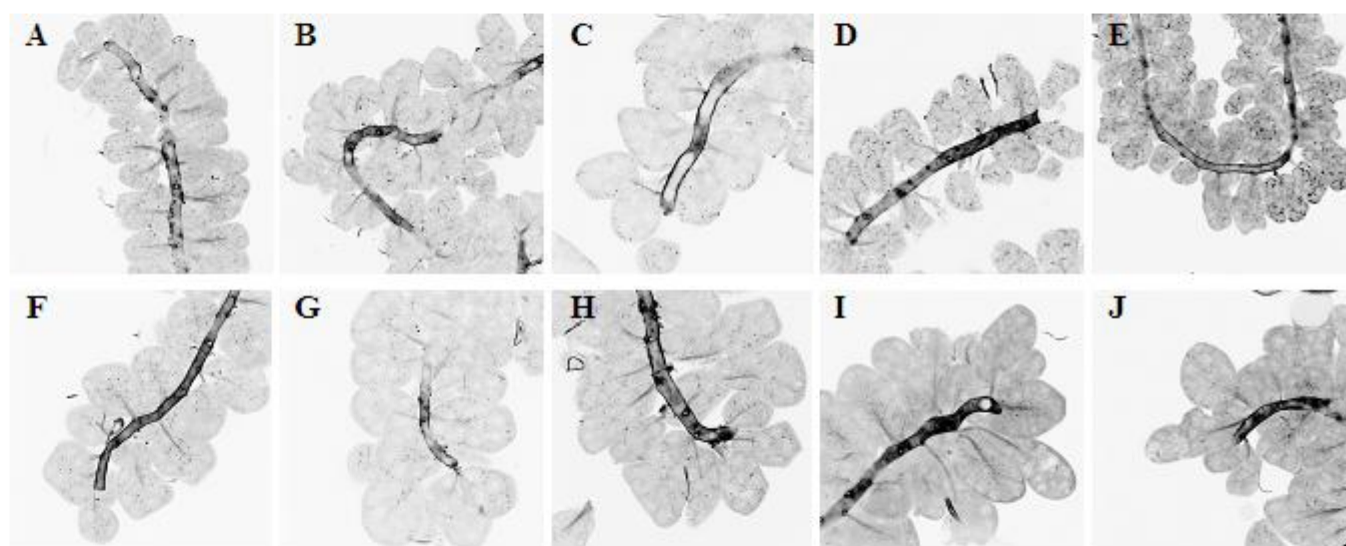

Fig 5: Image A-E represents the acini size of foragers from each age groups (day1- day17)
Image F-J represents the acini size of nurse from each age group (day1-day17)

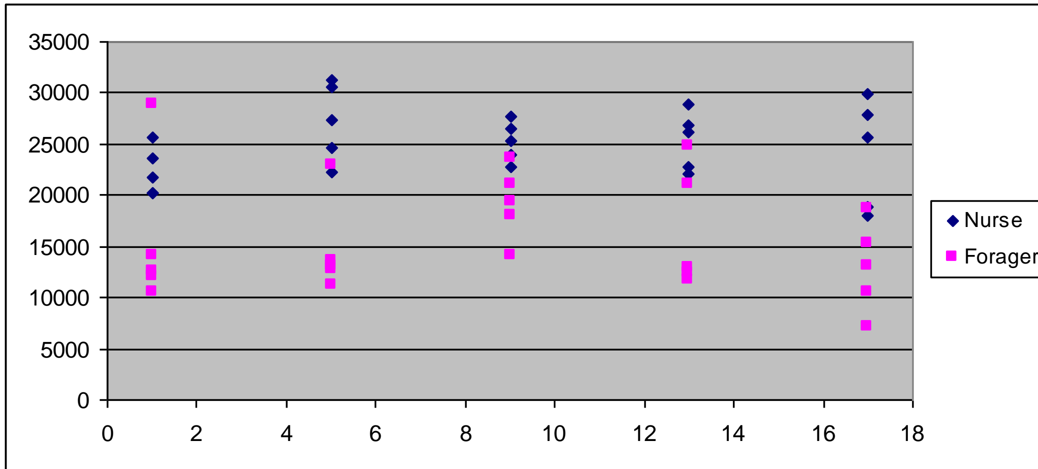

Fig 6: represents the median value of the acini size. The y values are given in µm, and the x represents days of foraging

Fig 5 A-E shows no clear difference between foragers (A and E) in regards to the acini size over time. There is also no clear difference among nurse bees belonging to different age groups (F-J). But a notable difference between E and J can be observed. Fig 6 represents the median acini size of each individual, and shows that there is little difference between the different age groups of foragers. This can also be seen among the nurse bees. But in general, a bigger acini size among the nurse bees can be observed.

Statistics:

A two-factorial ANOVA with the median value of the acini size as a dependent variable, and sub cast (forager/nurse) and age difference as independent factors, showed significant effects for caste, but not for age difference and the interaction between both factors (F_{caste}=47,26, P<0.01; $F_{\Delta age}$=1,43, P=0.24; $F_{caste\ x\ \Delta age}$=1,17, P=0,3384)

This was also supported by using linear regression analysis; n=25, R square= 0.00868013, and a P= 0.65779994 (foragers) and of n=25, R square= 0.00416074, and a P= 0.75935629 (nurse), indicating that the acini size did not change significantly over time among the different worker type.

Then I tested for possible differences between groups of age matched nurse and foragers. These tests showed that there was a significant difference between foragers and nurse on day 1 ($P_{F1 \text{vs. N1}}P$<0.01), day 5 ($P_{F5 \text{vs. N5}}P$<0.01), day 13($P_{F13 \text{vs. N13}}P$<0.01) and on day 17 ($P_{F17 \text{vs. N17}}P$<0.01) This data suggests that the size of the acini will be different between the nurse and foragers already from day one, but will not continue to differentiate significantly over this time period of 17 days.

Similar statistical tests for both lipofuscin granule size, lipofuscin granule distance and acini-size were also performed using the 1. and 3. quartile values from the image analysis data. But these tests had similar outcome as the median value, and are therefore not presented here. The values of 1. 2. and 3. quartile from the image analysis are presented in Appendix A and B.

4 Discussion

4.1 Results

This study showed an effect of foraging activity both for lipofuscin granule size, lipofuscin granule density, and acini size.

For the lipofuscin granule size, there were no significant difference between nurse bees from age group 1, and nurse bees from age group 17. Among the foragers, no significant differences were detected between foragers from age group 1 and foragers from age group 9. But foragers from age group 13 and 17 were both significant different from younger forager bees and the nurse bees. These results indicates that the size of the lipofuscin granules depends on worker sub caste, instead of just chronological age. But the time spent as a forager also reflects on the lipofuscin granule size.

Either an increased or a decreased density of lipofuscin granules, could give information about how the lipofuscin granules evolves aver time, but my results showed neither of these. Instead, only a difference in the density of lipofuscin granules between foragers and nurse bees from day one was observed, and no further change was found during the time of the study.

In this study, it was observed that the size of the acini decreased when the bee went from nursing to foraging. But as the bee continued its foraging activity, no further significant decrease in acini

size were observed. There were also not observed any significant changes in the acinisize of the nurse bees over time.

My data illustrates the strong effect of these social factors among honey bees. Age related accumulation was detected, when comparing foragers with only 8 days difference in foraging duration (9 to 17 days flight experience). That points to an extremely rapid accumulation pattern among bees once this process has started.

These results are in line with previous studies on age related lipofuscin in a wide variety of organisms, which showed an increased accumulation of lipofuscin over time.(Katz 2002; Szweda, Camouse et al. 2003; Terman and Brunk 2004; Fonseca, Sheehy et al. 2005) But our results also showed that this accumulation could occur at different stages of life, and thereby not solely be dependent on the chronological age of the organism. This correlates well with previous studies on the plasticity of honey bee aging (Rueppell, Bachelier et al. 2007; Rueppell, Christine et al. 2007; Munch and Amdam 2010)

As a biomarker of aging, lipofuscin is considered to have a nearly linear dependency of increasing age.(Munnell and Getty 1968; Brunk and Terman 2002) But several studies suggested that signs of senescence don't necessarily correlates with increased chronological age.(Corona, Velarde et al. 2007; Rueppell, Christine et al. 2007) The main goal for an organism is to reproduce, and the term "essential lifespan" for an organism is the time it takes for it to be able to do just that. Biological aging or senescence is considered to occur mainly when an organism exceeds this essential life span(Rattan 2006) Whether an organism is young or old often depends on this expected lifespan of this organism. Among mammals, the sexual maturity or puberty is a considered the starting point of the aging process(Nakano and Gotoh 1992). A twelve year old human is considered young while a dog with same chronological age is considered old.

4.2 Lipofuscin

As a factor of aging, the build up of lipofuscin would thereby be expected to correlate to the relative age of an organism according to its expected lifespan.

A study performed on long- and short-lived monkeys showed the same rate of lipofuscin accumulation at given life stages. These stages (puberty, middle age, old age) occurred at different chronological age among the monkeys, but still showed similar amounts of lipofuscin(Nakano, Mizuno et al. 1993)

Among eusocial animals like the honey bee, its expected lifespan varies accordingly to its social role in the hive. Bees like the foragers that are exposed to predators or is subject to a disease might be expected to have a shorter life span than other bees. But experiments were these

extrinsic mortality factors were eliminated, showed that there were still a difference in the life spans of the bees living in the same hive.(Rueppell, Bachelier et al. 2007)
The social role of a bee alone could thereby indicate the expected lifespan of the bee. According to the theory of lipofuscin being a biomarker of expected lifespan, a forager with shorter lifespans than a nurse bee, would then accumulate lipofuscin at an earlier chronological age.

If two granules of lipofuscin increases in size, but do not move, the distance between them will decrease.
So if any given number of granules and their position is fixed in a cell, and the size of each granule increases, the density will likely also increase without the appearance of new granules. If the density decreases and the size of each lipofuscin granules increase, it could be an indication of lipofuscin granules fusing together. This would not indicate an increased formation of lipofuscin, but rather a rearranging of the granules already present.
My data does not give much information about how the accumulation of lipofuscin occurs, but can instead support the theory that the increased size of lipofuscin granules is not just a result of already existing granules fusing together, since this would probably lead to a decreased density which is not observed.

4.3 Hypopharyngeal gland

The tissue that was used to observe lipofuscin accumulation was from the hypopharyngeal glands of the bees. Studies have showed that these hypopharyngeal glands will start to degenerate when bees becomes a forager(Deseyn and Billen 2005)
These gland are known to be undeveloped in newly emerged bees (Malone, Todd et al. 2004), but will continue to grow and be fully developed after six to twelve days(Holliday 1997)
When the bee is performing its in-hive activity, its believed that the presence of larvae will cause an elevated hypopharyngeal gland activity.(Holliday 1997) And as the bees goes from nursing activity to foraging activity, the hypopharyngeal gland will undergo changes where the total activity of the glands is redused. Enzymes that hydrolysis sucrose to glucose and fructose has been detected and the size of the glands is redused.(Kubo, Sasaki et al. 1996; Ohashi, Sasaki et al. 2000) Furthermore, a reduction in the vitellogenin synthesis rate as well as the total haemolymph protein concentration is also observed in the hypopharyngeal glands of older foragers.(W. Rutz 1976)
Major proteins required for the gland cellular functions, that are expressed by the cells in the glands, also changes when the bee goes from nursing to foraging(Kubo, Sasaki et al. 1996) And

symptoms of apoptosis, necrosis and a vacuolar form of programmed cell death in the hypopharyngeal glands have been observed(Silva de Moraes and Bowen 2000)

All these changes in the hypopharyngeal glands are believed to be the result of the social role of the bee, and not as an age-dependent development. This is supported by the observation of bees being able to maintain its gland function over longer than normal time, and redevelop degenerated glands when reverting from foraging back to nursing(Kubo, Sasaki et al. 1996; Ohashi, Sasaki et al. 2000)

In my study, a redused hypopharyngeal glands size were observed among the foragers, but this size did not continue to decrease significantly, neither among the foragers nor the nurse bees during the time of the study.

However, there are apoptotic processes in place relatively early in the hypopharyngeal glands among the foragers(Silva de Moraes and Bowen 2000), but these do not seem to interfere with the accumulation of lipofuscin in the foragers that were examined. My data reveals significant accumulation only in the oldest forager groups, which would not be expected if this accumulation would be a result of the apoptotic processes already in progress. This may suggest that lipofuscin accumulation is indeed strongly associated with senescence, which is further supported by studies on mortality(Dukas 2008)and functional senescence(Behrends, Scheiner et al. 2007; Williams, Roberts et al. 2008) that likewise show significant decline patterns only for older forager groups after about 2 weeks of foraging.

Furthermore, studies have showed that accumulation of lipofuscin can result in the inhibition of proteasomes and the destabilization of the mitochondria. In turn, this can initiate an apoptotic cascade as a result of dysregulation of several proapoptotic proteins(Suter. M, Reme. C et al. 2000; Powell, Wang et al. 2005). Lipofuscin-laden lysosomes has also been found to contain markers associated with apoptosis(Soleiman. A, Lukschal. A et al. 2008) These studies indicates that the accumulation of lipofuscin might be a factor that contributes to the apoptotic process, instead of being a result of this process in the cell.

4.4 Methodological considerations

When acquiring the samples from the hives, it is crucial to not collect a nurse bee, and mistaking it for a forager or vice versa. It is thereby important to avoid collecting foragers during orientating flight, and be able to distinguish between forager and nurse behaviour when collecting nurse bees from inside the hive.

Having a large number of samples will strengthen the study, and reducing the impact of a wrongly collected sample. But this will also increase the amount of time required to prepare and analyse the samples. Software that can perform the image analyses would greatly reduce the time

required to perform this, and also exclude the possibility of any bias caused by potential day-to day technical or human variations. This could also enable the possibilities of including more lipofuscin granules from each individual, to asses both the size and the distance between them. To include more samples or granules per individual without a software, would lead to an increased workload that have to be performed on the same day, and could probably lead to an increased chance of variations in the analysis caused by human errors.

4.5 Possible effects of lipofuscin

As a marker of chronological age, lipofuscin has in many cases been considered a harmless wear-and-tear product. But there is a growing evidence that lipofuscin may have multiple negative cellular effects.(Terman and Brunk 2004; Double, Dedov et al. 2008)

Studies have shown that lipofuscin does not affect the surface area of rough endoplasmic reticulum, protein synthesis, or the formation of neurosecretory granules.(Davies, Fotheringham et al. 1983; Ferland, Audet et al. 1992)

But lipofuscin can have an affect on proteasomal and lysosomal degradation within a cell(Terman and Brunk 2004).

Lysosomes usually contain and degrade many components of a cell. This is an important mechanism for the turnover of proteins and other macromolecules in the cell. As a non-degradable substance, the lipofuscin will fill up these lysosomes. These lysosomes that are fully loaded with lipofuscin, will still receive lysosomal enzymes from the trans-golgi area, but will contain no other proteins or macromolecules. These lipofuscin filled lysosomes will thereby function as a sink for these enzymes, and prevent them from having any useful function.(Terman and Brunk 2004) Furtermore, lipofuscin can contain toxic compounds such as the amphiphilic pyridinium bisretinoid, A2E, and it also have a very chemically reactive surface, which can be deleterious to cell function, and disturb the cellular metabolism.(Fonseca, Sheehy et al. 2005) By its ability to bind metals, lipofuscin also causes a toxic effects within a cell.(Jung, Bader et al. 2007)

Observation of bees being able to redevelop degenerated glands when reverting from foraging back to nursing(Kubo, Sasaki et al. 1996; Ohashi, Sasaki et al. 2000) opens up the possibilities that this could also affect the accumulation of lipofuscin. If lipofuscin has serious deleterious effects on cell function, and could be able to initiate apoptosis in cells, regenerating gland must have a way of counteract this problem, and re-establish its normal cellfunctions. This might not be possible when the amount of lipofuscin has reached a certain limit, but reversion experiments regarding lipofuscin accumulation could prove useful when assessing cells ability to proliferate and thereby diluting the lipofuscin already present. Using the same techniques that were used in

this study, it will be possible to observe a possible gland regenerating (acini size) and how lipofuscin accumulates in foragers that revert back to nursing.

If lipofuscin has a negative or harmful effect on cells, it would prove useful to study how and why it accumulates. This study has showed that the accumulation of lipofuscin can be a regulated process, and begs the question if and how it is possible to slow down the accumulation of this material.

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Appendix

Appendix A

Lipofuscin granule size and distance data

This data shows 1. quartile, 2. quartile and 3. quartile of the data collected in the image analysis. This values are given in µm^2 for the lipofuscin granule size, and µm for the distance.

Numbers of foraging days, and individual nr.1-5 from each group Forager (F) or Nurse (N)	Liposize 1.quart	Liposize Med.	Liposize 3.quart	Lipodist. 1.quart	Lipodist. Med	Lipodist. 3.quart
Day 1 F1	4.8535	6.613	9.527	2.2675	4.2355	6.80375
Day 1 F2	3.9365	5.916	7.4005	4.597	7.219	10.284
Day 1 F3	3.349	4.5215	6.491	2.941	6.382	12.733
Day 1 F4	3.04225	4.21	6.1135	3.688	7.725	13.816
Day 1 F5	4.1245	6.167	11.506	2.516	4.618	6.789
Day 1 N1	3.8525	5.382	7.6735	3.43975	6.578	14.37125
Day 1 N2	4.632	6.205	8.433	4.127	11.794	21.093
Day 1 N3	2.9715	4.057	6.0975	3.1645	8.645	16.9805
Day 1 N4	2.7955	3.662	7.2175	3.861	10.69	19.302
Day 1 N5	3.6555	5.183	9.0385	3.401	11.2355	18.6685
Day 5 F1	4.68	6.793	11.296	2.546	4.042	8.468
Day 5 F2	4.9685	7.628	14.6265	2.15225	4.4085	7.5385
Day 5 F3	3.45275	5.2635	8.20025	2.33575	5.129	10.90525
Day 5 F4	3.92525	6.904	11.941	2.08175	4.183	8.8945
Day 5 F5	4.235	6.067	9.409	3.606	6.8425	13.56725
Day 5 N1	3.437	4.602	6.2845	3.208	7.72	14.72
Day 5 N2	3.504	4.79	6.281	3.1985	7.205	14.435
Day 5 N3	4.43175	5.933	8.098	3.178	8.182	15.494
Day 5 N4	4.2565	5.922	7.64525	2.41375	6.819	17.165
Day 5 N5	4.052	5.631	10.507	2.29525	4.7315	8.178
Day 9 F1	3.68225	4.895	7.29875	4.6855	8.936	13.602
Day 9 F2	4.724	6.73	10.251	3.306	6.06	11.012
Day 9 F3	3.80975	5.404	8.62325	4.338	9.222	18.7395
Day 9 F4	3.143	4.267	7.203	2.651	5.767	13.2865
Day 9 F5	4.6615	6.6535	8.9975	3.323	7.347	15.639
Day 9 N1	3.5285	5.153	8.0985	2.344	4.632	10.684
Day 9 N2	3.456	5.116	7.541	3.067	6.9535	16.165
Day 9 N3	3.1445	4.212	6.712	4.375	10.406	17.37425
Day 9 N4	4.4165	5.992	7.81075	7.431	16.519	26.5555
Day 9 N5	3.5885	4.2485	4.85175	4.271	9.543	19.853
Day 13 F1	3.892	5.493	7.7025	2.45975	4.964	9.478
Day 13 F2	4.29175	6.945	11.28625	2.578	4.966	9.557
Day 13 F3	5.42925	8.7095	14.48625	2.334	4.225	7.1795
Day 13 F4	5.774	9.302	15.753	2.68475	4.398	7.9495
Day 13 F5	9.9235	15.071	23.021	2.304	4.365	8.4645
Day 13 N1	3.844	6.5865	9.21575	2.58825	5.799	14.65325
Day 13 N2	3.36725	4.728	6.6955	4.872	8.7285	18.69575
Day 13 N3	3.66275	5.037	7.6705	6.41275	11.089	17.79
Day 13 N4	4.1	5.702	7.9135	3.2145	6.399	13.227

Day 13 N5	5.0425	8.0645	15.1095	2.022	4.794	12.228
Day 17 F1	7.935	12.613	28.116	1.666	3.005	5.652
Day 17 F2	6.7375	10.516	19.1825	2.847	5.271	8.449
Day 17 F3	5.76275	8.793	17.7235	1.912	3.781	6.88375
Day 17 F4	5.291	8.5455	16.2355	2.427	4.8035	8.375
Day 17 F5	7.485	11.907	21.4275	2.409	4.489	7.025
Day 17 N1	3.584	5.313	9.1865	2.909	6.057	13.194
Day 17 N2	3.4995	4.728	7.65775	4.71375	9.003	15.18825
Day 17 N3	3.902	5.153	7.66575	4.093	10.561	16.11775
Day 17 N4	3.14825	4.2355	6.71325	4.6095	10.447	26.199
Day 17 N5	3.335	5.022	6.535	5.095	8.897	16.3975

Table A1: datavalues from each individual used in the statistical analysis.

Appendix B

Acinisize

These data shows 1. quartile, 2. quartile and 3. quartile values of the data collected in the image analysis. The values are presented in µm.

Numbers of foraging days, and individual nr.1-5 from each group Forager (F) or Nurse (N)	Acinisize 1.quart	Acinisize Med	Acinisize 3.quart
Day 1 F1	7895.488	10505.742	13634.6665
Day 1 F2	9936.664	12497.943	14581.5065
Day 1 F3	22663.1545	28919.2865	33305.3658
Day 1 F4	9997.667	12137.079	13581.396
Day 1 F5	11149.8565	14077.155	16311.652
Day 1 N1	19669.2675	23618.0135	31943.6748
Day 1 N2	16752.279	21822.856	26081.0575
Day 1 N3	17992.6795	20186.077	26162.5388
Day 1 N4	22557.9023	25679.8105	29277.1438
Day 1 N5	17902.606	20139.68	22883.969
Day 5 F1	9603.007	11237.781	15110.488
Day 5 F2	11473.059	13027.2115	14614.0128
Day 5 F3	10188.983	12670.356	14944.376
Day 5 F4	10627.0323	13540.1545	14761.652
Day 5 F5	14309.4265	22923.206	26672.3313
Day 5 N1	20009.5115	24566.572	29186.2118
Day 5 N2	17507.9465	22232.695	26115.712
Day 5 N3	28205.4328	31264.6195	38147.8253
Day 5 N4	20361.3545	27432.295	33538.4963
Day 5 N5	23590.2328	30622.7965	35359.5738
Day 9 F1	16059.3328	18072.728	21355.3068
Day 9 F2	20453.0023	23534.958	29676.3858
Day 9 F3	15473.787	19384.156	22305.584
Day 9 F4	15819.0425	21105.136	26022.489

Day 9 F5	23214.4755	24732.398	28184.0965
Day 9 N1	23792.5748	26518.964	31375.3138
Day 9 N2	21856.078	23918.448	29816.006
Day 9 N3	19223.342	25241.618	25241.618
Day 9 N4	22726.7355	27611.0085	34084.3753
Day 9 N5	19527.356	22716.425	26539.0123
Day 13 F1	10111.368	11787.097	14579.9313
Day 13 F2	16080.9558	21102.5585	27295.2523
Day 13 F3	10103.2058	12886.5885	16046.731
Day 13 F4	9558.0425	12389.684	14414.2485
Day 13 F5	20699.307	24895.073	27734.448
Day 13 N1	18576.22	22737.905	26529.2745
Day 13 N2	16666.6453	22131.5955	25012.068
Day 13 N3	23746.751	26220.5345	32524.4955
Day 13 N4	21464.855	28827.924	32927.747
Day 13 N5	21945.7215	26767.56	34383.234
Day 17 F1	15396.6023	18754.0745	19694.6143
Day 17 F2	8652.7305	10588.225	12438.0855
Day 17 F3	11946.336	15230.776	18332.493
Day 17 F4	10469.0825	13016.328	16066.3495
Day 17 F5	6225.773	7119.343	8551.3445
Day 17 N1	21158.693	27827.241	36715.393
Day 17 N2	15841.955	18921.905	23629.756
Day 17 N3	23842.5515	29859.2525	32254.9928
Day 17 N4	13931.5205	17964.182	20961.077
Day 17 N5	18536.983	25603.628	29435.093

Table B1: datavalues from each individual used in the statistical analysis.

Appendix C

Regression analysis

Regressionanalysis were made using the median values.

Regresjonsstatistikk	
Multippel R	0.66277438
R-kvadrat	0.43926988
Justert R-kvadrat	0.41489031
Standardfeil	2.09092438
Observasjoner	25

Variansanalyse

	fg	*SK*	*GK*	*F*	*Signifkans-F*
Regresjon	1	78.7738416	78.7738416	18.0179498	0.00030594
Residualer	23	100.555189	4.37196476		
Totalt	24	179.329031			

	Koeffisienter	*Standardfeil*	*t-Stat*	*P-verdi*	*Nederste 95%*	*Øverste 95%*
Skjæringspunkt	4.612945	0.78583715	5.87010301	5.5432E-06	2.98731701	6.23857299
X-variabel 1	0.313795	0.07392534	4.24475557	0.00030594	0.16086878	0.46672122

Table C1: Results of regressionanalysis of forager lipofuscin size

Regresjonsstatistikk	
Multippel R	0.09810739
R-kvadrat	0.00962506
Justert R-kvadrat	-0.0334347
Standardfeil	0.94672288
Observasjoner	25

Variansanalyse

	fg	*SK*	*GK*	*F*	*Signifkans-F*
Regresjon	1	0.2003445	0.2003445	0.22352787	0.64082073
Residualer	23	20.6145369	0.89628421		
Totalt	24	20.8148814			

	Koeffisienter	*Standardfeil*	*t-Stat*	*P-verdi*	*Nederste 95%*	*Øverste 95%*
Skjæringspunkt	5.083895	0.35580914	14.2882641	6.3063E-13	4.34784772	5.81994228
X-variabel 1	0.015825	0.03347171	0.47278734	0.64082073	-0.0534165	0.0850665

Table C2: Results of regressionanalysis of nurse lipofuscin size

Regresjonsstatistikk	
Multippel R	0.34499241
R-kvadrat	0.11901977
Justert R-kvadrat	0.08071628
Standardfeil	1.55216629
Observasjoner	25

Variansanalyse

	fg	*SK*	*GK*	*F*	*Signifkans-F*
Regresjon	1	7.48612818	7.48612818	3.10728267	0.0912278
Residualer	23	55.4120646	2.4092202		
Totalt	24	62.8981928			

	Koeffisienter	*Standardfeil*	*t-Stat*	*P-verdi*	*Nederste 95%*	*Øverste 95%*
Skjæringspunkt	6.325975	0.5833544	10.844137	1.6219E-10	5.11921449	7.53273551
X-variabel 1	-0.096735	0.05487737	-1.7627486	0.0912278	-0.2102575	0.01678748

Table C3: Results of regressionanalysis of forager lipofuscin distance

Regresjonsstatistikk	
Multippel R	0.06130217
R-kvadrat	0.00375796
Justert R-kvadrat	-0.0395569
Standardfeil	2.78618693
Observasjoner	25

Variansanalyse

	fg	*SK*	*GK*	*F*	*Signifkans-F*
Regresjon	1	0.67349618	0.67349618	0.08675902	0.77098068
Residualer	23	178.545264	7.76283759		
Totalt	24	179.218761			

	Koeffisienter	*Standardfeil*	*t-Stat*	*P-verdi*	*Nederste 95%*	*Øverste 95%*
Skjæringspunkt	8.798255	1.04713935	8.40218166	1.8301E-08	6.63208224	10.9644278
X-variabel 1	-0.029015	0.09850658	-0.2945488	0.77098068	-0.2327914	0.17476139

Table C3: Results of regressionanalysis of nurse lipofuscin distance

Regresjonsstatistikk	
Multippel R	0.09316723
R-kvadrat	0.00868013
Justert R-kvadrat	-0.0344207
Standardfeil	5469.8697
Observasjoner	25

Variansanalyse

	fg	*SK*	*GK*	*F*	*Signifkans-F*
Regresjon	1	6025517.96	6025517.96	0.20139117	0.65779994
Residualer	23	688147915	29919474.6		
Totalt	24	694173433			

	Koeffisienter	*Standardfeil*	*t-Stat*	*P-verdi*	*Nederste 95%*	*Øverste 95%*
Skjæringspunkt	16591.4492	2055.75431	8.07073547	3.6867E-08	12338.7975	20844.101
X-variabel 1	-86.786505	193.389098	-0.4487663	0.65779994	-486.84233	313.269322

Table C4: Results of regressionanalysis of forager acini size

Regresjonsstatistikk	
Multippel R	0.06450378
R-kvadrat	0.00416074
Justert R-kvadrat	-0.0391366
Standardfeil	3661.5441
Observasjoner	25

Variansanalyse

	fg	*SK*	*GK*	*F*	*Signifkans-F*
Regresjon	1	1288360.49	1288360.49	0.09609679	0.75935629
Residualer	23	308358819	13406905.2		
Totalt	24	309647180			

	Koeffisienter	*Standardfeil*	*t-Stat*	*P-verdi*	*Nederste 95%*	*Øverste 95%*
Skjæringspunkt	24456.1705	1376.12694	17.7717402	6.2427E-15	21609.435	27302.9059
X-variabel 1	40.13042	129.455133	0.30999481	0.75935629	-227.66792	307.928765

Table C5: Results of regressionanalysis of nurse acini size