Impact of brain performance on stress-handling in aged honeybees (Apis mellifera)

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Abstract

Elderly humans run a risk of weakening or losing parts of their cognitive ability after surgery. Traditional risk factors like surgical, anesthetic and environmental influences fail to explain the cause of this. However, the highly variable cognitive aging found in humans, might be related to why some handle damage, while others do not. Studies show that there exists a correlation between IQ score at a young age and brain function in old age. This is commonly explained with highly connected environmental influences, like socio-economic status, education and nutrition. Yet, a part of the correlation remains when these factors are statistically adjusted for, leading to a complementary hypothesis; that a biological mechanism can explain part of this connection.

Honeybees (*Apis mellifera*) serve as an invertebrate model to understanding more complexly organized brains, for example those found in mammals. It is shown that learning performance and metabolic stress resilience is positively associated in nursing bees. This finding resembles the positive IQ-score and brain function correlation found in old humans and suggests that this biological mechanism may exist in honeybees as well. This lay the foundation of what is further investigated in this study. I wanted to examine if brain performance could predict the outcome of learning, mortality and long-term memory after a surgical insult in old honeybees.

I documented that long-term memory in honeybees seems to be unaffected by surgical insult. This preservation of long-term memory is also found among elderly humans with cognitive decline. I also found a positive correlation between brain performance and survival of the surgical insult. The second finding indicates that brain performance in honeybees might be used to predict the outcome of a surgical trauma in this species – and perhaps in others.

Humans and honeybees are naturally different, so the positive correlation between brain performance and stress resilience are necessarily not caused by a common functional principle. However, research on this possible biological mechanism can provide valuable insight in how mechanisms in biology influence lifespan. This insight might also turn out valuable to humans.

Sammendrag

Eldre mennesker risikerer å få svekket eller miste deler av sine kognitive evner etter en operasjon. Vanlige risikofaktorer, som kirurgiske, anestetiske og miljømessige påvirkninger, forklarer ikke årsaken til dette. Imidlertid, kan det at mennesker viser svært varierende kognitive aldringen, være knyttet til hvorfor noen håndterer kirurgisk skade bedre enn andre. Studier har vist at det finnes en positiv korrelasjon mellom IQ-score i ung alder og hjernefunksjon når man blir gammel. Vanligvis forklares dette med interrelaterte miljømessige påvirkninger, som sosioøkonomisk status, utdanning og ernæring. Likevel, når disse faktorene blir korrigert for statistisk, eksiterer korrelasjonen fremdeles. Dette gir opphav til en komplementær hypotese, at det finnes en biologisk mekanisme som kan forklare deler av denne sammenhengen.

Honningbier er virvelløse dyr, men fungerer likevel som en veletablert modell for å forstå mer komplekst organiserte hjerner, som for eksempel hos pattedyr. Studier har vist at det eksisterer en positiv sammenheng mellom læringsevne og stresshåndtering i ammebier. Dette funnet gjenspeiler sammenhengen mellom IQ-score i ung alder og hjernefunksjon i alderdommen funnet i mennesker, og antyder at en lignende biologisk mekanisme også kan eksistere i honningbier. Det er dette som legger grunnlaget for hypotesen som undersøkes i dette studiet. Jeg ønsket å se om hjerneytelse før et kirurgisk inngrep kunne forutsi utfallet av læring, dødelighet og langtidsminne i eldre honningbier etter et kirurgisk inngrep.

Jeg dokumenterte at langtidsminne i honningbier later til å være upåvirket av kirurgisk skade. Bevaring av langtidsminne ser man også blant eldre mennesker med reduserte kognitive evner. Jeg fant også en positiv sammenheng mellom høy hjerneytelse og overlevelse etter det kirurgiske inngrepet. Dette indikerer at hjerneytelsen i honningbier kan brukes til å forutsi utfallet av en kirurgisk skade hos denne arten – muligens også i andre arter.

Mennesker og bier er selvsagt veldig ulike, så den positive korrelasjonen mellom hjerneytelse og stresshåndtering må ikke skyldes en felles biologisk funksjon. Likevel, forskning på en mulig biologisk mekanisme kan gi verdifull innsikt i hvordan slike mekanismer påvirker livsløpet. Denne innsikten kan også bli verdifull for mennesker.

List of symbols and abbreviations

CS conditioned stimulus

GRS gustatory response score

MWU Man-Whitney U test

PER proboscis extension response

POCD postoperative cognitive dysfunction

US unconditioned stimulus

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1. Introduction

As we grow older, natural signs of aging are seen in most parts of the human body. Visible signs like grey hair and wrinkles are seen in most elderly. More hidden signs of aging, like aged vasculature, decline in bone and muscle strength, and reduced vision and hearing is also common (1) (2). Still, the most feared consequence of aging for most people is age-related cognitive decline, which may affect perception, memory and information processing (2).

As we age, an increased susceptibility to damage influences is observed. This can be seen in elderly who go through surgery. After surgery, they run a higher risk of weakening or losing their cognitive ability compared to youths (3). One common complication of surgery in elderly is postoperative cognitive dysfunction (POCD), which is defined as the decline of cognitive processes and an abnormality on neuropsychological testing (4) (5). This condition is present for weeks or months postoperatively and afflicts up to 14% of patients over 70 years. In many cases POCD is overlooked or marginalized, and there is little knowledge about and research on the condition (3). To date, traditional risk factors like surgical, anesthetic and environmental influences fail to explain the condition (5).

Why some elderly handle damage, while others do not, might be related to the highly variable cognitive aging in humans. Some individuals have an early onset of cognitive deficits, as opposed to subjects who maintain notably high cognitive functions until very old age (6). This heterogeneity, which represents an increase relative to younger segments of populations, is not well understood, but a combination of genetic and environmental factors seem to contribute to this diversity (7). It is shown that regular exercise, moderate alcohol intake and a healthy diet have a positive impact on brain aging (1). A high educational level or occupational attainment also seems to be protective (1). Furthermore, studies show that there is a positive correlation between IQ score at a young age, brain function in old age and lifespan (8) (9). This cognition-survival correlation is commonly explained by highly connected environmental influences, such as socio-economic status, education and nutrition. When markers on fetal development and parental social status are statistically adjusted for, the cognition-survival correlation remains (10). This leads to a complimentary explanation, that a biological mechanism can explain the connection between IQ-score at a young age and brain function in old age. This claim however, is debated and poorly understood.

By the year 2050 it is expected that 30% of the total population will be over 65 years of age, resulting in an increasing focus on understanding how environmental factors and biological mechanisms affect health, longevity and resilience to surgical stress (11). In particular the patterns of age-related cognitive decline have become a growing clinical and social issue. As more and more people live longer, an increasing number of individuals will require surgery during old age. This might result in a higher number of POCD incidents, making research on this condition even more important. Since expected risk factors fail to explain the outcome of surgery, it may be necessary to use controversial methods to identify patients who risk developing POCD after surgery. One solution might exist in the potential link between cognitive ability and metabolic biology. In this case, research on animal models can provide critical biological knowledge on factors that may have a positive or negative influence on cognitive abilities throughout life and show how such factors connect to resilience to surgery (7).

Honeybees (*Apis mellifera*) serve as a well-established invertebrate model to understand more complexly organized brains, for example mammals. Honeybees live in colonies, consisting of one queen, up to 50 000 sterile female workers and a few hundred males (drones). The number of drones however, might vary with hive size and throughout the season (12). Honeybees are social animals with a highly sophisticated community structure. Their social system is characterized by division of labor among workers. Young workers are mostly found inside the hive nursing, protecting and feeding larvae. The older workers have foraging tasks, collecting either pollen or nectar from flowers. (13) (14). The foraging and exploratory behavior places high demands to their navigation system. To secure a safe return to their nest site they need to learn, memorize and discriminate both celestial cues, like the sun's position and sky patterns of polarized light, and terrestrial cues, including landmarks like shapes, patterns, odors and colors (15).The honeybees also have abstract communication about food sources. They communicate direction and distance with ritualized body movements, called waggle dance (14).

All this requires sophisticated cognitive abilities like extinction learning, stimulus categorization and rule learning. Among insects, they represent one of the most advanced restraint models of learning and memory at an individual level (16) (13).

In honeybees, brain performance can be measured in protocols for associative learning performance. Their learning and memory can be evaluated by associative olfactory condition on restrained honeybees using Pavlovian classical conditioning of the proboscis extension response (PER) (17) (18). When the antennas of a hungry honeybee get in contact with sucrose (unconditioned stimulus, US), it reflexively extends its proboscis to feed. Neutral odors presented to the antennas do not normally release this reflex in naive animals. However, if an odor (conditioned stimulus; CS) is presented and immediately followed by sucrose reward, bees can learn this association.

Earlier studies of aging honeybees show that elementary principles of mammalian behavioral aging can be modeled in insects, and that honeybees show functional decline patterns during aging comparable to that found in mammals (19).Individuals committed to foraging show reduction in olfactory memory acquisition after 15 days, compared to bees involved in nest tasks (20). It is also shown that learning performance and metabolic stress resilience is positively associated in nursing bees (21). This finding resembles the positive correlation between IQ and preserved cognitive function found in humans. This correlation is interesting in relation to POCD. If a similar decline of cognitive processes after surgical insult exists in honeybees, it would yield an excellent opportunity to study how the brain performance before surgical insult influences the outcome of the surgery.

This positive correlation between IQ-score and preserved cognitive function in elderly establish the foundation of my two main hypotheses in this study. First, I hypothesized that a surgical insult will affect the learning, mortality and long-term memory in honeybees. Second, I hypothesized that brain performance, measured prior to surgery, can predict the influence that the surgical insult has on learning, mortality and long-term memory.

My results argue that surgical trauma in foragers, of 14 days or more, affects the learning performance, but not long time memory. This finding indicates that the ability to process new information is affected by surgical insult in old invertebrates, in contrast to the long-term memory which stays intact. This resembles what we see in elderly humans.

I also found that associative learning performance prior to damage treatment can be used to predict mortality rate. This finding indicates that brain performance in honeybees might be used to predict the outcome of a surgical trauma in this species – and perhaps in others.

Knowledge about the link between cognitive ability and brain resilience may elucidate how biology contributes to differences in cognitive health in elderly. Mapping the relationship between cognition and the outcome of surgery might facilitate developing a reliable estimate of clinical risk of POCD for the individual patient.

2. Materials and Methods

2.1 Animals

The experiments were conducted during the summer 2011 at the Norwegian University of Life Sciences (UMB) in Ås, Norway. The honeybees used in this study were at the facilities of the UMB. To control for hive specific effects, individuals from two different colonies were used.

The different social tasks in the hive, i.e. nest and foraging tasks, affect behavioral aging in honeybees. Individuals committed to foraging show reduced olfactory memory acquisition after 15 days, compared to bees involved in nest tasks (20). To assure aging individuals, only foragers of 14 days or more were used in the experiment. This was acquired by catching foragers of random age at the entrance of the hive. They were marked with a felt-tip on the dorsal thorax and released for another 14 days of foraging. To ensure the right age and hive, different color-codes were used during marking.

The day before the first learning test, the marked honeybees were collected at the entrance of the hives. They were placed overnight in wooden boxes in high humidity and 30°C, with unlimited access to water and 30 % sucrose solution.

2.2 General setup

The condition experiment started the day after collection (see figure 1 for overview). To avoid compromising the learning performance by satiation state, the individuals where starved for four hours. After starvation, they were immobilized on ice so they could be individually strapped in small plastic tubes. When strapped in the tubes, they were only able to move their mouthpart and antennas. To lower the mortality rate, the bees were then force-fed with 1µL of 30% sucrose solution without touching the antenna.

Next, the bees were trained in a differential learning paradigm (see section 2.6) and their learning score were noted (see appendix 1 for form used). After the first learning test they were put in separate numbered cages, with unlimited access to 30 % sucrose solution and put in an incubator overnight.

On day two, the bees were split into two similar groups, damage and a control group. (For details, see section 2.4). The damage group was immobilized on ice, injected with 3µL of Millie-Q water and put back in separate cages in the incubator.

On the third day both damage and control group went through a memory retrieval test; with the odors from learning test one, followed by a new differential learning test with two new odors.

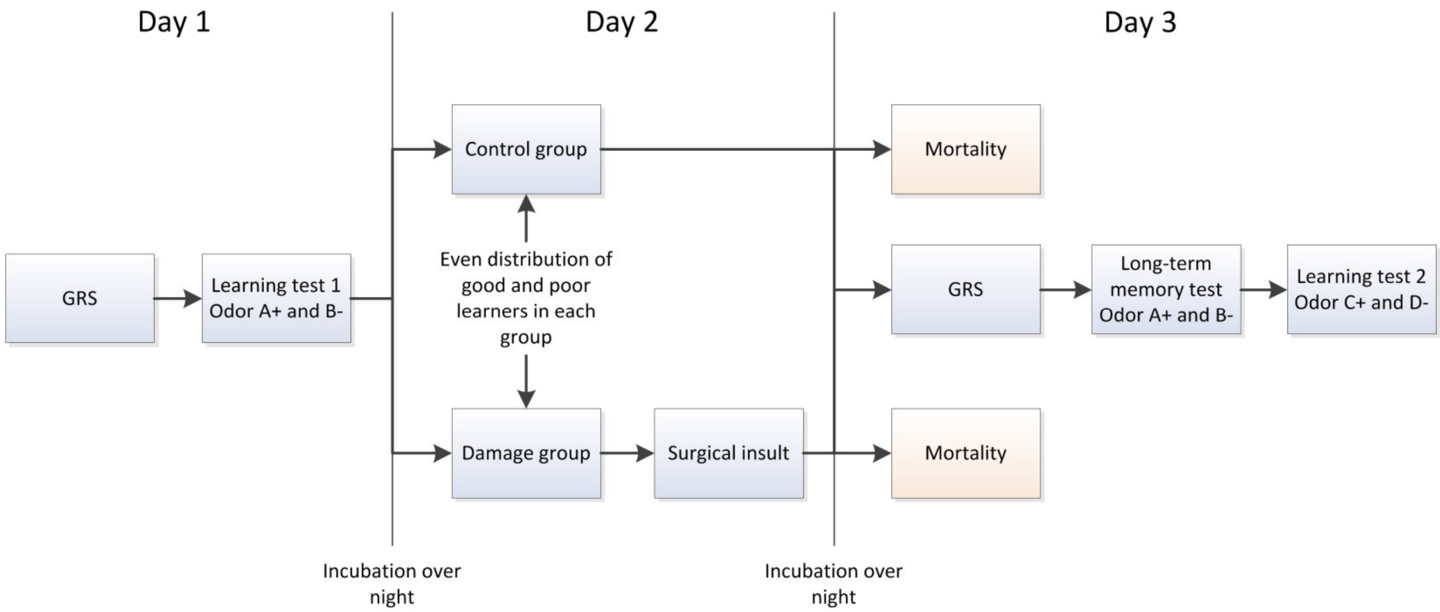

Figure 1: Overview of the experimental set-up in the laboratory.

2.3 Odors

The honeybee olfactory sense is able to distinguish between wide range of odors (22). It was important that the bees were able to differentiate between the odors and that the odors had no similarities with floral species currently foraged. The four odors were carefully selected based on the article of Guerrieri et. al. and a pilot study (22). The pilot study controlled the honeybees' response to different potential odors. To ensure that the odor were novel for the honeybees, the odors not inducing high levels of spontaneous proboscis extension response were selected.

2.4 Treatment groups

For the surgical insult it was important to form two identical treatment groups, one serving as the control group and the other as the damage group. The bees were divided according to the learning score of the rewarded odor in learning test one. Individuals with a similar learning score were divided between the two groups, ensuring an equal distribution of good and poor learners. Sorting the groups on the basis of the rewarded odor alone, were justified with the existence of a negative correlation between the learning score of the rewarded and the punished odor i.e. bees that display a high learning score of the rewarded odor tend to show a lower score of the punished odor(Appendix 2). Hence, the groups would have turned out similar, whether they were sorted according to the punished odor or the rewarded odor.

2.5 Surgical treatment

A pilot study was run to optimize the surgical lesion technique by monitoring the mortality. An impact on mortality was desired, yet it was necessary to maintain enough individuals alive in order to collect a sufficient data set. It was found that piercing the dorsal thorax followed by an injection of 3µL of Millie-Q water into the flight muscle, without disturbing vital internal organs, was the optimal approach.

2.6 Olfactory conditioning

2.6.1 Learning test one, day one, of odors A and B.

The training procedure applied in this study was differential learning of the PER (17) (23). Before the training procedure was applied, the gustatory response score (GRS) was measured by monitoring the proboscis extension response to 20% sucrose solution. This was done by gently touching the antenna with 2µL of 20% sucrose solution, not followed by feeding. GRS measures the bee's subjective value of the sucrose solution. Since only bees that respond to sucrose can be rewarded by it, none responders were left out of the first learning test.

A differential learning paradigm was used. In this procedure bees were trained to associate odor A (CS+) with the reward sucrose solution and odor B (CS-) with the punishment 3M NaCl solution. The differential conditioning was done with two different odors, 2-Octanone and Hexanal. The two odors were counter balanced over days to prevent any odor-specific effect (See table 1 for overview).

All in all 12 trials were done per individual, six with both the rewarded and the unrewarded odor (A and B). The odor sequence was pseudo-randomized and equal for each animal in the test (ABBABAABABBA).

Table 1: Overview of odors used for each group in learning test 1 and 2

Group	Learning test 1		Learning test 2	
	Odor A (CS+)	*Odor B (CS-)*	*Odor C (CS+)*	*Odor D (CS-)*
1	2-Octanone	Hexanal	2-Nonanol	1-Hexanol
2	Hexanal	2-Octanone	1-Hexanol	2-Nonanol
3	2-Octanone	Hexanal	2-Nonanol	1-Hexanol
4	Hexanal	2-Octanone	1-Hexanol	2-Nonanol
5	2-Octanone	Hexanal	2-Nonanol	1-Hexanol
6	Hexanal	2-Octanone	1-Hexanol	2-Nonanol
7	2-Octanone	Hexanal	2-Nonanol	1-Hexanol

A conditioning trial began with placing the bee in front of an exhaust fan (10 cm diameter) for 10 seconds. The bee was then accustomed to the airflow before being exposed to CS and US. The odor was manually delivered with a 10 mL syringe containing 2 µL of pure odorant on a paper. CS was presented for 5 seconds, with US applied in addition after 3 seconds (see figure 2). The US was given by touching the antenna and mouthparts with 30% sucrose solution or the 3M NaCl solution. Bees that showed proboscis extension was allowed to feed (approximately 1µL). To ensure proper memory formation, there was at least a 10 minute interval between conditioning trials (24).

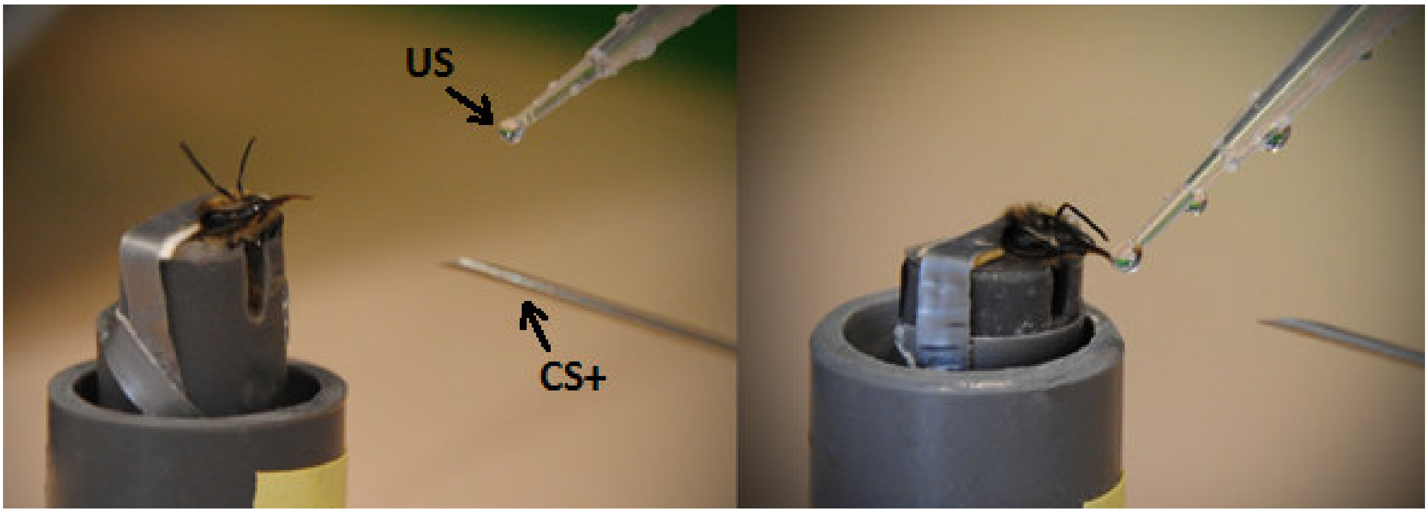

Figure 2: Conditioning trial. Picture to the left shows a honeybee that has learned to associate the odor with sucrose reward. Picture to the right shows a honeybee feeding on the sucrose solution.

2.6.2 Memory retrieval, day 3, of odors A and B.

Bees where again starved for 4 hours, paralyzed on ice, strapped in individual plastic tubes and checked for GRS. The memory retrieval test of odor A+ and B- was done by presenting the odor, without the US. Animals that responded, i.e. extended proboscis, got a score 1 and non-responders a score of 0.

2.6.3 Learning test 2, day 3, of odors C and D.

After the memory retrieval, bees went through a new differential learning test, similar to day 1, but with two new odors: 2-Nonanol and 1-Hexanol (abbreviated C+ and D-) (See table 1 for overview).

2.7 Data and Statistical analyses

The total number of individuals used in the first learning test was 254, with 128 individuals in the control group (n_c= 128) and 126 in the damage group (n_d=126). After the general handling in the lab, the surgical insult and removal due to low GRS the number of individuals decreased in the second learning test to a total number of 141. There were 76 individuals in the control group and 65 in the damage group. Of the individuals in the second learning test,100 classified as good learners (n_g=100). Within this group 55 were in the control group ($n_{g,c}$=55) and 45 in the damage group ($n_{g,d}$=45). Another 41 individuals classified as poor learners, within this group 21 were in the control group ($n_{p,c}$=21) and 20 were in the damage group ($n_{p,d}$=20).

STATISTICA was used for all the statistical analysis in this experiment. A significance level of 5 % ($p<0,05$) was used.

The learning score, i.e. number of times each subject responded to an odor (i.e. A+, B-, C+ and D+) during each conditioning phase was summed up. The possible score ranged from zero to six. The distribution of the learning score data is highly skewed, in other words it was not normally distributed; therefore the non-parametric Mann Whitney U(MWU) test was used to compare groups. This test was used to compare the control and the damage group, and it was also used to compare good and poor learners. Correlation between the learning score in learning test 1 and learning test 2 was analyzed with a spearmen rank correlation test. To assess damage effect on mortality and long-term memory the Chi-square test was used.

3. Results

This result sections consists of two main parts. The first part covers the general effect of damage treatment on the different parameters measured in this experiment. The second part assesses if learning performance, as measured in the learning test prior to surgery, can be used to predict the effects that surgical insult causes the measured parameters.

3.1 Damage effect on learning, mortality, spontaneous response and long-term memory.

3.1.1 Damage effect on learning

The first learning test was done to separate individuals into control and damage group, with equal representation of bees with different learning score in each group (Figure 3 and 4, panel A). After damage, the learning was tested again to assess how surgical insult affects learning in a discrimination learning assay, i.e. for rewarded and punished odors (Figure 3 and 4, panel B).

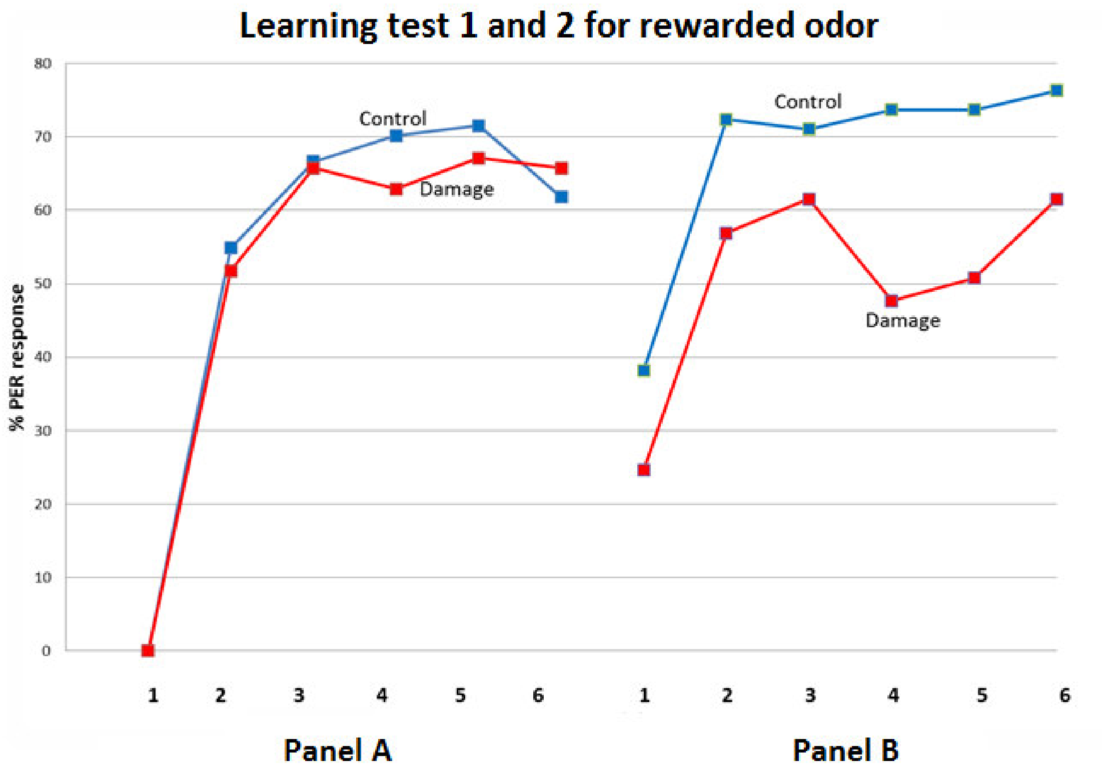

Figure 3: Learning performance of the rewarded odor, measured in % PER response, before and after damage. Panel A refers to performance in learning tests before damage, and panel B refers to learning performance after damage. The learning curves give the percentage of individuals with positive PER to the rewarded odor for each learning trial. Separate curves are shown for the two groups tested, i.e. the control and the damage group. An individual's performance in the learning tests was expressed as learning scores. After surgical insult, these learning scores were significantly reduced in the damage group, as compared to the control.

Using the resulting data, I first analyzed whether damage influenced the learning performance of the rewarded odor (Figure 3, panel B). I found that the damage group scored significantly lower than the control group. (MWU-test: Z=3,14 , p<0,001, df=1, n_C=76, n_d=65). This finding supports the assumption that surgical insult has a negative impact on learning.

Next, I examined if the surgical insult had an impact on aversive learning of the punished odor (figure 4, panel B). In theory, a good aversive learner will show a low learning score of the punished odor. The MWU-test showed that the damage group scored significantly lower than the control group (Z=2,25, p<0,024, df=1, n_c=76, n_d=65). This result suggests that damaged individuals are better at aversive learning than control individuals. Thus, in contrast to reward learning, this finding does not support the presumption that damage has a negative effect on aversive learning.

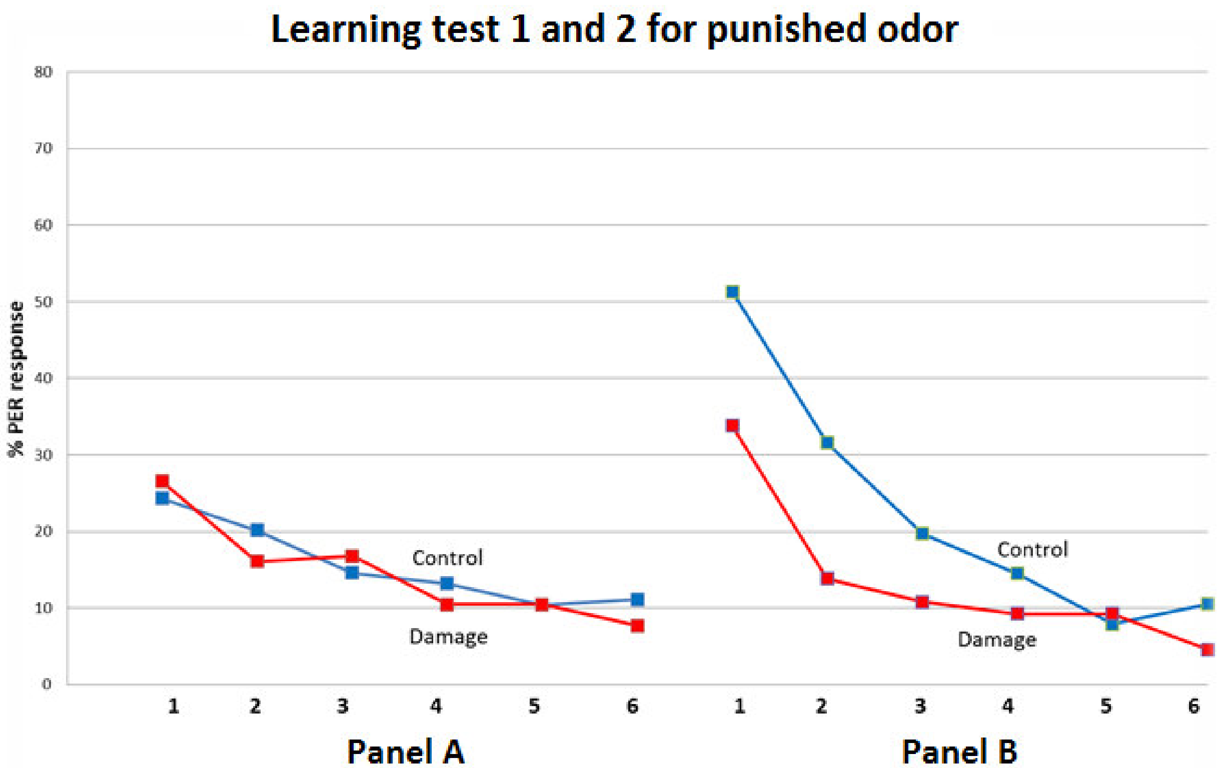

Figure 4: Learning score for the punished odor (aversive learning), measured in % PER response, before and after damage. Panel A refers to learning score before damage and panel B refers to learning score after surgical damage. Learning was measured in two groups, control and damage treated group. Learning score after surgical insult was significantly lower in the damage group, compared to the control group.

3.1.2 Damage effect on mortality

To see how surgical insult affects the mortality, an assessment of the total mortality numbers in the control and the damage group was done. This Chi square test was not significant (X^2=2,64, p=0,104, df=1, n_c=123, n_d=126). Yet, during handling honeybees might die of random causes, introducing noise to the experimental data, and thereby masking mortality between the treatment groups. The total mortality includes both deaths during incubation and during mounting, and might not give a true representation of the damage effect. A new analysis was therefore performed, leaving out the individuals that died during mounting, i.e. most likely due to handling errors, and not due to surgical insult. The Chi-square test of the adjusted mortality shows a significant difference between the damage and the control group (X^2=9,02, p=0,003, df=1, n_c=98,n_d=112), with more deaths occurring in the damage group. This result shows that surgical insult increases mortality.

3.1.3 Damage effect on spontaneous response to odors

In the second learning test, bees frequently displayed a 'spontaneous' PER to the novel odors, i.e. when odors first were presented to them to determine the learning score. A spontaneous response, thereby, is PER to an odor that the bee is naïve for. Many spontaneous responses were not expected in this experiment, since honeybees can discriminate between the odors used in the first and second test (compare materials and methods). However, further analyses provided possible explanations for the increase in this response.

The spontaneous individuals from the first learning test were left out of the dataset. Hence the spontaneous response in this test was 0%. When comparing the number of spontaneous responses in the first versus second learning test; in other words, before and after damage, a significant increase in spontaneous responses was detected. The damaged individuals showed an increase in spontaneous responses from 0,0 % to 24,6 % (Chi square: X^2=56,94, p<0,001, df=1, n_d=128 in LT1, n_d=76 in LT2) while the increase for the control group was 0,0 % to 38,0 % (Chi square: X^2=33,85 , p<0,001, df=1, n_c=126 in LT1, n_c=65 in LT2). The same trend is also observed when the spontaneous individuals from the first learning test were included in the data set. The increase of spontaneous response were significant both in the control (Chi square: X^2=29,52 , p<0,000, df=1, n_c=160 in LT1, n_c=87 in LT2) and the damage group(Chi square: X^2=6,21 , p<0,013, df=1, n_c=160 in LT1, n_c=74 in LT2).

Data from the previous section indicated that the number of spontaneous responses in the second learning test was influenced by the treatment. To directly test this suggestion the difference in spontaneous response between the control and the damage groups was compared (Figure 5). The histograms display the distributions of non-responders versus spontaneous individuals in the two groups, with almost twice as many individuals with spontaneous response occurring in the control group. However, the Chi-square test did not reveal a significant difference, but a trend was supported (X^2=2,98, p=0,085, df=1, n_c=76, n_d=65).

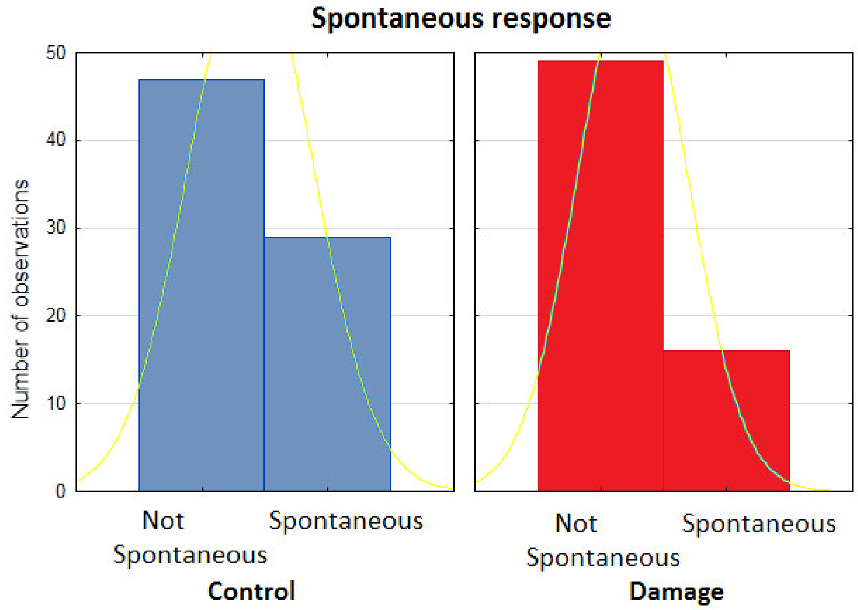

Figure 5: Overview of the spontaneous response in the control and damage group after surgical insult. The individuals not being spontaneous were approximately similar in control and damage group, while the spontaneous response was almost twice as high in the control, compared with the damage group.

These results demonstrate that the spontaneous response increases significantly from the first to the second learning test, both in the control and the damage group. The trend that fewer individuals in the damage group responded spontaneously in the second learning test might indicate that spontaneous response is influenced by surgical insult.

Since the spontaneous response is so prevalent in the second learning test, it plays an important role for my interpretation of the outcome of this test. Its contribution was revealed when the spontaneous individuals were left out of the data set from the second learning test. Without the spontaneous individuals, the comparison of learning scores between the control and the damage group did not show a significant difference in learning of the rewarded odor (MWU: Z=-1,32, p=0,186, df=1, n_c=53, n_d=57). A similar trend is seen in aversive learning (MWU: Z=-0,45, p=0,652, n_c=53,n_d=57).

3.1.4 Damage effect on long-term memory

Next, I examined if surgery had an influence on long-term memory, as measured 48 hours after the initial learning trial. The Chi-square test of memory retrieval of the rewarded odor yielded no significant difference between the surgically treated group and the control group (X^2=1,73, p=0,189 , df=1, n_c=76,n_d=65). Neither did the test of memory retrieval of the punished odor (X^2=1,41 , p=0,235, df=1, n_c=76, n_d=65). Both results suggest that long-term memory is not affected by the surgical insult and supports the assumption that long-term memory is differently affected by surgical insult than processes involved in acquisition of new memory.

3.2 Can brain performance predict the outcome of the surgery?

I wanted to explore if performance in learning tests, as measured in the learning test prior to surgery, could predict the effects that surgery had on learning, spontaneous response, long-term memory and mortality. For this analysis, individuals were grouped according to learning score on day 1. They were classified as good learners if their learning scores were 4-6, while individuals with a learning score of 0-3 were rated as poor learners. In the following, the terms “good learners” and “poor learners” always refer to the individual’s performance on day1.

To easier show the development from the first to the second learning test, for the good and poor learners, the histogram with the learning scores from the first learning test is included (figure 6).

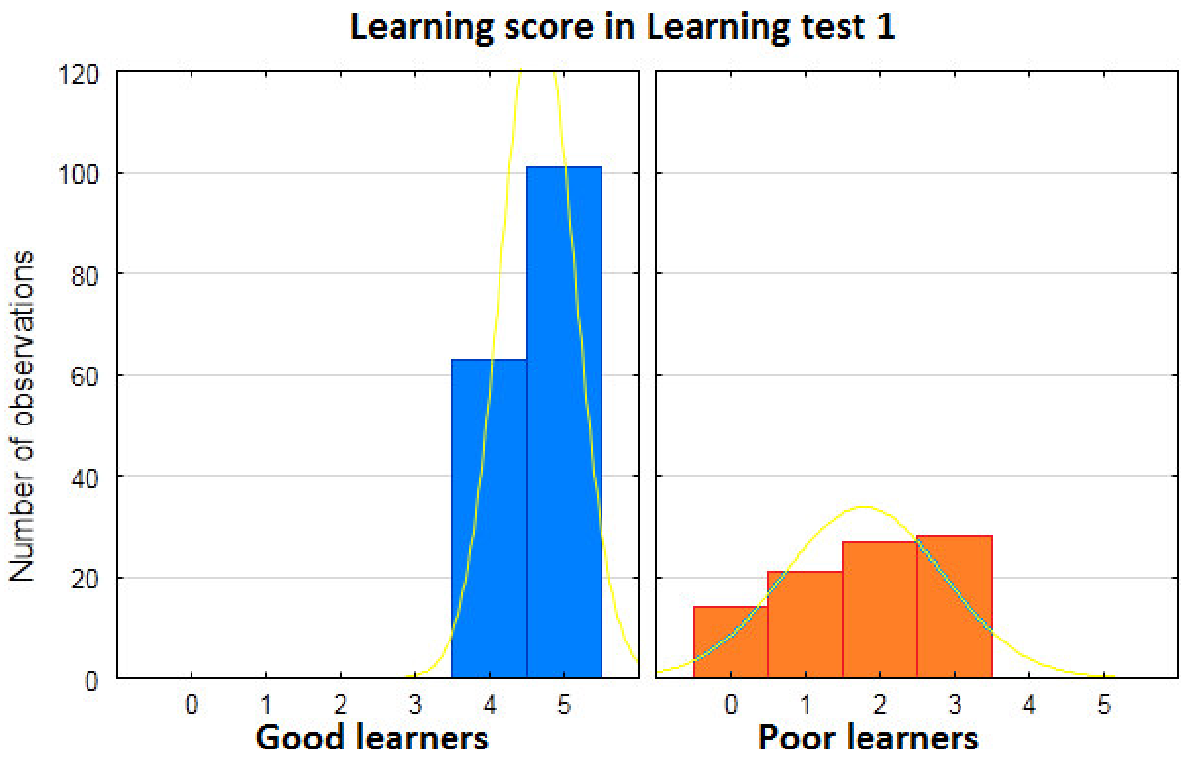

Figure 6 Learning score from the first learning test. The poor learners' score between 1-3 and good learners score 4 or 5. Since the spontaneous individuals are excluded from this group no individuals have the score of 6.

3.2.1 Can brain performance predict the damage effect on learning

First, I examined whether or not the learning scores prior to surgery could predict the learning score after surgical insult. Respective data is given first for reward learning and second for aversive learning.

3.2.1.1 Reward learning

Good and poor learners were studied separately, each group with surgical treatment compared to its respective control group. First the analysis of the good learners will be presented, followed by the analysis of the poor learners.

Good learners: control vs. damage

The histograms in Figure 7 show how the good learners responded to surgical insult. The control group scores mainly around 5 and 6, while the damage group scores are more equally distributed although many individuals still score a 5. The MWU-test revealed a significant difference in learning score between the two groups (Z= 2,86, p=0,004, df=1, $n_{g,c}$=55, $n_{g,d}$=45). This result shows that good learners are negatively affected by the surgical insult.

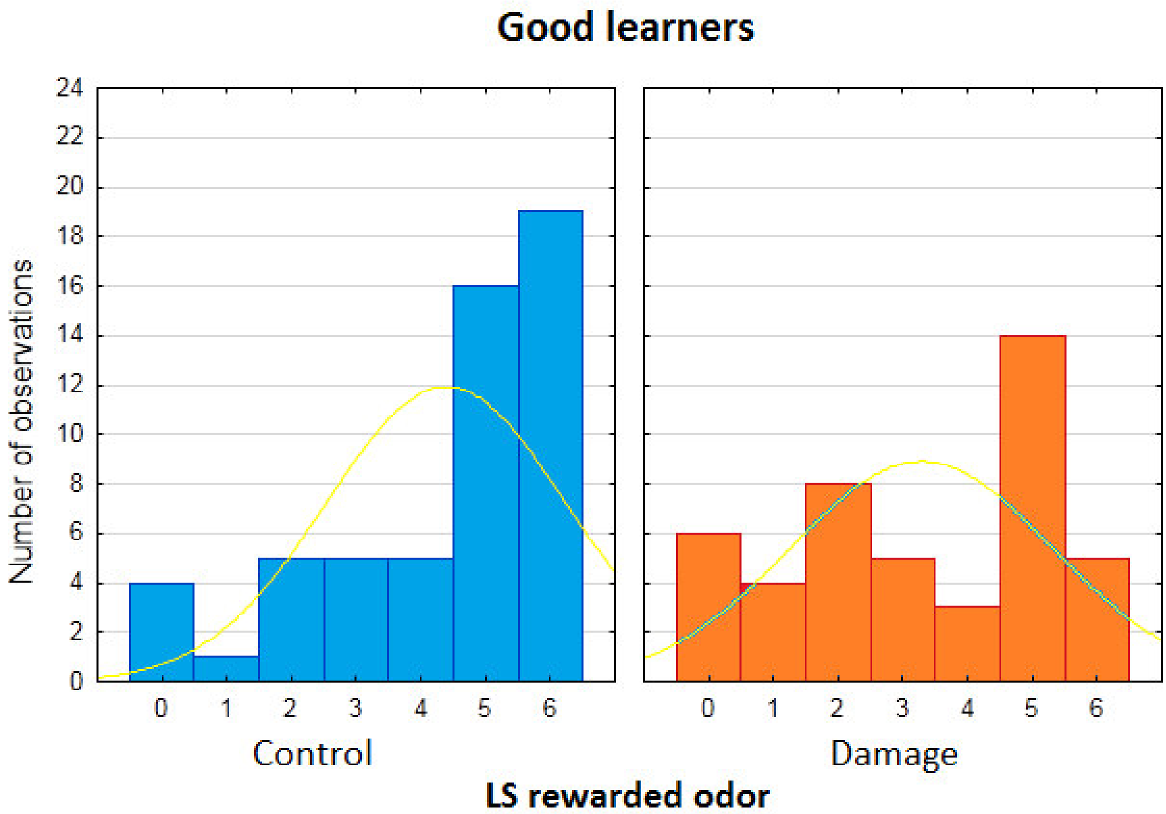

Figure 7: Categorized histograms of learning score for good learners in the second learning test. Learning score to the rewarded odor was measured in two groups, control and damage group. The control group scored significantly higher than the treated group after damage.

A spearman rank analysis was done to study if there were any correlation between the learning performance in the first and second learning test. (R=0,06, t(N-2)=0,66, p=0,509, n=100). The test revealed a non-significant result, indicating that learning performance in learning test 1 cannot predict the outcome of learning test 2 for the good learners.

Poor learners: control vs. damage

For poor learners, the histograms in Figure 8 display similar learning score distributions for both the damaged and control group. The MWU-test did not reveal a significant difference between the two groups (Z=1,26, p=0,206, $n_{p,c}$= 21, $n_{p,d}$=20). Thus, in contrast to the good learners, the performance of the poor learners did not seem to change after surgical insult. In fact, both the control and the damage group increase their learning score compared to learning test 1, where they all scored between 0-3.

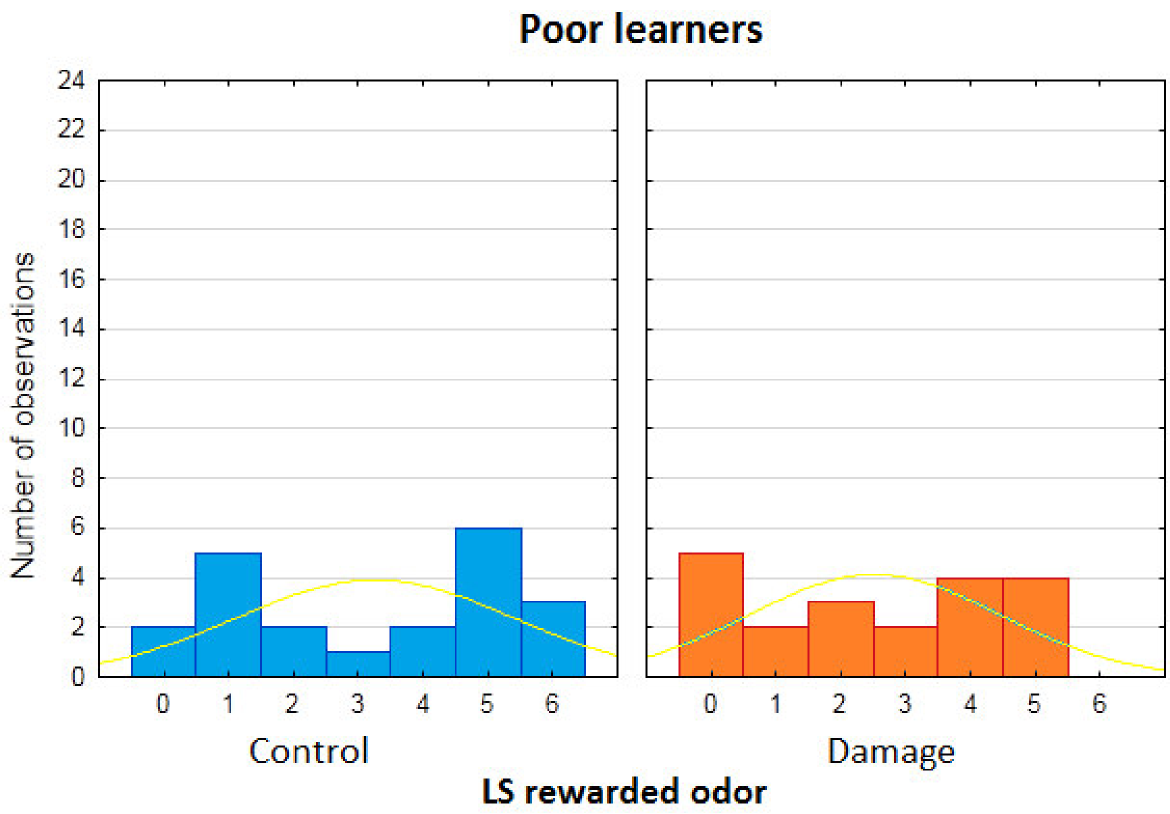

Figure 8: Histograms of learning score for poor learners in the second learning test after damage. There is a similar distribution of both control and damage group and no significant difference between were detected.

A spearmen rank analysis did not reveal any correlation between the first and the second learning test in the poor learning group, arguing that the learning performance in learning test 1 cannot predict the outcome of learning test 2 for poor learners. (R=-0,05, t(N-2)=0,34, p=0,739, n=41).

3.2.1.2 Aversive learning

To assess if performance in the first learning test affects the aversive learning after damage, the good and the poor learners were studied separately.

Good learners: control vs. damage

How the surgical insult affects the aversive learning in good learners is shown in Figure 9. The histograms indicate that the control individuals scored higher in aversive learning tests than the damaged ones. This observation is corroborated by a MWU test (Z=2,46, p=0,014, df=1, $n_{g,c}$=55, $n_{g,d}$=45). In other words, as a good aversive learner will show a low learning score of the punished odor, the damage treatment appeared to improve the good learner's performance.

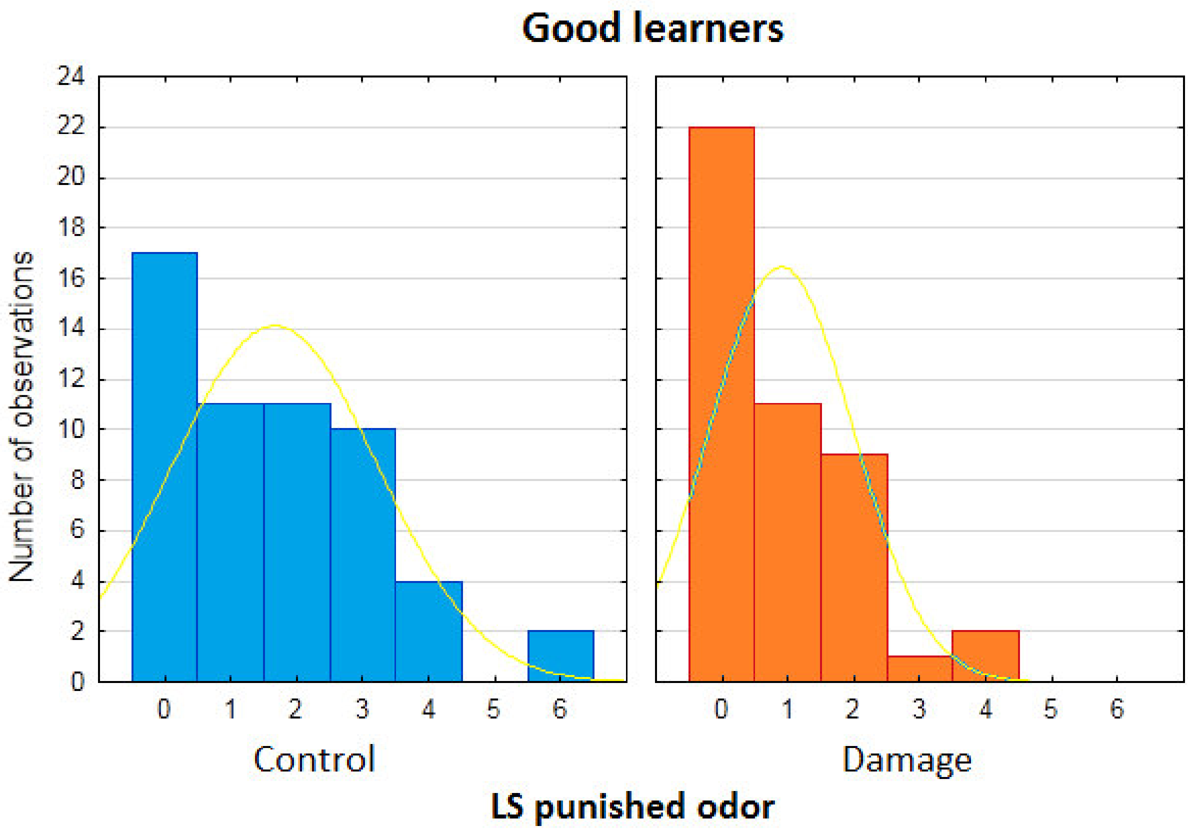

Figure 9: The good learners' performance in aversive learning (second learning test). Learning score of the punished odor was measured in two groups, control and damage group. The damage group scored significantly lower in aversive learning as compared to the control group.

Poor learners: control vs. damage

The poor learners, on the other hand, did not show a significant difference in learning score between the control and the damage group (MWU: Z=0,50, p=0,620, df=1 $n_{p,c}$=21, $n_{p,d}$=20). These results indicate that a surgical insult might not influence how the poor learning individuals cope with aversive learning after damage (figure 10).

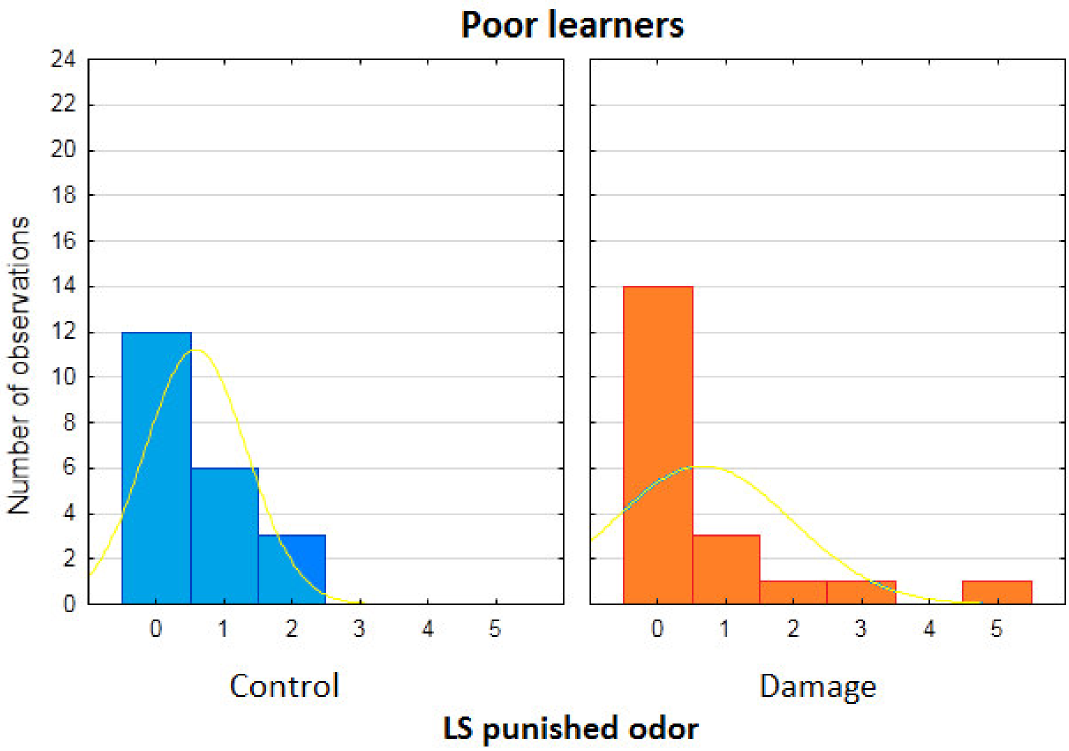

Figure 10 **The poor learners' performance in aversive learning (second learning test).** Learning score of the punished odor was measured in two groups, control and damage group. No significant difference is found between the control and the damage group.

3.2.2 Influence of brain performance on mortality

The surgical insult shows an effect on mortality (section 3.1.2). I now tested if performance prior to surgery is associated with the mortality outcome. When comparing the mortality among good and poor learners, a remarkably high survival among good learners was observed (figure 11, next page). Accordingly, the Chi-square test verified a highly significant difference in the death rate between the good and the poor learners (X^2=7,03, p=0,008, df=1, n_g=159, n_p=90). This finding indicates that performance can indeed predict survival capacity.

I investigated this further by studying the good and poor learning groups separately. According to a chi-square test of the death rate versus survival rate of the good learning individuals, there was a close to significant difference between the control and the damage group (Chi-square: X^2=3,04, p=0,081, df=1, n_c=79, n_d=80). The same test for the poor learners yielded no significant difference between the groups (Chi-square: X^2=16, p=0,686, df=1, n_c=44, n_d=46). This finding seemingly contradicts the conclusions of the previous tests.

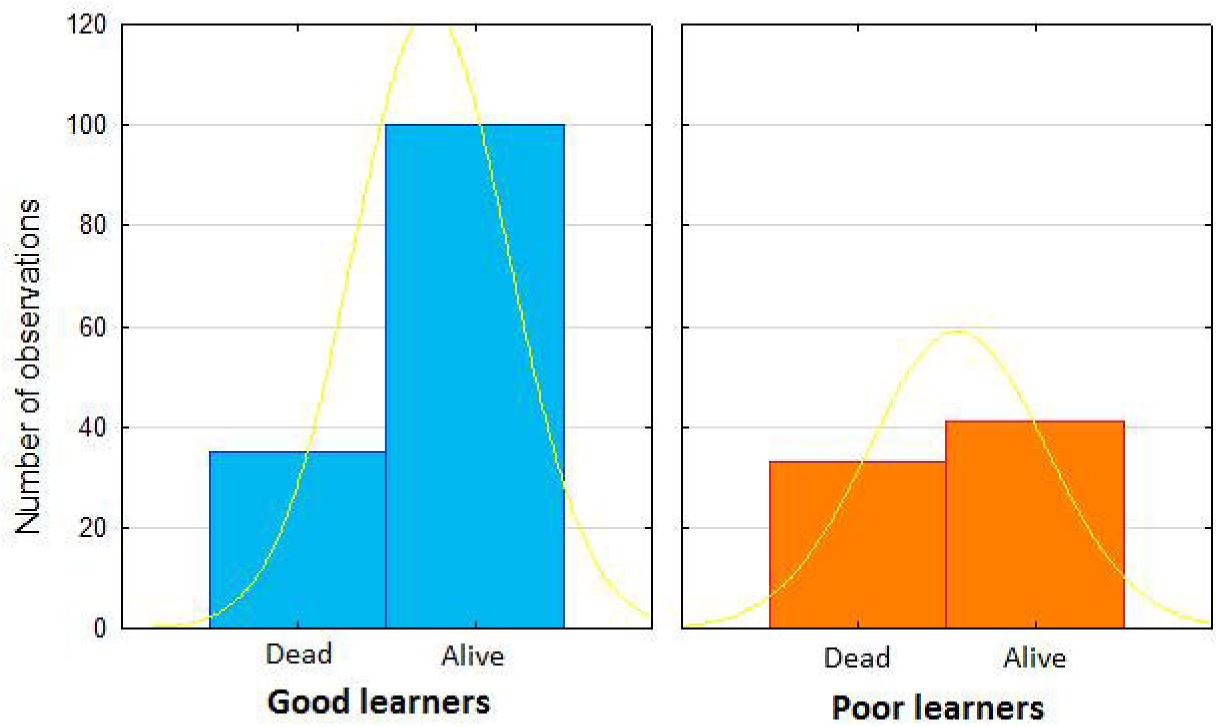

Figure 11. Overview of mortality and survival during incubation among good and poor learners. The good learners have significant higher survival than the poor learners

3.2.3 Influence of learning performance on spontaneous response to odors

A damage effect has been shown for the spontaneous response and section 3.1.3 above demonstrates that this response is important for interpreting the results. Next, I wanted to examine if the spontaneous response was equally present among the good and the poor learners. The good learners showed a spontaneous response of 38, 0 % while the poor had a spontaneous response of only 17, 1 % (figure 12, next page). A Chi-square test confirmed a significant difference between the groups (X^2=5,86, p=0,016, df=1, n_g=100, n_p=41). This demonstrates that a higher level of spontaneous response is characteristic of the good learners.

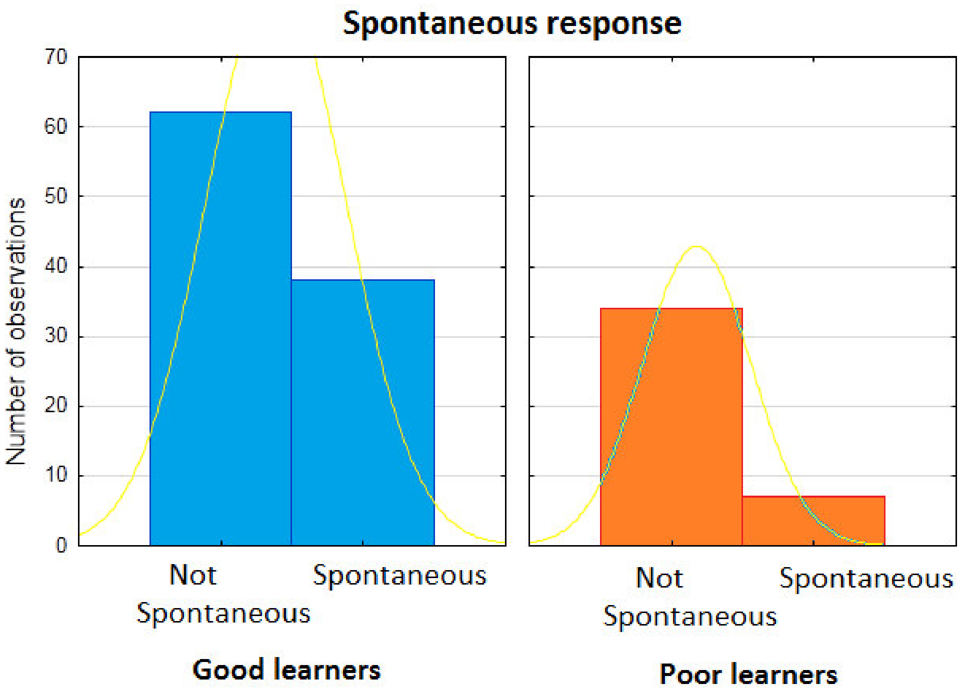

Figure 12: Overview of spontaneous activity among the good and the poor learners. The good learners show a significantly higher spontaneous response than the poor learners.

3.2.4 Influence of learning performance on long-term memory

Surgical insult did not affect the long-term memory, as shown before. Yet, it is interesting to study if the performance, as measured in the first learning test influences the score in the memory test.

First, long-term memory for the rewarded odor was tested. The Chi-square test reveals a highly significant difference between the good and poor learners (X^2= 12,37, p<0,000, df=1, n_g=100, n_p=41). This result indicates that the good learners more often showed a consolidated memory two days after training.

Next, the long-term memory for the punished odorant was tested. In contrast to long-term memory of the rewarded odor, Chi-square test could not detect any significant difference in long-term memory between the good and the poor learners in aversive learning (X^2= 0,02, p=0,885, df=1, n_g=100, n_p=41).

The analysis of the good and poor learners showed that good learners remember the rewarded odor better than the poor learners. On the other hand, in aversive learning no significant difference between the groups was detected.

4. Discussion

My study evaluates how damage affects rewarded and aversive learning, mortality, spontaneous response and long-term memory. It also assesses if learning performance can predict how individuals cope with stress-handling after a surgical insult, in relation to learning, mortality, spontaneous response and long-term memory. Like in the result part, these two main sections will be discussed separately, in respective order.

4.1 Damage effect on learning, spontaneous response, mortality and long term-memory

The analyses show that damage does affect reward learning, mortality rate and the amount of spontaneous response. However, the long-term memory and aversive learning of the punished odor, does not seem to not be affected.

4.1.1 Effect of damage on learning and spontaneous response

Since the damage effect on learning and spontaneous response are highly interconnected they will be discussed in the same chapter.

The analyses showed that damage treatment had an influence on memory acquisition in old foraging honeybees. The effect was found for both in reward learning and aversive learning. However, the pattern of the effect was different than hypothesized. As expected, the surgically insulted bees show decline in learning of the rewarded odor. On the other hand, the effect of damage on aversive learning was not as anticipated. The surgically insulted bees were better at aversive learning than the control bees. These results can be interpreted in different ways.

One explanation might be that the results are influenced by how the damage affects the responsiveness to the unconditioned stimulus, for example a lower responsiveness to the US would entail a poorer learning performance. Earlier research has shown that stress increases the response-threshold (25). The fact that surgically insulted individuals have to cope with extra stress-handling might be responsible for the observed results. If the threshold for responding to sucrose increases after damage, the surgically insulted group will display a lower response to the rewarded odor than the control group, yielding them a lower learning score. This will have the opposite effect on aversive learning of the punished odor. Low learning score characterizes a good aversive learner, so the lower response in the damage group, make them appear as better aversive learners than the control group.

Gradual individual differences in the threshold to sucrose response, are usually measured in a full test of gustatory responsiveness, where the response to different concentrations of sucrose solutions are measured. However, for practical reasons, only the gustatory response to 20% sucrose solution was tested in this experiment. Consequently, only individuals that showed a response to this specific solution are included in the data set. Therefore, if there was a more subtle difference in the threshold to respond to sucrose, between the control and the damage group, this would remain undetected.

An alternative explanation for damage effect on learning could be the presence of spontaneous response in the second learning test. When the spontaneous activity in the control and the damage group was compared, the damage group was characterized by a close to significant decline in this response. This may explain the learning results for both the rewarded and the punished odorant. Since the damage group show little spontaneous response to the rewarded odor, they will yield a lower learning score than the control group and appear to be poorer learners in the second learning test. On the other hand, when responding to the punished odor, the lack of spontaneous response after surgical lesion, will make damaged individuals appear better at aversive learning. This assumption is supported when the spontaneous individuals are removed from the data set. Then, neither the learning score of the rewarded, nor the punished odor show any significance between the control and the damage group. The fact that these results are no longer significant when the spontaneous individuals are removed is not unexpected. Bees displaying a spontaneous response also tend to yield an overall high learning score, as almost 40% of these bees with the highest learning scores are removed from the control group, the difference between the control and damage group disappear.

It is not feasible to conclude what causes the effect on the damaged individuals. It could be that the results are influenced by how the damage affects the responsiveness to sucrose, or it could be that the control group displays a considerably higher presence of spontaneous response than the damage group. However, these two hypotheses do not stand in contrast to each other; hence the damage effect could be due to one of the hypothesis or there could be an interaction between them.

Further, I will discuss possible explanations related to the increase in spontaneous response. Since honeybees that expressed spontaneous response in the first learning test was left out, this response developed from the first to the second learning test. This increase could have several explanations.

First, I would like to present the precautions done in the experimental set-up, that possible could counteract in abnormal increase in spontaneous response. When setting up the experiment I assured that honeybees were able to discriminate between the chosen odors. Therefore it is unlikely that this effect is caused by lack of ability to discriminate between the odors in the first and the second learning test. In addition, a test was done to control if the bees had any preferences for one of the two odors used. No such preference was found; therefore it is unlikely that this response was caused by the odors themselves (Appendix 3). Another factor triggering the spontaneous response could be that the odors existed in the honeybees' natural environment, like in flowers they forage on. However, this seems to be highly unlikely since these odors are found mostly in orchids, which do not exist in Ås.

There could be biological explanations to the increase in spontaneous response. A significant correlation was revealed between the long-term memory of the rewarded odor and spontaneous response (Appendix 4). In other words, the bees remembering the rewarded odor in the first learning test, was more likely to respond spontaneously, than the bees not remembering. It seems like a positive memory of the context in the first learning test increases the spontaneous response to the novel odor. The transmission of the learned response from the stimulus in the first learning test, to the similar stimulus in the second learning test, can be viewed as generalization. The honeybees generalize between the two learning tests and therefore respond the novel stimuli. The hypothesis of bees generalizing in the second learning test is supported by the findings of Mota and Giurfa in their experiment with multiple reversal learning in honeybees. They showed that after four learning tests in a row, the individuals did not improve their discrimination, but rather tended to generalize (26). If the damage treatment influences the ability to generalize between the first and the second learning test, it will give the control bees an advantage, making them better learners than the damaged ones.

The analysis of the spontaneous response in the control and the damage group, display a close to significant difference between the groups. The lower response among the damaged individuals indicates that spontaneity might be influenced by surgical insult. Since long-term memory is unaffected by the damage treatment, this effect is most likely due to something other than the bees not remembering the context. One explanation for decrease in spontaneous response might be a pessimistic cognitive bias after damage. Bateson et. al. showed that agitated bees were more likely to predict punishment from an ambiguous stimulus (27). The first trial in the second learning test might be viewed as an ambiguous stimulus, since the bees do not know if it will be followed by reward or punishment. If the damaged bees display a pessimistic cognitive bias, they are less likely to respond spontaneously.

4.1.2 Damage effect on mortality

I hypothesized that damage treatment would have an effect on the honeybees. The analysis done without individuals that died of handling effect, revealed a significant difference in mortality between the control and the damage group, hence supporting the hypothesis. This result is an important confirmation that the surgical insult results in impairment that concurrently affects mortality and behavioral function in the honeybees.

4.1.3 Damage effect on long-term memory

In contrast to the memory acquisition, where there was a significant difference between the damage and the control group, no effect of the surgical insult was observed in long-term memory. This disproves my hypothesis that damage would have an effect on long-term memory. To my knowledge, no earlier research has compared long-term memory in surgically insulted honeybees with control animals. However, age-related long-term memory differences between young and aged bees have been studied both with olfactory learning and tactile memory. In olfactory learning Münch et. al. found no age related difference in retention and extinction of consolidated memory, indicating no age related difference in long-term memory retention (19). Nor did Scheiner and Amdam find any differences between young and aged bees in long-term memory retention when testing tactile memory (28). This demonstrates that long-term memory is not only preserved in elderly honeybees, it is also preserved after a surgical insult, and therefore seems to be more robust than behaviors related to acquisition of new memory.

The preservation of long-term memory is also found among elderly humans. Anterograde amnesia is one of the most common forms of memory loss in humans, resulting in loss of short term memory and the ability to form new memories. This form of memory loss is associated with dementia and Alzheimer's disease and is also a common effect of injury among elderly humans (29). The fact that memory acquisition in aging honeybees is affected by damage treatment, while long-term memory is not, resembles the anterograde amnesia found in humans. This indicates that aging brains in honeybees and humans may have some common features.

4.2 How does brain performance influence the outcome of the damage treatment?

I explored if the learning performance measured in learning test 1, prior to surgery, could predict the effects that surgery had on learning, mortality, spontaneous response and long-term memory. As mentioned in the result part, individuals with learning score of 4-6 classified as good learners, while individuals with learning score of 0-3 classified as poor learners.

My experiment shows that brain performance is a good predictor of mortality rate, long-term memory and spontaneous response, but does not seem to be suitable for predicting rewarded and aversive learning after damage.

4.2.1 Influence of brain performance on learning after damage

I will start with discussing the results of rewarded learning followed by the results of aversive learning.

4.2.1.1 Rewarded learning

Analysis done on learning of the rewarded odor revealed that good learners seem to be significantly influenced by the surgical insult, while the poor learners are not. I did hypothesize that brain performance could predict the influence of surgical insult, however I expected this relation to be the other way around.

The fact that the poor learners do not seem to be influenced by the surgical insult is an interesting finding. My immediate interpretation of this was that the poor learners must somehow be physically superior to the good learners, for instance by having a better immune system. However, this theory is contradicted by the mortality numbers (see section 4.2.3), where the poor learners are overrepresented. Another explanation for why the poor learners' performance is not affected by surgical insult might be that the high mortality yields an uneven removal of individuals from the poor learner group, leaving only the less frail individuals. This may conceal the effect of the damage treatment. This hypothesis presupposes a gap in constitution between poor learners that die and poor learners that survive. In other words, the surviving poor learners have such a good constitution, that they are unaffected by the surgical insult. However, it seems unlikely that there exists such a gap in constitution.

A final explanation could be that the poor learners are so frail and the general handling in the lab is so stressful for them, that any additional stress (damage treatment), will not affect the results. In other words, the reason for why the effect of damage treatment is not detected could be that the control group experienced approximately the same level of stress as the damage group, making the damage effect hard to detect. This hypothesis also corresponds to the high mortality in the poor learner group.

4.2.1.2 Aversive learning

The analysis of the good learners revealed that damage treatment appeared to improve performance in aversive learning. Once again, my hypothesis is disproved; I did not expect any positive influences of the surgical insult. The improvement might be explained by the general decrease in response to sucrose among the damaged individuals. A lower response to sucrose would yield the damage group a lower learning score, and make them appear as better aversive learners than the control group.

Another explanation of this finding might be caused by a higher tendency among the control individuals to generalize (discussed in the first part). If the control individuals tend to generalize more than the damaged individuals, this would yield them a higher score, making them worse aversive learners than the damage group.

In the poor learner group there were no differences between the control and damage group in aversive learning. One explanation for this could be that the individuals in the control group have little memory of the first learning test and therefore do not tend to generalize, yielding them a similar score as the damage group.

Regardless of what causes this difference between good and poor learners, in reward and aversive learning, it seems like the brain performance measured prior to damage is not a good predictor of performance in a discrimination learning assay after damage. However, olfactory conditioning is only one part of the wide specter of learning protocols used in honeybee research, therefore I cannot exclude that this prediction is possible in other experimental methods.

4.2.2 Influence of brain performance on spontaneous response

The spontaneous response increased from the first to the second learning test in both groups. Furthermore, it was shown that good learners had a significantly higher average spontaneous response in the second learning test, compared to the poor learners. Earlier I suggested that spontaneous activity was due to the bees' ability to generalize. These results conform to the explanation that good learners remember more of the context in the first learning test, than the poor learners. Therefore they tend to generalize more i. e. show an average higher spontaneous response. The fact that the spontaneous activity was more prevalent among the good learners, implies that there is a correlation between good brain performance and the ability to generalize. This corresponds to the view that generalization is more complex than elemental forms of learning (15). Generalization might therefore be reserved for those with higher brain performance.

4.2.3 Influence of brain performance on mortality

As mentioned before, the poor learners showed a significantly higher mortality compared to the good learners. It conforms to my hypothesis that brain performance can predict the outcome of surgical insult. The finding indicates that good learners have an overall better constitution than the poor learners. Hence, a high brain performance seems to correlate with survival after damage. Amdam et. al. (21) have shown a similar correlation in young bees, when studying associative learning performance and survival time in hyperoxia. This indicates that there is a relation between brain performance and the ability to handle stress for both young and old honeybees.

As mentioned in the introduction, a similar relation between IQ-score and preservation of cognitive ability in old age is found in humans. This relation is commonly explained by environmental influences. However, some researches claim that mechanisms in physiology also may explain some of this relationship, but this is challenging to study in humans (10).

In this study, most of the environmental factors were controlled for. Bees used were from two different hives in the same location that had a similar developmental stage and size. Furthermore, the honeybees were all approximately the same age and performed the same task in the hive, foraging. The individuals within each hive are all siblings or half siblings, i.e. genetic heterogeneity is reduced. Since most of the environmental factors are similar for bees in this experiment, the higher survival rate among the good learners could be due to a biological mechanism that links high brain performance and good constitution. What kind of biological mechanism this could be is not feasible to address based on this experiment, but further research on this topic could be very interesting. Revealing a such biological mechanism in honeybees, could lay the foundation for research on a similar mechanism existing in humans. If a relationship between cognition and the outcome of surgery is found, it might be attainable to develop solutions for preventing or reducing the patterns of age related cognitive decline. For instance, it might facilitate in developing a reliable estimate of clinical risk of POCD for the individual patient.

4.2.4 Influence of brain performance on long-term memory

The damage treatment showed no effect on long-term memory, therefore only the differences in consolidated memory between good and poor learners will be discussed. The analysis of the good and poor learners showed that good learners remember the rewarded odor better than the poor learners. Hence, supporting my hypothesis that brain performance could predict consolidated memory. The most likely explanation, of the difference between good and poor learners, is that the poor learners did not manage to associate the odor with the sucrose reward in the first learning trial and therefore have little consolidated memory of this odor.

The memory test of aversive learning, i.e. learning of the punished odor, showed no difference between good and poor learners. However, I can only speculate about whether or not the poorer learners actually learned to avoid the punished odor, or if they have no memory of this odor and therefore did not respond to it.

5. Conclusion and perspectives

5.1 Damage effect on learning, spontaneous response, mortality and long term-memory

My study shows that surgical damage affects various behavioral traits differently, i.e. learning of new memory can be affected, while consolidated memory is not. Consolidated memory seems to be preserved both during aging and after damage treatment. The same trend is also seen in humans, indicating that there might be some common features in the human brain and the honeybee brain during aging.

Mortality rate and learning performance show that the individuals are influenced by the surgical insult. This could have a general significance for the methods used in honeybee research. Intramuscular injection is a common approach in many methods in this research field. Methods used are similar to my damage approach, where the dorsal thorax is pierced followed by an injection of substance in the flight muscle. However, intramuscular injections do not aim to surgically insult bees, but rather look for an effect of the injected substance. My finding, that damage affects the learning performance and mortality, might be important in relation to this method. Because, results found using intramuscular injections might be biased by the effect of the injecting procedure itself. The method used in this experiment, however, differs at some points from other methods where intramuscular injection is done. First, the injection was 3µL of Millie-Q water. This is more than in other methods, where usually 1-2µL is injected. Secondly, to evoke an immune activation in the honeybees, this procedure was not performed sterile. Therefore, the results in my experiment may only be partly transferable to other methods using this approach. Nevertheless, to disprove any effect of the injecting procedure, it would be useful to do further research on such treatment effects.

5.2 How does learning performance influence the outcome of the damage treatment?

My study also assesses if learning performance can predict how individuals cope with stress-handling after a surgical insult.

Learning performance is a good predictor of mortality rate, since the good learners showed a significantly higher survival rate than the poor learners. This finding is most relevant, as it can indicate that there is a relation between brain performance and the ability to handle stress in old honeybees. This finding argues that brain performance in honeybees might be used to predict the outcome of a surgical trauma in this species – and perhaps in others.

Most environmental factors in this study were controlled for, indicating that the relation between brain performance and stress resilience could be linked to a biological mechanism. Knowledge about this link may elucidate how biology contributes to differences in cognitive health in elderly. Mapping the relationship between cognition and the outcome of surgery might facilitate developing a reliable estimate of clinical risk of POCD for the individual patient. I am not claiming that the positive correlation between brain performance and good constitution in humans and honeybees is due to a common functional principle. However, I believe that further research on this possible biological mechanism can provide useful insight in how metabolic biology influences lifespan. This insight may also be highly valuable for understanding such links in humans.

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Appendix 1

Gustatory response (GRS) and learning score (LS)

Date ____________

Notes ____________

Hive ID ____________

running nr	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Average score pr/trial
GRS sucrose(20%)															
LS															
Tr. 1 A+															
Tr. 2 B-															
Tr. 3 B-															
Tr. 4 A+															
Tr. 5 B-															
Tr. 6 A+															
Tr. 7 A+															
Tr. 8 B-															
Tr. 9 A+															
Tr. 10 B-															
Tr. 11 B-															
Tr. 12 A+															
Notes															
LS Odor A(sum ca 1-6)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
LS Odor B(sum ca 1-6)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

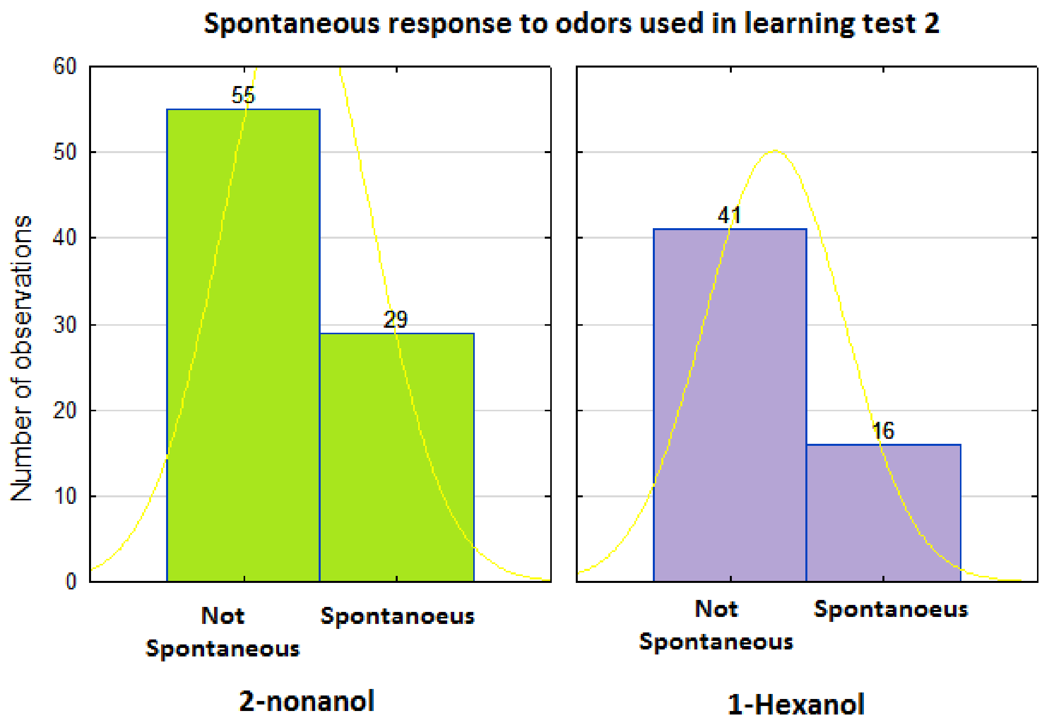
Appendix 2

Correlation between the learning score of the rewarded and the punished odor in learning test 1:

Pair of Variables	Valid N	Spearman R	t(N-2)	p-value
LS Odor A & LS Odor B	254	0,312661	5,225315	0,000000

Appendix 3

No significant difference in spontaneous response to the two odors used in learning test 2 was found (Chi square: X2=0,65, df=1, p=0,420, $n_{2\text{-nonanol}}$=84, $n_{1\text{-Hexanol}}$=57)



Appendix 4

Correlation between the long-term memory of the rewarded odor and spontaneous response:

Pair of Variables	Valid N	Spearman R	t(N-2)	p-value
Spontaneous response & Long term memory	141	0,279238	3,428548	0,000799