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Faculty of Veterinary Medicine and Biosciences
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60 credits

Studying Effects of Valerian Compounds (Valproic acid and Isovaleric acid) and Sodium butyrate on the Lifespan and Global DNA methylation in the Honey Bee Model.

Effektene av legevendelrot komponenter og natrium butyrat på levelengden og lobal DNA metylering i honningbier.

Ingrid Kjelsberg Pedersen

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Ingrid Kjelsberg Pedersen.

Abstract

Epigenetic modifications have been linked to age related decline. These modifications are changes in the DNA that does not alter the sequence itself, but still affect gene expression. Epigenetic modifications include post translational histone modifications, DNA methylation and chromatin remodeling. Bioactive food compounds and different nutrients have been found to affect these mechanisms in various model organisms. By inducing epigenetic modifications when ingested, some of these compounds have shown to extend lifespan. The honey bees short lifespan and its possession of an epigenetic machinery homologous to that found in humans, can make it a suitable model organism for studying these mechanisms.

The main goal of this study was to investigate the effects of sodium butyrate, and two compounds of the valerian plant extracts (valproic acid and isovaleric acid), on the lifespan of the honey bee. By conducting ELISAs and Western blots, I aimed to test if levels of DNA methylation and histone acetylation are affected by these nutrients.

Here I show that low doses of isovaleric acid extend the lifespan of honey bees. To my best knowledge, this represents the first evidence of isovaleric acid exerting life-extending effects, in any animal or human. I also show that increasing concentrations of this compound reduce the bees' food intake. High concentrations of valproic acid showed life shortening effects, and sodium butyrate showed minor effects only. The ELISAs did not reveal any modifications in global DNA methylation, and the differences in histone acetylation could not be tested, as Western blotting was not feasible.

This study provides good prospects for future studies on how isovaleric acid, valproic acid and sodium butyrate can affect lifespan and aging. In particular, the results from testing isovaleric acid, may inspire future work to test if potential health promoting effects can be found in other organisms as well, and to address the mechanisms through which lifespan extension is achieved.

Sammendrag

Epigenetiske modifikasjoner har blitt koblet til funksjonsnedgang relatert til alder. Disse er endringer som oppstår i DNAet uten å påvirke selve sekvensen, men som likevel påvirker genuttrykket. Epigenetiske modifikasjoner inkluderer histon modifikasjoner, DNA metylering og kromatin remodellering. Noen næringsstoffer og bioaktive komponenter i mat, har blitt funnet til å påvirke disse mekanismene i ulike modell organismer. Ved å indusere epigenetiske modifikasjoner når disse inntas, har noen vist seg å forlenge levetiden. Honningbienes korte levetid, og det at den har et epigenetisk maskineri som er homologt til det som finnes i mennesker, kan gjøre den til en god modell organisme for studier som omhandler disse mekanismene.

Hovedmålet i denne studien var å undersøke effektene av natrium butyrat, og to komponenter av legevendelrotens planteekstrakt (valproic acid og isovaleric acid), på levelengde i honningbien. Ved å utføre ELISA og Western blot, ville jeg teste om nivåene av DNA metylering og histon acetylering i biene ble påvirket av næringsstoffene.

I denne studien blir det vist at lave doser av isovaleric acid forlenger levetiden til honningbiene. Så langt min kunnskap rekker, utgjør dette det første beviset på at isovaleric acid utøver en livs forlengende effekt i noe dyr eller menneske. Det blir også vist at økende konsentrasjoner av isovaleric acid, reduserer bienes inntak av mat. Høye konsentrasjoner av valproic acid viste en reduksjon i bienes levetid, mens behandlingen med natrium butyrate kun viste mindre effekter. ELISA analysene viste ingen forandring i global DNA metylering. Ulikheter i histon acetylering kunne ikke bli testet, da Western blottingen ikke var gjennomførbar.

Denne studien gir gode utsikter for videre forskning på hvordan isovaleric acid, valproic acid og natrium butyrat kan påvirke levetid og aldring. Særlig resultatene for isovaleric acid kan inspirere videre forskning til å finne ut om potensielle helsefremmende effekter også kan observeres i andre organismer, og hvilke mekanismer som påvirkes og fører til den økte levetiden.

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Abbreviations

5-mC	5-methyl Cytosine
Bi-seq	Bisulfite sequencing
BSA	Bovine serum albumine
CI	Choroform:Isoamylalcohol
CpG	Cytosine-phosphate-guanine
dH_2O	Distilled water
DNMT	DNA methyltransferase
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme immunosorbant assay
h	Hour
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HRP	Horse raddish peroxidsase
µg	Microgram(s)
µl	Microliter(s)
kDa	Kilo dalton
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)
mM	Milli molar
MS	Mass spectrometry
NaAc	Sodium acetate

nm	Nano meter(s)
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCI	Phenol:Chloroform:Isoamyl-alcohol
PVDF	Polyvinylidene fluoride
rpm	Revolutions per minutes
RT	Room temperature
SAM	S-adenosyl methionine
sec	Second(s)
SDS	Sodium dodecyl sulfate
V	Volt(s)

1. Introduction

An undeniable fact of life is that we are all growing old. From the day we are born we are destined to age, whether we like it or not. The possibilities of postponing or slowing down aging have always been a topic of interest, across many research fields. Due to modern technology, high developed medicines and generally better knowledge about how to treat different diseases, the world's population is growing older. At the same time also the amount of older people is increasing. The United Nations homepage, have predicted the world's population over 60 years old, to triple in number from 2007 to 2050 (United Nations Global Issues n.d). They also predict the world's population over 80 will reach 400 million by 2050. With old age follows age-related diseases. The risk of developing neurodegenerative diseases like Parkinson and Alzheimer increases, but also the risks of developing different cancers increases as we get older. On the basis of this, research is needed to acquire more knowledge about the decline that is related to old age, and also the mechanisms that affect it. The development of some of these diseases is believed to originate from epigenetic alternations in the patients DNA. Epigenetics are defined as modifications to the DNA that does not alter the sequence itself, but rather alternates the gene expression by making various DNA locations more or less accessible (Watson et al. 2008).

Bioactive food components are natural components that are shown to have an effect on health in humans or animals (Biesalski et al. 2009). The mechanisms, by which these compounds work, are debated and they often differ from one another. Different compounds can affect the epigenetic machinery in different ways, and are therefore worth being studied further (Milner 2004). Also, new products are constantly emerging on the market (especially in health stores), promising positive effects on both health and lifespan. Whether these products actually work or how they exert their function is not always proven.

In this thesis the honey bee, *Apis mellifera*, was used as a model organism to study lifespan effects and epigenetic modifications as results of treatment with nutrients and plant compounds.

1.1 Honey Bees

The bees' relatively short generation cycle makes them suitable to be used as model organisms. The honey bees are much easier to handle compared to other popular model organisms, like mice and rats. Their small size makes it possible to obtain numerous sample individuals without occupying too much space. Also, since they normally live inside the confined space of a hive, it does not seem to be a major problem for them to be kept inside cages. By caging them it is easy to control their test environment (temperature and humidity), and to make precise food blends for treatments. This also makes working with bees inexpensive. Because of their short lifespan, one does not meet many ethical issues, like one often does with other model organisms.

Bees are eusocial insects possessing the curious ability to switch back and forth between short- and long-lived sub castes (Dolezal & Toth 2013). This makes them especially interesting for studies related to aging (Münch et al. 2013a). Results from studies conducted on bees, can be used for understanding the aging process in other animals, including humans (Ford 2013). As the bees' epigenetic machinery is somewhat similar to that found in mammals (Lyko et al. 2010), using them as models for different neurological diseases is therefore a great possibility. In turn, this also makes them good models for studying age related diseases like Alzheimer, Parkinsons disease and Huntingtons disease (neurological diseases related to age). The increased amount of possible genomic tools makes the study of the bees a possible task, as the honey bees' genome is sequenced (Weinstock et al. 2006).

There are several different phenotypes of honey bees. Even though all the bees in one colony are half sisters with a very similar genome, the various phenotypes of bees can be quite different (Chittka & Chittka 2010). A bee colony mainly consists of one queen, male drones and a female worker caste (Seeley 1995). The workers are further divided into three sub-castes; nurses, foragers and winter bees, who all carry out different tasks in order to optimize the life of the beehive. The nurse- bee's main tasks are to take care of new brood, feeding and cleaning combs. The nurses are relatively long lived, with a maximum life expectancy of 50 days (Remolina et al. 2007). Most newly emerged bees start with performing typical nursing tasks inside the hive. After some time, these nurses can develop to become foragers (Münch & Amdam 2010). When nurses become over-abundant in the hive, some of them will start foraging. Foragers can, however, also develop directly after hatching (Huang & Robinson 1992).

Foragers perform their main tasks outside the hive (Seeley 1995). They collect pollen, nectar and water which they bring back to the nest workers who in turn, will process it and feed it to the larvae and queen. Compared to nurse bees, foragers have a much shorter lifespan, with an estimated life expectancy of less than 2 weeks after the onset of foraging (Münch & Amdam 2010). Thus, the aging process accelerates after the nurse – forager transformation. Former nurses also possess the exceptional ability to "switch" back from the foraging stage to the nursing stage, thereby slowing down the ageing process (Herb et al. 2012; Münch et al. 2013a). This kind of retransformation is seen for foragers returning to a weak hive with a reduced amount of nurse bees. This technique has been used by scientists, as a manipulative method for studying the age plasticity in the honey bees. Though, it is important to emphasize that this is not a typical transformation, but rather an example of extreme aging plasticity.

During their early stages of life, the queen and worker larvae receive the same diet called royal jelly, which is rich in proteins, sugars and fatty acids (Chittka & Chittka 2010). Whilst the queen is continuously fed with the royal jelly throughout and beyond the larval stage, the workers are switched to a diet consisting of gland secretions and pollen only three days after hatching (Ford 2013). The phenotypical difference between nurses and queens are therefore thought to originate from the different diets they receive through the stages of their development. There is also a huge difference in life expectancy when comparing queens and nurses. As mentioned earlier, nurses can live up to 50 days, while queen bees can live for several years (Münch & Amdam 2010; Remolina et al. 2007).

The bees' body can be divided into three different parts; the head, the thorax and the abdomen (Seeley 1995). The mouth, sensory organs (antenna and eyes) and brains are located in the head of the bee. The thorax is a connector between the head and the abdomen. It is also the bee's main locomotory center, containing muscles that control the general movement of its wings, legs and the rest of the body. The abdomen contains the bee's digestive system, and thereby houses most of the bee's organs in addition to its stinger and poison glands. An illustration of the honey bee is shown in figure 1.

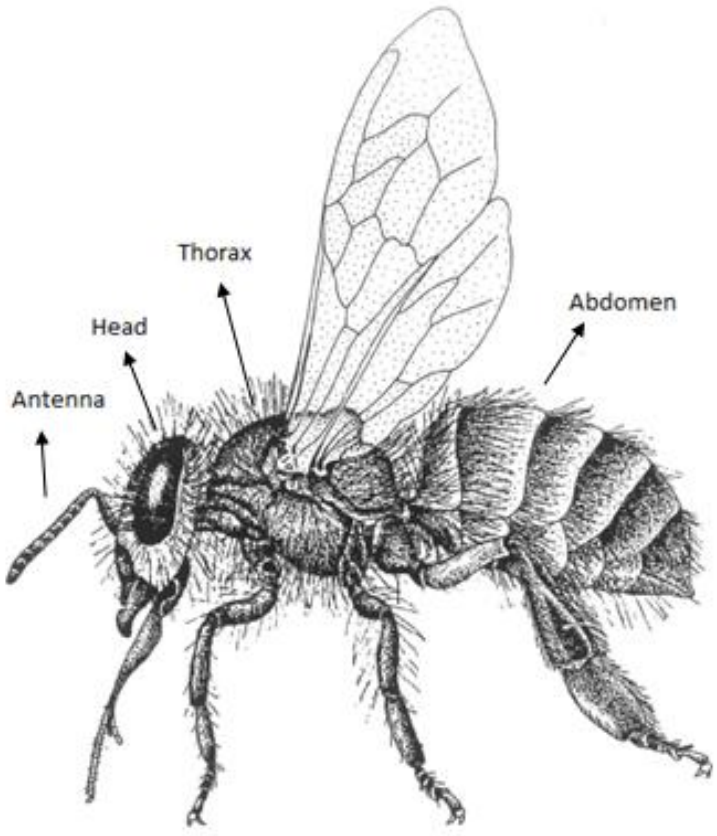

Figure 1: The anatomy of the honey bee (Winston 1987).

Another long-lived phenotype of bees, are the winter bees, *diutinus* (Seeley 1995). Winter bees can survive up to ten months, without any major signs of aging (Münch et al. 2013b). This phenotype of bees, develop when there is little or no brood in the hive, or when the need of nursing is low (like it would be in the wintertime). To secure energy for maintenance of the life inside the hive throughout the winter, winter bees possess well developed food production and storage organs in their abdomen (Arrese & Soulages 2010). Hence their main storage is bigger than what one could find in the shorter lived bee phenotypes (Seeley 1995).

In this project, the bee abdomens were dissected and DNA was extracted from cells and tissue found inside the walls of the abdomen, covering the inner organs. These tissues consists largely of trophocytes, oenocytes and fat cells (Hsieh & Hsu 2011). As mentioned above, the amounts of these tissues are much greater in the winter bee phenotype compared to the other phenotypes. Also brains were dissected for use in some of the assays conducted in this thesis.

1.2 Epigenetics

Epigenetics are defined as changes in gene expression without alternations of the genetic codes (the DNA sequence) (Watson et al. 2008). Through different mechanisms, epigenetic events can make various DNA, histone or chromatin modifications which, in some cases, can be inherited from one generation to the next. These modifications are frequently related to the packing of the DNA into chromosomes. The local packing of a chromosome is important for expression or repression of the associated genes. A tightly packed DNA is harder to access for the transcription machinery, than a loosely bound DNA. Examples of epigenetic modifications are DNA methylation, posttranslational histone modifications and chromatin remodeling (Choi & Friso 2010). Different modifications can also work as binding-sites for proteins like transcription factors, and thereby alter the downstream events of the DNA. Different modifications can also crosstalk and influence each other (Izzo & Schneider 2010).

1.2.1 Histone modifications

DNA is organized into nucleosomes; where 147 base pairs of the DNA are coiled around an octamer, consisting of the histone proteins H2A, H2B, H3 and H4 (Watson et al. 2008). First the H3 and H4 histones forms a tetramer which binds to specific sites on the DNA, then two H2A*H2B dimmers bind, forming the octamer. The nucleosomes are then coiled, and packed together into a structure called chromatin. Amino acid extensions, also known as histone "tails", protrude from the core histones through the nucleosomes. As these tails protrude the nucleosomes, they are accessible for recognition and modifications by different enzymes, which can alter the nucleosomes activity. Depending on the modification, the DNA can become either more or less condensed (tightly packed). Such modifications can also act as binding sites for other proteins and transcription-factors and determine the activity of specific genes located in the area adjacent to the modification.

The histone tails can be modified in several ways (Berger 2002). The amino acids located in the histone tails can be acetylated, methylated, ubiquitinylated or phosphorylated (other modifications are also possible). The different modifications affect different amino acids in the histone tails. Post translational modifications on histones in the nucleosomes can work together, and coordinate the downstream events of the DNA, i.e. expression or repression of adjacent genes (Ford 2013). They can also form binding sites for different proteins involved in the regulation of genes.

Histone acetylation, which will be the main focus in this thesis (regarding histone modifications), occurs on lysine residues in the histone tails (Watson et al. 2008). This acetylation is catalyzed by histone acetyltransferases (HAT's), which places the acetyl groups ($COCH_3$) on the lysine residues, using acetyl-CoenzymeA (Galdieri & Vancura 2012). The removal of these groups is catalyzed by histone deacetylases (HDAC). The acetylation of the histones causes them to lose their positive charge and, in turn, make them less bound to the negatively charged phosphate groups in the DNA (Watson et al. 2008). The histone acetylation makes the DNA more loosely packed (at the site, and adjacent to the modification), and thus more accessible for the transcription machinery. Generally, acetylation increases gene expression while deacetylation silences them (the reaction is shown in figure 2).

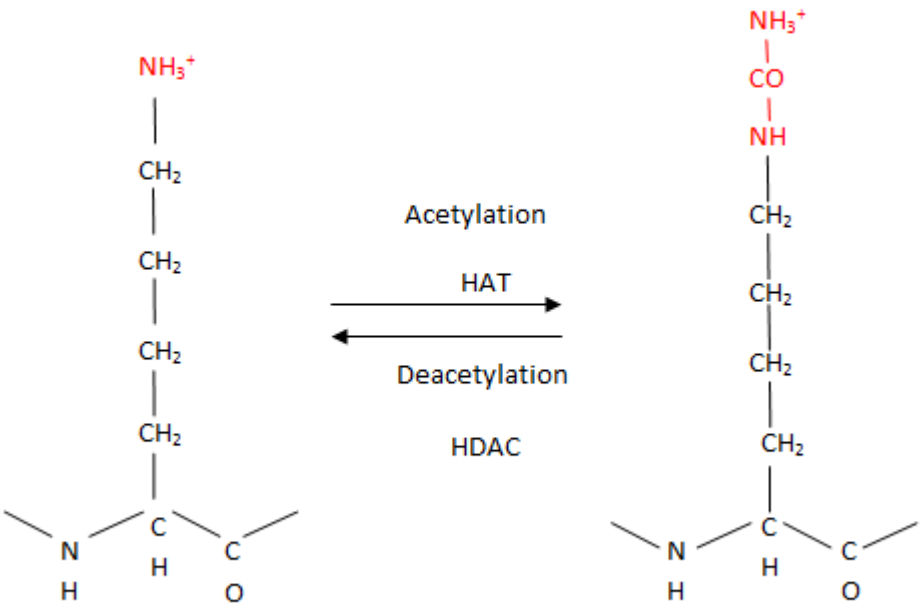

Figure 2: Acetylation and deacetylation of a lysine residue, catalyzed by HAT and HDAC respectively.

Assays have found through sequencing, that there are conserved sequences of histone modifications shared between bees and humans (Weinstock et al. 2006).

1.2.2 DNA methylation

Another modification that modifies the DNA itself (not altering the sequence) is DNA methylation. In this process, methyl-groups get attached to cytosine nucleotides in the DNA (Watson et al. 2008) by DNA methyltransferases (DNMT) using the methyldonor S-adenosymlethionine (SAM). Most often, it is the fifth carbon atom in the cytosine base that receives this modification (reaction shown in figure 3). S-adenosylmethionine is formed from a reaction between ATP and methionine, which makes a ion that has a high tendency to transfer its methylgroup (Mathews et al. 2000). This type of modification, is most frequently observed in cytosine-phosphate-guanine (CpG) dinucleotides in mammals (Jones & Takai 2001). CpG sites are commonly associated with genes, i.e. they are found in promoter regions and at exon/intron boundaries. The DNA methylation patterns are inherited through cell division.

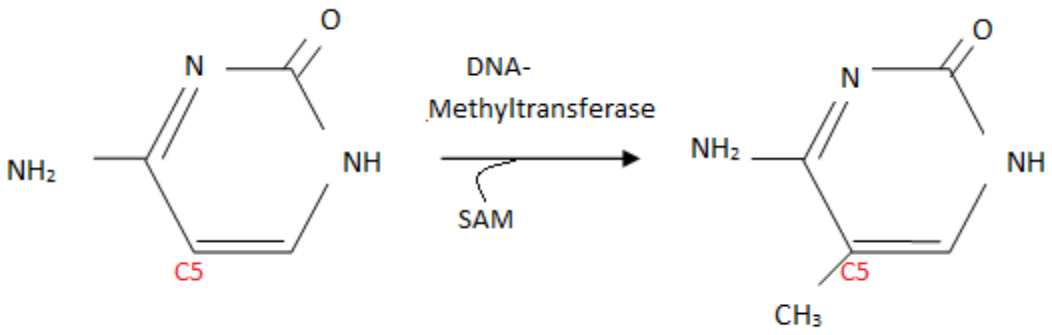

Figure 3: DNA methyltransferase methylates the 5' carbon in the cysteine base (marked red) in the DNA with the help of S-adenosylmethionine (SAM) working as a methyl donor.

After each cell division, the hemimethylated sites in the daughter cells are re-methylated by maintenance DNMT's (DNMT1), which is recruited to the newly replicated DNA (Watson et al. 2008). This mechanism ensures that the methylation patterns are conserved throughout cell divisions. Though, this DNMT is not responsible for the *de novo* methylation of the DNA. It has been shown that additional DNMTs (DNMT3a and DNMT3b) are necessary for this type of methylation (Okano et al. 1999). This was detected by knockdown of DNMT3 in mice. This genetic knockdown did not have an effect on the maintenance of the imprinted methylation pattern, but no *de novo* methylation was observed. An illustration of de novo methylation followed by maintainence methylation after cell replication is seen in figure 4.

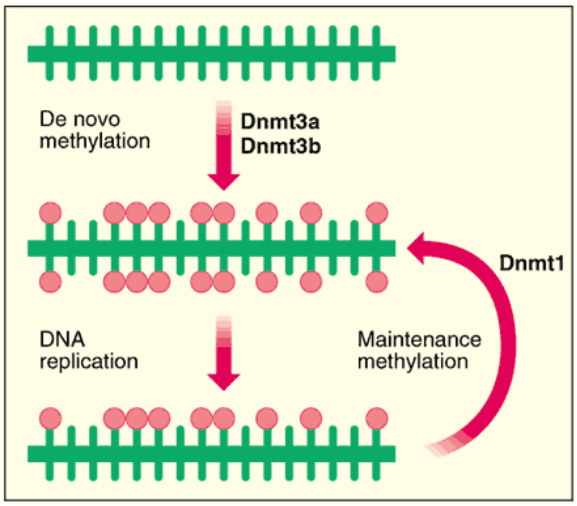

Figure 4: *De novo* methylation by DNMT3a and DNMT3b, followed by replication and maintenance methylation after replication (Bird 1999).

DNA methylation is usually linked to repression of genes, i.e. gene silencing, and the differentiation between cells (McGowan et al. 2008). The methylation then either serves as an obstacle for the binding of transcription factors, or it can affect the chromatin structure by making the DNA less accessible (Weber et al. 2005).

The methylation pattern of the DNA may also affect the acetylation patterns found in histones (Chittka & Chittka 2010), and vice versa (McGowan et al. 2008). After replication, histone deacetylases or acetyltransferases recognizes methylated or non-methylated sites on the DNA. Further they deacetylates or acetylates histones associated with the specific DNA sequence, deciding the activity of the adjacent genes. Thus, the different mechanisms of epigenetics (DNA methylation, histone acetylation/deacetylation) are interrelated. Methylated sites in the DNA bind methylbinding proteins which in turn can recruit HDAC's which demethylates the histones.

The honey bees possess somewhat the same DNA methylation machinery as mammals (Ford 2013). The DNMTs found in the honey bee and mammals are homologous, as the bees too have three DNA methyl transferases, which exert the same functions. In 2010 it was found that the honey bee is dependent on DNMT3 function for memory (Lockett et al. 2010). In the honey bee, it is also the fifth carbon atom in the cytosine in CpG dinucleotides that is modified by methylation (Lyko et al. 2010), however, the ammount of methylated CpGs in

the honey bee is much smaller than that in humans. Nevertheless, this feature makes the honey bee a more suitable model for studying epigenetic patterns, as opposed to *Drosophila melanogaster* (*D. melanogaster*) in which methylation occurs in other sites, or *Caenothabditid elegans* (*C. elegans*) which does not show any DNA methylation at all (Bird 2002). Methylated CpG's seem to accumulate in exons (coding) and some at intron (noncoding) sites in the honey bees, thus indicating that they are involved in the splicing mechanism when genes are transcribed and assembled (Lyko et al. 2010). Methylation may, therefore, have an effect on both gene expression and variance in splicing products.

1.2.3 Nutritional Epigenetics, health and aging

Epigenetic patterns can be altered by environmental factors such as nutrition and diet (Choi & Friso 2010). Bioactive food-components can, either directly or indirectly, change the epigenetic patterns by affecting the DNAs own maintenance machinery. Certain components can, as an example, act as methyl donors and are therefore bioactive. By altering the availability of methyl donors, they can affect the activity of DNMTs in the cells (Jiménez-Chillarón et al. 2012). A diet lacking methyl-donors may lead to DNA hypomethylation in individual locations of the DNA, and thereby alter the expression of genes located in these sites.

Other bioactive food components may impact the acetylation pattern of the histones by acting as HDAC inhibitors (Ford 2013). This can lead to hyper-acetylation of histones, which in turn may affect the activity of the genes adjacent to the modification. These compounds are natural, and can be found in plants and other natural products. Different HDAC inhibitors have been studied, and in some cases they are shown to exert positive effects on both health and lifespan (Evason et al. 2008; Kang et al. 2002). As the different mechanisms of epigenetic modification interrelated, bioactive food compounds can work both directly and indirectly on modifications in the epigenetic patterns.

The epigenetic patterns are also related to aging (D'Aquila et al. 2013). Thus, the rate and quality of aging can quite possibly be affected by bioactive compounds. Certain natural HDAC inhibitors have been found to have an effect on the development of the age related neurodegenerative disease Parkinson (Yuan et al. 2014). Aging is an unavoidable process in which the organism gets more fragile to environmental stress, and more prone to different diseases. Even though aging is something all organisms go through, the longevity is species

specific. However, variation within species is also observable, suggesting that environment and genetics are interrelated. In twin studies, for example, it has been shown that methylation patterns differ more and more as the individuals grow older, suggesting that maybe diet and environment plays a role in the epigenetic modifications (Fraga et al. 2005). It has been observed a global loss of DNA methylation as organisms are growing older (Calvanese et al. 2009). Also histone modifications are alternated during aging. On the background of this knowledge, it is possible that certain bioactive food components somehow can affect these mechanisms and delay the onset of senescence.

Some bioactive food components may even be effective in preventing development of cancer cells (Knowles & Milner 2001). Allyl sulfides, found in garlic (among others) have shown efficiency in preventing cancer cells from proliferating, through inducing apoptosis (cell death). Other substances have also been studied for their effect on health and lifespan. As an example, a substance found in the skin of red grapes (resveratrol), have been found to extend the lifespan of the nematode *C. elegans*, *D. melanogaster* and honey bees (Rascón et al. 2012; Wood et al. 2004). Other studies have also revealed a positive effect of resveratrol on the lifespan of mice (Baur et al. 2006). Phenylbutyrate, a HDAC inhibitor, has been found to extend the lifespan of *D. melanogaster* by re-expressing previously silenced genes (Kang et al. 2002), in studies related to Parkinson's disease. Phenylbutyrate is also a component of the royal jelly, which is the diet queen bees or larvae destined to become queen bees are fed (Lyko et al. 2010). The substance have also shown a positive effect in a mouse model of Alzheimer's disease (Ricobaraza et al. 2009). The plant *Rhodiola rosea* have also been found to effect the lifespan of bees in a dose dependant manner (Rojahn 2013). In low doses, the plant acts as an antioxidant protecting the bee from reactive oxygen species. The effects of caffeine have also been studied for its effect on the lifespan in bees (Yusaf 2012). Caffeine was then found to show a harmful effect on lifespan in high doses, but at lower doses, it seemed as though Caffeine had a positive effect.

1.3 The tested substrates

Bioactive food compounds are defined as “Essential or non-essential components that occur in nature, are part of the food chain, and can be shown to have an effect on human health” (Biesalski et al. 2009). As mentioned above, some of these substances have shown a positive effect on lifespan of different model organisms, which in turn have led to an increased interest in studying such compounds. The substances tested in this project are presented in further detail below.

1.3.1 Sodium butyrate

Sodium butyrate is the salt of the butyric acid, and has the molecular formula $Na(C_3H_7COO)$. Butyrate is a short chain fatty acid, which is naturally produced by colon bacteria when metabolizing different fibers (Zimmerman et al. 2012). The structure of sodium butyrate is shown in figure 5.

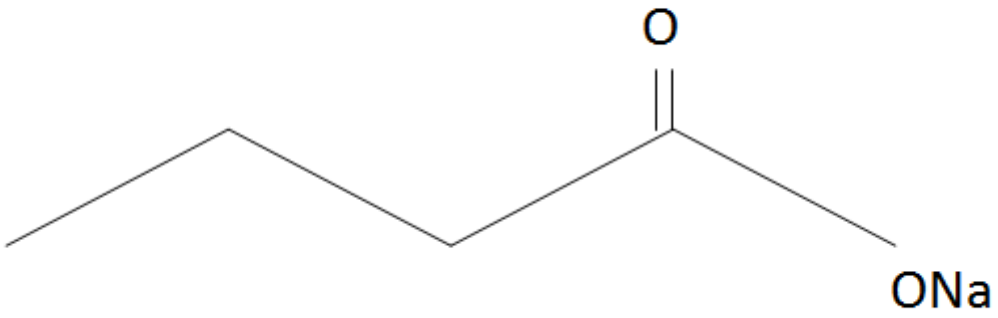

Figure 5: Sodium butyrate structure.

Sodium butyrate is a well known histone deacetylase inhibitor (Kruh 1981). Treatment with sodium butyrate can lead to inhibition of proliferation, induction of differentiation, and expression or repression of different genes. These are reactions that typically result from hyperacetylation of histones due to inhibition of histone deacetylases (Davie 2003). In relation to aging and treatment of aging symptoms, sodium butyrate has been studied for its effect on Parkinson disease symptoms (a neurodegenerative disease) (St. Laurent et al. 2013). In that

project, they studied its effect on longevity of *Drosophila melanogaster* (*D. melanogaster*) used as models by inducing Parkinson-like symptoms using the pesticide rotenone. The result from this study showed that an exposure to sodium butyrate after rotenone treatment, gave a significant effect on extending mortality and improving locomotive improvement. The substance have also shown an interesting effect in treatment of different cancer cells, by inducing apoptosis (Kuefer et al. 2004). Also, in combination with another bioactive molecule (epigallocatechin gallate – a component found in green tea) which have a DNMT inhibitor effect, sodium butyrate have been found to be effective in inducing apoptosis and cell cycle arrest of cancer cells (Saldanha et al. 2014). Studies in rats have also showed positive effects of treatment (Sun et al. 2013).

1.3.2 *Valeriana officinalis* (valerian)

The valerian is an herb, originally found in Europe and northern Asia, but is nowadays widely spread over most of the world. The Valerian is present in Volume 1 of WHO monographs on selected medicinal plants, which aims to give scientific information on safety, quality control and efficiency of plants used for medicinal purposes (WHOpublications 1999).

Extracts from the valerian roots and rhizomes have for centuries been used as an anticonvulsant and as a treatment for epileptic seizures (Eadie 2004). The plant extracts are also sold in health food stores, and are thought to have a sedative effect which in turn may help people who have trouble sleeping (Fernández-San-Martín et al. 2010). Extracts of the valerian roots and rhizomes became popular in medicinal use in the mid-1800, and were a remedy used by both ordinary people and physicians. Its use for medicinal purposes, however, can be traced back to the ancient Greece (Patočka & Jakl 2010). In addition to treating epilepsy, the valerian extracts also were used in treatment of headaches, menstrual cramps and childhood behavior problems. Even though it was the 10th most popular herbal based medicine in 1998 in the US, it is not clear which components of the extracts gives the effects or how.

In this study, two components of the *V. officinalis* extract were tested. These were Valproic acid and isovaleric acid.

Valproic acid, also called valproate (IUPAC name is 2-propylpentanoic acid), has the molecular formula $C_8H_{16}O_2$ and is a short chain fatty acid (structure shown in figure 6).

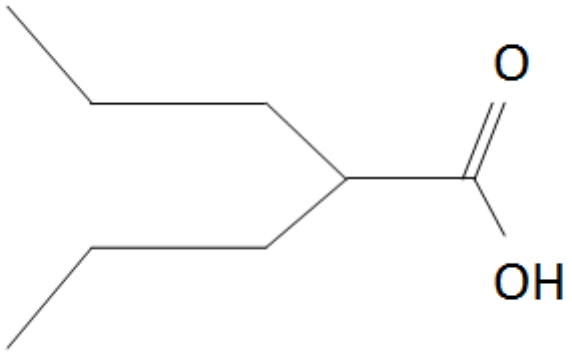

Figure 6: Valproic acid structure

Valproic acid is a well known HDAC inhibitor (Göttlicher et al. 2001), by binding to the catalytic seat of the HDAC enzyme. As the valproic acid is a compound found in the extracts of the valerian plant, it has also been used in treatment of epilepsy, which is a neurodegenerative disorder (Phiel et al. 2001). Valrpoic acids effect in treatment is believed to be connected to the regulation of different proteins. As an HDAC inhibitor, the valproic acid prevents the deacetylation of histones, which in turn may lead to hyper-acetylation. The valproic acid thereby leaves the DNA loosely packed and the previously silenced genes available for transcription. Even though valproic acid is used as a therapeutic drug, its precise method of action is uncertain (Singh et al. 2014). Valproic acid has been found to cause hyperacetylation of H3 and H4 core histones, by inhibiting class 1 and 2 histone deacetylases, and through inhibition of these causes differentiation between cells (Gurvich et al. 2004).

Isovaleric acid (Figure 7), or 3-methylbutanoic acid is also a component of the valerian extracts.

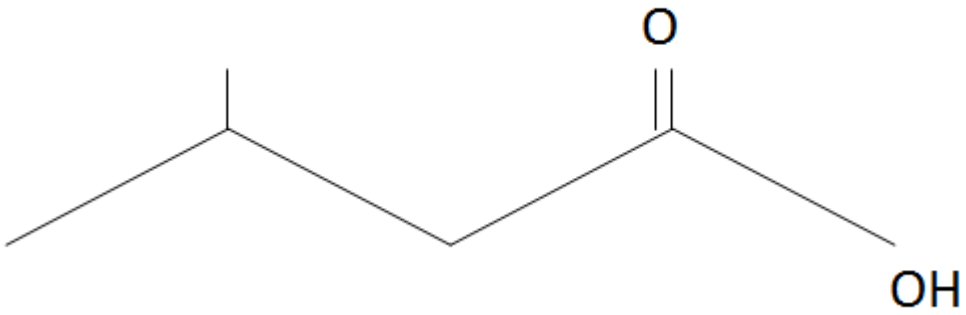

Figure 7: Isovaleric acid structure

Isovaleric acid is known for its unpleasant odor, which can be characterized as somewhat cheesy. The acid can also be produced by skin bacteria that metabolize the amino acid leucine (Ara et al. 2006), but it is also found in the extract from the valerian plant. It is debated whether the isovaleric acid is a natural compound of the extract, or if it is just a byproduct from the extraction (Eadie 2004). The isovaleric acid has also been assumed to be the anticonvulsant component in valerian extracts. Nevertheless, because of its smell the isovaleric acid has not been a popular remedy in this context (Patočka & Jakl 2010).

1.4 Aim of study

The focus in this study will be to examine the lifespan and epigenetic (DNA methylation) effects of sodium butyrate, valproic acid and isovaleric acid when these are fed to honey bees.

This information could then contribute to the understanding of how these compounds exert their functions, and in turn how they might affect aging. Since the bees' epigenetic machinery is somewhat similar to that of humans, it is possible that compounds that show a positive effect in bees might also show positive effects in humans.

2. Materials

Table 1: Chemicals and equipment.

Chemical/equipment	Purity/ concentration	Catalog number	Lot number	Supplier
Food blends				
Grace's amino acids, solution-modified for TC-100	10x		57H2348	SIGMA-ALDRICH
Bifor		Bar code: 27310340 112873		Nordic sugar
Isovaleric acid	99 %		BCBG1133V	SIGMA-ALDRICH
L-alanine	≥98 %		BCBL0498V	SIGMA-ALDRICH
L-histidine	≥99 %		SLBC6818V	SIGMA-ALDRICH
Lipide mixture	1000 x		SLBF7419	SIGMA-ALDRICH
RPMI 1640 AMINO ACIDS SOLUTION	50 x		RNBC8950	SIGMA-ALDRICH
Sodium butyrate	98 %		MKBL5008V	SIGMA-ALDRICH
Valproic acid sodium salt	≥98 %		SLBC9758V	SIGMA-ALDRICH
Western blot				
Acetic acid	>99 %			MERCK
BSA	>98 %			SIGMA-ALDRICH

Brillian blue R			20K1534	SIGMA-ALDRICH
Immun-Blot® PVDF Membrane for protein blotting		162-0177		BIO-RAD
Laemmli sample buffer		161-0737		BIO-RAD
Methanol	99 %			SIGMA-ALDRICH
Mini-protean TGX gels 4-20 %		456-1096		BIO-RAD
Phosphate buffered saline (PBS) tablet		9171S	SLBF5741V	SIGMA-ALDRICH
Primary antibody, H3K23ac polyclonal antibody			A615 – 001	Diagenode
Precision Plus Protein Standards, unstained		161-0363		BOI-RAD
Secondary antibody, Goat-anti rabbit Cy5				Jackson immunoresearch
SYPRO Ruby Protein Blot stain	1 x	170-3127		BIO-RAD
Tris/Glycine buffer	10 x	161-0771		BIO-RAD
Tris/Glycine/SDS buffer	10 x	161-0772		BIO-RAD
Tween® 20				SIGMA-ALDRICH
Typhoon 8600 variable mode imager				Molecular dynamics

DNA extraction and Enzyme immunosorbant assay (ELISA)				
Absolute ethanol	100 %			
Buffer ATL, Tissue lysis buffer			139271811	QIAGEN
Chloroform:Isoamyl alcohol			SLBD9562V	SIGMA-ALDRICH
Linear acrylamide			1207020	Life Technologies
Proteinase K, From Tritirachium album, crude lyophilized powder.	20 mg/ml		026K8607	SIGMA-ALDRICH
Pure link RNAase A			1413314	Invitrogen, Life Technologies
Sodium acetate solution. BioUltra, for molecular biology	3 M in dH_2O		BCBJ2797V	SIGMA-ALDRICH
Ultra Pure™ Phenol:Chloroform:Isoamyl Alcohol	25:24:1		1162C081	Invitrogen, Life Technologies
ELISA Kit			ZRC175732	ZYMO RESEARCH
SPECTRO star^{Nano}				BMG LABTECH
Qubit® 2.0 Fluorometer				Invitrogen, Life technologies

3. Methods

3.1 Experimental setup

The experiments were conducted during the fall of 2013 and the winter of 2014, at the Norwegian University of Life Sciences (NMBU) in Ås, Norway. A total of approximately 3200 European honey bees (*Apis mellifera carnica* Pollmann) were used throughout the whole study, covering both the lifespan studies and the molecular assays. The bees were sampled from NMBU's own bee facilities. Winter bees were used for all the assays conducted in this study to ensure similar characteristics between the bees.

To obtain winter bees in fall (September/October), the queen bees were caged for 2-4 weeks before sampling. When the queen is caged no new brood is produced, and the workers transform to the winter phenotype, like explained in the introduction.

3.2 Lifespan studies.

The sampling of bees was arranged in the same way for all the substances tested. To allow testing for differences due to other factors than treatment, such as hive effects or sampling dates, bees were collected from two separate hives, 2-3 days apart. The first day of sampling, 8 cages were sampled for each substance tested (marked in green in figure 8). 4 of the cages were sampled from hive 1 (H1), and the other four from hive 2 (H2), hence, producing hive replicates. Each round gave 2 cages per concentration treatment (control/ treatment1/treatment2/treatment3) originating from different hives (hive replicates). 2-3 days later, 8 new cages were sampled (from the same two hives as in the first round) to produce round replicates (marked red in figure 8). While sampling, it could be difficult to keep count due to sampling conditions, but approximately 50 bees were sampled in each cage, one by one.

The general setup for all the three substances tested is shown in the figure below:

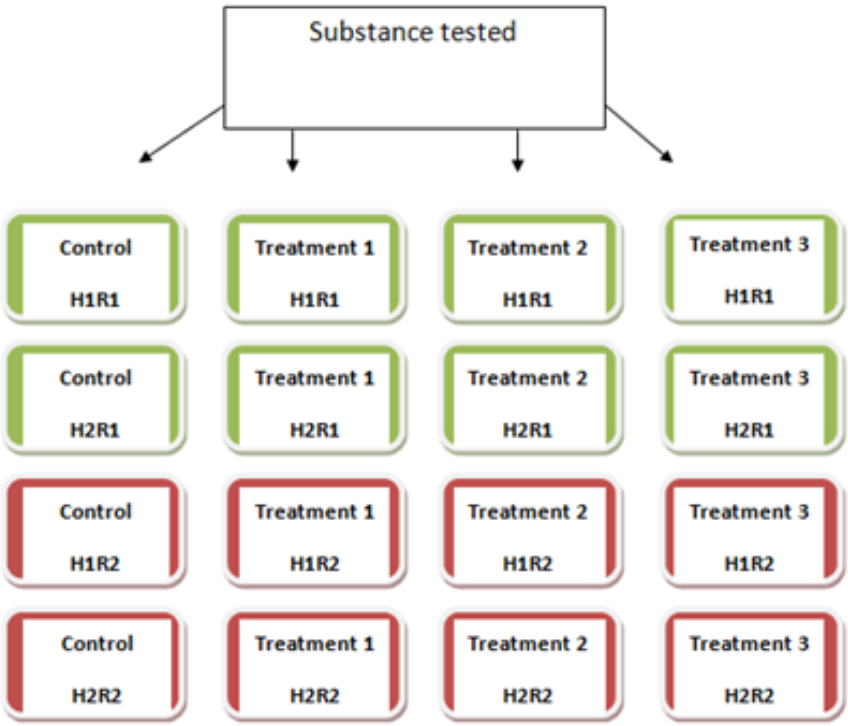

Figure 8: The general setup for the lifespan study. The green boxes represent round 1, and the red round 2. R = round and H = hive.

Before sampling, cages had to be prepared. The plastic boxes, used as cages, were purchased at a local store (Europris). To convert the boxes into more suitable cages for the bees, two larger holes were carved out in the front and the back. To make the bees' easily observable while inside the cage, these were covered with a plastic lattice. In addition, two smaller holes on the top and one on the lower side were drilled out. The two on the top were made for placement of food and water tubes, while the one on the lower side was made for the easy removal of dead bees.

Inside the cages, a steel mesh was placed near the bottom to prevent bees from sticking to it and drowning, if food or water were to drip from the tubes (this usually occurred under the daily changing of tubes). Foam rubber was placed to cover the edges between the mesh and the cage walls, to keep bees from climbing under and getting stuck. To make the environment more familiar for the bees, one wall inside the cage was covered with beeswax. An example of one cage is shown in figure 9.

Figure 9: Picture of one of the cages used in the experiment, with one food and one water tube on the top.

The original recipe for the bees' control food is listed below. This recipe was obtained from a previous master thesis (Rojahn 2013). The food solutions containing the substances were made by replacing some of the water with a given volume of a stock solution of the relevant substance (powder resolved in distilled water) to achieve wanted concentration. For the isovaleric acid, there was no need to make a stock solution, since the substance was liquid.

- 50 % Bifor (75 % sugar (37 % sucrose, 19 % glucose, 19 % fructose) 25 % water).
- 2 % Grace amino acid mix/ RPMI 1640 amino acid solution
- 1 % lipid mix
- 47 % distilled water

The different concentrations to be used were chosen based on relevant reference literature dealing with lifespan studies, preferably with insects or animals similar to bees, with the same or similar substances. From this search, three different treatment concentrations were chosen for each of the 3 substance (Sodium butyrate, Valproic acid and Isovaleric acid).

When preparing the treatment solutions with the valproic and isovaleric acid, the "Grace amino acid mix" was no longer distributed. Thus, another amino acid mix was used in making

the food. Since this mix (RPMI 1640 amino acid solution) lacked, or had insufficient amounts of some amino acids present in the Grace amino acid mix (L-Alanine and L-Histidine), these had to be added separately to make sure that all of the food blends had a comparable composition.

Table 2 shows the content of the amino acids L-histidine and L-alanine in both the Grace amino acid mix, and the alternative amino acid mix. The other table (table 3), show the treatment concentrations used for each substance. As seen from this table, the same concentrations were used for both isovaleric acid and valproic acid. To cover a reasonable range of treatment concentrations, the concentrations were separated from each other by a factor of ten.

Table 2: The content of amino acids in the Grace amino acid mix and the RPMI 1640 amino acid solution.

Amino acid	Grace amino acid mix	RPMI 1640 amino acid solution
L- histidine	2,25 g/L	0 g/L
L-alanine	25 g/L	0,75 g/L

Table 3: Treatment concentrations for the survival studies of sodium butyrate, valproic acid and isovaleric acid.

Substance tested and treatment	Concentration
Sodium butyrate, treatment 1	1.0 mg/ml
Sodium butyrate, treatment 2	0.1 mg/ml
Sodium butyrate, treatment 3	0.01 mg/ml
Valproic/isovaleric acid treatment 1	10.0 mg/ml
Valproic/isovaleric acid treatment 2	1.0 mg/ml
Valproic/isovaleric acid treatment 3	0.1 mg/ml

After preparation, the food was poured into 15 ml tubes. 4 holes were drilled near the bottom of each tube, so that the bees could easily access the food by sticking out their proboscis (“tongue”). Approximately 12 ml was poured into the marked tubes before they were flipped

upside-down a few times, to prevent leakage later on. The food tubes were then stored at -20 ºC.

The temperature and humidity inside the cabinet where the bee cages were kept, was monitored and recorded daily. At the same time, the volume of consumed food and the amount of dead bees inside the cages were registered. To ensure similar time intervals, this procedure was done at approximately in the same period of time every day for the duration of the experiment. Dead bees were removed, new food and fresh water was exchanged daily. Other relevant observations, like escaped individuals or potential incidents due to handling, were noted.

For the sodium butyrate lifespan studies, the first round of bees was collected on September 4, 2013. The second round was collected on September 6, 2013. To account for the bees that might be injured or weak due to sampling, all the cages were put on the control diet for two days before starting the treatment (this acclimatization period was also applied for the bees used in the valproic and isovaleric acid lifespan study). The experiment was terminated when the last bee in the last cage died on October 19, 2013.

The bees for the valproic acid and isovaleric acid experiments were collected on the same two days and the experiments were run in parallel. Bees for round one were collected on November 8, 2013, while the second round of bees was collected on November 11, 2013, separating the two rounds by three days. The experiment went on until December 20, 2013, and terminated by placing the last surviving bees at -20 ºC. The amount of bees left at this date, was so small that terminating the study at this time point, did not seem to affect the statistical power of the results in a negative way.

3.3 Statistics for the lifespan study

The survival data from the lifespan studies were listed in spreadsheets, where each individual bee was registered. The spreadsheet gave information about how long the bee had survived, which treatment it had been given, which hive it came from, and in which round it had been collected in. The survival statistics used a censoring scheme in which bees that either escaped, or died by accident not caused by the treatment itself, were registered as censored individuals.

The finished spreadsheets were exported to the statistical software STATISTICA. First, a Kaplan Meier analysis was conducted for the overall data, from each substance tested. As a result, a graph showing the statistics of survival was given together with corresponding p-values and chi^2s. The p-value revealed whether any effect of treatment was observed or not. The significance level was set to be 0.05.

If a treatment effect in the overall data was observed, two-sample cox-F tests were conducted, comparing each concentration treatment with the controls. This comparison was done to see which one of the concentration treatments that showed a significant effect lifespan compared to the control. After performing these tests on the overall data, two-sample cox-F tests were run to observe if there were any effects of replicate, i.e. effect of round or hive (if bees collected from different hives, or in different rounds responded differently to the treatments). If round or hive effects were detected, data from the respective hives or rounds were studied further separately. These data were processed in the same way as for the pooled data, by first running a Kaplan Meier test, then pairwise cox-F tests.

Some cages went through unforeseen incidents. Cages where the bees had escaped early in the lifespan study could affect the social environment, and stress the bees inside the cage for the rest of the experiment. Also, cages that suffered from starvation due to lack of holes in the food tubes, causing the bees to weaken were excluded from the statistical analyses. The reason for removing these cages was to eliminate all possible effects on lifespan that did not correspond to the treatments.

3.4 Screening for epigenetic modifications

3.4.1 Preparation of bees for epigenetic testing.

The bees used for the molecular tests were collected in one round on January 15, 2014 (after the lifespan studies were terminated). As in the lifespan study, 50 bees per cage were collected and put on a control diet the first 2 days of captivity (Hive replicates were sampled, but round replicates were not conducted for these bees). For the treatment of these bees, only two of the three concentrations of each substance were chosen. To select which concentrations to use for treatment, the statistical information from the lifespan studies was used (results from Kaplan Meier and the cox-F tests). For the choice of concentrations, these three rules were applied:

1. The concentration to be used must show significant effect in the lifespan studies.
2. If there was no significant effect, use the two highest concentrations.
3. Do not use concentrations with a death rate exceeding 50% within the first 10 days.

The bees were then kept on the selected diet for 10 days (12 including the first two days with control treatment). Like in the lifespan studies, dead bees were removed from the cages, water and food tubes were replaced and the consumption, temperature and humidity were listed daily. This way, comparison of the behavior and environment of the molecular bees to the lifespan bees was possible. Potential differences could then be taken into account when discussing the results.

3.4.2 Western blot

To observe potential histone acetylation modifications, resulting from treatment with any of the three substances, western blots were meant to be conducted. To provide tissue for the analyses, bees had to be dissected. To test the protocol, non-significant bees (random bees) were used. These bees were obtained from the supervisor.

For the western blot, both tissues from the abdomen and the brain were used. After dissection, the brains were put in 25 µl homogenizing buffer (PBS with 1mM disodium EDTA and complete protease inhibitor) in 1.5 ml Eppendorf tubes from Axygen (one brain in each tube), before they were homogenized by using a pistil.

The abdomens were prepared by removing the mid-gut (intestines) and placing the remains (exoskeleton containing the "fat body" tissue) in 100 µl homogenizing buffer (the same as used for the brains). The abdomens were homogenized with a pistil before they were spun at 10 000 x g for 20 min. To get rid of the exoskeleton, the supernatant was taken out and transferred to new tubes. The prepared samples were then stored at -20 ºC until sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was conducted.

Two gels with the exact same samples were run in parallel. One of them was meant for western blotting, while the other one was stained with a coomassie brilliant blue dye.

SDS-PAGE: For the gel electrophoresis, 15 well precast polyacrylamide gels from BIO-RAD were used. The samples were diluted 1:1 with 2x Laemmli buffer and heated for 5 min at 95 ºC to denature the proteins. Then 12 µl of each sample were loaded onto each well. The

remaining wells were filled with distilled water (dH_2O) and laemmli buffer to avoid, or reduce, a possible "smiley"-effect on the gel. Also, a molecular weight standard (ladder) was added to the first well, so that later it was possible to estimate the size of the proteins separated on the gel.

The gels were first run for 5 min at 75 V, and then for another 1 h and 15 min at 100 V. The laemmli buffer contains SDS, which binds to protein and gives them a negative charge (Brown 2007). As an electric current is added to the system, the proteins will travel towards the positive pole (anode). By using an electric field one can separate proteins from each other, based on their size. Smaller proteins run further through the gel (since they are more easily able to migrate through the pores), while larger proteins don't migrate as far. Figure 10 illustrates the principle of gel electrophoresis.

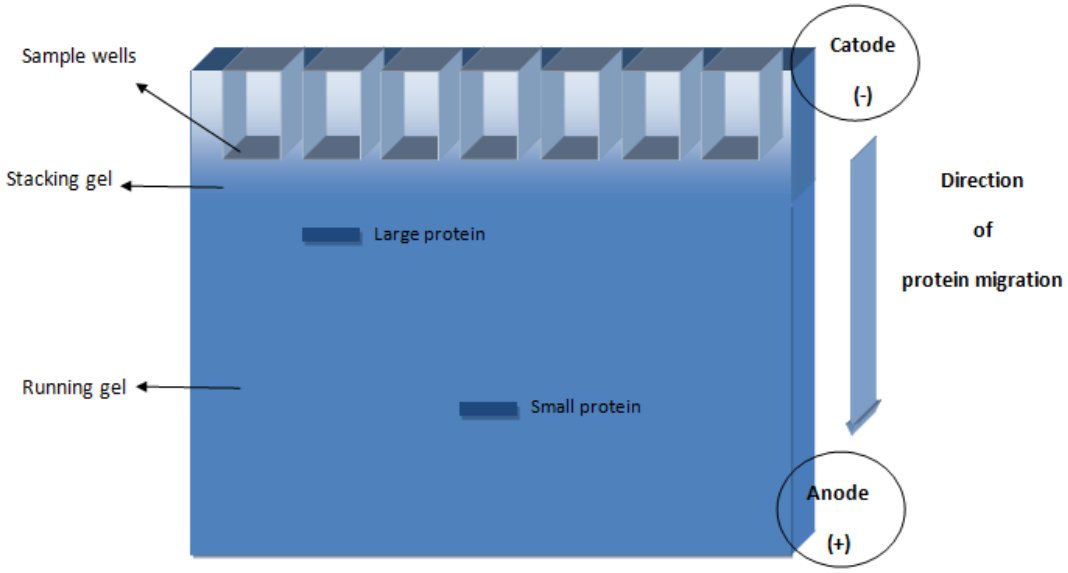

Figure 10: The principle of gel electrophoresis. Smaller proteins migrate further than the larger ones because of the pores in the polyacrylamide gel. The proteins will migrate towards the anode, once the current is applied to the system.

Coomassie staining: After the gel electrophoresis, the gel meant for coomassie staining was removed from the plastic plate and placed in a premade coomassie brilliant blue dye. This solution consisted of 0.2 % Coomassie brilliant blue R-250, 50 % methanol and 7.5 % acetic acid (Rasmussen 2011). The gel was microwaved until boiling point, and incubated at room temperature with shaking for 30 min. Coomassie dye binds unspecificly to all proteins

separated, which makes it possible to visualize all the protein bands on the gel. To remove the unbound dye, the gel was washed once with distilled water, before it was left in destaining solution (containing 5% methanol and 7% acetic acid). The gel was then again heated in the microwave until boiling point, and left on a shaker at room temperature for 2 h. To reduce the destaining time, a paper cloth was put in the destaining solution beside the gel, to soak up the excess dye.

Western blotting: The other gel was used for Western blotting (wet transfer). A polyvinylidene difluoride (PVDF) membrane was presoaked in methanol for activation, and left in blotting buffer before use. The blotting cassette was assembled and run at 100 V for 2 h with a frozen cooler, to prevent the gel from sticking to the membrane. After 1 h, the frozen cooler was replaced by a new one. As for the gel electrophoresis, an induced electrical field made the proteins separate. As they separate, they transfer from the gel to the membrane (which is closest to the positively charged electrode), and get attached to it.

Immuno detection: After the blotting step, the membrane was left in blocking buffer (BSA) for 2.5 h with shaking at room temperature, to avoid unspecific binding of the primary or secondary antibodies when they were added, i.e. blocking epitopes. After this, the primary antibody (H3K23ac rabbit polyclonal antibody) was added (1:250). The selected antibody was polyclonal, thus binding various epitopes, but stated (by the manufacturer) to be specific for the H3K23ac protein (Histone 3 with an acetyl group at its 23d lysine residue). The antibody was diluted directly in the blocking buffer. To prevent contamination, the membrane was incubated over night at 4 ºC with gentle shaking. The next day, the gel was first washed twice with 1x PBS-T (0.5% tween-20), and left for 30 min with PBS-T to remove the excess unbound antibodies. Finally it was washed twice, 15 min each, at room temperature.

After these washing steps, the secondary antibody (goat-anti-rabbit, Cy 5) was diluted 1:250 in 10 ml blocking buffer. Since the primary antibodies that were used, were produced in rabbit, the secondary were an anti-rabbit antibodies. Thus, the secondary antibodies were produced in rabbit-antigen-immunized goats, to recognize the epitopes of the primary antibody from the rabbit (Lea 2008). A fluorescent molecule covalently bound to the secondary antibody, made it possible to observe the protein bands when the membrane was scanned.

The box was then wrapped in foil, and incubated for 1 h at room temperature with gentle shaking. Finally the membrane was washed in the same way as described above with PBS-T

to get rid of the excess secondary antibody before scanning. The scanner that was used was: Typhon 8600 Variable mode imager. From previous tests, the best pictures were given using these settings: cy5 filter; 670-633 nm; 400 V; normal.

Unspecific protein staining: After the scanning, the membrane was submerged in 15 ml of 7% acetic acid and 10 % methanol for 15 min, and washed 4 times for 5 min each, with dH_2O. The membrane was then incubated with SYPRO Ruby stain reagent for 15 min for non-specific protein staining. Before scanning, the membrane was washed twice, two minutes each, with dH_2O. The scanning settings used for this scan was: Rox filter; 610-523 nm; 400 V; normal.

3.4.3 DNA extraction

To observe changes in the global DNA methylation levels in bees receiving the different substances, a 5-mC Enzyme linked immuno-sorbant assay (ELISA) was conducted. To perform ELISA on the bees, DNA had to be extracted. For this, the abdomens of the honey bees were used. The procedure started with dissection of the abdomen. To avoid contamination with bacterial DNA (from the gut), the bees' intestines were removed by using a pair of tweezers, leaving an "empty" abdomen. The abdomens were then cut in two, from the posterior to the anterior end, and put in separate marked Eppendorf tubes (Axygen) together with 200 µl tissue lysis buffer (ATL buffer). A pistil was used to homogenize the samples, and to break the cell walls. Finally 20 µl proteinase K was added to the tubes, before they were incubated at 56 ºC overnight (16 h) with shaking at 400 rpm.

The next day, the samples were cooled down at room temperature for 5-10 min. The supernatants were transferred to new Eppendorf tubes (to avoid possible leakage due to the heating of the tubes), and dH_2O was added to reach a final volume of 550 µl. To degrade the proteins (Sambrook & Russel 2001), an equal volume of phenol:chloroform:isoamyl-alcohol (25:24:1) (PCI), 550 µl, was added to each tube. PCI binds to and removes proteins from the DNA nucleic acids (i.e. histones), and leaves the DNAin the aqueous phase. Phenol, being non-polar, makes the proteins fold their non-polar residues inside out, thus denaturing the protein and holding it in the organic phase (illustrated in figure 11). The DNA is left in the aqueous phase.

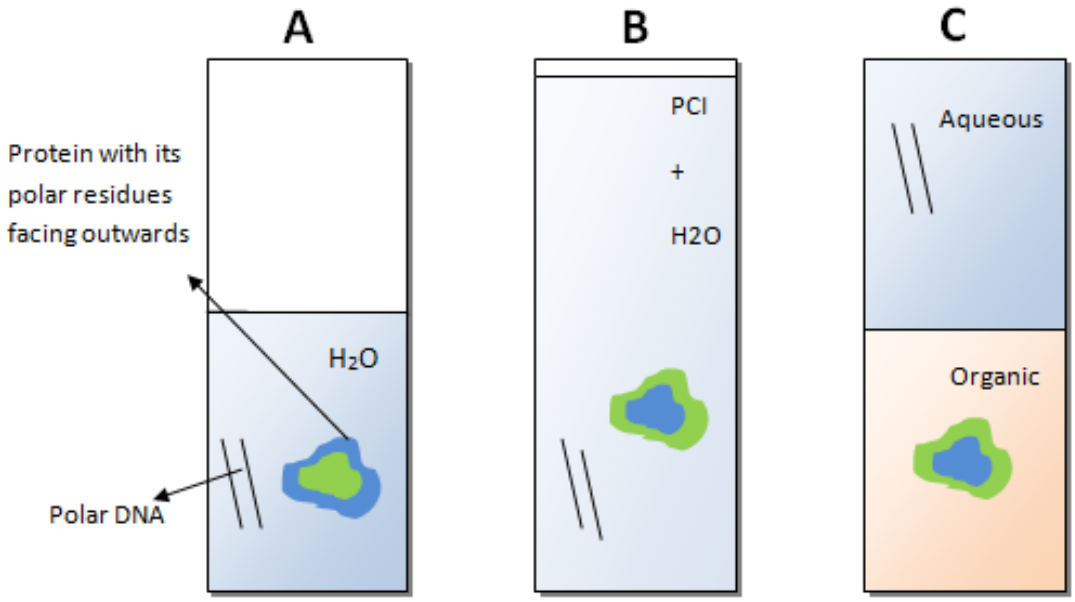

Figure 11: The principle of DNA extraction using PCI. PCI makes the protein flip its less polar residues outwards (B), and remain in the organic phase after spinning, while the polar DNA molecule is found in the aqueous phase (C).

The tubes were vortexed 3 x 5 sec and centrifuged for 5 min at RT and 15 000 x g. The upper aqueous phases, containing the DNA, were transferred to new tubes, while the organic phases holding most of the degraded proteins were discarded. To remove RNA from the samples, 20µl of RNAase A (20 mg/ml) was added, before incubation at 37 ºC for 30 min and 550 rpm. After incubation, the samples were chilled for 2-5 min before the supernatants again were transferred to new tubes. Eequal volumes of PCI were then added to the samples a second time, before they were vortexed and centrifuged in the same way as described above. The aqueous phase, still containing the DNA, was transferred to new tubes while the proteinaceous organic phases were discarded. Next, equal volumes of chloroform:isoamylalchohol (24:1) (CI) were added to the samples, before they were spun as above and the aqueous phase were transferred to new tubes (maximum 400µl). The reason for adding CI was to remove excess phenol from the aqueous phase. Phenol is a very toxic substance, and could have interfered in the later reactions.

Finally, the DNA was precipitated with 1/10 volume of 3M NaAc (40 µl), 5 µl linear acrylamide and 2.5 x ice-cold absolute ethanol. The tubes, containing the DNA were vortexed, and incubated at -80 ºC overnight. Sodium acetate is used for neutralizing the

negative charge in the DNA's phosphate backbone, in turn, making the DNA less soluble in water. The alcoholic environment makes the interaction between the salt (Na^+) and the negative phosphate groups stronger, thus precipitating the DNA.

The next day, after defrosting the samples on ice, the tubes were spun at 20 000 x g at 4 ºC for 15 min, to pellet the DNA. The pellet was then washed twice with 1 ml 70% ethanol and spun at 20 000 x g at 4 ºC for ≥8 min, to eliminate the remaining salt residues. The following step was to remove as much of the ethanol as possible, and leave the pellet to dry with the tube cap open (10-15 min).

When the DNA pellet was dry, it was dissolved in 40 µl dH_2O. A Qubit fluorometer was used to check the DNA yield. Finally, the samples were stored at -20 ºC.

3.4.4 Enzyme linked immunosorbent assay (ELISA).

To investigate 5-mC levels in the bees DNA, ELISAs were conducted. The instruction manual for the 5-mC DNA ELISA kit, from ZYMO RESEARCH, was used to perform these assays (listed in appendix 1).

To decide which samples to be used for the ELISA, the concentrations given from the Qubit fluorometer were used. Samples with DNA concentrations < 4 ng/µl were discarded, as their concentrations were too low.

Each sample consisted of extracted DNA from one bee's abdomen. The first plate was filled with DNA samples extracted from the molecular bees fed with the two concentrations of sodium butyrate, and the control containing the Grace amino acid mix.

4 samples were applied in 4 wells on the plate, from each concentration of the substances that were tested (the two concentration treatments, plus the controls). Each sample consisted of DNA extracted from one single bee given one specific treatment. Each sample was run in duplicates (technical duplicates) on the plate, giving a total of 4 biological replicates, in 8 wells per concentration treatment. The bees used for dissection and in the ELISA assay, were randomly chosen from each specific cage. As mentioned above, 4 bees were chosen from one treatment. In the feeding setup for the bees meant for the ELISA, the bees were collected from two separate hives (like in the survival study). Thus two of these bees were collected from one cage, the other two from the other (hence Hive 1 and Hive 2). For the 3 treatments of each

substance (control/ treatment 1/treatment 2) a total of 24 wells were filled with samples of extracted DNA including the duplicates. In addition, 7 wells were filled with standards (0%, 5%, 10%, 25%, 50%, 75% 100% 5-mC DNA) and one blank (dH_2O). Each well was filled with 100 ng DNA.

Before the samples could be applied to the plate, the DNA had to be denatured. The samples were first transferred to Eppendorf tubes and brought to a final volume of 100µl, with 5-mC coating buffer. The DNA was then denatured at 98 ºC for 5 min. After denaturing, the samples were put directly on ice for 10 min to keep the DNA from renaturing, before they were transferred to the wells. For a more reliable reading, the samples were applied pseudo-randomly. Thus, if there was something wrong with the reading, or the wells on the plate, this would not impact all the samples from one category. After application, the plate was covered with foil and incubated for 1 h at 37 ºC, constituting the DNA coating step. In this step, the DNA was coated onto the well surface. After 1 h, the coating buffer was discarded from the wells, before they were washed three times with ELISA-buffer. 200 µl of ELISA buffer was then added to each well, before the plate again was incubated at 37 ºC for 30 min for blocking.

After 30 min the buffer was removed from the wells, and 100 µl of an antibody mix consisting of ELISA buffer, anti-5-methylcytocine and secondary antibody was added to each well. The plate was, yet again, incubated at 37 ºC for 1 h. In this step, the primary and secondary antibodies attached to their epitopes. The primary antibody (anti-5-methylcytocine) attached to 5-mC DNA sites in the single stranded DNA, while the horseradish peroxidase (HRP)-conjugated secondary antibody attached itself to the primary antibody.

After incubation, the antibody mix was discarded from the plate, and the wells were again washed three times with ELISA-buffer. Finally, 100 µl HRP developer was added to each well, and color was allowed to develop for 45 min (from previous test runs with ELISA, 45 min seemed to be a sufficient time span for color development) at room temperature. The HRP – developer contains the horseradish peroxidases substrate which, when catalyzed by the enzyme conjugated to the secondary antibody, develops a color. The more 5-mC present in the single stranded DNA from the bees, the more antibodies bind, and the color becomes stronger. Finally an ELISA plate reader (SPECTRO $star^{Nano}$) was used for measuring the absorbance at 405-450 nm.

The exact same procedure was used for the second plate, where the samples from bees fed with valproic acid, isovaleric acid and the controls (RPMI amino acid mix) were applied.

4. Results

4.1 The lifespan studies

The first experiments conducted, were based on the hypothesis that certain plant compounds or natural substances can affect the lifespan of honey bees. Separate experimental groups were given different concentrations of each substance. The reason for testing different concentrations was to see if dose had an effect, and to estimate which dose was optimal if the effect was positive. To identify the effect of the given substance, one group for each substance tested was given a control diet consisting only of sugars, lipids and amino acids. As mentioned in the methods part, Kaplan Meier analyses were conducted on the overall survival-data from each of the three lifespan studies, to detect significant effects of treatment. As a general rule, the significance level for all of the statistics was set at 0.05.

Two horizontal lines are drawn in the survival graphs for each substance, one at 0.5 and the other at 0.1 on the Y-axis. These lines represent the bees' median and maximum lifespan respectively (hence 50% and 10% survival). These terms will from now on be used when presenting the results, and when comparing treatment concentrations.

4.1.1 Sodium butyrate:

Three concentrations of Sodium butyrate were used as treatment for the bees; 1.0 mg/ml, 0.1 mg/ml and 0.01 mg/ml. The results from a Kaplan Meier analysis on the overall lifespan data, showed that treatment with sodium butyrate did have significant overall effect on lifespan ($N_{control/\ 0.01mg/ml/\ 0.1mg/ml/\ 1.0\ mg/ml}$ = 198/209/154/209; Chi^2 =16.97; $p<0.001$). One cage was excluded from the analysis due to a substantial loss of individuals early in the study (day 3). Thus, the middle concentration with 0.1 mg/ml sodium butyrate is represented with 3 cages instead of 4.

The graph below shows the effect of each treatment from the overall data from the sodium butyrate lifespan study. When studying the graph, there does not seem to be any major

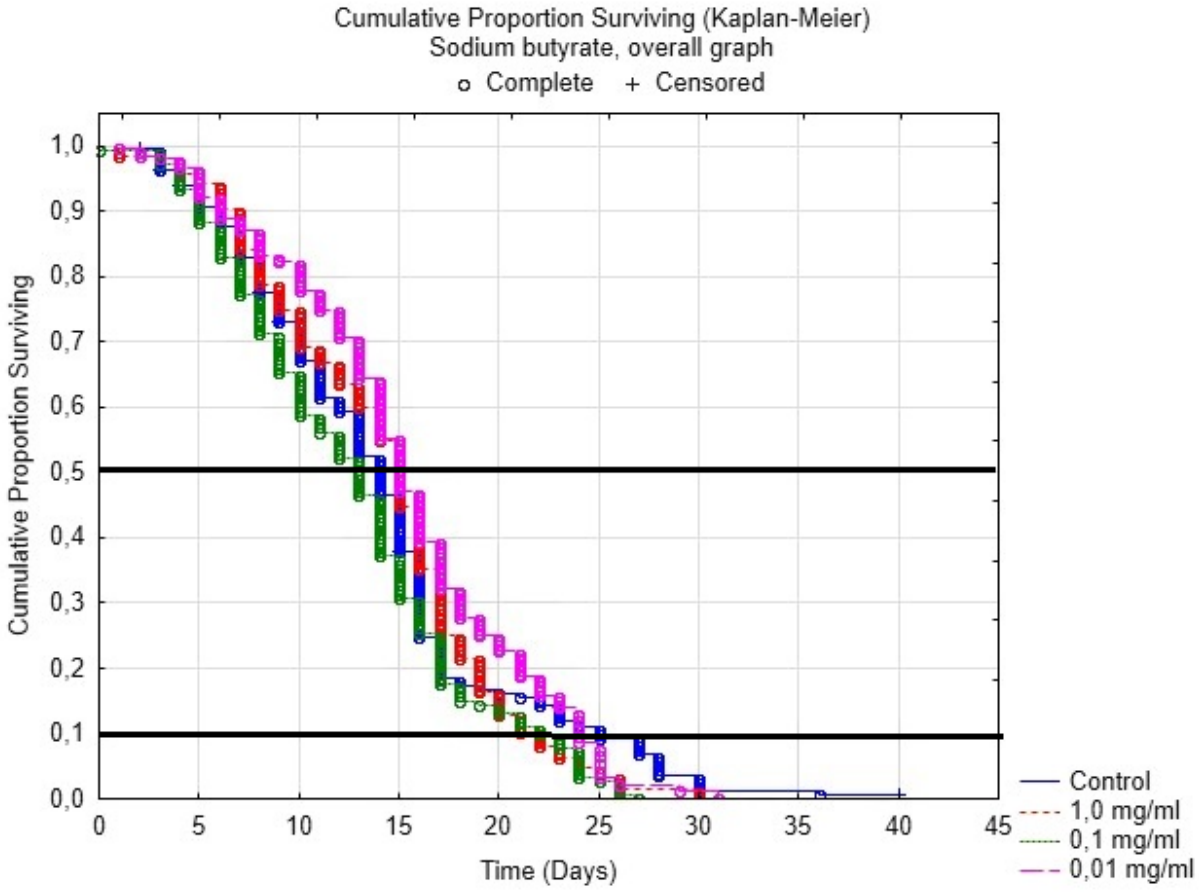

Figure 12: Treatment effect of sodium butyrate on lifespan (overall data). The blue graph shows the survival-curve for the control, the pink is the 0.01 mg/ml, the green the mid 0.1 mg/ml and red is the 1.0 mg/ml concentration treatment with sodium butyrate. The two lines at 0.5 and 0.1 represent the bees' median and maximum lifespan.

differences between the control and the three treatments at median lifespan. When considering maximum survival, they seem to differentiate a bit more from one another. At the maximum survival rate, the 1.0 mg/ml and the 0.1 mg/ml concentration treatments of sodium butyrate reduce the bees' lifespan. These two treatments reach their maximum lifespan 4 days before the control treatment (day 21 for the 0.1 and 1.0 mg/ml treatments, day 25 for the control).

To be certain if there was a statistical significant difference between the treatments, and to find which treatment or treatments that gave the effect, pairwise cox-F tests were conducted. Each test compared the treatments with the control separately. The result from these tests showed effect in the two highest concentrations (1.0 mg/ml and 0.1 mg/ml), and can be seen from the listing of p- and F-values in table 4.

Table 4: Pairwise cox-F tests on the effects of sodium butyrate treatment.

Pairwise Cox-F test	Statistics
Control vs. 1.0 mg/ml sodium butyrate.	$N_{Control/1.0\ mg/ml}$ =198/209; F=1.28; p<0.01
Control vs. 0.1 mg/ml sodium butyrate.	$N_{control/0.1\ mg/ml}$ = 198/154; F=1.52; p<0.001
Control vs. 0.01 mg/ml sodium butyrate.	$N_{control/0.01\ mg/ml}$ = 198/209; F=1.03; p=0.400

To reveal potential lifespan effects that were dependent on other factors (replicate effects), rather than direct effects of treatment, pairwise cox-F tests were conducted comparing the hives and rounds with each other separately. These tests could then detect effects that otherwise might mask minor effects of the sodium butyrate treatment.

The cox-F tests revealed a significant effect of hive ($N_{Hive1/Hive2}$ = 356/414; F=1.145; p<0.05), but no round effect was detected ($N_{Round1/Round2}$ = 356/414; F=1.110; p=0.0782). Each hive was then analyzed separately. The Kaplan Meier analysis revealed a significant treatment effect in hive 1 ($N_{control/\ 1,0\ mg/ml/\ 0,1\ mg/ml/\ 0,01\ mg/ml}$ = 98/102/51/105; Chi^2=41.48; p<0.001), but not in hive 2 ($N_{control/\ 1,0\ mg/ml/\ 0,1\ mg/ml/\ 0,01\ mg/ml}$ = 100/107/103/104; Chi^2=4.404; p=0.2211). The pairwise cox-F test showed that only the middle concentration of sodium butyrate (0.1 mg/ml) in hive 1 had an effect (see table 5 for statistics). The survival graphs from the two hives are shown in figure 13.

Table 5: Statistics for pairwise cox-F tests in hive 1.

Pairwise Cox-F test Within Hive 1.	Statistics
Control vs. 1.0 mg/ml sodium butyrate	$N_{Control/1.0\ mg/ml}$ =98/105; F=1.170; p=0.143
Control vs. 0.1 mg/ml sodium butyrate	$N_{control/0.1\ mg/ml}$ = 98/51; F=2.274; p<0.001
Control vs. 0.01 mg/ml sodium butyrate	$N_{control/0.01\ mg/ml}$ = 98/102; F=1.111; p=0.249

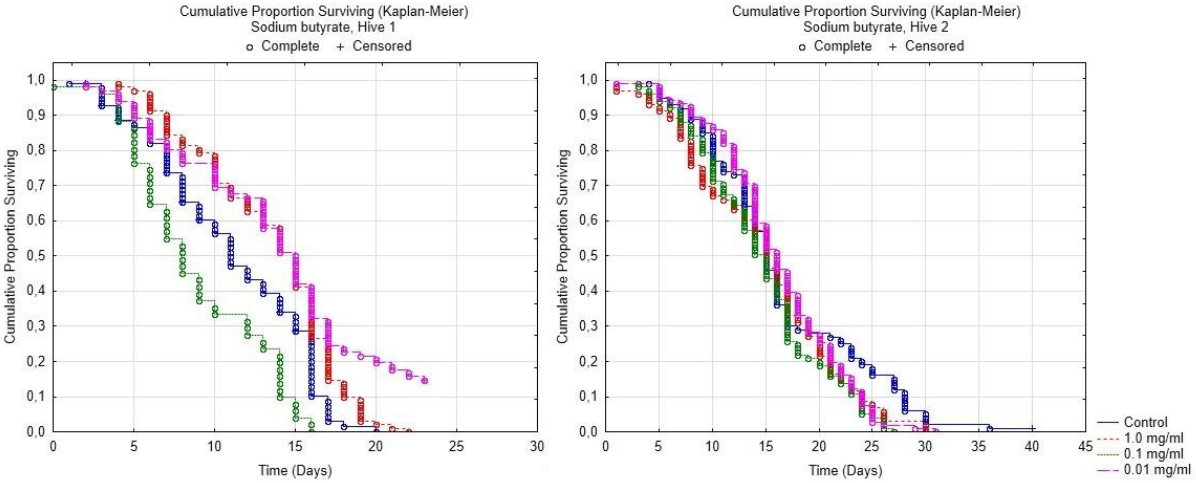

Figure 13: The survival curves for the two separate hives in the sodium butyrate study, with hive 1 on the left, and 2 on the right. The blue curves show the survival for the control, the pink is the 0.01 mg/ml, the green is the 0.1 mg/ml and red is the 1.0 mg/ml concentration treatment with sodium butyrate.

The survival graphs for hive 1 and 2 from the sodium butyrate study, shows that the bees sampled from hive 2 generally lived longer than the bees from hive 1.

The sodium butyrate treatment showed an effect in reducing the maximum lifespan of the bees treated with the highest and the middle concentration (1.0 mg/ml and 0.1 mg/ml respectively) when considering the overall data. When comparing replicates (hive/round effects), these effects vanished. Only one hive (hive 1) showed an effect of treatment with sodium butyrate. Within hive 1 however, only the middle concentration was shown to have an effect on lifespan.

4.1.2 Valproic acid

The valproic acid was given in these three concentrations: 10.0mg/ml, 1.0 mg/ml and 0.1 mg/ml. The Kaplan Meier analysis on the overall data, did detect a significant effect of treatment ($N_{\text{control/ 0.1 mg/ml/ 1.0 mg/ml/ 10.0 mg/ml}} = 209/204/203/206$; $Chi^2=513.33$; $p<0.001$).

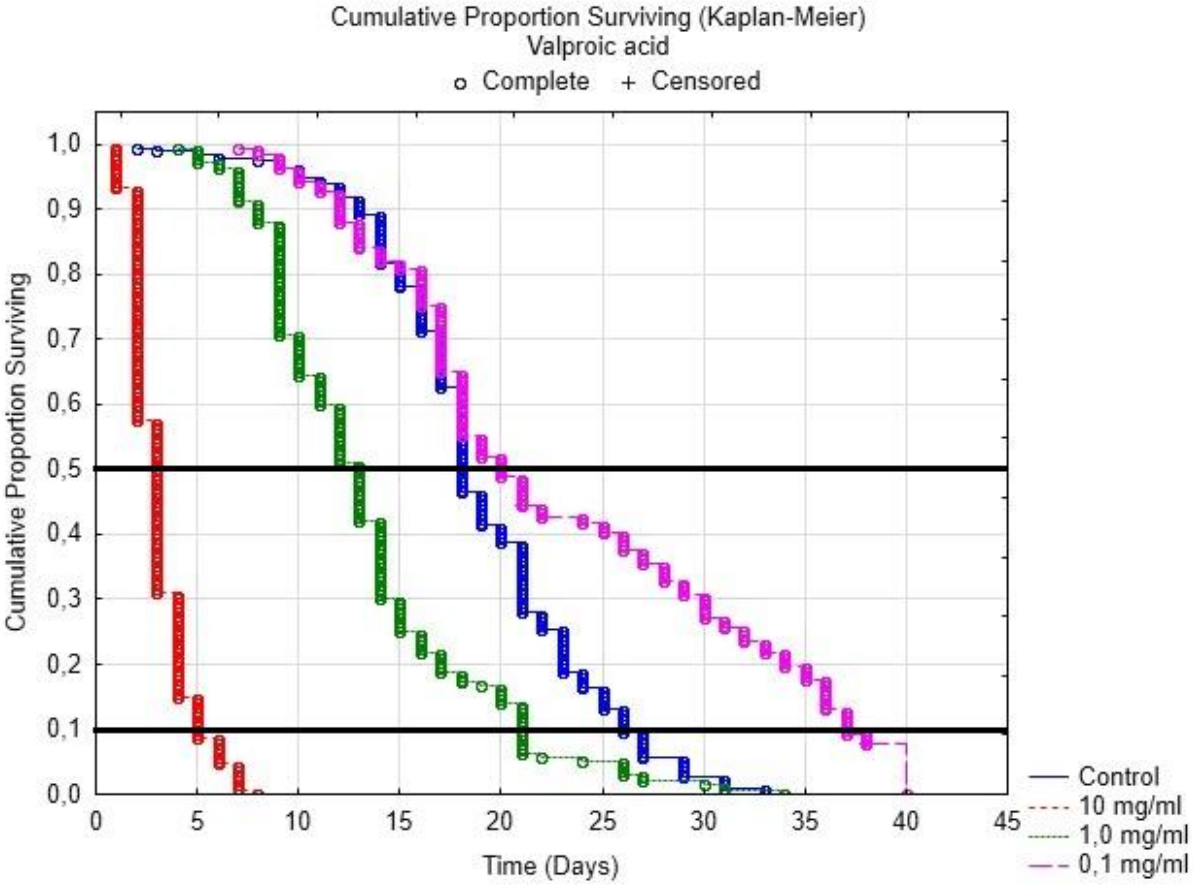

Figure 14: The overall graph for the survival study with valproic acid treatment. The two lines drawn represent the bees' median (0.5) and maximum (0.1) lifespan. The blue graph show the survival curve for the control, the pink is the 0.1 mg/ml, the green is 1.0 mg/ml and the red is the 10.0 mg/ml concentration treatment with valproic acid.

When studying the survival curves in figure, the two highest concentrations (10.0 mg/ml and 1.0 mg/ml) both clearly differ from the control at median lifespan. The survival of the bees receiving the highest concentration of valproic acid (10.0 mg/ml) dropped to 50 %, after only 3 days of treatment. In contrast, the control bees reached median survival after 18 days of treatment, giving a difference of 15 days. The bees fed with the middle concentration of valproic acid (1.0 mg/ml) hit the same rate 4 days before the control (after 14 days of

treatment). Even though the two highest concentrations do, the lowest concentration of valproic acid (0.1 mg/ml) does not seem to differ much from the control when comparing their median lifespan (only 2 days). When considering maximum lifespan however, a larger difference is observed. The bees receiving the lowest concentration of valproic acid (0.1 mg/ml) live 11 days longer than the control, before reaching maximum lifespan (day 37 for the low concentration and 26 for the control). Also, the low concentration group outlives the high concentration group (10.0 mg/ml) with a total of 32 days, at maximum survival.

The pairwise cox -F tests, comparing the different treatments with the control, showed a significant effect on lifespan in all concentration treatments ($p<0.001$). The statistics are shown in table 6. As can be seen from the overall graph (figure 14) the two highest concentrations, 10.0 mg/ml and 1.0 mg/ml (red and green) show a significant effect of reducing the bees lifespan, while the low concentration (0.1 mg/ml (pink)), shows a positive effect of extending the lifespan in the honey bees when comparing with the control (blue).

Table 6: Results from tests of treatment effect (Cox F test with the overall data) of Valproic acid.

Pairwise Cox-F test	**Statistics**
Control vs. 10.0 mg/ml valproic acid.	$N_{Control/10.0\ mg/ml}$ =209/206; F=1.85; p<0.001
Control vs. 1.0 mg/ml valproic acid.	$N_{Control/1.0\ mg/ml}$ = 209/203; F=2.17; p<0.001
Control vs. 0.1 mg/ml valproic acid.	$N_{Control/0.1\ mg/ml}$ = 209/204; F=5.40; p<0.001

For the valproic acid study, a hive effect was observed ($N_{Hive\ 1/Hive\ 2}$=399/423; F=1.85; p<0.001), but there was no effect of round ($N_{Round\ 1/Round\ 2}$ = 404/418; F=1.05; p=0.2599). Furher analyzes of the separate hives, found a significant effect of treatment in both hive 1 and hive 2 ($N_{control/\ 0.1\ mg/ml/\ 1.0\ mg/ml/\ 10.0\ mg/ml}$ = 101/101/100/97; Chi^2=274.80; p<0.001 and $N_{control/\ 0.1\ mg/ml/\ 1.0\ mg/ml/\ 10.0\ mg/ml}$ = 108/103/103/109; Chi^2 =307.92; p<0.001 respectively).

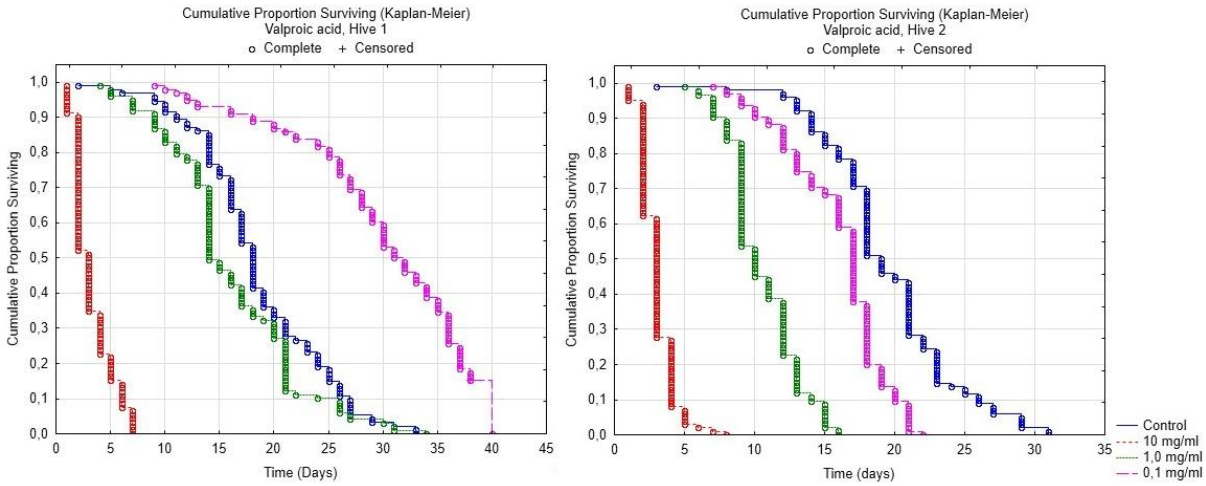

Figure 15: Comparison of hive 1 and 2 for the valproic acid treatments. hive 1 is on the left, and hive 2 is on the right. The blue survival curves represent the controls, the pink is the 0.1 mg/ml, green 1.0 mg/ml and red is the 10.0 mg/ml treatment concentration with valproic acid.

When studying figure 15, the effects of the lowest concentration differ between the two hives. In hive 1, the lowest concentration (0.1 mg/ml) clearly shows a positive effect on lifespan. In contrast, the same concentration treatment in hive 2 shows the opposite effect, hence shortening the bees' lifespan compared to the control. The statistics from the pairwise cox-F tests conducted on the two hives separately are shown in table 7.

Table 7: Pairwise cox F tests in the separate hives for the Valproic acid treatment.

Pairwise cox F test	**Hive 1**	**Hive 2**
Control vs. 10.0 mg/ml valoproic acid	$N_{Control/10\ mg/ml}$ = 101/98; F=5.32; p<0.001	$N_{Control/10\ mg/ml}$ = 108/109; F=5.41; p<0.001
Control vs. 1.0 mg/ml valproic acid	$N_{Control/1.0\ mg/ml}$ = 101/100; F=1.35; p<0.05	$N_{Control/1.0\ mg/ml}$ = 109/103; F=4.93; p<0.001
Control vs. 0.1 mg/ml valproic acid	$N_{Control/0.1\ mg/ml}$ = 101/101; F=3.97; p<0.001	$N_{Control/0.1\ mg/ml}$ = 101/103; F=2.37; p<0.001

All of the treatments with valproic acid, showed a significant effect on lifespan. High concentrations of valproic acid reduced the bees' lifespan. The low concentration did show a positive effect in prolonging lifespan in bees from one hive replicate, while in the other replicate, it was seen that the same concentration treatment significantly shortened lifespan.

4.1.3 Isovaleric acid

The three concentrations used for isovaleric acid treatment were; 10.0 mg/ml, 1.0 mg/ml and 0.1 mg/ml. The Kaplan Meier analysis confirmed that treatment with isovaleric acid show a significant effect on lifespan ($N_{control/\ 0.10\ mg/ml/\ 1.0\ mg/ml/\ 10.0\ mg/ml}$ = 211/198/208/203; Chi^2=85.43; p<0.001).

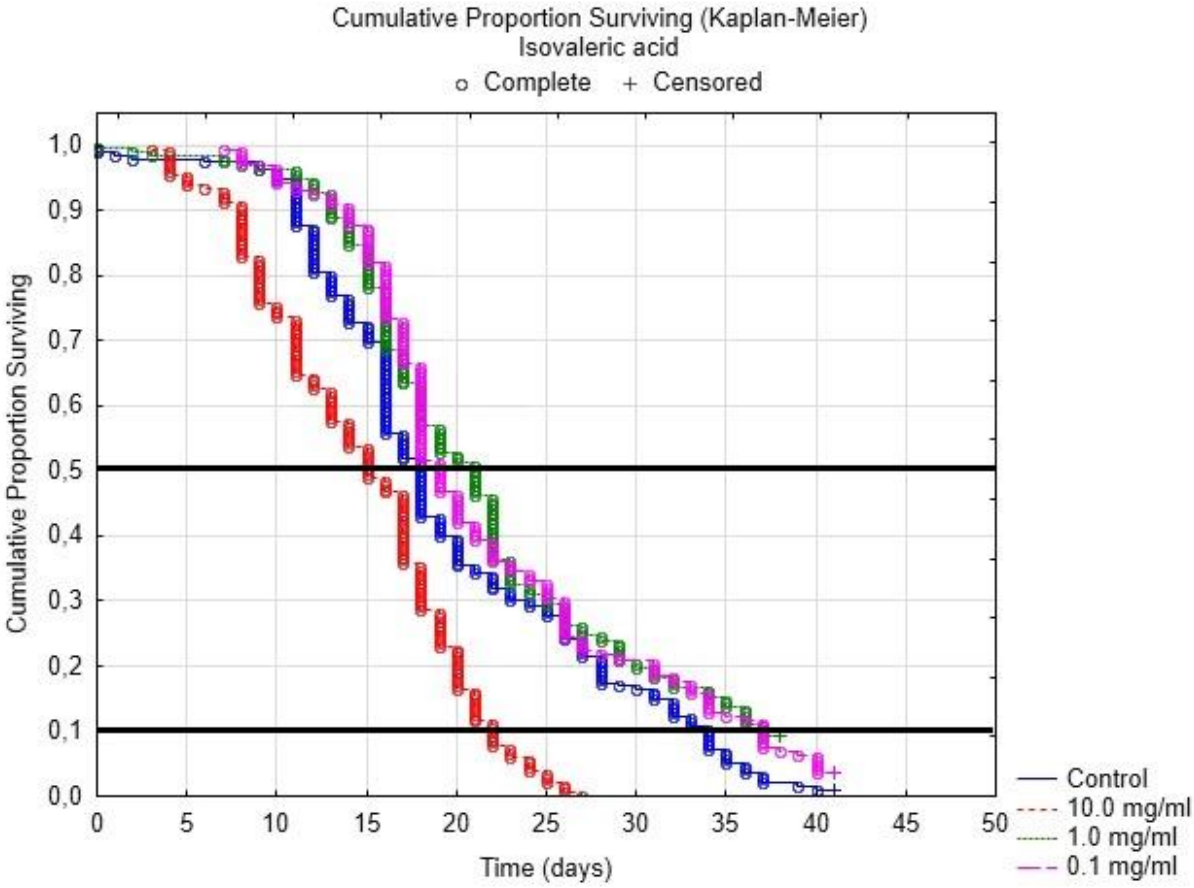

Figure 16: The overall graph for the lifespan study with the feeding with isovaleric acid. The drawn lines represent median (0.5) and maximum (0.1) lifespan. The blue survival curve represents the control, the pink is the 0.1 mg/ml, the green is the 1.0 mg/ml and red is the 10.0 mg/ml concentration treatment with isovaleric acid.

Figure 16 show that the treatment group fed with the highest concentration of isovaleric acid (10 mg/ml) reaches median lifespan 15 days after starting treatment. The control encounters median lifespan at day 18 of treatment, giving a 3 day difference. The low concentration group (0.1 mg/ml isovaleric acid) reaches median lifespan at day 19, and the middle concentration (1.0 mg/ml) at day 21, giving 1 and 3 days difference from the control respectively. Comparing maximum survival, however, show a larger difference between the control and the high concentration treatment. The 10.0 mg/ml treatment group, reach maximum survival after 22 days of treatment, hence 12 days earlier than the control reaching maximum lifespan at day 34. The middle and low concentration treatments (1.0 and 0.1 mg/ml) both reach their maximum lifespan at day 37, i.e. outliving the control and the high concentration group by 3 and 15 days respectively.

All the concentrations tested did show statistically significant effect on lifespan (statistics shown in table 8). The highest concentration (10 mg/ml), show a life shortening effect when looking at the graph for the overall data. The middle and low concentration however, shows a positive effect on lifespan.

Table 8: Results from tests of treatment effect of isovcaleric acid.

Pairwise cox-F test	Statistics
Control vs. 10.0 mg/ml Isovaleric acid.	$N_{Control/10.0\ mg/ml}$ =211/203; F=11.96; p<0.001
Control vs. 1.0 mg/ml Isovaleric acid.	$N_{Control/1.0\ mg/ml}$ = 211/208; F=1.35; p<0.01
Control vs. 0.1 mg/ml Isovaleric acid.	$N_{Control/0.1\ mg/ml}$ = 211/198; F=1.27; p<0.05

The pairwise cox-F tests revealed a hive effect ($N_{Hive1/Hive2}$ = 406/414; F=3.39; p<0.001), but no round effect was observed ($N_{Round1/Round2}$ = 353/467; F=1.0311; p=0.3401). (Round 2 is represented by more individuals than round one, due to removal of one cage from round 1, and re-sampling of this cage in round 2).

Further, tests were conducted on the two hives separately. These tests showed that the treatments had an effect in both hive 1 and 2 ($N_{control/\ 0.1mg/ml/\ 1.0\ mg/ml/\ 10.0\ mg/ml}$ = 106/102/101/97; Chi^2 =67.45; p<0.001 and $N_{control/\ 0.1\ mg/ml/\ 1.0\ mg/ml/\ 10.0\ mg/ml}$ = 105/96/107/106;

Chi^2 =106.41; p<0.001 respectively). The graphs for the survival in hive 1 and 2 are shown in figure 17.

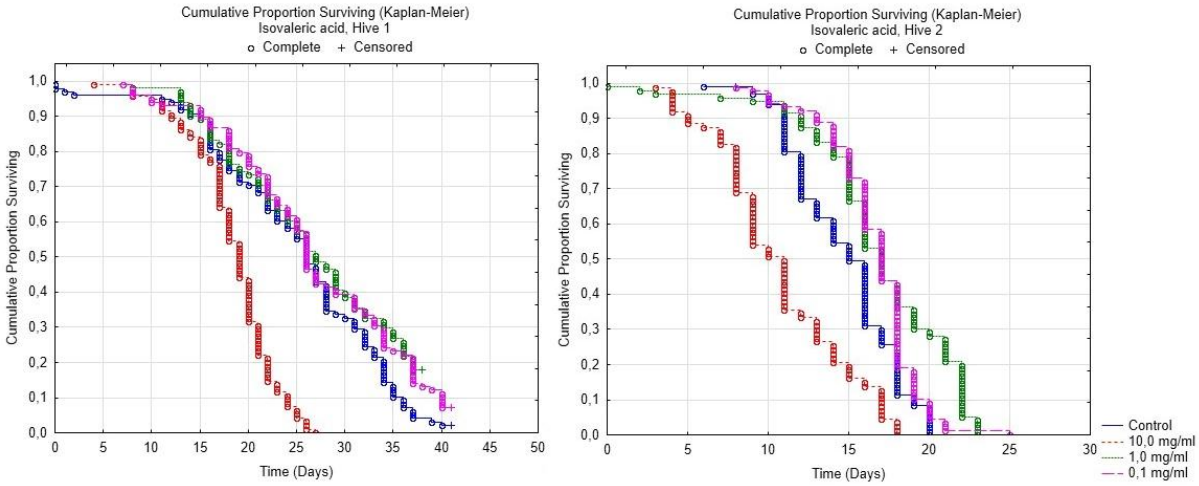

Figure 17: The effect of isovaleric acid treatment in the two separate hives, with hive 1 on the left, and 2 on the right. The blue curve represents the control, the pink is the 0.1 mg/ml, green is the 1.0 mg/ml and the red curve is the 10.0 mg/ml concentration treatment.

Pairwise cox-F tests were also conducted, comparing each concentration treatment with the control, within the two hives. The results are shown in the table below.

Table 9: Pairwise cox-F tests comparing treatments with control in the two separate hives.

Pairwise cox F test	**Hive 1**	**Hive 2**
Control vs. 10.0 mg/ml isovaleric acid	$N_{Control/10\ mg/ml}$ = 106/97; F=2.81; p<0.001	$N_{Control/10\ mg/ml}$ = 105/106; F=2.44; p<0.001
Control vs. 1.0 mg/ml isovaleric acid	$N_{Control/1.0\ mg/ml}$ = 106/101; F=1.51; p<0.005	$N_{Control/1.0\ mg/ml}$ = 105/107; F=1.80; p<0.001
Control vs. 0.1 mg/ml isovaleric acid	$N_{Control/0.1\ mg/ml}$ = 106/102; F=1.34; p<0.05	$N_{Control/0.1\ mg/ml}$ = 105/96; F=1.43; p<0.05

Isovaleric acid did show an effect of treatment for all the three concentrations tested in this study. The highest concentration (10.0 mg/ml) resulted in a shortening of lifespan, but the middle and the lowest concentrations (1.0 and 0.1 respectively) showed a slightly positive

effect on lifespan. When comparing hives, a replicate effect was observed. The graphs in figure 17, and the statistics in table 8 and 9 show the same effects for each treatment in both hives (i.e. the highest concentration shortens, while the middle and low concentration extends lifespan). However, figure 17 also show that the bees in hive 1 1ive longer than the bees in hive 2, regardless of treatment.

4.2 Consumption data

As mentioned in the methods part, was the consumption of food monitored daily. The average daily consumption per bee for the first 10 days of treatment, was calculated to test if there were any difference in the consumption between the concentration treatments and the control (for the three separate substances).

The reason for analyzing the consumption data was to see if the bees' food intake was affected in any way due to the treatment. If the bees from treatment groups ate less than the control bees, it may explain the treatment effects seen on lifespan. For example; a significantly low consumption compared to a control treatment, could lead to death by starvation. Low consumption, or restricted dietary intake, have also been found to expand lifespan (Rascón et al. 2012). On the background of this, a low food intake could either result in reduction or increasing lifespan, without being a direct effect of the substance itself.

The one way ANOVA analysis conducted on the average consumption in the sodium butyrate study, did not show any statistically significant difference in consumption between the four treatments (the three concentrations + the control) ($N_{control/\ 1,0\ mg/ml\ /0,1\ mg/ml\ /0,01\ mg/ml}$= 4/4/3/4; F=0.76; p=0.5405).

For the valproic acid, the 10 mg/ml concentration group reached maximum lifespan after only 5 days. Since data for the first 10 days of treatment was to be used, the consumption for the bees receiving this concentration was disregarded. Thereby, only the middle (1.0 mg/ml) and lowest (0.1 mg/ml) concentration groups' consumption data were taken into account. The ANOVA analysis did not show any significant difference in consumption between the treatments with valproic acid ($N_{control\ /1,0\ mg/ml\ /0,1\ mg/ml}$=4/4/4; F=0.25; p=0.7832).

For the Isovaleric acid on the other hand, a significant difference in consumption was detected between the groups. ($N_{control\ /\ 10\ mg/ml\ /\ 1,0\ mg/ml/\ 0,1\ mg/ml}$=4/4/4/4; F=29.76; p<0.001).

The graph showing the mean and the standard deviation of consumption per concentration treatment including the control is seen in figure 18.

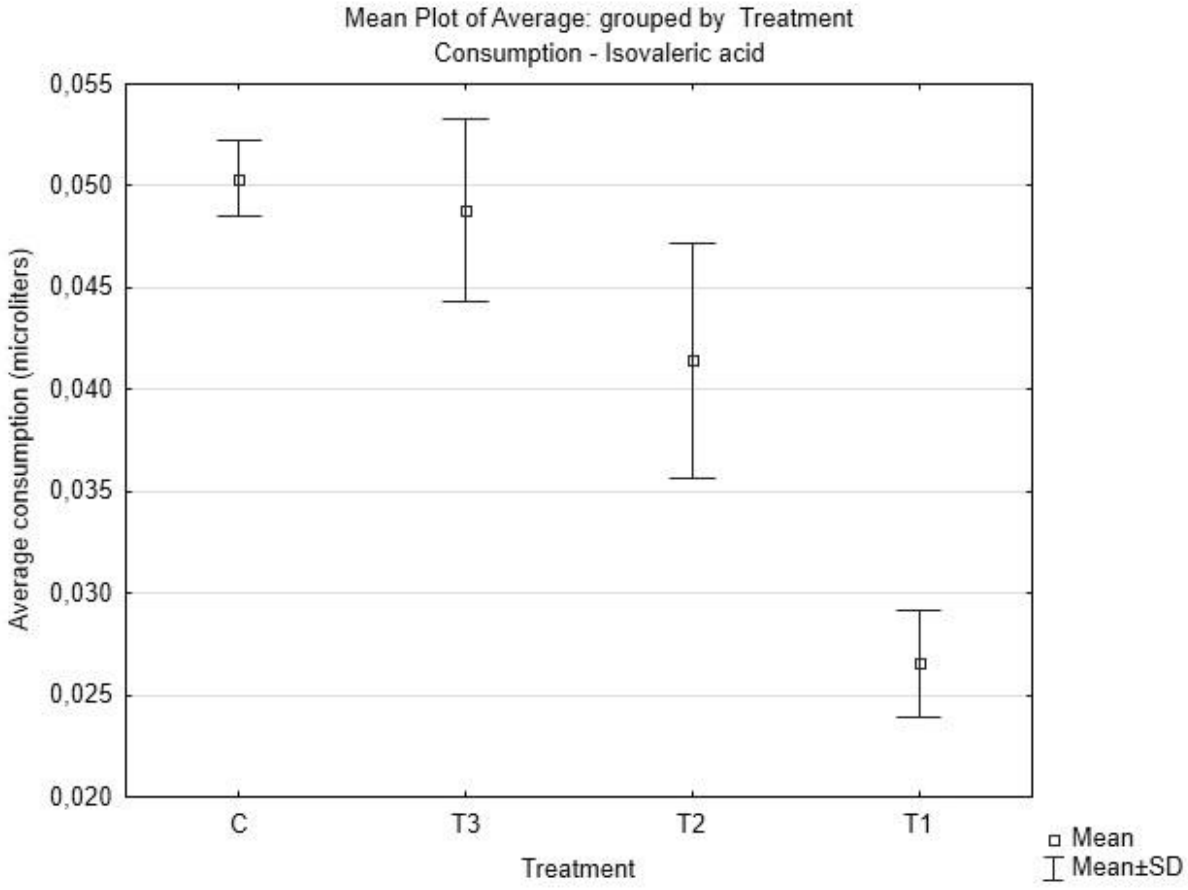

Figure 18: The mean and standard deviation of the average consumption per bee/day (µl) for each treatment of isovaleric acid. (C=control, T3=0.1mg/ml, T2=1.0 mg/ml, T1=10.0 mg/ml concentration treatment).

The 10 mg/ml treatment of isovaleric acid shows a clear reduction in consumption compared to the control. The high concentration treatment consumption also differs from the other isovaleric acid treatments. To test which consumptions differed among the three treatments of isovaleric acid, as well as from the control, a Fisher LSD post-hoc test was conducted. The results from the test are shown in table 10.

Table 10: Results of the Post Hoc Fisher LSD test conducted on the isovaleric acid consumption data (p-values) .

Treatment	Control	0.1 mg/ml	1.0 mg/ml	10.0 mg/ml
Control		p=0.5936	p<0.05	p<0.001
0.1 mg/ml	p=0.5936		p<0.05	p<0.001
1.0 mg/ml	p<0.05	p<0.05		p<0.001
10.0 mg/ml	p<0.001	p<0.001	p<0.001	

The table above shows that the high and middle concentrations (10.0 mg/ml and 1.0 mg/ml) differ both between each other, but also from the other treatments. The consumption graph shows that the bees receiving the high concentration of isovaleric acid consumes, on average, half the amount of food compared to the control. The consumption of control and the low concentration of isovaleric acid (0.1 mg/ml) do not differ significantly from one another.

4.3 Screening for epigenetic effects

As described in the introduction, different natural substances can be bioactive and affect the epigenetic pattern in the genome of different organisms. In this project, the focus was mainly directed towards changes in methylation of the DNA (5-mC) and the acetylation of histones, as results of treatment with sodium butyrate, valproic acid and isovaleric acid. Several other changes can, however, arise from changes in the epigenome of an organism.

4.3.1 Histone acetylation modifications

A Westen blot was conducted to find potentially acetylated histone proteins. An immunohistone blotting procedure was used to identify the protein of interest. Immuno histone blotting, is based on using antibodies specific for the protein/antigen which one wants to detect. In this case, the antigen of interest was the H3K23ac (H3 histone protein with an acetyl modification on its 23 lysine residue). This lysine residue, among others, has previously been shown to be a subject for acetylation in the honey bee (Dickman et al. 2013).

Several tests with Western blotting were conducted, however, the method did not seem to be a good predictor of histone acetylation in this project. Diagenode (the manufacturer of the polyclonal primary antibody used in this thesis) specified the band size of the H3K23ac protein as 14.4 kDa. This band was not observed on the membrane after incubation with the antibodies in any of the test runs.

To find the possible source of error, two gels were run in parallel in the gel electrophoresis step. One of these gels proceeded with the blotting step, while the other was stained with a Coomassie brilliant blue dye. The reason for running the two gels in parallel was to investigate the specificity of the antibody.

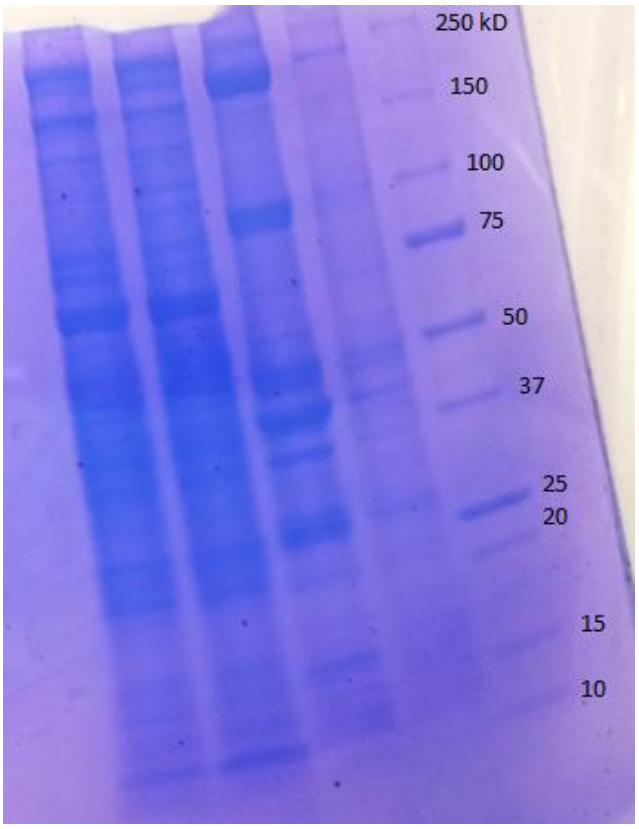

Figure 19: The gel stained with coomassie blue dye. The molecular weight standard is on the right, and the size of the bands (kilo Dalton) is shown. The two lanes next to the ladder were samples taken from brains, and the next two were obtained from the bees' abdomen.

Figure 19 shows the gel stained with the Coomassie brilliant blue dye, with the molecular weight standard (ladder) to the right giving the protein sizes in kilo Dalton (kDa). The two lanes next to the ladder were filled with homogenized brain tissue, while the two on the left were samples from the bees' abdomen. The bees that were used for these analyzes were random bees that were not included in the feeding experiment.

In the Coomassie stained gel, several bands were observed at different protein sizes, implying that the protein separation by gel electrophoresis was not a confounding factor. Bands are also observed in the wanted size-range on the gel (14.4 kDa).

The other gel was used for immuno-blotting. In this step, the proteins separated on the gel were transferred to a membrane and incubated with H3K23ac antibodies. After addition and incubation with the secondary antibody, the membrane was scanned.

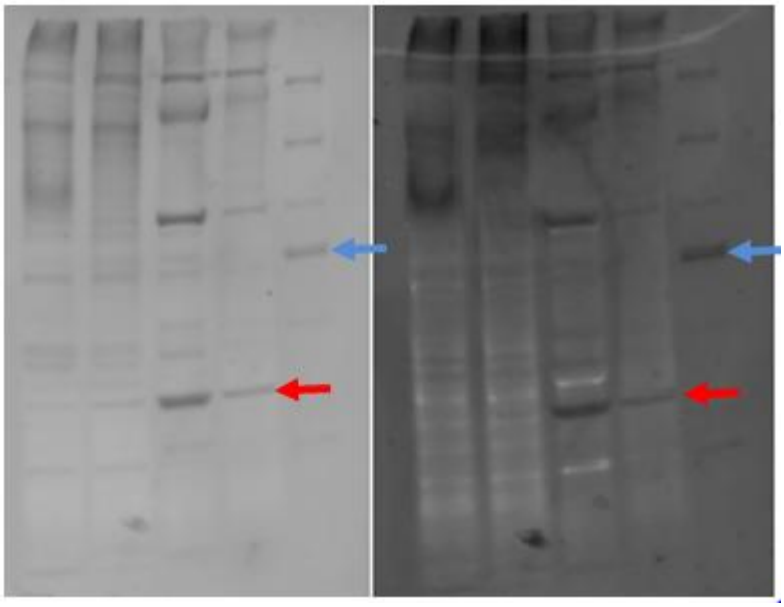

Figure 20: Pictures of the membrane stained after antibody incubation (left) and after staining with SYPRO Ruby stain (right).

The secondary antibody, labeled with a fluorescent molecule (Cy5), made it possible to observe the bound antibodies when scanning the membrane. Hence, by incubation with the secondary antibody, it would bind to the already bound primary antibodies and thereby, visualize the bands of the antigen/protein that had been separated on the gel and transferred to the membrane (picture to the left in figure 20). After the first scanning, the membrane was stained with SYPRO Ruby dye, and scanned anew (picture to the right in figure 20). Neither of the two scans shows a protein band at the wanted size. The last observable band for the molecular weight standard is observed at the size of of 75 kD (marked with the blue arrows). The smallest proteins transferred from the gel to the membrane are observed in the size range of 30 – 37 kDa (marked by the red arrows) in the brain tissue samples.

4.3.2 DNA methylation detection

As mentioned in the methods, only two concentrations in addition to the control were used for treatment of the bees analyzed for DNA methylation by ELISA. Three rules were applied in the choosing of these treatments (listed in the methods). The sampled bees meant for these assays, received the certain treatment concentrations for 10 days prior to collection and freezing with liquid nitrogen. The choices of concentrations are listed in table 11.

Table 11: Choice of concentration treatments for bees meant for DNA methylation assays.

Concentration/ substance	High concentration	Mid concentration	Low concentration
Sodium butyrate	**X (1.0 mg/ml)**	**X (0.1 mg/ml)**	
Valproic acid		**X (1.0 mg/ml)**	**X (0.1 mg/ml)**
Isovaleric acid	**X (10.0 mg/ml)**	**X (1.0 mg/ml)**	

The methylation patterns in the DNA are subjects to modifications. These modifications are shown to be affected by diet and different food compounds (McGowan et al. 2008). The methylation pattern is also shown to change as one ages (Fraga et al. 2005). To detect possible differences in DNA methylation between bees receiving different concentration treatments of the substances studied, 5-mC ELISA's were conducted on DNA extracted from the bees' abdomens.

By measuring the absorbance in the standard wells (at 405 nm), a standard curve was made. The standard curve was then used to estimate the percentage of methylated CpGs in the unknown DNA samples from the bees. This was calculated using the equation; $e^{((\text{absorbance} - y - \text{intercept})/\text{slope})}$, given in the ELISA instruction manual from ZYMO RESEARCH. The equation was used together with the average absorbance from the duplicate wells. The information from these calculations was processed in STATISTICA to look for statistically significant differences in the amount of 5-mC DNA between treatment concentrations.

The one-way ANOVA analysis conducted on the results from the ELISA, did not detect a significant difference in 5-mC DNA for any of the three substances tested (statistics are shown in table 12).

Table 12: Statistics for the ANOVA analysis of the ELISA results.

Substance tested	Statistics
Sodium butyrate	$N_{Control/0.1\ mg/ml/1.0\ mg/ml}$ = 4/4/4; F=1.1672; p=0.3543
Valproic acid	$N_{Control/0.1\ mg/ml/1.0\ mg/ml}$ = 4/4/4; F=1.0820; p=0.3792
Isovaleric acid	$N_{Control/1.0\ mg/ml/10.0\ mg/ml}$ = 4/4/4; F=1.2634; p=0.3289

The graphs below show the mean and standard deviation of the % 5-mC for the different concentration treatments for each substance.

Figure 21 shows the 5-mC ELISA results from the bees treated with 0.1 and 1.0 mg/ml sodium butyrate. The control shows a very high standard deviation compared to the samples from the 0.1 and 1.0 mg/ml sodium butyrate treated bees.

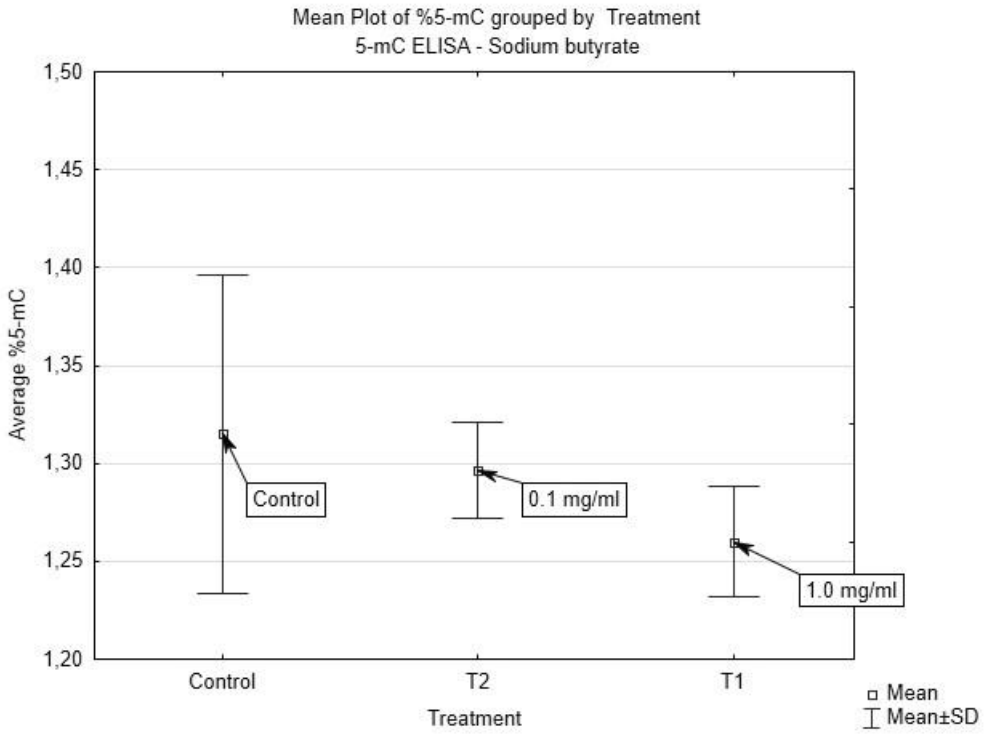

Figure 21: The mean and standard deviation of % 5-methyl cytosine in the bees treated with sodium butyrate. (T2 = 0.1 mg/ml, T1=1.0 mg/ml)

Figure 22 shows the 5-mC ELISA results of the samples obtained from the bees that were treated with 1.0 and 0.1 mg/ml valproic acid. The samples from the T1 concentration (1.0 mg/ml) shows a very high standard deviation compared to the control and T2 (0.1 mg/ml) treatment.

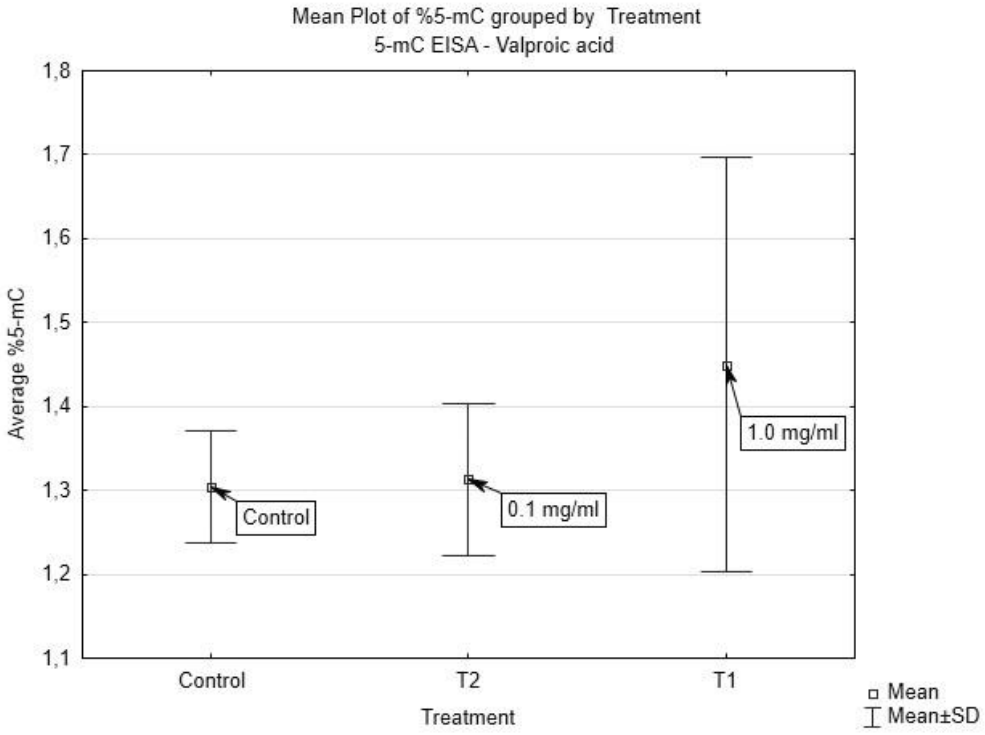

Figure 22: Mean and standard deviation of % 5-mC, in bees treated with valproic acid. T2 = 0.1 mg/ml, T1= 1.0 mg/ml.

Figure 23 shows the mean and standard deviation of the percentage of 5-mC DNA in the samples from the bees treated with 10.0 mg/ml (T1) and 1.0 mg/ml (T2) isovaleric acid. As can be seen from the graph, the result from the treatment with the highest concentration of isovaleric acid shows a large standard deviation in the percent amount of 5-mC DNA.

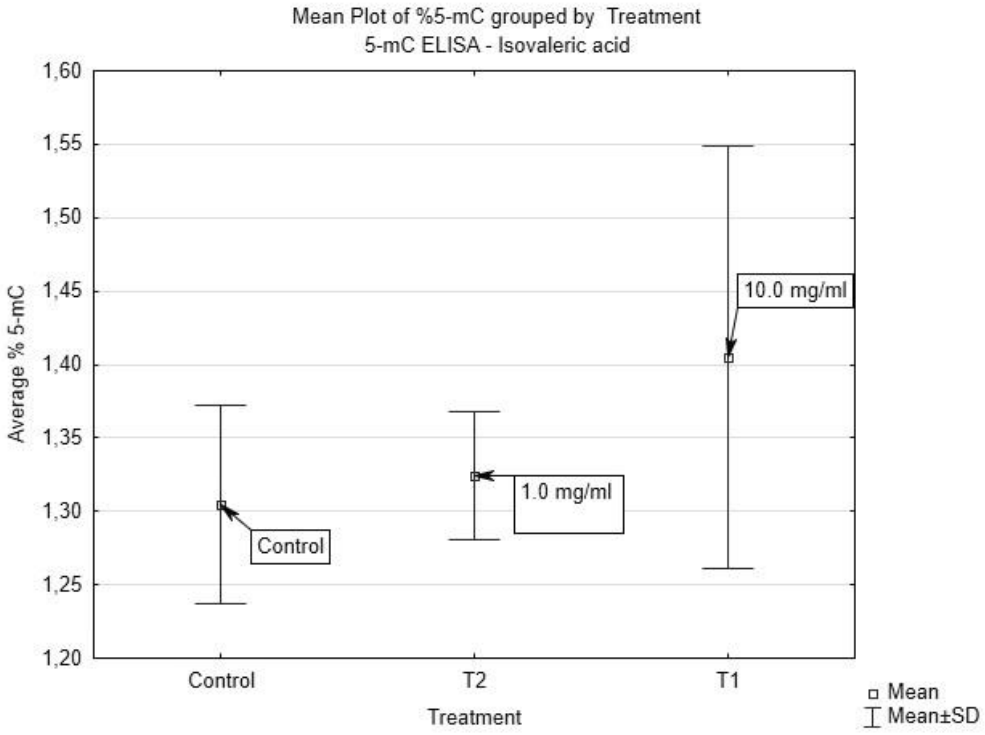

Figure 23: The mean and standard deviation of % 5-mC DNA in the bees treated with isovaleric acid. T2 = 1.0 mg/ml, T1= 10.0 mg/ml.

The results from the ELISAs did not show significant difference in DNA methylation, for any of the three substances tested. Another thing, that is also important to note, is that the standard deviation is very large for some of the treatments. This is especially true for the control for the sodium butyrate, and the two highest concentrations concentration treatments of valproic and isovalerc acid.

5. Discussion

In this study, the effects of three natural substances (valproic acid, isovaleric acid and sodium butyrate), were tested on the winter phenotype of the honey bee (*A. mellifera*). By first conducting a lifespan study, it was possible to see if any of the substance treatments were capable of affecting the bees' longevity. The study also aimed to reveal potential alternations in the bees' global DNA methylation state and histone acetylation as a consequence of treatment with any of the substances.

The isovaleric acid, showed a reduction of lifespan for treatment with the highest concentration (10.0 mg/ml). The two lower concentrations however, resulted in a robust significant extending effect on lifespan. To my best knowledge, this represents the first evidence that treatment with isovaleric acid exerts an extending effect on lifespan in honey bees. The sodium butyrate study did not show any robust effects of treatment, as the apparent variance vanished when the hive replicates were analyzed in separate. The valproic acid primarily showed an effect on shortening lifespan. The low concentration (0.1 mg/ml) however, seemed to have a prolonging effect, though this effect was only seen in one hive replicate.

To study the effects on DNA methylation, ELISAs were conducted. These assays did not reveal any significant effects on global DNA methylation in any of the treatment concentrations, of the three substances.

To detect potential variance in histone acetylation, a Western blot was meant to be conducted. However, no specific staining was observed when using the antibodies, which is why quantification of histone acetylation was not feasible in this study. The Western blot presented in this thesis, were conducted with random bees, provided by the supervisor.

5.1 The Sodium butyrate study

In the present study, the sodium butyrate study did not show any robust effect of treatment. This indicates that sodium butyrate might only exert minor effects on lifespan in honey bees. In the overall data treatment did show effect, however, when separated by hive these effects vanished. The ELISA assay aimed for detection of variance in global DNA methylation, did not reveal any variance in methylation state between any of the treatment groups.

Sodium butyrate is a well known HDAC inhibitor (Davie 2003). A similar substances called phenyl butyrate have been suggested to affect the DNA methyltransferases in bees, indirectly by inhibiting HDACs (Chittka & Chittka 2010; Lyko et al. 2010). Knowing this possibility, it can be suggested that treatment with sodium butyrate also might cause alternations in the bees' methylation pattern in addition to increasing the amount of acetylated histones.

Based on a literary search, the three concentrations chosen for the sodium butyrate treatment of the honey bees were: 1.0, 0.1 and 0.01 mg/ml. The studies used for the determination of these, all found an effect of treating *D. melanogaster* with 10 mM (McDonald et al. 2013; St. Laurent et al. 2013), which would correspond to a 1.1 mg/ml concentration. One study also found that concentrations over 20 mM phenyl butyrate, caused harmful effects (Kang et al. 2002). As all of these studies detected an effect on *D. melanogaster*, it was reasonable to expect observational effects of concentrations within the same range.

A study showed that, feeding *D. melanogaster* 4-phenylbutyrate throughout adulthood, resulted in an extension of lifespan (Kang et al. 2002). The treatment also showed a positive effect when given in limited periods of life. 10 mM concentrations of phenyl butyrate extended both the maximum and median lifespan, while concentrations over 20 mM showed a harmful effect by reducing lifespan compared to controls. Kang et al. also suggested that the concentration effect of the substance could be related to the individual's own genetic background. The same study also showed an increase in acetylation of the H3 and H4 histone proteins in relation to the phenyl butyrate treatment.

Another study, testing for sodium butyrate effects in *D. melanogaster*, studied the effect when treatment was administered at different stages of life (McDonald et al. 2013). Two concentrations of sodium butyrate were tested (100 mM and 10 mM, hence 11.0 mg/ml and 1.11 mg/ml). An increase in lifespan was shown when treatment was administered in the late

aging phases. In contrast, when they were treated throughout their adult life, a reduction of lifespan was shown. This suggests that the effect of sodium butyrate is influenced by the specific individuals' stage of life, when treatment is administered (hence; Sodium butyrate may therefore exert stage specific effects). It was suggested that different target molecules for the sodium butyrate, might be expressed in various amounts at different stages of life. Thereby; an effect of a substance might be observed at one specific stage of life in one individual, but absent or alternated in another individual that is of another age.

The effects of sodium butyrate have also been studied on a rotenone induced *D. melanogaster* model (St. Laurent et al. 2013). The rotenone pesticide was administered to create Parkinson-like symptoms in the flies. Rotenone has the effect of lowering the locomotive activity and degenerating specific neurons, which are also typical symptoms in a Parkinson patient. St.Laurent et al. (2013) found that models treated with sodium butyrate, after being exposed to the rotenone pesticide, showed a reduction of symptoms. Groups receiving sodium butyrate also showed a delay in the early mortality that was observed in a control group. Individuals which did not express HDACs (knockdowns) also showed a delay in early mortality, and minor loss of motion function. This suggests that treatment with sodium butyrate might be effective in treatment of neurodegenerative diseases including Parkinsons disease and Alzheimer or other diseases that are associated with old age.

When the bees were sampled for this study, their age was not known, though they were all adults. Even though they were of the same phenotype (*diutinus*), they could still be of different chronological age. If sodium butyrate exerts a life-stage specific effect, like suggested in the studies mentioned above, it could explain why robust significant treatment effects of lifespan was not observed. One of the studies showed a decrease in lifespan when the model organisms (*D. melanogaster*) were fed sodium butyrate through their adult lifespan. When treatment was introduced at senescent phases of their lifetime however, both their maximum and median lifespan increased (McDonald et al. 2013). This might suggest that, if treatment was administered at specific stages of the bees' life, significant lifespan effects could have been shown.

The pairwise tests, comparing replicates (hive and round), detected a significant effect of hive. Tests performed on each hive in separate, detected significant effect of treatment in just one of the two. This could indicate that sodium butyrate only exerts a minor effect on lifespan in bees. The overall data shows effect of the two highest concentrations, but when analyzing

the hives separately, these effects disappear. When studying the graphs in figure 12, the lifespan curves are very close to each other, and a clear effect cannot be observed from the graph itself, like in the graphs for the valproic and isovaleric acid lifespan graphs (figure 14 and 16 respectively). When studying the replicate results further, hive 1 (the hive showing the effect), is represented by fewer individuals due to the removal of one cage from the statistical test (this cage was excluded from the analysis due to significant loss of bees on day 1 of treatment). Further, hive 1 only revealed an effect of middle concentration treatment (0.1 mg/ml). This can be explained by that the middle concentration was represented by only 51 individuals compared to the control which was represented by 98, as one of the cages receiving this treatment was excluded prior to the statistical tests. When comparing the lifespan in the two hives (figure 13), it is clear that the bees sampled from hive 1, in general, had a shorter lifespan than the bees from hive 2.

When testing consumption, no statistical significance between the treatments, hence sodium butyrate does not alter the bees' food intake.

The bees that used for the molecular test (ELISA) were treated with the two highest concentrations. Since the sodium butyrate treatments only showed minor effects, it was speculated that a potential effect on DNA methylation most likely would be observed in bees treated with the highest concentrations. The results from the ELISA however, did not reveal any significant effect of sodium butyrate on either increasing or decreasing the amount of 5-methylated cytosines in the bees.

From what is known about sodium butyrate's effect on epigenetic mechanisms, is that it inhibits HDACs from removing acetyl groups on histones (Kang et al. 2002). In turn, this leads to an increased number of acetylated histones associated with the DNA. Because the Westen blot analysis was not conducted on the molecular bees receiving sodium butyrate, observing a potential increase in histone acetylation was not possible. As the mechanisms of histone acetylation and DNA methylation are interrelated, I would have expected a decrease in DNA methylation from this treatment.

5.3 Valproic acid

The lifespan study with the valproic acid treatment, showed that high concentration treatments cause a severe reduction of the bees lifespan. The low concentration treatment (0.1 mg/ml) showed a life extending effect in the overall survival data. When studying the two hive-replicates separately, only one of them (hive 1) showed this positive effect of life extension. The other hive (hive 2), showed a significant reduction of lifespan with the low concentration treatment. No alternations in global DNA methylation were found in the bees treated with valproic acid.

Based on the literary search, the three concentrations chosen for the valproic acid study were; 10.0 mg/ml, 1.0 mg/ml and 0.1 mg/ml. Studies conducted on the nematode *C. elegans* have previously shown effect of a 6 and 3 mM concentration treatment (Evason et al. 2008; Kautu et al. 2013). A concentration of 6 mM, would correspond to a 0.9 mg/ml treatment. As *C. elegans* is a much smaller organism than the honey bee, it was suggested that the honey bee would tolerate higher concentrations.

As mentioned in the introduction, is the valproic acid a compound found in extracts from the valerian plant. These extracts have, like the study with sodiumbutyrate (St. Laurent et al. 2013), been studied on rotenone induced *D. melanogaster*, to examine its effect on reducing the symptoms caused by the pesticide (Sudati et al. 2013). This study found that the aqueous extracts from the valerian plant (10 mg/ml) could protect against the toxicity caused by rotenone.

A study conducted on the nematode *C.elegans* showed that treatment with valproic acid extended their lifespan, and delayed the onset of aging (Evason et al. 2008). With this result, they suggested that compounds which show an effect of increasing longevity, might also show positive effects in treatments of age-related neurodegenerative diseases like Alzheimer and Parkinson's disease. Since the valproic acid act as a HDAC inhibitor, it might help with the expression of genes that have been silenced due to age-related epigenetic changes. They found that a treatment dose of 6 mM valproic acid showed the longest extension of lifespan in the nematode. Lower doses showed smaller effects while higher doses shortened lifespan. The same study, suggested that the valproic acid does not exhibit its function at different developmental stages, but rather perform its function on postponing degeneration in adult individuals.

Another study conducted on *C.elegans* showed that Valproic acid (Kautu et al. 2013), improve the dopaminergic neurodegeneration caused by a toxic factor (alpha-synuclein, a mutated protein found in populations of patients with familial Parkinsons disease). In that study, they used concentrations of 1, 2, 3 and 4 mM valproic acid for treatment, and the 3 mM seemed the best treatment, giving the best and most significant results. The study also suggested valproic acid to be a protective agent against neurodegeneration.

The results presented in this thesis, might suggest that the valproic acid exerts its function in the later stages of life, delaying the development of the physiological disadvantages that follows with growing old, although the study mentioned above suggests the opposite (Evason et al. 2008). When studying the effect of the low concentration in figure 14, the survival curve differentiates from the control at the median lifespan. However, before reaching this survival rate, the two graphs are quite similar.

For both hive 1 and 2 a significant negative effect of the two higher treatment concentrations (10.0 mg/ml and 1.0 mg/ml) was detected, however, for the 0.1 mg/ml treatment the results differed between the two hives. When studying the survival curves for the lowest concentration treatments (0.1 mg/ml) in figure 15, hive 1 shows a significant effect on extending, while the same treatment in hive 2 shows a significant effect of shortening lifespan. In hive 1, the group receiving the low concentration treatment (0.1 mg/ml) outlives the control by 11 days, before reaching maximum lifespan. In hive 2, however, the same group reaches their maximum lifespan 6 days before the control group. As mentioned in the sodium butyrate discussion, the effect of treatment might be related to the individuals genetic background (Kang et al. 2002), which can also be relevant for the results seen in these two separate hives. The bees inside one hive all originate from the same queen, making them share much of the same genome. Bees from a different hive, originates from a different queen and other drones giving them another genetic background. This could explain why a positive effect is observed in hive 1, but not in hive 2.

Consumption data were also processed for the valproic acid lifespan study. Since the high concentration group (10 mg/ml) showed such high mortality, and all individuals within this group were dead before 10 days of treatment, the consumption data from this group was excluded from the statistical trials. This because, the consumption might give false predictions of what the daily average consumption per bee really was (many outliers). The statistical tests

conducted on the consumption data for the two remaining concentrations, did not show any statistical significant difference between the treatments with valproic acid.

One review article have discussed the adverse reactions of valproic acid treatment in patients (Nanau & Neuman 2013). This article addressed some of the reactions that have been observed in relation to valproic acid treatment. In humans, it has been reported that valproic acid could lead to severe liver damage. It has also been reported to cause reactions like neural and mitochondrial toxicity. This review also suggest that the genetic background and different environmental factors could be decisive for how disposed the patients are for these reactions. These effects can thereby be suggested as an explanation for why the bees died at such a high rate when receiving the high concentrations of valproic acid.

Since the valproic acid is a well known histone deacetylase inhibitor, treatment may have increased the degree of acetylated histones, also in bees. This could have been shown with a Western blot, however, this method was excluded in this project.

The results from the ELISA run on samples from bees treated with valproic acid, did not show any significant effect of valproic acid alternating the amount of methylated DNA (5-mC DNA).

5.4 Isovaleric acid

For all the concentrations of isovaleric acid tested in this thesis, a significant effect on lifespan was detected. As seen from the graph in figure 16 the highest concentration (10.0 mg/ml) of isovaleric acid shows a negative effect on lifespan. This result suggests that isovaleric acid, in high concentrations, may cause harmful effects in honey bees. The middle and low concentration (1.0 mg/ml and 0.1 mg/ml respectively), on the other hand, exerts a positive effect by extending the bees' lifespan.

Because no previous studies on isovaleric acid were found in the literary search, the same concentrations as the valproic acid study, was chosen for treatment.

An effect of hive replicates was also observed in the lifespan study with isovaleric acid. Significant treatment effects were detected for all concentration treatments in both hive 1 and 2. The effects of treatment (expanding/reducing lifespan) were the same for all treatment concentrations, in both hives. When studying the graphs for the two hives in figure 17, it

seems as though the individuals in hive 2 are less tolerant to the isovaleric acid than the bees in hive 1. The bees in hive 2 are showing high mortality, before the bees in hive 1 does. As an example; the control bees in hive 1 live 46 % longer than the bees from hive 2, before reaching their maximum lifespan. The same trend is seen in the three concentration treatments. Like for the other substances above, this observation might be explained by the suggestion that the genetic background, or other colony specific factors, might be decisive for the observed hive effect.

The isovaleric acid is, as the valproic acid, found in the plant extracts of the valerian. The effect of this substance is less known, however it has been speculated that the isovaleric acid is not found in the valerian plant itself, but is rather a product from the methods of processing or storage conditions (Patočka & Jakl 2010). The isovaleric acid has been suggested as one of the compounds that are the effective factors related to treatment with valerian extracts. Perhaps, due to the isovaleric acid's very unpleasant smell, there are very few studies that have been preformed to map its effects in medicinal treatment.

When processing the consumption data from the isovaleric acid study, a clear statistical significance was found. The results showed that the amount of food consumed decreased with the higher concentrations of isovaleric acid (see figure 18 and table 10). A reason for this could be that the unpleasant smell of the isovaleric acid reduced the bees' motivation to feed. The bees receiving the high concentration ate, on average, 48 % less than the control group. As the bees receiving this treatment showed a shortening in lifespan compared to the control, one can assume that these bees died from starvation rather than the physiological effects of the isovaleric acid itself. This assumption is reinforced by the fact that the middle concentration group (also showing a significant reduction of consumption compared to the control), showed an extension of lifespan. Caloric restriction (or reduced energy intake) has been known to delay senescence of many model animals (Masoro 2005). The effects of caloric restriction might also serve as an explanation for the observed extending effect on lifespan, which is observed for the middle concentration treatment (1.0 mg/ml) of isovaleric acid. However, the mechanisms in which caloric restriction works, are uncertain.

A similar effect of consumption seen in this project, was observed in a study where honey bees were fed with resveratrol (Rascón et al. 2012). The resveratrol treatment extended the lifespan of the bees; however, their consumption of food was significantly lower than that of the controls. The bees receiving this treatment, also responded less to sugar solutions.

Resveratrol is regarded as a caloric restriction mimic, hence affecting mechanisms that are related to caloric restriction.

5.5 Methodological considerations

5.5.1 The lifespan studies

The sampling of bees was a simple task, since they were sampled directly into the premade cages. However, some bees seemed more agitated during sampling, which could have affected their longevity and the results of the lifespan studies. This was particularly evident for the valproic acid and isovaleric acid study. When sampling these bees, it was observed that the bees from hive 2 seemed more agitated and aggressive. When sampling from this hive, the bees tended to fly up and defending their hive, which led to them sting both each other and the boxes they were put in. In turn, this could explain the observation of the bees from hive 2 being weaker than the bees sampled from hive 1, which is seen in both the isovaleric acid and valproic acid study. As mentioned in the discussion for the isovaleric acid, the bees receiving the control treatment from hive 1 lives 46 % longer than the bees sampled from hive 2. The sampling of these bees occurred in November, and the weather was very cold. During the sampling, some of the bees froze to death, while others were weakened by the cold. This could also have led to all the bees being generally weak from the beginning of the study.

As mentioned in the discussion above, the effects of treatment can also be dependent on the individuals' specific life-stage when treatment is administered. When the bees were sampled for the lifespan study in this project, their age was not known (all adults). Even though all of the bees were of the winter phenotype (*diutinus*), their chronological age could still be different. Hence, if some of the substances tested exert life stage dependant effects, it would be difficult to identify these in this study as some of the sampled bees might be old while others were young. Also, the observation that some bees lived longer than others, could be because these bees were of a younger age when they were sampled.

Even though the handling of bees during the lifespan studies went relatively smoothly, some incidents did occur, causing loss of bees. Mostly, these were only minor losses (1-2 bees) caused by escapes during the removal of dead bees (these bees were registered as "censored" individuals). Other factors that resulted in censored bees were incidents where bees climbed

under the mesh and were unable to get back. These bees eventually died, and were registered as censored the day they were observed as dead. The reason for registering these bees as censored individuals was that they did not die directly from treatment. The hive effect found in the sodium butyrate study might be explained by such incidents. When sampling bees the sodium butyrate study, an extreme difference in hive behavior, like that seen when sampling for the other two substances, was not observed. However, some incidents did occur through the lifespan study with some of the cages sampled from hive 1 (escape of bees, getting stuck under cages).

Other incidents, where a substantial amounts of bees were lost due to either starvation (lack of holes in the food tubes), or escape resulted in the removal of the affected cage, or re-sampling if this was a possibility.

5.5.2 The epigenetic assays

The ELISAs conducted in this project, did not detect any variance in DNA methylation in the concentration treatments for any of the three substances. One suggestion could be that increasing the number of replicates per treatment on the ELISA plate, might show a significant effect if there really was a difference in the global 5-mC content between treatment and controls. Even though the samples were applied pseudo randomly, scanning the ELISA plate could still produce potential outliers which, in turn, could affect the statistical result (especially since the numbers of samples were so few). This can also be seen from the graphs in figure 21, 22 and 23. In all of the graphs, a large standard deviation is seen (for at least one of the treatments). The sodium butyrate results show a large standard deviation in the control, while in the isovaleric acid and valproic acid a large standard deviation is seen for the high concentrations (T1 in the graph). Conducting the same assays with more samples may lower the standard deviation in the different groups, and reveal a variance in % 5-mC if the substance really exerts an effect on the DNA methylation mechanisms. With a bigger sample size, one can remove potential outliers, without losing too much statistical power.

The Western blot was in this project supposed to be used for detection of difference in histone acethylation in the bees DNA. However, this method was excluded from being applied to the bees treated with any of the substances. In this study, a H3 histone protein with an acetyl-modification at its 23 lysine residue (H3K23ac) was the protein to be detected. For the detection of this protein, an immune-detection procedure was conducted, using an antibody

specific for the antigen (H3K23ac). For visualization, a secondary antibody bound to a fluorescent molecule was used. After incubation with the primary and secondary antibody, no band was observed at the size stated in the datasheet form Diagenode (14.4 kDa). When studying the membrane scans in figure 20, there is no difference between the membrane scans after incubation with antibodies and after staining it with the SYPRO Ruby stain. The primary antibody that was used, was a polyclonal antibody, which may result in some unspecific binding (Lea 2008). However, the scans do not indicate any specificity of the antibody at all. When studying the Coomassie stained gel additional bands are observed, also in the wanted size-range of 14.4 KDa (figure 19), which reinforces the assumption that the antibody had low specificity and bound to several other epitopes on the membrane. In the datasheet for the primary antibody (Diagenode), it is stated that the antibody is positive for human samples, but not tested on samples obtained from other species. Thereby, the antibody might not be specific for honey bee histones, or the amount of these proteins is too small and therefore not observed on the gel when using an immunodetection procedure.

6. Conclusion

- Treatment with low doses of isovaleric acid extends the lifespan of the honey bee.
- The bees' consumption is significantly reduced with higher concentrations of isovaleric acid.
- High doses of valproic acid reduce the honey bees' lifespan.
- Sodium butyrate only showed minor effects on lifespan.
- No significant effect on DNA methylation by any of the substances tested was observed in this study.
- Western blotting was not feasible, hence observations of histone acetylation effectswas not possible.

7. Future perspectives

As mentioned in the discussion for isovaleric acid, the quantity of consumed food lowered significantly as the concentrations increased. The observed increased lifespan for the middle and low concentration treatments could be a result of isovaleric acid working as a caloric restriction mimetic. To investigate whether the reduced food intake was a consequence of the unpleasant smell, or if the isovaleric acid otherwise reduces consumption, gustatory response tests could have been conducted to quantify an individual's food response. Such tests examine the bees' response to stimulation on the antenna with different sucrose solutions (high and low concentrations). A similar test was also conducted in the study with resveratrol (Rascón et al. 2012). The principle of a future experiment, could be to first conduct a feeding trial with the substance for 10 days, and then measure the bees' gustatory response. If the bees treated with the high concentration of isovaleric acid were to show reduced gustatory response score (only responding to food high in sugar) compared to the controls, it could be concluded that the isovaleric acid treatment causes a lower food intake by changing physiological mechanisms linked to food intake. Accordingly, the isovaleric acid could, for example cause a reduction in the bees' taste sensitivity or satiation state, and thereby reduce their motivation to feed (long term effect).

On the other hand, to find if the direct contact with isovaleric acid immediately reduces the gustatory response, another experiment can be conducted. For this experiment, no prior feeding trials are required. By testing random bees' gustatory response to sugar solutions, with isovaleric acid added in different concentrations, one can find if the smell is what keeps the bees from eating. If the isovaleric acid is directly repellant to the bees, they will better respond to the sugar solutions with low concentration or zero added isovaleric acid.

I think it would also be interesting to see if any of the substances show a different or stronger effect on bees of other phenotypes (nurses, foragers and queens). As mentioned above, the effects of a substance can be different, when administered at different stages of life. For example, one could monitor the bees' age from the larval stage, and induce treatment at specific ages. Another interesting study could be to induce treatment directly after hatching, and throughout life, and study the effects.

No epigenetic modifications (global DNA methylation/histone acetylation) were detected in this study, however, it would be interesting to conduct investigate these possibilities further. Especially in relation to treatment with isovaleric acid, it would be interesting to test other

methods for observing epigenetic effects, since little is known about the effects of treatment with this substance.

As mentioned above, even though the band it not observed on the membrane from the western blot, it doesn't mean that the samples did not contain these acetylated histone proteins. A method that can be used to detect the acetylated H3 histone protein, is mass spectrometry (MS) (Brown 2007).

By cutting out the wanted bands on the gel (14.4 kDa), extracting the proteins and treating them with a sequence specific proteinase, a MS analysis could have been conducted to identify the proteins (Lesk 2010). The general principle of mass spectrometry is that the peptides (made from the proteinase treatment) are separated based on their mass, and each protein gets a "peptide-fingerprint". These fingerprints are then compared with theoretical peptide fingerprints from a database, and get a score for similarity (The more similar, the higher the score). Thereby, the protein in the database giving the highest score is most likely the same as the protein separated on the gel. However, mass spectrometry is a qualitative method, and will not give any information of how much of the protein the samples contain.

It would also be interesting to conduct an ELISA with larger sample sizes from each substance. If a difference in DNA methylation between the concentration groups were to be observed, a sequencing analysis could be conducted. Mapping of the bees' DNA methylation patterns might reveal a difference in splicing products when comparing treated bees with controls. A method that is used for mapping the methylated cytosine residues in a genome, is bisulfite sequencing (bi-seq), originally developed in 1992 (Frommer et al. 1992). This method has since then been frequently used, and is constantly developing to become more sensitive (Li & Tollefsbol 2011). By treating the DNA with bisulfite, all unmethylated cytosines are converted to the urasil base which, in PCR is recognized as thymine residues. Hence, a cytosine peak in the sequencing results indicates a methylation in the genome. The variance in methylation pattern can be determined by comparing the sequencing results from the bi-seq with the original DNA sequence (Li & Tollefsbol 2011).

For the mapping the methylated CpG dinucleotides in specific sites in the bees genome, specific restriction enzymes could be used to cut the DNA into pieces and sequence the wanted DNA restriction sequence (Boyle et al. 2012). Whole genome methylation sequencing is also a possible to assess the methylation pattern throughout the whole genome (Lyko et al. 2010).

To see if the observed effect on lifespan can be explained by modified DNA pattern, one could compare annotated transcrips from treated bees with control bees. If some of the genes that are found to be modified by methylation is already known, one could also screen for different spice variants between them. Since the methylation machinery in honey bees, is similar to that found in humans. The effects of diet on methylation patterns, can be studied in honey bees and mammal cell lines in parallel (Ford 2013). Both of these models have their own restrictions, however, these can cancel each other out when studying them together. To determine if a change in DNA methylation really do affect aging, and not an effect of just "growing old", is still a challenge. But, manipulation of these models can help reveal links between DNA methylation and aging, and how these mechanisms may be affected by different bioactive compounds.

8. References

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Appendix 1

ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

5-mC DNA ELISA Kit

Catalog Nos. **D5325** & **D5326**

Highlights

- For high-throughput, detection of global 5-methylcytosine (5-mC) in DNA.
- The streamlined workflow can be completed in less than 3 hours.

Contents

ZYMO RESEARCH CORP.

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Satisfaction of all Zymo Research products is guaranteed. If you should be dissatisfied with this product please call 1-888-882-9682.

Product Contents

5-mC DNA ELISA Kit (Kit Size)	D5325 (1 x 96 wells)	D5326 (2 x 96 wells)	Storage Temperature
5-mC Coating Buffer	15 ml	15 ml x 2	4 °C
5-mC ELISA Buffer	250 ml	250 ml x 2	4 °C
Anti-5-Methylcytosine (1 µg/µl)	15 µl	30 µl	-20 °C
Secondary Antibody (1 µg/µl)	15 µl	30 µl	4 °C
HRP Developer	15 ml	15 ml x 2	4 °C
Negative Control (100 ng/µl)	50 µl	50 µl	- 20 °C
Positive Control (100 ng/µl)	50 µl	50 µl	- 20 °C
96-well plate (12 x 8-well Strips)	1 plate	2 plates	Room Temp.
Protocol	1	1	-

Note - Integrity of kit components is guaranteed for up to up to six (6) months from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

Specifications

Sample Sources – Purified genomic DNA, plasmid DNA, PCR amplification products, or DNA fragments in water, Tris-EDTA, or similar.

DNA Quantity – This protocol is optimized for 100 ng input DNA/well. Compatible with DNA in the range of 10-200 ng.

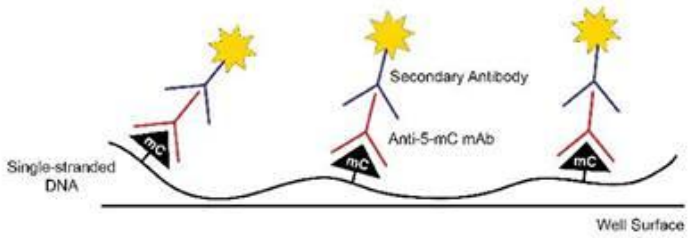
Detection – ≥ 0.5% 5-methylcytosine (5-mC) per 100 ng single-stranded DNA.

Equipment Required – Incubator and ELISA plate reader. A multi-channel pipettor is recommended. An automated plate washer may be used for blocking and wash steps.

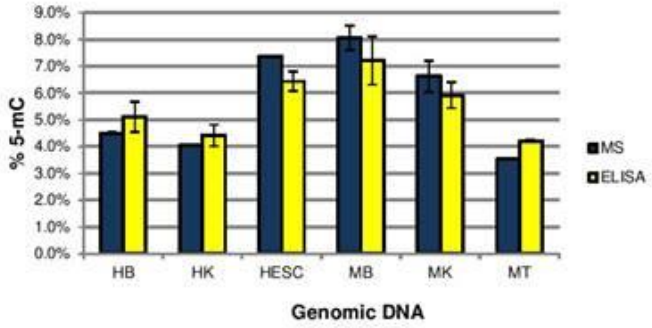
Product Description

The ability to efficiently detect and quantify DNA methylation (i.e., 5-methylcytosine) has become essential for epigenetic-based research. To date, a number of methods have been developed for this purpose including high-performance capillary electrophoresis, bisulfite sequencing, and methylated DNA immunoprecipitation.

The **5-mC DNA ELISA Kit** is a convenient and powerful tool that allows the researcher to accurately quantitate 5-mC in *any* DNA sample in less than 3 hours. The kit features a unique **Anti-5-Methylcytosine** monoclonal antibody that is both sensitive and specific for 5-mC. The assay is compatible with a wide range of input DNA from vertebrate, plant, and microbial sources as well as PCR amplicons and fragmented DNA. Percent 5-mC in a DNA sample can be accurately quantified from a standard curve generated with specially designed controls included with the kit. Also, the fast, streamlined workflow is ideal for high-throughput analyses.



The 5-mC DNA ELISA Kit utilizes the indirect ELISA technique in its workflow. Denatured, single-stranded DNA samples are coated on the well surfaces in **5-mC Coating Buffer**. **Anti-5-Methylcytosine** monoclonal antibody (Anti-5-mC mAb) and the HRP-conjugated **Secondary Antibody** are prepared in **5-mC ELISA Buffer** and added to the wells. Detection of 5-mC occurs after addition of the **HRP Developer**.



The 5-mC DNA ELISA Kit can quantify 5-mC in numerous DNA samples with close correlation to LC-MS/MS-MRM analysis. 100 ng of genomic DNA from human brain (HB), human kidney (HK), human embryonic stem cell (HESC), mouse brain (MB), mouse kidney (MK), and mouse testes (MT). Percent 5-mC was calculated using the second-order regression equation of the standard curve that was contructed with the **Negative Control** and the **Positive Control** (see Appendix, page 5). The percent 5-mC detected in DNA samples by **5-mC DNA ELISA Kit** (ELISA) strongly correlates to mass spectrometry (MS) data of 5-mC found in the respective gDNA sample.

For **Assistance**, please contact Zymo Research Technical Support at 1-888-882-9682 or e-mail tech@zymoresearch.com.

Experimental Considerations

- All DNA *must* be denatured (single-stranded) prior to use with the kit. The protocol is optimized for the detection of 5-mC in 100 ng of single-stranded DNA per well. However, depending on your experimental design, 10 to 200 ng of sample DNA can be used in the assay.
  Note: When using inputs other than 100 ng per well, the amount of control DNA used must be adjusted to equal the amount of sample used. This will ensure accurate % 5-mC quantification.

- The **Negative** and **Positive Controls** consist of double stranded DNA at a concentration of 100 ng/µl, and can be used for the detection/quantification of 5-mC in DNA. For 5-mC detection, both controls should be assayed. For 5-mC quantification, the **Negative Control** should be mixed with the **Positive Control** at different ratios to construct a standard curve (see Appendix, page 5).

- **Secondary Antibody** is a horseradish peroxidase (HRP) conjugate, and supplied at a concentration of 1 µg/µl.

Buffer Storage

- ✓ **5-mC Coating Buffer** is stable at room temperature or 4 °C for extended periods of time.

- ✓ **5-mC ELISA Buffer** should be storage at 4°C and used within 6 months. Alternatively, the buffer may be dispensed into multiple aliquots and kept at -20°C for long term storage. Avoid repeated freeze/thaw cycles.

- ✓ **HRP Developer** must be stored at 4 °C and used within 6 months. Do not freeze. For more rapid color development, bring to room temperature before adding to the wells.

Protocol

This protocol is optimized for 100 ng of DNA per well.
***Duplicate** samples are recommended for accurate 5-mC detection and quantification.*

DNA Coating:

1. Remove the necessary number of well strips[1] to assay DNA samples and controls[2].
2. Add 100 ng of each DNA[3] to a PCR tube and bring the final volume to 100 µl with **5-mC Coating Buffer**.

   Example: If the DNA concentration is 20 ng/µl, add 5 µl of DNA to 95 µl of **5-mC Coating Buffer** for a final volume of 100 µl.
3. Denature the DNA at 98°C for 5 minutes in a thermal cycler. After denaturation, transfer immediately to ice for 10 minutes.
4. Add the denatured DNAs to the wells of the plate, cover with foil, and incubate at 37 °C for 1 hour.

Blocking:

1. Discard the buffer from the wells[4].
2. Wash each well 3 times with 200 µl of **5-mC ELISA Buffer**. *Discard the buffer after each wash.*
3. Add 200 µl of **5-mC ELISA Buffer** to each well. Cover the plate with foil and incubate at 37 °C for 30 minutes.

Antibody Addition:

1. Discard buffer from the wells.
2. Prepare an antibody mix[5] consisting of **Anti-5-Methylcytosine** and **Secondary Antibody** in **5-mC ELISA Buffer** according to the following table:

	Dilution	Volume (µl)	Example (18 wells)
5-mC ELISA Buffer	N/A	(# wells + 2) 100	2,000 µl
Anti-5-Methylcytosine	1:2,000	Buffer Vol. / 2,000	1 µl
Secondary Antibody	1:1,000	Buffer Vol. / 1,000	2 µl

3. Add 100 µl of this antibody mix to each well. Cover the plate with foil and incubate at 37°C for 1 hour.

Color Development:

1. Discard the antibody mix from the wells.
2. Wash each well 3 times with 200 µl of **5-mC ELISA Buffer**.
3. Add 100 µl of **HRP Developer** to each well. Allow color to develop for 10-60 minutes[6] at room temperature.
4. Measure absorbance at 405-450 nm using an ELISA plate reader.

Notes:

[1] The well strips should be stored in a clean, dry, dark place for later use.

[2] For more information regarding 5-mC detection and quantification using the **Negative** and **Positive Controls**, refer to the Appendix, page 5.

[3] Make sure that the volume of the DNA added to the **5-mC Coating Buffer** does not exceed 20% of the final volume.

[4] Tap out any remaining buffer onto a paper towel after emptying a well.

[5] The antibody mix can be prepared during the blocking step and kept on ice until it is needed.

[6] The development time will depend on the temperature of the **HRP Developer** (see p. 3).

Appendix - Analysis with Negative and Positive Control DNAs

Notes:

[1] The **Negative** and **Positive Controls** must be included on the same plate as the DNA samples for each assay.

[2] A new standard curve should be generated for each assay.

[3] The number of standard curve mixtures for 5-mC quantification can vary. In the example given in the table, seven mixtures were prepared. Leftover mixtures can be frozen at or below -20 °C for future use.

For 5-mC Detection:

The presence or absence of 5-mC can be determined by comparing the absorbance of samples to **Negative** (0% methylation) and **Positive** (100% methylation) **Controls**[1].

For 5-mC Quantification:

To quantify the percentage of 5-mC in a DNA sample, a standard curve[2] must be generated. This is done by preparing mixtures[3] of the **Negative Control (100 ng/µl)** and **Positive Control (100 ng/µl)** to generate standards of known 5-mC percentage (see table below). These must be prepared *prior to denaturation* and assayed in parallel with the samples. Add 1 µl (i.e., 100 ng) of each mixture to a PCR tube and bring the final volume to 100 µl with **5-mC Coating Buffer**. Proceed with Coating Step 3 of the protocol (p. 4).

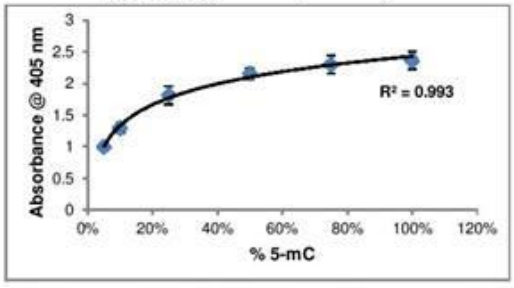
% 5-mC	Negative Control (100 ng/µl)	Positive Control (100 ng/µl)
0%	10.0 µl	0 µl
5%	9.5 µl	0.5 µl
10%	9.0 µl	1.0 µl
25%	7.5 µl	2.5 µl
50%	5.0 µl	5.0 µl
75%	2.5 µl	7.5 µl
100%	0 µl	10.0 µl

Table highlights the preparation of seven mixtures using the **Negative Control** and **Positive Control** to be used to generate a standard curve. Total volume of each is 10 µl at a concentration of 100 ng/µl.

Note: The **Positive** and **Negative Control DNAs** consist of *Escherichia coli* gDNA. The **Positive Control DNA** has been treated with CpG Methylase (Catalog # E2010/11). The density of CpG dinucleotides varies between species and to accurately quantitate the %5-mC simply multiply the calculated %5-mC by the fold difference in CpG density between *E. coli* and the sample species. For example, *E.coli* CpG density/gemone length is 0.075 and mouse CpG density/genome length is 0.0081, therefore, the fold difference between *E. coli* and mouse CpG density is 9.22.

The absorbance for each mixture must be plotted as a function of Absorbance @ 405 nm (Y-axis) vs. % 5-mC (X-axis). Using the equation below, derived from the logarithmic second-order regression, determine the 5-mC percentage for DNA samples (unknowns) based on their absorbance.

$$\%\ 5\text{-mC} = e^{\left\{\frac{(\text{Absorbance} - \text{y-intercept})}{\text{Slope}}\right\}}$$



Standard curve generated with DNA mixtures. The curve was using the absorbance values of the mixtures indicated in the table above. A logarithmic relationship was observed with a correlation of 0.99.

Ordering Information

Product Description	Catalog No.	Kit Size
5-mC DNA ELISA Kit	D5325 D5326	1 x 96 wells 2 x 96 wells

For Individual Sale	Catalog No.	Amount
5-mC Coating Buffer	D5325-1-15	15 ml
5-mC ELISA Buffer	D5325-2-250	250 ml
Anti-5-Methylcytosine (1 µg/µl)	A3001-15 A3001-30	15 µl 30 µl
Secondary Antibody (1 µg/µl)	D5325-3-15 D5325-3-30	15 µl 30 µl
HRP Developer	D5425-4-15	15 ml
Negative Control (100 ng/µl)	D5325-5-1	50 µl
Positive Control (100 ng/µl)	D5325-5-2	50 µl
96-well ELISA plate (12 x 8-well Strips)	C2020	1 plate

Related Products for 5-mC Analysis:

Additional Products for

X

Epigenetics Research:

Product Name	Size	Catalog No.
Methylated-DNA IP Kit	10 Rxns.	D5101
OneStep qMethyl™ Kit	1 x 96	D5310
OneStep qMethyl™-Lite	1 x 96	D5311
Zymo*Taq*™ DNA Polymerase	50 Rxns. 200 Rxns.	E2001 E2002
Zymo*Taq*™ PreMix	50 Rxns. 200 Rxns.	E2003 E2004
EZ DNA Methylation™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5001 D5002 D5003 D5004
EZ DNA Methylation-Gold™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5005 D5006 D5007 D5008
EZ DNA Methylation-Direct™ Kit	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5020 D5021 D5022 D5023
EZ DNA Methylation-Startup™ Kit	50 Rxns.	D5024
EZ Bisulfite DNA Clean-up Kit™	50 Rxns. 200 Rxns. 2 x 96 2 x 96	D5025 D5026 D5027 D5028
Universal Methylated DNA Standard	1 set	D5010
Universal Methylated Human DNA Standard	1 set	D5011
Universal Methylated Mouse DNA Standard	1 set	D5012
Human HCT116 DKO Methylation Standards	1 set	D5014
Human HCT116 DKO Non-methylated DNA Standard	5 µg	D5014-1
Human HCT116 DKO Methylated DNA Standard	5 µg	D5014-2
Bisulfite Converted Universal Methylated Human DNA Standard	1 set	D5015
E. coli Non-methylated Genomic DNA	5 µg	D5016
ChIP DNA Clean & Concentrator™	50 50	D5201 D5205
Anti-5-Methylcytosine Monoclonal Antibody (clone 10G4)	50 µg 200 µg	A3001-50 A3001-200
CpG Methylase (M.SssI)	200 units 400 units	E2010 E2011
5-Methyl dCTP [10 mM]	1 µmol	D1035
5-Methylcytosine dNTP Mix [10 mM]	2.5 µmol	D1030

Product Name	Size	Catalog No.
Quest 5-hmC™ DNA ELISA Kit	1x96	D5425
	2x96	D5426
Anti-5-Hydroxymethylcytosine Polyclonal Antibody	50 µg	A4001-50
	200 µg	A4001-200
Quest 5-hmC™ DNA Enrichment Kit	25 Preps.	D5420
	50 Preps.	D5421
Quest 5-hmC Detection Kit™	25 Preps.	D5410
	50 Preps.	D5411
Quest 5-hmC Detection Kit™-Lite	25 Preps.	D5415
	50 Preps.	D5416
Quest *Taq*™ PreMix	50 Rxns.	E2050
	200 Rxns.	E2051
Human Matched DNA Set	2 x 5 µg	D5018
Mouse ^{5hm}C & ^{5m}C DNA Set	4 x 5 µg	D5019
5-Methylcytosine & 5-Hydroxymethylcytosine DNA Standard Set	3 x 2 µg	D5405
DNA Degradase™	500 units	E2016
	2,000 units	E2017
DNA Degradase Plus™	250 units	E2020
	1,000 units	E2021
5-hmC Glucosyltransferase	100 units	E2026
	200 units	E2027
5-Hydroxymethyl dCTP [100 mM]	10 µmol	D1045
5-Hydroxymethylcytosine dNTP Mix [10 mM]	2.5 µmol	D1040

THE Epigenetics COMPANY™

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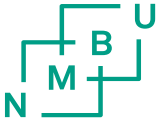

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