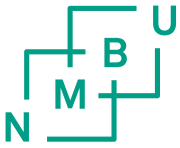
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Abstract

The physiological and social changes that follows aging have long been of interest for scientists around the world, and the effect of diet on aging and longevity through epigenetic mechanisms has become highly topical.

Different natural food components, such as plant extracts and biochemical compounds, have the ability to change epigenetic traits, including DNA methylation and histone modifications, and may in turn affect aging and lifespan. Epigenetic traits include biochemical alterations to the DNA sequence and to the tails of the histones that do not alter the DNA sequence, but have the ability to alter gene expression. Changes in such epigenetic traits are thus linked to various diseases, and provide great prospects for future medical research. The honey bee (*Apis mellifera*) serves as a promising and suitable model organism to address such aspects due to its short lifespan and the fact that it has an epigenetic machinery homologous to humans and other mammals.

This thesis aim to test the three substances curcumin (from the turmeric plant), folic acid (vitamin B9), and cyanocobalamin (vitamin B12) on longevity, food consumption, and epigenetic traits such as DNA methylation and histone acetylation in the honey bee. This will be conducted by thoroughly monitoring the bees during the lifespan studies in regards to food consumption, and by using DNA extraction, western blot and ELISA to look for changes in epigenetic traits.

The results showed a significant impact on lifespan for all three substances, but no significant differences in global DNA methylation levels. No results were obtained for histone acetylation due to shortcomings with the method. Moreover, no differences in food consumption were observed. These results provide opportunities for future studies on epigenetic states using curcumin, folic acid, and cyanocobalamin, and also give prospects for future studies related to the effects of purified biochemical compounds. To my best knowledge, this is the first time life-extending effects from curcumin have been found in the honey bee.

Sammendrag

De fysiologiske og sosiale forandringene som følger aldring har lenge vært av interesse for forskere verden rundt, og effekten av diett på aldring og levetid gjennom epigenetiske faktorer har blitt svært dagsaktuelt.

Forskjellige matkomponenter, som planteekstrakter og andre biokjemiske komponenter, har evnen til å endre epigenetiske trekk som DNA metylering og histonmodifikasjoner, og slik påvirke aldring og levetid. Med epigenetiske trekk menes biokjemiske endringer på DNA sekvensen og på histonhalene som ikke påvirker sammensetningen av selve DNA sekvensen, men som har evnen til å endre genuttrykk. Endringer i slike epigenetiske trekk er ofte forbundet med forskjellige sykdommer, og gir store muligheter for fremtidig medisinsk forskning. Honningbien (*Apis mellifera*) tjener som en lovende og velegnet modellorganisme for å adressere slike aspekter grunnet dens korte levetid, og det faktum at den har et epigenetisk maskineri homologt med mennesker og andre pattedyr.

Denne oppgaven har som mål å teste de tre stoffene curcumin (fra gurkemeie), folsyre (vitamin B9), og cyanokobalamin (vitamin B12) på levetid, matinntak og epigenetiske trekk som global DNA metylering og histonacetylering i honningbier. Registrering av matinntak vil gjennomføres ved daglig oppfølging og overvåking av biene gjennom overlevelsesstudiene, mens eventuelle endringer i epigenetiske trekk vil undersøkes ved hjelp av DNA ekstraksjon, western blot og ELISA.

Resultatene i denne oppgaven viste at alle tre stoffene hadde signifikant effekt på levetid, men ikke på nivåer av global DNA metylering. Ingen resultater var oppnådd ved undersøkelser for endringer i histon acetylering, dette på grunn av svakheter ved metoden. Videre ble det ikke funnet forskjeller i matinntak mellom noen av behandlingene. Disse resultatene gir utsikter for fremtidige studier av forandringer på epigenetiske trekk ved bruk av curcumin, folsyre og cyanokobalamin. Resultatene gir også prospekter for fremtidige studier relatert til effekten av rensede biokjemiske forbindelser som kosttilskudd. I henhold til min kunnskap er dette første gang curcumin har vist forlengelse av levetid i honningbier.

List of abbreviations

Ado-B12	=	5'-deoxyadenosylcobalamin
BSA	=	Bovine serum albumin
CH_3-B12	=	Methylcobalamin
CI	=	Chloroform:isoamylalcohol
CpG	=	Cytosine-phosphate-guanine
dH_2O	=	Distilled water
DNMT	=	DNA methyltransferase
ELISA	=	Enzyme-linked immunosorbent assay
HAT	=	Histone acetyltransferase
HDAC	=	Histone deacetylase
HMT	=	Histone methyltransferase
H3K23ac	=	Acetylated lysine 23 on histone 3
kDa	=	Kilodalton
MALDI-TOF	=	Matrix-assisted laser desorption/ionization time-of-flight
mC	=	Methylated cytosine
mg	=	Milligram
mL	=	Milliliter
mM	=	Millimolar
mTHF	=	Methyltetrahydrofolate
OD	=	Optical density
ON	=	Over night
PCI	=	Phenol:chloroform:isoamylalcohol
PTM	=	Posttranslational modifications
Rpm	=	Revolutions per minutes
RT	=	Room temperature
SAH	=	S-adenosylhomocysteine
SAM	=	S-adenosylmethionine
SDS-PAGE	=	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
V	=	Volts
µg	=	Mikrogram
µL	=	Mikroliter

5-mC	=	5-methyl cytosine
5-mTHF	=	5-methyltetrahydrofolate
% 5-mC	=	% of total 5'methylation on cytosine

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1. Introduction

Aging, and the neurodegenerative diseases that follows with it, is one of the greatest fields of interest for researches worldwide. The human society, with its growing population of elderly people, has turned to dietary supplements and other medications as a way of dealing with the illnesses that follows with it. Innovative medical research has ensured a prolonged lifespan compared to that of a hundred years ago, which again reveals new challenges considering human health and disease. The discovery of epigenetic patterns as an important link between diet and age-related diseases has become highly topical, and more and more are being discovered in this field every year.

1.1 – Epigenetics

Aging and longevity have been accepted as associated to gene regulation through changes in epigenetic patterns. Epigenetics is often defined as a state of gene expression that is due to changes in chromatin structure or other DNA modifications, but without affecting the DNA sequence (Sang-Woon Choi 2010). Epigenetic modifications are chemical modifications attached to the bases in the DNA sequence, or to amino acids on the histone tails, that alters the chemical structure of the nucleosome as well as the availability of the DNA for proteins and modulators (Fig. 1). Such epigenetic traits can be inherited from generation to generation (Rakyan & Beck 2006), or through cell division, which is often called epigenetic regulation (Watson et al. 2008). The epigenetic traits discussed in this thesis include DNA methylation, and histone modifications such as acetylation and methylation.

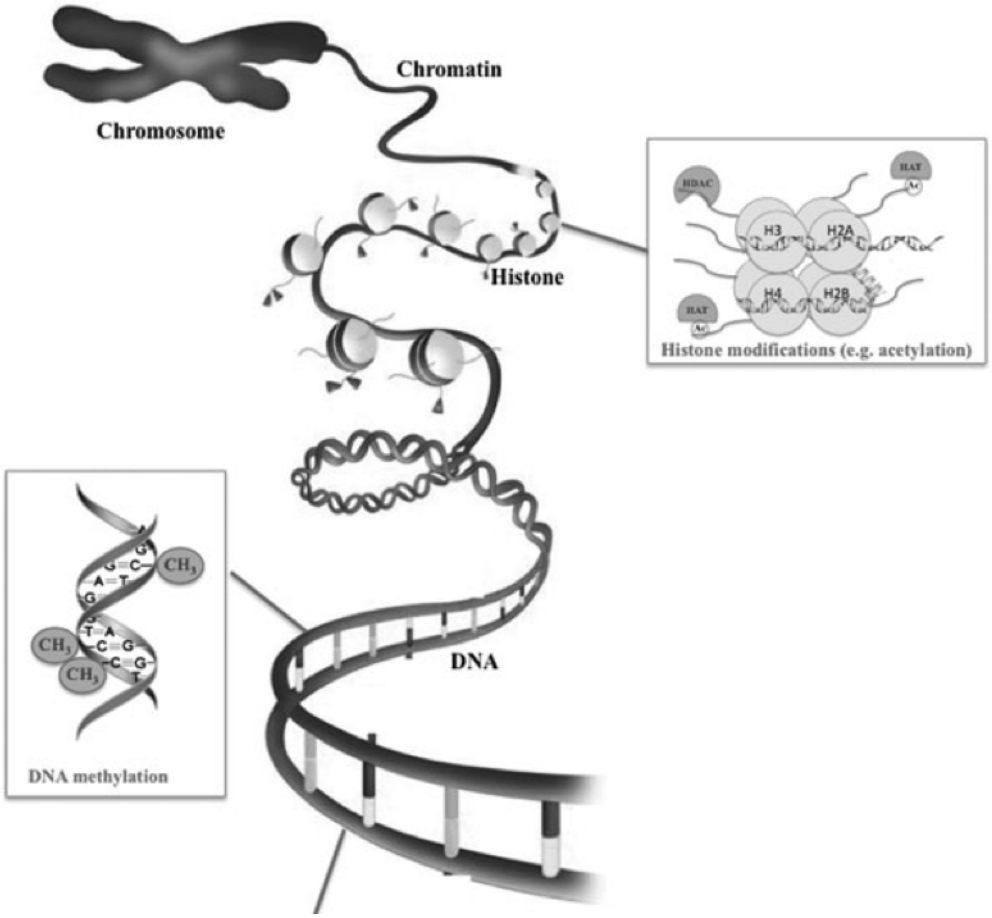


Figure 1: Epigenetic traits, including histone modifications and DNA methylation (Marie-Helène Teiten et al. 2013).

1.1.1 – DNA methylation

DNA methylation is a biochemical process involving the addition of a methyl group to the 5' position of a cytosine base (Fig. 1), and is catalyzed by DNA methyltransferases (DNMT's) (Sang-Woon Choi 2010). Methyl groups can also be added to the adenine nucleotide, though the addition to a cytosine base is more common. In mammals, methylation primarily occurs at cytosine-phosphate-guanine (CpG) sites. These sites are not evenly distributed throughout the genome but can be found in CpG-poor (areas of repeated DNA) and CpG-rich areas (CpG islands), normally in promoter regions (Fig. 2) (Huidobro et al. 2013).

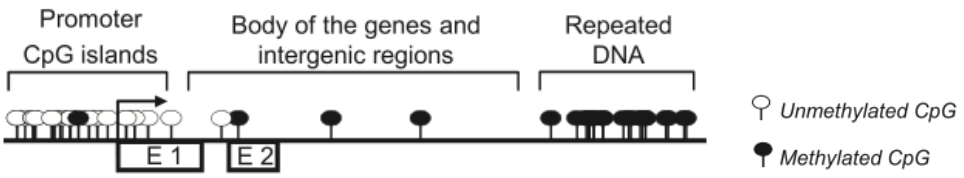


Figure 2: Distribution of methylation of CpG's in normal mammalian cells (Huidobro et al. 2013).

CpG-poor sites are usually methylated in normal cells while CpG sites in CpG islands are not, but during aging this can often get reversed and one can find that global DNA methylation decreases whilst CpG islands become hypermethylated (Fig. 3) (Huidobro et al. 2013).

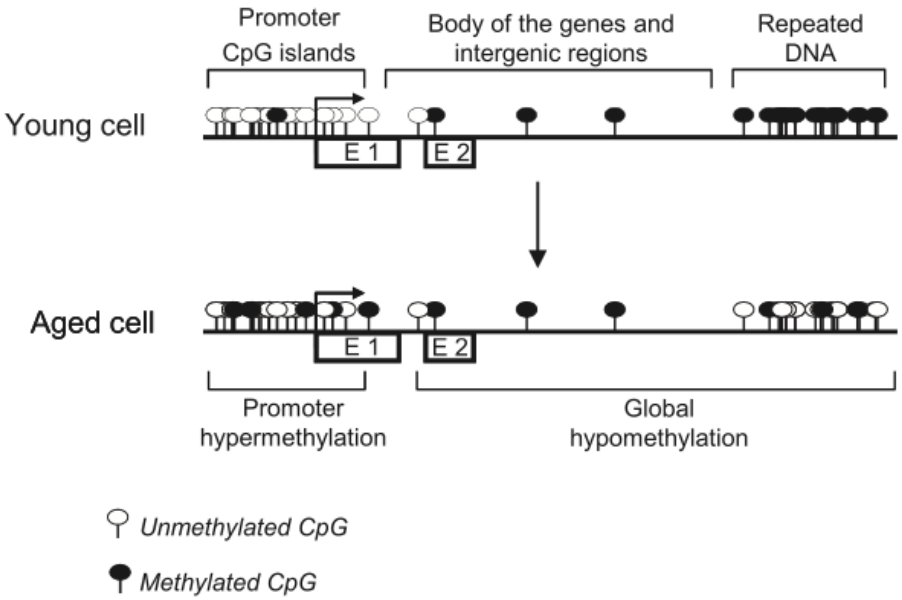


Figure 3: The change in methylation patterns at CpG sites with aging (Huidobro et al. 2013).

In honey bees, genes are not evenly targeted with methylation, but it appears to be modulating globally expressed genes (Lockett et al. 2010). Methylation in honey bees is, though not very abundant, mostly found in exons and is considered specific due to the fact that it recognizes intron-exon boundaries (Flores & Amdam 2011). The accumulation of methylated sites in honey bee exons indicate a correlation between methylation and gene variants of splicing (Lyko et al. 2010). And, as in humans, the 5' carbon of the cytosine base in CpG dinucleotides is the site that is methylated in honey bees.

DNMTs catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) which is the successor of methionine (Patrick O. McGowan 2008). There are three DNMTs both in humans and in honey bees, and they hold the same *in vivo* properties (Kim et al. 2009). The synthesis of SAM is dependent on amongst others the presence of methionine, vitamin B12, and dietary folates, and SAM has been shown to both stimulate methylation and to inhibit demethylation. DNA methylation can alter gene expression and local chromatin state,

and can have both harmful and beneficial effects (Sang-Woon Choi 2010). It is often linked to gene repression, and has the ability to alter transcription as it can change whether or not the DNA stays available for RNA polymerase II (Patrick O. McGowan 2008), and other proteins (Watson et al. 2008).

DNA methylation patterns, as other epigenetic modifications, can be inherited through cell division (Watson et al. 2008). In addition to the methylation of unmodified DNA by DNMT 3a and -3b, maintenance DNMTs (DNMT1) can modify hemimethylated DNA, which is how methylated DNA appears after replication. These hemimethylated sites are recognized by the maintenance methylases and then remetylated into fully methylated sites.

1.1.2 – Histone modifications and histone acetyltransferases

Another epigenetic trait is histone modifications, which are posttranslational modifications (PTMs) to the tails of histones (Fig. 1). Histone modifications are biochemical alterations to the tails of histones, for example the addition or removal of a methyl- or acetyl group (Watson et al. 2008). These modifications can affect the accessibility of the DNA and thus alter replication, transcription and translation. Modifications including methyl groups are more specific than those involving acetylation, but both modifications affect the nucleosomes ability to form higher order of chromatin, along with gene expression.

The histone tail amino acid most often modified by acetylation and methylation is Lysine. Acetylation in general will reduce the overall positive charge of the nucleosome, while acetylation of Lysine, in particular, will neutralize the positive charge. This change in the charge will affect the nucleosomes ability to stay in its compact form and the DNA will thus be more susceptible to modifications like methylation. In this way, histone modifications are linked to DNA methylation and gene expression.

Modifications to the histone tails are catalyzed by the histone-modifying enzymes histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (Watson et al. 2008). HATs are modifiers that add acetyl groups to the tails of histones while HMTs add methyl groups to the histone tails. The deacetylases and demethylases remove acetyl groups

and methyl groups respectively. This, in turn, alters the interaction between histone tails and the nucleosomes, as it has the ability to change the overall charge of the nucleosome, and can thus make the DNA strand more or less available for modifications. One way of obtaining or interfering with such modifying enzymes is through diet by different nutrients.

1.1.3 – Nutrition and epigenetics

Theoretically, any nutrient that can affect the methyl donor SAM, or S-adenosylhomocysteine (SAH), which is a product inhibitor of methyltransferases, can alter DNA methylation and histone methylation (Sang-Woon Choi 2010), and thus alter DNA replication and cell cycle. This is because both are metabolites of the one-carbon metabolism, which is a part of DNA replication. One can also affect the machinery responsible for removing epigenetic marks by affecting deacetylases and demethylases. In addition, one can affect these epigenetic changes by hindering the necessary enzymes, by for example ingesting inhibitors of HATs and DNMTs.

It is known that nutrition affect epigenetic patterns. Different dietary substances contain different metabolites necessary for biological pathways and thus have the ability to change these pathways based on their availability. An example is the one-carbon metabolism and folate/folic acid.

1.1.4 – One-carbon metabolism

One-carbon metabolism is a biosynthetic pathway and a part of DNA synthesis. The primary function of this pathway, when located in the mitochondria, is to generate 1-carbon units in the form of formate to enter 1-carbon metabolism in the cytoplasm (review article: (Stover 2009)). In the cytoplasm folate-activated 1-carbon units participate in a network of three biosynthetic pathways: *de novo* purine biosynthesis, *de novo* thymidylate biosynthesis, and the remethylation of homocystein to methionine. This last pathway requires 5-methyl-tetrahydrofolate (5-mTHF), which is a successor of folic acid. This means that if there is a deficit in folate or folic acid levels, enough 5-methyl-THF cannot be generated and the remethylation of homocystein to methionine will stop.

SAM is both produced in the one-carbon metabolism, and donates its methyl group in this pathway (Stephanie A. Tammen 2012). The pathway involves both vitamin B12

and vitamin B9 along with others as precursors. A defect in the one-carbon metabolism, for example due to insufficient amounts of folate/folic acid, can alter both DNA methylation and histone modifications and consequently lead to disease (Stephanie A. Tammen 2012).

Epigenetic changes can happen due to external environmental factors such as diet (Sang-Woon Choi 2010), and intrinsic factors meaning genetics (Huidobro et al. 2013). The experiments conducted in this thesis aim to investigate longevity and the epigenome of European honey bees (*Apis mellifera)*, by feeding them with substances previously shown to have epigenetic effects either *in vitro* or in other animal models.

1.2 – The substances

This thesis discusses three different natural substances that have been fed to honey bees and tested through lifespan studies and molecular tests. The substances include curcumin, folic acid (vitamin B9), and cyanocobalamin (vitamin B12). These are all nutrients with known epigenetic effects, which have been chosen based on the mechanisms they affect. All three have been shown to affect DNA methylation, and are tested here with suspected effects on global DNA methylation levels in the honey bee, but also with the expectation that they can affect histone acetylation. This is due to the fact that honey bees have the molecular machinery to process these substances. The substances are also of importance for humans, both in medicines and as food supplements, and they work through studied mechanisms. Any findings in the honey bee would be of interest for future research regarding humans.

Food consumption will also be measured throughout the lifespan studies as some substances can increase lifespan through dietary restriction (Rascón et al. 2012), whilst other substances may reduce lifespan if the bees do not want to eat them, and thus starve.

1.2.1 – Curcumin

Curcumin (diferuloylmethane), from the turmeric plant (*Curcumma longa*), is a small polyphenol (Fig. 4) and a principle part of the Indian spice curry. It is known to have health benefits, such as anti-inflammatory effects, and the ability to act as an antioxidant (Li-Rong Shen 2012). It is also studied for its chemo preventive effects (Vivian Hsiu-Chuan Liao 2011).

Curcumin is thought to be able to affect amongst others both inflammatory cytokines and protein kinases (Vivian Hsiu-Chuan Liao 2011), and also to inhibit histone acetyltransferases (HATs) (Sang-Woon Choi 2010) and DNA methyltransferases (DNMTs) (Marie-Helène Teiten et al. 2013), which makes it a very interesting substance when it comes to epigenetic studies. This because HATs are important enzymes that are generally thought of as transcriptional activators (Stephanie A. Tammen 2012), in addition to being important in regulation of different diseases (Balasubramanyam et al. 2004). DNMTs catalyze the transfer of a methyl group from the methyl donor SAM, and are thus important for gene expression. Curcumin is known for being a potent DNA hypomethylating agent both *in vitro* and *in vivo* (Marie-Helène Teiten et al. 2013), and is therefore tested in this study in hope of seeing lifespan- and epigenetic effects on bees after treatment.

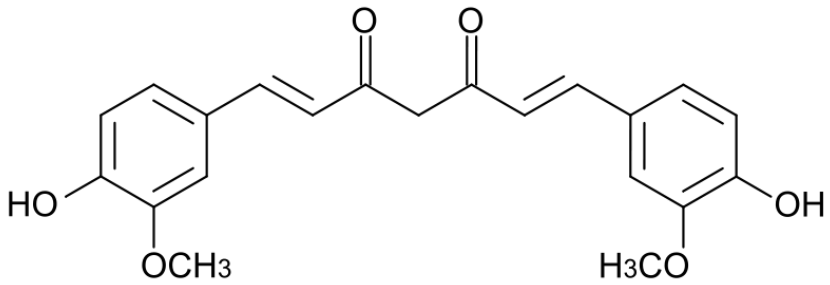


Figure 4: The chemical structure of curcumin in keto-form (Marie-Helène Teiten et al. 2013).

1.2.2 – Folic acid (vitamin B9)

Folic acid (vitamin B9), the synthetic form of folate, is a water-soluble polyphenol (Fig. 5) found in dietary supplements (review article (Crider et al. 2012)). Folate, the salt form, is found in selected foods, mostly fruits and vegetables. Vitamins are organic molecules that are required for normal growth and function of all cells (Armstrong 1978), but cannot be synthesized in efficient amounts by the organism. Dietary intake is thus needed to gain sufficient amounts of the substance.

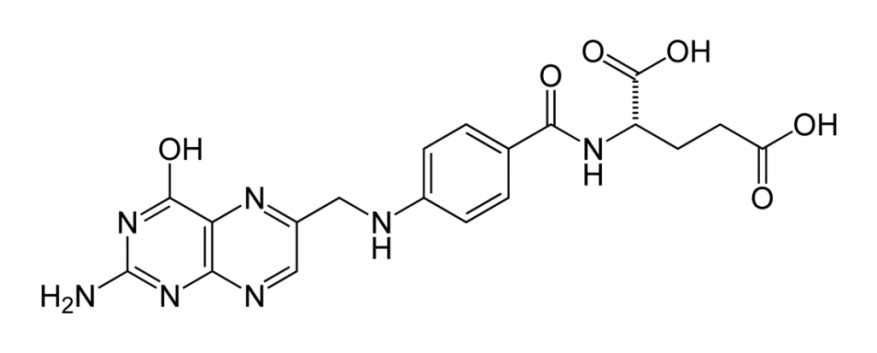


Figure 5: The chemical structure of folic acid (Wikipedia 2014).

Folate carries and donates a methyl group and thus has an effect on DNA methylation as a hypermethylating agent (Zhang et al. 2008). Folic acid primarily gets converted to 5-methyl-THF in the intestines and after that behaves the same way as folate (Crider et al. 2012). Both plays a role in the reduction and oxidation, and the activation of single carbons, and are therefore important substances in biosynthetic pathways such as the one-carbon metabolism, which is necessary for both DNA synthesis and DNA methylation amongst others. Figure 6 shows folic acid metabolism, and demonstrates how folic acid and/or folate is needed in different syntheses, and that a lack of one of them can result in limiting one or more pathways necessary for DNA synthesis.

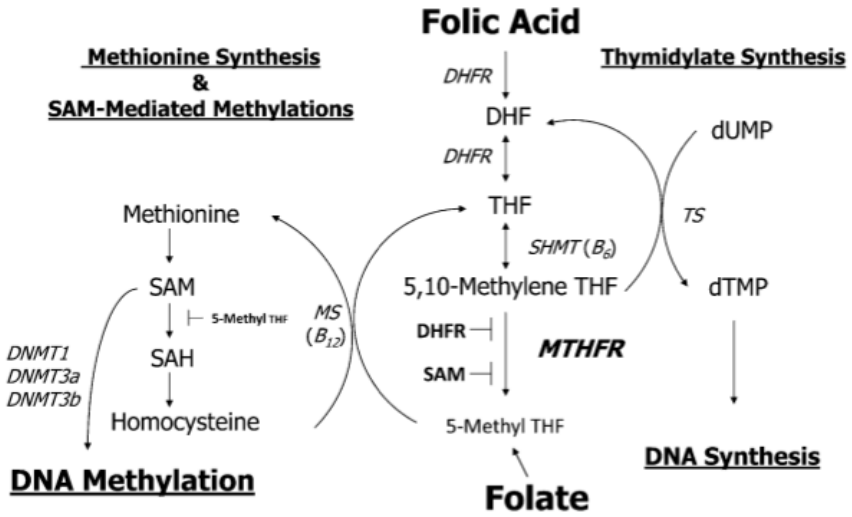


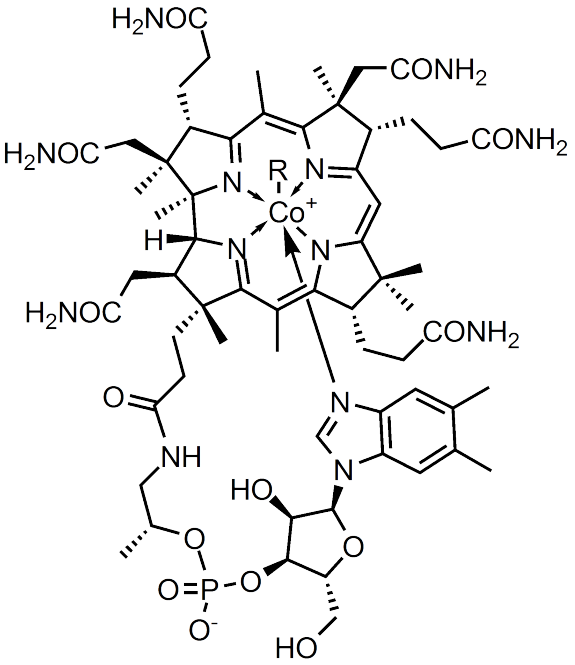
Figure 6: Folic acid metabolism (Crider et al. 2012).

Folic acid is for these reasons tested as a dietary supplement in honey bees in this thesis to look for changes in lifespan and epigenetic traits.

1.2.3 – Cyanocobalamin (vitamin B12)

Cyanocobalamin, vitamin B12, is a large polyphenol and the largest of the 13 vitamin molecules (Fig. 7) (Bito et al. 2013). It is a very complex molecule synthesized by only certain bacteria, and it is most abundant in carnivores. The main source of vitamin B12 for humans is food of animal origin. Cyanocobalamin plays an essential role in the catalytic cycle, as it is both a methyl donor and –acceptor (Gueant, J. L. et al. 2013).

When vitamin B12 is taken up in the cell it is converted into the two coenzymes 5'-deoxyadenosylcobalamin (Ado-B12) and methylcobalamin (CH_3-B12) (Bito et al. 2013). The first is the cofactor of methylmalonyl coenzyme A mutase located in the mitochondrion and plays a role in metabolism by being a part of the oxidative degeneration of amino acids (Gueant, J. L. et al. 2013). The latter is necessary for methionine synthase (Bito et al. 2013). It catalyzes the transfer of a methyl group from methyltetrahydrofolate (mTHF) to homocystein, which then forms methionine (the remethylation of methionine), the precursor of SAM. This reaction is important for both normal DNA- and histone methylation, and also DNA replication. In addition, the remethylation of homocystein to methionine is important to prevent accumulation of homocystein (Zhuo & Pratico 2010). This is because an increase in homocystein levels can alter DNA methylation, and cause inflammation-like conditions, and thus cause disease.



R = 5'-deoxyadenosyl, Me, OH, CN

Figure 7: The chemical structure of cyanocobalamin (vitamin B12) (Wikipedia 2010).

A deficiency in vitamin B12 is associated with different kinds of diseases in both humans and other animals (Bito et al. 2013). These include both growth retardation and metabolic disorders, and this substance is thus tested in this thesis in hope of seeing effects on lifespan, along with epigenetic traits.

1.3 – Honey bees

Eusocial honey bees live in complex societies, or colonies, where each and every one has its task that contribute to the community. These hives normally consists of more than 10 000 bees, and most of them are female worker bees with a lifespan of just weeks. The nurse bees, for example, can live up to 50 days in the hive before becoming foragers, and foragers normally die approximately two weeks after the onset of foraging (Munch & Amdam 2010). In addition a hive also has some male drones with a main purpose of fertilizing the queen. Bees that are born late summer – early autumn, so called winter bees, have a lifespan of up to ten to twelve months (Munch et al. 2013). Normally, the female bees' work tasks depend on their age, and thus change during their lifespan. The youngest ones start off as nurses taking care of the brood and conducting other tasks in the hive, and then move on to foraging outside of the hive later on (Adam G. Dolezal 2013). This transition from nurse bee to forager can be seen in the brain gene expression, along with changes in the epigenetic

patterns (Munch & Amdam 2010). This includes DNA methylation, which is found to be very different from nurse bees to foragers, but it is known to be reversible (Herb et al. 2012). This means that if a forager reverses its transition and again becomes a nurse bee, for example due to a lack of nurse bees in the hive, the earlier epigenetic alteration will also change, and this can thus perhaps increase lifespan (Munch & Amdam 2010). In addition to the worker bees each hive also has a queen. She is the only fertile bee, and she is normally both bigger and live longer than the rest (Adam G. Dolezal 2013).

This study uses winter bees (*Apis mellifera diutinus*) as they live longer than the bees born in spring and early summer (Seeley 1995). Winter bees are, in addition to being born late summer – early autumn, developed when the amount of brood becomes scarce in the hive, and brood pheromones disappear (Munch et al. 2013).

Honey bee anatomy, like in all other insects, consists of three main parts: the head, the thorax, and the abdomen (Fig. 8) (Seeley 1995). The head holds the eyes, two antennas that are used for smelling, tasting and touching, along with the brain and the glands for producing food. The thorax holds the bee's "machinery", meaning the muscles that are used for controlling the abdomen and head, along with the wings and the legs. The abdomen contains the stinger and all of the bee's vital parts; organs such as the heart, intestines, stomach and honey sack. The abdomen also contains the fat body, a layer of fat cells outside of the organs, next to the exoskeleton. The fat body, which is normally enlarged in winter bees, is the tissue being used to check for global methylation effects after treatment, in this thesis. The fat body's functions are similar to that of the liver in mammals (Maleszka 2008).

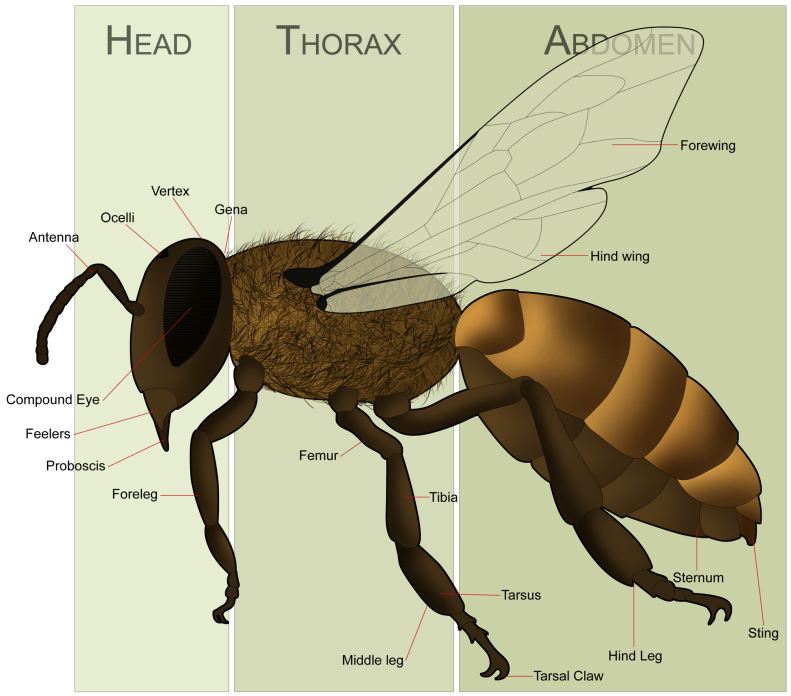


Figure 8: Honey bee anatomy showing head, thorax and abdomen (Wikipedia 2007).

Studies on insects are conducted in controlled environments and are thus easy to monitor, and compared to other animal models, such as mice, they are easier to cage due to their small size. The foods given to insects are precisely formulated, so one knows exactly what they ingest. In addition one does not encounter many ethical problems by studying insects, probably due to their way of life and short lifespan; being used to the confined space of a hive they do not seem to mind being caged.

When working with honey bees it is easy to create an environment similar to that of the hive in regards to temperature, humidity, and population density. Also, acquiring the equipment necessary for such experiments are relatively cheap, and the cages and bees does not occupy much space. The honey bees' short lifespan makes it easy to measure longevity, and the fact that they have an epigenetic machinery homologous to mammals, in contrast to other animal models such as *Drosophila melanogaster* and *Caenorhabditis elegans*, make honey bees suitable to address the aspects in this thesis (Maleszka 2008). In addition, the fact that changes in aging patterns can be linked to the bee worker task, makes the honey bee an excellent model organism to study how social behavior and environment influences aging (Münch & Amdam 2013).

1.3.1 – Epigenetic effects on lifespan and aging

Senescence is often described as "a gradually reduced ability to cope with physiological challenges" (Huidobro et al. 2013), a inner functional decline (Munch & Amdam 2010). In honey bees aging and longevity is connected to social task in the hive. The queen fed on royal jelly lives up to several years, while worker bees normally have a lifespan of weeks. Winter bees again can live up to ten months, and aging, along with the change in epigenetic traits associated with it, can be reversed if the bee changes its work task to a task related with earlier age; for example a transition from forager to nurse bee.

1.4 – Molecular analyses

Different molecular tests were used to check for epigenetic patterns in bees fed the three different substances and the control bees. These tests include western blot, DNA extraction and ELISA (enzyme-linked immunosorbent assay).

Western blot is a protocol used for separating and identifying proteins based on their size. It first uses gel electrophoresis to separate the proteins in a gel according to size. These proteins are then blotted onto a membrane, which becomes a replica of the gel, where they can be identified using specific antibodies.

DNA extraction is done prior to ELISA, which is an assay here used for determining percent of global methylation in a sample. The method uses the principle of specific antibody binding to antigens; known or unknown antigens from the sample are adhered to the wells in the plate. Then specific antibodies are added that will bind to the said antigen. This specific antibody is linked to an enzyme which later, when adding the enzyme's substrate, will produce a color signal that can be registered and measured using OD (optical density). The measured value can then be used to calculate percent of methylation when compared to a known standard.

1.5 – A brief outlook

With this thesis I aim to see changes in lifespan in the honey bee after treatment with the different substances. Next, I aim to see differences in epigenetic traits, such as global DNA methylation levels and histone acetylation, in the honey bee abdomen after treatment, and when comparing different concentrations of each substance.

The results in this thesis provide prospects for future studies both when it comes to the use of these substances in treatment of age related challenges in humans, such as cancer, and as well as for potential risks associated with therapeutic use of purified biochemical compounds.

2. Materials and methods

The experiment was conducted in 2013 and 2014 at the Norwegian University of Life Sciences (NMBU) in Ås, Norway. It was conducted in two steps starting with lifespan studies on honey bees followed by molecular tests of bees subjected to different treatments. The three bioactive compounds curcumin, folic acid (vitamin B9) and cyanocobalamin (vitamin B12) were tested on approximately 800 bees each (*Apis mellifera carnica* Pollmann). The bees sampled for these experiments were all winter bees (*Apis mellifera diutinus*). This is to have a homogenous group of bees with a similar behavioral phenotype and physiological traits. Collecting winter bees in September was accomplished by keeping the queen caged for a minimum of 4 weeks prior to sampling, so that she would not lay eggs, and the hive would be broodless, which is a key feature of long-lived winter bees (Munch et al. 2013). All bees were collected from hives containing between 6000 and 10.000 bees. To control for hive specific effects in the results, bees were sampled from two different hives each time (replicate: hive). Also, to control for any effects of the sampling date and weather on individuals, bees were collected on two different days for each sampling (replicate: round). Each cage was designed as viewed in the figure below (Fig. 9), and was used both to sample the bees in, and throughout the experiments for holding the bees. Each cage contained approximately 50 bees. Explained briefly, the cages consisted of simple plastic containers with two holes on the top for feeding and drinking tubes; one hole on the side for collecting dead bees; a mesh inside for the bees to stand on, so that they would not drown in any leakage from the tubes; foam along all four sides of the mesh, so that the bees would not clime underneath it, and to keep the mesh in place; and holes covered with mesh on the front and back for breathing, as well as easy observation of the bees.

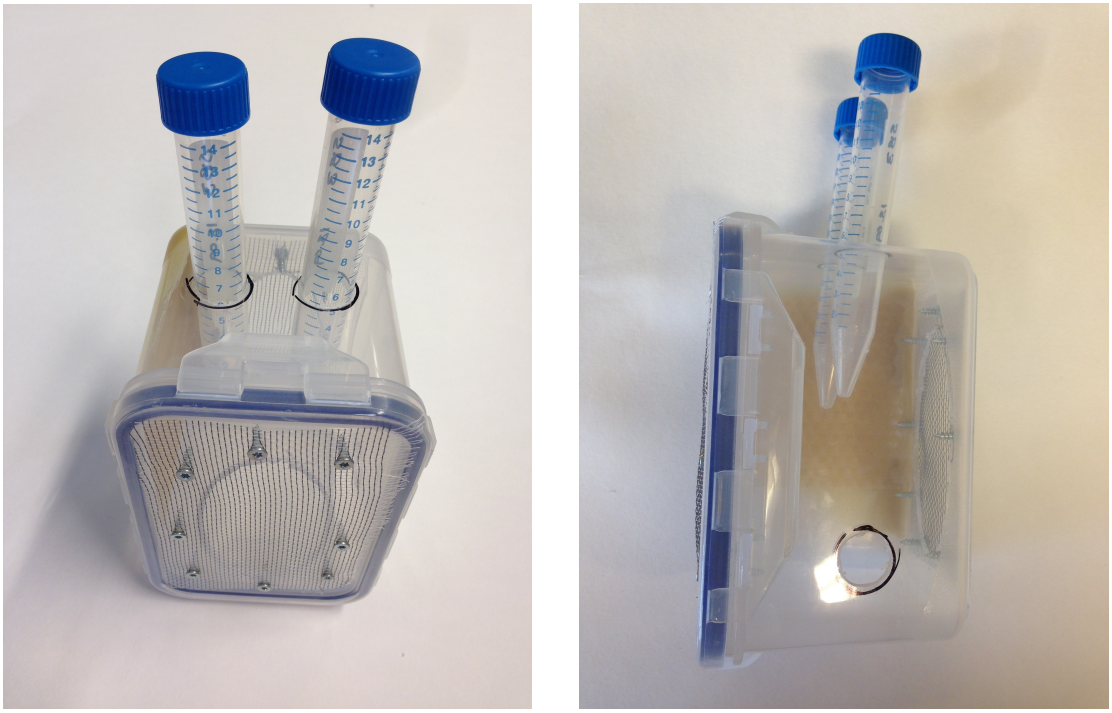


Figure 9: These pictures show the setup of the cages used throughout the lifespan experiments. The picture to the left shows the front of the cage with a mesh over an air-hole. On the top there are two holes: one for a feeding tube and one for a drinking tube. The picture to the right shows the cage from the side with a hole for collecting dead bees and sample bees. The cage has one side covered with bees wax.

The bees were checked at approximately the same time every day (between 10 am and noon); the amount of food consumed was registered, new food and water provided, and dead bees were taken out of the cage. Survival was continuously determined by registering the number of dead bees every day.

The different concentrations of treatment fed to the bees were calculated based on previous studies done *in vitro* or in other animal models; values were calculated on the basis of the honey bees body mass (200 mg; D. Münch pers. com.).

Maximum lifespan was defined as the point where 10 % of the bees had survived, and is described as long-term survival effects (on the graphs: cumulative proportion surviving of 0.1). Median survival was defined as the point where 50 % of the bees had survived (on the graphs: cumulative proportion surviving of 0.5).

2.1 – Curcumin

The phenol curcumin was tested on bees sampled on September 4th and September 6th 2013. All bees were placed in 16 cages in total. Out of those 16 cages 4 were control cages (C) and 12 were treated with curcumin at three different concentrations: 4 cages

were given 0.1 mg/mL (T1); 4 cages 0.01 mg/mL (T2); 4 cages 0.001 mg/mL (T3). See table 1 for general setup.

Table 1: The table shows the general setup of cages for the substances.

		Control cages	Treatment 1	Treatment 2	Treatment 3
Round 1	**Hive 1**	Control 1	Replicate 1	Replicate 1	Replicate 1
	Hive 2	Control 2	Replicate 2	Replicate 2	Replicate 2
Round 2	**Hive 1**	Control 3	Replicate 3	Replicate 3	Replicate 3
	Hive 2	Control 4	Replicate 4	Replicate 4	Replicate 4

The bees on treatment were fed with a mixture containing Bifor (50%), lipid mix (1 %), Grace's amino acid mix (2 %), dH_2O (46 %), and stock solution containing curcumin and ethanol (1 %). The stock solution consisted of 10 mg/mL curcumin in absolute ethanol. Ethanol was used as a solvent because curcumin could not be dissolved in dH_2O, but dissolved completely in ethanol. The food given to the control cages consisted of Bifor (50 %), lipid mix (1 %), Grace's amino acid mix (2 %), dH_2O (46 %), and ethanol (1 %). A concentration of 1 % ethanol was maintained for all treatments. The basic diet, excluding the substances and the ethanol, was made based on a recipe previously used by another master student working with honey bees (Rojahn 2013).

2.2 – Folic acid (vitamin B9)

The substance folic acid (vitamin B9) was tested on bees sampled on November 8th and November 11th 2013. All bees were placed in 16 cages in total. Out of those 16 cages 4 were control cages (C) and 12 were treated with folic acid at three different concentrations: 4 cages were given 500 µg/mL (T1); 4 cages 50 µg/mL (T2); 4 cages 5 µg/mL (T3). See table 1 for general setup.

The bees given folic acid were fed with a mixture containing Bifor (50 %), lipid mix (1 %), RPMI 1640 amino acid mix (2 %), dH_2O (47 %), and folic acid in powder form (0.5 – 0.05 – 0.005 mg/mL). Because the product Grace's amino acid mix was discontinued, a new amino acid mix was used with the folic acid- and the cyanocobalamin mediated diets. To ensure that the food mixes had similar

composition it was necessary to supplement this amino acid mix with L-Histidine and L-Alanine (see table 2).

Table 2: Amount of L-Histidine and L-Alanine in Grace's amino acid mix and RPMI 1640 amino acid mix.

Amino acid	Grace's amino acid mix	RPMI 1640 amino acid mix
L-Histidine	2.25 g/L	0
L-Alanine	25 g/L	0.75 g/L

2.3 – Cyanocobalamin (vitamin B12)

The substance cyanocobalamin (vitamin B12) was tested on bees sampled on November 8th and November 11th 2013. All bees were placed in 16 cages in total. Out of those 16 cages 4 were control cages (C) and 12 were treated with cyanocobalamin at three different concentrations: 4 cages were given 2000 ng/mL (T1); 4 cages 200 ng/mL (T2); 4 cages 20 ng/mL (T3). See table 1 for general setup.

The bees given cyanocobalamin were fed with a mixture containing Bifor (50 %), lipid mix (1 %), RPMI 1640 amino acid mix (2 %), dH_2O (47 %), and a stock solution consisting of 1 mg/mL cyanocobalamin in dH_2O. The stock solution replaced some of the dH_2O in the food mix; the appropriate amount of dH_2O that was to be removed was calculated on the basis of how much food that was to be made.

The bees that were still alive when terminating the lifespan studies with folic acid and cyanocobalamin were snap frozen using liquid N_2 and registered as "censored".

2.4 – Bees for molecular analyses

The bees for molecular testing were given the three substances at two different concentrations, including controls (see table 3 for general setup). The two concentrations for each substance were selected on the basis of the earlier statistics. As a general rule, the concentrations for which statistical significant effects were observed were the once being tested also on the bees for molecular analyses. If there were no significant effects found, the highest concentration would be used. Also, concentrations that had >50 % mortality within 10 days was not selected to ensure that enough bees could be retrieved after sampling. The bees for molecular testing were sampled on January 15, 2014. In total 18 cages were filled with 50 bees each,

giving 900 bees in total. The bees were sampled from two different hives to control for hive specific effects.

Table 3: The general setup of cages used for bees for molecular testing. Each concentration and control tested consisted of 2 cages.

Substance tested	First concentration tested	Second concentration tested	Controls	
Curcumin	T1	T2	With ethanol, with Grace's amino acid mix	Without ethanol, with Grace's amino acid mix
Folic acid (B9)	T1	T3	With amino acid mix (RPMI 1640)	
Cyanocobalamin (B12)	T2	T3	With amino acid mix (RPMI 1640)	

The experiment to generate bees for molecular testing was performed until January 27, 2014. At that day the bees were snap frozen with liquid N_2 and stored in tubes at -80 degrees for later analysis.

2.5 – Molecular analyses

To address the possible epigenetic mechanisms molecular analyses were used to test for nutritional effects on global DNA methylation changes and histone acetylation. Different protocols were used, including DNA extraction, ELISA and western blot. All protocols were tested with random bees before testing on own bees to ensure that the protocols worked properly.

For molecular testing four bees were randomly chosen from each treatment, two from each cage, generating a biological duplicate per bee per cage. All bees, giving 32 in total, were dissected and the fat body was collected for DNA extraction, and later ELISA.

2.5.1 – DNA extraction

DNA extraction was done with phenol:chloroform:isoamylalcohol, a liquid-liquid extraction where proteins are separated from nucleic acids; the proteins will move to the organic phase while nucleic acids remain in the aqueous phase. Honey bee

abdomens were homogenized in 200 µL ATL buffer, and 20 µL proteinase K was added to each sample to degrade proteins and remove any contamination from the nucleic acid samples. Samples were then incubated at 56 ^{o}C over night (ON) (max 16 hours) with shaking at 400 rpm. After incubation, samples were chilled at room temperature, dH_2O was added to a final volume of 550 µL, and an equal volume of PCI (25:24:1) was added. The samples were vortexed 5 seconds 2-3 times, then centrifuged at 15 000 x g for 5 minutes at room temperature (RT). The aqueous phase containing the DNA was transferred to a clean Axygen tube. 15 µL of RNase A (20 mg/mL) was added for the isolation of RNA-free DNA, and the samples were incubated at 37 ^{o}C with 550 rpm shaking for 30 minutes, and then chilled in RT. An equal volume of PCI was added, the samples were vortexed and centrifuged as above, and the aqueous phase was transferred to a clean tube. An equal volume of chloroform:isoamylalcohol (24:1) (CI) was added to further separate proteins and polysaccharides from the nucleic acids. The samples were vortexed and centrifuged as above. The aqueous phase was collected (max 400 µL). The DNA was then precipitated with 1/10 volume of 3M sodium acetate (NaAc) (40 µL), 5 µL linear acrylamide, and 2.5 x ice-cold (-20 ^{o}C) absolute ethanol (1 mL) at -80 ^{o}C ON. NaAc was added to increase the number of ions so that the ethanol can precipitate the DNA; linear acrylamide is a neutral carrier of nucleic acids and was added to visualize the pellet; and ethanol was added to remove any salts. The samples were vortexed before incubation. Samples were thawed on ice, then centrifuged at 20 000 x g at 4 ^{o}C for 15 minutes to pellet the DNA. The pellets were then washed two times with 1 mL 70 % ethanol, each time centrifuged at 20 000 x g at 4 ^{o}C for ≥8 minutes. Slow breaking settings were used (break settings at 4). As much as possible of the ethanol was removed and the pellets were left to dry with the tube-cap open for about 10-15 minutes in RT. This was to prevent the ethanol from hindering subsequent applications. The DNA was dissolved in 40 µL dH_2O, the yield was checked using Qubit flourometer, and total nucleic quantities were checked using a nanodrop spectrophotometer. Samples were then stored at -20 ^{o}C.

Samples of DNA extractions used for the ELISA protocol were selected based on their values; minimum yield was set to 4 ng/µL.

2.5.2 – ELISA

ELISA was used to identify and quantify DNA methylation, using the 5-mC DNA ELISA Kit from Zymo Research (see appendix 1), catalog numbers: D5325 & D5326, version 1.2.0. Lot number: ZRC175732.

DNA-samples, including the controls, were added into PCR tubes together with the buffer. The samples were denatured at 98 ^{o}C for to get single stranded DNA, and then put on ice immediately to avoid annealing of the strands. The DNA samples were then added to the wells of the plate, and underwent a series of incubation- and washing steps with the antibody mix and buffer respectively. HRP developer was added to each well and left at room temperature for 45 minutes for the color to develop. Absorbance was measured at 405 nm using an ELISA plate reader. Percent of 5-mC was calculated by using the second-order regression equation from the standard curve, generated on basis of the negative and positive controls consisting of *Escherichia coli* gDNA.

2.5.3 – Western blot

The western blot histone protocol was conducted in several steps. Sample preparation was done by dissecting honey bee brains or abdomens. The brains were put in 25 µL homogenizing buffer, while abdomens were put in 100 µL PBS with a protease inhibitors cocktail, both in 1.5 mL Axygen tubes. The proteinase inhibitor cocktail was used to protect the integrity of the proteins during extraction. The samples were then homogenized in homogenizing buffer (complete protease inhibitor cocktail homogenized mixture) using pistils. Abdomens were centrifuged at 10 000 x g at 4 ^{o}C for 20 minutes to get rid of exoskeletons, and the supernatant was taken up. Brain samples were not centrifuged.

SDS-PAGE was then conducted to separate the proteins in the samples. Pre-prepared gels from BioRad were used. Samples were diluted 1:1 with 2x Laemmli buffer. These were next boiled for 5 minutes at 95 ^{o}C, and then vortexed and spun. Molecular standard (5 µL) and samples (12 µL) were loaded, and the gels were run at 75 V for 5 minutes, then at 100 V for another 85 minutes in 1x TGS buffer at ambient temperature until the marker had left the gel. Some gels were also run at 100 V for 75 minutes instead of 85 minutes to check whether the run time could be of some effect on the results. A gel for coomassie staining was run at the same time with the same

samples. This to visualize the proteins separated by SDS-PAGE as the coomassie dye binds the proteins unspecifically. After the electrophoresis the gel was submerged in a staining solution consisting of 0.2 % (w/v) Coomassie Brilliant Blue R-250, 50 % (v/v) methanol, and 7.5 % (v/v) acetic acid, and heated to boiling point in a microwave. This was done to increase the staining rate. Then, the gel in the solution was incubated for 30 minutes with moderate shaking. After incubation, the gel was washed once with dH_2O to remove unbound dye, and then submerged in a destaining solution consisting of 5 % (v/v) methanol and 7 % (v/v) acetic acid. This was then heated to the boiling point in a microwave to increase the destaining rate, and then incubated for 2 hours with moderate shaking. A small piece of paper tissue was put into the solution to further increase the destaining rate, and this was changed once every hour.

Western blot was conducted by removing the gel. A PVDF membrane sheet was cut similar in size to the gel and presoaked in methanol for 3 minutes (until translucent), and then in blotting buffer for 5 minutes (until sinking). Gel and blot was assembled. The power supply was set to 100 V constant for two hours. The frozen cooler was changed after one hour, as it was not frozen any more. After the two hours the blotting was terminated.

The immunodetection of the blot was done by incubating the membrane in blocking/dilution buffer (bovine serum albumin (BSA)) for 2.5 hours with gentle shaking. The 1^o antibody (polyclonal antibody from rabbit) was diluted 1:250 in blocking buffer and incubated ON at 4 ^oC with gentle shaking. The membrane was then rinsed two times with 1x PBS-T, washed once with 1x PBS-T for 30 minutes, and then twice for 15 minutes each, all at RT. The 2^o antibody (goat anti-rabbit Cy5), labeled with the fluorescent molecule Cy5 for detection, was diluted 1:250 in blocking/dilution buffer (BSA), and the membrane was incubated in the solution. The box was wrapped in a foil to keep the light out. The incubation was 1 hour, 18-25 ^oC, with gentle shaking. The membrane was then washed again just like after the incubation with the 1^o antibody.

The membrane was scanned with the following settings: filter: Cy5 (670 nm); 400 V; Normal. This was based on the secondary antibody.

After scanning the membrane was stained with SYPRO Ruby to stain all proteins. First the membrane was left in dH_2O, next the membrane was incubated in 15 mL 7 % acetic acid and 10 % methanol (rest dH_2O) for 15 minutes. Then, floated for five minutes each in four changes of dH_2O. Next, the membrane was floated in SYPRO Ruby stain reagent for 15 minutes. Last, the membrane was washed two times for one minute in dH_2O before scanned again. Scanning settings were set to: Rox filter; 532 nm; 610 BP; 400 V; normal.

Western blot was run several times to prepare for the analysis of the molecular bees, and was supposed to be used for identification of the 14,4 kilo Dalton (kDa) protein H3K23ac (acetylated lysine 23, histone 3) (Diagenode 2010), but due to weak results and a lack of time the protocol had to be dropped.

2.6 – Statistical analyses

Statistical analyses were performed using the program Statistica (StatSoft 2014). For analyzing the lifespan data, primary data containing information on survival, hive, round, cause of death, start date, type of bee, treatment, substance tested, and cage during experiment were assembled using Excel and then processed using Statistica. The Kaplan-Meier test was used to obtain statistical data on the bees; it provided data on the overall statistics, and graphs. Post-hoc analyses, two-sample tests, were conducted using the Cox's F-test.

For analyzing the consumption data, datasheets were prepared containing average consumption per bee per cage per day for the 10 first days of the lifespan studies. The datasheet also contained an average and the median for all 10 days. Using the statistical program the average of all four replicates for each treatment was calculated and plotted in a diagram with the standard deviation.

For analyzing the data from the ELISA protocol datasheets were prepared containing information on cage number, treatment, percent of global methylation and replicate number. The data was plotted into Statistica, and average percentage of methylation (% 5-mC) of all four replicates was calculated for each treatment with the standard deviation. The results were then plotted into graphs. Statistical tests were conducted to look for significant effects in methylation between treatments.

2.7 – Reagents

For a complete list of reagents used throughout these experiments see appendix 2.

3. Results

The first three sections describe the effects of curcumin, folic acid, and cyanocobalamin on survival. The next sections describe the consumption data and the results from the molecular tests. All substances in this thesis were tested at three different concentrations and with one control group, except from the bees for the molecular tests, which were tested at two concentrations and with one control group (see chapter 2.4).

3.1 – Curcumin

The graph below show survival curves from the lifespan studies with the curcumin-mediated diet for all three concentrations of curcumin and for the control (Fig. 10). The effect of the highest concentration (T1: 0.1 mg/mL) can be seen as the red curve. The graph shows longest survival for treatment T1, but for all treatments I observed a higher survival than for the control when considering median survival. When looking at maximum survival, the graph clearly shows a difference between T1 and the rest as it can be read that 90 % of the bees from T1 were dead by day 22, whilst for the rest 90 % mortality hit in at approximately day 15 to 18.

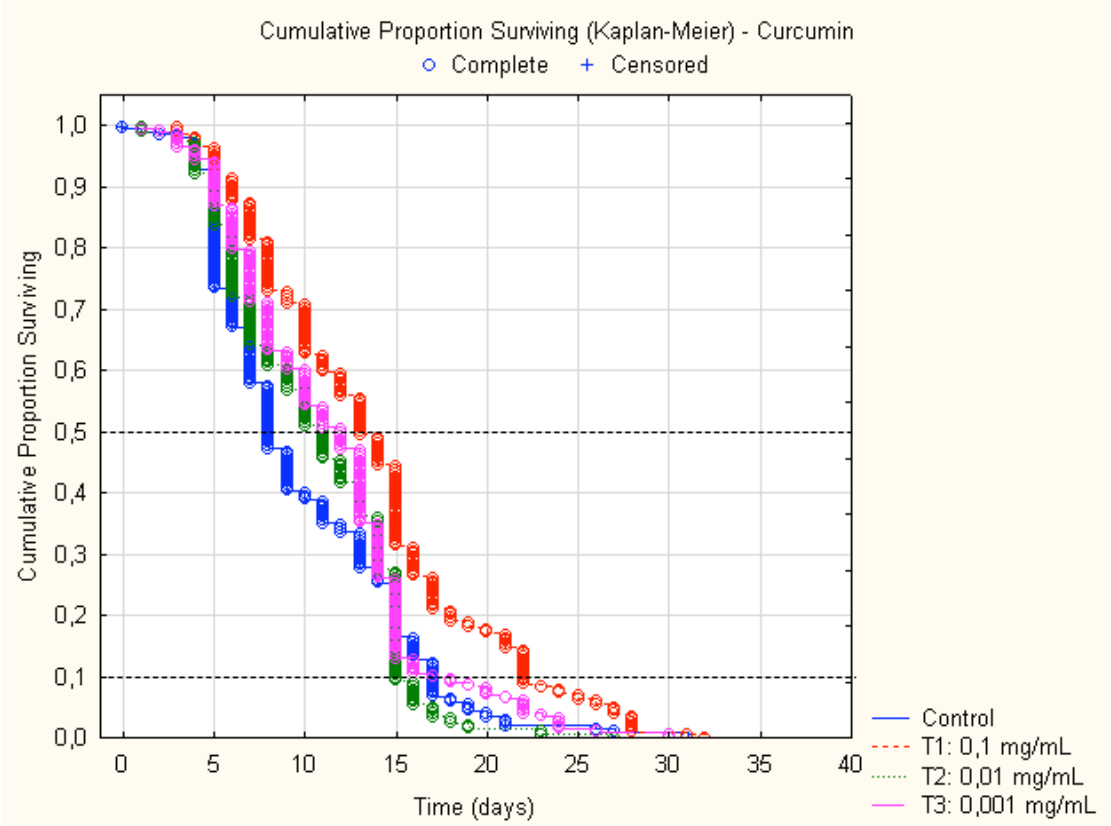


Figure 10: The graph shows the overall lifespan in days for all bees in the experiment given a curcumin-mediated diet. Dashed lines at cumulative proportion survival of 0.5 and 0.1 indicate median survival and maximum survival respectively.

The results from the statistical analyses support the observations from the graph, and showed an overall significant effect of treatment. This was confirmed with Kaplan-Meier statistics ($N_{C/T1/T2/T3}$ 208/204/204/201; $p < 0.001$; $Chi^2 = 41.9776$). The pairwise comparison using the Cox's F post-hoc test revealed significant differences for T1 as compared to the control ($p < 0.001$; $F = 1.5762$).

Statistical analysis with the Cox's F-test also revealed a hive effect ($N_{H1/H2}$ 408/409; $p < 0.001$; $F = 1.3289$), which led to separate analysis of the data from the two hives. This comparison was done because differences between replicates might have masked minor differences in treatment effects. This approach applies to all three substances. Conducting statistical tests on the two hives separately showed that the two highest concentrations of curcumin (T1 and T2) had a significant effect on lifespan. Hive 1 also showed a significant effect for the lowest concentration (T3), but hive 2 did not (table 4). Overall statistical results from the two hives separately showed that there was a significant effect on lifespan in both (hive 1: $p < 0.001$; $Chi^2 = 24.8071$)(hive 2: $p < 0.001$; $Chi^2 = 41.9724$).

Table 4: N, p- and F-values from two-sample tests between control and treatments for curcumin, done on the two hives separately, and overall N, p- and Chi^2-values from Kaplan-Meier tests for each hive.

Hive	Overall (Kaplan-Meier)	Control vs. T1 (Cox's F-test)	Control vs. T2 (Cox's F-test)	Control vs. T3 (Cox's F-test)
1	N = 408; p <0.001; Chi^2 = 24.8071	$N_{C/T1}$ = 106/104; p <0.001; F = 1.5765	$N_{C/T2}$ = 106/102; p <0.05; F = 1.2887	$N_{C/T3}$ = 106/96; p <0.01; F = 1.4965
2	N = 409; p <0.001; Chi^2 = 41.9724	$N_{C/T1}$ = 102/100; p <0.001; F = 1.5955	$N_{C/T2}$ = 102/102; p <0.05; F = 1.3768	$N_{C/T3}$ = 102/105; p = 0.1794; F = 1.1365

Taken together I found that curcumin affects lifespan, with a strong lifespan extension for the highest concentration (T1: 0.1 mg/mL).

3.2 – Folic Acid (vitamin B9)

The effect of the folic acid-mediated diet can be seen on the overall graph (Fig. 11). The graph shows a decrease in survival for treatment T3 (5 µg/mL). This is evident when looking at maximum survival where 90 % of the bees from T3 were dead at day 27 whilst 90 % of the control bees were dead at day 35. When looking at the median survival the differences between the treatments are less clear.

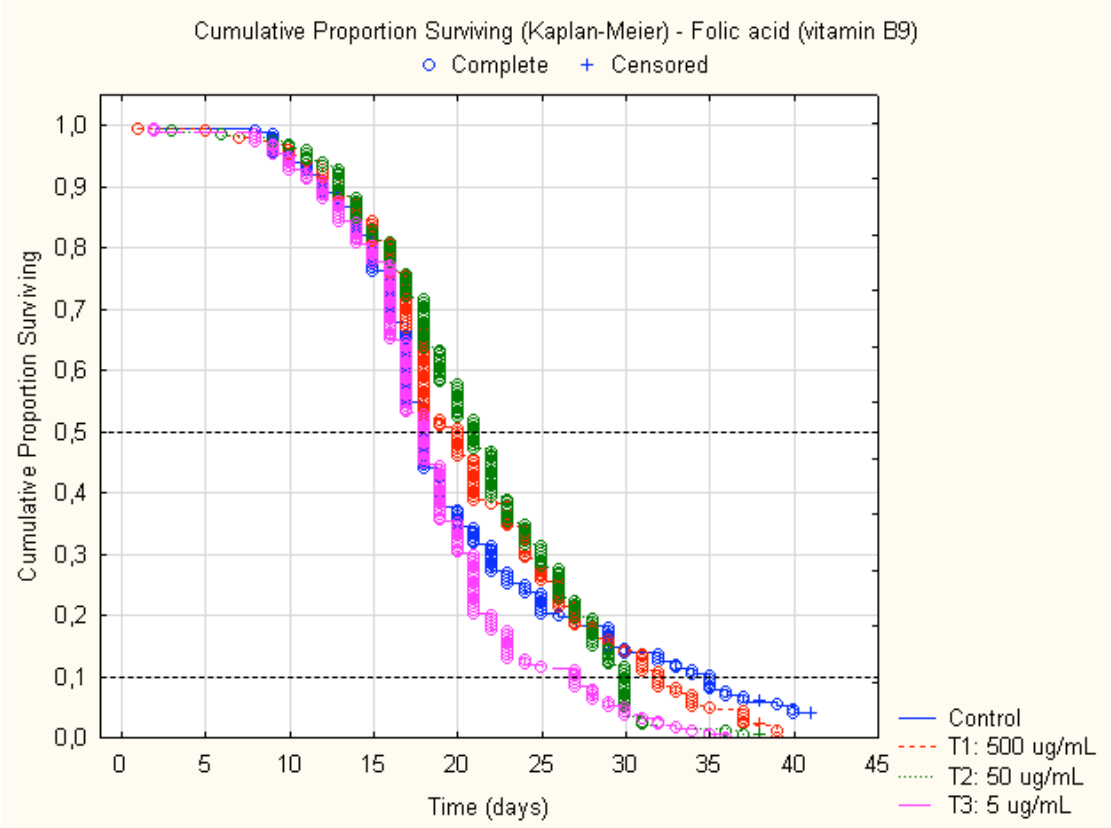


Figure 11: Overall lifespan in days for all bees in the experiment given a folic acid mediated diet. Dashed lines at cumulative proportion survival of 0.5 and 0.1 indicate median survival and maximum survival respectively.

The results from the statistical analyses support the observations from the graph, and revealed an overall significant effect of the folic acid treatment. This was confirmed with Kaplan-Meier statistics ($N_{C/T1/T2/T3}$ 210/205/202/203; $p < 0.001$; $Chi^2 = 21.80079$). The Cox's F post-hoc test conducting the pairwise comparison between the control and the different treatments revealed significant lifespan shortening for T3 ($p < 0.01$; $F = 1.3591$).

Statistical analysis with the Cox's F-test also revealed a hive effect ($N_{H1/H2}$ 413/407; $p < 0.001$; $F = 2.2972$) and a round effect ($N_{R1/R2}$ 409/411; $p < 0.01$; $F = 1.1875$), which led to separate analysis of the data from the two hives and rounds. Overall statistical results from the two hives separately showed that there was a significant effect on lifespan in both (hive 1: $p < 0.001$; $Chi^2 = 17.3848$)(hive 2: $p < 0.001$; $Chi^2 = 31.5800$). However, statistical results on the two rounds separately showed that significant effects on lifespan were only found in round 2 ($p < 0.001$; $Chi^2 = 24.3623$). Pairwise comparison tests from both hives revealed that the two lowest concentrations (T2 and T3) had significant lifespan shortening effects. Hive 2 also showed a significant effect for the highest concentration (T1), but hive 1 did not. Pairwise comparison tests from

the two rounds showed that only the lowest concentration (T3) shortened lifespan in a significant manner in both rounds, and that the middle concentration (T2) had significant effect in round 1 (table 5).

Table 5: N, p- and F-values from two-sample tests between control and treatments for folic acid, done on the two hives and rounds separately, and overall N, p- and Chi^2-values from Kaplan-Meier tests for each hive and round.

	Overall (Kaplan-Meier)	**Control vs. T1 (Cox's F-test)**	**Control vs. T2 (Cox's F-test)**	**Control vs. T3 (Cox's F-test)**
Hive 1	N = 413; p <0.001; Chi^2 = 17.3848	$N_{C/T1}$ = 107/103; p = 0.10884; F = 1.1944	$N_{C/T2}$ = 107/104; p <0.01; F = 1.4613	$N_{C/T3}$ = 107/99; p <0.001; F = 1.9057
Hive 2	N = 407; p <0.001; Chi^2 = 31.5800	$N_{C/T1}$ = 105/99; p <0.001; F = 1.7864	$N_{C/T2}$ = 105/102; p <0.001; F = 2.3968	$N_{C/T3}$ = 105/101; p <0.05; F = 1.3931
Round 1	N = 409; p = 0.3630; Chi^2 = 3.1917	$N_{C/T1}$ = 104/103; p = 0.1527; F = 1.1569	$N_{C/T2}$ = 104/101; p = 0.0504; F = 1.2639	$N_{C/T3}$ = 104/101; p <0.01; F = 1.5302
Round 2	N = 411; p <0.001; Chi^2 = 24.3623	$N_{C/T1}$ = 106/102; p = 0.08515; F = 1.2241	$N_{C/T2}$ = 106/101; p = 0.0904; F = 1.2158	$N_{C/T3}$ = 106/102; p <0.05; F = 1.2767

In all, I found that folic acid caused lifespan shortening for the lowest concentration (T3: 5 µg/mL), which suggests a harmful effect.

3.3 – Cyanocobalamin (vitamin B12)

The effect of the cyanocobalamin-mediated diet can be seen on the overall graph (Fig. 12). The graph shows a decrease in survival for treatment T2 (200 ng/mL) and T3 (20 ng/mL). This is evident when looking at maximum survival where 90 % of the bees from T2 and T3 were dead at day 27-30 whilst 90 % of the control bees and bees from T1 were dead at day 32-34. When looking at median survival the differences between the curves are less clear, but the same tendency can still be observed.

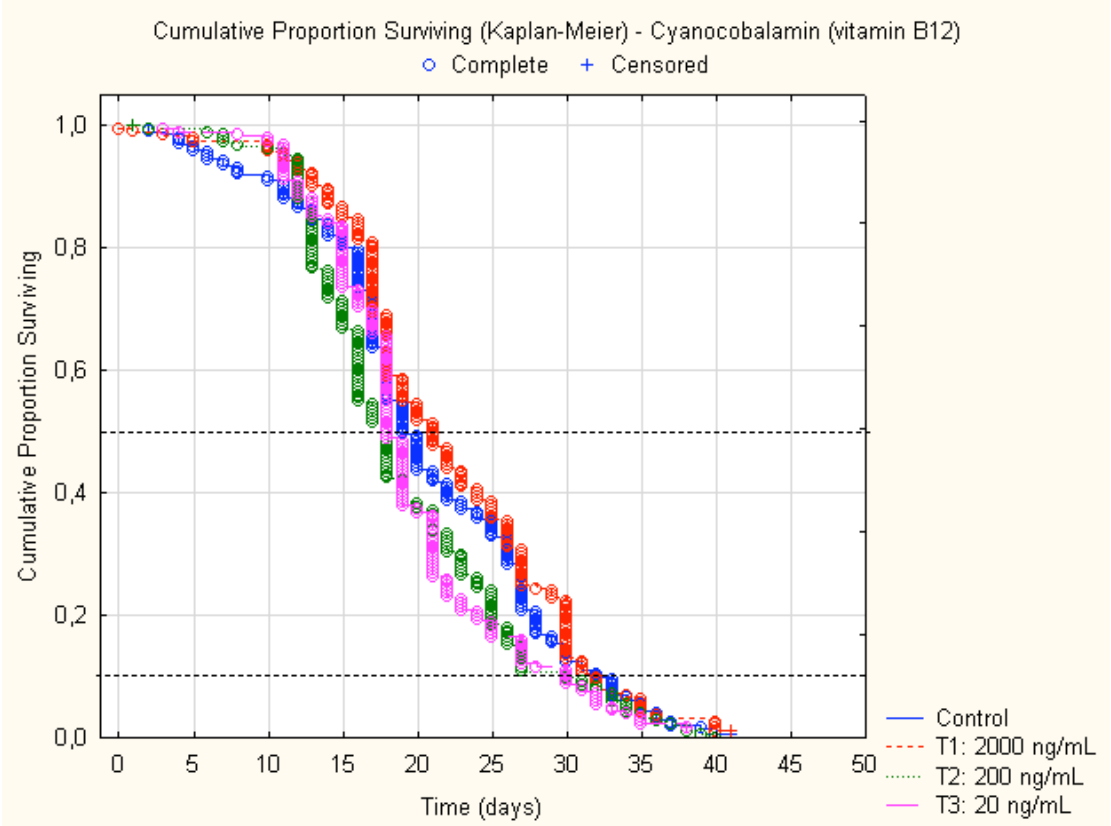


Figure 12: Overall lifespan in days for all bees in the experiment given a cyanocobalamin mediated diet. Dashed lines at cumulative proportion survival of 0.5 and 0.1 indicate median survival and maximum survival respectively.

The results from the statistical analyses support the observations from the graph, and revealed an overall significant effect of the cyanocobalamin-mediated diet. This was confirmed with Kaplan-Meier statistics ($N_{C/T1/T2/T3}$ 204/209/193/214; $p < 0.001$; $Chi^2 = 20.85720$). The Cox's F post-hoc test conducting the pairwise comparison between the control and the different treatments revealed significant lifespan shortening for both T2 and T3 (T2: $p < 0.05$; $F = 1.2395$)(T3: $p < 0.05$; $F = 1.2104$).

Statistical analysis with the Cox's F-test also revealed a hive effect ($N_{H1/H2}$ 411/409; $p < 0.001$; $F = 2.3077$) and a round effect ($N_{R1/R2}$ 407/413; $p < 0.001$; $F = 1.3183$), which led to separate analyses of the data from the two hives and rounds. Overall statistical results on the two hives separately showed that there was a significant effect on lifespan only in hive 1 ($p < 0.001$; $Chi^2 = 22.3220$). However, statistical results from the two rounds separately showed that there was a significant effect on lifespan in both (round 1: $p < 0.05$; $Chi^2 = 8.3500$)(round 2: $p < 0.001$; $Chi^2 = 36.7384$).

Pairwise comparison tests from both hives separately revealed that in hive 1 only T2 significantly shortened lifespan, whilst in hive 2 only T3 showed this effect. Pairwise

comparison tests from both rounds, on the other hand, showed that in round 1 only T3 reduced lifespan in a significant manner, whilst in round 2 both T1 and T2 significantly did (table 6).

Table 6: N, p- and F-values from two-sample tests between control and treatments for cyanocobalamin, done on the two hives and rounds separately, and overall N, p- and Chi^2-values from Kaplan-Meier tests for each hive and round.

	Overall (Kaplan-Meier)	**Control vs. T1 (Cox's F-test)**	**Control vs. T2 (Cox's F-test)**	**Control vs. T3 (Cox's F-test)**
Hive 1	N = 411; p <0.001; Chi^2 = 22.3220	$N_{C/T1}$ = 107/102; p = 0.0821; F = 1.2182	$N_{C/T2}$ = 107/101; p <0.05; F = 1.3443	$N_{C/T3}$ = 107/101; p = 0.1310; F = 1.1779
Hive 2	N = 409; p = 0.0837; Chi^2 = 6.6558	$N_{C/T1}$ = 100/105; p = 0.0748; F = 1.2322	$N_{C/T2}$ = 100/92; p = 0.0945; F = 1.2158	$N_{C/T3}$ = 100/112; p <0.01; F = 1.4731
Round 1	N = 407; p <0.05; Chi^2 = 8.3500	$N_{C/T1}$ = 102/104; p = 0.3954; F = 1.0383	$N_{C/T2}$ = 102/90; p = 0.4960; F = 1.0019	$N_{C/T3}$ = 102/111; p <0.001; F = 1.5522
Round 2	N = 413; p <0.001; Chi^2 = 36.7384	$N_{C/T1}$ = 102/105; p <0.05; F = 1.3866	$N_{C/T2}$ = 102/103; p <0.001; F = 2.0249	$N_{C/T3}$ = 102/103; p = 0.2086; F = 1.1292

Taken together I found that cyanocobalamin caused lifespan shortening for the middle (T2: 200 ng/mL) and the lowest (T3: 20 ng/mL) concentrations, suggesting a harmful effect.

3.4 – Consumption data

To check whether there were any differences in consumption between the different treatments, consumption data was calculated, and analyzed using Statistica. Consumption was calculated as average consumption per bee per cage per 24 hours, from the ten first days of the experiment. This was to exclude any effects from sick and dying bees on the last days of the lifespan studies. The average was then calculated on the basis of the ten days, giving one average per cage per day. These calculations showed that bees fed the curcumin-mediated diet consumed on average 40 µL each per day, whilst bees fed the folic acid and cyanocobalamin-mediated diets consumed on average 50 µL each per day.

Statistical analyses were conducted using one-way ANOVA, and the results showed that there are no significant differences in consumption between the different treatments. This was true for all three substances (table 7).

Table 7: Results from the one-way ANOVA analyses of the consumption data. Values were calculated on average consumption grouped by treatment.

Substance	N	p-value	F-value
Curcumin	$N_{C/T1/T2/T3}$: 4/4/4/4	0.4623	0.9163
Folic acid	$N_{C/T1/T2/T3}$: 4/4/4/4	0.5637	0.7114
Cyanocobalamin	$N_{C/T1/T2/T3}$: 4/4/4/4	0.9244	0.1551

The graphs below (Fig. 13, 14, 15) show average consumption, with standard deviation, grouped by treatment for all three substances.

From the figures one can observe only small differences in consumption between the different treatments, and as seen on the graphs the standard deviations are also large for all treatments in all graphs, except for treatment 0.1 mg/mL in figure 13.

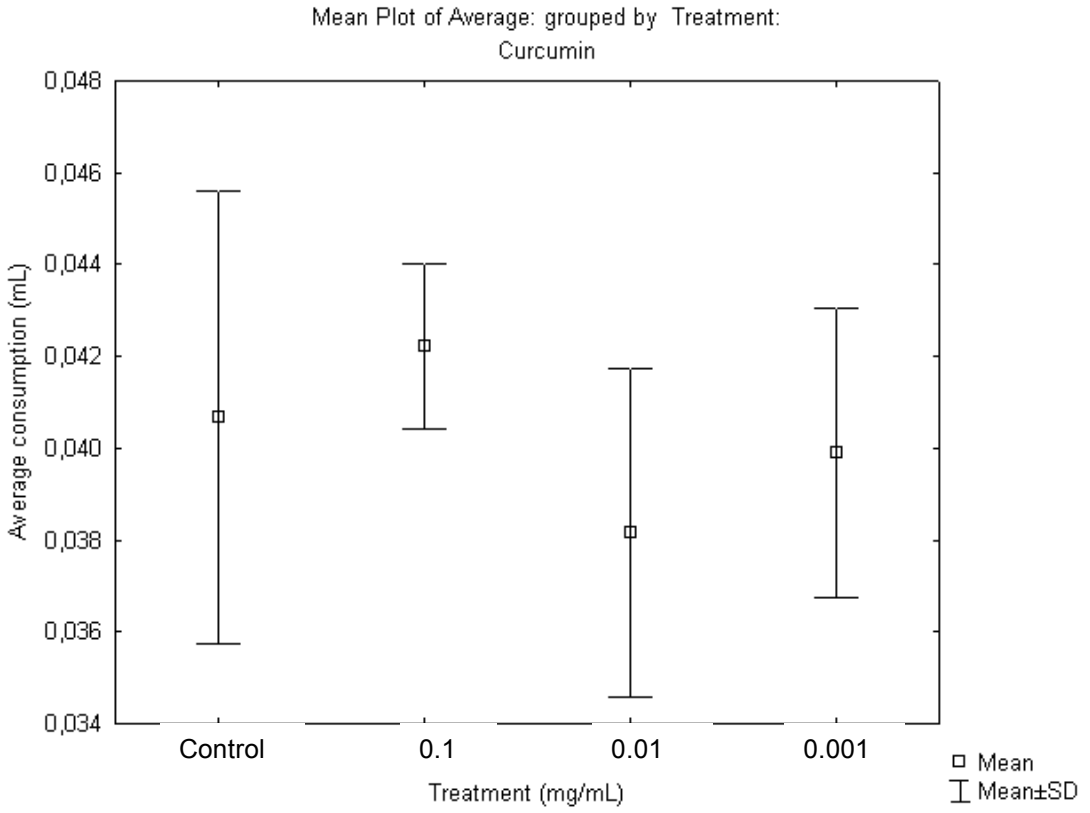


Figure 13: The graph shows average consumption, with standard deviation, grouped by treatment for curcumin.

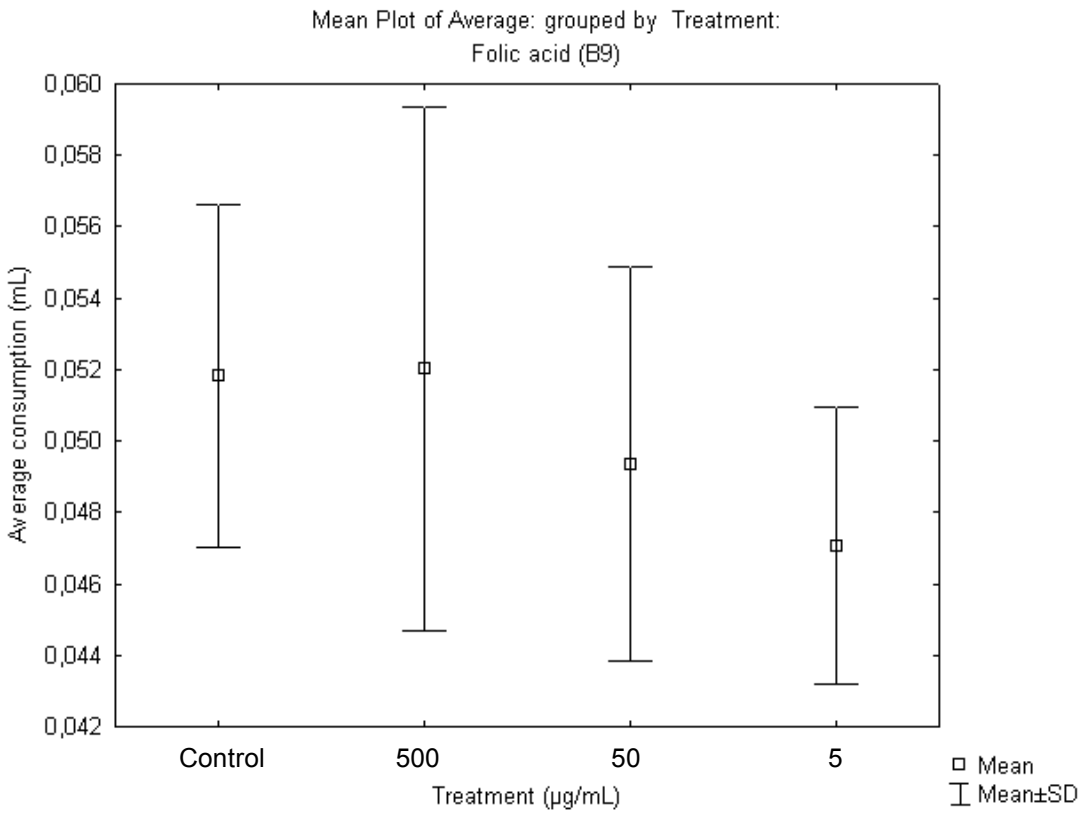


Figure 14: The graph shows average consumption, with standard deviation, grouped by treatment for folic acid.

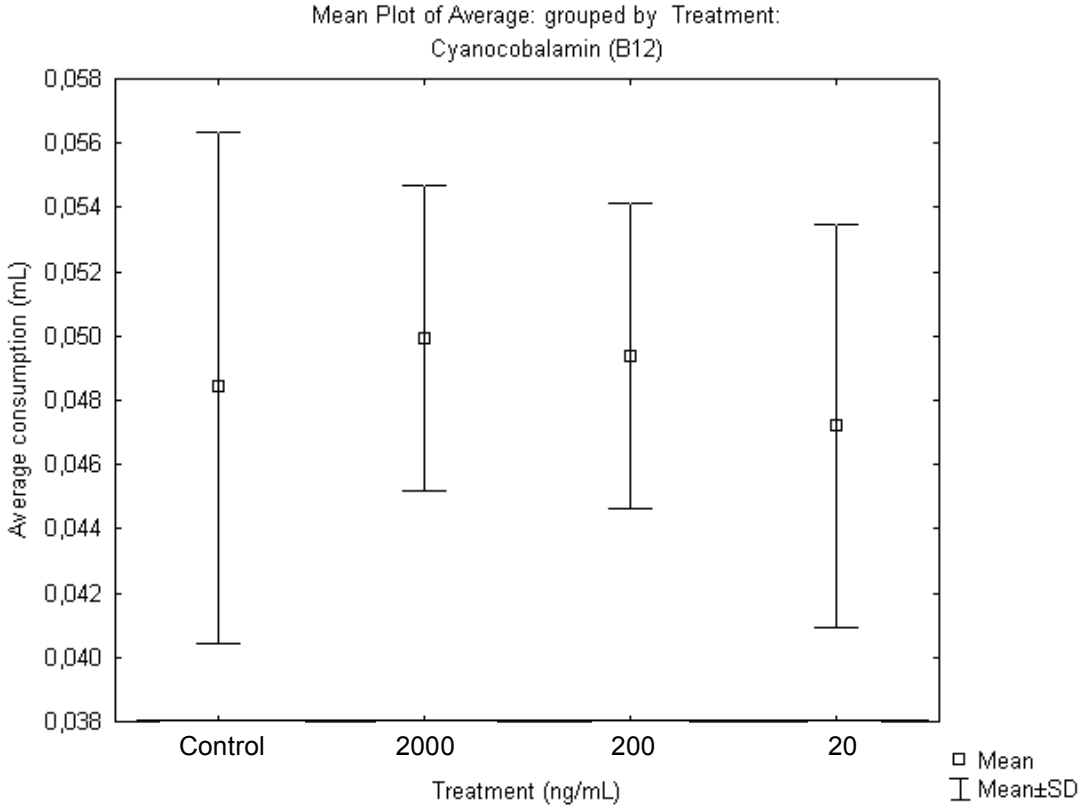


Figure 15: The graph shows average consumption, with standard deviation, grouped by treatment for cyanocobalamin.

3.4 – Molecular tests

The following sections describe the results from the DNA extractions, ELISA, and western blot.

3.4.1 – DNA extraction

The extractions went well, and gained samples from two bees from each cage creating one biological replicate per sample, and giving 32 samples in total.

3.4.2 – ELISA

ELISA was performed to test for global DNA methylation changes in the honey bee fat body in all treatments. This is due to the fact that we found a higher percentage of total methylation (% 5-mC) in the fat body than in the brain during test runs of ELISA (data not shown).

The ELISA plates were prepared according to the protocol, and each well was filled with 100 ng DNA, in addition to seven standards and one well with dH_2O. Each sample of isolated DNA was filled into two wells, generating one technical duplicate per sample. The samples and duplicates were applied in a pseudo-randomized order onto the plate to prevent any errors in the scanner from affecting all samples from one treatment. After scanning an average was made from the sample and the duplicate, and percentage of methylation was calculated using a standard curve.

The results provide information on total methylation (% 5-mC) per 100 ng DNA. Statistical analyses were conducted using one-way ANOVA on the results from the ELISA scan, and showed that there are no statistical significant differences between any of the treatments. This goes for all three substances (table 8).

Table 8: Results from the one-way ANOVA analyses of the results on global DNA methylation from the ELISA scan. Values were calculated on % 5-mC versus treatment.

Substance	N	p-value	F-values
Curcumin	$N_{C/T1/T2}$: 4/4/4	0.1786	2.0992
Folic acid	$N_{C/T1/T3}$: 4/4/4	0.1778	2.1052
Cyanocobalamin	$N_{C/T2/T3}$: 4/4/4	0.1033	2.9520

The results can also bee seen in the graphs below (Fig. 16, 17, 18). Figure 16 shows the average of global DNA methylation in percentage per 100 ng DNA, for bees fed a

curcumin-mediated diet, grouped by treatment. Stressing that there are no statistical significant results I would like to point out that a reduction in global methylation in T1 as compared to the control can be observed. Figure 17 shows the average of global DNA methylation in percentage per 100 ng DNA, for bees fed a folic acid-mediated diet, grouped by treatment. While underlining that there are no statistical significant results I would like to highlight that an increase in global methylation in both T1 and T3 as compared to the control can be observed. Figure 18 shows the average of global DNA methylation in percentage per 100 ng DNA, for bees fed a cyanocobalamin-mediated diet, grouped by treatment. Still while stressing that there are no statistical significant results on differences in global DNA methylation levels between treatments, I observed a trend in increased methylation between treatments and control. This suspicion was further increased with statistical significant results from the Fisher LSD post-hoc test between T2 and Control ($N_{C/T2}$ 4,4; $p < 0.05$).

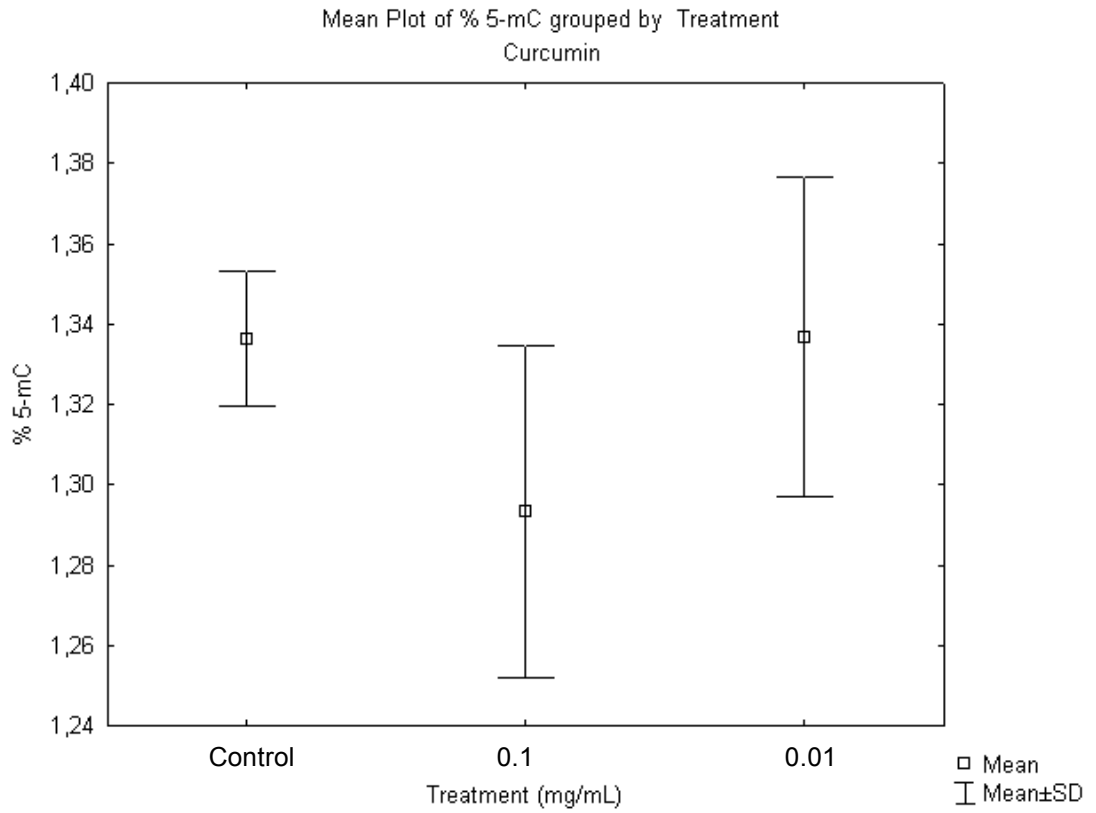


Figure 16: The average of global DNA methylation (% 5-mC) with standard deviations, per bee tested, for bees fed curcumin, grouped by treatment.

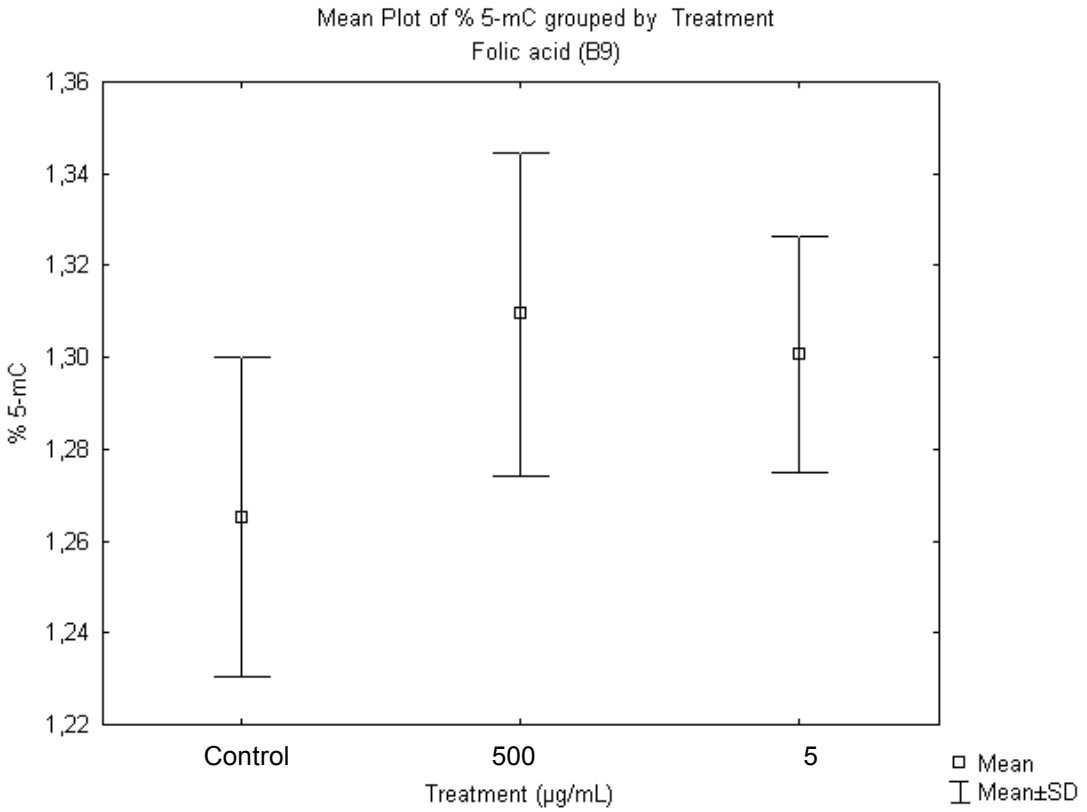


Figure 17: The average of global DNA methylation (% 5-mC) with standard deviations, per bee tested, for bees fed folic acid, grouped by treatment.

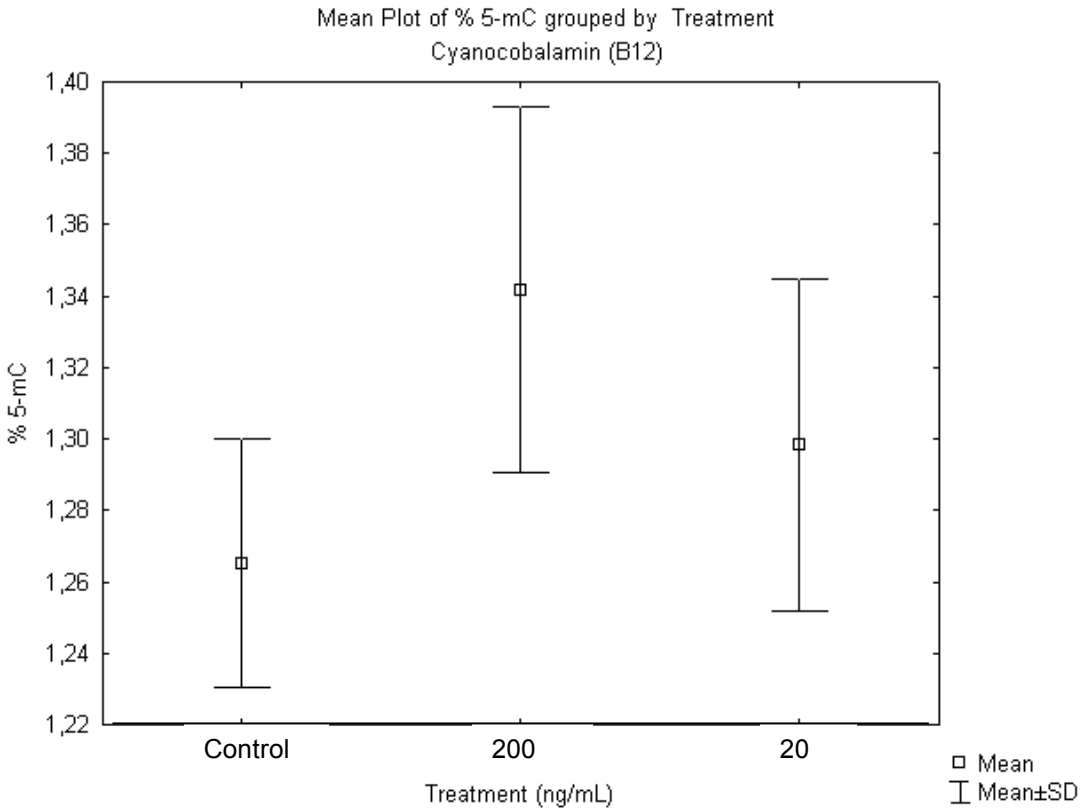


Figure 18: The average of global DNA methylation (% 5-mC) with standard deviations, per bee tested, for bees fed cyanocobalamin, grouped by treatment.

3.4.3 – Western blot

Western blots were performed to separate and identify the proteins in honey bee brain- and abdomen samples based on their size, with the purpose of identifying acetylated lysine 23 on histone 3 (H3K23ac) by using a polyclonal antibody. This peptide has previously been shown to be acetylated in honey bees (Dickman et al. 2013). Changes in histone acetylation patterns were expected for all substances as an epigenetic effect of treatment, but mostly for curcumin as it is a known inhibitor of HATs. Staining with Brilliant Blue (coomassie) was done to detect the separated proteins – to see if the desired protein was present in the gel.

Gels were run to separate the proteins, the separated proteins were blotted onto a membrane, and blots were stained with the antibodies (Fig. 19A) to assess its specificity for the protein (14,4 kDa). To evaluate electrophoretic separation of proteins, blots were stained with a protein stain (SYPRO Ruby, Fig. 19B), and gels with Coomassie Brilliant Blue (Fig. 19C). Fig. 19A, when compared to Fig. 19B, shows that the antibody binding was unspecific. The two pictures show little difference, even though the antibody should stain the H3K23ac-peptide (the antigen) specific while SYPRO Ruby stains all proteins. Neither of the blots shows bands in the desired area (around 14 kDa), and the smallest observable bands are found around 35 kDa. Fig. 19C show that the Coomassie staining gave good results; on the picture one can observe several bands of peptides of different sizes throughout the gel. One can also observe bands in the area of interest, around 14 kDa. This indicates that the gel-electrophoresis (separation of proteins) was not compromised.

In conclusion, these data do not support that this antibody specifically stains the acetylated lysine 23 on histone 3 in honey bees. Therefore the protocol was discontinued.

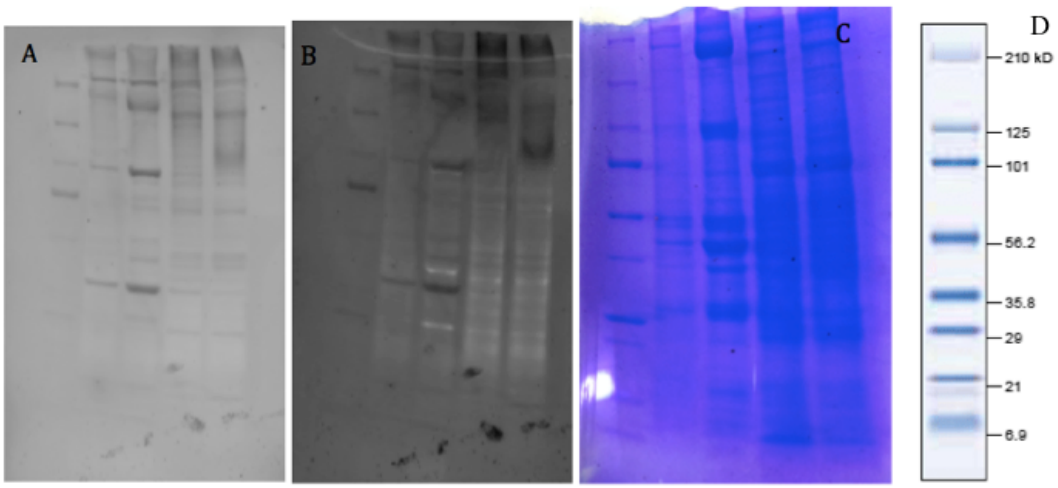


Figure 19: Picture A shows the membrane after antibody staining; Picture B shows the membrane after SYPRO Ruby staining; Picture C shows the parallel gel after Coomassie staining; Picture D shows a SDS-PAGE molecular weight standard equivalent to the lanes on the far left on picture A, B, and C. On all pictures, except from D, the lanes contain (from the left): molecular weight standard, two brain-samples, and two abdomen-samples.

4. Discussion

This study investigated the effect of the three different natural substances curcumin, folic acid (vitamin B9) and cyanocobalamin (vitamin B12) on longevity and epigenetic traits in honey bees. Each substance was tested in three different concentrations, with a control group, based on other studies. The concentrations were set with a tenfold increase/decrease between them to gain a broader range of treatments. I found that all the three substances affect lifespan in honey bees, and in a dose dependent manner. The experiments also show that there were no significant differences in levels of global methylation per 100 ng DNA in the honey bee abdomen between treatments for any of the substances. In addition I show that there were no differences in consumption between any of the treatments for any of the substances.

4.1 – The effect of curcumin on longevity and global DNA methylation levels

The substance curcumin from the turmeric plant was tested on honey bees in the three concentrations 0.1, 0.01, and 0.001 mg/mL, in addition to one control. The concentrations were chosen using a range of studies as references; studies showing both positive and negative effects were considered to ensure concentrations with good prospects for lifespan effects in the honey bees. In the present study, curcumin showed a statistical significant effect on lifespan for the highest concentration (T1: 0.1 mg/mL) as compared to the control. This is evident in the graph (Fig. 10), which clearly shows an increase in survival for T1 both for maximum survival and median survival. This indicates that the curcumin-mediated diet had an impact on survival, and increased longevity in the honey bees. From the results I observed a hive effect, and thus analyzed the two hives separately. This revealed that all the concentrations had a significant effect on lifespan in hive 1, and that the highest and middle concentrations had significant effect on lifespan in hive 2 (see table 4 and methodological considerations chapter 4.4). This effect can be observed when looking at median survival on the graph (Fig. 10). However, at maximum survival it becomes hard to separate the middle and lowest concentrations from the control, and the only treatment standing out is T1. Still, the most robust effects are the effects seen in the pooled data, and any effects from separate analyses are likely to bee less strong. To

my best knowledge, this is the first time a curcumin-dependent lifespan extension has been found in honey bees.

The observed increase in longevity is in accordance with studies done on both *D. melanogaster* (Li-Rong Shen 2012) and *C. elegans* (Vivian Hsiu-Chuan Liao 2011) which both show an increase in longevity with curcumin-mediated diets. The lifespan study done on *D. melanogaster* tested curcumin dissolved in ethanol, and used curcumin standards at concentrations of 23.1, 46.2, 92.5, and 185 µg/mL dissolved in 2 mL ethanol and added in the diet to final concentrations of 0.5 and 1.0 mg/g (Li-Rong Shen 2012). This corresponds to approximately 0.5 and 1.0 mg/mL respectively, and is equivalent to the concentrations chosen for this thesis. The study found that these curcumin-mediated diets significantly increased mean lifespan in *D. melanogaster*. The other study, done on *C. elegans* on media, tested the two concentrations 20 and 200 µM (0.007 and 0.07 mg/mL respectively) of curcumin dissolved in dimethylsulfoxide on longevity and aging (Vivian Hsiu-Chuan Liao 2011). The lowest concentration showed a statistically significant increase in lifespan compared to untreated ones, while the highest concentration did not. A study reporting negative effects of curcumin on lifespan in transgenic *D. melanogaster* used concentrations of 1, 10, and 100 mg curcumin per gram yeast paste from a stock solution consisting of 10 mg/ml curcumin in 95 % ethanol (Caesar I et al. 2012). The highest and middle concentrations reduced lifespan in one of the five lines of transgenic flies. Two other lines showed an increase in survival for the lowest and middle concentration, while one line of transgenic flies showed an increase in longevity for all concentrations. The last line showed no effect from treatment with the two lowest concentrations, but showed a decrease in survival time with the highest concentration.

When comparing studies done on different organisms it is important to keep in mind that different species have different metabolisms, and it is therefore problematic to do a direct comparison. This is true for all three substances tested in this thesis.

To explain the lifespan-effects found for curcumin I investigated changes in global DNA methylation levels in the honey bees' abdomen. I expected a global decrease in percentage of methylation for both treatments tested (T1 and T2) as compared to control due to the fact that curcumin is a hypomethylating agent that inhibits DNMTs

(Marie-Helène Teiten et al. 2013). Curcumin at a level of 3 μM, or 0.001 mg/mL, is known to be able to induce a decrease in global DNA methylation of 15-20 % (Liu et al. 2009). Still, statistical data showed that there were no significant differences between the treatments. However, I did observe a lower percentage of methylation with T1 as compared to the control (Fig. 16), and I hypothesize that the low number of samples used in the ELISA protocol might be the reason for lack of statistical significance. An increase in number of samples tested might increase the possibility of detecting possible effects. This was not tested due to the fact that this was initial studies, and too many samples and replicates would not have been possible.

When dissecting the bees fed the curcumin-mediated diet (control bees included) I observed less intestinal contents when compared to bees fed folic acid and cyanocobalamin (including control bees). There was a decrease in amount of food consumed of approximately 10 μL in average per bee per cage per 24 hours for all curcumin treatments, as compared to bees fed folic acid and cyanocobalamin; The bees fed a curcumin-mediated diet (controls included) consumed on average approximately 40 μL each per day, and the bees fed folic acid- and cyanocobalamin-mediated diets (controls included) consumed on average approximately 50 μL each per day (rough calculations). The reason for this might be that both curcumin (Vivian Hsiu-Chuan Liao 2011), and the ethanol used as solvent (Julie A. Mustard et al. 2008), can have the ability to reduce the motivation to eat, although it has been shown that honey bees readily consumes food blends with ethanol. To check if this is affecting the consumption it could be of interest to do a gustatory responsiveness test with the different treatments, including the control and another control without ethanol. Here the bees would be fed the different treatments for approximately 10 days, and then tested for gustatory response (Rascón et al. 2012). It can also be a question of whether they liked the smell of food or not, due to the curcumin and/or the ethanol. This could be examined by testing the response of random bees, not subjected to previous feeding with the treatments, to sucrose-blends and blends with ethanol and/or curcumin.

During the lifespan studies I also noted that the bees fed a curcumin and ethanol mediated diet were less active compared to the bees fed folic acid and cyanocobalamin. They did not fly much, they walked less, and some were lying on their backs not being able to get up. This happened after only days on treatment. I

speculate that the ethanol might be the reason for this reduced mobility. The fact that the bees in the control cage behaved similar to the cages fed curcumin in different concentrations further confirms this theory. Moreover, studies has also shown that ethanol reduces locomotion in honey bees in a dose and time dependent manner (Maze et al. 2006). However, the concentration of ethanol used in this thesis (1 %) is considerable lower than the ones showing an effect on locomotion in the mentioned study (10, 25, 50, 75 %), thus nothing supports the notion that the ethanol used with the curcumin-mediated diet should have an inhibitory effect on mobility; these two studies are not directly comparable.

To test whether there were any differences in consumption between the four treatments of curcumin, statistical analyses, using ANOVA, were done on the consumption data. The reason for analyzing the consumption data was to see if it could explain any of the effects found in the lifespan studies due to for example a significant change in consumption for bees fed treatments as compared to the control. This is based on the notion that calorie restriction has been found to increase lifespan (Rascón et al. 2012), whereas severe starvation could serve as an explanation for any decrease in longevity. This goes for all three substances.

Analyses of the consumption data revealed that there were no significant differences in consumption between any of the treatments, indicating that the bees had no preferences for the concentration of curcumin in the diet. I was not able to test if there were any significant differences in consumption between bees with ethanol in their diet and bees without ethanol in their diet since all treatments, including the control, had 1 % ethanol added. To test whether the ethanol might have affected their motivation to eat it could have been an idea to include a control set without ethanol. The fact that there were no significant differences in consumption between any of the curcumin treatments, or between the control and the treatments, indicate that curcumin does not have a significant effect on motivation to eat, and implies that any reduction in motivation might then be due to the ethanol.

4.2 – The effect of folic acid (vitamin B9) on longevity and global DNA methylation levels

Folic acid was tested in the three concentrations 500, 50, and 5 µg/mL in addition to one control. As for curcumin, the concentrations were chosen using other studies as

references. In this thesis, folic acid showed a statistical significant effect on lifespan for the lowest concentration (T3: 5 µg/mL) as compared to the control. This is evident in the graph (Fig. 11), which clearly shows a decrease in maximum survival for T3 as compared to control; when comparing maximum survival between the treatments and control one can see that bees fed the control diet lived about eight days longer than the bees fed the lowest concentration of folic acid. This indicates that the lowest concentration had a harmful impact on survival, and decreased longevity in the honey bees. Looking at median survival one does not see much difference between treatments, which indicates that any effects appear after time. In the graph one can also observe an indication of a lifespan increasing effect from the middle concentration (T2), especially around median survival. Statistical analyses, however, did not confirm this. Statistical analyzes also revealed both a hive and a round effect (see table 5 and methodological considerations chapter 4.4). As for curcumin, the most robust effects are the effects seen in the pooled data.

The effects of the concentrations used in this thesis are somewhat inconsistent with other studies done on folic acid. A study done on transgenic mice showed that a dose of 4 mg/kg/day of folic acid had a significant effect in increasing lifespan compared to the control group (Zhang et al. 2008). Calculated according to the body weight of a honey bee, which is approximately 0.2 g (D. Münch pers. com.), this would be an amount of 0.8 µg/day/bee. According to my studies, a bee eats about 45 µL food per day, so this would give a concentration of approximately 18 µg/mL which lies between the lowest and middle concentrations used in my study (5 and 50 µg/mL respectively). A study done on *D. melanogaster* tested the effect of phenol and folic acid on development (Askin et al. 2007). Phenol is a commonly found organic compound, which in the mentioned study showed harmful effects on development. Folic acid was added in concentrations from 0.25-2.50 mg/kg, and was found to eliminate the negative effects caused by phenol, and in addition showed no negative effects. It is known that folic acid is important for development and DNA synthesis, and especially during pregnancy. It could be that effects are best seen during early developmental stages. Another study, done on *D. melanogaster*, tested the effect of dietary folic acid on body folate, development and fitness using five different concentrations: 0.1, 0.9, 3.0, 9.0 and 60.0 µg/mL (Blatch et al. 2010). 0.1 µg/mL represents the diet prepared without folic acid and is considered 0 % (the control

group). They found that increasing concentrations showed increasing positive effects on all matters, with the exception that the larvae fed nearly zero folic acid (0.1 µg/mL) developed faster. They also wanted to test whether the fruit fly itself produce folate if needed, or if they have to obtain it through diet. The results indicate that the gut bacteria of the flies can provide the necessary folates for development if not obtained through diet. When regarding honey bees, there is not much data on whether their gut microbiota provides the necessary folate or if they have to obtain it through diet. What was perceived as unusual with the folic acid treatments in my thesis is that the bees fed the highest and middle concentrations lived longer than bees fed the lowest concentration, but still shorter than the control. I speculate that one explanation could be related to the findings in this study done on *D. melanogaster* (Blatch et al. 2010). The study used a somewhat comparable setup as I did, and their results are somewhat similar. They found that the lowest concentration of folic acid (0.9 µg/mL) gave the lowest fitness (larval growth rate, development, viability etc.) when compared to both higher concentrations and control. These surprising results were explained by the gut microbiota of the larvae, which compensated for the lack of folate in the control group. This means that larvae fed no folic acid ended up with the same amount of folic acid as the high concentrations due to the gut bacteria. The larvae fed the lowest concentration still had folic acid in their diet, so it could be that the gut microbiota did not produce any additional folate, and the larvae ended up with only the small amount obtained through diet.

Another explanation for the somewhat inconsistent findings in this thesis could be that folic acid, or folate, is a substance important for early developing states (Gueant, J. L et al. 2013). It might have an effect that this study was conducted using grown bees.

As with curcumin, no effect was detected when looking for changes in global DNA methylation levels. I expected a global increase in percentage of methylation for both treatments tested (T1 and T3) as compared to control due to the fact that folic acid is a methyl carrier and donor (Zhang et al. 2008), and thus can be considered as a hypermethylating agent. Still, statistical data showed that there were no significant differences between the treatments. However, on the graph (Fig. 17) one can observe a slight increase in methylation for T1 and T3 as compared to the control. As with

curcumin I hypothesize that an increase in sample numbers might increase the likelihood of detecting possible effects.

For the consumption data, statistical analyses were done the same way as for curcumin and no significant differences were found between any of the treatments. This indicates that the bees had no preferences for concentration of folic acid in the diet, and also that there were no preference in diets with and without folic acid (the treatment without folic acid being the control diet).

4.3 – The effect of cyanocobalamin (vitamin B12) on longevity and global DNA methylation levels

The last substance, cyanocobalamin, was tested in the three concentrations 2000, 200, and 20 ng/mL in addition to one control. Just as for the other substances the concentrations were chosen using other studies as references. In the present study, I found that the middle (T2) and the lowest concentrations (T3) had an effect on lifespan as compared to the control, which can be seen on the graph (Fig. 12). When looking at median survival the effects are less clear, but after median survival and down to maximum survival one can observe a separation between T1 and control versus T2 and T3 with a longevity difference of approximately 2-5 days when reaching maximum survival. This indicates that the middle and the lowest concentrations had a harmful impact on survival, and decreased longevity in the honey bees.

This observed decrease in longevity is inconsistent with other studies done on cyanocobalamin (vitamin B12). A study done on transgenic mice showed that a dose of 0.2 mg/kg/day of cyanocobalamin had a significant effect in increasing lifespan compared to the control group when in a mix with folic acid (Zhang et al. 2008). Calculated according to the body weight of a honey bee, as done above for folic acid, this would be an amount of 0.04 µg/day/bee, and based on the estimated consumption of one bee per day this would give a concentration of approximately 0.89 µg/mL, or 889 ng/mL. Another study showed that an amount of 0.2 µg/g wet weight of B12-deficient *E. coli* cells grown on B12 (100µg/L)-supplemented medium resulted in B12 deficiency and thus decreased lifespan in *C. elegans* (Bito et al. 2013). They reported an amount of 11.1 µg/g wet weight as being a sufficient concentration of B12 to sustain normal growth and lifespan. A study done on the red flour beetle *Tribolium*

castaneum also showed that B12 deficiency decreased lifespan, while a complete diet with a B12-concentration of 1.35 mg/100 lbs, equivalent of 1.35 mg/45.4 kg or 30 ng/g, increased lifespan in comparison (Armstrong 1978). Based on theses studies I expected an increase in lifespan for at least one of the concentrations of cyanocobalamin chosen for this thesis.

As for folic acid I observed both a hive and a round effect from the results on cyanocobalamin (see table 6 and methodological considerations chapter 4.4), but as for the other substances I stress that the most robust effects are the effects seen in the pooled data.

As for the other substances, no effect was detected when looking for changes in global DNA methylation levels. I expected a global increase in percentage of methylation for both treatments tested (T2 and T3) as compared to control due to the fact that cyanocobalamin is a methyl donor and –acceptor (Gueant, J. L. et al. 2013), and thus can be considered as a hypermethylating agent. Still, statistical data showed that there were no significant differences between the treatments. However, as mentioned in the results I observed a trend in increased methylation between the control group and T2, which was further confirmed using a Fisher LSD test ($p < 0.05$). Due to this I would suggest that it would be useful to repeat the ELISA experiment with more replicates, and I hypothesize that this would increase the likelihood of detecting possible effects. Just as for the other substances, this was not tested due to the fact that these were initial studies (see chapter 4.4.2).

As for the consumption data, statistical analyses were done the same way as for the other two substances and no significant differences were found between any of the treatments. Just as for folic acid, this indicates that the bees had no preferences for concentration of cyanocobalamin, and also that there were no preference in diets with and without cyanocobalamin (the control diet).

4.4 – Methodological considerations

4.4.1 – Lifespan studies

All together, the lifespan studies went well. A shortcoming is, however, the lack of a control group without ethanol for the curcumin-mediated diet to test whether the ethanol might have an impact on consumption and locomotion in the honey bees.

Also, the observed negative effects on lifespan for folic acid and cyanocobalamin could be due to the concentrations; it is a possibility that other concentrations might have given other effects. For future studies it could be of interest to test the substances using other concentrations.

Moreover, the encountered hive and round effects are considered shortcomings due to the fact that they masked effects from the lifespan data. The observed hive effects are most likely due to one hive being weaker than the other, for example due to a sick and dying queen or a low number of bees in the hive, and this could then bee the reason for the masked effects in the overall statistics. When sampling bees for the curcumin treatments in September I did not encounter any problems, and the observed hive effects are thus most likely due to one of the reasons just mentioned. However, when sampling bees for the folic acid and cyanocobalamin treatments in November I noticed that one hive (hive 2) was behaving somewhat agitated. The bees sampled from this hive attacked both each other and the sampling cages and some died during sampling. They also died much sooner than the bees from the other hive throughout the lifespan studies. This is most likely the reason for the observed hive effect for both folic acid and cyanocobalamin. As for the round effects found with folic acid and cyanocobalamin, it is most likely explained by the differences in conditions between the two sampling days. On the first sampling day everything went ok, and the weather was good. The second sampling day the weather was very cold and some of the bees froze and died during sampling. They behaved agitated and sampling took much longer time. Those that survived sampling were probably affected by these conditions, and that likely caused the observed round effect. However, I emphasis that the most robust effects are the effects seen in the pooled statistical data, and the effects seen from the separate analyses are likely to bee less strong.

After sampling for the folic acid and cyanocobalamin treatments our beekeeper observed small clusters of brood in one of the hives. This could be one explanation for the observed hive effects, however the amount was so small that it is not very likely. Although winter bees can produce small amounts of brood, a defining feature is that they have low to no amounts of brood. Still, what could have caused a small difference in this case is that one hive had small clusters of brood while the other had none.

4.4.2 – ELISA

The ELISA protocol was used to test for changes in global DNA methylation levels in the honey bee abdomen, but an encountered problem was the lack of time to do anything but the initial studies. Due to many substances, of four treatments each, I had to cut down on sample numbers and this, I suspect, has affected the statistical results. The low number of 4 samples from each treatment was probably not enough to state significance. A solution would be to increase the number of samples. This would probably increase the possibility of detecting effects. Another approach could be to test for global DNA methylation levels by using bisulfite DNA sequencing (Herb et al. 2012), first developed by Frommer et. al. in 1992 (Frommer et al. 1992). With this technique, one could analyze DNA sequences from the treated bees for methylated cytosine (mC) in both a quantitative and qualitative way. Desired sequences are treated with bisulfite, which converts cytosine to uracil, but 5-methylcytosine remains unchanged. The sequences are then amplified using PCR. Next, the products are sequenced and the results can be read; all cytosine bases represent methylated sites in the genome (unmethylated cytosine, converted to uracil by bisulfite, has been amplified to thymine).

Another shortcoming with the ELISA was the large standard deviations observed in the graphs (Fig. 16, 17, 18); these could indicate that the ELISA-scan generated outliers. An increase in sample numbers could apply as a solution also to this matter, and removing possible outliers then, would not reduce the statistical power too much.

4.4.3 – Western blot

The western blot had the purpose of identifying acetylated lysine 23 on histone 3 by using a polyclonal antibody. This protocol was used because changes in histone acetylation patterns were expected for all substances, as an epigenetic effect of treatment, but results did not show any binding of the antibody to the desired protein in any of the blots. I speculate that this was because the binding of the antibody was unspecific (Fig. 19A and B). The fact that the desired protein appeared on the coomassie stained gel (Fig. 19C) indicates that the separation of the proteins was uncompromised, and that any shortcomings in the blotting could be due to the antibody being unspecific. This theory was further confirmed by the information found in the antibody's datasheet, which states that the antibody has only been tested

on humans (Diagenode 2010). It could be that because of the lower amount of acetylated lysine found in honey bees compared to humans the antibody is not compatible with bees; cannot bind properly. An alternative approach could be to analyze the samples using mass spectrometry (Dickman et al. 2013). Several different mass spectrometry-methods can be used to identify proteins from SDS-PAGE, but most have a common procedure. The desired bands are cut out from the gel, and the proteins are extracted and treated with a specific proteinase. The peptides are separated according to their mass, and the mass fingerprints from each peptide are compared against a database for identification. A suitable mass spectrometry-technique after separation with SDS-PAGE is Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) (here described by Marvin *et al*. (Marvin et al. 2003)). Mass spectrometry was not conducted due to the high costs of running mass spectrometry on so many samples.

5. Conclusion and outlook

Curcumin, folic acid (vitamin B9), and cyanocobalamin (vitamin B12) all have dose dependent effects on lifespan in honey bees. While curcumin increased lifespan, folic acid and cyanocobalamin had harmful effects on longevity. To my best knowledge, this is the first time lifespan-extending effects have been found for curcumin in honey bees. Neither of the substances gave significant changes in global DNA methylation levels, though the low number of samples might mask any effects, and repeating the experiment with more samples could be useful as a way of controlling these findings. The experiments also showed that none of the substances showed significant differences in food consumption between any of the treatments.

The results provide prospects for the use of these substances in treatment of age related challenges in humans, such as cancer and degenerative diseases, as well as for potential risks associated with therapeutic use. There are not enough studies done on purified biochemical compounds to know the effects they will have on humans or in other animal models, both short-term and long-term. Some might have toxic effects. Here I show that purified forms of both folic acid (vitamin B9) and cyanocobalamin (vitamin B12) can have harmful effects on honeybees, and thus perhaps also in humans. Abrupt changes in DNA methylation are often associated with age-related diseased such as cancer, and substances that can cause such changes might either serve as a potential risk or as therapeutic measures.

My study underline that the honey bee serve as a very promising model organism both for longevity studies and to study epigenetic mechanisms. Moreover, the fact that studies that are done on honey bees can be transferred to humans due to similar epigenetic machineries make them excellent model organisms for medical research.

For future studies I recommend testing for changes in levels of histone acetylation using mass spectrometry, and also to test for changes in global DNA methylation levels using bisulfite DNA sequencing (see chapter 4.4.2 and 4.4.3). In addition it would be interesting to test the correlation between curcumin and lipofuscin levels in the honey bee. Lipofuscin is a material that accumulates in aging, a so called “universal aging symptom”, and curcumin has been shown to reduce lipofuscin levels in *C. elegans* (Vivian Hsiu-Chuan Liao 2011). Lipofuscin is found to accumulate in

honey bees in aging (Munch et al. 2013), and it would therefore be interesting to test if similar associations between lipofuscin accumulation and curcumin are present in the bee model. Lastly, I would find it interesting to study the correlation between curcumin and SIR-2.1, a histone deacetylase. Curcumin-mediated lifespan extensions are believed related to dietary restriction-like states of metabolism, and such states of metabolism are again believed modulated by SIR-2.1 (Vivian Hsiu-Chuan Liao 2011).

6. Acknowledgments

I am very grateful for valuable comments, guidance and help on this thesis and on the laboratory projects provided by my supervisors Dr. Daniel Münch and PhD student Erik Rasmussen. I would also like to thank Claus Kreibich for guiding, support, and help with sampling and handling of the honey bees. Last, I would like to show my appreciation towards the rest of the ″Aging and Nutritional Physiology in Animal Models”-group for general help and guidance.

7. Appendix

Appendix 1: 5-mC DNA ELISA Kit from Zymo Research.

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

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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.

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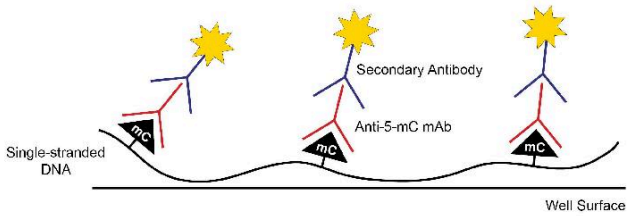
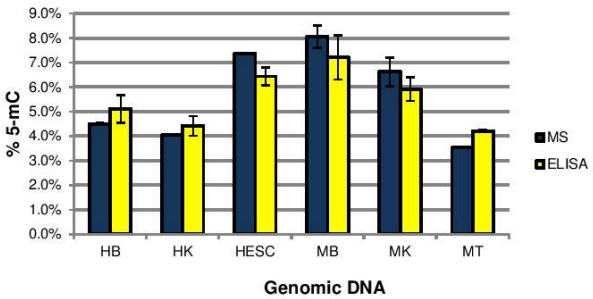
Product Description

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 ºC 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 ºC 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 37ºC 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).

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.

Appendix - Analysis with Negative and Positive Control DNAs

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).

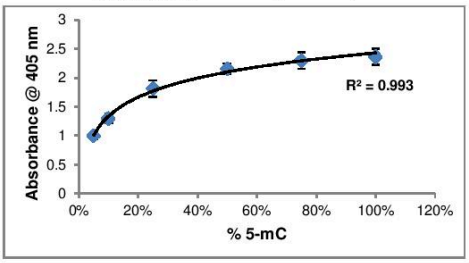
% 5-mC	Negative Control (100 ng/µl)	Positive Control (100 ng/µl)
0%	10.0 µl	0 µl
5%	9.5 µl	0.5 µl
10%	9.0 µl	1.0 µl
25%	7.5 µl	2.5 µl
50%	5.0 µl	5.0 µl
75%	2.5 µl	7.5 µl
100%	0 µl	10.0 µl

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:

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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

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Appendix 2: Complete list of reagents used throughout the experiments.

Substance/chemical	Purity/ concentration	Supplier	LOT-No.
For the diets:			
Curcumin	70 %	Sigma	SLBD0850V
Cyanocobalamin (vitamin B12)	≥ 98 %	Sigma	SLBC7794V
Folic acid (vitamin B9)	≥ 97 %	Sigma	SLBG8380V
Lipid Mixture	1000X	Sigma	SLBF7419
L-Alanine	≥ 98 %	Sigma	BCBL0498V
L-Histidine	≥ 99 %	Sigma	SLBC6818V
RPMI Amino acid solution	50X	Sigma	RNBC8950
Grace's Amino acid solution	10X	Sigma	57H2348
Bifor	75 %	Nordic Sugar	27310340112873 (bar code)
For DNA extraction:			
Phosphate buffered saline (PBS) buffer	-	Sigma	SLBF5741V
ATL buffer (tissue lysis buffer)	-	Qiagen	139271811
AL buffer (lysis buffer)	-	Qiagen	136266789
Phenol:chloroform:isoamylalcohol (PCI)	25:24:1 v/v	Invitrogen	0493C455
Chloroform:isoamylalcohol (CI)	24:1 v/v	Sigma	SLBD9562V
Linear acrylamide	5 mg/mL	Life Technologies	1207020
Proteinase K (Tritirachium album, crude lyophilized powder)	20 mg/mL	Sigma	026K8607
RNase A	20 mg/mL	Invitrogen & Life Technologies	1413314
Absolute Ethanol	100 %	-	-
Sodium acetate (NaAc) 3M	3M in dH_2O	Sigma	BCBJ2797V
For ELISA:			
5-mC DNA ELISA Kit from Zymo Research		Zymo Research	ZRC175732
For western blot:			
Homogenizing buffer (PBS with 1mM dinatrium EDTA and complete protease inhibitor (Roche) added)	-	-	-
PBS buffer	-	Sigma	SLBF5741V
Polyacrylamide gels	4-20 %	BioRad	456-1096 (catalog no)
Laemmli buffer	-	BioRad	161-0737 (catalog no)
TGS buffer	10X	BioRad	161-0772 (catalog no)
Tris-glycine buffer	10X	BioRad	161-0772

			(catalog no)
Methanol	99 %	Sigma	9171S
Blocking buffer (Bovine Serum Albumin)	≥ 98 %	Sigma	-
Primary antibody (rabbit), polyclonal, H3K23ac	Whole antiserum	Diagenode	A615-001
Secondary antibody (goat anti rabbit), Aleyfluon 647, Cy5, Gar	-	Jackson Immuno Research	-
Tween (for PBS-T)	-	Sigma	-
Acetic acid	> 90 %	MERCK	-
SYPRO Ruby stain reagent	1X	BioRad	170-3127 (catalog no)
Brilliant Blue (coomassie) R.250	-	Sigma	20K1534

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