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elegans“

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TABLE OF CONTENTS

1 Introduction	1
2 Literature.....	3
2.1 <i>Caenorhabditis elegans</i>	3
2.1.1 General characterisation of the model organism	
<i>Caenorhabditis elegans</i>	3
2.1.2 <i>C. elegans</i> as a laboratory model organism.....	3
2.1.3 Anatomy of an adult <i>C. elegans</i>	5
2.1.4 Development and reproduction of <i>C. elegans</i>	6
2.2 Folic acid and folate	10
2.2.1 Metabolism of folates	11
2.2.2 Effects of folate deficiency	13
2.2.3 Folate reaction of interfering drugs	14
2.2.4 Folic acid metabolism in <i>C. elegans</i>	14
3 Materials and Methods.....	17
3.1 Materials.....	17
3.1.1 Instruments	17
3.1.2 Chemicals and solutions	18
3.1.3 <i>E. coli</i> bacteria	20
3.2 Methods	20
3.2.1 General <i>C. elegans</i> handling.....	20
3.2.2 Freezing of bacteria	21
3.2.3 Folic acid supplementation.....	21
3.2.4 Synchronisation of a <i>C. elegans</i> population	21
3.2.5 Bacterial growth curve with SMX treatment	21
3.2.6 Number of progeny of <i>C. elegans</i>	23
3.2.7 Life span of <i>C. elegans</i>	23
4 Results.....	25
4.1 Comparison of brood size and life span of wild type <i>C. elegans</i>	
with living or dead bacteria as food source	25

TABLE OF CONTENTS

4.1.1 Brood size and life span of wild type <i>C. elegans</i> fed with living and dead OP50 <i>E. coli</i>	25
4.1.2 Brood size and life span of wild type <i>C. elegans</i> fed with living and dead MH828 and MH951 <i>E. coli</i>	27
4.2 Folate depletion induced by feeding dihydrofolate reductase deficient <i>E. coli</i> to wild type <i>C. elegans</i>	27
4.2.1 Brood size of wild type <i>C. elegans</i> fed with OP50, MH828 and MH951 <i>E. coli</i> bacteria	28
4.2.2 Life span of wild type <i>C. elegans</i> fed with OP50, MH828 and MH951 <i>E. coli</i> bacteria.	28
4.3 Folate depletion in wild type <i>C. elegans</i> induced by feeding OP50 <i>E. coli</i> with inhibited folate biosynthesis	30
4.3.1 Inhibition of folate biosynthesis in OP50 <i>E. coli</i>	30
4.3.2 Brood size of wild type <i>C. elegans</i> fed with OP50 bacteria with inhibited folate biosynthesis.....	31
4.3.3 Life span of wild type <i>C. elegans</i> fed with OP50 bacteria with inhibited folate biosynthesis.....	32
4.4 Phenotypic characteristics of folate supplementation concerning the number of progeny and life span of wild type <i>C. elegans</i>	34
4.4.1 Brood size of folate depleted wild type <i>C. elegans</i> with folic acid supplementation.....	35
4.4.2 Life span of folate depleted wild type <i>C. elegans</i> with folic acid supplementation.....	39
5 Discussion	43
5.1. Two ways to induce a folate deficiency in <i>C. elegans</i>	43
5.2 Folate deficiency partially reduces reproduction which can be rescued by folic acid supplementation	45
5.3 Folate deficiency influences life span which can be rescued by folic acid supplementation	47
6 Abstract.....	49
Zusammenfassung	51
7 List of References	53

8 Appendix.....	63
8.1 List of figures.....	63
8.2 List of abbreviations	65
8.3 List of tables	67

1 INTRODUCTION

Folic acid is responsible for various functions throughout several species, e.g. mammals, nematodes, insects and also in bacteria. For *in vivo* studies with humans the folate metabolism is too complex and too difficult to obtain reproducible and reliable results. Hence, common model organisms like *Caenorhabditis elegans* [HERRINGTON and CHIRWA, 1999], *Escherichia coli* [ROLAND et al, 1979], *Drosophila melanogaster* [BAUER et al, 2006] or mammals like mice [CHAN et al, 2010], rats or rabbits [HELM et al, 1976] are used to get deeper into the whole function and metabolism of folic acid.

Insights of pregnant women, which have an inadequate supply of folate during the preconception period, are more susceptible to bearing progeny with a number of congenital malformations, e.g. the neural tube defect [reviewed by BLOM and SMULDERS, 2011]. Other studies show ineffective haematopoiesis of folate deficient mice [BILLS et al., 1992], impact of folate deficiency on fertility and fetal development of rats and rabbits [HELM F. et al., 1976] and platal fusion in mice following dietary folic acid deficiency [MALDONADO et al., 2011]. Beneath influences on fertility and fetal development, a correlation between folate and life span is predicted in several model organisms like mice, rats [BAUER et al., 2006] and *Drosophila* [MILLER et al., 2005]. Hence, a supplementation of folic acid before and during pregnancy is recommended [MILUNSKY et al., 1989]. Studies have also proven that supplementation cause a reduction of the risk of congenital heart defect, cleft lips, limb defect and urinary tract anomalies [GOH and KOREN , 2008].

In this thesis folate deficiency was induced in the model organism *C. elegans* to investigate main functions of folic acid and folate in the nematode. Until now only little is known about folate metabolism in *C. elegans*. Previously it was shown that three orthologues to mammalian reduced folate carrier (RFC) exists in *C. elegans* namely FOLT-1, FOLT-2 and FOLT-3 and that a folate deficiency causes an articulately reduced germline and an infrequent production of functional oocytes [AUSTIN et al., 2010]. Herrington has induced a folate deficiency in worms by feeding *folA* (dihydrofolate reductase) deficient *E. coli* bacteria

INTRODUCTION

(MH829), although it was proven that these bacteria have the ability to produce folate by a separate pathway [HERRINGTON and CHIRWA, 1999]. The aim of this work was to establish a new possibility to induce a folate deficiency in *C. elegans* by feeding folate depleted *E. coli* bacteria besides feeding *E. coli* with *folA* knockout. The effects of both kinds of folate depletion on reproduction and life span of the worms were investigated and compared. To ensure that the observed effects were dependent on folate depletion, folic acid supplementation studies were performed as well.

2 LITERATURE

2.1 *Caenorhabditis elegans*

2.1.1 General characterisation of the model organism *Caenorhabditis elegans*

The transparent nematode *Caenorhabditis elegans* (*C. elegans*) is a representative of Rhabditia and has a length of about 1.2 mm and a diameter of 65 μm .

Kingdom:	Animalia
Phylum:	Nematoda
Class:	Secernentea
Order:	Rhabditida
Family:	Rhabditidae
Genus:	<i>Caenorhabditis</i>
Species:	<i>C. elegans</i>
Binominal name:	<i>Caenorhabditis elegans</i>

[MAUPAS, 1900]



Fig. 1: A wild type *C. elegans* hermaphrodite.

It is living on an agar plate with *E. coli* and shown at magnification of 40 fold [provided by J. Benner].

The free living nematodes prefer a temperate soil environment. The optimal temperature is between 4°C and 30°C. Their food sources are primary bacteria, which catabolise organic material, but also fungi [WOOD, 1988a].

2.1.2 *C. elegans* as a laboratory model organism

C. elegans is a favoured model organism for a variety of reasons. The simplicity of this multicellular eukaryotic organism is one of the reasons why it is studied in great detail [STRANGE, 2003; STRANGE, 2000]. There are several knockout strains available which are cheap to breed and there is no complicated effort for long-

LITERATURE

term storage, because worms can be stored in a 15% glycerol solution at -80°C [STIERNAGLE, 2006].

In addition, this model organism has a host of unique features that include simple anatomy, transparency and the facility of studying cellular differentiation and further developmental processes in an intact organism. Adult hermaphrodites have a total of 959, while adult males consist of 1031 somatic cells [KIMBLE and HIRSH, 1983; SULSTON and HORVITZ, 1977]. Normally, these patterns of cell lineage are widely constant between individuals, in contrast to mammals, where it depends mostly on cellular cues. The whole anatomical characterisation and its complete cell lineage have already been established [WOOD, 1988a; LEWIS and FLEMING, 1995]. *C. elegans* also stand out as a common model organism because of its defined genome, ease of maintenance and growth, a defined life cycle and a short life span of 2-3 weeks. Notably it was shown that a lot of human physiological functions seem to have analogs in *C. elegans* and a huge amount of human genes have orthologs in the genome of this nematode, e.g. studies indicated that 28.4% of the worm genome has one or more human orthologs and 83% of the worm proteins have domains with remarkable similarity to human proteins [O' BRIEN and SONNHAMMER, 2005; THE *C. ELEGANS* SEQUENCING CONSORTIUM, 1998]. *C. elegans* also consists of differentiated tissues, e.g. a nervous system which is responsible for several interesting behaviours, e.g. chemotaxis, thermotaxis, mechanotransduction and also male mating behaviour.

A specific characteristic of *C. elegans* is the possibility of an easy knockdown of specific genes by using RNA interference (RNAi). This extensively used method can be done by simple feeding of transgenic bacteria, which are expressing dsRNA complementary to the gene of interest. Hence, the strategy of gene loss enables scientists to knock down 86% of approximately 20.000 predicted genes in the nematode and to provide functional associations for 9% of the genome [KAMATH et al., 2003].

C. elegans was the first multicellular organism, whose genome was completely sequenced in 1998. This achievement was owed to Sydney Brenner, H. Robert

Horvitz and John Sulston, who received the Nobel Prize in Physiology or Medicine for their work on the genetics of organ development and programmed cell death in *C. elegans*, in 2002. The Prize was also awarded, in 2006, to Andrew Fire and Craig C. Mello for their discovery of RNA interferences in *C. elegans* [FIRE et al., 1998].

2.1.3 Anatomy of an adult *C. elegans*

In 1974, investigations into molecular and developmental biology of *C. elegans* were started by Sydney Brenner and since then it has been used extensively as a model organism [BRENNER S., 1974]. In respect to reproduction a distinction is drawn between self-fertilising hermaphrodites (Fig. 2 A) and males (Fig. 2 B)

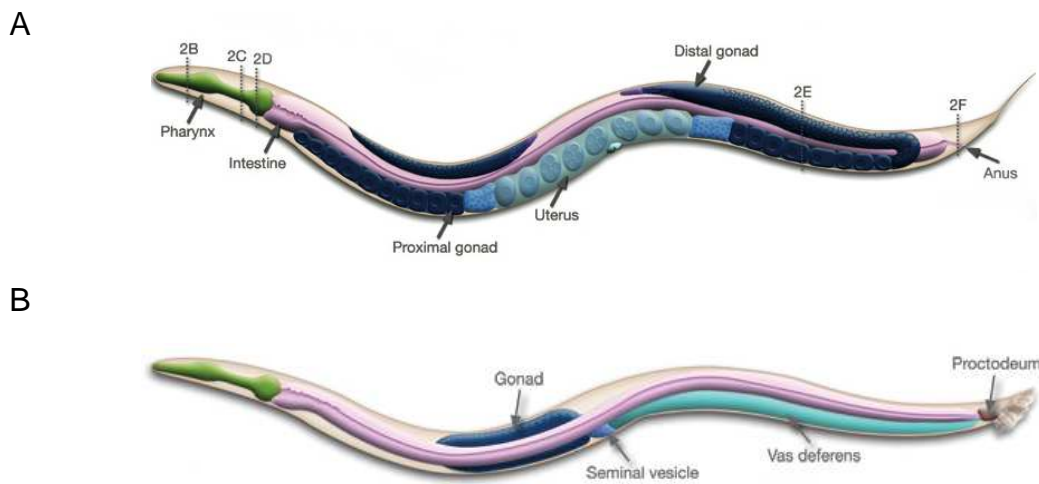


Fig. 2: *Caenorhabditis elegans* hermaphrodite and male

(A) Schematic picture of the major anatomical features of a *C. elegans* hermaphrodite. (B) Diagram of the major anatomical differences in *C. elegans* males. Left = anterior part, right = posterior part (modified from Wormatlas).

The Intestinal System of *C. elegans* consists of 20 large epithelial cells and has various functions. It is comparable to the intestine of higher eukaryotes, is responsible for digestion of food, absorption of processed nutrients, synthesis and storage of macromolecules, initiation of an innate immune response to pathogens and it produces yolk for germ cells [KIMBLE and SHARROCK, 1983; SCHULENBURG et al., 2004; PAULI et al., 2006; MCGHEE, 2007]. The intestine possesses a

LITERATURE

large number of nutrient transporters, including FOLT-1, FOLT-2 and FOLT-3, which are responsible for folic acid and folate uptake [BALAMURUGAN et al., 2007].

The Epithelial System is made up of the hypodermis, which constitutes two general categories of cells, the hypodermis and specialised epithelial cells. Those specialised cells act as support cells for neuronal receptors and as linker cells to attach the hypodermis to internal tissues, while forming various holes in the cuticle [JOHNSTONE and BARRY, 1996; MICHAUX et al., 2001; WHITE, 1988].

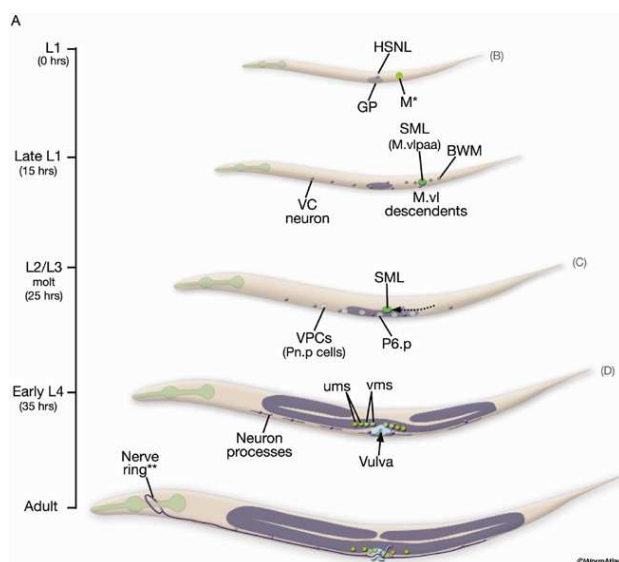
The Nervous System is primarily segmented into the ganglia in the head and into the tail. Two distinct and independent nervous systems were differentiated [Ward et al., 1975]. While the small pharyngeal nervous system consists of only 20 neurons, the large somatic one counts 282 neurons [Sulston and Horvitz, 1977]. Overall the adult hermaphrodite has 302 neurons [White et al., 1986].

The muscle system of the nematode *C. elegans* contains 95 muscle cells. A basal membrane always separates the muscles of underlying nervous tissue and hypodermis [Sulston and Horvitz, 1977].

2.1.4 Development and reproduction of *C. elegans*

Development and reproduction of *C. elegans* are already well investigated and simplify further studies.

A



B

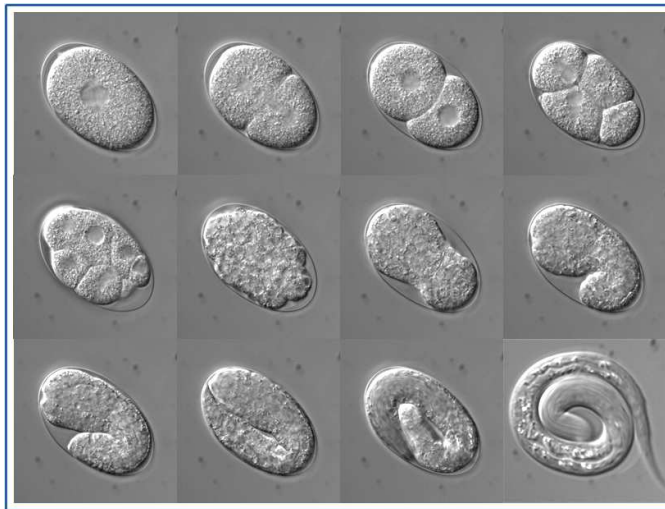


Fig. 3: Embryonic development of *C. elegans*

(A) Photo of *C. elegans* hermaphrodite in different larval stages (L1 to L4) up to an adult worm, also an adult male [WORMATLAS]. (B) Picture of the embryogenesis of *C. elegans* [WORMATLAS].

Almost all individuals (99.95 %) are self-fertilising hermaphrodites (XX) who are characterised by two ovaries, oviducts, spermatheca and a single uterus (Fig. 2 A) [BRENNER, 1973]. The ability of self-fertilization of the hermaphrodites allows homozygous worms to generate genetically identical progeny. The percentage of male (XO) individuals varies from 0 to as high as 22% of the total population depending on their geographical habitat [ANDERSON et al., 2010]. They are characterised by a single-lobed gonad, vas deferens and a tail specialized for mating (Fig. 2 B) [SULSTON et al., 1983; WOOD, 1988a]. Males arise infrequently by spontaneous non-disjunction in the hermaphrodite germ line and at higher frequency, up to 50 % through mating [LEWIS and FLEMING, 1995]. Hermaphrodites are responsible for reproduction. After development in the egg (embryonic development) and hatching they pass through four juvenile stages, L1 to L4 (post-embryonic development), until they reach adulthood and lay eggs (Fig. 3 A). Each end of a larval stage is marked by a molt, where a new phase-specific cuticle is synthesised [BYERLY et al., 1976].

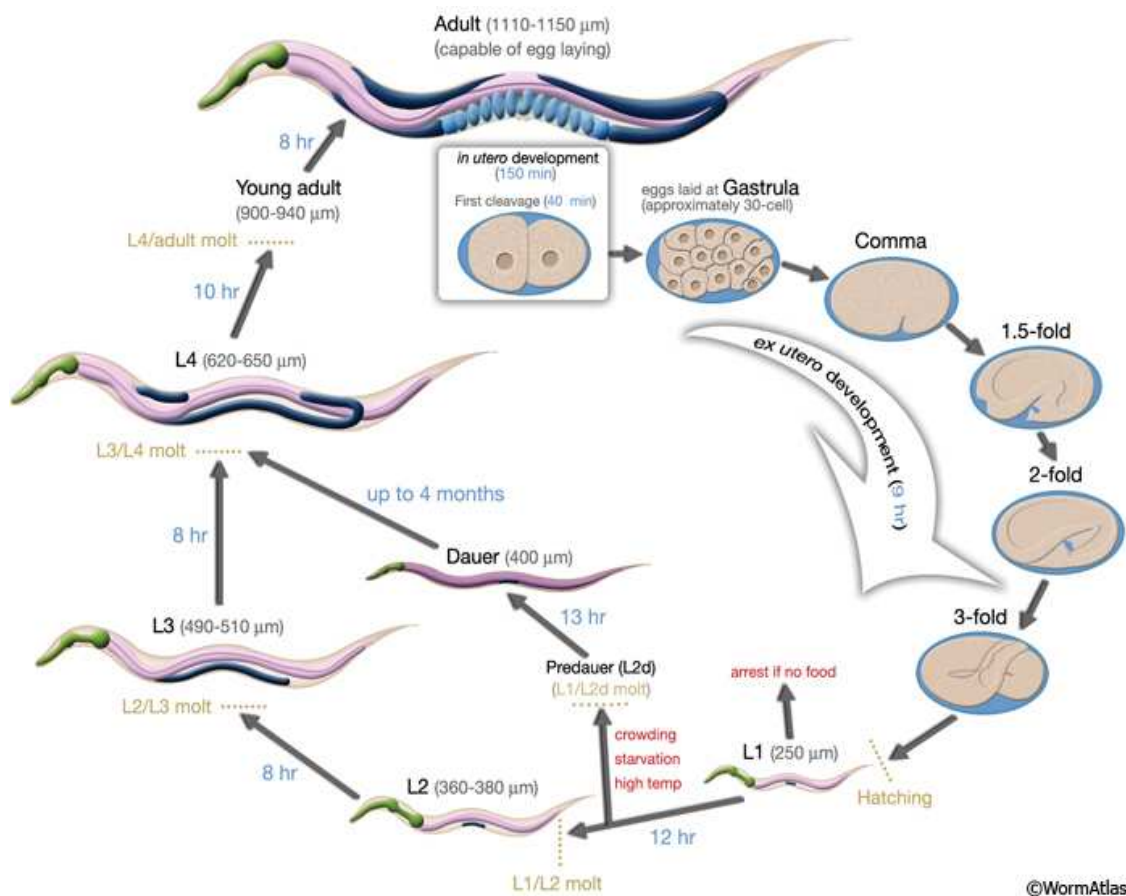


Fig. 4: Life cycle of *C. elegans*.

The picture shows the development from the egg to an adult. Blue numbers indicate the hours from one to another larval stage [WORMATLAS].

Embryo

Two stages of embryogenesis were differentiated in *C. elegans*, first the proliferation and secondly the organogenesis/morphogenesis [SULSTON et al., 1983]. The process of proliferation comprehends a cell division of one single cell to approximately 550 essentially undifferentiated cells [VON EHRENSTEIN and SCHIERENBERG, 1980; WOOD, 1988b]. During the phase of organogenesis/morphogenesis (5.5-6 to 12-14 hr) several cell divisions let the embryo grow and elongate threefold. Three hours after hatching, the post-embryonic development begins, which is activated by the presence of food [AMBROS, 2000].

L1 Larvae

This stage contains a further developing of the nervous system, which contains a generation of five of the eight classes of motor neurons by 13 precursors in the end of this stage [SULSTON and HORVITZ, 1977, WHITE et al., 1986]. In the second half of L1 the reproductive system starts with developing of 12 cells (hermaphrodite), which also begins with dividing. The division of germline precursors occur continuously from L1 through adulthood [KIMBLE and HIRSH, 1979].

L2 Larvae

The nervous system will be enlarged by postdeirid sensilla and two ventral ganglion neurons [WHITE et al., 1986]. Moreover, the germ cell division continues. The general organization of the future gonad will be established by rearranging somatic and germ cells during L2/L3 stage, whereupon the gonade begins to elongate [KIMBLE and HIRSH, 1979].

Dauer Larvae

In case of inauspicious environmental conditions, worms at the end of the L2 stage can enter an arrested state called dauer larvae. A high density of population, food deficiency or high temperatures act as signals [RIDDLE, 1988]. The dauer is a non-aging state and also characterized by an indefinitely feeding and a remarkable reduction of locomotion. After one hour of favourable environmental conditions and in presents of food the worm exits in the state of dauer larvae [CASSADA and RUSSELL, 1975; SULSTON, 1988].

L3 Larvae

Over the whole stage of L3 and at the beginning of L4 the somatic precursors produce the final total cells, which form the anterior and posterior gonadal sheaths, the spermathecae and the uterus [KIMBLE and HIRSH, 1979]. During this stage of development the two sex myoblasts divide to generate 16 sex muscles

LITERATURE

and the vulval precursor fates are already specified, which committed cells divide to generate vulval terminal cells by early L4 stage [BURDINE et al., 1998].

L4 Larvae

The gonadogenesis which starts 7 hours after hatching is fully completed in this stage [ANTEBI et al., 1997]. In the proximal arms of gonad the meiosis in the germ line begins and the germ cells differentiate into mature sperm. In the later L4 stage the sperm production stops and the oocytes production begins. [GREENWALD, 1997].

Adult

After approximately 45 to 50 hours after hatching at 22°C to 25°C a hermaphrodite lays its first eggs, thus completing its 3 to 4 day reproductive life cycle [BYERLY et al., 1976; LEWIS and FLEMING, 1995]. The average maximal life span is about 15 to 20 days and the self-fertilization of a hermaphrodite is limited to about 300 progeny, because of the defined production of sperm. The number of progeny can be increased to 1200 to 1400 with a male participant. Males are able to produce 3000 progeny with hermaphrodites during their six fertile days [HODGKIN, 1988].

2.2 Folic acid and folate

Folic acid (vitamin B₉, vitamin B_c or folacin) and folate (natural occurring derivatives) are variants of the water-soluble vitamin B₉. Pteroyl-L-glutamic acid, pteroyl-L-glutamate, and pteroylmonoglutamic acid are synonymous forms of vitamin B₉ [BAILEY and AYLING, 2009]. It can be found in several leafy vegetables, legume, egg yolks, baker's yeast, fortified grain products and in sun flower as well in liver and kidney in relatively high concentration [KWAZULU-NATALI, 2010].

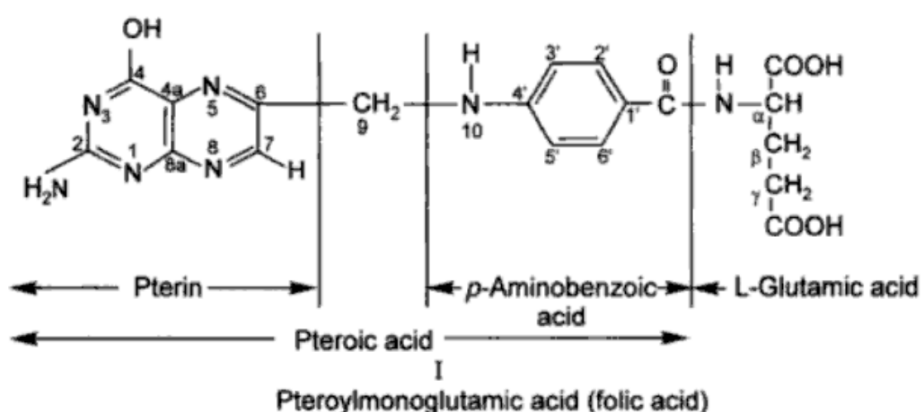


Fig. 5: Chemical structure of folic acid [BALL, 1998].

Generally folic acid itself is not biologically active and has to pass several metabolic pathways in human liver [BAILEY and AYLING, 2009]. For the human body vitamin B₉ (folic acid and folate) is essential to synthesise, repair and methylate DNA and it is also involved as cofactor in biological reactions [WEINSTEIN et al., 2003]. This essential vitamin is particularly relevant for cell division and growth, such as in infancy and pregnancy and also a growth factor in some cancers. Concerning the cancer genesis natural folates in high amounts decrease the risk of cancer, while several investigations indicated that a high intake of folate from supplementations may promote some cancers [KAMEN, 1997]. For a successful prevention of anemia and to synthesize healthy red blood cells folic acid will be required for both adults and children [FENECH et al., 1998].

2.2.1 Metabolism of folates

Folates act in a large number of metabolic reactions in case of one carbon transformation and results in activation of other molecules as well as oxidations and reductions of single carbons. Result of those specific pathways is the synthesis of DNA, RNA and also membrane lipids and neurotransmitter. The metabolism of folic acid starts with a reduction to dihydrofolate (DHF) and continues with a further catalysed reduction of DHF, by dihydrofolate reductase, to tetrahydrofolate (THF). NADPH acts as a cofactor for both reactions.

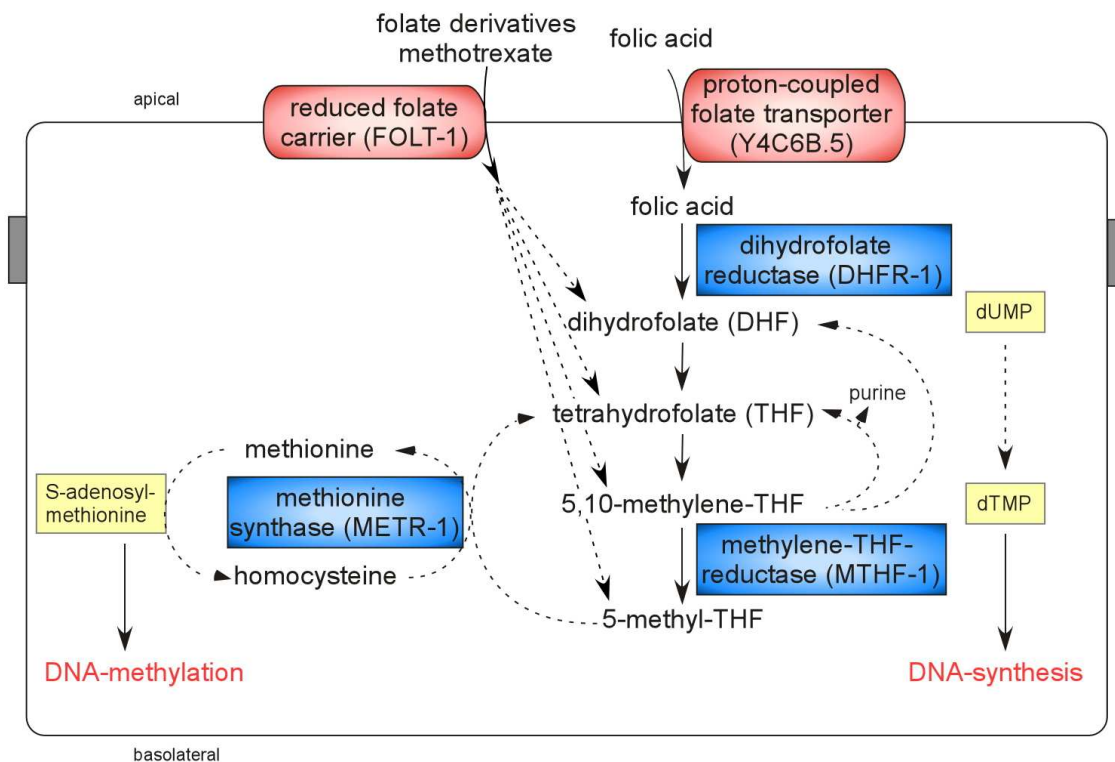


Fig. 6: Metabolic pathway of folic acid and folate [provided by J. Benner].

The graph shows the pathway of folic acid and its transformation into new products which finally cause the DNA-methylation and DNA-synthesis. Enzymes (DHFR-1, METR-1, DHFR-1), reduced folate carrier (FOLT-1) and the proton coupled folate transporter (Y4C6B.5) adwert to wildtype *C. elegans*.

Obtained THF acts as an acceptor of one carbon groups. It typically accepts methyl groups from serine. Hence, the main compound of the one carbon metabolism, described as N5,N10-methylene-tetrahydrofolate (Methylene-THF), will be synthesised. The chemical reaction of an addition of methylene groups from formaldehyde of serine or glycine to THF causes the development of methylene-THF [LEHNINGER, 2005]. The folic acid metabolism continues either with a directly donation of the single carbon group of methylene-THF or it can also be oxidised by NADP, which causes the methenyl form or reduced by NADH which leads to the methyl form [SALWAY, 2004].

Depending on the biosynthetic pathway defined metabolic products are able to donate their one-carbon group to an acceptor, which causes the synthesis of different essential compounds in the living organism. During the process of thymidine nucleotide synthesis the methyl group of methylene will be donated

for DNA synthesis, while donation of the methenyl form occurs during the purine biosynthesis [LEHNINGER, 2005]. The methyl form donates its methyl group to sulfur in case of methionine formation [SALWAY, 2004].

The synthesis of methyl THF is accomplished by a reduction with NADPH of the methylene group of methylene THF. Formyl THF or folinic acid can be formed on the one hand as a result of an oxidation of methylene THF [LEHNINGER, 2005] and on the other hand by donating the formyl group to THF [SALWAY, 2004]. Finally a single carbon can be donated to THF from histidine which causes the synthesis of methenyl THF [LEHNINGER, 2005, SALWAY, 2004].

2.2.2 Effects of folate deficiency

A folate deficiency can be caused by an increased demand for folate [PIETRZIK and THORAND, 1997] or an inadequate uptake [MCNULTY, 1995]. There exist also several medications that interfere with the organisms ability to absorb folate or increase the requirement for this vitamin [STOLZENBERG, 1994].

Human symptoms of a folate deficiency are loss of appetite and a possible weight loss. Further weakness, sore tongue, headaches, heart palpitations, irritability and also behavioral disorders can be caused by a folate deficiency [HASLAM and PROBERT 1998]. A folate depletion of pregnant women causes a higher risk to bear progeny with a number of congenital malformations, e.g. the neural tube defect [SHAW et al., 1995]. In other assays it was investigated that folate deficient mice show an ineffective haematopoiesis. There were also alterations, concerning the hematopoietic organs, detectable, which seem to be similar to those seen in folate deficient humans [BILLS et al., 1992]. Helm et al. used rats and rabbits and showed an impact of a folate deficiency on fertility and fetal development. The depletion of folate causes malformations like cleft palates, rarely cleft lips micrognathies and shortening of limbs, decreases in the number of pups and an increase in the number of absorption sites. Further the litter size and litter weight were reduced [HELM et al., 1976]. Besides influences on fertility and fetal development, a correlation between folate and extended life span could be assessed in *D. melanogaster* [BAUER et al., 2006].

2.2.3 Folate reaction of interfering drugs

The biosynthesis of THF (the biological active form of folate) can be affected by a number of drugs. Among them is the relatively large group of sulfonamides. One common example for such sulfonamide is sulfamethoxazole (SMX) [HERRINGTON and CHIRWA, 1999; ROLAND et al., 1979; SHIROKY, 1997].

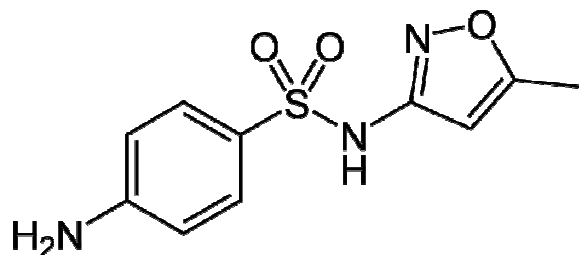


Fig. 7: Chemical structure of Sulfamethoxazole (SMX) [ACAR et al., 1973]. SMX is a bacteriostatic and antibiotic sulfonamide and also named 4-amino-N-(5-methylisoxazol-3-yl)-benzenesulfonamide [GARG. et al., 1986, MA et al., 2007].

Sulfamethxazole (SMX, SMZ) has bacteriostatic and antibiotic characteristics. [GARG. et al., 1986, MA et al., 2007]. SMX is often used in a synergistic combination with trimethoprim for antagonising against susceptible forms of *Streptococcus*, *Staphylococcus aureus* (including MRSA), *Escherichia coli*, *Haemophilus influenzae*, and oral anaerobes [MAY T. et al., 1994]. SMX competitively inhibits the dihydropteroate synthase, the first step of bacterial folate biosynthesis and secures a competition between p-amino benzoic acid (p-ABA) and SMX. In case of binding SMX no THF can be build. Hence, SMX treatment in bacteria leads to a concentration dependent reduction of intracellular folate, when there is no available folate outside the cell [ROLAND S. et al., 1979]. Thus, SMX can be used as an antibiotic, caused by its selective toxicity to bacterial cells.

2.2.4 Folic acid metabolism in *C. elegans*

Generally, three different systems are currently known in mammals to be responsible for folate uptake. The folate receptor family (FOLR) [TAPARIA S, 2007], which are found in mammals only, the reduced folate carrier (RFC, SLC19A1) [SIROTNAK and TOLNER, 1999] and the proton coupled folate

transporter (PCFT, SLC46A1) [QIU et al., 2006]. Balamurugan et al. have investigated homologous folate uptake systems in *C. elegans*. They found three genes, namely *folT-1*, *folT-2*, *folT-3* which might express proteins orthologous to the human reduced folate carrier (hRFC) which share an identity to hRFC sequence of 40, 31 and 20% respectively. The genes *folT-1* and *folT-2* were expressed in heterologous expression systems, but only FOLT-1 was shown to be an active folate transporter, mainly expressed at L1 larvae in the pharynx and the posterior portion of intestine and decline further with maturation. The uptake of folic acid via FOLT-1 could be competitively inhibited by structural analogues like folinic acid or 5-formyltetrahydrofolate (5-MFTHF) and also by methotrexate (MTX). Worms with *folT-1* knockout or RNAi knockdown have a significantly reduced folate uptake and the knockout leads to a frequent sterility of hermaphrodites and nearly one third of those have a small progeny, which are themselves sterile [AUSTIN et al., 2010]. The FOLT-1 uptake system is acidic pH dependent and has the similar affinity to oxidised, reduced and substituted folate derivatives comparable to the mammalian system RFC [BALAMURUGAN et al., 2007].

After treatment with an anti-inflammatory drug named sulfasalazine, an increase of expression of *folT-1* was detectable. By contrast an over supplementation with high pharmacological doses of folic acid leads to a significant decrease in the level of mRNA expression of *folT-1* and was then associated with a specific decrease in the level of folic acid uptake by wild type *C. elegans*. A folate deficiency was induced by feeding folate depleted MH829 *E. coli*, which correspond to MH951 *E. coli* [BALAMURUGAN et al., 2007]. MH828 bacteria are characterised through a *thyA* deficiency, so they are not able to synthesise thymine and thymidine. The process of synthesising thymidylat is necessary for recycling the dihydrofolate. Following by inhibition of this process no dihydrofolate can be recycled and used for the folate metabolism. MH951 bacteria have a *thyA* and additionally a *folA* deficiency. The coding region of *folA*, dihydrofolate reductase gene was substituted by a kanamycin resistance gene [HERRINGTON and CHIRWA, 1999].

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Instruments

Table 1: Instruments

Instruments	Description	Company
Pipettes	Eppendorf Reference variabel (10µl, 100µl und 1000µl)	Eppendorf AG (Hamburg, Germany)
Vortex	MS 1 Minishaker	IKA Werke GmbH und Co. KG (Wilmington, USA)
Inkubator	Thermomixer 5436	Eppendorf AG (Hamburg, Germany)
Centrifuge	Jouan BR4i multifunction centrifuge	Thermo Electron Corporation Key Write DTM (France)
Water quench	GFL-Inkubations-/Inaktivierungsbad 1002	Gesellschaft für Labortechnik GmbH (Burgwedel, Germany)
Scale	Satorius ISO 9001 Modell KB BA 100	Satorius AG (Göttingen, Germany)
pH-meter	Seven Easy TM, pH-Meter S20 with Inlab Routine per glass electrode	Mettler Toledo GmbH (Schwerzenbach, Switzerland)
Photometer	Cary 50 scan UV visible Spectrophotometer	Varian Medical Systems Gesellschaft m.b.H (Brunn am Gebirge, Austria)
Autoclav	Table Autoclav	CERTOclav (Austria)

MATERIALS AND METHODS

3.1.2 Chemicals and solutions

Table 2: Chemicals

Chemicals	Company
Glucose	Roth
Kanamycine	Roth
Sulfamethoxazole	Roth
Thymine	Roth
Folic acid	Sigma Aldrich

Table 3: Buffers and solutions

Bleaching Solution		
Substances	Amount	Company
NaOCl	0.5 ml	
5M KOH	1.25 ml	
adjust to 25 ml with distilled water		

DYT-Medium		
Substances	Amount	Company
Pepton	16.0 g	Roth
Bacto Yeast Extract	10.0 g	Roth
NaCl	5.0 g	Roth
adjust to 1000 ml with distilled water; sterilize by autoclaving		

M9 Buffer		
Substances	Amount	Company
Na ₂ HPO ₄ - 7H ₂ O	6.0 g	Roth
KH ₂ PO ₄	3.0 g	Roth
NaCl	5.0 g	Roth
adjust to 1000 ml with distilled water; sterilize by autoclaving		
1M MgSO ₄		Roth

M9 Minimal Medium with Glucose		
Substances	Amount	Company
M9 salt	200 ml	
1M MgSO ₄	2 ml	Roth
20% Glucose	20 ml	
1M CaCl ₂	100 µl	Roth
Mix all substances in 700 ml distilled water; adjust to 1000 ml with distilled water; agitate until all substances are fully dissolved		

M9 salt		
Substances	Amount	Company
Na ₂ HPO ₄ - 7H ₂ O	64.0 g	Roth
KH ₂ PO ₄	15.0 g	Roth
NaCl	2.5 g	Roth
NH ₄ Cl	5.0 g	Roth
Mix all substances in 800 ml distilled water; adjust to 1000 ml with distilled water; sterilize by autoclaving		

NGM-Agar		
Substances	Amount	Company
NaCl	3.0 g	Roth
Pepton	2.5 g	Roth
Agar	17.0 g	Roth
add 1000 ml dH ₂ O, after autoclaving add:		
1 M CaCl ₂	0.5 ml	Roth
1 M MgSO ₄	1.0 ml	Roth
Potassium phosphate buffer	25.0 ml	
Nystatin	4.0 ml	
Cholesterin	1.0 ml	Roth
for MH828 and MH951 bacteria 10 µl per ml NGM-Agar		
for MH951 bacteria 10µl kanamycin per ml NGM-Agar		

Nystatine		
Substances	Amount	Company
Nystatine	4.0 g	Roth
Solution A		
Ammoniumacetate	115.62 g	Roth
adjust to 200 ml with destilled water		
Solution B		
EtOH (96%, absol.)	200 ml	Roth
use a glass bottle and put in the Nystatine; use a magnetic stirrer with 50°C; add solution A and B carfully and slowly		

Potassium phosphate buffer		
Substances	Amount	Company
KH ₂ PO ₄	108.6 g	
K ₂ PO ₄	35.28 g	
adjust to 1000 ml with distilled water; use pH-meter and 5 M KOH to set the pH-value = 6		

MATERIALS AND METHODS

3.1.3 *E. coli* bacteria

Table 4: Used Escherichia coli bacteria

Bacteria			
bacteria	phenotype	description	purchased from
OP50	<i>ura⁻</i>	an uracil auxotroph strain of <i>E.coli</i>	CGC
MH828	thyA (ts) art3 RNA	<i>E. coli</i> bacteria with inhibited folate biosynthesis	donated by M. Herrington [HERRINGTON and CHIRWA, 1999]
MH951	thyA (ts) art3 RNA delta FoIA:kan3	<i>folA</i> (dihydrofolate reductase) deficient <i>E.coli</i> bacteria	donated by M. Herrington [HERRINGTON and CHIRWA, 1999]

3.2 Methods

3.2.1 General *C. elegans* handling

Only N2 var. Bristol wild type strain of *C. elegans* was used, which was fed with different *E. coli* bacteria like OP50, MH828 and MH951. The NGM agar plates were inoculated with fresh overnight culture a day before using. Then some worms from an old plate were transferred to a new plate. This procedure was repeated twice a week. The reference stocks were collected in a box and stored at -20°C.

Table 5: Overnight-cultures of bacteria

Overnight culture of OP50	
Substances	Amount
DYT-Medium	
OP50 bacteria	

Overnight culture of MH828	
Substances	Amount
LB-Medium	
thymine (5 mg/ml conc.)	10 µl/ml
MH828 bacteria	
Add MH828 bacteria to DYT-Medium with thymine and incubate overnight at 37°C	

Overnight culture of MH951	
Substances	Amount
LB-Medium	
thymine (5 mg/ml conc.)	10 µl/ml
kanamycin (3 mg/ml conc)	10 µl/ml
MH951 bacteria	
Add MH951 bacteria to DYT-Medium with thymine and kanamycin and incubate overnight at 37°C	

3.2.2 Freezing of bacteria

For all used *E. coli*. bacteria several glycerol-stocks were made, by mixing 1 vol fresh bacteria over night culture with 1 vol glycerol and stored at -20°C .

3.2.3 Folic acid supplementation

For the supplementation assays NGM agar plates were inoculated with 0.1 mM (final concentration) of folic acid, while the OP50, MH828 and MH951 bacteria were added onto the plates at the same day of usage. In case of MH951 the kanamycin was added a day before usage.

3.2.4 Synchronisation of a *C. elegans* population

Agar plates with a mixed population of young adult worms were washed with M9 buffer and centrifuged for 2 minutes at 2500 rpm at room temperature. The worms were washed several times with M9 buffer until the supernatant was completely clear. Afterwards, 3 ml of bleach solution were added (mixture 1:1), followed by 5 minutes of intensive shaking. The washing process with M9 buffer and centrifugation was repeated for 4 times. After the final washing the falcons were incubated over night to allow hatching of the L1 larvae. The following day the L1 worms were placed on fresh seeded NGM agar plates and grown until they reached L4 larval stage. These synchronised L4 worms were used for brood size and life span experiments.

3.2.5 Bacterial growth curve with SMX treatment

Generally a bacterial growth curve consists of 4 specific phases. The first phase is called lag phase, where bacteria prepare for further maturation and

MATERIALS AND METHODS

segmentation. In this experiment the lag phase lasts considerably longer than average with a duration of about 16 hours, because of using M9 minimal medium as food source. This phase is followed by the log phase (also exponential phase). During the log phase the bacterial reproduction happens exponentially until it reaches the stationary phase. The stationary phase is characterised by an equilibration of reproduction and dying of bacteria. The last phase finalises the bacterial growth curve, where bacteria die of an inadequate accommodation of nutrients.

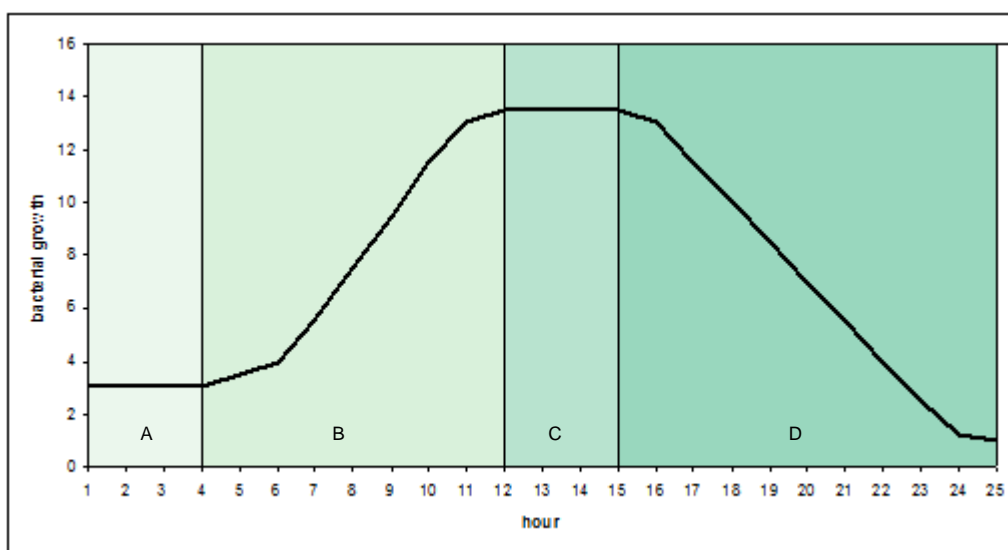


Fig. 8: Bacterial growth curve.

The graph shows the four different phases of a bacterial growth curve: lag phase (A), log or exponential growth phase (B), stationary phase (C) and death or logarithmic decline phase.

The bacterial growth curve with SMX-treatment was made to obtain the optimal SMX concentration, which inhibits the *de novo* folate synthesis of *E. coli* bacteria. This restricted folate synthesis let the bacteria barely grow and causes finally a folate depletion in wild type *C. elegans*. In this experiment we were seeking for the SMX concentration which drastically reduces reproduction of the bacteria (reduced log phase) as an indicator of folate deficiency, but did not completely inhibit growth, because certain amount of bacteria is needed to feed the worms.

For this experiment different concentrations of 0 μ M, 0.1 μ M, 1 μ M, 10 μ M and 100 μ M SMX in M9 minimal medium with glucose were used. To eliminate an additional folate source, DYT medium was substituted for M9 minimal medium with glucose.

For sample preparation 5 ml of an overnight bacterial OP50 culture was pipetted into a falcon and finally filled up with 25 ml M9 minimal medium with glucose. After incubating over night the samples grow further in the water bath at 37°C.

The first measure was made at hour zero. The lag phase took 16 hours. So further measurements started with hour 16 and were repeated every hour until hour 24. All measurements were made with the photometer at 578 nm.

3.2.6 Number of progeny of *C. elegans*

The brood size experiment was performed to identify the influence of folate depletion or folate supplementation on the reproduction rate of single wild type *C. elegans*. Therefore at least 36 synchronised L4 larvae were singled on 12-well agar plates seeded with corresponding bacteria. During the egg laying period (approximately 4 days) the worms were once transferred to fresh 12-well plates. The number of progeny of each worm was counted. The experiment was performed independently at least two times. Statistical analysis was performed with GraphPad Prism 5 demo.

3.2.7 Life span of *C. elegans*

To investigate the influences of folate depletion and folate supplementation on the life span of wild type *C. elegans* the life span experiment was accomplished. For this purpose approximately 35 synchronized L4 larvae per group were transferred on three NGM agar plates, which were seeded with bacteria as food. During the first week the worms were moved to fresh agar plates daily, afterwards every two or three days. For analysing the life span, the living and dead worms were counted every day. The experiment was performed

MATERIALS AND METHODS

independently at least two times. For the statistical analysis Graph Pad Prism 5 demo and Excel 2003 were used.

4 RESULTS

This thesis was performed to analyse phenotypic characteristics of a folate depletion in *C. elegans*. The lack of folate could be induced by feeding folate depleted *E. coli* bacteria, as previously shown [HERRINGTON and CHIRWA, 1999] or with a new method using sulfamethoxazole (SMX), an inhibitor of folate *de novo* biosynthesis in bacteria. Provided that the lack of folate induces differences in brood size and/or life span further investigations will be performed to find out, whether the effect of a folate depletion could be compensated by folic acid supplementation.

4.1 Comparison of brood size and life span of wild type *C. elegans* with living or dead bacteria as food source

The first step in this thesis was to decide, whether dead bacteria could be used as a food source. Dead bacteria should be used for following supplementation studies, due to its disability to take up folate and metabolise it. Hence, it can be assumed that the supplemented folate could be only taken up by the worms.

To use dead bacteria it was necessary to assess, whether dead *E. coli* as food source have any affects on wild type *C. elegans*, concerning their number of progeny and life span.

4.1.1 Brood size and life span of wild type *C. elegans* fed with living and dead OP50 *E. coli*

To find out, if there are phenotypic differences between wild type worms, which are fed with living or with dead OP50 *E. coli* a brood size (Fig. 9) and a life span (Fig. 10) assay were performed. To kill the bacteria they were heated up to 60°C for 1 hour.

RESULTS

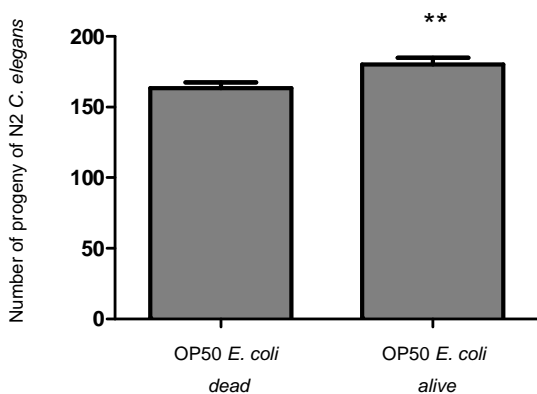


Fig. 9: Brood size of wild type *C. elegans* fed with living and dead OP50 *E. coli* bacteria.

The number of progeny of *C. elegans* fed with living or heat killed OP50 *E. coli* of 25-35 worms per experiment was counted. The experiment was performed with four biological replicates and the statistical analysis was performed by a Student's *t*-Test with significance denoted as ** $p < 0.01$.

Wild type *C. elegans* fed with living OP50 *E. coli* have a brood size of 163 ± 4 hatched larvae, whereas it was increased to 180 ± 5 when N2 worms were grown on heat killed OP50 *E. coli*. Hence, there is a significant difference caused by alteration in food source.

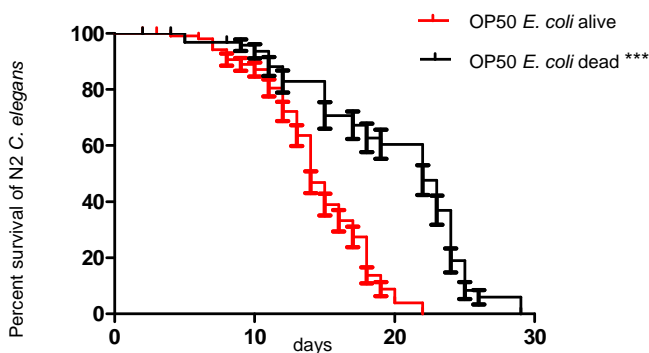


Fig. 10: Life span of wild type *C. elegans* fed with living and dead OP50 *E. coli* bacteria.

The life span experiment of *C. elegans*, grown on living and heat killed OP50 *E. coli* was performed with two biological replicates. Each investigation included three replicates with 30-40 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test. The significance is denoted as *** $p < 0.001$.

Wild type *C. elegans* fed with living OP50 *E. coli* demonstrate a median survival of 14 days and a maximal survival of 22 days [HODGKIN, 1984, BALAMURUGAN et

al., 2007]. By contrast, the median survival of worms fed with dead OP50 is 22 days and 29 days maximal survival. Therefore feeding heat killed OP50 *E. coli* significantly increase the number of progeny and also life span of wild type *C. elegans* compared with control worms.

4.1.2 Brood size and life span of wild type *C. elegans* fed with living and dead MH828 and MH951 *E. coli*

Obtained results confirmed a significant difference in brood size and life span of *C. elegans* grown on living and dead OP50 *E. coli*. Hence, it was also investigated if there were similar effects on the number of progeny and life span of *C. elegans* fed with living and dead MH828 and MH951 *E. coli*.

Both bacterial strains have a *thyA* deficiency and the MH951 *E. coli* have an additional *folA* knockout. Neither a brood size nor a life span experiment with heat-killed MH828 and MH951 *E. coli* could be performed, because wild type worms grown on these dead bacteria show a larval arrest or develop to dauer larvae. Due to this knowledge and to obtain comparable results it was decided that all following assays were solely performed with living bacteria.

4.2 Folate depletion induced by feeding dihydrofolate reductase deficient *E. coli* to wild type *C. elegans*

To cause folate depletion in wild type *C. elegans* two strains of *E. coli* bacteria were used. They are called MH828 and MH951. Both have a *thyA* (thymidylate synthase) deficiency and additionally the coding region of the *folA* (dihydrofolate reductase) gene of MH951 *E. coli* was replaced by a kanamycin resistance. MH951 *E. coli* are genetically identical to MH829 used by Balamurugan et al. (personal communication to M. Herrington). Hence, it is predicted that feeding MH951 *E. coli* to *C. elegans* induce a folate deficiency which was also predicted by another study [BALAMURUGAN et al., 2007].

RESULTS

4.2.1 Brood size of wild type *C. elegans* fed with OP50, MH828 and MH951 *E. coli* bacteria

In this assay it was assessed, if there are significant differences in the number of progeny between wild type *C. elegans* fed with OP50, MH828 and MH951 *E. coli*.

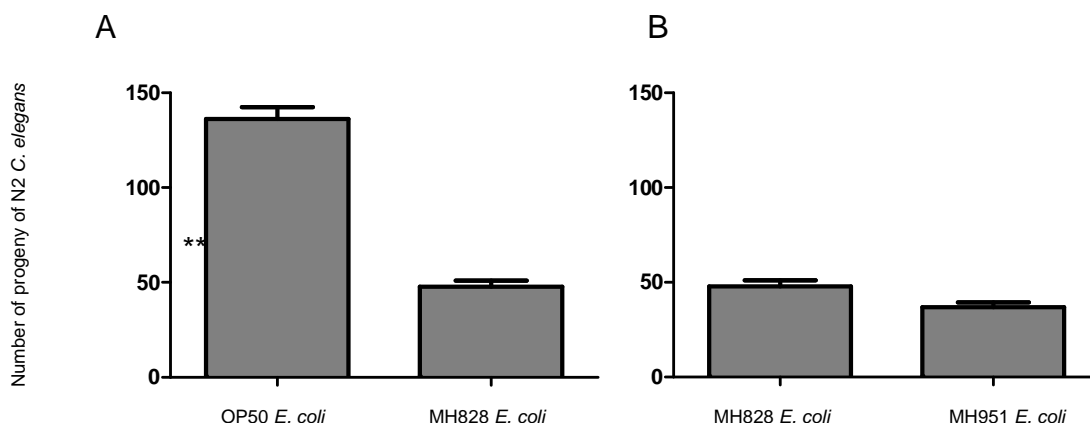


Fig. 11: Brood size of wild type *C. elegans* fed with OP50, MH828 and MH951 *E. coli*

The number of progeny of *C. elegans*, fed with (A) OP50 and MH828 *E. coli* and (B) MH828 and MH951 *E. coli* of 24-34 worms per experiment was counted. The experiment was performed with two biological replicates and the statistical analysis was performed by a Student's *t*-Test with significance denoted as *** $p < 0,001$

Wild type *C. elegans* grown on OP50 *E. coli* had a brood size of 136 ± 6 whereas the number of progeny of MH828 fed worms was significantly decreased (50 ± 3) (Fig. 11 A). The comparison of the number of hatched larvae between nematodes fed with MH828 and MH951 (37 ± 3) *E. coli* (Fig. 11 B) show that there was no significant effect induced by folate depletion.

4.2.2 Life span of wild type *C. elegans* fed with OP50, MH828 and MH951 *E. coli* bacteria.

It was determined, whether there were significant differences in life span between wild type *C. elegans* fed with OP50 *E. coli* bacteria and worms grown on MH828 (Fig. 12) and MH951 *E. coli* (Fig. 13)

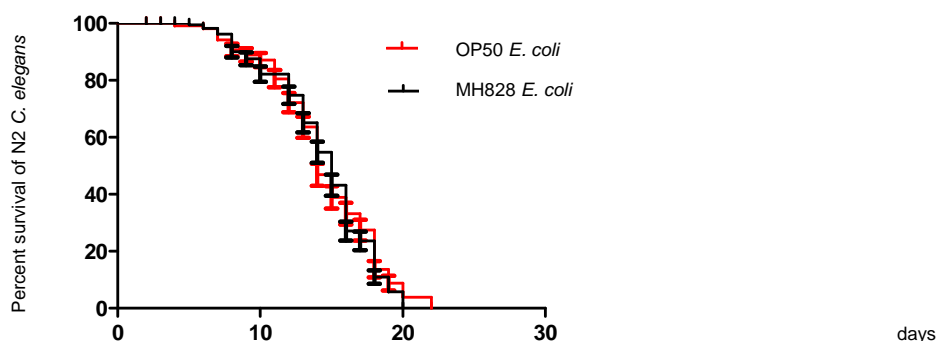


Fig. 12: Life span of wild type *C. elegans* fed with OP50 and MH828 *E. coli*.

The life span experiment of *C. elegans* grown on OP50 (red line) or MH828 (black line) bacteria was performed with two biological replicates. Each investigation included three replicates with 24-30 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test.

The median survival of *C. elegans* grown on OP50 *E. coli* was 16 days, while nematodes developed on MH828 *E. coli* had a median survival of 15 days. No significant difference between *C. elegans* fed with OP50 and MH828 *E. coli* was remarkable.

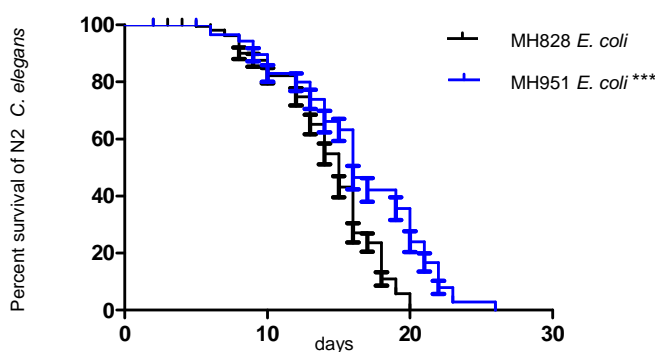


Fig. 13: Life span of wild type *C. elegans* fed with MH828 and MH951 *E. coli*.

The life span experiment of *C. elegans*, grown on MH828 (black line) and MH951 (blue line) bacteria was performed with two biological replicates. Each investigation included three replicates with 24-30 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test. The significance is denoted as *** $p < 0.001$.

The survival curve shows a median survival of 15 days for *C. elegans* fed with MH828 *E. coli*, while it was significantly increased to 16 days for worms grown on MH951 *E. coli*. Also maximal life span was extended from 20 days to 26 days for MH951 fed worms compared with MH828 control *C. elegans*. Hence,

RESULTS

folate depletion seems to increase median as well as maximal life span in *C. elegans*.

The number of progeny of nematodes grown on MH828 and MH951 bacteria was drastically reduced compared with the brood size of worms grown on the normal food source OP50, whereby folate depletion by feeding MH951 bacteria had no additional effect. By contrast, the change in bacterial food source had no effect on life span, but folate depletion seemed to elongate life span of *C. elegans*.

4.3 Folate depletion in wild type *C. elegans* induced by feeding OP50 *E. coli* with inhibited folate biosynthesis

Besides using *folA* knockout bacteria, inhibition of folate *de novo* biosynthesis in *E. coli* and feeding these bacteria to *C. elegans* is a new possibility to induce a folate deficiency.

4.3.1 Inhibition of folate biosynthesis in OP50 *E. coli*

A new way to induce a folate depletion in worms was established, whereby *E. coli* with inhibited folate biosynthesis were fed to wild type worms. The lack of folate in the bacteria was induced with the use of the sulfonamide sulfamethoxazole (SMX). SMX competitively inhibits the dihydropteroate synthase, the first step of bacterial folate biosynthesis and secures a competition between p-amino benzoic acid (p-ABA) and SMX. In case of binding SMX no folate can be build. Hence, SMX treatment in bacteria leads to a concentration dependent reduction of intracellular folate, when there is no available folate outside the cell. It was shown previously that this lack of folate inhibits bacterial growth [ROLAND S. et al., 1979]. To assess which concentration of SMX is necessary to clearly reduce growth of OP50 *E. coli* as an indicator of folate deficiency, but does not completely inhibit it, a bacterial growth curve experiment with different SMX concentration was performed.

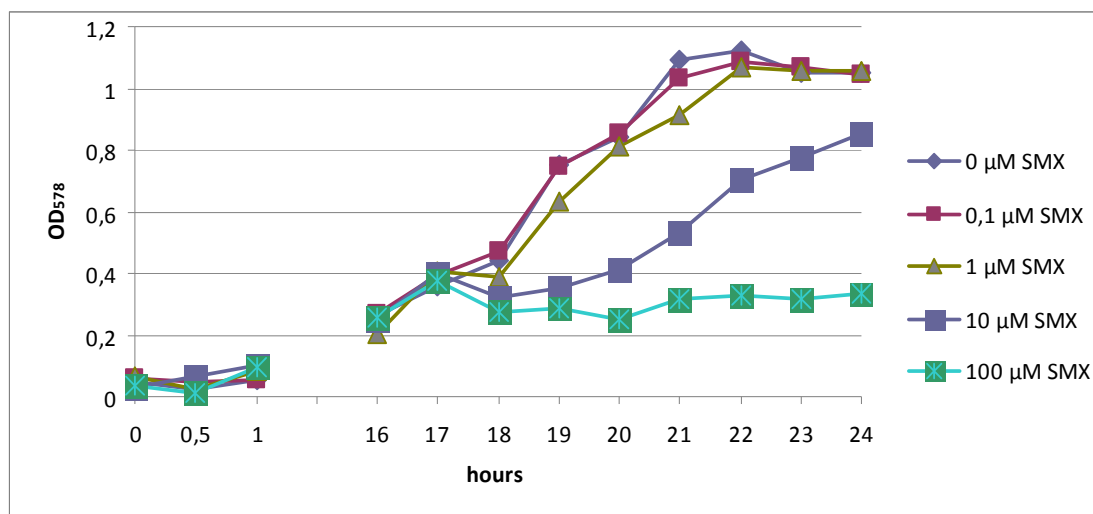


Fig. 14: Growth curve of OP50 *E. coli* treated with different SMX concentrations.

The graph shows growth curves of OP50 bacteria in M9 minimal medium with glucose with 0 μM (control), 0.1 μM , 1 μM , 10 μM and 100 μM SMX measured over a period of 24 hours. For every time point two replicates per group were measured at 578 nm. The experiment was performed independently 3 times.

This assay was performed with minimal medium instead of DYT medium, because DYT contains much folate which could be taken up by the bacteria and counteract endogenous folate depletion. The growth curve for OP50 bacteria treated with 10 μM SMX shows the favoured effect, namely a reduced growth, induced by folate depletion. By contrast a concentration of 100 μM SMX demonstrates a completely inhibited growth, whereas lower concentration did not affect bacterial growth.

4.3.2 Brood size of wild type *C. elegans* fed with OP50 bacteria with inhibited folate biosynthesis

Previous results showed a decreased bacterial growth when incubated with 10 μM SMX and therefore predicted folate depletion. Hence, bacteria treated with 10 μM SMX were used as food for *C. elegans* to induce a folate depletion in all following experiments like brood size (Fig. 15) and life span (Fig. 16, Fig. 17).

RESULTS

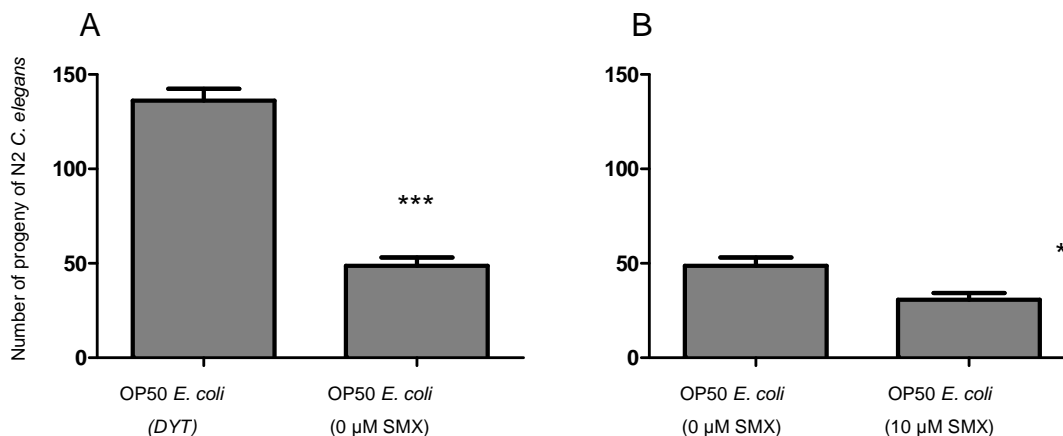


Fig. 15: Brood size of wild type *C. elegans* fed with SMX-treated *OP50 E. coli*.

The number of progeny of *C. elegans* fed with (A) *OP50 E. coli* (DYT) grown in full medium and *OP50 E. coli* (0 μ M SMX) grown in minimal medium without SMX and (B) *OP50 E. coli* (0 μ M SMX) = control and *OP50 E. coli* (10 μ M SMX) grown in minimal medium with 10 μ M SMX were investigated. The number of progeny of *C. elegans* of 21-27 worms per group was counted. The experiment was performed with two biological replicates and the statistical analysis was performed by a Student's *t*-Test with significance denoted as * p <0.05, *** p <0.001.

The results show that wild type worms fed with *OP50 E. coli* grown in minimal media (0 μ M SMX) have a significantly reduced reproduction of 49 ± 4 hatched larvae compared with *C. elegans* grown on *OP50* in full media (DYT) (Fig. 15 A). In contrast to the brood size experiment of *C. elegans* fed with *OP50 E. coli* grown in minimal media (0 μ M SMX), the number of progeny of folate depleted nematodes (10 μ M SMX) display a significant decline with 31 ± 4 hatched larvae (Fig. 15 B).

4.3.3 Life span of wild type *C. elegans* fed with *OP50* bacteria with inhibited folate biosynthesis

Besides reproduction in a next experiment the influence of SMX-treated *OP50* bacteria on median and maximal life span of wild type *C. elegans* was investigated (Fig 16 and Fig. 17).

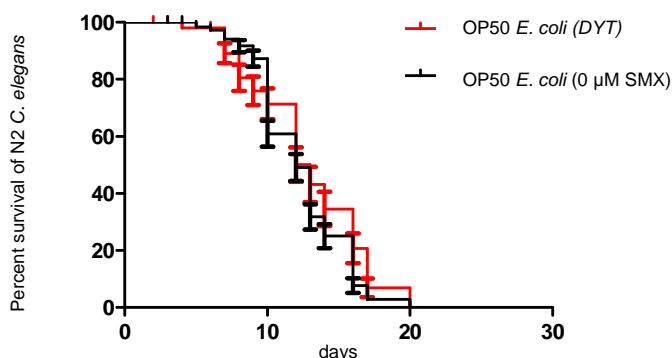


Fig. 16: Life span of wild type *C. elegans* fed with OP50 *E. coli* (DYT) and OP50 *E. coli* (0 μ M SMX).

The life span experiment of *C. elegans*, grown on OP50 *E. coli* (DYT) (red line) or OP50 *E. coli* (0 μ M SMX) (black line) was performed with two biological replicates. Each investigation included three replicates with 32-56 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test.

The median survival of the nematodes developed on OP50 *E. coli* grown in full media was 13 days with a maximal survival of 20 days, while 50 % of the worms grown on OP50 *E. coli* (0 μ M SMX) were dead within 12 days. The maximal survival in this group was 20 days. Hence, there were no significant differences in life span observable.

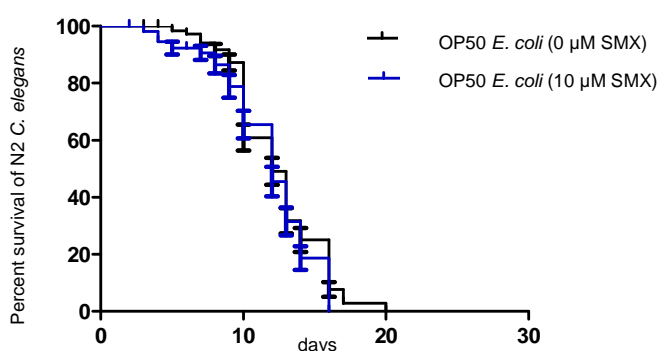


Fig. 17: Life span of wild type *C. elegans* fed with OP50 *E. coli* (0 μ M SMX) and OP50 *E. coli* (10 μ M SMX).

The life span experiment of *C. elegans*, grown on OP50 *E. coli* (0 μ M SMX) (black line) and OP50 *E. coli* (10 μ M SMX) (blue line) was performed with two biological replicates. Each investigation included three replicates with 47-56 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test.

RESULTS

Feeding of OP50 *E. coli* with 0 and 10 μ M SMX had no influence on life span in wild type *C. elegans*. The median survival for both groups was 12 days. Only maximal life span was a little bit longer (20 days) when worms were fed with OP50 (0 μ M SMX) compared with 16 days during feeding OP50 *E. coli* (10 μ M SMX).

These findings suggest that there is no significant difference in life span of wild type *C. elegans* fed with OP50 *E. coli* (DYT), OP50 *E. coli* (0 μ M SMX) and OP50 *E. coli* (10 μ M SMX) in contrast to the number of progeny, which is significantly decreased in case of a folate depletion.

4.4 Phenotypic characteristics of folate supplementation concerning the number of progeny and life span of wild type *C. elegans*

As described above, there are two possible ways to induce a folate depletion in wild type *C. elegans*. On the one hand feeding *thyA* deficient MH828 *E. coli* and *thyA*, *folA* deficient and kanamycin resistant MH951 *E. coli* bacteria and on the other hand feeding OP50 bacteria with SMX-inhibited folate *de novo* biosynthesis.

The following experiments determine the influences of compensating effects of folic acid supplementation on life span and brood size of *C. elegans*, while an exogenous induced folate depletion exist.

In a preliminary experiment it was tested, whether the folic acid solvent potassium hydroxide (final conc. 50 mM KOH) per se had an influence on life span of *C. elegans* (Fig. 18).

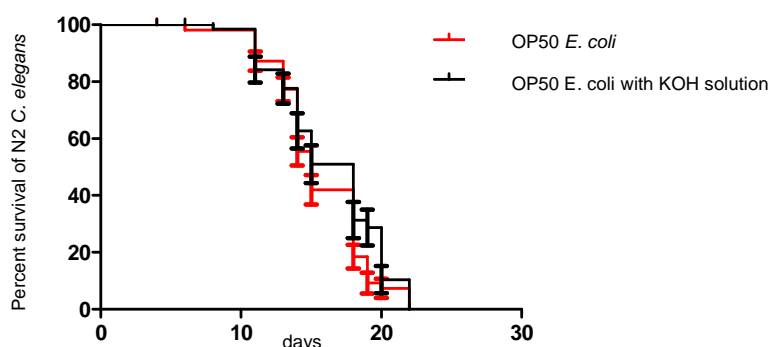


Fig. 18: Life span of wild type *C. elegans* fed with OP50 *E. coli* and OP50 *E. coli* with 50 mM KOH solution.

The life span experiment of *C. elegans*, grown on OP50 *E. coli* (red line) and OP50 *E. coli* with 50 mM KOH solution (black line) was performed with two biological replicates. Each investigation included three replicates with 35-46 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test.

Wild type *C. elegans* grown on OP50 *E. coli* demonstrate a median survival of 15 days, while it was 14 days for worms fed with OP50 *E. coli* with KOH solution. No difference shows the maximal life span with 22 days for both cases. Therefore no significant differences in life span can be explored in this experiment and KOH as a solvent can be used for following investigations.

4.4.1 Brood size of folate depleted wild type *C. elegans* with folic acid supplementation

To obtain data about the number of progeny of folate depleted wild type *C. elegans* living under supplemented conditions (0.1 mM folic acid) brood size experiments were performed. A comparison of OP50 *E. coli* with and without folic acid supplementation (Fig. 19), differences between supplemented and non-supplemented MH828 and MH951 (Fig. 20) and also between supplemented and non-supplemented OP50 treated with SMX (Fig. 21) were explored.

RESULTS

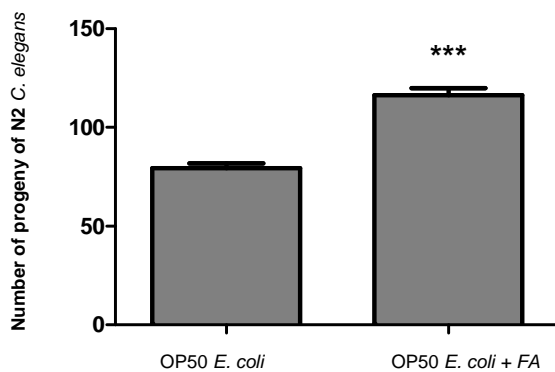


Fig. 19: Brood size of wild type *C. elegans* fed with OP50 *E. coli* and OP50 *E. coli* with 0.1 mM folic acid.

The number of progeny of *C. elegans* fed with OP50 *E. coli* without and with 0.1 mM folic acid (FA) supplementation of 25-29 worms per experiment was counted. The experiment was performed with two biological replicates and the statistical analysis was performed by a Student's *t*-Test with significance denoted as *** $p < 0.001$.

Wild type *C. elegans* fed with OP50 *E. coli* bacteria had a brood size of 79 ± 2 hatched larvae. By contrast, the number of progeny of worms grown on OP50 *E. coli* supplemented with 0.1 mM folic acid, show a significant increase in reproduction (116 ± 4). These results must be observed with caution, because the number of progeny of not supplemented *C. elegans* showed a lower quantity compared with those in previous experiments and compared with values in the literature [HODGKIN, 1984].

In a next experiment the influence of folic acid supplementation on reproduction of worms grown on MH828 and MH951 bacteria were investigated (Fig. 20).

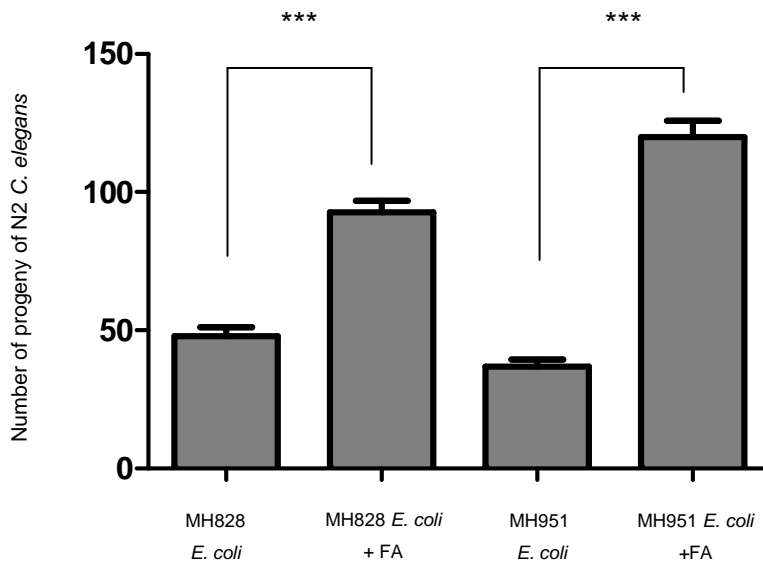


Fig. 20: Brood size of wild type *C. elegans* fed with MH828 *E. coli* and MH951 *E. coli* bacteria with and without folic acid supplementation.

The number of progeny of *C. elegans* fed with MH828 and MH951 *E. coli* with and without 0.1 mM folic acid (FA) supplementation with 20-33 worms per group was counted. The experiment was performed with two biological replicates and statistically analysed by an One-Way-ANOVA. The significance is denoted as *** $p < 0.001$.

The comparison of *C. elegans* grown on MH828 *E. coli* with 48 ± 3 hatched larvae and supplemented worms fed with MH828 *E. coli* with 93 ± 4 , showed a significant increase in brood size. The same effect was also observable in the case of MH951 with a progeny of 37 ± 3 without and 120 ± 6 with folic acid supplementation.

RESULTS

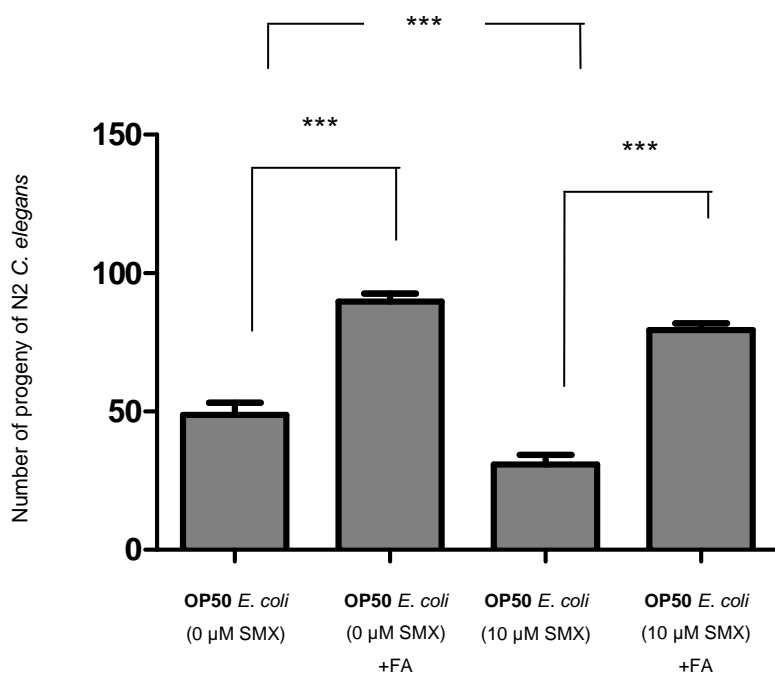


Fig. 21: Brood size of wild type *C. elegans* fed with SMX-treated OP50 *E. coli* with and without folic acid supplementation.

The number of progeny of *C. elegans*, fed with OP50 *E. coli* treated with 0 and 10 μM SMX with and without supplementation of 0.1 mM folic acid (FA) of 13-28 worms per experiment was counted. The experiment was performed with two biological replicates and the statistical analysis was performed by an One-Way-ANOVA and Turkey Post-Test with significance denoted as *** $p < 0.001$.

C. elegans fed with OP50 *E. coli* (0 μM and 10 μM SMX) demonstrates a progeny of 49 ± 4 and 31 ± 4 respectively. Supplementation with 0.1 mM folic acid leads to a considerable increase of the brood size of *C. elegans*. In case of 0 μM SMX the progeny raise up to 90 ± 3 larvae and of 10 μM SMX an increase to 79 ± 4 hatched larvae is observable.

Although folic acid deficiency induced by feeding MH951 bacteria did not influence reproduction, folic acid supplementation caused in both cases (MH828 and MH951) an obvious increase in the number of progeny. Folate depletion evoked by feeding SMX-treated bacteria lead to a decreased number of larvae, whereas folic acid supplementation also over-compensated this effect.

4.4.2 Life span of folate depleted wild type *C. elegans* with folic acid supplementation

Life span investigations were performed to analyse, if any compensational effects through supplementation of folic acid on wild type *C. elegans* with an induced folate deficiency, could be observed. First the life span of worms fed with OP50 *E. coli* with and without exogenous folate source (Fig. 22), secondly with MH828 or MH951 *E. coli* with and without supplementation (Fig. 23 and Fig. 24), third with OP50 *E. coli* incubated with 0 μ M and 10 μ M SMX with and without additional folic acid (Fig. 25 and Fig. 26) was determined.

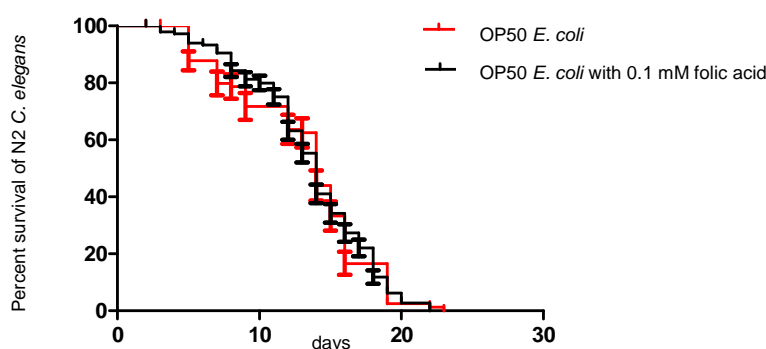


Fig. 22: Life span of wild type *C. elegans* fed with OP50 *E. coli* and OP50 *E. coli* with 0.1 mM folic acid.

The graph shows the survival curve of wild type *C. elegans* grown on OP50 *E. coli* (red line) and OP50 *E. coli* with 0.1 mM folic acid dissolved in 50 mM KOH (black line). The assay was performed twice in triplicates, each time with 25-43 worms per triplicate. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test.

Wild type *C. elegans* grown on OP50 *E. coli* demonstrate a maximal life span of 22 days, while worms supplemented with 0.1 mM folic acid was 23 days. No difference shows the median survival with 14 days for both cases. Hence, no significant differences in maximal and median life span were observable.

RESULTS

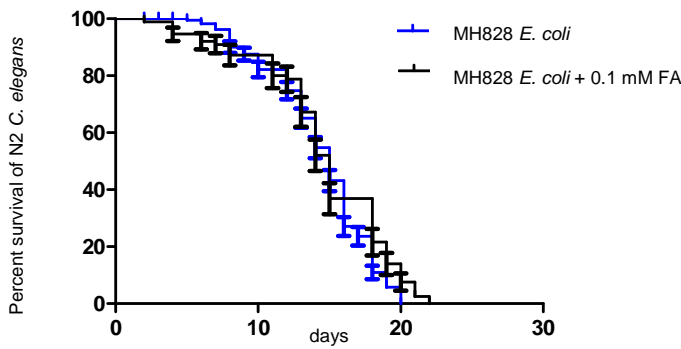


Fig. 23: Life span of *C. elegans* fed with MH828 *E. coli* with and without 0.1 mM folic acid.

The life span experiment of *C. elegans*, grown on MH828 *E. coli* (blue line) and MH828 *E. coli* with 0.1 mM folic acid (FA) (black line) was performed once in case of MH828 + 0.1 mM FA and twice in the other case. Each investigation included three replicates with 40-48 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test.

The survival curve demonstrates a median survival of 15 days for *C. elegans* fed with MH828 *E. coli* either with or without supplemented folic acid. Only the maximal life span elongates under supplementation from 20 days to 22 days. Hence, folate depletion has neither a significant influence on median survival nor on maximal life span.

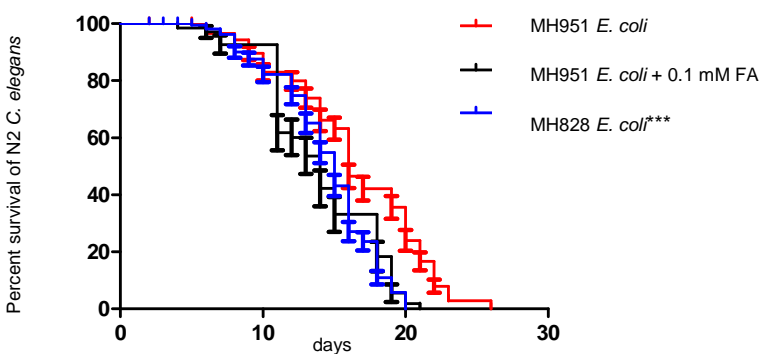


Fig. 24: Life span of *C. elegans* fed with MH828 and MH951 *E. coli* with and without 0.1 mM folic acid.

The life span experiment of *C. elegans*, grown on MH951 *E. coli* (red line), MH951 *E. coli* with 0.1 mM folic acid (black line) and MH828 *E. coli* (blue line) was performed once in case of MH951 *E. coli* + 0.1 mM FA and twice in the other both cases. Each investigation included three replicates with 27-73 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test. The significance is denoted as *** $p < 0.001$.

Wild type *C. elegans* grown on MH951 *E. coli* display a median survival of 16 days, while worms fed with additionally 0.1 mM folic acid live in average only 14 days. The maximal life span decreases under supplemented conditions from 26 days without supplementation to 21 days with supplementation. Both the median survival and the maximal life span of MH951 fed worms decline significantly and reach a value comparable to MH828 control. Therefore a compensating effect of supplementation of *C. elegans* fed with MH951 *E. coli* was observable.

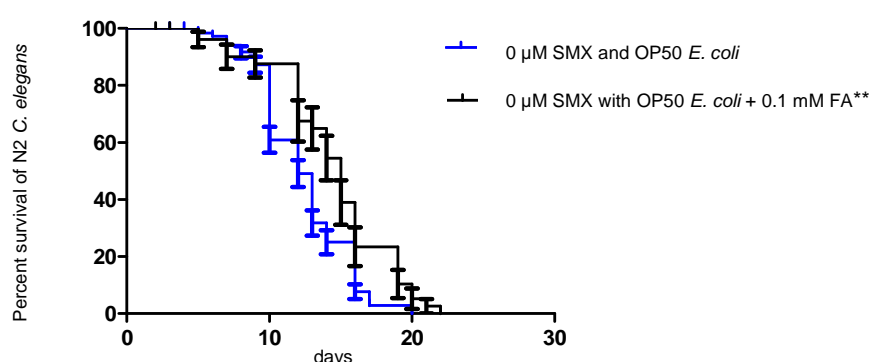


Fig. 25: Life span of *C. elegans* fed with 0 μ M SMX-treated OP50 *E. coli* with and without 0.1 mM folic acid.

The life span experiment of *C. elegans*, grown on OP50 *E. coli* with 0 μ M SMX (black line) and 0 μ M SMX + 0.1 mM folic acid (FA) (blue line) was performed once in case of OP50 (0 μ M SMX) + 0.1 mM FA and twice in the other case. Each investigation included three replicates with 20-56 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test. The significance is denoted as ** $p < 0.01$.

Wild type worms fed with OP50 *E. coli* (0 μ M SMX) with and without supplementation of folic acid show significant differences in median survival, which was 12 days and 15 days respectively. The maximal life span of *C. elegans* elongates from 20 to 22 days under supplemented conditions. Thus, a significant difference in life span of *C. elegans* grown on OP50 *E. coli* (0 μ M SMX) with and without additional folic acid is observable in this investigation.

RESULTS

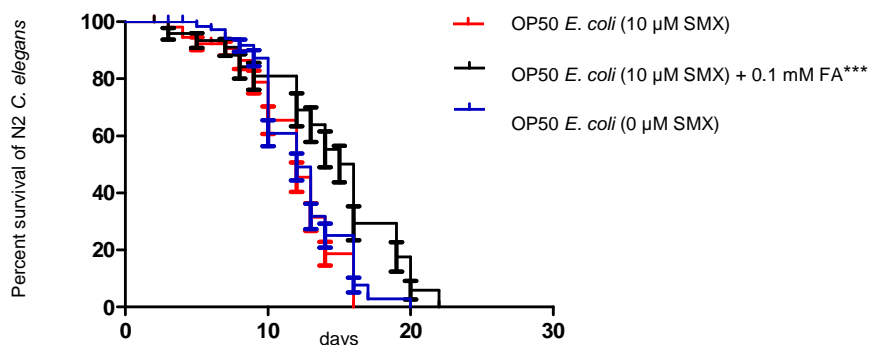


Fig. 26: Life span of wild type *C. elegans* fed with 0 and 10 μ M SMX-treated OP50 *E. coli* with and without 0.1 mM folic acid.

The life span experiment of *C. elegans*, grown on OP50 *E. coli* (10 μ M SMX) (red line) and OP50 *E. coli* (10 μ M SMX) with 0.1 mM folic acid (black line) and OP50 *E. coli* (0 μ M SMX) was performed once (OP50 *E. coli* (10 μ M SMX) + 0.1 mM FA) and twice in the other both cases with 28-47 worms per group. Denoted is the Kaplan-Meier-Survival-Curve and the significance was statistically analysed with a Log-Rank-Test. The significance is denoted as *** $p < 0.001$.

Significant differences of the median survival between OP50 *E. coli* (10 μ M SMX) with (16 days) and without (12 days) 0.1 mM folic acid, are noticeable. Also the maximal life span is extended from 16 days under non-supplemented conditions to 22 day during folic acid supplementation. Therefore, a remarkable increase in both, the median survival and maximal life span, are confirmed. The graph also demonstrates a regressive tendency of life span, comparing *C. elegans* fed with OP50 *E. coli* (10 μ M SMX) with 0.1 mM folic acid and OP50 *E. coli* (0 μ M SMX).

In conclusion, the elongated life span induced by feeding MH951 *E. coli* bacteria (folate deficiency) could be rescued by adding exogenous folic acid to the control level (MH828). By contrast, folate deficiency induced by feeding SMX-treated *E. coli* causes no alterations in life span, whereby folic acid supplementation in both cases increases life span to the same extent.

5 DISCUSSION

Folic acid is an essential vitamin for human metabolism and will be taken up in a biologically inactive form. After activation in the liver [BAILEY and AYLING, 2009] it acts as a cofactor in several biological reactions [WEINSTEIN et al., 2003]. To avoid negative effects caused by a folate deficiency it is important to investigate interrelated pathways and mechanisms, but the current state of science is not able to fully explore them in the human organism, because of its complexity. Hence, scientific research started with model organisms like *C. elegans*.

In *C. elegans* only little is known about folate uptake and folate metabolism. It was previously shown that FOLT-1 is an orthologous protein to the human reduced folate carrier (hRFC) and shares a 40% identity. The expression of mammalian RFC as well as *folt-1* are reduced by high extracellular substrate levels. Comparable to mammalian RFC, FOLT-1 is acidic pH dependent and has a similar affinity to oxidised, reduced and substituted folate derivatives. Further, a gene knockout or RNAi knockdown of the *folt-1* gene induces a remarkable inhibition of folate uptake system by intact living *C. elegans* [BALAMURUGAN et al. 2007].

Blast results suggest that there exist homologues of all key enzymes of mammalian folate metabolism, e.g. dihydrofolate reductase (*dhfr-1*), methylenetetrahydrofolate reductase (C06A8.1) in *C. elegans*, but their function and especially the influence of a folate deficiency in worms was not investigated in detail.

5.1. Two ways to induce a folate deficiency in *C. elegans*

A folate deficiency may have various negative effects on unborn humans and animals. Folate deficient pregnant women have a higher risk of bearing progeny with different congenital malformations, e.g. the neural tube effect [SHAW G. M. et al., 1995]. It is also suggested that a folate deficiency has an impact on life span of several model organisms like mice, rats and drosophila [BAUER et al., 2006, MILLER et al., 2005]. Hence, a folate deficiency has a gravid effect on progeny of human and animals. To avoid that large number of malformations, diminishing

DISCUSSION

effects on fertility and fetal development and partly reduced life span, it is important to investigate folate dependent mechanisms in detail. Hence, this thesis investigates the influence of two ways of folate depletion on reproduction and life span in *C. elegans*.

Induction of a folate deficiency in *C. elegans* was performed by using the modified *E. coli* strains MH828 and MH951. MH828 bacteria have a *thyA* deficiency, so they are not able to synthesise thymine and thymidin. The process of synthesizing thymidylat is nessecary for recycling the dihydrofolate. Because of the knockout of *thyA* no dihydrofolate can be recycled and used for the folate metabolism. To counteract thymine and thymidine deficiency, thymine was added into the medium and on NGM-Agar plates. MH951 bacteria have a *thyA* and additionally a *folA* deficiency. The coding region of *folA*, encoding the dihydrofolate reductase, was substituted by a kanamycin resistance gene. Hence, the MH951 bacteria are not able to synthesise THF (tetrahydrofolate) within the framework of *folA* [HERRINGTON and CHIRWA, 1999].

In cell growth of *E. coli*, the genes *folA* and *thyA*, have a key role and are strongly involved in the biosynthesis of folate [NAGHAVI, 2010]. Folates are used as cofactors, e.g. in the formation of methionine [DUTHIE, 1999], thymidylate [HERRINGTON and CHIRWA, 1999], purine [FIGUEIREDO et al., 2009], glycine [HOFFBRAND and WEIR, 2001], pantothenate and formyl-methionyl tRNA^{met} [HERRINGTON and CHIRWA, 1999]. When there is a folate deficiency all mentioned substances are predicted to be also inadequately synthesised.

In case of an absence of *folA*, like in MH951 *E. coli*, the synthesis of THF is reduced. Hence, feeding this bacteria is expected to induce a folate deficiency in *C. elegans*, as previously used in the studies of Balamurugan et al. [BALAMURUGAN, 2007]. But nevertheless it was postulated that the folate depleted *E. coli* strains have an alternative pathway to synthesise THF, hence, it is not certainly clarified if and to what extent a folate deficiency in *C. elegans* can be caused by those bacteria [HERRINGTON and CHIRWA 1999]. Thus, a second model was developed and established.

Instead of using MH828 or MH951 bacteria for inducing a folate deficiency, sulfamethoxazole, a p-amino benzoic acid analogue, was added in the growth media of *E. coli*. This chemical substance interferes in folate *de novo* synthesis. It competes with p – aminobenzoic acid for dihydropteroate synthase and is predicted to cause a folate depletion in *E. coli* bacteria and when fed to *C. elegans* also in these worms [ROLAND et al., 1979; BERMINGHAM and DERRICK, 2002].

Numerous studies showed an inhibitory effect of sulfonamides on steady state growth of *E. coli* bacteria which was manifested in a decreased growth curve. As SMX acts only bacteriostatically in very high concentrations [SEYDEL et al., 1972], it is often used in combination with trimethoprim (TMP, inhibitor of DHFR). In our case TMP was not used, because DHFR seems to be present in *C. elegans* as well and we did not want to interfere directly in worm metabolism. In this study a folate deficiency in *C. elegans* was induced in two different ways. Either by feeding a *folA* deficient *E. coli* strain (MH951), or by feeding *E. coli* with inhibited folate biosynthesis via SMX and the influences on reproduction and life span were investigated. In both cases the severity of the induced folate deficiency should be proven by measuring the concentration of different folate derivatives via LC-MS.

5.2 Folate deficiency partially reduces reproduction which can be rescued by folic acid supplementation

Previous studies have shown that the nematode *C. elegans* has a dietary choice behaviour [Osborne and Mendel, 1918]. It differentiates food sources because of their quality. Some food species (bacteria) are easy to consume and support the nematodes growth quite well, while others do not. So generally OP50 *E. coli* grown in full media present a good food source for *C. elegans* [SHTONDA and AVERY 2005], while OP50 *E. coli* grown in minimal media and MH828 *E. coli* were demonstrated here to be an inadequate food source with a diminished reproduction rate per se. Brooks et. al have also documented that a correlation exists between the number of progeny and used bacteria strain. This

DISCUSSION

study used HT115 RNAi *E. coli* as a diet, which caused a reduced progeny of *C. elegans* [BROOKS et al., 2009], the same effect as seen for MH828 *E. coli*. Also a considerably reduced progeny was determined by feeding worms with OP50 grown in minimal media. This kind of media does not contain the whole nutrient palette like DYT media, which is the standard media for OP50 *E. coli*. In summary, OP50 grown in minimal media and MH828 *E. coli* seem to be less nutritive for the worms than OP50 grown in full media.

A folate deficiency induced by feeding MH951 bacteria did not influence the number of progeny, compared with feeding MH828 control bacteria. Due to those results it can be assumed that the used *E. coli* strain is able to synthesise sufficient amounts of THF to sustain an adequate level of folate in *C. elegans*. By contrast, SMX treated bacteria as food source caused a reduced number of progeny of *C. elegans*. These results suggest that a severe folate deficiency exists in the worms, which was already investigated in rats and rabbits. Those folate depleted rats and rabbits showed a negatively affected fertility and fetal development [HELM et al., 1976]. Human studies showed a higher risk of bearing progeny with a number of congenital malformations, e.g. the neural tube defect, if pregnant women suffer from a folate deficiency [SHAW et al., 1995]. It was shown previously that folic acid acts in form of THF in various biological reaction as a cofactor, e.g. in methionine pathway. An existing folate deficiency might cause an inadequate synthesis of methionine and elevated levels of homocysteine, as well. Studies determined that methionine is an essential amino acid for developing and growth in humans [REFSUM et al., 1998], several animals, e.g. rats [OZ et al., 2008] and also lots of plants [RAVANEL et al., 2004]. Investigations with the model organism *Drosophila melanogaster* showed that only methionine was sufficient to stimulate fertility without a reduction of life span [GRANDISON et al., 2009]. Hence, an absence of methionine, which might be initiated by a folate deficiency, might be the cause for the reduced number of progeny in folate depleted *C. elegans*.

In a next step the effect of an exogenous folic acid supplementation was assessed. In all four cases (MH828, MH951, both SMX-treated OP50) the

supplementation led to a significantly increased reproduction, approximately from under 30 % up to 80 % compared to control worms. Helm et al. used two groups of mice, which were fed with a folate free, amino acid-based diet supplemented with either 0 or 2 mg folic acid/kg for 35 to 48 days. Mice fed with the folate free diet showed a declined folate concentration in the serum, erythrocytes, liver and kidney. In contrast, folate deficient mice had anemia, reticulocytopenia, thrombocytopenia and leukopenia. These symptoms of a folate deficiency could be reversed after supplementation of folic acid [HELM et al., 1976]. Similar effects could be seen in folate deficient *C. elegans* supplemented with folic acid.

In summary, the brood size experiment on *C. elegans* with an induced folate deficiency through 10 μ M SMX showed a significant decrease in number of progeny compared with no folate deficiency. By adding external folic acid the declining effect can be compensated.

5.3 Folate deficiency influences life span which can be rescued by folic acid supplementation

As already mentioned above, studies described methionine as an essential amino acid with regard to reproduction and life span. Explorations with *Drosophila melanogaster* displayed that only methionine affects an ascending fertility without a reduction of life span. Hence, an absence of methionine, causes an extended maximal lifespan in the fruit fly [MILLER et al., 2005]. The folate deficiency induced by feeding MH951 bacteria might also cause a methionine deficiency with an elongated median and maximal life span, observed for those worms. As described above, *C. elegans* has a dietary choice behaviour [SHTONDA and AVERY, 2005] and MH bacteria seem to be less nutritive. Hence, there might be a dietary restriction, which is associated with an elongated life span [LEE et al., 2006]. Purines are important for several essential functions in humans and animals [MORIWAKI et al., 1999]. The purine synthesis is dependent on THF, which acts as a one carbon carrier. Aside from DNA and RNA, purines are biochemically significant components in a number of other

DISCUSSION

important biomolecules, such as ATP, GTP, cyclic AMP, NADH, and coenzyme A [KWON et al., 2010]. Therefore a folate depletion in *C. elegans* might have an influence on purine metabolism which might effect growth and fertility. Previously it was shown that a folate deficiency, induced by a lack of the folate transporter FOLT-1 in *C. elegans* affects both the soma and the germline [AUSTIN et. al., 2010]. Worms with a *fol-1* knockout or RNAi knockdown show the same results of a diminished progeny like hermaphrodites in this study. Hence, the effects of a reduced folate uptake might be comparable with the effects induced by a folate depleted diet.

Hermaphrodites with a *fol-1* knock out or knock down have a slower defecation, are characterised through a diminished germline proliferation, have a significantly shorter life span, produce a small amount of sperm and are oogenesis defective. The defecation, which is associated with the energy metabolism, was also measured and resulted in a decrease. Thus, *fol-1* knockout mutants have a reduced metabolism and following a significantly shorter life span compared to normal wild type *C. elegans*. Many mutations, which are characterised through a slow metabolism, tend to expand lifespan, but there are also metabolic mutations, including *fol-1* mutation, which show a contrary effect [AUSTIN et. al., 2010].

In case of MH828 and MH951 *E. coli* the folate depletion elongates life span significantly, maybe because of dietary restriction, retarded metabolism or other currently unknown reasons. The observed effects could be rescued by folic acid supplementation. In case of SMX no alterations in life span could be determined, which cannot be explained so far.

6 ABSTRACT

A folate deficiency can be caused by an increased demand for folate [PIETRZIK and THORAND, 1997], an inadequate uptake [MCNULTY, 1995] or interfering medications [STOLZENBERG R., 1994]. Mammals which suffer from a folate depletion are often concerned by typical symptoms, like a diminished fertility, altered life span, irritability and also behavioural disorders [HASLAM and PROBERT 1998].

To investigate the effects of a folate deficiency the model organism *Caenorhabditis elegans* was used in this thesis. The intention was to compare two different induced folate deficiencies and to assess their influences on reproduction and life span. One previously described way was feeding *folA* (dihydrofolatereductase) depleted *E. coli* to the worms and the second new developed way was feeding *E. coli* with inhibited folate *de novo* biosynthesis (inhibition with sulfamethoxazole, SMX). Feeding *folA* knockout *E. coli* did not affect reproduction, but elongated life span significantly, whereas the effect was completely opposite in worms fed with SMX treated *E. coli*. Here a decline in reproduction could be observed and no effect on life span was assessed.

Reproduction as well as life span might be influenced by decreasing concentration of THF (tetrahydrofolate), which acts as cofactor in several pathways, amongst others for methionine [GRANDISON et al., 2009] and purines [KWON et al., 2010]. Investigations showed an influence on fertility and growth caused by an inadequate synthesis of purines [WALKER et al., 2005], while reproduction was reduced by a methionine deficiency [GRANDISON et al., 2009].

It was not surprising that there were different effects on reproduction and lifespan observable, caused by a folate deficiency induced through two different ways. Further studies are required to explain these differences.

ZUSAMMENFASSUNG

Ein Folsäuremangel bei höheren Tieren und Menschen wird oft von Symptomen wie verminderter Fruchtbarkeit, verminderter Lebensdauer und Reizbarkeit, begleitet [HASLAM and PROBERT 1998].

Ziel der hier vorgestellten Studie war ein Vergleich zweier Methoden, die auf unterschiedlichem Wege eine Folsäuredefizienz in *C. elegans* erzeugen. Eine bereits beschriebene Methode ist die Verfütterung folsäurearmer *E. coli*, die eine Deletion in dem Dihydrofolat-Reduktase-Gen enthalten. Die zweite, neu entwickelte Methode beschreibt die Verfütterung von folsäurearmen *E. coli*, deren Folsäure *de novo* Synthese mit Hilfe von Sulfamethoxazole (SMX) inhibiert wurde. Der Einfluss des Folsäuremangels auf die Reproduktion und die Lebensspanne bei Wildtyp *C. elegans* wurde untersucht und verglichen.

Die Induktion der Folsäuredefizienz bei *C. elegans* mit Hilfe genetisch veränderter Bakterien hatte keinen weiteren Einfluss auf die Reproduktion, führte allerdings zu einer signifikant verlängerten Lebensspanne. Wohingegen die Verfütterung von Bakterien mit gehemmter *de novo* Synthese eine Verringerung der Nachkommenschaft verursacht hat und keinen Effekt auf die Lebensspanne hatte. Durch die Supplementation von Folsäure konnten alle resultierenden Effekte umgekehrt werden. Folglich scheinen beide Methoden einen Folsäuremangel bei *C. elegans* erfolgreich zu induzieren, wenn auch die phänotypische Ausprägung unterschiedlich ist.

Studien an anderen Organismen, zB. an Ratten oder Kaninchen [HELM F. et al., 1976] haben gezeigt, dass sowohl Lebensspanne wie auch Reproduktion von einem ausreichenden Vorhandensein von THF (Tetrahydrofolsäure), welche als Cofaktor, zB. im Purinstoffwechsel [KWON et al., 2010] oder Methioninstoffwechsel [GRANDISON et al., 2009] dient, abhängig ist. Die verringerte intrazelluläre Konzentration von THF kann auch bei *C. elegans* unter Folsäuremangel dafür verantwortlich sein, dass diese Parameter negativ beeinflusst werden. Nachdem die Folsäuredefizienz auf zwei unterschiedlichen Wegen induziert wurde, ist es nicht verwunderlich, dass unterschiedliche Effekte auf

ZUSAMMENFASSUNG

Reproduktion und Life span beobachtet werden konnten. Um genauere Aussagen treffen zu können, sollten weitere Untersuchungen folgen.

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8 APPENDIX

8.1 List of figures

Fig. 1: A wild type <i>C. elegans</i> hermaphrodite.	3
Fig. 2: <i>Caenorhabditis elegans</i> hermaphrodite and male.....	5
Fig. 3: Embryonic development of <i>C. elegans</i>	7
Fig. 4: Life cycle of <i>C. elegans</i>	8
Fig. 5: Chemical structure of folic acid	11
Fig. 6: Metabolic pathway of folic acid and folate	12
Fig. 7: Chemical structure of Sulfamethoxazole	14
Fig. 8: Bacterial growth curve.....	22
Fig. 9: Brood size of wild type <i>C. elegans</i> fed with living and dead OP50 <i>E. coli</i> bacteria.....	26
Fig. 10: Life span of wild type <i>C. elegans</i> fed with living and dead OP50 <i>E. coli</i> bacteria.....	26
Fig. 11: Brood size of wild type <i>C. elegans</i> fed with OP50, MH828 and MH951 <i>E. coli</i>	28
Fig. 12: Life span of wild type <i>C. elegans</i> fed with OP50 and MH828 <i>E. coli</i>	29
Fig. 13: Life span of wild type <i>C. elegans</i> fed with MH828 and MH951 <i>E. coli</i>	29
Fig. 14: Growth curve of OP50 <i>E. coli</i> treated with different SMX concentrations.	31
Fig. 15: Brood size of wild type <i>C. elegans</i> fed with SMX-treated OP50 <i>E. coli</i>	32
Fig. 16: Life span of wild type <i>C. elegans</i> fed with OP50 <i>E. coli</i> (DYT) and OP50 <i>E. coli</i> (0 μ M SMX).	33
Fig. 17: Life span of wild type <i>C. elegans</i> fed with OP50 <i>E. coli</i> (0 μ M SMX) and OP50 <i>E. coli</i> (10 μ M SMX).	33

APPENDIX

LIST OF FIGURES

Fig. 18: Life span of wild type <i>C. elegans</i> fed with OP50 <i>E. coli</i> and OP50 <i>E. coli</i> with 50 mM KOH solution.....	35
Fig. 19: Brood size of wild type <i>C. elegans</i> fed with OP50 <i>E. coli</i> and OP50 <i>E. coli</i> with 0.1 mM folic acid.	36
Fig. 20: Brood size of wild type <i>C. elegans</i> fed with MH828 <i>E. coli</i> and MH951 <i>E. coli</i> bacteria with and without folic acid supplementation.	37
Fig. 21: Brood size of wild type <i>C. elegans</i> fed with SMX-treated OP50 <i>E. coli</i> with and without folic acid supplementation.	38
Fig. 22: Life span of wild type <i>C. elegans</i> fed with OP50 <i>E. coli</i> and OP50 <i>E. coli</i> with 0.1 mM folic acid.	39
Fig. 23: Life span of <i>C. elegans</i> fed with MH828 <i>E. coli</i> with and without 0.1 mM folic acid.	40
Fig. 24: Life span of <i>C. elegans</i> fed with MH828 and MH951 <i>E. coli</i> with and without 0.1 mM folic acid.	40
Fig. 25: Life span of <i>C. elegans</i> fed with 0 μ M SMX-treated OP50 <i>E. coli</i> with and without 0.1 mM folic acid.....	41
Fig. 26: Life span of wild type <i>C. elegans</i> fed with 0 and 10 μ M SMX-treated OP50 <i>E. coli</i> with and without 0.1 mM folic acid.....	42

8.2 List of abbreviations

°C	Degree celcius
μM	Mycromolar
5-MFTHF	5-methyltetrahydrofolate
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>
DHF	Dihydrofolate
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
g	Gram
h	Hour
hRFC	Human reduced folate carrier
L1	Larval stage 1
L2	Larval stage 2
L3	Larval stage 3
L4	Larval stage 4
LC-MS	Liquid chromatography–mass spectrometry
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
MTX	Methotrexate
NADH	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
nm	Nanometre
p-ABA	Para aminobenzoic acid
PCFT	Proton coupled folate transporter
RFC	Reduced folate carrier
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
rpm	Revolutions per minute

APPENDIX

LIST OF ABBREVIATIONS

SMX	Sulfamethoxazole
THF	Tetrahydrofolate
thyA	Thymidylate synthase
TMP	Trimethoprim

8.3 List of tables

Table 1: Instruments	17
Table 2: Chemicals	18
Table 3: Buffers and solutions.....	18
Table 4: Used Escherichia coli bacteria	20
Table 5: Overnight-cultures of bacteria	20

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Sprachen

Deutsch: Muttersprache
Englisch: sehr gute Kenntnisse
Französisch: gute Kenntnisse

Interessen

Ernährung
Sport
Kunst