

**Life- and healthspan extension by
marine natural products in
*Drosophila melanogaster***

Dissertation

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Summary

Organisms experience aging at cellular and organismal levels. In this inevitable consequence of life, the organs, tissues, and cells undergo functional decline stemming from the accumulation of cellular damage, e.g., genomic instability, telomere attrition, stem cell exhaustion, epigenetic alteration, dysregulated proteostasis, and deregulated nutrient sensing pathways. Due to the increase in life expectancy worldwide and its economical and medical health challenges, it is necessary to quest interventions that extend the life- and healthspan. Fortunately, aging seems to be modifiable and strategies such as exercise and diet show promising results to combat negative aspects of aging. However, these approaches are not sufficient to slow aging and interventions with less complexity and ease of implementation are required. In this regard, an alternative could be the development of drugs and the use of natural products with antiaging effects that can be used daily without adverse effects. Marine species have been used in drug development research to fight different diseases; however, their potential as antiaging agents have been mostly overlooked. In the current study, I studied the geroprotective effects of the marine brown alga *Eisenia bicyclis* and the halophyte plant *Salicornia europaea*. I discovered that the aqueous extracts of these species extended the lifespan of *Drosophila melanogaster* in a sex-specific manner that could be reproduced for different fly strains. Both extracts influenced the metabolism of flies by altering the glucose and TAG levels. I showed that in the flies fed with *Eisenia bicyclis* extract, the adverse effects of high-sugar and high-fat dieting on lifespan were rescued with greater effects on high-sugar diets indicating antidiabetic effects of the extract. Interestingly, even late administration of the *Eisenia bicyclis* extract was beneficial on a high-sugar diet. On the other hand, *Salicornia europaea* extract was beneficial on a high-fat diet but not a high-sugar diet which revealed the lipid-lowering effects of the extract. Additionally, these flies were more sensitive to starvation conditions. I found that interwoven insulin/insulin-like and Tor signaling pathways are required to extend the lifespan of flies, since the modification of these signaling pathways led to the hindrance of the beneficial effects of *Eisenia bicyclis* extracts. Moreover, the extract upregulated the expression of the pro-longevity gene *Sirtuin 6*. However, *Salicornia europaea* extract failed to extend the lifespan of *Tor* mutant flies whereas it was effective in lifespan extension in *foxo*-deficient animals. These results suggest *Salicornia europaea* mainly modifies the Tor signaling pathway. I also revealed that RNAi-depletion of *Tor* downstream effector S6

Kinase in the fat body abrogated the lifespan-extending effects of *Eisenia bicyclis* whereas this manipulation in the enterocytes and skeletal muscle cells did not block the positive effect of the extract suggesting the fat-storing organ is the target organ of the *Eisenia bicyclis* extract. Similarly, manipulation of Tor signaling in the fat body by driving Tor dominant negative alleles in this fat-storing organ, abolished the extension in the lifespan of flies fed with *Salicornia europaea* extract. Moreover, I showed that the enterocytes (EC), intestinal stem cells (ISCs), and enteroblasts (EB) are not targets of the extract. I found that three weeks of treatment with the *Salicornia europaea* extract downregulated the expression of *impl2*, *foxo*, and *glut1*. In summary, *Eisenia bicyclis* and *Salicornia europaea* are two species with bioactive compounds that can be used as health-promoting food supplements.

Zusammenfassung

Biologische Organismen unterliegen dem Alterungsprozess auf zellulärer und organischer Ebene. In dieser unvermeidlichen Folge des Lebens kommt es zu einem Funktionsverlust von Organen, Geweben und Zellen, der auf die Anhäufung zellulärer Schäden zurückzuführen ist, z. B. genomische Instabilität, Telomerabnutzung, Stammzellenerschöpfung, epigenetische Veränderungen, gestörte Proteostase und deregulierte Nährstofferkennungswege. Angesichts der weltweit beschleunigten Alterungsrate und der damit verbundenen wirtschaftlichen und medizinischen Herausforderungen ist es notwendig, nach Maßnahmen zu suchen, die die Lebens- und Gesundheitsspanne verlängern. Glücklicherweise scheint das Altern veränderbar zu sein, und Strategien wie Bewegung und Ernährung zeigen vielversprechende Ergebnisse bei der Bekämpfung des Alterns. Diese Strategien reichen jedoch nicht aus, um den Alterungsprozess zu verlangsamen, und es werden Maßnahmen benötigt, die weniger komplex und einfacher umzusetzen sind. Ein wichtiges Thema in diesem Zusammenhang ist die Entwicklung von Arzneimitteln und Naturprodukten mit Anti-Aging-Wirkung, die ohne Nebenwirkungen täglich angewendet werden können. Ein gutes Beispiel für solche natürlichen Produkte sind marine Arten, die viele bioaktive Verbindungen mit nützlichen biologischen Aktivitäten enthalten. Sie wurden in der Arzneimittelforschung zur Bekämpfung verschiedener Krankheiten eingesetzt; ihr Potenzial als Anti-Aging-Mittel wurde jedoch übersehen. In der vorliegenden Studie habe ich die geroprotektiven Wirkungen der marinen Braunalge *Eisenia bicyclis* und der Halophytenpflanze *Salicornia europaea* untersucht. Ich entdeckte, dass wässrige Extrakte dieser Arten die Lebensspanne von *Drosophila melanogaster* geschlechtsspezifisch und nicht stammspezifisch verlängern. Beide Extrakte beeinflussten den Stoffwechsel der Fliegen, indem sie den Glukose- und TAG-Spiegel veränderten. Ich konnte zeigen, dass die Fliegen, die mit *Eisenia bicyclis*-Extrakt gefüttert wurden, keine negativen Auswirkungen einer zucker- und fettreichen Diät auf die Lebensspanne mehr zeigten, wobei die Auswirkungen bei zuckerreicher Diät größer waren, was auf antidiabetische Wirkungen des Extrakts hindeutet. Interessanterweise war die späte Verabreichung des *Eisenia bicyclis*-Extrakts bei einer zuckerreichen Diät von Vorteil. Der Extrakt aus *Salicornia europaea* hingegen wirkte sich positiv auf eine fettreiche Diät aus, nicht aber auf eine zuckerreiche Diät, was die lipidsenkende Wirkung des Extrakts verdeutlicht. Diese Fliegen reagierten empfindlicher auf Hungerstress. Ich fand heraus, dass die

miteinander verwobenen Insulin/Insulin-ähnlichen und Tor-Signalwege erforderlich sind, um die Lebensspanne der Fliegen zu verlängern, da die Veränderung dieser Signalwege in den Fliegen dazu führte, dass die vorteilhaften Wirkungen von *Eisenia bicyclis* beeinträchtigt wurden. Darüber hinaus regulierte der Extrakt die Expression des lebensverlängernden Gens Sirtuin 6 hoch. *Salicornia europaea*-Extrakt verlängerte jedoch nicht die Lebensspanne von Tor-Mutanten, während er die Lebensspanne von Tieren mit FoxO-Mangel positiv beeinflusste. Diese Ergebnisse deuten darauf hin, dass *Salicornia europaea* hauptsächlich den Tor-Signalweg modifiziert. Ich habe auch gezeigt, dass der RNAi-Knockdown des nachgeschalteten Tor-Effektors S6-Kinase im Fettkörper die lebensverlängernde Wirkung von *Eisenia bicyclis* aufhob, während diese Manipulation in den Enterozyten und Skelettmuskelzellen die positive Wirkung des Extrakts nicht blockierte. Das deutet darauf hin, dass das fettspeichernde Organ das Zielorgan des *Eisenia bicyclis*-Extrakts ist. In ähnlicher Weise wurde durch die Manipulation der Tor-Signalübertragung im Fettkörper durch die Aktivierung von *Tor^{DN}* in diesem fettspeichernden Organ die Verlängerung der Lebensspanne von Fliegen, die mit *Salicornia europaea*-Extrakt gefüttert wurden, aufgehoben. Darüber hinaus konnte ich zeigen, dass Enterozyten, intestinale Stammzellen (ISCs) und Enteroblasten keine Ziele des Extrakts sind. Ich fand heraus, dass eine dreiwöchige Behandlung mit dem *Salicornia europaea*-Extrakt die Expression von *impl2*, *foxo* und *glut1* herunterreguliert. Zusammenfassend lässt sich sagen, dass *Eisenia bicyclis* und *Salicornia europaea* zwei Arten mit bioaktiven Verbindungen sind, die als gesundheitsfördernde Nahrungsergänzungsmittel verwendet werden

Abbreviations

Akt	Protein kinase B
AMP	Antimicrobial peptide
AMPK	AMP-activated protein kinase
CCA	Coupled colorimetric assay
CD	Control diet
cDNA	Complementary DNA
CM	Concentrated medium
CR	Caloric restriction
d	Days
Da	Dalton
DAM	<i>Drosophila</i> activity monitor
Dilps	<i>Drosophila</i> insulin-like peptides
EtOH	Ethanol
FB	Fat body
DNA	Desoxyribonucleic acid
DSS	Dextran Sulfate Sodium
EB	Enteroblast
EBE	<i>Eisenia bicyclis</i> extract
EC	Enterocyte
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
Esg	Escargot
Fkh	Forkhead
Fig	Figure
FoxO	Forkhead box class O
gDNA	Genomic desoxyribonucleic acid
Glut1	Glucose transferase 1
h	Hours
H ₂ O	Water
HFD	High fat diet
HSD	High sugar diet
HPLC	High pressure liquid chromatography
IIS	IGF/insulin signaling
IGF	Insulin-like growth factor
IGFBPs	Insulin-like growth factor binding proteins
IMD	Immune deficiency
Imp-L2	Imaginal morphogenesis protein-Late 2
InR	Insulin-like receptor
IPCs	Insulin producing cells
IR	Insulin receptor

IRS1	Insulin receptor substrate 1
ISC	Intestinal stem cell
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
min	Minutes
ml	Milliliter
μl	Microliter
NAD	Nicotinamide adenine dinucleotide
NM	Normal medium
PBS	Phosphate buffered saline
PI3K	Phosphoinositid 3 kinase
Ras	Rat sarcoma
RNA	Ribonucleic acid
Rpl32	60s ribosomal protien L32
RTK	Receptor tyrosine Kinase
s	Seconds
SD	Standard deviation
SEE	Salicornia europaea extract
SEM	Standard error of the mean
SGK	Serum and glucocorticoid-inducible kinase
Sir2	Silent information regular 2
STAT	Signal transducer and activator of transcription protein
SREBP1	Sterol regulatory element binding protein 1
S6K1	P70-S6 kinase 1
TAG	Triacylglycerol
TCA	Tricarboxylic acid
TSC1/2	Tuberous sclerosis complex 1/2
Tor	Target of rapamycin
UAS	Upstream activating sequence
Upd	Unpaired
V	Volume
W	Weight
4EBP	eIF4E binding protein

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1. Introduction

Aging is an inevitable fate of biological organisms. At organismal levels, aging is characterized by a progressive functional decline in the organs, tissues, and cells resulting in loss of function that can lead to a couple of chronic diseases such as stroke, sarcopenia, neurodegenerative diseases, renal failure, osteoporosis, heart failure, atherosclerosis, diabetes, and metabolic syndrome and finally results in mortality. The occurrence of these chronic diseases exponentially increases with age and most elderly people experience a combination of age-related conditions after the age of 65 [1]. The prevalence of sarcopenia, the decline in structure and function of muscle, is 5-13 % in people aged 60-70 years and this number sharply raises to 11-50 % in subjects over 80 years old [2]. The same pattern is found in the prevalence of osteoporosis that the prevalence of it in women aged 60, 70, 80, and 90 is 10 %, 20 %, 40 %, and 66 %, respectively [3], [4]. Similarly, the number of people with neurodegenerative diseases is increasing with age. In 2022, 5 % of people aged 65-74, 13.1 % of people aged 75-84, and 33 % of people aged 85 and older were diagnosed with Alzheimer's disease [5]. 2-3 % of the world population aged over 65 was calculated to suffer from Parkinson's disease in 2017 [6]. At cellular levels, aging is a process of losing the division potential of somatic cells by cellular damage accumulation. These cellular damages are collectively called the hallmarks of aging [7].

1.1 Hallmarks of aging

1.1.1 Genomic instability and telomere attrition

It has been shown that during aging genomic stability is compromised by a variety of factors including damage caused by intrinsic and extrinsic factors to DNA although the cell has a DNA-repair toolbox to circumvent the damage of DNA [8]. The terminal DNA-protein complex called telomere is an important player in aging. The telomere progressively shortens after each DNA replication cycle in somatic cells, a cellular process called telomere exhaustion. In addition to this, due to their connection to telomere binding protein, telomeres are not visible to the DNA repair system of the cell. Thus, the errors in telomeres are not repaired and lead to cellular damages that eventually cause cellular senescence [9]–[12]. Hyper-long telomeres in mice show no deleterious effects on mice and these mice were long-lived and had less DNA damage during aging. Moreover, these animals showed lower cholesterol and LDL levels and higher

insulin tolerance [12]. Short telomeres in different genetic backgrounds in mice lead to decreased viability and it was suggested that the telomere shortening might be associated with several disease states such as abnormal blood cell counts and atrophy of the gut [13]. However, the effect of telomere length on aging and lifespan is not always straightforward. In *Drosophila melanogaster*, telomeres are composed of three *non-Long Terminal Repeat* (non-LTR) retrotransposons elements, *Healing Transposon* (HeT-A), *Telomere Associated Retrotransposon* (TART), and *Telomere Associated and HeT-A Related* (TAHRE) and their length varies in different fly stocks [14]. While there is evidence showing that telomere length is associated with aging, it does not affect the lifespan of *Drosophila*. Increasing the copy number of non-LTR retrotransposons elements of fruit flies does not change the lifespan of flies; however, it affects the flies' fecundity and development [15].

1.1.2 Mitochondrial dysfunction

Mitochondria play substantial roles in energy metabolism, homeostasis, and aging. Mouse models expressing a proof-reading-deficient version of the nucleus-encoded catalytic subunit of mitochondrial DNA (mtDNA) polymerase have increased levels of mtDNA point mutations as well as deleted mtDNA. These somatic mtDNA mutations result in reduced lifespan and the emergence of conditions associated with aging including osteoporosis, anemia, reduced fecundity, and weight loss [16]. These age-related disorders resulting from mtDNA mutations are uncoupled from reactive oxygen response (ROS) or oxidative stress and they are likely to occur due to increased apoptosis in tissues [17]. Moreover, decreased lifespan through Insulin receptor substrate 2 (IRS2) increased level is linked with mitochondrial dysfunction and oxidative stress [18]. In flies, the inactivation of the mitochondrial electron transport chain (ETC) function extends the lifespan of flies. These animals with reduced expression of the genes encoding the mitochondrial respiratory complexes did not exhibit reduced assembly of respiratory complexes or reduced ATP levels [19]. Beforehand, the involvement of the mitochondrial electron transport chain in longevity was demonstrated in *Caenorhabditis elegans* (*C. elegans*). A mutation in iron-sulfur protein (isp-1) of mitochondrial complex III in *C. elegans* results in lifespan extension, low oxygen consumption, and decreased sensitivity to reactive oxygen species (ROS) [20].

1.1.3 Nuclear pore complexes architecture

The nuclear pore complexes (NPCs) alteration might be another hallmark of aging since their

quality is subjected to changes during aging [21], [22]. While they function as a mediator between the nucleus and the cytoplasm, recently their role in aging has attracted the attention of researchers. Hutchinson-Gilford progeria syndrome (HGPS) is a genetic disease with mutations in the LMNA gene encoding lamin A/C proteins that are constituents of NPCs [23], [24]. Patients suffering from HGPS show premature symptoms of aging such as osteoplasia, absence of subcutaneous fat, and premature atherosclerosis, and the median age at death is 13.4 years [24]. In yeast, the extrachromosomal rDNA circles (ERCs) and DNA circles' attachment to NPCs contributes to the aging process. The attachment might reorganize NPCs leading to perturbations such as losing their nucleus basket and cytoplasmic complexes and activating a regulatory mechanism pertinent to aging. Interestingly, these alterations in NPCs are not subjected to the NPC quality control machinery [22]. During cell aging, the abundance of some proteins involved in the assembly of NPCs, and proteins that assist in this assembly, decreases leading to misassembled NPCs. The misassembled NPCs are not able to engage in the exchange between the cytoplasm and the nuclear and are covered by the nuclear membrane [21]. The deregulated NPCs and nucleoporins assembly results in the loss of the nuclear membrane barrier. This, in turn, leads to protein leaking from the cytoplasm to the nucleus, a condition so-called nuclear leakiness that deteriorates during cell aging [25].

1.1.4 Epigenetic alterations

It is well-known that the epigenome contributes to gene expression regulation and cell fate. Moreover, recent studies have shown that epigenetic alterations including DNA methylation, histone modification, and chromosome remodeling are an important tier of the aging process and can influence life- and healthspan [26]–[29]. During aging, while Sir 2 which is an NAD⁺-dependent deacetylase with a role in histone modification decreases, histone H4 lysine 16 acetylation increases, and these events are accompanied by loss of histones in subtelomeric regions in old yeast cells [30]. In addition to this, histone biosynthesis is compromised during aging and its mechanism probably involves epigenetic alteration in the telomeric region that results in the activation of DNA damage response (DDR) [31]. Among histone modifications, histone methylation is particularly relevant. Depletion of ASH-2 trithorax complex, which functions to trimethylate histone 3 lysine 4, leads to the extension of lifespan in *C. elegans* [32]. However, the role of other histone modifications in aging is not as straightforward to interpret and these modifications are usually context dependent. For example, Histone H3

lysine 9 trimethylation in heterochromatin regions in human senescent mesodermal cells decreases whereas in fruit fly heads the H3K9 trimethylation increases [33], [34]. Similarly, chromatin modifications in human immune cells display cell-type and hematopoietic-lineage-specific patterns and heterogeneity among individuals and cell-to-cell variability in chromatin modification elevates with age [28]. Along with DNA methylation and histone modifications, chromatin remodeling contributes to the aging process. Heterochromatin protein 1 α (HP1- α) is quintessential in chromatin condensation and along with other factors such as poly comb proteins, participate in chromatin remodeling and architecture and ultimately in chromatin alterations during aging. Studies show levels of both HP1- α and poly comb proteins reduce with age [11], [35], [36].

1.1.5 Loss of proteostasis

The old proteins and the proteins that lost their stability and function due to cellular damage throughout cell life tend to aggregate and in consequence interfere with several cellular processes. Consequently, cells need to surveillance the proteins status and degrade misfolded proteins through proteostasis. Proteostasis comprises two distinct stages, one for the maintenance of stable proteins by chaperones and another for the degradation of proteins through the proteasome and the lysosome [37], [38]. Nevertheless, aging affects proteostasis and in this condition, aberrant protein aggregation increases that in consequence exhausting the proteostasis network machinery [37], [39]. During aging, the number of chaperones undergoes drastic changes. For example, in the human brain 332 genes act in concert to safeguard the proteome from challenges to protect its functionality. However, aging influences this gene network in a way that the expression of 32 % of chaperone genes is downregulated and of 19.5 % is upregulated [40]. The effect of proteostasis via proteasome activity on aging can be a long-range response through inter-organ communication. A recent study on *Drosophila* shows that moderate manipulation of the proteasome in the skeletal muscle reduces age-related aggregation of poly-ubiquitinated proteins in the central nervous system and thus preserving proteostasis during aging [41]. In *C. elegans* a causal relationship between ubiquitination and aging was shown by assessing ubiquitin signatures in the proteome. This study shows a global loss of ubiquitination with age that stems from enhanced de-ubiquitinase activity, and this process can be ameliorated by pro-longevity interventions such as dietary restriction and disrupting the insulin signaling pathway. Moreover, it was

shown that decreased ubiquitination causes alterations in proteasomal targets that their levels' reduction favors longevity and preventing their degradation is pro-aging [42].

1.1.6 Deregulated nutrient sensing

Nutrient sensing links aging to nutrients and other environmental factors and genetic or pharmacological manipulations of evolutionary conserved nutrient-sensing pathways are associated with life- and healthspan alterations [43]–[46]. It was first discovered in *C. elegans* that genetic manipulation in different components of the Insulin/IGF signaling pathway can extend the lifespan. Mutations in the gene *daf-2*, which codes for an insulin-like growth factor receptor, can extend the lifespan at least 2-fold [47], [48]. These findings were confirmed in other organisms including *Drosophila*, mice, and humans [45], [49]–[52]. Fork head box O (FoxO) proteins are central to IIS (insulin/insulin-like growth factor signaling) pathways. FoxO proteins are transcription factors acting downstream of IIS and are highly conserved along evolution. While invertebrates have only one *FoxO* gene (*foxo* in *Drosophila*, *daf-16* in *C. elegans*), vertebrates have 4 *FoxO* genes (*FoxO1*, *FoxO3A*, *FoxO4*, and *FoxO6*) [53]. *FoxO* is essential for longevity-related effects of IIS alterations [48], [54], [55]. When the IIS is activated, FoxO is phosphorylated by the serine/threonine kinase AKT and this phosphorylation retains FoxO in the cytoplasm. Upon inactivation of IIS, FoxOs translocate into the nucleus and trigger the transcription of genes relevant to longevity [56]–[58].

The mechanistic target of rapamycin (mTOR) signaling pathway is another nutrient signaling pathway that its perturbation is linked to longevity [59]–[62]. Tor is comprised of two protein complexes, TORC1, and TORC2, that are involved in regulating cell growth, metabolism, and aging [63], [64]. Inhibition of this amino acid sensing signaling pathway via genetic or pharmacological interventions prolongs lifespan in different animal models. For example, genetic downregulation of distinct components of Tor signaling pathways including overexpression of tuberous sclerosis complex genes 1 and 2 (TSC1 and TSC2) that inhibit TOR or the dominant negative form of Tor or dS6K can prolong the lifespan of flies [65]. Similarly, reduction in Tor signaling by administration of rapamycin could extend the longevity of flies at both median and maximum lifespan levels [60]. In addition to flies, late administration of rapamycin could extend the lifespan of genetically heterogeneous mice at both median and maximum lifespan levels in both sexes [66]. Inhibition of the Tor signaling pathway via genetic manipulations on TORC1 extends the lifespan of female mice and deficiency in Tor

downstream effector S6K1 had analogous effects on the longevity of flies [67], [68].

Unlike IIS and Tor signaling pathways that are activated by the abundance of nutrients such as glucose and amino acids, AMP-activated protein kinase (AMPK) senses the paucity of nutrients and signals catabolism [38], [46]. This energy-sensing pathway has established roles in the regulation of carbohydrate, lipid metabolism, and mitochondrial function [69]–[71] and its activation is associated with longevity in different model organisms [72], [73]. The lifespan-extending effects of metformin are attributed to the activation of AMPK [74], [75]. Despite of ineffectiveness of metformin in lifespan extending effects in flies, it activates AMPK signaling [76]. Resveratrol and glucosamine are other compounds with geroprotective effects that mediate their effects through the activation of AMPK [77], [78].

1.1.7 Cellular senescence

The life of a cell is finite and as cells divide and grow, their ability to proliferate reduces and finally the cell cycle arrests. Although these cells are still viable, they cannot divide, grow and proliferate in response to nutrients and growth factors, a process that is called cellular senescence [79]. Cellular senescence stems from stressors such as telomere shortening, non-telomeric DNA damage, and excessive mitogenic signals that lead to oncogene activation [80], [81]. It provokes metabolic and functional shifts including resistance to apoptosis, secretion of proinflammatory molecules and proteases. This phenomenon is called senescence-associated secretory phenotype (SASP) and stems from increased protein synthesis, and perturbation in mitochondrial functions [82], [83]. Senescent cells accumulate in tissues and organs with age and contribute to aging by recruiting inflammatory responses because of their SASP. For example, expression of p16(Ink4a), a biomarker for senescence, is increased during aging in mammalian tissues. Its inactivation in mouse models attenuates the senescence phenotype in the tissues with high levels of p16(Ink4a), and its removal delays onset of age-related pathologies [84], [85]. In addition to p16, expression of the *p21* gene is elevated during aging in skeletal muscles. Cells with high expression of *p21* showed senescence-related signaling pathways activation namely, p53, NF- κ B, cytokine signaling pathways as well as senescence-related genes that further supports the p21 involvement in the cellular senescence [86]. Another marker of cellular senescence is senescence-associated β -galactosidase (SABG), which elevates its levels in senescence macrophages. However, its application as a cellular senescence marker is limited because many senescence cells

undergoing apoptosis are SABG positive; moreover, SABG staining might be affected by cell density producing false positive results [87].

1.1.8 Stem cell exhaustion

Stem cells and their progenitors facilitate tissue renewal to maintain tissue homeostasis; however, this process is compromised during aging and tissues lose their regenerative potential [88], [89]. One major challenge of stem cells during aging is the decline of their ability to generate differentiated cells to keep the function of the tissue. Single-cell RNA sequencing of neurogenic niches in mice showed a reduction in activated neural stem cells, endothelial cells, microglial alterations, and T-cells infiltration in old neurogenic niches that consists of neural stem cells and other cell types. T-cells in old brains may hinder neural stem cells by secreting interferon- γ [90]. Regulatory cells in stem cell niches can influence the stem cell repertoire during aging. The mouse fibro-adipogenic progenitors (FAPs) function deteriorates during aging due to loss of WNT1 Inducible Signaling Pathway Protein 1 (WISP1) that is FAP-derived extracellular matrix signal and consequently impairs the myogenic potential of muscle stem cells (MSCs) [91]. Likewise, hair follicle stem cells (HFSCs) are influenced by their microenvironment and their number drops with age. Interestingly, the neonatal dermis can rejuvenate HFSCs implicating the importance of stem cell niches during aging [92]. Mice hematopoietic stem cells show an increase in their number during aging, whereas their function and clonal complexity decline with age [93], [94]. Moreover, old HSCs produce fewer lymphoid cells and have a propensity to myeloid lineage and generally, the aged HSCs lose the ability of multilineage hematopoiesis, which means their differentiation capacity is reduced [95]. Furthermore, the heterogeneity of stem cells increases with age. Although the number of old quiescent NSCs in the brain elevates, their activation drops down and it might be due to a reduction in symmetrical divisions and a rise in asymmetrical divisions [90], [96]. Similarly, skeletal muscle stem cells quiescence state diminishes with age in an Akt/FoxO-dependent manner [97].

In addition to stem cells, their niche cells undergo age-dependent alterations such as niche composition changes and transcriptomic shifts [89], [98]. For example, the niche and cell surface receptors on MSCs such as integrins, Lrp1, Egfr, and Cd44 undertake drastic perturbations during aging. Furthermore, Smoc2 as a secreted protein from MSCs accumulates with age and its accumulation contributes to Integrin beta-1 (Itgb1)/mitogen-

activated protein kinase (MAPK) signaling perturbations leading to impaired functionality of muscle stem cells [99].

1.1.9 Altered Intercellular Communication

To maintain homeostasis in tissues and organs, cells need to communicate with each other and convey the received messages from different sources. Cells commonly transfer these messages to neighboring cells by soluble factors that modify the function or status of these cells. During aging, intercellular communication is compromised. One characteristic of compromised intercellular communication is a dysfunctional inflammatory response termed inflammaging. In this condition, the senescence cells accumulate and show a senescence-associated secretory phenotype (SASP) with age, and the cells with a defective immune system are not capable of removing the senescence cells resulting in a pro-inflammatory phenotype [100]. The emerging intercellular communication means comprise extracellular vesicles (EVs) that are membrane vesicles containing cytoplasmic DNA and cellular particles [101]. The EVs' number increases with age and they transmit the senescence message to neighboring cells in a paracrine manner [102].

1.2 Reasons to study aging

Growing older and maintaining health is an old dream of mankind [103]. Beside increasing lifespan, the increase of healthspan is not addressed to the same level, although the prevalence of diseases such as neoplasms, cardiovascular diseases, and neurodegenerative diseases is surging with age [104]. Studying aging und understanding the underlying processes is mandatory, as the number of people aged over 65 is increasing with time and this number is exceeding the number of children aged 5 and younger. People over 65 will comprise 20 % of the world's population while 70 years ago this number was 5 % [103]. In addition to the beneficial effects of aging studies on human life- and healthspan, aging studies can have a fundamental impact on the economy. Delayed aging can have about \$7.1 trillion in economic value for society over 50 years, thus it is understandable why the private sector is investing in aging studies [105].

1.3 Current Strategies to slow aging

Aging is a complicated and complex phenomenon, and its study shows both genetic and environmental factors are playing roles in its manifestation. However, the role of genetic is not

as significant as the environmental impact. Twin studies have shown a variable impact of genetics on lifespan ranging from 1 % to 27 % and these studies estimate about 25 % heritability of lifespan on average in humans [106], [107]. A population pedigree study consisting of millions of individuals revealed that the heritability of lifespan of humans is 12 % and the genetic influence on lifespan is mainly an additive genetic effect and is not dominantly inherited [108]. However, there are only few genes related to longevity and these loci are involved in cellular senescence, lipid metabolism, inflammation, and cardiovascular diseases [109]. Reduced frequency of an allele of the apolipoprotein E (*ApoE*) gene was reported in centenarians [110]. The role of this allele in lifespan determination was further confirmed in a genome-wide association studies (GWAS) and here, it was the only gene with genome-wide significance [111]. The *ApoE* gene is not the sole gene correlated to longevity and lifespan. A population study in long-lived Japanese men showed variations in the *FOXO3A* gene in nested case-control analyses. Moreover, in this study, three single nucleotide polymorphisms (SNPs) were assessed and all showed a correlation with lifespan [112]. Another group replicated this finding in German centenarians of both sexes. Evaluation of 16 SNPs in *FOXO3A* revealed the association of *FOXO3A* polymorphisms with longevity in this population [113]. Overall, these studies emphasize that lifespan and longevity are to a certain extent genetically influenced, but the environment and lifestyle are apparently more important regulators of this complex trait of humans and other organisms.

Up to now, caloric restriction, dietary restriction, and exercise are the lifestyle interventions with positive effects on the lifespan in both animal models and humans [114]–[118].

1.3.1 Exercise

Exercise is a set of physical activities that are planned, have a special structure, and are done on a regular basis to improve physical fitness [119]. Aerobic exercise or endurance exercise slows down the decline in the cardiovascular system and dysfunction in the autonomic system [120], [121]. Regular exercise, even at old age (e. g., above age 70), has the potential of slowing down age-related muscle dystrophy and improving muscle mass and function in older adults [122], [123]. Moreover, Physical activity targets different organs and increases mitochondrial biogenesis, antioxidant capacity, effector, and regulatory T-cell activity [124]. It has been suggested that exercise mediates its effects by attenuating the hallmarks of aging [125]. For example, proteostasis is compromised in aging leading to the accumulation of damaged

proteins. Exercise attenuates this aggregation by induction of autophagy in the brain, muscles, and heart [126]–[128]. Exercise modulates the age-related deregulation of IIS/TOR signaling and improves muscle protein synthesis in old subjects [129]. In line with this, physical activity defined as 25 km weekly running for eight years restored the age-dependent decline in growth hormones in women [130], [131]. Mitochondrial dysfunction is another hallmark of aging influenced by exercise. Five months of endurance exercise elevated mitochondrial biogenesis, oxidative capacity, and respiratory chain assembly, and hindered mitochondrial DNA depletion and mutations in mice [132].

1.3.2 Caloric restriction

Caloric restriction (CR) was the first investigated nutritional intervention that extended the lifespan with delayed development in rats [133]–[135]. It is characterized by a daily reduction in food and calorie intake by 20-40 % with constant ratios of macronutrients and without malnutrition [115]. Subsequently, the observed effects of caloric restriction were replicated in animal model organisms, namely yeast, *C. elegans*, and flies [136]–[138]. It is suggested that there is a linear relationship between the level of reduction of caloric intake and lifespan, and it causes a switch from glycolysis to beta-oxidation. The time of intervention initiation is critical, i. e., the extension of lifespan and the positive effect of CR is more pronounced when the CR regimen begins early in life compared to late administration of CR [139], [140]. CR not only positively affects the longevity of animal models, but also improves the health of these animals in a variety of ways. Studies on mice models showed protection against carcinogenesis via activation of the NF-E2-related factor 2 (Nrf2) pathway [141]. Further studies suggested CR limits tumor proliferation and angiogenesis along with reduced levels of insulin pathway-related metabolites such as insulin, insulin-like growth factor 1, and leptin. These studies revealed that the inhibitory effect of CR on tumor growth is more pronounced in comparison with fasting-mimicking diets [142]–[144]. It has also been suggested that CR was associated with a lower risk of breast cancer in women [145] and is associated with improved insulin sensitivity and metabolic state [146], [147].

1.3.3 Dietary restriction

Later research findings suggested that the beneficial effects of CR are not due to decreased calories but the absence of some specific nutrients are quintessential of CR's mode of action. In other words, its dietary restriction increases lifespan [148], [149]. Although dietary

restriction and caloric restriction are often used synonymously, the dietary restriction has a broader scope of nutritional interventions that share a reduction of a specific macro- or micronutrient such as protein restriction (PR) [150], methionine restriction (MR) [151], [152], time-restricted feeding (TRF) [153], [154], time-restricted eating (TRE)[155], [156], intermittent fasting (IF) [157], periodic fasting (PF) [154], fasting-mimicking diet (FMD) [158], ketogenic diet (KD) [159], [160].

Different dietary restriction strategies can extend the lifespan in distinct animal models and have positive effects on different measures of health resulting in a decrease in the incidence of chronic diseases [115], [116]. A reduction in glucose concentration or restriction of amino acids is associated with lifespan extension of the budding yeast *S. cerevisiae* [136], [161]. Similarly, dietary restriction administered by different methods such as diluting bacteria or reduced peptone concentration is sufficient to increase the lifespan in *C. elegans* [162], [163]. Another invertebrate model that confirmed the effectiveness of dietary restriction regarding longevity is *Drosophila melanogaster*. Separate studies revealed that the yeast concentration as the source of protein in the flies' food has a critical role in the lifespan of flies and a reduction in the concentration of yeast increases the longevity of flies [65] [148], [164].

1.3.3.1 Protein and amino acid restriction

A low-protein diet in middle-aged mice and a moderate or high-protein diet in old age can balance the lifespan and healthspan of mice [165]. Moreover, the increased lifespan effect of protein restriction is attributed to the protein to carbohydrate ratio and its more dependent on the restriction of specific amino acids such as methionine and glutamate [166]–[169] . Likewise, the restriction of dietary protein and specifically dietary amino acids can promote the lifespan of flies at cost of reduced fecundity [170]. In yeast, the composition of amino acids is the regulator of lifespan extension by protein restriction and a diet of low methionine or high glutamic acid extends the lifespan; furthermore, a combination of low methionine and high glutamic acid has an additive effect on the chronological lifespan of yeast [171]. Methionine restriction also is a regulator of lifespan under a low amino-acid condition in *Drosophila* and this restriction is associated with a trade-off between longevity and fecundity reminiscent of protein restriction. However, under a high amino-acid condition the lifespan extension effect of methionine restriction is abolished and the compromised fecundity is rescued [152]. Strikingly, genetic manipulation of methionine metabolism in *Drosophila* by

expressing bacterial *methioninase* in the whole body or tissue-specific has a similar effect of methionine restriction irrespective of amino acid status [172]. However, these were not the first studies depicting the effect of methionine restriction on longevity. Earlier, it was shown in rats that an 80 % reduction in methionine concentration in the diet increases the lifespan by 30 % [151]. Subsequently, another study provided insight into the effect of methionine restriction in mice. This study reported an increase in the maximal lifespan of mice along with a decrease in glucose, insulin, and IGF-1 levels in mice fed with a methionine-deficient diet [173]. Tryptophan is another amino acid that its restriction has the potential to extend the lifespan and reduce the body weight in rats [174].

1.3.3.2 Fasting

Fasting is another dietary restriction approach that can affect the different aspects of the metabolism and physiology of organisms leading to longevity benefits. Fasting strategies can be categorized into two main groups including intermittent fasting (IF) and periodic fasting (PF) [115], [175]. IF covers a variety of eating patterns such as water-only fasting every other day, a 70 % reducing energy intake every other day, and time-restricted feeding [135], [176]–[178]. On the other hand, periodic fasting (PF) comprises a longer time and includes prolonged-fasting and fasting-mimicking diets (FMD)[175], [179]. It has been shown that switching the stationary phase of yeast from an ethanol-containing medium to water extends their lifespan 2-fold [180]. IF induces longevity in *C. elegans* by 56.6 % when it is applied every two days along with improved resistance to heat, and oxidative stresses, and promoted locomotor activity of the nematode during aging [181]. This finding recapitulates the earlier studies on rats showing an 83 % increase in the mean lifespan of male rats on an every other day feeding diet initiated early in life [182]. However, the magnitude of the effect is reduced to 30-40 % when IF treatment is administered on middle-aged animals [183]. Conversely, every other day feeding regimens in flies did not increase the lifespan of flies [184], [185]. Interestingly, another IF approach called 2:5 intermittent fasting characterized by two days ad libitum eating and five days fasting was pro-longevity and extended the lifespan when it was administered at the beginning of adulthood for 30 days. Early-life fasting was sufficient to improve health and age-related measurement such as resistance to stresses, lipid content, and gut barrier function [157]. Different inbred mice strains showed increased median and maximum lifespan along with reduced body weight in response to IF when it was

administrated early in life. Late administration of the regimen influenced the body weight and maximum lifespan of the animal like early administration; however, it did not affect the median lifespan of mice. Moreover, the effects of the regimen were dependent on the genotype of the mice [186].

One major difference between IF and periodic fasting (PF) is the period of applying the intervention. Unlike IF, where the fasting time is short and frequent, and water-only time in PF last between two to seven days depending on the organism and protocol [175], [187]. Although PF has several beneficial effects on the health of organisms such as improved metabolism, increased insulin sensitivity, protection of hematopoietic cells against chemotoxicity, and promotion of hematopoietic stem cell self-renewal through inhibition of IGF-1/PKA pathway [154], [188], it might give rise to several side effects and it is difficult to maintain due to its long period of fasting. These limitations led to the development of diets that mimic PF containing low protein, low sugar, and high unsaturated fat [175]. A periodic fasting mimicking diet (FMD) administrated on mice twice per month for four days each had positive effects on the survival of the animals and it reduced visceral fat and cancer incidence. In addition to that, the diet showed a rejuvenation effect on the immune system as well as retardation of bone mineral density loss [176]. A hypocaloric FMD containing high levels of fat, low levels of protein, and carbohydrates rescued the deleterious effects of a high-fat, high-caloric diet (HFCD) when it was administrated in female mice five days per month for two years. It prevents the accumulation of visceral and subcutaneous fat, hyperglycemia, and hypercholesterolemia mediated by HFCD. Moreover, the reduced lifespan by HFCD was reversed to some extent and the median survival for the mice increased by about 28.5 % [189].

1.3.3.3 Ketogenic diets

Ketogenic diets comprising very low levels of carbohydrates and high levels of fat are additional nutritional interventions associated with longevity and improved healthspan. These diets mimic the dietary restriction approach by shifting the metabolism to fatty acid oxidation and generating ketone bodies [115], [190], [191]. A ketogenic diet containing less than 1 % sugar and 89 % fat revealed a reduction in midlife mortality while it did not affect the maximum lifespan in mice. Furthermore, feeding the mice every other day with the ketogenic diet prevented the accumulation of fat and these mice were not obese compared to their control counterparts. Gene expression analysis depicted an upregulation of genes involved in

fatty acid oxidation and a downregulation of genes related to fatty acid synthesis, Tor signaling, and glucose and insulin metabolism [160]. Interestingly, another study confirmed these results showing the increased median lifespan of male mice and preservation of physiological functions such as motor activity and memory. In line with the aforementioned study, it was shown that the ketogenic diet mediates its effects through Tor signaling [159].

1.4 Limitations of current antiaging approaches

Although current antiaging approaches exhibited promising results in animal models and humans, a couple of constraints will ensue. Most of the aforementioned approaches are complicated and arduous as they require care and precise and individualized program. Exercise reduces the risk of morbidity and mortality; nonetheless, it fails to extend the maximum lifespan in mice or humans [192], [193]. Moreover, the duration and frequency of exercise should be individualized, since excessive exercise might intensify the rate of mortality in the elderly [117]. Malnutrition is the main concern in caloric restriction diets. Furthermore, the lifespan-extending effects of caloric restriction are dependent on the time of the administration of the intervention. On one hand, when it is initiated early in life it increases the lifespan in rodents while late administration does not show the same effect [194]. On the other hand, earlier initiation of CR might compromise bone marrow density, cortical and trabecular bone mass and elevate bone marrow adiposity in mice implicating detrimental effects on cortical and trabecular bone mass [195], [196]. CR might have negative effects on wound healing [197], [198] as it might influence collagen production as a substantial factor of wound healing in a dose-dependent manner in a brief period of intervention. Likewise, it decreases the noncollagen protein production [198][199]. Finally, CR-treated rodents are more susceptible to infection. CR-treated mice have a reduced natural killer (NK) activity and dendritic cells and their T-cell response to West Nile virus was diminished [194], [200]. Different dietary strategies represent similar limitations. For example, a 12-week protein restriction impaired wound healing in rats [201]. Fasting strategies are required to be coupled with diets improving health to circumvent possible side effects. Besides, their administration for children, pregnant women, underweight people, and individuals predispose to chronic diseases might be harmful [117]. Both caloric and dietary restrictions are associated with reduced fertility.

1.5 Antiaging drugs and natural products

Owing to the aforementioned limitations, it seems the current strategies to slow down aging are not sufficient. One promising strategy to circumvent the limitations of these approaches is to develop drugs and natural products that have geroprotective effects and lack the disadvantages of current strategies. A myriad of antiaging concepts has been introduced and here the most promising ones will be discussed.

Rapamycin is an antiaging agent produced by the bacterium *Streptomyces hygroscopicus* that showed antifungal effects in earlier studies [202]. Moreover, these studies suggested rapamycin has the potential of an immunosuppressant [203]. Investigating the effect of rapamycin on the lifespan of flies showed that it functions by interacting with the TORC1 branch of the Tor pathway influencing autophagy and protein translation through it [60]. These results confirmed previous studies that showed rapamycin inhibits Tor signaling in *Saccharomyces cerevisiae* and increase the chronological lifespan of the yeast [204]. It has also shown life-extending effects in genetically heterogeneous mice as well as for different times of administration [66], [205], [206] .

Metformin is an antidiabetic drug and was synthesized from a compound derived from *Galega officinalis* [103]. It increases the median lifespan of *C. elegans* and has positive effects on the healthspan of the nematode. In addition, the locomotor activity of *C. elegans* is improved during aging by the administration of metformin, independent of insulin signaling. It was suggested that metformin mimics dietary restriction by the activation of the AMPK signaling pathway [75]. Metformin alters the microbial folate and methionine metabolism by inducing a methionine restriction status in the host [207]. The promotion of lifespan in *C. elegans* is also attributed to mitohormesis and improved lysosomal pathway activity [208][209]. Similarly, metformin increased the mean and maximum lifespan of female HSR mice by 37.8 % and 20.8 % respectively [210]. While a lower dose of metformin (0.1 %) in middle-aged mice increased the median and maximum lifespan, at higher concentrations (1 %) it showed toxicity. like in *C. elegans*, It was shown that metformin activates AMPK signaling pathway and mimics dietary restriction along with improved antioxidant protection in mice [211].

Senolytics are a group of antiaging drugs that chemically eliminate senescent cells that are resistance to apoptosis. Pro-longevity pathways such as BCL-2 and FoxO are in active mode in

senescent cells and targeting them with chemical agents is a way to remove senescent cells. A cocktail of senolytics including dasatinib and quercetin diminished the senescent cell numbers and the secretion of proinflammatory cytokines and increased the survival of naturally aged mice and senescent cell-transplanted young mice by inhibiting BCL-2 action [212]. This combination also improves physical function and prevents age-related uterine dysfunction and fibrosis in mice [212], [213]. Another pathway involved in apoptosis resistance in senescence cells is a Heat shock protein 90 (HSP90); thus, therapeutic approaches with the ability to inhibit its action are potential senolytic drugs and can remove senescent cells. One example of such a drug is 17-DMAG (alvespimycin) that has beneficial effects on healthspan and reduces the expression of *p16^{INK4a}* as a marker of senescence [214]. Another natural product with senolytic properties is fisetin. It shows a stronger effect on several cultured cell types compared to quercetin and its late administration in mice extends the median and maximum lifespan. Moreover, treatment of wild-type and progeroid mice with fisetin diminishes SASP in multiple tissues [215]. Cardiac glycosides are a category of senolytics that inhibit Na⁺/K⁺ ATPase pumps in the plasma membrane and consequently lower the pH of these cells. The senescence cells have a low pH thus lowering the pH results in more sensitivity to apoptosis in these cells [216].

Spermidine is another natural product with geroprotective effects and modulates mechanisms such as gene expression, autophagy, apoptosis, and cell growth and proliferation [103]. During aging, the levels of spermidine decline, and adding spermidine to the food has been shown to increase lifespan via induction of autophagy [217]. As mentioned, most geroprotective drugs and products are naturally based. Therefore, investigating natural products for antiaging effects is a promising approach to slow aging. I investigated two extracts from marine species for their antiaging effects using *Drosophila melanogaster* as a model.

1.6 *Drosophila melanogaster* as a research model

Drosophila has been a research model in the lab for over 100 years since Thomas Morgan used it in his genetic studies [218]. Since then, it has played a significant role in distinct research fields such as development, physiology, and molecular and cell biology [219]. Due to their short life cycle and a large number of offspring, the experiments are performed rapidly with a rigorous sample size. Given its small size and the variety of existing methods such as the binary Gal4/UAS system, manipulation and observation of the cells and tissues are easy

[220]. The *Drosophila* genome considerably resembles the human genome. For example, 77 % of disease-related genes have a counterpart in *Drosophila* [221]. Finally, physiological and metabolic pathways show considerable conservation between flies and vertebrates; therefore, the research findings in *Drosophila* can be translated into a vertebrate context. Moreover, these pathways are less complicated compared to vertebrates and thus it is easier to study them in flies [222]. For example, the nutrient-sensing Tor signaling pathway is highly conserved between flies and humans and it is well-studied in flies [62]. Likewise, IGF/IIS signaling pathway in flies shares its central components with mammalian counterparts [223].

1.7 *Eisenia bicyclis*

Eisenia bicyclis is a brown marine alga of the family *Laminariaceae* found in kelp forests in Korea and Japan and grows at a range of temperatures from 5° to 30° C [224]. *E. bicyclis* contains bioactive compounds that mediate several beneficial biological effects. Eisenin is a compound found in the brown alga with the ability to enhance the cytotoxicity of lymphocytes in peripheral blood thus showing anti-inflammatory effects [225]. Oxylipins are another category of natural products found in *E. bicyclis* and they are secreted in response to inflammation and infection. Thus, *E. bicyclis* provides the cells with higher levels of oxylipins and finally improves the anti-inflammation and anti-infection responses [226], [227]. Phlorotannins are polymerized phloroglucinol with different biological activities. Dieckol, a hexameric phlorotannin, inhibits the production of nitric oxide and prostaglandin E2 in lipopolysaccharide (LPS)-stimulated murine BV2 microglia and suppresses expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). Moreover, it reduces the production of proinflammatory cytokines and the activation of nuclear factor κ B (NF- κ B) and p38 mitogen-activated protein kinases (MAPKs), thus it presents anti-inflammatory effects and might be a possible treatment for neurodegenerative diseases [228]. Dieckol and eckol extracted from *E. bicyclis* might entail antidiabetic effects [229]. Methanolic extract of *E. bicyclis* exhibited *in vitro* antiviral effects against infectious hematopoietic necrosis virus (IHNV) and it was suggested that the extract mediates its antiviral effects via chlorophyll c2 derivative lacking the metal ion Mg^{2+} [230]. Another study evaluated the antibacterial effects of *E. bicyclis* extract reporting the anti-infection activity of *E. bicyclis* against Methicillin-resistant *Staphylococcus aureus*. This finding was further investigated by analyzing the effect of six phlorotannins, among them, the phlorotannin phlorofucofuroeckol-A had the highest

inhibitory effect [231], [232]. *E. bicyclis* extract possesses antioxidant activity and fractionating of the extract revealed that the ethyl acetate fraction of the extract exhibits the highest activity. Further isolation and purification of the ethyl acetate fraction suggested that dieckol and phlorofucofuroeckol bear the strongest antioxidant activities [233]. Laminaran and fucoidan, two polysaccharides from *E. bicyclis* suppressed colony formation of human melanoma SK-MEL-28 and colon cancer DLD-1 cells thus they might potentially have anti-tumor activities. However, these polysaccharides did not show cytotoxicity to these cells [234]. Fucofuroeckol-A and eckol from *E. bicyclis* suppress the gelatinases MMP-2 and 9 that are required for the metastasis of cancer cells [235].

1.8 *Salicornia europaea*

Salicornia europaea is a salt-resistant plant belonging to the family *Amaranthaceae* and the subfamily *Chenopodiaceae*. Its genus is widely distributed in Eurasia, North America and South Africa [236]. It is often found in harsh environments such as salt marshes and muddy seashores and has several biological activities such as being anti-oxidant, anti-inflammatory, anti-hyperglycemic, and anti-hyperlipidemic [237]. Concerning the anti-oxidant activity, different fractions of *S. europaea* showed significant effects in xanthine oxidase and DPPH assay with the strongest effect in the ethyl acetate fraction. The same fraction of the extract hinders LPS-induced NO production in macrophages and reduces the nitric oxide synthase (iNOS) in these cells. Therefore, it put forwards an anti-inflammatory effect of the succulent plant [237]. 50 % ethanol extracts of *S. europaea* showed anti-hyperglycemic and anti-hyperlipidemic effects in rats on a high-fat diet. It also reduced the body weight of mice and targeted lipogenesis-related genes by modulating the expression of SREBP1a, FAS GAPT, PEPCCK, and glucose 6-phosphatase in the liver [238]. *S. europaea* is enriched with several bioactive compounds such as oleanane triterpenoid saponins, flavonoids and flavanones, chromones, and sterols and it might mediate its beneficial effects through these bioactive compounds [239]. In addition to positive effects on health, *Salicornia* species have been subject to phytoremediation studies and shown to absorb Pb, Zn, and Ni in polluted soil [240].

1.9 Aims of this study

The aim of this work was to quantify the lifespan and healthspan prolonging effects of two extracts of different origins and to elucidate the underlying molecular mechanisms. The focus was on the aqueous extract of a brown alga (*Eisenia bicyclis*) and of the halophyte plant *Salicornia europaea* that for both the preliminary tests indicated a life-prolonging potential.

The major questions addressed in this study were:

1. Do the aforementioned extracts have pro-longevity effects on flies?
2. What is the influence of these extracts on the metabolism of flies?
3. How do these extracts exert their effects on the life- and healthspan of flies?
4. Which organ is the target organ of these extracts to implement their effects?

2. Materials and Methods

2.1 Devices

Analytical balance (ABS)
 Balance (MXX-412)
 Bead ruptor 24
 Centrifuge (5415 D)
 Centrifuge (5417 R)
 Electrophoresis chambers
 Incubators (WB250K, WB120K)
 IKA analytical mill type A11 basic
 Invitrogen™ Mini-Cell Blot
 Magnetic stirrer (RET)
 Magnetic stirrer (MR3001)
 pH 340/ION
 Power supply (EV245)
 StepOne Real-Time PCR System
 Stereo microscope (MZ10F)
 Stereo microscope (S6E)
 Stereo microscope (Stemi 506)
 Spectrophotometer (DS-11)
 SureCast™ Gel Handcast System
 Tecan plate reader (Infinite M200 Pro).
 Thermocycler (Labcycler)
 Thermomixer comfort

Kern & Sohn GmbH(Balingen, Germany)
 Denver Instrument GmbH (New York, USA)
 Omni International (Kennesaw, USA)
 Eppendorf (Hamburg, Germany)
 Eppendorf (Hamburg, Germany)
 Biometra GmbH (Göttingen, Germany)
 Heinrich Eimecke GmbH (Kiel, Germany)
 IKA GmbH & CO. KG, Staufen, Germany
 Thermo Fisher Scientific Inc, Waltham, USA
 IKA®(Staufen, Germany)
 Heidolph (Schwabach, Germany)
 WTW (Weilheim, Germany)
 ConsortNT (Nürnberg, Germany)
 Applied Biosystems (Foster City, USA)
 Leica Microsystems (Wetzlar, Germany)
 Leica (Wetzlar, Germany)
 Zeiss (Oberkochen, Germany)
 DeNovix® (Wilmington, USA)
 Thermo Fisher Scientific Inc., Waltham, USA
 Tecan (Männedorf, Switzerland)
 SensoQuest GmbH (Göttingen, Germany)
 Eppendorf (Hamburg, Germany)

2.2 General materials

Ceapren stopper (Ø 50 mm)
 Cellulose stopper (Ø 29 mm)
 Cover slips (24 x 50 mm)
 Drosophila food vial (50 ml)
 Drosophila food vial (175 ml)
 Falcon tubes (15 ml, 50 ml)
 Filter tips (10, 100, 1000 µl)
 Forceps
 MicroAmp® Fast Optical 48-Well Reaction
 Plate (0.1 ml)
 Petri dishes, plastic
 Reaction tubes, low binding (0.2, 0.5, 1.5, 2 ml)
 Reaction tube, screw, 2 ml
 Serological pipettes (5 ml, 10 ml, 25 ml)
 Slides
 Tips, low-binding (10, 100, 1000 µl)

Greiner Bio-One (Kremsmünster, Austria)
 nerbe plus GmbH (Winsen, Germany)
 Carl Roth (Karlsruhe, Germany)
 nerbe plus GmbH (Winsen, Germany)
 Greiner Bio-One (Kremsmünster, Austria)
 nerbe plus GmbH (Winsen, Germany)
 nerbe plus GmbH (Winsen, Germany)
 neoLab® (Heidelberg, Germany)

Applied Biosystems (Foster City, USA)
 nerbe plus GmbH (Winsen, Germany)
 nerbe plus GmbH (Winsen, Germany)
 Sarstedt (Nümbrecht, Germany)
 nerbe plus GmbH (Winsen, Germany)
 Carl Roth (Karlsruhe, Germany)
 nerbe plus GmbH (Winsen, Germany)

Well plates (12-well, 96-well)

Sarstedt (Nümbrecht, Germany)

2.3 General chemicals

Ammonium persulfate (APS)

Bacto™ yeast extract

Brewer's yeast

Brilliant Blue FCF food dye; E133

Bromocresol Purple

Coconut fat

Cornmeal

Dithiothreitol (DTT)

dNTPs

Ethanol

Glycin

KCl

Molasses

Methanol

Methyl 4-Hydroxybenzoate

NaCl

Propionic acid

reagentplus® (nipagin)

ROTIPHORESE®Gel 40 (29:1)

SDS pellets

Soda lime

Sugar beet syrup

TEMED

triglycerides

trioleate standard

Tris

Tween-20

Yeast extract

2-Mercaptoethanol

Carl Roth (Karlsruhe, Germany).

Becton, Dickinson and Company

Leiber (Bramsche, Germany)

Ruth GmbH & Co.KG)

Sigma Aldrich (Munich, Germany)

Palmin

Mühle Schlingemann (Waltrop, Germany)

Thermo Fisher Scientific (Waltham, USA)

Promega (Madison, USA)

Carl Roth (Karlsruhe, Germany).

Carl Roth (Karlsruhe, Germany).

Merck

Biohof Heidelberg (Gerdau, Germany)

Carl Roth (Karlsruhe, Germany).

Carl Roth (Karlsruhe, Germany).

Carl Roth (Karlsruhe, Germany).

Sigma Aldrich (Munich, Germany)

Carl Roth (Karlsruhe, Germany).

Carl Roth (Karlsruhe, Germany).

Merck (Darmstadt, Germany)

Kanne Brottrunk (Selm-Bork, Germany)

Carl Roth (Karlsruhe, Germany).

Thermo Fisher Scientific (Waltham, USA)

Sigma Aldrich (Munich, Germany)

Carl Roth (Karlsruhe, Germany).

Carl Roth (Karlsruhe, Germany)

Carl Roth (Karlsruhe, Germany)

Carl Roth (Karlsruhe, Germany)

2.4 Enzymes and kits

PureLink™ RNA Mini Kit

Pierce™ BCA Protein Assay Kit

qPCRBIO SyGreen Mix Hi-Rox

Hexokinase Assay Kit

Clarity Western ECL Substrate

Life Technologies (Carlsbad, CA, USA)

Thermo Fisher Scientific (Waltham, USA)

PCR Biosystems Ltd (London, UK)

Sigma-Aldrich (St. Louis, USA)

Bio-Rad (Feldkirchen, Germany)

2.5 Recipe

RIPA buffer	150mM NaCl, 0.1% Triton X-100, 0.5% Sodium deoxy cholate, 0.1% SDS, 50mM Tris-Hcl pH 8 or 25mM pH 7.6, EDTA 5mM, Protease inhibitor
2X Laemmli buffer	100mM Tris pH 6.8, 20% Glycerol, 4% SDS, Protease inhibitor, 5% β -mercaptoethanol
Running buffer	25 mM Tris base, 192 mM Glycine, 0.1 % SDS, pH 8.3
Transfer buffer (semi-dry)	48 mM Tris base, 39 mM Glycine, 20% methanol, 0.04% SDS
TBST	20 mM Tris, 150 mM NaCl, 0.1% (w/v) tween 20
Blocking buffer	3-5% non-fat milk + TBST
Trehalase Buffer (TB)	5 mM Tris pH 6.6, 137 mM NaCl, 2.7 mM KCl

2.6 Oligonucleotides

Table 2.1 Primer and primer sequences used for qRT-PCR

Primer	sequence (5' → 3')
dTor_F	GCCAGCAGTTCGTCGAGTAT
dTor_R	CGGTCGTCAAGCAGCTCATA
Glut1_F	GTCAACTGGATGGCCAACTT
Glut1_R	CAGTTGACCTACCGGTTGAA
Impl2_F	GCCGAGAGTATAACTGCAACTG
Impl2_R	CGGCTCTCATATTGACGTTGAC
bmm_F	CACCAGCACAGCCACAATAC
bmm_R	GTGGTCGTGTCGGTGTTATG
foxo_F	GCCTCGTTATTGAGCACCTC
foxo_R	CTCCCTGAACACGTACAGCA
Sirt6_F	TGGATTGTCAGCCTACGACA
Sirt6_R	ACCTAACAGTCGGATGCTGT
Puck_F	CATCTCGCCCAATCTGAACT
Puck_R	GTAGAGCGGGTTAGACTTGA
4ebp_F	CTCCTGGAGGCACCAAACCTT
4ebp_R	GAGGACCTCCGTGGTTTGAA
tobi_F	GTTACACATTGTGCCAGCATC
tobi_R	ACCTCTCCTAAGTCGCACGTA
OligodTT7	GAGAGAGGATCCAAGTACTAATACGACTCA
	CTATAGGGAGATTTTTTTTTTTTTTTTTTTTTT

2.7 Extraction from *Eisenia bicyclis* and *Salicornia europaea*

Eisenia bicyclis extract was prepared according to the method described by Onur et al. (Onur et al., 2013). The dried algal material was ground with an analytical mill (IKA Type A11 basic). 3 grams of ground alga were transferred into a test tube containing 30 ml of boiling double distilled water. After slight stirring, the suspension was sonicated for 1 min and centrifuged for 2 min at 2000 g. The supernatant was filtered and the soluble extract was freeze-dried and stored frozen until use. For *Salicornia* extract, first fresh *Salicornia europaea* leaves were dehydrated using lyophilization and next the dried leaves were used for extraction in the same manner described above.

2.8 Fly husbandry

Flies were cultivated on the normal medium (NM) in mm fly vials at room temperature. 3–5-day-old flies were anesthetized using nitrogen gas and 10 flies were placed in each experimental vial. The experimental groups were kept at 25°C, a light-dark cycle of 12h:12h, and 60 % humidity. For crossing fly lines, virgin female flies of interest were mated with male flies for 2 days and the offspring of this crossing (F1) were used in the experiments.

2.9 Addition of extracts to the fly food

To supplement the food with the algal extract, the aqueous extract was added on top of the food in the experimental vials at a final concentration of interest. Then, the water is allowed to evaporate, and the food becomes dry again.

2.10 Addition of mifepristone to the fly food

For the experiments using the GeneSwitch system, mifepristone was added to the food at a final concentration of 200 μ M on the day of the experiments. For the control group, 70 % ethanol was added on top of the food.

Table 2.2 *Drosophila* culture medium ingredients

	Normal medium	Concentrated medium	High sugar diet	High fat diet
Cornmeal (W/V)	6.25 %	8.60 %	8.60 %	8.60 %
brewer yeast (W/V)	6.25 %	-	-	-
Yeast extract (W/V)	-	5 %	5 %	5 %
Glucose monohydrate	2 %	-	-	-
Sucrose (W/V)	-	5 %	30 %	5 %
Agar-agar (W/V)	1 %	1 %	1 %	1 %
Coconut fat (Palmitin)	-	-	-	20 %
Molasses (W/V)	3 %	-	-	-
Syrup (W/V)	3 %	-	-	-
Nipagin (V/V)	0.30 %	0.30 %	0.30 %	0.30 %
Propionic acid (V/V)	0.10 %	0.10 %	0.10 %	0.10 %

2.11 Normal medium

The ingredients for normal medium (NM) are presented in the table 2.2. To prepare 1 L normal medium, 1 L double distilled water was added to 62.5 g Cornmeal, 62.5 g brewer yeast, 20 g glucose monohydrate, and 10 g agar-agar and boiled for 15 minutes. Then, 30 g molasses and 30 g Syrup were added to it. The medium was autoclaved for 20 minutes and cooled down to 60 °C. After this, 10 ml propionic acid (10 % V/V) and 30 ml nipagin (10 % V/V) were added. 5 ml medium was distributed into each empty drosophila vial and the vials were kept at room temperature overnight. Finally, the vials were stored at 4 °C the next day.

2.12 Concentrated medium

The ingredients of concentrated medium (CM) are presented in the table 2.2. To prepare 1 L

of CM, 1 L of double distilled water was added to 86 g Cornmeal, 50 g yeast extract, 50 g sucrose, and 10 g agar-agar and boiled for 15 minutes. The medium was autoclaved for 20 minutes and cooled down to 60 °C. After this, 10 ml propionic acid (10 % V/V) and 30 ml nipagin (10 % V/V) were added. 5 ml medium was distributed into each empty drosophila vial and the vials were kept at room temperature overnight. Finally, the vials were stored at 4 °C the next day.

2.13 High sugar diet

The ingredients of the high-sugar diet (HSD) are presented in the table 2.2. To prepare 1 L HSD, 1 L double distilled water was added to 86 g Cornmeal, 50 g yeast extract, 300 g sucrose, and 10 g agar-agar and boiled for 15 minutes. The medium was autoclaved for 20 minutes and cooled down to 60 °C. After this, 10 ml propionic acid (10 % V/V) and 30 ml nipagin (10 % V/V) were added. 5 ml medium was distributed into each empty drosophila vial and the vials were kept at room temperature overnight. Finally, the vials were stored at 4 °C the next day.

2.14 High-fat diet

The ingredients of the high-fat diet (HFD) are presented in the table 2.2. To prepare 1 L HFD, 1 L double distilled water was added to 86 g Cornmeal, 50 g yeast extract, 50 g sucrose, and 10 g agar-agar and boiled for 15 minutes. Next, 20 g of coconut fat was included. The medium was autoclaved for 20 minutes and cooled down to 60 °C. After this, 10 ml propionic acid (10 % V/V) and 30 ml nipagin (10 % V/V) were added. 5 ml medium was distributed into each empty drosophila vial and the vials were kept at room temperature overnight. Finally, the vials were stored at 4 °C the next day.

2.15 Survival

The mated female or male flies were separated into experimental groups and kept on the food described above. For each group, 8 to 10 replicates with 10 flies in each replicate were used. A total of at least 100 animals were used at a minimum per experiment and each lifespan experiment was repeated 2-3 times. Vials were regularly changed every 2 days and dead flies were counted every day.

2.16 Fecundity

Virgin females of wild-type flies were transferred to individual vials containing CM with the

extract or without extract and 3 male flies were placed in each vial to mate for 2 days. After the removal of male flies, the number of laid eggs was counted every day for 10-14 days.

2.17 *Drosophila* fly lines

Table 2.3 *Drosophila* lines used in this study

Bloomington	Genotype	Source
yw	<i>y[1]w[1118]</i>	Bloomington #6598
<i>w</i> ¹¹¹⁸	<i>w[1118]</i>	Bloomington #5905
foxo-deficient	<i>yw;*,foxo21/foxo21</i>	M. Tatar, R. Yamamoto, Brown University, USA
Tor-deficient	<i>y[1] w[67c23]; P{w[+mC]=lacW}Tor[k17004]/CyOP{w[+mC]=lacW}Tor[k17004]/CyO</i>	Bloomington #11218
Sirt1 KO	<i>w</i> ¹¹¹⁸ ; <i>Sir2</i> ^{2A-7-11}	Bloomington #8838
InR[E19]	<i>InR[E19]/TM2</i>	Bloomington #9646
chico-deficient	<i>cn[1] P{ry[+t7.2]=ry11}chico[1]/CyO; ry[506]</i>	Bloomington #10738
tubP-Gal4	<i>y1 w*</i> ; <i>P{tubP-GAL4}LL7/TM3, Sb1</i>	Bloomington #5138
P(Switch)106	<i>w</i> ¹¹¹⁸ ; <i>P{Switch1}106</i>	Ronald Kühnlein
NP1-Gal4	<i>NP1-Gal4</i>	D. Ferrandon, Straßburg
NP1-Gal4	<i>NP1-Gal4;tubPGal80ts</i>	D. Ferrandon, Straßburg
esg-Gal4	<i>esg-Gal4, UAS-GFP; tub-Gal80</i>	
ppl-Gal4	<i>w</i> ¹¹¹⁸ ; <i>P{w[ppl-Gal4]}</i>	P. Leopold
Mhc-Gal4	<i>P{w[+mC]=Mhc-GAL4.K}1, w[*]/FM7c</i>	Bloomington #55132
UAS-Sir2	<i>w;UAS-sir2(2)/CyO</i>	Kynung-Tai Min
UAS-Tor.WT	<i>y[1] w[*] P{ry[+t7.2]=hsFLP}12; P{w[+mC]=UAS-Tor.WT}III</i>	Bloomington #7012
UAS-Tor.TED	<i>y1 w*</i> ; <i>P{w[+mC]=UAS-Tor.TED}II</i>	Bloomington #7013
UAS-S6K-RNAi	<i>y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02267}attP2</i>	Bloomington #41702
Upd2 deficient	<i>w[*] upd2[Delta]</i>	Bloomington #55727

2.18 Survival under desiccation and starvation stress

The flies were treated with food of interest for 2 weeks. Next, the resistance of female flies to desiccation stress was tested in empty standard *Drosophila* vials. 10 biological replicates with 10 mated female flies were implemented for each treatment. The survivorship of flies was recorded every two hours at 25° C and 60 % humidity and a 12-hour light-dark cycle. In order to assess flies' resistance to starvation, flies were transferred to standard drosophila vials

containing 1 % agar after feeding with the food of interest for 2 weeks. 10 biological replicates with 10 mated female flies were implemented for each treatment. The survivorship of flies was recorded every two hours at 25° C and 60 % humidity and a 12-hour light-dark cycle.

2.19 Food intake

The food consumption of flies was measured using the consumption-excretion method by Shell et al[241]. First the 0.5 % (w/v) blue dyed (Brilliant Blue FCF food dye; E133) was prepared. The concentrated medium (CM) or blue dyed medium (0.5% (w/v) Brilliant Blue FCF food dye; E133) was dispensed into caps of 2 ml screw cap vials. For adaptation, individual flies were transferred to 2 ml screw cap vials with CM. After a few hours of feeding on the concentrated medium, the flies were transferred to 2 ml vials with blue-dyed food. After 24 hours of feeding on blue-dyed food three ceramic beads and 500 µl H₂O were added to the vials. The samples were homogenized using a bead ruptor (OMNI International, OMNI Bead Ruptor 24) for 90 sec at 3.25 m/s. The homogenized samples were centrifuged at 3000 rpm for 3 min to deposit the tissue debris. Then 200 µl of the supernatant was added to clear 96-well-plates. The absorbance of wells containing the samples was measured utilizing a microplate reader at 630 nm. Two technical replicates were used for each biological replicate were used. Using the standard curve of a dilution series of the blue food, the amount of ingested food was measured.

2.20 Puromycin assay

Young adult flies were starved overnight and transferred to a vial containing concentrated medium and puromycin (10 µg/ml). After 1 hour of treatment with the food, the flies were homogenized in 200 µl RIPA buffer using a Bead Ruptor apparatus (BioLab Products, Bebensee, Germany) for 2 minutes at 3.25 m/s. The protein concentration was quantified using BCA Protein Assay Kit (Thermofisher, Karlsruhe, Germany).

2.21 Western Blotting

40 µg of protein was loaded on SDS-PAGE gel and the protein was resolved through electrophoresis. The proteins were transferred to a PVDF membrane using semi-dry transfer for 1 hour. To block unspecific binding, the membrane was treated with 5 % non-fat dry milk powder in TBST for 1.30 hours. The membrane was incubated with an anti-puromycin (3RH11) antibody (Kerafast, Boston, USA, EQ0001) over night at 4°C. After 3 times washing with TBST,

HRP-coupled anti-rabbit secondary antibody was used. The bands were visualized using Clarity™ Western ECL substrate and the ChemiDocImager (BioRad, Feldkirchen, Germany).

2.22 Body fat quantification

The whole-body triacylglycerol (TAG) content was measured using a coupled colorimetric assay (Hildebrandt et al., 2011). Samples were collected in 2 ml screw-cap tubes (5 female flies per sample) and weighted. 1 ml fresh 0.05 % PBS/tween-20 was added to samples and homogenized in a bead ruptor (BioLab Products, Bebensee, Germany) for 2 minutes at 3.25 m/s using 3 ceramic beads. The homogenates were heat-inactivated at 70° C for 5 minutes and centrifuged for 3 minutes at 2500 g. 50 µl of each sample was added to a 96-well plate and the absorbance was measured at 500 nm (T0) using a microplate reader (LEDetect 96, Labexim products). 200 µl prewarmed (37 °C) Thermo Infinity triglyceride reagent was added to each well and incubated for 30 minutes at 37° C with mild shaking. The absorbance was again measured at 500 nm (T1). Two technical replicates for the samples and the standards were used. The TAG concentration was determined by subtracting T0 from T1. The TAG content was quantified using a triolein-equivalent 2-fold dilution series standard curve from 100 µg to 3.125 µg. Two technical replicates of standards and biological replicates were measured.

2.23 Glucose measurement

The glucose levels of the body were determined using a Glucose (HK) Assay Kit (GAHK20, Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's instructions with minor modifications. Samples were collected (5 female flies per sample), weighted, and homogenized in PBS using a bead ruptor. The samples were kept cold to avoid glycogen degradation. The supernatant was heated for 10 minutes at 70° C and then centrifuged for 3 minutes at maximum speed at 4° C. The supernatant was transferred to a new 1.5 ml tube.

To prepare glucose standards, 16 µl of 1 mg/ml glucose with 84 µl PBS was diluted to produce 0.16 mg/ml glucose standard. 2-fold serial dilutions in PBS were made to obtain 0.01, 0.02, 0.04, 0.08, and 0.16 mg/ml glucose standards.

30 µl of glucose standards and a PBS blank were added to the first row of the 96-well plate. Each diluted supernatant (1:4-1:8 with PBS) was added to the next row of the plate. 100 µl of

the HK reagent was added to each well and the plate was incubated at room temperature for 15 minutes. Then, the absorbance was measured at 340 nm. The glucose content was calculated using a glucose standard curve.

2.24 Trehalose measurement

The glucose levels of the body were determined using a Glucose (HK) Assay Kit (GAHK-20, Sigma-Aldrich, Taufkirchen, Germany) according to the manufacturer's instructions with minor modifications. Samples were collected (5 female flies per sample), weighted, and homogenized in 100 μ l cold Trehalase Buffer (TB) (5 mM Tris pH 6.6, 137 mM NaCl, 2.7 mM KCl). To avoid enzymatically degradation into free glucose by endogenous enzymes, the homogenized samples. The supernatant was heated for 10 minutes at 70° C and then centrifuged for 3 minutes at maximum speed at 4° C. The supernatant was transferred to a new 1.5 ml tube. The Trehalase Stock (TS) was prepared by adding 3 μ l porcine trehalase in 1 ml TB. Next, the glucose and trehalose standards are generated. To prepare glucose standards, 16 μ l of 1 mg/ml glucose with 84 μ l TB was diluted to produce 0.16 mg/ml glucose standard. 2-fold serial dilutions in TB were made to obtain 0.01, 0.02, 0.04, 0.08, and 0.16 mg/ml glucose standards. To prepare trehalose standards, 16 μ l of 1 mg/ml trehalose with 50 μ l TS and 34 μ l TB was diluted to produce 0.16 mg/ml trehalose standard. 2-fold serial dilutions in a 1:1 mix of TB and TS were made to obtain 0.01, 0.02, 0.04, 0.08, and 0.16 mg/ml trehalose standards. 30 μ l of TB blank or glucose standards was added to 30 μ l TB in 1.5 ml tubes.

For the trehalose standards, two sets of 1.5 ml tubes were used. In one set, for the determination of free glucose 30 μ l of TB was added and in the second set 30 μ l of TS was added to convert trehalose to glucose. In the same manner as trehalose standards, two sets of tubes are prepared for the trehalose measurement in the samples. In the first set, 30 μ l of TB was added to measure the free glucose and in the second set 30 μ l of TS was added to convert trehalose to glucose. All the samples and standards were incubated at 37 °C for 18-24 hours. The samples and standards were centrifuged at maximum speed for 3 minutes. Then, 30 μ l of each sample and standard was transferred into a well of a 96-well plate. 100 μ l of the HK reagent was added to each well and the plate was incubated at room temperature for 15 minutes. Then, the absorbance was measured at 340 nm. The glucose content was calculated using the glucose standard curve. To measure the trehalose concentration, the absorbance of not digested samples (the first set) was subtracted from the absorbance of the samples that

were treated with TS (the second set). Finally, the trehalose concentration of the samples was calculated using the trehalose standard curve.

2.25 Protein content measurement

Samples were collected (5 female flies per sample) and weighted. 1 ml 0.05 % PBS/tween-20 was added to samples and homogenized in a bead ruptor (BioLab Products, Bebensee, Germany) for 2 minutes at 3.25 m/s. Then, the samples were centrifuged for 1 min at 1000 rpm at 4°C. The supernatant was transferred to a new 1.5 ml tube and centrifuged again at 6000 rpm, at 4° C for 10 min. The supernatant was again transferred to new tubes and centrifuged at maximum speed, at 4° C for 10 min. The protein content was measured using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions. In short, the working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. 25 µl of each sample (it can be diluted) or standard was added into an individual well of a 96-well plate. Two technical replicates for the samples and the standards were used. 200 µl of the working reagent was added to each well and thoroughly was mix. The plate containing the samples and standards was covered and incubated at 37 °C for 30 minutes. The plate was kept at room temperature to cool it. Then, the absorbance was measured at 562 nm on a plate reader and the concentration was calculated using the BSA standard curve.

2.26 Activity and sleep analysis

Activity and sleep were monitored using the *Drosophila* Activity Monitor (DAM2) system and analyzed by ShinyR. The mated female flies were allowed to feed on the food of interest for 2 weeks; then, single flies were placed into tubes containing concentrated medium (CM) and the tubes were sealed with cotton wool. The tubes were horizontally loaded into the DAM system and maintained at constant conditions (25°C, 60 % humidity, and 12/12-hour light-dark cycle). The first two days were spared for acclimatization and the data were collected on the third day.

2.27 Metabolic rate

The metabolic was measured according to Yatsenko et al [242]. To prepare Respirometers the 1,000 µl pipette tips were cut and inserted into the 50 µl capillary micropipette. One piece of foam was placed into the pipette and pushed downed at the bottom of the pipette tips. 3 pieces of soda limes were added to the pipette tip and a second piece of foam was placed on

top of the soda limes. 3 flies were put in each respirometer and the respirometers were sealed with clay. The metabolic rate chamber containing 1:1 water and Brilliant Blue FCF food dye; E133 were prepared and several respirometers with flies and 2 empty respirometers as controls were hanged in the chamber in a way that the micropipette tip down into the chamber and its tip submerged into the colored solution. The chamber was closed. After 15 minutes a photograph was taken from the respirometers inside the chamber and a scale outside. After 2 hours, a second photograph was taken from the respirometers and the scale.

$$\frac{(\pi * R^2) * (\Delta d - \Delta c) * 1000}{n * h}$$

Next, the result was analyzed according to the following formula:

R = radius of micropipette tube in centimeters

Δd = distance the liquid has moved up in the micropipette of test samples measured in centimeters

Δc = distance the liquid has moved up in the micropipette of the negative control sample (without flies)

n = number of flies used

h = hours

2.28 Total RNA extraction

1 ml RNA Magic and 3 ceramic beads were added to 2 ml screw-cap tubes. 3 female flies were transferred into the tubes and homogenized in a bead ruptor (BioLab Products, Bebensee, Germany) for 2 minutes at 3.25 m/s. 200 μ l chloroform was added to the homogenized samples and the samples were shaken for 10 s and placed on ice for 5 minutes. A centrifugation was performed at 4°C and 12,000 x g for 15 min. 400 μ l of the upper phase of supernatant was transferred into a 1.5 ml reaction tube and RNA was purified according to the manufacturer's protocol. 30 μ l RNase-free elution of the samples was stored at -80. The concentration of the extracted RNA was measured by a spectrophotometer.

2.29 First strand DNA synthesis

To synthesize cDNA reverse transcription of mRNA using SuperScriptIV reverse transcriptase (ThermoFisher) was done.

Table 2.4 Components and temperatures for first strand cDNA synthesis

Component	Volume
RNA	5.75 μ l
Oligo(dT)18 Primer	0.5 μ l
dNTPs (10 mM)	0.5 μ l
Incubation 5 min at 65°C 2 min on ice	
5 x Buffer	2 μ l
Ribolock RNase Inhibitor	0.5 μ l
DTT (0.1 M)	0.5 μ l
SuperScriptIV Reverse Transcriptase	0.25 μ l

Next, the samples were incubated at room temperature for 10 min. Then, the temperature increased to 50°C and the samples were incubated at this temperature for 20 min. Finally, the samples were placed at 80°C and incubated for 10 min. At this stage, the products can be stored at -20°C.

2.30 Quantitative real-time PCR

StepOne Real-Time PCR System was used to perform Quantitative real-time polymerase chain reaction (qRT-PCR) using the qPCRBIO SyGreen Mix Hi-Rox, MicroAmp fast Optical 48-well Reaction plates (0.1 ml). The results were normalized to the reference gene *rp132* and a comparative $\Delta\Delta$ CT-Method was used to calculate the expression levels.

Table 2.5 qRT-PCR reaction mix for one reaction

Component	Volume [μ l]
Master mix	5
Forward primer	0.5
Reverse primer	0.5
cDNA	4 [5 ng/ μ l]
Total volume	10

Table 2.6 qRT-PCR program

Step	Temperature [°C]	Time
Initial denaturation	95	10 min
Amplification (40 cycles)		
1. Denaturation	95	15 sec
2. Annealing	60	20 sec
3. Elongation	72	35 sec
Melt curve stage	95	15 sec
	60	60 sec
	95	15 sec

2.31 Statistics

Statistical analysis and plotting figures were done by GraphPad Prism 7 and 8. For lifespan curves analysis Log-rank (Mantel-Cox) test was used. For other experiments, first the data was tested for normal Gaussian distribution by Shapiro Wilk normality test and then an unpaired t-test was used for normal distributed data and Mann-Whitney test was used for the rest of data.

3. Results

3.1 The *Eisenia bicyclis* extract extends the lifespan in a sex-specific manner

Eisenia bicyclis has previously been shown to have anti-bacterial, anti-viral, and anti-diabetic effects [243]–[245]. I asked whether *E. bicyclis* also has any beneficial effects on the longevity of *Drosophila melanogaster*. To investigate this, a lifespan experiment with 100 mated female wild-type flies (*w¹¹¹⁸*) was performed. An aqueous extract of the brown algae was prepared and added to a concentrated medium comprising 5 % yeast extract, 8.6 % corn meal, 1 % agar, and 5 % sucrose. I designed the experiment with two experimental groups, control and *E. bicyclis* extract (EBE) treated ones. First, I started the experiment with a low concentration of 0.05 % of *E. bicyclis* extract. The survival curves showed a highly significant increase in longevity of flies fed with 0.05 % algal extract (Fig. 3.1 A). The median lifespan of EBE-treated flies extended by about 40 %. Moreover, the increase in lifespan was not only seen for the median lifespan but also the maximal lifespan (10 % of animals with the highest lifespans) with 0.05 % EBE (56 to 63 days). To confirm the results, the lifespan assay was repeated three times and the finding was reproducible in these separate lifespan assays. Then, I asked whether a higher concentration of the extract results in toxicity. Thus, I doubled the concentration to 0.1 % and investigated the effect on the lifespan of flies. Monitoring the lifespan of flies demonstrated that the algal extract is not toxic at the higher concentration, and it is still able to extend the survival of flies (Fig. 3.1 A). The algal extract could increase the lifespan of flies by 39.5 %. Similar to the lower concentration, the higher concentration extended the maximum lifespan of mated female flies (51 to 65 days). To assess whether the life-prolonging effect of *E. bicyclis* is strain-specific, I conducted a lifespan experiment with another wild-type strain of *Drosophila*, namely *yw*. Flies fed with a CM containing 0.05 % algal extract significantly outlived the control group at both median and maximum lifespan (45 to 48.5 days) (Fig. 3.2 B). I also tested cohorts of mated males, but I did not see any lifespan-extending effect by application of the EBE (Fig. 3.2).

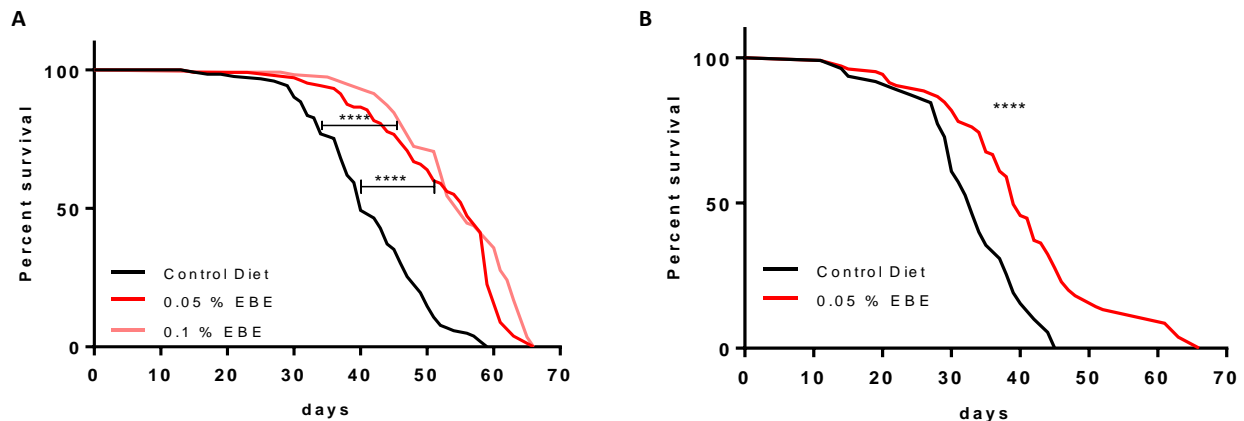


Figure 3.1. *Eisenia bicyclis* extract extended the lifespan of *Drosophila melanogaster*.

(A) The lifespan of *w¹¹¹⁸* wild-type animals increased in response to the various concentrations of *Eisenia bicyclis* extract. ($n = 180$, the median lifespan of flies treated with 0.05 % EBE = 56, the median lifespan of flies treated with 0.1 % EBE = 53, median lifespan of control group = 40). (B) The lifespan of *yw* animals increased significantly in response to 0.05 % EBE. ($n > 100$, the median lifespan of EBE-treated flies = 39, the median lifespan of control flies = 33). EBE: *Eisenia bicyclis* extract, **** $p < 0.0001$.

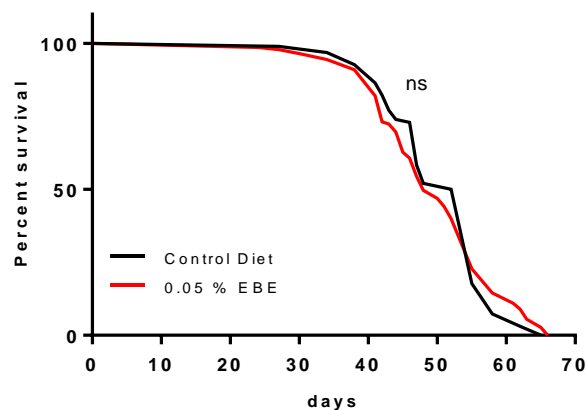


Figure 3.2. The lifespan of male *w¹¹¹⁸* flies in response to the *Eisenia bicyclis* extract.

The *Eisenia bicyclis* extract failed to increase the lifespan of male flies ($n > 100$, the median lifespan for EBE-treated = 51, and the median lifespan of the control group = 53.5). Ns: not significant, EBE: *Eisenia bicyclis* extract.

3.1.1 The EBE does not affect the food intake and fecundity

To exclude indirect effects caused by reduced uptake due to changes in food taste or odor and therewith leading to an induced caloric restriction with its lifespan prolonging effects, I quantified the nutritional intake using the consumption-excretion assay. Here, no statistically significant differences in food consumption during a 24 h period were observed (Fig. 3.3 A).

The pharmacological and genetic interventions that increase lifespan often compromise the

fecundity of animals. For example, the administration of rapamycin prolongs the lifespan of flies but reduces fecundity [60]. Similarly, the downregulation of IGF/Insulin signaling by genetic manipulation that extends the lifespan decreases the fecundity of animals [246], [247]. Therefore, I assessed the possibility of a tradeoff between longevity and fecundity. However, counting the eggs laid over the course of 7 days did not show compromised fecundity by the extract (Fig. 3.3 B).

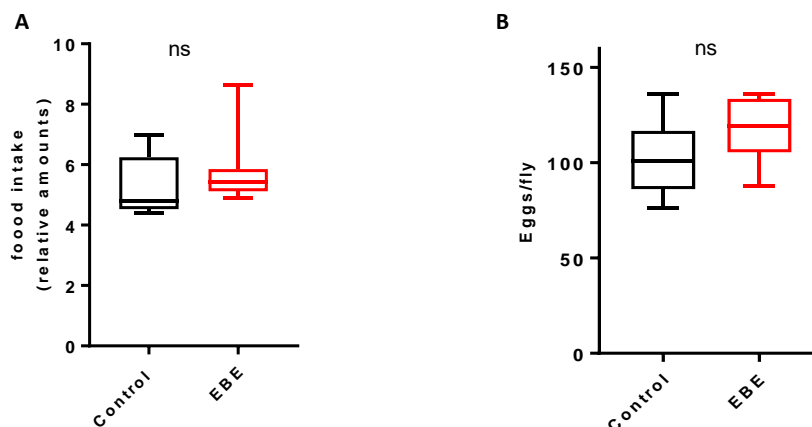


Figure 3.3. The food intake and fecundity of wild-type animals.

(A) The extract did not negatively affect the feeding behavior of animals (n = 20). (B) The fecundity of animals was not compromised by the extract (n = 8). Ns: not significant, EBE: *Eisenia bicyclis* extract.

3.1.2 The *Eisenia bicyclis* extract reduces the protein content

I asked whether treating flies with 0.05 % EBE affects the body composition. To address this question, several physiological parameters were tested including body weight, protein content, triacylglycerol levels, and glucose content. Regarding body weight, the experimental groups did not show any difference (Fig. 3.4 A). I examined lipid storage and carbohydrate content by measuring triglyceride (TAG) and glucose levels, respectively. TAG levels and glucose content of the whole flies fed with the extract showed no significant changes after two weeks of treatment with the diet of interest (Fig. 3.4 B, C). I repeated the experiment after three weeks of treatment with the extract. Interestingly, the flies fed with the extract had lower fat content compared to the control group (Fig. 3.5 A). Since the extract caused a reduction in the whole body of flies fed with the extract, I wondered whether the reduction in TAG levels is due to the triglyceride lipase activity. To test this hypothesis, I quantified the expression levels of the main lipase in *Drosophila* Brummer. Strikingly, the qPCR results

showed an upregulation in the expression of *bmm* (Fig. 3.5 D). In addition to the whole-body glucose levels, the hemolymph glucose and trehalose content were measured. Nonetheless, the hemolymph glucose and trehalose levels did not differ significantly compared to their control counterparts (Fig. 3.6 A, B). It has been shown that the aging process decreases proteostasis and leads to the assemblage of protein aggregates [248]. Since the administration of EBE improved the longevity of flies, I postulated that the protein content of these long-lived flies is lower than the control group. Assessing the protein content of EBE-treated flies showed a significant reduction compared to the control group (Fig. 3.2 D). I tested this hypothesis that the reduced protein level is due to reduced protein synthesis using a puromycin assay was performed and the signal density of newly synthesized proteins was quantified. Analyzing the data showed no significant changes in the protein synthesis of flies (Fig. 3.7 A). Repeating the experiment after three weeks of treatment showed that the flies fed with the extract showed lower protein production however this result was not statistically significant (Fig. 3.7 B).

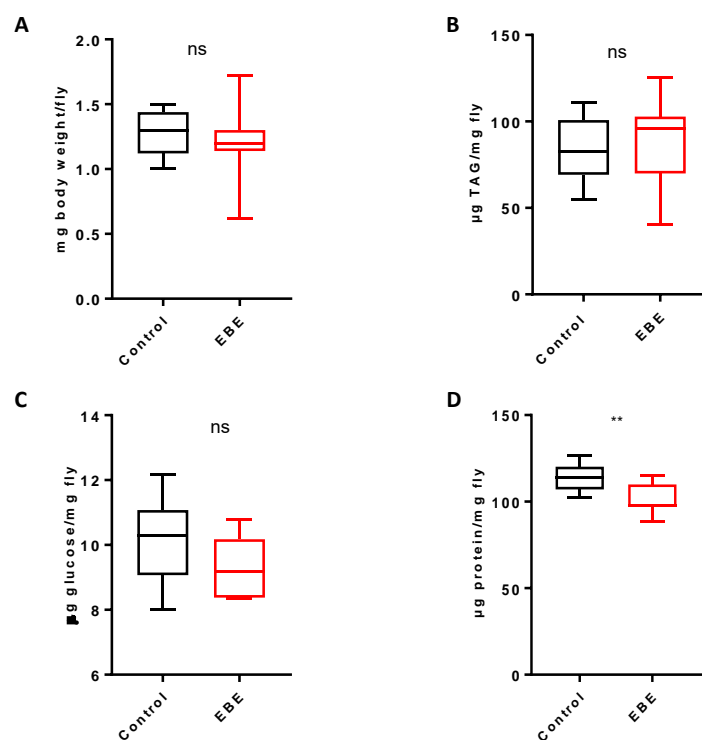


Figure 3.4. The effects of *Eisenia bicyclis* extract on physiological parameters of female *Drosophila*.

(A-C) The body weight, the TAG, and the glucose levels of flies fed with the extract did not change significantly (n = 10). (D) The extract decreased the protein content of flies (n = 10). ns: not significant, **p < 0.01.

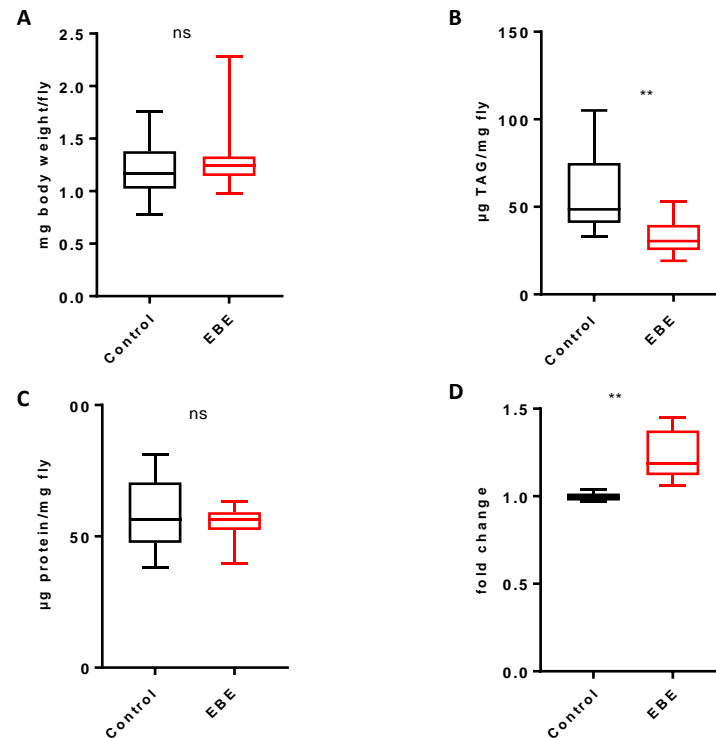


Figure 3.5. Flies fed with EBE showed a reduction in their TAG levels after 3 weeks treatment.

(A) Body weight was not affected after 3 weeks treatment (n = 10) (B) The TAG levels in flies fed with the extract reduced significantly (n = 10) (C) The protein content did not differ between the experimental groups after 3 weeks (n = 10) (D) The expression of the main lipase in flies (Brummer) was upregulated upon treatment with the extract (n = 10). ns: not significant, TAG: triacylglycerol, **p < 0.01.

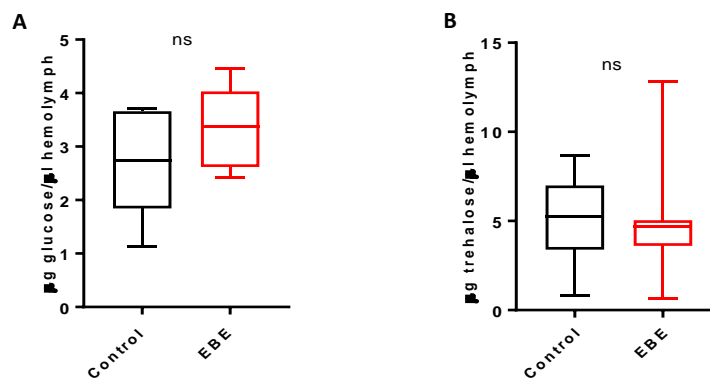


Figure 3.6. The amount of nutritive carbohydrate in the hemolymph was not altered by the administration of the extract.

(A) Glucose content (n = 6) and (B) trehalose content (n = 8) of hemolymph did not show significant alterations. Ns: not significant

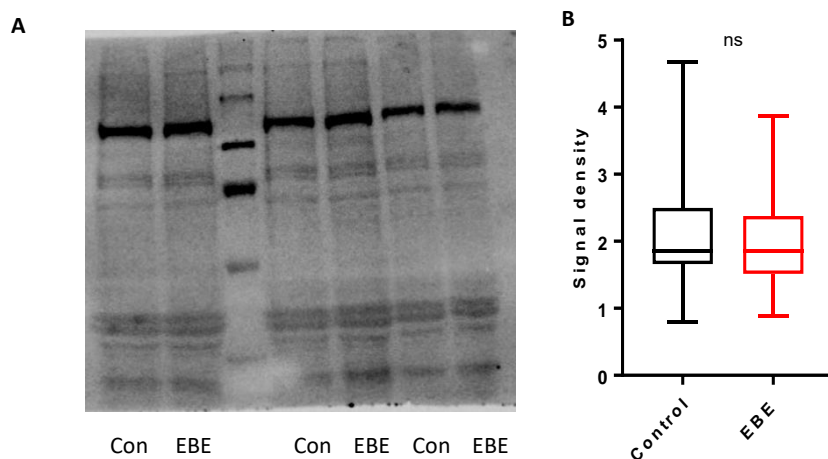


Figure 3.7. The extract does not affect the protein synthesis in flies.

(A, B) Western blot analysis of samples from control flies (Con) and EBE-treated ones (EBE) of puromycin treated flies and the quantification of band intensities did not show significant alteration in the newly synthesized proteins.

3.1.3 The sleep patterns and locomotor activities are not altered by EBE

Several studies have investigated the correlation between sleep and longevity in a wide variety of evolutionary taxa, demonstrating the significance of the quantity and quality of sleep [249]–[252]. I asked whether applying the algal extract to *Drosophila* food affects its circadian rhythm and locomotor activity. I analyzed the sleep and locomotor activity pattern of flies (Fig. 3.8 A, B) fed on food with algal extract over a course of 24 hours with a 12-hour light/dark cycle. I also quantified the locomotor activity. While flies had two distinct times of increased activity, the locomotor activity did not show any significant alterations in EBE-fed flies in comparison with the control flies (Fig. 3.8 C). Although daytime sleep was not significantly altered (Fig. 3.8 D), the EBE-treated flies showed a sleep pattern toward the increasing amount of sleep during the nighttime (Fig. 3.8 E). Moreover, these flies had longer sleep bouts at nighttime (Fig. 3.8 F).

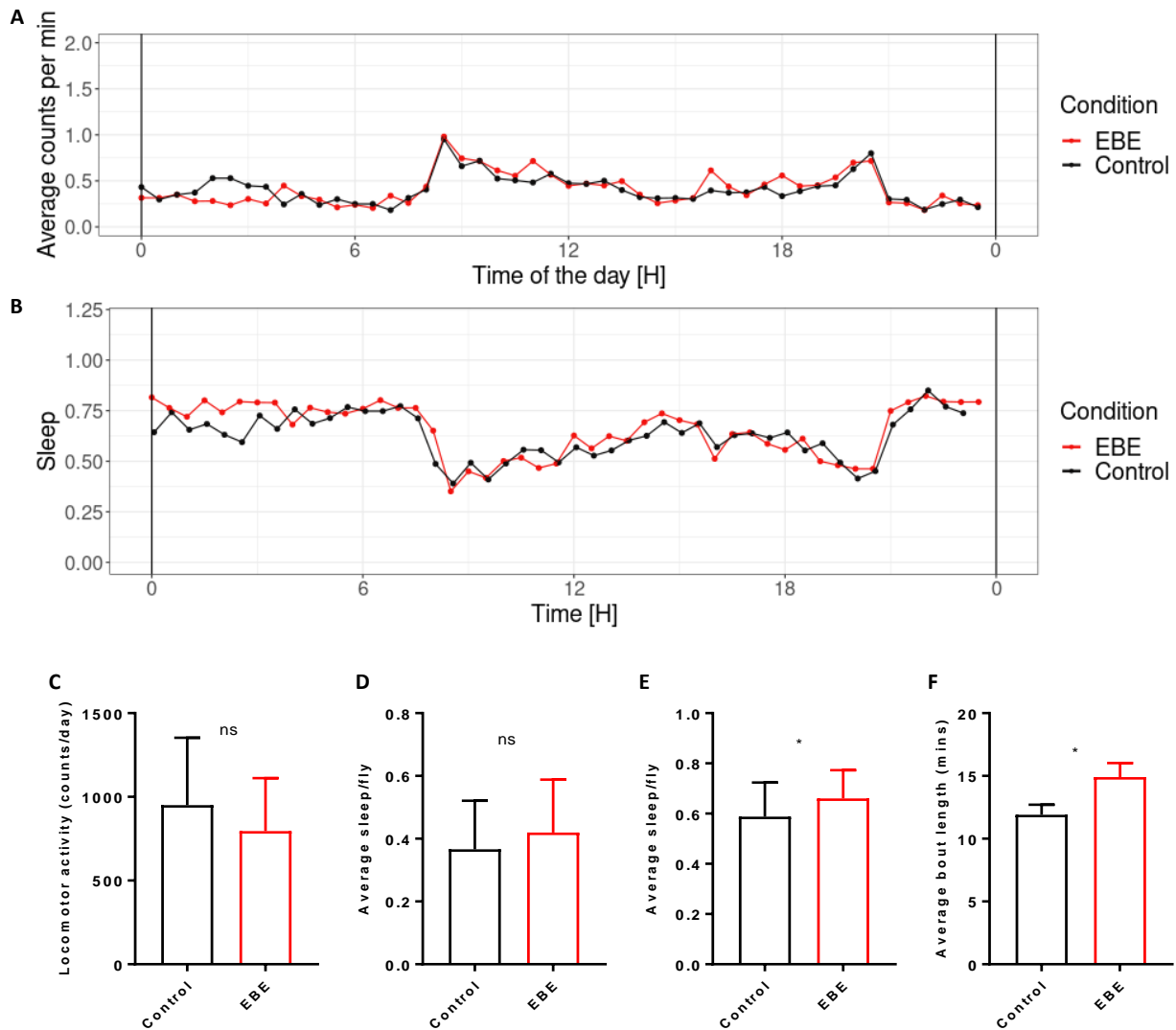


Figure 3.8. EBE improved the quality of sleep of flies.

(A) Daily locomotor activity of flies showed similar pattern. (B) The sleep pattern of flies showed similar pattern. (C) The locomotor activity measurement did not reveal significant alteration (D) The daily sleep of EBE-treated flies did not differ compared to the control group (E) The flies fed with the extract had longer sleep night. (F) The flies fed with the extract had longer sleep bouts compared to the control group. N = 32, EBE: *Eisenia bicyclis* extract, ns: not significant, *p < 0.05.

3.1.4 *E. bicyclis* has beneficial effects in response to nutritional stressors

It is known that the pro-longevity interventions in flies such as rapamycin treatment and downregulation of insulin signaling are associated with more resistance to stresses [45], [253]. Therefore, I tested EBE-treated flies for survival under starvation and desiccation stress. I first assessed resistance to starvation by placing control and EBE-treated flies on agar only to prevent energy intake, while sufficient water is supplied for two weeks. Under these conditions, the median lifespan was 72 h for the EBE-treated and 70 h for the control group. However, the difference was not statistically significant (Fig. 3.9 A). In contrast, adding EBE to the flies' food increased the resistance to desiccation. The median lifespan for the control

group was 24 h, whereas this increased to 25 h in EBE-treated flies (Fig. 3.9 B).

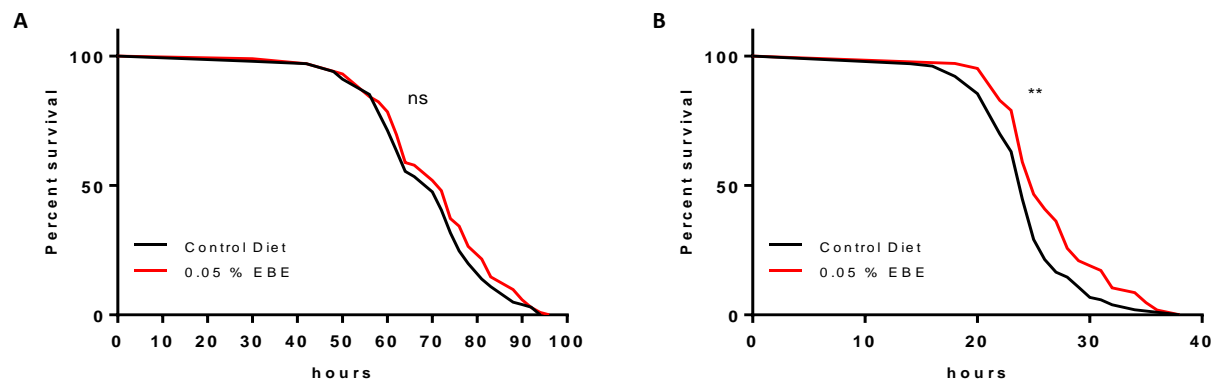


Figure 3.9. The effect of EBE on the survival of female flies under stress conditions.

(A) The survival of EBE-treated flies is not significantly altered ($n > 100$, the EBE-treated median survival = 72 h, the control median survival = 70 h). (B) The EBE-treated flies outlived the control group under desiccation stress ($n > 100$, EBE-treated median survival = 25 h, the control group median survival = 24 h). EBE: *Eisenia bicyclis* extract, ns: not significant, ** $p < 0.01$.

I continued with testing the effect of the extract on nutritional stressors such as a high-fat diet and a high-sugar diet. For survival under a high-fat diet, 20 % palm oil was added to the flies' food and to one of the experimental groups' food EBE was added. As the lifespan curves show the high-fat diet decreased the survival of flies compared to the control diet, reiterating the negative effects of high-fat diets. However, adding the extract to the flies' food slightly increased the flies' survival and the median lifespan of flies fed on a high-fat diet containing EBE increased by 3.5 % (from 29 to 30 days; Fig. 3.10. A). The effect of the extract was more pronounced at the maximum level of lifespan and the maximum lifespan of flies fed with the extract extended by 8.3 %. Next, a survival assay was performed on a diet containing 30 % sucrose. Like the high-fat diet, increasing the sugar content of the food negatively affects flies' survival, and flies lived much shorter on this diet. Interestingly, treating flies with EBE under this condition could extend the lifespan of flies almost to the level of flies on the control diet and even their median lifespan was higher than the control diet (46 versus 40 days; Fig. 3.10 B). The extract was beneficial at both median and maximum levels of the lifespan of flies. However, the effect on the median lifespan (by 30 % increase) was much stronger than the effect on the maximum lifespan where the extension in lifespan was by 8 % (Fig. 3.10 B).

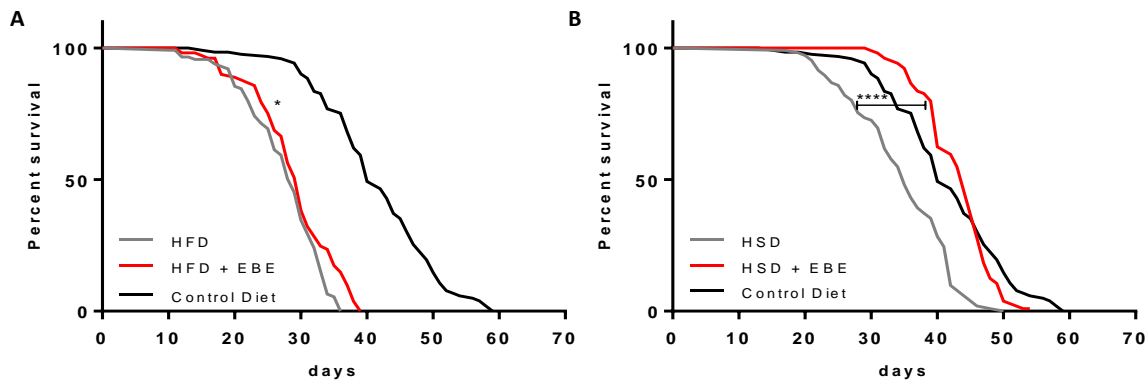


Figure 3.10. The influence of EBE on the nutritional stresses.

(A) The brown alga extract had positive effects on a high-fat diet ($n = 100$, the median lifespan of EBE-treated = 30, the control flies' median lifespan = 29). (B) The extract exhibited strong effect on a 30 % high-sugar diet ($n = 100$, the median lifespan of EBE-treated = 46, the control flies' median lifespan = 35). HFD: high-fat diet, HSD: high-sugar diet, EBE: *Eisenia bicyclis* extract, * $p < 0.05$, **** $p < 0.0001$.

Next, I asked whether treatment with the extract later in the life of flies on a 30 % sucrose diet is beneficial. To test this, flies were kept on a control diet for 20 days without EBE treatment. Then, one group of flies was transferred to a high-sugar diet containing 30 % sugar, and another group was transferred to the 30 % high-sugar diet plus 0.05 % EBE. In this experimental design, the flies fed with EBE were more resistant to the high-sugar diet and the median lifespan was increased by 5 % (Fig. 3.11 A). Next, I wondered if the extract could bestow the flies with higher resistance to a high-sugar diet when the concentration of sugar is increased. In the same manner as in a 30 % high-sugar diet, the flies treated with the extract were substantially more resistant to the 40 % sucrose in the food (Fig. 3.11 B). While the median survival of flies without the extract decreased to 21 days, the median lifespan of flies with the extract was similar to the flies on a control diet. Comparing the median lifespan of flies on a 40 % high-sugar diet with and without the extract revealed a 95 % increase in the survival of flies with the extract. Like the median lifespan, the maximum lifespan of flies on a 40 % sugar diet significantly increased by 40 %.

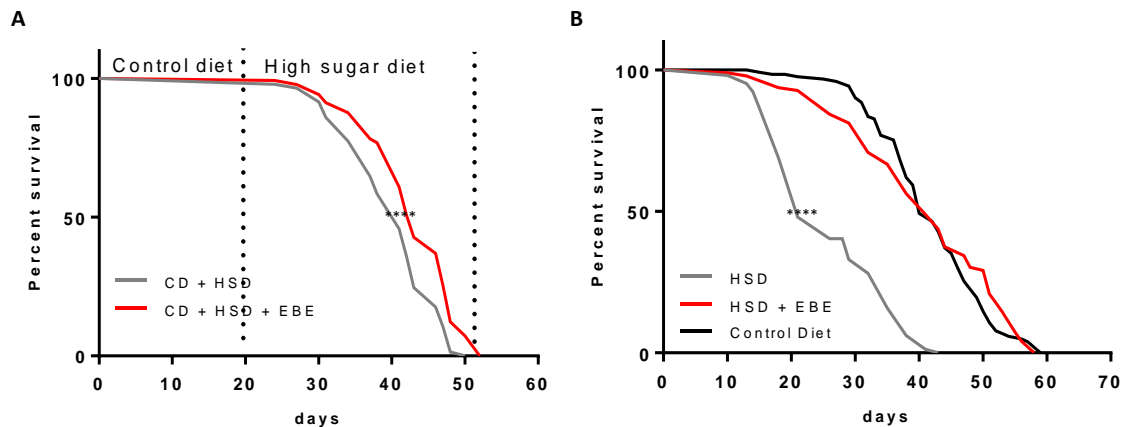


Figure 3.11. The effect of EBE upon higher sugar concentration and late administration.

- (A) EBE rescued the flies from the detrimental effects of 40 % sucrose in the diet on the lifespan ($n > 100$, the median lifespan of EBE-treated = 42, the median lifespan of control group = 21). (B) The extract could improve the lifespan of flies treated with a 30 % sucrose diet ($n > 100$, the median lifespan of EBE-treated = 43, the median lifespan of control group = 41). CD: control diet, HSD: high-sugar diet, EBE: *Eisenia bicyclis* extract, **** $p < 0.0001$.

3.1.5 The *Eisenia bicyclis* extract reduces the glucose content on HSD

Since the extract had such a strong effect on high-sugar diets, I measured the body composition and the effect of the extract on *Drosophila's* metabolism upon treatment with a diet containing 30 % sucrose. Like in the control diet (Fig. 3.4), the body weight and TAG levels did not change by supplementing flies' food with EBE for two weeks (Fig. 3.12 A, B). However, the reduced protein level in the control diet was not observed in the high-sugar diet. Interestingly, the glucose levels were lower in the flies fed with EBE on the high-sugar diet, suggesting the extract has an anti-hyperglycemic effect (Fig. 3.12 C). Besides, the expression of *glucose transferase 1 (glut1)* was upregulated in the whole body of the female wild-type flies (Fig. 3.12 D).

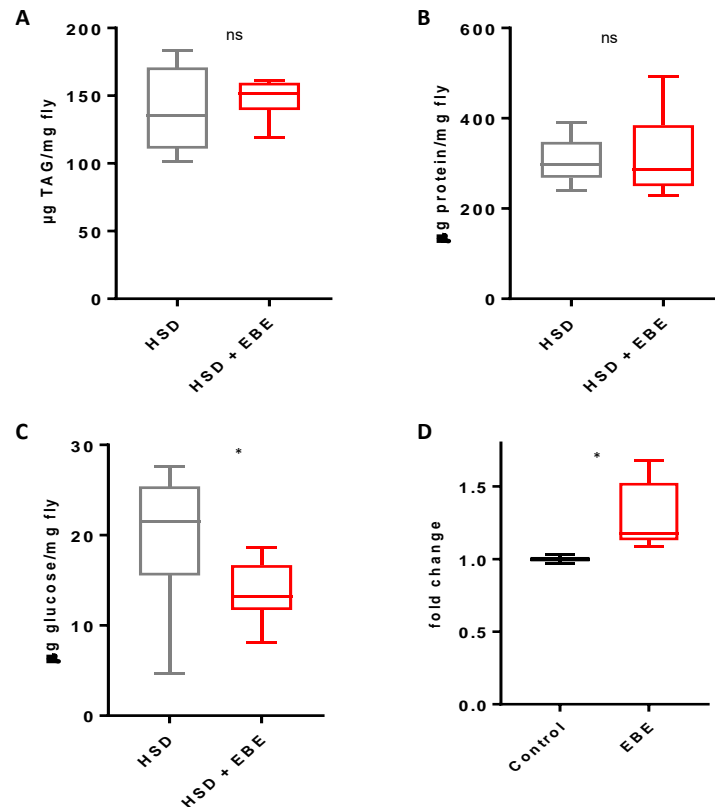


Figure 3.12. The body composition of the flies treated with EBE on a 30 % sucrose diet.

(A-B) The TAG and Protein levels did not alter on the 30 % sugar diet ($n = 10$). (C) The flies fed with the extract had lower content of glucose on the high-sugar diet ($n = 10$). (D) The glucose transporter 1 (*glut1*) expression reduced in the whole body of flies on the control diet ($n = 5$). TAG: triacylglycerol, EBE: *Eisenia bicyclis* extract, ns: not significant, $*p < 0.05$.

3.2 The mode of action of *Eisenia bicyclis* aqueous extract

Next, I asked what the mode of action of *Eisenia bicyclis* is. To this end, I investigated three evolutionary conserved signaling pathways that are associated with the regulation of lifespan in different model organisms including the sirtuin family, insulin/IGF signaling (IIS), and the mechanistic target of rapamycin (mTOR) signaling pathways.

3.2.1 EBE might influence longevity through sirtuins

Sirtuins are NAD^+ -dependent histone deacetylases and thus are involved in epigenetic alterations. Moreover, this family has distinct roles in metabolism particularly fat and glucose metabolism and influences energy hemostasis [254]. While the sirtuin family has seven members in mammals, it comprises five proteins in flies including Sirt1, Sirt2, Sirt4, Sirt6 and Sirt7 [255]. The initial studies reporting the association of sirtuins in lifespan was that overexpression of the *Sir2* gene in *Saccharomyces cerevisiae* extended the lifespan of the

animals and *Sir2* mutant animals had a reduced lifespan [256]. Later it was demonstrated that the extension in lifespan by an increase in *Sir2* is not specific to yeast and increasing the *Drosophila* *Sir2* represented the same phenotype. Also it was shown that the mechanism of lifespan extension by caloric restriction requires *Sir2* in flies [257]. To test the hypothesis that the extract exerts its effect through *Sirt1* signaling, I employed *Sirt1* KO flies and did a survival assay. Consistent with existing literature, *Sirt1* knockout flies lived shorter than their genetic control group. However, the administration of the extract in the flies' food elevated the median and maximum lifespan of the *Sirt1* KO animals (Fig. 3.13 A). Another family member of sirtuins with roles in metabolism and aging is *Sirt6*. Studies in mice and flies have shown that overexpression of *Sirt6* produces a significant elevation in the longevity of animals [258], [259]. I, therefore, tested the impact of the EBE on the levels of *Sirt6* expression using quantitative polymerase chain reaction (qRT-PCR). The flies were fed with the extract for two weeks then the qRT-PCR test was performed. Analyzing the results revealed an upregulation of *Sirt6* expression in flies fed with the extract (Fig. 3.13 B).

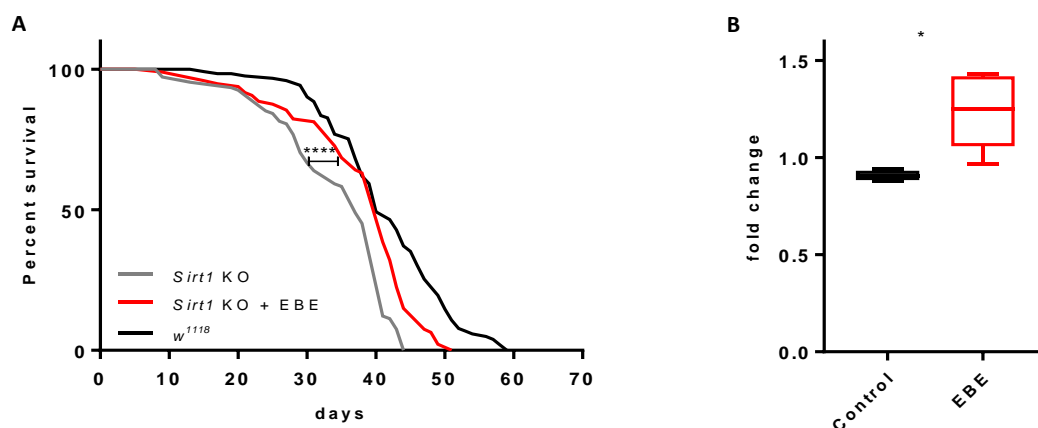


Figure 3.13. EBE might have positive effects on the sirtuin family.

(A) The extract rescued the reduced lifespan in *Sirt1* KO animals ($n = 120$, the median lifespan of EBE-treated *Sirt1* KO flies = 41, the median lifespan of *Sirt1* KO flies = 37). (B) The expression of *Sirt6* in wild-type animals increased in response to the extract ($n = 5$). EBE: *Eisenia bicyclis*, * $p < 0.05$, **** $p < 0.0001$.

3.2.2 IIS signaling is required for the mode of action of EBE

The IIS pathway is the key regulator of mechanisms such as stress resistance, metabolism, reproduction, and growth. It is composed of insulin in mammals and insulin-like peptides (Ilps) in *Drosophila*, the tyrosine kinase receptor known as insulin receptor in mammals and insulin-like receptor in flies, insulin receptor substrate (IRS) known as chico in flies, the downstream

effectors [44], [260]. It is well-known that the downregulation of insulin signaling pathways at distinct levels could extend the lifespan of various organisms [47], [261]. Therefore, I assessed the effect of EBE on this nutrient and energy-sensing signaling. To this end, I started with the evaluation of the FoxO transcription factor as a proxy of IIS signaling in the mode of action of the extract. When the IIS is activated, FoxO localizes in the cytoplasm and upon the inactivation of insulin signaling, FoxO translocates into the nucleus and triggers the transcription of genes that lead to longevity. I implemented flies with a point mutation in *foxo*, which causes a deficiency in FoxO, and did a survival analysis. As expected, the downregulation of *foxo* led to a shortening of the lifespan of *foxo*-deficient flies. I administered the extract in the food of one experimental group; however, it did not result in an extension in the lifespan of flies (Fig. 3.14 A). Further, I evaluated the role of FoxO in the observed phenotype caused by the extract by analyzing the expression of *foxo* after two weeks of treatment with the extract in the wild-type animals and the quantification of *foxo* expression revealed an upregulation in *foxo* in flies fed with the extract (Fig. 3.14 B).

To further illuminate the EBE's mode of action and the role of IIS in it, I asked whether disrupting the IIS at the receptor level could block the effect of EBE. IGF/insulin receptor is a receptor tyrosine kinase that in *Drosophila* is called *Drosophila* insulin-like receptor (InR) and its knockout leads to lifespan extension. As expected, *InR* KO animals outlive the wild-type group and while the median lifespan of flies with the extract slightly increased (from 33 to 35 days) is not significantly extending the lifespan of insulin-like receptor knockdown animals (Fig. 3.14 C). Chico is another component of insulin signaling with known roles in longevity phenotypes in flies. A homozygote mutation in flies in the *chico* gene extends the median lifespan of flies by 48 % and the heterozygote animals had a 36 % increase in their lifespan [45]. Thus, I investigated whether this insulin-like receptor substrate is required for the lifespan-extending effect of the extract. I implemented female flies with a loss of function allele of *chico* (*P{ry11}chico[1]*) in a lifespan assay. Assessing the lifespan curves exhibited that the mutant animals outlived their genetic background in wild-type animals and the *chico* mutant animals fed with the extract showed an extension in their lifespan compared with the mutant animals without the extract (Fig. 3.14 D).

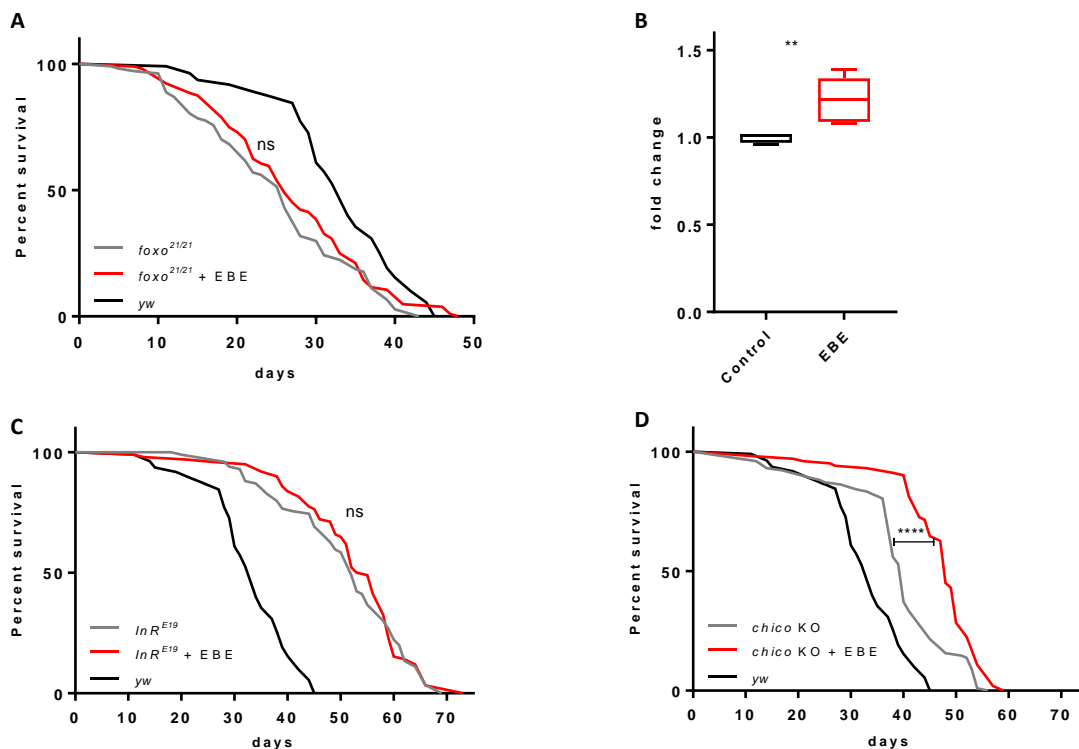


Figure 3.14. The beneficial effect of EBE was abolished by disruption of Insulin/insulin-like signaling pathway.

(A) The lifespan extending effect of the extract was blocked in *foxo* deficient flies ($n < 100$, the median lifespan for both treated and untreated *foxo*-deficient flies was 26). (B) The expression of *foxo* was upregulated in *w¹¹¹⁸* flies fed with the extract ($n = 5$) (C) Effective insulin-like receptor is necessary for the action of the extract ($n = 100$, the median lifespan of *InR*^{E19} + EBE = 55, the median lifespan of *InR*^{E19} = 52) (D) The insulin receptor substrate *chico* might not be involved in the mode of action of the extract ($n = 100$, the median lifespan of *chico* KO + EBE = 48, the median lifespan of *chico* KO = 40). EBE: *Eisenia bicyclis* extract, ns: not significant, ** $p < 0.01$, **** $p < 0.0001$.

3.2.3 The Tor signaling pathway is required to extend the lifespan by EBE

Since the Tor and IIS signaling pathways are interwoven and crosstalk occurs between them in most of the biological mechanisms, I went on to test the requirement of Tor protein for the beneficial effects of EBE. Tor pathway is modulated by a variety of signals such as amino acids, cellular energy, and growth factors through its upstream protein TSC complex. Upon activation of TOR complex 1, mechanisms such as pyrimidine synthesis, ribosomal biogenesis, lipogenesis, translation, and autophagy are affected by its downstream effectors including S6K1, 4E-BP, ULK1, and lipin 1 [46], [262]. As mentioned before, many anti-aging agents exert their beneficial effects through the Tor signaling pathway and the existing literature shows downregulation of Tor signaling extends the lifespan [65], [66], [68], [263]. Thus, I analyzed the lifespan-extending effect of flies in animals with a defective Tor protein. I postulated if the EBE targets tor signaling, the effect in the animals with a defective tor would be blocked. Lifespan curves analysis showed that whereas animals with downregulation of *Tor* outlived

the wild-type group, the EBE did not elevate the lifespan of the treated group in comparison with *Tor* mutant without the extract (Fig. 3.15 A). To evaluate this finding more, a ubiquitous driver line was implemented to drive downregulation of *Tor* in the whole body using the Gal4/UAS system. Unlike the *Tor* mutant animals, the extract increased the lifespan of these flies, particularly their maximum lifespan (Fig. 3.15 B).

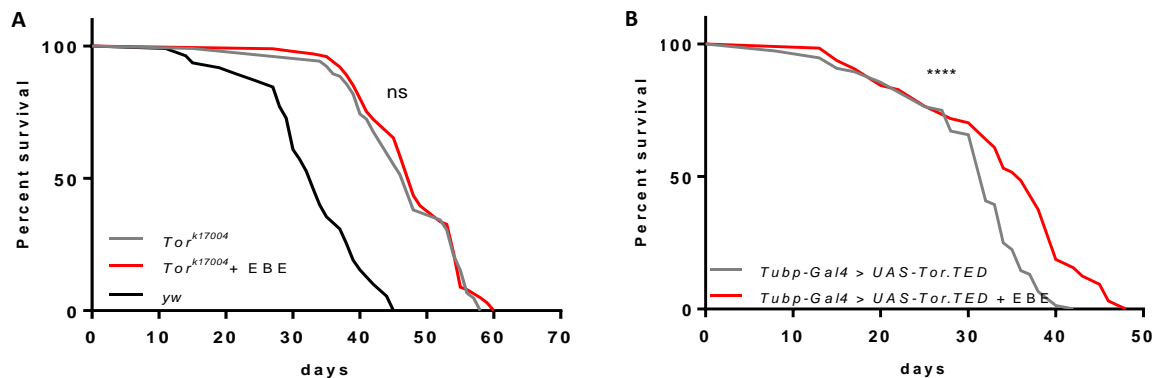


Figure 3.15. EBE probably requires effective TOR signaling to mediate the extension in lifespan.

(A) The extract failed to extend the lifespan of *Tor* mutant flies ($n = 180$, the median lifespan of EBE-treated *Tor* mutants = 48, the median lifespan of *Tor* mutants = 48). (B) Ubiquitous driving *Tor* dominant negative did not block the lifespan extension effect of the extract ($n = 70$, the median lifespan of treated flies = 36, the median lifespan of untreated flies = 32). Ns: not significant, **** $p < 0.0001$.

3.2.4 What is the target organ of *Eisenia bicyclis*?

To answer the question whether the extract targets a specific organ to mediate its positive effects, I investigated the main tissues and organs regulating metabolism and lifespan in flies including the fat body, the intestine, and the skeletal muscles. A reverse genetic approach was implemented by modulating the signaling pathways involved in lifespan regulation using binary Gal4/UAS gene expression system in the tissue or organ of interest.

3.2.5 EBE might target the *Tor* signaling pathway in the fat body

The fat body is the main location of storing the excess energy in form of triglycerides and glycogen and it is central to flies' metabolism and nutrient sensing equivalent to the liver and adipose tissue in mammals [264]. It communicates metabolism and nutrient status by secretion of systemic factors such as unpaired-2, eiger, growth-blocking peptides, CCHamide-2, dawdle, ImpL2, peptidoglycan recognition proteins, and Dilp6 [265]. First, I used the fat body-specific driver *P(GS)106* to induce genes of interest in the fat body. In this system, the driver line requires a drug called mifepristone or RU486 to drive the expression of the gene of

interest in the responder line in the offspring of the crossing between the driver and responder lines. I induced the expression of a dominant negative form of Tor in the fat body using the GeneSwitch system ($P(GS)106 > UAS-Tor.TED$). Induction of the dominant negative form of Tor in the fat body extended the lifespan of flies slightly but not significantly in comparison with non-induced flies. Moreover, the administration of the EBE in the food of induced flies did not extend their lifespan significantly (Fig. 3.16 A). Using the same GeneSwitch system, the *Tor* was overexpressed in the fat body ($P(GS)106 > UAS-Tor.WT$). The median and maximum lifespan of induced and non-induced flies did not change and the lifespan extension by the extract was blocked (Fig. 3.16 B). To further investigate the role of Tor signaling on the positive effects of the extract, I downregulated the nutrient-sensing pathway by RNAi depletion of the downstream effector of Tor in the fat body using the GeneSwitch system ($P(GS)106 > UAS-S6K-RNAi$). Like the downregulation via the dominant negative form of Tor, RNAi depletion of *S6K* abolished the lifespan extension caused by the extract and I did not observe any extension either at minimum or at maximum levels of the lifespan of flies (Fig. 3.16 C). I went on with the inhibition of *sir2* in the flies' fat bodies using a *UAS-Sir2* line. Inducing overexpression of *UAS-Sir2* did not extend the lifespan compared to the non-induced flies. Nonetheless, EBE promoted the lifespan of induced flies (Fig. 3.16 D).

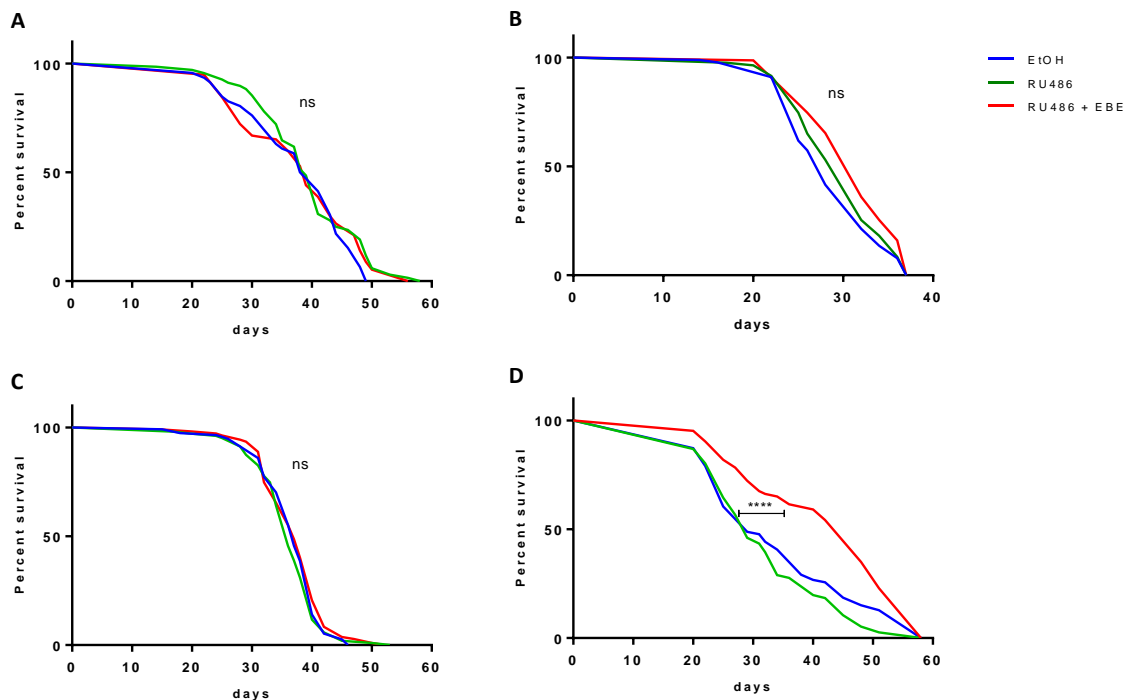


Figure 3.16. Manipulation of the Tor signaling in the fat body blocked the lifespan extending effects of EBE.

(A) Overexpression of the Tor signaling in the fat body ($P(GS)106 > UAS-Tor.WT$) resulted in blocking the effect of the extract ($n = 60$, the median lifespan for uninduced flies = 39.5, the median lifespan of induced flies = 39, the median lifespan of induced flies fed with the extract = 39). (B) Extension of lifespan by the extract was abolished in flies with dominant negative form of TOR in their fat body ($P(GS)106 > UAS-Tor.TED$) ($n = 80$, the median lifespan for uninduced flies = 28, the median lifespan of induced flies = 32, the median lifespan of induced flies fed with the extract = 32). (C) The RNAi depletion of S6K in the fat body ($P(GS)106 > UAS-S6K-RNAi$) led to abolish of positive effect of the extract ($n > 100$, the median lifespan for uninduced flies = 37, the median lifespan of induced flies = 36, the median lifespan of induced flies fed with the extract = 37). (D) The extract extended the lifespan of flies with downregulation of sirtuin 2 in their fat bodies ($P(GS)106 > UAS-Sir2$) ($n = 80$, the median lifespan for uninduced flies = 29, the median lifespan of induced flies = 29, the median lifespan of induced flies fed with the extract = 45). Ns: not significant, EBE: *Eisenia bicyclis* extract, **** $p < 0.0001$.

Since I observed the extract failed to increase the lifespan by the inhibition of Tor signaling either at the Tor level or the S6K level and that the induction of the downregulation of *Tor* in the fat body of these animals did not extend the lifespan in comparison with non-induced animals, I used another fat body-specific driver line to modulate the Tor signaling in the fat body. Unlike the GeneSwitch system, overexpression of *Tor* in the fat body by the *ppl-Gal4* driver did not abolish the extension in the lifespan of flies and the flies fed with the extract outlived the flies without the extract (Fig. 3.17 A). On the other hand, RNAi depletion of *S6K* in the fat body using this driver recapitulated the results of the GeneSwitch system. The EBE failed to extend the lifespan of flies with *S6K* down-regulation in their fat body whereas these flies outlived their genetic control flies (Fig. 3.17 B).

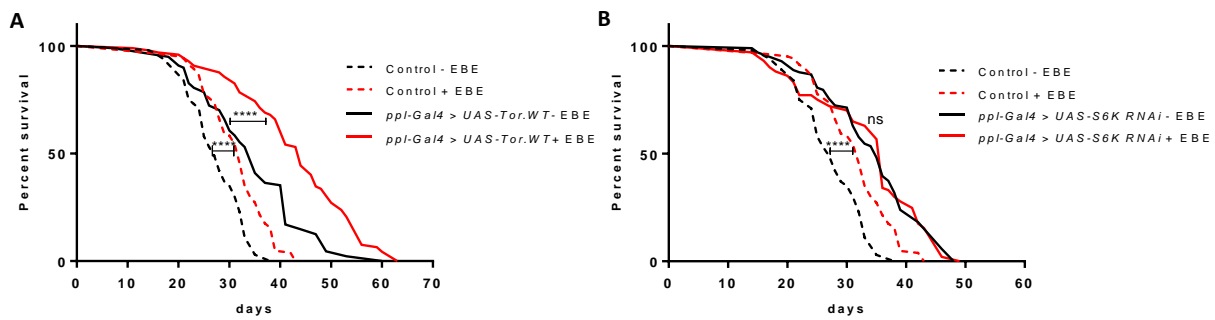


Figure 3.17. S6K is required for the mode of action of EBE.

(A) Overexpression of *Tor* in the fat body (*ppl-Gal4 > UAS-Tor.WT*) did not block the positive effect of the extract ($n = 100$, control - EBE median lifespan = 27, control + EBE = 32, *ppl-Gal4 > UAS-Tor.WT* - EBE = 35, *ppl-Gal4 > UAS-Tor.WT* + EBE = 44). (B) The RNAi depletion of S6K in the fat body using *ppl-Gal4* driver (*ppl-Gal4 > UAS-S6K RNAi*) hindered the lifespan-extending effect of the extract. ($n = 100$, control - EBE median lifespan = 27, control + EBE = 32, *ppl-Gal4 > UAS-S6K RNAi* - EBE = 35, *ppl-Gal4 > UAS-S6K RNAi* + EBE = 36). Ns = not significant, **** $p < 0.0001$.

3.2.6 Enterocytes are not the main target of EBE

Like mammals, the *Drosophila* intestine is the main site of digestion and absorption of nutrients. However, it is not the only function of the gut in flies, and it is central to signal the food intake status and consequently insulin-like peptides secretion from the brain and thus balancing energy homeostasis. In addition to signaling food intake, the gut plays an important role in physiology and metabolism by interacting with the flies' microbiota [264]–[266]. Although the gut is not directly linked to longevity in flies, due to its significant roles in physiology and metabolism, I investigated its involvement in the mode of action of EBE. I modulated the Tor signaling in the flies' enterocytes using a driver line specific to these cells (*NP1-Gal4*). Tor signaling was inhibited using the negative form of Tor in these cells (*NP1-Gal4 > UAS-Tor.TED*). Likewise, the extract was effective and the flies with the downregulation of *Tor* in their enterocytes outlived their genetic control without the extract by 28 % (Fig. 3.18 A). Then, I asked whether the downregulation of Tor signaling in enterocytes via its downstream effector S6K abolishes the lifespan extension effect of the extract. I crossed S6K-RNAi flies (*UAS-S6K-RNAi*) with an enterocyte-specific driver line and assessed the lifespan of the offspring. This manipulation did not block the positive effect caused by the extract either (Fig. 3.18 B). I showed global inhibition of *Sirtuin1* did not block the effect of the extract. I wondered if enterocyte-specific modulation of sirtuins represents the same observation. For this purpose, *sirt2* was overexpressed in flies' enterocytes and the extract was administrated to one group of overexpressed flies and the survival was compared. I found that like global modulation of *Sirt1*, enterocyte-specific modulation of *sir2* does not affect the lifespan

extension effect of EBE (Fig. 3.18 C).

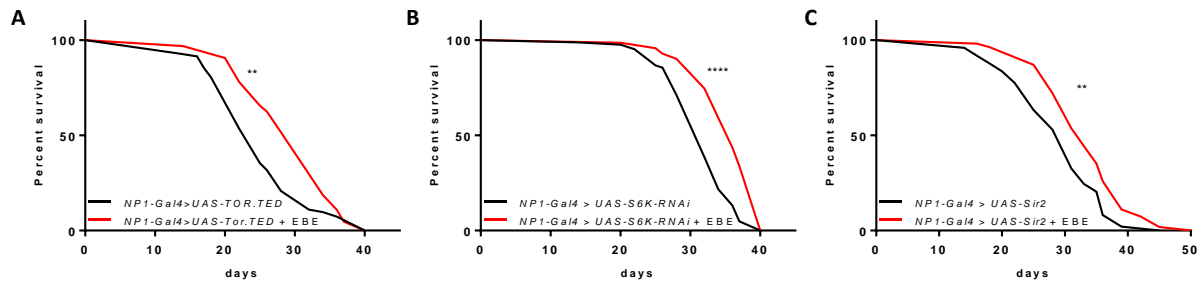


Figure 3.18. The enterocytes were not the main target of EBE to extend the lifespan of flies.

(A) Enterocyte specific downregulation of the TOR signaling pathway (*NP1-Gal4 > UAS-Tor.TED*) did not hinder the effect of the extract (n = 80, the median lifespan of EBE treated = 32, the median lifespan of untreated flies = 25). (B) EBE extended the lifespan of flies with RNAi depleted S6K in enterocytes (n = 80, the median lifespan of treated flies = 36, the median lifespan of untreated flies = 32). (C) Manipulation of Sirtuin 2 did not block the extension in EBE-treated flies (n = 50, the median lifespan of treated flies = 33, the median lifespan of untreated flies = 31).

3.2.7 The downregulation of Tor signaling in muscles did not abolish the lifespan extension by EBE

Whereas the gut digests and absorbs nutrients and the fat body stores excessive energy, the skeletal muscles are the major center of energy expenditure [264]. Moreover, skeletal muscles are linked to lifespan regulation since overexpression of *foxo* in muscles prevents age-related decline in skeletal muscle function. Moreover, the age-related protein aggregations in skeletal muscles that characterize muscle aging are removed by FoxO/4EBP signaling thus delaying muscle functional decay and *foxo* mutants have dysfunctional proteostasis [267]. This signaling in the skeletal muscles reduces the food intake of flies and inhibits insulin-like peptides from insulin-producing cells in the brain thus extending the lifespan of animals [267]. Therefore, I investigated whether the EBE is targeting the Tor signaling in the skeletal muscles to increase the lifespan of flies. A dominant negative form of Tor was driven in skeletal muscles (*Mhc-Gal4 > UAS.Tor.TED*). The flies were divided into two groups, EBE-fed and control. Assessing the lifespan curves revealed a 20.5 % lifespan extension of flies with the extract (Fig. 3.19).

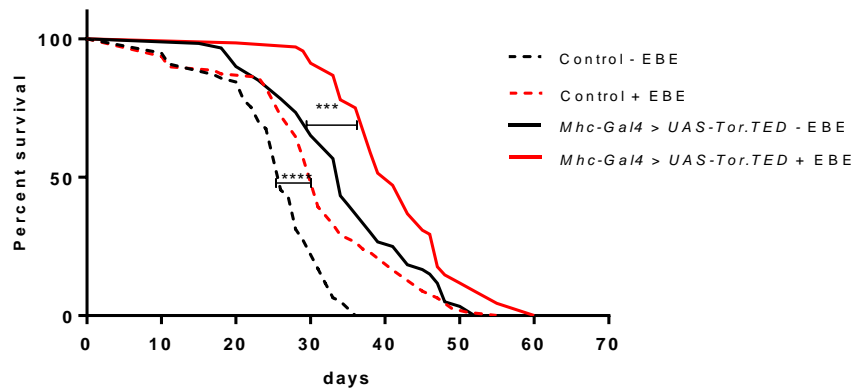


Figure 3.19. EBE extended the lifespan of flies with the downregulation of Tor signaling in their muscles.

The inhibition of Tor in the skeletal muscle cells extended the lifespan and it did not abrogate the lifespan extension by the extract. N = 70, the median lifespan of *Mhc-Gal4 > UAS.Tor.TED + EBE* = 41, the median lifespan of *Mhc-Gal4 > UAS.Tor.TED - EBE* = 34, the median lifespan of control + EBE = 31, and the median lifespan of control - EBE = 26. ***p < 0.001, ****p < 0.0001.

3.2.8 Gene expression changes in response to *Eisenia bicyclis*

Since I observed that EBE mediates the extension of lifespan through interwoven IIS/Tor signaling pathways, I evaluated the expression of genes involved in these pathways including *Tor*, *4ebp*, *impl2*, *tobi*, *atg8a*, and *puc* in wild-type female *w¹¹¹⁸* flies after two weeks of treatment with EBE. The qRT-PCR results revealed that only the expression of *atg8a* was differentially expressed and the transcription of *tor*, *4ebp*, *impl2*, *tobi*, and *puc* was not modified by EBE (Fig. 3.20).

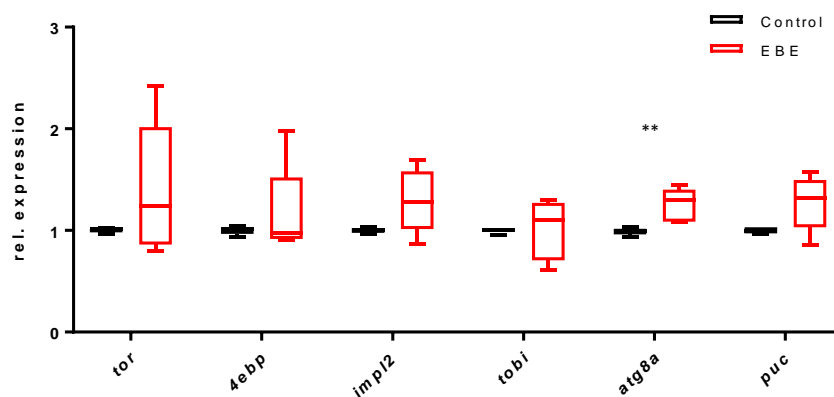


Figure 3.20. The relative expression of genes involved in IIS/Tor signaling pathways.

The autophagy-related gene *atg8a* was upregulated whereas the rest of the evaluated genes showed no significant differential expressions. The results were normalized to *rpl32* (n = 5). ** p < 0.01

3.3 *Salicornia* extract extends the lifespan of flies in a sex-specific manner

Salicornia species have been linked to some positive biological activities. Nonetheless, no study has been done on the geroprotective effects of *Salicornia*. Thus, I wondered whether *S. europaea* also has any beneficial effects on the longevity of *Drosophila melanogaster*. To investigate this, a life span experiment with 100 mated female wild-type flies (w^{1118}) was performed. An aqueous extract of the halophyte was prepared and added to a concentrated medium comprising 5 % yeast extract, 8.6 % corn meal, 1 % agar, and 5 % sucrose. I designed the experiment with two experimental groups, control, and *S. europaea* treated. First, I started the experiment with a low concentration of 0.05 % of the extract. The survival curves showed no significant extension in the lifespan of mated female flies (Fig. 3.21 A). Next, the concentration of the extract in the food of flies was increased to 0.2 % and the lifespan of female flies was assessed. The extract could extend the lifespan of flies at this concentration by 36 % (from 30 to 41 days) (Fig. 3.21 B). To confirm the results, the lifespan assay was repeated three times and the positive effect was reproducible in these separate experiments. The extract not only increased the lifespan of flies at median levels but also at maximum levels (from 45 to 50 days). I also tested cohorts of mated males, but I did not see any lifespan-extending effect by application of the EBE and the median and maximum lifespan for both experimental groups were 41 days and 48 days respectively (Fig. 3.21 C). This sexual dimorphism in lifespan extension is recapitulating the other nutritional and pharmacological studies showing a greater effect in female animals than in males [49], [60]. I continued with evaluating the effect on another wild type strain of *Drosophila* to exclude the possibility of a strain-specific influence. The lifespan assay was performed using female *yw* animals. Flies fed with a CM containing 0.2 % *S. europaea* extract (SEE) significantly outlived the control group by 16 % (Fig. 3.21 D). The maximum lifespan of these animals was extended by the extract as well (from 42 to 50 days).

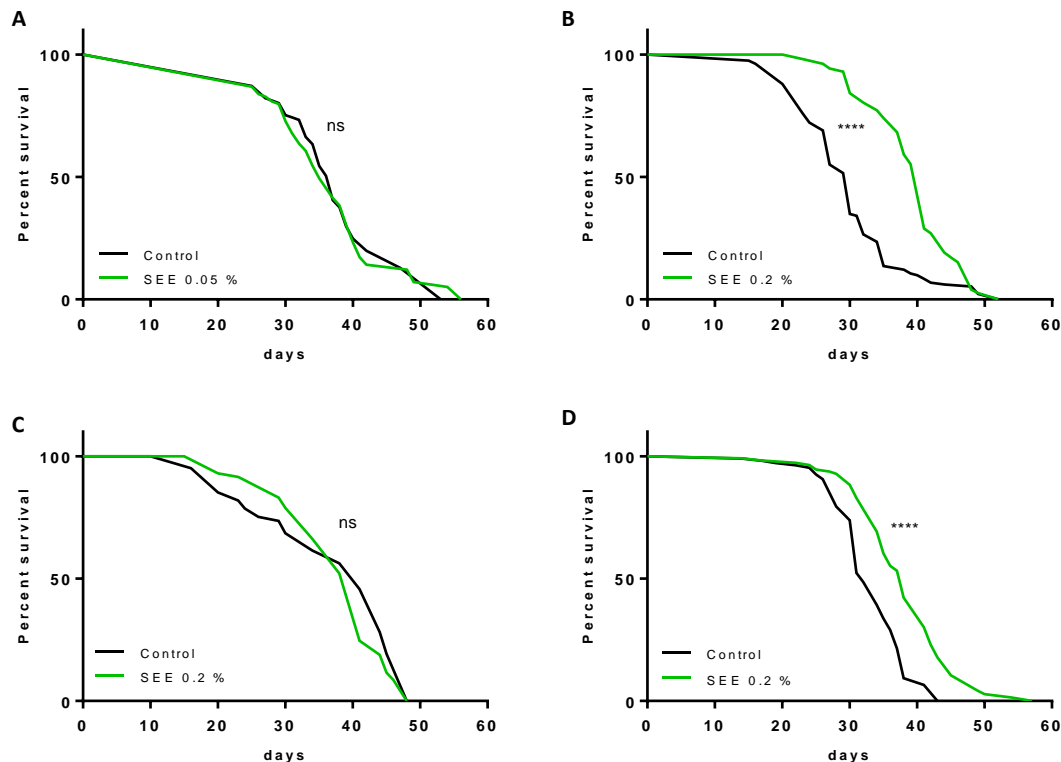


Figure 3.21. The effect of SEE on the lifespan of *Drosophila melanogaster*.

(A) 0.05 % of *Salicornia* extract did not extend the lifespan of w^{1118} female flies ($n = 100$, the median lifespan of SEE-treated flies = 35, the median lifespan of untreated flies = 37). (B) 0.2 % of the extract extended the lifespan of flies significantly ($n = 150$, the median lifespan of SEE-treated = 41, the median lifespan of the control group = 30). (C) The extract was not beneficial on the lifespan of w^{1118} male flies ($n = 100$, the median lifespan of both groups = 41). (D) SEE increased the lifespan of yw female flies ($n = 100$, the median lifespan of flies treated with SEE = 38, the median lifespan of control = 32). SEE: *Salicornia europaea* extract, ns: not significant, **** $p < 0.0001$.

3.3.1 The *Salicornia* extract improves the fecundity of flies

I wondered whether the effect of SEE on lifespan extension can stem from reduced food intake. To test this, food intake of flies upon administration of the extract in the food was examined. Here, no statistically significant differences in food consumption during a 24 h period could be observed (Fig. 3.22 A).

Fecundity is an important readout in longevity studies since the intervention that extend the lifespan are often associated with reduced fecundity [246], [247]. Then, I examined the hypothesis of a tradeoff between longevity and fecundity by a fecundity test on the female flies treated with the extract. However, counting the eggs laid over the course of 14 days revealed the extract not only does not compromise fecundity by the extract but also has beneficial effects on the fecundity of animals that had a higher number of eggs laid over this period (Fig. 3.22 B).

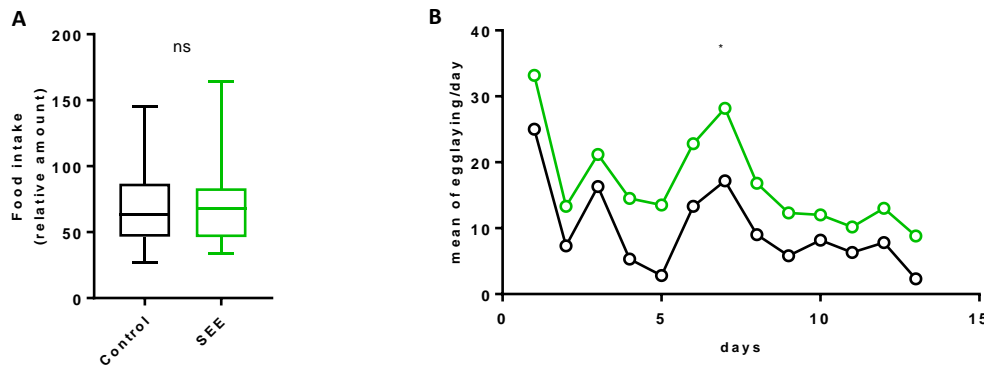


Figure 3.22. The effect of SEE on food intake and fecundity of *w¹¹¹⁸* female flies.

(A) The extract does not affect the food intake of female flies ($n = 15$). (B) The flies fed with SEE had the higher number of eggs per day over a course of 14 days ($n =$, the mean number of laid eggs per day for SEE-treated flies = 16.91, the mean number of laid eggs for the control flies = 9.7). SEE: *Salicornia europaea* extract, ns: not significant, $*p < 0.05$.

3.3.2 The *Salicornia* extract reduces the TAG and glucose levels

Then, I addressed the question whether treating flies with 0.2 % SEE affects the flies' metabolism and body composition by testing several physiological parameters including body weight, protein content, triacylglycerol levels and glucose content at two time points. Regarding body weight, the experimental groups did not show any difference at any time point (Fig 23 A). The lipid storage and carbohydrate content was examined by measuring triglyceride (TAG) and glucose levels, respectively. TAG levels and glucose content of whole flies fed with the extract for two weeks showed no significant changes (Fig. 3.23 B, C), whereas flies fed with the SEE showed lower amounts of both TAG levels and glucose levels after three weeks of treatment (Fig. 3.23 B, C). Interestingly, comparing the TAG levels between two-week treated and three-week treated animals shows a drastic reduction in their lipid content after three weeks on both experimental diets. Since I observed a reduction in fat and glucose content in flies fed with the extract, I examined the expression of *glucose transporter 1* (*glut1*) and found a significant downregulation of *glut1* expression in flies fed with the extract (Fig. 3.37). Concerning the whole-body protein content, the experimental groups did not show any significant difference (Fig. 3.23 D). However, the comparison of two-week and three-week treated animals revealed a significant increase in the protein content in three-week treated flies. Finally, I examined the water content of the whole body of female flies upon treatment with the diet of interest and found that the extract does not influence the water content of flies (Fig. 3.24 A).

Since I observed the reduced TAG and glucose levels in flies fed with the extract, I asked

whether these reductions are due to the increased metabolic status of flies. Thus, the metabolic rate of flies treated with the diet of interest for two weeks was measured. Comparing the results did not show an altered metabolic rate in the flies fed with the extract (Fig. 3.24 B).

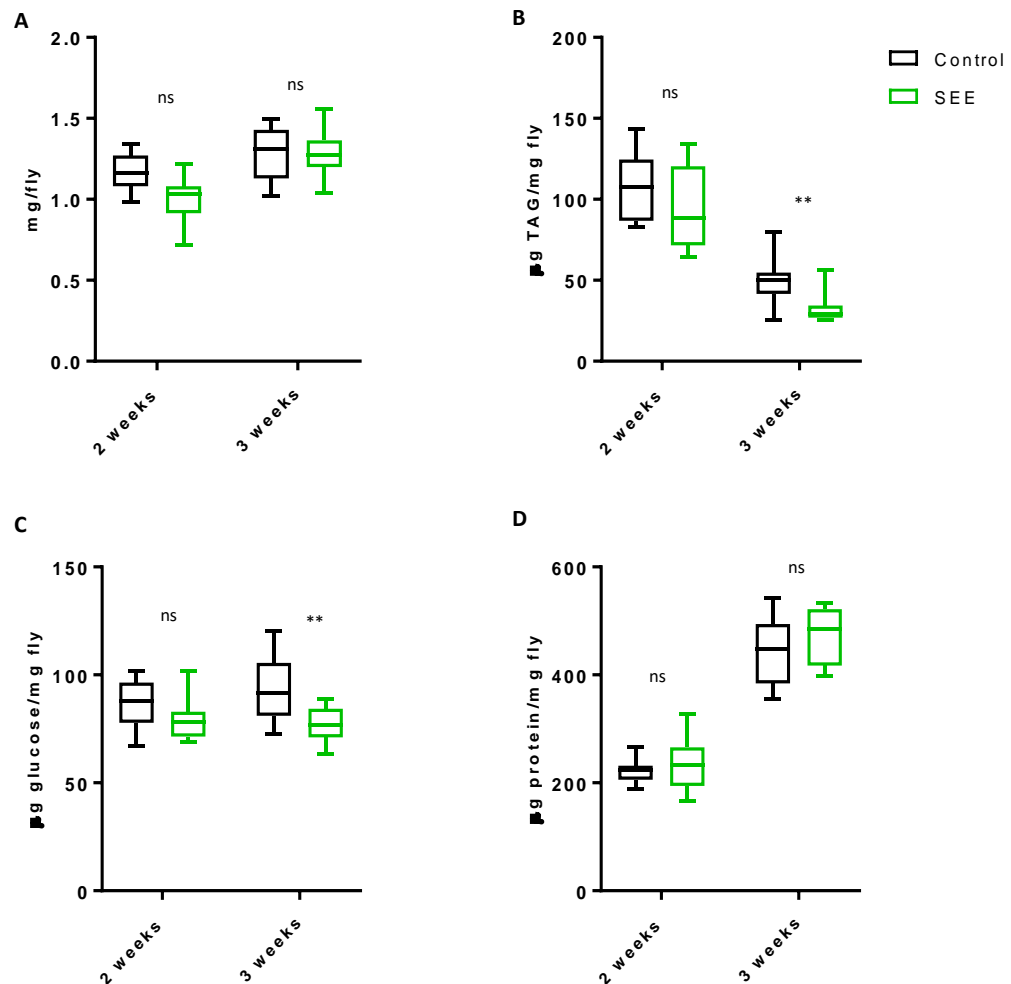


Figure 3.23. SEE modulated flies body composition.

(A) The body weight of flies did not differ in response to the extract. (B) The flies fed with the extract had lower content of TAG after three weeks of treatment. (C) SEE reduced the whole-body glucose levels after three weeks of treatment. (D) The protein content was not affected by the extract compared to the control counterparts (n = 10). SEE: *Salicornia europaea* extract, TAG: triacylglyceride, ns: not significant, **p < 0.01.

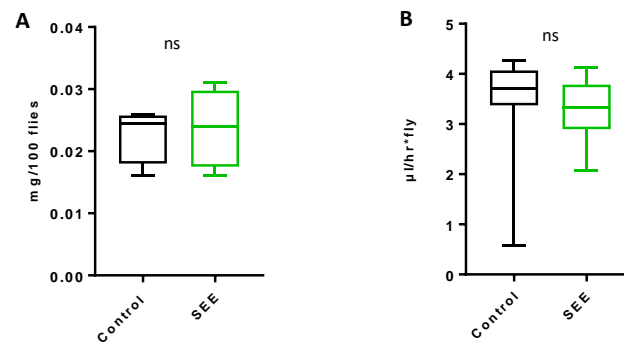


Figure 3.24. The water content and metabolic rate of flies did not differ by feeding with SEE.

(A) The water content of flies (n = 3). (B) The metabolic rate of flies (n = 10). Ns: not significant, SEE: *Salicornia europaea* extract.

3.3.3 The flies fed with SEE are more sensitive to starvation

It has been suggested that pro-longevity interventions benefit animals under stressful conditions [45], [268]. Thus, I wondered whether the SEE bestows the flies with more resistance to stress conditions. To test this, the flies were exposed to stress conditions such as desiccation, starvation, a high-sugar diet, and a high-fat diet and their survival was analyzed upon treatment with the diet of interest.

To investigate the survival under desiccation conditions, wild-type flies (w^{1118}) were treated with 0.2 % SEE for two weeks and then transferred to an empty vial to test their survival. Recording the number of dead flies every two hours revealed no beneficial effect by the extract and the median and maximum survival for the *Salicornia*-treated and control groups were the same under desiccation conditions (33 h) (Fig. 3.25 A). Next, the effect of the extract was tested under starvation conditions. The wild-type flies (w^{1118}) were reared on the diet of interest for two weeks and then transferred to vials containing 1.5 % agar to supply water for the flies. The survival curves showed the median and maximum lifespan of control and treated flies were the same, 159 and 200 h respectively (Fig. 3.25 B). Since I observed the alteration in the body compositions of flies after three weeks of treatment, the starvation resistance test was repeated after three weeks of treatment with the diet. Strikingly, the three-week treated flies showed more sensitivity to starvation conditions, and their survival drastically reduced compared to two-week treated flies (Fig. 3.25 B). This result is in line with the TAG level measurement showing lower TAG content in three-week treated flies since the TAGs are the

main source of energy under starvation conditions. Moreover, the flies fed with the SEE for three weeks, which had less fat content compared to the control group, were more sensitive to starvation as well (Fig. 3.25 B).

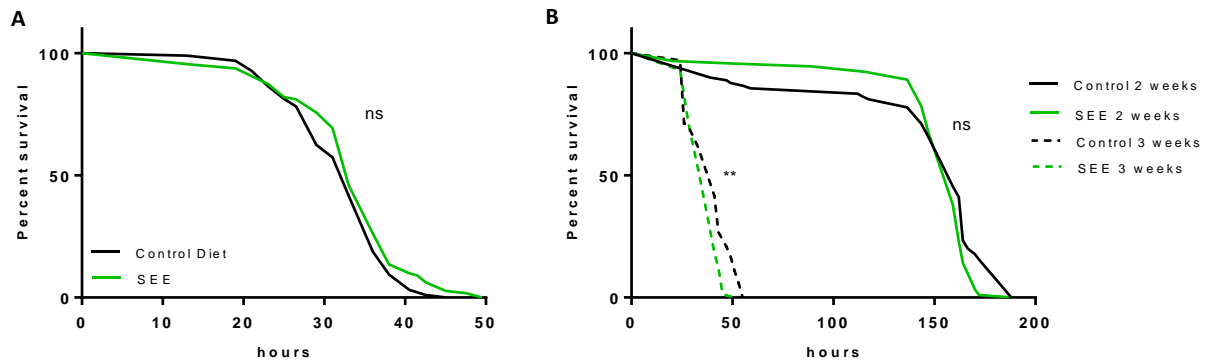


Figure 3.25. The flies fed with the extract were more sensitive to starvation.

(A) The effect of the extract under desiccation conditions ($n = 100$, the median survival of SEE-treated flies = 33 hours, the median survival of untreated flies = 33 hours). (B) The starvation resistance of the experimental group did not differ significantly after two weeks ($n > 90$, the median survival of flies = 159). However, the resistance to starvation reduced drastically in both experimental groups and the flies fed with the extract were more sensitive to starvation ($n > 100$, the median survival of three-week treated for SEE-treated flies = 37, the median survival of untreated flies = 41). SEE: *Salicornia europaea* extract, ** $p < 0.01$

3.3.4 *Salicornia europaea* extends the survival on a high-fat diet

I also investigated the survival of flies on a 30 % sucrose diet. The eggs and larvae were reared on a normal diet and hatching adults were transferred to a 30 % sucrose diet. As observed in the starvation and desiccation tests, the *S. europaea* extract did not increase the median or the maximum survival of flies on a high-sugar diet (Fig. 3.26 A).

Next, the effect of SEE on resistance to a high-fat diet containing 20 % coconut oil was examined. The eggs and larvae were reared on a normal diet until they emerged as adults when they were divided into two groups. One group was treated with a high-fat diet and the other group was fed with the high-fat diet plus the extract. The flies fed with the extract outlived their control counterparts and their median lifespan extended significantly by 16.6 % (from 30 to 35 days) (Fig. 3.26 B). Moreover, the *Salicornia*-treated flies had an increased maximum lifespan.

Finally, I assessed the effect of the extract on the resistance to paraquat as an oxidative stress inducer. The wild-type flies were pretreated with the extract for two weeks and then transferred to the food containing paraquat. The flies pretreated with the extract had increased median lifespan (one day). However, the statistical analysis did not show a

significant extension in survival under paraquat treatment (Fig. 3.26 C).

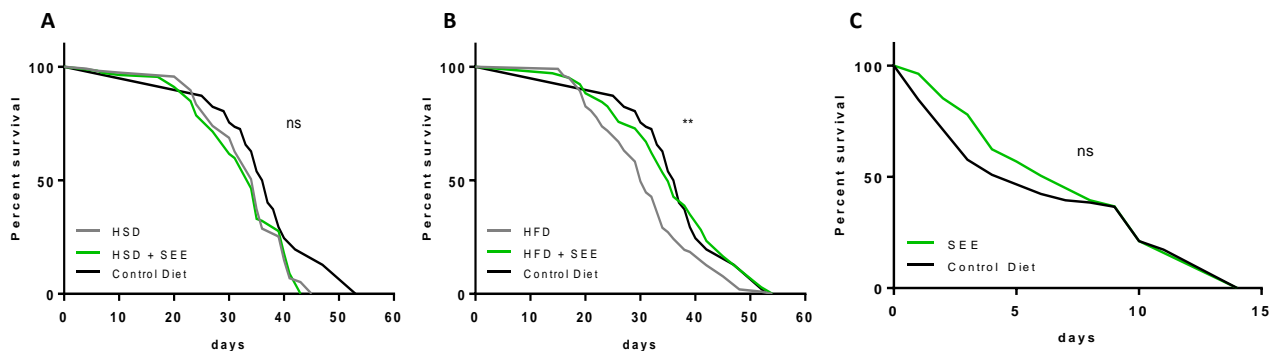


Figure 3.26. The effect of SEE on nutritional stresses.

(A) The extract did not extend the survival of flies under a high-sugar diet ($n > 110$, the median lifespan of SEE-treated = 34, the median lifespan of untreated = 35). (B) The survival of flies fed with SEE under a high-fat diet increased ($n = 100$, the median survival of SEE-treated flies under HFD = 35, the median survival of untreated flies under HFD = 30). (C) The median survival of flies under paraquat stress improved ($n > 100$, the median survival for SEE-treated flies = 7, the median survival of control flies = 6). SEE: *Salicornia europaea* extract, ** $p < 0.01$.

3.3.5 SEE does not affect the circadian rhythm and locomotor activity

To investigate the flies' locomotor activity and circadian rhythm upon treatment with the algal extract, the *Drosophila* activity monitoring system was used (Fig. 3.27 A and B). One group of flies was pretreated with the extract for 5 to 7 days and the control group was fed with the CM. The statistical analysis showed no significant alteration in the locomotor activity of flies fed with the extract (Fig. 3.27 C). The sleep patterns of experimental groups overlapped and its quantification showed that resting and sleeping patterns are not negatively affected by the extract (Fig. 3.27 D, E).

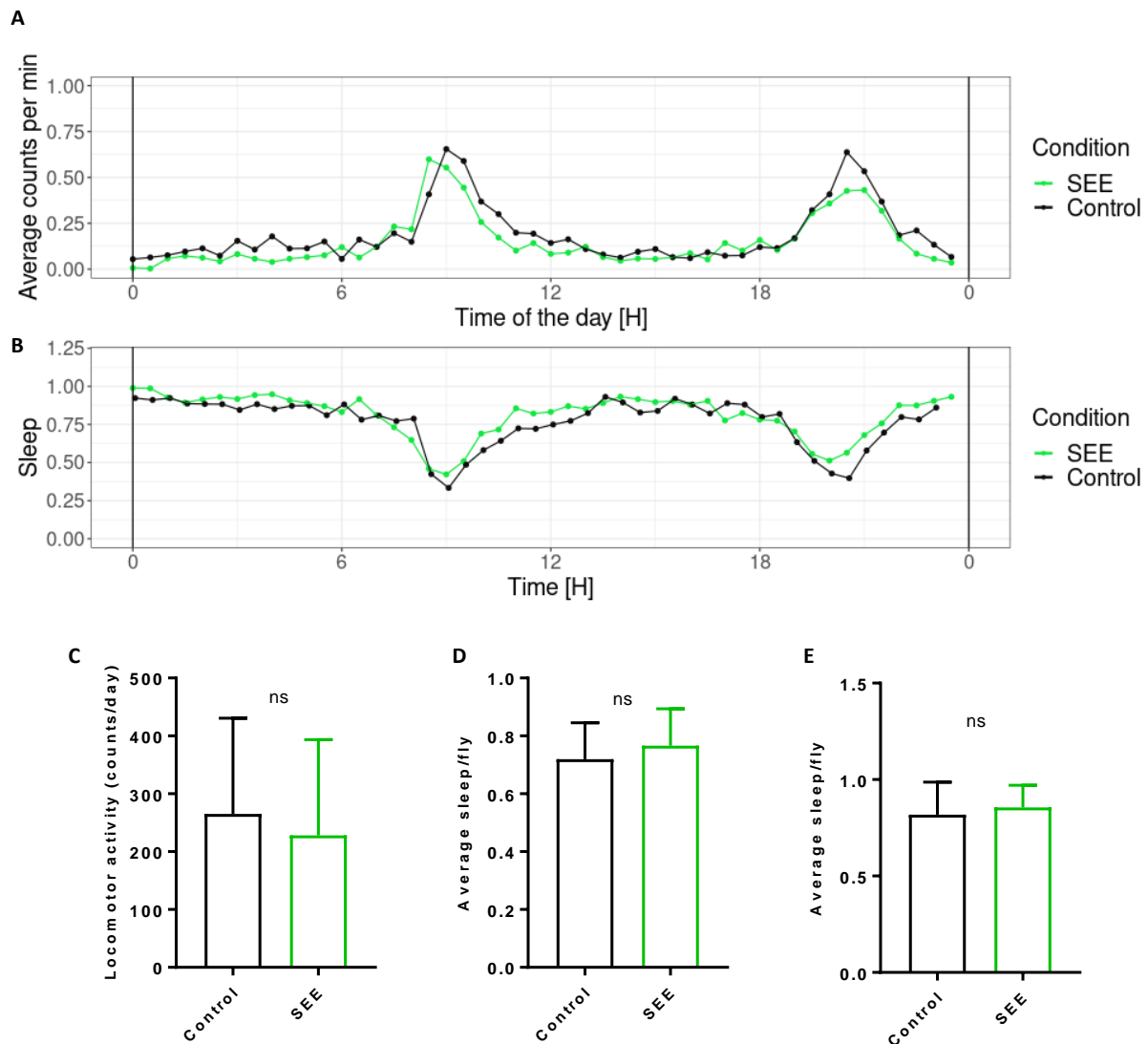


Figure 3.27. SEE did not affect the locomotor activity and circadian rhythm of flies.

(A) Daily locomotor activity of flies showed similar pattern. (B) The sleep pattern of flies showed similar pattern. (C) The locomotor activity measurement did not reveal significant alteration (D) The daily sleep of SEE-treated flies did not differ compared to the control group (E) The nighttime sleep of flies fed with the extract did not affect by the extract. N = 25, SEE: *Salicornia europaea* extract, ns: not significant.

3.3.6 The *Salicornia* extract improves the barrier function of the intestine

Impaired intestinal barrier function has been associated with aging in flies [269], [270]. Therefore, I wondered whether the increased lifespan in flies caused by the extract is also beneficial to gut integrity. To test this, I perform a so-called smurf assay at two time points. The flies were treated with a diet of interest for 20 days and were then transferred to food containing blue dye. If the digestive tract of flies is healthy and its integrity is intact, the blue food remains in the gut, whereas in the case of dysfunctional barrier function, the blue dye is observed in the other parts of the body and the flies are referred to as smurf flies. Evaluation

of the animals after 20 days of treatment showed about 10 % flies with the smurf phenotype in the control group while this number in treated flies was with 1 % significantly reduced (Fig. 3.28 A). Then, flies were transferred to the diet of interest and treated for an additional week. The smurf assay was repeated after 27 days of treatment. While still, 10 % of animals in the control group displayed the smurf phenotype, the number of animals with impaired barrier function in the treated group increased to about 7 %. The statistical analysis showed no significant difference at the second time point (Fig. 3.28 B).

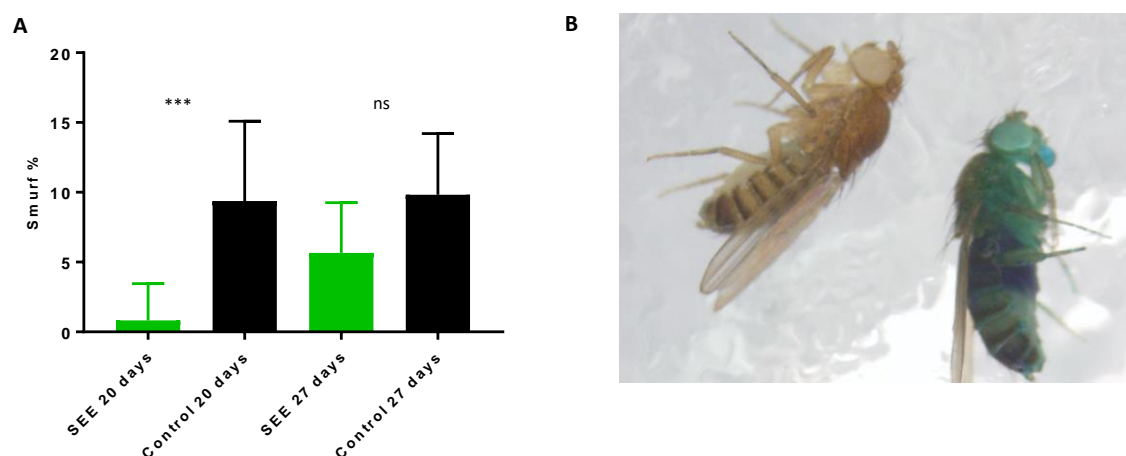


Figure 3.28. SEE improves the intestinal integrity of flies.

(A) The flies fed with the extract showed a lower percentage of smurf individuals ($n = 100$, the percentage of smurf animals in SEE-treated flies after 20 days = 1 %, the percentage of smurf animals in control flies after 20 days = 10 %, the percentage of smurf animals in SEE-treated flies after 27 days = 6.5 %, the percentage of smurf animals in control flies after 27 days = 10 %). (B) A picture of smurf and non-smurf animals. Ns: not significant, *** $p < 0.001$.

3.4 The mode of action of *Salicornia europaeae* aqueous extract

The same approach that I used to investigate the mode of action of EBE, I applied to examine the involvement of the canonical signaling pathways mediating lifespan extension including sirtuins, FoxO, and Tor signaling pathways using the mutant animals.

3.4.1 The *Salicornia* extract extends the lifespan of *Sirt1* KO flies

As mentioned before, sirtuins are conserved proteins functioning as nicotinamide adenine dinucleotide-dependent deacetylases and are serving as mono-ADP-ribosyltransferases with established roles in the longevity of organisms of diverse evolutionary taxa [254], [255]. I asked whether *Sirt1* is required for the effect of the extract. Thus, *Sirt1* KO female flies were

implemented in a lifespan assay and divided into two groups. One group was fed with CM plus the extract and the other group as the control only with CM. The lifespan curves revealed the extract could extend the lifespan of flies in the absence of *sirt1*, thus the effect of the extract is uncoupled from this member of the sirtuin family (Fig. 3.29 A). I tested the effect of the extract on the expression of *sirt6* using qPCR and did not find a significant alteration in the expression of this member of sirtuins (Fig. 3.29 B).

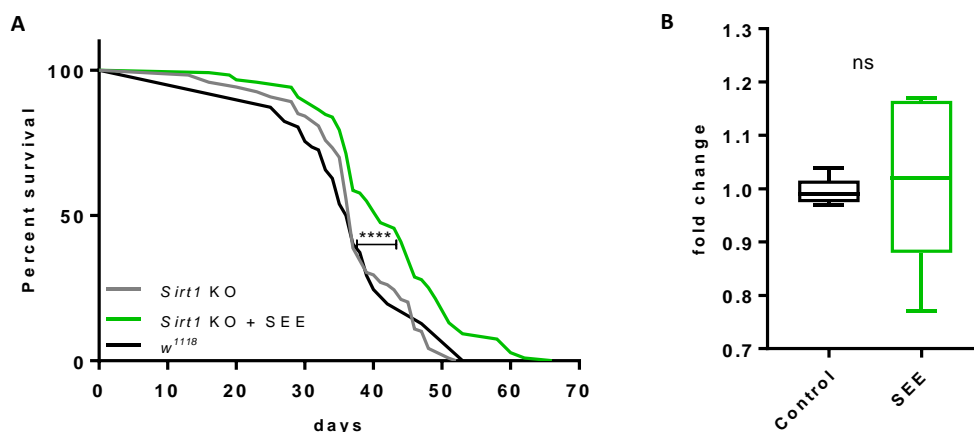


Figure 3.29. The influence of SEE on the sirtuin family.

(A) The lifespan of *Sirt1* KO flies was extended in response to SEE in the diet ($n = 110$, the median lifespan of SEE-treated *Sirt1* KO = 41, the median lifespan of *Sirt1* KO flies = 37). (B) The expression of *Sirtuin 6* did not differ upon treatment with the extract ($n = 5$). SEE: *Salicornia europaea* extract, ns: not significant, **** $p < 0.0001$.

3.4.2 The *Salicornia* extract extends the lifespan of *foxo*-deficient flies

Next, I asked whether FoxO is necessary for the lifespan-extending effect of the SEE. To test this hypothesis, I used *foxo*-deficient flies that had a point mutation in the coding region of the gene, leading to an early stop of translation. One group of *foxo*-deficient flies was treated with the extract and their lifespan was compared with the control group that was fed with only CM. As expected [54], the mutation in the *foxo* gene reduced the lifespan of flies significantly compared to their genetic control flies. Moreover, the median lifespan of *foxo*-deficient flies increased in the SEE-treated group significantly (from 21 to 24 days) (Fig. 3.30 A). This result suggests that FoxO transcription factor is not required for the extension of lifespan by the extract. However, the extension in lifespan by the extract in *foxo* mutants is not as pronounced as in wild-type animals. To further investigate the role of FoxO in the mode of action of the extract, the expression of the transcription factor was analyzed. Surprisingly,

the qRT-PCR results revealed the downregulation of *foxo* in the whole body of female flies upon pretreatment with the extract.

Another component of the IIS pathway in flies is a set of insulin-like peptides (Dilps) produced in a distinct spatiotemporal pattern with distinct roles in metabolism, growth, reproduction, and lifespan [271]. I investigated the necessity of Dilps that are secreted from the insulin-producing cells in the flies' brains and have established roles in the regulation of lifespan in the effect of the SEE. For this, *Dilp2-3, 5* knockout animals were used and divided into two experimental groups. One group was fed with the SEE throughout the lifespan experiment. I observed the downregulation of the IIS signaling pathway did not hinder the lifespan-extending effect of the extract, thus these insulin-like peptides are probably not the target of the extract (Fig. 3.30 B).

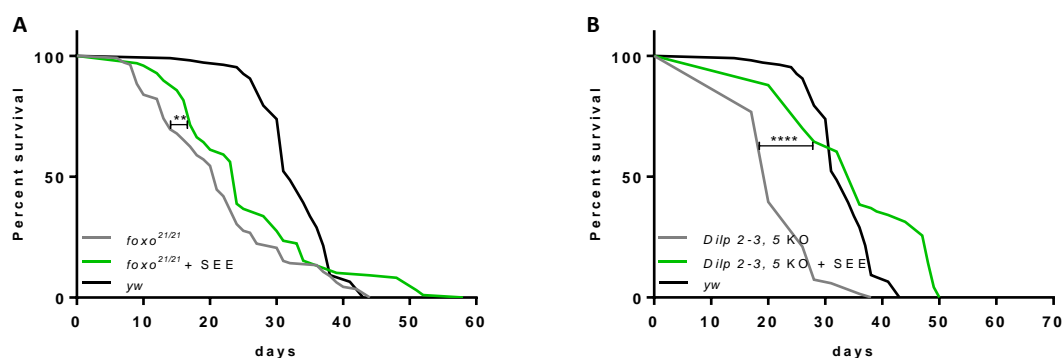


Figure 3.30. The IIS pathway might be uncoupled from the mode of action of SEE.

(A) The extract slightly increased the median lifespan of *foxo*-deficient flies ($n = 100$, the median of SEE-treated *foxo*-deficient flies = 24, the median lifespan of *foxo*-deficient flies = 21). (B) The median and maximum lifespan of *Dilp 2-3, 5 KO* flies significantly increased by the extract ($n > 70$, the median of SEE-treated *Dilp2-3, 5 KO* flies = 36, the median lifespan of *Dilp 2-3, 5 KO* flies = 20). ** $p < 0.01$, **** $p < 0.0001$

3.4.3 The *Salicornia* extract fails to extend the lifespan of *Tor* mutant flies

Since the nutrient-sensing Tor signaling pathway is an important component of many antiaging agents, I wondered whether the *S. europaea* extract mediates its lifespan-extending effect through this pathway. To test this possibility, I implemented flies with a defective form of Tor protein in a lifespan experiment. After emerging as adults, these flies were divided into two experimental groups, and 0.2 % SEE was added to the food of one group while the other group was fed with CM as the control group. Analyzing the lifespan curves showed an extension of lifespan in flies with a defective Tor compared with the wild-type animals (median

lifespan increased by 33.3 %). Interestingly, comparing the lifespan curves of the *Tor* mutant groups shows no extension of the lifespan of the SEE-treated *Tor* mutant animals (Fig. 3.31), and the median and maximum lifespan of groups were almost equal, suggesting Tor is required for the action of the extract.

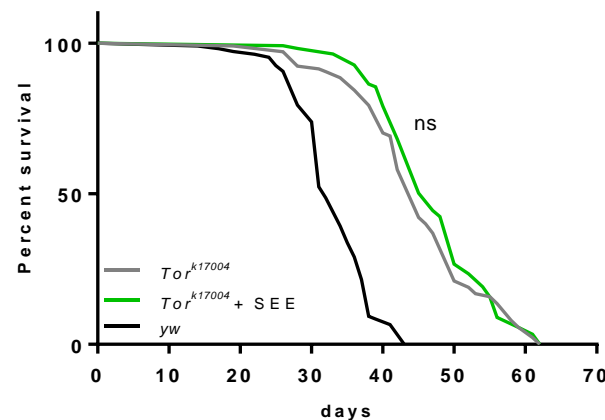


Figure 3.31. A functional form of Tor is required for the positive effects of SEE.

The lifespan-extending effects of SEE were blocked in flies with a defective form of Tor ($n = 100$, the median lifespan of *Tor*^{K17004} flies = 45, the median lifespan of *Tor*^{K17004} flies fed with SEE = 46). SEE: *Salicornia europaea* extract, ns: not significant

3.4.4 Downregulation of the Tor signaling in the fat body abolishes the lifespan extension effect

I asked whether the SEE targets a specific organ to mediate its effects. To this end, I implemented a binary Gal4/UAS system to modify the expression of the gene of interest in the specific cells or tissues. The GeneSwitch system with an abdominal fat body-specific driver line (*P(GS)106*) was used to manipulate Tor and FoxO signaling in this nutrient-storing organ. After emerging as adults, the offspring of the driver line and UAS line were divided into three groups. One group was fed with CM. In the second group, mifepristone, which is required to induce gene expression, was administered on the CM. The third group was the induced flies that were fed with the SEE on their CM.

First, the overexpression of *foxo* in the fat body led to an increase in the median lifespan of induced flies compared to uninduced animals (31 vs 35 days). Comparing the lifespan curves of the induced *Salicornia*-treated flies with induced flies revealed a significant increase in the lifespan of flies with the extract (Fig. 3.32 A). Since I observed global downregulation of Tor signaling blocked the lifespan extension effect of the extract, I inhibited this signaling pathway

in the abdominal fat body using a dominant negative form of Tor downstream of UAS. Whereas the inhibition of Tor signaling in the fat body did not increase the lifespan of flies in comparison with uninduced flies, the SEE extended the lifespan of the *Tor^{DN}* flies significantly (Fig. 3.32 B). I also investigated the role of S6K in the mode of action of the SEE. Using the same GeneSwitch system, S6K was RNAi depleted in the fat body. Similar to inhibition of Tor signaling via *TOR^{DN}*, inducing the RNAi-depletion of S6K in the fat body did not have a positive effect on the lifespan of flies compared to uninduced flies that had the normal form of S6K, and like *Tor^{DN}* the extract significantly prolonged the lifespan of flies in comparison with RNAi S6K-uninduced and -induced (Fig. 3.32 C). Next, these two experiments were repeated using another fat body-specific driver line (*ppl-Gal4*) that did not need mifepristone to induce the gene of interest and the gene of interest was expressed in the offspring of Gal4 > UAS crossing from early stages of life. RNAi depletion of S6K in the fat body using the *ppl-Gal4* line did not abolish the extension in the lifespan of flies via the extract and the flies with the extract significantly outlived the control group by 67.7 % (Fig. 3.33 A). This is supporting the previous experiment's results (Fig. 3.31 C). I went on to inhibit Tor signaling via driving *Tor^{DN}* in the fat body by the *ppl-Gal4* line. Unlike driving the *Tor^{DN}* using *P(GS)106*, inhibition of Tor by targeting the fat body via *ppl-Gal4* abolished the lifespan extension effect of the extract indicating the extract mediates lifespan extension by targeting the Tor signaling and inhibiting it in the fat body (Fig. 3.33 B).

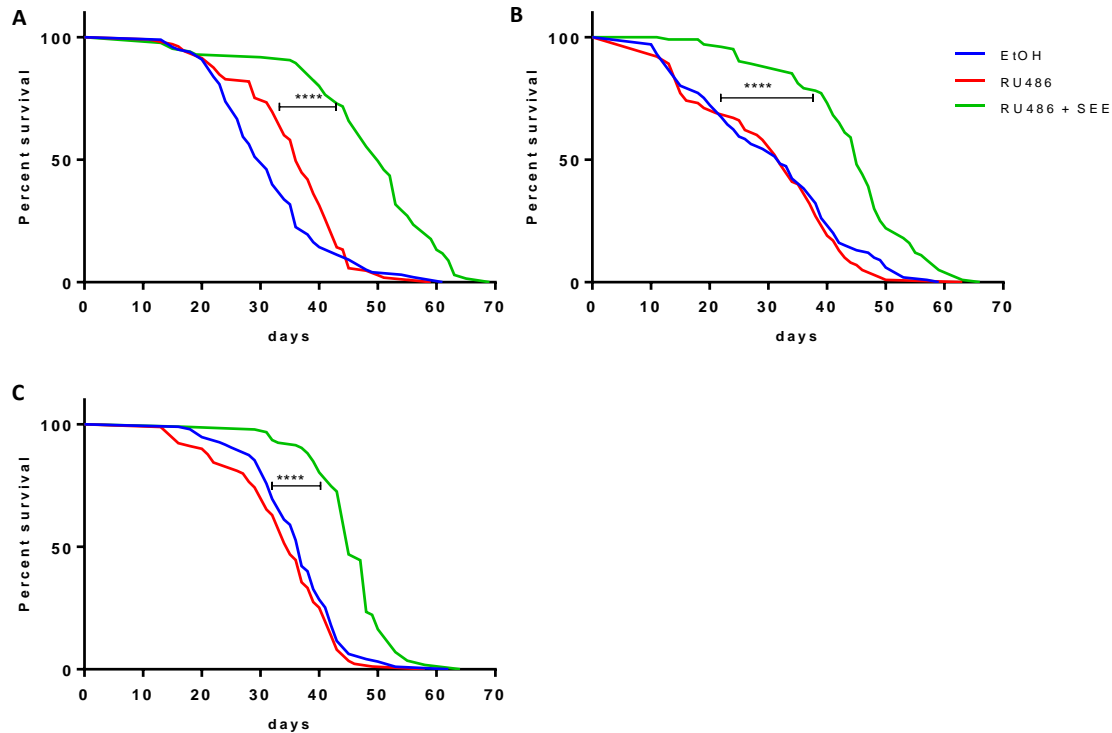


Figure 3.32. The manipulation of the IIS and TOR signaling pathways in the fat body using the GeneSwitch system.

(A) The overexpression of *foxo* in the fat body ($P(GS)106 > UAS-foxo$) extended the lifespan of flies and it did not block the effect of the extract ($n = 100$, the median lifespan of uninduced flies = 31, the median lifespan of induced flies = 36, the median lifespan of induced flies fed with the extract = 50). (B) The reduction of Tor signaling using Tor^{DN} ($P(GS)106 > UAS-Tor.TED$) did not block the extension of lifespan of induced flies by the extract ($n = 100$, the median lifespan of uninduced flies = 32, the median lifespan of induced flies = 32, the median lifespan of induced flies fed with the extract = 45). (C) The flies fed with SEE outlived the control flies when RNAi depletion was induced in the fat body ($P(GS)106 > UAS-S6K-RNAi$) ($n > 80$, the median lifespan of uninduced flies = 37, the median lifespan of induced flies = 35, the median lifespan of induced flies fed with the extract = 45). SEE: *Salicornia europaea* extract, **** $p < 0.0001$.

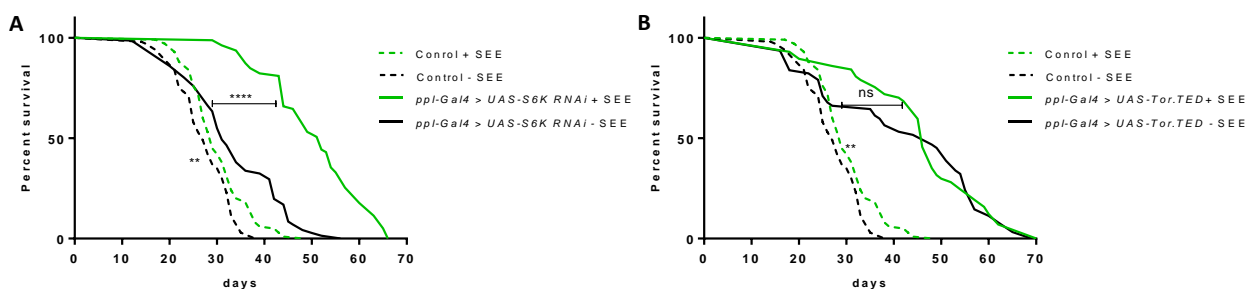


Figure 3.33. The fat body might be the target organ of SEE.

(A) The lifespan of flies increased by the extract when S6K was depleted in the fat body ($ppl-Gal4 > UAS-S6K-RNAi$) ($n > 70$, the median lifespan of $ppl-Gal4 > UAS-S6K-RNAi$ = 31, the median lifespan of $ppl-Gal4 > UAS-S6K-RNAi + SEE$ = 52). (B) The lifespan of flies with a dominant negative form in their fat body ($ppl-Gal4 > UAS-Tor.TED$) did not significantly increase by the extract ($n > 60$, the median lifespan of $ppl-Gal4 > UAS-Tor.TED$ = 46, the median lifespan of $ppl-Gal4 > UAS-Tor.TED + SEE$ = 48). Ns: not significant, ** $p < 0.01$, **** $p < 0.0001$.

3.4.5 ISCs and EBs might be the targets of SEE

As already mentioned, the *Drosophila* intestine has central functions in the metabolism and physiology of flies. Thus, I evaluated the role of different intestinal cells in the effect of the *S. europaea* extract. First, the Tor signaling was inhibited in the enterocytes using a specific driver line that induced the expression of *Tor^{DN}* in these cells. The flies were divided into the control group and the treated group that was fed with CM and the 0.2 % extract. The treated group outlived the control by 7 % and the extension in lifespan was statistically significant (Fig. 3.34 A). However, the extension in lifespan is not as great as in wild-type animals, thus it might have a partial effect on the Tor signaling pathway in the enterocytes. Next, *Tor* was upregulated in these cells and the lifespan curves showed increased lifespan in response to the extract (Fig. 3.34 B). I went on to test the RNAi-depletion of S6K in flies' enterocytes. Monitoring the lifespan of flies in this condition revealed a similar phenotype and the extract extended the median and maximum lifespan of flies with S6K RNAi-depletion in the enterocytes (Fig. 3.34 C).

Next, I examined the effect of downregulation of Tor signaling in intestinal stem cells (ISCs) using *Tor^{DN}* and S6K RNAi. I used a driver line that targets intestinal stem cells and enteroblasts (EBs) (*esg-Gal4*) to express the gene of interest. The extract could extend the lifespan of flies in both conditions of inhibition of Tor signaling including *Tor^{DN}* and RNAi-S6K in the ISC and EB, suggesting the mode of action of the extract might be uncoupled from these cells (Fig. 3.35 A, B). However, the effect of the extract on lifespan extension in flies with RNAi depletion of S6K in their ISCs and EBs is not as pronounced as in flies with *Tor^{DN}* in these cells. The extract extended the lifespan by about 20 % in flies with *Tor^{DN}* in ISCs and EBs whereas the median lifespan of flies with RNAi S6K in these cells did not extend, and only the extension was observed at maximum lifespan. These results further put forward the possibility of a partial pleiotropic effect by the extract.

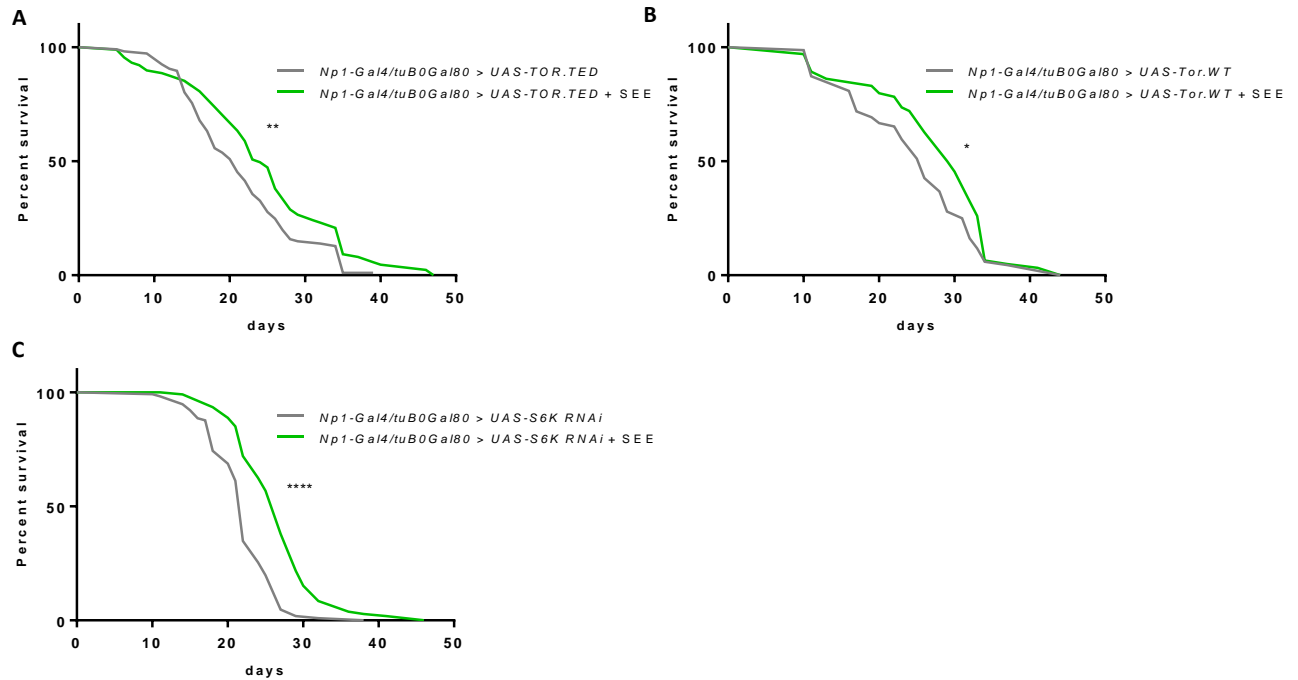


Figure 3.34. The enterocytes are not primary target of SEE.

(A) Reduction of the Tor signaling pathway in the enterocytes (*Np1-Gal4/tuBGal80 > UAS-Tor.TED*) is not sufficient to abolish the positive effect of SEE ($n > 80$, the median lifespan of *Np1-Gal4/tuBGal80 > UAS-Tor.TED + SEE* = 24, the median lifespan of *Np1-Gal4/tuBGal80 > UAS-Tor.TED* = 21). (B) The overexpression of the *Tor* in the enterocytes (*Np1-Gal4/tuBGal80 > UAS-Tor.WT*) attenuated the effect of SEE ($n > 60$, the median lifespan of *Np1-Gal4/tuBGal80 > UAS-Tor.WT + SEE* = 30, the median lifespan of *Np1-Gal4/tuBGal80 > UAS-Tor.WT* = 26). (C) The reduction of S6K in the enterocytes (*Np1-Gal4/tuBGal80 > UAS-S6K-RNAi*) did not hinder the effect of the extract ($n > 100$, the median lifespan of *Np1-Gal4/tuBGal80 > UAS-S6K-RNAi + SEE* = 27, the median lifespan of *Np1-Gal4/tuBGal80 > UAS-S6K-RNAi* = 22). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$

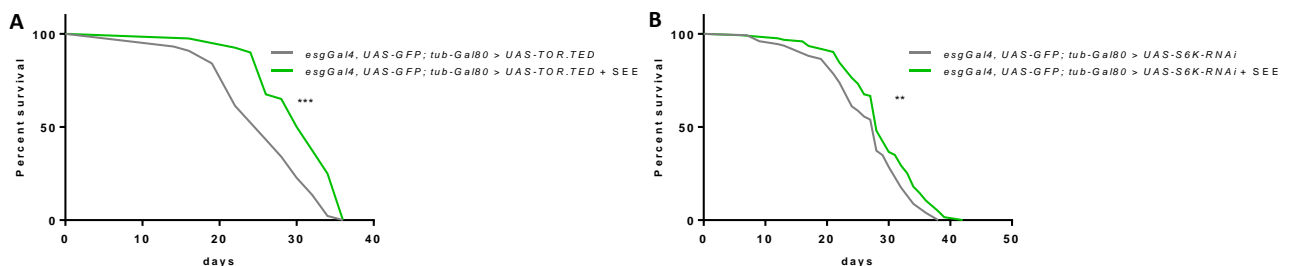


Figure 3.35. The downregulation of TOR signaling in the intestinal stem cells and enteroblasts did not block the effects of SEE.

(A) SEE extended the lifespan of flies with dominant negative form of Tor in the ISCs and EBs (*esgGal4, UAS-GFP; tub-Gal80 > UAS-Tor.TED*) ($n = 45$, the median lifespan of *esgGal4, UAS-GFP; tub-Gal80 > UAS-Tor.TED + SEE* = 31, *esgGal4, UAS-GFP; tub-Gal80 > UAS-Tor.TED* = 26). (B) SEE increased the lifespan of flies with RNAi-depleted S6K in ISCs and EBs ($n > 120$, the median lifespan of *esgGal4, UAS-GFP; tub-Gal80 > UAS-S6K-RNAi + SEE* = 28, the median lifespan of *esgGal4, UAS-GFP; tub-Gal80 > UAS-S6K-RNAi* = 28). ** $p < 0.01$, *** $p < 0.001$

3.4.6 Upd2 is not required for the positive effect of SEE

Since the inhibition of Tor in the fat body blocked the lifespan-extending effects of the extract, I examined the lifespan of flies upon mutation of the JAK/STAT cytokine unpaired2 (*Upd2*) which is a fat body and intestine systemic factor secreted in response to excessive fat or sugar in the diet. *Upd2*, in turn, induces the secretion of *Drosophila* insulin-like peptides (Dilps) from insulin-producing cells in flies' brains [265]. I hypothesized that the extract targets the fat body and inhibits the Tor pathway in the fat body thus the fat body reduces the secretion of *Upd2* and in consequence, the secretion of Dilps is diminished leading to a shift from anabolism to catabolism. I used *Upd2* deficient animals in a lifespan experiment to evaluate the SEE effect on the lifespan of flies in the absence of *Upd2* thus miscommunication of the fat body and IPCs. The lifespan curves analysis showed that *Upd2* is not essential for the lifespan-extending effect of the extract and the *Upd2* deficient animals fed with the extract outlived the *Upd2* deficient animals fed the CM (Fig. 3.36). I further tested the involvement of the fat body-driven factors in the positive effects of the SEE by analyzing the expression of *ImpL2*. Imaginal morphogenesis protein-Late 2 (*ImpL2*) is a homolog of insulin-like growth factor binding proteins (IGFBPs) of mammals that binds to Dilps and dampens the IIS pathway. The wild-type animals were treated with the diet of interest and the RNA was extracted from the whole body of the animals. Surprisingly, the quantification of mRNA levels of *ImpL2* revealed a significant reduction in the mRNA levels of *ImpL2* in the animals treated with the extract (Fig. 3.37 B).

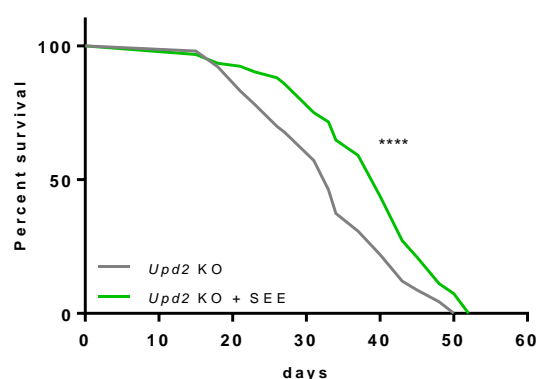


Figure 3.36. SEE extended the lifespan of *Upd2* KO flies.

The lifespan-extending effect of SEE was not abrogated in *Upd2* Knock out flies. (n = 100, the median lifespan of *Upd2* KO flies = 33, the median lifespan of *Upd2* flies fed with SEE = 40). SEE: *Salicornia europaea* extract, *Upd2*: Unpaired2, ****p < 0.0001.

3.4.7 Differentially expressed genes in response to *S. europaea*

Since the flies fed with the extract showed lipid-lowering and anti-hyperglycemic effects, I evaluated the expression of genes related to lipid and carbohydrate metabolism particularly genes regulating IIS/Tor signaling pathways. The RNA was extracted after two and three weeks of treatment with the extract. The qPCR results showed no significantly altered expression in genes evaluated after two weeks of treatment (Fig. 3.37 A). Strikingly, the expression of *foxo*, *glut1*, *impl2*, and *atg8a* was significantly downregulated (Fig. 3.37 B).

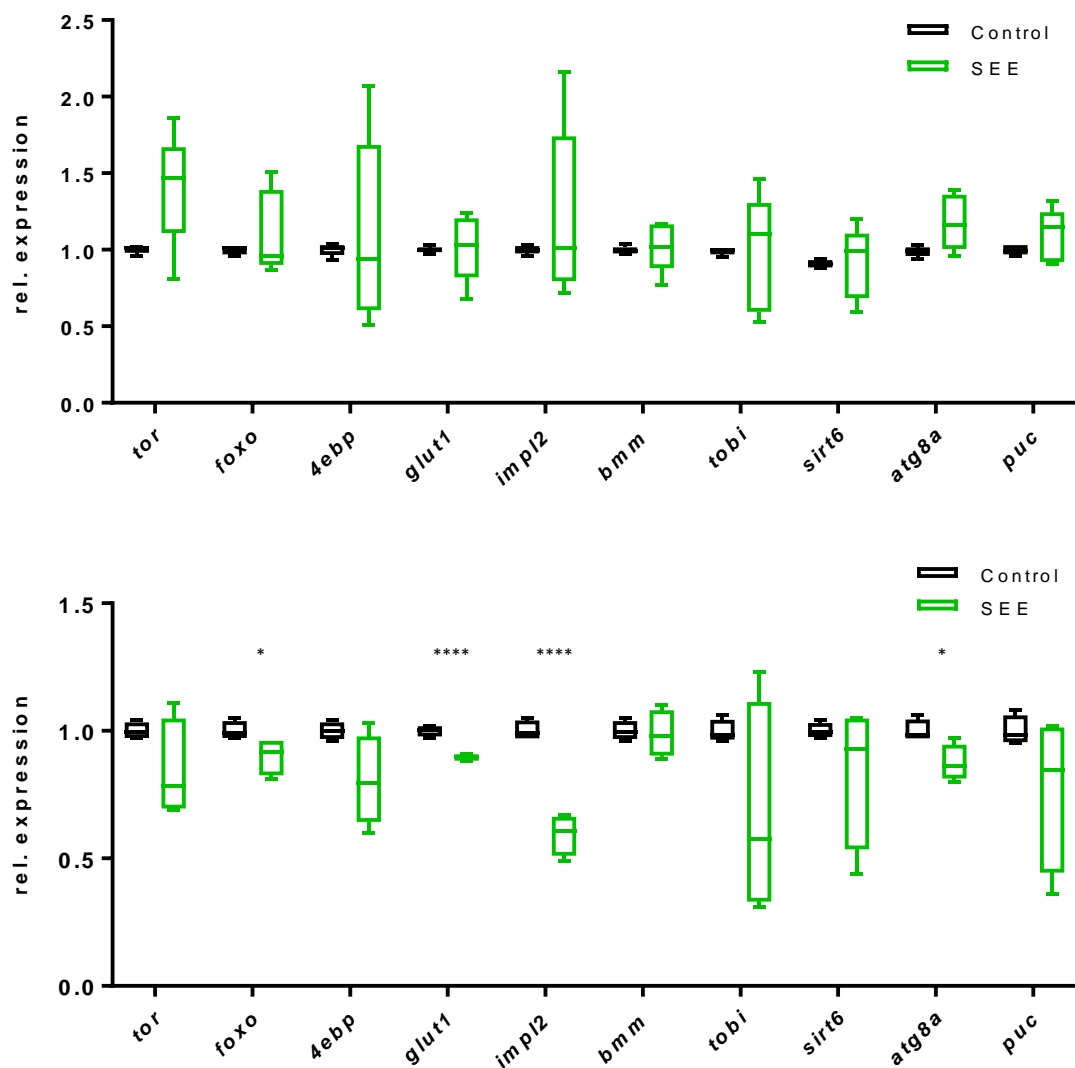


Figure 3.37. The regulation of genes related to IIS/Tor signaling pathways.

(A) The expression of none of the evaluated genes was differentially regulated in response to the extract after two weeks treatment. (B) *foxo*, *glut1*, *impl2*, and *atg8a* were differentially expressed in response to the extract after three weeks treatment. The results were normalized to *rpl32* (n = 5). * p < 0.05, *** p < 0.001, **** p < 0.0001.

3.5 Bioactive compounds in *Eisenia bicyclis* and *Salicornia europaea*

Finally, in collaboration with Roman Lang and Sebastian Dirndorfer of the Leibnitz Institute for Food Systems Biology at the TU Munich, a qualitative HPLC-MS analysis was performed and metabolites specifically occurring in the *Eisenia bicyclis* and *Salicornia europaea* extracts were determined. Both extracts were enriched with bioactive compounds such as amino acids, vitamins, nucleotides and nucleosides, and polyphenols. A few of the metabolites in the extracts are listed in table 3.1.

Table 3.1 The metabolites with differential occurrence in the extracts

<i>Eisenia bicyclis</i>	<i>Salicornia europaea</i>
Isocitric acid	Isocitric acid
Itaconic acid	Pantothenic acid
Methylthioadenosine	Methylthioadenosine
Cytidine	Cytidine
Eugenin	L-tryptophane
Biotin	Biotin
Erythraline	Thymine
L-isoleucine	L-isoleucine
Uridine	Uridine
Guanosine	Guanosine
Thiamine monophosphate	Trans-fumaric acid
Eugenin	Cinnamic acid
Imidazoleacetic acid riboside	N-acetylglutamic acid
1-benzazepine	Coumaroylquinic acids
Pyroglutamylvaline	L-phenylalanine
Biotin	Biotin
Colchicine	Coumaroyl-D-glucose
Piperine	Argininosuccinic acid
Enkephalin L	Succinyl adenosine
Caffeic acid quinone	Benzoic acid
7-phloroeckol	2-Aceto-2-hydroxybutanoic acid
Fucosterol	Oxidized glutathione

4. Discussion

4.1 Natural products are potential antiaging supplements

Aging is often associated with the increasing occurrence of diseases and elderly individuals experience one or a combination of chronic diseases. For example, the incidence of diseases such as neoplasms, dementias, and cardiovascular diseases sharply increases after the age of 60, irrespective of the income of the countries and the sex of the person. Within 30 years the proportion of people aged 65 and over will consist of 20 % of the world's population while this was 10 % in 2010 [103]. Thus, aging will be an issue in the immediate future and will put forward the challenges associated with aging societies and their health issues that are necessary to be addressed. Studies have shown that lifestyle is more impactful than genetics on aging. Twin studies showed a variation of genetic factors in aging processes and its influence on aging is 25 % on average [106]–[108]. Therefore, lifestyle including diet and exercise is more critical, and most antiaging approaches are engrossed in the interventions that modify it. Exercise is one of the geroprotection approaches that hinder the hallmarks of aging including deregulated nutrient sensing pathways, dysfunctional proteostasis, and mitochondria. Moreover, it restores the age-related undermined cardiovascular and autonomic systems and muscle dystrophy [122], [125], [126]. Caloric restriction is another pillar of geroprotection interventions with proven effects on the lifespan of different animal models such as mice, yeast, *C. elegans*, and flies via manipulating the IGF/Insulin signaling pathway and thus improving insulin sensitivity and metabolic state [136]. Similarly, dietary restriction extends the life- and healthspan of different animal models and it includes distinct nutrients restriction and different fasting strategies [138], [151].

Due to the aforementioned limitations, these approaches are not sufficient to defy the looming changes in the age composition of the world and new approaches are required to circumvent the current approaches' limitations [195], [198], [201], [272]. One auspicious treatment to contend with aging is drugs and natural products that can be repurposed as geroprotective agents that have shown promising results in research animals [103], [173], [203], [207], [208].

To this end, I investigated the effects of a plant and an alga species on the lifespan of *Drosophila melanogaster* as a research mode that due to its short life cycle is better suited for

aging studies.

4.2 *Eisenia bicyclis* extends the lifespan of *Drosophila melanogaster* in a sex-specific manner

I used *Drosophila melanogaster* as my research model since it is an established model with fantastic genetic and genomic tools and powerful methodologies at hand. It has functionally equivalent organs to humans such as a well-developed brain, liver/adipose tissue (fat body), digestive tract, kidney (the Malpighian tubules), and heart. Moreover, many signaling pathways regulating lifespan are well-conserved between humans and flies [223], [264], [265]. Interestingly, pro-longevity interventions such as caloric restriction, dietary restriction, rapamycin and spermidine addition, that extend the lifespan in mice have similar effects in flies. Moreover, deleterious diets such as a high-fat diet or a high-sugar diet show similar detrimental effects. Thus, the extension in the lifespan of flies by 0.05 % and 0.1 % EBE is suggesting the extract could impact the lifespan in higher animals as well. The beneficial influence of the lifespan of flies is in line with the HPLC-MS study showing the extract is enriched with bioactive compounds harboring positive biological activities such as eisenin, phlorotannins, and oxylipins [225], [226], [228], [229]. Thus, the observed effect might be due to a synergic effect of all these compounds enhancing different aspects of health and collectively inhibiting the hallmarks of aging. One of these compounds is the polyphenol antioxidant caffeic acid quinnone. Caffeic acid is found in plants such as blueberries, kiwis, coffee, cherries, apples, oils, and tea and shows in addition to antioxidant activity anti-inflammatory, antibacterial, and antiviral properties [273]. Moreover, recent studies proposed that caffeic acid and its derivatives might harbor anticancer properties [274]. Dihydrocaffeic acid (DHCA) inhibits ischemia-induced neuronal damage and brain edema [275] and this compound showed similar inhibitory effects on HepG2 cells by inhibiting induced cytotoxicity and macromolecular damage [276]. Furthermore, it has been shown that caffeic acid harbors pro-longevity and stress resistance effects. In addition to extending lifespan and enhancing stress resistance, it regulates the amino acid metabolism and mitochondria function in *Drosophila* [277].

Another compound with higher abundance in the extract is 7-phloroeckol belonging to the family phlorotannins and being associated with antioxidant, antibacterial, and antiviral

properties that might improve the anti-infective capacity of the flies and thus have a positive effect on the lifespan. Isocitric acid is also abundant in the extract. Isocitric acid formed from citrate is a major player in the tricarboxylic acid cycle and therefore is an essential substrate for energy metabolism. While there is no literature on the effect of isocitric acid on longevity in flies, the supplementation of citrate in the food extended the lifespan of flies and improved the metabolism of flies and mice [278]. This finding is consistent with my results showing increased lifespan by EBE which is enriched with isocitric acid. Moreover, the flies fed with 1 % supplementary citrate had increased activity and fecundity. Conversely, higher concentrations of isocitrate in human plasma are associated with lower longevity and cardiovascular complications [279]. The discrepancies in the effect of TCA cycle metabolites on the lifespan was addressed in *C. elegans* studies. A study reported that the supplementation of α -ketoglutarate, succinate, or pyruvate extended the lifespan of *C. elegans* by inhibiting ATP synthase subunit β in a Tor-dependent manner, whereas citrate and isocitrate did not affect it [280]. The effect of isocitrate on the lifespan was contradicted in another study showing isocitrate increased the lifespan of *C. elegans* at 5 mM concentration and the higher concentration of 10 mM harmed the longevity of the animals [281]. This discrepancy may be due to the different media used in the studies, as well as the different concentrations of the compounds [278]. However, both studies suggest that intermediate metabolites of the TCA cycle are pro-longevity.

The interventions that extend the lifespan often have a greater effect on female animals than on males. For example, the deletion of ribosomal S6 protein kinase 1 (S6K1) could increase the lifespan of female mice but it failed to extend the lifespan of male mice either at the median or at maximum levels [68]. Targeting the insulin receptor substrate to lower the insulin signaling in mice also exhibited a greater effect in female individuals [282]. Similarly, in flies targeting insulin signaling, rapamycin treatment, and dietary restriction are more effective in female flies [45], [60], [283]. Consistent with these, I did not observe any extension in the lifespan of male flies. In *C. elegans* reducing the Tor signaling either at Tor level or at S6K levels and consequently increasing lifespan led to reduced fecundity in animals [284]. Such interventions in flies exhibited the same phenotype and a trade-off between longevity and fecundity upon treatment with rapamycin or reducing insulin signaling through mutations in *Dilps* represented the reduced fecundity [60], [285]. In contrast to these pro-longevity

interventions, EBE did not negatively affect the fecundity of flies that puts forward independent underlying mechanisms of lifespan extension by the marine brown alga.

4.3 *Eisenia bicyclis* modifies flies' metabolism and increases stress resistance

One of the hallmarks of aging is the loss of proteostasis and dietary restriction and inhibition of the IIS pathway restore the deregulated proteostasis [38], [42]. I observed that the flies fed with the extract had a lower content of protein after two weeks of treatment, suggesting the improvement of protein degradation since the protein synthesis in these flies was not affected.

Aging also modulates sleep, leading to reduced nighttime sleep and sleep fragmentation that results in less sleep consolidation. Furthermore, it dampens the circadian rhythm and the quality of sleep [252], [286]. Similarly, in flies, aging is associated with compromised sleep and aberrant circadian rhythm and activity during aging [251], [287], [288]. Studies in flies have shown that the regulation of circadian rhythm is IIS- and Tor signaling-dependent and reducing these pro-longevity evolutionarily conserved pathways lead to increased sleep quality and time [249]. Strikingly, in both flies and humans, anti-longevity diets such as high-fat diets and high-sugar diets deregulate sleep and diminish its consolidation [289], [290]. In this study, I demonstrated the *Eisenia bicyclis* extract improves the quality of sleep of flies by increasing nighttime sleep and sleep bouts leading to sleep consolidation. Thus, EBE shares a lifespan-extending effect and improved sleep quality phenotype with the pro-longevity interventions such as pharmacologically or genetic inhibition of IIS and Tor signaling pathways. Moreover, in the HPLC study of EBE, I observed the extract was abundant with Benzazepines, a group of natural products with pharmaceutical potential used in the treatment of depression, insomnia, severe anxiety, and hypertension [291].

High-caloric diets are a major challenge in modern societies and they are central risk factors in conditions such as obesity, metabolic syndrome, cardiovascular diseases, and reduced lifespan [292], [293]. In mice and rats, the high caloric diets cause accumulation of fat and cholesterol, insulin resistance, and shorten lifespan and fasting-mimicking diets can rescue these deleterious effects [115], [116]. Flies subjected to a diet containing a high amount of fat lead to shortening lifespan decreased sleep consolidation and accelerated decline of climbing ability [263], [294], [295]. Here I showed that the aqueous extract of *Eisenia bicyclis* extract is

beneficial on a 20 % coconut fat diet and slightly increased the lifespan of flies. This observation was consistent with the lowered TAG level after three weeks of treatment with the extract on a control diet. Since the expression of the lipase *brummer* was upregulated in response to the extract, presumably the extract increases the lifespan of the flies by mobilization of TAGs and improving the lipid metabolism on the high-fat diet. Moreover, several anti-adipogenic bioactive compounds were found in the extract. It has been suggested that benzazepines enriched in the extract are anti-obesity by acting as modulators of 5-HT_{2C} receptors and attenuating fat accumulation [291]. Fucosterol is another anti-adipogenic metabolite found in *Eisenia bicyclis* and *Ecklonia cava* macroalgae [296], [297]. It is involved in cholesterol homeostasis and reduces intracellular lipid accumulation in 3T3-L1 adipocytes via downregulation of PI3/Akt and ERK signaling pathways and reducing the expression levels of transcription factors such as proteins peroxisome proliferator-activated receptor γ (PPAR γ), CCAAT/enhancer-binding protein α (C/EBP α) and SREBP-1. Moreover, fucosterol upregulates *Sirt1* expression in the cells and activates FoxO1 [298], [299].

In addition to the high-fat diets, consuming diets containing a high amount of carbohydrates leads to serious consequences to human health including type 2 diabetes and obesity resulting in reduced life expectancy of individuals [300], [301]. Similarly, flies fed with a high-sugar diet display complications such as hyperglycemia, obesity, and a shortened lifespan [302], [303]. I found the flies on a 30 % or 40 % sucrose diet had a drastic reduction of lifespan and adding the extract to these diets could compensate for the shortening lifespan to the level of the flies on a control diet. This finding supports previous studies on the anti-hyperglycemic and anti-diabetic effects of phlorotannins extracted from *Eisenia bicyclis*. It was demonstrated in enzymatic assays that fucofuroeckol-A and dioxinodehydroeckol from *E. bicyclis* inhibit the action of α -glucosidase and α -amylase enzymes [245]. These results were confirmed in another study reporting α -glucosidase inhibitory effects of *E. bicyclis* and its phlorotannins including phlorofurofueckol-A, dieckol, and 7-phloroeckol [304]. Interestingly, it is reminiscent of the mode of action of the antiaging agent acarbose that inhibits α -glucosidases in the intestine and thereby reducing glucose intake [305]. Another enzymatic study exhibited the inhibitory effects of eckol and dieckol against α -amylase. Moreover, these phlorotannins hindered glycation and thus reducing the advanced glycation end products (AGEs) that cause diabetic complications [229]. I also found the glucose content of flies reared on the 30 %

sucrose diet was lowered compared to the control flies similar to the effect of supplementation of citrate in flies and mice [278]. As mentioned before, the extract is enriched with caffeic acid derivative and fucosterol both are associated with antidiabetic and anti-obesity effects[296], [306]. In diabetic rats, applying fucosterol lowered serum glucose and accumulation of sorbitol [307] and molecular docking studies showed like phlorotannins, it shows inhibitory effects against rat lens aldose reductase (RLAR), human recombinant aldose reductase (HRAR), protein tyrosine phosphatase 1B (PTP1B), and α -glucosidase [297]. I also demonstrated that late administration of the extract on a high-sugar diet could be beneficial and extend the survival of the animals in these conditions. This finding is of note since the deleterious effects of a high-sugar diet on lifespan and other measures of health are irreversible when the flies feed on a high-sugar diet early in life [308].

4.4 The IIS and Tor signaling pathways are required for the effects of EBE

The underlying mechanisms of the lifespan-extending effect of *E. bicyclis* were one major objective of this study. To this end, I investigated key proteins and signaling pathways regulating aging including the sirtuin family, IIS pathway, and Tor signaling pathway [44], [46], [309]. Overexpression of *sir2* in animal models such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila* can extend the lifespan [310]. In mice, the longevity effect of different members of sirtuins is controversial but it has been shown that Sirtuin 6 regulates the lifespan in male mice [258]. From five members of the sirtuin family in flies, I investigated the involvement of two members of the family including Sirt1 and Sirt6 in the mode of action of the *E. bicyclis* extract. In a lifespan assay, I showed that *Sirt1* knock-out flies had a shortened lifespan compared to *w¹¹¹⁸* control flies and the extract extended the lifespan of these animals in comparison with the untreated genetic control animals, indicating another underlying mode of action of the extract. However, the magnitude of the extension was not as much as the wild-type animals (40 % versus 10 %) and thus it is conceivable that the extract partially boosts up the NAD⁺-dependent deacetylase Sirtuin 1. I also showed that the expression of the *sirt6* gene enhances in wild-type animals fed with *E. bicyclis* extract. This further suggests that the extract might target the sirtuin family. Overexpression of *sirt6* in mice showed improved healthspan and the animals had lower fasting glucose levels [311]. Similarly, I found lowered glucose levels of flies fed with the extract on the concentrated medium and 30 % sucrose, in the latter the result was statistically significant. In agreement

with the results in mice, overexpression of *sirt6* extends the lifespan of *Drosophila*. Moreover, the extension in lifespan did not compromise the fertility of the animals contrary to the effects of inhibition of Tor and insulin signaling pathways [259], which is in line with my result on the effects of *E.bicyclis* extract on lifespan and fecundity of flies. Altogether, these findings raise the possibility of regulation of lifespan by *E.bicyclis* through sirtuins.

Modulation of different components of the IIS pathway regulate lifespan and it shows a high degree of conservation among taxa [48], [49], [261], [312]. In *C. elegans*, mutations in *age-1* and *daf-2* increase the lifespan [48], [313]. In mice, mutations in the somatotrophic axis including growth hormone (GH) and insulin-like growth factor (IGF) decrease the IIS leading to an extension in lifespan [314]. A similar phenotype was observed in insulin receptor substrate 1 knockout (*Irs1*^{-/-}) mice [282]. Impairing IIS in flies through *Drosophila* insulin-like peptides (Dilps), the insulin receptor, and the insulin receptor substrates chico increase the lifespan [45], [253], [315]. Here I reported that mutation in insulin-like receptor (*dInR*) abolished the positive effect of *E. bicyclis*, indicating the necessity of the IIS pathway in the mode of action of the brown alga extract. Conversely, the extract exhibited the lifespan prolonging effect in *chico*-deficient animals and the lifespan increased by 20 %. Nonetheless, compared to the 40 % extension in the lifespan of wild-type flies the effect diminished. One reason for this observation might be the involvement of other downstream mediators of insulin-like receptor. For examples, Lnk is an orthologue of intracellular adaptors the SH2B proteins that play critical roles in protein-protein interaction downstream of receptor tyrosine kinases such as insulin receptor. It has been shown that similar to *dInR* and *chico*, *Lnk* loss of function extend the lifespan of flies. Moreover, it is able to directly modulate FoxO upon activation of IIS pathway [316]. Therefore, I assume the extract blocks the activation of the *dInR* leading to inactivation of *Lnk*. This event results in translocation of FoxO into the nucleus as I showed FoxO is a target of EBE. It has been shown that the inhibition of IIS in the renal tubules' principal cells through the reduction of *Dilp5* or RNAi depletion of *dInR* increased resistance to desiccation [317]. Interestingly, I observed the flies fed with the extract lived longer under starvation conditions. This is further suggestive of inhibition of the IIS pathway by the extract. Another line of evidence supporting the regulation of IIS by *E. bicyclis* is the aforementioned rescuing of the shortening lifespan on high-sugar diets. In mammals, insulin signaling is a central player in the regulation of carbohydrate metabolism and carbohydrate-

rich meals lead to an increase in blood glucose, which in turn stimulates insulin secretion from beta cells of the pancreas. In response to insulin, the liver, muscles, adipose tissue, neurons, macrophages, endothelial cells, and pancreatic beta cells uptake glucose and metabolize it, resulting in lowering blood glucose levels [260]. Since the IIS pathway is evolutionary conserved, it also maintains carbohydrate homeostasis in flies, and insulin homologs are secreted from IPCs leading to sugar clearance and shifting the metabolism toward anabolism processes upon feeding on dietary sugars [318]. For that reason, a diet of excessive sugar activates insulin/insulin-like signaling in flies, and the constant activation of the IIS pathway results in a shortened lifespan [319]. I suggest that the increased survival on the high sugar diet caused by the extract is due to the inhibition of insulin/insulin-like signaling pathway.

Another player in the IIS signaling pathway is the transcription factor FoxO that is highly conserved from *C. elegans* to human and plays important roles in mechanisms such as autophagy, stem cell function, stress resistance, nutrition, and aging [53], [56], [320]. It has been demonstrated that in flies, high sugar diets induce the IIS pathways by repressing FoxO and consequently its downstream target genes [321]. I observed deficiencies in *foxo* drastically reduced the lifespan of the animals compared to the wild-type and the administration of the extract in the food of these animals was not sufficient to recover the shortened lifespan caused by the deficiency. Furthermore, analyzing the expression of *foxo* in the whole body of wild-type animals displayed an upregulation of *foxo*. Interestingly, the *E. bicyclis* extract is enriched with fucosterol that downregulates IIS pathway and activates FoxO [299]. All in all, my study strongly implicates the regulation of the lifespan of flies by the extract through inhibition of evolutionary conserved IIS pathway and shifting the flies' metabolism toward catabolic processes.

Tor signaling is another nutrient-sensing pathway with established roles in aging and metabolism among different evolutionary taxa [59], [223], [322]. It is comprised of TORC1 and TORC2 with TORC1 having been linked to the aging functions of Tor [63], [323]. Pharmacological and genetic interventions repressing Tor extend the lifespan in animal models such as yeast, flies, and mice [60], [68], [284]. While rapamycin is the most studied geroprotective natural product reducing Tor signaling pathway, it is not the only one and a couple of new natural products with the potential functions to inhibit Tor have been introduced. An extract from *Celastrus orbiculatus* plants induces autophagy and inhibits Tor

signaling pathway in HT-29 cells [324]. Salidroside, a polyphenol glycoside, exhibited similar effects in these cells [325]. Oridonin is a diterpenoid isolated from *Rabdosia rubescens* that showed inhibitory effects against Tor *in vitro* and mice models [326]. Afrocyclamin A, arctigenin and, vitexin are other examples of natural products that have shown mTOR inhibitory effects [327][328][327]–[329]. An aqueous extract of marine brown alga *Saccorhiza polyschides* extends the lifespan of flies by dampening Tor signaling pathway in the fat body [330]. In my study, I showed that Tor is a target of *E. bicyclis* extract since the life-prolonging effect of the extract was not observed in flies with a mutation in the *Tor* gene. As mentioned before, the extract was not extending the lifespan in male animals, a phenotype reminiscent of genetic and pharmacological inhibition of the Tor signaling pathway [68], [284]. In addition to IIS, a high-sugar diet fosters systemic Tor activation in flies. Dietary sugars trigger the secretion of Dilp3 from IPCs and Dilp3 promotes Tor signaling to synthesize proteins and TAGs [331]. Because of this, amending the detrimental effects of the high-sugar diet by the extract is indicative of repression of the Tor signaling pathway as well. Moreover, Tor signaling through its S6K axis downregulates autophagy, which is a catabolic mechanism providing cells by an alternative source of nutrients through the degradation of cellular debris [46]. Here, I showed that the expression of the autophagy-related gene *Atg8* is promoted upon treatment with the extract suggesting the extract inhibits the interwoven IIS/Tor signaling pathways leading to the activation of autophagy. Another similarity between the pro-longevity effects of *E. bicyclis* extract and inhibition of IIS and Tor signaling pathway is their effects on the quality of sleep. As mentioned above, the flies fed with the extract had improved sleep consolidation recapitulating the effect of dampening the receptor tyrosine kinases (RTKs) signaling pathways such as IIS, Tor, and anaplastic lymphoma kinase (Alk) [249], [251]. However, the extract did not show the classic trade-off between longevity and fecundity caused by the pro-longevity interventions such as rapamycin treatment, dietary restriction, or *Dilps* deletion [60], [285] asserting combinatorial targeting by the extract and distinct underlying mechanisms to extend the lifespan.

The *Eisenia bicyclis* extract targets the Tor signaling in the fat body to induce its positive effects, whereas manipulation of the *foxo* and *sir2* in the fat body did not seem to modify the lifespan of flies upon treatment with the extract. Overexpression of *foxo* in the fat body of adult flies represses the expression and secretion of *Dilp2* leading to the inhibition of the IIS

pathway and extending the lifespan [56]. In agreement with this, long-lived IIS mutant flies exhibited enhanced expression of mitochondrial electron transport chain proteins in the fat body resulting in increased lifespan [332]. Likewise, targeting Tor signaling pathway in the fat body through the overexpression of tuberous sclerosis complex genes 2 (*dTsc2*), which inhibits Tor, the dominant negative form of Tor or the dominant negative form of S6K represented a similar phenotype and the lifespan of the animals was extended [65]. Sirtuins are other aging regulators that extend the lifespan through the fat body and it has been suggested that fat body-specific overexpression of *Sir2* or *Sirt6* increases the lifespan of flies [259], [333]. Genetic manipulations are not the only interventions that implement their positive effects through the fat body. Citrate treatment in flies extends the lifespan by targeting the Tor and AMPK signaling pathways in the fat body [278]. The marine brown alga *Saccorhiza polyschides* is another example of nutritional intervention targeting the fat-storing organ [330]. I used two driver lines for RNAi depletion of S6K in the fat body and in both conditions, the genetic manipulation of S6K abrogated the lifespan-extending effects of *E. bicyclis*. However, *Tor*^{DN} overexpression in the fat body using the two distinct driver lines gave inconsistent results. While overexpression of *Tor* in the fat body using the GeneSwitch system blocked the extension of the lifespan by the extract, the marine brown alga extract extended the lifespan in the fat body-specific overexpression of *Tor*^{DN} using *ppl-Gal4*. In my study, the extract did not seem to target the enterocytes or skeletal muscles with respect to lifespan extension.

4.5 *Salicornia europaea* extends the lifespan of *Drosophila melanogaster*

The second marine-related species that I investigated on the potential beneficial effects on the life- and healthspan of flies was the halophyte *S. europaea*. I tested two concentrations of *Salicornia europaea* extract (SEE) and found that the extract exhibited lifespan-extending effects in a dose-dependent manner. The lower concentration of 0.05 % did not increase the lifespan of the animals, whereas increasing the concentration to 4-fold had positive effects and increased the lifespan by 36 %. This is the first study reporting the lifespan-increasing effect of *S. europaea*. I suggest the pro-longevity effects of *S. europaea* is due to the abundant bioactive metabolites in the extract. Like the *E. bicyclis*, *S. europaea* extract is enriched with isocitrate, a tricarboxylic acid metabolite that might have pro-longevity effects. Fumaric acid was another TCA intermediary metabolite highly occurring in the extract. Interestingly, the dietary supplementation of fumarate increased the lifespan in *C. elegans* and activated the

nucleus translocation of daf16/FoxO, suggesting it might inhibit the IIS pathway [334]. One important category of metabolites highly enriched in the extract was “nucleosides and nucleotides” including guanosine, uridine, methyl adenosine, and thymine. Nucleosides are compounds composed of a nucleotide and ribose and their supplementation in the diet can boost the immune system, resistance to infection, and growth and development in human and animals models [335]–[338]. Moreover, dietary nucleotides might promote intestinal growth in piglets by diminishing intestinal inflammation and oxidative stress [339]. In rats, nucleotide supplementation hindered the progression of hepatocyte steatosis and restored the alcohol-induced alteration in alanine aminotransferase, aspartate aminotransferase, and oxidative stress [340]. Nucleotides and nucleosides have also been a subject in aging studies. It has been suggested that a mixture of nucleotides and nucleosides might prevent the age-induced deterioration of brain morphology and memory tasks of mice [341]. In addition, feeding rats with a diet containing supplementary nucleotides increased the medium and maximum lifespan of the animals and prevented the age-related decrease in the activities of antioxidant enzymes dose-dependently [342]. Fibroblast metabolite profiling among mammals revealed a positive correlation between several nucleotides/nucleosides including ADP, GDP, and adenosine, and longevity [343]. Conversely, in flies, supplementary adenine blocked the lifespan extension caused by dietary restriction or in *Adenylosuccinate Synthetase* deficient animals [344]. *S. europaea* is abundant with minerals and vitamins with beneficial effects on different measures of health. Three examples of such vitamins are Pantothenic acid (B5), thiamine (B1) derivative thiamine monophosphate, and biotin. It has been shown that supplementary calcium pantothenate could increase the mean lifespan of mice [345]. This result confirmed the previous study on *Drosophila*, reporting the longevity effects of Pantothenic acid [346]. The HPLC study of the extract showed an abundance of amino acids such as L-phenylalanine, L-tryptophan, cysteine, and Isoleucine. Phenylalanine is an essential amino acid and a precursor to tyrosine. It has been suggested that phenylalanine has protective effects against free radicals in rat brains [347]. However, the existing literature on the effect of phenylalanine on aging is contradictory. An association between the telomere shortening and increased phenylalanine levels of human serum during aging has been reported. However, no mechanistic link was suggested [348], although phenylalanine showed a positive correlation with longevity traits in a cell culture-based-metabolite profiling [343]. Tryptophan is another essential amino acid in the extract with indecisive effects on aging

processes. On the one hand, temporary tryptophan restriction extended the lifespan in mice [174]. On the other hand, it was reported that long-lived flies had higher levels of tryptophan [349]. These findings suggest that tryptophan, per se, might not be a lifespan-limiting amino acid but its degradation in the kynurenine pathway to the metabolites kynurenine and quinolinic acid is anti-longevity and reduces the lifespan. In line with this, the inhibition of tryptophan degradation or its cellular uptake showed lifespan-extending effects [350]. This was confirmed by showing inhibition of tryptophan 2,3-dioxygenase, the first enzyme in degradation of tryptophan in kynurenine pathway, extends the yeast lifespan [351]. However, degradation of tryptophan in kynurenine pathway results in production of pro-longevity metabolite NAD⁺ [103], [352] and thus, it might have pro-longevity effects.

4.6 SEE modifies the lipid metabolisms of flies

In this study, the effect of the *S. europaea* on the lifespan of male *w¹¹¹⁸* was shown and like *E. bicyclis* the aqueous extract of *S. europaea* did not affect the males' lifespan. I also showed that the extract not only does not negatively affect the fecundity of the flies, but it improves it. This finding is uncommon in the pro-longevity interventions that show a trade-off between longevity and fecundity indicating a distinct mechanism of actions and general enhancement of the metabolic state of the flies. This is consistent with the positive effect of citrate on the lifespan and fecundity of flies [278] and interestingly the extract had a high content of isocitrate and fumaric acid and therefore, it is presumable that by promoting TCA intermediary metabolites the metabolic state of flies improved, resulting in increased fecundity. In addition, argininosuccinate, a precursor of arginine and fumarate, was a frequently occurring compound in the extract. This raises the possibility that this compound is degraded to fumarate and arginine and subsequently fumarate incorporates into the TCA cycle, whereas arginine enhances the fertility of the animals, since it has been reported that arginine increases the sperm count and motility in humans [353].

In agreement with other studies reporting lipid-lowering effects of *Salicornia* species in cell culture-based studies [239], [354], I found the extract decreased the TAG levels of flies after three weeks of treatment and I hypothesize cinnamic acid and its derivatives in the extract might be responsible for the lipid-lowering effects. In a cell culture-based study, it was shown that cinnamic acid derivatives such as *o*-coumaric acid decreased intracellular triglyceride and inhibited glycerol-3-phosphate dehydrogenase (GPDH) activity. Furthermore, these

compounds reduced the expression of PPAR γ , C/EBP α , and leptin [355]. In the murine preadipocyte cell line 3T3-L1 and in bone marrow cultures, p-hydroxycinnamic acid inhibits adipogenesis and in the latter, it simulates osteoblastogenesis and mineralization [356]. Moreover, I showed that the extract extended the survival of *w¹¹¹⁸* female flies on a 20 % coconut fat diet by 17% further supporting an anti-hyperlipidemic effects of *Salicornia*. The flies treated with the extract were more sensitive to starvation after three weeks of treatment, confirming the result for TAG levels alteration upon the treatment owing to notable roles of TAGs in energy metabolism during starvation. My study also provided evidence for the anti-hyperglycemia effect of the extract. Similar to the effect on the TAGs levels, *S. europaea* reduced the whole-body glucose level of the female flies after three weeks of treatment and it could be that cinnamic acid found in the extract is involved in this effect, since cinnamic acid and its derivatives have been demonstrated to show anti-hyperglycemic and anti-diabetic effects. These compounds inhibit the activation of several enzymes responsible for insulin resistance and carbohydrate absorption. Protein tyrosine phosphatase 1B (PTP1B) activation is a negative regulator of insulin and leptin signaling and leads to insulin resistance and termination of GLUT4 translocation to the plasma membrane [357]. It has been suggested that the inhibition of PTP1B is a promising target to treat diabetes. Cinnamic acid isolated from *Cinnamomum cassia* exhibited inhibitory effects against PTP1B and consequently increased *glut4* expression in L6 myotube cells [358]. Conversely, my study showed downregulation of *glucose transporter 1 (glut1)* expression in the whole-body of flies after treatment with the extract. It might be due to the inhibitory effect of cinnamic acid against PPAR γ that its expression leads to increased glucose uptake [359]. Thus, it could be that the mode of action of the extract for the anti-hyperglycemic effect is not only targeting PTP1B and PPAR γ cinnamic acid derivatives, but also inhibiting digestive α -amylase and α -glycosidase and therefore they reduce the postprandial glucose increase by diminishing the degradation of dietary carbohydrates leading to reduced glucose uptake [360]. I propose that the *S. europaea* extract could act through cinnamic acid and its derivatives to impede carbohydrate and lipid digestion in the intestine resulting in reduced intestinal glucose and fatty acid uptake and lowered their levels in the hemolymph. Since the dietary sugars in the hemolymph are sensed by IPCs and these cells recruit glucose transporters on the plasma membrane in these conditions, the lowered glucose content in the hemolymph leads to reduced expression of *glut1* in the IPCs.

4.7 SEE targets the Tor signaling pathway in the fat body

In a mechanistic approach, I demonstrated that Sirt1 and Sirt6 are not the main targets of the extract to extend the lifespan of flies since the *Sirt1*-deficient flies retained their responsiveness to the extract and their lifespan increased. Additionally, the expression of *sirt6* did not alter in wild-type animals upon treatment. However, it could not be ruled out that slight modulations of sirtuins are induced by the extract. First, the effect of the extract in *Sirt1*-deficient animals is not as large as in the wild-type animals, and thus it is conceivable that the extract has pleiotropic effects. Second, it is known that sirtuins regulate lipid metabolism [254], [361] and we observed marked alteration in lipid metabolism by the extract. Third, the extract might provide the cells with NAD⁺ precursors such as tryptophan and therefore it promotes the activation of NAD⁺-dependent protein deacetylases. All in all, the effect on sirtuins should be further investigated especially their influence on the metabolism particularly the mitochondrial Sirtuin 4 [254]. Similarly, I showed that *foxo* is not required to extend the lifespan of the flies since *foxo*-deficient animals replicated the positive effects of the extract in wild-type flies, indicating other mechanisms to extend the lifespan. Nonetheless, this does not exclude the possibility of IIS involvement in the mode of action of the extract and the extract might target the other components of the IIS pathway, e. g., Dilps, dInR, and IRS (*chico*) similarly as DR does. In *C. elegans*, DR extends the lifespan of animals independent of *daf16*, whereas the effects of DR is abrogated in mice with defective growth hormone receptor [362], [363]. Similarly, in flies, DR extends the lifespan in the absence of *foxo* while fat body-specific overexpression of *foxo* alters the effect of DR on the lifespan of the animals [364]. Moreover, the observed extension in the lifespan of *foxo*-deficient animals is not highly pronounced (8 %). Surprisingly, the expression of a negative regulator of *Drosophila* insulin-like peptides Imaginal morphogenesis protein-Late 2 (*ImpL2*) was downregulated in flies treated with the extract. *ImpL2* is a homolog of vertebrate IGF-binding protein 7 that binds to Dilp2 and Dilp5 and its ubiquitous or fat body-specific overexpression leads to the extension of lifespan in flies [365]. This finding seems contradictory to the pro-longevity effects of the extract. However, it has been demonstrated that there is a negative feedback system between the expression of *ImpL2* and Dilps, e.g., the expression of *ImpL2* was downregulated in long-lived *Dilp2–3, 5* mutants [253]. The downregulation of *ImpL2* upon treatment with the extract is consistent with the result of starvation conditions. *ImpL2* has a

critical role in starvation resistance by dampening the IIS and its mutation leads to severe sensitivity to starvation conditions [366]. Here I observed a correlation between reduced expression levels of *ImpL2* and starvation sensitivity. Due to a negative feedback regulation system between IIS and *ImpL2*, the extract presumably inhibits the IIS pathway by targeting different components of the pathway, e.g., Insulin-like receptor or chico. For that reason, the function of *ImpL2* is not required and the cell prevents further inhibition of the signaling pathway to maintain the necessary threshold of function of IIS pathway.

I showed the *S. europaea* requires Tor signaling pathway to mediate the longevity phenotype. Flies with a defective form of Tor treated with the extract did not show the lifespan extension phenotype, suggesting the extract inhibits Tor signaling. It has been shown that citrate the first intermediary metabolite in the TCA cycle reduces the energy status and extends the lifespan of flies through inhibition of the Tor signaling pathway [278], recapitulating the impact of DR on the animals [17], [153], [367]. Besides, it has been suggested that the TCA cycle intermediary metabolites supplementation decreases ATP/ADP and increases the NAD⁺/NADH ratio, i.e., mitochondrial respiration increases when the TCA cycle rate decreases that serves two purposes. First it signals a low energy status and second provides NAD⁺ for pro-longevity NAD⁺-dependent proteins [334], [368], [369].

Intestine-specific modification in the expression of lifespan regulatory genes is an important intervention to mediate lifespan extension in research animals. In *C. elegans*, overexpression of *daf-16* in the intestine rescued the shortened lifespan in the *daf-16(-)* germline-deficient animals and increases the lifespans of *daf-16(-)* insulin/IGF-1-pathway mutants [370]. In addition, ectopically expressed SKN-1, a proxy of reduced IIS, in the intestine of these animals increased the lifespan and stress resistance [371]. Similarly, Intestinal stem cell-specific inhibition of IIS through different components of the pathway such as IR and Akt revealed longevity phenotype in flies [372]. However, I did not find solid evidence for intestinal-specific mediation of lifespan extension via targeting the Tor signaling pathway by the *S. europaea* extract, although global blocking of the Tor signaling abrogated the lifespan extension by the extract, indicating it mediates the positive effects through Tor signaling pathway. I showed neither *Tor^{DN}* nor *S6K* RNAi could abolish the extension in lifespan. These results might be explained in light of the finding that the downregulation of Tor signaling in the intestinal stem cells and enteroblasts is not pro-longevity, but reduces the TAGs and glycogen content of the

body [373]. Thus, it could be assumed that the extract targets this nutrient-sensing pathway in the intestinal stem cells leading to reduced TAG levels, but that this does not rule out the possibility of affecting the pathway in the other organs and tissues. In addition, I showed the extract could positively affect the lifespan while the *Tor* or *S6K* is reduced in the enterocytes.

The intestine not only plays important role as a signaling center for aging, but also its homeostasis and barrier function is of note in aging mechanisms [372], [374]–[376]. In *C. elegans*, an aging gut is associated with the degradation of intestinal microvilli and the size and shape alteration of the intestine [375]. In flies, aging causes the over-proliferation of ISCs, leading to dysplasia via activation of JNK signaling, and this is linked to the lifespan [372], [374]. Moreover, it has been shown that flies lose the barrier function of the intestine accompanied by elevated levels of antimicrobial peptides (AMPs) and aberrant IIS [377]. Similarly, in rodent studies, it has been demonstrated that the intestine loses the barrier function with age, and its permeability increases, whereas the ability of sugar and amino acid absorption is compromised [378], [379]. It has been proposed that the pro-longevity interventions in flies, e.g., reduced yeast concentration in the food or reduced environmental temperature reduces gut permeability [377]. In the present work, I revealed that the *S. euryphaea* extract's longevity benefits accompany improved intestinal barrier function, and the extract treatment reduced the number of flies with compromised intestinal integrity after 20 days of treatment. Owing to age-related increased ISC proliferation and mis- differentiation [374] and immunity-related gene expression in animals with loss of gut integrity [377], these results raise the possibility of balanced proliferative homeostasis of ISCs and improved organismal immunity in flies treated with the extract.

Finally, I suggest the fat body may be the target organ of the extract, since driving *Tor^{DN}* using the *ppl-Gal4* line in the fat body did not extend the lifespan significantly. Conversely, the fat body-specific downregulation of *Tor* using the GeneSwitch system driver *P(GS)106* did not abolish the lifespan extension of the flies fed with the extract. The observed discrepancy in the results of lifespan assays using two different driver lines for the fat body may arise from the fact that the *P(GS)106* is expressed not only in the fat body, but also in the intestine of the flies [380]. Thus, it is possible the extension in lifespan by the extract in flies with the downregulation of the *Tor* signaling pathway using *P(GS)106* is an additive effect of inhibition of *Tor* by the extract in the fat body and its genetic downregulation in the intestine. Regarding

the *ppl-Gal4* lifespan results, it should be noted that the experimental groups represented no differences in the maximum lifespan whereas the median lifespan of the flies fed with the extract improved. Thus, I do not exclude the contingency of another mode of action or target tissues.

Upon feeding, the fat body senses the nutritional status and secretes Upd2, a homolog of mammalian leptin, provoking Dilps secretion from IPCs [264]. In this study, I showed the lifespan extension by the extract is not due to canonical cross-talk between the fat body and the IPCs in response to the dietary nutrients and the extract could extend the lifespan of *upd2*-knock-out flies. Unlike mammals that have one insulin hormone, *Drosophila* has eight insulin-like peptides (Dilps) with distinct roles and functions in the growth, development, and lifespan of flies [253], [381]. IPCs-produced Dilp2, Dilp3, and Dilp5 and fat body-produced Dilp6 is known to regulate lifespan counterintuitively. Downregulation of *Dilp2*, *Dilp3*, and *Dilp5* extends the lifespan, whereas the overexpression of *Dilp6* in the fat body is pro-longevity [253], [382]. My results are suggestive of uncoupling the effects of the extract from IPCs secreted Dilp2, 3, and 5. However, further studies are needed to investigate the effects of the extract on other Dilps, specifically fat body-secreted Dilp6 that has a specific role in lipid storage and mobilization [383].

4.8 Future perspectives

I found both extracts used in this study extend the lifespan of flies and are beneficial upon nutritional stresses such as high-fat and high-sugar dieting. Thus, it is interesting to investigate whether a diet containing both extracts elicits additive effects on the lifespan of flies on a normal diet and detrimental diets, e. g., high-sugar or high-fat diets. Moreover, both extracts were enriched with bioactive compounds such as isocitric acid and nucleosides. Further research is required to investigate the effects of these compounds singularly on lifespan. I revealed that *Eisenia bicyclis* mediates the lifespan-extending effects through the IIS pathway and the extract was highly effective on high-sugar diets. For these reasons, the effects of the extract on the carbohydrate metabolism of flies should be studied in more details. To this end, major metabolites involved in carbohydrate metabolism such as glycogen, cholesterol, lipids, acetyl-CoA, ATP, NAD, and uric acid should be measured after treating the flies with the extract on a high-sugar diet. Furthermore, the amount of Dilp2, Dilp3, and Dilp5 that are secreted from insulin producing cells (IPCs) in the brain of flies are of note. Carbohydrate-responsive

genes are another target to study the effects of *Eisenia bicyclis* on the carbohydrate metabolism of flies. *Mondo-Mlx*, *sugarbabe* and *cabut* are major carbohydrate responsive genes that their alteration upon treatment with the extract can be evaluated.

In this study, I showed *S. europaea* has lipid-lowering effects at organismal levels, and flies fed with its extract were more resistant to a high-fat diet. Further research should be done on this effect of the halophyte plant. Moreover, the mode of action of the extract involved the fat body. Thus, the effect of *S. europaea* on lipid uptake, storage, and mobilization in this specialized nutrient-storing organ can be studied. Finally, it should be elucidated that the lipid-lowering effect of the extract is due to increased lipolysis or decreased lipogenesis. For this purpose, lipogenesis through fatty acid synthase 1 (FASN-1) in the fat body might be examined. It is also interesting to monitor the size of lipid droplets in the fat body upon treatment with *Salicornia europaea* extract.

5. Curriculum Vitae

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6. Publications

Sharifi-Rad M, Tayeboon GS, Miri A, Sharifi-Rad M, Setzer WN, Fallah F, Kuhestani K, **Tahanzadeh N**, Sharifi-Rad J. Mutagenic, antimutagenic, antioxidant, anti-lipoxygenase and antimicrobial activities of *Scandix pecten-veneris* L. Cell. Mol. Biol. 2016, 62 (6): 8-16.doi: 10.14715/cmb/2016.62.2

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8. Declaration

I declare that the dissertation titled “Life- and healthspan extension by marine natural products in *Drosophila melanogaster*” apart from my supervisor’s guidance and the listed references, is my own literary property and represents my own work. This dissertation has not been submitted elsewhere in order to obtain an academic degree and was prepared according to the *Rules of Good Scientific Practice* of the German research Foundation. An academic degree never was not withdrawn.

Hiermit erkläre ich, dass die vorliegende Dissertation mit dem Titel “ Life- and healthspan extension by marine natural products in *Drosophila melanogaster* ”, die ich unter der Betreuung durch meinen Supervisor Prof. Dr. Thomas Roeder und unter Zuhilfenahme der angegebenen Referenzen angefertigt habe, mein eigenes geistiges Eigentum ist und meine eigene Arbeit widerspiegelt. Die Arbeit wurde unter Berücksichtigung der *Regeln guter wissenschaftlicher Praxis* der Deutschen Forschungsgesellschaft angefertigt und an keiner weiteren Stelle im Rahmen eines Promotionsvorhabens eingereicht. Ein akademischer Grad wurde mir nicht entzogen.

Kiel, den

Navid Tahan Zadeh

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