

**Lifespan extension by nutritional
interventions in the fruit fly *Drosophila melanogaster***

DISSERTATION
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von
Yang Li
aus China

Kiel, 2016

Referent: Prof. Dr. Thomas Roeder

Koreferent: Prof. Dr. Holger Heine

Tag der mündlichen Prüfung: 5. Oktober 2016

Zum Druck genehmigt:

gez. Dekan:

Table of contents

List of tables	1
List of figures	2
List of abbreviations	5
1 Introduction	8
1.1 Overview of the mechanisms of aging	8
1.2 The function of dietary restriction on slowing aging and preventing metabolism diseases	10
1.3 Insulin/IGF signaling and its regulation	12
1.4 Limitations of Dietary restrict and the solutions	15
1.5 Advantage of <i>Drosophila</i> as a model organism	16
1.6 Aims of the study	19
2 Materials and Methods	20
2.1 Materials	20
2.1.1 <i>Drosophila</i> strains and husbandry	20
2.1.2 Fly mediums	21
2.1.3 Solutions	22
2.1.4 Antibodies	23
2.1.5 Oligonucleotides	24
2.1.6 Enzymes	24
2.1.7 Chemicals	24
2.1.8 Equipment	25

2.2 Methods	26
2.2.1 Lifespan measurement	26
2.2.2 Gal4/UAS crossing	27
2.2.3 Egg laying test	27
2.2.4 Body fat quantification	28
2.2.5 Hemolymph glucose measurement	29
2.2.6 CAFE assay	29
2.2.7 Metabolic rate assay	31
2.2.8 Locomotor assay	32
2.2.9 Molecular biological methods for relative quantification of qRT-PCR	33
2.2.10 Quantification of circulation and total DILP2 content	36
2.2.11 Statistical analysis	38
3 Results	39
3.1 Dietary restriction regulates lifespan and phenotypical peculiarities of <i>Drosophila melanogaster</i>	39
3.1.1 Dietary restriction is essential for lifespan	39
3.1.2 Dietary restriction affected fecundity	40
3.1.3 Dietary restriction regulated females' locomotor behavior and circadian rhythms but not that of males	41
3.1.4 Dietary restriction affected body fat	43
3.1.5 Dietary restriction influenced hemolymph glucose level	48
3.1.6 Metabolic rate was increased in the flies treated with high protein medium	49

3.2 Analyses of dietary restriction's influence on regulation of DILP2 secretion	52
3.2.1 Circulating as well as total DILP2 level were affected by protein intake	52
3.2.2 Dietary restriction influenced imp-l2 expression level	54
3.3 <i>Saccorhiza polyschides</i> is a safety anti-age food supplement	56
3.3.1 <i>Saccorhiza polyschides</i> extract increased lifespan of <i>Drosophila</i>	56
3.3.2 Lifespan was significantly increased by <i>Saccorhiza polyschides</i> extract diet for flies confronted with stressful conditions	57
3.3.3 Fly's fecundity remained unchanged by <i>Saccorhiza polyschides</i>	61
3.3.4 <i>Saccorhiza polyschides</i> extract affected fly's circadian rhythmicity but not locomotor behavior	62
3.3.5 <i>Saccorhiza polyschides</i> influenced the TAG content and body weight in males, but not in females	64
3.3.6 <i>Saccorhiza polyschides</i> altered the hemolymph glucose level	65
3.3.7 <i>Saccorhiza polyschides</i> failed to influence fly's food intake	66
3.3.8 The fly's metabolic rate was not altered by <i>Saccorhiza polyschides</i>	67
3.4 Characterization of <i>Saccorhiza polyschides</i> extract-mediated lifespan changes on mutant flies	69
3.4.1 <i>Saccorhiza polyschides</i> extract failed to extend lifespan of Tor mutant flies	69
3.4.2 <i>Saccorhiza polyschides</i> extract failed to extend lifespan of Sirt1 KO flies	70

3.4.3 Performance of <i>Saccorhiza polyschides</i> extract in FoxO, Sirt2 and Tor over expression lines	71
3.5 Characterization of <i>Saccorhiza polyschides</i> extract-induced gene expression changes	76
4 Discussion	81
4.1 Dietary restriction influences lifespan and associated phenotypes	82
4.2 Dietary restriction regulates insulin-like peptide release	85
4.3 <i>Saccorhiza polyschides</i> extracts influence lifespan and relevant phenotypical peculiarities in different ways	86
Summary	93
Zusammenfassung	95
Reference	97
Appendices	120
Acknowledgement	126
Curriculum vitae	127
Publications	128
Declaration	130

List of tables

Table 1. Ingredients and preparation process of mediums	20
Table 2. Ingredients and preparation process of mediums	21
Table 3. Oligonucleotides used for PCRs	23
Table 4. RT-PCR program	35
Table 5. Target genes for qRT-PCR.....	77
Table 6. Efficiency of primers utilized in this experiment.....	78
Table 7. Seaweeds have been reported to be essential for health promotion	87

List of figures

Figure 1. Photograph of <i>Saccorhiza polyschides</i>	16
Figure 2. The schematic diagram of GAL4/UAS system	18
Figure 3. Scheme of the CAFE assay	30
Figure 4. Scheme of setups for measurement of CO ₂ production	32
Figure 5. <i>Drosophila</i> activity monitors.....	33
Figure 6. Protein concentration of diet influenced lifespan in females and males ..	40
Figure 7. Dietary restriction influenced fecundity	41
Figure 8. Locomotor activity of flies treated with DR and CM medium	42
Figure 9. Circadian rhythm activity of flies treated with DR and CM medium	42
Figure 10. Over high protein diet inhibited TAG storage.....	44
Figure 11. The influence of protein intake on females' TAG storage was stronger..	45
Figure 12. The amount of protein intake increased flies body weight	46
Figure 13. The influence of protein intake on females' body weight was stronger..	47
Figure 14. Hemolymph glucose level was altered by dietary restriction	48
Figure 15. High protein diet increased CO ₂ production of flies	49
Figure 16. Longer time treatment of high protein diet affected more on CO ₂ production of flies	50
Figure 17. High level of protein intake increased circulating DILP2 content and decreased total DILP2 content	53
Figure 18. The expression level of <i>imp-L2</i> was reduced by the high protein diet.....	55

Figure 19. <i>Saccorhiza polyschides</i> increased fly's median lifespan obviously	56
Figure 20. <i>Saccorhiza polyschides</i> enhanced fly's resistance to drought environment	58
Figure 21. <i>Saccorhiza polyschides</i> increased fly's resistance to high fat diet	59
Figure 22. <i>Saccorhiza polyschides</i> extract promoted flies' tolerance to starvation ..	60
Figure 23. Fecundity assay for flies treated with <i>Saccorhiza polyschides</i> extract containing medium	61
Figure 24. Locomotor activity of flies treated with <i>Saccorhiza polyschides</i> extract medium	62
Figure 25. Circadian rhythm activity of flies treated with <i>Saccorhiza polyschides</i> extract medium	63
Figure 26. <i>Saccorhiza polyschides</i> treated flies stored less total TAG	64
Figure 27. <i>Saccorhiza polyschides</i> intake increased fly's body weight slightly	65
Figure 28. <i>Saccorhiza polyschides</i> significantly influences hemolymph glucose level	66
Figure 29. CAFE assay for <i>Saccorhiza polyschides</i> extract treated flies	67
Figure 30. CO ₂ production of flies treated by <i>Saccorhiza polyschides</i> extract	68
Figure 31. Median lifespan of Tor mutant flies was not obviously influenced by <i>Saccorhiza polyschides</i> extract	70
Figure 32. <i>Saccorhiza polyschides</i> extract failed to affect median lifespan of Sirt1 KO flies	71
Figure 33. <i>Saccorhiza polyschides</i> extract did not influence lifespan of Tor over expression flies	72

Figure 34. *Saccorhiza polyschides* extract decreased lifespan of Tor.TED expression flies73

Figure 35. Median lifespan of Sirt2 overexpression fly was not extended by *Saccorhiza polyschides* extract74

Figure 36. Median lifespan of FoxO overexpression females was extended by *Saccorhiza polyschides* extract75

Figure 37. Determination of differential expression levels using relative qRT-PCR.....79

List of abbreviations

aa	amino acids
AE	algae extract
AKH	Adipokinetic Hormone
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
bp	base pair
BSA	Bovine serum albumin
Ca ²⁺	calcium ion
CAFE	Capillary Feeder
cAMP	cyclic adenosine monophosphate
CCA	Coupled Colorimetric Assay
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CM	Concentration Medium
CNS	Central Nervous System
CO ₂	Carbon dioxide
d	day
DAG	Diacylglycerin
DAMS	Drosophila Activity Monitor System
DNA	Deoxyribonucleotideic acid
dNTPs	deoxyribonucleotide
DR	dietary restriction
EDTA	Ethylenediamine tetra acetic acid
EGFR	epidermal growth factor receptor
EtOH	Ethanol
FB	Fat Body
FSB	First strand buffer
g	gram
GFP	Green Flourescence Protein

GPCR	G-Protein Coupled Receptor
Grb2	growth factor receptor bound protein 2
GS	GeneSwitch
GTP	Guanosine-5'-triphosphate
h	hour
HF	High Fat
HL3	Hemolymph-like buffer
HPLC	High Pressure Liquid Chromatography
IGF	Serum insulin-like growth factor
IGFBP	Serum insulin-like growth factor binding protein
IgG	Immunoglobulin G
IIS	Insulin/IGF1-Signalling
ILP	Insulin-like peptide
Imp-L2	Imaginal morphogenesis protein-Late 2
InR	Insulin-like peptide receptor
IPCs	Insulin Producing Cells
JNK	Jun amino-terminal kinase
K ⁺	potassium ion
kb	kilobase pairs
L	Liter
Lgr3	Leucine-rich repeat-containing G protein-coupled receptor 3
LD	Light/Dark
mg	milligram
Mg ²⁺	Magnesium ion
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
Na ⁺	sodium ion
NAD	Nicotinamide adenine dinucleotide

NCBI	National Center for Biotechnology Information
ng	nanogram
O-GlcNac	O-Linked β -N-acetylglucosamine
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
pg	picogram
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RPD3 (HDAC3)	Histone deacetylase 3
Rpl32	60S ribosomal protein L32
RTK	receptor tyrosine kinase
rDNA	ribosomal deoxyribonucleotideic acid circles
rpm	rounds per minute
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
S.D.	Standard deviation
Sec	second
Sir2	Silent information regular 2
sNPF	short neuropeptide F
TAG	Triacylglycerin
Tor	Target of rapamycin
UAS	Upstream Activating Sequence
YE	Yeast extract
μ g	microgram
μ l	microliter
μ M	micromolar

1. Introduction

1.1 Overview of the mechanisms of aging

Aging, in a narrow sense, is a natural process of biological becoming older, it also refers to cellular aging or population aging in a broader sense. It is a process necessarily experienced by most organisms and leads to inconveniences for them. Taking humans as examples, many negative changes are brought by this process in physical, such as the decrease of reproduction ability, movement ability and metabolism functions, as well as the increase of the risk of suffering a number of age-associated diseases (Dillin A et al., 2014). The neurodegenerative diseases including the various dementias are mostly highly associated with age (Larson EB, 2013). It is reported that about 3% of the population in the age range of 65-74 have dementia, while this ratio sharply raises to 19% and about 50% for people between 75 and 84 and over 85 years, respectively (Umphred D, 2012). Besides dementia, metabolic diseases like high blood pressure and type II diabetes are also tightly associated with age. Moreover, communication barriers, due to hearing loss and visual impairment are also common among aged people (Worrall L et al., 2003).

Until now, the biological basis of aging is still not well understood. The factors proposed to influence biological aging fall into two main categories, programmed and damage related ones (Jin K, 2010). Programmed factors are believed to follow a biological timetable, such as the theory that telomeres are “molecular clocks”, which is based on the observation that telomeres become shorter after each successful cell division, and the shortened telomeres prevent further cell multiplication (Mikhelson V et al., 2013). Other theories, such as evolutionary theories, believe that lifespan is selected like other phenotypes (Williams GC, 1957), and reproductive-cell cycle theory, which claims aging is regulated by reproductive hormones promoting growth and development early in life but becoming dysregulated late in life (Atwood CS et al., 2011), also describe other possible

programmed factors such as DNA methylation (Berdyshev G et al., 1967; Christiansen L, 2015) and autoimmune problems. Additionally, the aging process induced by programmed factors normally depends on changes of gene expression. Previous work has demonstrated that different levels of expression or performance of some genes, such as FoxO3A (Willcox BJ, 2008), mTor (Fontana L et al., 2010; Johnson SC et al., 2013) and Growth hormone (GH)/Insulin Growth Factor-1 (IGF1) (Junnila RK et al., 2013; Guarente LP et al., 2008), are tightly associated with the aging process. Compared with endogenous programmed factors, damage-related factors are environmental assaults to living organisms that induce cumulative damage at various levels. The DNA damage theory describes that aging is caused by abnormal mRNA synthesis (Callegari AJ, 2016) and DNA replication happening in each cell division (Robert L et al., 2010). The genetic instability theory implies that genetic damage (particularly gene loss) is almost certainly (or probably the) the central cause of aging (Strehler BL, 1986), as it has been supported by the observation that approximated 3.3% of DNA is lost in the dog's heart muscle every year. Meanwhile, accumulation of waste and errors, cross-linkage compounds and free-radicals in cells are also believed to be damage-related factors. (Gavrilov LA, 2006; Bernstein C, 1991; Bjorksten J, 1990; Schulz TJ, 2007; Harman D, 1981).

Population aging, which is mainly contributed by the life expectancy and declining fertility rate, has become one of the greatest world challenges in 21st century. According to a report from United Nations (UN), the world has never seen as aged a population as currently exists globally. The number people of 60 years old or above have tripled from 200 million in 1950 (7.9%) to 600 million in 2000 (9.8%), and surpassed 700 million in 2006 (10.6%), while it is predicted this number will reach 2 billion in 2050 (20.6%) (Chucks J, 2010; Alhassan Issahaku, P 2013; the number in brackets presents the population portion over 60). In advanced countries, this problem is even more urgent. In the USA the population portion over 60 has reached 19.7% in 2013, while this portion is 27.1% in Germany and 32.4% in Japan. The median age of advanced countries has increased from 28 in 1950 to 40 in 2010, while the corresponding figure worldwide is 24 in 1950 and 29

in 2010. Population aging would bring a series of serious social problems, such as the pressure of pension support, the lack of labor supply and the availability of medical resources. As the trend of population aging is hard to reverse, to decrease the incidence of age-related diseases and promote health condition and life quality of aged people, so as to reduce the burden of the society and youth, are the problems, which are urgent to be solved. So researches on diet restriction, which might have the function of slow aging, were in the focus of this work.

1.2 The function of dietary restriction on slowing aging and preventing metabolism diseases.

Dietary restriction (DR), or calorie restriction, is a dietary regime that reduces calorie intake while avoiding malnutrition. It is believed to slow aging and extend lifespan. About 100 years ago, Osborne and colleagues demonstrated that restricting the food intake of female rats extended their lifespan (Mitchell SE et al., 2016). Since then, it has been demonstrated that the median and maximal lifespan of many species, such as yeast (Kaeberlein M et al., 2007), various invertebrates (Piper MD et al., 2008) and mammals (Weindruch R et al., 1988), can be prolonged by dietary restriction. The mechanism of dietary restriction that slow aging is still under exploration. Reducing DNA damage is one of the explanations, which describes that dietary restriction reduces production of reactive oxygen species (ROS) (Sohal RS et al., 1994; Gredilla R et al., 2001), a metabolic waste product that causes several types of DNA damage including 8-hydroxy-2'-deoxyguanosine (8-OHdG) formation (Hamilton ML et al., 2001; Wolf FI et al., 2005). Mitochondrial hormesis is another explanation, which believes dietary restriction extends lifespan by increasing oxidative stress to stimulate the organism to launch an effective anti-oxidative response (Schulz TJ et al., 2007). Besides, there are some other possible explanations for this, such as reduced core body temperature (Conti B et al., 2006),

reduced cell division, reduced free radicals (Sohal RS et al., 1994) and lower metabolic rates. Moreover, it has been demonstrated that the silent information regular 2 (Sir2) family of proteins mediates lifespan extension from dietary restriction in many relevant studies. Sir2 is a highly conserved NAD⁺ - dependent histone deacetylase (Frankel S et al., 2004) and has been identified in a wide range of organisms, from bacterial to mammals as being involved in regulation of lifespan (Kaeberlein M et al., 1999). Its overexpression is sufficient to prolong lifespan in many species (Schumpert CA et al., 2016). It is found that dietary restriction failed to induce longevity in Sir2 mutant yeast (Lin SJ et al., 2000). Also, Sir2 regulates silencing of the homologous recombination at the extra-chromosomal ribosomal DNA circles (rDNA) loci (Brachmann CB et al., 1995), the accumulation of rDNA is resistant to dietary restriction mediate longevity (Sinclair DA et al., 1997; Guarente L et al., 2005). Moreover, dietary restriction increases the activity of Sir2 *in vivo* (Lin SJ et al., 2002).

Dietary restriction is benefit for preventing metabolic diseases, many of which are age-related, such as cancer, type II diabetes, obesity, and cardiovascular diseases. Dietary restriction in rodents decreases serum insulin-like growth factor (IGF-1) concentration (Fontana L et al., 2016). Data from experimental and epidemiological studies indicate that IGF-1 and its binding proteins play a role in the biology of aging and in the pathogenesis of several common cancers (Yu H et al., 2000; Renehan AG et al., 2004; Yakar S et al., 2005; Fontana L et al., 2010; Kopchick JJ et al., 2014). Another study on rat showed that moderate dietary restriction can substantially elevate glucose uptake by insulin-stimulated skeletal muscle from both young and old rats (Wang H et al., 2016). High IGF-1 level and low glucose uptake are risk factors for type II diabetes. Besides, some other risk factors such as c-reactive protein, serum triglycerides, blood pressure and fasting blood sugar which are associated with obesity and cardiovascular diseases are substantially brought down by diet restriction (Weiss EP et al., 2011). These effects suggest dietary restriction may have a major beneficial effect on health, lifespan, and quality of life.

It was found that nutrient imbalance, particularly amino-acid imbalance, also modulates aging (Lee BC et al., 2013). Some studies focused on identifying the exact nutrients influencing lifespan and aging. Fruit fly fed with DR medium have a longer median lifespan than those fed with full medium, while adding back vitamins, carbohydrate and lipids to DR median failed to affect the lifespan extension mediated by diet restriction. But in contrast to these nutrients, adding back amino acid to DR medium would decrease fly's lifespan to the level of full medium feeding (Grandison RC et al., 2009). Furthermore, it was shown that essential amino acids influence much stronger DR-mediate longevity than non-essential amino acids (Emran S et al., 2014). Additionally, among the essential amino acids, the restriction of methionine is essential for lifespan extension in many model organisms without energy restriction (Miller RA et al., 2005; Mclsaac RS et al., 2016), even for inhibiting aging-related diseases processes in mice (Sun L et al., 2009). It could be concluded that specific nutrient restriction, instead of energy or calorie restriction, is the main factor for DR-mediate anti-age and longevity.

1.3 Insulin/IGF signaling and its regulation

Metabolic regulation and physiological feedback systems play a central role to all aspects of postembryonic life, via balancing energy needs, influencing sexual maturation and adult fertility, as well as acting as a key determinant of aging. The conserved insulin/IGF pathways plays a central role in growth and metabolism in higher organisms (Baker KD et al., 2007). In mammals, IGFs primarily regulates regular growth and insulin sustains the glucose homeostasis, while in *Drosophila melanogaster* these two activities are unified to the insulin/IGF pathway. *Drosophila* has eight insulin-like peptides (DILP 1-8) but only two receptors (dInR and Lgr3) (Fernandez R et al., 1995; Brogiolo W et al., 2001; Garelli A et al., 2012; Nässel DR et al., 2016). Leucine-rich repeat-containing G protein-coupled receptor 3 (Lgr3) is a newly defined receptor for DILP8. It is a neuronal relaxin receptor

coupling growth to developmental timing. Lgr3 is expressed in the central nervous system (CNS) and essential for the DILP8-dependent development delayed (Garelli A et al., 2015). InR is a dimeric glycosylated receptor tyrosine kinase (RTK) that triggers signaling pathways targeting the activity of phosphatidylinositol-3-kinase (PI3K) and further downstream components to regulate a great variety of processes (Teleman AA, 2010). The insulin/IGF activates InR at tyrosine residues and initiates a cascade of intracellular events mediated by conserved components of the insulin/IGF pathways (Oldham S et al., 2003), recruiting various SH₂-containing signaling proteins, including p85 (the regulatory subunit of PI3-kinase), growth factor receptor bound protein 2 (Grb2), SH₂-containing phosphatase-2, (SHP), isoforms of SH₂-containing protein (Shc), and c-Cbl-associated protein (CAP) (Chang YJ et al., 2016). Via these distinct adaptor molecules, insulin/IGF-1 signaling triggers signaling cascades that are initiated by PI3-kinase, small GTPase Ras, and c-Cbl (Saltiel AR et al., 2001; Baumann CA et al., 2000; Giorgetti S et al., 1993; Backer JM et al., 1992; Myers MG Jr et al., 1994). In *Drosophila*, much progress has been made to understand the role of the insulin receptor signaling pathway and its interaction with other pathways, as well as its role in growth, protein synthesis and metabolism (Saucedo et al., 2003; Cheng et al., 2011).

For the DILPs, three of the DILPs, DILP2, DILP3 and DILP5 seem to be independently regulated at the level of transcription (Grönke S et al., 2010; Ikeya T et al., 2002; Rulifson EJ et al., 2002). All of them are highly expressed in insulin producing cells (IPCs) that play the same roles as pancreatic beta cells in mammals. DILP6 is IGF-like; structurally and functionally (Slaidina M et al., 2009; Okamoto N et al., 2009), while DILP7 and DILP8 have been proposed to be relaxin-like (Colombani J et al., 2012; Garelli A et al., 2012). Only DILP7 seems to have conserved orthologs in insects outside the *Drosophila* (Veenstra JA, 2014). Apparently, DILP production is regulated by neurohormones, neuropeptides, O-GlcNac and microRNA (Lee KS et al., 2008; Sekine O et al., 2010; Varghese J et al., 2010). A key trigger of DILP release from IPCs appears to be the intake, with many fat body derived nutritional signaling compounds mediating these effects. It is reported that DILP6 expressed in fat body

inhibits DILP2 release from the brain, which is beneficial for lifespan extension (Bai H et al., 2012). The adiponectin receptor was identified in *Drosophila* IPCs and shown to be relevant for DILP2 release, which defines the regulatory mechanism of IPCs by adipokine signaling in *Drosophila* (Kwak SJ et al., 2013). The expression of Myc, a protein that controls fundamental processes in the cell-autonomous regulation of growth, in the *Drosophila's* fat body diminishes the ability to retain DILP2 in the brain during starvation (Parisi F et al., 2013). GABA and its specific receptor are reported to inhibit IPCs and DILP release in response to metabolic stress (Enell LE et al., 2010). It was found that the fat body-secreted factor unpaired 2 (Upd2), which is a functional homolog of leptin in mammals, activates the IPCs through relieving the inhibitory tone of the GABAergic neurons (Rajan A et al., 2012). Short neuropeptide F (sNPF) has been proposed with respect to the role in regulation of growth, feeding and lifespan, as well as for insulin production (Lee KS et al., 2008; Hong SH et al., 2012). sNPF and its co-expressed neuropeptide corazonin affect carbohydrate and lipid levels via acting in different ways on the IPCs. sNPF expressed in neurons influences the transcript level of DILP2 and DILP5, while corazonin appears to be released into the hemolymph to act on the fat body (Kapan N et al., 2012). Additionally, the GTPase nucleostemin 3 (NS3), expressed in serotonergic neurons, regulates insulin signaling and thereby control of organismal growth via the serotonin 5-HT1A receptors on IPCs, which directly connects serotonergic neurons and IPCs (Kaplan DD et al., 2008; Luo J et al., 2012). A mammalian IGF binding proteins 7 (IGFBP7) tumor suppressor homologue, Imaginal morphogenesis protein-Late 2 (Imp-L2), which is secreted in *Drosophila*, is capable of binding DILP2, DILP3 and DILP5, revealing a feedback circuitry comprising the fly gut and/or fly fat body (Alic N et al., 2010). Ablation of IPCs or other genetic manipulations that diminished insulin signaling results in growth diapause, extended lifespan, increased resistance to oxidative and starvation stress, elevated carbohydrates and triacylglycerol storage, reduced fecundity and increased feeding under poor nutritional condition (Broughton SJ et al., 2005).

1.4 Limitations of Dietary restrict and the solutions

As it is mentioned above, dietary restriction without malnutrition extends lifespan of many species from yeast to human. One of the most important limitations of dietary restriction is the diminished fecundity (Partridge L et al., 2005), which is for instance seen in the nematode *C. elegans* (Klass MR, 1977), fruit fly (Chapman T, Partridge L, 1996) and rodents (Selesniemi K et al., 2008). This phenomenon is explained as an evolved response to food shortages (Harrison DE et al., 1989). Somatic maintenance and reproduction compete for nutrients, in the condition of abundant nutrition, reproduction is prioritized, while the somatic maintenance only allocated the nutrients necessary for survival during the reproductive period. So the actual lifespan is much shorter than the intrinsic potential lifespan, because of the extrinsic hazards in the wild (Williams GC, 1966). For the food shortage period, reproduction is dangerous for parents and offspring normally survive poorly, so the nutrients are hence reallocated to somatic maintenance, which increases the chances of the parents to survive and reproduce successfully when the food supply returns (Kirkwood TB, 1977). In this scenario, high survival, associated with dietary restriction, and high reproductive rates, associated with full feeding, are mutually exclusive. In addition, dietary restriction also might cause tissue loss when energy storage is insufficient, as well as slow growth and low body temperature. Even some people have been reported that their mood alters in a negative way when their energy intake goes too low. Natural health care products and natural dietary supplements are widely accepted as the means to slow aging and prevent metabolism diseases.

Algae are important natural source of health care products and dietary supplements for it contains many biologically active compounds that could be used as functional ingredients (Plaza M et al., 2008). They are claimed to contain low cholesterol levels, fight obesity, reduce blood pressure, tackle free radicals and promote healthy digestion (Plaza M et al., 2008); furthermore, some of them have been demonstrated to carry biological properties such as antibacterial and

anticoagulant activities (Alghazeer R et al., 2013, Chandía NP et al., 2008). *Saccorhiza polyschides* (Fig.1) is a large brown algae often found in the lowest part of the shore and it is the largest seaweed found in Europe. *Saccorhiza polyschides* is rich of polysaccharose, the main representative of which are alginate and fucoidans that have various biological activities and it is widely used by humans (Mak W et al., 2013; Mak W et al., 2014). Besides, ingredient analysis showed that it is also has a low-fat content (Rodrigues D et al., 2015). The presence of these polysaccharides upholds the interest in investigating *Saccorhiza polyschides* for applications in health-related fields. In this work, *Saccorhiza polyschides* is employed as an additive of the diet of a model organism *Drosophila melanogaster* to evaluate its potential to be utilized as an anti-age dietary supplement.



Figure 1. Photograph of *Saccorhiza polyschides*. (Source: en.wikipedia.org)

1.5 Advantages of *Drosophila* as a model organism

The fruit fly *Drosophila melanogaster* has been used as a model organism for over 100 years since Thomas Hunt Morgan introduced fruit flies in experimental studies of heredity in 1910. As one of the first model organisms used for genetic analysis, *Drosophila* has a great number of advantages compared with other invertebrate models. It has a very short life circle and a high fecundity, which could accelerate

the progress of lifespan assays. The husbandry of fruit flies requires little equipment and little space, so the overall cost is low. Meanwhile, it has only four pairs of chromosomes (three pairs of autosomes and one pair of sex chromosome), and the large polytene chromosomes allow genes to be mapped easily. Moreover, the utility of balancer chromosomes carried invisible genetic markers allows passing on of recessive in a heterozygous state, often lethal genes and induced mutations. Most importantly, its complete genome was sequenced in 2000 (Adams et al., 2000), and the approximately 14,000 genes contained in the fruit fly genome share a high degree of homology with the corresponding human genes (Chen D et al., 2008). Since the last century, enormous resources for public research have been accumulated. A large collection of *Drosophila* stocks including P-element insertions (Bellen HJ et al., 2004), genomic gene-defined deficiencies (Parks AL et al., 2004; Thibault ST et al., 2004), induced mutations and RNA interference mediated gene-specific knockdown lines (Dietzl G et al., 2007) are mostly available from the Bloomington Stock Centre or the Vienna *Drosophila* Resource Centre (VDRC). In addition, Flybase, a database of *Drosophila* genes and genomes, provides a comprehensive knowledge and information online.

In addition, another unique advantage for using *Drosophila* as model organism is the powerful binary and ternary expression systems are available for it, such as GAL4/UAS system, LexA/LexAop system or QF/QUAS system (Gnerer JP et al., 2015; Silbering AF et al., 2012; del Valle Rodríguez A et al., 2012; Diegelmann S et al., 2008; Potter CJ et al., 2010; Potter CJ et al., 2010). It allows researchers to ectopically overexpress or silence genes of interest in different spatial and temporal patterns. Among these systems, the GAL4/UAS expression system is the most widely used (Brand AH et al., 1993). GAL4 encodes a protein of 881 amino acids, identified in the yeast *Saccharomyces cerevisiae* as a regulator of genes induced by galactose (Laughon A et al., 1984; Oshima Y, 1982). Gal4 regulates the transcriptions by directly binding to four related 17bp site located in these loci (Giniger E et al., 1985), which defines upstream activating sequence (UAS) element and is essential for the transcriptional activation of these Gal4-regulated genes

(Duffy JB, 2002). GAL4 and UAS are separately inserted into two transgenic fly lines. The driver line is created by inserting the GAL4 gene at the downstream of an endogenous gene promoter, while the UAS is inserted at upstream of a gene of interest to create responder line. When the male driver line is crossed with the female responder line, the GAL4 gene is expressed under the control of the upstream endogenous gene promoter, then the UAS is activated by GAL4 gene and expresses, which leads the gene of interest is expressed in a specific cell or tissue defined by the GAL4 line of their progeny (Fig. 2). In order to the accesses spatial control, many extremely versatile updates of GAL4/UAS system have been achieved by using as temporal control, such as GeneSwitch and TARGET systems, which are activated by chemical agent and by permissive temperature respectively (Osterwalder T et al., 2001; Suster ML et al., 2004). Due to these advantages, *Drosophila* has been studied extensively for investigating developmental and biological processes such as growth, cell death, metabolic disorders, learning and memory, sleep and awake, circadian rhythms and flight. Modeling of fruit fly physiological development has made important contributions to the understanding of developmental processes in humans.

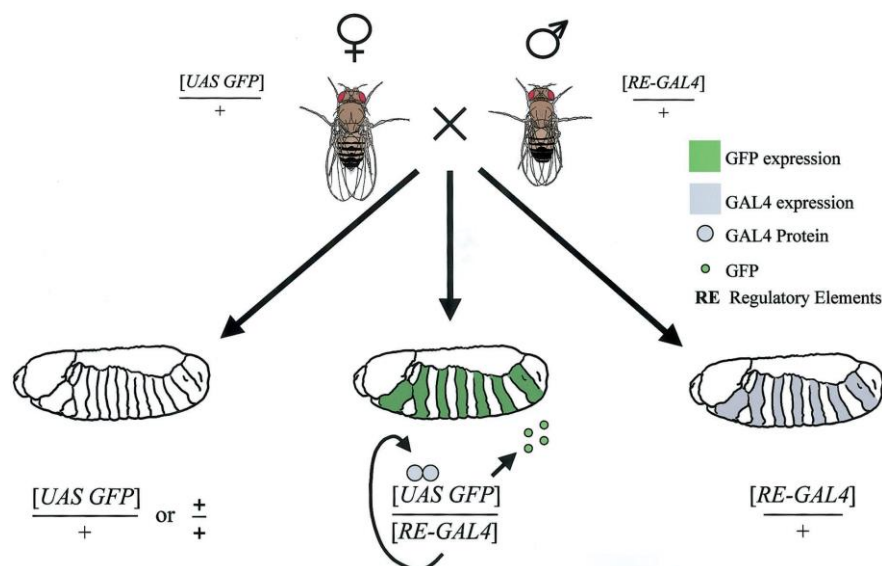


Figure 2. The schematic diagram of GAL4/UAS system. After GAL4 driver line crosses with UAS responder line, the target gene is expressed in a specific cell or tissue defined by the GAL4 line of their progeny. Adopted from (Duffy JB, 2002).

1.6 Aims of this study

The major aim of this work is to study the influence of dietary restriction and *Saccorhiza polyschides* extract intake on lifespan, metabolism, as well as on some behaviors in *Drosophila melanogaster*, in order to understand the potential of these treatments to be utilized as anti-ageing interventions.

The first part focused on the roles of dietary restriction in affecting lifespan extension and some other phenotypical peculiarities: fecundity, body weight, lipid content, hemolymph glucose concentration and metabolic rate by incubating the flies in a series of medium with different yeast extract concentration. Meanwhile, the locomotor behavior of the flies was also measured to characterize energy-demanding behaviors. After that, in order to elucidate the influence of dietary restrictions on insulin/IGF signaling, the DILP2 content and relative *imp-L2* expression level of flies treated by dietary restriction (DR) medium and concentration (CM) medium were measured by ELISA and relative quantitative real-time PCR (qRT-PCT) in the second part.

In the third part, the lifespan of flies treated by *Saccorhiza polyschides* extract was measured, as well as the phenotypical peculiarities mentioned in the first part, to check if the *Saccorhiza polyschides* extract has the same effects on flies as dietary restriction. Lifespan in drought environment, high fat diet and starvation condition was also calculated to examine the function of this algae extract in strengthening stress resistance.

In the last two parts, the molecular mechanism of these phenotypical peculiarities changes mediated by *Saccorhiza polyschides* extracts were studied with the help of transgenic lines and qRT-PCT respectively.

2 Materials and Methods

2.1 Materials

2.1.1 Drosophila strains and husbandry

Without any special statements, all flies are stocked and raised in a chamber which keeps 65% humidity under a 12 h light/dark cycle. Temperature is kept at 25°C in light stage while at 20°C in dark stage in the chamber. All new born flies are incubated in standard medium for 3 days at density of 25-30 adults in each vial before utilized for assays.

Table 1. Fly stock, genotype and donator

stock name	genotype	donator
w ¹¹¹⁸	w1118	Bloomington 5905
Sirt1 KO	w ¹¹¹⁸ ; Tl{TI}Sirt1 ^{2A-7-11}	Bloomington 8838
Tor-mutant	y[1] w[67c23]; P{w[+mC]=lacW}Tor[k17004]/CyO P{w[+mC]=lacW}Tor[k17004]/CyO	Bloomington 11218
P(Switch)106	w1118; P(Switch)106	Ronald Kühnlein
UAS-Sirt2	w*;UAS-Sirt2/Cyo	Kyung-Tai Min
UAS-Tor.WT	yw*;P{ry[+t7.2]=hsFLP}12; P{w[+mC]=UAS-Tor.WT}III	Bloomington 7012
UAS-Tor.TED	yw*;P{w[+mC]=UAS-Tor.TED}II	Bloomington 7013
UAS-FOXO in II	yw[*];P{w[+mC]=UAS-FoxO.P}2	Bloomington 9575
USA-GFP	w1118 ; P[UAS-Cameleon.2.1]82	Bloomington 6901
20xUAS-6xGFP	y1w*;wgSp-1/CyO,P{Wee-P.ph0} BaccWeeP20;P{20XUAS-6XGFP}attP2	Bloomington 52262
DILP2-HF	w1118;ilp2 ¹ gd2 HF(attp2)	Sangbin Park
Imp-L2-RA	yw ; + ; Imp-L2_RA-Gal4 / TM6B	gift

2.1.2 Fly medium

The ingredients and preparation process of mediums are showed in the table below.

Table 2. Ingredients and preparation process of mediums

	Standard medium	Algae extract medium	Protein control medium	High fat medium
Cornmeal (w/v)	6.25%	6.25%	7%	7%
Autolyzed yeast (w/v)	6.25%	6.25%	-	-
Bacto™ yeast extract (w/v)	-	-	FR**	5%
Glucose (w/v)	2%	2%	-	-
Sucrose (w/v)	-	-	5%	5%
Agar (w/v)	1%	1%	0.5%	0.5%
Molasses (w/v)	3%	3%	-	-
Syrup (w/v)	3%	3%	-	-

The mediums were dissolved in sterile water and autoclaved for 15 min. Following ingredients were added after the mediums cool down to 60°C.

Nipagin* (v/v)	3%	3%	3%	3%
Propionic* (v/v)	1%	1%	0.3%	0.3%
Algae extract (w/v)	-	FR**	-	-

*: Preservative

** : Following the requirement

2.1.2.1 Standard medium

The ingredient of the food comprised of 6.25% autolyzed yeast, 6.25% cornmeal, 1% agar powder, 2% glucose and 3% molasses and syrup. It was dissolved in sterile water and boiled for 15 minutes. After cooling, 3% nipagin and 1% propionic acid were added as preservatives.

2.1.2.2 *Saccorhiza polyschides* medium

The algae extract medium was prepared based on standard medium. Certain amount of algae extract (0.1% or 0.5%) was added in the medium after the preservatives was stirred uniform.

2.1.2.3 Protein control medium

The protein control medium was prepared with 7% cornmeal, 5% sucrose, 0.5% agar and certain amount of bacto yeast extract (following the requirement). Specifically, the protein control medium with 0.25% bacto yeast extract is defined as dietary restriction (DR) medium, while the one with 5% bacto yeast extract is defined as concentration (CM) medium.

2.1.2.4 High fat (HF) medium

The high fat fly food was based on a recipe of CM medium added 15% w/v of pure coconut fat. After loading the preservative, the diet was dispensed after cooling down to 45°C.

2.1.3 Solutions

Phosphate Buffered Saline (PBS): dissolve the following in 800 ml of distilled water, 9.07g NaCl, 0.21g KH₂PO₄, 0.49g Na₂HPO₄·2H₂O. Subsequently, the pH value was adjusted to 7.4 and additional distilled water was added up to 1 liter. The solution was autoclaved.

Phosphate Buffered Saline Tween-20 (PBST): 0.2% (v/v) or 2% (v/v) of Tween-20 was added in PBS solution for the preparation of 0.2% or 2% PBST.

Hemolymph-like saline (HL3): 4mM MgCl₂·6H₂O, 1.5mM CaCl₂·2H₂O, 70mM NaCl, 5mM KCl, 10mM NaHCO₃, 5mM trehalose, 5mM HEPES and 115mM sucrose were

dissolved in 800ml deionized water, adjusted pH to 7.2 and brought to the final volume of 1 liter. The buffer was sterile filtered and stored at 4°C.

Elisa coating buffer (0.2M sodium carbonate/bicarbonate buffer): dissolve 2.12g of Na₂CO₃ and 6.72g of NaHCO₃ in 500ml of distilled water. The pH value was adjusted to 9.4 and then the solution was filter-sterilized.

2.1.4 Oligonucleotides

Table 3. Oligonucleotides used for PCRs

Targets	Primers	Sequence (5'→3')	Product length*	Intron spanning	Intron size
Brummer	Sense	CACCAGCACAGCCACAATAC	93	Yes	97
	Anti-sense	GGCTACGTCGTGTAGTTCAGG			
Catalase	Sense	ATGTCACCAAGGTCTGGTCCG	193	Yes	97
	Anti-sense	TGGGTGTCCGAGTAGGAGAA			
dTor	Sense	GCCAGCAGTTCGTCCGAGTAT	164	Yes	191
	Anti-sense	TATGCGGACTCCAGTACGTTG			
EGFR	Sense	CTGCTACGTAAGCAATATCCG	175	Yes	67
	Anti-sense	CTTGCAGGTAAGGCACTGAT			
Glut1	Sense	GTCAACTGGATGGCCAACTT	196	Yes	78
	Anti-sense	CATACCTGCCATTGTTGTGC			
Ilp8	Sense	AGAAGGTCCATCGAGTTCGC	95	Yes	424
	Anti-sense	GGATAGCTGCTTCGGCTGAT			
Imp-L2	Sense	GCCGAGAGTATAACTGCAACTG	93	No	-
	Anti-sense	CTGGAGTTTCCGGTGGAAATG			
Puckered	Sense	GAAGCGCCGTGTCTTCTGTTA	119	Yes	2136
	Anti-sense	TCGTACATGCCAGATTCTCC			
RPD3	Sense	GCTGCTCAACTATGGGCTCT	111	Yes	78
	Anti-sense	GATCGCAAGAACCGGACGTA			
Rpl32	Sense	CCGCTTCAAGGGACAGTATC	196	No	-
	Anti-sense	GACAATCTCCTTGCCTTCT			
Sirt2	Sense	GTGGGAGCCGGCATATCTAC	98	Yes	56
	Anti-sense	GGATGTGGCAGCTCGTACTT			
Sirt-6	Sense	TGGATTGTCAGCCTACGACA	124	No	-
	Anti-sense	GACAACGTGTCCCGATTCT			
Slimfast	Sense	CCACAGGTGTTCTCGTTGGA	105	No	-
	Anti-sense	GTCACATCGGTCTCCACTG			
Oligo dT-7-1		GAGAGAGGATCCAAGTACTAATACGACTCACTATAGGGAGA(T) ₂₅			

*: Products without introns

2.1.5 Antibodies

Mouse monoclonal anti-Flag	Sigma-Aldrich, Steinheim, Germany
Mouse monoclonal anti-HA-Peroxidase	Sigma-Aldrich, Steinheim, Germany

2.1.6 Enzymes

DNase (RNase free 1U/ μ l)	Invitrogen, Darmstadt, Germany
RNase inhibitor (40U/ μ l)	Invitrogen, Darmstadt, Germany
Superscript III TM Reverse Transcriptase	Invitrogen, Darmstadt, Germany
Taq DNA Polymerase (5 U/ μ l)	Invitrogen, Darmstadt, Germany
2x qPCRBIOSyGreen Mix Hi-ROX	PCR Biosystems, London, UK

2.1.7 Chemicals

Agar-Agar	Carl Roth, Karlsruhe, Germany
Agarose	Biozym, Oldendorf, Germany
Bacto™ yeast extract	Becton, Dickinson and Company, Erembodegem, Belgium
Calcium chloride dehydrate	Carl Roth, Karlsruhe, Germany
Chloroform	Carl Roth, Karlsruhe, Germany
Coconut Fat	Peter Kölln KGaA, Elmshorn, Germany
di-Sodium hydrogen phosphate dihydrate	Carl Roth, Karlsruhe, Germany
dNTPs	Promega, Mannheim, Germany
Ethanol	Carl Roth, Karlsruhe, Germany
GeneRuler TM 100bp DNA Marker	Fermentas, St.Leon-Roth, Germany
GeneRuler TM 1kb DNA Marker	Fermentas, St.Leon-Roth, Germany
Glucose	Carl Roth, Karlsruhe, Germany
Glucose (HK) Assay Kit	Sigma-Aldrich, Steinheim, Germany
HEPES	Carl Roth, Karlsruhe, Germany
HPLC water	Carl Roth, Karlsruhe, Germany
Isopropanol	Carl Roth, Karlsruhe, Germany

Magnesium chloride hexahydrate	Carl Roth, Karlsruhe, Germany
Mifepristone	Sigma-Aldrich, Steinheim, Germany
Molasses	Kanne Brottrunk GmbH & Co. KG, Selm-Bork, Germany
Nipagin	Sigma-Aldrich, Steinheim, Germany
Potassium chloride	Merck, Darmstadt, Germany
Potassium dihydrogen phosphate	Carl Roth, Karlsruhe, Germany
Propionic acid	Carl Roth, Karlsruhe, Germany
RNAmagic	Bio-Budget, Kredeld, Germany
Sodium bicarbonate	AppliChem, Darmstadt, Germany
Sodium carbonate	AppliChem, Darmstadt, Germany
Sucrose	Carl Roth, Karlsruhe, Germany
Sulfuric acid (98%)	Carl Roth, Karlsruhe, Germany
SYBR [®] Green	Invitrogen, Darmstadt, Germany
Syrup	Kanne Brottrunk GmbH & Co. KG, Selm-Bork, Germany
Trehalose	Carl Roth, Karlsruhe, Germany
Triglyceride	Invitrogen, Darmstadt, Germany
Triolein	Sigma-Aldrich, Steinheim, Germany
TritonX-100	Sigma-Aldrich, Steinheim, Germany
Tween-20	Carl Roth, Karlsruhe, Germany
1-Step [™] Ultra TMB-ELISA Substrate Solution	Sigma-Aldrich, Steinheim, Germany

2.1.8 Equipment

Agarose Gel electrophoresis unit	Biometra GmbH, Göttingen, Germany
Centrifuge 5415D	Eppendorf, Hamburg, Germany
Centrifuge 5417R	Eppendorf, Hamburg, Germany
Drosophila Activity Monitor DAM2	Trikenetics, Waltham, USA
EV 245 Electrophoresis power supply	Consort, Belgium
Leica TCS SP1 confocal Microsystems	Leica, Heidelberg, Germany
NanoDrop (ND-1000UV)	Peqlab, Erlangen, Germany
Olympus SZX12 microscope	Olympus GmbH, Hamburg, Germany

Omni Bead Ruptor 24	Omni International Inc, Kennesaw, USA
PCR amplification	Bio-Rad Laboratories GmbH, München
Spectrophotometer	Multiscan MCC/340P version 2.32
StepOnePlus™ System	Applied Biosystems, Life Technologies
TB1 Thermoblock	Whatman Biometre GmbH, Göttingen, Germany
Thermo electron corporation	Thermo Electron LED GmbH, Langenselbold, Germany
Vortex-2 Genie	Scientific Industries, INC., Bohemia, N.Y., USA
Waterbath WNB 10	memert GmbH+Co.KG, Schwabach, Germany

2.2 Methods

2.2.1 Lifespan measurement

Without otherwise noted, adult female and male flies were cultured in standard medium together at the rate of 1:1.5 for 3 days after eclosion to make sure all of the flies were mated. Subsequently females and males were separated and cultured in medium of interest (according to the experimental program). No more than 30 flies were kept in one vial to avoid a too high density.

For each group (different genotypes or nutritional conditions), four or five replicate vials were combined to analyze the different survival between different groups. The flies were transferred to fresh medium every three days, and the number of dead flies was recorded every day.

2.2.1.1 Drought environment resistance

During this experiment, all flies were cultured at 25°C for 3 days, and then they were transferred to a chamber in which the humidity was between 20%-30%, while the other parameters were kept constant.

2.2.1.2 High fat diet resistance

This work includes two parts. In the first part, flies were divided into 2 groups and cultured in HF medium or HF medium contain 0.1% AE (HFAE medium), respectively, and then the lifespan was monitored. In the second part, flies were cultured in standard medium or 0.1% AE medium for 10 days first, and then all of these flies were transferred to HF medium and cultured in it until all the flies were dead.

2.2.1.3 Starvation resistant

In this assay, flies were separated into 3 groups and cultured in standard medium, 0.1% AE medium or 0.5% AE medium, respectively for 3 days and then transferred to starvation medium which contained 1% agar only. This assay was performed employing more than 100 flies per group. The number of dead flies was counted every 2h until all the flies were dead.

2.2.2 Gal4/UAS crossing

Adult male Gal4 lines and female UAS lines flies were separated within 8 h after eclosion and cultured with standard medium to make sure unmated male flies and virgin female flies were utilized for crossing. They were allowed to mate 3 days later, and the F1-generation was used for corresponding experiments.

2.2.3 Egg laying test

Mated females were collected and cultured in fresh vials. Twenty females in one vial were fed with the medium of interest (according to experimental program) and the number of laid eggs was counted every 24 h. At least five vials per group were

analyzed. Statistical analyses were performed using the unpaired two-tailed Student's t-test. All data are presented as mean values \pm S.D..

2.2.4 Body fat quantification

A Coupled Colorimetric Assay (CCA) method (Hildebrandt et al., 2011) was employed to determine total triacylglycerol (TAG) levels of flies. Glyceryl trioleate was utilized as TAG standard to establish a standard curve, which was diluted in a series of 2-fold dilution from starting quantity of 100 μ g to the final concentration of 3.125 μ g. Specifically, 5 female or 8 male flies were used as one biological replicate, which were transferred to a 2 ml screw-cap tube and weighted. Added 1 ml freshly diluted 0.05% Tween-20 into screw-cap tubes and homogenized in bead ruptor at 3.25m/s for 2 min with the help of 4 ceramic beads. Homogenates were heat-inactivated at 70°C for 5 min and followed centrifugation for 3 min at 3000 rpm. Transferred 50 μ l of sample supernatant to a 96 well microtiter plate. The various concentrations of TAG standard were treated in an identical way.

The absorbance of samples was measured at 562 nm using a microplate reader (T_0 values). After additional 200 μ l of pre-warmed (37°C) Thermo Infinity Trig. solution into each well and incubating the samples for 30 min at 37°C with mild shaking (120 rpm) the absorbance was read at 562 nm again (T_1 values). The calculated standard curve was established using the ΔT - S_0 -values ($\Delta T = T_1 - T_0$; $S_0 = T_1 - T_0$ from 0 μ l Triglycerides standard from standard curve). ΔT values of the samples were calculated by T_1 subtracted from T_0 . The final TAG quantity of samples was determined per mg of fly weight based on the standard curve. Each experimental group was measured in triplicate, while duplicates were used for the standards.

2.2.5 Hemolymph glucose measurement

To enable an accurate and reproducible glucose measurement, good quality of hemolymph isolates from fruit fly is most important. Only pure hemolymph extracts from peripheral blood could accurately reflect the state of circulatory carbohydrates. A novel technique was utilized in this project, which has advantages over traditional ways of isolation, such as avoiding the contamination of intestinal contents to the droplet samples, and minimizing any fat body tissue exuding to the hemolymph sample. Moreover, this method is easy for operation.

In brief, before hemolymph isolation, the flies were starved for 3h. Then a dissecting needle was used to gently puncture the thorax of each fly, and these flies were transferred to a 500 μ l centrifuge tube with a small hole created by a syringe needle at the bottom. Then, the lids of these tubes were covered and placed in a 1.5 ml centrifuge tube. This package was then centrifuged at 5000 \times g for 5 min and the hemolymph was gathered at the bottom of the outer tube.

Usually, not less than 0.5 μ l of hemolymph was isolated from 25 adult flies. The sample was diluted 1:10 in deionized water. A standard curve was established with a glucose solution with known glucose concentrations. The glucose standard solution was diluted in 2-fold serial dilutions starting from 1 mg/ml to 0.0625 mg/ml with deionized water, 3 μ l of each diluted solution was loaded in 50 μ l glucose assay reagent (which was supplied in the glucose assay kit). The same volume of water was added to the 50 μ l glucose assay reagent was set as a blank. Mixed tubes and incubated them for 15 min at RT (25°C). Subsequently, the absorbance was measured at 340 nm with a Nanodrop 1000.

2.2.6 CAFE assay

The Capillary Feeder (CAFE) assay was performed as previously described with minor modifications (William WJ et al., 2007). Briefly, a container (84 \times 30 mm) with

a cap was employed. A hole was drilled in the cap where a yellow pipette (100 μl) tip was inserted, and some distilled water was added at the bottom of the container to supply adequate internal humidity. A medium-sized tube (84 \times 16 mm) was installed into the container. The tube had several fitted holes to ensure humidity coming in instead of flies crawling out. For each container, a 5 μl glass capillary was applied with liquid food which consisted of 5% (w/v) sucrose and 5% (w/v) autolyzed yeast. About 0.5 μl mineral oil was sucked in by capillary action to minimize evaporation. The capillary filled with liquid was inserted through the tip that placed 5 mm below the cap, so the flies could approach the food easily (Fig. 3). For each assay, 2 seven-day-old flies were loaded in the internal tube and allowed to recover for one day prior to the measurement. The experiment was carried out under constant environmental conditions in the standard chamber described above. The descent of the top meniscus of liquid was monitored for 24 h, and then one photo was taken and the length of food consumption was measured by the stereo microscope and evaluated using the *cellA* program, two blank tubes without flies were set up as evaporation controls, the final consumption was calculated as the test consumed volume subtracts the evaporated volume.

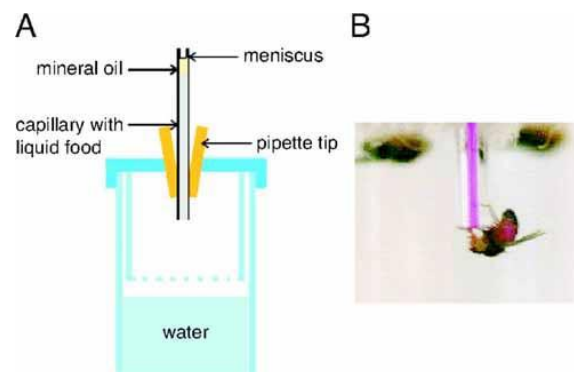


Figure 3. Scheme of the CAFE assay. (A) A 5 μl glass capillary with liquid food was held in place by a pipette tip. Mineral oil was introduced to minimize evaporation. Flies were housed in a pierced inner chamber. Water in an outer chamber provides humidity. (B) Fly feeding could be visualized with a red dye added to the liquid food. Source is from Ja et al., 2007.

2.2.7 Metabolic rate assay

The protocol of metabolic rate assay was described recently (Yatsenko et al., 2014) and used with some modifications. The schematic picture of the respirometer for CO₂ consumption measurement is shown (Fig. 4). To construct a respirometer, the tip of a 1,000µl pipette was cut with a razor blade to allow insertion of the 50µl capillary micropipette, trying to get the pipette tip as straight as possible. Then a piece of foam was placed into the pipette and pushed down into the pipette tip. Soda lime, the CO₂ absorbent, was added into the pipette tip and contained by a second piece of foam. Glue was applied at the place where the micropipette was inserted into the pipette tip to make sure this section was air tight. 3-5 adult flies of interest were placed in the pipette tip. Plasticine was used to seal the respirometer tightly at the top. Two respirometers without flies were used as the atmospheric controls. Then a chamber, with blue dye solution at the bottom was added to make the liquid visible, was prepared. The respirometers were placed straight into the chamber with the micropipette tips submerged into the colored solution. The chamber was covered with glass to prevent it from temperature and pressure fluctuations. Before assay started, the system must be allowed to equilibrate for 15 min, and a photograph of the chamber was taken. The assay was performed for 2h, then another photograph was taken. The amount of CO₂ production (µl/h/fly) was calculated with the following formula:

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

R: inner radius of capillary micropipette (mm).

Δd: height of the liquid in experimental samples far from the colored solution (mm).

Δc: height of the liquid in negative controls far from the colored solution (mm).

n: number of flies used.

h: hours of assay performed.

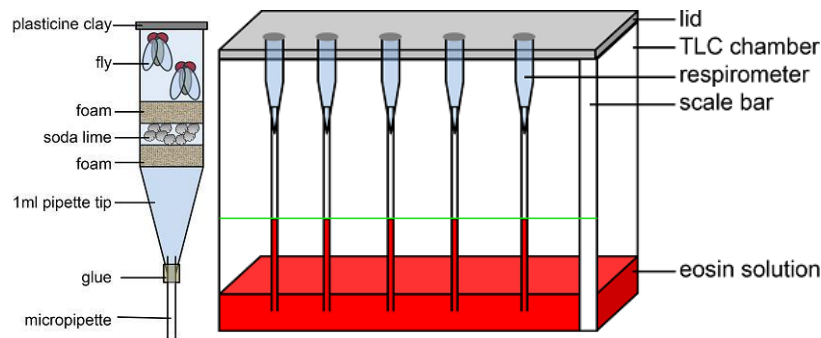


Figure 4. Scheme of setups for measurement of CO₂ production. The construction of the individual respirometer and the chamber show details. Eosin solution was alternatively replaced by blue dye (FD&C Blue No.1). Source is adopted from Yatsenko et al., 2014.

2.2.8 Locomotor activity

Locomotor activities were monitored using the *Drosophila* Activity Monitoring System (DAMS) (Fig. 5). To assemble the monitor, 10 adult flies for the big monitors and one individual fly each for the small monitors were put into the glass tubes which contained roughly one-sixth of the entire volume of fly food in one end. The activity monitors carrying the glass tubes containing fly and food were housed in a thermostatic incubator at 25°C and entrained for 7 d under a 12 h light/dark (LD) cycle condition. When flies crossed an infrared beam, the DAMS would record the sum of movement automatically every 2 min during the testing period. Locomotor activity was quantified as the sum of total movement records during a phase period. Individual periods for days 2–6 were taken and determined using histogram analysis.

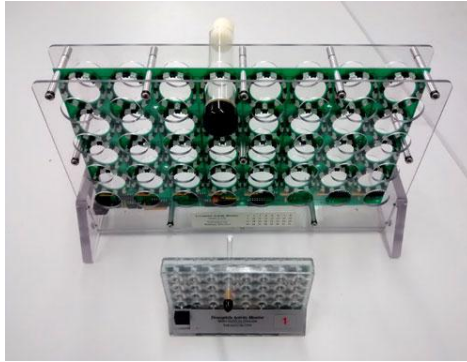


Figure 5. *Drosophila* activity monitors. The big monitor (upper) holds 32 tubes (2.5 cm × 12.5 cm) for groups of flies, the small one (lower) holds 32 tubes (0.5 cm × 8 cm) for individual fly.

2.2.9 Molecular biological methods for relative quantification of qRT-PCR

2.2.9.1 Total RNA extracting

Tissues of interest were dissected in HL-3 buffer on ice, and transferred to the centrifuge tubes containing HL-3 buffer to be protected. Then the tubes were centrifuged 10 min at 15,000×g and the supernatant was discarded. Addition of 1 ml RNA magic to the sample was followed by shock freezing in liquid nitrogen and thawed on ice. This freeze/thaw cycle was repeated several times. Following addition of 200 µl of chloroform, vortexing and incubation on ice was done for 10 min, the mixture was centrifuged for 5 min at 15,000×g, 4°C. The RNA contained in the upper aqueous phase was carefully transferred into a fresh tube without touching the interphase. This upper phase was mixed with an equal volume of isopropanol and incubated at -20°C overnight. The mixture was centrifuged for 60 min at 17,000×g at 4°C, and then the supernatant was discarded. The RNA pellet was washed with 500µl cold 70% ethanol, centrifuged at 17,000×g at 4°C 10 min, and repeated. Finally, all aqueous phase materials were discarded and air-dried, the RNA resolved in RNase-free water. RNA 1µl of each sample was run in a 1%

agarose gel for quality control, while the RNA concentration was measured by the NanoDrop photometer. RNA was stored at -80°C if it was not used immediately.

2.2.9.2 Genomic DNA (gDNA) digestion

This step was employed only if gDNA contamination was detected in samples during the process of RNA quality control. In particular, no more than $1\mu\text{g}$ of RNA was mixed with $1\mu\text{l}$ $10\times$ reaction buffer with MgCl_2 , 1U of DNase I (RNase-free) and $0.25\mu\text{l}$ of RNase inhibitor. HPLC water was added up to a total volume of $10\mu\text{l}$. The mixture was incubated 30 min at 37°C , and then it was incubated in 65°C for another 10 min with $1\mu\text{l}$ of 50mM EDTA to prevent the RNA hydrolyzing.

Repeating the step RNA quality control again was done to check if the gDNA was completely digested. Only the RNA without any gDNA contamination could be utilized in the next steps.

2.2.9.3 First-Strand cDNA synthesis

In this procedure, mRNA was converted into first-strand complementary DNA. The following components were used: 250 to 500ng of total RNA, $0.5\mu\text{l}$ Oligo-dT7 I (10pmol), $0.5\mu\text{l}$ dNTPs mix (10mM), add HPLC water up to $6.75\mu\text{l}$. The mixture was incubated at 65°C for 5 min, then placed on ice for at least 1 min. The following components were added in the mixture in the indicated order: $2\mu\text{l}$ $5\times$ FSB buffer, $0.25\mu\text{l}$ RNase Inhibitor (10U), $0.5\mu\text{l}$ DTT (0.1M) and $0.5\mu\text{l}$ Superscript III Reverse Transcriptase (100U). Gently mixed and incubated at room temperature for 5 min, and then synthesized at 50°C for 1 h. Finally, the reaction was terminated at 70°C for 15 min to inactivate the transcriptase. Then it was chilled on ice for immediate usage or stored at -20°C .

2.2.9.4 Primers and cDNA quality control

In this procedure, freshly prepared cDNA was amplified by matching primers with the method of polymerase chain reaction (PCR). In brief, the following components were added in the mixture in the indicated order: 19.75 μ l HPLC H₂O, 2.5 μ l AV Buffer (10 \times), 0.5 μ l dNTP-mix (10mM), 0.5 μ l sense primer (10 μ M), 0.5 μ l antisense primer (10 μ M), 0.25 μ l Taq polymerase and 1 μ l cDNA template. The program was processed according to table 4. Subsequently, the PCR products were checked by 1.5 % agarose gel electrophoresis.

Table 4. RT-PCR program.

Window	Denarutataion 95°C	Primer-Annealing 60°C	Elongation 72°C	Cycles
1	1 min	-	-	1
2	30s	30s	1 min	32
3	-	-	5 min	1
4	Cool down 4°C			

2.2.9.5 Relative quantification of qRT-PCR

For relative quantitative PCR, the reaction was performed using the StepOnePlus™ Real-Time PCR System for the primer efficiency check and relative expression level of gene of interest determination. For primer efficiency check, the template cDNA was treated as a series of 2-fold dilution from starting quantity of 50ng/ μ l to the final of 3.125ng/ μ l, while for the gene expression level determination template cDNA was diluted to 20ng/ μ l. Then 5 μ l 2x qPCRBIOSyGreen Mix Hi-ROX, 0.5 μ l sense primer (5 μ M), 0.5 μ l anti-sense primer (5 μ M), 1 μ l template cDNA and 3 μ l HPLC H₂O were mixed for one reaction. The amplification was run as below:

95°C	10 min	}	40 cycles
95°C	15 sec		
60°C	20 sec		
72°C	35 sec		
95°C	5 min	}	Melting Curve
60°C + 0.3°C until 95°C			
95°C	15 sec		

The primer efficiencies were calculated according to $E=10^{-1/\text{slope}}$ formula (Pfaffl, 2001). Thus, the relative expression ratio (fold change) of a target gene was determined based on E and C_T values in comparison to a reference gene. In triplicates, the fold changes for each group were calculated by the formula below and represented as mean \pm S.D. for statistical analysis.

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_{P_{\text{target}}(\text{control-sample})}}}{(E_{\text{ref}})^{\Delta C_{P_{\text{ref}}(\text{control-sample})}}}$$

In this case, *rpl32* was served as a reference gene for all of assays.

2.2.10 Quantification of circulation and total DILP2 content

The circulation and total DILP2 content of flies were measured by sandwich ELISA. DILP2-HF fly line, the DILP2 of which is tagged by both, FLAG (immunogen sequence DYKDDDDK) and HA (human influenza virus hemagglutinin, immunogen sequence YPYDVPDYA), was employed for this immunological work. In particular, 100 μ l of 2.5 μ g/ml anti-FLAG antibody in coating buffer (0.2M sodium carbonate/bicarbonate buffer, pH 9.4) was added in each well of a 96-well microtiter plate, which was then covered with a lid and incubated overnight at 4°C. Then washed the plate with PBS with 0.2% Tween 20 (PBST w0.2%) twice, and blot the plate on

paper towels followed by adding 350 μ l of PBS with 2% BSA (filter-sterilized) to block the wells. The plate was incubated overnight at 4°C.

For sample preparation, the posterior end of female and male flies' abdomen was dissected away. 10 dissected female or male bodies were transferred into a centrifuge tube containing 60 μ l of PBS, which was vortexed using a micro-tube holder at the max speed for >10 minutes, and then centrifuged at 1,000 \times g for 1 min. Transferring (avoid crashing heads) 50 μ l of supernatant into PCR strips, which was utilized for circulating DILP2-HF measurement. The remaining flies were transferred to 2 μ l screw-cap tubes, 500 μ l of PBS with 1% Triton-X100 was added to the tubes and homogenized in the bead ruptor at 3.25m/s for 2 min with the help of 4 ceramic beads. Vortexing the tubes using micro-tube holder at the max speed for >5 minutes was done, followed by centrifugation at max speed for 5 min. 50 μ l of supernatant was given into PCR strips, which was utilized for total DILP2-HF measurement. FLAG(GS)HA peptides (Peptide was synthesized in LifeTein LLC: DYKDDDDKGGGGSYPYDVPDYA, Amidated, 1-4mg, crude (57.8%) purity at \$87, MW=2412.42 Daltons) was prepared at a 2-fold gradually dilute concentration (from 10pg/50 μ l on, 7 points totally), including a blank point, to 50 μ l of PBS or PBS with 1% Triton-X100 in PCR strips for standard curve establishment.

The blocked microtiter plate was washed with PBST three times, and blotted the plate on paper towels. Transferring the prepared samples to microtiter plate, was done before sealing the plate with sealing tape, and incubating the plate in a humid chamber (with a wet paper towel) overnight at 4°C. Then, liquid was removed and plates were washed six times with PBST. During each wash, the buffer was flicked off, and the plate was blotted on paper towels before refilling. Addition of 100 μ l 1-Step Ultra TMB-ELISA Substrate per well was done, followed by incubation for 30 min at room temperature. The reaction was stopped by adding 100 μ l 2M sulfuric acid into each well. Finally, measuring the absorbance at 450nm using a microplate reader was done immediately. Data were analyzed in triplicates and shown as mean \pm S.D. for statistical analysis.

2.2.11 Statistical analysis

In all experiments, GraphPad Prism version 5 was mainly used for statistical analysis. The comparison between two groups, the unpaired two-tailed Student's *t*-test was used, if multiple comparisons were done, one-way ANOVA with Tukey Post-hoc test were performed, while two-way ANOVA was employed at the conditions with two different categorical independent variables on one continuous dependent variable. All histograms were represented as means \pm S.D.. The survival curves of lifespan were analyzed with the Log-rank (Mantel-Cox) test. Statistical analysis of significant difference were defined as n.s. not significant, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

3 Results

3.1 Dietary restriction regulates lifespan and phenotypical peculiarities of *Drosophila melanogaster*

3.1.1 Dietary restriction is essential for lifespan

For most organisms, the genotype dramatically influences lifespan. Reduced insulin/IGF receptor of *C. elegans* or target of rapamycin (Tor) signaling can extend lifespan in *Drosophila* significantly (Kenyon C et al., 1993; Lapierre LR et al., 2012; Kapahi P et al., 2004; Jia K et al., 2004). Additionally, numerous studies indicated that nutritional conditions are also essential factors for slow aging. It was shown that reducing high calorie food intake helps the organisms to extend lifespan (Magwere T et al., 2004; Bass TM et al., 2007). Most importantly amino acid intake accounts for the dietary restriction effect (Lee BC et al., 2013; Grandison RC et al., 2009; Emran S et al., 2014). Nevertheless energy as well as essential amino acids are indispensable for somatic maintenance of organisms and an over restricted diet leads to malnutrition and is harmful for them. Thus in order to characterize the concentration of dietary restricted, which is still beneficial for lifespan extension, wild type flies (w^{1118}) were treated with a series of protein control medium with different yeast extract concentration and lifespan monitoring was done.

Both females and males treated with strong restricted diet or with extremely rich diet had a decreased lifespan. In females, the flies fed with 1% yeast extract (YE) medium displayed the longest median survival, which was 85.3% (Fig. 6, Log-rank test; $P < 0.001$) and 75.0% (Fig. 6, Log-rank test; $P < 0.001$) higher compared with the flies fed with the lowest YE content medium and the highest one, respectively. The requirement of YE for males was less pronounced than for females, as those treated with 0.25% YE medium showed the longest median survival. Meanwhile, the gap between longest median survival group and the shortest one was not as

large as in females (about only 47.8% increased). Thus, the effectiveness and the diet composition that result in optimal lifespan extension differ between females and males.

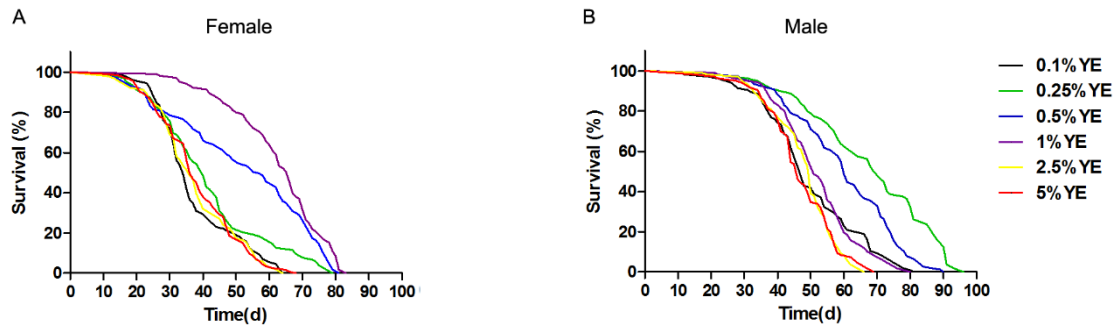


Figure 6. Protein concentration of diet influenced lifespan in females and males. Lifespan was assayed using wild type flies (w^{1118}). In females (A), median survival time were 34 days ($n=126$) for 0.1% YE treated group, 40 days ($n=105$) for 0.25% YE treated group, 58 days ($n=105$) for 0.5% YE treated group, 63 days ($n=105$) for 1% YE treated group, 36 days ($n=103$) for 2.5% YE treated group, and 36 days ($n=113$) for 5% YE treated group. In males (B), median survival time were 47 days ($n=105$) for 0.1% YE treated group, 68 days ($n=100$) for 0.25% YE treated group, 60 days ($n=106$) for 0.5% YE treated group, 51 days ($n=107$) for 1% YE treated group, 50 days ($n=105$) for 2.5% YE treated group, and 46 days ($n=106$) for 5% YE treated group. Log-rank test was used for analysis.

3.1.2 Dietary restriction affected fecundity

For most organisms, reproduction require a large amount of nutrients and fecundity should be significantly influenced by dietary restriction. Thus, the same experimental setup as described above (3.1.1) was used to quantify fecundity of mated females used an egg laying test. The number of eggs produced by the flies treated with medium containing no more than 0.25% YE had no obvious difference (unpaired two-tailed Student's t-test). But the egg production ability of the flies was sharply enhanced with the increase of YE in their diet. The number of eggs laid by the flies treated with 5% YE medium was about 8 times as those laid by flies

treated with 0.5% YE medium, which shows that fecundity is dramatically influenced by dietary restriction.

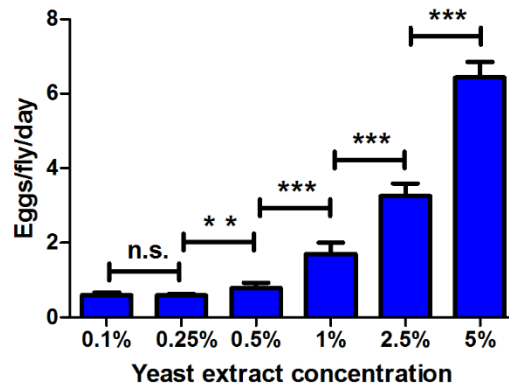


Figure 7. Dietary restriction influenced fecundity. Fecundity was assayed using wild type (w^{1118}) mated female flies. High YE concentration medium increases flies fecundity. Egg production were 0.60 ± 0.07 per fly/day ($n=126$) for 0.1% YE treated group, 0.59 ± 0.05 per fly/day ($n=105$) for 0.25% YE treated group, 0.79 ± 0.14 per fly/day ($n=105$) for 0.5% YE treated group, 1.70 ± 0.30 fly/day ($n=105$) for 1% YE treated group, 3.26 ± 0.33 per fly/day ($n=103$) for 2.5% YE treated group, and 6.43 ± 0.42 per fly/day ($n=113$) for 5% YE treated group. Data are presented as mean \pm S.D.; the unpaired two-tailed Student's t-test was used for analysis, n.s. not significant, ** $p < 0.01$, *** $p < 0.001$.

3.1.3 Dietary restriction regulated females' locomotor behavior and circadian rhythms but not that of males

The nutritional condition is an essential factor affecting locomotor behavior of most animals (Bruni JF et al., 1977; Mulinacci F et al., 2013; Brown-Borg HM et al., 2014). To investigate if the yeast extract content in diet affected flies' locomotor behavior or not, locomotor assay was operated for flies treated with CM and DR medium. Females of CM medium group displayed significantly lower physical activity than that of DR medium (Fig. 9). Specifically, the activity of flies in all groups

showed no obvious differences during the initial 48h. But for the CM medium group, flies' activity was reduced more dramatically than for those flies on DR medium after 48h, which showed significantly lower evening peaks. In contrast to the females, the males' activity tendency was almost identical. It was also noticed that, for DR medium groups, the activity of females was almost the same as males, while females' activity was significantly lower than the males for CM medium groups. Meanwhile, it could be concluded the flies of all groups had regular circadian rhythms (Fig. 10), a physiological processes under the control of an internal timing-clock system.

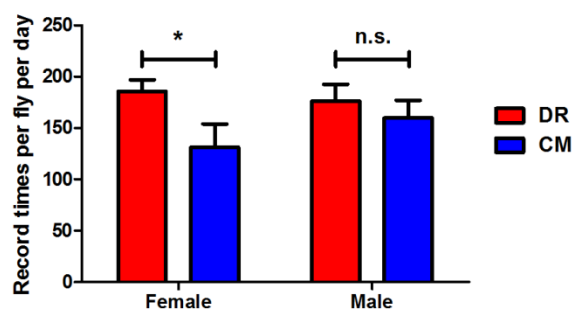
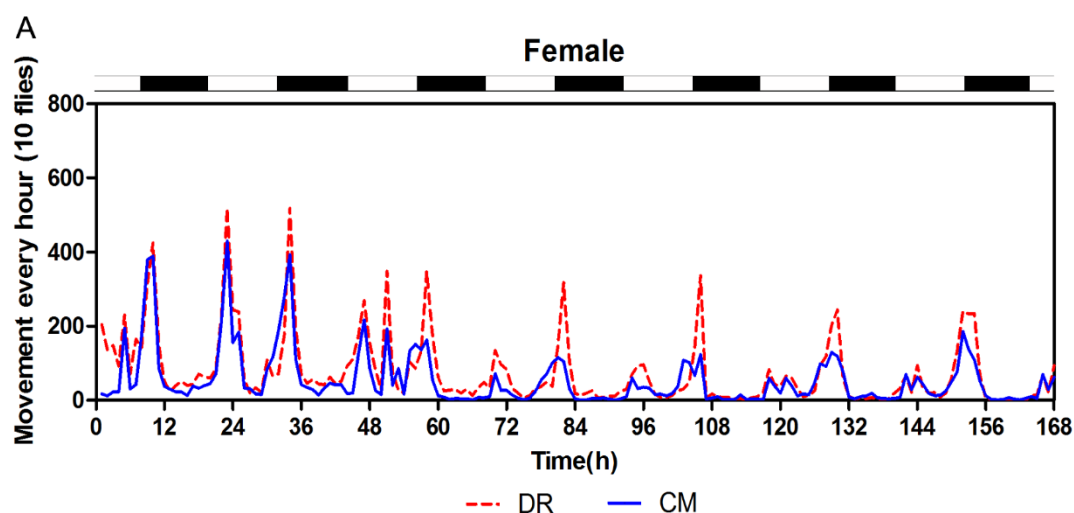


Figure 8. Locomotor activity of flies treated with DR and CM medium. This histogram show the average activity counts of flies fed with DR and CM medium for 7 days in LD condition. There was a significant raising of activity in DR group females but only slightly increase in males. Data are presented as mean \pm S.D.; the unpaired two-tailed Student's t-test was used for analysis, n.s. not significant, * $p < 0.05$.



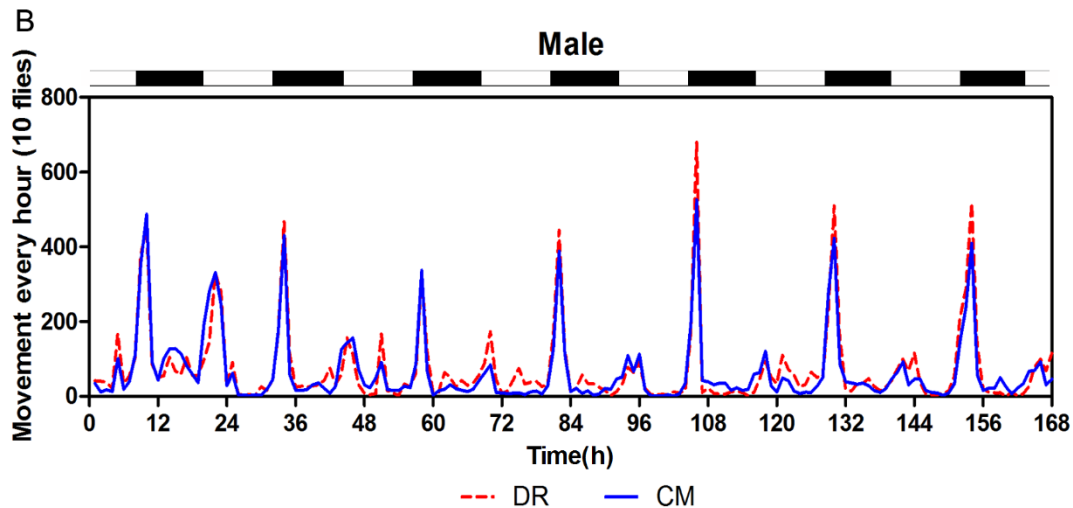


Figure 9. Circadian rhythm activity of flies treated with DR and CM medium. Females (A) fed with DR or CM medium exhibited high level of morning and evening peaks during the first 48h, then the circadian rhythm went regular during the rest of time. The females of DR medium group maintained relevant high level of evening peaks compared with CM medium after 48h. The variation of males (B) was not as obvious as females.

3.1.4 Dietary restriction affected body fat

In *Drosophila*, energy accumulation is strongly associated with intervention that regulates lifespan and stress resistance (Djawdan et al., 1998). The levels of triglycerides (also called triacylglycerol, TAG), the major form of lipid droplet storage in insect, of flies fed with 0.25% YE, 1% YE and 5% YE medium was measured every day for four days to check whether dietary restriction plays a role in modulating metabolism. Whole body TAG levels were measured in wild type (w^{1118}) adult flies. The TAG content of 5% YE group flies was significantly lower than the other two groups in both females and males (Two-ways ANOVA, $p < 0.01$). For females, the TAG content of 1% YE group flies was much higher than 0.25% YE group (Two-ways ANOVA, $p < 0.001$). For males, the TAG content of 0.25% YE group and 1% YE group flies had no obvious differences (Two-ways ANOVA, $p > 0.05$).

Moreover, the TAG content of females was more than males under the same nutritional condition.

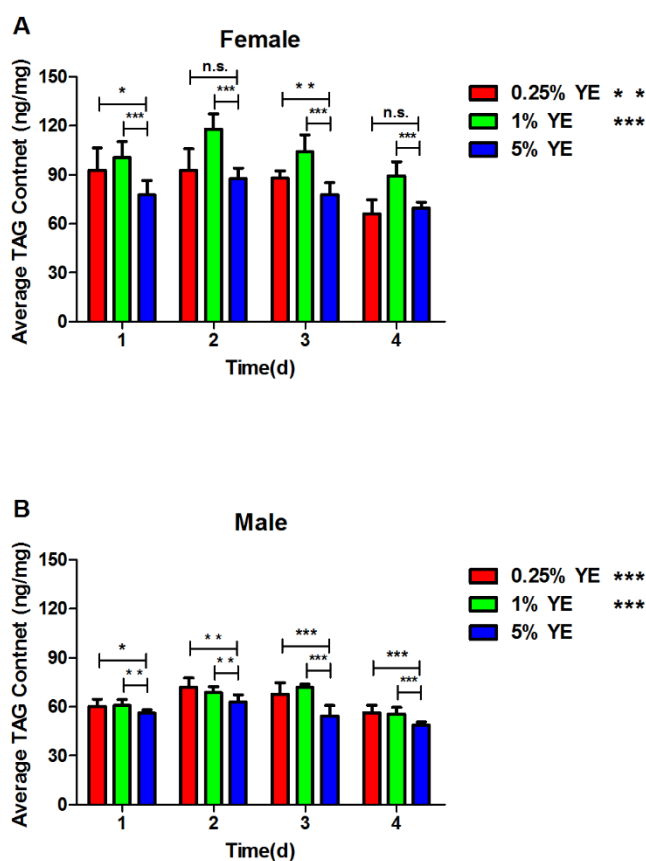


Figure 10. Over high protein diet inhibited TAG storage. Total TAG levels were measured in wild type flies (w^{1118}) in 0.25% YE, 1% YE and 5% YE medium feeding condition. 5% YE medium group flies had significantly low TAG levels, both in females and males. For females ($n=45$), 1% YE medium was most beneficial for TAG storage. For males ($n=72$), the TAG content of 0.25% YE and 1% YE group were probably the same. Data are presented as mean \pm S.D.; the unpaired two-tailed Student's t-test and two-ways ANOVA were used for analysis, n.s. not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Besides, the flies treated in these media for one, two, three and four days were all transferred to standard medium and incubated for 3 days, then the TAG content was measured. The result showed the influence of protein content in diet on females was stronger than that on males, for the 1% YE group flies still had highest

TAG content (Two-ways ANOVA, $p < 0.001$), followed by 0.25% YE group and 5% YE group. For males, only long time treatment groups (3 days and 4 days) can sustain the original trend, 0.25% YE group flies had relevant higher TAG content (Two-ways ANOVA, $p < 0.001$).

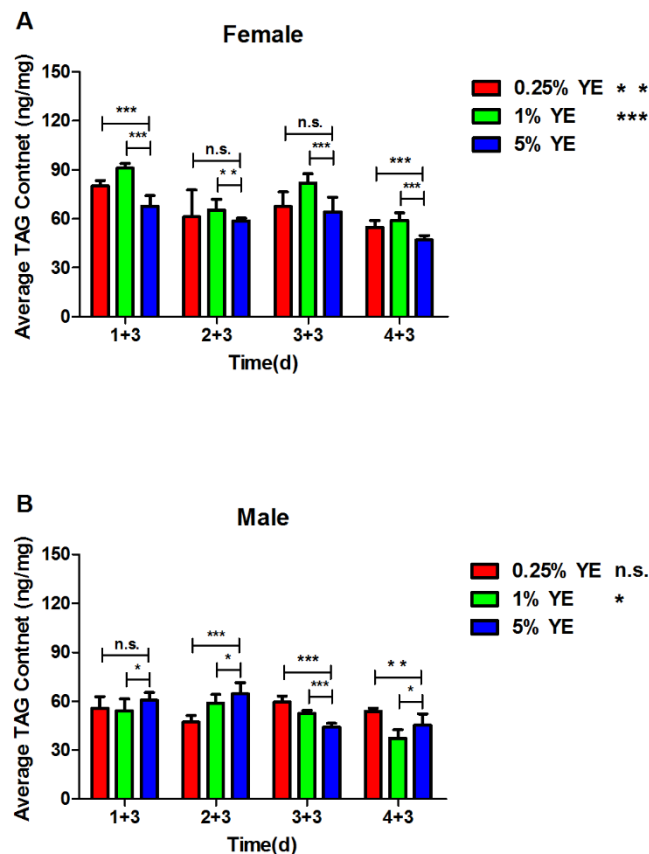


Figure 11. The influence of protein intake on females' TAG storage was stronger. Total TAG levels were measured after 3 days' treatment in standard medium of all group of flies. For females ($n=45$), 5% YE medium group flies still had the lowest TAG levels, while 1% YE group flies had the highest. For males ($n=72$), 5% YE group flies had higher TAG content in short time treatment groups (1 day and 2 days), while 0.25% YE group flies had higher TAG content in long time treatment groups (3 day and 4 days). Data are presented as mean \pm S.D.; the unpaired two-tailed Student's t-test and two-ways ANOVA were used for analysis, n.s. not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

What is more interesting was that the average body weight of flies treated with 5% YE medium was obviously more than the other two groups (Two-ways ANOVA, $p < 0.001$), this phenomenon was more obviously in females. No significant differences were detected between the average body weight of 0.25% and 1% YE medium flies (Two-ways ANOVA, $p > 0.05$).

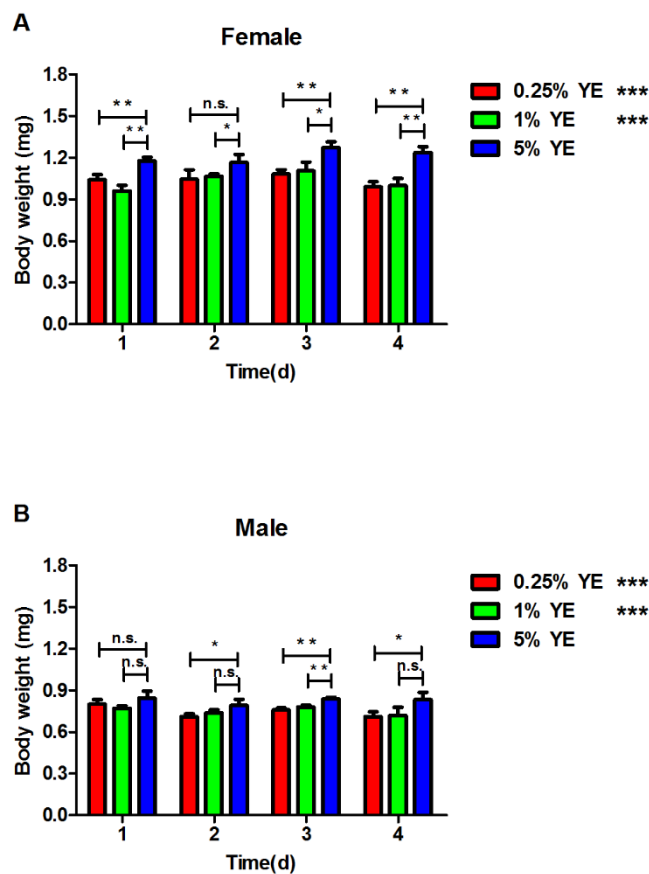


Figure 12. The amount of protein intake increased flies body weight. The body weight of flies treated in 0.25% YE, 1% YE and 5% YE medium for one, two, three and four days was measured respectively. For both, females ($n=45$) and males ($n=72$), the average body weight of 5% YE group flies was significantly higher than other two groups, the average body weight of which was almost the same. The influence of high protein diet on females was stronger than that on males. Data are presented as mean \pm S.D.; the unpaired two-tailed Student's t-test and two-ways ANOVA were used for analysis, n.s. not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Similar as described for TAG content measurement, body weight of all these three groups of flies was also measured after 3 days treatment in standard medium. High protein diet still showed stronger influence on females, as the flies of 5% YE group had highest body weight (Two-ways ANOVA, $p < 0.001$). For males, the body weight of 0.25% YE group and 5% YE group flies had no obvious differences (Two-ways ANOVA, $p > 0.05$). Moreover, the body weight disparity between the 5% YE group flies and other two groups became smaller after the 3 days treatment in standard medium in both females and males.

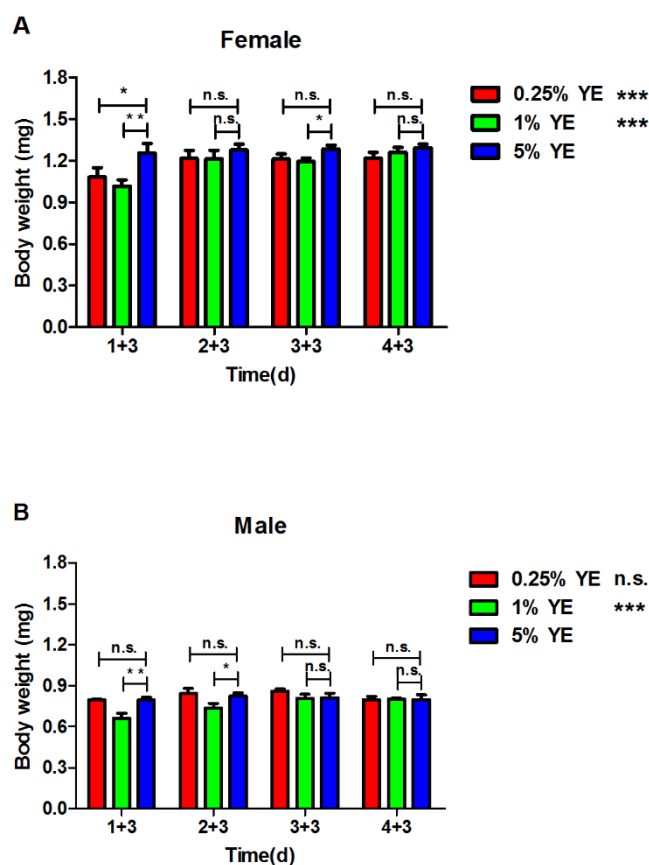


Figure 13. The influence of protein intake on females' body weight was stronger. Body weight of all groups of flies was measured after 3 days' treatment in standard medium. For females ($n=45$), 5% YE medium group flies still had the highest body weight, but the disparity became smaller. The average body weight of the other two groups was still almost the same. For males ($n=72$), average body weight of 0.25% YE group and 5% YE group flies had no obvious differences, while for 1% YE group, only short time treatment groups (1 day and 2 days) had lower average body weight. Data are presented as mean \pm

S.D.; the unpaired two-tailed Student's t-test and two-ways ANOVA were used for analysis, n.s. not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.1.5 Dietary restriction influenced hemolymph glucose level

Besides the TAG content, the hemolymph glucose content, similar to blood sugar of mammals, is another important parameter to evaluate metabolic homeostasis of flies. Glucose is one of the primary sources of energy in the body and, levels outside a normal range may cause metabolic diseases such as diabetes. Compared with CM group, DR group flies had higher hemolymph glucose content, both in females (Student's t-test, $P < 0.001$) and males (Student's t-test, $P < 0.001$). The influence of the nutritional condition on females was much stronger than on males. The low TAG storage and low hemolymph glucose level of CM group flies may be caused by the high metabolic rate.

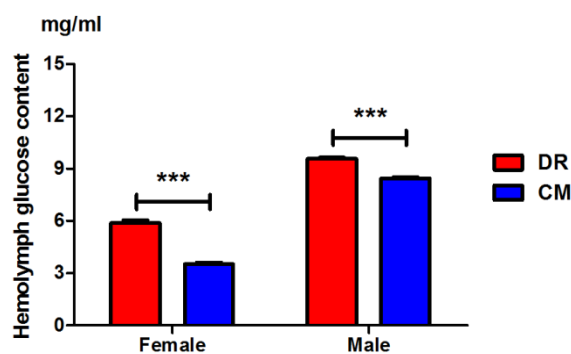


Figure 14. Hemolymph glucose level was altered by dietary restriction. Females were more sensitive to nutritional condition in this aspect. In females, hemolymph glucose content were $5.89 \pm 0.46 \text{ mg/ml}$ for DR group ($n=225$) and $3.52 \pm 0.29 \text{ mg/ml}$ for CM group ($n=225$). In male, hemolymph glucose content were $9.58 \pm 0.22 \text{ mg/ml}$ for DR group ($n=225$) and $8.44 \pm 0.21 \text{ mg/ml}$ for CM group ($n=225$). Data are presented as mean \pm S.D.; unpaired two-tailed Student's t-test was used for analysis, *** $p < 0.001$.

3.1.6 Metabolic rate was increased in the flies treated with high protein medium

Three groups, flies fed with protein control medium contain 0.25% YE, 1% YE and 5% YE, were established for this assay. All of the flies were incubated for four days in the corresponding medium, and the metabolic rate was measured every day at the same time of the day. For females, the 5% YE group flies showed significantly higher metabolic rates than the other two groups, with the metabolic rate of the 1% YE group flies was slightly higher than seen in the 0.25% YE group. Similar to females, metabolic rate of the 5% YE group males also showed the highest values among the three groups. But the metabolic rate diversity between 1% YE group and 0.25% YE group was larger as the treatment lasted longer. This is consistent with the result of lifespan assay, which showed that females fed with 1% YE medium had the longest median lifespan.

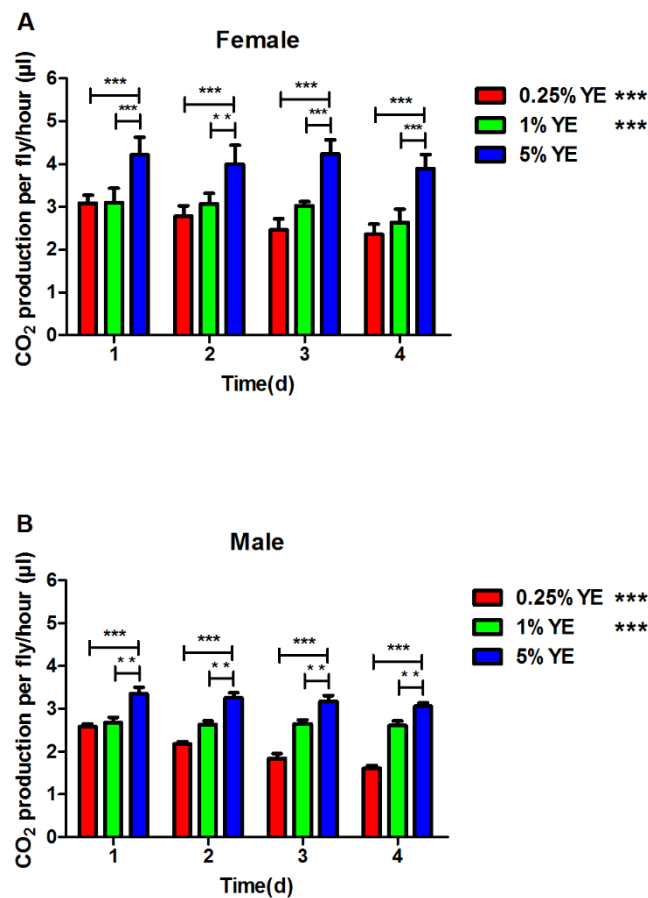
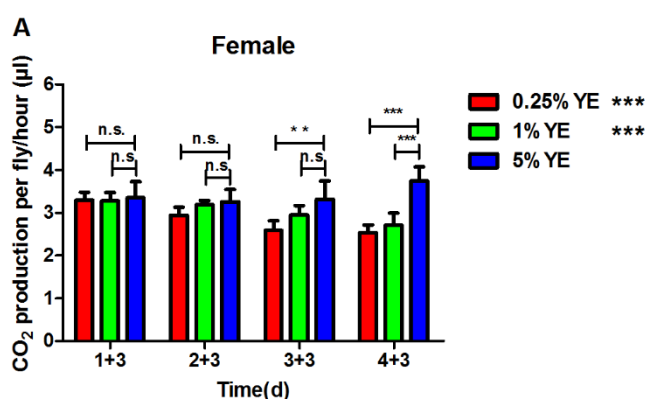


Figure 15. High protein diet increased CO₂ production of flies. CO₂ production of flies treated with 0.25% YE, 1% YE and 5% YE medium was measured. For females (A), 0.25% YE group flies (n=24) and 1% YE group flies (n=24) produced same amount of CO₂ after 1 day's treatment, then the CO₂ production of 0.25% YE group flies was obviously lower than that of 1% YE group flies after longer time treatment (2 days, 3 days and 4 days). The CO₂ production of 5% YE group (n=24) was significantly higher than the other two groups from the first day to the fourth day. For males (B), 0.25% YE group (n=24) flies also produced least CO₂. Similar to females, the 5% YE (n=24) group flies had the highest CO₂ production, but this gap between 5% YE (n=24) group and 1% YE (n=24) group was not as large as females. Data are presented as mean \pm S.D.; the unpaired two-tailed Student's t-test and two-ways ANOVA were used for analysis was used for analysis, **p<0.01, ***p<0.001.

To further investigate the effect of treatment time to metabolic rate and the persistence of this effect, the flies of all groups were transferred to standard medium after the first metabolic rate assay and incubated for another 3 days, then utilized for the second metabolic rate assay. Both females and males of 5% YE medium group still showed highest metabolic rate, this phenomenal appeared to be more obviously with the increase of treatment time (Fig. 16). For the other two groups, similar result as the first metabolic rate assay was shown, 1% YE medium was over high protein content medium for males, as the males treated with 1% YE medium showed obviously higher metabolic rate than 0.25% YE group, but the effect of this diet was not so strong for females.



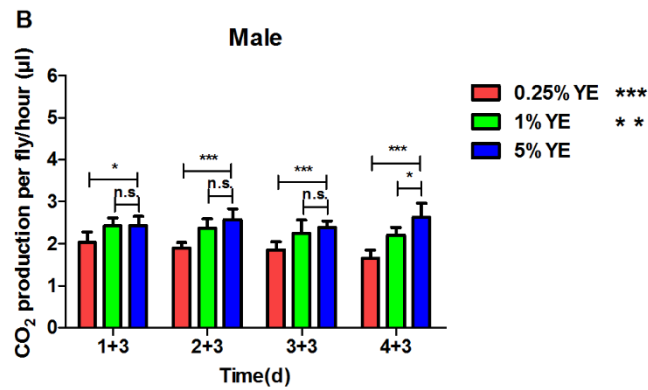


Figure 16. Longer time treatment of high protein diet affected more on CO₂ production of flies. CO₂ production of flies of all groups treated 3 more days in standard medium was measured. More treatment time in 5% YE medium drove flies producing more CO₂ in both females (A, n=24) and males (B, n=24), and for 1% YE group and 0.25% YE group, the CO₂ production reduced as the treatment lasted long, as it was seen that flies produced almost the same amount of CO₂ after no more than 3 days' treatment in 1% YE or 5% YE medium. Similar to the result of first metabolic rate assay, 1% YE group flies produced obviously more CO₂ than 0.25% YE group in males (B, n=24), while the CO₂ production of these two groups were almost the same in females (A, n=24). Data are presented as mean ± S.D.; the unpaired two-tailed Student's t-test and two-ways ANOVA were used for analysis, n.s. not significant, n.s. not significant, *p<0.05, **p<0.01, ***p<0.001.

3.2 Analyses of dietary restriction's influence on regulation of DILP2 secretion

Insulin/insulin like growth factor (IGF) plays an important role as a systemic regulator of metabolism in multicellular organisms (Sato-Miyata Y et al., 2015). It is able to promote glucose uptake in muscles and adipose tissues, induce cell growth and proliferation, and stimulate glyconeogenesis, lipogenesis and protein synthesis (Saltiel AR et al., 2001). It is evolutionally conserved throughout the animal kingdom (Skorokhod A et al., 1999) and *Drosophila* possesses eight insulin-like peptides (DILPs) (Brogiolo W et al., 2001). Among these peptides DILP2 is the most highly expressed and has the highest potential for promoting tissue growth (Broughton SJ et al., 2005; Ikeya T et al., 2002; Rulifson EJ et al., 2002). It has been demonstrated that DILP2 regulates glucose homeostasis, for the reduction of DILP2 in hemolymph increase the content of glucose and trehalose in it, as well as increase fly's lifespan (Broughton S et al., 2008). In contrast, high circulating insulin levels are often associated with hypoglycemia, insulin resistance, type II diabetes and some other impaired physiological conditions. As DILP2 is highly related to fly's metabolism and lifespan, the DILP2 content in flies treated with DR and CM medium was measured, in order to explore the mechanism of nutritional condition mediate phenotypical peculiarities in hormone regulation level.

3.2.1 Circulating as well as total DILP2 level were affected by protein intake

As it was described in method 2.2.10, the flies, whose posterior end was removed, were immersed in PBS buffer and vortex to isolate their total hemolymph, which contained flies' circulating DILP2. Then the rest of flies was homogenized utilizing the beads ruptor and vortexed in PBS buffer with 1% Triton-X100, an agent for cell lysis, to break the cells and allowed the protein including total DILP2 contained in cells to be releasing into the buffer. The circulating and total DILP2 content of flies

treated with DR and CM medium for four days were all measured to examine the influence of nutritional condition to DILP2 production and secretion. The result showed that CM medium group flies secreted more circulating DILP2 into the hemolymph than the DR medium group, both in females (Student's t-test, $P < 0.001$) and in males (Student's t-test, $P < 0.001$). In contrast to circulating DILP2, the total DILP2 content of DR medium group flies was more than that of CM medium group, which was more significant in females (Student's t-test, $P < 0.001$) than males (Student's t-test, $P < 0.01$).

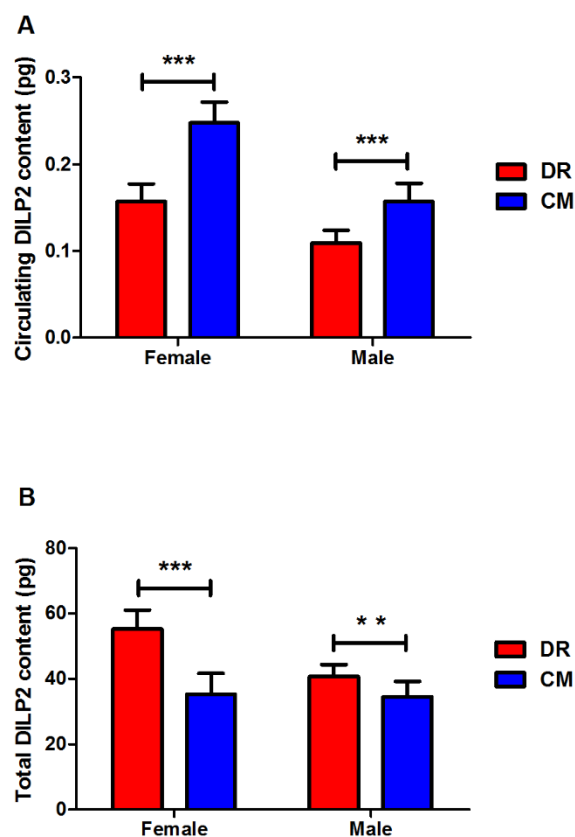


Figure 17. High level of protein intake increased circulating DILP2 content and decreased total DILP2 content. For females, the circulating DILP2 content of CM medium group (0.25 ± 0.02 pg, $n=60$) was 57.8% more than DR medium group (0.16 ± 0.02 pg, $n=60$). In contrast, DR medium group's total DILP2 content (55.27 ± 5.92 pg, $n=90$) was 44.0% more than CM medium group (35.29 ± 6.44 pg, $n=90$). This situation was the same for males, CM medium group's circulating DILP2 content (0.16 ± 0.02 pg, $n=80$) was 44.0% more than DR medium group (0.11 ± 0.01 pg, $n=80$), while DR medium group's total DILP2 content (40.77 ± 3.61 pg, $n=80$) was 44.0% more than CM medium group (34.56 ± 4.68 pg, $n=70$). Data

are presented as mean \pm S.D.; unpaired two-tailed Student's t-test was used for analysis, ** $p < 0.01$, *** $p < 0.001$.

3.2.2 Dietary restriction influenced *imp-L2* expression level

Imp-L2 (Imaginal morphogenesis protein-Late 2) is a homologous protein of mammal's IGFBP7 (insulin growth fact binding protein 7) present in *Drosophila*. It resembles IGFBP7 in sequence and appears to be equivalent to an IGFBP in function, acting as a negative regulator of IIS (insulin/insulin like growth fact signaling) during development, regulating growth cell-non-autonomously and antagonizing DILP2 in genetic assays (Honegger B et al., 2008). It is also found when the germline is ablated late in fly development, the level of Imp-L2 mRNA increases, which contributes to the lifespan extension (Flatt T et al., 2008). It has been indicated that one of the roles of Imp-L2 is to mediate a lifespan expending signal emanating from the gonad to IIS in the soma (Alic N et al., 2011). To further investigate the mechanism of the circulating DILP2 level raised by high protein diet, relative real-time quantification PCR (qRT-PCR) was adapted to examine expression levels of *imp-L2* of flies treated with DR medium and CM medium. Fat bodies, the largest metabolism tissue of *Drosophila*, of flies treated in DR medium and CM medium for 4 days were dissected. Following the protocol described in the methods section, the total RNA of fat body were extracted and reverse transcribed to cDNA, which was finally used for qRT-PCR. The results showed the *imp-L2* expression level of CM group was lower than DR group, both in females (31.0%) and males (17.4%).

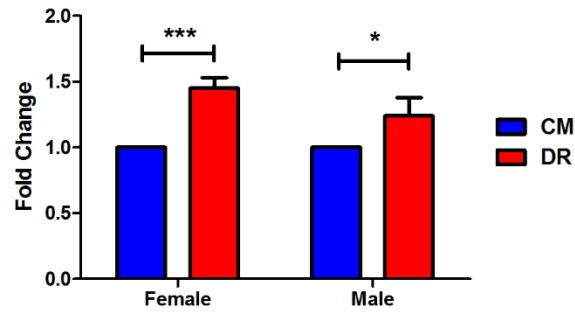


Figure 18. The expression level of *imp-L2* was reduced by the high protein diet. The expression level of *imp-L2* of CM medium group females (n=45) was 31.0% less than that of DR medium group (n=45), while for the males, CM medium group (n=45) was 17.4% less than DR medium group (n=45). All values represent as mean \pm S.D., unpaired two-tailed Student's t-test was used for analysis, * $p < 0.05$, *** $p < 0.001$.

3.3 *Saccorhiza polyschides* is a safety anti-age food supplement

In addition to dietary restriction, other methods that increase lifespan are highly attractive. Thus I focus in this part on the extract of *Saccorhiza polyschides* in order to utilize it as a food supplement added to the fly's medium.

3.3.1 *Saccorhiza polyschides* extract increased lifespan of *Drosophila*

The lifespan of flies (w^{1118}) fed with standard medium and standard medium with 0.1% (w/v) *Saccorhiza polyschides* extract (0.1% AE medium) was quantified. 0.1% AE medium significantly extended flies lifespan, both in females (7.7%, log-rank test, $P < 0.001$) and males (10.5%, log-rank test, $P < 0.001$). To investigate if higher algae extract intake could enhance its effect, lifespan of flies incubated in standard medium with 0.5% algae extract (0.5% AE medium) was also measured. But the extra algae extract failed to promote its lifespan extension effect, which was similar to the effect observed in the 0.1% AE medium group, both in females (3.1%, log-rank test, $P < 0.05$) and males (12.3%, log-rank test, $P < 0.001$). The result indicated that, 0.1% algae extract is sufficient to mediate lifespan extension.

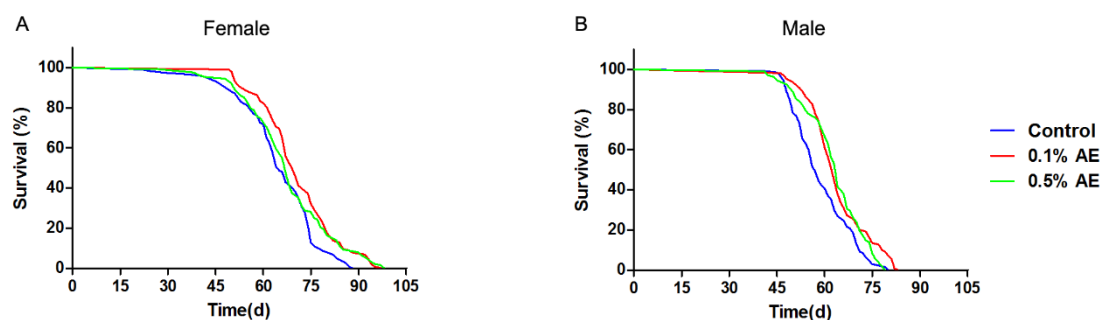


Figure 19. *Saccorhiza polyschides* increased fly's median lifespan obviously. Lifespan was assayed by wild type (w^{1118}) flies. In females (A), median survival time were 65 days (n=117)

for control group, 70 days (n=102) for 0.1% AE treatment group and 67 days (n=127) for 0.5% AE treatment group. In males (B), median survival time were 57 days (n=130) for control group, 63 days (n=110) for 0.1% AE treatment group and 64 days (n=108) for 0.5% AE treatment group. Log-rank test was used for analysis.

3.3.2 Lifespan was significantly increased by *Saccorhiza polyschides* extract diet for flies confronted with stressful conditions

Considering observation that longer lifespan is often coupled with higher stress resistance, three stress conditions, a drought environment, a high fat diet and starvation, were applied to flies (w^{1118}), to evaluate the effects of *Saccorhiza polyschides* extracts on lifespan extension under stress condition.

A chamber sustaining a humidity of approximately 20% was utilized to simulate drought environment, in which the lifespan assay was performed. The result displayed the lifespan extension mediated by *Saccorhiza polyschides* extracts in drought environment was substantially higher than that seen under normal condition (65% humidity). For females, the median lifespan of 0.1% AE medium group was 17.3% longer (log-rank test, $p < 0.001$) than in the controls, the 0.5% AE enhanced lifespan by 13.5% (log-rank test, $p < 0.01$). In males, the increase of median lifespans in the 0.1% AE medium group and in the 0.5% AE medium group were 3.4% (log-rank test, $p < 0.001$) and 8.5% (log-rank test, $p < 0.001$), respectively. It should be noticed that, compared to the groups incubated in normal condition, the median lifespan of all groups subjected to drought environment were shorter for females, while no significant differences were observed for males. Moreover, it has been confirmed that drought environment could eliminated calorie restriction diet mediate lifespan extension (William WJ et al., 2009). This result also gave some evidence that *Saccorhiza polyschides* extracts mediate lifespan extension in a different way than dietary restriction does.

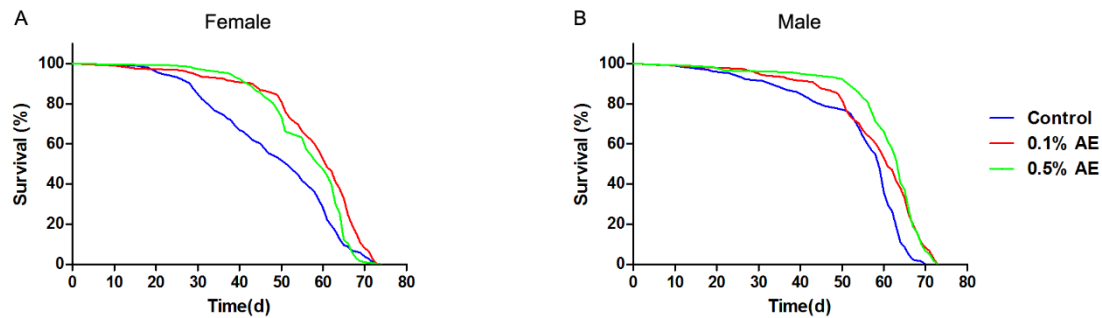


Figure 20. *Saccorhiza polyschides* enhanced fly's resistance to drought environment. Median lifespan is increased by *Saccorhiza polyschides* extract diet in drought environment. For females, median lifespan were 52 days (n=103) for control group, 61 days (n=112) for 0.1% AE treatment group, 59 days (n=123) for 0.5% AE treatment group. In males (B), median survival time were 59 days (n=128) for control group, 61 days (n=121) for 0.1% AE treatment group, 64 days (n=119) for 0.5% AE treatment group. Log-rank test was used for analysis.

High fat (HF) diet is also a stress condition for most animals, for it has been indicated that excessive dietary accumulation of lipids is a high risk factor of causing deleterious effect on metabolism and heart function and has been strongly linked to the progression of heart disease and type II diabetes (Szendroedi J, 2009; Van Herpen NA, 2008). It was also found that flies treated with HF diet exhibit increased triglyceride fat and alterations in insulin/glucose homeostasis. In this part, wild type (w^{1118}) flies were utilized and raised in HF medium (15% coco nut fat) or HF medium mix with 0.1% *Saccorhiza polyschides* extract (HFAE). The result of the lifespan assay showed that the median lifespan of flies treated with HFAE medium was significantly longer than those treated with HF medium only, both in females (19.2%) and males (20.0%) (Log-rank test, $P < 0.001$). I used an alternative design, where flies were incubated in 0.1% AE medium or standard medium respectively for 10 days first, then all flies were transferred to HF medium without AE and incubated until death. The median lifespan of 0.1% AE medium treatment flies was still longer than the control group, both in female (37.0%) and males (39.3%) (Log-

rank test, $P < 0.001$), which showed that *Saccorhiza polyschides* improved the fly's resistance to high fat diet.

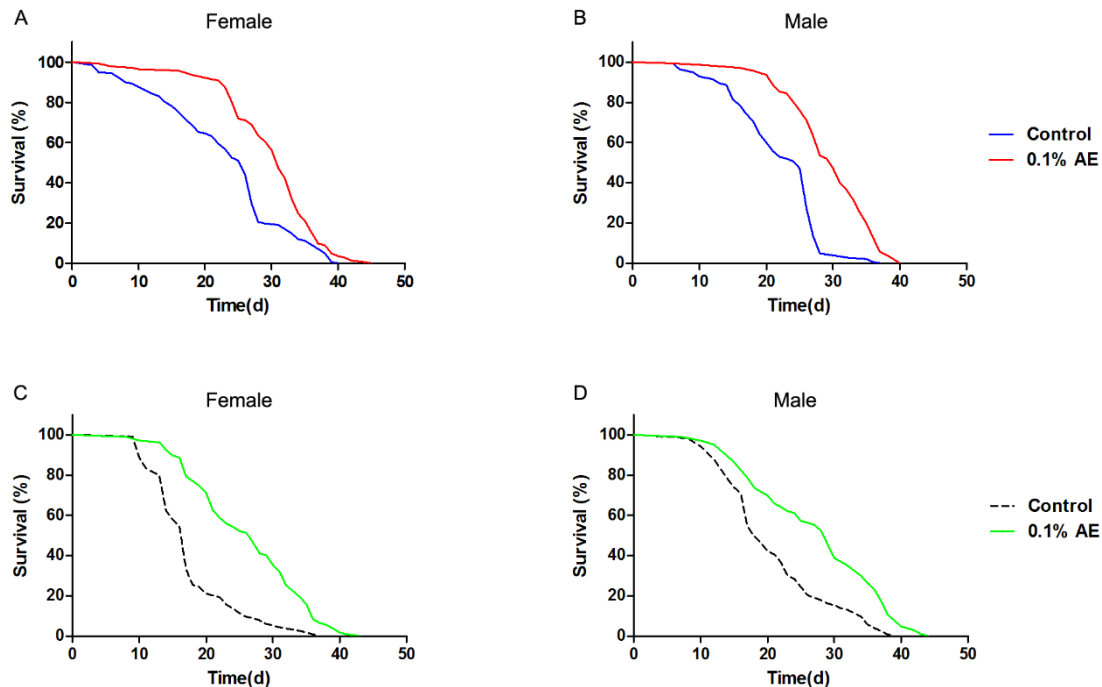


Figure 21. *Saccorhiza polyschides* increased fly's resistance to high fat diet. Two experiments were designed in this part: flies were treatment with HFAE medium and HF medium (served as control), respectively (A and B), as well as flies were treatment with 0.1% AE medium and standard medium (served as control) for ten days first, and then were cultured in HF medium (C and D). HFAE group flies had longer median lifespan than HF group (A and B). For females (A), median lifespan were 26 days ($n=141$) for control group and 31 days ($n=142$) for 0.1% AE treatment group. For males (B), median survival time were 25 days ($n=140$) for control group and 30 days ($n=142$) for 0.1% AE treatment group. Algae extract extended median lifespan of flies treated with HF diet (C and D). For females (C), median lifespan were 27 days ($n=114$) for control group and 37 days ($n=107$) for 0.1% AE treatment group. For males (D), median survival time were 28 days ($n=114$) for control group and 39 days ($n=103$) for 0.1% AE treatment group. Log-rank test was used for analysis.

At last, the effect of *Saccorhiza polyschides* extract for fly's resistance to starvation was also examined. Wild type (w^{1118}) flies were divided into 3 groups and incubated in standard medium, 0.1% AE medium and 0.5% AE medium respectively. After 7

days incubation, all the flies were transferred to starvation medium, in which only 1% agar (w/v) was available, survival of each group was measured every 2h. In females, this algae extract obviously enhanced fly's resistance to starvation. Both the mean lifespan and the maximum lifespan were significantly prolonged by *Saccorhiza polyschides* extract. Moreover, the mean and maximum lifespan of 0.1% AE group (Log-rank test, $P < 0.001$) and 0.5% AE group (Log-rank test, $P < 0.001$) were almost identical. Similar to females, this algae extract was also able to promote males' resistance to starvation, but the effect was smaller. The mean lifespan of males treated with this algae extract was only slightly prolonged, so was the maximum lifespan. The effect of 0.1% AE medium (Log-rank test, $P < 0.01$) was seemed to be better than 0.5% AE medium (Log-rank test, $P < 0.05$). Overall, *Saccorhiza polyschides* extract treatment flies showed an increase lifespan under starvation condition, which demonstrated that this algae extract could promote flies tolerance to starvation.

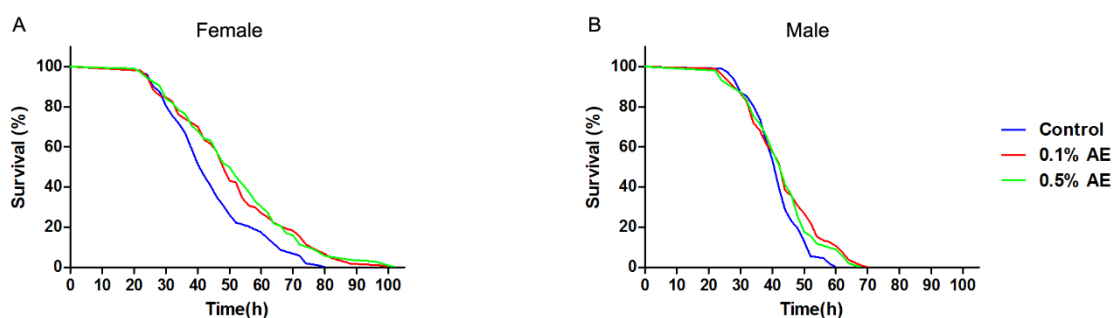


Figure 22. *Saccorhiza polyschides* extract promoted flies' tolerance to starvation. *Saccorhiza polyschides* extract mediated starvation resistance promotion of females was stronger than of males. For females (A), median lifespan were 42 hours (n=103) for control group, 49 hours (n=104) for 0.1% AE treated group and 51 hours (n=106) for 0.5% AE treated group. For males (B), median lifespan were 42 hours (n=108) for control group, 44 hours (n=104) for 0.1% AE treated group and 44 hours (n=112) for 0.5% AE treated group. Log-rank test was used for analysis.

3.3.3 Fly's fecundity remained unchanged by *Saccorhiza polyschides*

It has been confirmed that the protein content of the diet significantly influenced the fly's fecundity (3.1.2), which showed that higher concentrations of protein in diet stimulated to flies producing more eggs. In this part, the egg production ability of flies treated with AE medium was examined in order to evaluate its influence on the fly's fecundity, which could also verify if the mechanism of lifespan extension mediated by AE medium was the same as seen with dietary restriction. Flies treated with 0.1% AE medium laid more eggs (19.4%) than control, but when the AE concentration was raised to 0.5%, the egg production decreased to the same level as seen in the control group. This trend was consistent with the lifespan assay, which displayed the median lifespan of 0.1% AE medium group was higher than in 0.5% AE medium group. This result indicated that *Saccorhiza polyschides* could mediate lifespan extension without decreasing the fly's fecundity.

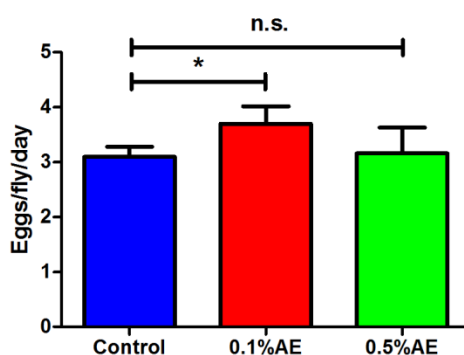


Figure 23. Fecundity assay for flies treated with *Saccorhiza polyschides* extract containing medium. Wild type flies (w^{1118}) were utilized for this assay. Egg production were 3.10 ± 0.18 per fly/day ($n=117$) for the control group, 3.70 ± 0.32 per fly/day ($n=102$) for the 0.1% AE medium group, 3.16 ± 0.47 per fly/day ($n=127$) for the 0.5% AE medium group. Data are presented as mean \pm S.D.; the unpaired two-tailed Student's t-test was used for analysis, n.s. not significant, $*p < 0.05$.

3.3.4 *Saccorhiza polyschides* extract affected fly's circadian rhythmicity but not locomotor behavior

The locomotor behavior of flies treated with *Saccorhiza polyschides* extract was monitored using the *Drosophila* activity monitor. Flies were divided into 3 groups and incubated in the monitor tubes supplying standard medium (control), 0.1% AE medium and 0.5% AE medium, respectively for a period of 7 days. In females, the activation of control group flies was higher than the other two groups, the average activation of, which was almost at the same level. But two-tailed t-test showed that the variation between the control and the AE groups was not significantly different. Similar to females, the locomotor activity of AE groups has no significant differences compared with the controls. In general, *Saccorhiza polyschides* extract did not affect the fly's locomotor activity. Moreover, the results of the circadian rhythm assays displayed flies of all groups had regular behaviors in general. Nevertheless males show some differences in more subtle details. The evening peaks of 5% AE group males always came earlier than the other two groups and end at the same time point after 24h treatment, though they have relevant lower peaks. It means their high activation period lasted longer in evening, which led to the higher average activation of 5% AE group males. It could be concluded that high concentration *Saccorhiza polyschides* extract influences male fly's circadian rhythmicity.

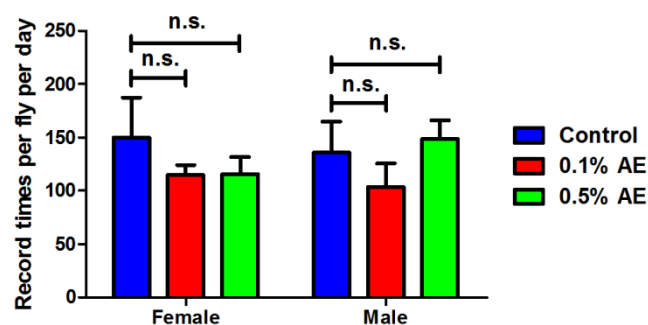


Figure 24. Locomotor activity of flies treated with *Saccorhiza polyschides* extract medium.

This histogram show the average activity counts of flies treated with standard, 0.1% AE and 0.5% AE medium for 7 days in LD condition. This algae extract did not influence flies activation obviously, both in females and males. Data are presented as mean \pm S.D.; the unpaired two-tailed Student's t-test was used for analysis, n.s. not significant.

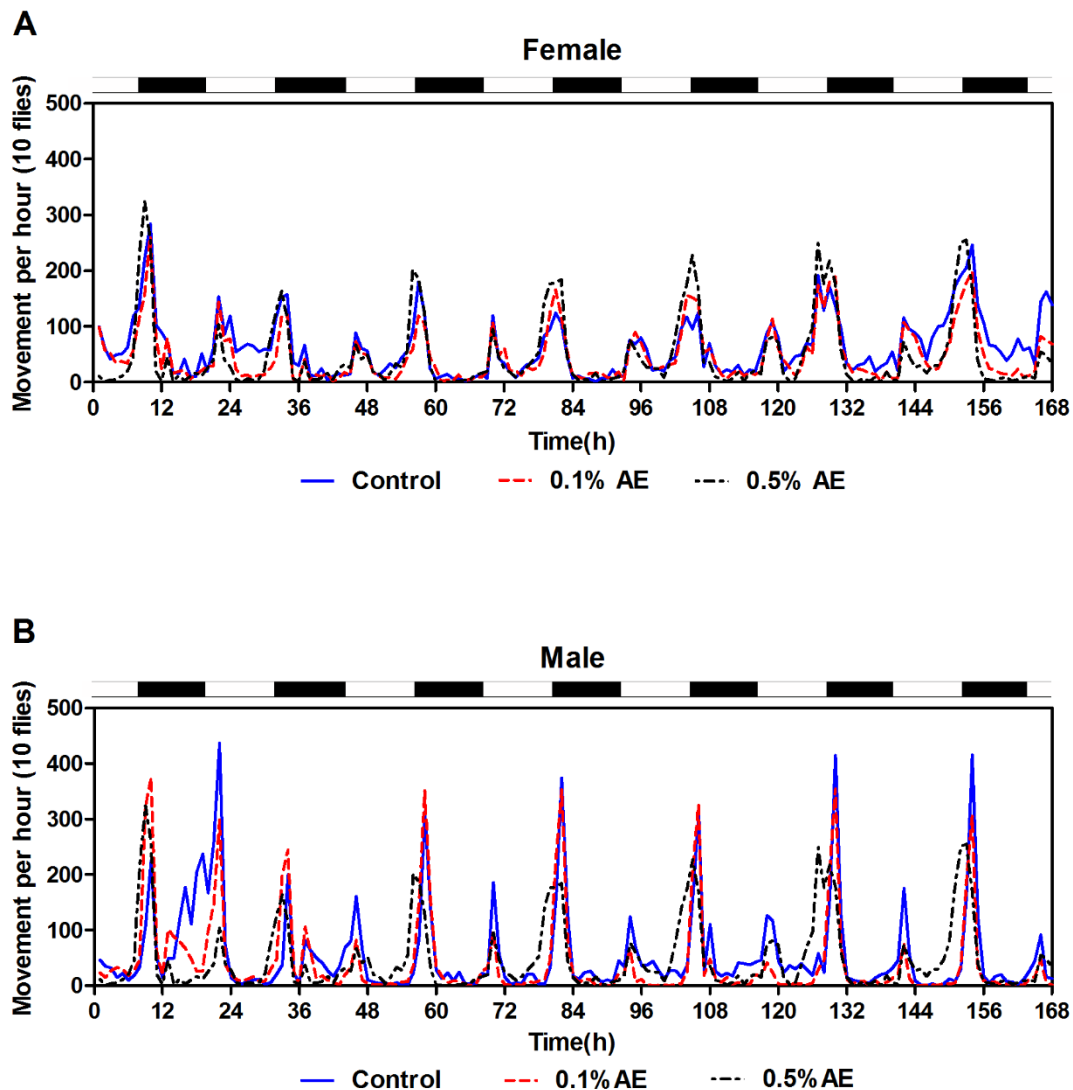


Figure 25. Circadian rhythm activity of flies treated with *Saccorhiza polyschides* extract medium. Both females (A) and males (B) displayed regular circadian rhythm in general. Overall, males' peaks were sharper than females, which means the high activation period of females last longer than males, but not so intensity as males. The control males showed relevant higher level of peaks compared with the males of AE medium group during the first 24h. But the evening peaks of 5% AE group males came obviously earlier than the other two groups.

3.3.5 *Saccorhiza polyschides* influenced the TAG content and body weight in males, but not in females

TAG storage of flies treated by *Saccorhiza polyschides* extract was measured, because it is an important parameter to evaluate insect energy storage, which is relevant to lifespan regulation and stress resistance. After 7 days of treatment, *Saccorhiza polyschides* extract treated flies exhibited less lipid content relative to fly weight, which was only significant males (two-tailed Student's t-test; 16.77% and 29.18% less than control group).

Moreover, the average body weight of all groups was also measured. Although males' lipid content was decreased by algae extract, the body weight of 0.5% AE medium males was high than in controls. It is supposed that this algae extract might inhibit the synthesis of storage lipid in males, which caused males treated with it showed relevant low lipid content. Similar to the fly's TAG content, body weight of females was also not significantly influenced by *Saccorhiza polyschides* extract.

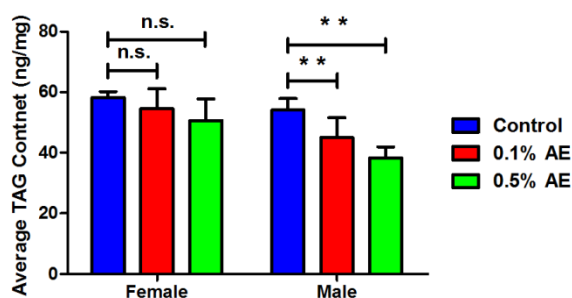


Figure 26. *Saccorhiza polyschides* treated flies stored less total TAG. Total TAG levels were measured in wild type flies treated with *Saccorhiza polyschides* extract. In females, the TAG content were 58.18 ± 1.96 ng per fly body weight ($n=80$) for control group, 54.47 ± 6.70 ng per fly body weight ($n=80$) for 0.1% AE group and 50.55 ± 7.22 ng per fly body weight ($n=80$) for 0.5% AE group. In males, the TAG content was 54.08 ± 3.75 ng per fly body weight ($n=128$) for control group, 45.01 ± 4.49 ng per fly body weight ($n=128$) for 0.1% AE group and 38.30 ± 3.66 ng per fly body weight ($n=128$) for 0.5% AE group. Data are presented as mean \pm S.D.; unpaired two-tailed Student's t-test was used for analysis, n.s. not significant, ** $p < 0.01$.

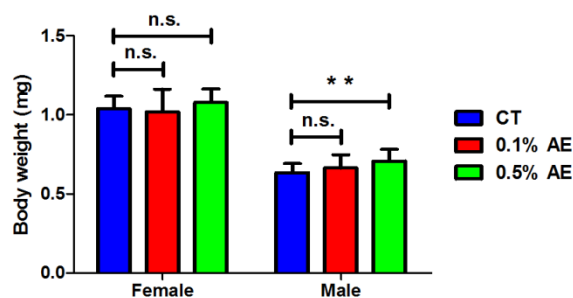


Figure 27. *Saccorhiza polyschides* intake increased fly's body weight slightly. 7 days-old adult flies were weighted. The body weight of both females and males was slightly influenced by algae extract. In females, the body weight were 1.04 ± 0.08 mg (n=80) for control group, 1.02 ± 0.14 mg (n=80) for 0.1% AE group and 1.08 ± 0.09 mg (n=80) for 0.5% AE group. In males, the body weight were 0.63 ± 0.06 mg (n=128) for control group, 0.66 ± 0.09 mg (n=128) for 0.1% AE group and 0.71 ± 0.08 mg (n=128) for 0.5% AE group. Data are presented as mean \pm S.D.; unpaired two-tailed Student's t-test was used for analysis, n.s. not significant, **p<0.01.

3.3.6 *Saccorhiza polyschides* altered the hemolymph glucose level

Besides TAG storage, hemolymph glucose content of flies treated by *Saccorhiza polyschides* extract was also measured. This algae extract had opposite effects on females and males. In females, the hemolymph glucose content was raised by algae extract, which increased 21.63% in 0.1% AE group and 11.22% in 0.5% AE group. In contrast to females, the males' hemolymph glucose content was obviously reduced by algae extract, the reduction rate were 11.34% and 22.06% in 0.1% AE group and 0.5% AE group respectively.

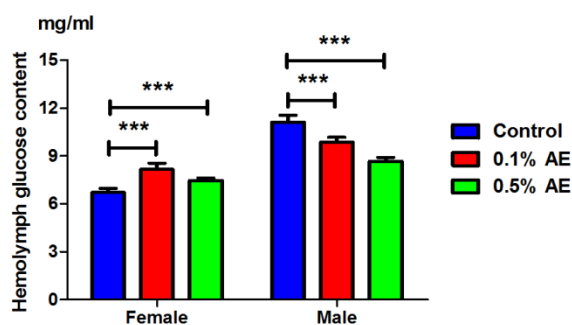


Figure 28. *Saccorhiza polyschides* significantly influences hemolymph glucose level. *Saccorhiza polyschides* extract performances differently in females and males. In females, hemolymph glucose content were 6.72 ± 0.25 mg/ml for control group (n=225), 8.17 ± 0.39 mg/ml for 0.1% AE group (n=225) and 7.47 ± 0.16 mg/ml for 0.5% AE group (n=225). In male, hemolymph glucose content were 11.12 ± 0.44 mg/ml for control group (n=225), 9.86 ± 0.32 mg/ml for 0.1% AE group (n=225) and 8.67 ± 0.25 mg/ml for 0.5% AE group (n=225). Data are presented as mean \pm S.D.; unpaired two-tailed Student's t-test was used for analysis. ***p<0.001.

3.3.7 *Saccorhiza polyschides* failed to influence fly's food intake

It is known that energy storage in organisms is closely related to the food intake. To test whether *Saccorhiza polyschides* extract functions in metabolism by affecting food intake, a measurement by a standard system of capillary feeder (CAFE) assay was performed (William WJ et al., 2007). This assay followed the method described in 2.2.5 and lasted for 9 days. Three groups were established, the flies of the control group were fed with solution consisted of 5% (w/v) sucrose and 5% (w/v) autolyzed yeast, while 0.1% AE (w/v) and 0.5% AE (w/v) were added to this solution as the food for 0.1% AE group and 0.5% AE group. Data from the second day to the ninth day was collected to allow the flies to adapt new environment. The result showed no significant differences on total food consumption among the three groups, both in females and males, which indicated that low TAG content in flies treated with *Saccorhiza polyschides* extract was not induced by food intake reduction.

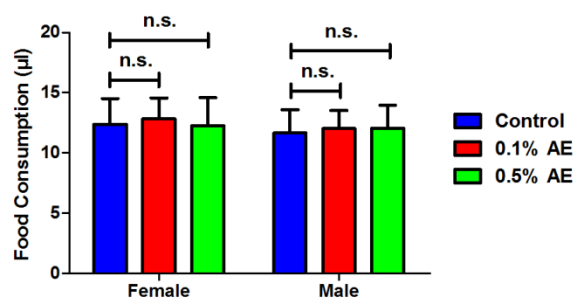


Figure 29. CAFE assay for *Saccorhiza polyschides* extract treated flies. *Saccorhiza polyschides* had no obvious influences on fly's food consumption both in females and males. In females, the total food consumption was $12.39 \pm 2.13 \mu\text{l}$ ($n=18$) for control group, $12.82 \pm 1.76 \mu\text{l}$ ($n=18$) for 0.1% AE treated group, and $12.24 \pm 2.36 \mu\text{l}$ ($n=18$) for 0.5% AE treated group. In males, the total food consumption was $11.64 \pm 1.96 \mu\text{l}$ ($n=18$) for control group, $12.02 \pm 1.48 \mu\text{l}$ ($n=18$) for 0.1% AE treated group, and $12.04 \pm 1.93 \mu\text{l}$ ($n=18$) for 0.5% AE treated group. Data are presented as mean \pm S.D.; unpaired two-tailed Student's t-test was used for analysis, n.s. not significant.

3.3.8 The fly's metabolic rate was not altered by *Saccorhiza polyschides*

To further investigate if the reduction of the TAG content in flies treated by *Saccorhiza polyschides* extract is caused by the elevating of the metabolic rate, this parameter was measured. The measurement was performed over a period of 9 days, with every day data collection. The metabolic rate was also measured before the flies were divided into treatment groups, which served as day 0. Similar to the result of the CAFE assay, this algae extract did not significantly influence the flies' metabolic rate, both in females and males.

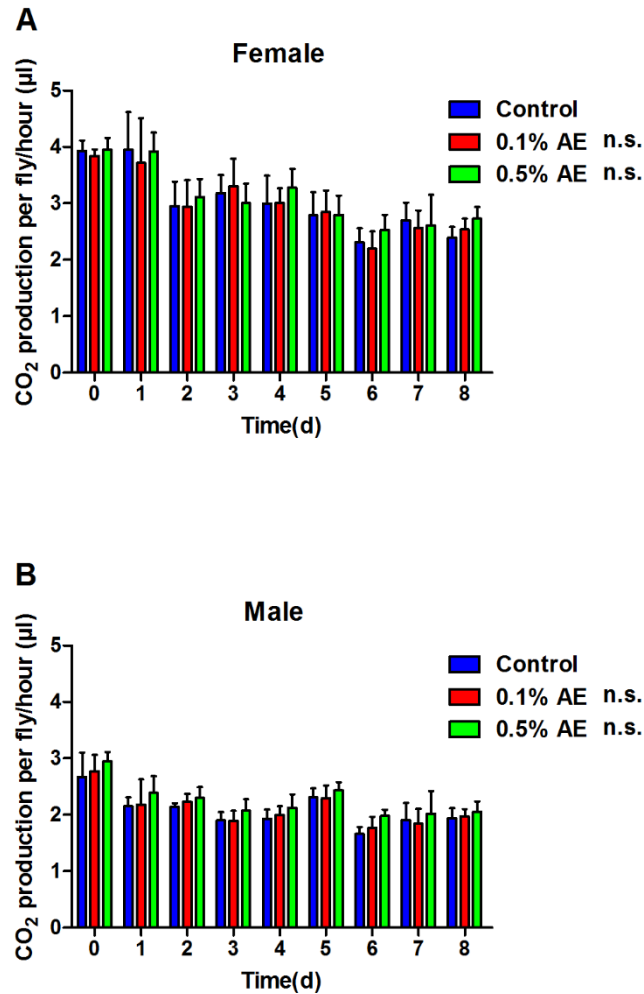


Figure 30. CO₂ production of flies treated by *Saccorhiza polyschides* extract. CO₂ production was not obviously influenced by *Saccorhiza polyschides* extract. Two-ways ANOVA showed no differences were detected in different treatment groups, while the CO₂ production was only affected by fly's age, both in females (A) and males (B). In addition, the interaction of *Saccorhiza polyschides* extract treatment and fly's age influenced little on CO₂ production either. Data are presented as mean \pm S.D.; two-ways ANOVA was used for analysis, n.s. not significant.

3.4 Characterization of *Saccorhiza polyschides* extract-mediated lifespan changes on mutant flies

Besides the phenotypical peculiarities, lifespan extension also complied with alternations in nutrient-pathways. In order to investigate the influences of *Saccorhiza polyschides* extract on nutrient-pathways, several mutant lines were employed and lifespan monitoring was done.

3.4.1 *Saccorhiza polyschides* extract failed to extend lifespan of Tor mutant flies.

The target of rapamycin (TOR) pathway is a major nutrient-sensing pathway, which regulates growth and metabolism in response to growth factors, amino acids, various stresses, and changes in cellular energy status (Bjedov I, 2010). It has also been implicated in the control of protein translation and ribosome biogenesis, upregulation of which is required for growth (Wullschleger S et al., 2006). Moreover, the TOR pathway has emerged as an important modulator of aging, an effect firmly established in several invertebrate models (Kaeberlein M et al., 2008). To confirm if the *Saccorhiza polyschides* extract-mediate lifespan extension related to the Tor pathway, Tor deficient flies were employed and treated with 0.1% AE medium and standard medium (control). The median lifespan of Tor deficient flies showed no differences between 0.1% AE medium group and control group, both in females and males ($P > 0.05$, Log-rank test). Additionally, both female and male Tor deficient flies exhibited significantly longer median lifespan than the wild type. It indicated that Tor mutant could indeed extend the flies' lifespan, as well as the lifespan extension mediated by *Saccorhiza polyschides* extract should be related to Tor signaling, for it was absent in Tor deficient flies.

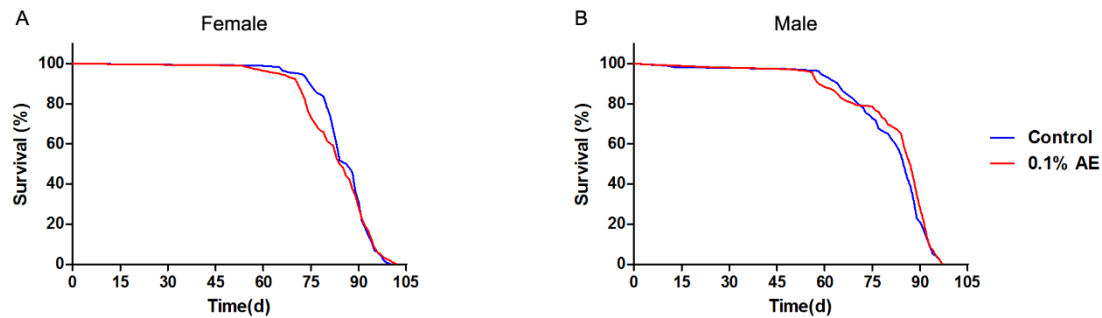


Figure 31. Median lifespan of *Tor* mutant flies was not obviously influenced by *Saccorhiza polyschides* extract. In females (A), median survival time were 86.5 days (n=116) for control group and 84.5 days (n=120) for 0.1% AE treatment group. In males (B), median survival time were 85 days (n=114) for control group and 87 days (n=112) for 0.1% AE treatment group. Log-rank test was used for analysis.

3.4.2 *Saccorhiza polyschides* extract failed to extend lifespan of Sirt1 KO flies

The silent information regulator 2 (Sir2) family of proteins is highly conserved serving as nicotinamide adenine dinucleotide (NAD⁺)–dependent protein deacetylases and mono-ADP-ribosyltransferases (Imai SI and Guarente L, 2010; Haigis MC and Sinclair DA, 2010), which is known as Sirt1 (NAD-dependent deacetylase sirtuin-1) in *Drosophila*, and has been implicated overexpression of them in the extension of longevity for several species (Frankel S, 2011), such as yeast, worms and flies (Kaeberlein M, 1999; Tissenbaum HA et al., 2001; Rogina B et al., 2004; Bauer JH, 2009). Sirt1 KO flies were employed and treated by *Saccorhiza polyschides* extract to investigate the function of Sirt1 on this algae extract mediate-lifespan extension. Similar to *Tor* mutant flies, this algae extract failed to significantly change median lifespan of Sirt1 KO flies, both in females and males ($P > 0.05$, Log-rank test). The Sirt1 KO fly's median lifespan was indeed decreased compared to the wild type, especially for males, about 30% of which died in 20 days. This result indicated that Sirt1 influenced AE mediate-lifespan extension.

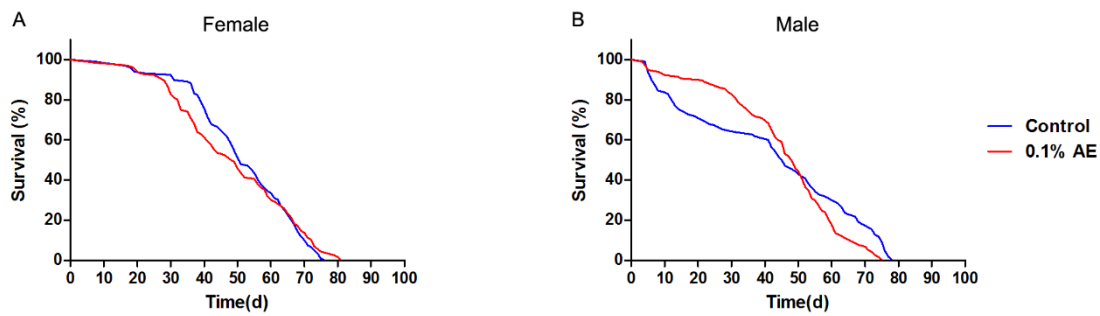


Figure 32. *Saccorhiza polyschides* extract failed to affect median lifespan of Sirt1 KO flies. In females (A), median lifespan were 51 days (n=119) for control group and 48.5 days (n=116) for 0.1% AE treatment group. In males (B), median survival time were 46 days (n=110) for control group, 48 days (n=117) for 0.1% AE treatment group. Log-rank test was used for analysis.

3.4.3 Performance of *Saccorhiza polyschides* extract in FoxO, Sirt2 and Tor over expression lines

To further investigate the role of insulin/IGF pathway in *Saccorhiza polyschides* extract in mediating lifespan regulation, we genetically manipulated expression of Tor, Sirt2 and FoxO using the Geneswitch (GS) system. The P(Switch)106 was used to drive Tor, Sirt2 and FoxO under transcriptional control of an UAS promoter in the fly's abdominal fat body. This GS system needs the drug RU486 (mifepristone) as stimulus, for a chimeric regulator of nuclear progesterone, which can only be activated by RU486, is combined with Gal4 (Wang Y, 1994). For every experimental, the median lifespan of crossing flies treated with standard medium with RU486 (Gal4>UAS RU group) and 0.1% AE median with RU486 (Gal4>UAS RUE group) were measured. RU486 was dissolved in 90% ethanol solution (400 μ M), so standard medium containing same volume of 90% ethanol was employed to treat crossing flies as control (EtOH group). Meanwhile, offspring of wild type (w¹¹¹⁸) crossing with UAS lines (WT>UAS RU) and Gal4 line crossing with wild type

(Gal4>WT RU) were also served as control. These flies were treated with standard medium contained RU486 only.

3.4.3.1 *Saccorhiza polyschides* extract had no effect on Tor overexpression flies

It has been indicated that *Saccorhiza polyschides* extract failed to mediate lifespan extension of Tor mutant flies. In this part, UAS-Tor line was employed to investigate if this algae extract would implicate any changes in the lifespan of Tor overexpression flies. The result showed that overexpression of Tor in abdomen fat body only induced lifespan reducing in males, but not in females, for the median lifespan of male RU group was 7.5% less than EtOH control group ($P < 0.01$, log-rank test), while no significant differences were detected between these two groups in females ($P > 0.05$, log-rank test). It might be caused by the higher metabolic rate and Tor expression level of females than males, which made females not be so sensitive to the raise of such amount of Tor expression level as males. Meanwhile, the median lifespan of RUAE group flies was probably the same with the RU group, both in female and male. The overexpression of Tor concealed the lifespan extension mediated by *Saccorhiza polyschides* extract.

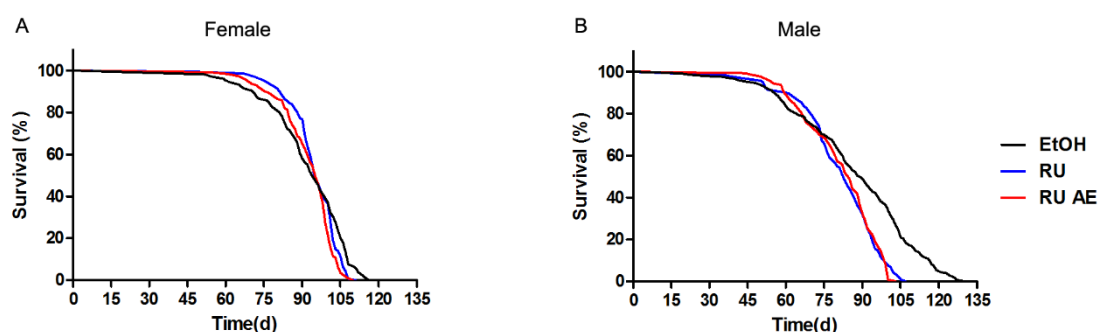


Figure 33. *Saccorhiza polyschides* extract did not influence lifespan of Tor overexpression flies. Lifespan of P(GS)106>Tor flies was measured. In females (A), the median lifespan were 95 days (n=142) for RU group, 95.5 days (n=112) for RUAE groups and 94 days (n=112) for EtOH control group. In males (B), the median lifespan were 83 days

(n=116) for RU group, 85 days (n=127) for RUAE groups and 89.5 days (n=206) for EtOH control group. Log-rank test was used for analysis.

To further confirm if the *Saccorhiza polyschides* extract caused lifespan extension is related to Tor signaling, a 754 amino acid central region (toxic effector domain, TED), which inhibits cell growth and arrests cell in G1 when over expressed in yeast (Vilella-Bach et al., 1999) and produces dominant-negative effects of Tor is constructed as UAS line (UAS-Tor.TED) (Hennig KM, 2002). Then the lifespan of P(GS)106>Tor.TED crossing flies was measured. The expression of Tor.TED reduced median lifespan of females (P<0.01, log-rank test), but not males (P>0.05, log-rank test). In addition, *Saccorhiza polyschides* extract decreased the median lifespan of Tor.TED expression flies, both females and males (P<0.01, log-rank test).

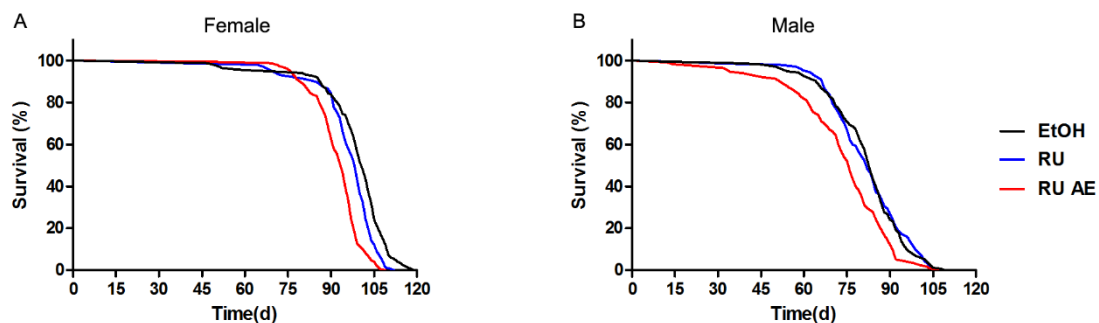


Figure 34. *Saccorhiza polyschides* extract decreased lifespan of Tor.TED expression flies. Lifespan of P(GS)106>Tor.TED flies was measured. In females (A), the median lifespan were 99 days (n=147) for RU group, 94 days (n=147) for RUAE groups and 101 days (n=166) for EtOH control group. In males, the median lifespan were 82 days (n=145) for RU group, 76 days (n=115) for RUAE groups and 83 days (n=175) for EtOH control group. Log-rank test was used for analysis.

3.4.3.2 *Saccorhiza polyschides* extract failed to mediate lifespan extension of Sirt2 overexpression flies

In this part, UAS-Sirt2 line was utilized to be crossed with the P(GS)106 line. Both Sirt2 over express females and males had significantly longer survival times compared to the EtOH control ($P < 0.01$, log-rank test). Meanwhile, the P(GS)106>Sirt2 flies of the RUAE group did not show longer lifespan than those of RU group. In females, median lifespan of RUAE group was 26.8% shorter than in the RU group ($P < 0.01$, log-rank test), while in males the median lifespan of the two groups was almost the same ($P > 0.05$, log-rank test).

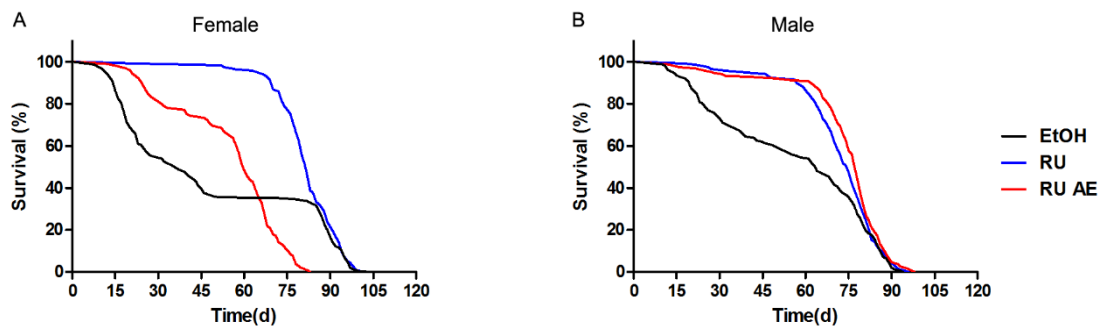


Figure 35. Median lifespan of Sirt2 overexpression fly was not extended by *Saccorhiza polyschides* extract. Lifespan of P(GS)106>Sirt2 flies was measured. In females (A), the median lifespan were 82 days (n=114) for RU group, 60 days (n=105) for RUAE groups and only 37 days (n=205) for EtOH control group. In males (B), the median lifespan were 75 days (n=118) for RU group, 78 days (n=151) for RUAE groups and 65 days (n=152) for EtOH control group. Log-rank test was used for analysis.

3.4.3.3 *Saccorhiza polyschides* extract extended median lifespan of female FoxO overexpression flies, but not in males.

The FoxO (forkhead box, sub-group "O") transcription factors regulate cellular processes under conditions of low levels of insulin signaling (Kramer JM, 2003). FoxO was first identified in the worm, *C. elegans* as and named Daf-16, the corresponding deletion mutant can suppress the increased lipid levels and

enhanced longevity caused by loss of *Daf-2*, the worm InR ortholog (Luong N, 2006; Lin et al., 1997; Ogg S et al., 1997). To investigate if the algae extract-mediate lifespan extension is related to *FoxO*, a UAS-*FoxO* line was utilized to cross with P(GS)106 and lifespan of this crossing line was measured. Female and male P(GS)106>*FoxO* flies exhibited totally reverse result in lifespan assay. For females, lifespan of RU group flies was significantly shorter than in the EtOH control group ($P < 0.001$, log-rank test), while this algae extract extended lifespan of these *FoxO* overexpression flies, but still shorter than the EtOH control. For males, the overexpression of *FoxO* increased the median lifespan ($P < 0.001$, log-rank test), which was not significantly affected by the algae extract ($P > 0.05$, log-rank test).

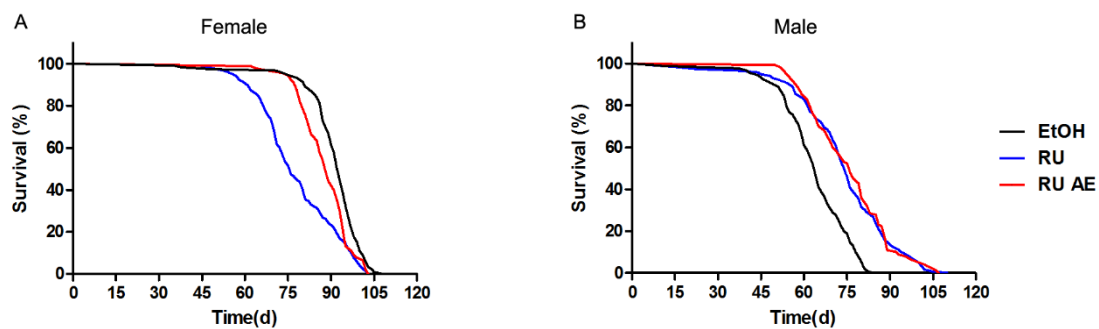


Figure 36. Median lifespan of *FoxO* overexpression females was extended by *Saccorhiza polyschides* extract. Lifespan of P(GS)106>*FoxO* flies was measured. In females (A), the median lifespan were 76 days (n=118) for RU group, 88 days (n=105) for RUAE groups and 93 days (n=119) for EtOH control group. In males (B), the median lifespan were 74.5 days (n=118) for RU group, 76 days (n=147) for RUAE groups and 67 days (n=121) for EtOH control group. Log-rank test was used for analysis.

3.5 Characterization of *Saccorhiza polyschides* extract-induced gene expression changes

It has been demonstrated that the *Saccorhiza polyschides* extract contributes to the lifespan extension of *Drosophila melanogaster*, especially in challenging conditions like drought environment and high fat diet. Further work indicated the lipid content of males treated by this algae extract was obviously decreased. Algae extract treated females had lower hemolymph glucose level, while algae extract treated males had higher hemolymph glucose level than the control. No differences were detected in food intake and metabolic rate between algae treated flies and the controls. Thus, the discrepancy has drawn my attention to the changes in the expression level of some genes relevant to metabolic pathways, especially the insulin/IGFs pathway, in response to *Saccorhiza polyschides* extract treatment.

The insect fat body plays an essential role in energy storage and utilization. In addition to the role as a central storage depot for excess nutrients, the fat body is also an issue of great biosynthesis and metabolic activity (Law JH, 1989), not only for the synthesis of fat and glycogen, but also for most of the hemolymph protein and circulating metabolites. Moreover, the fat body is a target of several hormones (Gäde G, 2004; Schooley DA, 2005), which helps the fat body to integrate signals from other organs. Overall, fat body is a key tissue for insects' metabolism, which couples a number of metabolic pathways. So the gene expression changes of the flies' fat body was analyzed, in order to clarify the mechanism of lifespan extension mediated by *Saccorhiza polyschides* extract. To achieve this aim, 11 target genes were selected and assessed using qRT-PCR with gene-specific primers for each target, which is shown in the table below.

Table 5. Target genes for qRT-PCR

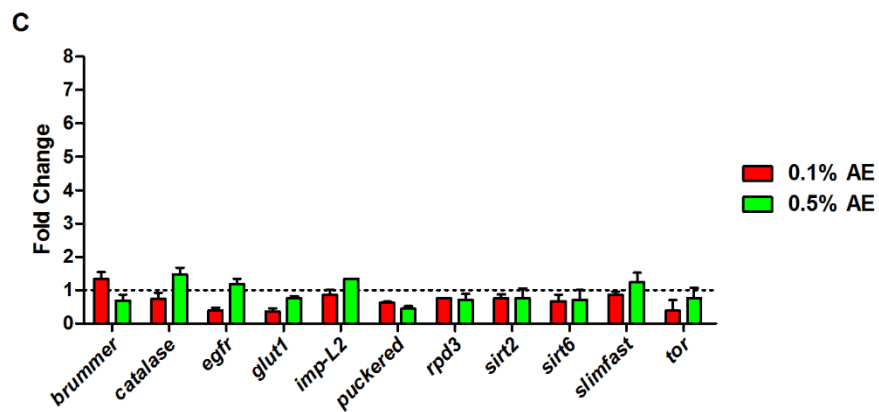
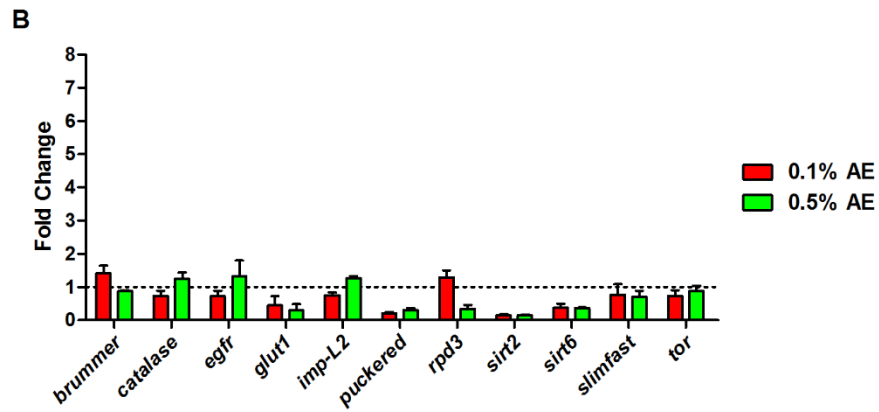
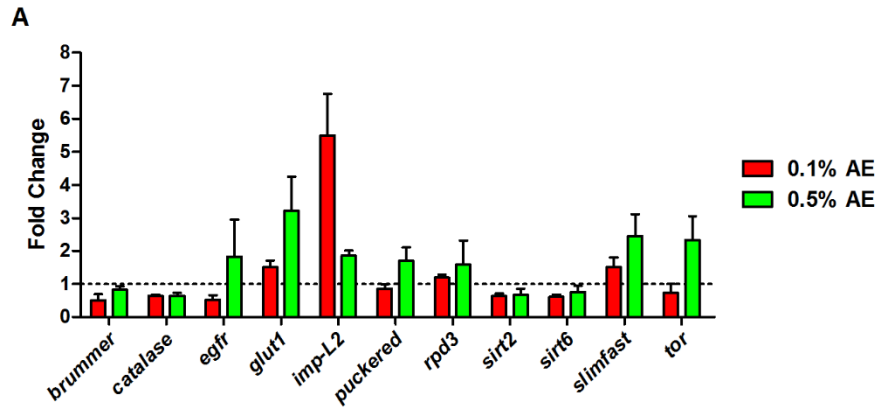
Target	Gene ID	Molecular function
<i>brummer</i>	39611	Adipocyte triglyceride lipase
<i>catalase</i>	40048	Catalyzing the decomposition of H ₂ O ₂ to H ₂ O and O ₂
<i>egfr</i>	37455	Cell-surface receptor for member of epidermal growth factor
<i>glut1</i>	38109	Facilitating transport of glucose across the plasma membrane
<i>imp-L2</i>	38513	Insulin-like growth factor binding
<i>puckered</i>	40958	Acting as JUN kinase phosphatase
<i>rpd3</i>	38565	Histone deacetylase 3, modulate cell growth and apoptosis
<i>rpl32</i>	43573	Ribosomal protein L32, reference gene
<i>sirt2</i>	42414	NAD-dependent protein deacetylase
<i>sirt6</i>	41254	Stress responsive protein deacetylase
<i>slimfast</i>	40510	Catalysis of the basic amino acid trans-membrane
<i>tor</i>	47396	Serine/threonine protein kinase

Specifically, female and male wild type (w^{1118}) flies were divided into three groups and incubated in standard medium, 0.1% AE medium and 0.5% AE medium respectively. At the time point of 7 days (Fig. 36 A and B) and 14 days (Fig.36 C and D), flies of all groups were dissected and their abdomen fat body was isolated. Then the RNA was extracted following the method described in method 2.2.9 for qRT-PCR further processed. Before the primers listed in 2.1.5 were utilized for gene expression level measurement, the efficiency of these primers was examined to make sure the primers can combine with the sample cDNA efficiently. The result of primer efficiency is shown in the table below. The efficiency of all these primers exceed 85% and are qualified for qRT-PCR.

Table 6. Efficiency of primers utilized in this experiment

Target	Slope	R ²	Efficiency (%)
<i>brummer</i>	-3.456	0.995	94.731
<i>catalase</i>	-3.716	0.992	85.857
<i>egfr</i>	-3.214	0.981	104.71
<i>glut1</i>	-3.457	0.998	94.656
<i>imp-L2</i>	-3.320	0.984	100.081
<i>puckered</i>	-3.342	0.998	99.169
<i>rp3</i>	-3.418	0.997	96.141
<i>rpl32</i>	-3.284	0.998	101.608
<i>sirt2</i>	-3.387	0.988	97.254
<i>sirt6</i>	-3.666	0.987	87.403
<i>slimfast</i>	-3.445	0.998	95.108
<i>tor</i>	-3.578	0.983	90.321

In general, the result showed that algae extract did not massively change the transcription level of the target genes. In females, only the expression level of *tor*, *imp-L2*, *glut1* and *egfr* was significantly affected by *Saccorhiza polyschides* extract (Fig.36 A and C), whereas the expression level *sirt2*, *sirt6*, *rp3*, *puckered*, *imp-L2*, *glut1*, and *brummer* was obviously changed in males (Fig.36 B and D). It seemed that males were more sensitive to this algae extract than females, especially during the earlier time point (Fig.36 B). However, the expression of some genes in 0.1% AE group and 0.5% AE group showed opposite directions.



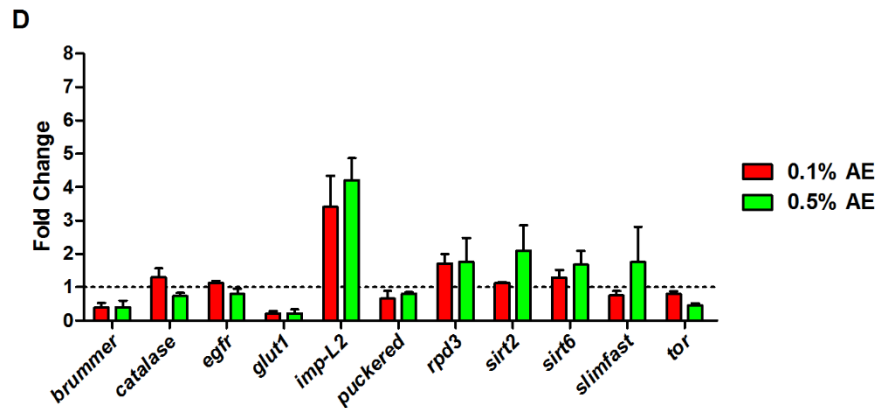


Figure 37. Determination of differential expression levels using relative qRT-PCR. The relative fold change of eleven selected individuals in wild type incubated by three different medium was normalized by an endogenous reference gene of *rp132*. The red bars and green bars indicate gene expression levels in 0.1% AE group and 0.5% AE group, while the control group was defined as 1. All values represent as mean \pm S.D. from three independent replicates.

4. Discussion

Dietary restriction has the potential to extend lifespan and youthful health (health span) in numerous eukaryotes ranging from yeast to humans (Kaeberlein M et al., 2007; Piper MD, Bartke A, 2008; Weindruch R et al., 1988). But it comes along with some negative effects, such as low fecundity, slow growth, or even depression of moods (Partridge L et al., 2005; Williams GC, 1966; Chapman T et al., 1996). So natural diet supplements, which have the potential to slow aging, while avoiding serious negative effects are highly attractive. In this project, the fruit fly *Drosophila melanogaster* was utilized as a simple model in order to study the influence of dietary restriction on lifespan and some other phenotypical read-outs. Taking advantage of the wealth of genetic tools available in *Drosophila*, such as the GAL4/UAS system, RNAi and gene disruption technologies, the various roles of dietary restriction in metabolism were dissected. Moreover, extracts of *Saccorhiza polyschides*, a large brown algae, were supplied as a diet supplement and their effects on phenotypical peculiarities and metabolism were also examined. Using *Drosophila* for this type of studies is highly recommended as the fruit fly is not only an almost perfect simple model organism that is amenable to a huge variety of different genetic interventions, flies and mammals share also very similar organ compositions. For example, the fat body and oenocytes of flies are functional analogues to adipose tissue and liver of mammals, respectively (Gutierrez E et al., 2007). In addition, the homologues of many mammalian genes could be found in the *Drosophila* genome, making it an adaptable model for biomedically relevant research (Chen D et al., 2008). Evaluation of the changes in transcript levels of these target genes in response to dietary restriction and *Saccorhiza polyschides* extract treatment should increase our understanding of the underlying mechanisms and may pave the way to utilize this highly attractive dietary supplement in order to increase health or even lifespan in humans.

4.1 Dietary restriction influences lifespan and associated phenotypes

Overall, the lifespan experiments I presented here, where flies were treated with media containing different protein concentrations showed that reducing protein content in the diet is beneficial for lifespan extension, but an over-restricted diet with too low protein concentrations has drawbacks, thus representing malnutrition. I could also show that the protein requirements differ substantially between females and males. It is assumed that females require much energy and protein for egg production, as large amounts of nutrients are allocated in eggs to ensure the development of their offspring, so the limited nutrient supplied by low protein content medium is insufficient for somatic maintenance of females, followed by the damage of somatic tissue and acceleration of aging (Heck MJ et al., 2016; Tatar M, 2001). The fecundity test verifies this, the results show that the high protein medium significantly increases egg production of female flies. This result also indicates that although dietary restriction induces lifespan extension, it is unfavorable for the reproduction rate of organisms (Piper DW et al., 2007; Heininger K, 2012; Nakagawa S et al., 2012; Tatar M, 2007). It was explained that life extension reallocates ingested nutrients away from reproduction and towards somatic investments, which allows enhanced maintenance of somatic tissues, thereby increasing life span while reducing reproduction (Heck MJ et al., 2016; Kirkwood TB, 1977; Kirkwood TB et al, 1991).

Besides lifespan, many phenotypical peculiarities are tightly connected with nutritional conditions. In the locomotor behavior assay, the physical activity of flies treated with DR medium is much higher than seen in the other two groups, which holds especially true for females. As already mentioned, females request more nutrients to cover the energy requirement of somatic maintenance and egg production, so their activation elevation is more obvious than in males under dietary restriction conditions. Dietary restriction does not influence the flies' circadian rhythmicity. The activation of the flies in all the three groups has obviously high evening peaks, as well as relevant low morning peaks. It has been

demonstrated that disruption of circadian rhythms (also defined as chrono-disruption) is the cause of many diseases, such as cardiovascular diseases and obesity (Erren TC et al, 2009; Turek FW et al., 2005; McAlpine CS et al, 2016). Thus, regular circadian rhythms are the basic insurance of health and longevity for most organisms. Neither dietary restriction nor high protein diet should not change this phenotype too much.

Lipids are mainly stored as TAGs (triacylglycerides), which reflects energy storage in the body. Adequate TAG storage can supply energy in the period of fasting or for the activity phases, such as flight, that require large amounts of energy (Arrese EL et al, 2010). My results show that dietary restriction promotes the TAG storage of flies, which is counterintuitive. The content of carbohydrate, an important source of the storage TAG, is probably the same among the three kinds of medium. So the TAG storage variation should be due to the suitable protein content in the medium maintain the metabolic rate in a reasonable degree, which helps that more ingested carbohydrate is stored in the form of TAG. The result is consistency with the lifespan measurement, 1% YE group females store more TAG and had the longest lifespan, while for males 0.25% YE group flies have most TAG and longest lifespan. More TAG storage does not contribute to higher body weight. The body weight of 5% YE group flies is obviously higher than 0.25% YE and 1% YE group, which indicates that protein consumption is a main source leading to changes in body weight. Moreover, it appears that flies first use their protein stores as energy store before they start to mobilize their fat stores, which is supported by the finding that the body weight is decreased while the relative fat content is increased upon DR (Owen OE et al., 1967; Owen OE et al., 1969).

Hemolymph glucose, similar to blood sugar of mammals, is one of the primary sources of energy in the body. To keep the hemolymph glucose concentration within a certain range is essential for metabolic homeostasis, otherwise diseases, such as diabetes, will occur (Uqrakar R et al., 2015). The lower hemolymph glucose content observed in CM fed flies suggests that high protein diet leads to higher metabolic rates, which reduces both storage and circulating glucose. The

significant decrease seen in the CM group females indicates that high protein diet sharply increases energy consumption, an interpretation that is strengthened by the results obtained in the egg production assay. Alternatively, or in addition, high circulating DILP2 content may also cause the low hemolymph glucose content of CM treated flies (Broughton S et al., 2008; Sato-Miyata Y et al., 2015).

Potential drawbacks of high energy content diets that are believed to be the cause of premature death are often explained by the production of reactive oxygen species (ROS) in the mitochondria. While under normal conditions ROS could be detoxified in cells easily, conditions of high metabolic activities are characterized by very high ROS production levels. Higher metabolic rates would lead to increased ROS production and accumulation, which, if the speed of production and accumulation exceeds the cells' ability of self-clear, would in turn damage cells (especially mitochondria) by the oxidative stress, and the process of cellular senescence is promoted (Balaban RS et al., 2005; Schieber M et al., 2014, Bohovych et al., 2016; Takauji Y et al., 2016). High protein concentration diets significantly elevate metabolic rates, which may be another reason for the short lifespan brought by high protein dieting. Males seem to be more sensitive to high protein diets than females: 1% yeast extract diet could sharply increase metabolic rate of males, while only 5% yeast extract diet could raise metabolic rates of females significantly. This phenomenon also explains the result of the lifespan assays, females treated with 1% yeast extract diet has the longest lifespan, while 0.25% yeast extract diets are most preferable for males.

4.2 Dietary restriction regulates insulin-like peptide release

Insulin-like peptides play an important role in regulation of the flies' metabolism (Sato-Miyata Y, 2014). It promotes glucose uptake in muscles and adipose tissues, induces cell growth and proliferation, and stimulates glyconeogenesis, lipogenesis and protein synthesis (Saltiel AR, 2001). Eight different insulin-like peptides are present in *Drosophila* (DILP1- DILP8; Nässel DR et al., 2016). DILP2 is apparently the most important ILP that is expressed in IPCs of the *Pars intercerebralis* and it has the highest potential for promoting tissue growth as it has been demonstrated to be essential for glucose homeostasis regulation. High protein diets raise circulating DILP2 levels, which may be one reason of the high metabolic rate brought by high protein diet (Broughton SJ et al., 2005; Géminard C et al., 2009). Meanwhile, total DILP2 content measurement shows the flies of the DR group produced more DILP2 than the control group. It indicates that dietary restriction does not inhibit the DILP2 production in IPCs. Instead, dietary restriction could reduce DILP2 secretion to the hemolymph effectively. It is known that appropriate reduction of DILP2 in the hemolymph increases its glucose and trehalose content and extends lifespan, while high level of circulating DILP2 often couples with a series of impaired physiological conditions such as hypoglycemia, insulin resistance and type II diabetes (Pasco MY et al., 2012). In this aspect, dietary restriction is beneficial for lifespan extension.

Regulation of hemolymph glucose levels mainly depend on DILP levels, but additional regulators are known. Imp-L2, a homolog of the mammalian IGFBP7, acts as a negative regulator of insulin-like peptide signaling during development, regulates growth cell-non-autonomously and antagonizes DILP2 in genetic assays (Alic N, 2011). It is reported the increase of *imp-L2* mRNA level contributes to lifespan extension (Alic N, 2011). Dietary restriction increases the transcription level of *imp-L2* in the fly's fat body, the most important metabolism tissue.

4.3 *Saccorhiza polyschides* extracts influence lifespan and relevant phenotypical peculiarities in different ways

In the previous parts, I described that dietary restriction is beneficial for lifespan extension, as it increases the fly's locomotor activity and TAG storage as well as it decreases metabolic rates. Meanwhile, it inhibits the secretion of DILP2 to the hemolymph, and it fosters expression of *imp-L2*, a negative regulator of insulin-like peptide signaling. But dietary restriction still has some relevant limitations such as low fecundity and, even more importantly, DR is hard to follow for a longer period of time. Thus, other means which could promote health conditions and slow aging, while bringing few or no side effects are highly attractive. The utilization of natural products on the treatment of a broad range of diseases has been documented extensively (De Corte BL, 2016; Newman DJ et al., 2012; Kinghorn AD, 2001; Butler MS, 2004). Plant derived natural products, which were normally used in crude form as extracts and oils, in particular have been used for centuries to treat a wide variety of ailments (Farnsworth NR et al., 1985; Cragg GM et al., 2009). Marine macroalgae (seaweeds) are rich in bioactive compounds that could potentially be exploited as functional ingredients for both human and animal health applications (Gupta S et al., 2011), as they are known for their richness in polysaccharides, minerals and certain vitamins, but they also contain bioactive substances like polysaccharides, proteins, lipids and polyphenols, with antibacterial, antiviral and antifungal properties, as well as many other biologically active components (Arasaki S et al., 1983; Kumar CS et al., 2008; Holdt SL et al., 2011). Two kinds of brown seaweeds, *Turbinaria ornata* and *Padina pavonia*, have been shown decrease azoxymethane-induced hepatic repercussions of mice through up-regulation of peroxisome proliferator-activated receptor gamma and attenuation of oxidative stress (Abdella EM et al., 2016). A dulse (*Palmaria palmate*) and three kelp (*Laminaria setchellii*, *Macrocystis intergrifolia*, *Nereocystis leutkeana*) extracts were reported to reduce the risk of cancer, as the proliferation rate of HeLa cells was significantly inhibited by these extracts (Yuan YV et al., 2006). Moreover, *Fucus*

vesiculosus, *Laminaria ochroleuca*, *Undaria pinnatifida*, *Chondrus crispus* and *Porphyra umbilicalis* could scavenge free radicals (2,2-diphenyl-1-picrylhydrazyl, DPPH-), induce ferric-reduction and inhibit copper-catalyzed *in vitro* human low-density lipoprotein (LDL) oxidation, thus reducing oxidative stress of cells (Jiménez-Escrig A et al., 2001). More seaweeds have been reported to be essential for health promotion are shown in table 7. In all, seaweeds are important sources of natural health products, known for their excellent performances on antioxidants, anticancer as well as lifespan extension (Snare DJ et al., 2013). Thus, *Saccorhiza polyschides* extracts were added to the fly medium in this work to evaluate its potential to be used as diet supplement by comparing its influence on lifespan and relevant phenotypical peculiarities with those induced by dietary restriction.

Table 7. Seaweeds have been reported to be essential for health promotion

	Possible health effect	References
<i>Ascophyllum nodosum</i>	Anti-diabetic	Paradis ME, 2011
<i>Chondrus crispus</i>	Prevention of cardiovascular disease	Berteau O et al., 2003
<i>Ecklonia cava</i>	Anti-cancer	Kong CS et al., 2009
<i>Eisenia arborea</i>	Anti-allergy	Sugiura Y et al., 2007
<i>Fucus evanescens</i>	Anti-tumor and anti-metastatic	Alekseyenko TV et al., 2007
<i>Fucus vesiculosus</i>	Anti-diabetic	Paradis ME, 2011
<i>Haematococcus pluvialis</i>	Anti-oxidants	Kurashige M, 1989
<i>Ishige okamurae</i>	Anti-diabetic	Heo SJ et al., 2010
<i>Laminaria japonica</i>	Anti-apoptotic	Kim KH et al., 2006
<i>Pelvetia siliquosa</i>	Anti-diabetic	Lee et al., 2004
<i>Pittosporum eugenoides</i>	Anti-oxidants	Elansary HO et al., 2016
<i>Saccharina japonica</i>	Anti-diabetic	Kim MS et al., 2008
<i>Sargassum vulgare</i>	Anti-tumor	de Souza APA et al., 2007
<i>Spiraea nipponica</i>	Anti-oxidants	Elansary HO et al., 2016
<i>Spirulina platensis</i>	Anti-oxidants	Khan M et al., 2006
<i>Undaria pinnatifida</i>	Anti-viral	Hemmingson JA et al., 2006

Similar to dietary restriction, *Saccorhiza polyschides* extract supplementation is beneficial for lifespan extension in suitable conditions, though its effect is not as strong as that seen following dietary restriction. When flies are kept under stressful conditions, the influence of this algae extract on lifespan is massively enhanced. In drought environment, the lifespan extension mediated by this algae extract is highly significant, although it has been shown that drought environment could eliminate dietary restriction-mediated lifespan extension (William WJ et al., 2009), which implies that the mechanism underlying lifespan extension mediated by *Saccorhiza polyschides* extracts is different compared with that induced by dietary restriction. High fat diet is usually stressful for most animals, as the excessive lipids accumulation brings a high risk of deleterious effect on metabolism and heart function as well as on type II diabetes. *Saccorhiza polyschides* extract feeding extends the lifespan of flies raised under high fat diet significantly. However, it might simply result from the fat in medium being decomposed by some ingredients of this algae extract. The flies pre-treated 10 days by *Saccorhiza polyschides* extract still have longer lifespan than the control, which indicates that this algae extract indeed promotes the fly's resistance to high fat diet and this effect is sustainable. The tolerance to starvation is also an important index, for it reflects an important aspect of the energy homeostasis of organisms. This algae extract enhances the starvation tolerance of both females and males. It could be concluded that the *Saccorhiza polyschides* extract mediated lifespan extension does not share the same mechanism with dietary restriction. *Saccorhiza polyschides* extract could also rise the fly's stress resistance, which indicates that it is able to eliminate the negative effects brought by over diet and sustain the energy homeostasis of flies. As already mentioned, dietary restriction inhibits egg production. It is one of the limitations of dietary restriction, which is adverse to reproduction. *Saccorhiza polyschides* extract does not have a negative effect on egg production, in contrast, small amount of algae extract slightly promotes the fly's fecundity. This result indicates that the *Saccorhiza polyschides* extract overcomes one limitation of

dietary restriction, and further implies that this algae extract mediates lifespan extension in different ways.

Unlike dietary restriction, *Saccorhiza polyschides* extract does not influence the fly's locomotor activity, though a slight decrease was observed in algae treated animals. The result of the circadian rhythm assays shows that flies treated with algae extract also have regular biological clock activity, which is characterized by obvious daytime peaks and evening peaks. But it has to be noticed that the evening peaks of male flies treated with 0.5% AE start earlier than the other two groups while they end at almost the same time. Males are more sensitive to high concentration of *Saccorhiza polyschides* extract compared with females in this aspect.

Another physiological index differently influenced by *Saccorhiza polyschides* extract and dietary restriction is the TAG content. Flies treated with DR medium store more TAG in their body, while the algae extract treatment fails to increase the TAG storage of flies. In contrast, the males treated by algae extract have less TAG content than the controls. Moreover, the *Saccorhiza polyschides* extract had only a minor influence on flies' body weight. The low TAG storage of males coupled with decrease of hemolymph glucose content. Unlike high protein diet, *Saccorhiza polyschides* extract mediate low hemolymph glucose content is not due to high metabolic rate. This algae extract might inhibit the absorbance of nutrients. Compared with males, the metabolism of females seems to be less influenced by *Saccorhiza polyschides* extract.

One major reason that leads to decreased TAG storage is low food consumption. The CAFE assay was utilized to check if *Saccorhiza polyschides* extracts reduced the flies' food consumption. The result of the CAFE assay points into a different direction. Beside the food consumption, *Saccorhiza polyschides* extracts do not significantly influence the metabolic rate either, which indicates *Saccorhiza polyschides* extract works in a different way on lifespan extension compared with dietary restriction. This is insofar highly important, as many extracts known to have lifespan prolonging effects simply taste bad, which leads to reduced food intake and therewith to a dietary restriction effect.

One possible target for the action of *Saccorhiza polyschides* extracts is the Tor pathway. Tor (target of rapamycin) was first identified in yeast in 1991, as Tor deficient *Saccharomyces cerevisiae* were rapamycin resistant (Heitman J et al., 1991). Rapamycin is an immunosuppressant macrolide isolated from the bacterium *Streptomyces hygroscopicus* first discovered in the 1970s on Rapa Nui, one of the Easter Islands (Vezina C, 1975). This compound arrests fungal activity at the G1 phase of the cell cycle (Dobashi Y, 2011). Rapamycin inhibits Tor by association with its intracellular receptor FKBP12 (Huang S et al., 2001; Huang S et al., 2003). The FKBP12-rapamycin complex binds directly to the FKBP12-Rapamycin Binding (FRB) domain of Tor, thus inhibiting its activity (Huang S et al., 2003). Tor is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, transcription (Hay N et al., 2004). Taken together, the Tor pathway is a major nutrient-sensing pathway. Mechanistically, Tor-signaling appears to be of central importance in order to understand the mechanisms underlying the lifespan elongating effects of *Saccorhiza polyschides* extract. This algae extract failed to extend lifespan of Tor-mutant flies. The expression of Tor toxic effector domain (Tor-TED) in abdomen fat body only reduces females' lifespan and the lifespan of both females and males of this line is decreased by this algae extract. As the treatment with the *Saccorhiza polyschides* extract does not influence lifespan of Tor-deficient animals, it can be concluded that this extract acts via interference with Tor-signaling.

Sirt1 (NAD-dependent deacetylase sirtuin 1), a member of the sirtuin family of proteins, is highly conserved in serving as NAD⁺-dependent protein deacetylases and mono-ADP-ribosyltransferases, homologs of the Sir2 gene in *S. cerevisiae* (Imai SI et al, 2010; Haigis MC et al, 2010). The overexpression of *sirt1* is implicated to extend longevity for several species. *Saccorhiza polyschides* extract also fails to influence longevity of *sirt1* knock out line. Sirt2 is one of the ortholog of Sirt1 protein family, it regulates microtubule acetylation, myelination in nervous system as well as cell reproduction and DNA damage repair. Overexpression of *sirt2* in the abdominal fat body significantly increases lifespan in both females and males

(Frankel S, 2011, Hoffmann et al. 2013). The treatment of *Saccorhiza polyschides* extract decreases the lifespan of *sirt2* overexpression females. Normally Sirt2 induces the response to stress, such as autophagy in cells, which acts opposite to Tor. So this is consistent with the results of lifespan assay of Tor-TED line. FoxO (forkhead box, sub-group "O") transcription factors regulate cellular processes under conditions of low levels of insulin signaling (Kramer JM, 2003). The active of FoxO requires the decarboxylation of Sirt2 (Srivastava S et al.; 2011). Overexpression of *foxo* mediates short longevity of females while extends the lifespan of males. *Saccorhiza polyschides* extract treatment on this line extends females' lifespan, while affects nothing on males. It indicates that the *Saccorhiza polyschides* extract is not in the same pathway as *foxo* overexpression.

The data of qRT-PCR also shows confidence to these results. Over all, *Saccorhiza polyschides* extracts does not influence the transcription level of most target genes dramatically. The transcription level of *tor* is down regulated by *Saccorhiza polyschides* extract, which is consistent with the result of lifespan assay. Epidermal growth factor receptor (*egfr*), which promotes the phosphorylation of *tor* (Guertin DA et al., 2005), is also down regulated. Opposite to *egfr*, transcription levels of *imp-L2* are up regulated, which would cause the decrease of functional insulin-like peptide signaling (Alic N, 2011). The transcription of *sirt2* is downregulated. Another ortholog of sirtuin family, *sirt6*, which is a stress responsive protein deacetylase and functions in DNA repair, telomere maintenance, glycolysis and inflammation (Michishita E et al., 2008), is also down regulated. But the transcription of a JNKs inhibitory factor, *puckered*, which activates the phosphatase of JNKs on its Thr-Pro-Tyr sites (Martín-Blanco E et al., 1998), is also down regulated. The expression level of glucose transporter 1 (*glut1*) and amino acid transmembrane carrier (*slimfast*) are all decreased in males. For females, it raises at early stage, and is downregulated later on.

Taken together, the *Saccorhiza polyschides* extract reduces *tor* as well as *egfr* transcript levels. Transcription of *sirt2*, *sirt6* and *puckered* are also downregulated. Opposite to these targets, transcription of *imp-L2* is upregulated. The

transmembrane carriers of glucose and amino acid are all downregulated after a long term treatment. *Saccorhiza polyschides* extract is a kind of safety diet supplement for its mild influences on most of the target genes.

Summary

Population aging has become one of the greatest worldwide challenges. To conquer this problem, studies on slowing aging as well as prevention and treatment of the diseases brought by aging, such as type II diabetes, cancer and cardiovascular diseases, have been largely expanded during recent years. Dietary restriction is believed to have anti-aging and life span extension effects for a large range of organisms ranging from yeast to humans. Unfortunately, this strategy also has some negative effects, such as low fecundity, slowing development and mental problems. Even more importantly, it is hard to follow for a longer period of time. In this study, *Saccorhiza polyschides* extracts were utilized as a diet supplement to treat the model organism *Drosophila melanogaster*. The influences of this algae extract on fly's lifespan, fecundity, circadian rhythm, metabolism and metabolic genes transcription was analyzed. By comparing these parameters of *Saccorhiza polyschides* extracts with those of dietary restriction, the potential of this algae extract to be utilized as a kind of anti-age diet supplement was evaluated. *Saccorhiza polyschides* extract induced life span extensions both, in male and female flies. Unlike DR, this algae extract did not limit fecundity of flies; in contrast, a small amount of this algae extract could promote egg production. Moreover, this algae extract strengthened the fly's stress resistance in drought environment, under high fat diet and in starvation conditions. Locomotor activity was not significantly influenced by this algae extract, nor was the circadian rhythm. Females and males show different phenotypical peculiarities to the treatment of this algae extract. For females, TAG content and body weight were not changed, whereas the hemolymph glucose level was increased. For males, TAG content and hemolymph glucose level were reduced, only the body weight was slightly increased. Food intake and metabolic rate were not altered, both, in females and males. Compared to dietary restriction, *Saccorhiza polyschides* extract changed the physiological parameters in a different way. Algae extracts reduced *tor* and *sirt2* and *egfr*

transcription levels. The transcript level of insulin signaling antagonist *imp-L2* was upregulated. Genetic studies could show that this algae extract acts via the Tor-signaling pathway.

In the second part of this thesis, the role of dietary restriction applied in different temporal regimes was studied. Appropriate reduction of protein intake was beneficial for lifespan extension. Flies treated with low protein diet (DR), showed phenotypical peculiarities including increased locomotor activity, TAG storage, hemolymph glucose level, decreased body weight and metabolic rate. On the other hand, egg production of females was indeed reduced by low protein diet. Insulin-like peptides (ILPs) act as key effectors regulating lifespan, metabolism, growth, development, and reproduction. DR increased total dILP2 content while it decreased circulating dILP2 levels. Expression of Imp-L2, an antagonist of circulating dILP2, was increased by DR. Thus, limitation of circulating dILP2 is one way for dietary restriction to mediate lifespan extension and the phenotypical peculiarities observed here.

Taken together, *Saccorhiza polyschides* extract mediate lifespan extension presumably via the Tor-pathway, which would not reduce fecundity as dietary restriction does. Moreover, *Saccorhiza polyschides* extracts have the potential to be adopted as an anti-age diet supplement.

Zusammenfassung

Das Altern ganzer Populationen gehört zu den größten, weltweiten Herausforderungen, denen sich Gesundheitssysteme stellen müssen. Um dieser Problematik gerecht zu werden, sind Studien, die Mechanismen einer verzögerten Alterung bzw. einer Verlängerung des sog. „Health-spans“ zum Ziel haben notwendig, um altersbedingte Erkrankungen wie z.B. Typ2 Diabetes, Krebs und Kardiovaskuläre Erkrankungen zu bekämpfen. Kalorische Restriktion gehört zu den wenigen natürlichen Interventionen, denen eine Lebensspannen-Verlängerung zugeschrieben werden kann, ein Befund, der von der Hefe bis zum Menschen aufzutreten scheint. Allerdings hat eine lebenslange kalorische Restriktion auch negative Effekte, wie reduzierte Fruchtbarkeit, verlangsamte Entwicklung und auftretende mentale Beschwerden. Außerdem ist es sehr schwierig, eine derartige Diät lebenslang durchzuhalten. In dieser Studie habe ich Extrakte des Sackwurzeltangs *Saccorhiza polyschides* eingesetzt, um ihre Effekte am Beispiel des Modells *Drosophila melanogaster* zu analysieren. *Saccorhiza polyschides* Extrakte verlängerten die Lebensspanne von männlichen und weiblichen Fliegen. Im Gegensatz zu kalorischer Restriktion führte diese Intervention zu keiner reduzierten Fruchtbarkeit. Außerdem konnte die Stressresistenz bei Trockenstress, Hoch-Fett-Diäten und bei Hunger substantiell gesteigert werden. Weder die Bewegungsaktivität noch der zirkadiane Rhythmus wurden beeinflusst. Weibchen zeigten erhöhte Hämolymp-Glukose Spiegel, während Männchen verringerte Hämolymp-Glukose Spiegel sowie einen verringerten Fettgehalt zeigten. Es scheint, dass die Sackwurzeltang-Extrakte die physiologischen Parameter anders beeinflussen als es durch kalorische Restriktion geschieht. Die Transkription der Lebensspannen assoziierten Gene *sirt2*, *tor* und *egfr* wird verringert, während die Transkription des Insulin-Antagonisten *imp-L2* erhöht wird. Mit Hilfe genetischer Studien konnte ich zeigen, dass der Sackwurzeltang über den Tor-Signalweg wirkt.

Im zweiten Teil dieser Studie habe ich die Bedeutung kalorischer Restriktion untersucht, die im Rahmen unterschiedlicher Nahrungsregime eingesetzt wurde. Die kalorische Restriktion war im Modell *Drosophila* Lebensspannen-verlängernd. Die Fliegen zeigten eine Reihe physiologischer Anpassungen, zu denen eine erhöhte Bewegungsrate, ein höherer Fettspeicher, höhere Hämolymp-*Glukose* Spiegel, sowie reduzierte Körpergewichte und geringere metabolische Raten gehörten. Auf der anderen Seite war die Eiproduktion substantiell reduziert. Außerdem erhöhte eine kalorische Restriktion die Konzentration sogenannter Insulin-like peptides (ILPs), während der Hämolymp-Spiegel dieser Insuline reduziert war. Außerdem war die Expression des Insulin-Antagonisten Imp-L2 erhöht, was darauf hinweist, dass die Fliegen ein drastisch reduziertes insulin-*Signaling* zeigen.

Zusammengefasst kann gesagt werden dass Sackwurzeltang-Extrakte eine Lebensspanne-Verlängerung induzieren, die wahrscheinlich über den Tor-Signalweg erfolgt. Das ist nicht assoziiert mit einer verringerten Fruchtbarkeit. Deshalb könnte der Sackwurzeltang-Extrakt in Zukunft als Nahrungsergänzungsmittel zur Verlängerung der Lebens- und/oder Gesundheits-Spanne eingesetzt werden.

Reference

- Abdella, E. M., Mahmoud, A. M., & El-Derby, A. M. (2016). Brown seaweeds protect against azoxymethane-induced hepatic repercussions through up-regulation of peroxisome proliferator-activated receptor gamma and attenuation of oxidative stress. *Pharmaceutical biology*, 1-9.
- Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F. & George, R. A. (2000). The genome sequence of *Drosophila melanogaster*. *Science*, 287(5461), 2185-2195.
- Alekseyenko, T. V., Zhanayeva, S. Y., Venediktova, A. A., Zvyagintseva, T. N., Kuznetsova, T. A., Besednova, N. N., & Korolenko, T. A. (2007). Antitumor and antimetastatic activity of fucoidan, a sulfated polysaccharide isolated from the Okhotsk Sea *Fucus evanescens* brown alga. *Bulletin of experimental biology and medicine*, 143(6), 730-732.
- Alghazeer, R., Whida, F., Abduehrman, E., Gammoudi, F., & Azwai, S. (2013). Screening of antibacterial activity in marine green, red and brown macroalgae from the western coast of Libya. *Natural Science*, 5(01), 7.
- Alhassan Issahaku, P., & Neysmith, S. (2013). Policy implications of population ageing in West Africa. *International Journal of Sociology and Social Policy*, 33(3/4), 186-202.
- Alic, N., Hoddinott, M. P., Vinti, G., & Partridge, L. (2011). Lifespan extension by increased expression of the *Drosophila* homologue of the IGF1P7 tumour suppressor. *Aging cell*, 10(1), 137-147.
- Anderson, R. M., Shanmuganayagam, D., & Weindruch, R. (2009). Caloric restriction and aging: studies in mice and monkeys. *Toxicologic pathology*, 37(1), 47-51.
- Arasaki, S., & Arasaki, T. (1983). Low calorie, high nutrition: vegetables from the sea; to help you look and feel better.

- Atwood, C. S., & Bowen, R. L. (2011). The reproductive-cell cycle theory of aging: an update. *Experimental gerontology*, *46*(2), 100-107.
- Backer, J. M., Myers Jr, M. G., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., P., Margolis, B., Skolnik, E. Y. & Schlessinger, J. (1992). Phosphatidylinositol 3'-kinase is activated by association with IRS-1 during insulin stimulation. *The EMBO journal*, *11*(9), 3469.
- Bai, H., Kang, P., & Tatar, M. (2012). Drosophila insulin-like peptide-6 (DILP6) expression from fat body extends lifespan and represses secretion of Drosophila insulin-like peptide-2 from the brain. *Aging cell*, *11*(6), 978-985.
- Baker, K. D., & Thummel, C. S. (2007). Diabetic larvae and obese flies—emerging studies of metabolism in Drosophila. *Cell metabolism*, *6*(4), 257-266.
- Balaban, R. S., Nemoto, S., & Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell*, *120*(4), 483-495.
- Bass, T. M., Grandison, R. C., Wong, R., Martinez, P., Partridge, L., & Piper, M. D. (2007). Optimization of dietary restriction protocols in Drosophila. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *62*(10), 1071-1081.
- Bauer, J. H., Morris, S. N. S., Chang, C., Flatt, T., Wood, J. G., & Helfand, S. L. (2009). dSir2 and Dmp53 interact to mediate aspects of CR-dependent life span extension in D. melanogaster. *Aging (Albany NY)*, *1*(1), 38.
- Baumann, C. A., Ribon, V., Kanzaki, M., Thurmond, D. C., Mora, S., Shigematsu, S., Bickel, P. E., Pessin, J. E. & Saltiel, A. R. (2000). CAP defines a second signaling pathway required for insulin-stimulated glucose transport. *Nature*, *407*(6801), 202-207.
- Bellen, H. J., Levis, R. W., Liao, G., He, Y., Carlson, J. W., Tsang, G., Evans-Holm, M., Hiesinger, P. R., Schulze, K. L., Rubin, G. M. & Hoskins, R. A. (2004). The BDGP Gene Disruption Project Single Transposon Insertions Associated With 40% of Drosophila Genes. *Genetics*, *167*(2), 761-781.

- Berdyshev, G. D., Korotaev, G. K., Boiarskikh, G. V., & Vaniushin, B. F. (1967). Nucleotide composition of DNA and RNA from somatic tissues of humpback and its changes during spawning. *Biokhimiia (Moscow, Russia)*, 32(5), 988.
- Bernstein, C., Kacelnik, A., & Krebs, J. R. (1991). Individual decisions and the distribution of predators in a patchy environment. II. The influence of travel costs and structure of the environment. *The Journal of Animal Ecology*, 205-225.
- Berteau, O., & Mulloy, B. (2003). Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology*, 13(6), 29R-40R.
- Bjedov, I., Toivonen, J. M., Kerr, F., Slack, C., Jacobson, J., Foley, A., & Partridge, L. (2010). Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*. *Cell metabolism*, 11(1), 35-46.
- Bjorksten, J., & Tenhu, H. (1990). The crosslinking theory of aging—Added evidence. *Experimental gerontology*, 25(2), 91-95.
- Bohovych, I., Kastora, S., Christianson, S., Topil, D., Kim, H., Fangman, T., Zhou, Y. J., Barrientos, A., Lee, J., Brown, A. J. and Khalimonchuk, O. (2016). Oma1 links mitochondrial protein quality control and TOR signaling to modulate physiological plasticity and cellular stress responses. *Molecular and Cellular Biology*, MCB-00156.
- Brachmann, C. B., Sherman, J. M., Devine, S. E., Cameron, E. E., Pillus, L., & Boeke, J. D. (1995). The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. *Genes & development*, 9(23), 2888-2902.
- Brogiolo, W., Stocker, H., Ikeya, T., Rintelen, F., Fernandez, R., & Hafen, E. (2001). An evolutionarily conserved function of the *Drosophila* insulin receptor and insulin-like peptides in growth control. *Current Biology*, 11(4), 213-221.
- Broughton, S., Alic, N., Slack, C., Bass, T., Ikeya, T., Vinti, G., Tommasi, A. M., Drieger, Y., Hafen, E. & Partridge, L. (2008). Reduction of DILP2 in *Drosophila* triages

- a metabolic phenotype from lifespan revealing redundancy and compensation among DILPs. *PloS one*, 3(11), e3721.
- Broughton, S. J., Piper, M. D., Ikeya, T., Bass, T. M., Jacobson, J., Driege, Y., Martinez, P., Hafen, E., Withers, D. J., Leever, S. J. & Partridge, L. (2005). Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. *Proceedings of the National Academy of Sciences of the United States of America*, 102(8), 3105-3110.
- Brown-Borg, H. M., Rakoczy, S. G., Wonderlich, J. A., Rojanathammanee, L., Kopchick, J. J., Armstrong, V., & Raasakka, D. (2014). Growth hormone signaling is necessary for lifespan extension by dietary methionine. *Aging cell*, 13(6), 1019-1027.
- Bruni, J. F., Van Vugt, D., Marshall, S., & Meites, J. (1977). Effects of naloxone, morphine and methionine enkephalin on serum prolactin, luteinizing hormone, follicle stimulating hormone, thyroid stimulating hormone and growth hormone. *Life Sciences*, 21(3), 461-466.
- Butler, M. S. (2004). The role of natural product chemistry in drug discovery. *Journal of natural products*, 67(12), 2141-2153.
- Callegari, A. J. (2016). Does transcription-associated DNA damage limit lifespan?. *DNA repair*, 41, 1-7.
- Chandía, N. P., & Matsuhira, B. (2008). Characterization of a fucoidan from *Lessonia vadosa* (Phaeophyta) and its anticoagulant and elicitor properties. *International Journal of Biological Macromolecules*, 42(3), 235-240.
- Chang, Y. J., Zhou, L., Binari, R., Manoukian, A., Mak, T., McNeill, H., & Stambolic, V. (2016). The Rho Guanine Nucleotide Exchange Factor DRhoGEF2 Is a Genetic Modifier of the PI3K Pathway in *Drosophila*. *PloS one*, 11(3), e0152259.
- Chapman, T., & Partridge, L. (1996). Female fitness in *Drosophila melanogaster*: an interaction between the effect of nutrition and of encounter rate with males. *Proceedings of the Royal Society of London B: Biological Sciences*, 263(1371), 755-759.

- Chen, D., Ahlford, A., Schnorrer, F., Kalchhauser, I., Fellner, M., Viràgh, E., Kiss, I., Syvänen, A. C. & Dickson, B. J. (2008). High-resolution, high-throughput SNP mapping in *Drosophila melanogaster*. *Nature Methods*, 5(4), 323-329.
- Cheng, L. Y., Bailey, A. P., Leever, S. J., Ragan, T. J., Driscoll, P. C., & Gould, A. P. (2011). Anaplastic lymphoma kinase spares organ growth during nutrient restriction in *Drosophila*. *Cell*, 146(3), 435-447.
- Chuks, J. (2010). Population Ageing in Ghana: Research Gaps and the Way Forward. *Journal of Aging Research*, 8.
- Colombani, J., Andersen, D. S., & Léopold, P. (2012). Secreted peptide DILP8 coordinates *Drosophila* tissue growth with developmental timing. *Science*, 336(6081), 582-585.
- Conti, B., Sanchez-Alavez, M., Winsky-Sommerer, R., Morale, M. C., Lucero, J., Brownell, S., Fabre, V., Huitron-Resendiz, S., Henriksen, S., Zorrilla, E.P., & de Lecea, L. (2006). Transgenic mice with a reduced core body temperature have an increased life span. *Science*, 314(5800), 825-828.
- Cragg, G. M., Grothaus, P. G., & Newman, D. J. (2009). Impact of Natural products on developing new anti-cancer agents†. *Chemical reviews*, 109(7), 3012-3043.
- de Sousa, A. P. A., Torres, M. R., Pessoa, C., de Moraes, M. O., Rocha Filho, F. D., Alves, A. P. N. N., & Costa-Lotufo, L. V. (2007). In vivo growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed *Sargassum vulgare*. *Carbohydrate polymers*, 69(1), 7-13.
- del Valle Rodríguez, A., Didiano, D., & Desplan, C. (2012). Power tools for gene expression and clonal analysis in *Drosophila*. *Nature methods*, 9(1), 47-55.
- Diegelmann, S., Bate, M., & Landgraf, M. (2008). Gateway cloning vectors for the LexA-based binary expression system in *Drosophila*. *Fly*, 2(4), 236-239.
- Dietzl, G., Chen, D., Schnorrer, F., Su, K. C., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oettel, S., Scheiblauer, S. & Couto, A. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature*, 448(7150), 151-156.

- Dillin, A., Gottschling, D. E., & Nyström, T. (2014). The good and the bad of being connected: the integrons of aging. *Current opinion in cell biology*, 26, 107-112.
- Djawdan, M., Chippindale, A. K., Rose, M. R., & Bradley, T. J. (1998). Metabolic reserves and evolved stress resistance in *Drosophila melanogaster*. *Physiological and Biochemical Zoology*, 71(5), 584-594.
- Dobashi, Y., Watanabe, Y., Miwa, C., Suzuki, S., & Koyama, S. (2011). Mammalian target of rapamycin: a central node of complex signaling cascades. *Int J Clin Exp Pathol*, 4(5), 476-495.
- Duffy, J. B. (2002). GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *genesis*, 34(1-2), 1-15.
- Elansary, H. O., Skalicka-Woźniak, K., & King, I. W. (2016). Enhancing stress growth traits as well as phytochemical and antioxidant contents of *Spiraea* and *Pittosporum* under seaweed extract treatments. *Plant Physiology and Biochemistry*.
- Emran, S., Yang, M., He, X., Zandveld, J., & Piper, M. D. (2014). Target of rapamycin signalling mediates the lifespan-extending effects of dietary restriction by essential amino acid alteration. *Aging (Albany NY)*, 6(5), 390.
- Enell, L. E., Kapan, N., Söderberg, J. A., Kahsai, L., & Nässel, D. R. (2010). Insulin signaling, lifespan and stress resistance are modulated by metabotropic GABA receptors on insulin producing cells in the brain of *Drosophila*. *PLoS One*, 5(12), e15780.
- Erren, T. C., & Reiter, R. J. (2009). Defining chronodisruption. *Journal of pineal research*, 46(3), 245-247.
- Farnsworth, N. R., Akerele, O., Bingel, A. S., Soejarto, D. D., & Guo, Z. (1985). Medicinal plants in therapy. *Bulletin of the world health organization*, 63(6), 965.
- Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., & Schlessinger, J. (1995). The *Drosophila* insulin receptor homolog: a gene essential for embryonic

- development encodes two receptor isoforms with different signaling potential. *The EMBO journal*, *14*(14), 3373.
- Flatt, T., Min, K. J., D'Alterio, C., Villa-Cuesta, E., Cumbers, J., Lehmann, R., Jones, D. L. & Tatar, M. (2008). *Drosophila* germ-line modulation of insulin signaling and lifespan. *Proceedings of the National Academy of Sciences*, *105*(17), 6368-6373.
- Fontana, L., Partridge, L., & Longo, V. D. (2010). Extending healthy life span—from yeast to humans. *Science*, *328*(5976), 321-326.
- Fontana, L., Villareal, D. T., Das, S. K., Smith, S. R., Meydani, S. N., Pittas, A. G., Klein, S., Bhapkar, M., Rochon, J., Ravussin, E. & Holloszy, J. O. (2016). Effects of 2-year calorie restriction on circulating levels of IGF-1, IGF-binding proteins and cortisol in nonobese men and women: a randomized clinical trial. *Aging cell*, *15*(1), 22-27.
- Frankel, S., Ziafazeli, T., & Rogina, B. (2011). dSir2 and longevity in *Drosophila*. *Experimental gerontology*, *46*(5), 391-396.
- Gäde, G. (2004). Regulation of intermediary metabolism and water balance of insects by neuropeptides. *Annual Reviews in Entomology*, *49*(1), 93-113.
- Garelli, A., Gontijo, A. M., Miguela, V., Caparros, E., & Dominguez, M. (2012). Imaginal discs secrete insulin-like peptide 8 to mediate plasticity of growth and maturation. *Science*, *336*(6081), 579-582.
- Garelli, A., Heredia, F., Casimiro, A. P., Macedo, A., Nunes, C., Garcez, M., Dias, A.R.M., Volonte, Y.A., Uhlmann, T., Caparros, E. & Koyama, T. (2015). DILP8 requires the neuronal relaxin receptor Lgr3 to couple growth to developmental timing. *Nature communications*, *6*.
- Gavrilov, L. A., & Gavrilova, N. S. (2006). Reliability theory of aging and longevity. *Handbook of the Biology of Aging*, *6*, 3-42.
- Géminard, C., Rulifson, E. J., & Léopold, P. (2009). Remote control of insulin secretion by fat cells in *Drosophila*. *Cell metabolism*, *10*(3), 199-207.
- Giniger, E., Varnum, S. M., & Ptashne, M. (1985). Specific DNA binding of GAL4, a positive regulatory protein of yeast. *Cell*, *40*(4), 767-774.

- Giorgetti, S., Ballotti, R., Kowalski-Chauvel, A., Tartare, S., & Van Obberghen, E. (1993). The insulin and insulin-like growth factor-I receptor substrate IRS-1 associates with and activates phosphatidylinositol 3-kinase in vitro. *Journal of Biological Chemistry*, *268*(10), 7358-7364.
- Gnerer, J. P., Venken, K. J., & Dierick, H. A. (2015). Gene-specific cell labeling using MiMIC transposons. *Nucleic acids research*, *43*(8), e56-e56.
- Grandison, R. C., Piper, M. D., & Partridge, L. (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in *Drosophila*. *Nature*, *462*(7276), 1061-1064.
- Gredilla, R., Barja, G., & López-Torres, M. (2001). Effect of short-term caloric restriction on H₂O₂ production and oxidative DNA damage in rat liver mitochondria and location of the free radical source. *Journal of bioenergetics and biomembranes*, *33*(4), 279-287.
- Grönke, S., Clarke, D. F., Broughton, S., Andrews, T. D., & Partridge, L. (2010). Molecular evolution and functional characterization of *Drosophila* insulin-like peptides. *PLoS Genet*, *6*(2), e1000857.
- Guarente, L. P., Partridge, L., Wallace, D. C. (2008). *Molecular Biology of Aging*. New York: Cold Spring Harbor. 347–362.
- Guarente, L., & Picard, F. (2005). Calorie restriction—the SIR2 connection. *Cell*, *120*(4), 473-482.
- Guertin, D. A., & Sabatini, D. M. (2005). An expanding role for mTOR in cancer. *Trends in molecular medicine*, *11*(8), 353-361.
- Gupta, S., & Abu-Ghannam, N. (2011). Bioactive potential and possible health effects of edible brown seaweeds. *Trends in Food Science & Technology*, *22*(6), 315-326.
- Gutierrez, E., Wiggins, D., Fielding, B., & Gould, A. P. (2007). Specialized hepatocyte-like cells regulate *Drosophila* lipid metabolism. *Nature*, *445*(7125), 275-280.
- Haigis, M. C., & Sinclair, D. A. (2010). Mammalian sirtuins: biological insights and disease relevance. *Annual review of pathology*, *5*, 253.

- Hamilton, M. L., Guo, Z., Fuller, C. D., Van Remmen, H., Ward, W. F., Austad, S. N., Troyer, D.A., Thompson, I., & Richardson, A. (2001). A reliable assessment of 8-oxo-2-deoxyguanosine levels in nuclear and mitochondrial DNA using the sodium iodide method to isolate DNA. *Nucleic Acids Research*, *29*(10), 2117-2126.
- Hamilton, M. L., Van Remmen, H., Drake, J. A., Yang, H., Guo, Z. M., Kewitt, K., Walter, C.A., & Richardson, A. (2001). Does oxidative damage to DNA increase with age?. *Proceedings of the National Academy of Sciences*, *98*(18), 10469-10474.
- Harman, D. (1981). The aging process. *Proceedings of the National Academy of Sciences*, *78*(11), 7124-7128.
- Harrison, D. E., & Archer, J. R. (1989). Natural selection for extended longevity from food restriction. *Growth, development, and aging: GDA*, *53*(1-2), 3.
- Hay, N., & Sonenberg, N. (2004). Upstream and downstream of mTOR. *Genes & development*, *18*(16), 1926-1945.
- Heck, M. J., Pehlivanovic, M., Purcell, J. U., Hahn, D. A., & Hatle, J. D. (2016). Life-extending Dietary Restriction Reduces Oxidative Damage of Proteins in Grasshoppers but Does Not Alter Allocation of Ingested Nitrogen to Somatic Tissues. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, glw094.
- Heitman, J., Movva, N. R., & Hall, M. N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science*, *253*(5022), 905-909.
- Hemmingson, J. A., Falshaw, R., Furneaux, R. H., & Thompson, K. (2006). Structure and antiviral activity of the galactofucan sulfates extracted from *Undaria pinnatifida* (Phaeophyta). *Journal of Applied Phycology*, *18*(2), 185-193.
- Hennig, K. M., & Neufeld, T. P. (2002). Inhibition of cellular growth and proliferation by dTOR overexpression in *Drosophila*. *Genesis*, *34*(1-2), 107-110.
- Heininger, K. (2012). The germ-soma conflict theory of aging and death: Obituary to the "evolutionary theories of aging". *WebmedCentral AGING*, *3*.

- Heo, S. J., Ko, S. C., Kang, S. M., Cha, S. H., Lee, S. H., Kang, D. H., Jung, W. K., Affan, A., Oh, C. & Jeon, Y. J. (2010). Inhibitory effect of diphloretohydroxycarmalol on melanogenesis and its protective effect against UV-B radiation-induced cell damage. *Food and Chemical Toxicology*, 48(5), 1355-1361.
- Hildebrandt, A., Bickmeyer, I., & Kühnlein, R. P. (2011). Reliable Drosophila body fat quantification by a coupled colorimetric assay. *PLoS One*, 6(9), e23796.
- Holdt, S. L., & Kraan, S. (2011). Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology*, 23(3), 543-597.
- Honegger, B., Galic, M., Köhler, K., Wittwer, F., Brogiolo, W., Hafen, E., & Stocker, H. (2008). Imp-L2, a putative homolog of vertebrate IGF-binding protein 7, counteracts insulin signaling in Drosophila and is essential for starvation resistance. *Journal of biology*, 7(3), 1.
- Hong, S. H., Lee, K. S., Kwak, S. J., Kim, A. K., Bai, H., Jung, M. S., ... & Yu, K. (2012). Minibrain/Dyrk1a regulates food intake through the Sir2-FOXO-sNPF/NPY pathway in Drosophila and mammals. *PLoS Genet*, 8(8), e1002857.
- Huang, S., Bjornsti, M. A., & Houghton, P. J. (2003). Rapamycins: mechanisms of action and cellular resistance. *Cancer biology & therapy*, 2(3), 222-232.
- Huang, S., & Houghton, P. J. (2001). Mechanisms of resistance to rapamycins. *Drug resistance updates*, 4(6), 378-391.
- Ikeya, T., Galic, M., Belawat, P., Nairz, K., & Hafen, E. (2002). Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in Drosophila. *Current Biology*, 12(15), 1293-1300.
- Imai, S. I., & Guarente, L. (2010). Ten years of NAD-dependent SIR2 family deacetylases: implications for metabolic diseases. *Trends in pharmacological sciences*, 31(5), 212-220.
- Jia, K., Chen, D., & Riddle, D. L. (2004). The TOR pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism and life span. *Development*, 131(16), 3897-3906.

- Jiménez-Escrig, A., Jiménez-Jiménez, I., Pulido, R., & Saura-Calixto, F. (2001). Antioxidant activity of fresh and processed edible seaweeds. *Journal of the Science of Food and Agriculture*, *81*(5), 530-534.
- Jin, K. (2010). Modern biological theories of aging. *Aging and disease*, *1*(2), 72.
- Johnson, S. C., Rabinovitch, P. S., & Kaeberlein, M. (2013). mTOR is a key modulator of ageing and age-related disease. *Nature*, *493*(7432), 338-345.
- Junnila, R. K., List, E. O., Berryman, D. E., Murrey, J. W., & Kopchick, J. J. (2013). The GH/IGF-1 axis in ageing and longevity. *Nature Reviews Endocrinology*, *9*(6), 366-376.
- Kaeberlein, M., Burtner, C. R., & Kennedy, B. K. (2007). Recent developments in yeast aging. *PLoS Genet*, *3*(5), e84.
- Kaeberlein, M., McVey, M., & Guarente, L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes & development*, *13*(19), 2570-2580.
- Kaeberlein, M., & Kennedy, B. K. (2008). Protein translation, 2008. *Aging cell*, *7*(6), 777-782.
- Kapahi, P., Zid, B. M., Harper, T., Koslover, D., Sapin, V., & Benzer, S. (2004). Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway. *Current Biology*, *14*(10), 885-890.
- Kapan, N., Lushchak, V., Luo, J., & Nässel, D. R. (2012). Identified peptidergic neurons in the *Drosophila* brain regulate insulin-producing cells, stress responses and metabolism by coexpressed short neuropeptide F and corazonin. *Cellular and Molecular Life Sciences*, *69*(23), 4051-4066.
- Kaplan, D. D., Zimmermann, G., Suyama, K., Meyer, T., & Scott, M. P. (2008). A nucleostemin family GTPase, NS3, acts in serotonergic neurons to regulate insulin signaling and control body size. *Genes & development*, *22*(14), 1877-1893.
- Kenyon, C. (2005). The plasticity of aging: insights from long-lived mutants. *Cell*, *120*(4), 449-460.

- Khan, M., Varadharaj, S., Shobha, J. C., Naidu, M. U., Parinandi, N. L., Kutala, V. K., & Kuppusamy, P. (2006). C-phycoyanin ameliorates doxorubicin-induced oxidative stress and apoptosis in adult rat cardiomyocytes. *Journal of cardiovascular pharmacology*, *47*(1), 9-20.
- Kim, K. H., Kim, Y. W., Kim, H. B., Lee, B. J., & Lee, D. S. (2006). Anti-apoptotic activity of laminarin polysaccharides and their enzymatically hydrolyzed oligosaccharides from *Laminaria japonica*. *Biotechnology letters*, *28*(6), 439-446.
- Kim, M. S., Kim, J. Y., Choi, W. H., & Lee, S. S. (2008). Effects of seaweed supplementation on blood glucose concentration, lipid profile, and antioxidant enzyme activities in patients with type 2 diabetes mellitus. *Nutrition research and practice*, *2*(2), 62-67.
- Kinghorn, A. D. (2001). Pharmacognosy in the 21st century. *Journal of Pharmacy and Pharmacology*, *53*(2), 135-148.
- Kirkwood, T. B. (1977). Evolution of ageing. *Nature*, *270*(5635), 301-304.
- Kirkwood, T. B., & Rose, M. R. (1991). Evolution of senescence: late survival sacrificed for reproduction. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *332*(1262), 15-24.
- Klass, M. R. (1977). Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span. *Mechanisms of ageing and development*, *6*, 413-429.
- Kong, C. S., Kim, J. A., Yoon, N. Y., & Kim, S. K. (2009). Induction of apoptosis by phloroglucinol derivative from *Ecklonia cava* in MCF-7 human breast cancer cells. *Food and Chemical Toxicology*, *47*(7), 1653-1658.
- Kopchick, J. J., List, E. O., Kelder, B., Gosney, E. S., & Berryman, D. E. (2014). Evaluation of growth hormone (GH) action in mice: discovery of GH receptor antagonists and clinical indications. *Molecular and cellular endocrinology*, *386*(1), 34-45.

- Kumar, C. S., Ganesan, P., Suresh, P. V., & Bhaskar, N. (2008). Seaweeds as a source of nutritionally beneficial compounds-a review. *Journal of Food Science and Technology*, *45*(1), 1-13.
- Kurashige, M., Okimasu, E., Inoue, M., & Utsumi, K. (1989). Inhibition of oxidative injury of biological membranes by astaxanthin. *Physiological chemistry and physics and medical NMR*, *22*(1), 27-38.
- Kwak, S. J., Hong, S. H., Bajracharya, R., Yang, S. Y., Lee, K. S., & Yu, K. (2013). Drosophila adiponectin receptor in insulin producing cells regulates glucose and lipid metabolism by controlling insulin secretion. *PLoS One*, *8*(7), e68641.
- Lapierre, L. R., & Hansen, M. (2012). Lessons from *C. elegans*: signaling pathways for longevity. *Trends in Endocrinology & Metabolism*, *23*(12), 637-644.
- Larson, E. B. (2013). The Body-Mind Connection in Aging and Dementia. *Journal of the American Geriatrics Society*, *61*(7), 1210.
- Laughon, A. L. L. E. N., & Gesteland, R. F. (1984). Primary structure of the *Saccharomyces cerevisiae* GAL4 gene. *Molecular and cellular biology*, *4*(2), 260-267.
- Law, J. H., & Wells, M. A. (1989). Insects as biochemical models. *J. biol. Chem*, *264*(28), 16335-16338.
- Lee, K. S., Kwon, O. Y., Lee, J. H., Kwon, K., Min, K. J., Jung, S. A., ... & Yu, K. (2008). *Drosophila* short neuropeptide F signalling regulates growth by ERK-mediated insulin signalling. *Nature cell biology*, *10*(4), 468-475.
- Lee, Y. S., Shin, K. H., Kim, B. K., & Lee, S. (2004). Anti-Diabetic activities of fucosterol from *Pelvetia siliquosa*. *Archives of pharmacal research*, *27*(11), 1120-1122.
- Lin, K., Dorman, J. B., Rodan, A., & Kenyon, C. (1997). *daf-16*: An HNF-3/forkhead family member that can function to double the life-span of *Caenorhabditis elegans*. *Science*, *278*(5341), 1319-1322.
- Lin, S. J., Defossez, P. A., & Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science*, *289*(5487), 2126-2128.

- Lin, S. J., Kaeberlein, M., Andalis, A. A., Sturtz, L. A., Defossez, P. A., Culotta, V. C., Fink, G.R., & Guarente, L. (2002). Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature*, *418*(6895), 344-348.
- Luo, J., Becnel, J., Nichols, C. D., & Nässel, D. R. (2012). Insulin-producing cells in the brain of adult *Drosophila* are regulated by the serotonin 5-HT1A receptor. *Cellular and Molecular Life Sciences*, *69*(3), 471-484.
- Luong, N., Davies, C. R., Wessells, R. J., Graham, S. M., King, M. T., Veech, R., Bodmer, R. & Oldham, S. M. (2006). Activated FOXO-mediated insulin resistance is blocked by reduction of TOR activity. *Cell metabolism*, *4*(2), 133-142.
- Mikhelson, V., & Gamaley, I. (2012). Telomere shortening is a sole mechanism of aging in mammals. *Current aging science*, *5*(3), 203-208.
- Magwere, T., Chapman, T., & Partridge, L. (2004). Sex differences in the effect of dietary restriction on life span and mortality rates in female and male *Drosophila melanogaster*. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *59*(1), B3-B9.
- Mak, W., Hamid, N., Liu, T., Lu, J., & White, W. L. (2013). Fucoïdan from New Zealand *Undaria pinnatifida*: Monthly variations and determination of antioxidant activities. *Carbohydrate polymers*, *95*(1), 606-614.
- Mak, W., Wang, S. K., Liu, T., Hamid, N., Li, Y., Lu, J., & White, W. L. (2014). Anti-proliferation potential and content of fucoïdan extracted from sporophyll of New Zealand *Undaria pinnatifida*. *Frontiers in nutrition*, *1*.
- Martín-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A. M., & Martínez-Arias, A. (1998). *puckered* encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes & development*, *12*(4), 557-570.
- McAlpine, C. S., Swirski, F. K. (2016). Circadian Influence on Metabolism and Inflammation in Atherosclerosis. *Circulation Research*, *119*(1), 134-141.
- McIsaac, R. S., Lewis, K. N., Gibney, P. A., & Buffenstein, R. (2016). From yeast to human: exploring the comparative biology of methionine restriction in

- extending eukaryotic life span. *Annals of the New York Academy of Sciences*, 1363(1), 155-170.
- Michishita, E., McCord, R. A., Berber, E., Kioi, M., Padilla-Nash, H., Damian, M., Cheung, P., Kusumoto, R., Kawahara, T. L., Barrett, J. C. and Chang, H. Y. (2008). SIRT6 is a histone H3 lysine 9 deacetylase that modulates telomeric chromatin. *Nature*, 452(7186), 492-496.
- Miller, R. A., Buehner, G., Chang, Y., Harper, J. M., Sigler, R., & Smith-Wheelock, M. (2005). Methionine-deficient diet extends mouse lifespan, slows immune and lens aging, alters glucose, T4, IGF-I and insulin levels, and increases hepatocyte MIF levels and stress resistance. *Aging cell*, 4(3), 119-125.
- Mitchell, S. E., Delville, C., Konstantopoulos, P., Derous, D., Green, C. L., Wang, Y., Han, J.J., Promislow, D.E., Douglas, A., Chen, L. and Lusseau, D. (2016). The effects of graded levels of calorie restriction: V. Impact of short term calorie and protein restriction on physical activity in the C57BL/6 mouse. *Oncotarget*.
- Mulinacci, F., Poirier, E., Capelle, M. A., Gurny, R., & Arvinte, T. (2013). Influence of methionine oxidation on the aggregation of recombinant human growth hormone. *European Journal of Pharmaceutics and Biopharmaceutics*, 85(1), 42-52.
- Myers, M. G., Grammer, T. C., Wang, L. M., Sun, X. J., Pierce, J. H., Blenis, J., & White, M. F. (1994). Insulin receptor substrate-1 mediates phosphatidylinositol 3'-kinase and p70S6k signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation. *Journal of Biological Chemistry*, 269(46), 28783-28789.
- Nakagawa, S., Lagisz, M., Hector, K. L., & Spencer, H. G. (2012). Comparative and meta-analytic insights into life extension via dietary restriction. *Aging cell*, 11(3), 401-409.
- Nässel, D. R., & Broeck, J. V. (2016). Insulin/IGF signaling in *Drosophila* and other insects: factors that regulate production, release and post-release action of the insulin-like peptides. *Cellular and Molecular Life Sciences*, 73(2), 271-290.

- Newman, D. J., & Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of natural products*, 75(3), 311-335.
- North, B. J., Marshall, B. L., Borra, M. T., Denu, J. M., & Verdin, E. (2003). The human Sir2 ortholog, SIRT2, is an NAD⁺-dependent tubulin deacetylase. *Molecular cell*, 11(2), 437-444.
- Oldham, S., & Hafen, E. (2003). Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control. *Trends in cell biology*, 13(2), 79-85.
- Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., & Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. *Nature*, 389(6654), 994-999.
- Okamoto, N., Yamanaka, N., Yagi, Y., Nishida, Y., Kataoka, H., O'Connor, M. B., & Mizoguchi, A. (2009). A fat body-derived IGF-like peptide regulates postfeeding growth in *Drosophila*. *Developmental cell*, 17(6), 885-891.
- Oshima, Y. (1982). Regulatory circuits for gene expression: the metabolism of galactose and phosphate. *Cold Spring Harbor Monograph Archive*, 11, 159-180.
- Osterwalder, T., Yoon, K. S., White, B. H., & Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. *Proceedings of the National Academy of Sciences*, 98(22), 12596-12601.
- Owen, O. E., Felig, P., Morgan, A. P., Wahren, J., & Cahill Jr, G. F. (1969). Liver and kidney metabolism during prolonged starvation. *Journal of Clinical Investigation*, 48(3), 574.
- Owen, O. E., Morgan, A. P., Kemp, H. G., Sullivan, J. M., Herrera, M. G., & Cahill Jr, G. F. (1967). Brain metabolism during fasting. *Journal of Clinical Investigation*, 46(10), 1589.
- Paradis, M. E., Couture, P., & Lamarche, B. (2011). A randomised crossover placebo-controlled trial investigating the effect of brown seaweed

- (*Ascophyllum nodosum* and *Fucus vesiculosus*) on postchallenge plasma glucose and insulin levels in men and women. *Applied Physiology, Nutrition, and Metabolism*, 36(6), 913-919.
- Parisi, F., Riccardo, S., Zola, S., Lora, C., Grifoni, D., Brown, L. M., & Bellosta, P. (2013). dMyc expression in the fat body affects DILP2 release and increases the expression of the fat desaturase *Desat1* resulting in organismal growth. *Developmental biology*, 379(1), 64-75.
- Parks, A. L., Cook, K. R., Belvin, M., Dompe, N. A., Fawcett, R., Huppert, K., Tan, L. R., Winter, C. G., Bogart, K. P., Deal, J. E. & Deal-Herr, M. E. (2004). Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. *Nature genetics*, 36(3), 288-292.
- Partridge, L., Piper, M. D., & Mair, W. (2005). Dietary restriction in *Drosophila*. *Mechanisms of ageing and development*, 126(9), 938-950.
- Pasco, M. Y., & Léopold, P. (2012). High sugar-induced insulin resistance in *Drosophila* relies on the lipocalin Neural Lazarillo. *PLoS One*, 7(5), e36583.
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research*, 29(9), e45-e45.
- Piper, M. D., & Bartke, A. (2008). Diet and aging. *Cell metabolism*, 8(2), 99-104.
- Piper, M. D., & Partridge, L. (2007). Dietary restriction in *Drosophila*: delayed aging or experimental artefact?. *PLoS Genet*, 3(4), e57.
- Plaza, M., Cifuentes, A., & Ibáñez, E. (2008). In the search of new functional food ingredients from algae. *Trends in Food Science & Technology*, 19(1), 31-39.
- Potter, C. J., & Luo, L. (2011). Using the Q system in *Drosophila melanogaster*. *nature protocols*, 6(8), 1105-1120.
- Potter, C. J., Tasic, B., Russler, E. V., Liang, L., & Luo, L. (2010). The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. *Cell*, 141(3), 536-548.
- Rajan, A., & Perrimon, N. (2012). *Drosophila* cytokine unpaired 2 regulates physiological homeostasis by remotely controlling insulin secretion. *Cell*, 151(1), 123-137.

- Renehan, A. G., Zwahlen, M., Minder, C., T O'Dwyer, S., Shalet, S. M., & Egger, M. (2004). Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. *The Lancet*, *363*(9418), 1346-1353.
- Robert, L., Labat-Robert, J., & Robert, A. M. (2010). Genetic, epigenetic and posttranslational mechanisms of aging. *Biogerontology*, *11*(4), 387-399.
- Rodrigues, D., Freitas, A. C., Pereira, L., Rocha-Santos, T. A., Vasconcelos, M. W., Roriz, M., Rodríguez-Alcalá, L. M., Gomes, A. M. & Duarte, A. C. (2015). Chemical composition of red, brown and green macroalgae from Buarcos bay in Central West Coast of Portugal. *Food chemistry*, *183*, 197-207.
- Rogina, B., & Helfand, S. L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(45), 15998-16003.
- Rulifson, E. J., Kim, S. K., & Nusse, R. (2002). Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. *Science*, *296*(5570), 1118-1120.
- Saltiel, A. R., & Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*, *414*(6865), 799-806.
- Sato-Miyata, Y., Muramatsu, K., Funakoshi, M., Tsuda, M., & Aigaki, T. (2015). Overexpression of DILP2 causes nutrient-dependent semi-lethality in *Drosophila*. *The Coming of Age of Insulin-Signalling in Insects*, 130.
- Saucedo, L. J., Gao, X., Chiarelli, D. A., Li, L., Pan, D., & Edgar, B. A. (2003). Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nature cell biology*, *5*(6), 566-571.
- Schooley, D. A., Horodyski, F. M., & Coast, G. M. (2012). Hormones controlling homeostasis in insects. *Insect endocrinology*, 366-429.
- Selesniemi, K., Lee, H. J., & Tilly, J. L. (2008). Moderate caloric restriction initiated in rodents during adulthood sustains function of the female reproductive axis into advanced chronological age. *Aging cell*, *7*(5), 622-629.
- Schieber, M., & Chandel, N. S. (2014). ROS function in redox signaling and oxidative stress. *Current Biology*, *24*(10), R453-R462.

- Schulz, T. J., Zarse, K., Voigt, A., Urban, N., Birringer, M., & Ristow, M. (2007). Glucose restriction extends *Caenorhabditis elegans* life span by inducing mitochondrial respiration and increasing oxidative stress. *Cell metabolism*, *6*(4), 280-293.
- Schumpert, C. A., Anderson, C., Dudycha, J. L., & Patel, R. C. (2016). Involvement of *Daphnia pulex* Sir2 in regulating stress response and lifespan. *Aging (Albany NY)*, *8*(2), 402.
- Sekine, O., Love, D. C., Rubenstein, D. S., & Hanover, J. A. (2010). Blocking O-linked GlcNAc cycling in *Drosophila* insulin-producing cells perturbs glucose-insulin homeostasis. *Journal of Biological Chemistry*, *285*(49), 38684-38691.
- Silbering, A. F., Bell, R., Galizia, C. G., & Benton, R. (2012). Calcium imaging of odor-evoked responses in the *Drosophila* antennal lobe. *JoVE (Journal of Visualized Experiments)*, (61), e2976-e2976.
- Sinclair, D. A., & Guarente, L. (1997). Extrachromosomal rDNA circles—a cause of aging in yeast. *Cell*, *91*(7), 1033-1042.
- Skorokhod, A., Gamulin, V., Gundacker, D., Kavsan, V., Muller, I. M., & Muller, W. E. (1999). Origin of insulin receptor-like tyrosine kinases in marine sponges. *The Biological Bulletin*, *197*(2), 198-206.
- Slaidina, M., Delanoue, R., Gronke, S., Partridge, L., & Léopold, P. (2009). A *Drosophila* insulin-like peptide promotes growth during nonfeeding states. *Developmental cell*, *17*(6), 874-884.
- Snare, D. J., Fields, A. M., Snell, T. W., & Kubanek, J. (2013). Lifespan extension of rotifers by treatment with red algal extracts. *Experimental gerontology*, *48*(12), 1420-1427.
- Sohal, R. S., Ku, H. H., Agarwal, S., Forster, M. J., & Lal, H. (1994). Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mechanisms of ageing and development*, *74*(1), 121-133.

- Srivastava, S., & C Haigis, M. (2011). Role of sirtuins and calorie restriction in neuroprotection: implications in Alzheimer's and Parkinson's diseases. *Current pharmaceutical design, 17*(31), 3418-3433.
- St Johnston, D. (2002). The art and design of genetic screens: *Drosophila melanogaster*. *Nature reviews genetics, 3*(3), 176-188.
- Strehler, B. L. (1986). Genetic instability as the primary cause of human aging. *Experimental gerontology, 21*(4), 283-319.
- Sugiura, Y., Matsuda, K., Yamada, Y., Nishikawa, M., Shioya, K., Katsuzaki, H., Imai, K. & AMANO, H. (2007). Anti-allergic phlorotannins from the edible brown alga, *Eisenia arborea*. *Food science and technology research, 13*(1), 54-60.
- Sun, L., Akha, A. A. S., Miller, R. A., & Harper, J. M. (2009). Life-span extension in mice by preweaning food restriction and by methionine restriction in middle age. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences, glp051*.
- Suster, M. L., Seugnet, L., Bate, M., & Sokolowski, M. B. (2004). Refining GAL4-driven transgene expression in *Drosophila* with a GAL80 enhancer-trap. *genesis, 39*(4), 240-245.
- Szendroedi, J., Chmelik, M., Schmid, A. I., Nowotny, P., Brehm, A., Krssak, M., Moser, E. and Roden, M. (2009). Abnormal hepatic energy homeostasis in type 2 diabetes. *Hepatology, 50*(4), 1079-1086.
- Takauji, Y., En, A., Miki, K., Ayusawa, D., & Fujii, M. (2016). Combinatorial effects of continuous protein synthesis, ERK-signaling, and reactive oxygen species on induction of cellular senescence. *Experimental Cell Research*.
- Tatar, M. Senescence. In: Fox CW, Roff, D. A., Fairbairn, D. J., eds. *Evolutionary Ecology: Concepts and Case Studies*. Oxford: Oxford University Press; 2001:128–141.
- Tatar, M. (2006). Diet restriction in *Drosophila melanogaster*. In *Mechanisms of dietary restriction in aging and disease* (Vol. 35, pp. 115-136). Karger Publishers.

- Teleman, A. A. (2010). Molecular mechanisms of metabolic regulation by insulin in *Drosophila*. *Biochemical Journal*, *425*(1), 13-26.
- Thibault, S. T., Singer, M. A., Miyazaki, W. Y., Milash, B., Dompe, N. A., Singh, C. M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H. L. & Ryner, L. (2004). A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. *Nature genetics*, *36*(3), 283-287.
- Tissenbaum, H. A., & Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature*, *410*(6825), 227-230.
- Turek, F. W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., Laposky, A., Losee-Olson, S., Easton, A., Jensen, D. R. and Eckel, R. H. (2005). Obesity and metabolic syndrome in circadian Clock mutant mice. *Science*, *308*(5724), 1043-1045.
- Ugrankar, R., Berglund, E., Akdemir, F., Tran, C., Kim, M. S., Noh, J., Schneider, R., Ebert, B. and Graff, J. M. (2015). *Drosophila* glucone screening identifies Ck1alpha as a regulator of mammalian glucose metabolism. *Nature communications*, *6*.
- Umphred, D., (2012). Neurological rehabilitation (6th ed.). St. Louis, Mo.: Elsevier Mosby. 838.
- Van Herpen, N. A., & Schrauwen-Hinderling, V. B. (2008). Lipid accumulation in non-adipose tissue and lipotoxicity. *Physiology & behavior*, *94*(2), 231-241.
- Varghese, J., Lim, S. F., & Cohen, S. M. (2010). *Drosophila* miR-14 regulates insulin production and metabolism through its target, sugarbabe. *Genes & development*, *24*(24), 2748-2753.
- Veenstra, J. A. (2014). The contribution of the genomes of a termite and a locust to our understanding of insect neuropeptides and neurohormones. *Frontiers in physiology*, *5*, 454.
- Vezina, C., Kudelski, A., & Sehgal, S. N. (1975). Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *The Journal of antibiotics*, *28*(10), 721-726.

- Vilella-Bach, M., Nuzzi, P., Fang, Y., & Chen, J. (1999). The FKBP12-rapamycin-binding domain is required for FKBP12-rapamycin-associated protein kinase activity and G1 progression. *Journal of Biological Chemistry*, 274(7), 4266-4272.
- Wang, H., Arias, E. B., & Cartee, G. D. (2016). Calorie restriction leads to greater Akt2 activity and glucose uptake by insulin-stimulated skeletal muscle from old rats. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, ajpregu-00449.
- Wang, Y., O'Malley, B. W., & Tsai, S. Y. (1994). A regulatory system for use in gene transfer. *Proceedings of the National Academy of Sciences*, 91(17), 8180-8184.
- Weindruch, R., & Walford, R. L. (1988). *Retardation of aging and disease by dietary restriction*. CC Thomas.
- Weiss, E. P., & Fontana, L. (2011). Caloric restriction: powerful protection for the aging heart and vasculature. *American Journal of Physiology-Heart and Circulatory Physiology*, 301(4), H1205-H1219.
- Willcox, B. J., Donlon, T. A., He, Q., Chen, R., Grove, J. S., Yano, K., Masaki, K. H., Willcox, D. C., Rodriguez, B. and Curb, J. D., (2008). FOXO3A genotype is strongly associated with human longevity. *Proceedings of the National Academy of Sciences*, 105(37), 13987-13992.
- William, W. J., Carvalho, G. B., Mak, E. M., Noelle, N., Fang, A. Y., Liong, J. C., ... & Benzer, S. (2007). Prandiology of Drosophila and the CAFE assay. *Proceedings of the National Academy of Sciences*, 104(20), 8253-8256.
- William, W. J., Carvalho, G. B., Zid, B. M., Mak, E. M., Brummel, T., & Benzer, S. (2009). Water-and nutrient-dependent effects of dietary restriction on Drosophila lifespan. *Proceedings of the National Academy of Sciences*, 106(44), 18633-18637.
- Williams, G. C. (1966). Natural selection, the costs of reproduction, and a refinement of Lack's principle. *The American Naturalist*, 100(916), 687-690.

- Williams, G.C., (2001). Pleiotropy, natural selection, and the evolution of senescence. *Science's SAGE KE*, 2001(1), 13.
- Wolf, F. I., Fasanella, S., Tedesco, B., Cavallini, G., Donati, A., Bergamini, E., & Cittadini, A. (2005). Peripheral lymphocyte 8-OHdG levels correlate with age-associated increase of tissue oxidative DNA damage in Sprague–Dawley rats. Protective effects of caloric restriction. *Experimental gerontology*, 40(3), 181-188.
- Worrall, L., Hickson, L.M., (2003). Theoretical foundations of communication disability in aging, 32–33 in Linda E. Worrall & Louise M. Hickson(Eds.). *Communication disability in aging: from prevention to intervention*. Clifton Park, NY: Delmar Learning.
- Wullschleger, S., Loewith, R., & Hall, M. N. (2006). TOR signaling in growth and metabolism. *Cell*, 124(3), 471-484.
- Yakar, S., LeRoith, D., & Brodt, P. (2005). The role of the growth hormone/insulin-like growth factor axis in tumor growth and progression: lessons from animal models. *Cytokine & growth factor reviews*, 16(4), 407-420.
- Yatsenko, A. S., Marrone, A. K., Kucherenko, M. M., & Shcherbata, H. R. (2014). Measurement of metabolic rate in *Drosophila* using respirometry. *JoVE (Journal of Visualized Experiments)*, (88), e51681-e51681.
- Yu, H., & Rohan, T. (2000). Role of the insulin-like growth factor family in cancer development and progression. *Journal of the National Cancer Institute*, 92(18), 1472-1489.
- Yuan, Y. V., & Walsh, N. A. (2006). Antioxidant and antiproliferative activities of extracts from a variety of edible seaweeds. *Food and Chemical Toxicology*, 44(7), 1144-1150.

Appendices

Table A1. Lifespan of protein control medium treatment flies

	Female		
	Mean Lifespan (d)	Maximum Lifespan (d)	n
0.1% YE	34	64	126
0.25% YE	40	79	105
0.5% YE	58	81	105
1% YE	63	83	105
2.5% YE	36	64	103
5% YE	36	67	113

	Male		
	Mean Lifespan (d)	Maximum Lifespan (d)	n
0.1% YE	47	81	105
0.25% YE	68	96	100
0.5% YE	60	90	106
1% YE	51	80	107
2.5% YE	50	66	105
5% YE	46	69	106

Table A2. Lifespan of *Saccorhiza polyschides* extract medium treatment flies

	Female		
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control	65	88	117
0.1% AE	70	97	102
0.5% AE	67	98	127

	Male		
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control	57	80	130
0.1% AE	63	83	110
0.5% AE	64	79	108

Table A3. Lifespan of *Saccorhiza polyschides* extract medium treatment flies in drought environment

Female			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control	52	73	103
0.1% AE	61	73	112
0.5% AE	59	74	123

Male			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control	59	70	128
0.1% AE	61	73	121
0.5% AE	64	73	119

Table A4. Lifespan of *Saccorhiza polyschides* extract medium treatment flies in high fat diet

Female			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control HFD	26	40	141
0.1% AE HFD	31	45	142
Standard -> HFD	27	37	114
0.1% AE -> HFD	37	43	107

Male			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control HFD	25	37	140
0.1% AE HFD	30	40	142
Standard -> HFD	28	39	114
0.1% AE -> HFD	39	44	102

Table A5. Lifespan of *Saccorhiza polyschides* extract medium treatment flies in starvation condition

Female			
	Mean Lifespan (h)	Maximum Lifespan (h)	n
Control	42	80	103
0.1% AE	49	100	104
0.5% AE	51	102	106

Male			
	Mean Lifespan (h)	Maximum Lifespan (h)	n
Control	42	62	108
0.1% AE	44	70	104
0.5% AE	44	68	112

Table A6. Lifespan of *Saccorhiza polyschides* extract medium treatment Tor-mutant flies

Female			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control	86.5	100	116
0.1% AE	84.5	102	120

Male			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control	85	97	114
0.1% AE	87	97	112

Table A7. Lifespan of *Saccorhiza polyschides* extract medium treatment Sirt1-KO flies

Female			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control	51	76	119
0.1% AE	48.5	81	116

Male			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
Control	46	78	110
0.1% AE	48	75	117

Table A8. Lifespan of *Saccorhiza polyschides* extract medium treatment P(GS)106>UAS-Tor flies

Female			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
RU	95	111	142
RU AE	95.5	110	112
EtOH	94	116	112
WT>UAS	101	116	109
Gal4>WT	75	99	110

Male			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
RU	83	107	116
RU AE	85	103	127
EtOH	89.5	130	206
WT > UAS	75	99	107
Gal4 > WT	73	93	105

Table A9. Lifespan of *Saccorhiza polyschides* extract medium treatment P(GS)106>UAS-Tor.TED flies

Female			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
RU	99	112	147
RU AE	94	109	147
EtOH	101	119	166
WT>UAS	88	100	108
Gal4>WT	75	99	110

Male			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
RU	82	108	145
RU AE	76	107	115
EtOH	83	109	175
WT>UAS	79	103	109
Gal4>WT	73	93	105

Table A10. Lifespan of *Saccorhiza polyschides* extract medium treatment P(GS)106>UAS-Sirt2 flies

Female			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
RU	82	102	114
RU AE	60	83	105
EtOH	37	104	205
WT>UAS	80	95	108
Gal4>WT	75	99	110

Male			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
RU	75	96	118
RU AE	78	98	151
EtOH	65	94	152
WT>UAS	64	92	111
Gal4>WT	73	93	105

**Table A11. Lifespan of *Saccorhiza polyschides*
extract medium treatment P(GS)106>UAS-FoxO flies**

Female			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
RU	76	103	118
RU AE	88	103	105
EtOH	93	108	119
WT>UAS	83	99	105
Gal4>WT	75	99	110

Male			
	Mean Lifespan (d)	Maximum Lifespan (d)	n
RU	74.5	110	118
RU AE	76	107	147
EtOH	64	84	121
WT>UAS	67	88	110
Gal4>WT	73	93	105

Acknowledgement

My deepest gratitude goes first and foremost to Prof. Dr. Thomas Roeder, my supervisor, for his constant support and guidance throughout the program. He has walked me through all the stages of performance of the experiments and the writing of this thesis. I appreciate all his valuable ideas and encouragement that made my PhD experience plentiful. I would like to thank Britta Laubenstein and Christiane Sandberg for excellent technical assistance. I deeply appreciate to Dr. Christine Fink, Dr. Julia Hoffmann and Dr. Yong Li for guiding me into the experimental work. And my special thanks go to Dr. Christine Fink for the stuff ordering and organizing. Many thanks also here go to Gu Tian, Yuanyuan Ma who have already been with me for nice office life. Then I am very grateful to all the other friendly members of our department of Zoophysiology I and II for their direct and indirect help to me.

Curriculum vitae

Personal data:

Name: Yang Li

Gender: Male

Data of birth: June 02, 1987

Place of birth: Shandong, China

Marital status: Single

Email: baskerville_cap@hotmail.com

Education:

05.2013-data: PhD at the Institute of Zoophysiology II, Faculty of mathematics and natural science, Christian–Albrechts University, Kiel, Germany. Title of my thesis: “Lifespan extension by nutritional interventions in the fruit fly *Drosophila melanogaster*”.

09.2009-06.2012: M.Sc. in Marine Biology, Department of Marine Biology, Ocean University of China, China. Thesis: “Preparation and application of a kind of novel layer-by-layer alginate bead”.

09.2005-06.2009: B.Sc. in Biology Science, Department of Marine Biology, Ocean University of China, China. Thesis: “The character of tissue expression dynamics of sulfide: Quinone oxidoreductase (SQR) from *Urechisunicinctus* in rich-sulfide environment”.

Publications

- [1] **Yang Li**, Ming Kong, Chao Feng, Wei Fang Liu, Ya Liu, Xiao Jie Cheng*, Xi Guang Chen*. Preparation and property of layer-by-layer alginate hydrogel beads based on multi-phase emulsion technique. *Journal of Sol-Gel Science and Technology*, 62(2012)2: 217-226.
- [2] Chao Feng, Ruixi Song, Guohui Sun, Ming Kong, Zixian Bao, **Yang Li**, Xiaojie Cheng, Dongsu Cha, Hyunjin Park*, Xiguang Chen*. Immobilization of Coacervate Microcapsules in Multilayer Sodium Alginate Beads for Efficient Oral Anticancer Drug Delivery. *Biomacromolecules*, 2014, 15 (3): 985–996.
- [3] Liu Y, Kong M, Yang KK, **Li Y**, Su J, Cheng XJ , Park HJ*, Chen XG*. The safety, cellular uptake and biodistribution of the polymeric amphiphilic nanoparticles as oral drug carriers. *Colloids and surfaces B: Biointerfaces*, 103(2013): 345-353.
- [4] Feng C, Li J, Kong M, Liu Y, Cheng XJ, **Li Y**, Park HJ*, Chen XG*. Surface charge effect on mucoadhesion of chitosan based nanogels for local anti-colorectal cancer drug delivery. *Colloids and surfaces B: Biointerfaces*, 128(2015): 439-447.
- [5] Chao Feng, Zhiguo Wang, Changqing Jiang, Ming Kong, Xuan Zhou, **Yang Li**, Xiaojie Cheng*, Xiguang Chen*. Chitosan/o-carboxymethyl chitosan nanoparticles for efficient and safe oral anticancer drug delivery: In vitro and in vivo evaluation. *Colloids and surfaces B: Biointerfaces*, 103(2013)1: 158-167.
- [6] Wei Fang Liu, Hui Di Zang, Xuan Zhou, Chuan Zhen Kang, **Yang Li**, Jing Li, Qi Feng Dang, Xiao Jie Cheng* , Xi Guang Chen*. The primary culture and subculture of lymphoid cells from shrimp, *Penaeus chinensis* on thermo-sensitive CS/ α , β -GP hydrogel. *Aquaculture Research*, 45(2014)2: 334-340.
- [7] Wei-Fang Liu, Chuan-Zhen Kang, Kong Ming, **Yang Li**, Jing Su, An Yi, Xiao-Jie Cheng*, Xi-Guang Chen*. Controlled release behaviors of Chitosan/ α , β -GP hydrogel-glycerophosphate thermo-sensitive hydrogel. *Frontiers of material science*, 6(2012)3: 250-258

[8] Yong Li, Julia Hoffmann, **Yang Li**, Flora Stephano, Iris Bruchhaus, Christine Fink, Thomas Roeder. Octopamine controls starvation resistance, life span and metabolic traits in *Drosophila*. (submitted)

Declaration

I, Yang Li, here declare that my doctoral thesis entitled “Lifespan extension by nutritional interventions in the fruit fly *Drosophila melanogaster*” and the work presented in it are my own work apart from supervisor’s guidance and design of the paper. This dissertation has not been submitted for the award of doctoral degree to another examining body and was prepared according to the Rules of Good Scientific Practice of the German Research Foundation.

Erklärung

Hiermit erkläre ich, Yang Li, dass diese Dissertation mit dem Titel: “Lifespan extension by nutritional interventions in the fruit fly *Drosophila melanogaster*”, sowie die darin beschriebenen Arbeiten, abgesehen von der Beratung durch meinen akademischen Lehrer, nach Inhalt und Form meine eigene Arbeit ist. Diese Arbeit wurde weder im Ganzen noch zum Teil an anderer Stelle im Rahmen eines Promotionsvorhabens eingereicht. Sie wurde nach den Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft angefertigt.

Kiel,

Yang Li