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MASTERARBEIT

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„Influence of sugar on the fat metabolism and the insulin signaling pathway in *Caenorhabditis elegans*“

verfasst von

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2 INTRODUCTION

In this master thesis that was supported by the “Hochschuljubiläumsstiftung” in the framework of the project “H-293911/2014 Fruktose-induzierte Veränderungen im Fett- und Zuckerstoffwechsel – Metabolische Auswirkungen eines übermäßigen Zuckerkonsums“ the nematode *Caenorhabditis elegans* is used to study the influences of different sugars (glucose, fructose, sucrose, 50:50/glucose:fructose) on fat metabolism and the insulin signaling pathway. *C. elegans* is an ideal model to study these pathways as they are conserved in mammalian organisms.

The study is conducted within the framework of the “fructose project” of the working group “Emerging Focus Nutrigenomics”, which already achieved results concerning the influence of different sugars on gluconeogenesis, stress response and lipogenesis. These results were based on gene expression studies and life span analyses. In brief, they could show that fructose increases the expression of genes of the insulin signaling pathway (*sir-2.1* and *daf-16*). This induced an increased expression of genes of the gluconeogenesis (*pck-1* and *pyc-1*). Furthermore fructose led to a slight increase in oxidative stress resistance including an increased expression of *sod-3*, a gene of stress response. An influence of sugars on two genes of lipogenesis (*sbp-1*, *pod-2*) could not be shown.

These results aroused further interest in investigating the influence of sugars on the insulin signaling pathway and whether the fat metabolism may be influenced by other pathways than the ones observed. Thus gene expression studies of genes of the fat metabolism and insulin signaling pathways are conducted. Furthermore protein levels of SIR-2.1 and DAF-16 are analyzed to see whether they are similarly influenced by sugar than mRNA levels, which were already investigated.

The outcomes of this thesis could provide interesting approaches for further investigations on animals or humans and improve our understanding of effects on sugar ingestion on a molecular basis.

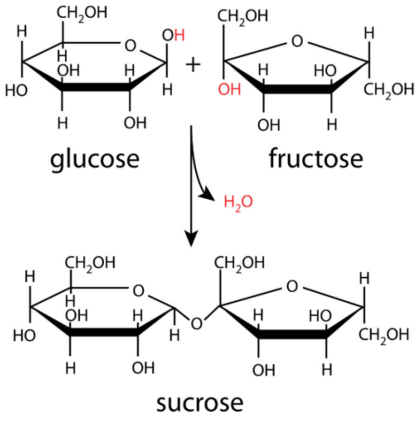
3 THEORETICAL BACKGROUND

3.1 Sucrose, glucose, fructose – what are the differences?

In the human diet sugar is consumed mainly as the disaccharide sucrose, the ordinary table sugar, which consists of the two monosaccharides glucose and fructose in the ratio of 1:1 (Figure 1). During digestion it is cleaved into glucose and fructose.

In the usual human diet pure glucose and pure fructose are not consumed separately in a considerable degree (Rippe 2013). Fructose naturally exists in honey, fruits and some vegetables. Today the relevant amount of ingested fructose originates from commercial products such as soft drinks, sweetened beverages, and high-fructose corn syrup (HFCS) (Kelishadi, Mansourian et al. 2014).

Figure 1: Structure of glucose, fructose and sucrose



3.1.1 Absorption

Fructose and glucose are absorbed from the gastrointestinal tract by different mechanisms (Figure 2).

Fructose is transported across the intestinal epithelium by GLUT-5, or together with glucose by GLUT-2. GLUT-2 is a facilitative transporter for both, fructose and glucose. GLUT-5 is the only transporter that is specific for fructose with no

ability to transport glucose. The expression of GLUT-5 is highest in the small intestine, which regulates fructose absorption from dietary sources and thus the availability of fructose to other tissues.
Across the basolateral membrane of gastrointestinal epithelial cells fructose is transported again by GLUT-5 or GLUT-2. GLUT-2 is also involved in fructose uptake across the hepatic plasma membrane into the liver (Douard and Ferraris 2008).
Glucose is transported into enterocytes by SGLT-1 (Na^+/glucose cotransporter) or GLUT-2 (Kellett and Brot-Laroche 2005). Glucose uptake into cells is mediated by a family of highly related facilitative glucose transporters. Important transporters are for example GLUT-4 that mediates glucose uptake in response to acute insulin stimulation or GLUT-2 (Karnieli and Armoni 2008).

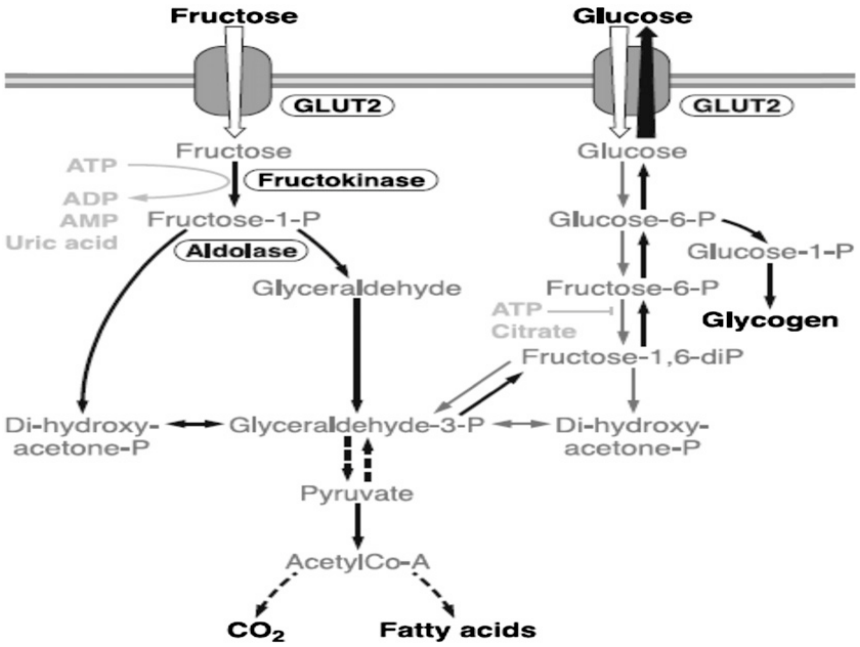
3.1.2 Metabolism in the liver

The liver metabolizes fructose and glucose in a different way (Figure 2).
More than 90% of absorbed fructose are metabolized on first pass by the liver (Rippe 2013). The ability of the liver to extract that much of fructose that passes through it, is due to the high activity of fructokinase. This enzyme phosphorylates fructose to form fructose-1-phosphate. This process bypasses the rate-limiting step in glycolysis, which is catalyzed by phosphofructokinase. Fructose-1-phosphate is split by aldolase B into glyceraldehyde and dihydroxyacetone phosphate. Dihydroxyacetone phoshphate is an intermediate in glycolysis: aldolase B also splits fructose-1,6-bisphospate, which originates from glucose digestion, to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. A triokinase catalyzes the phosphorylation of glyceraldehyde to form glyceraldehyde-3-phosphate, which is another intermediate in glycolysis. Thus, the pathways of glucose and fructose metabolism in the liver are connected at some points (Mayes 1993).
More than 50% of fructose is metabolized into glucose, approximately 15% into glycogen, 25% into lactate and a few percent into carbon dioxide. Various studies show that 1-5% of fructose consumed can be converted into

triglycerides in the process of de novo lipogenesis (Lim, Mietus-Snyder et al. 2010).

Glucose is phosphorylated to glucose-6-phosphate by liver glucokinase, which is the rate limiting enzyme for hepatic glucose utilization. Dependent on the metabolic state, glucose-6-phosphate is further processed in glycolysis or utilized for glycogen synthesis. In glycolysis glucose is metabolized to pyruvate with a net gain of two ATP and two NADH molecules per glucose molecule. In glycogen synthesis glucose-6-phosphate is converted to UDP-glucose. Glycogen synthase converts UDP-glucose into a polymeric chain for storage as glycogen (Bechmann, Hannivoort et al. 2012).

Figure 2: Metabolism of fructose and glucose in the liver (Rippe 2013)

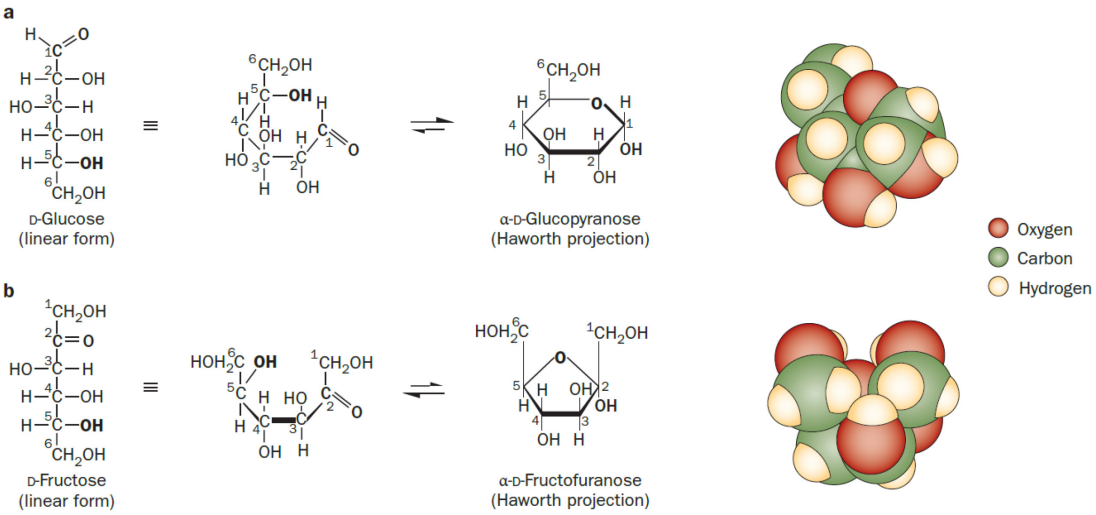


3.1.3 Reactivity

Another difference between glucose and fructose is their favored molecular form and therefore their reactivity. In the human body the majority of fructose exists in the linear form, which has a reactive ketal group. Therefore it is more reactive to proteins than glucose that remains in the ring form which is

molecularly stable and nonreactive (Figure 3). Each protein fructosylation reaction releases a superoxide radical and is more rapid than protein glycation with glucose as the substrate. Fructose generates 100 times more reactive oxygen species (ROS) than glucose (Lim, Mietus-Snyder et al. 2010).

Figure 3: Isomers of glucose and fructose (Lim, Mietus-Snyder et al. 2010)



In the linear form glucose and fructose possess a reactive aldehyde or ketal moity, which can bind to freely available amino groups of proteins. At conditions that are found in the human body, the majority of fructose exists in the linear form and is more reactive to proteins than glucose, which exists mostly in the cyclic isomer state.

3.2 When did we start to sweeten our food with sugar?

3.2.1 The introduction of sugar

Today sugar is consumed with almost every meal. But sugar was introduced into our diet relatively recent. For a long time honey had been the primary sweetener, but as it was relatively rare and not mass produced, the majority of people, especially the poorer ones, had no sweeteners at all in their normal diet. Sugar produced from sugar cane was first developed in New Guinea and India and was introduced into Europe during the Middle Ages. During this time sugar was a luxury article. By the late 1400s the Spanish and Portuguese began

growing sugar cane, e.g. in the Canary Islands. The discovery of the New World lead to expanding sugar production. Sugar plantations were established in the Caribbean islands, Brazil, in the Guiana coasts and also in the Southern United States. Initially sugar was imported to Europe, mainly by Spain and Portugal and later on also England.
Sugar was mainly used as an expensive medicine. The primary diet was based on barley, wheat, oats and rye. But as sugar production increased people´s diet changed. Sugar intake in England was 1.8 kg per capita in 1700 and 8.1 kg in 1800.
England blocked the export of sugar after 1700, which caused a sugar shortage in Europe. Thus techniques were developed to extract sugar from beets. The sugar beet industry was flourishing in Germany, France and Austria by the beginning of the 18th century (Johnson, Segal et al. 2007).

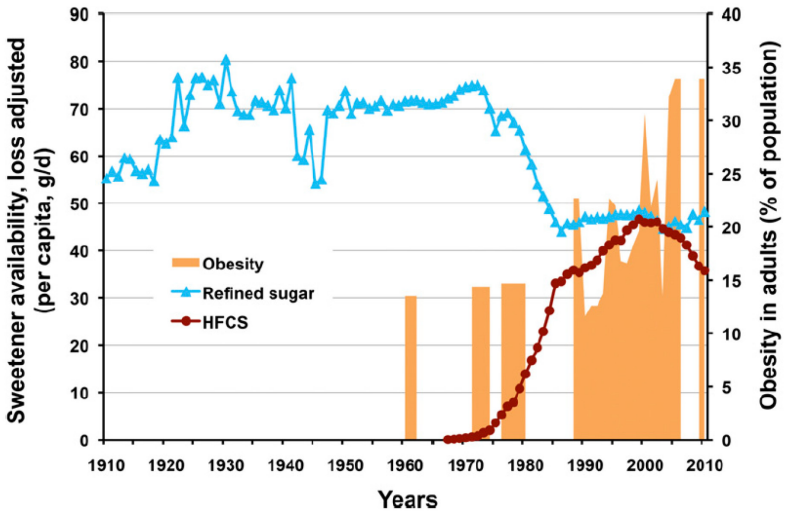
Between 1910 and 1921 sucrose consumption increased by 40% in the US. Then it remained rather constant for more than 50 years, except for the supply interruption during World War II (Figure 4).

3.2.2 The introduction of high fructose corn syrup (HFCS)

In the late 1960s HFCS was introduced to the food and beverage industry as a liquid alternative to sucrose (White 2013). For the production of HFCS starch is extracted from corn and hydrolyzed to monomer glucose. In an isomerization step glucose is converted to fructose. Commercially available HFCS contains 42% (HFCS 42) or 55% (HFCS 55) of fructose (Hanover and White 1993).
Over the next 20 years HFCS rapidly gained market share at the expense of sucrose. It replaced almost half of the available sucrose. There are several reasons for its success: HFCS is similar to the composition, sweetness and caloric content of sucrose, can easily be added to food products, is consistently available and cheaper than sucrose. But it must be pointed out that the use of HFCS peaked in 1999 and has been declined for more than a decade. Use levels of 2010 were the same than in 1989. Notably during this decade of HCFS

decline obesity rates continued to climb (Figure 4). This indicates that there has been no positive association between HFCS and obesity for 13 years. There is equally no association with other diet-related chronic diseases that have increased over the past decade (White 2013).

Figure 4: Historical trends in sucrose and HFCS availability versus rates of obesity in adults in the US (White 2013)



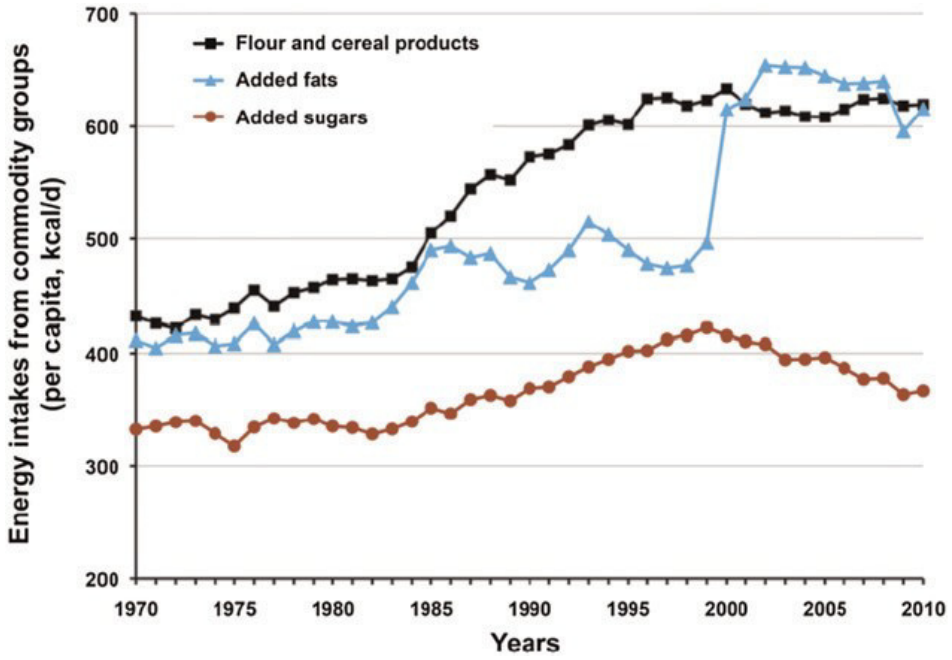
From WHO Global Database on BMI and USDA Economic Research Service per capita consumption data, adjusted for loss.

3.3 How did sugars participate to the rise in consumption of calories?

During the last 4 decades sugar consumption has not augmented disproportionately with the rise in consumption of calories in the modern diet. Conferring to United States Department of Agriculture (USDA) data, mean energy intake in the US increased by 449 kcal/d between 1970 and 2014. Surprisingly increased energy from caloric sweeteners was minor: only 8% of this increase resulted from added sugars. Whereas energy from flour-cereal

products and added fats accounted for the vast majority (nearly 90%) (Figure 5) (Rippe 2013).

Figure 5: Energy intake from commodity groups in the US, 1970-2000 (Rippe 2013)



From USDA Economic Research Service average daily per capita energy intakes from commodity groups, adjusted for loss.

3.4 What about Europe and HFCS?

The United States are the world´s largest producers and consumers of HFCS. In 2002 HCFS had a market share of 43% of the total sugar production, which decreased to 36% in 2011 (in a shrinking total caloric sweetener market). This reflects the decreasing HFCS consumption (Burrel, Himics et al. 2014).

HFCS is an American definition. In Europe the term isoglucose or glucose-fructose syrup refers to a liquid sweetener that consists of mainly glucose and fructose in varying compositions (Sluik, Engelen et al. 2015). Market share of isoglucose is about 5% in the EU, which indicates that the use of isoglucose as

a replacement for sucrose in foods and beverages is not widespread in Europe. This market share reflects the relative size of the production quotas for sucrose and isoglucose and various trade restrictions rather than technical limitations or consumer preferences. As quotas are going to be removed for both products in 2017, there is much speculation about how market shares might change (Burrel, Himics et al. 2014).

3.5 Sugar consumption patterns

The most used sweeteners in the human diet contain fructose and glucose in basically the same proportions: Sucrose and honey contain the same amount of glucose and fructose, HFCS-42 contains 42% fructose and 58% glucose, HFCS-55 55% fructose and 45% glucose. In this context *high* fructose corn syrup seems a poor name because *medium* fructose would be more in line with its composition (White 2013). USDA caloric sweetener availability data between 1966 and 2002 show that glucose is the dominant sugar in our diet. It exceeded fructose in added sugars by 1.3:1 This ratio was unchanged by the introduction of HFCS (Forshee, Storey et al. 2007). Another calculation was done for the period of 1970-2010 by White 2013. Here loss-adjusted per capita commodity group energy availability and specific sugar composition data for all dietary sources of glucose and fructose were used. The glucose-to-fructose ratio was calculated by >5:1. This higher ratio seems more representative for real-world diets, especially if the increasing energy contributions of glucose-based flours and cereal grain products, starches, dextrose, maltodextrins and corn syrups are considered (Figure 5) (White 2013).

3.5.1 How much sugar do we consume?

In the US the total energy from added sugars (i.e. caloric sweeteners added in the processing or preparation of foods and beverages) was 14.6% from 2007-

2008. This indicates a decrease, as it was 18.1% E in 1999-2000 (Welsh, Sharma et al. 2011).

Conferring to the Austrian nutrition report of 2012 the sucrose consumption in adults is within the tolerable range of under 10% E (percentage of total energy). Children consume 11% E but with shrinking tendency, as sucrose intake was 14% E in 1998 (Elmadfa, Hassenegger et al. 2012).

3.5.2 How much fructose do we consume?

Conferring the National Health and Nutrition Examination Survey (NHANES) 1999-2006, mean fructose consumption was 59 g/d (10% E) among adolescents (12-18 y) and 48 g/d (8% E) among adults in the US. But in many non US populations the actual fructose consumption is not clear. Nevertheless there is a worldwide scientific and media attention concerning potential health effects of fructose. A recent Dutch study by Sluik, Engelen et al. (2015) used data from the Dutch national food consumption survey 2007-2010 to estimate the fructose consumption in the Netherlands. In this representative sample of the Dutch population median fructose consumption was 46 g/d (9% E), so slightly lower than most recent figures from the US. Fructose intake was highest among the younger age groups and mainly (about two third) consumed as sucrose. Main food sources of fructose were soft drinks, juices, fruits, cakes and cookies and dairy products (Sluik, Engelen et al. 2015).

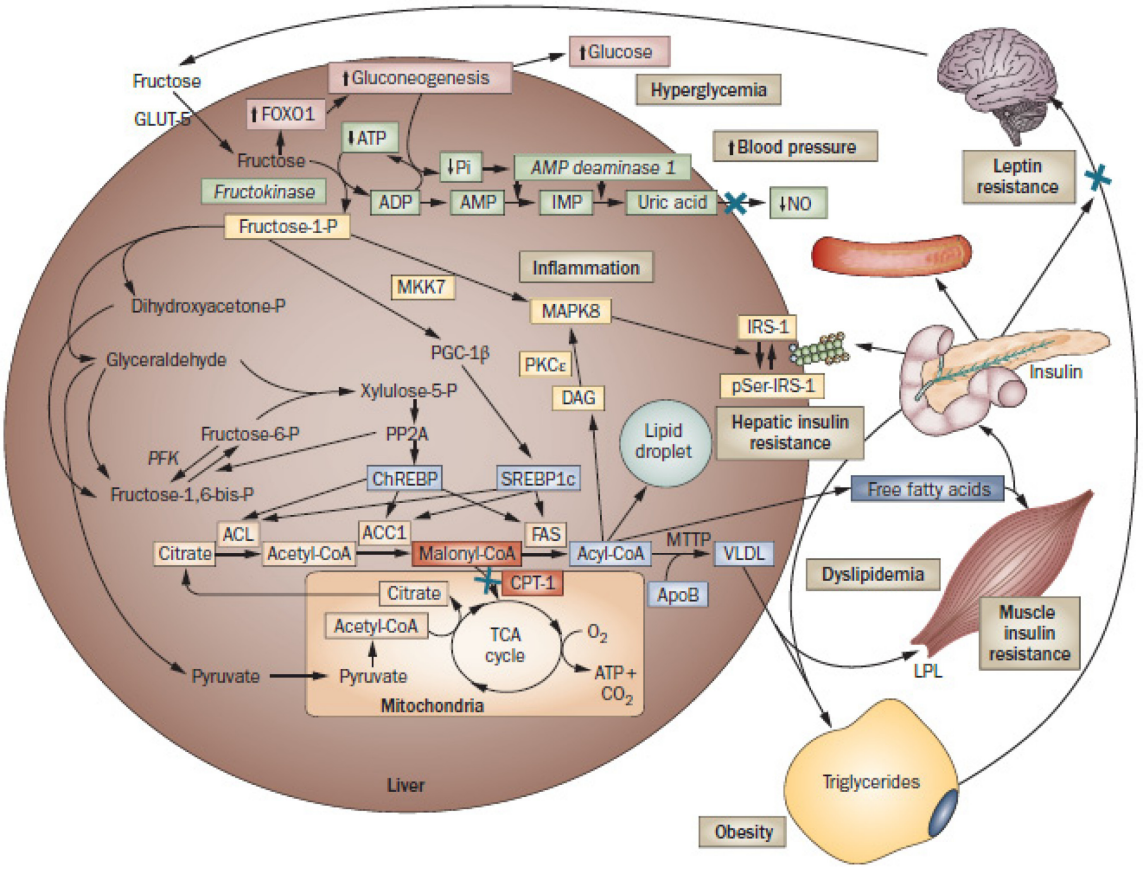
3.6 Does sugar make us sick? Potential detrimental effects of sugar

There are various studies on humans and animals that investigate the association between sugar, especially fructose, and chronic diseases (CVD, hypertension, diabetes, cancer). However, their findings are controversial.
Studies on animals enable us to investigate metabolic pathways that change under sugar feeding. This can shed light on how sugar may contribute to

various diseases. Anyway the drawing of conclusions about influences on a more complex organism like the human body, has to be done with caution. Intervention studies on humans have the disadvantage of limited time as experiments on humans can be conducted only in a short timeframe and not lifelong as in animal models. Epidemiological studies that deal with associations between fructose and disease outcomes, did not find direct causal links. Most of such studies have been cross sectional or based on passive inaccurate surveillance (Rizkalla 2010).

The claimed detrimental effects of fructose are mainly due to its metabolism in the liver which is summarized in Figure 6.

Figure 6: Hepatic fructose metabolism (Lim, Mietus-Snyder et al. 2010)



Fructose induces:

…substrate-dependent phosphate depletion, which rises uric acid and contributes to hypertension through inhibition of endothelial nitric oxide synthase and reduction of NO (green)

…excess formation of citrate that serves as the substrate for de novo lipogenesis (orange)

…excess formation of malonyl-CoA, which prevents β-oxidation (red)

…hepatic lipid droplet formation and steatosis; activation of MAPK8 and PKCε which contributes to serine phosphorylation of IRS-1 and hepatic insulin resistance, which in turn stimulates hyperinsulinemia and influences substrate deposition into fat (yellow)

…export of free fatty acids which leads to VLDL formation and muscle insulin resistance (light blue)

…augmented synthesis of FOXO-1, which promotes gluconeogenesis and hyperglycemia (pink)

…CNS hyperinsulinemia, which antagonizes central leptin signaling and stimulates continued energy intake

In the following some mechanism of how sugars may contribute to the development of chronic diseases are explained.

3.6.1 Nonalcoholic fatty liver disease (NAFLD)

A recent review by Lim, Mietus-Snyder et al. (2010) postulates that fructose may play a role in the pathogenesis of NAFLD and the metabolic syndrome. The underlying mechanisms are explained as follows: NAFLD is characterized by two steps of liver injury (intrahepatic lipid accumulation and inflammatory progression to nonalcoholic steatohepatitis) where fructose is involved in both. In the first step hepatic metabolism of fructose promotes de novo lipogenesis and intrahepatic lipid formation, inhibition of mitochondrial β-oxidation of long-chain fatty acids, triglyceride formation and steatosis, hepatic and skeletal muscle insulin resistance and hyperglycemia. In the second step fructose promotes protein fructosylation and formation of reactive oxygen species (Lim, Mietus-Snyder et al. 2010) (Figure 6).

3.6.2 Blood pressure, serum lipids

A recent systematic review and meta-analysis by Te Morenga, Howatson et al. (2014) examined the effects of the modification of dietary free sugars on blood pressure and fasting lipids. Free-sugars are all monosaccharides and disaccharides added to foods by the manufacturer, cook or consumer plus sugars naturally present in honey, syrups and fruit juices. The authors conclude from their study that higher compared with lower intakes of sugars are associated with increased concentrations of triglycerides, total and LDL cholesterol and blood pressure (for systolic blood pressure this effect was only significant in studies of a longer duration). These observations were seen in studies in which attempts were made to achieve an isocaloric exchange or when no difference in weight change between interventions was reported. Thus, they cannot be explained by altered weight or energy intake. The authors see

the most likely explanation for the effect of free sugars on blood pressure and lipids in the fructose component of sugars (Te Morenga, Howatson et al. 2014).

As illustrated in Figure 6 the effect of fructose on blood pressure is explained by fructose induced substrate-dependent phosphate depletion. This increases uric acid and contributes to hypertension through inhibition of endothelial nitric oxide synthase and reduction of NO, which results in vasoconstriction (Lim, Mietus-Snyder et al. 2010). Uric acid may also directly stimulate the renin-angiotensin system to increase blood pressure (Te Morenga, Howatson et al. 2014).

Another recent systematic review and meta-analysis by Kelishadi, Mansourian et al. (2014) showed that consumption of fructose was inversely associated with an increase in HDL and positively associated with increased triglycerides and systolic blood pressure. But after excluding studies that led to heterogeneity, these adverse effects were not statistically significant (Kelishadi, Mansourian et al. 2014).

3.6.3 Body weight

Te Morenga, Mallard et al. (2013) conducted another review that confirmed the relation between sugar intake and body weight. It is important to mention that the effect of sugars on weight probably results from the extent to which sugar consumption influences energy intake. Isoenergetic replacement of dietary sugar with other macronutrients did not alter weight. It appears that whether sugars are consumed in a liquid (e.g. sugar sweetened beverages) or solid form does not influence these observations. Furthermore these findings propose that energy imbalance is a major cause for dietary sugars to influence body fatness (Te Morenga, Mallard et al. 2013).

However, other less direct mechanisms independent of energy balance have been suggested. Especially fructose is suspected to cause obesity via several different mechanisms. A clinical study found that fructose may not cause the

level of satiety equivalent to that of a glucose-based meal. The mechanism was connected to the inability of fructose to acutely stimulate insulin and leptin and to inhibit ghrelin. All these factors affect the satiety center in the central nervous system (Johnson, Segal et al. 2007).

3.7 Do we really have to be worried? Limitations of studies that predict health outcomes

According to reviews from the annual meeting symposiums of the American Society for Nutrition (ASN) 2012 and 2013, studies that show detrimental effects of sugars should be taken with great caution. They challenge the disrepute of sugars, especially fructose, by comparing normal US levels and patterns of fructose intake with current experimental models and looking for cause-and-effect evidence from real-world diets.

They conclude that fructose intake at normal population levels and patterns do not cause biochemical outcomes significantly different from other dietary sugars. Furthermore extreme experimental models that feature overdosing or significantly alter the typical dietary glucose-to-fructose ratio are not predictive of usual human outcomes.

As mentioned in chapter 3.2.2 during the decade of HFCS decline obesity rates continued to climb, which indicates that there has been no positive association between HFCS and obesity. Furthermore the share in increased energy from caloric sweeteners was minor, whereas energy from flour-cereal products and added fats accounted for the vast majority.

Numerous studies that reported differences in metabolic outcomes compared fructose alone and glucose alone which does not reflect a typical human diet. It namely contains over five times more glucose than fructose. Furthermore human and animal subjects in studies reporting adverse effects are routinely given extreme fructose doses. In many human studies doses exceed even 95th population percentile intakes by 1.5- to 3-fold. Fructose doses given to animals are even more extreme, exceeding the 95th human percentile intake by 4 to 5

times. Fructose overfeeding studies are obviously not physiological and are likely to provoke abnormal metabolism through radical shifts in glucose-to-fructose ratios. They cannot be relied on to assess human health risks (Rippe 2013; White 2013). There are several recent meta-analyses that put human fructose-alone experiments into a dose-dependent perspective:
Isocaloric comparisons of fructose with other carbohydrates (HFCS, sucrose, lactose, starch) were found not to affect weight gain (Sievenpiper, de Souza et al. 2012) or blood pressure (Ha, Sievenpiper et al. 2012). Furthermore there was no effect on uric acid in nondiabetic and diabetic subjects (Wang, Sievenpiper et al. 2012). In the previous mentioned meta-analyses some differences were observed with hypercaloric feeding trials, but that was likely due to confounding from extra calories rather than fructose. Two other meta-analyses by Dolan et al. concluded that fructose does not cause significant changes in triglycerides or body weight when consumed at levels approaching the 95th percentile (Dolan, Potter et al. 2010a; Dolan, Potter et al. 2010b).

Epidemiological studies have low evidentiary value. A review that includes the epidemiological literature, which deals with associations between fructose and various disease outcomes, concluded that they have not established direct causal links. This is because most of such studies have been cross-sectional or based on passive inaccurate surveillance (Rizkalla 2010).

As there are all these conflicting data, it is obvious that there is a need for further trials. Randomized controlled trials exploring real-world conditions in humans are urgently needed. Studies on animal models, e.g. *C. elegans,* can substantially contribute to investigate metabolic effects of sugars as many essential metabolic pathways in mammals are well conserved in animal models.

3.8 What is *Caenorhabditis elegans* and why work on it?

The nematode *Caenorhabditis elegans* is an ideal model to study conserved pathways within a whole animal, because it shares many molecular and cellular mechanisms with mammalian organisms (Imanikia and Stürzenbaum 2013). A comparison of the human and the *C. elegans* genome showed that the majority of human disease pathways and disease genes are present in *C. elegans* (Kaletta and Hengartner 2006).

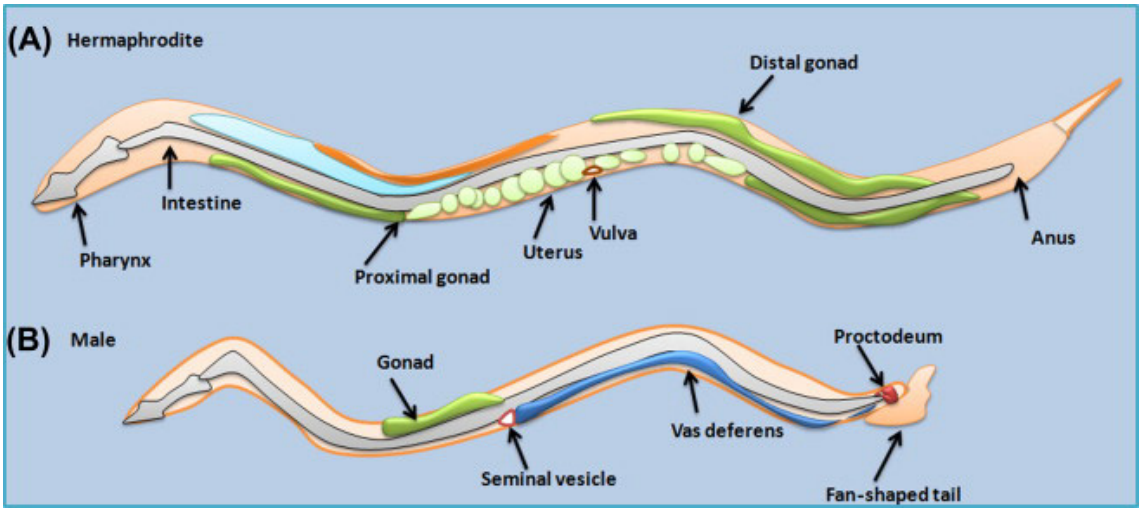
C. elegans is an omnipresent free-living soil-dwelling roundworm that feeds on bacteria. It can be raised easily in the laboratory on a diet of Escherichia coli.

An adult hermaphrodite is about 1 mm in length and produces 250-300 eggs. Less than 0.2% of a wild-type population raised at 20 °C are male. Within 3 days, at 20 °C and food *ad libitum,* the L1 larva grows into a fully developed gravid adult (Imanikia and Stürzenbaum 2013). This short generation time and the high number of produced eggs enable a large-scale production of a very high number of animals per day (Kaletta and Hengartner 2006).

Under optimal conditions the life span varies between 14 and 21 days. Under extreme conditions e.g. stress or food deprivation the L1 larva undergoes a protection phase and enters “dauer”. This stage allows the worm to survive up to 4-5 months. If external conditions improve the dauer worm develops into an L4 worm (Figure 8).

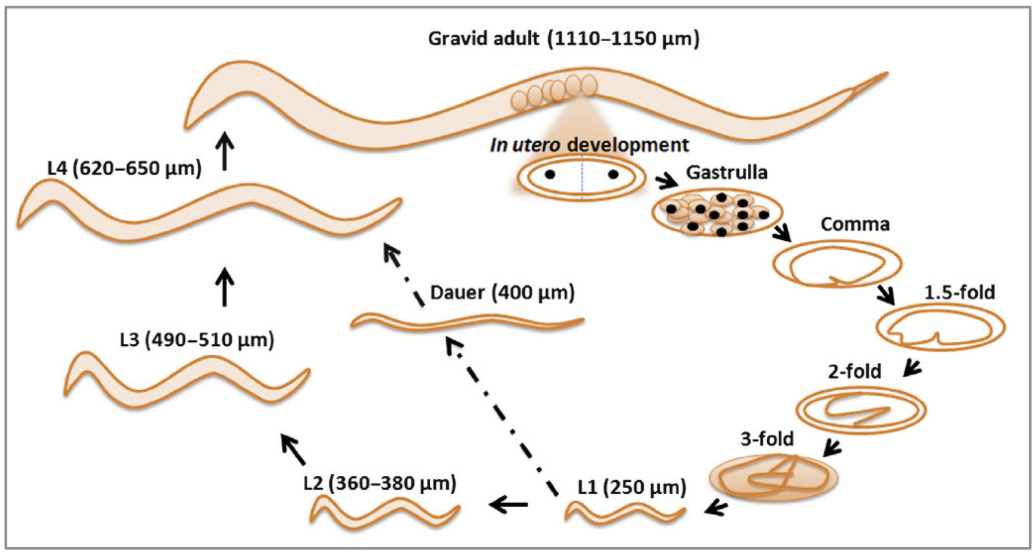
An adult hermaphrodite consists of a defined number of somatic cells (959 of which 302 are neurons and 95 are muscle cells). As an invertebrate *C. elegans* lacks bon tissue and blood but has tissues and organs that are comparable to those of higher organisms e.g. muscle cells, neurons, epidermis and a large gastrointestinal tract that occupies nearly the entire body (Imanikia and Stürzenbaum 2013).

Figure 7: Schematic overview of the hermaphrodite and male *C. elegans* (Imanikia and Stürzenbaum 2013)



(A) The hermaphrodite has two gonads (distal and proximal) and is, in the absence of males, self-fertilizing. The adult is about 1 mm long and consists of 959 cells. (B) The male nematode is smaller and more active (compared to its hermaphroditic counterpart). The posterior end of the male worm comprises a well-defined fan-shaped tail, which is required for the mating process with hermaphrodites. The male *C. elegans* comprises 1031 cells.

Figure 8: *C. elegans* life cycle (Imanikia and Stürzenbaum 2013).



The egg is laid at gastrula stage. When exposed to undesirable conditions, the first larval stage enters “dauer”. This allows the worm to survive up to 4-5 months. When conditions improve, the dauer animal develops into an L4 worm and oogenesis begins. Under ideal growth conditions, the entire life cycle (egg to egg) is completed within 3 days.

3.9 How does *C. elegans* metabolize fat?

Many essential metabolic pathways found in mammals are well conserved in *C. elegans*. These include pathways for fatty acid synthesis, elongation and desaturation and β-oxidation of fatty acids (Mullaney and Ashrafi 2009). Therefore this simple organism has become a popular model for exploring the genetic basis of fatty acid synthesis and regulation of fat storage (Watts 2009).
C. elegans stores fats as triglycerides, similar to higher organisms. Triacylglycerides (TAGs) are stored in lipid droplets or yolk and serve as energy reservoirs. Fat is stored either in intestinal cells or under the skin-like epidermis. TAGs are utilized during food shortage, embryogenesis and in the dauer stage, in which nematodes stop feeding. *C. elegans* contains saturated, monounsaturated and polyunsaturated fatty acids as well as a wide spectrum of elongases and desaturases, which are also found in other animals and plants. This ensures a supply of essential linoleic and linolenic acids. Lipases and peptidases are secreted by intestinal cells. *C. elegans* is cholesterol auxotroph, which means that it requires cholesterol in its food (Imanikia and Stürzenbaum 2013).

In this thesis the influence of sugar consumption on fat metabolism was investigated. Therefore a gene expression study of genes that are involved in lipid metabolism was conducted (Table 1).

Table 1: Orthology of *C. elegans* genes (fat metabolism)

Gene in *C. elegans*	**Human orthologue gene product**	**Function in human**
nhr-49	PPARα	regulation of lipid metabolism
lpd-2	C/EBP	control of adipogenesis
fasn-1	FASN	synthesis of fatty acids
elo-2	ELOVL-5	elongation of PUFA

3.9.1 *nhr-49* (nuclear hormone receptor)

The gene *nhr-49* encodes a nuclear hormone receptor (NHR). In *C. elegans* NHR-49 regulates multiple genes in the fat metabolism. Upon food deprivation it induces β-oxidation genes which promote fat breakdown and conversion to sugars (Pathare, Lin et al. 2012). In fed animals it activates FAT-7, a stearoyl-CoA desaturase (Δ^9D) that converts stearic acid (C18:0) to oleic acid (C18:1n9). Thus *fat-7* ensures that the relative levels of saturated fat do not become too high and prevents excess accumulation of saturated fats (Van Gilst, Hadjivassiliou et al. 2005). Other targets of NHR-49 are sphingolipid processing and lipid remodeling genes (Pathare, Lin et al. 2012). Suppression of *nhr-49* in *C. elegans* results in increased fat stores and shortened life span (Elle, Olsen et al. 2010; Imanikia and Stürzenbaum 2013).

Interestingly, NHR-49 has sequence homology to the human hepatocyte nuclear factor 4 (HNF4) (Brandstadt, Schmeisser et al. 2013). The ligand-binding domain of NHR-49 is structurally similar to that of HNF4 receptor, which can directly bind fatty acids. Despite this homology, *nhr-49* is assumed to act as the functional orthologue of the mammalian “lipid sensing” peroxisome proliferator-activated receptor alpha (PPARα), since it shares most of the biological activities with it (Van Gilst, Hadjivassiliou et al. 2005). After binding to their respective response elements (PPREs), PPARs regulate expression of genes involved in development, metabolism and cellular differentiation. PPARs also influence fat metabolism as they express genes of intra- and extracellular lipid metabolism, especially genes involved in β-oxidation.
PPARα is specialized on the regulation of lipid metabolism. PPARα induces the expression of the liver-X-receptor and ABCA1, a transporter that mediates cholesterol efflux from macrophages. Furthermore it controls adaptive response to caloric restrictions as it is able to activate ketogenesis (Brandstadt, Schmeisser et al. 2013).

3.9.2 *lpd-2* (LiPid Depleted)

lpd-2 is a homolog of the mammalian gene C/EBP (CCAAT/enhancer binding protein transcription factor) that controls mammalian adipogenesis as it regulates the expression of a number of lipogenic enzymes. RNA mediated interference (RNAi) targeting *lpd-2* results in pale, skinny larval arrested worms. These worms lack fat stores and have reduced expression of lipogenic enzymes. The same effects are observed for the mammalian SREBP-1 (sterol regulatory element binding protein) which is a homologue of the *C. elegans sbp-1* (=*lpd-1*). *lpd-2* RNAi changes the expression levels of *sbp-1*, but the contrary is not true. This supports the notion that in worms SREBP functions downstream of C/EBP. This was also proposed for mammals (McKay, McKay et al. 2003).

3.9.3 *fasn-1* (Fatty Acid SyNthase)

fasn-1 encodes a fatty acid synthase that is orthologous to human FAS (Wormbase 2011). FAS is a cytosolic enzyme complex that synthesizes fatty acids of up to 16 carbons (palmitic acid) in length. Synthesis of fatty acids by FAS is initiated by the elongation of a primer (i.e. acetyl) with two carbon units provided from malonyl-CoA and uses NADPH as reductant in the elongation reaction. In order to ultimately produce palmitic acid this reaction has to be repeated seven times (Jakobsson, Westerberg et al. 2006). Upon starvation *fasn-1* transcription is moderately activated in L1 or L2 larvae. The activation is lower in later larvae or adults (Wormbase 2011). *fasn-1* RNAi leads to small and low in fat worms (Pino, Webster et al. 2013).

3.9.4 *elo-2* (fatty acid ELOngation)

elo-2 encodes a palmitic acid elongase that is homologous to polyunsaturated fatty acid (PUFA) elongases such as ELOVL-5 (elongation of very long chain

fatty acid; previously known as HELO-1). ELOVL-5 is required for normal rapid growth, fertility and normal large body size (Wormbase 2011).

ELO-2 is an active enzyme involved in FA elongation. Together with ELO-1 it functions in PUFA biosynthesis. At least one of these enzymes is necessary for C20 PUFA production. *elo-2* is most strongly expressed in intestinal cells, as well as other tissues such as pharyngeal muscles, the ventral nerve cord, tail and uterus. A loss of ELO-2 function results in a significant accumulation of palmitate that directly or indirectly causes multiple phenotypic defects in reproduction, growth and physiological rhythms. *Elo-1*/*elo-2* mutants are unable to synthesize C20 PUFAs and are barley viable (Kniazeva, Sieber et al. 2003).

There are certain transcription factors that regulate elongases as well as desaturases, e.g. SREBP-1 (SBP-1 in *C. elegans*) and PPARα (NHR-49 in *C. elegans)*. Also enzymes such as insulin or leptin, and nutrients such as glucose and fat control the hepatic elongase and desaturase gene expression, fatty acid elongase activity, and lipid composition (Wang, Botolin et al. 2006).

3.10 Insulin signaling pathway in *C. elegans*

Fat metabolism in *C. elegans* is influenced by the insulin/insulin-like growth factor-1 (IGF-1) signaling pathway. Beside fat storage this pathway controls many biological processes such as life span, dauer diapause, reproduction and stress response. This pathway is comprised of many genes including the insulin/IGF-1 receptor (DAF-2) that down-regulates DAF-16, a forkhead transcription factor (FOXO) (Mukhopadhyay, Oh et al. 2006). SIR-2.1 (protein deacetylase) and HCF-1 (transcription regulator) physically associate and antagonize each other to correctly regulate DAF-16-mediated gene expression. The functional relationships among these three proteins are conserved in mammals (Rizki, Iwata et al. 2011). A loss of insulin/insulin like signals (Ils) leads to longevity in *C. elegans* which is at least partly dependent on elevated HSP expression. Possibly Ils leads to enhanced binding of DAF-16 and HSF

(heat shock factor) to promoter regions of HSP-encoding genes (Swindell 2009).

The influence of different sugars on gene expression of *sir-2.1* and *daf-16* was investigated in this working group previously. In this thesis protein levels of SIR-2.1 and DAF-16 were investigated as well as the expression of *hcf-1* and *hsp-1*. The following passages give more detailed information of these four genes/proteins.

Table 2: Orthology of *C. elegans* genes (insulin signaling pathway)

Gene in *C. elegans*	Human orthologue gene product	Function in human
sir-2.1	SIRT-1	protein deacetylase (promotes FOXO activation)
daf-16	FOXO	FOXO transcription factor
hcf-1	HCF-1	transcription regulator (cell cycle progression, histone modification)
hsp-1	Hsp70A	stress response, protein folding

3.10.1 *hcf-1* (host cell factor)

C. elegans hcf-1 encodes the ortholog of human host cell factor (HCF-1). HCF-1 is a transcription regulator that plays a role in cell cycle progression and associates with histone modification. HCF-1 is a protein that is ubiquitously expressed and localizes to the nucleus. In *C. elegans hcf-1* influences the regulation of cell division and mitotic histone modification (Wormbase 2009). HCF-1 associates with DAF-16 in the nucleus and limits its access to a subset of target gene promoters (Rizki, Iwata et al. 2011). Inactivation of *hcf*-1 leads to an increased access of DAF-16 to its target gene promoters and enforces DAF-16-mediated regulation of selective target genes. This probably contributes to the prolonged life span and enhanced stress resistance in *hcf-1* mutants (Li, Ebata et al. 2008).

3.10.2 daf-16 (abnormal DAuer Formation)

DAF-16 is the *C. elegans* FOXO transcription factor that is controlled by the activity of DAF-2 (Murphy 2006). DAF-2 is an ortholog of the mammalian insulin and IGF-1 receptor. DAF-2 represses DAF-16 activity through a phosphorylation cascade including phosphorylation by the AKT protein kinase. Phosphorylation by AKT prevents DAF-16 to translocate into the nucleus and bind to its target genes. Interestingly, phosphorylation by JNK-1 positively influences nuclear translocation of DAF-16 (Mukhopadhyay, Oh et al. 2006).
Under low insulin-like signaling conditions, DAF-16 is not phosphorylated by AKT and accumulates in the nucleus. Nuclear localization of DAF-16 can also be caused by stress. Following stress, SIR-2.1 binds DAF-16 in the nucleus in a 14-3-3-dependent manner. 14-3-3 proteins may promote the interaction between SIR-2.1 and DAF-16. 14-3-3 proteins play another antagonistic role in DAF-16 regulation. Namely they bind to phosphorylated DAF-16 to promote its retention in the cytoplasm (Berdichevsky, Viswanathan et al. 2006).
Upon entering the nucleus, DAF-16 binds to and activates/represses various target genes involved in life span regulation, metabolism, fat storage, dauer formation and stress response. In mammals there are several known “targets” of FOXO including genes involved in the oxidative stress response, DNA repair, cell-cycle arrest and apoptosis. These genes are activated when insulin or IGF-1 levels are reduced. The final outcome of a DAF-16-depentent phenotype depends on a complex regulation of downstream genes (Mukhopadhyay, Oh et al. 2006). However it seems that DAF-16 activation upregulates genes, that contribute to specific protective mechanisms, which may increase life span (Murphy 2006).

3.10.3 *sir-2.1* (silent information regulator)

Sir-2.1 encodes for the ortholog of human SIRT-1. SIRT-1 is an NAD^+-dependent protein deacetylase that promotes forkhead box O (FOXO) activation. In *C. elegans* genetic studies and expression analyses suggest that

sir-2.1 and *daf-16* play overlapping and distinct roles in life span regulation (Wang and Tissenbaum 2006).
Sir-2.1 overexpression extends life span by functioning through the *daf-2* insulin-like signaling pathway. This extension is completely suppressed by mutation in *daf-16*, which indicates that life span extension by overexpression of *sir-2.1* requires *daf-16*. SIR-2.1 is also required for life span extension by caloric restriction which is independent of the insulin/IGF-1 signaling pathway (Mukhopadhyay, Oh et al. 2006).

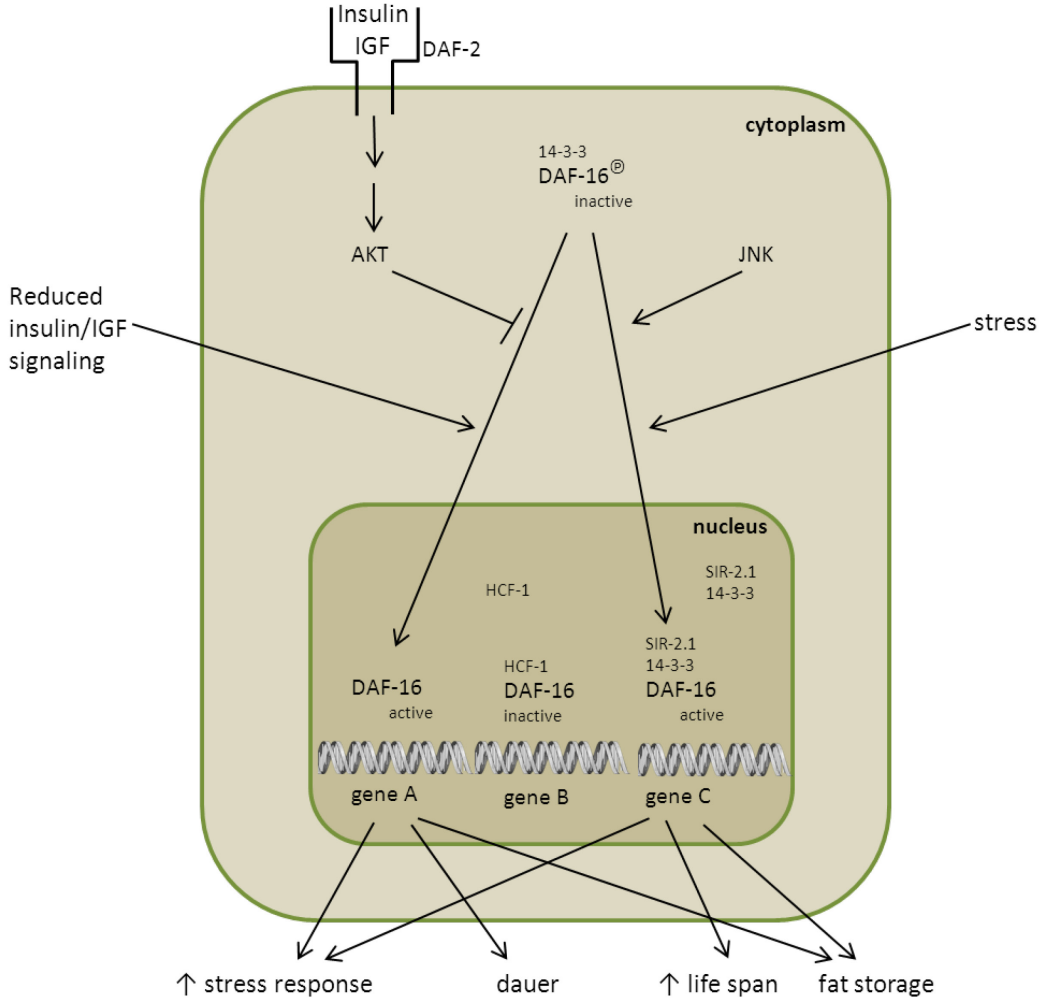
Figure 9 shows the interaction of the explained proteins HCF-1, DAF-16 and SIR-2.1.

3.10.4 *hsp-1* (heat shock protein)

Hsp-1 encodes Hsp70A, a member of the heat shock family of proteins. It is expressed throughout development and upon heat shock its expression is enhanced 2-6 fold. RNAi of *hsp-1* results in a small reduction in the life span of an *age-1* mutant (*age-1* is a crucial component of the insulin signaling pathway in *C. elegans*). This indicates that *hsp-1* may play a role in regulating longevity (WormBase 2004). Indeed, in *C. elegans daf-2* (insulin receptor) mutants, longevity resulting from a loss of insulin signaling is at least partly dependent on elevated *hsp* expression. Overexpression of HSP-encoding genes increases life span in *Drosophila*, as well as *C. elegans*. In *C. elegans*, loss of Ils increases expression of HSPs, possibly by enhancing binding of DAF-16 (FOXO) and HSF (heat shock factor) to promoter regions of HSP-encoding genes. HSPs are critical for recovery from stress and cellular insults, but they also have diverse important functions independent of external stress (e.g. cellular transport, peptide synthesis and modulation of key cell signaling pathways) (Swindell 2009).

Figure 9: Model for the roles of SIR-2.1, HCF-1 and 14-3-3 in DAF-16 regulation of stress resistance, life span and fat storage

adapted from Mukhopadhyay, Oh et al. (2006), Berdichevsky, Viswanathan et al. (2006) and Li, Ebata et al. (2008)



Insulin and IGF bind to DAF-2 receptor and activate a pathway composed of kinases, such as AKT. This pathway negatively regulates DAF-16 by phosphorylation which sequesters it in the cytoplasm. Phosphorylation by JNK positively influences DAF-16 nuclear translocation. Under low insulin-like signaling conditions, DAF-16 is not phosphorylated, dissociates from 14-3-3 and accumulates in the nucleus. In the nucleus, it binds a large number of target promoters directly, either acting as an activator or a repressor of transcription. Therefore it does not need cofactors for activation. But following stress binding to some genes may require additional cofactors such as SIR-2.1. It binds DAF-16 in the nucleus in a 14-3-3–dependent manner. The resulting complex participates in transcriptional activation of DAF-16 target genes. HCF-1 associates with a part of DAF-16 in the nucleus and limits the binding of some DAF-16 to its target gene promoters. The final outcome of a DAF-16-dependent phenotype depends on a complex regulation of the downstream genes.

4 MATERIAL AND METHODS

4.1 Chemicals, consumable material, equipment

Table 3: Chemicals

Chemicals	Manufacturer
Agar-Agar	Roth
Ammonium acetate	Roth
Antibody DAF-16 (ce-300): sc-33738	Santa Cruz Biotechnology
Antibody donkey anti-rabbit IgG (H+L)	Thermo Fisher Scientific
Antibody goat anti-rabbit IgG-HRP: sc-2004	Santa Cruz Biotechnology
Antibody SIR-2.1	Thermo Fisher Scientific
Bromophenol blue sodium salt	Roth
$CaCl_2$ 1M	Roth
Cholesterol	Roth
DEPC H_2O	Invitrogen
Ethanol 96%	Roth
Fructose	Roth
Glucose	Roth
Glycerin	Roth
Glycine	Roth
HCl	Roth
Clarity Western ECL substrate	Bio-Rad
KH_2PO_4	Roth
KOH 5M	Roth
LB Basis	Roth
Mercaptoethanol	Roth
Methanol	Roth
$MgSO_4$ 1M	Roth
Milli-Q H_2O	LS Orbital
Na_2HPO_4	Roth

NaCl	Roth
Sodium hypochlorite	Roth
Nystatin	Roth
Peptone	Roth
Skim milk powder	Heirler
Sodium lauryl sulfate (SDS)	Roth
Sucrose	Roth
TaqMan Gene Expression Master Mix	Life Technologies
TaqMan RT-qPCR Probes	Life Technologies
Tris	Roth
Tween	Roth

Table 4: Consumable material

Consumable material	**Manufacturer**
Amersham ECL gel 10%, 10 wells	GE Healthcare Life Sciences
Cryotubes	Roth
Culture flasks	Sarstedt
Cuvettes disposable (1.5 ml semi-micro)	Brand
Falcon tubes	Sarstedt
Filter paper	Bio-Rad
Glass beads (Ø 325-600 µm)	Sigma-Aldrich
Immun-Blot PVDF membrane (0.2 µm)	Bio-Rad
Insulin syringes (500 µl)	Terumo
Microcentrifuge tubes (1.5 ml)	Eppendorf
Parafilm	American National Can
PCR tube strips with single flat caps	Peqlab
Petri dishes (90 mm)	Roth
Pipette tips (10 µl, 200 µl, 1000 µl)	Sarstedt
QuantiTec Reverse Transcription Kit	Qiagen
RNeasy MiniKit	Qiagen
Syringe filters (21 µm)	Roth

Syringes	Sarstedt
Weighing boats	Roth

Table 5: Equipment

Equipment	**Manufacturer**
Amersham ECL Gel Box	GE Healthcare Life Sciences
Autoclave CV-EL 12L/18L	CertoClav
Beadbeater (precellys 24 lysis & homogenization)	Bertin technologies
Block Heater SBH200D	Stuart
BR4I Multifunction Centrifuge	Jouan
Chemidoc station	Bio-Rad
Magnetic stirrer RCT Basig	IKA
Micro scale MC410S	Sartorius
Microscope CH2	Olympus
Microscope Wild M3C	Wild Heerbrugg
MS1 Minishaker	IKA
Picodrop Picopet01	Picodrop Ltd
Realtime PCR 7300	Applied Biosystems
Table centrifuge Sprout	Biozym
Thermocycler primus 25	Peqlab
Transfer-Blot SD, Semi-dry transfer cell	Bio-Rad
Ultrasonic bath Sonorex Digitec	Bandelin
UV-Visible Spectrometer Cary 50 Scan	Varian
Water bath	GFL

4.2 Bacteria, nematodes

Table 6: Bacteria

Bacteria	Manufacturer
Escherichia coli OP 50	Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, USA)

Table 7: Nematodes

Nematodes	Manufacturer
Bristol N2 (wild type) *Caenorhabditis elegans*	Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, USA)

4.3 Solutions and reagents

All solutions were prepared with Milli-Q H_2O.

Table 8: 10x Phosphate Buffered Saline (PBS)

Reagents	Amount	Preparation
NaCl	80 g	The reagents were dissolved in 800 ml H_2O and pH was adjusted to 7.4 by adding HCl. The volume was brought to 1 l and sterilized by autoclaving.
KCl	2 g	
Na_2HPO_4	14.4 g	
KH_2PO_4	2.72 g	
HCl	as required	

Table 9: 2x SDS-PAGE gel-loading buffer (sample buffer)

Reagents	Amount	Preparation
SDS	2.5% (w/v)	SDS was dissolved in glycerol and Tris-HCl. Before adding bromophenol blue the pH was adjusted to 6.8.
Glycerol	25%	
Tris-HCl	125 mM (pH 6.8)	
bromophenol blue	0.01%	

Table 10: Transfer Buffer

Reagents	Amount	Preparation
Tris base	25 mM	Tris base and glycine were dissolved in H_2O and methanol was added. The pH was adjusted to 8.5.
Glycine	0.2 M	
Methanol	20%	

Table 11: Sugars 5.4%

Reagents	Amount	Preparation
Glucose	5.4%	The sugars were dissolved in H_2O and sterilized by filtration.
Fructose	5.4%	
Sucrose	5.4%	
Glucose/Fructose (50:50)	2.7%/2.7%	

Table 12: Cholesterol

Reagents	Amount	Preparation
Cholesterol	0.5 g	Cholesterol was dissolved in ethanol and stored at – 20 °C.
Ethanol 100%	100 ml	

Table 13: Nystatin (Fungicidin)

Reagents	Amount	Preparation
Ammonium acetate	11.562 g	Ammonium acetate was dissolved in H_2O slowly. Nystatin was dissolved in ethanol and the ammonium acetate solution using a magnetic stirrer at 50 °C.
H_2O	20 ml	
Nystatin	0.4 g	
Ethanol 96%	20 ml	

Table 14: M9 Buffer

Reagents	Amount	Preparation
KH_2PO_4	3 g	KH_2PO_4, Na_2HPO_4 and NaCl were dissolved in 1,000 ml H_2O and autoclaved. Then 1M $MgSO_4$ was added.
Na_2HPO_4	6 g	
NaCl	5 g	
1 M $MgSO_4$	1 ml	

Table 15: Bleach Solution

Reagents	Amount	Preparation
NaOCl	3 ml	NaOCl and 5 M KOH were mixed with H_2O.
5 M KOH	2 ml	
H_2O	20 ml	

Table 16: Freezing Solution for *C. elegans* glycerol stock

Reagents	Amount	Preparation
1 M NaCl	20 ml	1 M NaCl, 1 M KH_2PO_4, glycerol and H_2O were mixed and autoclaved. Then 0.1 M $MgSO_4$ was added. 2.5 ml of this solution were added to 2.5 ml M9 buffer containing L1 larvae. 1 ml aliquots were frozen in cryo tubes at – 80 °C.
1 M KH_2PO_4	10 ml	
Glycerol	60 ml	
H_2O	110 ml	
0.1 M $MgSO_4$	0.6 ml	

Table 17: Potassium-phosphate Buffer (pH= 6.0)

Reagents	Amount	Preparation
KH_2PO_4	108.6 g	KH_2PO_4 and K_2HPO_4 were dissolved in H_2O and autoclaved.
K_2HPO_4	35.28 g	
H_2O	500 ml	

Table 18: Nematode Growth Medium (NGM)

Reagents	Amount	Preparation
NaCl	3 g	NaCl, peptone and agar-agar were weighed out and brought to 1,000 ml. Then it was autoclaved and cooled down to 60 °C. 1 M $CaCl_2$, 1 M $MgSO_4$, potassium-phosphate buffer, nystatin and cholesterol were added. The medium was poured into 90 mm petri dishes for nematode cultivation.
Peptone	2.5 g	
Agar-agar	8.5 g	
1 M $CaCl_2$	0.5 ml	
1 M $MgSO_4$	1 ml	
Potassium-phosphate buffer	25 ml	
Nystatin	3 ml	
Cholesterol	1 ml	

Table 19: LB Medium

Reagents	Amount	Preparation
LB basis	20 g	LB basis was dissolved in H_2O and autoclaved.
H_2O	500 ml	

4.4 Methods

4.4.1 Handling of bacteria

Escherichia coli (E. Coli) OP50 were used as a food source for *C. elegans*. A starter culture of *E. coli* OP50 was obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, USA) and stored as glycerol stocks at – 80 °C. For bacterial growth bacteria were removed from the glycerol stock using an inoculating loop. They were transferred into a falcon tube containing 5-10 mL LB medium (Table 19) and inoculated overnight at 37 °C. Photometric measurement at 600 nm was used to control bacterial growth. If OD_{600} was between 0.7 and 0.9 100 µl bacteria culture were pipetted on each NGM plate and spread using a Drigalski spatula. At higher OD_{600} levels the bacteria culture was diluted with fresh LB medium until OD_{600} decreased to 0.7-0.9. To avoid contamination with other bacteria the seeding was performed under a disinfected hood. Furthermore the Drigalski spatula was flamed before and after spreading the bacteria and stored in 20% ethanol in between. Seeded plates were dried in the hood for 30 minutes. If stored at 4 °C they are usable for several weeks.

4.4.2 Nematodes and culture conditions

Bristol N2 (wild type) *C. elegans* were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, USA). These strains were grown on nematode growth medium (NGM)-plates (90 mm diameter) (Table 18) in a 20 °C incubator. To provide a natural food source the NGM-plates were seeded with *E. coli* strain OP50.
For long time storage at -80 °C *C. elegans* glycerol stocks were prepared (Table 16). Before usage, worms of the glycerol stock were pipetted on NGM plates. After incubating them overnight worms were washed with M9 buffer to remove the glycerol. To make sure there is no impairment of the worms due to glycerol storage, they were allowed to reproduce for some generations before used for

experimental treatment. Therefore a few of them were transferred onto new plates every 2-4 days. For the transfer a sterilized scalpel was used to move a chunk of agar, upside-down, from an old plate to a fresh plate. The worms were crawling off the chunk and spreading onto the bacterial lawn of the new plate.

4.4.2.1 Egg preparation

Egg preparation was used to attain a large synchronized population of worms. This is desirable for gene expression studies as gene expression is influenced by the age of nematodes. For egg preparation 2-3 90 mm plates with many young adults that have already started to lay eggs were used. Worms were washed off the plates with 2.5 ml M9 buffer (Table 14) 2 times and transferred into a 15 ml falcon tube. For 10-20 min the tube was placed on ice to let the worms settle. To remove bacteria from the solution the supernatant was discarded. 5 ml M9 buffer were added to the worm pellet before centrifuging for 2 min (3,000 rpm, 4 °C). If the supernatant was still not clear this step was repeated. To bleach all worms that were not protected by the egg shell 7 ml of bleach solution (Table 15) were added and the tube was vortexed 12-15 min. After centrifuging again the bleach solution was removed. Four washing steps with 5 ml M9 buffer each followed to ensure there is no bleach solution left. In the end the worm pellet was dissolved in 10 ml M9 buffer and transferred into a culture flask. Larvae were allowed to hatch overnight to 2 days at 20 °C in the shaking culture flask.

The synchronized larvae were transferred into a 15 ml falcon tube again, centrifuged and the supernatant was discarded. The remaining worm pellet was dissolved with slightly shaking the tube. These L1-larvae were distributed equally to the NGM plates prepared with different kind of sugars (Table 11).

4.4.2.2 Sugar supplementation

To investigate any influences of sugar consumption on gene expression and protein synthesis worms were fed with different kind of sugars at a concentration of 5.4% (Table 11). A control group received only the bacteria as a standard food source and no sugar. The sugar solutions were sterilized by filtration. Under a disinfected hood 300 µl were pipetted onto the OP50 seeded plates and dried. The L1-larvae, obtained from egg preparation, were distributed equally to the plates. Larvae lived on the sugar plates for 50 hours at 20 °C. The obtained L4-larvea/young adults were used for all experimental treatments.

4.4.3 Gene expression analysis

To analyze gene expression RNA of the different fed worms was purified and extracted. In order to amplify the single stranded mRNA using real-time qPCR (RT-qPCR) it was transcribed to double stranded cDNA.

4.4.3.1 Preparation of worm extracts

Worms were washed off the sugar NGM plates 2 times using 2.5 ml M9 buffer and transferred into a 15 ml falcon tube. For 10-20 min the tube was placed on ice to let the worms settle. To remove bacteria from the solution the supernatant was discarded. 5 ml M9 buffer were added to the worm pellet before centrifuging for 2 min (3,000 rpm, 4 °C). This washing step was repeated 2 times and worm pellets were snap frozen in liquid nitrogen immediately. Snap freezing is necessary to achieve proper cell disruption. After this step worms can be stored at – 20 °C until usage.

4.4.3.2 RNA purification and extraction

For total RNA Extraction RNeasy MiniKit (Qiagen) was used.

Worms were thawed on ice, 500 µl buffer RLT were added and transferred into a cryotube containing glass beads. As a stabilizer 10 µl β-Mercaptoethanol per 1 ml buffer RLT were added before use. A bead beater was used 3 times for 20 s (5500 rpm). During bead-beating the beads collide with the sample and lyse and homogenize the worms. The samples were placed on ice between each bead-beating interval for 1 min. This is necessary to prevent damage to the heat sensitive RNA.

After bead-beating the cryotube was centrifuged at full speed (12,000 rpm) for 3 min. The supernatant was transferred into a new microcentrifuge tube using an insulin syringe. 200 µl buffer RLT were added to the beads in the cryotube, vortexed and centrifuged again full speed for 1 min. This was done to avoid RNA leftovers in the cryotube. The obtained supernatant was mixed with the other supernatant and centrifuged again full speed for 1 min. Then 500 µl of ethanol were added.

Up to 700 µl of the samples, including any precipitates that may have formed, were transferred to an RNeasy spin column placed in a 2 ml collection tube. The tube was centrifuged 15 s (10,000 rpm) and the flow through was discarded (RNA was hold back by the membrane of the column).

700 µl buffer RW1 were added to the spin column and centrifuged (15 s, 10,000 rpm). Again the flow through was discarded. 500 µl buffer RPE were added and centrifuged (15 s, 10,000 rpm) and the flow through was discarded. Again 500 µl buffer RPE were added but centrifuged longer (2 min) in order to dry the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions. The flow through was discarded.

Then the spin column was placed in a new 2 ml collection tube and centrifuged full speed for 1 min. This step eliminates any possible carryover of buffer RPE, or if residual flow-through remained on the outside of RNeasy spin column after the previous step.

For RNA elution the spin column was placed in a new 1.5 ml collection tube. 30 µl Rnase-free water were pipetted directly to the spin column membrane and centrifuged (1 min, 10,000 rpm). The eluate contained purified RNA.

RNA concentrations were measured using a Picodrop and lied between 100-500 ng/µl.

4.4.3.3 Reverse transcription

To amplify single stranded RNA using PCR it is crucial to transcribe it to double stranded cDNA. Furthermore cDNA is more stable than RNA and therefore more suitable for long term storage.
Reverse transcription was done using a QuantiTect Reverse Transcription Kit (Qiagen). This kit contains reverse transcriptase, the enzyme that allows transcription from RNA to cDNA. 1 µg RNA and 2 µl gDNA wipeout buffer were used for reverse transcription. The total reaction volume of 14 µl was reached by adding the appropriate volume of RNase-free water. This solution was incubated for 2 min at 42 °C and placed on ice immediately. Then a master mix containing 1 µl RT primer mix, 4 µl quantiscript RT buffer and 1 µl quantiscript reverse transcriptase was added. The master mix was prepared on ice and the reverse transcriptase added in the very end, as it is a very thermolabile enzyme. The tube with a final volume of 20 µl was placed in a thermocycler and incubated for 15 min at 42 °C and for 3 min at 95 °C (to inactivate quantiscript reverse transcription). The received cDNA was used for RT-qPCR immediately or stored at – 20 °C before use.

4.4.3.4 Real Time qPCR

To achieve a relative quantification of mRNA levels of the treated worms relative to control worms a RT-qPCR was performed. mRNA levels of one gene were normalized to housekeeping genes and to mRNA levels of the same gene in the control group. This was calculated by the ddCt-method (relative quantification method) following the equation of the Qgene software REST-384.

The stability of all housekeeping genes was checked by BestKeeper software routinely. All measurements were done in duplicates with 3-9 independent experiments for each condition.

The genes of interest were the following: *nhr-49* (nuclear hormone receptor), *lpd-2* (LiPid Depleted), *fasn-1* (Fatty Acid SyNthase), *elo-2* (fatty acid ELOngation), *hsp-1* (heat shock protein) and *hcf-1* (host cell factor). As housekeeping genes *pmp-3* (peroxisomal membrane protein related), *csq-1* (CalSeQuestrin) and *Y45F10D.4* (iron-sulfur cluster assembly enzyme) were used. For all genes TaqMan probes were used.

All working steps were performed under a disinfected hood and, whenever possible, on ice.
Before utilization the cDNA was diluted 1:2.5 with DEPC H_2O. On the one hand this ensures enough volume for the RT-qPCR, on the other hand it leads to an optimal cDNA-concentration to achieve a high resolution of the measurement.
The master mix contained 12.5 µl TaqMan Gene Expression Master Mix, 8.75 µl DEPC H_2O and 1.25 µl TaqMan probe. For each probe it was prepared 12 times (2x5 treatments, 1 no template control, 1 backup).
The no template control (NTC) contained only the master mix and no cDNA. It served as a control of the purity of master mix and probes. Furthermore it was the blank value for calculations with the PCR software.
22.5 µl master mix and 2.0 µl cDNA were pipetted into PCR tube strips with single flat caps according to Table 20.

Table 20: Scheme of pipetting (example)

Gene	**Treatment**										
	CTL	CTL	GLU	GLU	FRU	FRU	SUC	SUC	50/50	50/50	NTC
pmp-3											
csq-1											
Y45F											
elo-2											
fasn-1											
nhr-49											
lpd-2											
hsp-1											

Before inserting the tube strips into the heat cycler they were centrifuged. This removes air from the liquid and merges cDNA and master mix.

The PCR studies were carried out on an Applied Biosystems 7300 Real-time PCR System. The relative quantification method (ddCt-method) was chosen and the scheme of pipetting was entered. The temperature was optimized for the probes to reach their plateau phase. The program concludes the following phases: initiation (50 °C, 2 min, 93 °C, 5 min – activation of the hot start polymerase), denaturation (93 °C, 15 s), annealing and elongation (60 °C, 1 min). The number of cycles was 49 and sample volume 25 µl.
After running the program the Ct (cycle threshold) values were calculated by the Applied Biosystems 7300 software and exported as an Excel file for further calculations.

4.4.4 Western blot analysis

Western blot analysis was conducted to see whether mRNA products, the proteins, are influenced by sugar consumption in a similar way.

4.4.4.1 Preparation of worm extracts

Worms were washed off the sugar NGM plates and prepared for freezing in liquid nitrogen as described in chapter 4.4.3.1. After thawing the samples on ice 100 µl 2x sample buffer (blue) (Table 9) were added. To release the proteins from the cell the membrane was disrupted by sonicating the samples for 2 min. Then they were put into a heating block at 95 °C for 5 min to inhibit proteases.

4.4.4.2 SDS PAGE

For gel electrophoreses a 10% Amershan ECL gel was used. 90 ml of 1x running buffer was added to each tank of the Amersham ECL Gel Box. The gel cassette was rinsed with distilled water (d.H_2O) and placed in the gel box so that the well side of the cassette faced toward the cathode and the other cassette leg faced toward the anode. After placing the safety lid on top of the gel box it was connected to the power supply and pre-ran for 12 min in 160 V. After the pre-run the comb had to be removed carefully to make the wells available for sample loading. Before loading, 6 ml of 1x running buffer were added to the well container. 20 µl marker and 40 µl of each sample were loaded into the wells. The gel was run 60 min in 160 V. To remove the gel from the cassette the edge of the comb was inserted in the slot opposite the sample wells and twisted. The top plate from the gel cassette was removed and the gel could be cut out and removed from the cassette.

4.4.4.3 Coomassie staining

For Coomassie staining one gel was incubated in staining solution for 30 min then another 30 min in de-staining solution and in the end in d.H_2O overnight. Coomassie dye binds to proteins through ionic interactions between dye sulfonic acid groups and positive protein amine groups as well as through Van der Waals attractions (Merril 1990). It was used to check whether the same amount of protein was used for each sample. This is essential to make the results of immunoblotting comparable.

4.4.4.4 Immunoblotting

Another gel was used for immunoblotting. In this step the protein extracts from the gel were transferred onto a polyvinylidene diflourid (PVDF) membrane. This transfer was done using an electric field oriented vertical to the surface of the gel.

Therefore the PVDF membrane was equilibrated in methanol for 1 min. Then the gel, PVDF membrane and 2 filters were soaked in transfer buffer (Table 10) for 20 min. For the semidry protein transfer filter, membrane, gel and another filter were laid on top of each other. This transfer sandwich was built on a transfer apparatus. Transfer was done 12 min in 15 V. Then the membrane was washed with d.H_2O and a washing buffer (PBST, phosphate buffered saline + tween).

4.4.4.5 Blocking and antibody incubation

The membrane was blocked with 5% milk (skim milk powder) in PBST for 1 hour. Blocking is an important step of western blotting, as it prevents antibodies from binding to the membrane nonspecifically (Mahmood and Yang 2012).

The 1st antibody (Table 21) in 5% milk in PBST was added afterwards and incubated at 4 °C on a shaker overnight. The next day the membrane was

washed 3-5 times with PBST and the 2nd antibody (Table 21) in 5% milk in PBST was added for 2 hours at room temperature. Again 3-5 washing steps followed. Washing is essential as it minimizes background and removes unbound antibody. However, the membrane should not be left in the washing solution a long time, as it can also reduce the signal.

Table 21: Antibody dilutions

		Antibody	**Dilution (in 5% milk in PBST)**	**Incubation time, temperature**
DAF-16	1st antibody	Antibody DAF-16 (ce-300): sc-33738	1:500	o/n, 4 °C
	2nd antibody	Antibody donkey anti-rabbit IgG (H+L)	1:5.000	2 h, RT
SIR-2.1	1st antibody	Antibody SIR-2.1	1:2.000	o/n, 4 °C
	2nd antibody	Antibody goat anti-rabbit IgG-HRP: sc-2004	1:400	2 h, RT

Before detection Clarity Western ECL substrate was added to the membrane and incubated for 2 min. This substrate is detected by the signal it produces corresponding to the position of the target protein. This signal was captured using a Bio-Rad Chemidoc station.

5 RESULTS

5.1 Gene expression data from RT-qPCR

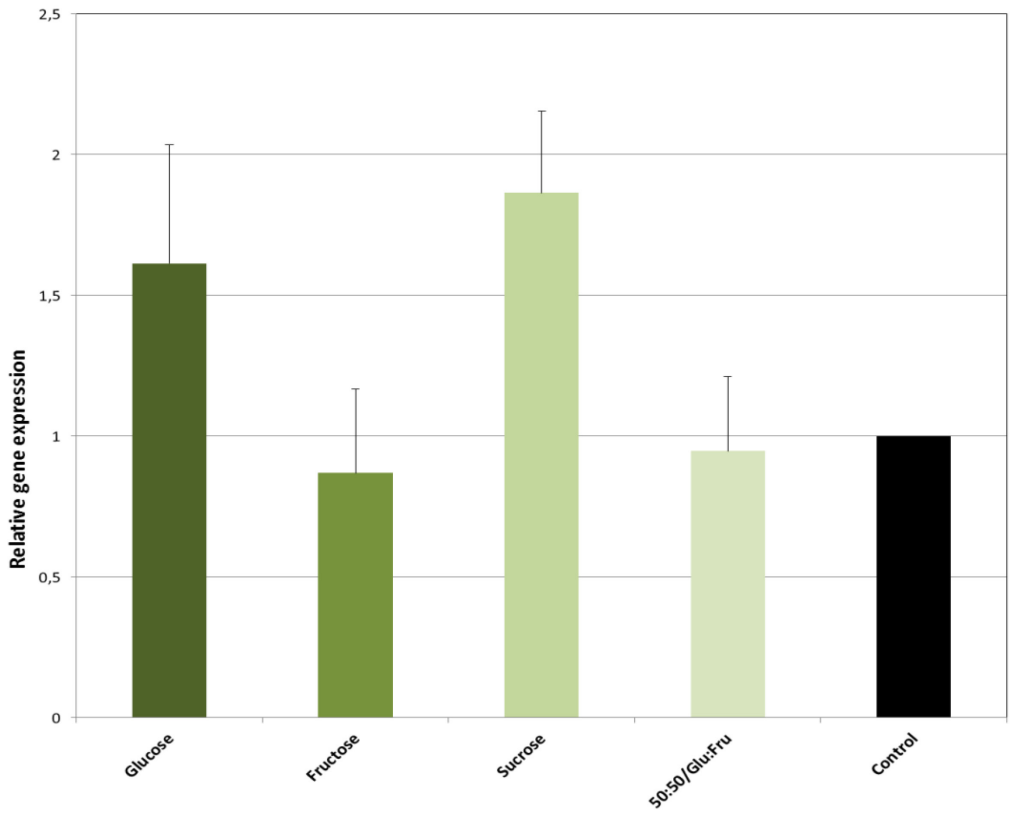
In the following, mRNA levels are presented in figures that were created using Excel. All measurements were done in duplicates with 3-9 independent experiments for each condition. The figures represent mRNA levels of treated worms (fed with glucose, fructose, sucrose or 50:50/glucose:fructose) relative to the mRNA level of the control group (no sugar supplementation). The height of the bars represents the mean value of all conducted measurements. The error bar specifies the standard error, which results from standard deviation. T-tests were conducted with a significance level of $\alpha = 0.05$ (5%). A result is significant if the t-value is lower than the chosen significance level. Significant results are marked with a star (*).

5.1.1 Genes involved in insulin signaling

5.1.1.1 *Hcf-1* (host cell factor)

The gene *hcf-1* encodes the ortholog of human HCF-1. HCF-1 associates with DAF-16 in the nucleus and limits its access to a subset of target gene promoters. *Hcf-1* expression is increased in response to glucose and sucrose. Fructose feeding led to a slight decrease. A 50:50 mixture of glucose and fructose did not influence it.

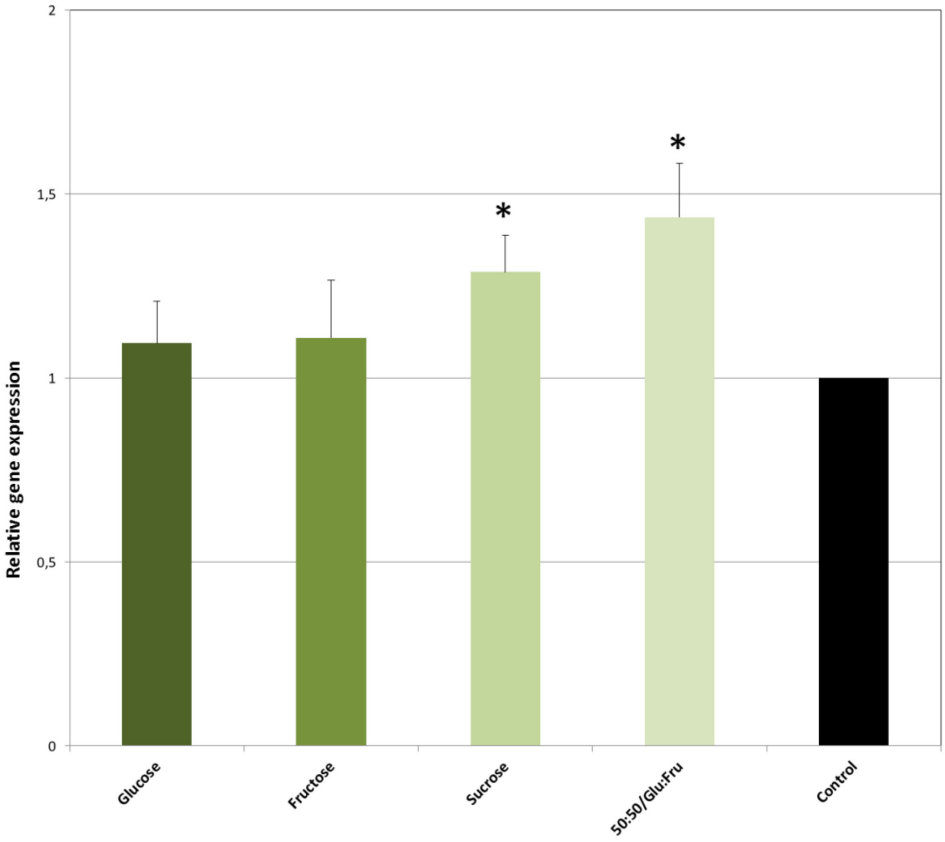
Figure 10: Relative mRNA levels of *hcf-1*



5.1.1.2 *Hsp-1* (heat shock protein)

Hsp-1 encodes Hsp70A, a member of the heat shock family of proteins. A loss of insulin/insulin-like signaling increases expression of HSPs, possibly by enhancing binding of DAF-16 (FOXO) and HSF (heat shock factor) to promoter regions of HSP-encoding genes. Glucose and fructose alone did not alter *hsp-1* expression. Interestingly, when glucose and fructose were given bound in sucrose or in a 50:50 mixture the expression increased slightly but significantly.

Figure 11: Relative mRNA levels of *hsp-1*

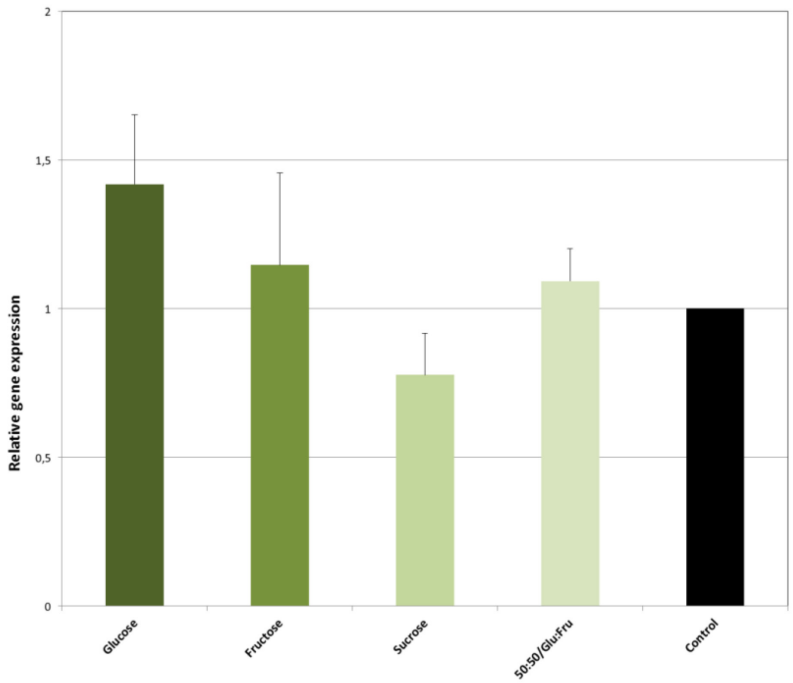


5.1.2 Genes of the fat metabolism

5.1.2.1 *nhr-49* (nuclear hormone receptor)

The gene *nhr-49* encodes a nuclear hormone receptor that regulates multiple genes in fat metabolism (β-oxidation genes and stearoyl-CoA desaturase). NHR-49 is assumed to act as the functional orthologue of the mammalian "lipid sensing" PPARα. Expression of *nhr-49* was slightly influenced by sugar consumption. Glucose feeding led to increased expression, whereas sucrose decreased it. Fructose and the glucose-fructose mixture had no effect on *nhr-49* expression. As the expression of *nhr-49* is higher under glucose than fructose feeding it seems that glucose alone may increases the influence of *nhr-49*. Interestingly when glucose was consumed bound in sucrose or combined with free fructose it did not increase *nhr-49* expression. On the contrary sucrose consumption even decreased it. As the differences between treatment groups and control group were not significant it seems that sugars do not affect *nhr-49* in an extent that changes β-oxidation or levels of saturated fat.

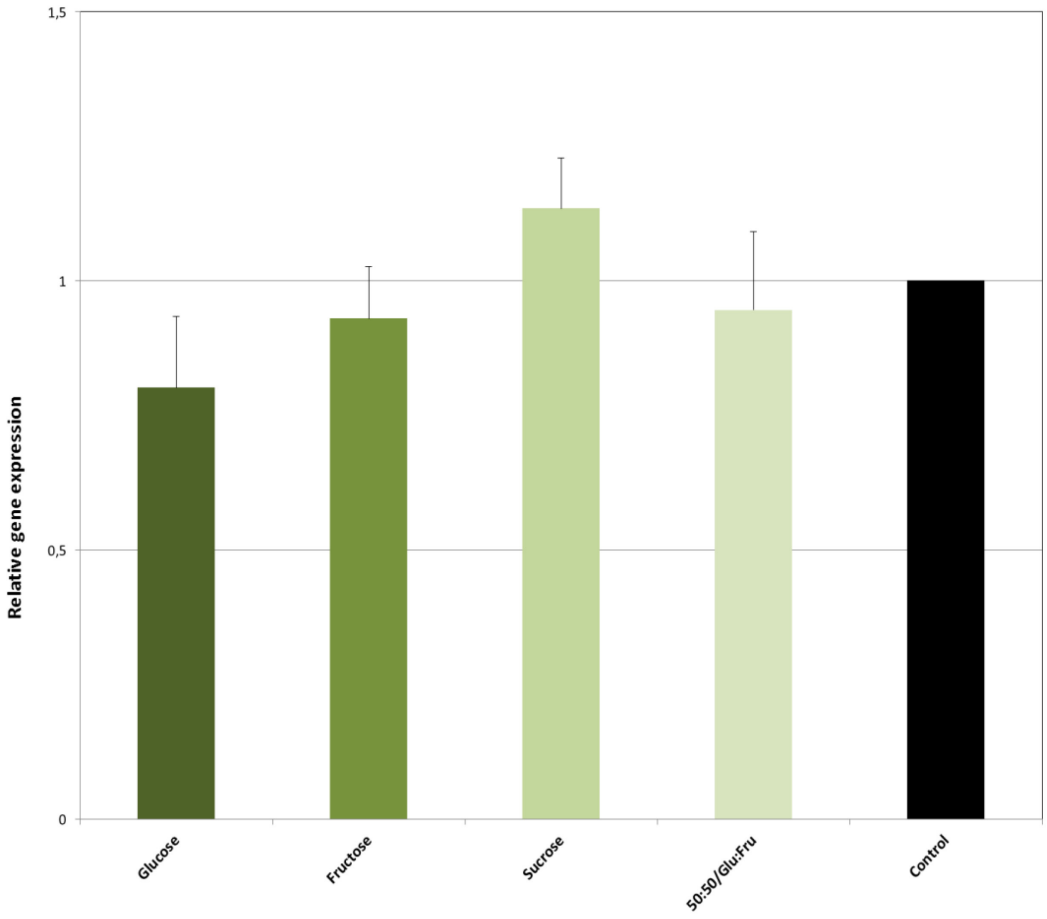
Figure 12: Relative mRNA levels of *nhr-49*



5.1.2.2 *lpd-2* (LiPid Depleted)

Lpd-2 is a homolog of the mammalian gene C/EBP, which is crucial for the formation of fat. mRNA levels of *lpd-2* were slightly decreased when glucose was fed, and slightly increased during sucrose feeding. But differences were not significantly among the different fed worms. As investigated previously in this working group, the same is true for *sbp-1*, which functions downstream of *lpd-2*. As sugars did not influence *spb-1* or *lpd-2* it can be assumed that they do not have an influence on lipid storage and metabolism in the SREBP/C/EBP pathway.

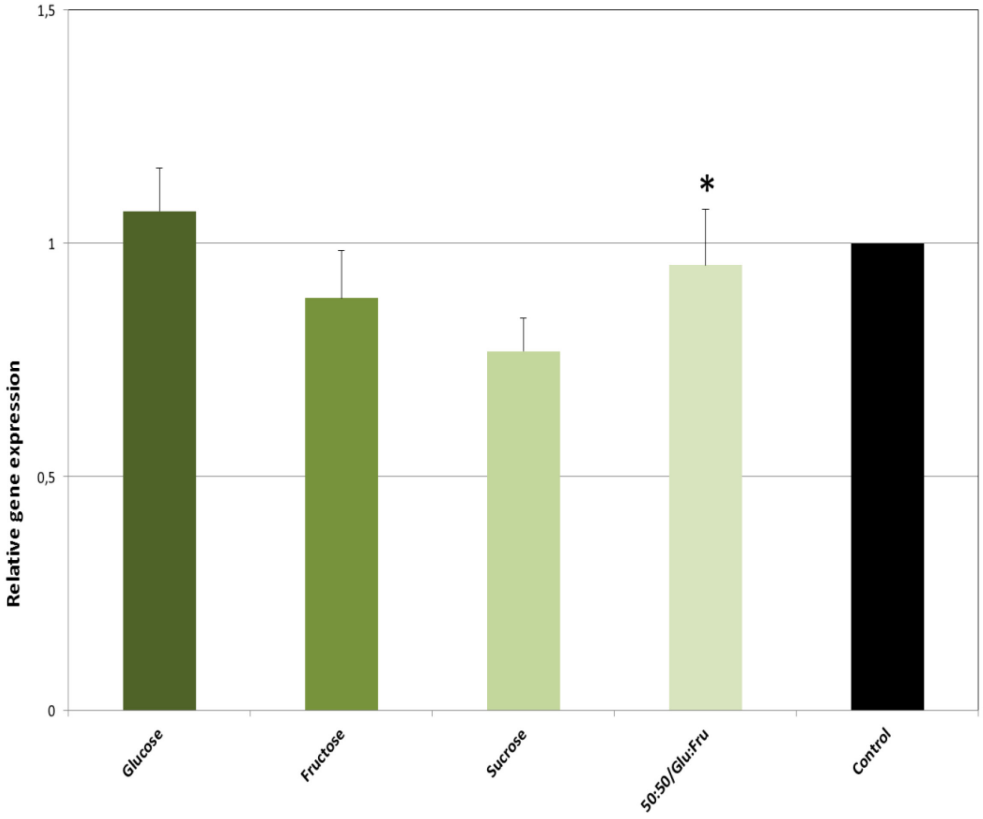
Figure 13: Relative mRNA levels of *lpd-2*



5.1.2.3 *fasn-1* (Fatty Acid SyNthase)

fasn-1 encodes a fatty acid synthase that is orthologous to human FAS. FAS is a cytosolic enzyme complex that synthesizes fatty acids of up to 16 carbons (palmitic acid) in length. The feeding of glucose and 50:50/ glucose:fructose did not influence the expression of *fasn-1*. There was only a slight decrease in *fasn-1* expression when worms were supplemented with fructose or sucrose. This indicates that these sugars do not promote fatty acid synthesis by FASN-1.

Figure 14: Relative mRNA levels of *fasn-1*



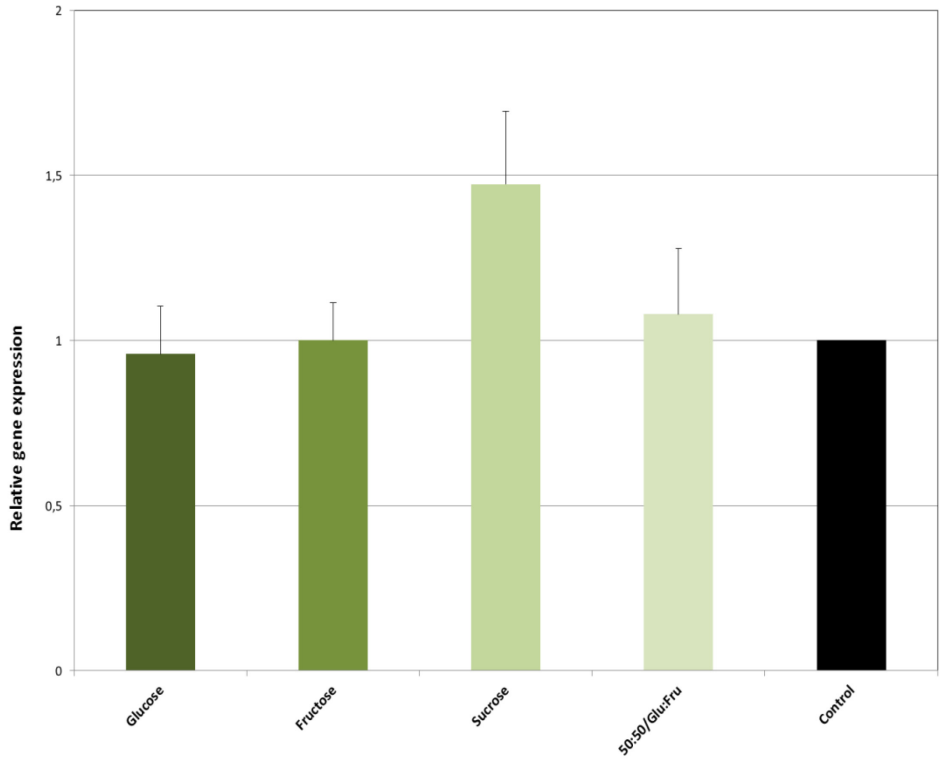
5.1.2.4 *elo-2* (fatty acid ELOngation)

Elo-2 encodes a palmitic acid elongase that is homologous to PUFA elongases such as ELOVL-5. It has been established that specific hepatic fatty acid elongases as *elovl-5 (elo-2)* and *elovl-6 (elo-3)* are regulated in the liver by nutrients (glucose and fat), hormones (insulin), and nuclear receptor agonists (e.g., PPARα agonists) (Wang, Botolin et al. 2006).

This is conformed partly by the results of this study as fructose, which is metabolized independently of insulin, did not change *elo-2* expression. But unexpectedly this was also true for glucose. In this study only sucrose did change *elo-2* expression as it is expressed almost 1.5 fold in sucrose fed worms compared to control worms.

Anyhow, the differences are not statistically different, which indicates that sugars do not have an influence on fatty acid elongation by *elo-2* in *C. elegans*.

Figure 15: Relative mRNA levels of *elo-2*



5.2 Western blots

Western blot analysis of SIR-2.1 and DAF-16 was performed in order to show whether protein levels are consistent with altered gene expression levels. For each protein 3 independent experiments were conducted. The presented figures show bands corresponding to the target protein. Their identity is confirmed by comparison to molecular weight markers (for size). Data produced with a Western blot is considered to be semi-quantitative. It provides a relative comparison of protein levels of the control group (only bacteria as food source) and treatment groups (bacteria and different sugars as food source).

5.2.1 Proteins involved in insulin signaling

5.2.1.1 SIR-2.1

The Western blot results of this study show increased protein levels of SIR-2.1 after fructose feeding. The previous conducted RT-qPCR study supports this result as it showed increased mRNA levels of *sir-2.1* in response to fructose. When glucose or a mixture of glucose and fructose was ingested, SIR-2.1 levels did not change. Sucrose led to an increase in SIR-2.1 levels similarly to fructose (the shown band for sucrose shows not the real extent of increase, as more protein was used, what was seen in coomassie staining, Figure 17).

Figure 16: Immunoblot of SIR-2.1

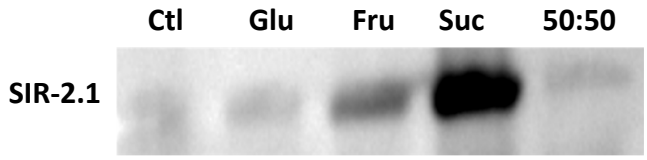
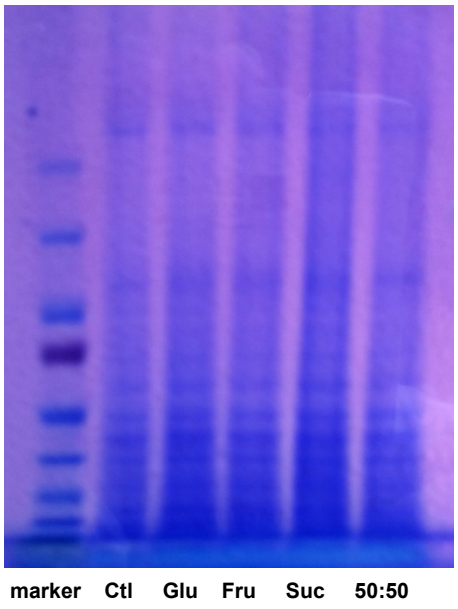


Figure 17: Coomassie gel corresponding to Figure 16



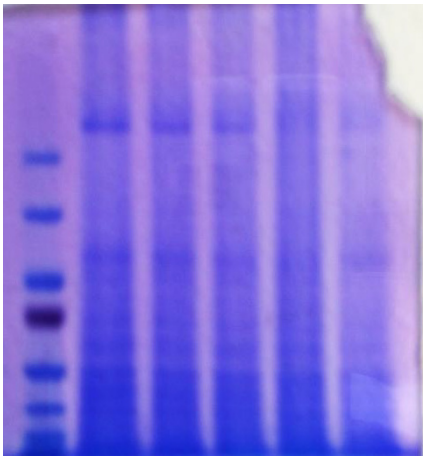
5.2.1.2 DAF-16

Western blot analysis showed that feeding of 5.4% glucose, fructose and sucrose decreased DAF-16 levels compared to the control group. Feeding of a 50:50 mixture of glucose and fructose did not alter DAF-16 levels.

This result was unexpected as earlier conducted RT-qPCR analysis showed increased expression of *daf-16* in response to sucrose, 50:50/glucose:fructose and especially fructose.

Figure 18: Immonublot for DAF-16

Figure 19: Coomassie gel corresponding to Figure 18



6 DISCUSSION

6.1 Insulin signaling pathway

In this thesis the influences of different sugars on genes and proteins of the insulin signaling pathway were investigated. Some effects that were already observed in this working group could be further established, whereas conclusions that were made due to other results have to be attenuated and reviewed because of new observations.

It could be shown that *hcf-1* expression increased in response to glucose and sucrose whereas fructose slightly decreased it. *Hcf-1* encodes the ortholog of human HCF-1 which limits the access of DAF-16/FOXO to its target gene promoters. Thus the results of the conducted gene expression study indicate that glucose and sucrose indirectly inhibit DAF-16 via increased *hcf-1*.
It was previously shown that fructose increases *daf-16* expression. It seems that this activation is further stimulated as fructose slightly decreased *hcf-1* expression. Thus DAF-16 inhibition by *hcf-1* was low or absent under fructose alone feeding.

Glucose and fructose alone did not alter *hsp-1* expression whereas sucrose and the mixture of glucose and fructose increased it slightly. It is assumed that a loss of insulin/insulin-like signaling increases the expression of HSPs in *C. elegans*. This relation could not be confirmed by this study. As fructose does not stimulate insulin signaling it would have been expected that it increases *hsp-1* expression to a certain extent, which could not be observed. Furthermore glucose and sucrose increase insulin signaling which would have been expected to decrease *hsp-1* expression. Also this was not observed. Interestingly, only the combination of fructose and glucose, either bound in sucrose or a mixture of their free forms, could influence *hsp-1* expression. This indicates that it makes a different for metabolic pathways in *C. elegans* whether

the monosaccharides glucose and fructose are ingested isolated or in combination. Anyway, differences among the treatment groups are not major which indicates a negligible effect of sugars on *hsp-1* expression.

This study showed increased protein levels of SIR-2.1 after fructose feeding. The previous conducted RT-qPCR study supports this results as it showed increased mRNA levels of *sir-2.1* in response to fructose. *sir-2.1* encodes for the ortholog of human SIRT-1. SIRT-1 is an NAD^+-dependent protein deacetylase that promotes FOXO activation. *Sir-2.1* overexpression extends life span, which requires daf-16. SIR-2.1 is also required for life span extension by caloric restriction, which is independent of the insulin/IGF-1 signaling pathway. Sucrose led to an increase in SIR-2.1 levels similarly to fructose, whereas in *sir-2.1* gene expression there was a difference between fructose and sucrose feeding. Expression was only slightly increased by sucrose i.e lower than after fructose feeding. As sucrose had the same effect on SIR-2.1 protein levels than fructose the RT-qPCR results are attenuated. It is possible that the higher mRNA levels in response to fructose are partly degraded before translation into protein.

Feeding of 5.4% glucose, fructose and sucrose decreased DAF-16 levels compared to the control group. But it has to be taken into consideration that DAF-16 is only active after translocation into the nucleus (in its phosphorylated form) and that the relation of active to inactive DAF-16 is not known. DAF-16 is the *C. elegans* FOXO transcription factor that is controlled by the activity of DAF-2 (insulin receptor), which represses DAF-16 activity. DAF-16 binds to and activates/represses various target genes involved in life span regulation, metabolism, fat storage, dauer formation and stress response. It seems that DAF-16 activation upregulates genes, that contribute to specific protective mechanisms, which may increase life span.
The result of this study was unexpected as earlier conducted RT-qPCR analysis showed increased expression of *daf-16* in response to sucrose, 50:50/glucose:fructose and especially fructose. This may be explained by a

degradation of augmented mRNA before the translation to protein or also degradation of protein before its level was analyzed. Thus previously suggested conclusions that fructose activates DAF16/FOXO transcription factor and therefore induces metabolic changes in glucose and lipid metabolism as well as in stress response have to be attenuated.

To sum up it could be shown that fructose influences the insulin signaling pathway by a decrease in *hcf-1* expression which indicates that under fructose alone feeding the inhibition of DAF-16 by *hcf-1* is low or absent. This is in consistence with a previous conducted gene expression study that showed increased *daf-16* expression under fructose feeding. Against expectation protein levels of DAF-16 were not augmented under fructose feeding. Furthermore protein levels of SIR-2.1 were not higher under fructose than sucrose feeding. As protein levels were not in consistence with elevated mRNA levels it seems that mRNA is degraded before its translation into proteins. Sugars had a negligible effect on *hsp-1* expression. It may can be concluded that the circumstance that only the combination of fructose and glucose, either bound in sucrose or a mixture of their free forms, could influence *hsp-1* expression indicates a difference for metabolic pathways in *C. elegans* whether the monosaccharides glucose and fructose are ingested isolated or in combination.

6.2 Fat metabolism

It was one aim of this thesis to investigate potential effects of sugars on fat metabolism. Therefore a gene expression study of four genes (*nhr-49*, *lpd-2*, *fasn-1* and *elo-2*) involved in fat metabolism, was conducted. A closer look on acquired results follows:

Expression of *nhr-49* was slightly influenced by sugar consumption. The gene *nhr-49* encodes a nuclear hormone receptor that regulates multiple genes in fat metabolism (β-oxidation genes and stearoyl-CoA desaturase). It acts as the functional orthologue of the mammalian “lipid sensing” PPARα. As the

differences between treatment groups and control group are not significant it seems that sugars do not affect *nhr-49* in an extent that changes β-oxidation or levels of saturated fat. The effects of NHRs on animal physiology are only partly understood and wide-ranging. The biological activities of NHRs that are conserved in mammals, influence development, dauer formation, and sex determination (Van Gilst, Hadjivassiliou et al. 2005). This indicates that *nhr-49* regulates more than genes in fat metabolism, which may be a reason for absent effects of sugars on its expression.

Lpd-2 is a homolog of the mammalian gene C/EBP and *spb-1* of SREBP. Both are crucial for the formation of mammalian fat. *Spb-1* functions downstream of *lpd-2* (McKay, McKay et al. 2003). Neither *lpd-2* nor *spb-1*, which was investigated in a previous study by this working group, were influenced by sugar ingestion. An influence on *lpd-2* would probably have affected *spb-1* in a similar way. As sugars did not influence *spb-1* or *lpd-2* it can be assumed that they do not have an influence on lipid storage and metabolism in the SREBP/C/EBP pathway.

Fasn-1 encodes a fatty acid synthase that is orthologous to human FAS. FAS is a cytosolic enzyme complex that synthesizes fatty acids of up to 16 carbons (palmitic acid) in length. As shown in Figure 6 in mammal liver FAS is activated by SREBP1c. It is assumed that SREBP1c is activated by fructose over several steps. In this thesis it could not be confirmed that this is true for the nematode *C. elegans* as well. Neither *fasn-1* nor *spb-1* expression could be increased by feeding of 5.4% fructose or other sugars. The contrary was true as *fasn-1* expression was slightly decreased under fructose and sucrose treatment. *Spb-1* expression was slightly decreased in glucose fed worms. These differences were not statistically significant, therefore it is suggested that the fed sugars do not influence fatty acid synthesis via a *spb-1/fasn-1* pathway in *C. elegans*.

Elo-2 encodes a palmitic acid elongase that is homologous to PUFA elongases such as ELOVL-5. It has been established that specific hepatic fatty acid

elongases as *elovl-5* are regulated by nutrients (glucose and fat), hormones (insulin), and nuclear receptor agonists (i.e. PPARα agonists) (Wang, Botolin et al. 2006). This is conformed partly by the results of this study as fructose, which is metabolized independently of insulin, did not change *elo-2* expression. But unexpectedly this was also true for glucose. Anyhow, the differences are not statistically different, which indicates that sugars do not have an influence on fatty acid elongation by *elo-2* in *C. elegans*.

Taken together, feeding of 5.4% sugars did not alter gene expression in an extent that permit drawing a conclusion concerning an influence of sugars on fat metabolism in the examined pathways. Differences between the control group and treatment groups were mostly minor and statistically not significant. Despite these results it cannot be excluded that there may be other pathways in fat metabolism that are influenced by sugars.

7 CONCLUSION

It can be concluded that the feeding of 5.4% fructose influences the insulin signaling pathway of *C. elegans* by a decrease in *hcf-1* expression. This indicates that under fructose alone feeding the inhibition of DAF-16 by *hcf-1* is low or absent. Against expectation protein levels of DAF-16 were not augmented under fructose feeding. Furthermore protein levels of SIR-2.1 were not higher under fructose than sucrose feeding. As protein levels were not in consistence with elevated mRNA levels it seems that mRNA is degraded before its translation into proteins. As only the combination of fructose and glucose, either bound in sucrose or a mixture of their free forms, could influence *hsp-1* expression it might be concluded that it makes a difference in the metabolism of *C. elegans* whether the monosaccharides glucose and fructose are ingested isolated or in combination.

The conducted gene expression study of fat genes do not allow to draw a conclusion concerning an actual influence of sugars on fat metabolism in the examined pathways. Differences between the control group and treatment groups were mostly minor and statistically not significant. Despite these results it cannot be excluded that there may be other pathways in fat metabolism that are influenced by sugars.

Considering all results of the “fructose project” it seems that influences of sugars on insulin signaling pathway where first assumed to be greater than they really are. This is suggested because levels of protein products of the studied genes did not correspond to mRNA levels. Nevertheless, genes of the gluconeogenesis were induced in response to fructose. This cannot be supposed concerning lipogenesis.

8 SUMMARY

8.1 Background

There is a worldwide scientific and media attention concerning potential health effects, for example overweight, of sugar, especially fructose. Various studies on humans and animals investigate the association between sugar consumption and chronic diseases. However, their findings are controversial, which indicates a need for further trials. Studies on animal models, e.g. the nematode *C. elegans*, can substantially contribute to investigate metabolic effects of sugars as many essential pathways in mammals are well conserved in animal models.

Sugar is consumed mainly as the disaccharide sucrose, which consists of the two monosaccharides glucose and fructose. There are differences between them, concerning their absorption, their metabolism in the liver and their reactivity. This master thesis investigated the influences of glucose and fructose alone, sucrose and a 50:50 mixture of glucose and fructose on genes of the fat metabolism (*nhr-49*, *lpd-2*, *fasn-1*, *elo-2*) and genes (*hcf-1*, *daf-16*, *sir-2.1*, *hsp-1*) and proteins (DAF-16, SIR-2.1) of the insulin signaling pathway in *C. elegans*.

8.2 Methods

Bristol N2 (wild type) *C. elegans* were grown on *E. coli* seeded nematode growth medium-plates in a 20 °C incubator. Synchronized larvae were fed with different kind of sugars at a concentration of 5.4%. A control group received only the bacteria and no sugar. Larvae lived on the sugar plates for 50 hours at 20 °C and were used for all experimental treatments. For gene expression analysis RNA of the different fed worms was purified and extracted using an RNeasy MiniKit (Qiagen). Before amplifying the single stranded mRNA using RT-qPCR it was transcribed to double stranded cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). mRNA levels of one gene were normalized

to housekeeping genes and to mRNA levels of the same gene in the control group using the ddCt-method. For Western blot analysis the proteins of the worm samples were separated by molecular weight using SDS PAGE. The protein extracts from the gel were transferred onto a PVDF membrane, which was blocked with 5% milk in PBSt and incubated with 2 specific antibodies. The signal was captured using a Bio-Rad Chemidoc station, after incubating the membrane with a substrate that produces a signal corresponding to the position of the target protein.

8.3 Results

Hcf-1 expression is increased in response to glucose and sucrose. Fructose feeding led to a slight decrease. Glucose and fructose alone did not alter *hsp-1* expression. When glucose and fructose were given bound in sucrose or in a 50:50 mixture the expression increased slightly but significantly. Fructose and sucrose feeding increased protein levels of SIR-2.1. Feeding of glucose, fructose and sucrose decreased DAF-16 levels compared to the control group. Genes of the fat metabolism (*nhr-49*, *lpd-2*, *fasn-1*, *elo-2*) were not influenced by sugars in a way that would change their effects on metabolism.

8.4 Conclusion

It can be concluded that the feeding of 5.4% fructose influences the insulin signaling pathway of *C. elegans* by a decrease in *hcf-1* expression. This indicates that under fructose alone feeding the inhibition of DAF-16 by *hcf-1* is low or absent. As levels of DAF-16 and SIR-2.1 were not in consistence with elevated mRNA levels it seems that mRNA is degraded before its translation into proteins. As only the combination of fructose and glucose, either bound in sucrose or a mixture of their free forms, could influence *hsp-1* expression it might be concluded that it makes a difference in the metabolism of *C. elegans* whether the monosaccharides glucose and fructose are ingested isolated or in combination. The conducted results do not allow to draw a conclusion

concerning an actual influence of sugars on fat metabolism in the examined pathways.

9 ZUSAMMENFASSUNG

9.1 Hintergrund

Der Einfluss von Zucker, besonders von Fruktose, auf die menschliche Gesundheit, beispielsweise das Körpergewicht, gelangte in den letzten Jahren in den Fokus von Wissenschaft und Medien. Eine Vielzahl an Studien an Menschen und Tieren untersucht den Zusammenhang zwischen Zuckerkonsum und chronischen Krankheiten. Deren Ergebnisse sind jedoch kontrovers, weswegen es weiterer Untersuchungen bedarf. Studien an Modellorganismen, wie dem Fadenwurm *Caenorhabditis elegans* (*C. elegans*), können einen wertvollen Beitrag zur Erforschung von metabolischen Effekten von Zucker leisten, weil viele essentielle Stoffwechselwege im Menschen konserviert sind.
Zucker wird hauptsächlich als Disaccharid Saccharose konsumiert. Saccharose besteht aus den beiden Monosacchariden Glukose und Fruktose, die sich bezüglich Absorption, Metabolismus in der Leber und Reaktivität unterscheiden. Diese Masterarbeit beschäftigt sich mit den Einflüssen von Glukose und Fruktose allein, sowie Saccharose und einer 50:50 Mischung aus Glukose und Fruktose auf Gene des Fettstoffwechsels (*nhr-49*, *lpd-2*, *fasn-1*, *elo-2*) und Gene (*hcf-1*, *daf-16*, *sir-2.1*, *hsp-1*) und Proteine (DAF-16, SIR-2.1) des Insulinsignalwegs von *C. elegans*.

9.2 Methoden

Bristol N2 (Wildtyp) *C. elegans* wurden auf *E. coli* beimpften NGM-Platten in einem 20 °C Inkubator gezüchtet. Synchronisierte Larven wurden mit

unterschiedlichen Zuckerlösungen mit einer Konzentration von 5,4 % gefüttert. Die Kontrollgruppe erhielt nur die Bakterien und keinen Zucker als Futter. Die Larven lebten 50 h bei 20 °C auf den Zuckerplatten und wurden für alle Experimente verwendet. Für die Genexpressionsanalysen wurde mithilfe eines RNeasy Minikits (Qiagen) die RNA von den unterschiedlich gefütterten Würmern gereinigt und extrahiert. Vor der Amplifikation der einzelsträngigen RNA mittels RT-qPCR wurde sie mittels eines QuantiTect Reverse Transcription Kits (Qiagen) zu doppelsträngiger cDNA transkribiert. Mittels der ddCt-Methode (relative Quantifizierung) wurden die mRNA Levels von einem Gen zu den mRNA Levels desselben Gens in der Kontrollgruppe und zu den Houskeeping Genes normalisiert. Für die Western Blot Analysen wurden die Proteine der Wurmproben nach Molekulargewicht mittels SDS PAGE (Gelelektrophorese) aufgetrennt. Die Proteinextrakte wurden von dem Gel auf eine PVDF-Membran transferiert, mit 5 % Milch in PBST geblockt und mit 2 spezifischen Antikörpern inkubiert. Vor der Detektion wurde die Membran mit einem Substrat, das ein Signal an der Stelle des Zielproteins produziert, inkubiert. Das Signal wurde mittels einer Bio-Rad Chemidoc Station detektiert.

9.3 Ergebnisse

Zu einer erhöhten Expression von *hcf*-1 kam es nach Glukose- und Saccharosefütterung. Fruktose verringerte sie leicht. Glukose und Fruktose allein veränderten die *hsp-1* Expression nicht. Wenn sie allerdings gebunden in Form von Saccharose oder in der 50:50-Mischung gegeben wurden erhöhten sie die Expression leicht, aber signifikant. Die Fütterung von Fruktose und Saccharose erhöhten den Proteingehalt von SIR-2.1. Glukose, Fruktose und Saccharose erniedrigten den DAF-16 Gehalt im Vergleich zur Kontrollgruppe. Die untersuchten Gene des Fettstoffwechsels (*nhr-49*, *lpd-2*, *fasn-1*, *elo-2*) wurden durch die Fütterung von Zucker nicht in einem Ausmaß, welches ihren Einfluss auf den Stoffwechsel ändern würde, beeinflusst.

9.4 Fazit

Die verminderte *hcf-1*-Expression nach Fruktosefütterung lässt auf einen Einfluss von Fruktose auf den Insulinsignalweg von *C. elegans* schließen. Die Hemmung von DAF-16 durch *hcf-1* ist demnach unter Fruktosefütterung vermindert oder abwesend. DAF-16- und SIR-2.1-Levels stimmten nicht mit erhöhten mRNA-Levels überein, was auf einen Abbau der mRNA vor der Translation in Protein hindeutet. Die *hsp-1*-Expression konnte nur durch eine Kombination von Fruktose und Glukose, in Form von Saccharose oder als Mischung ihrer freien Formen, beeinflusst werden. Das könnte darauf hinweisen, dass die isolierte bzw. kombinierte Aufnahme der Monosaccharide Glukose und Fruktose den Stoffwechsel von *C. elegans* unterschiedlich beeinflussen.

Die erzielten Ergebnisse erlauben keine Schlussfolgerungen auf einen tatsächlichen Einfluss von Zucker auf die untersuchten Fettstoffwechselwege.

10 APPENDIX

10.1 References

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10.2 Table of figures

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10.4 Abbreviations

ABCA1	ATP-binding cassette transporter (member 1 of human transporter sub-family ABCA)
AKT = PKB	protein kinase B
ASN	American Society of Nutrition
ATP	adenosine triphosphate
C. elegans	*Caenorhabditis elegans*
C/EPB	CCAAT/enhancer binding protein transcription factor
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
csq-1	calsequestrin
Ct	cycle threshold
ctl	control
CVD	cardiovascular diseases
daf-16	abnormal dauer formation type 16
daf-2	abnormal dauer formation type 2
DEPC H_2O	diethylpyrocarbonate water
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
elo-2	fatty acid elongation
ELOVL-5	elongation of very long chain fatty acid
FA	fatty acid
FAS	fatty acid synthase
fasn-1	fatty acid synthase
FOXO-1	forkhead box 'other'
Fru	fructose
gDNA	genomic deoxyribonucleic acid
Glu	glucose
GLUT	glucose transporter
H_2O	water
hcf-1	host cell factor 1

HCl	hydrochloric acid
HDL	high density lipoprotein
HELO-1	human fatty acid elongation
HFCS	high fructose corn syrup
hsp-1	heat shock protein 1
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin G
Iis	insulin/insulin like signals
IRS-1	insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
KCl	potassium chloride
KH_2PO_4	potassium hydrogen phosphate
KOH	potassium hydroxide
L1 (2,3,4)	larval stage
LB medium	lysogeny broth medium
LDL	low density lipoprotein
lpd-2	lipid depleted
malonyl-CoA	malonyl coenzyme A
MAPK8 = JNK1	mitogen-activated protein kinase 8
$MgSO_4$	magnesium sulfate
mRNA	messenger ribonucleic acid
Na_2HPO_4	sodium hydrogen phosphate
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phopshate
NAFLD	nonalcoholic fatty liver disease
NaOCl	sodium hypochlorite
NGM	nematode growth medium
NHANES	National Health and Nutrition Examination Survey
nhr-49	nuclear hormone receptor
NO	nitric oxide
NTC	no template control
OD	optical density

PBST	phosphate buffered saline + tween
pck-1	phophophenolpyruvat carboxykinase
PCR	polymerase chain reaction
PKCε	Protein kinase C epsilon type
pmp-3	peroxisomal membrane protein related
pod-2	polarity and osmotic sensitivity defect
PPARα	peroxisome proliferator-activated receptor alpha
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene diflourid
pyc-1	pyruvate carboxylase
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RNase	ribonuclease
ROS	reactive oxygen species
rpm	rounds per minute
RT-qPCR	real-time quantitative polymerase chain reaction
sbp-1	sterol regulatory element binding protein
SDS	sodium lauryl sulfate
SGLT	sodium/glucose cotransporter
sir-2.1	silent information regulator
sod-3	superoxiddismutase
SREBP-1	sterol regulatory element binding protein
Suc	sucrose
TAG	triacylglyceride
UDP	uridine diphosphate
VLDL	very low density lipoprotein
Y45F10D.4	iron-sulfur cluster assembly enzyme

10.5 Gene expression data

Table 22: Relative mRNA levels of *hcf-1*

Date of measurement	Glucose	Fructose	Sucrose	50:50/Glu:Fru	Control
2015-02-18	0,9481	0,8244	1,6527	0,8841	1
2015-02-21	1,4932	1,4051	2,4370	1,4302	1
2015-03-05	2,3949	0,3745	1,5010	0,5253	1
arithmetic mean	**1,6121**	**0,8680**	**1,8636**	**0,9465**	**1**
standard deviation	0,7307	0,5167	0,5024	0,4557	
standard error	0,4219	0,2983	0,2900	0,2631	
T-value	0,2839	0,7014	0,0967	0,8577	

Table 23: Relative mRNA levels of *hsp-1*

Date of measurement	Glucose	Fructose	Sucrose	50:50/Glu:Fru	Control
2014-06-03	0,7412	0,6256	0,9501	1,1059	1
2014-06-12	0,6615	0,6778	1,6668	2,3414	1
2014-09-01	1,2770	1,3618	1,2948	1,5151	1
2014-09-03	1,4715	1,8056	1,6107	1,4776	1
2015-02-13	0,9463	1,1560	1,4232	1,4119	1
2015-02-18	1,0419	1,1210	1,0779	1,3062	1
2015-02-21	1,0523	0,6203	0,9180	0,9257	1
2015-03-15	1,5652	1,5038	1,3626	1,4110	1
arithmetic mean	**1,0946**	**1,1090**	**1,2880**	**1,4368**	**1**
standard deviation	0,3242	0,4417	0,2845	0,4166	
standard error	0,1146	0,1562	0,1006	0,1473	
T-value	0,4364	0,5078	0,0242	0,0209	

Table 24: Relative mRNA levels of *nhr-49*

Date of measurement	Glucose	Fructose	Sucrose	50:50/Glu:Fru	Control
2014-06-12	2,2454	2,5614	1,0667	1,0612	1
2014-07-29	1,4000	0,9677	1,1228	1,3597	1
2014-08-09	1,4328	1,3085	0,8503	1,0027	1
2014-09-01	1,8321	0,9939	0,8812	1,1147	1
2014-09-03	0,8870	0,6032	0,4923	0,6459	1
2014-10-08	0,7118	0,4456	0,2530	1,3725	1
arithmetic mean	**1,4182**	**1,1467**	**0,7777**	**1,0928**	**1**
standard deviation	0,5723	0,7576	0,3392	0,2679	
standard error	0,2336	0,3093	0,1385	0,1094	
T-value	0,1335	0,6553	0,1694	0,4350	

Table 25: Relative mRNA levels of *lpd-2*

Date of measurement	Glucose	Fructose	Sucrose	50:50/Glu:Fru	Control
2014-06-03	0,5380	0,8084	1,4739	0,5555	1
2014-07-29	1,0850	1,1124	1,2024	0,9732	1
2014-08-06	0,6551	0,8247	1,1647	1,1196	1
2014-09-01	1,2986	1,2953	1,1922	1,4149	1
2014-09-03	0,7597	0,8933	0,9828	1,1106	1
2014-10-08	0,4695	0,6463	0,7901	0,5018	1
arithmetic mean	**0,8010**	**0,9301**	**1,1343**	**0,9459**	**1**
standard deviation	0,3257	0,2343	0,2305	0,3543	
standard error	0,1330	0,0956	0,0941	0,1446	
T-value	0,1947	0,4974	0,2128	0,7238	

Table 26: Relative mRNA levels of *fasn-1*

Date of measurement	Glucose	Fructose	Sucrose	50:50/Glu:Fru	Control
2014-06-03	0,8036	0,4823	0,8169	0,5511	1
2014-07-29	1,1199	0,8106	0,9311	1,4634	1
2014-08-06	1,2601	1,2988	1,1076	1,3004	1
2014-09-01	0,9582	0,8308	0,7978	0,7792	1
2014-09-03	1,1946	1,1037	0,7237	0,9444	1
2014-10-08	0,5876	0,5883	0,5708	0,5166	1
2015-02-13	1,5717	1,3419	0,9035	1,2113	1
2015-02-18	1,0779	0,8587	0,6846	1,2202	1
2015-02-21	1,0413	0,6249	0,3843	0,5889	1
arithmetic mean	**1,0683**	**0,8822**	**0,7689**	**0,9528**	**1**
standard deviation	0,2790	0,3072	0,2117	0,3595	
standard error	0,0930	0,1024	0,0706	0,1198	
T-value	0,4836	0,2833	0,0113	0,7042	

Table 27: Relative mRNA levels of *elo-2*

Date of measurement	Glucose	Fructose	Sucrose	50:50/Glu:Fru	Control
2014-06-03	0,5167	0,6709	0,7786	0,7871	1
2014-06-12	0,8855	0,9413	1,9431	1,5804	1
2014-07-29	1,0768	1,1268	1,0612	1,6695	1
2014-08-06	1,8264	1,1016	2,9360	2,1316	1
2014-09-01	1,2024	1,3434	1,2302	0,6760	1
2014-09-03	1,4913	1,4396	1,6188	1,6188	1
2014-10-08	0,3926	0,4074	0,5348	0,2903	1
2015-02-13	0,6618	0,5973	1,7444	0,2781	1
2015-02-18	1,0203	0,9283	1,0423	0,8556	1
2015-02-21	0,5110	1,4431	1,8459	0,9075	1
arithmetic mean	**0,9585**	**1,0000**	**1,4735**	**1,0795**	**1**
standard deviation	0,4623	0,3596	0,6970	0,6316	
standard error	0,1462	0,1137	0,2204	0,1997	
T-value	0,7828	0,9998	0,0602	0,6998	

10.6 CV

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