Impact of high-fat diet on lifespan, metabolism, fecundity and behavioral senescence in *Drosophila*

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Abstract

Excess consumption of high-fat diet (HFD) is likely to result in obesity and increases the predisposition to associated health disorders. *Drosophila melanogaster* has emerged as an important model to study the effects of HFD on metabolism, gut function, behavior, and ageing. In this study, we investigated the long-term effects of HFD on physiology and behavior of female flies. We found that HFD decreases lifespan, and also with age leads to accelerated decline of climbing ability and increased sleep fragmentation. Furthermore, long-term exposure to HFD results in elevated adipokinetic hormone (AKH) transcript levels and an increased crop with massive lipid stores. We detected no long-term effects of HFD on body mass, or levels of triacylglycerids, glycogen or glucose, although fecundity was diminished. Finally, we investigated the role of AKH and *Drosophila* insulin like peptides (DILPs) in regulating effects of HFD during aging. Both with normal diet (ND) and HFD, *Akh* mutant flies displayed increased longevity compared to control flies, both mutants and controls showed shortened lifespan on HFD compared to ND. Triple mutants of *dilp2,3,5* are characterized by long lifespan on ND, but HFD drastically shortened longevity. Thus, long-term exposure to HFD impairs lifespan and fecundity and augments age-related behavioral senescence.

1. Introduction

Intake of high-fat diet (HFD) is likely to result in obesity, which in turn increases the predisposition to cardiovascular disease, diabetes, cancer, and other metabolic disorders (Bray and Popkin, 1998; Dietrich et al., 2013; Hill et al., 2000; O'Brien and Dixon, 2002; Szendroedi and Roden, 2009; van Herpen and Schrauwen-Hinderling, 2008). Obesity is also linked to increased risk of cognitive impairment (Hwang et al., 2010; Liu et al., 2015; McNay et al., 2010), especially during aging (Duffy et al., 2019; Freeman et al., 2014).

The genetically tractable fly *Drosophila* is an excellent organism to study effects of diet on metabolism, behavior, aging and lifespan, due to its short life cycle and the ease by which large number of animals can be reared (Baker and Thummel, 2007; Bellen et al., 2010; Birse et al., 2010; Fontana et al., 2010; Heier and Kühnlein, 2018; Owusu-Ansah and Perrimon, 2014;

Padmanabha and Baker, 2014; Rajan and Perrimon, 2013). *Drosophila* has also been employed to model certain human diseases based on the fact that about 65% of human disease-related genes have functional orthologs in flies [see (Ugur et al., 2016; Yamamoto et al., 2014)]. Recently studies have emerged on the effect of HFD on health and physiology of *Drosophila* (Birse et al., 2010; Driver and Cosopodiotis, 1979; Heier and Kühnlein, 2018; Heinrichsen and Haddad, 2012; Huang et al., 2020; Jung et al., 2018; Musselman and Kühnlein, 2018; Rivera et al., 2019; Stobdan et al., 2019; Tatar et al., 2014; Toprak et al., 2020; Trinh and Boulianne, 2013; von Frieling et al., 2020; Woodcock et al., 2015). Most of these studies have investigated effects of relatively short exposure to HFD, ranging between one and three weeks, although some determined the effect of HFD on total lifespan (Heinrichsen and Haddad, 2012; Rivera et al., 2019; Woodcock et al., 2015).

It is clear that HFD significantly increases the mortality of both male and female flies and thereby shortens lifespan (Heinrichsen and Haddad, 2012; Rivera et al., 2019; Woodcock et al., 2015), and has detrimental effects on climbing behavior, short-term phototaxis memory, and behavioral responses to odor (Jung et al., 2018; Rivera et al., 2019). Even as short an exposure as one week to HFD had adverse effects on negative geotaxis, heart function, as well as insulin and glucose homeostasis (Birse et al., 2010) and gut physiology (von Frieling et al., 2020). Five days of exposure to HFD is sufficient to render flies more sensitive to starvation, seen as an enhancement of the starvation induced hyperactivity (Huang et al., 2020). Furthermore, the HFD was shown to affect the transcription of genes associated with memory, metabolism, olfaction, mitosis, cell signaling, and motor function (Rivera et al., 2019).

We were interested in determining additional long-term effects of HFD on lifespan and physiology in *Drosophila*. We found that both virgin and mated female flies exposed to HFD exhibit decreased lifespan, accelerated decline of climbing ability with age, and three-week-old virgin females display an increased sleep fragmentation. Furthermore, long-term exposure to HFD leads to increased crop size and elevated adipokinetic hormone (AKH) gene transcript levels in addition to reduced fecundity. Surprisingly, we found no long-term effects of HFD on body mass, or levels of triacylglycerids (TAG),

glycogen or glucose. Finally, we investigated the role of AKH in regulating the effects of HFD during aging. *Akh* mutant flies display increased longevity compared to control flies, in general, but HFD shortens lifespan for both genotypes.

2. Materials and Methods

The *Drosophila melanogaster* strain w^{1118} (from the Bloomington Drosophila Stock Center (BDSC), Bloomington, IN) was used in most experiments. The AKH mutant flies akh^{AP} and its control w^{1118} (Galikova et al., 2015) were kindly provided by Dr. Ronald Kühnlein (Graz, Austria). The triple *dilp2,3,5* mutants and its control w^{1118} (Grönke et al., 2010) were kindly provided by Dr. Sebastian Grönke (Cologne, Germany). All the *Drosophila* lines were reared at 25°C, and 12h light/dark cycle. An agar based normal diet (ND) with sugar (10%), yeast (5%) and agar (0.9%) was used for rearing and keeping flies. In all experiments presented in this paper we used female flies.

2.1. High-fat diet feeding regime

In the bulk of the experiments in this study we used the same HFD food protocol and two major experimental designs (Fig. 1). To prepare the high-fat diet (HFD), we added 10% or 30% (volume) of coconut oil to food containing sugar (10%), yeast (5%), and agar (1.2%). Thus, our HFD has the same ratio of sugar (10%), and yeast (5%) as the ND. In a few experiments (shown in Supplementary Figures) we prepared the HFD slightly differently either to change the ratios of lipid to carbohydrate and yeast (HFD-2), or to match specific experiments performed previously (Birse et al., 2010). To prepare the HFD-2, ND was prepared first, and then 30% of coconut oil was added. The third high fat diet (Bloomington high-fat diet; BHFD) was utilized by (Birse et al., 2010) and is based on a standard Bloomington food recipe (https://bdsc.indiana.edu/information/recipes/bloomfood.html) to which 30% coconut oil was added. Thus, in the HFD-2 and BHFD recipes, the ratios of sugar and yeast differ from those of the ND.

To test the effects of HFD on flies, we used two different feeding regimes; the HFD treatment started either within one day of eclosion for virgin females (R1), or 4-5 days after eclosion for mated females (R2). To prevent

the flies from getting stuck and drown in the oily HFD food, a piece of moist filter paper was attached to the wall of the food vial, and the food vials were kept in a horizontal position. Flies were sampled at different time points for assays as shown in Fig. 1.

2.2. Negative geotaxis assay and locomotor activity assays

Negative geotaxis (climbing ability) of female flies was measured as described previously (Liao et al., 2017), based on (Gargano et al., 2005). Briefly, flies were kept in upright 35 ml plastic vials. After tapping flies to the bottom of the vial the speed of climbing back was monitored. Flies were allowed 10 seconds to climb back up, at which point a photo was taken. The scoring of climbing ability was performed in two ways, using different flies for the two experiments. (1) The percentage of flies climbing over 5 cm within 10 s was scored (three technical repeats, using six biological replicates) and (2) the height climbed by individual flies was scored from the photo taken after 10 seconds. This was repeated in three technical repeats and the average height each fly had climbed was used for the analysis. Three biological replicates were used for this assay.

The *Drosophila* Activity Monitoring System (DAMS) (Trikinetics, Waltham, MA, USA) was used to analyze locomotor activity and sleep. Female flies were kept on the different diets for three or four weeks. Thereafter, single flies were placed in glass tubes that in one end had food containing 5% sucrose and 2% agar (sealed with parafilm to prevent desiccation). A small piece of sponge was inserted in the other end of the tube. The tubes with flies were placed into the DAMS within 2 hours before the dark phase. Thus we tracked the activity also during the first night for analysis of individual 24 h periods. For analysis of sleep, locomotor activity was monitored under 12L:12D for 7 days. Only the data collected from the first to the third day were used for the sleep analysis.

2.3. Determination of carbohydrates and triacylglycerids (TAG)

Before measuring the concentration of carbohydrates and TAG, the body weight (wet weight) of single adult flies was determined using a Mettler Toledo MT5 microbalance (Columbus, USA). Whole flies were homogenized in PBS

+ 0.05% Triton-X 100 using a tissuelyser II from Qiagen Glycogen. Samples were then heat-treated at 37°C for 10 min. After centrifugation at 16000g, 4°C for 10 min, the supernatants were used to determine the concentration of carbohydrates, TAG and protein levels as described below. The supernatants were converted to glucose with 0.5 mg/ml amyloglycosidase from Aspergillus niger (Sigma #10115). Glucose levels were measured with a glucose assay kit with glucose oxidase and peroxidase (Liquick Cor-Glucose diagnostic kit, Cormay, Poland) following the manufacturer's guidelines. Sample absorbance was measured at 500 nm with a micro-plate reader (Thermo scientific). Glucose concentration was estimated with a linear regression coefficient from a standard curve made from original concentrations of 1.5–15 mg of glucose. Glycogen content was quantified by subtracting free glucose concentration from the total glycogen + glucose levels. The amount of TAG was determined with a Liquick Cor-TG diagnostic kit (Cormay, Poland) using a linear regression coefficient from a standard curve made from an original concentration of 2.2 µg/µl of TAG standard (Cormay, Poland). Sample absorbance was measured at 550 nm with a micro-plate reader (Thermo scientific). Protein levels were determined using a Bradford assay according to (Diop et al., 2017). The concentrations of carbohydrates and TAG were normalized to protein levels, and the relative levels were used for generating graphs.

2.4. Immunocytochemistry and imaging

Drosophila brain, crops and muscle tissue from flies of different age were dissected out and used for immunostaining. The dorsal longitudinal muscles 1d or 1e according to (Fabian et al., 2016) were analyzed in this study. After being dissected out, the tissues were first fixed for 4 h in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) on ice with gently shaking. After washing with PBS, the tissues were incubated with primary antiserum for 48 h at 4°C, followed by four rinses in PBS with 0.5% Triton-X100 (PBS-Tx). Tissues were then incubated with secondary antibody for 48 h at 4°C. After washes in PBS-Tx for 1 hour, the samples were mounted in 80% glycerol in 0.01 M PBS. The following primary antisera were used: Rabbit anti-DILP2 (1:2000) from J.A. Veenstra, Bordeaux, France (Veenstra

et al., 2008), rabbit anti-AKH (1:1000) from M.R. Brown, Athens, GA, a monoclonal antibody to mono- and polyubiquitinylated conjugates (FK2, 1:200; from AH diagnostics, Solna, Sweden) [see (Demontis and Perrimon, 2010)], and mouse anti-tyrosine hydroxylase (anti-TH; 1:200) from Incstar Corp., Stillwater, MO, USA (Nässel and Elekes, 1992). Rhodamine-phalloidin (1:1000; Invitrogen) was used to label the muscle fibers of the crop. DAPI (1:2000; Sigma) was used for staining nuclei and Nile red (1:1000; Invitrogen) was used to stain lipids.

As secondary antisera, we used goat anti-rabbit Alexa 546, and goat anti-mouse Alexa 488 antiserum, both from Invitrogen and used at a dilution of 1:1000). All the tissues were scanned with a Zeiss LSM 780 confocal microscope (Jena, Germany) using 10×, 20× or 40× oil immersion objectives. For each experiment, we used identical laser intensities and scan settings. The staining intensity of regions of interest and the nearby background area were measured using Fiji (https://imagej.nih.gov/ij/). Mean staining intensity of the structure was determined by subtracting the background intensity of the same sample. The outline of the crop was extracted manually to determine the crop size (calculated as area).

2.5. Capillary feeding (CAFE) assay

The CAFE assay (Ja et al., 2007) was used for measuring food intake and water consumption. Single female flies were placed in 1.5-ml Eppendorf micro centrifuge tubes with an inserted capillary tube (5 µl, Sigma). For the food intake assay, the capillary tube was filled with liquid food containing 5% sucrose, 2% yeast extract and 0.1% propionic acid. To test water consumption relative to feeding, flies were given a capillary tube filled with milli-Q water, in addition to the capillary tube containing food. Eppendorf tubes with food and water capillaries, but without flies, were used as controls for evaporation. The final food intake or water consumption was determined by calculating the decrease in food or water levels minus the average decrease in the control capillaries. Food consumption was measured daily and calculated cumulatively over four consecutive days. We used 8-10 flies in each of three biological replicates in this assay.

2.6. Fecundity

To determine whether HFD affects fecundity, w^{1118} mated flies were kept on ND and 30% HFD for either one or three weeks. After the diet treatment, individual females were transferred to ND and number of eggs laid were counted after 24 h. Three replicates with 19-21 flies in each were tested for each genotype and treatment. To investigate the role of AKH and HFD on fecundity, four-day-old mated *Akh* mutant flies and their w^{1118} controls were transferred to ND or 30% HFD for one or three weeks. We counted the number of eggs older than stage 10 [according to (Kubrak et al., 2014; Saunders et al., 1989; Shimada et al., 2011) in the ovaries of each fly.

2.7. Chill coma recovery

To determine the fitness and stress tolerance of the flies exposed to HFD we tested chill coma recovery. We used w^{1118} virgin flies exposed to ND, 10% HFD or 30% HFD. A total of 80-94 flies per treatment divided in three biological replicates were used. After one week on the respective diets, groups of four-five flies were placed in empty vials that were placed on ice in a 4°C room. After 130 min on ice, the vials were taken out to room temperature where the behavior of the flies was video recorded for 90 min. The time it took for the flies to recover from the chill coma, defined using righting behavior, was scored from the videos.

2.8. Quantitative real-time PCR (qPCR)

Relative expression of genes of interest and the reference gene ribosome protein 49 (rp49) were determined with qPCR. Total RNA was extracted using Trizol-chloroform (Sigma-Aldrich) from three independent biological replicates. The RNA concentration was measured by NanoDrop 2000 spectrophotometer (Thermo Scientific), the mRNA concentration was adjusted to 400 ng/µl. cDNA was synthesized using random hexamer primer (Thermo Scientific) and RevertAid reverse transcriptase (Thermo Scientific). The cDNA products were diluted 10 times and used for qPCR using a StepOnePlusTM instrument (Applied Biosystem, USA) and SensiFAST SYBR Hi-ROX Kit (Bioline) following the manufacturer's guidelines. The mRNA levels were normalized to rp49 levels in the same replicate. Relative expression values were determined

by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The primer sequences used are:

dilp1 F: CGGAAACCACAAACTCTGCG

dilp1 R: CCCAGCAAGCTTTCACGTTT

dilp2 F: AGCAAGCCTTTGTCCTTCATCTC

dilp2 R: ACACCATACTCAGCACCTCGTTG

dilp3 F: TGTGTGTATGGCTTCAACGCAATG

dilp3 R: CACTCAACAGTCTTTCCAGCAGGG;

dilp5 F: GAGGCACCTTGGGCCTATTC

dilp5 R: CATGTGGTGAGATTCGG;

dilp6 F: CCCTTGGCGATGTATTTCCCAACA

dilp6 R: CCGACTTGCAGCACAAATCGGTTA

akh F: GCGAAGTCCTCATTGCAGCCGT

akh R: CCAATCCGGCGAGAAGGTCAATTGA

bmm F: GGTCCCTTCAGTCCCTCCTT

bmm R: GCT TGTGAGCATCGTCTGGT

rp49 F: ATCGGTTACGGATCGAACAA

rp49 R: GACAATCTCCTTGCGCTTCT

2. 9. Statistics

For statistics and graphs we used Graph Pad Prism version 6.00 (La Jolla, CA, USA2). Results are presented as means ± SEM or means ± range. We initially tested normality of data using Shapiro–Wilk's normality test. Student's t-test was used for comparing two groups. For comparing more than two groups, we used one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test, or two-way ANOVA followed by Sidak's multiple comparisons test. Lifespan data were subjected to survival analysis using a log rank Mantel-Cox test and presented as survival curves. See figure legends for further details.

3. Results

We employed two regimes (R1 and R2) for feeding female flies (w^{1118}) high-fat diet (HFD) (Fig. 1). The first (R1) was designed according to an earlier study on aging and diapause (Liao et al., 2017) and employed virgin female

flies exposed to the experimental diets one day after hatching. The second (R2) was designed to resemble an earlier study where mated females were first put on standard diet for several days and then transferred to the experimental diet. More specifically in R1 the one-day-old virgin female flies were transferred to vials with standard food (see methods) mixed with 10% or 30% coconut oil (HFD), whereas control flies were kept on normal diet (ND) throughout. The R2 flies were first kept for four days on ND and thereafter mated female flies were transferred to food enriched with 30% coconut oil (Fig. 1). This latter protocol allows flies to adjust to adult metabolism on standard food the first four days after hatching. It is known that the first few days after hatching there is residual larval fat body which serves as a nutrient resource while the fly commences feeding and adjusts to its adult metabolism (Aguila et al., 2007). Flies exposed to HFD directly after hatching might thus experience a “dietary stress” during this maturation phase (including ovary maturation). We first describe data obtained with the virgin flies (R1 protocol).

3. 1. HFD effects on lifespan, negative geotaxis, metabolism and fecundity

In tests of lifespan of virgin female flies that were exposed to the HFD food from day one after hatching, both total and median lifespan was shortened with food enriched with 10% lipid, compared to ND controls, and the 30% lipid diet further diminished lifespan (**Fig. 2** A).

During normal aging, flies perform progressively worse at negative geotaxis (climbing ability) (Gargano et al., 2005). Flies kept in an empty food vial normally sit on the wall towards the top of the vial. After tapping the vial, flies that fall to the bottom rapidly climb upwards. This negative geotaxis response deteriorates with age. Previous studies showed that exposure to HFD speeds up this deterioration (Jung et al., 2018; Rivera et al., 2019). We performed this climbing assay for virgin flies fed 10% and 30% lipid and noted that after four weeks on the different diets the climbing ability diminished with age, both for ND and HFD, and that HFD aggravated this deterioration in a dose-dependent manner (**Fig. 2B**).

To determine whether the different diets affected body mass the flies were weighed at four different time points over one week on the respective diets. Virgin flies exposed to 30% HFD and ND displayed similar body weights

at the different time points (**Fig. 2C**). A dose-dependent increase of TAG levels after exposure to HFD has been reported (Birse et al., 2010; Diop et al., 2017). We therefore monitored the TAG levels in our virgin flies. To our surprise, the flies on 30% HFD did not differ significantly from those on ND over a week (**Fig. 2D**). There is an elevated TAG level in flies kept for five and seven days on HFD compared to the levels observed on day 1 and day 3; however, an increase was also observed in ND flies at this time (**Fig. 2D**). Next, we monitored the glucose and glycogen levels, and found that there is no significant difference between HFD and ND (**Fig. 2E and 2F**).

We also determined the body weight and nutrient levels in mated female flies exposed to HFD (R2 protocol). In contrast to the virgin flies (R1 protocol), mated flies fed 30% HFD displayed decreased body weight compared to ND flies from three days to three weeks of diet treatment (**Supplemental Fig. 1A**). Similar to the virgin flies, HFD has no significant effect on TAG, glycogen or glucose levels (**Supplemental Fig. 1B-D**). To further compare the R2 and R1 protocols, we also monitored the negative geotaxis of aging flies exposed to HFD (**Supplemental Fig. 1E**). At three and four weeks of age, we saw a similar decline in climbing activity in HFD flies for both regimes. However, in older flies (5 w), which were only tested for R2, there was no difference between ND and HFD (**Supplemental Fig. 1E**).

Insects, including *Drosophila*, respond to prolonged cold by entering a coma-like state and after transfer to normal temperature they recover (Overgaard and MacMillan, 2017; Sinclair et al., 2013). The time it takes to recover from chill coma is used as a measure of cold tolerance and general stress resistance (MacMillan et al., 2018; Overgaard and MacMillan, 2017; Terhzaz et al., 2015). We monitored the recovery time from chill coma in mated female flies after eight days of exposure to 10% and 30% HFD and ND (**Supplemental Fig. 1F**). Surprisingly, 30% HFD flies displayed significantly faster recovery from chill coma. Thus, short-term exposure to HFD seems to be beneficial for cold tolerance.

Fecundity in *Drosophila* females depends on nutrient availability and diet composition (Armstrong, 2020; Camus et al., 2019; Hartman et al., 2013; Musselman and Kühnlein, 2018; Partridge et al., 1987). Therefore, we asked whether the fecundity of the mated flies is affected by HFD. We exposed one-

and three-week-old to 30% HFD and ND for one or two weeks and then monitored the number of eggs laid over 24 h. Both after one and three weeks, the HFD results in a significantly decreased number of eggs laid (**Fig. 2G**).

Since we observed no difference in TAG levels in virgin and mated flies with the feeding regimes used, we asked whether this was due to the age of the flies or the different food recipe used in our study compared to those used in other studies. Thus, we tested a food composition where the ratio between lipid and carbohydrate/yeast content was altered to yield more lipid (HFD-2 in Methods). Here we used 10-day-old flies kept on ND that were subsequently transferred to HFD-2 for another 5 days. Again, these HFD-2 flies displayed no increase in body mass or TAG levels, but instead the TAG levels decreased (**Supplemental Fig. 2A** and **2B**).

We next applied a feeding protocol similar to Birse and coworkers (Birse et al., 2010) to test TAG levels. This employed standard Bloomington food (BLD) according to BDSC (see Methods) with 30% lipid (BHFD). We used 14-day-old flies that had been kept on BLD and transferred to BHFD food for one day only. Flies kept on the BHFD for one single day exhibited decreased body weight and increased TAG content compared to the flies kept for 15 days on BLD (**Supplemental Fig. 2A** and **2B**). Thus, short-term exposure to 30% lipid on this diet results in TAG levels similar to previously published data (Birse et al., 2010).

3.2. HFD, ageing and behavioral senescence

Both locomotor activity and sleep patterns change with ageing in *Drosophila* (Liao et al., 2017; Luo et al., 2012). Thus, we tested the long-term effects of HFD on locomotor activity and sleep. Virgin flies (R1 protocol) were kept on the different diets (ND, 10% HFD or 30% HFD) for one or three weeks, whereafter locomotor activity and sleep were monitored using the *Drosophila* Activity Monitor System (DAMS, Trikinetics). The average activity histograms across the first three days are shown in **Fig. 3A and 3B**. Note that during the first night after transfer to the DAMS, the flies kept for three weeks in 30% HFD exhibited hyperactivity (**Fig. 3B**, arrow). This is consistent with a recent study that demonstrated that HFD enhanced starvation induced hyperactivity (Huang et al., 2020). Next we analyzed the average activity (using 5 min bins)

of flies fed ND, 10% HFD and 30% HFD for one or three weeks. The 30% HFD flies (both after one and three weeks of diet) displayed less activity than ND flies during the light period, but 10% HFD did not affect activity (**Supplemental Fig. 3A-D**). The average total activity during 24 h calculated over three days indicates that the 30% HFD flies are less active than the ND flies both after one and three weeks of feeding the diet (**Supplemental Fig. 3 E**).

Since ageing impairs sleep patterns in *Drosophila* (Luo et al., 2012; Umezaki et al., 2012), we next monitored the total amount of sleep and the number of sleep bouts over 24 h in virgin flies kept for one or three weeks on the diets. Regardless of diet, the total amount of sleep decreased with age (**Fig. 3C**). After three weeks of 30% HFD flies slept more than the ND flies, whereas flies on 10% HFD are not significantly different from ND or 30% HFD (**Fig. C**). The number of sleep bouts (day and night; 24 h) is significantly higher in 30% HFD flies compared to ND fed flies both after one week and after three weeks (**Fig. 3D**). When we break up the sleep data into daytime and nighttime sleep (light and dark period), we see that the total nighttime sleep decreases with age and that the flies fed 10% and 30% HFD sleep more than ND flies (**Fig. 3E**). The daytime sleep does not change with age, but flies kept three weeks on the 30% HFD diet sleep more than the ND flies (**Fig. 3E**). During nighttime, the number of sleep bouts increased after three weeks, compared to one week in all three diets (**Fig. 3F**). This indicates an increase in sleep fragmentation during aging, as described before (Liao et al., 2017; Metaxakis et al., 2014; Williams et al., 2016). During daytime, the 30% HFD flies display a higher number of sleep bouts compared to both ND at 3 weeks and compared to 10% HFD flies at one week (**Fig. 3F**). This suggests that 30% HFD impairs sleep quality (increases sleep fragmentation), but the effect of ageing does not worsen after one week.

3.3. HFD effects on aging of neurons and muscle

The dopaminergic system in *Drosophila* plays a critical role in learning and memory, as well as in regulating locomotor behavior, aggression, arousal, food search, and stress (Alekseyenko et al., 2013; Aso and Rubin, 2016; Ichinose et al., 2017; Landayan et al., 2018; Petruccelli et al., 2020;

Riemensperger et al., 2013; White et al., 2010). It has been shown that dopamine (DA) levels decrease with age in brain neurons of *Drosophila* (Liao et al., 2017; Neckameyer et al., 2000; White et al., 2010). This was demonstrated by immunocytochemistry with antiserum to the rate limiting biosynthetic enzyme tyrosine hydroxylase (TH). Here, we monitored the TH-immunoreactivity in a cluster of brain DA neurons of flies kept for one and three weeks on ND and three weeks of 30% HFD, using mated flies (R2) (**Supplemental Fig. 4A, B**). Using the protocerebral posterior lateral (PPL1) cluster of DA neurons (Nässel and Elekes, 1992) we found that there is no significant difference in TH immunolabeling in PPL1 neurons after three weeks of aging under ND conditions (**Supplemental Fig. 4A, B**). However, three weeks of 30% HFD led to strongly decreased TH levels in PPL1 neurons compared to one and three weeks of ND (**Supplemental Fig. 4A, B**).

Another marker for ageing in flies is an increased deposition of aggregates containing polyubiquitinated proteins in muscles (Demontis and Perrimon, 2010). We compared the anti-polyubiquitin immunoreactivity levels in abdominal muscles of normally aging female flies and flies fed 30% HFD (**Supplemental Fig. 4C-F**). Our data show an increase in number and size of polyubiquitin particles over five weeks for both ND and HFD, but HFD in itself has no obvious effect on the accumulation of polyubiquitinated particles in muscle (**Supplemental Fig. 4C-F**).

3.4. HFD effects on crop size and food ingestion

Adult flies use the crop, a diverticulum from the foregut, for temporally storing nutrients, primarily carbohydrates (Stoffolano and Haselton, 2013). In our experiments, we observed that the crop is dramatically enlarged in flies kept on HFD for three weeks compared to ND flies (**Fig. 4A-E**). Using Nile red to stain lipids, we found that the enlarged crop is filled with aggregates of lipid (**Fig. 4E**).

Since the crop size increased after feeding flies HFD, we were interested testing the effect of HFD on feeding rate itself. Food consumption (using ND food) was measured using the CAFE assay. Flies fed HFD for seven days ingest significantly less standard food (ND) during the first day after transfer to the CAFE assay than control flies kept for the same time on

ND (**Fig. 5A**). Since flies kept in the CAFE assay obtain no separate water for drinking (they only obtain water available in the food), we next tested whether the increased food intake is due to thirst or hunger. Thus, we exposed the experimental flies to a food and water choice experiment. In a CAFE assay set-up, we inserted one capillary tube with water and another tube containing food. Flies kept for seven days on 30% HFD were monitored for 24 h. Our data show that the flies kept on HFD consume more food and drink less water than flies kept on ND; they rather drink less (**Fig. 5B, C**). These findings suggest that flies kept on HFD experience hunger, at least in the short-term.

3.5. HFD effects on hormonal signaling

Lipid and carbohydrate storage and metabolism are regulated hormonally. Among the key hormones are adipokinetic hormone (AKH), the functional analog of mammalian glucagon, (Bharucha et al., 2008; Grönke et al., 2007; Kim and Rulifson, 2004; Lee and Park, 2004) and *Drosophila* insulin-like peptides (DILPs) (Broughton et al., 2005; Grönke et al., 2010; Rulifson et al., 2002; Zhang et al., 2009). Thus, we investigated the effects of HFD on transcript levels of *Akh, dilp1*, *dilp2*, *dilp3*, *dilp5,* and *dilp6*. Flies were kept on ND and 30% HFD for one or five weeks to test effects of diet and ageing. At these time points, the transcript levels of several genes of interest were determined by qPCR. The *akh* transcript level is higher in the five-week-old HFD flies compared to those kept for one week on both diets, and compared to five-week-old flies on ND (**Fig. 6A**). The lipase Brummer (encoded on *Bmm*), which is a homolog of the mammalian adipose triglyceride lipase, is critical for lipid metabolism and mobilization in *Drosophila* (Grönke et al., 2007). We found that *Bmm* levels increased significantly only in flies kept five weeks on ND with no effect of HFD (**Fig. 6B**). Levels of *dilp1*, *dilp3, dilp5,* and *dilp6* were not found significantly different between diets and no effects of ageing were observed, with the exception of a slight increase in *dilp2* transcript after 5 weeks on ND (**Fig. 6C-G**). Moreover, we monitored the AKH peptide levels by immunohistochemistry in the adult corpora cardiaca, and found no changes in AKH levels with diet or aging (**Fig. 6H**). By contrast, the DILP2 peptide levels in the insulin producing cells (IPCs) were found to be reduced as an effect of both diet and age. Flies fed HFD for one week

exhibited much lower DILP2 levels compared to ND flies (**Fig. 6I**). This suggests that HFD may affect DILP2 release from IPCs. Flies fed ND and HFD both displayed decreased DILP2 levels after five weeks compared to younger ones (**Fig. 6I**). In HFD flies, the DILP2 levels were low already at one week of exposure and remained low until five weeks of exposure. This effect of ageing on DILP2 levels was also noted in a previous study (Liao et al., 2017).

3.6. Adipokinetic hormone and insulin signaling and effects of HFD

Increased AKH signaling is known to extend lifespan in *Drosophila* (Post et al., 2018; Waterson et al., 2014). In contrast, decreased insulin signaling can extend *Drosophila* lifespan (Broughton et al., 2005; Clancy et al., 2001; Tatar et al., 2001; Tatar et al., 2014). Thus, we next asked whether lifespan is affected by HFD when AKH and DILP signaling are disrupted. We examined the lifespan of *Akh*- and *dilp2,3,5* triple mutant flies kept on ND and HFD. Interestingly, we found that under both ND and HFD conditions, *Akh* mutants live longer than control flies on the same diets (**Fig. 6J**). Hence, in our experiments, diminished AKH signaling increases longevity of the flies, regardless of diet. Furthermore, HFD shortened the lifespan of both *Akh* mutants and w^{1118} flies compared to the same genotypes on ND (**Fig. 6J**). The lifespan of the *dilp2,3,5* triple mutants also decreased drastically under HFD conditions compared to flies kept in ND conditions (**Fig. 6K**).

It has been proposed that AKH is important in mobilizing energy to support oocyte growth (Lorenz and Gäde, 2009). We therefore asked whether HFD and AKH signaling have an effect on oogenesis. Thus, we exposed four-day-old mated *Akh* mutant and w^{1118} flies to ND and HFD for one week and monitored the number of mature eggs in their ovaries. We found that under ND conditions, the ovaries of *Akh* mutant flies contained much fewer eggs than controls (**Fig. 7A**). This supports the importance of AKH signaling for lipid mobilization and oogenesis. Moreover, the number of eggs is drastically lower in HFD flies for both the *Akh* mutants and w^{1118} (**Fig. 7A**), suggesting that increased lipid content is detrimental for fecundity. We also analyzed *Akh* mutant and w^{1118} flies exposed to ND and HFD for three weeks (Fig. 7B). The results are similar to those from one-week exposure, except that ovaries of

Akh mutant flies contains a larger number of eggs at three than at one week of ND. These findings of reduced egg numbers in HFD flies agree well with our results in a previous section that show that flies kept on HFD lay a reduced number of eggs (**Fig. 2G**).

4. Discussion

In this work, we recorded physiological effects of acute as well as chronic exposure to HFD in *Drosophila*. We demonstrate that both virgin and mated female flies exposed to HFD exhibit decreased lifespan, accelerated decline of climbing ability with age, and increased sleep fragmentation in three-week-old flies. Another long-term effect of HFD was a decline in TH immunolabeling in dopaminergic brain neurons. Furthermore, exposure to HFD for three weeks or longer lead to increased size of the crop and an elevation of *Akh* transcript levels. However, surprisingly we detected no long-term effects of HFD on body weight, or levels of TAG, glycogen or glucose. We also found that exposure to HFD for one or three weeks reduced egglaying and decreased egg numbers in the ovaries. Since *Akh* transcript was upregulated after five weeks of HFD we analyzed the role of AKH signaling in longevity and fecundity under HFD conditions. *Akh* mutant flies displayed increased lifespan and reduced numbers of mature eggs in ovaries compared to controls both on ND and HFD.

Previous studies have also shown that HFD decreases lifespan and climbing ability (Jung et al., 2018; Rivera et al., 2019; Woodcock et al., 2015). Deleterious effects of HFD on several other behaviors during ageing have been reported, including memory and odor sensitivity (Jung et al., 2018; Rivera et al., 2019). Similar to humans, aging flies display decreased activity rhythms and increased sleep fragmentation (Koh et al., 2006; Metaxakis et al., 2014; Umezaki et al., 2012). In this study, we found that during nighttime, aging flies exhibited an increased number of sleep bouts, suggesting increased sleep fragmentation. We also noted a decrease in total duration of nighttime sleep. Additionally, we explored the effects of HFD and found that 30% HFD increased both total sleep amount and total number of sleep bouts in three-week-old flies compared to flies on ND. Thus, flies kept three weeks on 30% HFD sleep more than ND flies, but display increased sleep

fragmentation. Interestingly, we also found that flies kept for three weeks on 30% HFD displayed hyperactivity during the first night after transfer to the DAMS system. This observation is consistent with a recent study (Huang et al., 2020), in which the authors showed that HFD increased starvation-induced hyperactivity. In conclusion, long-term exposure to HFD has an adverse effect on sleep.

Earlier studies found that short-term HFD induces increased TAG levels in flies (Birse et al., 2010; Diop et al., 2017; Jung et al., 2018). In contrast to this, our data show that both virgin and mated females kept on HFD from an early age display no significant increase in TAG, glycogen or glucose levels. An exception is when we used older mated flies (14 d post eclosion) that were fed a standard Bloomington diet with 30% lipid for one day only in a protocol similar to (Birse et al., 2010); this leads to increased TAG. Thus, the food composition, duration of HFD exposure and age of flies seem to affect the TAG levels. The HFD used in previous studies (for instance (Birse et al., 2010; Diop et al., 2017; Jung et al., 2018)] contained less yeast (protein) and carbohydrates than in the majority of our experiments, and possibly these flies consumed more food to compensate. In our main diets, we keept the same amount of protein and sugar in ND and HFD.

In flies kept on 30% HFD for three weeks the crop is drastically enlarged and full of lipid. The crop is used for temporal storing of food, especially carbohydrates (Stoffolano and Haselton, 2013). Thus, it is important for maintaining carbohydrate homeostasis in flies. In our experiments, it seems that HFD causes a defect in crop emptying leading to excess storage of ingested food, especially lipids. Coconut oil consists mainly of saturated fat in the form of fatty acids like lauric or myristic acid (Birse et al., 2010). This fat seems to get trapped in the crop, which could explain why long-term intake of HFD does not appear to affect TAG levels. The crop malfunction may also affect intake of other nutrients and alter metabolism, although we noted no significant effects of HFD on body mass, glycogen or glucose levels. Possibly alterations in nutrient uptake and metabolism lead to reallocation of energy for somatic maintenance (Partridge et al., 1987), which could explain the decreased fecundity, detected as a decrease in mature eggs in the ovaries and reduced egglaying.

Long-term exposure to HFD resulted in increased *Akh* transcript levels, although no change was seen after short exposure, and the AKH peptide levels remain unaltered. No differences were detected for transcripts of five different insulin-like peptides (*dilp1-dilp3, dilp5,* and *dilp6*). AKH is known to induce mobilization of lipids in insects, including *Drosophila* (Gäde and Auerswald, 2003; Grönke et al., 2007; Musselman and Kühnlein, 2018; Toprak et al., 2020; Van der Horst, 2003), but also to allocate carbohydrate as energy to fuel food search in hungry flies (Bharucha et al., 2008; Isabel et al., 2005; Lee and Park, 2004; Yu et al., 2016). AKH signaling has also been shown to regulate lifespan in flies (Katewa et al., 2012; Post et al., 2018; Waterson et al., 2014). More specifically, overexpression of *Akh* (Katewa et al., 2012), mutation of the water sensing protein pickpocket 28 (*ppk28*) (Waterson et al., 2014) or mutation of *dilp2* (Post et al., 2018) lead to increased *Akh* levels and a prolongation of life span. The lifespan effect of ectopic overexpression of *Akh* has been observed under dietary restriction (Katewa et al., 2012) and the *ppk28* mutants displayed altered lipid and water metabolism in addition to elevated *Akh* transcript (Waterson et al., 2014). In our work, we analyzed female *Akh* mutant flies and found that both on ND and HFD the mutants lived longer than w^{1118} controls, although HFD shortened lifespan both for mutants and controls. A previous analysis of *Akh* mutant flies detected no significant effect on lifespan in females or males (Bednářová et al., 2018). Thus, there are different effects of *Akh* mutation and *Akh* upregulation on lifespan in these studies. Possibly some of the differences can be attributed to the genetic background of the flies used, but also variations in experimental protocol and diets. In this context, it can be noted that *Akh* mutant flies live longer when exposed to starvation (Isabel et al., 2005).

AKH is required for mobilization of energy substrates, notably lipid and trehalose, during energy-demanding exercise, such as flight and locomotion, including starvation-induced food seeking (Gäde and Auerswald, 2003; Isabel et al., 2005; Lee and Park, 2004; Van der Horst, 2003; Yu et al., 2016). *Akh* expression increased almost four-fold after five weeks of HFD compared to ND and short-term exposure to HFD. This may suggest that five weeks of HFD renders flies that experience nutrient deficiency. In fact, when flies are

transferred from three weeks of HFD to activity monitors, they display increased activity the first 12 hours (**Fig. 3B**), which may represent food seeking behavior (subsequently the flies feed on carbohydrate in the monitor tubes).

Although several of the DILPs are involved in lipid metabolism in flies (Broughton et al., 2005; Musselman and Kühnlein, 2018; Toprak et al., 2020), we found that transcript levels of *dilp1-dilp3*, *dilp5,* and *dilp6* displayed no significant changes with short of long-term exposure to HFD. Similar findings were presented in previous reports (Jung et al., 2018; Woodcock et al., 2015). However, we observed a significant decrease in DILP2 peptide levels in the IPCs after one week of HFD, but not after five weeks, suggesting short-term effects on DILP2 release. Diminished insulin signaling increases lifespan in *Drosophila* (Broughton et al., 2005; Grönke et al., 2010; Tatar et al., 2001). The IPCs produce DILP1-3 and 5 (Brogiolo et al., 2001; Liu et al., 2016; Rulifson et al., 2002) and these cells are known to be critical for the longevity phenotype (Broughton et al., 2005). Interestingly, we found that *dilp2,3,5* triple mutant flies display drastically shortened lifespan when put of HFD compared to ND. Thus, the diminished insulin signaling does not protect flies from aversive effects of HFD in the context of longevity.

What are the mechanisms behind the shortened lifespan and behavioral senescence in flies kept on HFD? An excess of dietary lipids affects several tissues such as the intestine, the fat body, the central nervous system, and the heart, but also results in global effects on metabolism and the expression of a multitude of genes (Birse et al., 2010; Huang et al., 2020; Jung et al., 2018; Musselman and Kühnlein, 2018; Rivera et al., 2019; von Frieling et al., 2020; Woodcock et al., 2015). Some effects of HFD seem to be tissue specific, others are more global.

The heart function in *Drosophila* deteriorates already after short exposure to HFD with appearance of lipid accumulation, reduced contractility and structural changes in heart muscle (Birse et al., 2010). It therefore seems that HFD has direct lipotoxic effects on the heart muscle, but these HFD effects can be alleviated by both local and systemic reduction of activity in the insulin-TOR (target of rapamycin) pathway (Birse et al., 2010). This suggests that HFD causes a deregulation of the insulin-TOR pathway, at least in the

short-term. At the gut level, HFD alters the microbiota, increases intestinal stem cell activity, and increases the number of enteroendocrine cells in the midgut (von Frieling et al., 2020). In this study, it was shown that the HFD directly activates JNK signaling in enterocytes that in turn signal with the cytokine unpaired 3 (upd3) to activate JAK/STAT signaling in the stem cells. Especially in the long-term this signaling is dependent on presence of microbiota. The HFD induced changes in the gut function are likely to alter both intestinal and organismal metabolic homeostasis (due to peptide hormones in enteroendocrine cells) and also ageing of the gut. Another study implicated HFD-activated upd3 and JAK/STAT signaling in macrophage activation and inflammation in the shortened lifespan and altered metabolism of *Drosophila* (Woodcock et al., 2015).

Other studies have suggested that HFD alters mitochondrial respiration in cells, including neurons, that may result in behavioral senescence (Cormier et al., 2019), or induces effects on specific neurons via the lipoprotein LPT and its receptor LpR1 to induce behavioral changes (Huang et al., 2020). Two studies investigated HFD-induced alterations in gene expression that affected olfactory behavior, metabolism, and stress responses (Jung et al., 2018; Rivera et al., 2019). It is also clear that HFD and obesity influences ovary development and fecundity (Musselman and Kühnlein, 2018). Thus, in summary, there are several mechanisms that can explain the effects of HFD on longevity, fecundity, and behavioral senescence.

5. Conclusions

In summary, our data suggest that long-term exposure to HFD not only reduces lifespan, it also leads to hyperactivity that indicates food seeking, reduced fecundity, and increased food intake when transferred to ND. Mated females, but not virgins, exposed to HFD display decreased body weight compared to controls. The reduced number of mature eggs in the ovaries of HFD flies can possibly explain this weight difference. Furthermore, *Akh* expression is elevated in HFD flies suggesting increased energy mobilization. Lifespan is extended in *Akh* mutant flies kept on HFD compared to control flies on the same diet, but it is shortened compared to mutants kept on ND. Similarly *dilp2,3,5* triple mutant flies display shortened lifespan on HFD

compared to ND. Thus, AKH signaling plays a role in the response to HFD. The enlarged, lipid-filled crop observed in flies on HFD, is remarkable and suggests a malfunction in nutrient allocation in the fly. It is, however, not clear how the crop influences the phenotypes we found in this study.

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Figure legends

Fig. 1. Experimental design. **A.** Regime 1: Virgin female flies were collected after eclosion, and were thereafter kept on normal diet (ND) or food containing 10% coconut oil (HFD 10%) or food containing 30% coconut oil (HFD 30%). **B.** Regime 2: Flies were allowed to mate on ND for 4 days after eclosion, thereafter these flies were transferred to ND or HFD 30%. The time line is shown as a horizontal bar, where Ecl depicts collection of newly eclosed flies and 0 the transfer to experimental diet. The effects of HFD on lifespan of virgin and mated females were determined, and assays were performed at the sample times indicated.

Fig. 2. Effects of HFD on lifespan and climbing ability, body weight and levels of TAG, glycogen, and glucose of virgin female flies. **A.** Survival curve of virgin females exposed to ND, 10% HFD and 30% HFD. The survivorship decreased in flies fed with 10% HFD and 30% HFD compared to ND, and the lifespan is further decreased in flies with 30% HFD compared to 10% HFD. [***$p < 0.001$ as assessed by log-rank Mantel–Cox test, n = 298–320 flies per treatment, from three independent replicates]. **B.** HFD decreased the climbing ability of four-week-old flies fed 10% and 30% HFD compared to ND. Data are presented as means ± S.E.M, n = 8–19 flies for each group from three

independent replicates (*p < 0.05, ***p < 0.001, two-way ANOVA followed by Sidak's multiple comparisons test). **C - F.** Flies fed with 30% HFD for one to seven days displayed no effects on body weight, TAG levels, glycogen levels and glucose levels compared to ND. Note that TAG levels increased at day 5 and 7 comparing to day 1 in the flies fed with HFD, but were not significantly different from levels seen with ND. Data are presented as means ± S.E.M, n = 6–7 replicates with 5 flies in each replicate for each group (*p < 0.05, **p < 0.01, two-way ANOVA followed Sidak's multiple comparisons test). **G.** Flies kept on 30% HFD for one and three weeks laid significantly fewer eggs compared to flies kept on ND. Data are presented as means ± S.E.M, n = 19–21 flies for each group from three independent replicates (***p < 0.001, one-way ANOVA followed by Tukey's multiple comparisons).

Fig. 3. HFD leads to hyperactivity and sleep fragmentation in virgin female flies. **A.** Average activity histograms over three days during light and dark phase from flies fed with ND, 10% HFD and 30% HFD for one week (R1 protocol). Activity monitoring started after 1 week of HFD, and flies obtained food containing 5% sucrose during monitoring. **B.** Activity histograms from flies fed with ND, 10% HFD and 30% HFD for three weeks (otherwise as in A). Note that the flies fed HFD for 3 weeks before activity monitoring displayed increased activity during the first night (arrow). **C.** The total sleep time is decreased in three-week-old flies compared to one-week-old flies, and flies fed 30% HFD for 3 weeks displayed increased amount of sleep compared to ND. **D.** Average number of sleep bouts per 24 h. The number of sleep bouts averaged over three 24 hour periods is higher in flies exposed to 30% HFD both after 1 week and 3 weeks. **E.** The total sleep time shown separately for the light and dark phases. Aging decreased sleep time during dark phase. Total sleep time decreased during the dark phase in aging flies, and flies fed HFD for 3 weeks showed increased sleep time during both the light and dark phases. **F.** The number of sleep bouts during light and dark phases of flies fed with ND and HFD for 1 and 3 weeks. HFD and aging increase the number of sleep bouts. During the light period 30% HFD increased sleep bouts both after one and three weeks of the diet. Data in graphs are presented as means ± S.E.M, n = 46-48 flies for each treatment

from three replicates (*p < 0.05, **p < 0.01, ***p < 0.001, as assessed by one-way ANOVA followed by Tukey's multiple comparisons).

Fig. 4. The crop is enlarged in flies fed with HFD. Flies were kept on ND for 11 days, 25 days or transferred to 30% HFD at 4 days of age where after they were kept on the HFD for 21 days (reaching a total age of 25 days). The morphology of the crops was investigated after staining with rhodamine-phalloidin (red) and DAPI (blue). **A.** The morphology of the crop and crop duct of flies kept for 11 days on ND. **B.** Crop from a fly kept for 25 days on ND. **C.** Flies kept for 4 days on ND and then 21 days on 30% HFD displayed a drastically enlarged crop. **D.** Size quantification (total area) of fly crops after 11 days ND, 25 days ND and 4 days ND plus 21 days HFD. Data are presented as means ± S.E.M, n = 8-9 flies for each treatment from three replicates (***p < 0.001, compared to one-week-old ND flies, as assessed by one way ANOVA followed by Tukey's multiple comparisons). **E.** Nile Red staining reveals that the crop of a fly fed with HFD for 21 days is loaded with lipid aggregates (e. g. at arrows).

Fig. 5. HFD affects feeding and drinking. **A.** Food intake increased in HFD lies only during the first day after transfer to the CAFE assay (where ND was provided). Data are presented as mean ± S.E.M, n = 24-30 flies for each treatment from three replicates. (**p < 0.01, unpaired Student's t-test). **B and C.** When given a choice between food and water for 24h in the CAFE assay, flies kept for 7 days on 30% HFD consumed more food (**B**) but less water (**C**) compared to ND flies. Data are presented as mean ± range, n = 30 flies for each treatment from three replicates (**p < 0.01, ***p < 0.001, unpaired Student's t-test).

Fig. 6. The effects of aging and HFD influence transcripts of genes related to lipid metabolism and lifespan of mutant flies. Four-day-old female flies were transferred to ND or 30% HFD. Gene expression and protein levels were monitored after 1 and 5 weeks on the diets. **A.** *Akh* expression increased after 5 weeks on HFD. **B.** A slight increase in *bmm* levels is seen after 5 weeks on

ND, but no effect of HFD. **C-G.** The levels of different *dilp* transcripts *dilp2* do not change with HFD. A slight increase is seen for *dilp2* five-week-old flies on ND. The data in A-G are presented as means ± S.E.M.; three replicates with 10 flies in each replicates were analyzed (*$p < 0.05$, as assessed by one-way ANOVA followed by Tukey's multiple comparisons). **H.** AKH immunolabeling in corpora cardiaca does not change with age or diet. Data are presented as means ± S.E.M, n = 10-11 flies from three replicates for each treatment. Differences among groups were assessed by one-way ANOVA followed by Tukey's multiple comparisons). **I.** DILP2 immunolabeling decreased after one week on HFD compared to ND. After 5 weeks, a decrease in DILP2 levels was observed in both treatment groups compared to one-week-old flies kept on ND. However, after 5 weeks no difference is seen between ND and HFD. Note that the asterisks indicate significance levels compared to one week on ND. Data are presented as means ± S.E.M, n = 11 flies for each treatment from three replicates (**$p < 0.01$, ***$p < 0.001$, as assessed by one way ANOVA followed by Tukey's multiple comparisons). **J.** Survival curves of *Akh* mutants and its control w^{1118} under ND and HFD. *Akh* mutant flies live longer than w^{1118} flies under both ND and HFD conditions and HFD diminished the life span of both *akh* mutant and w^{1118} flies. (***$p < 0.001$ for all comparisons as assessed by log-rank Mantel–Cox test, n = 124–136 flies per treatment from three independent replicates). **K.** HFD leads to decreased life span of *dilp2,3,5* mutant flies. (***$p < 0.001$ as assessed by log-rank Mantel–Cox test, n = 150–157 flies per treatment from three independent replicates).

Fig. 7. HFD for one or three weeks affects fecundity. **A.** HFD for one week decreased egg numbers in ovaries compared to ND for both *Akh* mutants and w^{1118} controls, and *Akh* mutants have fewer eggs compared to w^{1118} controls. **B.** Also after three weeks on HFD egg numbers are decreased in both *Akh* mutants and controls compared to ND. However, in *Akh* mutant flies three weeks on ND results in significantly more eggs than in control flies on the same diet. Data are presented as means ± S.E.M, n = 11-14 flies for each treatment from three replicates (**$p < 0.01$, ***$p < 0.001$, as assessed by one way ANOVA followed by Tukey's multiple comparisons).

Supplementary material figures

Supplementary figure 1. HFD leads to decreased body weight but has no effects on metabolism of mated females. Four-day-old mated flies were fed either ND or 30% HFD for 3 weeks. Body weight, TAG, glycogen and glucose levels were monitored at day 1, 3, 5, 7, and 3 weeks of age. **A.** Body weight is significantly diminished in flies on HFD at all time points after 3 days of age. **B-D.** The TAG, glycogen and glucose levels displayed no significant changes in flies with ND and 30% HFD. Data in **A-D** are presented as means ± S.E.M, n = 7 replicates with 5 flies in each replicate for each group were tested (*p < 0.05, **p < 0.01, **p < 0.001, two-way ANOVA followed by Sidak's multiple comparisons test). **E.** Test of negative geotaxis (climbing assay). Flies kept for 3 to 4 weeks on HFD displayed decreased climbing ability compared to ND controls. Data are presented as means ± S.E.M, n = 6 replicates with 10 flies in each replicate for each group (*p < 0.05, two-way ANOVA followed by Sidak's multiple comparisons test). **F.** Chill coma recovery in flies on HFD. Flies kept for one week on 30% HFD recovered more rapidly from chill coma compared to ND controls. Data are presented as means ± S.E.M., 80-94 flies per treatment divided in three biological replicates were used group (***p < 0.001, one-way ANOVA followed by Tukey's multiple comparisons).

Supplementary figure 2. The effects of different high fat diets on body weight and TAG levels. **A.** Flies fed one day on standard Bloomington food (BLD) containing 30% coconut oil (BHFD) displayed decreased body weight, whereas flies kept on HFD-2 (see methods) for 5 d did not. Data are presented as means ± S.E.M.; 4-6 replicates with 5 flies in each replicate were analyzed and the average bodyweight of flies in each replicate is displayed in the figure (**p < 0.01, unpaired Student's t-test). **B.** 10-day-old flies fed with HFD-2 for 5 days displayed decreased TAG level compared to ND fed flies. However, 14-day-old flies that had been kept in BLD and then transfered to BHFD plus 30% coconut oil showed increased TAG levels compared to flies kept on BLD for 15 days. Data are presented as means ± S.E.M.; 4-6 replicates with 5 flies in each replicates were monitored and the

average bodyweight of flies in each replicate is displayed in the figure (**p < 0.01, ***p < 0.001, unpaired Student's t-test).

Supplementary figure 3. The average locomotor activity over 3 days of virgin female flies fed with ND and HFD (Regime 1; see Methods). Flies fed with 10% HFD display no change in locomotor activity after 1 week (**A**) nor 3 weeks (**C**) on the diet. However, flies fed 30% HFD decreased their locomotor activity both after 1 (**B**) and 3 weeks (**D**) on the diet, especially during the light phase (**D**). **E.** Summary of data depicted in A-D shown as total activity/day (average activity per 24-hour period across three days). The 30% HFD flies decreased total activity compared to ND flies at both 1 and 3 weeks of treatment. Data are presented as means ± S.E.M, n = 46-48 flies for each treatment from three replicates (*p < 0.05, as assessed by one-way ANOVA followed by Tukey's multiple comparisons).

Supplementary figure 4. Effects of HFD on the aging of dopamine neurons and muscle. **A.** Representative images of TH immunolabeling in dopaminergic protocerebral posterior lateral (PPL1) neurons after 1 week ND, 3 weeks ND and 3 weeks HFD. **B.** Tyrosine hydroxylase (TH) immunolabeling diminished in PPL1 dopamine neurons in flies kept 3 weeks on HFD compared to 1 and 3 weeks on ND. Data are presented as means ± S.E.M, n = 8-10 flies for each treatment from three replicates (***p < 0.001, as assessed by one-way ANOVA followed by Tukey's multiple comparisons). **C.** Images depicting polyubiquitin immunolabeling in abdominal muscles of flies fed ND and 30% HFD for 1 and 5 weeks. **D-F.** Quantification of polyubiquitin immunolabeling. The number, average size and the percentage area of polyubiquitin stained particles were quantified. HFD does not affect the accumulation of polyubiquitin immunolabeling, only age. Data are presented as means ± S.E.M, n = 10-11 flies for each treatment from three replicates (*p < 0.05, **p < 0.01, ***p < 0.001, as assessed by one-way ANOVA followed by Tukey's multiple comparisons).

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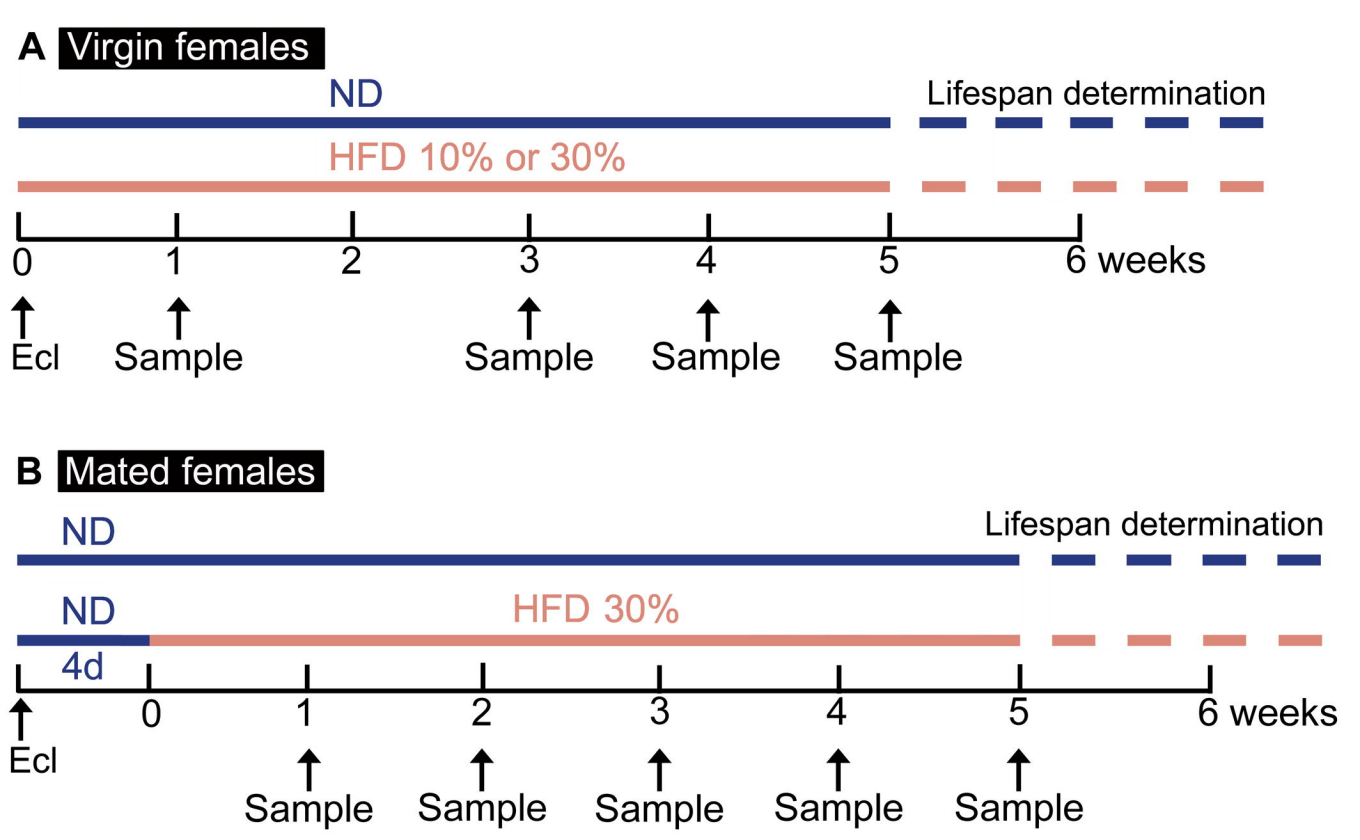
A Virgin females
ND
Lifespan determination
HFD 10% or 30%
0
1
2
3
4
5
6 weeks
Ecl
Sample
Sample
Sample
Sample
B Mated females
ND
Lifespan determination
ND
HFD 30%
4d
0
1
2
3
4
5
6 weeks
Ecl
Sample
Sample
Sample
Sample
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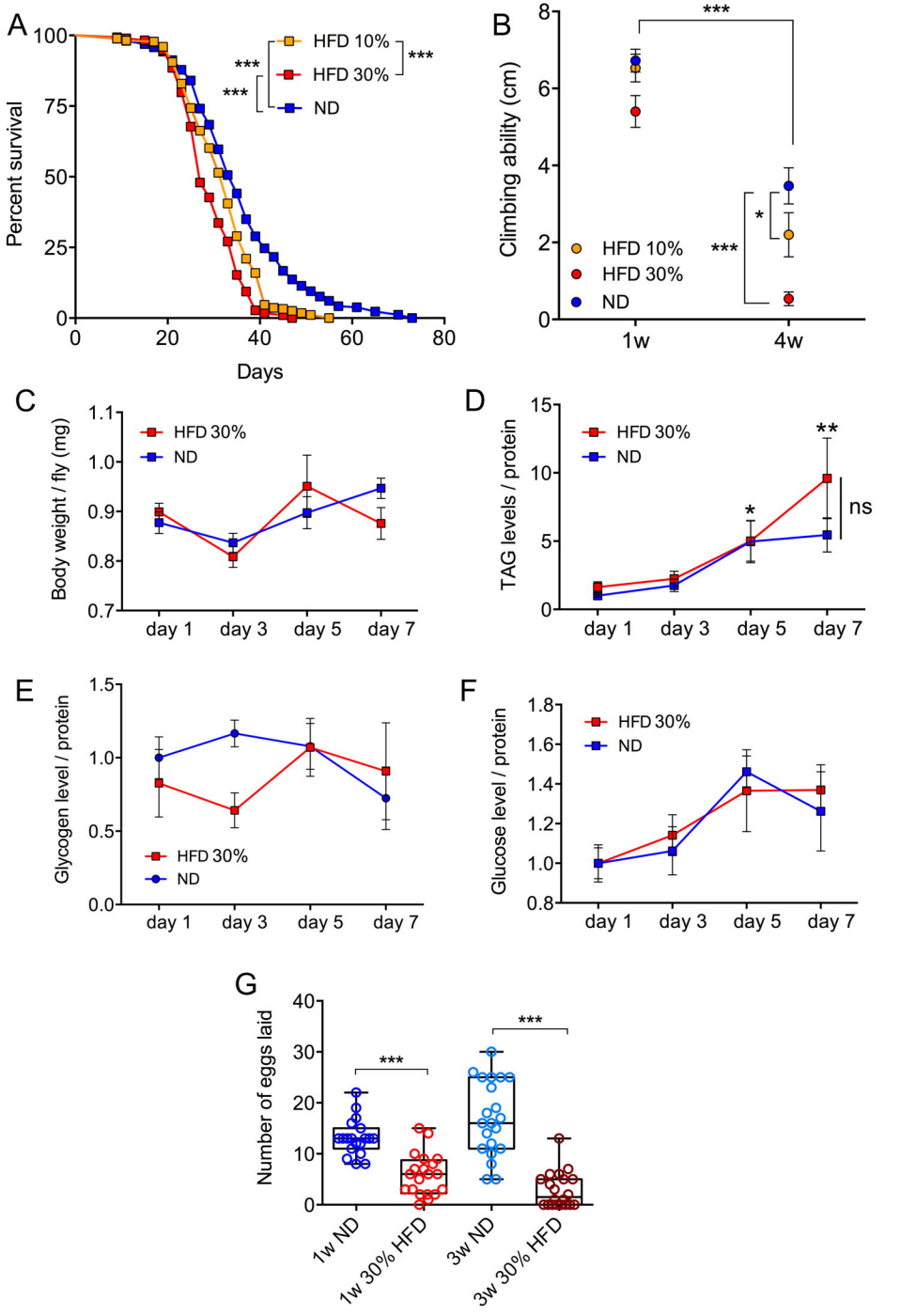
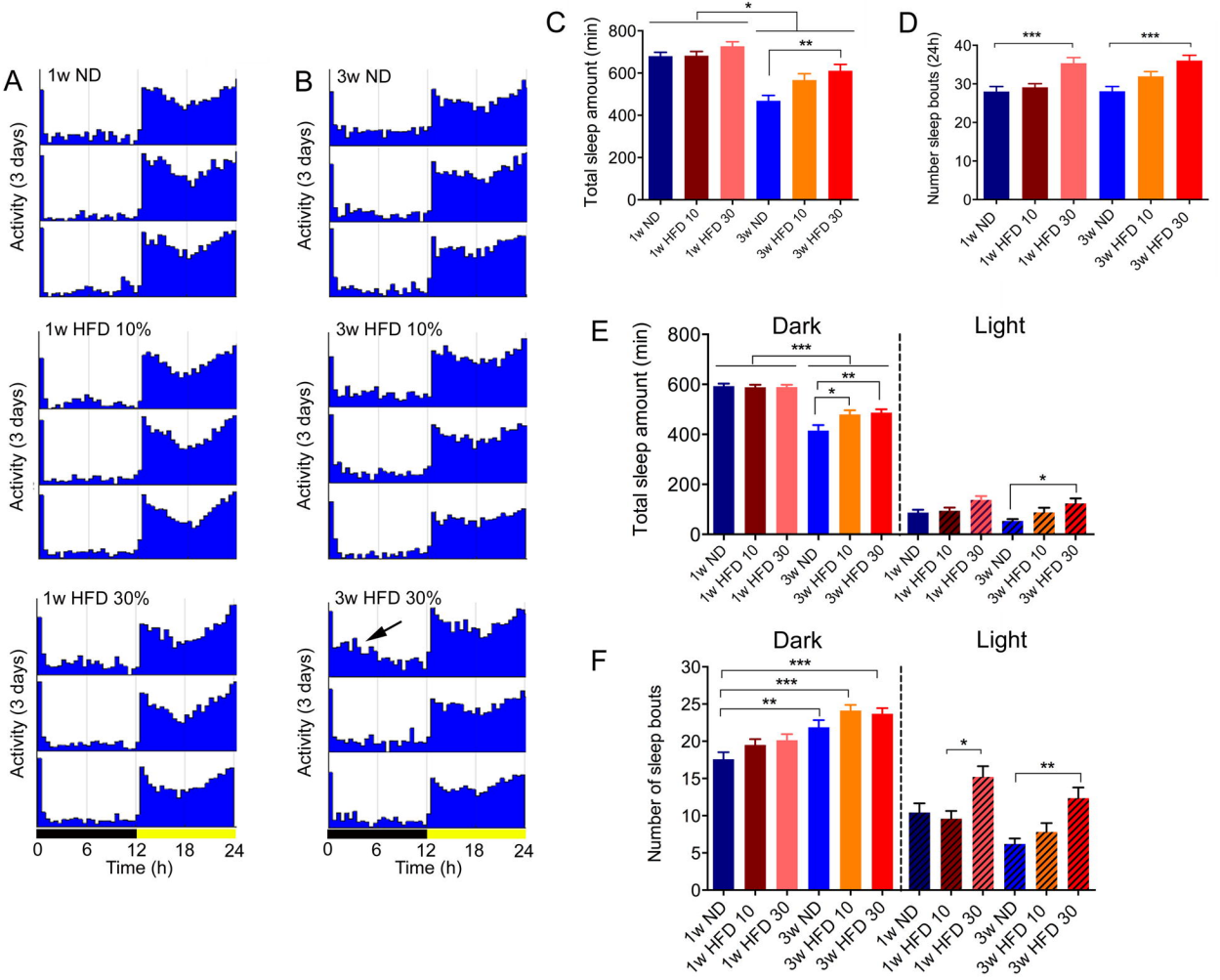
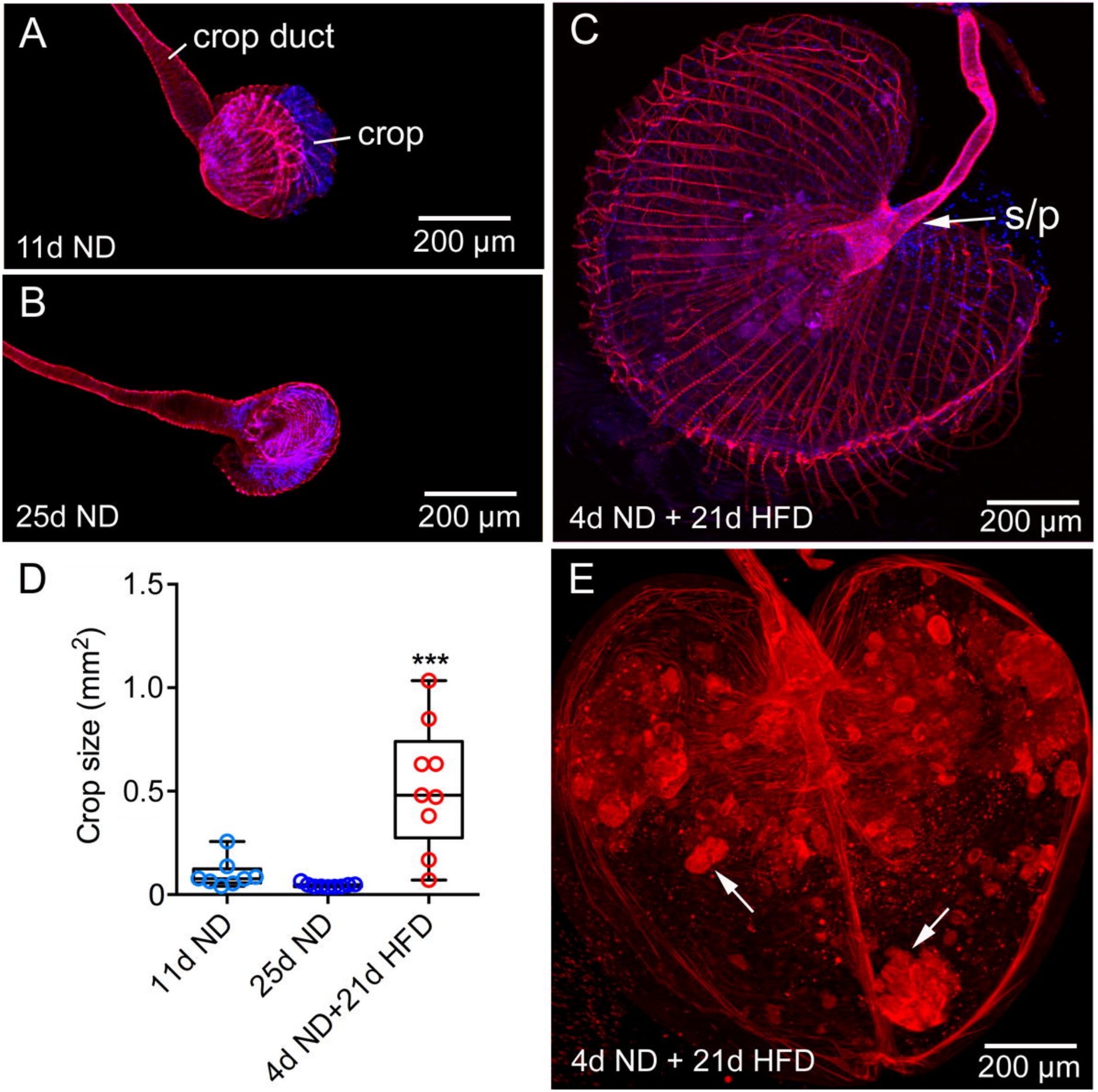
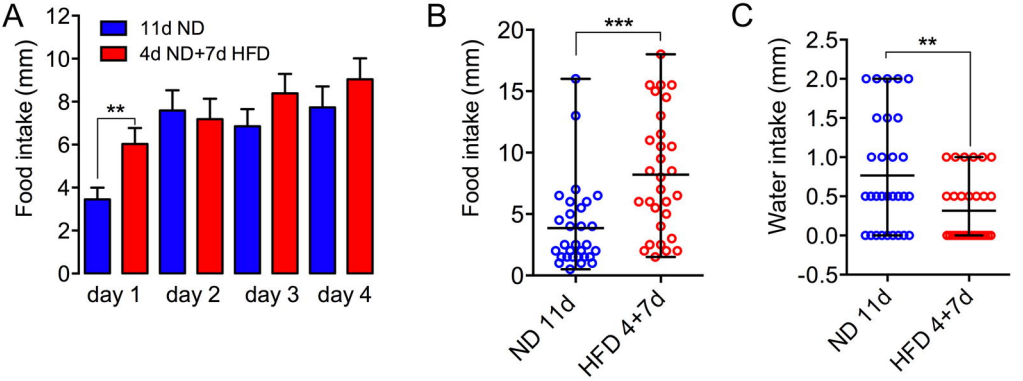
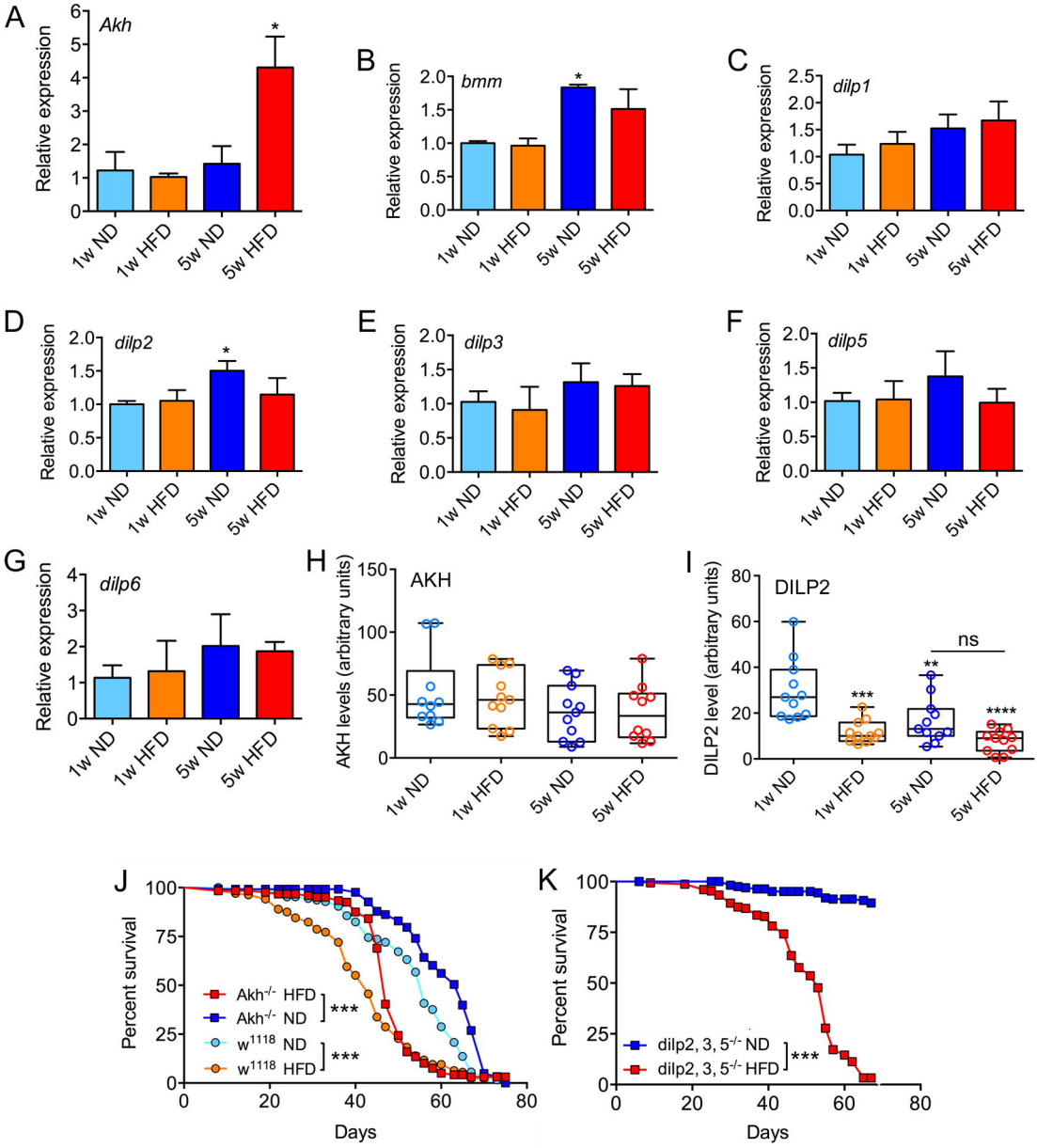
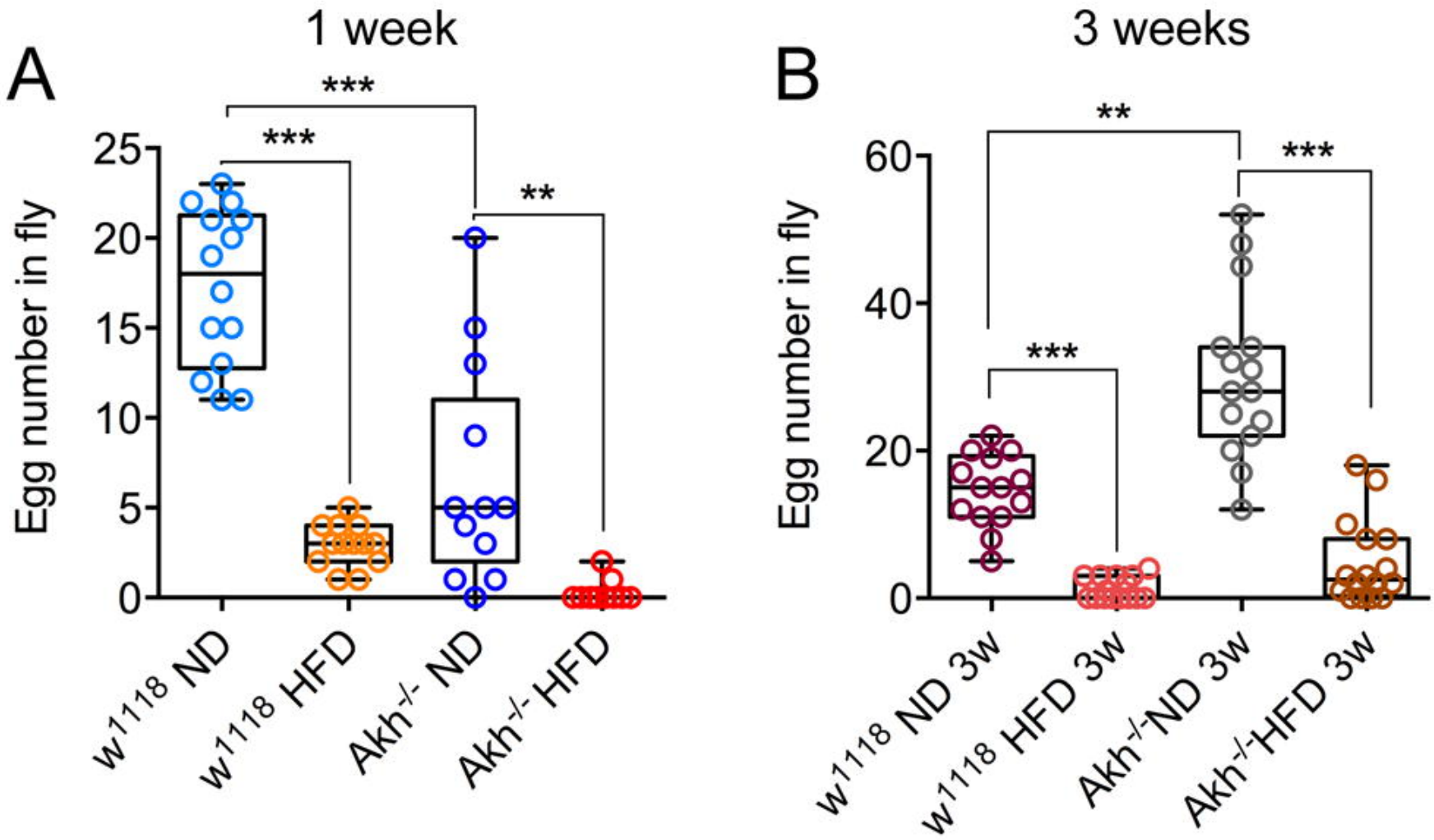

A
Percent survival
Days
HFD 10%
HFD 30%
ND
***
B
Climbing ability (cm)
1w
4w
HFD 10%
HFD 30%
ND
C
Body weight / fly (mg)
HFD 30%
ND
day 1
day 3
day 5
day 7
D
TAG levels / protein
HFD 30%
ND
ns
E
Glycogen level / protein
HFD 30%
ND
F
Glucose level / protein
HFD 30%
ND
G
Number of eggs laid
1w ND
1w 30% HFD
3w ND
3w 30% HFD


A
1w ND
1w HFD 10%
1w HFD 30%
Activity (3 days)
0
6
12
18
24
Time (h)
B
3w ND
3w HFD 10%
3w HFD 30%
C
Total sleep amount (min)
1w ND
1w HFD 10
1w HFD 30
3w ND
3w HFD 10
3w HFD 30
D
Number sleep bouts (24h)
E
Dark
Light
F
Number of sleep bouts


A
crop duct
crop
11d ND
200 µm
B
25d ND
200 µm
C
s/p
4d ND + 21d HFD
200 µm
D
Crop size (mm2)
1.5
1.0
0.5
0
***
11d ND
25d ND
4d ND+21d HFD
E
4d ND + 21d HFD
200 µm


A
11d ND
4d ND+7d HFD
Food intake (mm)
**
day 1
day 2
day 3
day 4
B
Food intake (mm)
***
ND 11d
HFD 4+7d
C
Water intake (mm)
**
ND 11d
HFD 4+7d


A
Akh
Relative expression
1w ND
1w HFD
5w ND
5w HFD
B
bmm
C
dilp1
D
dilp2
E
dilp3
F
dilp5
G
dilp6
H
AKH
AKH levels (arbitrary units)
I
DILP2
DILP2 level (arbitrary units)
ns
J
Percent survival
Days
Akh-/- HFD
Akh-/- ND
w1118 ND
w1118 HFD
K
dilp2, 3, 5-/- ND
dilp2, 3, 5-/- HFD


A
1 week
***
***
**
Egg number in fly
25
20
15
10
5
0
w1118 ND
w1118 HFD
Akh-/- ND
Akh-/- HFD
B
3 weeks
**
***
***
Egg number in fly
60
40
20
0
w1118 ND 3w
w1118 HFD 3w
Akh-/-ND 3w
Akh-/-HFD 3w