Title: Diet and heat - one neuronal subset two responses

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ABSTRACT

The Insulin signal cascade is one of the best studied metabolic circuits, and shows a remarkable high molecular and functional conservation across the animal kingdom. Insulin-producing cells respond directly to nutritional cues in circulation and receive modulatory input from connected neuronal networks. Neuronal control is rapid and integrates a wide range of variables including dietary change or environmental temperature. However, despite various detailed studies that demonstrated the potential of neuronal regulation the physiological relevance of this circuit remains elusive.

In *Drosophila*, Insulin-like peptide 7 (dIlp7) -producing neurons are wired with Insulin-producing cells. We found a dual role for this neuronal subset: a.) activated dIlp7-producing neurons are required to facilitate development at high temperatures, and if confronted with calorie-rich food that represses neuronal activity b.) their product, dIlp7, regulates Insulin signalling levels. Our work shows that Insulin-producing cells not simply integrate signals from circulating nutritional cues and neuronal inputs, but switch to neuronal control in response to dietary composition.

INTRODUCTION

Thriving in new ecological systems, *Drosophila* learned to utilize local food. Such resources vary in their composition in response to environmental temperature changes (1–3). Wild fruit flies feed on rotting fruits *i.e.* a composite diet of microbes and plants. As such food sources are erratic, adult fruit flies also feed on alternative carbohydrate sources, such as yeast-free pollen (4,5). Pollen represents a type of calorie-rich plant food characterized by unsaturated fatty acids and phytosterols (6,7). Previously, it has been shown that *Drosophila* struggle to survive on corresponding experimental diets at high temperatures (1) and that wild flies seek yeast-based food in the summer (3). Although global warming is the main problem our society is facing in this 21st century, its consequence on the animal kingdom is not understood (8,9). In this paper, we provide new insights in the dual response of one neuronal subset to diet and changes of environmental temperatures. With increasing temperatures, fruit flies accelerate their metabolism to acclimatize. By feeding on yeast, *Drosophila* can maintain high Insulin signaling (IS) levels required to shuttle efficiently nutritional cues (such as sugars in circulation) into cells (1,10–12). *Drosophila* produces seven different Insulin-like peptides (dIlps) in response to dietary signals. The structure and function of dIlps, as well as the putative precursor protein, are similar to human Insulin and Insulin-like growth factor (IGF) peptides or pre-pro-Insulin, respectively; thus, implying an analogous organization of the final secreted peptide (12–14). Some dIlps play exclusive developmental roles, such as dIlp1 (15–17), dIlp 4

(12) and dIlp8 (18–21), and their production is restricted in time. Others, such as dIlp6 are associated with nutritional disorders and counteract the group of canonical dIlp peptides (22). Adult flies express four neuronal dIlps: dIlp2,3,5 and 7. dIlp2,3 and 5, produced by neuronal Insulin-producing cells (IPCs), are secreted into circulation (14,23) and resemble the functional role of the vertebrate Insulin, secreted by the pancreatic Beta cells (14). dIlp7 is produced by specialized dIlp7-neurons (D7Ns) and is the only dIlp peptide showing structural conservation across species (13,24,25). Some D7Ns are wired with IPCs whereas others terminate in the periphery (23,26–29). Therefore, dIlp7 may regulate IS at multiple levels, by directly controlling IPC activity or stimulating the IS in the periphery (23,27).

The intracellular IS cascade is conserved and consists of molecular components known from the vertebrate pathway (13,30). Activated Insulin receptor (InR) recruits its adaptor protein, Chico - the functional counterpart of vertebrate Insulin receptor substrate (IRS) proteins - which conveys the signal into either the Ras- or PI3K-dependent pathway (31). The Ras-dependent pathway promotes mitogenic metabolism characterized by catabolic cellular turnover rates (32). PI3K signaling promotes anabolic processes that buildup cellular energy stores (33).

Here, we investigate IS in the scope of different diets and changing environmental temperatures. We show that yeast products can compensate for the loss of dIlp2, 3 and 5 at moderate temperatures. However, to endure heat stress, all three dIlps and yeast are required. In addition, we show that high intracellular Calcium (Ca^{2+}) levels in D7Ns are essential to develop at high temperatures. On yeast-free diets, D7Ns have low Ca^{2+} levels and in consequence, *Drosophila*, on such diets, do not develop.

We found that dIlp7 is essential to survive on yeast-free diet and identified the leucine-rich repeat-containing G protein-coupled receptor 3 (Lgr3) as its potential target. We speculate that dIlp7/Lgr3 signalling is maintaining systemic IS levels by regulating dIlp2 and 5 activity. Taken together, we propose that D7Ns form a neurosecretory network essential to sustain *Drosophila* in absence of dietary yeast and changing environmental conditions.

RESULTS

Yeast food is not sufficient to replace IPC activity at high temperatures

Drosophila larvae kept on Yeast food (YF) show high systemic Insulin signalling (IS) levels, and can endure heat stress. It was shown that yeast lipids facilitate the secretion of *Drosophila* Insulin-like peptides (dIlps) but it remained unclear if high dIlp levels are essential to thrive at high temperatures (1). To test if Insulin-producing cell (IPC) activity is required for flies to survive in warm environments, we have silenced or hyper-activated IPCs by expressing either the inward rectifier potassium channel Kir2.1 (34) or the thermo-sensitive transient receptor potential cation channel, TRPA1 (35,36). As expected, we found that on YF, at 28°C, IPC activity is essential for larval survival (Supplementary Figure S1). To investigate if IPC activity is required throughout larval development, we have induced IPC silencing at different larval stages. At high temperatures (28°C), IPC activity is essential for larval survival, whereas continuous high calcium (Ca^{2+}) levels in these cells are of no consequence (Supplementary Figure S1). To investigate if IPC activity is required throughout larval development, we have induced IPC silencing at different larval stages. At 28°C, high Ca^{2+} levels in IPCs are essential for larval survival at any tested time point (Supplementary Figure S1). To evaluate the contribution of individual dIlps, we have tracked the development of different *ΔdIlp* mutants at comfortable temperatures (20°C) or upon heat stress (28°C). At 20°C, all tested mutants, including *ΔdIlp*², *ΔdIlp*³, *ΔdIlp*⁵, *ΔdIlp*²⁻³ or *ΔdIlp*^{2-3,5}, survive (at 20°C, $n_{\text{larva}}=132$, control (*Cntr*)=132 ±0% and $n_{\text{larva}}=140$, *ΔdIlp*^{2-3,5}=72.1 ±4.3%, $p=****$); although the developmental

rate of $\Delta dIlp^{2-3,5}$ is much slower and produces small larvae and pupae with respect to controls (Figure 1 and Supplementary Figure S1 and (37,38).

At 28°C, tested $\Delta dIlp^2$, $\Delta dIlp^3$ or $\Delta dIlp^5$ larvae, kept on YF, show no developmental defects (Supplementary Figure S1). However, we found that heat stress slows the developmental rate and reduces survival of $\Delta dIlp^{2-3}$, and that a majority of $\Delta dIlp^{2-3,5}$ animals perish (Figure 1).

Yeast-free food fed animals have limited IPC activity, low systemic IS levels and show decreased survival at high temperatures (1). To rule out the effects of yeast products, we have repeated all experiments using Corn food (CF), a yeast-free diet that resembles the ingredient proportions found in YF ((39); Table 1 and Table 2, respectively). At 28°C, controls are unable to survive on CF. Furthermore, induced expression of dIlp2 in IPCs is only in part promoting larval development (Supplementary Figure S1). Taken together, to mount an effective heat response, *Drosophila* relies on dietary yeast products and its complement of IPC dIlp peptides.

DIlp7-neurons are required to provide heat resistance in *Drosophila*

DIlp7- producing neurons (D7Ns) are wired with IPCs in larval and adult stages and have a modulative function (23,26,27). To test if D7N activity influences the resistance to high temperatures, we have silenced these cells and tracked larval development on YF at 28°C. We found that low D7N activity results in reduced larval survival (Figure 1). However, the developmental speed of surviving animals is comparable to controls (Supplementary Figure S1). *Drosophila* kept on yeast-free food show low IS levels and are sensitive to heat stress (1). Therefore, we speculated that D7N, on such yeast-free diets, might be inactive and that induced D7N activity could increase heat resistance. To test our idea, we expressed the fluorescent Ca^{2+} reporter, GCaMP, in D7Ns in larval brain tissues (10). We found that D7N never show high Ca^{2+} levels on CF contrary to flies kept on YF (Figure 1). To increase Ca^{2+} yields in D7Ns, we expressed the temperature-sensitive Ca^{2+} channel, TRPA1, and assayed the survival of larvae kept on yeast-free CF ((39) and Table recipes). Interestingly, survival rates of $dIlp^7 >> TRPA1$ (*TRPA1*) larvae increased significantly (Figure 1). To investigate if dIlp7 is essential to provide heat resistance, we tracked $\Delta dIlp^7$ larvae at 20°C and 28°C. We found that $\Delta dIlp^7$ kept on YF show no phenotypes; however, $\Delta dIlp^7$ are unsuccessful on CF (Figure 2 and Supplementary Figure S2). We conclude that D7Ns are required to acclimatize at high temperatures. In addition, although not involved in temperature response, dIlp7 peptide produced by D7Ns is essential to promote survival on yeast-free diets.

On yeast-free food, dIlp2 and dIlp7 are negative regulators of dIlp5

To investigate the role of dIlps in animals feeding on yeast-free diets, we performed a metabolic screen of $\Delta dIlp$ mutants on CF at 20°C. We found that, in addition to $\Delta dIlp^7$, three other mutants struggle to thrive on CF, including $\Delta dIlp^2$, $\Delta dIlp^3$ and $\Delta dIlp^5$ (Figure 2). To evaluate potential genetic interactions between dIlp7 and each of these candidates, we have created $\Delta dIlp^{2,7}$, $\Delta dIlp^{3,7}$ and $\Delta dIlp^{5,7}$ double mutants. Astonishingly, the absence of dIlp2 and dIlp7 restored survival rates close to numbers shown by controls. In contrast, $\Delta dIlp^{3,7}$ and $\Delta dIlp^{5,7}$ animals did not perform better and $\Delta dIlp^{2,3}$ were unable to develop on CF (Figure 2; Supplementary Figures S1 and S2). To test, if the loss of dIlp2 and dIlp7 hyper-activates dIlp3 or dIlp5, we assayed the development of $\Delta dIlp^{2-3,5}$ and $\Delta dIlp^{2-3,7}$ triple mutants. In contrast to $\Delta dIlp^{2-3,5}$, $\Delta dIlp^{2-3,7}$ were able to survive on CF alike $\Delta dIlp^{2,7}$ or control larvae (Figure 2).

The presence of dIlp7 is required for survival on CF but not for individuals kept on YF. Hence, we tested the idea that yeast products compensate for the regulative role of dIlp7. To do so, we reared all created $\Delta dIlp$ mutants onto YF. We found that at 20°C, $\Delta dIlp^{5,7}$ are less successful, but did not reduce their developmental speed (Supplementary Figure S2). The

lethality of $\Delta dIlp^{2-3,5}$ is comparable to $\Delta dIlp^{5,7}$; however, these triple mutants develop slower (Figure 1; Supplementary Figures S1 and S2). Although delayed in development, the survival of $\Delta dIlp^{2-3,7}$ is not affected (Figure 2). Pupae of both triple mutants are small in size (Supplementary Figure S1).

In conclusion, the data from our work indicate that on CF, dIlp5 can compensate for the loss of all other neuronal dIlps expressed in larval stages. Our findings crystallize dIlp2 and dIlp7 as negative regulators of dIlp5.

dIlp7 is instructive for larval and adult feeding behavior on CF

Feeding behavior and intestinal food transit are two connected variables that can modulate the absorption efficiency of food compounds. It was shown that *Drosophila*, with genetically silenced D7Ns, change their feeding behavior with respect to controls (27). To test the feeding behavior of $\Delta dIlp$ mutants, we have measured larval mouth-hook contractions (40), and the appearance or disappearance of ingested color in the adult intestinal tracts (27). Transferred on CF, mouth-hook contractions of all tested $dIlp$ mutants appeared slightly faster with respect to controls. Nevertheless, we found that ingestion of stained food in $\Delta dIlp^7$ mutant larvae takes longer compared to controls. (Supplementary Figure S2). To test if $\Delta dIlp^7$ adults show the same feeding behavior on CF, we recorded how fast colored food is consumed. $\Delta dIlp^7$ flies feed faster, but need more time to excrete material compared to controls (Figure 3). Interestingly, we have discovered a similar behavior for $\Delta dIlp^2$ mutants feeding on CF (Supplementary Figure S2). Taken together, these data show that dIlp7 and dIlp2 are important for larva and adult feeding behavior on yeast-free diet.

dIlp7 and not dIlp2 regulates systemic Insulin signaling on CF

Food transition time might be critical for efficient absorption of nutrients, therefore we predicted that $\Delta dIlp^2$ and $\Delta dIlp^7$ mutants will show similar activity of metabolic circuits such as the IS cascade. The phosphorylation state of AKT and MAPK allows to quantify the activity of anabolic and mitogenic IS activity, respectively. Therefore, we probed the phosphorylation state of AKT⁸⁵ and MAPK in adults, fed on CF, by western-blotting. We found that the AKT^{85-Thr342} phosphorylation state remains unchanged in all tested genotypes (Supplementary Figure S3). In contrast, we detected reduced phosphorylation at the AKT^{85-Ser505} site of all tested $\Delta dIlp^7$ mutant genotypes and $\Delta dIlp^{2-3}$ mutants, while the AKT⁸⁵ activity of $\Delta dIlp^2$, $\Delta dIlp^3$ and $\Delta dIlp^5$ mutants remains unchanged (Figure 3). In addition, we detected reduced MAPK phosphorylation for all $\Delta dIlp$ loss-of-function genotypes (Supplementary Figure S3). For comparison we repeated experiments performed on CF with YF to compare the activity of AKT⁸⁵ and MAPK. We did not detect any meaningful phenotypes in most of $\Delta dIlp$ mutant regarding larval development and adult $\Delta dIlp$ mutants thrive on YF like controls (Supplementary Figure S3). Thus, we expected that neither $\Delta dIlp$ mutant shows differences in the activity of either enzyme compared to controls. We found that all tested genotypes kept on YF show similar AKT or MAPK phosphorylation levels (Supplementary Figure S3). Taken together, we conclude that YF compensates for the loss of individual dIlps. In contrast, on yeast-free diet (CF), adult flies rely on dIlp7 to adjust their feeding behavior and to maintain basic AKT and MAPK activity required to survive (data not shown).

Lgr3 represents a potential target for dIlp7 on Insulin-producing cells

We speculated that on yeast-free food, dIlp7 regulates IPC activity by activating one of its three predicted candidate receptors: the *Drosophila* Insulin-like receptor (dInR) (12), or one of the two predicted G-protein coupled receptors (GPCR), Leucine-rich repeat-containing G protein-coupled receptor 3 (Lgr3) and 4 (Lgr4) (41,42). We decided to target each receptor in IPCs, by RNA interference (RNAi) using published validated genetic tools (43). The reduction

of the dInR (InR^{KD}) or Lgr4 (Lgr4^{KD}) did not produce any phenotypes. InR^{KD} and Lgr4^{KD} larvae developed as fast as controls; however, InR^{KD} individuals show a slight decrease in survival on both YF and CF compared to controls (Supplementary Figure S4). In contrast, we found that the loss of Lgr3 (Lgr3^{KD}) slowed the development on YF and decreased pupariation success on CF (Figure 4).

One accessible marker for stored cellular energy is the Triacylglyceride (TAG) level stored in lipid droplets. High IS levels correlate with high TAG yields. To test the idea that loss of Lgr3 reduces IS and hence, produces fewer TAGs, we have analysed lipid profiles of adult flies kept on YF. We found, that Lgr3^{KD} store less lipids with respect to controls (Supplementary Figure S4) indicating lower anabolic turnover rates. To investigate if Lgr3 modulates systemic IS, we probed the phosphorylation of AKT⁸⁵ and MAPK in Lgr3^{KD} flies. We found that AKT⁸⁵ phosphorylation of Lgr3^{KD} adults kept on YF at 20°C remained unchanged (Figure 4). On CF, the AKT^{85-Ser505} phosphorylation was reduced to levels assimilable to tested $\Delta dIlp^7$ mutant backgrounds (Figures 3 and 4). However, Lgr3^{KD} MAPK activity remained comparable to controls (Figure 4) indicating that dIlp7 should regulate MAPK by other means than Lgr3 expressed by the IPCs. Although of indirect evidence, our data lead us to the conclusion that on yeast-free diets, systemic IS is regulated by Lgr3 expressed in the IPCs.

DISCUSSION

Drosophila expresses four neuronal Insulin-like peptides (dIlps) and we analyzed here the physiological role of dIlp7 produced by the dIlp7-neurons (D7Ns). We found that active D7Ns facilitate Insulin signalling (IS) and therefore, represent one component of a high-temperature-sensitive neuronal network similar to already described regulative circuits (44–46). Nonetheless, $\Delta dIlp^7$ mutants thrive at high temperatures similar to respective controls confirming that dIlp7 peptide is not crucial to withstand thermal treatment. We found that dIlp7 is essential to modulate IS in flies feeding on yeast-free diet, a food that allows only for low D7N activity and changes flies heat sensitivity.

Adult flies switch their feeding behavior in response to temperature and prefer yeast-free plant food during periods of cold temperatures (1,3). Moreover, *Drosophila* kept on plant food are unable to endure heat stress. We show that on corn food (CF), D7Ns have low calcium (Ca²⁺) levels and induced activity of these cells enables more animals to survive heat. Interestingly, we found high dIlp7 levels in D7Ns regardless of the dietary input (Supplementary Figure S5). Hence, we wondered if dIlp7 plays any role for *Drosophila* feeding on yeast-free food. We show that the developmental success of $\Delta dIlp^7$ is strongly reduced on CF with respect to controls. It was shown that dIlp7 expression is strongly dependent on nutritional variables including protein:sugar ratio and caloric load (47). CF is rich in calories, its protein:sugar ratio is optimized and contains about 10% lipids ((39) and Table 1). Nevertheless, on CF, systemic Insulin levels are low and wild-type larvae struggle to complete their development (39,48). Astonishingly, we found that the absence of both dIlp2 and dIlp7 improved larval survival on CF up to numbers shown by controls. dIlp2 and dIlp5 have been described as highly redundant and target identical dIlp binding proteins (13). Nevertheless, the survival rate of $\Delta dIlp^{5,7}$ mutants kept on CF is low; thus, implicating that dIlp5 plays a different biological role than dIlp2 (49,50). In addition, $\Delta dIlp^{2-3,7}$ mutants are as successful as $\Delta dIlp^{2,7}$; thus, ruling out dIlp3 as a determining factor. Moreover, our results position dIlp5 as sufficient to rescue development since $\Delta dIlp^{2-3,5}$ and $\Delta dIlp^{2-3}$ hardly grow on CF. Taken together, on yeast-free food, larval development is controlled by dIlp7, which may regulate the activity of dIlp2 and 5.

To better understand how possibly dIlp7 regulates the activity of the IPC-produced dIlp2 and dIlp5, we have investigated three predicted targets of dIlp7 including the *Drosophila* Insulin-like receptor (dInR) and the two G protein-coupled receptors GPCRs, Lgr3 and 4 (12,41,42). We found that the knock-down of Lgr3 in IPCs results in lower larval survival on CF. It is shown that Lgr3 is targeted by dIlp8 and plays an important role in larval development (18,21). However, $\Delta dIlp^8$ mutants performed as controls on CF (Supplementary Material/Excel sheet/dIlp mutants_Survival_CF20) and AKT^{85-Ser505} phosphorylation of adult Lgr3^{KD} is reduced to numbers shown by all tested $\Delta dIlp^7$ background mutants. On the other hand, measured MAPK activity of Lgr3^{KD} is comparable to controls. This result is contrasting findings made with $\Delta dIlp^7$ mutants, and we propose that secreted dIlp7 enters circulation (23,26) and regulates the mitogenic branch of the IS cascade. Nevertheless, future studies are required to demonstrate direct dIlp7/Lgr3 interaction at the molecular level.

Neuronal innervation of IPCs is established in many animals and modulates metabolic signals (23,45,51,52). Our findings indicate that food products can overwrite neuronal stimulation of IPCs. In *Drosophila*, we found a dual role for D7Ns: i.) these neurons facilitate the heat response of flies feeding on yeast and ii.) they form a metabolic circuit that enables adult flies to thrive on yeast-free diets if required. In mice and humans, pancreatic islets are directly innervated (51,52); however, the role of this neuronal stimulation in response to dietary cues is not understood. We have identified D7Ns and their product, dIlp7, important to regulate IS independently from the provided high caloric load and abundance in dietary sugars or proteins. Our findings provide new insights into the neuronal stimulation of Insulin-producing cells within a given ecological context and open the perspective to screen for nutritional cues capable to convey dietary signals to the brain.

MATERIALS AND METHODS

Stocks

If not stated, stocks were raised on normal food at 20-22°C (Table 3).

The following lines were used:

OregonR (#5), *w;foxo[mCherry]* (#80565) were provided by Bloomington Drosophila Stock Center (BDRC). Isogenized *Canton S* were shared by J.C. Billeter. *w¹¹¹⁸* and *yw* were provided by S. Eaton's laboratory.

Drosophila Insulin-like peptide mutants: $\Delta dIlp^1$ - $\Delta dIlp^8$, $\Delta dIlp^{2-3}$ and $\Delta dIlp^{2-3,7}$ ((13); from S. Grönke); $\Delta dIlp^{2-3,5}$ from R. Kühnlein.

Gal4 lines: *w;dilp2-Gal4* ((14); #37516 from BDRC); *w;dilp7-Gal4* ((27); gift from I. Miguel-Aliaga); *w;rab3-Gal4* and *w;moody-Gal4* (from S.Eaton); *yw;actin-Gal4* (#4414, from BDRC) UAS-lines: *w;UAS-TrpA1* (#26264, from BDRC); *w;UAS-Kir2.1-egfp,tub80ts* (from S.Eaton); *yw;UAS-Dilp2* ((11); #80936 from BDRC); *w;UAS-GcAMP* (#32116 from BDRC); *yw;UAS-Dilp3* and *yw;UAS-Dilp5* (from S.Eaton); *w;UAS-Dilp7* (gift from I. Miguel-Aliaga); *yw;UAS-IR^{DN}* (#8253, from S.Eaton); *w;UAS-Lgr4RNAs* (v102681 and v108915; Vienna Drosophila Resource Center (VDRC)); *w;UAS-Lgr3shRNA* (v330603; from VDRC).

Diets

Normal food was adapted from <https://bdsc.indiana.edu/information/recipes/bloomfood.htm.1>. (Table 3)

Yeast food was prepared as published by Brankatschk et al., 2018 (1) (Table 2).

Corn food recipe is described in Table 1 and in Trautenberg et al. 2020 (39).

Development tracking experiments

Embryos were collected overnight on apple-juice agar plates (25% apple-juice, 1.5% agar, 0.4% p-Hydroxybenzoic acid methyl ester (nipagin)), bleached in 80% bleach/water for 30 seconds, rinsed and kept for 24 hours on starvation plates (25% apple juice, 10% Glucose, 1.5% agar, 0.4% nipagin) at 20-22°C. First-instar larvae were pipetted in 10µL 0.00005% Triton-X100/PBS solution and transferred in 96 well-plates containing food. One larva per well (containing 200µL of food) was deposited. Later, the plates were sealed with a poked ventilated plastic film and put in incubators set at the temperature of interest. Both developmental speed/rate (how many days each larva took to reach the pupal stage) and success (how many larvae succeed to reach the pupal stage on the total number of larvae plated on one plate).

Induction experiments: The egg progeny of crosses between *UAS-Kir2.1* or *Canton S* and *dilp2-Gal4*, *dilp7-Gal4* or *Canton S* were collected. Larvae and plates were prepared as described above. Plates were kept at 20°C for 24, 72 or 96 hours and then transferred to the 28°C-incubator.

Statistics

Developmental success: For 20°C or 28°C/induction experiments, only experiments with minimum ten or six larvae tracked, respectively, were taken into account in the pool of data. Experiments out of the range (*i.e.* Mean + or - Standard deviation) were excluded of the normalized data. Mann-Whitney or Kruskal-Wallis tests were performed as statistical tests (PRISM Graphpad software).

Developmental speed: only speed data from experiments taken into account in the normalized data of the survival rates were picked. Mann-Whitney or Kruskal-Wallis tests were performed as statistical tests (PRISM Graphpad software).

Immunohistochemistry

The larvae were dissected, turned as socks in fix solution and kept in fix solution for 20 minutes. The carcasses were stained by HrpCy5 (HRP-S5-1; NANOCS) or with DAPI (mounted in Vectashiel medium containing DAPI, vectorlab). The central nervous systems were dissected from the carcasses and mounted in 50% Glycerol/PBS. Confocal microscopy was performed on a Zeiss confocal laser scanning microscope LSM 700 of the Light Microscopy Facility, a Core Facility of the CMCB Technology Platform at TU Dresden. Antibody used: anti-dIlp7 (gift from Irene Miguel-Aligua, (27))

GCaMP experiments

Sample collection

The egg progeny of crosses between *UAS-GCaMP* or *Canton S* and *dilp2-Gal4* or *dilp7-Gal4* were collected. Larvae were prepared as described above in “developmental tracking part”. Three larvae were pipetted in 10µL 0.00005% Triton-X100/PBS solution and transferred in 24 well-plates containing food. Three larvae per well (containing 1mL of food) were deposited. Fed larvae were collected after being reared for 6 or 22 days on yeast food or corn food, respectively.

Immunostaining

Performed as described in “Immunohistochemistry” part. Quantification

The total number of *dilp2*- or *dilp7*-GCaMP-positive (*i.e.* green-fluorescent cells) were counted in each brain imaged. The percentage of cells ON was then calculated on the total number of *dilp2*-positive cells (*i.e.* 8 per hemisphere (14) or *dilp7*-positive cells (*i.e.* 12 (26)). Statistics, Wilcoxon’s test.

Larva and adult weight

To determine the weight of larvae or adults reared on yeast food, a determined number of third-instar larvae or adults were put in Eppendorf 2mL tubes. Mass of one larva or one adult (mg/larva or mg/adult, respectively) = (Mass of the tube containing x number of larvae or adults (mT+xL or mT+xA, respectively) – mass of the tube (mT))/x number of larvae or adults contained in the tube (xL or xA, respectively).

Pupa area

Pupa arising from larvae reared in 96-well plates containing yeast food at 20°C were placed on microscope slides where a 5mm-scale bar was drawn. Pictures were taken with a camera and processed by using Fiji (53). By using the “freehand” tool, the area of each pupa was measured. Kruskal-Wallis tests were performed as statistical tests.

Feeding behavior

Larva mouth contraction

Third-instar larvae fed on normal food were rinsed and transferred to three Petri dishes containing 0.5%Bromophenol-Blue colored yeast food or corn food (27). On each plate, 10 larvae were placed. The number of mouth contractions were counted within 30 seconds for each larva. Kruskal-Wallis tests were performed as statistical tests.

Larva food intake

Third-instar larvae fed on normal food were rinsed and transferred to three Petri dishes containing 0.5%Bromophenol-Blue colored yeast food or corn food (27). On each plate, 10 larvae were placed. Every 5 minutes, the number of larvae with blue guts were counted. Finally, the time to reach 100% of the larvae with blue guts per plate was plotted. Kruskal-Wallis tests were performed as statistical tests.

Adult food intake

Seven-to-ten days old adults starved for 24 hours were transferred in vials containing 5mL 0.5%Bromophenol-Blue colored yeast food or corn food (27). The male:female ratio per vial was 1:3. Every 30 minutes, the number of females with blue guts were counted. Finally, the percentage of females with blue guts after 4 hours on the diet was plotted. Statistics, experiments out of the range (*i.e.* Mean + or - Standard deviation) were excluded of the normalized data. Kruskal-Wallis tests were performed as statistical tests.

Adult transit

Seven-to-ten days old adults kept for 24-48h into vials containing 5mL 0.5%Bromophenol-Blue colored yeast food or corn food (27) were transferred into vials containing starvation diet (25% apple juice, 10%Glucose, 1.5% agar, 0.4% nipagin). The male:female ratio per vial was 1:3. Every 30 minutes, the number of females without blue guts were counted. Finally, the percentage of females without blue guts after 4 hours on starvation was plotted. Statistics, experiments out of the range (*i.e.* Mean + or - Standard deviation) were excluded of the normalized data. Kruskal-Wallis tests were performed as statistical tests.

Biochemistry

AKT detection in yeast and corn-food fed adult heads

Sample preparation, western-blotting and quantification were performed as published by (48). In brief, flies were raised on normal food and 5d old adults transferred onto respective experimental foods for 7d at 20°C. Thereafter, flies were snap-frozen in liquid nitrogen and frozen heads from females collected for processing. Heads were homogenized on ice in 0.01% Tx100-PBS and subsequently cooked at 95°C for 5min. Polyclonal antibodies used to probe were Akt-pSer505 (Cell Signaling, 4054S), Akt-pThr308 (Invitrogen, 44-602G), Akt (Invitrogen, MAS14916), MAPK (Cell Signaling, p44/42 MAPK (Erk1/2) Antibody #9102),

P-MAPK (Cell Signaling, Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101).

Quantification

Pixel intensity of defined area (line) covering the signature (not saturated) center on photograph was measured using FIJI software (53). Intensity ratio between AKT85-Ser505 and panAKT85 or P-MAPK and MAPK was calculated and normalized to value obtained from controls.

Trehalose, protein and triacylglyceride quantification in larva or adult hemolymph

Sample collection and preparation

Larvae: third-instar larvae raised in 96-well plates containing YF were collected, rinsed, drained, transferred in 2ml Eppendorf tubes and short-frozen in liquid nitrogen. Each tube was filled with 20 larvae. To collect hemolymph, 60µL of ice-cold 1xPBS was added into the tube containing larvae. After spinning for 5 minutes at 500g, 50µL of the supernatant were pipetted and transferred into another Eppendorf tube.

Adults: eight-to-ten days old adults reared on yeast food were collected, transferred into 2mL Eppendorf tubes and short frozen in liquid nitrogen. For trehalose assay, 460 adults were required (male: female ratio $\approx$ 230:230); for protein quantification, 16 adults (male: female ratio $\approx$ 4:12) and for triacylglycerides, 9 adults were needed (male: female ratio $\approx$ 3:6). To collect hemolymph, 110µL of ice-cold 1xPBS was added to a tube containing sample flies. Samples were incubated on ice for 5 minutes and centrifuged for 10 seconds (benchtop centrifuge) two times, then 100µl of the supernatant was transferred to a fresh tube.....

Trehalose assay and quantification

Hemolymph samples were heated at 85°C for 15 minutes. Then samples were cooled down to room temperature, and trehalose was measured and quantified like recommended by the manufacturer (Trehalose Kit, Megazyme).

Protein assay and quantification

Proteins in hemolymph samples were concentrated by chloroform-methanol precipitation and re-dissolved in 0.1% TritonX100 in 1xPBS. Protein amount was measured and quantified like recommended by manufacturer (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific).

Thin layer chromatography (TLCs)

Lipid extraction were performed as described by Bligh and Dyer (54,55) and analyzed on TLC plates using published solvent protocols (polar lipids: CHCl₃ : CH₃OH : H₂O (75 : 25 : 2.5); neutral lipids: C₇H₁₆:(C₂H₅)₂O :CH₃COOH (70 : 30 : 1)) Plates were stained with CuSO₄ or Primulin and scanned using a Typhoon reader. Three homogenized fly heads per sample, 1µL lipid extract was pipetted on HPTLC Silica gel glass plates (Merck). Twenty larvae per sample, volume to resuspend the lipid pellets was normalized to the larval weight and 3µL lipid extract was pipetted on HPTLC Silica gel glass plates (Merck). Triacylglycerol (TAG) (10mg/ml) and Diacylglycerol (DAG) (1mg/ml) were used as standards.

Triacylglycerides quantification

Pixel intensity of defined area (round) covering triglycerides signatures on photograph was measured using FIJI software (53). Intensity was calculated and normalized to the blank value.

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COMPETING INTERESTS

The authors declare no competing or financial interests

AUTHOR CONTRIBUTION

Conceptualization: M.B., E.P.; Formal analysis: E.P.; Funding acquisition: M.B.; Investigation: E.P., J.K., L.C.T.; Methodology: E.P.; M.B.; Project administration: M.B., E.P.; Resources: E.P., M.B.; Supervision: E.P., M.B.; Validation: E.P.; Visualization: E.P.; Writing-Original Draft Preparation: M.B., E.P., J.K., L.C.T.

TABLES

Corn Food (CF) (1L)	Quantity	kcal	protein (kcal)	carbohydrates (kcal)	lipid (kcal)
Glucose (g)	30	116.1	0	114.24	0
Sorbitol (g)	18	43.2	0	43.2	0
Arabinose (g)	15	0	0	0	0
Yellow cornmeal (g)	65	224.9	20.8	182	17.55
Fructose (g)	20	80	0	79.2	0
Malt extract (g)	15	55.05	3.6	57.72	0
Soy pepton (g)	70	256.2	119	8.68	129.15
Sucrose (g)	15	60	0	60	0
Agar (g)	8	2.08	0.16	2.24	0
Water (mL)	fill until 1L				
0.1g/mL Nipagin/EtOH (mL)	40	Add at 60°C			
	kcal	837.53	143.56	547.28	146.7
	%		17%	65%	18%

P:C:Li ratio	1:4:1
P:C ratio	1:4

Table 1. Corn food recipe

Recipe for 1 liter of corn food. The calories (in kcal) brought by each ingredient have been also calculated. At the bottom, in black bold, the totals (in kcal) are shown. In blue are indicated the proportion (%) of kcal of protein, carbohydrates or lipids on the total of amount calories. In purple are shown the protein: carbohydrate: lipid and protein: carbohydrate ratios. Abbreviations: EtOH, ethanol; P, proteins; C, carbohydrates; Li, lipids.

Yeast Food (YF) (1L)	Quantity	kcal	protein (kcal)	carbohydrates (kcal)	lipids (kcal)
Yeast extract (g)	20	43.4	23.8	18.7	0.9
Soy pepton (g)	20	73.2	34.0	2.5	36.9
Sucrose (g)	30	120.0	0.0	120.0	0.0
Glucose (g)	60	232.2	0.0	228.5	0.0
Yeast (g)	80	321.6	144.0	112.0	43.2
Agar (g)	10	2.6	0.2	2.8	0.0
Water (mL)	fill until 1L				
0.1g/mL Nipagin/EtOH (mL)	40	Add at 60°C			
	kcal	793.0	202.0	484.5	81.0
	%		25%	61%	10%

P:C:Li ratio	3:6:1
P:C ratio	1:2

Table 2. Yeast food recipe

Recipe for 1 liter of corn food. The calories (in kcal) brought by each ingredient have been also calculated. At the bottom, in black bold, the totals (in kcal) are shown. In blue are indicated the proportion (%) of kcal of protein, carbohydrates or lipids on the total of amount calories. In purple are shown the protein: carbohydrate: lipid and protein: carbohydrate ratios. Abbreviations: EtOH, ethanol; P, proteins; C, carbohydrates; Li, lipids.

Normal Food (adapted from Indian Edu) (1L)	Quantity	kcal	protein (kcal)	carbohydrates (kcal)	lipid (kcal)
Sucrose (g)	22	88	0	88	0
Malt extract (g)	20	73.4	4.8	76.96	0
Yeast (g)	18	72.36	32.4	25.2	9.72
Soy pepton (g)	10	36.6	17	1.24	18.45
Yellow cornmeal (g)	80	276.8	25.6	224	21.6
Glucose (g)	20	77.4	0	76.16	0
Agar (g)	7	1.82	0.14	1.96	0
Water (mL)	fill until 1L				
0.1g/mL Nipagin/EtOH (mL)	40	Add at 60°C			
	kcal/L	626.4	79.9	493.5	49.8
	%		13%	79%	8%

P:C:Li ratio	2:10:1
P:C ratio	1:6

Table 3. Normal food recipe

Recipe for 1 liter yeast food. Quantities of each ingredient (in g or mL) are indicated. The calories (in kcal) brought by each ingredient have been also calculated. At the bottom, in black bold, the totals (in kcal) are shown. In blue are indicated the proportion (%) of kcal of protein, carbohydrates or lipids on the total of amount calories. In purple are shown the protein: carbohydrate: lipid and protein: carbohydrate ratios. Abbreviations: EtOH, ethanol; P, proteins; C, carbohydrates; Li, lipids

FIGURES

Figure 1. Activated D7Ns facilitate larval development at high temperatures

(A). Plotted is the developmental speed in days (d) to reach the pupal stage of *FOXO^{mCherry}* (Cntr, grey) and $\Delta dllp$ mutant larvae grown on YF at 20 or 28°C. Each black dot represents one tracked larva. Cntr (grey; median^{20°C}=11d; median^{28°C}=7d), $\Delta dllp^2$ (dark-blue; median^{20°C}=11d; median^{28°C}=8d), $\Delta dllp^{2-3}$ (light-blue; median^{20°C}=12d; median^{28°C}=10d) and $\Delta dllp^{2-3,5}$ (yellow; median^{20°C}=21d; median^{28°C}=19d). Statistics: Kruskal-Wallis test $p<0.0001$ ****; Dunn's multiple comparison test: Cntr vs $\Delta dllp^2$, $p^{20°C}>0.9999$ ns, $p^{28°C}=0.0001$ ***; Cntr vs $\Delta dllp^{2-3}$, $p^{20°C}=0.0159$ *, $p^{28°C}=0.0001$ ****; Cntr vs $\Delta dllp^{2-3,5}$, $p^{20°C}$, $p^{28°C}<0.0001$ ****.

(B). a.-d. Shown are representative pictures of *FOXO^{mCherry}* (a, Cntr), $\Delta dllp^2$ (b), $\Delta dllp^{2-3}$ (c) and $\Delta dllp^{2-3,5}$ pupae (d) raised on YF at 20°C. Scale bars=0.5mm. e. Plotted is the area of pupae grown on YF at 20°C. Each black dot represents one pupa. Cntr (grey; median=2.6), $\Delta dllp^2$ (dark-blue; median=2.6), $\Delta dllp^{2-3}$ (light-blue; median=1.9) and $\Delta dllp^{2-3,5}$ (yellow; median=1.8). Statistics: Kruskal-Wallis test $p<0.0001$ ****; Dunn's multiple comparison test: Cntr vs $\Delta dllp^2$, $p>0.9999$ ns; Cntr vs $\Delta dllp^{2-3}$, $p<0.0001$ ****; Cntr vs $\Delta dllp^{2-3,5}$, $p<0.0001$ ****. f. Plotted is the larva weight per larva calculated from n pool of larvae collected and grown on YF at 20°C. Each dot represents one pool. Cntr (grey; median=1.6), $\Delta dllp^2$ (dark-blue; median=1.0), $\Delta dllp^{2-3}$ (light-blue; median=1.1) and $\Delta dllp^{2-3,5}$ (yellow; median=0.8). Statistics: Kruskal-Wallis test $p<0.0001$ ****; Dunn's multiple comparison test: Cntr vs $\Delta dllp^2$, $p=0.0044$ **; Cntr vs $\Delta dllp^{2-3}$, $p=0.1488$ ns; Cntr vs $\Delta dllp^{2-3,5}$, $p<0.0001$ ****.

(C). Plotted is the pupariation rate (%) of larvae reared on YF or CF at 28°C. *FOXO^{mCherry}* (Cntr1, grey dots) and the $\Delta dllp^{2-3,5}$ (yellow dots), $dllp7-Gal4/+$ (Cntr2, grey triangles), $dllp7>>TRPA1$ ($TRPA1$, dark-pink triangles) and $dllp7>>Kir2.1,tub80ts$ ($Kir2.1$, light-pink triangles). Each mark represents one experiment; the median is depicted as a grey bar. Cntr1 (median=100%), $\Delta dllp^{2-3,5}$ (median=24.7%),

Cntr2 (YF, median=100%; CF, median= 16.65%), *TRPA1* (YF, median=100%; CF, median=32.45%), *Kir2.1* (YF, median=50%; CF, median=9.10%). Statistics: Mann-Whitney test: *Cntr1* vs $\Delta dIlp^{2-3,5}$, $p=0.0182$ *; Kruskal-Wallis test $p=0.0031$ **, Dunn's multiple comparison test: *Cntr2* vs *TRPA1*, $p=0.7091$ ns; *Cntr* vs *Kir2.1*, $p=0.0549$ ns (YF); Kruskal-Wallis test $p=0.0069$ **, Dunn's multiple comparison test: *Cntr2* vs *TRPA1*, $p=0.3138$ ns; *Cntr* vs *Kir2.1*, $p=0.9484$ ns (CF).

(D). **a.** Representative Z-projection confocal image of GCaMP-positive cells (green) in brains from larvae expressing GCaMP in IPCs (top, *dIlp2>GCaMP*; stack=47.5 μ m) or D7N (bottom, *dIlp7>GCaMP*; stack=36.1 μ m). Larvae were kept on YF at 20°C. All samples were stained with DAPI (grey). Scale bars=25 μ m. **b.** Plotted is the percentage of GCaMP-positive IPCs or D7Ns in larvae reared on YF (red) or CF (black) at 20°C. IPCs ($n_{YF}=8$; median=62.55%; $n_{CF}=23$; median=0%); D7Ns ($n_{YF}=8$; median=66.7%; $n_{CF}=20$; median=0%). Statistics, Wilcoxon-test, YF vs CF, $p=0.0078$ ** (IPCs, D7Ns).

Figure 2. On CF *dIlp7* peptide is instructive for IPCs

(A). Plotted is the pupariation rate in % (median=grey bar; each dot represents one experiment) of *FOXO^{mCherry}* (*Cntr*, grey), $\Delta dIlp^7$ (pink), $\Delta dIlp^{2-7}$ (blue) and $\Delta dIlp^{2-3,7}$ (green) larvae reared on CF at 20°C. *Cntr* (median=54.80%), $\Delta dIlp^7$ (median=11.25%), $\Delta dIlp^{2-7}$ (median=55.50%) and $\Delta dIlp^{2-3,7}$ (median=68.80%). Statistics: Kruskal-Wallis, $p<0.0001$ ****; Dunn's multiple comparison test, *Cntr* vs $\Delta dIlp^7$: $p=0.0183$ *, *Cntr* vs $\Delta dIlp^{2-7}$: $p>0.9999$ ns, *Cntr* vs $\Delta dIlp^{2-3,7}$: $p=0.8530$ ns

(B). Plotted are the pupariation rates in% (median=grey bar; each dot represents one experiment) of *FOXO^{mCherry}* (*Cntr*, grey), $\Delta dIlp^{2-3,5}$ (yellow) and $\Delta dIlp^{2-3,7}$ (green) larvae reared on YF at 20°C. *Cntr* (median=100%), $\Delta dIlp^{2-3,5}$ (median=69.40%) and $\Delta dIlp^{2-3,7}$ (median=84.60%). Statistics: Kruskal-

Wallis, $p < 0.0001$ ****; Dunn's multiple comparison test, *Cntr* vs $\Delta dIlp^{2-3,5}$: $p < 0.0001$ ****, *Cntr* vs $\Delta dIlp^{2-3,7}$: $p < 0.0001$ ****.

(C). Plotted is the developmental speed in days (d) to reach the pupal stage of *FOXO^{mCherry}* (*Cntr*, grey), $\Delta dIlp^{2-3,5}$ (yellow) and $\Delta dIlp^{2-3,7}$ (green) larvae reared on YF at 20°C. Each dot represents one tracked larva. *Cntr* (median= 11d), $\Delta dIlp^{2-3,5}$ (median=21d), $\Delta dIlp^{2-3,7}$ (median=20.5d). Statistics: Kruskal-Wallis, $p < 0.0001$ ****; Dunn's multiple comparison test, *Cntr* vs $\Delta dIlp^{2-3,5}$: $p < 0.0001$ ****, *Cntr* vs $\Delta dIlp^{2-3,7}$: $p < 0.0001$ ****.

(D). **a-b.** Representative pictures of *FOXO^{mCherry}* (a, Control, *Cntr*) and $\Delta dIlp^{2-3,7}$ (b) larvae reared on YF at 20°C. Scale bars=0.5mm. **c.** Area of *FOXO^{mCherry}* (Control, *Cntr*, grey) and $\Delta dIlp^{2-3,7}$ (green) larvae reared on YF at 20°C. Each dot represents one experiment. The area of pupae, grown on YF at 20°C, has been measured as depicted in Fig. 1Ba, dashed line. *Cntr* (median=2.6) and $\Delta dIlp^{2-3,7}$ (median=1.7). Statistics: Mann-Whitney test, $p < 0.0001$ ****. **d.** Plotted is the larva weight per larva calculated from n pool of larvae collected and grown on YF at 20°C. Each dot represents one pool. *Cntr* (grey; median=1.6) and $\Delta dIlp^{2-3,7}$ (green; median=0.850). Statistics: Mann-Whitney test, $p=0.0101$ *.

Figure 3. Dllp7 peptide regulates systemic IS on CF

(A). Plotted is the percentage of *CantonS* (grey, Cntr) and Δ dllp⁷ (pink) adult females with blue guts, 4 hours after their transfer from uncoloured starvation plates to blue-stained YF (red, left) or CF (black, right). Each black dot = one experiment. Cntr (YF, median=57.80%; CF, median=68.35%) and Δ dllp⁷ (YF, median=76.50%; CF, median=88.75%). Statistics: Mann-Whitney test, Cntr vs Δ dllp⁷: p_{YF} =0.0556 ns, p_{CF} =0.0095 **.

(B). Plotted is the percentage of *CantonS* (grey, Cntr) and Δ dllp⁷ (pink) adult females with blue guts 4 hours after their transfer from blue-stained YF (red, left) or CF (black, right) to uncoloured starvation

plates. Each black dot = one experiment. *Cntr* (YF, median=0%; CF, median=66.70%) and *Δdllp*⁷ (YF, median=51.65%; CF, median=100%). Statistics: Mann-Whitney test, *Cntr* vs *Δdllp*⁷: $p_{YF}=0.0079$ **, $p_{CF}=0.0317$ *.

(C). **a-e.** The phosphorylation states of AKT⁸⁵ (a-c; marked by arrow) and MAPK (d, e) in *Control* (*Cntr*, *FOXO*^{mCherry}) and *Δdllp* mutants kept on CF at 20°C were analysed by western blotting. Shown is the phosphorylation at the Thr³⁴² (a, AKT⁸⁵-Thr³⁴²), the Ser⁵⁰⁵ (b, AKT⁸⁵-Ser⁵⁰⁵) site and total amount of AKT⁸⁵ (c, Pan-AKT). In addition, phosphorylation (d.) and total yields (e.) of MAPK were detected. .
f. The activity at the AKT⁸⁵-Ser⁵⁰⁵ was quantified (in arbitrary units, A.U.) from 3 independent blots; each black dot=one experiment. Plotted is the relative activity of each genotype normalized to the control *FOXO*^{mCherry} (*Cntr*, grey, median=100%). *Δdllp*² (dark-blue, median=106.7%), *Δdllp*³ (blue, median=95.60%), *Δdllp*⁵ (dark-purple, median=91.10%), *Δdllp*⁷ (pink, median=81.40%), *Δdllp*^{2,7} (blue, median=80.10%), *Δdllp*^{3,7} (green-blue, median=82.40%), *Δdllp*²⁻³ (light-blue, median=79.90%), *Δdllp*^{5,7} (light-purple, median=81.80%). Statistics: Kruskal-Wallis test, $p=0.0120$ *; Dunn's multiple comparison test, *Cntr* vs *Δdllp*², *Δdllp*³, *Δdllp*⁵: $p>0.9999$ ns, *Cntr* vs *Δdllp*⁷: $p=0.1883$ ns, *Cntr* vs *Δdllp*^{2,7}: $p=0.0934$ ns, *Cntr* vs *Δdllp*^{3,7}: $p=0.5113$ ns, *Cntr* vs *Δdllp*²⁻³: $p=0.0596$ ns, *Cntr* vs *Δdllp*^{5,7}: $p=0.3579$ ns.

Figure 4. Dllp7 targets Lgr3 expressed by IPCs

(A, B). Plotted is the developmental speed in days (d, A) and the pupariation rate (% , B) of control *dllp2-Gal4/+* (*Cntr*, grey) and *dllp2>>Lgr3RNAi* (*Lgr3^{KD}*, light-blue) larvae reared on YF (A) or on CF (B) at 20°C. **A.** Each black dot = one larva, the median is indicated. *Cntr* (median=11d) and *Lgr3^{KD}* (median=12.5d). Statistics: Mann-Whitney test: $p < 0.0001^{****}$. **B.** Each dot = one experiment. The median is indicated. *Cntr* (grey, median=84.5%) and *Lgr3^{KD}* (light-blue median=62.5%).

(C). Shown are blots probed for AKT (a.) and MAPK (c.) of *Control* (*Cntr*, *FOXO^{mCherry}*) and *dllp2>>Lgr3RNAi* (*Lgr3^{KD}*) flies. Flies were raised on YF (a, b) or CF (c, d) at 20°C. The phosphorylation at the AKT^{85-Ser505} and P-MapK site was quantified and relative activity plotted (**b,d**), each black dot = one experiment. Statistics: Mann-Whitney test, *Cntr* vs *Lgr3^{KD}*, AKT^{85-Ser505}: $p = 0.1000$ ns; P-MAPK: $p = 0.7000$ ns. On CF (d), *Lgr3^{KD}* (n-w=3; AKT^{85-Ser505}, median=70.80%; P-MAPK, median=98.20%). Statistics, Mann-Whitney test, *Cntr* vs *Lgr3^{KD}*, AKT^{85-Ser505}: $p = 0.7000$ ns; P-MAPK: $p = 0.1000$ ns.

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