Somatic energy reserves are essential for reproductive success and can govern the onset of sexual maturation. Here, we present a toolkit to analyze the metabolic status of Drosophila larvae using an optimized NMR profiling assay in dissected tissues or whole animals, as well as a complementary protocol for the dissection and staining of key organs in nutrient sensing. This toolkit will aid investigations into critical body weight signaling and how it is sensed for maturation commitment in Drosophila.
Protocol
A toolbox to study metabolic status of Drosophila melanogaster larvae

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SUMMARY
Somatic energy reserves are essential for reproductive success and can govern the onset of sexual maturation. Here, we present a toolkit to analyze the metabolic status of Drosophila larvae using an optimized NMR profiling assay in dissected tissues or whole animals, as well as a complementary protocol for the dissection and staining of key organs in nutrient sensing. This toolkit will aid investigations into critical body weight signaling and how it is sensed for maturation commitment in Drosophila.

For complete details on the use and execution of this profile, please refer to Juarez-Carreño et al. (2021).

BEFORE YOU BEGIN
Preparation of fruit flies

© Timing: 2 weeks

1. Fly husbandry and harvest: Drosophila melanogaster strains were maintained on standard fly food medium at 25°C, 60% humidity, and 14 h/10 h light–dark cycles.
2. Cross 20–30 virgin females and 20–30 males:
   a. After 24–48 h, flies were transferred to grape juice agar plates with yeast paste and left for 4 h to allow egg deposition.
   b. Parental flies were removed. Surveys of the pupae were performed at 8-h intervals; 2–4 h after initiation of egg laying was considered time “0” and referred to as “after egg laying (AEL)”.
   c. Second-instar larvae were transferred onto 5 mL of Drosophila standard “Iberian” food (20 larvae per tube) and reared at 25°C.
3. Collect control (ppl> (Zinke et al., 1999) and phm> (Ono et al., 2006)) and non-pupating mutant (ppl>apolpp and phm>Sema1a) larvae at the stage of interest (Figure 1).

Preparation of metabolite extraction

© Timing: 10 min
4. Transfer methanol (MeOH, analytical grade), chloroform (CHCl₃, analytical grade), and deionized type I ultrapure water to small glass bottles and store in the fridge at 4°C.

Preparation of NMR spectrometer

- **Timing:** 3 h

5. Before starting sample measurement, calibrate the temperature at 27°C with a 99.8% deuterated methanol (MeOH-d₄) sample.

6. Optimize the parameters of the spectrometer to ensure optimal resolution and sensitivity (width at half-height < 1 Hz). Use a commercial standard sample (Bruker Cat#10246) containing 2 mM sucrose, 0.5 mM sodium trimethylsilylpropanesulfonate (DSS), 2 mM NaN₃ in 90% H₂O and 10% D₂O.

7. Prepare NMR analysis buffer by solubilizing 100 mM Na₂HPO₄ and 0.1 mM 3-(trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (TSP) in D₂O, and adjust the pH to 7.4 with a saturated NaOH solution in D₂O. The buffer can be stored for up to one year at 4°C.

| Reagent    | Final concentration | Amount  |
|------------|---------------------|---------|
| Na₂HPO₄    | 100 mM              | 142 mg  |
| TSP        | 0.1 mM              | 1.7 mg  |
| D₂O        | up to 100 mL        |         |

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| mouse anti-Dlg (Working dilution: 1/100) | DSHB | Cat#4F3; RRID: AB_528203 |
| rat anti-DE-Cad (1/50)    | DSHB   | Cat#DCAD2; RRID: AB_528120  |
| donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 (1/500) | Invitrogen | Cat#A-31570; RRID: AB_2536180 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| Description                                                                 | Source                          | Identifier       |
|-----------------------------------------------------------------------------|---------------------------------|------------------|
| donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (1/200) | Invitrogen                      | Cat#A-31571; RRID: AB_162542 |
| donkey anti-Rat IgG (H+L) Alexa Fluor 647 AffiniPure (1/200)               | Jackson ImmunoResearch          | Cat#712-605-153; RRID: AB_2340694 |

### Chemicals, peptides, and recombinant proteins

| Compound                                                                 | Source                          | Identifier       |
|--------------------------------------------------------------------------|---------------------------------|------------------|
| 3-(trimethylsilyl) propionic acid d4 sodium salt (TSP)                    | Eurisotop                       | Cat#D219PF       |
| Agar                                                                     | Millipore                       | Cat#9002-18-0    |
| Amyloglucosidase                                                         | Sigma-Aldrich                   | Cat#A1602        |
| Brown sugar                                                              | AB Azucarera Iberia SLU         | N/A              |
| CHCl₃                                                                    | Merck                           | Cat#650498-1L    |
| CHCl₃-d1                                                                 | Merck                           | Cat#151823-50G   |
| D₂O                                                                     | Eurisotop                       | Cat#D216L        |
| Gibco Schneider’s Drosophila Sterile Medium                               | ThermoFisher Scientific         | Cat#21720024     |
| KCl                                                                      | J.T. Baker                      | Cat#0208         |
| Instant yeast                                                            | Lesaffre Iberica S.A            | N/A              |
| Methyl 4-hydroxybenzoate                                                 | Sigma-Aldrich                   | Cat#H6654-1KG    |
| MeOH                                                                     | Merck                           | Cat#34860-1L-R   |
| MeOH-d4                                                                  | Sigma-Aldrich                   | Cat#441384-10X0.75ML |
| NaCl                                                                     | Sigma-Aldrich                   | Cat#59888        |
| Na₂HPO₄                                                                  | Sigma-Aldrich                   | Cat#53264        |
| NaH₂PO₄                                                                  | Sigma-Aldrich                   | Cat#71505        |
| Na₃                                                                     | Merck                           | Cat#52002-5G     |
| NaOH                                                                     | Merck Millipore                 | Cat#1.06498.1000 |
| Nile Red                                                                 | Sigma-Aldrich                   | Cat#72485        |
| NMR calibration sucrose sample                                           | Bruker                          | Cat#Z10246       |
| Paraformaldehyde 16% solution                                            | Electron Microscopy Sciences    | Cat#15710        |
| Power SYBR Green PCR Master Mix                                          | Applied Biosystems              | Cat#4367659      |
| Propionic acid                                                           | Scharlau                        | Cat#AC18911000   |
| SuperScript III First-Strand Synthesis System for RT-PCR                 | Invitrogen                      | Cat#18080-051    |
| Tetramethyilsilane                                                       | Deutero                         | Cat#10006-10mL   |
| Trehalase                                                                | Sigma-Aldrich                   | Cat#T8778        |
| Triton X-100                                                            | Sigma-Aldrich                   | Cat#648462-1KG   |
| Vectashield mounting medium with DAPI                                     | Vector Labs                     | Cat#H-1200       |
| Wheat flour                                                              | Gallo                           | N/A              |

### Critical commercial assays

| Assay Kit                                                                 | Source                          | Identifier       |
|--------------------------------------------------------------------------|---------------------------------|------------------|
| Amplex™ Red Cholesterol Assay Kit                                        | ThermoFisher Scientific         | Cat#A12216       |
| Glucose (H2O) Assay Kit                                                 | Sigma-Aldrich                   | Cat#GAHK20-1KT   |
| RNeasy-Mini Kit                                                          | Qiagen                          | Cat#74106        |
| RNase-Free DNase Set                                                     | Qiagen                          | Cat#79254        |
| Serum Triglyceride Determination Kit                                     | Sigma-Aldrich                   | TR0100-1KT       |

### Deposited data

| NMR                        | Juarez-Carreno et al., 2021 | https://doi.org/10.5281/zenodo.5520983 |

### Experimental models: Organisms/strains

| Organism/strain Description                                                                 | Source                          | Identifier       |
|--------------------------------------------------------------------------------------------|---------------------------------|------------------|
| D. melanogaster strain expressing GAL4 in larval prothoracic gland cells under the control of phm regulatory sequences. yw¹²²; Spr/CyO; phm¹⁵⁷-Gal4/TM6B,Tb | Kim Rewitz, Ono et al. (2006)   | N/A              |
| D. melanogaster strain expressing GAL4 in larval fat body cells under the control of ppl regulatory sequences. yw¹²²; ppl-Gal4/CyO; TM2/TM6B, Tb | BDSC                            | S8768            |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| D. melanogaster strain expressing CD8-tagged GFP under the control of UAS. GFP is targeted to the cell membrane. *yw*^{122}; P[w^{+mC}]=UAS-mCD8::GFP.L/LL5, P[UAS-mCD8::GFP.L]/CyO; TM2/TM6B, Tb | BDSC | 5137 |
| D. melanogaster strain expressing secreted GFP under the control of UAS. GFP is targeted to synaptic vesicles. *yw*^{122}; UAS-secGFP/CyO; TM2/TM6B, Tb | BDSC | 6925 |
| D. melanogaster strain expressing F-rp:GFP-tagged Sry1 under the control of UAS. GFP is targeted to label synaptic vesicles. *yw*^{122}; P[w^{+mC}]=UAS-syt.eGFP}/CyO; TM2/TM6B, Tb | BDSC | 39693 |
| D. melanogaster strain expressing endoplasmic reticulum membrane-localized tdTomato under UAS control. *yw*^{122}; P[w^{+mC}]=20XUAS-tdTomato-Sec61β}/CyO; TM2/TM6B, Tb | BDSC | 64747 |
| D. melanogaster strain expressing a transgenic RNAi construct to apolpp. *yw*^{122}; Sp/CyO; P[TRiP.HM05157]attP2/TM6B, Tb | BDSC | 28946 |
| D. melanogaster strain expressing a transgenic RNAi construct to Sema1a. *w^{111F}; P(GD2504)v4743 | VRDC | v4743 |

### Oligonucleotides

| RT-qPCR primers | Integrated DNA Technologies (IDT). See Table S1 | N/A |

### Software and algorithms

| Biorender | Biorender.com | N/A |
| Illustrator CS5 | Adobe | N/A |
| ImageJ/Fiji | NIH | N/A |
| Microsoft Excel 2016 | Microsoft Corporation | N/A |
| M nova12 | Mestrelab Research | N/A |
| Photoshop CS5 | Adobe | N/A |
| Prism 9 | GraphPad Software | N/A |
| Topspin3.2 | Bruker | N/A |
| ZEN blue 2.3 | Zeiss | N/A |

### Other

| 12 positions Barvap nitrogen evaporator | Glas-Col | Cat#CE-1200 |
| #22 Surgical blade | Na hita | N/A |
| #55 Forceps | Fine Science Tools | Cat#11255-20 |
| 5415 R centrifuge | Eppendorf | N/A |
| 5424 R centrifuge | Eppendorf | N/A |
| 96-well micro test plate | Sarstedt | Cat#B2.1581 |
| AB17500 apparatus | Applied Biosystems | N/A |
| BenchTop Pro Lyophilizer | VirTis | SP Scientific |
| Bruker Ultra shield Plus 600 MHz spectrometer | Bruker Corporation | N/A |
| Cover glasses (18 x 18-mm) | Menzel-Glaser | Cat#BB001800180 |
| Cover glasses (24 x 50-mm) | VWR | Cat#ECN 631-1574 |
| EZ Read 400 Microplate Reader | Biochrom | Cat#B-4001-40 |
| Infinite M200 Pro Microplate Reader | Tecan | N/A |
| Leica TCS SP2 confocal microscope | Leica | N/A |
| MicroAmp™ Optical 96-Well Reaction Plate with Barcode | Applied Biosystems | Cat#4306737 |

(Continued on next page)
MATERIALS AND EQUIPMENT

NMR spectrometer: We used an AVII Bruker 600 MHz spectrometer equipped with a Cryoprobe.

*Alternatives:* Similar results could be obtained with a 500 or 600 MHz spectrometer equipped with an ambient TXI or BBI probe, by acquiring more scans.

pH meter: The pH of the NMR buffer was adjusted with a calibrated pH meter (InoLab, Cat# WTW 720).

Vortex mixer: During metabolite extraction, samples were vortexed with a vortex mixer (VWR, Cat# 444-1372).

Centrifuge: For phase separation after metabolite extraction, samples were centrifuged in a refrigerated Eppendorf centrifuge for 1.5 mL tubes (Eppendorf, Cat#5424R).

Lyophilizer: The solvent of the aqueous extracts was evaporated with a lyophilizer VirTis BenchTop Pro (SP Scientific).

Nitrogen evaporator: The solvent of the organic extracts was evaporated under a nitrogen stream using a 12-position Barvap nitrogen evaporator (Glas-Col, Cat#CE-1200).

Dissecting microscopes: We used a Zeiss Stereo Discovery V12 microscope to dissect larval tissues.

Imaging microscope: To obtain super-resolution images of larval tissues, we used a Zeiss LSM 880 confocal microscope with Airyscan, equipped with LD LCI plan-Apochromat 25x (NA 0.8 and WD 0.57 mm) and plan-Apochromat 63x (NA 1.4 Oil and WD 0.14 mm) immersion objectives, and a Leica TCS SP2 confocal microscope equipped with an HC PL APO CS 10x (NA 0.4 and WD 2.2 mm) dry objective.

Quantitative real-time PCR was performed on an ABI 7500 system (Applied Biosystems).

Glucose, triglyceride, and cholesterol measurements: We used an EZ Read 400 Microplate Reader (Biochrom) for glucose and cholesterol-based absorbance assays, and an Infinite M200 Pro Microplate Reader (Tecan) for triglyceride measurements.

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Microscope slides   | Normax | Cat#5470317C |
| Minutien pins       | Fine Science Tools | Cat#26002-10 |
| pH meter            | InoLab | WTW 720 |
| Pin holders         | Fine Science Tools | Cat#26016-12 |
| Sylgard 184 Silicone Elastomer Kit | Dow Corning | Cat#000000818156 |
| Vortex Mixer        | VWR    | Cat#444-1372 |
| Zeiss LSM 880 confocal microscope with Airyscan | Zeiss | N/A |
| Zeiss Stereo Discovery V12 microscope | Zeiss | N/A |

Iberian fly food preparation

| Reagent            | Amount |
|--------------------|--------|
| Water              | 15 L   |
| Wheat flour        | 0.75 kg|
| Brown sugar        | 1 kg   |

(Continued on next page)
**STEP-BY-STEP METHOD DETAILS**

**Collection and freezing of larvae for metabolite extraction**

**Timing:** 1 day

1. Using forceps, collect 20 larvae at the stage of interest in a 1.5 mL Eppendorf tube, either from the walls of the vial or, in the case of non-pupating larvae (Figure 2), from the fly food. Alternatively, fill the vial with 50% glycerol diluted in 1/3 PBS: larvae will come up to the surface.

   **Note:** We use 20 larvae here, but this protocol can also be applied to 10 adult flies.

2. Weigh the animals with a precision scale.

3. Freeze on dry ice.

   **Pause point:** Store larvae at −80°C.

**Metabolite extraction**

**Timing:** 1 day

4. Allow twenty frozen larvae (−80°C) to thaw for 5 min on ice (Figure 3). Add a quality control (QC) sample consisting of a mixture of 5 representative metabolites, including the most unstable ones, for example ATP+ADP+histidine+methionine-sulfoxide+citrate. Perform exactly the same steps with the QC sample as with the larvae samples.

   **△ CRITICAL:** Samples and solvents have to be kept ≤ 4°C during the whole procedure to avoid metabolite degradation.
5. Add 240 μL of methanol, 48 μL of deionized water, and 200 μL of chloroform on ice.

CRITICAL: The solvents must be added to the larvae sequentially. The solvents cannot be mixed together in advance because, owing to the different polarities of the solvents, the mixture may not be uniform and different amounts of solvent could be added to different samples.

Note: No need to vortex samples after adding the solvents as the solvents will be mixed during the homogenization process with the pestle.

6. After 10 min, homogenize samples with a small teflon pestle for 2 min and resuspend with a pipette, on ice.

7. For uniform cell breakage, place the samples in liquid nitrogen for 1 min and then allow to thaw on ice for 2 min. Repeat this step two more times.

CRITICAL: Handle liquid nitrogen with care. Wear gloves and protective goggles.
8. Add 120 μL of deionized water and 120 μL of chloroform to each sample on ice and vortex for 5 s.

9. Centrifuge the samples at 10,000 g for 15 min at 4°C to allow phase separation into an aqueous (top) and an organic (bottom) phase, containing polar and non-polar compounds, respectively. The middle layer, containing mainly proteins and cell membranes, can be discarded, or stored at −80°C for quantification of the total protein in each sample. Troubleshooting 1.

10. Transfer each phase to a different 1.5 mL Eppendorf tubes on ice. Troubleshooting 2.

11. For lyophilization, make a hole with a needle in the caps of the tubes containing the aqueous phase, introduce the tubes into liquid nitrogen, and transfer them to the lyophilizer overnight to remove water and methanol.

12. To remove the solvents from the organic phase, tubes are placed in a fume hood and solvents evaporated under a gentle stream of nitrogen.

Pause point: Store extracts at −80°C.

NMR sample preparation

© Timing: 5 min per sample

13. Place extract samples on ice and allow to thaw for 5 min.

14. For aqueous extract: transfer 550 μL of NMR buffer (100 mM Na₂HPO₄, 0.1 mM TSP, pH 7.4, in 100% D₂O) to each sample and mix with a pipette. Transfer the samples into 5-mm NMR tubes on ice (Figure 4).

15. Organic extract: dissolve extracts in 600 μL of deuterated chloroform (CHCl₃-d1) with 0.0027% tetramethylsilane (TMS) as the internal standard. Vortex samples, transfer into 5-mm NMR tubes, and seal the tubes.

Pause point: Store samples up to 12 h at 4°C.

Note: ATP in samples can be stable during 24 h at 4°C, but as a precaution to the possible presence of less stable metabolites, it may be safer to reduce this delay.

△ CRITICAL: Small air bubbles inside the NMR tubes affect the resolution of the NMR spectra. Troubleshooting 3.

NMR spectra acquisition

© Timing: 20–50 min per sample

16. Introduce the sample into the NMR spectrometer.
17. Set the temperature to 27°C (aqueous phase) or 25°C (organic phase) and allow to stabilize for 5 min.

18. Aqueous extracts: record $^1$H 1D NOESY NMR spectra with 400 scans.

△ CRITICAL: To obtain good quality spectra, apply the following settings: 64k data points digitalized over a spectral width of 30 ppm for optimal baseline correction, a 4-s relaxation delay between FIDs and a water presaturation pulse of 25 Hz to minimize the water signal.

19. Organic extracts: record $^1$H 1D NOESY NMR spectra with 128 scans.

△ CRITICAL: To obtain good quality spectra apply the following settings: 64k data points digitalized over a spectral width of 30 ppm for optimal baseline correction and a 4-s relaxation delay between FIDs.

20. Acquire 2D experiments for selected representative samples to confirm metabolite assignment. Perform total correlation spectroscopy (TOCSY) and multiplicity heteronuclear single quantum correlation (HSQC) of the aqueous phase and the organic phase. For each of these experiments, use 256–512 $t_1$ increments and collect 32–96 transients. Set the relaxation delays to 1.5 s and acquire the experiments in the phase-sensitive mode. Record TOCSY spectra using a standard MLEV-17 pulse sequence with mixing times (spin-lock) of 65 ms.

NMR spectra processing and integration

© Timing: 3 min per sample

21. Perform Fourier transformation on each spectrum. Multiply the FID values by an exponential function with a 0.5-Hz line broadening factor.

22. Perform automatic phase correction on each spectrum. Troubleshooting 4.

△ CRITICAL: Adjust manually, if phase is not correct after automatic correction.

23. Reference the spectra of the aqueous extracts to the TSP peak (0.00 ppm) and the spectra of the organic phase to the TMS peak (0 ppm) using Mnova 12 or ChenomX.

24. Assign signals to metabolite identities by comparison to reference values for chemical shift and multiplicity, and spectra of pure compounds in the human metabolome database (Wishart et al., 2018). Troubleshooting 5.

25. Generate automatic integration regions in Mnova 12 or ChenomX, taking into account signal assignment.

26. Generate integration tables in Mnova 12 or ChenomX by applying automatic integration with the GSD deconvolution option.

27. Normalize integration tables to total intensity.

Quantitative RT-PCR of lipid- and carbohydrate-metabolism-related genes

© Timing: 3 days

28. To assess mRNA levels of lipid and carbohydrate-metabolism-related genes, extract the total mRNA from five Drosophila larvae using an RNeasy Mini Kit (Qiagen Cat#74106).

29. To remove contaminating DNA, treat RNA with an RNase-Free DNase Set (Qiagen Cat#79254).

30. Synthesize cDNA with the SuperScript III First-Strand Synthesis System for RT-PCR using random oligo-dT primers (Invitrogen Cat#18080-051).
31. Perform quantitative real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems Cat#4367659) with gene-specific primers and an ABI 7500 system (Applied Biosystems). For primers used, see Table S1.
32. Normalize to ß-actin.

Note: We perform comparative qPCRs in triplicate and calculate the relative expression with the comparative Ct method.

### Hemolymph sample preparation

© Timing: 20 min per sample

33. Rinse 15 larvae (three replicates per sample) in PBS and dry on tissue paper.
34. Immerse the larvae individually in Schneider’s Drosophila medium (ThermoFisher Scientific Cat# 21720024), a medium with L-glutamine and sodium bicarbonate suitable for insect cell culture. Carefully tear the cuticles to release the hemolymph.
35. In a 0.5 mL tube, puncture a cross using a #22 surgical blade and collect larvae in this tube.
36. To collect the hemolymph (Figure 5), place the 0.5 mL tube (with a cross-shaped hole in the lower part) inside a 1.5 mL tube. Then, spin using a 5415 R centrifuge (Eppendorf) 3 times for 3 s each time at room temperature and with the caps open.
37. Transfer 10 µL of the supernatant to a new 1.5 mL tube.

△ CRITICAL: To prevent melanization, the handling time should be as short as possible and proceed in cold conditions (i.e., keep on ice before and after larval cuticle breakage).

### Glucose, trehalose, triglyceride, and cholesterol measurements

© Timing: 2 days

38. Lipids are a heterogeneous group of organic compounds that are insoluble in water, and are used as cell membrane components, energy storage molecules, and hormones. Cholesterol is a precursor of steroid hormones and an essential structural component of animal cell
membranes. To determine cholesterol levels, use an Amplex Red Cholesterol Assay Kit (Thermo-
Fisher Scientific Cat#A12216, https://www.thermofisher.com/document-connect/document-
connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals% 2Fmp12216.pdf). To analyze triglycerides (TAG), the primarily nutrient store in the fat body,
use a Serum Triglyceride Determination Kit (Sigma-Aldrich Cat#TR0100-1KT, https://www.
sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/320/307/tr0100bul.pdf).

Troubleshooting 6.

39. Stored (glycogen) and circulating (glucose and trehalose) carbohydrates are essential energy
sources present in the larval hemolymph that, on cellular uptake, satisfy many of the energy
needs of cells. To determine glucose levels, use a GAHK20 Glucose Assay Kit (Sigma-Aldrich
Cat#GAHK20-1KT) for dissected tissues or whole larvae, according to the manufacturer’s in-
structions (https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/
211/614/gahk20bul.pdf). To determine trehalose levels, convert to glucose with porcine treha-
lase (Sigma-Aldrich Cat# T8778) incubating all standards and samples at 37°C for 18–24 h and
measure using the same kit described above. To determine glycogen levels, use amyloglucosi-
dase (Sigma-Aldrich Cat# A1602) to break down glycogen into molecules of free glucose, which
is then quantified using the GAHK20 assay.

⚠ CRITICAL: The amount of triglycerides in larval tissues is very high; depending on the larval
stage being analyzed, we use different numbers of individuals per sample (20 animals for
second-instar larvae, 6 for early third-instar larvae, 4 for mid third-instar larvae, and 2 for
late third-instar larvae). For glucose measurements and determination of cholesterol in he-
omymph we use 15 animals per sample.

Dissection, immunostaining, mounting, and super-resolution imaging of larval tissues

© Timing: 2 days

40. Using forceps, collect 10–20 larvae at the stage of interest, either from the walls of the vial or, in
the case of non-pupating larvae, from the fly food.

41. Place larvae in a petri dish layered with Sylgard 184 transparent resin (Dow Corning Cat#
000000818156) containing drops of cold 1× PBS (Figure 6A).

42. Remove the brain lobes (Figure 6B and Video S1), fat bodies (Figure 6C and Video S2), or imagi-
nal discs by holding the larval body with one pair of forceps and pulling from the larval mouth
hook with a second pair. Discard the larval body and remove excess unwanted tissue with fine
mounted pins.

43. Place clean fly tissues (brain lobes with intact prothoracic endocrine glands or imaginal discs or
fat bodies) in a glass well containing 150 μL of cold 1× PBS. Fix the larval tissues by adding 50 μL
of 16% paraformaldehyde (Electron Microscopy Sciences Cat#15710) for a final concentration of
4% paraformaldehyde. Incubate in the dark on an orbital shaker with gentle agitation for 20 min
at room temperature.

⚠ CRITICAL: Paraformaldehyde is an irritant. Avoid inhalation and wear gloves when
handling this reagent. After use, proper disposal according to appropriate guidelines
is mandatory.

44. Wash the fixed tissues three times with 1× PBS.

Note: Add 200 μL of blocking solution (5% normal goat serum) and incubate the fixed tissues
in the dark on an orbital shaker with gentle agitation for at least 30 min at room temperature.
For the majority of commercial antibodies, it is possible to omit the blocking step and incu-
bate the dissected tissues directly in primary antibodies overnight (10–12 h).
45. To label relevant architectural features, dilute primary antibodies (mouse anti-Dlg 4F3 (1/100, DSHB) or rat anti-DE-Cad DCAD2 (1/50, DSHB)) in 0.3% Triton in 1x PBS in 1.5 mL tubes (you will need 200 µL per well).

46. Incubate larval tissues with primary antibodies in the dark, overnight (10–12 h) at room temperature. **Note:** If the ambient temperature is very high, use a humidified chamber to avoid evaporation, adding moist papers to the chamber. For longer periods incubate at 4°C.

47. Wash the samples three times with PBS.

48. Dilute secondary antibodies (Alexa Fluor 555 (1/500) or 647 (1/200) for anti-Dlg 4F3 and Alexa Fluor 647 (1/200) for anti-DE-Cad) in 0.3% Triton in 1x PBS in 1.5 mL tubes (you will need 200 µL per well).

49. Incubate with secondary antibodies in the dark for 3 h at room temperature.

50. Wash the samples three times with PBS. **Note:** To label neutral lipids, after incubation with the corresponding secondary antibodies, rinse larval tissues three times with PBS and then incubate for 40 min with a 1:500 dilution.
Figure 7. Expected outcomes

(A) $^1$H NMR spectrum acquired at 600 MHz of an aqueous extract of Drosophila larvae. Signals correspond to the following metabolites: 1. valerate, 2. 2-hydroxyvalerate, 3. leucine, 4. valine, 5. isoleucine, 6. 2-oxobutyrate, 7. ethanol, 8. 3-hydroxyisovalerate, 9. lactate, 10. 2-phenylpropionate, 11. alanine, 12. lysine, 13. acetate, 14. glutamate, 15. glutamine, 16. methionine, 17. methionine-sulfoxide, 18. succinate, 19. citrate, 20. $\beta$-alanine, 21. malate, 22. aspartate, 23. asparagine, 24. PC, 25. GPC, 26. glucose, 27. methanol, 28. glycine, 29. trehalose, 30. proline, 31.
of PBS with 1 mg/ml Nile Red (Sigma-Aldrich Cat#72485) at room temperature. Troubleshooting 7.

51. Mounting: Add 4 μL of Vectashield mounting medium with DAPI (H-1200, Vector Labs) and use the bridge method (Figure 6D) to mount larval tissues and preserve their three dimensional configuration.

52. Use a Zeiss LSM 880 confocal microscope with Airyscan for super-resolution imaging of larval brains, fat bodies, and prothoracic gland cells. Airyscan is a module for super-resolution imaging that uses an array detector with laser scanning confocal microscopy.

Use ImageJ software to measure total fluorescence intensity or lipid droplet size in larval tissues.

EXPECTED OUTCOMES

This protocol provides a toolbox for determining the metabolic status of control and mutant larvae at different stages by quantifying the metabolites (Figure 7A) and lipids/carbohydrates in dissected tissues or whole animals. Furthermore, it allows correlation of the metabolic state with the secretory capacity of the prothoracic endocrine gland or fat body cells using secreted (secGFP; Entchev et al., 2000) or synaptotagmin (Syt::GFP) -GFP protein constructs (Figures 7B and 7C) and analysis of the disposition of lipids in the tissues of the larvae, including the cells of the fat body (Figures 7D and 7E). Additional reporters that could be used include mCD8::GFP (Lee and Luo, 2001) or PLCδ (phospholipase C delta)-PH (pleckstrin homology domain)-EGFP (enhanced green fluorescent protein) (Verstreken et al., 2009) to study the plasma membrane and Sec61β (Summerville et al., 2016) as an ER marker.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed in GraphPad 8.0 software with a 95% confidence limit (p < 0.05). The experimental data are presented as means ± SEM and the statistical tests used were one-way or two-way analyses of variance (ANOVA) followed by Bonferroni’s post hoc test for comparing more than two genotypes and time-points. An unpaired t test was used for comparisons between two genotypes or time-points.

LIMITATIONS

Although NMR is a very robust and simple technique, its main drawback is its relatively low sensitivity. This means that, while we can easily obtain a profile of the main metabolites that are present in Drosophila larvae, we may have to perform additional experiments with more sensitive techniques (e.g., mass spectrometry) if we want to quantify metabolites with very low abundance. Furthermore, concerning the analysis of the lipids present in larvae, NMR can easily detect the functional lipid groups (lipid chain, glycerides, phospholipids, etc.) that change under certain conditions, but cannot detect the exact mass of the lipid that is altered. Thus, in this case additional techniques are also needed.

TROUBLESHOOTING

Problem 1
Phases are not perfectly separated after centrifugation (step 9).
Potential solution
Centrifuge the samples again for 5 min under the same conditions. If the problem remains, add 50 μL of deionized water and centrifuge again.

Problem 2
Some solvent from the other phase is remaining (step 10).

Potential solution
Discard it carefully with a pipette.

Problem 3
Air bubbles can worsen spectral resolution (step 14).

Potential solution
Remove bubbles by gently knocking the NMR tube with your finger.

Problem 4
Phase is not well corrected after automatic phase correction (step 22).

Potential solution
Adjust phase manually.

Problem 5
Doubts in the assignment of peaks that are very close together (step 24).

Potential solution
Add 1 μL of a 100 mM standard solution of the metabolite in D₂O, and check if the additional signal matches the assigned signal perfectly.

Problem 6
Excessive amount of TAG is already in the control larvae (step 38).

Potential solution
Due to the high level of TAG in the samples, the resulting supernatant should be appropriately diluted (2, 4, and 8 times) to ensure that the concentrations of all samples are within the linear range of the assay.

Problem 7
Lipid coalescence (steps 46–49).

Potential solution
To avoid lipid coalescence, do all incubations, including those for primary and secondary antibodies, in 1× PBS and not in 0.3% Triton in 1× PBS.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Javier Morante (j.morante@umh.es).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This paper does not report original code.
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101195.

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AUTHOR CONTRIBUTIONS
Conceptualization and data analysis, J.M. and M.P.-S.; methodology, J.M. and M.P.-S.; investigation, J.M., J.C-V., J.G., S.C.-J., and M.P.-S.; writing – original draft, J.M. and M.P.-S.; writing – review & editing, J.M. and M.P.-S.; funding acquisition, J.M. and M.P.-S.; supervision, J.M.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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