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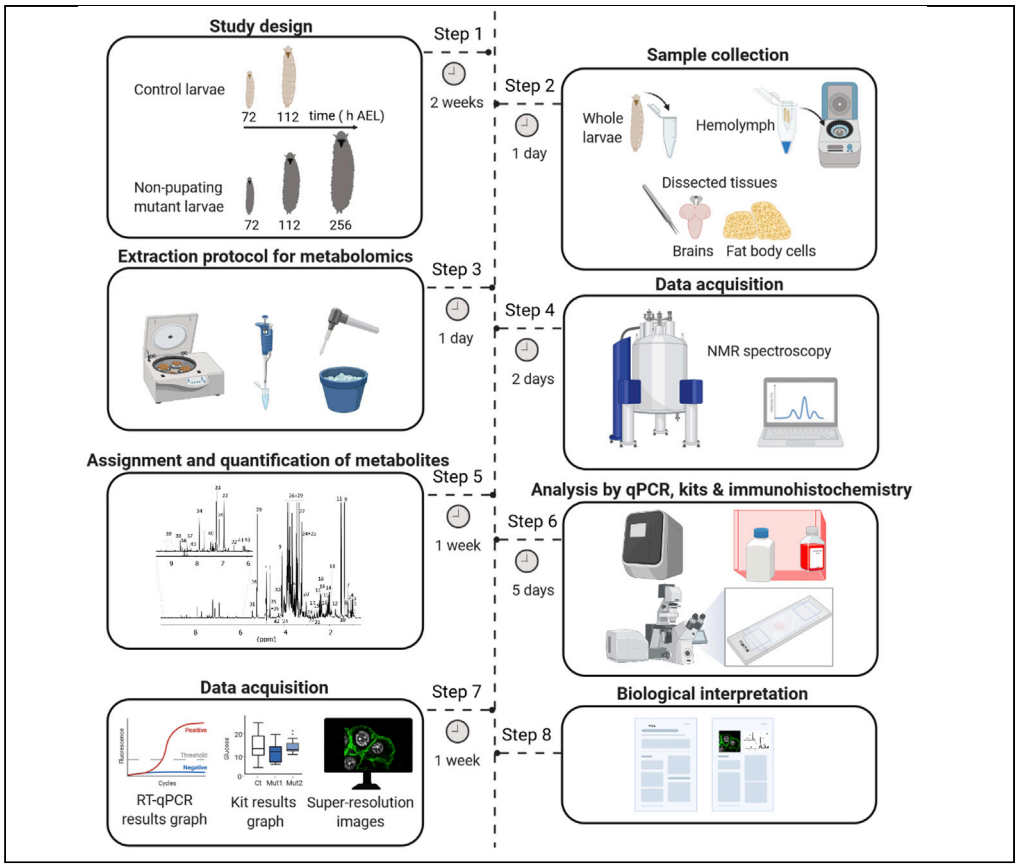
A toolbox to study metabolic status of *Drosophila melanogaster* larvae



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*.

Martina Palomino-Schätzlein, Juan Carranza-Valencia, Juan Guirado, Sergio Juarez-Carreño, Javier Morante

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Highlights

Single-assay quantification of metabolites in dissected tissues or whole animals

NMR-based profiling of polar and non-polar metabolites

Protocol for dissection and staining of brains and fat body cells

Metabolic status of control and obese Drosophila larvae

Palomino-Schätzlein et al., STAR Protocols *3*, 101195 March 18, 2022
https://doi.org/10.1016/j.xpro.2022.101195

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A toolbox to study metabolic status of *Drosophila melanogaster* larvae

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https://doi.org/10.1016/j.xpro.2022.101195

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*^{i} and *phm>Sema1a*^{i}) larvae at the stage of interest (Figure 1).

Preparation of metabolite extraction

Timing: 10 min

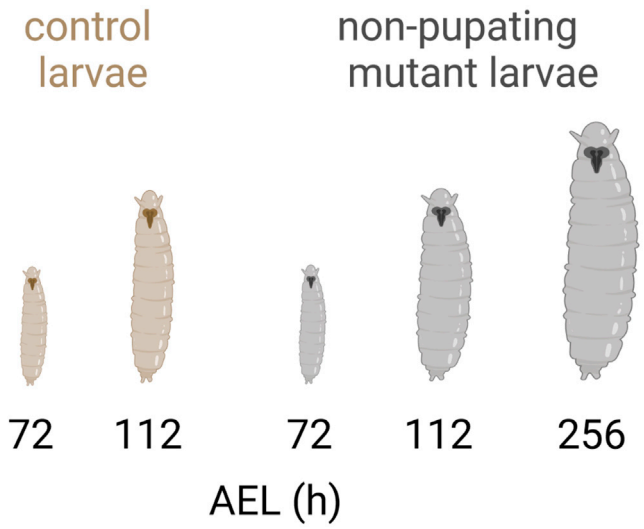

Figure 1. Scheme of the samples used in this study at the indicated ages

4. Transfer methanol (MeOH, analytical grade), chloroform ($CHCl_3$, 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 $\leq$ 1 Hz). Use a commercial standard sample (Bruker Cat#10246) containing 2 mM sucrose, 0.5 mM sodium trimethylsilylpropanesulfonate (DSS), 2 mM NaN_3 in 90% H_2O and 10% D_2O.
7. Prepare NMR analysis buffer by solubilizing 100 mM Na_2HPO_4 and 0.1 mM 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP) in D_2O, and adjust the pH to 7.4 with a saturated NaOH solution in D_2O. The buffer can be stored for up to one year at 4°C.

Reagent	Final concentration	Amount
Na_2HPO_4	100 mM	142 mg
TSP	0.1 mM	1.7 mg
D_2O		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

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Continued

REAGENT or RESOURCE	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		
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
$CHCl_3$	Merck	Cat#650498-1L
$CHCl_3$-d1	Merck	Cat#151823-50G
D_2O	Eurisotop	Cat#D216L
Gibco Schneider's *Drosophila* Sterile Medium	ThermoFisher Scientific	Cat#21720024
KCl	J.T. Baker	Cat#0208
Instant yeast	Lesaffre Ibérica S.A	N/A
Methyl 4-hydroxybenzoate	Sigma-Aldrich	Cat#H6654-1KG
MeOH	Merck	Cat#34860-1L-R
MeOH-d4	Merck	Cat# 441384-10X0.75ML
NaCl	Sigma-Aldrich	Cat#S9888
Na_2HPO_4	Sigma-Aldrich	Cat#S3264
NaH_2PO_4	Sigma-Aldrich	Cat#71505
NaN_3	Merck	Cat#S2002-5G
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
Tetramethylsilane	Deutero	Cat#10006-10mL
Trehalase	Sigma-Aldrich	Cat#T8778
Triton ×-100	Sigma-Aldrich	Cat#648462-1KG
Vectashield mounting medium with DAPI	Vector Labs	Cat#H-1200
Wheat flour	Gallo	N/A
Critical commercial assays		
Amplex™ Red Cholesterol Assay Kit	ThermoFisher Scientific	Cat#A12216
Glucose (HK) 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		
^{1}H NMR	Juarez-Carreño et al., 2021	https://doi.org/10.5281/zenodo.5520983
Experimental models: Organisms/strains		
D. melanogaster strain expressing GAL4 in larval prothoracic gland cells under the control of *phm* regulatory sequences. *yw^{122}; Sp/CyO; phm^{22}-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. *yw^{122}; ppl-Gal4/CyO; TM2/TM6B, Tb*	BDSC Zinke et al. (1999)	58768

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Continued

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*	Marcos Gonzalez-Gaitan Entchev et al. (2000)	N/A
D. melanogaster strain expressing GFP-tagged Syt1 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	6925
D. melanogaster strain expressing GFP-tagged pleckstrin homology domain from human PLCD for detecting phosphatidylinositol. *yw*^{122}; *Sp/CyO; P{w[+mC]= UAS-PLCδ-PH-EGFP}/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 RNA^{i} construct to *apolpp*. *yw*^{122}; *Sp/CyO; P{TRiP.HM05157}attP2/TM6B, Tb*	BDSC	28946
D. melanogaster strain expressing a transgenic RNA^{i} construct to *Sema1a*. w^{1118}; 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
Mnova12	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	Nahita	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#82.1581
ABI7500 apparatus	Applied Biosystems	N/A
BenchTop Pro Lyophilizer	VirTis	SP Scientific
Bruker Ultrashield Plus 600 MHz spectrometer	Bruker Corporation	N/A
Cover glasses (18 × 18-mm)	Menzel-Glaser	Cat#BBAD01800180
Cover glasses (24 × 50-mm)	VWR	Cat#ECN 631-1574
EZ Read 400 Microplate Reader	Biochrom	Cat#80-4001-40
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

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Continued

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

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 25× (NA 0.8 and WD 0.57 mm) and plan-Apochromat 63× (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 10× (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.

Iberian fly food preparation

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

(*Continued on next page*)

Continued	
Reagent	Amount
Yeast	0.5 kg
Agar	0.17 kg
5% Methyl 4-hydroxybenzoate/nipagin solution in ethanol	130 mL
Propionic acid	130 mL
Prepare fly food in advance and store at 4°C for a maximum of one week.	

⚠ CRITICAL: Nipagin and propionic acid are irritants. Wear gloves and mask when handling these reagents.

10×Phosphate-buffered saline (PBS) dilution		
Reagent	Final concentration	Amount
NaCl	137 mM	80 g
KCl	2.7 mM	2 g
Na_2HPO_4	10 mM	14.4 g
KH_2PO_4	2 mM	2.4 g
ddH_2O		up to 1 Liter
10× PBS stock dilution can be sterilized by autoclaving and stored indefinitely at room temperature (20°C–30°C), as long as sterility is maintained.		

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× 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.

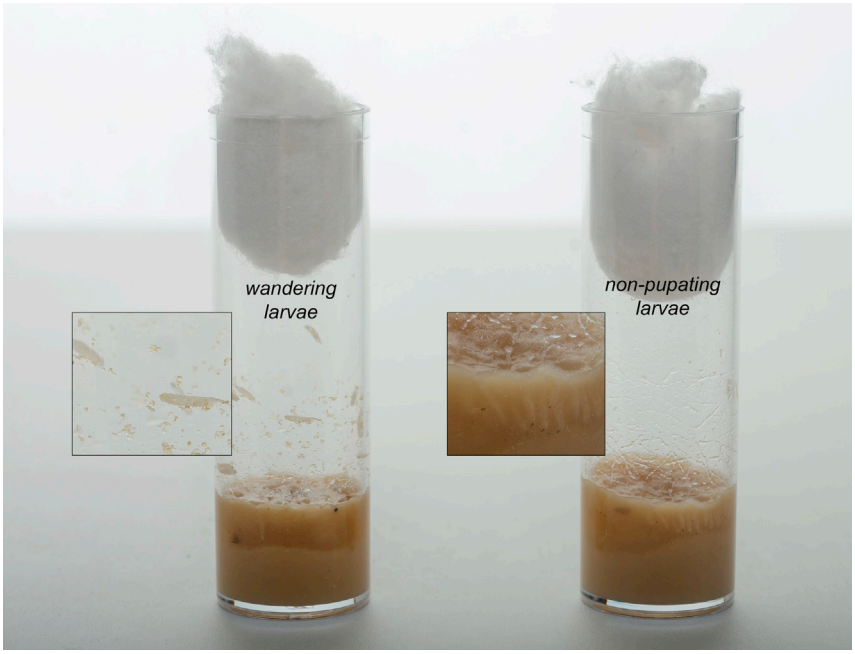

Figure 2. Larvae with knockdown of candidate factors controlling maturation commitment failed to wander out of the food

Insets are magnifications of control larvae and larvae with *RNA*^{i} of a hit gene.

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.

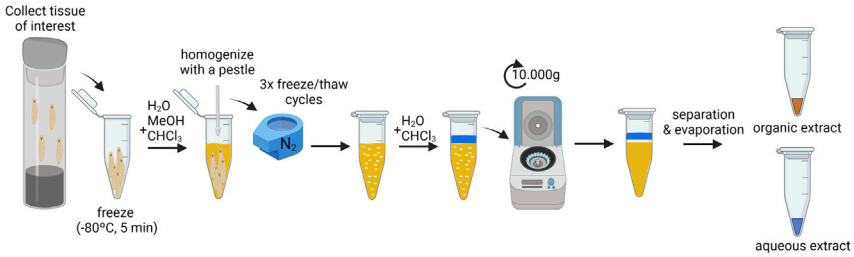

Figure 3. Metabolite extraction and NMR sample preparation

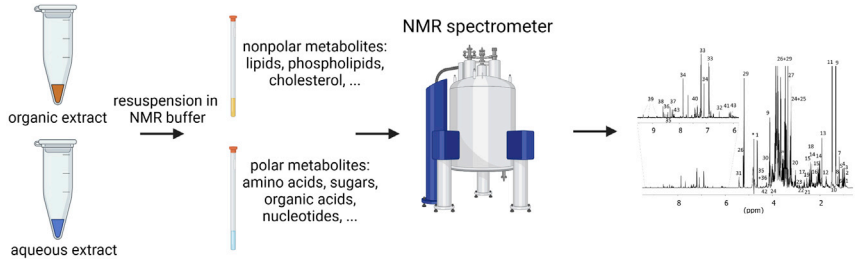

Figure 4. NMR spectrum acquisition

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 Na_2HPO_4, 0.1 mM TSP, pH 7.4, in 100% D_2O) 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 ($CHCl_3$-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 t1 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).

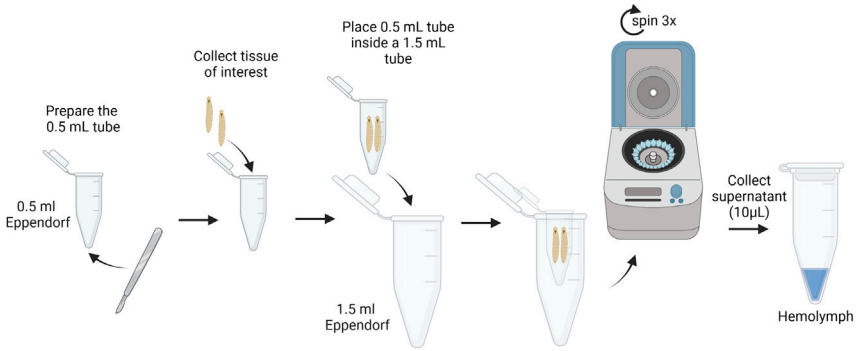

Figure 5. Collection of the hemolymph

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.

qPCR cycling conditions

Steps	Temperature	Time	Number of cycles
Pre-incubation	95°C	10 min	1
Amplification	95°C	15 s	40
Cooling	60°C	1 min	1

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 (ThermoFisher 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 instructions (https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/211/614/gahk20bul.pdf). To determine trehalose levels, convert to glucose with porcine trehalase (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 amyloglucosidase (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 hemolymph 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 imaginal 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 incubate the dissected tissues directly in primary antibodies overnight (10–12 h).

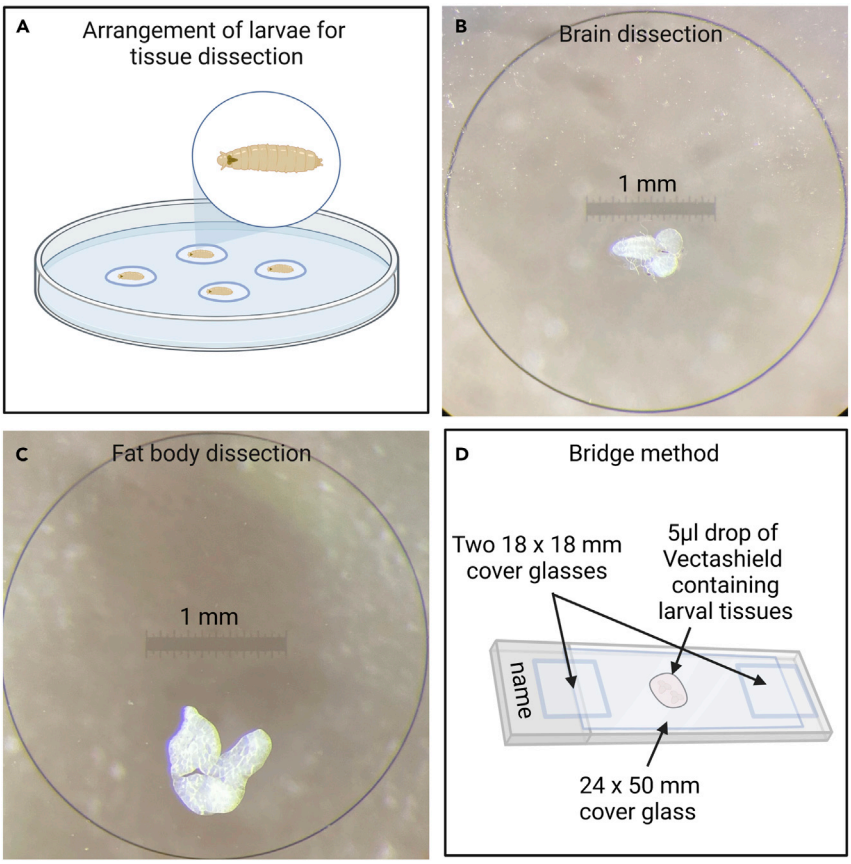

Figure 6. Dissection of the brains and fat bodies of the larvae

(A) Arrangement of larvae for tissue dissection.

(B) Detailed view of a dissected larval brain.

(C) Detailed view of a dissected larval fat body.

(D) Bridge method for mounting larval tissues. Scale bars, 1 mm in (B) and (C).

45. To label relevant architectural features, dilute primary antibodies (mouse anti-Dlg 4F3 (1/100, DSHB) or rat anti-*D*E-Cad DCAD2 (1/50, DSHB)) in 0.3% Triton in 1× 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- *D*E-Cad) in 0.3% Triton in 1× 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

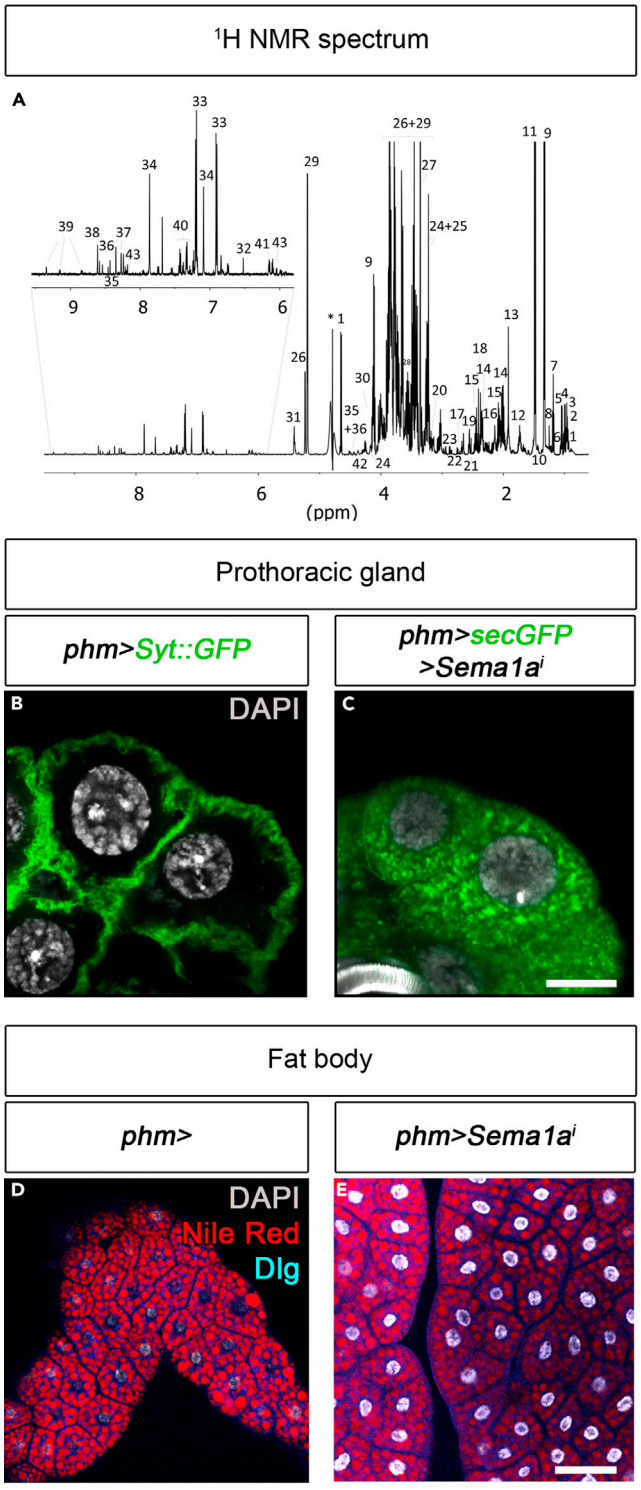

Figure 7. Expected outcomes

(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. β-alanine, 21. malate, 22. aspartate, 23. asparagine, 24. PC, 25. GPC, 26. glucose, 27. methanol, 28. glycine, 29. trehalose, 30. proline, 31.

Figure 7. *Continued*
glycogen, 32. fumarate, 33. tyrosine, 34. histidine, 35. ATP, 36. AMP, 37. ADP, 38. formate, 39. NAD, 40. phenylalanine, 41. UPD-derivatives, 42. threonine, 43. inosine.
(B) *Syt::GFP* in control prothoracic gland cells (*phm>Syt::GFP*).
(C) Cytoplasmic accumulation of Syt-positive vesicles in prothoracic gland cells from a *phm>Syt::GFP>Sema1a*^{i} larva at 112 h. Nuclei were stained with DAPI (gray).
(D and E) Whole-mount views of 112 h AEL larval fat body cells from *phm>* (D) and *phm>Sema1a*^{i} (E) larvae. Fat body cells were counterstained with anti-Dlg (blue) and neutral lipids were stained with Nile Red (red). (B and C) Adapted with permission from (Juarez-Carreño et al., 2021). Scale bars, 10 μm in (B and C) and 75 μm in (D and E).

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 (ANOVAs) 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 D_2O, 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.

ACKNOWLEDGMENTS

We thank S. Javaloy for helping with illustrations and video editing. All figures except Figure 2 and Figure 7 and the graphical abstract were created with BioRender.com. This work was supported by a Generalitat Valenciana Grant co-financed with the European Regional Development Fund (OP ERDF of Comunitat Valenciana 2014-2020) to M.P. and by grants BFU2016-76295-R and SEV-2017-0723 funded by MCIN/AEI/10.13039/501100011033 and by "ERDF A way of making Europe," a CSIC Grant (2019AEP181), and a Generalitat Valenciana Grant (PROMETEU/2021/027) to J.M. Grants BES-2017-081122 and BES-2013-064947 funded by MCIN/AEI/10.13039/501100011033 and by "ESF Investing in your future" to J.C-V. and S.J-C., respectively.

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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