Succinate Dehydrogenase-Regulated Phosphoenolpyruvate Carboxykinase Sustains Copulation Fitness in Aging C. elegans Males

HIGHLIGHTS
- C. elegans upregulates pck-1- and pck-2-encoded PEPCK during early adulthood
- Loss of PEPCK causes premature male copulatory behavior decline
- Epidermal PEPCK is required to sustain the copulatory fitness
- Subunit A of succinate dehydrogenase antagonizes PEPCK expression
Succinate Dehydrogenase-Regulated Phosphoenolpyruvate Carboxykinase Sustains Copulation Fitness in Aging C. elegans Males

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SUMMARY
Dysregulated metabolism accelerates reduced decision-making and locomotor ability during aging. To identify mechanisms for delaying behavioral decline, we investigated how C. elegans males sustain their copulatory behavior during early to mid-adulthood. We found that in mid-aged males, gluco-/glyceroneogenesis, promoted by phosphoenolpyruvate carboxykinase (PEPCK), sustains competitive reproductive behavior. C. elegans’ PEPCK paralogs, pck-1 and pck-2, increase in expression during the first 2 days of adulthood. Insufficient PEPCK expression correlates with reduced egl-2-encoded ether-a-go-go K+ channel expression and premature hyper-excitability of copulatory circuits. For copulation, pck-1 is required in neurons, whereas pck-2 is required in the epidermis. However, PCK-2 is more essential, because we found that epidermal PCK-2 likely supplements the copulation circuitry with fuel. We identified the subunit A of succinate dehydrogenase SDHA-1 as a potent modulator of PEPCK expression. We postulate that during mid-adulthood, reduction in mitochondrial physiology signals the upregulation of cytosolic PEPCK to sustain the male’s energy demands.

INTRODUCTION
Although reduction in decision-making and locomotor ability is an inevitable consequence of aging, many studies have suggested that nutrition and its effects on metabolism will modify the rate and manifestation of behavioral decline (Canevelli et al., 2016; Garland et al., 2016; Norton et al., 2012; Sanders et al., 2016; Smith and Blumenthal, 2016). Dysregulated cellular and mitochondrial metabolism, which can occur during an animal’s lifespan, has been demonstrated to reduce viability and synaptic and contractile functions of neurons and muscles (Baraibar et al., 2016; Bender et al., 2006; Camandola and Mattson, 2017; Kraytsberg et al., 2006; Saxton and Sabatini, 2017). The dysregulated metabolism can disrupt behavior by limiting ATP production or indirectly by generating excessive reactive by-products, such as reactive oxygen species and/or advanced glycation end products, which impair cellular and mitochondrial functions. Progressive accumulated damage can limit the regeneration of metabolic intermediates for sustaining neuronal and muscular performance (Allaman et al., 2015; Trushina and McMurray, 2007).

For adult males of the Caenorhabditis elegans laboratory N2 strain, reproductive-specific behaviors decline faster than general behaviors shared with hermaphrodites. When reared in groups, the average lifespan of well-fed adult males is 6–8 days, but their lifespan can be extended up to 15 days, if they are reared in solitude (Gems and Riddle, 2000; Shi et al., 2017). However, despite differences in lifespan and rearing conditions, copulatory behavior decays after 2 days of adulthood. The age-related decline in copulation efficiency is due to increases in muscle excitability, which lead to motor coordination defects during mating attempts (Guo et al., 2012).

The N2 male’s competitive copulation efficiency, during their first 2 days of adulthood, is affected by its nutritional status. For example, males that are transiently starved between the end of their last larval molt and up to the first 18 h of adulthood, but then refed, have (1) their copulation ability extended by an extra day (Guo et al., 2012) and (2) have enhanced meiotic activity of their germ cells (Chou et al., 2019). If the males continue to be starved, their copulation ability is attenuated (Honjoh et al., 2017). The hormesis response of transient starvation stress promotes calcium calmodulin kinase II
and ether-a-go-go-like K+ channel functions. These molecules mitigate the increases of age-related muscle excitability (Guo et al., 2012; LeBoeuf et al., 2011). In contrast to starved animals, if well-fed males are mutant for the NAD-dependent histone deacetylase gene sir-2.1, their copulation ability prematurely declines between days 1 and 2. The behavioral defects of young sir-2.1 mutant males resemble older wild-type males. Other general behaviors of the sir-2.1 mutant males, such as locomotion and feeding, do not differ from wild-type (Guo and García, 2014).

The sir-2.1-encoded NAD-dependent protein deacetylase monitors cellular NAD+ levels to regulate the expression of genes involved in metabolism and stress handling in C. elegans (Berdichevsky et al., 2006; Mouchiroud et al., 2013; Viswanathan et al., 2005; Wang et al., 2006). In sir-2.1 mutant adult males, catabolic genes involved in glycolysis and the tricarboxylic acid (TCA) cycle are upregulated, but reactive oxygen species (ROS) scavenger genes are down-regulated. The misregulated energy consumption in the sir-2.1 mutants abnormally increases muscle excitability and compromises the coordination of different sex-specific muscle contractions during the copulation step of male spicule penetration and sperm transfer. Other prematurely upregulated genes in sir-2.1 mutants are the anabolic genes pck-1 and pck-2 (Guo and García, 2014), implicated in gluconeogenesis and glyceroneogenesis (Yuan et al., 2012, 2016). A possibility arises that in the sir-2.1 mutant, the upregulation of these anabolic genes is a compensation response to consequences from abnormal upregulation of catabolic genes. Consistent with this idea, the mating defect of day 1 sir-2.1 mutant males is aggravated if they also contain a loss-of-function mutation in either pck-1 or pck-2. In wild-type males, pck-1 and pck-2 RNA levels naturally increase between days 1 and 2. This observation suggests that the role these molecules play in reducing the consequences of sir-2.1 deficiency in day 1 males might also occur to sustain mating fitness for wild-type day 2 males (Guo and García, 2014).

pck-1 and pck-2 both encode the enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Yuan et al., 2012, 2016). PEPCK phosphorylates and decarboxylates oxaloacetate (OAA), a TCA cycle intermediate, to form phosphoenolpyruvate (PEP). In mammalian cells, there are cytoplasmic and mitochondrial forms of the enzyme (Ballard and Hanson, 1967; Nordlie and Lardy, 1963). Depending on the cell’s immediate needs, PEP can be used for gluco-/glyceroneogenesis or to re-form pyruvate (Chakravarty et al., 2005). Consequently, the pyruvate can re-enter the TCA cycle, undergo a transamination reaction with glutamate to produce alanine and α-ketoglutarate or be reduced to lactate. In mammalian cells, if glucose levels get too high, cytoplasmic PEPCK can also be acetylated by the p300 acetyltransferase to cause the enzyme to catalyze the reverse reaction of generating OAA from PEP, presumably to feed the TCA cycle and promote further energy production. Mammalian SIRT1 NAD-dependent protein deacetylase has also been shown to deacetylate PEPCK to maintain the canonical cataplerotic function of generating PEP from OAA (Latorre-Muro et al., 2018).

Groups have reported that increased cytoplasmic PEPCK levels correlate with sustained or enhanced animal muscle function and longevity. For example, in rodents, artificial overexpression of cytoplasmic PEPCK in skeletal muscle increases behavioral activity, mitochondrial content, and the usage of fatty acids (Hakimi et al., 2007). Similarly, artificial overexpression of cytoplasmic PEPCK increases C. elegans’ lifespan, whereas elimination of cytoplasmic PEPCK reduces the worm’s longevity. In C. elegans body wall muscles, cytoplasmic PEPCK levels increase during normal aging and even more so during caloric restriction-induced lifespan extension (Yuan et al., 2012, 2016). In regard to C. elegans male mating behavior, the natural upregulation of pck-1 and pck-2 might enhance cellular function by either providing or shunting OAA to or from the TCA cycle, offsetting aging or sir-2.1 mutation-induced metabolic dysfunction.

In this work we investigated PEPCK’s metabolic contributions to the maintenance of N2 male’s copulation fitness. We examined why males upregulate their two cytoplasmic PEPCK-expressing genes during the first 2 days of adulthood and how pck-1 and pck-2 differentially promote copulation activity from different tissues. We also used forward genetics to identify metabolic factors that contribute to the up-regulation of PEPCK in both males and hermaphrodites, and we found that loss-of-function mutations in the mitochondrial succinate dehydrogenase subunit A of the electron transport chain complex II increase pck-1 and pck-2 expression. The succinate dehydrogenase complex participates in both the TCA cycle and the electron transport chain. We found that pharmacological perturbations of other electron transport chain components do not increase cytoplasmic PEPCK levels to levels similar to reducing complex II function, suggesting that C. elegans monitors the mitochondrial TCA cycle to adjust the abundance of PEPCK.
RESULTS

Expression of PCK-1 and PCK-2 in C. elegans Hermaphrodites and Males

C. elegans pck-1, pck-2, and pck-3 have sequence homology to PEPCK genes of other animal species. The amino acid sequences for PCK-1 and PCK-2 show 71% identity with each other and 58% identity to mammalian PEPCK orthologs (Figures S1 and S2). Both retain the conserved catalytic amino acids required for PEP formation, supporting their functionality (Figure S1) (Carlson and Holyoak, 2009; Sullivan and Holyoak, 2007). In contrast, PCK-3 has much lower similarity (23%–25% identity) to PCK-1 and PCK-2 and lacks some of the conserved catalytic amino acids (Figure S3). Yuan and colleagues showed that PCK-1 and PCK-2 account for all measurable OAA to PEP catalysis; cell extracts from pck-1(0); pck-2(0) double mutants lack detectable PEPCK activity, signifying that pck-3 might not function as a PEPCCK paralog (Yuan et al., 2016). Immunological staining of PCK-1, determined by antibodies to rat PEPC (Ballard and Hanson, 1969), suggested that PCK-1 is expressed in the cytoplasm of worm muscles, intestine, and to a lesser extent, the pharynx (Yuan et al., 2012, 2016); the expression of PCK-2 was not reported. However, due to the high amino acid identity, cross-reactivity of PCK1 and PCK2 to the rat antibody might complicate the expression profiles of the two paralogs. Therefore we studied the localization and expression of PCK-1 and PCK-2 by tagging a fluorescent protein to each gene.

To explore further the published pck-1 expression, we characterized yellow fluorescence protein (YFP) or Timer expression from transgenes that contained the 2.8-kb pck-1 promoter. YFP was fused downstream of just the promoter, to the promoter and a mini-pck-1 gene containing the first three introns, or to the promoter and intronless pck-1 cDNA. The three different constructs were made to test if pck-1 intronic and/or exonic sequences might contribute to the expression pattern. Consistent with the previous report on the hermaphrodites’ expression, we confirmed here that for both sexes, all three of pck-1 expressing reporters express in head and body wall muscles (Figure 1A). However, we found that the reporters also express in ventral cord and preanal neurons (Figure 1B) and ~20 nerve ring neurons per side in the head (Figures 1C and 1D). In the tail region, both larval males and larval/adult hermaphrodites express the transgenes in the dorsal rectal ganglion and three neurons per side in the tail. The adult male shows additional expression in sex-specific neurons of the ray, postcloacal, and spicule sensilla (Figures 1E and 1F). None of the constructs promote expression in male or hermaphrodite sex muscles, intestine, or pharyngeal muscles. The expression pattern suggests that PCK-1 contributes to the functional metabolism of C. elegans neurons and the muscles they innervate.

Previous work suggested that PCK-1 functions in the cytoplasm, whereas its paralog PCK-2 functions in the mitochondria (Yuan et al., 2016). YFP fluorescence from the pck-1::YFP cDNA fusion is found in the cytosol, confirming that PCK-1 is a cytoplasmic enzyme (Figures 1A–1F). Aside from large gene expression studies (Hunt-Newbury et al., 2007), no comprehensive expression pattern for PCK-2 has been published. To address this issue, we explored where PCK-2 is located in

PCK-2 Acts with PCK-1 to Sustain Male Copulation Fitness during First 2 Days of Adulthood

The expression of PCK-2::YFP suggests that this paralog of PEPC is cytosolic and functions in the epidermis, all muscles, and in the intestine. However, before characterizing the properties of the fusion
protein further, we tested if the YFP tag on PCK-2 disrupts the enzyme’s function or worm’s behavior. Previously, we reported that at 20°C, the ability for well-fed *C. elegans* N2 males to sire at least one progeny (the copulation potency assay) with 24-h adult hermaphrodites declines after 48 h of adulthood (Figure 2A). When pck-2 function is disrupted via the deletion allele (ok2586), referred to as pck-2(0), copulation decline is accelerated; the mutant’s ability to sire progeny decreases after 24 h of adulthood (Guo et al., 2012).
Figure 2. Male Mating Potency and Copulation Fitness

(A) Mating potency of wild-type and PEPCK mutant males determined at the newly molted adult stage and 24 and 48 h of adulthood. Single aged males were paired with single 24-h pha-1(ts) hermaphrodites for 5 days. Numbers of males assayed for each age group are listed at the bottom of the bars. The % potency values are listed at the top of the bars. p values were determined using Fisher’s exact test.

(B) Copulation competition; n = number of independent trials. The numbers within the colored bars are the percent victory trials for the specific genotype. 95% CI (confidence interval); p values were determined using Fisher’s exact test.

(C) Percentage of 24-h-old males with constitutively protracted spicules. Groups of 20–25 males were incubated with or without five 24-h-old wild-type hermaphrodites (Herm); vulvaless hermaphrodites contain the mutation let-23(sy1). Numbers of males assayed are listed at the bottom of the bars. The % spicule protracted values are listed at the top of the bars. p values were determined using Fisher’s exact test.

(D and E) Confocal images of EGL-2::YFP expression in one set of the bilateral SPD, SPV, and SPC spicule neurons; in the images, anterior region of the male tail is to the left and dorsal is to the top. Panel D depicts the tail of a wild-type male. Panel E depicts the tail of a pck-1(0);pck-2(0) double mutant male.

(F) Average fluorescence intensity of a region of interest (ROI) encompassing the SPD, SPV, and SPC spicule neurons on one side of a male. Fluorescence values are in arbitrary units (A.U). Bars and whiskers represent mean and standard deviation. Numbers of males quantified are listed at the bottom of the graph. Each dot represents an ROI of a single male. p value was determined using the Mann-Whitney test.

This figure is related to Figure S4.
accelerated decline is likely due to decrease of behavioral fitness rather than sperm quality, because reduced potency is not observed if pck-2(0) males copulate with older and easier-to-mate hermaphrodites (48 h of adulthood); 80% potent for 24-h pck-2(0) males, n = 50 and 84% for 24-h wild-type males, n = 50. If the CRISPR/Cas9 knock-in YFP tag disrupts the genomic PCK-2’s function in copulation, then aging pck-2::YFP males should display a reduced copulation potency similar to the pck-2(0) mutants. However, in the first 2 days of adulthood, males expressing PCK-2::YFP sired progeny with an efficiency similar to wild-type (Figure 2A). Thus the YFP tag on pck-2-expressed PEPCK does not obviously accelerate male impotence. In contrast to the pck-2 mutation, the pck-1 deletion allele (ok2098), referred to as pck-1(0), does not affect copulation kinetics of mutant males up till 48 h of adulthood (Figure 2A) (Guo and García, 2014). Although PCK-1 appears to be non-essential, the pck-1-encoded PEPCK is likely used for mating, because we observed that pck-1(0); pck-2(0) double mutant males display synthetically lower copulation efficiency right after L4 molt (~5 h) (Figure 2A).

The potency assay indicated that during early adulthood (~5 h after molt), the pck-1(0) and pck-2(0) single mutants are similar to wild-type, suggesting that the two PEPCK enzymes might compensate for each other. However, the compensation during this temporal window might not be equivalent. The potency assay addresses if wild-type and mutant males are competent to sire at least one progeny; however, it does not test if aging factors or genetic compensation affect competitive fitness. To determine fitness, we conducted a mating competition assay. In this assay, we paired two males (one containing a fluorescent marker) with one fog-2 mutant female on a 5-mm bacterial lawn and observed which male transfers sperm first; fluorescence status of the cross-progeny was used to validate the paternity. Results from the competition assay provided further insight into the differential requirement of PEPCK (Figure 2B). When ~5-h adult PCK-2::YFP and non-tagged wild-type males compete, 50% of the time either male transfers sperm first, reaffirming that the YFP tag does not interfere with mating behavior. The YFP tag allowed us to ask if copulation fitness is reduced in the first 48 h of adulthood. Surprisingly, the competition results between 24-h PCK-2 non-tagged and 48-h PCK-2::YFP wild-type adult males indicate that the differentially aged males have equivalent fitness. In contrast, although young adult pck-1(0) and pck-2(0) single mutants show similar mating ability in the potency assay (Figure 2A), pck-2(0) mutants are less fit than pck-1(0) mutants. When ~5-h adult pck-1(0) or pck-2(0) single mutants compete against ~5-h adult PCK-2::YFP wild-type males, the pck-1(0) males display similar fitness to the wild-type, but the pck-2(0) single males show reduced fitness. However, pck-1(0) males might not be entirely wild-type in behavior. When pck-1(0) and pck-2(0) males competed against each other, pck-1(0) males generally won, but unlike the competition between wild-type and pck-2(0) males, the margin was too low to be statistically different (Figure 2B). These data suggest that compared with pck-1, PEPCK expressed from pck-2 provides a larger contribution to the competitive fitness of early and mid-aged N2 male mating behavior.

Even though the copulation fitness was lower, casual mating observations of pck-2(0) single and pck-1(0); pck-2(0) double mutants did not identify any steps in copulation that were grossly defective; however, after 24 h, a proportion of pck-2(0) single and pck-1(0); pck-2(0) double mutant males displayed spontaneous irreversible spicule protractor muscle spasm (Figure 2C). The constitutively contracted protractor muscles cause the copulatory spicules to dangle out the cloaca, rendering the male impotent. This protraction constitutive phenotype (Prc) can be seen in old wild-type males (older than 3 days) and in mutants that are defective in regulating membrane excitability of the spicules’ sex-specific neurons and muscles (García and Sternberg, 2003; Jobson et al., 2015). Interestingly, we observed that extended copulation attempts with mating-reticent young hermaphrodites increased the frequency of the Prc phenotype in pck-2(0) single- and even more so in pck-1(0); pck-2(0) double mutants (Figure 2C). When we replaced those hermaphrodites with mutants that do not develop a functional vulva, the frequency of the Prc phenotype decreased (Figure 2C). These observations indicate that vulva insertion attempts exacerbate the pck-1(0); pck-2(0)-induced defect. Taken together, the synthetic pck-1(0); pck-2(0) phenotype suggests that for the pck-2(0) single mutant, PCK-1 provides partial compensatory PEPCK function up to the first 24 h of adulthood, but for the pck-1(0) single mutant, PCK-2 can provide compensatory PEPCK function at least up till the first 48 h of adulthood that we tested; the role for the two PEPCK likely involves sustaining differential regulation of neuron and muscle excitability in the copulation circuitry during adulthood.

In earlier reports, we observed the EGL-2 ether-a-go-go-like K+ channel expression in sex muscles increases during the first 48 h of male adulthood to regulate membrane excitability of copulation cellular components (LeBoeuf et al., 2011). Similar to the pck-2(0) phenotype, deletion of EGL-2 also results in
24–48 h premature decline of male mating potency (Guo et al., 2012). The studies indicated that the voltage-gated K+ channel is required to modulate male sex muscles’ membrane potential during aging and food stress; however, in those studies, neural expression from extrachromosomal EGL-2 transgenes was not consistent. To circumvent the expression variabilities with extrachromosomal transgenes, we used CRISPR/Cas9 to knock-in YFP into the terminus of the genome-encoded egl-2 gene. In the egl-2::YFP genomic recombinants, we found that in addition to male sex muscles, EGL-2::YFP is also expressed on the cell bodies, neural processes, and sensory endings of the male ray, post-cloacal, and spicule sensory neurons (Figure S4C).

To determine if the K+ channel’s expression in the sensory and motor neurons and protractor muscles used for spicule insertion behavior underlies the copulation defects, we crossed the genomic egl-2::YFP knock-in allele into pck-2(0) and pck-1(0); pck-2(0) mutants. We quantified EGL-2::YFP expression in the male’s protractor muscles and SPC, SPD, and SPV neurons (Figures S4D and 2D–2F). The spicule protractor muscles control the movement of the spicules. The SPC neurons are presumptive proprioceptive sensory motor neurons that sense the position of the spicules during copulation attempts and subsequently stimulate tonic contraction of the spicule protractor muscles to extend the spicules through the vulval opening. The SPD and SPV sensory neurons send their sensory processes through the shaft of the spicules, where their ciliated endings are exposed at the tips of the spicules. These sensory neurons presumably sense the hermaphrodite’s uterine environment and subsequently coordinate sperm transfer with the duration of spicule penetration (LeBoeuf et al., 2014). Previous work showed that the expression of a transcriptional egl-2 promotor reporter construct increased in wild-type sex muscles between 24 and 48 h of adulthood (LeBoeuf et al., 2011). Here we observed that the protractor muscles’ EGL-2::YFP levels also increased between 24 and 48 h in wild-type males (Figure S4D). However, aging did not significantly change the K+ channel levels in the three neurons we quantified (Figure 2F). In contrast to the wild-type and the pck-2(0) single mutant, at 24 h of adulthood, the EGL-2::YFP fluorescence in the muscles, as well as the three sex neurons, was lower in the pck-1(0); pck-2(0) mutant, consistent with their behavioral defect. However, as the animals aged by 48 h, the K+ channels eventually increased to wild-type levels (Figures 2D–2F). These results suggest that both PCK-1 and PCK-2 function promotes EGL-2 levels in the copulatory circuit early within the first 24 h of adulthood.

**PCK-1 Functions in Cholinergic Neurons and PCK-2 in Muscle and Epidermis to Promote Mating Success**

To understand how PCK-1 and PCK-2 impact mating behavior, we needed to determine where they were functioning. To address this question, we tested which tissue(s) require pck-1 and pck-2 to restore mating potency to 24-h adult pck-1(0); pck-2(0) double mutant males. We rationalized that restoring the different PEPCK gene to their relevant tissues should make the double mutant male behave similar to a PEPCK single mutant. The PEPCK double mutant displays a more severe defect than pck-1(0) or pck-2(0) alone, allowing for easier determination of tissue-specific function. Plasmids containing a pck-1 cDNA or a mini pck-2 gene (where the large second intron was removed) expressed by the pck-1 promoter, pan-muscle promoter (unc-103A promoter), the acetylcholine vesicular transporter promoter (unc-17 promoter), an epidermis promoter (dpy-7 promoter), intestinal promoter (gtl-1 promoter), or a sex muscle promoter (unc-103E promoter) were injected into the double mutant.

The mating potencies of transgenic males indicated that expressing pck-1 in cholinergic neurons and expressing pck-2 in either epidermis or muscle restored mating potential (Figure 3A). For pck-2, expression in epidermis provided higher rescue than pan muscle rescue, but the difference was not statistically significant. Behavioral rescue by restoring pck-1 in cholinergic neurons or pck-2 in all muscles were not surprising, because copulation requires neurons and muscles. However, the rescue from the epidermis-expressed pck-2 was not expected. The observation suggests that for wild-type pck-2 to compensate the pck-1(0) single mutant deficiency, the epidermis might require PEPCK to provide gluco-/glyceroneogenesis products to neighboring pck-1-deficient muscles and neurons.

**Supplementation of Glucose Partially Substitutes the Requirement of PEPCK for Male Copulation Efficiency in Early Adulthood**

We next asked how PEPCK from distinct tissues support copulation. Many groups have demonstrated various beneficial functions of PEPCK in well-fed animals. For example, transgenic over-expression of PEPCK provides increased muscle respiratory exchange, mitochondria biomass, behavioral activity, and lifespan extension in mammals and C. elegans (Franckhauser et al., 2002; Hakimi et al., 2007; Yuan et al., 2012, 2016). PEPCK is also essential for mammals and C. elegans larvae to survive short and long periods...
Figure 3. Male Mating Potency and Efficiency Rescue in PEPCK-Deficient Mutants

(A) Mating potency of 24-h pck-1(0); pck-2(0) adult males expressing transgenic tissue-specific-expressed pck-1:YFP or pck-2:YFP. Numbers of males assayed are listed at the bottom, and % potency values are listed at the top of the bars. p Values were determined using Fisher’s exact test. Error bars represent the 95% confidence intervals.
of fasting (Hibshman et al., 2017; Puigserver et al., 2003; Rothman et al., 1991). Enzymatically, PEPCK converts cytoplasmic or mitochondrial OAA into PEP. This reaction achieves two goals: (1) lowering potential mitochondrial ROS production by removing excess substrates from the TCA cycle and (2) diverting excess PEP to the anabolic generation of glucose and glycerol for stored sugar (glycogen and/or trehalose) and triglyceride synthesis (Owen et al., 2002). If the animal experiences fasting, the stored forms of sugar and triglycerides can then be catabolized for further energy usage and building materials. Thus we asked why adult C. elegans males require PEPCK for copulation behavior. We hypothesize that they could require PEPCK either for a metabolic protective role or to generate carbon intermediates for sustaining behavioral-related catabolic and anabolic needs.

To address this issue, we supplemented the diets of well-fed pck-1(0); pck-2(0) males with D-glucose. If in the context of wild-type behavior, PEPCK diverts excess substrates from the TCA cycle to maintain optimal mitochondrial function, then providing excess carbon into the TCA cycle, via artificial glucose supplementation, should be detrimental for the double mutant. However, if PEPCK is needed for anabolic processes to later distribute catabolic substrates for behavioral execution, then glucose supplementation should compensate for the double mutant’s metabolic deficiency.

To evaluate these possibilities, we tested how supplementing their already abundant food availability with D-glucose affects the mutant’s copulation ability. We tracked individual males to determine their kinetics for serially impregnating females over a course of 84 h (see Transparent Methods). For this experiment, an individual male was placed with 10 fog-2 mutant females. Every 12 h, mated egg-gravid females were counted, removed, and replaced with virgin females matched to the age of the male (Figures 3B–3E and S6). The assay allows for the temporal study of male reproductive endurance and behavioral deterioration. During the first 48 h, wild-type and pck-1(0) males were able to mate with ~3–4 partners every 12 h (Figures 3B–3E); however, this was not true for pck-2 mutant males. Wild type, pck-1(0) and pck-2(0) males had equivalent mating kinetics during the first 12 h of adulthood, indicating that the two PEPCK paralogs can effectively compensate for each other (Figure 3B). In contrast, the pck-1(0); pck-2(0) double mutant displayed a lower ability to serially mate with females. Between 12 and 24 h, pck-2(0) mutants began to lag behind wild-type and pck-1(0) males, suggesting that by this time, pck-1-expressed PEPCK alone can no longer meet the physiological demands of the male. After 24 h, the pck-2(0) single and pck-1(0); pck-2(0) double mutants were similarly defective for mating.

D-glucose supplementation has been shown to reduce the lifespan of wild-type C. elegans hermaphrodites and males (Lee et al., 2009; Schulz et al., 2007; Seo et al., 2018). Our experiments suggest that D-glucose could also have a small negative effect on reproductive-competent wild-type adult males; however, due to the sample size in our assay, the supplemented sugar does not have a statistical effect on their copulation ability (Figure 3B). In contrast, during the first 24 h of adulthood, glucose supplementation improved the copulation performance of pck-1(0); pck-2(0) double mutants; they were able to impregnate females at a rate similar to their wild-type counterparts (Figure 3C). After 24 h, sugar supplementation did not sustain the mutants’ mating behavior, but the supplementation also did not accelerate behavioral degradation either (Figures 3D and 3E). These observations suggest that early in the wild-type male’s adulthood, PEPCK-based gluconeogenesis provides energy to sustain copulation. However, later in the male’s adulthood, PEPCK, especially expressed by PCK-2, might be required for other processes such as glyceroiolgenesis/triglyceride synthesis to sustain copulation.

**PKC-1 and PKC-2 Expression Levels Are Coordinated and Increase during Aging**

Given the differential requirement for pck-1 and pck-2 in copulation behavior, we asked how PEPCK expression changes between young and mid-aged males. In our previous study, we conducted qRT-PCR analyses that indicated both pck-1 and pck-2 transcript levels increased between pooled day 1 and pooled...
day 2 wild-type males (Guo and García, 2014). Here, we asked if expression from the \( P_{pck-1}^{+}:\text{TIMER} \) reporter and PCK-2::YFP in individual males agrees with the earlier pooled qRT-PCR results. The fluorescent protein TIMeR can be used to estimate the timing of transcription in living cells. The fluorescent molecule initially emits fluorescence in the green wavelength, but after \( \sim 3 \) h, the molecule stably photoconverts its fluorescence emission to the red wavelength. The changes in green fluorescence intensity can give an approximation of promoter activity (Terskikh et al., 2000). As the green fluorescence emission is transient, the signal should represent actively transcribed transgenes. To quantify the \( P_{pck-1}^{+}:\text{TIMER} \) expression, we determined the average green fluorescence intensity across the whole ventral cord and ventral body wall muscle region of the male. To quantify total PCK-2::YFP levels, we determined the average yellow fluorescence intensity of the whole male. Consistent with our earlier qRT-PCR findings, \( P_{pck-1}^{+}:\text{TIMER} \) reporter and PCK-2::YFP expression increases during development and between 24 and 48 h of adulthood. However, by 72 h, the amount of \( P_{pck-1}^{+}:\text{TIMER} \) expression in males decreases and the accumulation of PCK-2::YFP does not significantly differ from 48-h adult males (Figures 4A and 4B). To isolate the specific tissues that upregulate PCK-2 during adulthood, we set regions of interest over different types of cells and quantified PCK-2::YFP levels. Consistent with its necessity in copulation, the epidermal, but not muscular PCK-2::YFP, level was higher at 48 h, compared with 24 h of adulthood. Interestingly, even though intestinal PCK-2 is not essential for copulation behavior, its expression was also high among different cell types and upregulated at 48 h of adulthood, suggesting additional function of intestinal PCK-2 (Figure S7).

Earlier, we inferred that PCK-2 must compensate for the loss of PCK-1 in the \( pck-1(0) \) males. PCK-1 is expressed in neurons and body wall muscles; PCK-2 is expressed in the body wall muscles, sex muscles, intestine, and epidermis. As cells likely provide metabolic substrates for each other, we entertained the possibility that changes in one cell might affect another cell’s metabolism through metabolite exchange. Thus we asked if differentially reducing PEPCK via mutation in one PEPCK gene will induce a corresponding expression change in its paralog, indicating the interdependency of different cells on their neighbor’s metabolic state.

\( P_{pck-1}^{+}:\text{TIMER} \) was integrated close to the pck-2 locus (~0.25 map unit), thus we were not able to cross-in the \( pck-2(0) \) mutation. Instead we used CRISPR/Cas9 to induce a frameshift/premature stop mutation in \( pck-2 \) into \( P_{pck-1}^{+}:\text{TIMER} \) males. We called this allelic \( pck-2(2(0)) \) to distinguish it from \( pck-2(0) \). During larval development, \( P_{pck-1}^{+}:\text{TIMER} \) expression was lower in \( pck-2(2(0)) \) compared with wild-type; however, in the first 48 h of adulthood, \( P_{pck-1}^{+}:\text{TIMER} \) expression for the \( pck-2(2(0)) \) males was equivalent to wild-type. After 48 h, \( P_{pck-1}^{+}:\text{TIMER} \) expression dropped slightly more in the \( pck-2(2(0)) \) than in the wild-type (Figure 4A). This result indicates that during wild-type larval development, \( pck-1 \) expression in neurons and muscles is affected by PCK-2 levels in the epidermis, muscles, and intestine, but this dependency is relaxed in adults.

In contrast, when \( pck-1(0) \) was crossed into \( pck-2::\text{YFP} \) adult hermaphrodites or males, the pattern of expression was reversed. PCK-2::YFP expression levels in \( pck-1(0) \) L2 to L4 larval animals were similar to wild-type, but during adulthood, PCK-2::YFP levels did not increase to levels of wild-type (Figure 4B). This observation was unexpected. As PCK-2 must compensate for the \( pck-1 \) deletion, we originally predicted \( pck-2 \) expression to be higher in its native tissues or expressed ectopically (such as in neurons). However, the lower expression suggests that in wild-type animals, adult tissues expressing PCK-2 readjust the levels of the enzyme in the direction of PEPCK levels in \( pck-1 \)-expressing tissues; and larval tissues expressing PCK-1 readjust the levels of the enzyme in the direction of PEPCK levels in \( pck-2 \)-expressing tissues.

To test further this idea, we over-expressed \( pck-1::\text{YFP} \), driven by its own promoter through a transgene; the high expression of \( P_{pck-1}^{+}:\text{YFP} \) led to aggregate formation (Figure 4C). We then asked which direction PCK-2::YFP levels change in the adult intestine, a tissue from which we can isolate fluorescence measurements from \( P_{pck-1}^{+}:\text{YFP} \). The over-expression of \( P_{pck-1}^{+} \) resulted in a coordinated increase in PCK-2::YFP levels (Figure 4D), consistent with the idea that \( pck-1 \) and \( pck-2 \) expression are metabolically coupled and regulated in the same direction.

**Mutation in Subunit A of Succinate Dehydrogenase Leads to Increased PEPCK Expression**

We hypothesized that \( pck-1 \) and \( pck-2 \) expression increases as a response to a change in the male’s physiology; however, we did not know what metabolic alterations would induce PEPCK levels to increase. To address this issue, we performed an ethyl methanesulfonate (EMS) mutagenesis on \( pck-2::\text{YFP} \) animals to identify what genetic changes could increase PCK-2::YFP expression. Our screen identified the \( rg550 \)
Figure 4. Interactions between PCK-1 and PCK-2 Expression

(A) Green emission fluorescence intensity of TIMER expressed from the pck-1 promoter.

(B) Yellow emission fluorescence intensity of whole-body PCK-2::YFP.

(C) Fluorescence micrograph of a 24-h pck-2::YFP male overexpressing pck-1::YFP from an extrachromosomal array. The overexpressed (OE) PCK-1::YFP forms aggregates in the motor neurons and body wall muscles. A pck-2::YFP male is also shown for comparison. Both males contain a pha-1 mutation and the pha-1(+)-rescuing plasmid in an extrachromosomal array.

(D) Yellow emission fluorescence intensity of intestinal PCK-2::YFP. (A, B, and D) Fluorescence values are in arbitrary units (A.U.). For each stage, the number of animals quantified is 10 per strain. Each symbol represents a single male of the specified age. Bars and whiskers represent mean and standard deviation. P values were determined using the Mann-Whitney test.

This figure is related to Figure S7.
Figure 5. *sdha-1*-Regulating Metabolic Genes Controlling Metabolic Flux of Mitochondria

(A) Fluorescence micrograph of PCK-2::YFP expression in 24-h *sdha-1*(rg550) and wild-type hermaphrodites. Below the micrograph is a cartoon showing the worms’ positions in the above micrograph.
Animals that are homozygous for the sdha-1(rg550) allele displayed multiple abnormal phenotypes (Figure 5B), similar to reported RNA interference (RNAi) phenotypes and mutations in other electron transport chain molecules (Huang and Lemire, 2009; Ishii et al., 1990; Mathew et al., 2016). We recorded the following defects in the sdha-1 mutants: their mitochondria were smaller and less networked than wild-type animals (Figures S8A and S8B); they consumed oxygen at a slower rate (Figure S8C), their larval developmental rate between L2 to L3 stage was slower (Figure S8D), they moved slower (Figure S8E), developing L4 males failed to remodel their anal depressor muscle and are not capable of copulation (Figures S8F and S8G), they were partially resistant to the reactive oxygen-producing poison paraquat (Figure S8H), and adult hermaphrodites retained eggs in their uterus (Figure S8I). These phenotypes indicate that mitochondrial function is compromised, detrimentally impacting development and behavior.

Hermaphrodites that are heterozygous for sdha-1 grossly appeared wild-type, suggesting that rg550 might be a recessive loss-of-function allele; however, as the sdha-1(rg550) allele is a missense mutation, the mutant phenotypes might be due to neomorphic functions caused by the amino acid change. We addressed this possibility using CRISPR/Cas9 to induce into wild-type sdha-1 an insertion/deletion mutation (rg448) in the general position of rg550. The rg448 lesion creates a premature stop, followed by a frameshift change lesion (Figure S8B). The developmental and behavioral defects induced by rg448 resembled animals containing the rg550 allele. Similarly, with respect to whole worm PCK-2::YFP increase, the rg448 and rg550 lesions promoted pck-2 expression through every stage of development (Figure 5E). Thus rg550-induced point mutation likely disrupts SDHA-1 function.

We also examined Ppck-1:TIMER expression in sdha-1(rg550) mutants, as the qRT-PCR experiment measuring pck-1 expression was slightly higher, albeit not statistically different between wild-type and mutant (Figure 5D). Similar to PCK-2::YFP, we found that Ppck-1:TIMER expression increased in adulthood (Figure 5F). These observations are consistent with the idea that the animal modulates PCK-1 and PCK-2 levels in the same direction. We also attempted to use CRISPR/Cas9 to generate a mutation in sdha-2, the paralog of sdha-1; however, we were unsuccessful at obtaining viable mutants. This might be because unlike sdha-1, sdha-2 is essential for the survival of the worms.

To verify that sdha-1 mutant phenotypes were not caused by an unknown mutation in our strains, we tested if the sdha-1(rg550) missense mutation can be rescued by an extrachromosomal array expressing sdha-1(+), and a separate SL2 splicing CFP from the sdha-1 promoter. A 500-bp region upstream of the first sdha-1 ATG start codon drives sdha-1 expression in the pharynx, epidermis, body wall muscles, and intestine; these are tissues that also express the pck-2 gene product (Figure 6A). From casual observation, we found...
Figure 6. SDHA-1 Inhibition on PCK-2 Expression in Hermaphrodites and Males

(A) Fluorescence micrographs showing a PCK-2-YFP male expressing sdha-1 and SL2 trans-spliced CFP from the sdha-1 promoter.

(B) Yellow emission fluorescence intensity of whole-animal PCK-2-YFP during different adult ages. Fluorescence values are in arbitrary units (A.U). Symbols represent individual wild-type, sdha-1(rg550), and transgenic sdha-1(rg550) transgenic males that express sdha-1(+) from its native promoter (sdha-1 promoter) or a muscle promoter (unc-103A promoter). For each stage, the number of animals quantified is 10 per strain. Each symbol represents a single male of the specified age. Bars and whiskers represent mean and standard deviation. p values were determined using the Mann-Whitney test.

(C) Fluorescence micrograph showing epidermal CFP-expressing mitochondria from a heat-shocked hermaphrodite that expresses CFP fused to SDHA-1.

(D) Yellow emission fluorescence intensity of whole animal PCK-2-YFP in 18- to 24-h adult males. Males were heat shocked at L4 stage to either induce transgenic sdha-1-CFP expression or test if heat shock artificially changed PCK-2-YFP levels. Fluorescence values are in arbitrary units (A.U). Symbols represent individual wild-type sdha-1(rg550) and transgenic wild-type and sdha-1(rg550) males that express sdha-1-CFP from a heat shock promoter (hsp-16 promoter). Numbers of animals quantified are listed at the bottom of the graph. Bars and whiskers represent mean and standard deviation. p values were determined using the Mann-Whitney test.
that expression from the extrachromosomal array complements the sdha-1 growth and behavioral defects in both sexes. Focusing on adult males, we also found that the extrachromosomal array reduces the sdha-1(rg550)-induced PCK-2 increase from L4 stage to the third day of male adulthood; in some mutant males, pck-2 expression was even lower than in wild-type (Figure 6B). These observations confirm that the rg550 missense mutation reduces the function of SDHA-1 and promotes PCK-2 expression.

We noticed that males that were mosaic for the transgene (as determined by patchy CFP expression throughout the worm) had uniformly lowered PCK-2::YFP expression in the mosaic tissue; this observation prompted us to hypothesize that tissues containing a functional mitochondrial Complex II system might partially modulate the metabolism of neighboring tissues. To test this possibility, we generated an extrachromosomal array that selectively expresses sdha-1(+) in the body wall and sex muscles (from the unc-103A promoter) of sdha-1(rg550) mutants. We found that similar to expression from its own promoter, sdha-1(+)-splicing rescued expression from the body wall muscle also had a cell non-autonomous effect on PCK-2::YFP levels in the epidermis, intestine, and pharyngeal muscles through several days of adulthood (Figure 6B). This result is consistent with our earlier experiment (Figure 4D) showing that altering PEPCK level in one tissue changes PEPCK levels in neighboring ones.

In the transgenic sdha-1(+) containing mutants, sdha-1(+) was chronically overexpressed throughout development. However, this experiment did not differentiate if the reduction in pck-2 expression was indirectly due to restoration of mitochondrial function at a specific stage in development, or due to an immediate acute response to expressed SDHA-1 levels. Thus we asked if acute expression of sdha-1(+) from a heat shock promoter at L4 stage can immediately alter PCK-2::YFP expression in early adult males. Instead of using the trans-splicing sdha-1(+)-SL2::CFP plasmids (Figures 6A and 6B), we constructed sdha-1(+)-directly fused to CFP at the C terminus, to visualize whether the expressed protein went to the mitochondria (Figure 6C). We found that heat shock expression of sdha-1(+)-CFP in L4 animals reduces PCK-2::YFP expression in 18- to 24-h adult wild-type and sdha-1(rg550) (Figure 6D). Thus, acute alteration of SDHA-1 levels results in the opposite change in PCK-2 levels; however, neither the heat shock nor the endogenous 500-bp sdha-1 promoter expressed sdha-1(+)-CFP rescue sdha-1 behavioral and developmental defects (casual observation). As the sdha-1(+)-CFP fusion from those constructs are stable and transgenically overexpressed, we reasoned that the failure of rescue is not likely from lack of production, instead the CFP, tagged to SDHA-1, likely interferes with a function of the subunit, which is independent of its role in modulating PEPCK levels.

PEPCK Regulates Fuel Usage and Energy Metabolism in sdha-1 Mutant and Wild-Type

As sdha-1 mutants display defective mitochondria and upregulate PEPCK, we hypothesized that genes, which divert substrates out of the oxidizing steps of TCA cycle and promote OAA production, should also be highly expressed in the mutant. pyc-1-encoded pyruvate carboxylase converts pyruvate to oxaloacetate, and the bifunctional icl-1(geo-7)-encoded isocitrate lyase/malate synthase enzyme promotes succinate and malate production directly from isocitrate; both these enzymes are expressed in the mitochondria (Liao and Freedman, 2001; Liu et al., 1995). Consistent with our hypothesis, qRT-PCR data indicated that sdha-1 mutants hyper-express pyc-1 and icl-1 (Figure 5D). However, although the anabolic genes pck-1, pck-2, icl-1, and pyc-1 are upregulated, sdha-1(rg550) mutants accumulated less glycogen than wild-type and the pck-2(0) mutant (Figures S9A and S9B). Moreover, the sdha-1(rg550) mutants also accumulated less intestinal neutral lipid droplets than wild-type, but more than the pck-2(0) mutant. Interestingly, under starvation conditions, the sdha-1(rg550) mutant retained more lipids than wild-type, and this property depended on functional pck-2 (Figure S9C). We hypothesize that instead of stockpiling lipids and carbohydrates, the sdha-1(rg550) mutants use PCK-1, PCK-2, ICL-1, and PYC-1 to generate the necessary fuel for immediate catabolism or to ration fuel under food-limiting conditions.

ATP Production in Wild-Type, sdha-1, and PEPCK Mutants

To further dissect the energy metabolism of aging animals, we determined the whole worm ATP content using a luciferase assay (Figure 7). Wild-type and pck-1(0) males maintained relatively constant ATP throughout the first 3 days of adulthood. As expected, disrupting mitochondrial function on day 1 via sdha-1 mutation or by acutely exposing day 1 and day 3 wild-type or day 1 pck-1(0); pck-2(0) double mutant males to the mitochondrial poison sodium azide (NaN₃) significantly lowered the ATP content; the remaining ATP was likely generated by substrate-level phosphorylation, such as glycolysis. NaN₃ inhibits ATP generation by blocking electron transfer between cytochrome c and complex IV (Figure 8A) (Palmieri and
Klingenberg, 1967; Wilson and Chance, 1966). In contrast to wild-type and \textit{pck-1(0)} males, day 1 \textit{pck-2(0)} single mutants showed significantly lower ATP content than wild-type, indicating that unregulated PCK-1 function, in the absence of \textit{pck-2}, abnormally lowers ATP levels (Figure 7).

Surprisingly, the \textit{pck-1(0)};\textit{pck-2(0)} double mutant had higher ATP content than wild-type, \textit{pck-1(0)} and \textit{pck-2(0)} single mutants (Figure 7). In addition, treating the double mutant with NaN$_3$ variably reduced the amount of ATP comparable to wild-type (wild-type, 90% G 10% reduction versus double mutant, 72% G 22% reduction) suggesting that either or both mitochondrial ATP production and substrate level phosphorylation are abnormally higher in the mutant. The contribution of substrate level phosphorylation on the double mutant’s ATP production is most evident on day 3, because NaN$_3$ did not significantly reduce the ATP levels. Possibly, the prolonged lack of accumulated gluco-/glyceroneogenesis products (i.e., fats) causes day 3 \textit{pck-1(0)};\textit{pck-2(0)} double mutant energy production to shift away from mitochondrial respiration.

If a function of PEPCK is to divert metabolic intermediates out of the mitochondria, then early-on, lacking both PEPCK paralogs could initially result in aberrant mitochondria with more TCA cycle intermediates, and thus more ATP production. In addition, compensation for the reduction in PEPCK could also result in increased substrate-level phosphorylation through altered catabolism of ingested or scavenged five-carbon sugars. However, these mechanisms to increase ATP production are not sufficient to offset defective gluco-/glyceroneogenesis, because dietary glucose supplementation is required to partially alleviate the double mutant’s copulation defect. Likely, the temporal and spatial production of energy cannot be fully restored by compensatory mechanisms.

**Electron Transport Chain Poisons Increase PEPCK Expression, but Less Than \textit{sdha-1} Deficiency**

In the \textit{sdha-1} male and hermaphrodite mutants, the increase in \textit{pck-1} and \textit{pck-2} expression could be due to a reduction of a specific function of succinate dehydrogenase, due to non-specific mitochondrial dysfunction, or due to a general response to mitochondrial stress, such as unfolded protein accumulation or amino acid limitation (Méndez-Lucas et al., 2014). We addressed these possibilities by asking if impairing other mitochondrial electron transport chain components can also increase PEPCK levels to the same level as the \textit{sdha-1} mutations. As the \textit{sdha-1} mutations affect PEPCK expression in both sexes, we simplified our quantification by just analyzing hermaphrodites. Hatched L1 hermaphrodite worms were grown until
Figure 8. Influences on PCK-2 Expression by Perturbing Mitochondrial Functions

(A) Cartoon of the mitochondrial electron transport system, and site of action for mutations and electron transport chain toxins.

(B–G) (B) Yellow emission fluorescence intensity of PCK-2::YFP in L4 and day 1 (D1) through day 3 (D3) hermaphrodites grown in the absence (−) or presence (+) of rotenone (B), azide (C), oligomycin (D), and malonate (G) or containing...
adulthood in the presence of the mitochondrial poison rotenone (blocks electron transfer between complex I and ubiquinone), NaN₃, or oligomycin (blocks complex V ATP synthase) (Figure 8A) at concentrations that slowed development and caused 5% to 10% lethality. Exposure to rotenone (Figure 8B), NaN₃ (Figure 8C), and oligomycin (Figure 8D) had modest variable effects on \textit{pck-2} expression over the first 3 days of adulthood.

Unlike the mitochondrial poisons used to disrupt the electron transport chain, the \textit{sdha-1} mutation also affects the TCA cycle by reducing the formation of fumarate from succinate. To specifically target the electron transport function of complex II, we tested if a loss-of-function point mutation in subunit C of complex II, mev-1\textit{(kn1)}, can also increase PCK-2::YFP expression. Subunit C participates with subunit D of complex II to reduce ubiquinone. Worms containing the mev-1\textit{(kn1)} point mutation have reduced mitochondrial respiration and are hypersensitive to the electron-accepting ROS generator paraquat. These defects indicate that the mev-1\textit{(kn1)} mutation disrupts electron flow from complex II and leaks electrons away from the electron transport chain (Ishii et al., 1990; Ishii et al., 1998; Yanase et al., 2002); however, similar to the electron transport chain poisons, the mev-1\textit{(kn1)} mutation (Figure 8E) does not increase \textit{pck-2} levels as high as the \textit{sdha-1(rg550)} allele (Figure 8F). This observation suggests that reduced succinate dehydrogenase’s catalytic activity in the TCA cycle might also contribute to increased PEPCK expression.

We use the competitor malonate to address whether interfering with the TCA cycle can also increase PCK-2::YFP levels. Malonate should reversibly compete with succinate for binding to the SDHA-1 and SDHA-2 active site on complex II; thus the competitor should interfere with the oxidation of succinate into fumarate (Krebs and Eggleston, 1940). When L3 worms were grown on UV-killed OP50, supplemented with 50 mM malonate, they expressed slightly more PCK-2::YFP than those fed with UV-killed OP50 (Figure 8G), but no substantial increase in \textit{pck-2} levels was observed (Figure 8F). The moderate malonate-induced increase in PCK-2::YFP expression could be due to insufficient competition between the exogenously added malonate with endogenous succinate levels. Nonetheless, taken with the results from the electron transport chain inhibitors, reducing succinate dehydrogenase’s activities to both the electron transport chain and TCA cycle contributes to increased PCK-2 levels.

As we showed that malonate can artificially increase PCK-2 in wild-type animals, we asked if the competitor can further increase PCK-2 levels in the \textit{sdha-1} mutant, by interfering with the SDHA-2 paralog. We casually observed that when L3 \textit{sdha-1(rg550)} mutants were grown on UV-killed OP50 supplemented with malonate, they developed 4–5 h slower than their control cohorts and many died by day 3 as either developmentally stalled L4 animals or as adults with internal hatched worms. Although malonate had a detrimental effect on succinate dehydrogenase activity in the \textit{sdha-1} mutant, PCK-2::YFP did not further increase on day 1 or on day 3; indeed, less PCK-2::YFP was measured on day 3, likely due to malonate toxicity on viability (Figure 8G). These observations indicate that the worm is limited by how much PEPCK can be expressed after insult to succinate dehydrogenase function.

**DISCUSSION**

In this work, we explore how N2 male \textit{C. elegans} copulation fitness is metabolically sustained during the first few days of adulthood. The copulation fitness of N2 males, raised at 20°C under abundant \textit{E. coli} food conditions, drops after 48 h of adulthood; by 96 h, most males are impotent (Guo et al., 2012; Hodgkin and Doniach, 1997). N2 males in laboratories are mainly maintained for use in occasional out-crossings. The amount of selection pressure for maintaining male-propagating lines likely differs from laboratory to laboratory, depending on idiosyncratic research demands. The abbreviated copulation window for males can be a consequence of decades-long relaxed laboratory conditions that researchers use to propagate the N2 strain (Hodgkin and Doniach, 1997). In the laboratory, worms are restrained to \textit{E. coli} lawns 3–4 cm in diameter. Under standard conditions, worms can develop from fertilize egg to reproducing adult in ~65 h (Byerly...
correlated with a decrease in ether-a-go-go K⁺ channel expression. Previous research demonstrated that transient starvation and aging can upregulate EGL-2 activity through an UNC-42/CAMKII- and DAF-16/FOXO-independent DAF-2/insulin receptor-like pathway (LeBoeuf et al., 2007, 2011; Reiner et al., 2006). The correlation between EGL-2 and PEPCK expression suggest that similar mechanisms might be adopted here as ether-a-go-go K⁺ channels regulate membrane thresholds as an adaptive response to age-related changes in the metabolism.

We find that PEPCK’s function is essential for ATP homeostasis, because pck-2 or pck-1; pck-2 mutations alter ATP contents and their response toward NaN₅. The higher-than-normal ATP level in the pck-1(0);
pck-2(0) double mutant suggests that the mutant does not suffer from overall ATP depletion, rather they are defective in utilizing metabolites or energy production at specific sites. We hypothesize that PEPCK facilitates redistribution among different tissues to accommodate such needs. This hypothesis is consistent with the benefit of glucose supplementation on increasing the double mutants’ copulation ability. Therefore, the possible requirement for PEPCK in day 1 and day 2 wild-type copulation behavior is to provide gluco-/glyceroneogenic substrates that ultimately sustain mitochondrial energy production for competitive fitness. PEPCK’s supportive role in maintaining mitochondrial function is also observed on day 3, because the pck-1(0); pck-2(0) mutant alters its reliance of ATP production from mitochondria.

The C. elegans epidermis is composed of several multinucleated tissues that are in contact with muscles and neurons. The epidermis secretes the worm’s external collagen-based cuticle that acts as the worm’s elastic exoskeleton and protects its internal cells from the outside environment. However, others have implicated that the epidermis is important in providing carbohydrates, amino acids, fatty acids, and growth signals to neighboring cells for promoting development through the different larval stages, for promoting recovery from starvation, and for extending lifespan (Dalton and Curran, 2018; Ewald et al., 2016; Fukuyama et al., 2015; Kaletsky et al., 2018; Kennedy et al., 2013; Saudenova and Wicky, 2018; Son et al., 2018). Our results suggest that at least between 24 and 48 h of adulthood, the epidermis plays a critical role in supplementing fuel to the neural musculature for the male to sustain reproductive behavior.

The intromission circuitry is composed of multiple interconnected sensory-motor neurons that induce fast and slow twitch-like responses in the copulatory spicule muscles. Upon contact with the vulva, the postcloacal sensory-motor neurons promote continuous high-frequency muscle contractions that produce repetitive spicule thrusts at the vulva. When the spicules partially penetrate the vulval slit, the SPC proprioceptive motor neurons induce sustained spicule muscle contraction, which forces the spicules through the vulva. The SPD and SPC sensory neurons, in conjunction with the SPC motor neurons, then regulate ejaculation (LeBoeuf et al., 2014). Despite the expression of pck-1 in neurons and pck-2 in muscles, we hypothesize that the male epidermis must provide additional metabolic investment for maintaining membrane thresholds used in triggering rapid on-and-off motor responses during extended stretches of copulation. Our data indicate that in pck-1 mutant males, epidermal PCK-2-mediated gluco-/glyceroneogenesis can provide fuel (either as glucose or the 1,1-glycosidic linkage disaccharide trehalose) to neurons and muscles for mating behavior. In contrast, cell-autonomous PCK-1-mediated processes in neurons or PCK-2-supplied PEPCK in the sex muscles cannot provide sufficient energy for maintaining behavioral fitness after 12–24 h of adulthood.

The rise in pck-1 and pck-2 expression, along with genes involved with glyoxylate, glycolytic, fatty acid oxidation, and fatty acid biosynthesis pathways, indicates a change in the 24- to 48-h well-fed adult male physiology (Guo and García, 2014). The decay in male copulation fitness can be delayed by raising males in antioxidants, suggesting that very early adult processes might already be stressed and metabolic alterations might be compensating for the decline (Guo and García, 2014). The EMS mutagenesis screen identified the mitochondrial succinate dehydrogenase subunit A as a potential molecule whose activity might be properly down-regulated or subtly be in decline in the early adult. Succinate dehydrogenase participates in both the TCA cycle and electron transport chain. Subunit A is covalently bound to FAD and couples the oxidation of succinate and the reduction of FAD. Electrons then move through Fe-S clusters in the B subunit, where they then reach the ubiquinone-binding site at the subunit C and D interface (Moosavi et al., 2019). Our observations that loss-of-function mutations in sdha-1 increase and sdha-1 overexpression decreases pck-2 expression suggest that for well-fed animals, the levels of anabolic processes might be inversely related to mitochondrial function. Others have observed that RNAi-mediated reduction of complex I NADH/ubiquinone oxidoreductase subunit V1 and complex II succinate dehydrogenase subunit B and D promoted pck-1 RNA expression (Zuryn et al., 2010). Similarly, we also noticed modest changes to pck-2 expression after incubation with mitochondrial electron transport chain energy poisons. However, as these effects are not as pronounced compared with the sdha-1 deficiency, we speculate that for wild-type animals, succinate dehydrogenase’s dual participation in the TCA cycle and electron transport chain play a more pivotal role in modulating anabolic and catabolic processes.

Animals containing the loss-of-function sdha-1 alleles are viable, but do have cell non-autonomous developmental and behavioral deficiencies. Viability of the sdha-1 mutant indicates that its sdha-2 paralog can provide function to the TCA cycle, albeit at reduced capacity. Similar to mutants that are defective in the
mitochondrial complex I and the ATP synthase, sdha-1 mutants developmentally stall at the L2/L3 stage (Rea et al., 2007; Tsang and Lemire, 2002; Tsang et al., 2001). Like other larval developmental genes (Fay and Han, 2000), developmental arrest could be due to depletion of maternal sdha-2. However, developmental arrest is temporary, and we speculate that the sdha-1 mutants can eventually express enough sdha-2 to progress the animals into fertile adulthood.

Our acute sdha-1 rescue experiments suggest that increased PEPCK expression is not due to enzyme accumulating during developmental stalling; however, we cannot rule out that the mechanism of increasing PEPCK expression via sdha-1 mutation is distinct from the increased PEPCK expression observed in aging wild-type males. Because of similarities between 48-h wild-type and sdha-1 mutant males, we favor the possibility that alteration in mitochondrial function is occurring in the former. Our previous study found that mRNAs that encode for multiple mitochondrial metabolic proteins, including the mev-1-encoded succinate dehydrogenase subunit C, are significantly less in 48-h-old wild-type males (supplementary file 1 in Guo and García, 2014). In contrast, the pyc-1-encoded pyruvate carboxylase (Liao and Freedman, 2001) and the bifunctional icl-1 encoded isocitrate lyase/malate synthase enzyme (Liu et al., 1995) are upregulated in 48-h wild-type males (Guo and García, 2014). Similarly, both genes are also increased in sdha-1 males. Pyruvate carboxylase catalyzes pyruvate into OAA, and isocitrate lyase participates in the glyoxylate pathway, which bypasses TCA cycle decarboxylation steps and promotes malate to OAA production. The coordinated increase of pck-1, pck-2, pyc-1, and icl-1 in wild-type and sdha-1 mutants suggests that anabolic processes are enhanced when mitochondrial processing of intermediates into the TCA cycle is altered.

Most studies concerning gluco-/glyceroneogenesis and the glyoxylate pathway in C. elegans have been in the context of environmental stress handling and postreproductive aging. The disaccharide trehalose is one of many possible end products of gluco-/glyceroneogenesis. Trehalose, internally synthesized or externally fed, has been shown to be a stress protectant against neurodegeneration, osmotic stress, temperature fluctuations, and dehydration, as well as being a mobile metabolizable sugar (Lee et al., 2018; Seo et al., 2018). The glyoxylate pathway genes and gluco-/glyceroneogenesis genes are highly expressed during early embryogenesis, larval starvation, dauer development, and dietary restriction (Castelein et al., 2008; Hibshman et al., 2017; Holt and Riddle, 2003; Liu et al., 1997). Common between these conditions is that the animals are deprived of external food and thus are metabolizing internal stores. In previous studies, we also showed that transient starvation between late L4 stage and 12 h of adulthood can reduce mutation-induced and aging-related neural muscular hyperexcitability (Gruninger et al., 2008; Guo et al., 2012; LeBoeuf et al., 2007). Transient starvation can also extend the sexual potency of a wild-type aging male. However, the mating extension comes at a fitness cost when the male is younger; a young well-fed male will successfully transfer sperm into a mate before an age-matched starved/refed male during a copulation competition (LeBoeuf et al., 2011). In this report, the 24- to 48-h males are not food-stressed, but the changes in the levels of their gluco-/glyceroneogenesis genes suggest that they are adapting some elements of a food-stressed metabolism to sustain competitive behavioral fitness. The activity profile of feeding behavior with respect to mating behavior is poorly studied in the C. elegans male, thus future studies can explore if the male modulates its feeding behavior to promote a caloric restriction-like metabolism. In addition, although our data suggest that these males require gluco-/glyceroneogenesis to maintain fuel for competitive copulation, we do not rule the possibility that trehalose production might provide catabolic-independent stress protective functions to sustain mating behavior.

Limitations of the Study
In this study, we report that 24- to 48-h adult male C. elegans requires the continual expression of neural and epidermal PEPCK to sustain competitive copulation behavior. Many of our interpretations hinge on the assumption that in the knock-in animals, fluorescence intensity of YFP-tagged PEPCK reflects similar PEPCK levels in non-tagged wild-type animals. We acknowledge that the YFP tag might interfere with PEPCK turnover and the increased fluorescent signal during aging might be due to accumulation of non-functional protein. Nonetheless, the continual accumulation of the fluorescently tagged YFP indicates that the older males are still synthesizing the enzyme. We do not interpret the increase in fluorescent signal as the males are participating in more gluco-/glyceroneogenesis than in any other life stage of the male.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100990.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.G., Y.W., X.G., and L.R.G.; Methodology, J.G., Y.W., X.G., K.R., and L.R.G.; Formal Analysis, J.G., Y.W., X.G., K.R., and L.R.G.; Investigation, J.G., Y.W., X.G., K.R., B.L., L.Z., K.E., and L.R.G.; Writing – Original Draft, L.R.G.; Writing – Review and Editing, J.G., Y.W., K.R., and B.L.; Visualization, J.G., Y.W., K.R., and L.R.G.; Supervision, L.R.G.; Funding Acquisition, L.R.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Succinate Dehydrogenase-Regulated Phosphoenolpyruvate Carboxykinase Sustains Copulation Fitness in Aging C. elegans Males

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### Supplemental Figures

**Figure S1.** Amino acid alignments of *C. elegans* PCK-1 and PCK-2, Related to Figure 1. The red boxes denote positions of amino acids that participate in oxaloacetate and GTP binding and phosphoenolpyruvate formation (Carlson and Holyoak, 2009; Sullivan and Holyoak, 2007).

|  | Sequence 1 | Sequence 2 | Additional Details |
|---|---|---|---|
| **PCK-1** | SLRN METDGFQVVTVETHKLHPIFKDFASLSFVKQRVAEKAELMNFAGIYICDGS | SLRQISEDFYYVNEWMRLGKAHPFPVQIAFKEAEMLMFSGRTFCIDGS | eukaryotic | 66 |
| | +F +V EVV | +L H+PI KDGP | 79 |
| **PCK-2** | SLRQISEDFYYVNEWMRLGKAHPFPVQIAFKEAEMLMFSGRTFCIDGS | SLRQISEDFYYVNEWMRLGKAHPFPVQIAFKEAEMLMFSGRTFCIDGS | 80 |
| | 127 | QKEYDIDVKLVERVIGLTPKAYNNLRCRTDPQVARVESTMXTDKVDYDSVCHTPDG | 126 |
| | 186 | V PIMGW++E ELDSFGCMAGR MYY+P+MMG+GGLSK G+LTDS YYVL | 186 |
| | 246 | V PIMG W++E ELDSFGCMAGR MYY+P+MMG+GGLSK G+LTDS YYVL | 199 |
| | 295 | CMRTMTRMGTVLEALGNDNFVICSHGVSGLRPVQKVQINVHWNCPENKVIAMRPKEREI | 246 |
| | 306 | V+MTR+V+ALG+DFVRCISVSGLRPVQKVQINVHWNCPENKVIAMRPKEREI | 259 |
| | 319 | SMRTMTRVNDVWDALGQNDFVRCISVSGLRPVQKVQINVHWNCPENLTAIRPREEI | 259 |
| **PCK-1** | W S F G S G G N S L G K C F A R I A C N I G R D E G W L A E H M L I M G V T N P E G E E K F I A A A F F R C | W S F G S G G N S L G K C F A R I A C N I G R D E G W L A E H M L I M G V T N P E G E E K F I A A A F F R C | 246 |
| | 256 | W S F G S G G N S L G K C F A R I A C N I G R D E G W L A E H M L I M G V T N P E G E E K F I A A A F F R C | 259 |
| **PCK-2** | G N T N L M L T P T P G W K V R V G D D I A W M K F G A D G R L Y A I N P E A G P G V A P T G S H K T N A M A | G N T N L M L P T P G W K V R V G D D I A W M K F G A D G R L Y A I N P E A G P G V A P T G S H K T N A M A | 246 |
| | 366 | G N T N L M L P T P G W K V R V G D D I A W M K F G A D G R L Y A I N P E A G P G V A P T G S H K T N A M A | 259 |
| **PCK-1** | E S C R A N T I F T N V A T A D E G Y F W E G L E K E L K E A K G Y T D E Q L K H L E I T N W I L G E R W H I G E G K | 426 |
| | 430 | A T F Q R I N S I F T N V A T A D E G Y F W E G L E K E L K E A K G Y T D E Q L K H L E I T N W I L G E R W H I G E G K | 430 |
| **PCK-2** | A H H P R S F T A P A K Q C P H D W E A P Q V G V I D A I V P G G R P E B G V L V F E S P S W H E G I L V G A | 486 |
| | 490 | A H H P R S F T A P A K Q C P H D W E A P Q V G V I D A I V P G G R P E B G V L V F E S P S W H E G I L V G A | 490 |
| **PCK-1** | L V K S T T T T A A E F T K G N M M A R P M M G Y N G Y K L E H W I K L G K A H P K I F H V N W F R | 546 |
| | 550 | L V K S T T T T A A E F T K G N M M A R P M M G Y N G Y K L E H W I K L G K A H P K I F H V N W F R | 550 |
| **PCK-2** | D S N K F L W P G F G D N I R V D L W I L W A G E E E I A I E A I G Y P K R G T I N L D G L P R I O W D N D L | 606 |
| | 609 | D S N K F L W P G F G D N I R V D L W I L W A G E E E I A I E A I G Y P K R G T I N L D G L P R I O W D N D L | 609 |
| **PCK-1** | T K D H K L W F P G F G D N I R V D L W I L W A G E E E I A I E A I G Y P K R G T I N L D G L P R I O W D N D L | 606 |
| | 651 | T K D H K L W F P G F G D N I R V D L W I L W A G E E E I A I E A I G Y P K R G T I N L D G L P R I O W D N D L | 651 |
| **PCK-2** | M S P A D Y W K Q Q A Q A R K F D Q V G E D L P + P R E D E K | 654 |
| | 654 | M S P A D Y W K Q Q A Q A R K F D Q V G E D L P + P R E D E K | 654 |
Figure S2. Amino acid alignments of *C. elegans* PCK-2 and rat PCK-1, Related to Figure 1. The red boxes denote positions of amino acids that participate in oxaloacetate and GTP binding and phosphoenolpyruvate formation.
Figure S3. Amino acid alignments of C. elegans PCK-2 and PCK-3, Related to Figure 1. The red boxes denote positions of amino acids that participate in oxaloacetate and GTP binding and phosphoenolpyruvate formation.
Figure S4. CRISPR/Cas9 engineered YFP-tagged PCK-2 and EGL-2, Related to Figure 1 and 2. (A) Cartoon recombination of *pck-2::YFP* C-terminus fusion into the endogenous genomic region of *pck-2* on chromosome I. Black bars depict exons of *pck-2*. Blue bars depict exons of the downstream gene *suro-1*. Grey bars depict introns. Cartoon introns and exons are drawn to relative scale. (B) Cartoon recombination of *egl-2::YFP* C-terminus fusion into the endogenous genomic region of *egl-2* on chromosome V. Black bars depict exons of *egl-2*. Grey bars depict introns. Cartoon introns and exons are drawn to relative scale. (C) Confocal images of 24 hour adult male tails that express EGL-2::YFP; in the images, anterior region of the male tail is to the left. (D) Average fluorescence intensity of a region of interest (ROI) encompassing the protractors on one side of a male. Fluorescence values are in arbitrary units (A.U). Bars and whiskers represent mean and standard deviation. Numbers of males quantified are listed at the bottom of the graph. Each dot represents on the ROI of a single male. P value was determined using the Mann-Whitney test.
Figure S5. Colocalization analysis of PCK-2::YFP and mitochondria, Related to Figure 1. (A-C) Fluorescent micrographs showing cells expressing CFP-tagged mitochondrial protein cyclophilin A (CYN-1::CFP) and PCK-2::YFP of a wild type animal. (A-B) Arrows indicate examples of mitochondria where high CYN-1::CFP is seen but PCK-2::YFP is low. White boxes at bottom-right show magnified regions surrounding the arrows. (D-F) Fluorescent micrographs showing cells expressing two fluorescent-tagged mitochondrial markers, mitoCFP and PDHA-1::YFP (pyruvate dehydrogenase E1 alpha 1 subunit). (C and F) White polygons indicates the regions of interest (ROIs) where both markers are expressed in the cells. (G-H) Scatter-plot of the fluorescent intensities of pixels within the ROIs in (C and F). Pearson’s correlation coefficient (PCC) and $R^2$ is calculated as Dunn et al., 2011. Red and green ovals are hypothetical clusters of the pixels. In (G) the pixels in the red or the green oval show little correlation between the CFP and YFP, signifying no colocalization, while in (G) the pixels in the green oval show strong correlation between the CFP and YFP.
Figure S6. Copulation endurance assay, Related to Figure 3. Grouped column plots of the data shown in Figure 3, depicting the number of females that individual males impregnated over 84 hours. The numbers on the x-axis are the ten individual male designations. The patterned segments within the columns depict the number of females each male impregnated during a specific time interval. Mean and standard deviation represent the number of females that was impregnated by the population of ten males during the 84 hrs assay.
Figure S7. Tissue specific changes in PCK-2::YFP expression with age, Related to Figure 4. Yellow emission fluorescence intensity of muscular, epidermal and intestinal PCK-2::YFP. Fluorescence values are in arbitrary units (A.U). Each symbol represents a single male of the specified age. Bars and whiskers represent mean and standard deviation. P values were determined using the Student’s T test.
Figure S8. Developmental and physiological phenotypes of the \textit{sdha-1(rg550)} mutant, Related to Figure 5. (A) Fluorescent micrographs of mitochondria in the lateral epidermis of a wild type and an \textit{sdha-1} mutant. The \textit{sdha-1} mutant’s mitochondria are smaller and more fragmented. The worms contain the integrated transgene \textit{rgls38} that express both mitochondrial targeted CFP (from the \textit{eft-3} promoter) and also the mitochondrial pyruvate dehydrogenase subunit A fused to YFP (from its native promoter). (B) Mitochondria size of wild type and \textit{sdha-1} mutant using mitochondrial targeted CFP in (A). Images were processed to remove background and highlight the mitochondria. Each mitochrondron was then quantified for its size by counting the number of pixels it covers and converting to \( \mu \text{m}^2 \) (see Transparent Methods). Columns and whiskers show mean and standard deviation of the mitochondria sizes from 247 and 283 mitochondria of wild type and \textit{sdha-1} mutant, respectively. P-value was calculated using the Mann-Whitney test. (C) Oxygen consumption of wild type and \textit{sdha-1(rg550)} hermaphrodites. Oxygen consumption was approximated using scratch built soda lime respirometers (Zhang et al., 2015). The data shown are the mean and standard deviations of three independent trials. Each trial contained 300 day 1 hermaphrodites. Statistical significance was achieved after 4 hours. P value was determined using Student’s T test. (D) Developmental time course of wild type and \textit{sdha-1(rg550)} hermaphrodites. The developmental morphology of the gonad was used as an approximation for the larval stage of the animal (Kimble and Hirsch, 1979). Some of the developmentally retarded \textit{sdha-1(rg550)} larva with an L2 gonad had P5.p, P6.p and P7.p descendants that had division patterns of an L3 animal. These animals were likely stalled at the L3 stage, but their gonad did not develop past the L2 stage. However by 55 hours, stalled larva progressed to L4 or adult stages. Numbers of hermaphrodites examined at each time point are listed at the bottom of the bars. (E) Number of body bends that 20 wild type and 20 \textit{sdha-1(rg550)} 24 hrs adult virgin males produced while thrashing for 1 minute in M9 buffer. The number of animals quantified is 10 per strain. Each symbol represents the number of body bends for 1 male. Bars and whiskers represent mean and standard deviation. P value was determined using the Mann-Whitney test. (F) DIC image of a 24 hrs adult wild type male expressing YFP from the \textit{aex-2} promoter. (F') Fluorescence image of the wild type male depicted in (F); the remodeled adult anal depressor expresses YFP. The sphincter and intestinal muscles also expresses YFP. (G) DIC image of a 24 hrs adult \textit{sdha-1(rg550)} male expressing YFP from the \textit{aex-2} promoter. (G') Fluorescence image of the \textit{sdha-1(rg550)} male depicted in (G); the \textit{sdha-1(rg550)} male anal depressor muscle did not remodel and has the appearance of a larval male’s anal depressor. (H) Survival of adult males grown on paraquat. Live males were determined by whether they thrash in M9 buffer for 10 seconds. Statistical significance was determined by the Mann-Whitney test. Error bars represent the 95% confidence intervals. Total number of animals assayed per strain is noted at the bottom of the column; the % of surviving males is listed at the top of the column. (I) Number of eggs laid by a 24 hrs adult wild type and \textit{sdha-1(rg550)} hermaphrodite. The number of animals quantified is 15 per strain. Each symbol represents the number of eggs laid for 1 hermaphrodite. Bars and whiskers represent mean and standard deviation. P-value was calculated using the Mann-Whitney test.
Figure S9. Glycogen and neutral lipid contents, Related to Figure 5. (A) Lugo’s staining of the isthmus region of pharynx of 24 hrs adult wild type, \textit{pck-2(0)}, \textit{sdha-1(rg550)}, \textit{pck-2(0); sdha-1(rg550)} hermaphrodites and males. Red dotted polygon outlines the isthmus region that is quantified. (B) Quantification of glycogen staining of animals in (A). Numbers of animals quantified are indicated in the brackets beside the strain names. Bars and whiskers show mean and standard deviation. P values were calculated by One-way ANOVA, Bonferroni’s multiple comparison test. (C) Nile red staining of fed and starved 24 hour wild type, \textit{pck-2(0)}, \textit{sdha-1(rg550)} and \textit{pck-2(0); sdha-1(rg550)} males. The y-axis represents the average pixel intensity within a series of rectangular ROI drawn over the Nile Red-stained intestine. Bars and whiskers show mean and standard deviation. P values were determined using Student’s T test. Numbers of animals quantified is listed at the bottom (top for WT starved) of the graph.
**Transparent Methods**

**Strains**

All animals, including the wild type, contain the **him-5(e1490)** allele (Hodgkin et al., 1979) and were grown at 20°C on NGM agar plates containing **E. coli** OP50 (Brenner, 1974). Additional strains used in the study contained the following alleles: **pck-2(ok2586)** (generated by the *C. elegans* Gene Knockout Consortium), **pck-2(rg551), pck-2(rg800, rg551)** (this work) on linkage group (LG) I; **let-23(sy1)** (Aroian and Sternberg, 1991) on LG II; **pck-1(ok2098)** (generated by the *C. elegans* Gene Knockout Consortium), **pha-1(e2123)** (Schnabel and Schnabel, 1990), **mev-1(kn1)** (Ishii et al., 1990) on LG III; **fog-2(q71)** (Schedl and Kimble, 1988), and **egl-2(rg444)** (this work) on LG V.

**Potency assay, spicule protraction assay, copulation fitness assay and copulation endurance assay.**

The potency assay was conducted as previously described (Guo et al., 2012). In groups of 10 to 15 animals, L4 males (at the developmental stage where the tail spike has retracted) from non-crowded cultures were separated from their hermaphrodite siblings. Every day, males were transferred to fresh NGM-OP50 plates to minimize their exposure to accumulated deleterious male secretions (Shi et al., 2017). **pha-1(e2123)** hermaphrodites were used as mating partners (Schnabel and Schnabel, 1990). **pha-1** cultures were propagated at 15°C (permissive temperature), but 24 hrs before the assay, L4 **pha-1** hermaphrodites were isolated from their siblings and cultured at 20°C (non-permissive temperature). To conduct the potency assay, one male of different ages (newly adult molted, 24 hour (hr) or 48 hr adult) was paired with one 24 hr adult **pha-1** hermaphrodite on a NGM plate containing a 5 mm diameter lawn of OP50. Matings were done at 20°C. Five days later, the mating was scored successful if the plate contains cross-progeny. **pha-1** embryos and L1 larva die at 20°C, but cross-progeny, which are heterozygous for **pha-1**, are viable and reproductive.

The spicule protraction assay was conducted as previously described (Garcia and Sternberg, 2003). Late L4 males (at a developmental stage close to molt) were isolated from hermaphrodites and kept together in groups of 20 to 25 males. In some trials, a group of 20 males was also incubated with five late L4 wild-type or **let-23(sy1)** vulvaless hermaphrodites. ~24 to 26 hours (hrs) later, using a stereo dissecting microscope at 56X magnification, males were scored as have the protraction constitutive phenotype (**Prc**) if they had one or both copulatory spicules dangling out their cloaca.

The copulation fitness assay was conducted as previously described (LeBoeuf et al., 2011). Briefly, L4 males from non-crowded cultures were separated from their hermaphrodite siblings. A day 1 virgin **fog-2(q71)** mutant female was used as the males’ mating partner. **fog-2** mutant females do not make sperm and requires male impregnation to produce progeny (Schedl and Kimble, 1988). One 24 hr adult male containing a genetically-integrated fluorescent marker and one unmarked 24 hr adult male were paired with the virgin **fog-2** mutant female on a 5 mm diameter OP50 lawn. Multiple copulations were periodically monitored every one to two minutes until one of the males was observed to transfer sperm. For the males used in this study, the mating competition lasts between 5 to 20 minutes. After insemination, both males were removed from the female. The next day, the all-or-none fluorescence status of the L1 progeny population was used to determine the paternity of the competing males. If the female produced a mixture of fluorescent and non-fluorescent progeny, then that competition trial was not counted, since both males in the competition mated with the female during a period that the copulation was not being observed.

The copulation endurance assay measures how many different partners a single male can impregnate. The assay is modified from Hodgkin and Doniach 1997 (Hodgkin and Doniach, 1997). Plates containing overnight lawns of OP50 were exposed to 1 Joule of UV, using a Stratagene UV-Stratalinker 1800. The killed bacteria were washed off the plates, concentrated and then spotted on NGM plates without or with D-glucose (Sigma) at a final concentration of 56 mM (~1% glucose). Mid-L3 males were then separated from hermaphrodites and grown on plates +/- glucose. One newly molted adult male and ten newly molted virgin **fog-2** mutant females were placed on a 5 mm diameter lawn of UV- killed OP50 ( +/- glucose). Every 12 hrs for 96 hrs, females that contained eggs in their uterus were counted and then...
removed from the assay. The male and remaining un-mated females were then moved to a fresh 5 mm diameter UV-killed OP50 lawn and new age-matched virgin fog-2 mutant females (virgin females were always the same age as the male) were added to replace the mated ones; thus the bacterial lawn always contain ten females.

Data analyses.

Statistical tests were performed using Graph Pad Prism 5.04 for Windows (Graphpad Software, San Diego, CA USA). Confidence intervals for the competition assay were calculated using the formula: 
$C = p \pm Z_{0.02} \sqrt{\frac{p(1-p)}{n}}$, where $p$ is the fraction of males that won the competition, $n$ is the number of independent competitions, and $Z_{0.02}$ is set at the 95% level, which is 1.96.

Construction of plasmids and transgenic strains

**pck-1 promoter and expression plasmid and transgenic strains.**

To generate the promoter for pck-1, a 2888 base pair (bp) DNA region between the upstream gene spt-4 and the first predicted pck-1 exon was PCR-amplified using the primers Pck-1attb1: GGGGACAAAGTTTGTACAAAAAGCTCGGCTAGATTTTCAAGACATCTCGCAATAGG and Pck-1attb2: GGGGACCACTTTGTACAAAGAAGCTCGGCTAGATTTTCAAGACATCTCGCAATAGG. The PCR product was recombined into the gateway entry vector pDG15 (Reiner et al., 2006) using BP clonase (Invitrogen) to generate pXG47. Using LR clonase (Invitrogen), the pck-1 promoter from pXG47 was then recombined with the DsRed1-E5-containing Gateway destination plasmid pLR186 (LeBoeuf et al., 2011) to generate the plasmid pXG55. 50 ng/µl pXG55 and 150 ng/µl pUC18 were then micro-injected into N2. After a stable transgenic line was obtained, the extrachromosomal array was integrated into the genome using trimethylpsoralen and UV to promote DNA breakage and repair (Anderson, 1995). The extrachromosomal array integrated into chromosome I to generate the transgenic allele rgl29.

To construct pck-1-containing plasmids, the pck-1 spliced variant cDNA W05G11.6a.1 (http://www.wormbase.org/db/get?name=WBGene00021043;class=Gene) was purchased as a G-block from IDT technologies. Using In-Fusion HD-cloning (Takara Bio), the cDNA was fused at the 3’end to YFP by cloning into the gateway plasmid pGW322YFP, to generate the plasmid pLR385 (LeBoeuf et al., 2011). LR clonase was used to recombine the unc-103A promoter (using the gateway entry plasmid pLR36 (Reiner et al., 2006)) and the unc-17 promoter (using the Gateway entry plasmid pBL228 (LeBoeuf et al., 2014)) into pLR385 to generate the plasmids pLR386 and pLR387, respectively. The pck-1 promoter from pXG47 was recombined with pLR385 using LR clonase to generate pLR388. A 1882 bp genomic region from pck-1, which contains the first three introns, was PCR- amplified from N2 DNA using the primers: ForPck1seq2: AGCTCGTCAGGCTGTTGCTTACand Revpck1seq5: GGGGACAAGTTTGTACAAGAAAAAGCTCGGCTAGATTTTCAAGACATCTCGCAATAGG. This region was recombined into pLR388 using In-Fusion HD-cloning to generate pLR389. Injection mixes, containing 20 ng/µl of pLR386, or 10 ng/µl pLR387 or 20 ng/µl of pLR388 and up to 180 ng/µl of pUC18 filler DNA, were injected into n2(ok2586);him-1(e2123);pck-1(attb1:G;1(ok298);pck-2(ok2586);him-5(e1490) hermaphrodites. Stable transgenic lines that heritably transmit YFP fluorescence were kept to generate the extrachromosomal arrays: rgEx842[P_pck-1::YFP], rgEx847[P_pck-1::YFP] and rgEx848[P_pck-1::YFP]. To over-expresses pck-1::YFP, an injection mix containing 50 ng/µl of pLR389, 50 ng/µl of the pha-1(+) rescuing plasmid pBX1 (Granato et al., 1994) and 100 ng/µl of pUC18 was injected into pck-2(rg551);pha-1(e2123);him-5(e1490). A stable transgenic line was kept to generate the extrachromosomal array: rgEx877[P_pck-1::YFP(CE);pha-1(+)]. A control injection, just containing 50 ng/µl pBX1 and 150 ng/µl pUC18, was injected into pck-2(rg551);pha-1(e2123);him-5(e1490) to generate the array rgEx877[pha-1(+)].

**pck-2 CRISPR/Cas9 plasmids, expression plasmids and transgenic strains.**

To generate the pck-2 CRISPR/Cas9 guide RNA plasmid pXG67, the 19 bp guide RNA sequence to the pck-2 3’ untranslated region (UTR) (5’ TTTTTCGCGCATTATTTCA 3’), was added to the CRISPR/Cas9 guide RNA/ enzyme plasmid pDD162 (Dickinson et al., 2013) using PCR and the primers, pck-2sgRNAFOR: TTTTTCGCGCATTATTTCAAGACATCTCGCAATAGG. To generate the CRISPR/Cas9 guide RNA plasmid
pYW52, the 19 bp guide RNA sequence to the pck-2 third exon region (5’AACGGAGAGTGACTTCTGGG 3’), was added to the CRISPR/Cas9 guide RNA/ enzyme plasmid pDD162 using PCR and the primers, pck-23drexgRNA: AACGGAGAGTGACTTCTGGGTTTTAGAGCTGAAATAGCAAGT sgRNA(universal):CAAGACATCTCGCAATAGG. To generate the egl-2 CRISPR/Cas9 guide RNA plasmid pJG3, the 19 bp guide RNA sequence to the egl-2 3’ untranslated region (UTR) (5’ CGAAGCTTGAAGCCCGCGAG 3’) was added to the CRISPR/Cas9 guide RNA/ enzyme plasmid pDD162 using PCR and the primers FeGl2cas9: CGAAGCTTGAAGCCCGCGAG 3’TTTTAGAGCTGAAATAGCAAGT and sgRNA(universal):CAAGACATCTCGCAATAGG.

The pck-2 repair template used for CRISPR/Cas9-mediated recombination was generated by PCR-amplifying from genomic DNA a ~ 3.7 kilo base pair (kb) fragment containing the terminal end of the pck-2 coding region, UTR and part of the downstream gene suro-1. This fragment was amplified using the primers pck-25’armfor: CACCAAGAAAGATGACACTGTCACAC and pck-23’armrev: TTTAAGCTGAAAAACAGGCTGTCG. The fragment was then inserted into pCRXLTOPO to generate pXG66. We amplified YFP from a stock YFP-containing plasmid (pGW322YFP) using the primers pck-2a::gfpFOR: GAGAGTGGGAGACATTTGTGTAAGAAGAAGACT TTC and pck-2a::gfpREV: GGTATAATAACTATTTGTATAGTTCATCCATG. We also amplified C. briggsae (Cb) unc-119 flanked with loxP sites using the primers unc-119(cb)FOR: AATTTGATTATTATACCTTCGTATGATACATTATAGAAGTTATTTGTAGGCAATTTATCCAAG and unc-119(cb)REV: TCATCAACGTGACTTAACTCCGTTTAATGCTGCTACATAGAAGTTATCTTGCTAGCTAGCTAGCTAGCTAGCTAGCT. In-Fusion HD-cloning was used to translate YFP to the last codon of pck-2 in the plasmid pXG66. Flanked loxP-Cb unc-119 was then added to the pck-2 3’UTR to make the final plasmid pXG68. Plasmids were sequenced and repaired for mutations that arose during the construction.

Plasmids used for conducting tissue-specific rescue of pck-2(0) were constructed as follows. Using inverse PCR, we removed unc-119(Cb) from the plasmid pXG68 with flanking primers. The resulting plasmid, pLR366 still contains a loxP site in the 3’ UTR. ~3.7 kb pck-2 DNA encompassing the third exon, third intron and part of the fourth exon was PCR-amplified using the primers AMP:pck2: GTGTGCGGTGACCTGTTTGAAGGATGTTTGCTGAGCTCAG and reverseAMP:pck2: CTTTGGGTGTTGACAGGAGTCATATCTTTTCTTTGTAGG. In-Fusion HD-cloning was used to fuse this fragment of DNA with pck-2 sequences in the plasmid pLR366 to generate pLR369. A second loxP site was introduced into the third intron. The large second intron of pck-2 contains multiple repeat sequences making it unstable, therefore we directly purchased the first exon, first intron and second exon sequences of pck-2 as a G-block from IDT technologies and using In-Fusion HD-cloning, fused the fragment into pLR369, to generate pLR373. The Invitrogen Gateway ATTR cassette B was cloned into the SalI site, immediately upstream of the pck-2’s first ATG to generate pLR374.

pLR374 is the pck-2::YFP expression vector that different tissue promoters were recombined with using Invitrogen LR cloning. For intestinal expression, the gateway entry plasmid pBL63 (LeBoeuf et al., 2007) was used to recombine in the gti-1 promoter (Teramoto et al., 2005) to generate pLR379. For body wall muscle, sex muscle, intestinal and anal depressor muscle expression, the gateway entry plasmid pLR36 (Reiner et al., 2006) was used to recombine in the unc-103A promoter (Reiner et al., 2006) to generate the plasmid pLR382. For sex muscle expression and seven pairs of head neurons, the gateway entry plasmid pBL458 (LeBoeuf and Garcia, 2017) was used to recombine the small unc-103E promoter (Reiner et al., 2006) to generate pLR380. For epidermal expression, the gateway entry plasmid pXG76 was used to recombine the dpy-7 promoter (Gilleard et al., 1997) to generate pLR381. For general worm-over-expression, the gateway entry plasmid pYW34 was used to recombine the egl-103 (also known as glp-3 and eel-1A) promoter (Newbury et al., 2007) to generate pLR375.

Injection mixes, containing 20 ng/µl of pLR379, pLR382, pLR380 or pLR381 and up to 180 ng/µl of pUC18 filler DNA, were injected into the germline of pck-1(ok2098); pck-2(ok2586); him-5(e1490) hermaphrodites. Stable transgenic lines that heritably transmit YFP fluorescence were kept to generate the extrachromosomal arrays: rgEx833[P_glt::pck-2::YFP], rgEx879[P_ung-103A::pck-2::YFP], rgEx834[P_ung-103E::pck-2::YFP] and rgEx837[P_glt::pck-2::YFP].
To generate mitochondrial targeted CFP vector, the F22B7.9 promoter was removed from the Andy Fire mitochondria-targeted CFP vector pPD135.41 (Addgene plasmid repository). T4 ligase cloning was used to add the Gateway cassette C.1 (Invitrogen) in front of the mitochondrial targeted protein to generate the Gateway entry vector pLR339. Using LR clonase, the eft-3 promoter from the plasmid pYW34 was recombined with pLR339 to generate the plasmid pJG34. The pdha-1 fragment in pJG34 was then recombined into the YFP-containing gateway destination vector pGW322YFP using LR clonase to generate pJG35. An injection mix containing 20 ng/µl of pJG35 and 20 ng/µl of pUC18 was injected into the germline of N2 hermaphrodites. After a stable transgenic line was obtained, the extrachromosomal array was integrated into chromosome V to generate rgl1s38[P:fut-3::mito-targeted CFP; Ppdha-1::pdha-1::YFP].

To generate cyclophylin A cyp-1 expression plasmids, the cyp-1 genomic sequence was PCR amplified from N2 DNA using the primers GW322Forcyc-1: TTGAGGGTACCGGTAGAAAAATGAAAAATTCTACTCGTGCTCTCA and RevCyn-1CFP: AAGGTCTCTTCCTTTAATCGGAGCGCCAGCA. Using In-Fusion HD-cloning, the 639 bp fragment was fused at the 3’end to CFP by cloning into the gateway plasmid pGW77CFP, creating the Gateway destination vector plasmid pSR20. LR clonase was used to introduce the heat shock promoter (hspos-16) from the plasmid pBL172 in front of cyp-1 of pSR20 to generate pSR20. An injection mix containing 20 ng/µl of pSR20 and 50 ng/µl of pBx1 and 100 ng/µl of pUC18 was injected into the germline of pha-1(e2123); him-5(e1490); lite-1(ce314) to generate the array rgeX773[P:hspos-16::cyp-1::CFP; pha-1(+)].

To generate pdha-1 expression plasmids, the pdha-1 genomic sequence was PCR-amplified from N2 DNA using the primers Sdha-1-cfP: GTACCGGTAGAAAAATGActCCCGAGCGCCAGCAAC and Sdha-1-cfpR: TTCTCTTTACTCATAGGAGCGGCACCTTTGCTGC. Using In-Fusion HD-cloning, the 2788 bp fragment was fused at the 3’end to CFP by cloning into the gateway plasmid pGW77CFP, creating the Gateway destination vector plasmid pXG110. LR clonase was used to introduce the heat shock promoter (hspos-16) from the plasmid pBL172 (Banerjee et al., 2015) in front of pdha-1 of pXG110 to generate pXG111. An injection mix containing 20 ng/µl of pXG111 and 50 ng/µl of pBx1 and 170 ng/µl of pUC18 was injected into the germline of pha-1(e2123); him-5(e1490); lite-1(ce314) to generate the array rgeX773[P:hspos-16::pdha-1(+):CFP; pha-1(+)]. The array was then crossed into pck-2(rg551):YFP; pha-1(e2123); him-5(e1490); sdha-1(rg550).

To generate the pdha-1 CRISPR/Cas9 guide RNA plasmid pXG128, the 19 bp guide RNA sequence to the 4th exon of pdha-1 (5’ TGGAGAAGGGAGGCTGTGT 3’), was added to the CRISPR/Cas9 guide RNA/ enzyme plasmid pD1662 using PCR and the primers, sdha1sgRNAFOR: ATGGAGAAGGGAGGCTGTGTGTTGAGACTGAATAGCAAG and sgRNA(universal)REV: CGAGATCTCCGAGCAGA. The CFP fusion to the C-terminus of SDHA-1 rescued the elevated PCK-2::YFP levels in pdha-1(rg550) animals, but did not rescue any other defects; we reasoned that the CFP likely interfered with Complex II function. Additional constructs were made to uncouple CFP from pdha-1 and also to add locP sites in front of pdha-1 and after CFP, to be used in future studies. Building on the plasmid pXG110, inverse PCR cloning and the primers UpForloxPsds1: GCATACTATACAGAAGTTACTCCCGTGAAGAAAAATGCTCGAGCGCCAGC and RevLoxPCFP: TATACGAAGTTACTCCTCTCCTCCTCCTAATTAGAAGACGCGCCGTC and
DG15 to generate the GACTGA. The hermaphrodites were microinjected with 50 ng/µl of the CRISPR/Cas9 plasmid pXG67 and 50 ng/µl the elements in its large second intron. The CRISPR/Cas9 from DNA sequences upstream of the first We used CRISPR/Cas9 integrated into chromosome II to generate 150 ng/µl of pUC18 into N2. After a stable transgenic line was obtained, the extrachromosomal array was recombined into pLR363 to generate the plasmids pJG47 and pLR376, respectively. 200 ng/µl injection mixes containing 20 ng/µl of pJG47 or 20 ng/µl of pLR376, and 50 ng/µl of pBX1 and 130 ng/µl of pUC18 were injected into the germline of phe-1(e2123); him-5(e1490); rgl5[+]. 20 ng/µl of pLR376 was injected into the germline of phe-1(e2123); him-5(e1490); sdha-1(rg550). sdha-1(rg550) is on LGX. In the possibility that the transgenic arrays might rescue the sdha-1(rg550) phenotype, rgl5[+] was used as a repulsive fluorescent marker to homozygous the sdha-1(rg550) allele.

A 4000 bp promoter region upstream of sdha-1 was PCR-amplified from N2 DNA using the primers ForATTBsdha-1Prom4kb:
GGGGACCAAGTTTGTACAAAAAGCAGGCTACAGTGGGTTTTTCCCTGAAAATCGATAAACGTGATATAC. Using BP clonase, the PCR fragment was then inserted into the Gateway entry vector pDG15 to generate the plasmid pJG43. Using primers that reduced the size of the promoter region, the 5000 bp promoter pLR377 was PCR-amplified from N2 DNA using the primers Phe-1Prom4kb:
GGGGACCAAGTTTGTACAAAAAGCAGGCTACAGTGGGTTTTTCCCTGAAAATCGATAAACGTGATATAC. Using BP clonase, the PCR fragment was then inserted into the Gateway entry vector pDG15 to generate the plasmid pJG43. Using primers that reduced the size of the promoter region, the 5000 bp promoter pLR377 was PCR-amplified from N2 DNA using the primers Phe-1Prom4kb:

**egl-2 CRISPR/Cas9 plasmids.**

The egl-2 repair template, used for CRISPR/Cas9-mediated recombination, was generated by PCR-amplifying from genomic DNA a 900 bp fragment containing the last two exons of egl-2 coding region and the 3’ UTR. This fragment was then inserted into the Gateway entry vector pDG15 to generate the plasmid pJG43. Using primers that reduced the size of the promoter region, the 5000 bp promoter pLR377 was PCR-amplified from N2 DNA using the primers Phe-1Prom4kb:

**Construction of Paex-2::YFP for visualizing the anal depressor.**

The plasmid containing the aex-2 promoter (pBL348) (LeBoeuf and Garcia, 2017) was recombined with pGW77YFP to generate pBL351. 20 ng/µl of pBL351 was inject with 30 ng/µl of pLR361[Pges-1:RFP] and 150 ng/µl of pUC18 into N2. After a stable transgenic line was obtained, the extrachromosomal array was integrated into chromosome II to generate rgl5[+].

**CRISPR/Cas9-mediated recombination of YFP into genomic pck-2 and egl-2**

We used CRISPR/Cas9 to explore the expression of PCK-2, since we could not get reliable expression from DNA sequences upstream of the first pck-2 ATG. Possibly, the pck-2 promoter has expression elements in its large second intron. The CRISPR/Cas9 plasmids and protocols established in Dickinson et al. 2013 were used in this report (Dickinson et al., 2013).

To generate the genomic pck-2::YFP knock-in, a hundred ~ 5 to 10 hour old adult unc-119(ed3) hermaphrodites were microinjected with 50 ng/µl of the CRISPR/Cas9 plasmid pXG67 and 50 ng/µl the pck-2::YFP-Cb unc-119 repair template pXG68. In the F2–F3 generation, non-paralyzed animals were
picked and analyzed by PCR and sequencing to verify homologous recombination between the repair template and the genomic pck-2 locus. Cb unc-119 was then excised from the animals by injecting germline expressing Cre recombinase from the plasmid pDD104 (Dickinson et al., 2013). The animals were then crossed with wild type (N2) to remove unc-119(ed3). The pck-2[rg551]:YFP knock-in allele is referred to as pck-2::YFP.

To generate the genomic egl-2::YFP knock-in, a hundred ~ 5 to 10 hour old adult N2 hermaphrodites were microinjected with 50 ng/µl of the CRISPR/Cas9 plasmid pJG3 and 50 ng/µl the egl-2::YFP repair template pJG2. In the F2 generation, single animals were separated to generate independent lines. Samples from each line were screened, using PCR, to determine if they had YFP integrated in their genome. The strain egl-2[rg444]:YFP was screened positive for YFP and was verified that YFP was physically linked with the native egl-2 loci on chromosome V. The K+ channel::YFP fusion was found to be expressed on the cell surface of neurons and muscles and the cilia endings of sensory neurons.

To generate hypodermal marker, the dpy-7 promoter from pXG76 was recombined upstream of DsRed in pGW322DsRed using LR clonase to generate pXG85. To distinguish individual muscle, injection mixes containing intestinal or hypodermal cell, 5 ng/µl muscle marker pLR132 (P_iex-11: DsRed) (Guo et al., 2012), 5 ng/µl hypodermal marker pXG85 and 190 ng/µl of pUC18 filler DNA, were injected into the germline of the pck-2::YFP strain.

**CRISPR/Cas9-mediated mutation of pck-2::YFP**

rgls29[P_pck-1::TIMER] is integrated on chromosome I near pck-2. To generate animals that contain rgls29[P_pck-1::TIMER] and a loss-of-function mutation in pck-2, we first constructed a rgls29[P_pck-1::TIMER] pck-2::YFP recombinant. We then used the CRISPR/Cas9 plasmid pYW52, which is directed to the third pck-2 exon, to generate an insertion/deletion mutation. We isolated pck-2[rg800, rg551] that disrupts pck-2::YFP expression; this allele is referred to as pck-2[lf]. CRISPR/Cas9 generated a stop mutation by changing the wild-type sequence GGA GAG TAG TTC TGG GAG to GGA GAG TA C TT. The CRISPR/Cas9 generated a stop mutation by changing the wild-type sequence GGA GAG TAG TTC TGG GAG to GGA GAG TA C TT. All animals containing pck-2::YFP[rgls29] were F3 generation, higher YFP fluorescent L4 hermaphrodites were picked using a dissecting scope. The sdha-1 region was sequenced and the CRISPR/Cas9 lesion changed the wild type sequence from GTC TGT GGA GTC ATC GCC to the mutant sequence GTC TGA A GTCT AGT CGG AGT CAT CGC C.

**Quantification of fluorescence**

Digital images of animals were obtained using a Hamamatsu ImagEm camera mounted to an Olympus BX51 microscope (Olympus Corporation, Tokyo Japan) or a Yokogawa CSU-X1 Spinning Disk Unit (Andor Technology, CT USA) mounted on an Olympus IX81 microscope. Animals were anesthetized using abamectin (Sigma-Aldrich) (Cully et al., 1994). The more commonly used anesthetic NaCN was not used, since we found that paralyzing levels of the compound caused PCK-2::YFP to highly aggregate in the intestine, epidermis and muscles; the aggregation was reversible if the animals were removed from NaCN. Acute paralytic levels of cyanide, rotenone and oligomycin also induced PCK-2::YFP aggregation; however, non-paralytic levels of these mitochondrial poisons caused much fewer aggregates to occur. PCK-1::YFP, expressed from transgenes, also aggregates under these conditions and when over-expressed. The aggregation, which confounded our data collection, might be related to the phenomenon where glycolytic enzymes accumulate at C. elegans synapses during energy stress (Jang et al., 2016). A 50 mg/ml DMSO stock of abamectin was diluted in M9 buffer to the working concentration of 250 µg/ml.
50 µl of the well-suspended colloidal abamectin solution was spotted on the surface of a 3 cm NGM plate containing OP50 bacteria. After the abamectin soaked into the agar, worms were then added to the abamectin-containing plate. Generally, 5 to 10 minutes elapsed before the worms became paralyzed. Independent animals were imaged at L1 through the 3rd day of adulthood. The adults were imaged using the 10X objective; L1 through L4 were imaged using a 20X objective.

Metamorph image software (version 7.8.0.0, Molecular Devices, Sunnyvale CA) was used to quantify average pixel intensity of a region of interest (ROI). To quantify P\textsubscript{pck-1}:\textit{TIMER}, a single line ROI was drawn along the whole ventral length of the animal. The ROI includes the ventral body wall muscle and ventral cord neurons. Average pixel intensity was determined for the line ROI. To quantify intestinal PCK-2::\textit{YFP} expression in \textit{pck-2(rg551); pha-1(e2123); him-5(e1490)} males over-expressing PCK-1::\textit{YFP}, a single line ROI was drawn along the animal’s intestine. To quantify wild type PCK-2::\textit{YFP} in different tissues, rectangular ROIs were drawn in accordance with the hypodermal and the muscle markers. Average pixel intensity was then determined for each ROI corresponding to PCK-2::\textit{YFP} level in the cell. To quantify PCK-2::\textit{YFP} expression in the whole animal, a series of rectangular ROIs were drawn that encompass the whole area of the worm. The ROI includes regions of the epidermis, pharynx, body wall muscles and intestine. Average pixel intensity was determined for each ROI and a single average of all ROIs was calculated for the whole worm. To quantify EGL-2::\textit{YFP}, during microscopy data acquisition, a stack of 15 images was taken. ROIs were drawn over the protractor muscles or the SPD, SPC and SPV neurons and the average intensity was quantified.

Subcellular colocalization analysis
MATLAB (version R2017a, The Mathworks, Natick MA) was used to quantify the colocalization between mitochondria and PCK-2::\textit{YFP}. Images containing cells expressing both mitochondrial CYN-1::\textit{CFP} and PCK-2::\textit{YFP} were selected from confocal image stacks. A polygon ROI was drawn over the cell to exclude other cells and background from interference. Then individual pixel intensity was quantified from the ROI for both CFP and \textit{YFP} fluorescence. MATLAB was then used to generate the scattered plot of the pixels’ intensities of the two channels, and calculate the Pearson’s correlation coefficient (PCC) and \(R^2\) (Dunn et al., 2011). As a positive control, wild type animals expressing two mitochondrial markers (mito\textit{CFP} and PDHA-1::\textit{YFP}) were used with the same procedures performed.

Quantification of mitochondria size
MATLAB (version R2017a, The Mathworks, Natick MA) was used to quantify mitochondria size from confocal images. Images containing mitochondrial tagged \textit{CFP} expressed in lateral epidermis were selected from confocal image stacks. To isolate the mitochondria signal from background noise, the pixels were filtered by cut off the intensity value with the local mean of 21 by 21 pixel region. Then, the grey-scale images were converted to black-white images with manually set thresholds so that the converted images were most consistent with the perceptions of the mitochondria signals. With an in-house script, connected white pixels reflecting mitochondria signals were quantified. Each cluster of connected white pixels represents individual mitochondrion, and the number of pixels were counted and converted to \(\mu m^2\) to represent the sizes of the mitochondria. Clusters with less than 10 pixels each were treated as random noise and excluded from quantification.

ATP content quantification
The ATP quantification method was adapted from previous methods (Palikaras and Tavernarakis, 2016). For each sample, 20 developmentally synchronized worms were picked using Tris buffer (10 mM Tris-Cl, pH=8.0). The bacteria on the worms were cleaned off by washing the worms on an unseeded NGM plate with the Tris buffer. The worms were then transferred to a 0.2 mL PCR tube with 20 µL of the Tris buffer and ~20 0.5 mm Zirconium Oxide beads (Next Advance). For NaN\textsubscript{3} treated samples, the Tris buffer was replaced with 20 µL of Tris buffer containing 10 mM NaN\textsubscript{3}. The worms were incubated in the NaN\textsubscript{3} containing Tris buffer for 10 minutes at 20°C before proceeding to the next step. The tube was then immersed and fast-frozen in the dry ice-ethanol bath for 5 minutes. After frozen, the tube was then heated to 95°C for 15 minutes in a PCR machine. To guarantee the release of ATP from all cells, tubes containing the Zirconium Oxide beads were put in a bullet blender (Next Advance) for 3 minutes at speed
setting level 8. The tubes were then spun down with a table top centrifuge and frozen at -80°C before the assay. On the day of the assay, the tubes were defrosted, and 180 µL Tris buffer was added into individual tubes to dilute the content by 10 fold. For ATP standards, 1 µM, 0.1 µM, or 0.01 µM ATP in the Tris buffer was used. The ATP content was determined using CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega) with a Synergy HT plate reader (BioTek Instrument) following the manufacturer’s instructions.

EMS mutagenesis

The sdha-1(rg550) allele, described in this study, was isolated from an ethyl methanesulfonate (EMS) screen for higher fluorescing PCK-2::YFP-containing worms. Worms were mutagenized following standard published methods (Anderson, 1995). ~5,000 hermaphrodites of the strain CG1367, which contain pck-2::YFP; him-5(e1490), were mutagenized with EMS. In the F2 generation, higher YFP-fluorescing L4 hermaphrodites were picked using a dissecting scope. The rg550 strain was outcrossed multiple times to the parental CG1367 strain to reduce background mutations. Whole genome sequencing was conducted by BGI Americas Corporation, in combination with further SNP mapping (Wicks et al., 2001), to locate the rg550 allele within the sdha-1 on chromosome X. The rg550 mutation changed the wild type sequence GTC TGT GTC G to the mutant sequence GTC TGT GTC GA to GGT to Gly.

Real-time PCR

The sequences for primers that anneal to pyc-1, icl-1, pck-1, and pck-2 were described previously (Castelein et al., 2008). Three biological replicates of 500 day 1 adult males were accumulated over a period of time. Total RNA was extracted with TRI Reagent (Sigma-Aldrich) as previously described (Correa et al., 2012). cDNA was then synthesized with SuperScript II (Invitrogen) according to the protocol using around 2 μg total RNA and a mixture of gene specific-primers targeting the 20 genes. The mixture of gene specific-primers contains 5 pmole of reverse primers for each of the 20 genes. The RT-qPCR reactions were performed using BIO-RAD CFX96 real-time system and SsoFast EvaGreen supermix. We picked 10 reference genes (act-1, ama-1, csq-1, tba-1, gpd-3, mdh-1, pat-10, mlc-3, mua-6 and unc-15) for the study. geNorm v3 (Vandesompele et al., 2002) was used to determine the most consistent reference gene from the 10 candidate reference genes. unc-15 was selected as the reference gene to normalize the expression of the metabolic genes.

Paraquat assay

Prepared NGM plates were allowed to soak with Paraquat CL tetrahydrate (Supelco) at a final concentration of 10 mM. Plates were then spotted with concentrated OP50 and allowed to dry overnight. L4 males were then placed on plates and allowed to grow for 24-48 hours. To assess survival, worms were suspended in M9 buffer for 10 seconds and monitored for thrashing. If worms trashed more than once then they were considered alive. If one or no thrashing events were observed worms were considered incapacitated or dead.

Nile Red staining and quantification

The following was adapted from (Pino et al., 2013). Briefly, 15-20 L4 males were isolated from hermaphrodites and incubated on NGM plates the night before staining. The next day, males were transferred using 150 µL PBS with 0.01% triton X-100 (Sigma-Aldrich) to a 1.5 mL Eppendorf tube, centrifuged and the supernatant was aspirated leaving the worm pellet. Animals were then fixed using 150 µL of 40% isopropanol and incubated at room temperature for 3 min. During this incubation, 1 mL of Nile Red (Invitrogen™ Molecular Probes™) stain was prepared using 6 µL Nile Red stock solution (0.5 mg/ml in acetone) per 1 mL of 40% isopropanol. Males were pelleted by centrifugation and the supernatant was aspirated. To each worm pellet, 150 µL of Nile red staining solution was added and males were stained in the dark for 2 hours. Males were then collected by centrifugation and supernatant was aspirated. Males were washed with 150 µL of PBS with 0.01% triton X-100 in the dark for 30
minutes. Males were centrifuged and carefully transferred to a glass slide with 2% noble agar pad before imaging.

Males were imaged on a Yokogawa CSU-X1 Spinning Disk Unit mounted on an Olympus IX81 microscope (514 nm laser) using a 10X objective. Metamorph imaging software was used to quantify the average intensity of intestinal neutral lipid stores. The ROI was drawn exclusively in the intestine using a series of 10 by 10 pixel rectangular ROI’s for males. Each rectangular ROI was used to calculate the average intensity in a segment of the intestine. Specific segments could then be averaged for each worm. Nile Red staining was occluded by hermaphrodite eggs therefore we treated hermaphrodites and males similarly by only calculating average intensity using the first five segmented ROI’s.

Oxygen consumption assay

Oxygen consumption was measured using lab-made respirometers (Zhang et al., 2015). A chunk of NGM agar, containing a 5 mm lawn of UV-killed OP50 and 300 day 1 wild type or sdha-1(rg550) hermaphrodites, was assembled into the respirometer. The respirometer contained soda lime (Sigma-Aldrich), which absorbs expelled CO₂ and thus decreases the pressure within the respirometer. The change in gas volume (decreased pressure) is measured by the rise of colored fluid in a glass capillary attached to the closed respirometer. The respirometers were kept in a constant temperature room (20°C). The assembled respirometer with the worms were allowed to adjust (balance) for 20 min before measurements were taken. The respirometers were not handled during the experiment; data were collected by taking digital photographs of the rising liquid in the capillary tube.

Measuring male body bends in M9 buffer

In groups of 10 animals, L4 males (either wild type or sdha-1(rg550)) from non-crowded cultures were separated from their hermaphrodite siblings. The next day, groups of 5 males were transferred to a 3-well glass Pyrex dish containing 1 ml of M9 salts buffer. Males were digitally recorded using an Olympus SZX16 stereomicroscope fitted with a Hamamatsu C4742-95 digital camera. Images were taken at a rate of 30 frames a second for 1 minute. On playback, the recordings were slowed down five to ten times to count the body bends, as the males thrashed in the M9 buffer.

Egg-Laying assays

To examine the effect of sdha-1 mutation on egg-laying behavior, L4 hermaphrodites were isolated 24 hours before the assay. For the assay, worms were individually transferred to new NGM plates seeded with OP50 and allowed to lay eggs for two hours. Number of eggs was then counted.

Glycogen staining

To stain glycogen, 1-day-old virgin males were stained in 100 μL of diluted (1:10) Lugo’s iodine solution (2% I₂ in 4% KI) in dark for 2 min (Frazier HN and MB., 2009). The worms were then transferred to microscope slides. 16-bit grey scale images of the isthmus region of pharynx were taken with Olympus BX51 compound microscope mounted with a Hamamatsu ImagEm camera and 520/30 nm light filter. The brown-red light of glycogen staining was absorbed by the light filter and darker in the image. ImageJ (Schneider CA et al., 2012) was used to quantify the mean grey level of the isthmus regions and the background. Relative absorbance of the glycogen staining was calculated using the formula: \[ A = \frac{TB - TI}{TB} \]
where Tb is the mean grey level of the background and Ti is the mean grey level of the isthmus region.

Growth of C. elegans in mitochondrial poisons

All reagents and plates were prepared freshly. Oligomycin (Sigma-Aldrich) and rotenone (Sigma-Aldrich) were dissolved in DMSO to make a stock concentration of 10 mg/ml and 100 mM, respectively. Sodium azide (EM Science) was dissolved in water to make a stock concentration of 100 mM. Stock solutions were diluted in M9 and added separately to NGM plates containing OP50 to make the final concentration 10 μg/ml of oligomycin, 10 μM of rotenone and 15 μM of sodium azide. For controls, the same volume of DMSO was diluted in M9 and then added to OP50-seeded NGM plates. Each concentration of
mitochondrial inhibitor was selected to induce slow developmental growth with ~10% lethality. 10 L4 pck-2::YFP were put onto each drug or control plate. In the next generation, ~40 L4 progeny were transferred to freshly prepared drug plates to be imaged. L4 worms were imaged on the same day, day 1 adult worms were imaged after 24 hrs, day 2 worms after 48 hrs and day 3 worms after 72 hrs. YFP fluorescent images were taken using a Hamamatsu ImageEm digital camera on an Olympus BX51 at 10X magnification.

For malonate treatment, plates were prepared similar to NGM plates, but without peptone. Additionally, OP50 was killed by UV exposure to prevent the bacteria from metabolizing malonate. Sodium malonate dibasic was dissolved in sterile water to make a stock concentration of 2 M. For the control, 4 ml of peptone-less agar media was poured into a 3.5 mm petri dish. To prepare the 50 mM malonate plates, 100 µl of 2M sodium malonate (Bean Town Chemicals) was mixed into of 4 ml molten peptone-less agar media and then poured into a 3.5 mm petri dish. After the agar solidified, UV-killed OP50 was added to the plates. Unlike the mitochondrial inhibitors, the malonate quickly lost its toxicity, thus ~30 L3 wildtype or sdha-1(rg550)I hermaphrodites were placed on each plate and after 24 and 72 hrs, YFP fluorescent images were taken.

To quantify PCK-2::YFP expression in the whole animal, a series of rectangular ROIs were drawn that encompass the whole area of the worm. The ROI includes regions of the epidermis, pharynx, body wall muscles and intestine. Average pixel intensity was determined for each ROI and a single average of all ROIs was calculated for the whole worm.

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