Sex differences in body composition, fat storage, and gene expression profile in *Caenorhabditis elegans* in response to dietary restriction

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The metabolic and health-promoting effects of dietary restriction (DR) have been extensively studied in several species. The response to DR with respect to sex is essentially unknown. To address this question, we used the model organism *Caenorhabditis elegans* to analyze body composition and gene expression in males and hermaphrodites in response to DR. Unexpectedly, DR increased the fat-to-fat-free mass ratio and enlarged lipid droplets in both sexes to a similar extent. These effects were linked to a downregulation of the lipase-like 5 (*lipl-5*) gene in both sexes at two developmental stages. By contrast, the reductions in body size, protein content, and total RNA content in response to DR were more pronounced in hermaphrodites than in males. Functional enrichment analysis of gene expression data showed a DR-induced downregulation of several embryogenesis-associated genes concomitant with an ongoing expression of sperm-associated genes in hermaphrodites. In conclusion, DR increases fat stores in both sexes of *C. elegans* in the form of large and possibly lipolysis-resistant lipid droplets and markedly alters the reproductive program in hermaphrodites but not in males.

dietary restriction; *Caenorhabditis elegans*; sex differences; fat metabolism; gene expression

DIETARY RESTRICTION (DR) IS a nutritional regime without signs of malnutrition. During DR, the intake of all macro- and micro-nutrients is reduced to ~20–50% compared with ad libitum feeding (96). The term caloric restriction is often used synonymously with DR. In this study, the term DR is used because a reduction in bacteria consumed is inevitably accompanied by a reduced intake of carbohydrate, protein, and/or fat. DR modulates a wide range of biological processes and particularly impacts aging and age-related disorders (reviewed in Ref. 96). It has been demonstrated, largely in rodent models, that DR affects lifespan (115), mortality (115), tumor progression (114), neurodegenerative disorders (1, 71), insulin sensitivity (10, 16), immune defense (99), physical activity (28), and reproduction (18). In particular, lifespan extension has been observed in a wide variety of species, such as Baker’s yeast Saccharomyces cerevisiae (51, 66), the nematode *Caenorhabditis elegans* (60), the fruit fly Drosophila melanogaster (22, 25), grasshoppers (42), spiders (6), hamsters (98), dogs (56), and even primates (27, 89).

While the life-prolonging effect of DR in *C. elegans* and the identification of genes and pathways involved have been in the spotlight in recent years (57), the effects of DR on metabolism and the mechanisms involved have not been studied extensively. Comparative genomic analyses and metabolic studies have demonstrated that the intermediary metabolism present among eukaryotes can also be found in *C. elegans* (see Worm-Base, KEGG, or Ref. 105). These reactions include the catabolism and anabolism of lipids, carbohydrates, and proteins in processes such as glycolysis, gluconeogenesis, β-oxidation or the citric acid cycle (17). *C. elegans* generally stores energy in the form of fat (mainly triglycerides) and carbohydrates (glycogen, trehalose, and glucose) (5, 17). The major fat storage compartments are intracellular lipid droplets (LD) (119), as is also observed in other species (39, 73). These droplets are found in intestinal and skin-like hypodermal cells (5, 43). The key metabolic pathways, especially with regard to lipid metabolism and homeostasis, are highly conserved in *C. elegans* (17, 53). Thus, this model is indeed suitable for analyzing metabolism. Currently, it is known that DR increases resistance to thermal and oxidative stress (47, 55, 68), elevates the activity of stress-defense enzymes (superoxide dismutase, catalase) (47, 48), reduces body size (102), and decreases ATP concentrations, but does not reduce the metabolic rate of *C. elegans* (48–50). Moreover, DR or starvation influences various behaviors such as locomotion (93), pharyngeal pumping (7), reproduction (13, 60), and olfaction (26).

Although the effects of DR have been extensively studied in several species, reports about sex differences in the response to DR are rare. In rats, males maintain a higher body weight under DR than females, whereas increased activity level, decreased size of the gonads, and improved cognitive performance are observed in females compared with males (72). Circulating lipids and energy-regulating hormones show similar responses to DR in both sexes (72). Additionally, a sex-specific DR effect on lifespan has been observed in *D. melanogaster* (69). It was demonstrated that female flies were more sensitive to DR and starvation than males, resulting in a greater lifespan extension. For *C. elegans*, a majority of studies analyzing the effects of DR used hermaphrodites. Some reports have shown that starvation or the administration of inedible food leads to changes in the mating behavior (40, 41, 63), physical activity, oxygen consumption, and body composition (40, 103) of males. However, it is essentially unknown how *C. elegans* males respond to DR. To gain insight into sex differences in response to DR in *C. elegans*, we compared the body size and composition, fat storage, and gene expression profile of males and hermaphrodites under ad libitum and DR conditions.

MATERIALS AND METHODS

Nematode cultivation and strains. *C. elegans* worms were maintained on nematode growth media (NGM) agar plates seeded with
The following mutants were used in this study: fog-2 (g71) V, him-8 (e1489) IV, him-5 (e1490) V and him-8 (e1489) IV; nls128 II [pkd-2::GFP; lin-15 (+)]. fog-2 hermaphrodites are functionally classed as females. All of the strains were obtained from the CGC (University of Minnesota), except for the him-8 (e1489) IV; nls128 II strain, which was generously provided by the Horvitz laboratory. All experiments were performed with synchronized eggs that were prepared from gravid worms by hypochlorite treatment.

**COPAS flow cytometric analysis.** Flow cytometry was used to sort a defined number of eggs to analyze worm characteristics and to separate males and hermaphrodites for worm extracts and RNA isolation. The instrument settings were previously described (59, 79). Individual worm attributes [time of flight (TOF), extinction (EXT), autofluorescence (green vs. yellow)] were automatically measured. Specific gating and sorting criteria were used, and the gain signals (extinction, green, yellow) were always set to 1.

**Separation of males by COPAS flow cytometry.** To separate males from hermaphrodites, we used the transgenic strain him-8 (e1489) IV; nls128 II as we have described elsewhere (79). Because the green fluorescent protein (GFP) signal is only expressed in male-specific neurons (9), males can be separated from hermaphrodites by a specific green fluorescence signal by flow cytometry. It was not possible to identify males before the young adult stage (~64–66 h) because the GFP signal is not expressed in earlier stages. Using this strain, we recovered males at a rate of nearly 100%.

**DR protocol.** The DR method was adapted from Klass et al. (60) and was recently developed in our laboratory (84). Worms were grown on NGM plates with (ad libitum feeding, control) or without bactopeptone (DR). Additionally, two bacterial dilutions [optical density (OD) 1.5 or 0.7] were used for DR-plates to create a dose dependency. Unless otherwise indicated, 600 eggs per plate were utilized, and worms were maintained on the same plates until they were harvested.

**BODIPY 493/503 fixative staining and fluorescence imaging.** BODIPY 493/503 fixative staining was performed as previously described (59). Briefly, worms were harvested, washed in M9, and incubated in paraformaldehyde (4%) for 15 min. After three cycles of freezing and thawing in liquid nitrogen, worms were washed with M9 and incubated in BODIPY 493/503 staining solution (1 μg/ml) for 1 h at room temperature. Worms were then washed with M9 and were used for microscopic analysis. BODIPY-stained worms were imaged with an inverted fluorescence microscope (Axio Observer D1; Zeiss, Jena, Germany) with an attached with a digital camera (AxioCam MRm camera, Zeiss). For comparisons, worms were photographed at a fixed exposure time. All illustrated pictures were improved according to their sharpness with an unsharp masking algorithm (Axio Vision Software, version 4.8, Zeiss). Body volume was determined from area and perimeter as previously described (8, 92) by bordering the worms in Axio Vision Software. Diameter of LD was quantified using the Axio Vision software.

**Worm extracts and biochemical assays.** Worm extracts were prepared as previously described (59, 79). Briefly, worms were collected by flow cytometry, resuspended in RLT buffer (RNeasy mini kit; Qiagen, Hilden, Germany), and homogenized with Precellys24 (1.4 mm ceramic beads, full speed, 15 s, 2 cycles) and QIAshredder columns (Qiagen). Total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. An optional DNA digestion was also performed (Qiagen). The RNA concentration and integrity were verified with a BioPhotometer (Eppendorf, Hamburg, Germany) and the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) using the RNA 6000 Nano Kit (Agilent Technologies). Each sample contained at least 10 μg of total RNA per μl.

**Gene expression analysis.** The microarray procedure and normalization of row data were carried out by imagenes/Source Bioscience (Berlin, Germany). Custom-designed Agilent gene expression microarrays, which were developed by Steffen Hennig (imagenes/Source Bioscience), were used. The array contained 61,643 oligonucleotides resulting in 26,843 genes. Quantile normalization was used on raw data using R-package (15). Three experiments were performed for each condition. To determine gene expression under DR, an average expression ratio of DR to ad libitum feeding was calculated for each gene. Gene ontology of DR-regulated genes was identified with WormBase (http://www.wormbase.org, WS229). The Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7, http://david.abcc.ncifcrf.gov/) was used for functional gene annotation to reveal overrepresented gene families. Enrichment scores and P values derived by the Benjamini multiple test correction were specified by DAVID. Gene expression data analyzed for the ad libitum condition have already been published (79).

**Statistical analysis.** The data are expressed as means ± standard deviation (SD). The data represent at least three independent experiments. For microscopic experiments, 20–50 worms were analyzed in each experiment. The statistical analysis was performed by Student's t-test. The variance homogeneity was always considered. The results were defined as significant at P < 0.05. All statistical analyses were performed with SPSS (version 11.0).

**RESULTS**

**DR-induced reductions in body size, protein content, and total RNA content are more pronounced in hermaphrodites than in males.** To compare the responses of males and hermaphrodites to DR, the him-8 (e1489) IV; nls128 II (abbreviated him-8 GFP) mutant strain was used. This strain expresses GFP under the control of the male-specific pkd-2 promoter (9), allowing an accurate separation of males and hermaphrodites by flow cytometry (79). Due to the him-8 background, this strain has a higher incidence of males compared with the N2 wild type. The morphology, body proportions, and body composition of him-8 GFP males and hermaphrodites are similar to those of N2 animals (79).

It has been shown that the body size of males is 30–50% smaller at adulthood compared with hermaphrodites (44, 79). To compare the effects of DR on body proportions between the sexes, TOF and EXT flow cytometry-based parameters were used to analyze a large number of worms. It has been shown that the TOF and EXT values are proxies for body length and body volume in *C. elegans* (59, 79). As shown in Fig. 1, both sexes have a reduced body length (TOF, Fig. 1A) and body volume (EXT, Fig. 1B) under DR at young adulthood (66 h), adulthood (76 h), and 1 day of adulthood (90 h). Interestingly, DR-induced reduction in body size was significantly more pronounced in him-8 GFP hermaphrodites (e.g., 76 h EXT: ~26.2%) than in males (e.g., 76 h EXT:
This observation was confirmed by body size analysis of microscopic images obtained from another male mutant (fog-2) subjected to two different DR conditions (Fig. 1C). Microscopic inspection of DR him-8 worms showed that both sexes are thinner than their ad libitum-fed counterparts with a somewhat higher effect in hermaphrodites (Fig. 1, D and E). Careful inspection of images revealed that DR him-8 hermaphrodites contained fewer eggs than ad libitum-fed him-8 hermaphrodites [ad libitum (n = 8) vs. DR (n = 10); 11.5 ± 2.7 (mean ± SD) vs. 6.7 ± 2.1].

Because body size may correlate with protein and total RNA content, these read-outs were compared between males and hermaphrodites in response to DR. As shown in Fig. 2A, the protein content per worm was reduced under DR in both sexes; however, this response was more pronounced in hermaphrodites (e.g., 90 h: -47.2%) than in males (e.g., 90 h: -36.5%, -7.9%).

Moreover, the total RNA content was reduced in hermaphrodites under DR at both developmental stages by factors of 1.9 (66 h) and 3.0 (76 h), respectively, whereas males exhibited no changes (Fig. 2, B and C). Together, measurements of body length, body volume, protein content, and RNA content under DR indicate that hermaphrodites exhibit a more pronounced response to DR than males.

DR enhances the fat-to-fat-free mass in both sexes to a similar extent. Next, the triglyceride content per worm and the triglyceride-to-protein ratio were determined in him-8 GFP males and hermaphrodites under ad libitum and DR conditions at young adulthood (66 h), adulthood (76 h), and 1 day of adulthood (90 h).
were statistically analyzed by Student’s t-test. Total RNA was isolated from young adult (66 h) and adult (76 h) him-8 GFP worms. The relative RNA contents, as a percentage of the total RNA content of ad libitum-fed worms, from hermaphrodites (B) and males (C) are presented. A significant difference between ad libitum and DR worms is indicated by asterisks (*P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test). The means ± SD from 3–5 experiments are presented. Absolute data for the ad libitum condition were previously published (79).

These body composition parameters represent the fat mass and the fat-to-fat-free mass ratio, respectively, in *C. elegans* (59). In both sexes, the triglyceride content per worm and the calculated triglyceride-to-protein ratio were increased under DR in all adult stages (Fig. 3, A and B; additional file 1). The extent of this effect was similar between the sexes.

**DR enlarges LD in both sexes.** Recently, it was discovered in our laboratory that DR enlarges LD in N2 hermaphrodites (84). Therefore, the effect of DR on lipid droplet size and distribution was compared between hermaphrodites and males. For this purpose, both sexes of him-8 mutant worms were cultivated under ad libitum and DR conditions until adulthood and were fixed and stained with the BODIPY 493/503 dye to visualize LD. As recently described, the accuracy of this staining method was confirmed by colocalization studies with other dyes, staining of lysosome-related organelles, TAG measurements, and CARS and Raman spectroscopy (59). Ad libitum-fed adult him-8 males and hermaphrodites had finely distributed LD of nearly the same size (Fig. 4, A and B). In contrast, DR males and hermaphrodites exhibited larger LD with a lower number of small droplets (Fig. 4, A and B). The enlarged LD were visible throughout the body in the intestinal and hypodermal regions of both sexes. Quantification of the proportion between medium (mLD, diameter 2–4 μm) and large-size LD (lLD, diameter >4 μm) revealed clear differences between ad libitum and DR fed him-8 males (*N* = 2, *n* = 5; ad libitum: 82.9 ± 6.0% mLD, 17.1 ± 6.0% lLD; DR0.7: 36.7 ± 19.0% mLD, 63.3 ± 19.0% lLD; DR0.7: 22.0 ± 17.6% mLD, 78.0 ± 17.6% lLD). To confirm this observation in other male mutants, him-5 and fog-2 mutants were also analyzed. As shown in Fig. 4 and additional file 2, him-5 and fog-2 worms also contained larger LD under DR in both sexes. Under a more intense DR condition (OD 0.7), the enlargement of LD seemed to be more enhanced in fog-2 males than in hermaphrodites (additional file 2). Overall, DR increases LD size in intestinal and hypodermal cells in both sexes. This effect seems to be more pronounced under harsh DR conditions, especially in males.

**DR reduces glucose and trehalose content in both sexes to a similar extent.** Previous studies from our laboratory revealed a 3.9- to 5.4-fold higher trehalose-to-glucose ratio in adult males compared with hermaphrodites under ad libitum feeding (79). Thus, the trehalose and glucose content of both sexes in response to DR was determined in him-8 GFP worms at young adulthood (66 h), adulthood (76 h), and 1 day of adulthood (90 h). As shown in Fig. 5, A and B, the glucose and trehalose content per worm were decreased under DR in both sexes to a similar extent (also see additional file 3). The resulting trehalose-to-glucose ratios under DR were similar to those found under ad libitum conditions.
Thus, DR reduces glucose and trehalose content in both sexes to a similar extent.

DR upregulates collagen genes and downregulates UDP-glucuronosyltransferase and cytochrome P450 genes in both sexes. To determine the impact of DR on gene expression in hermaphrodites and males, microarray-based gene expression profiling was performed. Isolated RNA from ad libitum-fed and DR (OD 1.5) him-8 GFP males and hermaphrodites at young adulthood (66 h) and adulthood (76 h) was used. To reduce the complexity of large data sets, genes regulated in both sexes (see Table 1 for cut-off criteria) and at both developmental stages were overlapped (Table 1, additional file 4). This overlap analysis resulted in 5 and 23 genes that were consistently up- and downregulated, respectively (Table 1). It should be noted that the metallothionein 2 (mtl-2) gene was identified as upregulated by 4.6- to 9.9-fold in both sexes at both stages (additional file 4). This gene functions in metal homeostasis and stress response (45) and is responsive to DR in a variety of murine tissues (100). Genes identified as consistently downregulated under DR are involved in ammonium transport, defense response, lipid metabolic processes, proteolysis inhibition, cell adhesion, and enzyme activity. Interestingly, the mRNA expression levels of lipase-like 5 (lipl-5) were 5.5- to 16.6-fold lower in both sexes at both adult stages (see additional file 4).

To identify DR-enriched gene families, functional annotation clustering analysis was performed using all of the genes whose expression was regulated by at least twofold (P < 0.05) under DR in both sexes and at both adult stages (Figs. 6A and 7A). This approach identified three gene families that were regulated under DR in both sexes simultaneously. Genes from the collagen family were enriched and showed an upregulation in response to DR in both males (Fig. 6B) and hermaphrodites (Fig. 6C) at young...
Regulated in hermaphrodite but not in male but not in female. Differential regulation between hermaphrodites and males may be related to detoxification and cuticle assembly.

**Table 1**. Numbers of DR-regulated genes in both sexes at both developmental stages

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Overlap Between Male and Hermaphrodite</th>
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<tr>
<td></td>
<td>Young Adult (66 h)</td>
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<tr>
<td>Downregulated in both sexes (ratio &lt; 0.5, P &lt; 0.05)</td>
<td>38</td>
</tr>
<tr>
<td>Upregulated in both sexes (ratio &gt; 2, P &lt; 0.05)</td>
<td>77</td>
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<tr>
<td>Differential regulation between both sexes (ratio &gt; 1.5 or &lt; 0.66, P &lt; 0.05)</td>
<td>8</td>
</tr>
<tr>
<td>Regulated in male but not in hermaphrodite (male: ratio &gt; 2 or &lt; 0.5, hermaphrodite: ratio between 0.66 and 1.5)</td>
<td>133</td>
</tr>
<tr>
<td>Regulated in hermaphrodite but not in male (hermaphrodite: ratio &gt; 2 or &lt; 0.5, male: ratio between 0.66 and 1.5)</td>
<td>399</td>
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DR, dietary restriction.

**Fig. 5**. Changes in trehalose and glucose content under DR (OD 1.5) in him-8 GFP males and hermaphrodites at different developmental stages. Changes in glucose content (A), trehalose content (B), and the resulting trehalose-to-glucose ratio (C) under DR in him-8 GFP males and hermaphrodites at young adulthood (66 h), adulthood (76 h), and 1 day of adulthood (90 h) were assessed. Relative changes under DR compared with ad libitum feeding were calculated. The absolute data for the ad libitum condition were previously published (79). The means ± SD from 3–5 experiments are presented. Differences in carbohydrate changes between sexes were statistically analyzed by Student’s t-test.

**Adulthood.** Collagens are ubiquitous structural proteins that are the major component of the nematode cuticle (83). Furthermore, genes belonging to the UDP-glucuronosyltransferase (ugt) and cytochrome P450 (cyp) families were downregulated under DR in both sexes at adulthood (Fig. 7, B and C). Members of these gene families are important components of the detoxification machinery of *C. elegans* (67). Overall, DR modulates genes in males and hermaphrodites that are linked to detoxification and cuticle assembly.

**DR regulates the C-type lectin, lipase (class 3), and saposin B genes in males but not in hermaphrodites.** Based on our overlap analysis of genes responsive to DR (Table 1), 11 genes [e.g., chitinase (cht-1), neuromedin U receptor (nmur-3), neuropeptide-like protein (nlp-4)] that were regulated under DR in males but not in hermaphrodites were identified (additional file 4). Moreover, the functional annotation clustering analysis revealed that several members of the C-type lectin gene family (clec) were affected by DR in males only (Figs. 6B and 7B). We found 15 and 17 clec genes upregulated under DR in males at young adulthood and adulthood, respectively. Additionally, 13 clec genes were downregulated in response to DR in adult males. Some members of the clec gene family play an essential role in defense-mediated reactions (94). Furthermore, members of the lipase (class 3) and saposin B gene families were downregulated in adult males but not in hermaphrodites. Lipases hydrolyze the ester linkages of triglycerides, performing an important step in fat catabolism. Saposins are small lysosomal proteins that remove lipid substrates from membranes and thus promote the activity of degradation enzymes (81). Taken together, DR induces a male-specific gene expression response, which indicates an altered immune defense and reduced fat catabolism.

**DR alters the expression of genes that represent several gene families, including the major sperm protein family in hermaphrodites but not in males.** The overlap analysis of gene expression data identified 73 genes whose expression was modulated under DR in hermaphrodites only (Table 1). Many of these genes (additional file 3) are part of the SKN-1-dependent zygotic transcript (sdz) and F-box protein gene families, which are known to be important for embryogenesis (11). The latter group of genes was also identified by our functional annotation clustering analysis. This approach revealed a hermaphrodite-specific response to DR at young adulthood and adulthood for 8 and 10 gene families, respectively (Figs. 6C and 7C). Young adult hermaphrodites showed an upregulation of genes belonging to the sterol-sensing domain and hedgehog gene family (Fig. 6C). Both gene families are required for normal cuticle assembly, growth, and the molting process (20, 37, 121). Nine protease inhibitor genes were also upregulated under DR in hermaphrodites but not in males at the young adult stage. A majority of these genes belong to the proteinase inhibitor I2 gene family, which inhibits proteases of the S1 family to prevent physiological unwanted proteolysis (62, 90).

In addition to Cyclin-like F-box, the T-box transcription factor and BTB/Poz fold protein domain gene families showed decreased expression under DR only in adult hermaphrodites (Fig. 7C). All three gene families are involved in embryonic development (11). Cyclin-like F-box proteins are associated with SKP1 in a protein complex that functions as an E3 ligase in the ubiquitin protein degradation pathway (85). Furthermore, it is known that BTB proteins merge the functional properties of SKP1 and F-box proteins (117). Members of the T-box family are transcription factors that are required for early cell-fate decisions (88). Furthermore, many protein ki-
nase and phosphatase-encoding genes belonging to four families were upregulated under DR in adult hermaphrodites but not in males (Fig. 7C). Genes that encode the major sperm protein (msp) family were also upregulated under DR exclusively in adult hermaphrodites. Msp genes function in sperm motility and influence oocyte maturation and sheath cell contraction (80). A detailed analysis of all msp genes revealed that these genes were either unchanged or downregulated in adult males (see Fig. 10). Thus, nearly all genes of the msp gene family seem to be upregulated under DR in adult hermaphrodites but not in males.

DR promotes an ongoing expression of sperm-associated genes from young adulthood to adulthood in hermaphrodites but not in males. Next, it was examined whether other genes associated with sperm function were also modulated in response to DR, especially in hermaphrodites. Genes belonging to the sperm-specific family class P (ssp), class Q (ssq), class S (sss), sperm-specific transcript (sst), defective spermatogenesis genes (spe), and other genes that encode a major sperm protein domain had significantly higher expression levels under DR in adult hermaphrodites, whereas adult males exhibited almost no changes (Fig. 8, additional file 6). At young adulthood, almost all sperm-associated genes were not regulated under DR in both sexes (Fig. 8, additional file 5). In hermaphrodites, the expression of msp genes dropped moderately (group 1) or considerably (group 2) between the young adult and adult stages under ad libitum feeding (Fig. 9). This decline was not present under DR, for which the levels remained nearly constant (group 2) or increased slightly (group 1) from the young adult to the adult stage. In males, the mRNA

Fig. 6. Functional annotation clustering of DR-regulated genes from him-8 GFP worms at young adulthood (66 h). A: the data show the number of significantly regulated genes ($P < 0.05$, at least 2-fold) under DR in both sexes at young adulthood, which were imported to the DAVID database (version 6.7, http://david.abcc.ncifcrf.gov/) for functional annotation analysis. DR-regulated genes in males (B) and hermaphrodites (C) that were overrepresented in their respective group (down- or upregulated) are clustered according to their biological significance (enrichment score). Only protein families, domains, and functional sites (InterPro term) from functional annotation analysis were extracted and represented. The number of genes in the gene family, percentage (involved genes/total genes), and corrected $P$ value (Benjamini correction) are also illustrated. Gene families in red were enriched in both sexes.
steady-state levels of the msp genes increased between the young adult and adult stages under ad libitum feeding and under DR conditions. The expression patterns of all other sperm-associated genes showed sex differences similar to those of the msp genes (Fig. 10). Thus, DR promotes an ongoing expression of sperm-associated genes in hermaphrodites but not in males between the young adult and adult stages.

DISCUSSION

Limited energy availability has lasting consequences for an organism that affect basal metabolism, physical activity, growth, and reproduction (111). In the present study, it was shown that DR during development from the egg to adulthood 1) enhances the fat-to-fat-free mass ratio, 2) enlarges lipid droplets, 3) reduces glucose and trehalose content, 4) upregulates collagen genes, and 5) downregulates UDP-glucuronosyltransferase and cytochrome P450 genes in both sexes of C. elegans. Measurements of body length, body volume, protein content, and RNA content indicate that hermaphrodites have a more pronounced response to DR than males. In hermaphrodites, DR leads to a downregulation of embryogenesis-associated genes and an upregulation of sperm-associated genes. In light of these findings, the sex-independent and sex-specific responses to DR in C. elegans will be discussed in the context of fat storage, lipid metabolism, developmental growth, and reproduction.

Influence of DR on fat storage and lipid metabolism in hermaphrodites and males. The results showed that both hermaphrodites and males respond to DR during development with a remarkable and similar adaptation with respect to fat metabolism and storage, whereby both sexes increased their fat content and fat-to-fat-free mass ratio. In line with this observation, it was found that inedible bacteria increase fat staining in C. elegans (40). By contrast, fat mobilization is induced in fasting worms, which results in a decreased fat content and a reduced number of LD (52, 77). Thus, fat metabolism seems to be inversely regulated in C. elegans during DR or fasting. Additionally, it is known that lipid storage is essential for metabolic adaptation to scarce food conditions in C. elegans (103). Therefore, worms may increase their fat stores under DR to survive a long-term food restriction. Moreover, gene expression analysis revealed that the lipase-like 5

Fig. 8. Differential expression of sperm-associated genes under DR (OD 1.5) in him-8 GFP males and hermaphrodites. Data from both sexes at young adulthood (66 h, left) and adulthood (76 h, right) are illustrated. Each row represents 1 gene with the corresponding average ratio (DR/ad libitum), which is indicated by a color. Orange (ratio between 1 and 2) and red (ratio > 2) represent an increased gene expression under DR, while light green (ratio between 0.5 and 1) and dark green (ratio < 0.5) represent a decreased gene expression under DR. Genes that were not significantly altered are shown in gray. See additional files 4 and 5 for full data showing genes and their expression values.
gene was dramatically reduced (5.5- to 16.6-fold) under DR in both sexes and at both adult stages, indicating that triglyceride degradation may be reduced. It should be noted that gene expression analysis detected no other genes involved in lipid metabolism that were consistently regulated under DR in both sexes and developmental stages. Thus, lipl-5 may have a specific role in mediating DR-induced increase in fat storage in both sexes of C. elegans.

An increase in the fat-to-fat-free mass ratio in response to DR indicated a reduced metabolic rate of the worms. In fact, this type of adaptation has also been reported in DR-treated mammals (12, 14), in the offspring of undernourished female rats during pregnancy (3, 106) and in growth-retarded human children during development (46, 74, 75). Furthermore, an increase in the fat-to-fat-free mass ratio in combination with a reduced metabolic rate was observed during recovery from DR (semistarvation) in growing rats (29, 30) and adult humans (31–33, 58). Therefore, we propose that this classical adaptive phenomenon in response to DR, also known as the catch-up fat phenomenon (34), was recapitulated in DR C. elegans hermaphrodites and males.

Another sex-independent response to DR is the downregulation of several UDP-glucuronosyltransferase and cytochrome P450 gene family members. This effect might reflect an ener-
gy-saving adaptation to DR because the enzymes encoded by these genes function in the energy-consuming reactions of the metabolism of xenobiotics (38, 76) and lipophilic compounds (70, 104). A similar response was found in rodents, in which protein energy malnutrition leads to a decrease in cytochrome P450 protein expression and enzyme activity (21, 24, 64, 120). Inverse regulation of these genes was found in dauer larvae and daf-2 mutants (76, 110). Together, DR-induced down-regulation of genes that are linked to detoxification and energy consumption in both sexes of C. elegans is a further indicator of a reduced metabolic rate.

As also observed in both sexes of C. elegans, increased triglyceride content under DR is associated with an enlargement of intestinal and hypodermal LD. This alteration of LD size was initially described only in hermaphrodites (84). Several studies in C. elegans have identified genetic pathways that seem to be essential for lipid homeostasis and LD size in C. elegans. Genes involved in peroxisomal β-oxidation (mao-1, dhs-28, and daf-22), intracellular fatty acid transport (acbp-1), and phospholipid synthesis (sams-l and pmt-1) have been shown to function in LD formation (35, 54, 65, 109, 119). None of these genes were detected in our gene expression analysis, indicating that other genes might be involved in DR-induced enlargement of LD. As the surface-to-volume ratio is reduced in larger LD, their degradation by lipases may be slowed down. Thus, the enlargement of LD and the reduced expression of the lip-5 gene under DR might be an adaptive phenomenon characteristic of both C. elegans sexes necessary for surviving long periods of reduced food availability. Supporting this hypothesis, enlarged LD in C. elegans were also observed during the dauer stage (82).

**Influence of DR on developmental growth and reproduction in hermaphrodites and males.** During development, considerable energy is needed for growth. When energy is limited, a developmental delay and/or reduced growth has been found across many species, including worms grown in chemically defined media (101) and rodents subjected to food restriction (87). This adaptation was also observed in this study, as DR during development led to a reduced body size in both hermaphrodites and males. In line with this finding, DR upregulates several collagen-encoding genes in both sexes, as well as hedgehog genes and sterol-sensing domain genes (20, 37, 121) in hermaphrodites. Because these genes are involved in molting, which occurs at the end of each larval stage (95), the reduced body size in response to DR might be caused by an altered molting process and may be more definite in hermaphrodites than in males.

With respect to sex differences, the body size-reducing effect of DR and the decreases in protein and total RNA content were more pronounced in hermaphrodites than in males. One reason for these sex differences could be that adult hermaphrodites are much larger in size than males. Thus, hermaphrodites need much more energy for growth and protein synthesis than males. From a physiological perspective, sex differences in response to DR might be associated with different reproductive roles. Hermaphrodites have to invest much more energy resources than males in producing progeny, as is also true for mammals (107, 108). Oocyte maturation, ovulation, and fertilization, as well as the synthesis of oocyte metabolites (e.g., yolk proteins), are very expensive energetically. In line with this observation, adult DR hermaphrodites have many fewer eggs than their age-matched, ad libitum-fed counterparts. This observation is linked to a downregulation of many embryogenesis-associated genes, including the cyclin-like F-box, T-box transcription factor, and BTB/Poz fold protein domain genes. It is known that DR reduces the fecundity of C. elegans (13, 60) and delays reproductive maturity in rats (78). Our phenomenon differs from the adult reproductive diapause described by Angelo and Van Gilst (2) in that more than two embryos were observed in our DR hermaphrodites.

Furthermore, an ongoing expression of sperm-associated genes was observed in DR hermaphrodites but not in males. Because males continuously produce sperm throughout adult life, it was not possible to observe differences between ad libitum-fed and DR males. It is known that msp and other sperm-associated genes are expressed solely in spermatocytes (4, 86, 112, 118), which are the last remaining cells that conduct protein biosynthesis in the process of spermatogenesis (61). Normally, spermatogenesis, simultaneously with spermiogenes, proceeds from 51 to 62–65 h after hatching at 20°C (113). Whether more spermatoocytes are present during the adult stage in DR hermaphrodites needs to be investigated further. In addition to sperm-associated genes, an increase in the expression of several genes that encode protein kinases and phosphatases was found in hermaphrodites but not in males. Data from Reinke et al. (91) showed that among sperm-enriched genes, genes that encode protein kinases and protein phosphatases were overrepresented. These authors postulated that these enzymes are necessary to modulate protein activity during spermiogenesis, which proceeds without additional protein biosynthesis. A recent report showed that the protein phosphatases GSP-3 and GSP-4 are required for sperm development and motility in C. elegans (116). These two genes were also more highly expressed in DR hermaphrodites (data not shown). Taking these results together, one can assume that spermatogenesis is prolonged in DR hermaphrodites, which may result in delayed embryogenesis as indicated by the downregulation of embryogenesis-associated genes. As reproduction requires considerable energy, DR hermaphrodites may delay their developmental progression to adulthood and suspend the onset of reproduction to save resources.

**Conclusions**

When food becomes limited during development, almost all organisms redistribute their energy resources between body maintenance, growth, and reproduction to ensure survival. This type of environmentally induced phenotypic plasticity is often accompanied by the establishment of an alternative “life history trait,” a term introduced by evolutionary biologists (97). In C. elegans, well-known alternative life-history traits under limited food conditions are the dauer stage, egg retention with internal hatching, and adult reproductive diapause (2, 23, 36). We surmise that DR during development may also lead to an alternative life-history trait characterized by a build-up of energy resources in the form of large and lipolysis-resistant LD in both sexes and a slowed reproductive program in hermaphrodites.

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AUTHOR CONTRIBUTIONS

Author contributions: C.M. performed experiments; C.M. analyzed data; C.M. and F.D. prepared figures; C.M. and F.D. drafted manuscript; F.D. conception and design of research; F.D. edited and revised manuscript; F.D. approved final version of manuscript.

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