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Involvement of Drosophila Uncoupling Protein 5 in Metabolism and Aging

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ABSTRACT

A novel uncoupling protein, UCP5, has recently been characterized as a functional mitochondrial uncoupler in Drosophila. Here we demonstrate that UCP5 knockout (*UCP5KO*) flies are highly sensitive to starvation stress, a phenotype that can be reversed by ectopic neuronal expression of UCP5. *UCP5KO* flies live longer than controls on low-calorie diets, have a decreased level of fertility, and gain less weight than controls on high-calorie diets. However, isolated mitochondria from *UCP5KO* flies display the same respiration patterns as controls. Furthermore, total ATP levels in both *UCP5KO* and control flies are comparable. *UCP5KO* flies have a lower body composition of sugars, and during starvation stress their triglyceride reserves are depleted more rapidly than controls. Taken together, these data indicate that UCP5 is important to maintain metabolic homeostasis in the fly. We hypothesize that UCP5 influences hormonal control of metabolism.

MITOCHONDRIAL uncoupling proteins (UCPs) affect oxidative phosphorylation by reducing the amount of ATP that can be generated from oxidative metabolism (Ricquier 2005). The existence of an evolutionarily conserved mechanism of energy loss through UCP activities in all four eukaryotic kingdoms suggests beneficial functional roles for these proteins in the regulation of energy metabolism (Ricquier and Bouillaud 2000). UCP studies using mammalian systems have been proven to be difficult and have yielded inconclusive results, mostly due to the complex physiological regulation of higher organisms. In this study we used *Drosophila melanogaster* to examine the effect of altering the activity of one of these proteins, UCP5, on the rate of aging, resistance to starvation, and other parameters that reflect energy production and use. Our data showed that eliminating UCP5 function has a great impact on fly metabolic homeostasis since these flies are highly sensitive to food deprivation, live longer than controls on low-calorie diets, have a decreased level of fertility, and gain less weight than controls on high-calorie diets.

The electron transport chain in the mitochondria uses the energy from high-energy electrons released during oxidative metabolism to establish a proton gradient across the inner mitochondrial membrane. In aerobic organisms, the vast majority of ATP is produced in the mitochondria via ATP synthase, which captures the energy of the protons as they diffuse across the membrane and uses it to synthesize ATP (Saraste 1999). UCPs generate a "proton leak" that allows the flow of protons across the mitochondrial membrane without the generation of ATP. Uncoupling due to UCPs is a ubiquitous process that occurs in all eukaryotic cells examined to date and can account for up to 20–30% of resting metabolic state (Brand 2000).

UCPs were originally discovered as heat dissipation catalyzers involved in the thermogenic capacity of brown adipose tissue (Nicholls and Locke 1984). However, the finding of homologs of the initial UCP (later renamed UCP1) has raised questions about the *in vivo* physiological roles of these proteins. The mammalian UCP family includes UCP1, UCP2, UCP3, UCP4, and UCP5/brain mitochondrial carrier protein 1 (BMCP1). While UCP1 expression is restricted to brown adipose tissue, the expression of the other homologs has been shown to be specific to other tissues and their function is not restricted to thermogenesis (Krauss *et al.* 2005). UCP overexpression and loss-of-function studies have demonstrated the importance of these proteins in metabolism. UCP2-deficient mice were shown to have higher islet ATP levels, which in turn increased glucose-stimulated insulin secretion in pancreatic β-cells (Zhang *et al.* 2001). Overexpression of UCP3 led to hyperphagic mice that were lean and exhibited fat mass reduction and increased glucose clearance rates (Clapham *et al.* 2000). Additional studies have pointed to a role of uncoupling in maintaining an adequate redox balance, which in turn would protect against the formation of free radicals during oxidative metabolism (Arsenijevic *et al.* 2000; Echtay *et al.* 2000, 2002).

UCP5, which was the last homolog of the UCP family to be identified, shares ~35–40% amino acid identity

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with the other UCPs (Sanchis *et al.* 1998). UCP5 expression was found to be highly enriched in the mammalian nervous system (Sanchis *et al.* 1998; Yu *et al.* 2000) and in the brain expression has been determined to be almost exclusively neuronal (Kim-Han *et al.* 2001). Because nutritional status and temperature affect *ucp5* transcript levels in the brain and in the liver of mice, UCP5 has been postulated to be involved in mediating metabolic adaptations (Yu *et al.* 2000). On the other hand, on the basis of their observation that UCP5 overexpression in neuronal cell lines decreases the levels of reactive oxygen species in mitochondria, Kim-Han *et al.* (2001) hypothesized that UCP5 plays an important role in regulating mitochondrial oxidant production by controlling mitochondrial respiratory efficiency.

Sequence homology analysis revealed the existence of four putative UCPs in Drosophila—UCP4a, UCP4b, UCP4c, and UCP5 (Jezek 2002)—that share 60–70% homology with their mammalian counterparts. Drosophila UCP5 uncoupling activity has been functionally characterized in the heterologous yeast system, where UCP5 expression reduces mitochondrial membrane potential and increases respiration rate. UCP5 action is governed by the mechanisms known to regulate the UCPs characterized to date, including fatty acid stimulation and GDP inhibition (Fridell *et al.* 2004). *ucp5* is expressed throughout Drosophila development but at higher levels in adults, where it is expressed most abundantly in the head (Fridell *et al.* 2004).

Although UCPs have been shown to be involved in multiple pathways related to metabolism, the biological role of the predominantly brain-expressed UCP5 is not understood. It is possible that due to its brain expression, UCP5 plays a systemic role in fine tuning organismal energy homeostasis. Alternatively, UCP5 may be involved in specific metabolic tasks in the neuronal tissues where its expression is restricted. Using a UCP5 knockout (*UCP5KO*) line, we sought to investigate the *in vivo* involvement of UCP5 mitochondrial uncoupling in metabolism and aging.

On the basis of the assumption that mitochondria in *UCP5KO* flies would produce more ATP per molecule of oxygen consumed during respiration, we predicted that *UCP5KO* flies would be more resistant to food deprivation and would gain more weight than controls. Surprisingly, *UCP5KO* flies were highly sensitive to starvation stress and gained less weight than controls on high-calorie diets. Moreover, *UCP5KO* flies exhibited a decreased level of fertility and lived longer than controls on low-calorie diets. The results show that UCP5 may play an important role in Drosophila metabolic homeostasis.

MATERIALS AND METHODS

Fly strains and genetic crosses: The *P*-element insertion $Bmcp^{BG02446}$ line was generated by the Berkeley Drosophila *P*-element gene disruption project and obtained from the Bloomington Drosophila Stock Center. Congenic *UCP5KO* and control lines were created by crossing $Bmcp^{BG02446}$ females to w^{1118} males for 10 generations. After 10 backcrosses, heterozygous *P*-insertion females were crossed to heterozygous *P*-insertion males to produce homozygous congenic *P*-insertion *UCP5KO* flies (*O-10*) or homozygous congenic UCP5 control flies (*W-10*). *P*-element excision experiments were performed by crossing 3612 transposase-producing females (Bloomington stock center) to $Bmcp^{BG02446}$ males. Male progeny were selected with either the transposase-producing $P(\Delta_{2,3})$ allele or the *TM6* balancer. Heterozygous $Bmcp^{BG02446}$ males were crossed to double balancer *TM3*/TM6B females. Following this cross, homozygous excision lines were established from individual males that had lost the mini-*white* marker. Additionally, homozygous control lines were established from individual males resulting from the $Bmcp^{BG02446}/TM6 \times TM3/TM6B$ cross.

To create transgenic *ucp5* lines, the full-length *ucp5* cDNA fragment fused at the 3′-end to the FLAG epitope tag (FT) was subcloned from the pRS426 vector (Fridell *et al.* 2004) into the pUAST transformation vector. The pUAST–*UCP5::FT* construct was injected into the germline of $w; \Delta_{2,3}/TM3$ flies. Independent homozygous *UCP5::FT* transgenic lines were established and backcrossed to w^{1118} flies for 10 generations. To generate a *UCP5::FT* line that was homozygous *UCP5KO*, a *UCP5::FT* transgenic line containing the *ucp5* transgene on the second chromosome was crossed to *w;Sco/+;TM3/+* flies to obtain *w;UCP5::FT/Sco;TM3/+* flies. In parallel, *w;CyO/+; UCP5KO/TM6B* males were generated and crossed to *w;UCP5::FT/Sco;TM3/+* females to ultimately obtain homozygous *w;UCP5::FT/ UCP5::FT;UCP5KO/UCP5KO* flies. Generation of *Elav-GAL4* driver flies that were homozygous *UCP5KO* was accomplished by first producing *Elav-GAL4* flies that were *TM3/TM6B* double balanced. From the progeny of the cross of *UCP5KO* males with *Elav-GAL4;+;TM3/TM6B* females, we selected *Elav-GAL4;+;UCP5KO/TM3* males, which in turn were crossed to *Elav-GAL4;+;TM3/TM6B* females. From the resulting progeny, we generated homozygous *Elav-GAL4;+;UCP5KO/UCP5KO* flies.

Drosophila maintenance: All flies were reared on standard cornmeal agar medium (Ashburner 1989). Flies were passed to fresh vials every 4–6 days and maintained in humidified temperature-controlled environmental chambers at 25° throughout development. Adult flies were sorted and collected under CO_2 anesthesia and allowed to recover for at least 48 hr prior to assays.

Life-span assays: Groups of 25 newly eclosed males and 25 newly eclosed females were placed together in each vial with a total of 10–12 vials per assay. Flies were transferred to fresh vials containing the different percentage of yeast–sucrose (Y–S) diet under study every other day, and the number of dead flies was scored. Flies were maintained at 25° with 60% humidity on a 12 hr:12 hr light:dark cycle. Different yeast–sucrose calorie diets were prepared as described (Magwere *et al.* 2004). Alternate low- and high-calorie diets were implemented using standard cornmeal agar medium with or without yeast added, respectively (Ashburner 1989). All life-span studies were performed using 450–600 flies per assay.

Fertility analysis: Ten to 20 newly eclosed flies were maintained in single pairs in vials containing the different percentages of yeast–sucrose diet. Flies were transferred to fresh vials every day, and the number of eggs was counted. Flies were maintained under standard conditions (see above).

Longitudinal weight analysis: Three groups of 30–40 newly eclosed males and females were collected and weighed to determine their initial body weight. Every 3–4 days, each group of flies was anesthetized under CO_2 and weighed using a Mettler Toledo AB54-S precision scale. The average weight of

the flies remaining alive was then calculated. Flies were maintained under standard conditions (see above).

Starvation stress assay: Flies were segregated by sex and groups of ~25 newly eclosed flies were maintained on standard food for ~10 days prior to assays. For starvation stress assays, flies were transferred to vials containing 2% agar. The number of dead flies in each vial was scored every 6–12 hr. All starvation assays were performed using 100–200 flies per line. Flies were maintained under standard conditions (see above).

Reverse transcription–PCR: Total RNA was isolated using TRIzol reagent (Invitrogen, San Diego) and subsequent reverse transcription (RT)–PCR experiments were performed as described (Fridell *et al.* 2004). Forward and reverse primers for *ucp5* amplification were 5′-ATACGAGGGCGTTCGTGG-3′ and 5′-GTACTTCTTTAGTTGTTCGTA-3′. Primers for the amplification of *rp49* were the same as described previously (Radyuk *et al.* 2003).

Genomic PCR: Fly genomic DNA preparations were performed as described (Ashburner 1989). Approximately 200 ng of genomic DNA preparations was used for PCR experiments following the manufacturer's recommendations. All reagents were purchased from Invitrogen. Primers used were 7314-X (5′-GCGATGGAATCCCAATAAAACTGC-3′), 7314-Y (5′-TGACCTTGGATTTGGAGGCG-3′), and EP-d (5′-CAAT CATATCGCTGTCTCACTCA-3′). Primers 7314-1 and 7314-FN are the forward and reverse primers used in the RT–PCR experiment, respectively.

Mitochondrial respiration: Heads and thoraxes of 7- to 14-day-old flies, which had been maintained on standard food upon eclosion, were dissected on ice and kept chilled for <30 min before mitochondria isolation was performed as described (Fridell *et al.* 2005). Following measurement of total mitochondrial protein, respiration of freshly isolated mitochondria was determined in a Clark-type oxygen electrode at 25° (Hansatech, Norfolk, United Kingdom). Mitochondria were suspended at a protein concentration of 150 μg/ml in electrode buffer containing 20 mm α-glycero-3-phosphate as substrate as described (Miwa and Brand 2003). ADP (1 mm), oligomycin (1 μg/ml), and GDP (0.5 mm) were added sequentially to record state 3, state 4, and GDP-sensitive mitochondrial respiration, respectively. The substrate α-glycero-3-phosphate, ADP, and GDP were dissolved in water, and oligomycin was dissolved in ethanol before adding to reactions. All chemicals were purchased from Sigma (St. Louis).

ATP measurements: Steady-state ATP content was measured using the sensitive luciferin–luciferase system (Manfredi *et al.* 2002). The principle of this assay is based on the fact that luciferase requires ATP for light production using luciferin as substrate. Total cellular extracts from heads and thoraxes were prepared using the guanidine hydrochloride method to prevent ATP degradation (Schwarze *et al.* 1998). Homogenates were added to reaction buffer containing luciferin and assayed using a TD-20/20 luminometer (Turner Designs).

Sugars, glycogen, and triglyceride body composition determination: Fly homogenates were prepared as described (Clark and Keith 1988). Groups of 10 flies were anesthetized under CO_2 and weighed prior to homogenization in 1 ml of chilled homogenization buffer (0.01 m KH_2PO_4, 1 mm EDTA pH 7.4). Homogenates were spun using a refrigerated microcentrifuge for 2 min at 2000 rpm and the supernatant was recovered. Triplicates with 25 μl of homogenate for each sample were aliquoted into 96-well titer plates. Total glucose was measured using the 510-A glucose determination kit (Sigma). Total amount of trehalose was measured by determining total amount of glucose after addition of 0.2 units/ml of trehalase (Sigma) for 1 hr. Glycogen determination was performed by incubating homogenate samples with 0.1 units/ml of amyloglucosidase as described (Clark and Keith 1988) and measuring the total amount of glucose. Glycogen composition was calculated by subtracting the total glucose composition without amyloglucosidase digestion from the total glucose composition after amyloglucosidase digestion. Triglyceride measurements were performed using the triacylglycerol hydrolysis kit 335-UV (Sigma). All results were normalized with fly weight.

Statistical analysis: Statistical analyses for life spans were performed using a log-rank test (StatView). Differences for body composition assays were analyzed using a paired Student's *t*-test.

RESULTS

UCP5 loss-of-function mutants: To study the *in vivo* metabolic effects of UCP5, we obtained flies with a *P*-element insertion, $Bmcp^{BG02446}$, which were predicted to disrupt UCP5 expression. Sequencing of the *ucp5* locus revealed this line to possess a 10-kbp *P* element inserted in between the AT and the G of the translational start codon of the *ucp5* gene (Figure 1A). RT–PCR analysis showed that, as predicted, *ucp5* expression is impaired in the $Bmcp^{BG02446}$ line (Figure 1B). Moreover, Southern hybridization analysis indicated the presence of only one *P*-element insertion in the $Bmcp^{BG02446}$ line (data not shown).

UCP5 loss-of-function mutant flies are starvation sensitive: In the absence of UCP5, mitochondrial respiration should be more coupled, thus producing more ATP per molecule of oxygen consumed. We thus predicted that *UCP5KO* flies would be more resistant to starvation stress than control flies. To test this hypothesis, we compared the length of time that controls and *UCP5KO* flies lived under starvation conditions. Contrary to our expectations, *UCP5KO* flies were highly sensitive to food deprivation when compared to control w^{1118} flies (Figure 2, A and B). However, although $Bmcp^{BG02446}$ flies were originally created in the w^{1118} background, making the latter the closest genetic match, genetic modifiers could have appeared over time. Therefore, to rule out potential genetic background differences, the $Bmcp^{BG02446}$ line was backcrossed for 10 generations to the w^{1118} stock used in our laboratory. As a result, two new congenic lines were created, *O-10* and *W-10*, with virtually the same genetic background except that the *O-10* line retained the *P*-element insertion at the *ucp5* locus. Performance of starvation stress assays using these closely matched lines confirmed that *UCP5KO* flies are highly sensitive to starvation (a 31.4 and a 40.1% increase in median lethality for males and females, respectively) when compared to the genetically matched *W-10* control flies (Figure 2, C and D). To further determine whether the *P*-element insertion in the *ucp5* gene is the cause of the starvation sensitivity, a *P*-element excision study was performed. $Bmcp^{BG02446}$ flies were crossed to transposase-producing flies to generate lines in which the *P* element was precisely and imprecisely excised. From the same

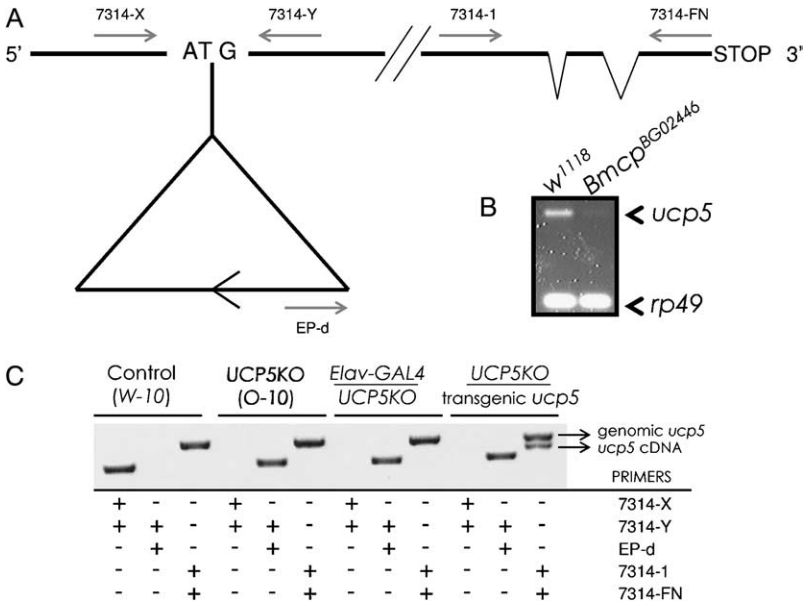

FIGURE 1.—*P*-element insertion in the *ucp5* gene leads to UCP5 loss of function. (A) Schematic of the *ucp5* locus illustrating the *P*-element insertion site and primers used in PCR experiments. (B) RT–PCR showing *ucp5* expression in control and *UCP5KO* male and female adult flies relative to the constitutively expressed gene *rp49*. (C) Genomic PCR confirming genotype of lines used for the ectopic expression of UCP5 in the fly nervous system.

crosses, control lines that had not inherited the transposase allele were established.

Precise and imprecise excisions were confirmed by sequencing genomic PCR. Each of the imprecise excision lines had either an ~30- to 50-bp insertion made up of a small duplication of *ucp5* sequence and *P*-element sequence or a partial *P*-element truncation between the AT and the G of the translational start codon. The sequence of each of the imprecise excision lines is predicted to prevent the expression of any UCP5 protein. Precise excision of the *P* element in the *ucp5* gene restored starvation resistance in the flies. However, imprecise excision of the *P* element produced flies that retained the starvation sensitivity as observed by the 42.9 and 36.2% difference in median lethality for males and females, respectively, with respect to the precise excision

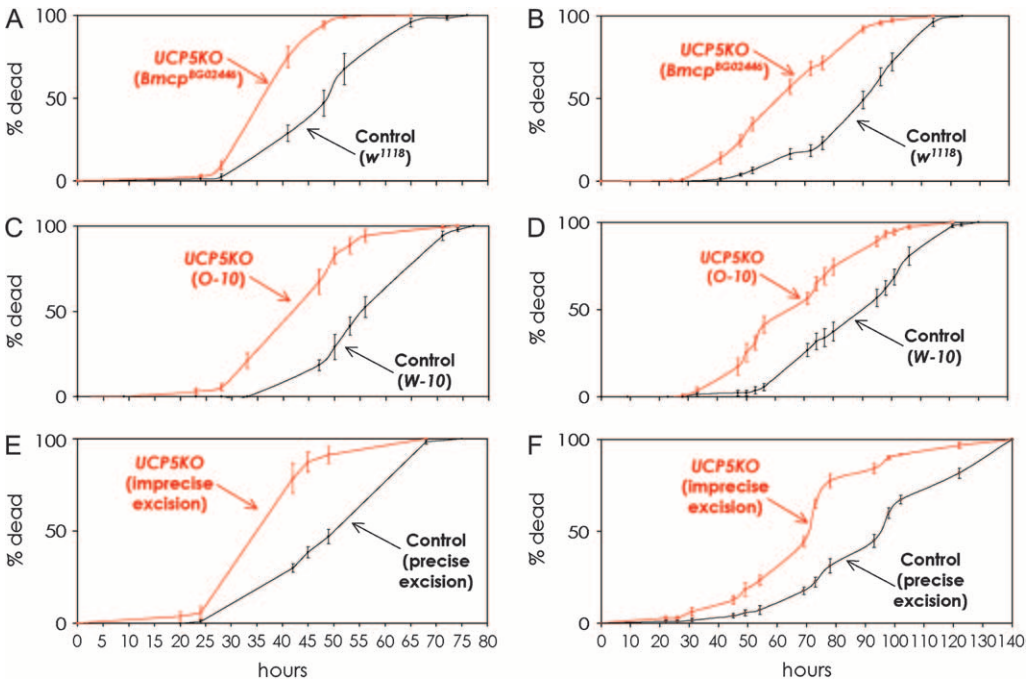

FIGURE 2.—Disruption of the *ucp5* locus results in starvation sensitivity. Comparison of starvation sensitivity for flies with loss of UCP5 activity and controls (A, C, and E are males; B, D, and F are females). (A and B) $Bmcp^{BG02446}$ (*UCP5KO*) and w^{1118} (control) flies. $Bmcp^{BG02446}$ (*UCP5KO*) flies are 35.8% (males) and 50.1% (females) more sensitive to starvation. (C and D) Congenic *O-10* (*UCP5KO*) and *W-10* (control) flies. Congenic *O-10 UCP5KO* flies are 31.4% (males) and 40.1% (females) more sensitive to starvation. (E and F) Imprecise (*UCP5KO*) and precise (control) *P*-element excision flies. *UCP5KO* imprecise excision flies are 42.8% (males) and 36.2% (females) more sensitive to starvation. A total of 100–200 flies were used to obtain each starvation curve. Each point is the average ±SEM of 5–10 separate vials of flies.

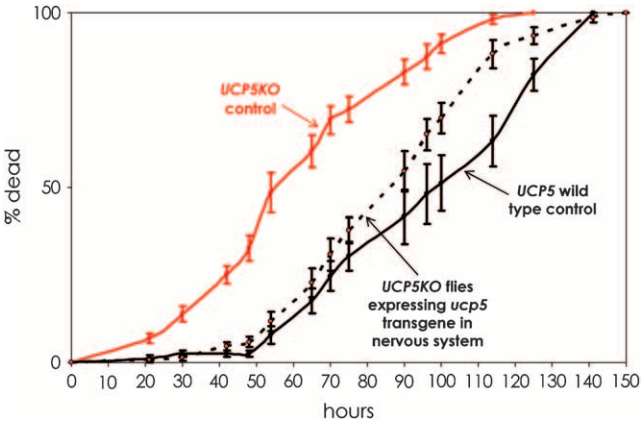

Figure 3.—Ectopic neuronal expression of UCP5 rescues starvation resistance. *UCP5KO* flies expressing a *ucp5* transgene in the nervous system driven by the *Elav-GAL4* driver restore wild-type starvation resistance. The *UCP5KO* control flies are deficient for UCP5 but have the *Elav-GAL4* driver. The UCP5 wild-type control flies also have the *Elav-GAL4* driver. Other controls, flies deficient for UCP5 but having the *ucp5* transgene, were starvation sensitive, while flies with wild-type UCP5 and the *ucp5* transgene, expressed or not expressed, have normal starvation sensitivity (data not shown). A total of 100–200 flies were used to obtain each starvation curve. Each point is the average ±SEM of 5–10 separate vials of flies. Data are shown for female flies. Male flies had a similar rescue of the starvation sensitivity with expression of UCP5 in the nervous system (data not shown).

controls (Figure 2, E and F). Similarly, control lines that had not been exposed to the transposase allele retained the *P* element and the starvation sensitivity as expected (data not shown). Taken together, these results demonstrate that loss of normal UCP5 function leads to flies that are highly sensitive to nutrient deprivation.

Ectopic neuronal expression of UCP5 rescues starvation resistance: *ucp5* transcript levels are higher in the head of adult flies than in the rest of the body (Fridell *et al.* 2004). Therefore, UCP5 might function in the nervous system and contribute to the regulation of metabolic homeostasis. To test this hypothesis, we generated *ucp5* transgenic flies to ectopically express UCP5 in the starvation-sensitive *UCP5KO* flies. An *Elav-GAL4* nervous system driver line, as well as a transgenic *ucp5* line, were crossed into the homozygous *UCP5KO* genetic background and starvation sensitivity was examined. The presence of the *P*-element insertion disrupting the *ucp5* gene was confirmed in the newly generated lines by genomic PCR (Figure 1C). Homozygous *UCP5KO* flies containing the *Elav-GAL4* driver element (Figure 3) or the *ucp5* transgene alone (data not shown), but not both, exhibited sensitivity to starvation as compared to their matched genetic controls. However, homozygous *UCP5KO* flies containing the *Elav-GAL4* driver element and the *ucp5* transgene restored flies to almost a wild-type starvation -resistant phenotype (Figure 3). In conjunction with the genetic studies above, these transgenic studies confirm that disruption of the *ucp5* gene is important in the normal response to starvation. Furthermore, the fact that restricting expression to the nervous system is sufficient to restore the starvation resistance to near-normal levels supports the hypothesis that UCP5 normally functions in the nervous system and may contribute to the regulation of metabolic homeostasis.

***UCP5KO* flies develop normally:** Lack of UCP5 expression throughout Drosophila development could cause metabolic alterations during development, which could lead to the starvation-sensitive phenotype observed in *UCP5KO* flies. However, no obvious morphological developmental abnormalities were observed in developing or mature *UCP5KO* flies. Furthermore, the cross of *UCP5KO* heterozygous females and males led to the predicted Mendelian progeny ratios of 1:2:1 for homozygous *UCP5KO*, heterozygous *UCP5KO*, and homozygous control flies, respectively. Therefore, the presence of the *ucp5* mutation does not reduce viability. Additionally, no retardation of developmental time was detected in *UCP5KO* flies as compared with controls when embryos were raised on two different types of diets, the richer diet, as expected, leading to a more rapid time to eclosion. After 11 days of development on the 5% yeast–sucrose low-calorie diet, 23.6 ± 2.9% *UCP5KO* and 20.2 ± 8.2% ($n = 40$ ± SEM) control flies had eclosed. Moreover, in the same time frame, the 15% yeast–sucrose diet yielded 46.3 ± 7.6% *UCP5KO* and 52.6 ± 10.8% ($n = 130$ ± SEM) control fully developed flies, respectively.

***UCP5KO* flies live longer than controls on low-calorie diets but not on high-calorie diets:** Aging and metabolism are tightly linked. An example illustrating the linkage between aging and metabolism is the recurrent phenotype of life-span extension across species upon limiting dietary calorie intake (Guarente and Picard 2005). Because UCP activity influences ATP production and metabolism, it is reasonable to expect that modulation of UCP activity will have an impact on longevity and consequently on aging. To test this prediction, life-span assays were performed using *UCP5KO* and control flies on diets ranging from severely calorie restricted to high-calorie content. *UCP5KO* flies had 10–30% shorter median life spans than controls on two different types of severely calorie-restricted diets: 1 and 1.5% Y–S. These results were expected since, as described above, *UCP5KO* flies are highly sensitive to food deprivation. Interestingly, upon addition of slightly more calories to their diet, 2% Y–S, *UCP5KO* flies had up to 18% longer median life spans than controls (Figure 4A and Table 1). Furthermore, on the still low-calorie 5% Y–S food, *UCP5KO* flies had a >30% increase in median life span as compared to controls. This phenotype suggests that *UCP5KO* flies are not merely sickly flies but instead may have an altered metabolic homeostasis. Gradual dietary calorie increases showed that *UCP5KO* flies live longer than controls on low-calorie diets (2 and 5% Y–S) but the

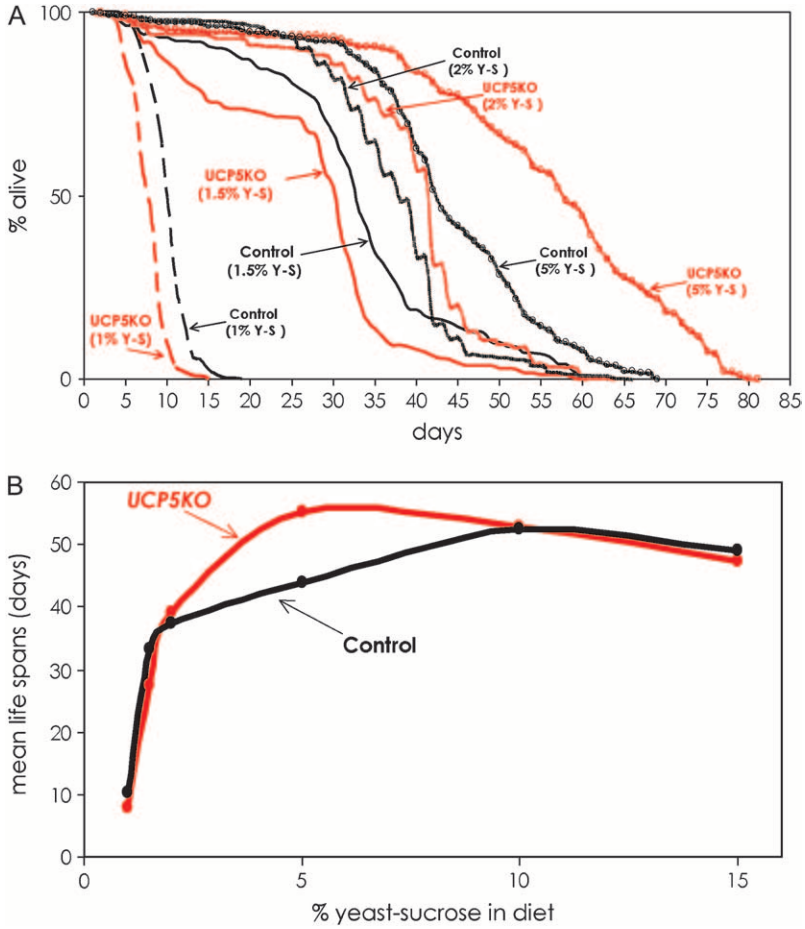

FIGURE 4.—*UCP5KO* flies live longer than controls on low-calorie diets, but not longer than controls on starvation or high-calorie diets. (A) Survivorship curves of male *UCP5KO* (*O-10*) and control (*W-10*) flies on starving (1% Y–S, and 1.5% Y–S), very low (2% Y–S), and low (5% Y–S) calorie diets. (B) Comparison of mean life spans of *UCP5KO* (*O-10*) and control (*W-10*) flies for each of six different calorie content diets. Each survivorship curve represents at least 250–300 male flies. Similar results are seen with females. Table 1 contains the mean, median, and maximal life spans for males and females.

same as controls on moderately higher (10% Y–S) or high (15% Y–S) calorie diets (Figure 4, B and C, and Table 1). Additional life spans were tested on alternative low- and high-calorie food diets, yielding very similar results (data not shown). Altogether, these results suggest that the highly starvation-sensitive *UCP5KO* flies have an altered metabolic homeostasis since, depending on the calorie intake, they can starve much faster than controls, live longer than controls on low-calorie content diets, or live for the same time as controls on high-calorie diets.

***UCP5KO* female flies are less fertile than controls:** It is well known that dietary calorie content, especially protein content, will affect metabolism by influencing vitellogenesis and ovoposition in Drosophila females (MAIR *et al.* 2004). The results from starvation and lifespan analyses described above suggest that *UCP5KO* flies have an altered metabolic homeostasis. Therefore, we decided to investigate the impact of the *ucp5* mutation on the fertility status of the fly. *UCP5KO* and control flies were reared on a very-low-calorie diet (2% Y–S), a low-calorie diet (5% Y–S), or high-calorie diet (15% Y–S) for 20 days and the number of eggs produced was counted daily. Both control and *UCP5KO* flies showed a direct correlation between calorie content and number of eggs laid. However, *UCP5KO* flies consistently laid fewer eggs than controls on every diet. When analyzing the cumulative number of eggs laid by *UCP5KO* flies as compared to controls, we observed that *UCP5KO* flies laid proportionally many fewer eggs than controls on low-calorie diets, 50.4% and 41.6% fewer for 2% Y–S (Figure 5A) and 5% Y–S (Figure 5B), respectively, than on the 15% Y–S high-calorie diet, where they laid only 11.4% fewer eggs than controls (Figure 5C).

***UCP5KO* flies gain as much weight as controls under low-calorie conditions, but they gain less weight on higher-calorie diets:** Once metabolic demands are met, energy excess is stored and body weight increases. Consistently, Drosophila fed on high-calorie diets experience increases in body weight over time. The amount of weight increase is directly correlated with the calorie content of the diet that the flies are fed (Figure 6A). With respect to resistance to starvation, life span, and fertility, *UCP5KO* flies displayed marked phenotypic differences compared to controls, depending on whether flies are exposed to low- or high-calorie conditions. Therefore, we sought to examine the weight gain rate of control and *UCP5KO* flies on different diets. On the 1% Y–S severely calorie restricted diet, both types of flies lost weight rapidly. After one week on this diet, *UCP5KO* flies continued losing more weight than controls up to the point where the assay was terminated because the flies

TABLE 1

Percentage of change in mean, median, and maximal life span of *UCP5KO* flies with respect to control flies on different types of caloric content diets

	Females		Males		Females		Males	
	UCP5KO	Control	*UCP5KO*	Control	*UCP5KO*	Control	*UCP5KO*	Control
	1% Y–S DIET				5%Y–S DIET			
Mean	10.5	11.4	8.0	10.2	52.9	38.5	55.2	43.7
Median	10.5	12.6	7.6	10.0	54.3	39.7	57.5	42.3
Maximal	15.0	16.0	10.0	12.6	70.0	53.1	74.4	58.0
% change mean	−8.9		−27.0		37.2		26.3	
% change median	−20.0		−31.6		36.8		35.9	
% change maximal	−6.7		−26.0		31.8		28.3	
Chi-square	44.59		148.94		199.03		159.70	
Probability	<0.0001		<0.0001		<0.0001		<0.0001	
N	319	302	310	294	315	302	302	301
	1.5% Y–S DIET				10%Y–S DIET			
Mean	34.0	37.3	27.3	33.1	47.8	46.3	53.0	52.5
Median	31.8	34.9	30.2	32.6	50.9	46.3	54.7	51.0
Maximal	58.0	59.0	36.6	49.0	68.0	65.1	74.0	70.9
% change mean	−10.0		−21.5		3.2		1.0	
% change median	−9.8		−8.0		9.9		7.3	
% change maximal	−1.7		−33.9		4.5		4.4	
Chi-square	11.42		42.33		8.36		0.04	
Probability	0.0007		<0.0001		0.0038		0.8469	
N	289	279	296	308	315	317	316	304
	2% Y–S DIET				15%Y–S DIET			
Mean	42.5	36.2	39.0	37.3	42.9	42.6	47.3	49.0
Median	41.9	35.5	41.2	37.7	43.9	43.1	49.0	51.4
Maximal	59.4	53.9	49.8	45.2	59.0	62.5	67.9	69.2
% change mean	17.4		4.8		0.8		−3.6	
% change median	18.0		9.3		1.9		−4.9	
% change maximal	10.2		10.2		−5.9		−1.9	
Chi-square	42.04		20.28		0.87		0.97	
Probability	<0.0001		<0.0001		0.3500		0.3245	
N	284	292	293	316	300	295	302	284

Percentage change is calculated as the percentage change between the *UCP5KO* (*O-10*) and the control (*W-10*) flies on each specific diet. Chi-square and probability (*P*-values) are calculated by log-rank test (StatView). Maximal life span is the maximum life span calculated as the median life span of flies remaining at 10% survivorship. *N*, number of flies in each life span.

presumably were dying from starvation (Figure 6B). On a 2% very-low-calorie diet, both *UCP5KO* and control flies maintained their baseline weight throughout the 3 weeks that the flies were monitored (Figure 6C). This trend began to change on the still low-calorie 5% Y–S diet, with controls gaining slightly more weight than *UCP5KO* flies (Figure 6D). Upon increasing the dietary calorie content, the weight gain rate differences between controls and *UCP5KO* flies gradually widened over time. Thus, fed moderately higher (10% Y–S) or high (15% Y–S) calorie diets, control flies exhibited up to a 16 and a 26% increase in body weight, respectively. The same types of diets increased *UCP5KO* body weight up to only 8 and 16%, respectively (Figure 6, E and F). In summary, *UCP5KO* flies gain as much weight as controls on low-calorie conditions, but with caloric increases they tend to gain less weight than controls. Taken together with the fact that *UCP5KO* and control flies weighed the same immediately after eclosion (data not shown), these data point to *ucp5* as an important Drosophila gene for regulating metabolic homeostasis in response to nutritional cues.

Isolated *UCP5KO* mitochondria have normal uncoupling levels: To investigate the physiology of the *UCP5KO* metabolic phenotype, mitochondria from both control and mutant *UCP5KO* flies were isolated to measure their physiological status. Mitochondria were isolated only from the head and the thorax of the animals to eliminate background effects produced by the eggs contained in the abdomen, as well as to avoid gut enzymes that could affect the integrity of the mitochondria. Interestingly, *UCP5KO* mitochondria showed the same oxygen consumption ratio, or state 3 respiration, as controls (data not shown). Using oligomycin, an inhibitor of ATP synthase, we determined the state 4 respiration of mitochondria, or the consumption ratio

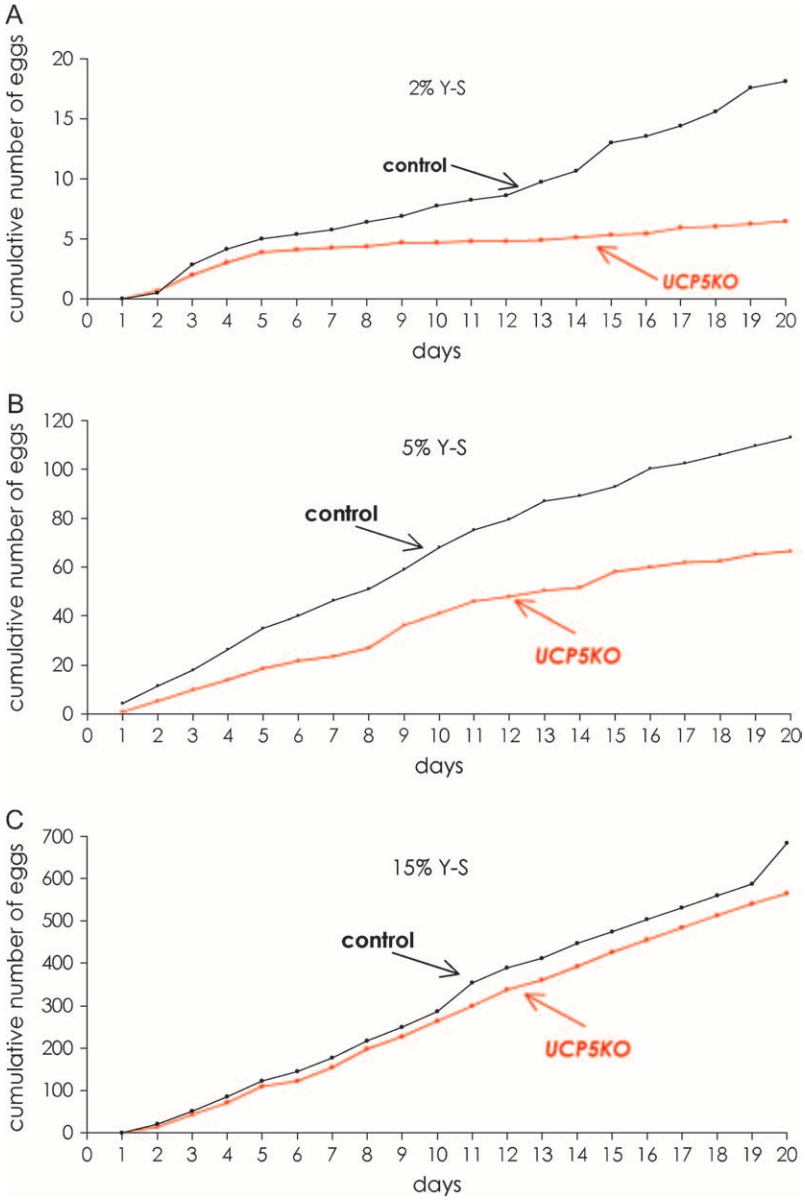

FIGURE 5.—Cumulative numbers of eggs laid by *UCP5KO* (*O-10*) and control (*W-10*) female flies on different calorie content diets. Cumulative numbers of eggs laid on (A) a very-low-calorie 2% Y–S diet; (B) a low-calorie 5% Y–S diet, and (C) a high-calorie 15% Y–S diet.

of oxygen after blocking ATP-synthase activity. The respiratory control ratio (RCR), or state 3/state 4 respiration ratio, which is a common parameter used when analyzing mitochondrial uncoupling levels (MIWA and BRAND 2003), showed that control and *UCP5KO* mitochondria were equally uncoupled (Figure 7A). The fact that the RCR is the same in both types of mitochondria could result from the lack of uncoupling being compensated by some other protein utilizing the mitochondrial proton gradient. Another possibility is that the lack of UCP5 function does indeed create an uncoupling difference but that this difference is present only in a subset of cells and, therefore, the overall RCR of the fly is not affected appreciably.

ATP levels in *UCP5KO* and control flies: UCPs uncouple oxidative phosphorylation of ADP into ATP. Therefore, we wished to investigate whether *UCP5KO* flies produced more ATP than controls. Cell lysates obtained from *UCP5KO* and control heads and thoraxes showed no differences in steady-state levels of ATP (Figure 7B). This result suggests the possibility that the UCP5 uncoupling is limited to a small population of cells and that its lack of function does not represent a major increase in the overall ATP production of the fly.

***UCP5KO* flies have lower sugar levels and upon starvation use their triglyceride reserves faster than controls:** UCP5 expression is impaired in *UCP5KO* flies, yet these animals are comparable to those of controls in overall mitochondrial uncoupling and total levels of ATP. Nonetheless, *UCP5KO* flies exhibit a metabolic homeostasis imbalance as manifested by the phenotypes described above. Therefore, a possibility to consider is that UCP5 acts in small subsets of cells and that these cells are important in mediating the homeostatic control of

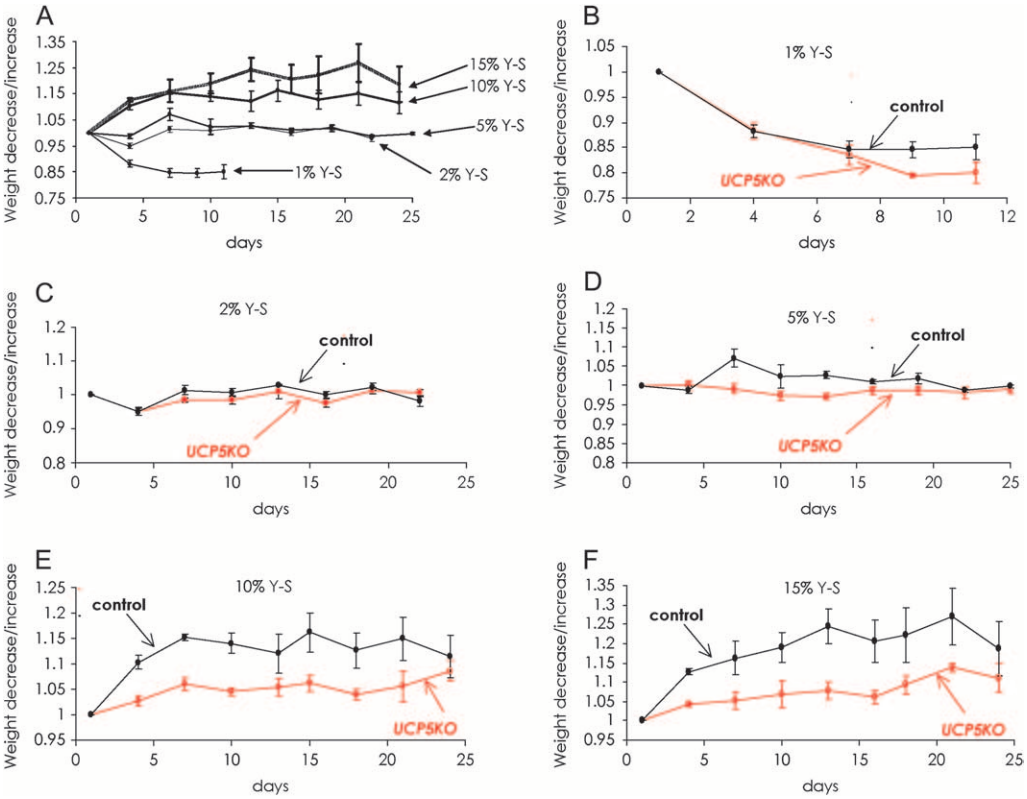

FIGURE 6.—Longitudinal weight comparisons of female *UCP5KO* (*O-10*) and control (*W-10*) flies on various calorie content diets. Data are expressed as fold decrease or increase in weight relative to weight at eclosion. (A) Longitudinal weights of control flies on different calorie diets show weight gain on the highest-calorie foods (10 and 15%) and weight loss on the lowest-calorie food (1%). (B–F) Comparisons between female control flies and *UCP5KO* flies on different calorie diets (B, 1% diet; C, 2% diet; D, 5% diet; E, 10% diet; F, 15% diet). In comparison to control flies, *UCP5KO* flies gained little weight on the higher-calorie diets. Each point represents the average ±SEM of 90–120 female flies.

metabolism. Mammalian UCP2 has been shown to negatively influence insulin secretion (ZHANG *et al.* 2001). Thus, expression of UCP2 in a very limited subset of cells, pancreatic β-cells, can greatly affect the endocrine control of metabolic homeostasis. In flies, the secretion of insulin-like peptides and adipokinetic hormone (the insect equivalent of glucagon) is controlled by clusters of neurosecretory cells in the brain (RULIFSON *et al.* 2002; KIM and RULIFSON 2004). Because Drosophila UCP5 expression has also been reported to be predominantly in the nervous system, a hormonal imbalance caused by less uncoupled neurosecretory cells could explain the observed phenotypes in the *UCP5KO* flies. To test this possibility, we compared the level of glucose and trehalose (a fundamental sugar for the fly) in control and *UCP5KO* flies fed normally or starved. We detected ~21% lower levels of total sugars in normally fed *UCP5KO* flies as compared to two *ucp5* wild-type controls, *W-10* and w^{1118}. We then subjected flies to starvation stress and observed that the total level of sugars remained at their respective baselines up to 18 hr before they started to slowly decrease (Figure 8A). In parallel to the sugar measurements, we analyzed the rate of use of the two major energetic reserves, glycogen and triglyceride (TAG). These two different metabolites were stored at the same level in fed *UCP5KO* and control flies. Once flies are starved, glycogen and TAG reserves decreased rapidly. However, while glycogen was depleted in all fly types during the first 18 hr of starvation, *UCP5KO* flies consumed their TAG reserves at a much faster rate than controls (Figure 8, B and C). These results are consistent with the possibility that *ucp5* mutant flies are hypoglycemic and that, upon starvation, for them to maintain their basal metabolism, they have to utilize their TAG reserves at a faster rate than controls. Then if starvation conditions persist, *UCP5KO* flies would exhaust their reserves faster than controls and consequently also would die faster than controls.

DISCUSSION

UCPs affect mitochondrial oxidative phosphorylation by reducing the amount of ATP that can be generated from oxidative metabolism. Therefore, modulation of uncoupling may be important in maintaining organismal metabolic balance. Several UCP homologs are expressed in a tissue-specific manner in species of all eukaryotic kingdoms (JARMUSZKIEWICZ *et al.* 2000). On the basis of work done primarily in mammalian systems, UCPs have been attributed different biological roles that appear to be in concordance with the type of tissue in which each UCP is expressed (KRAUSS *et al.* 2005).

Recently, our laboratory functionally characterized UCP5 as a *bona fide* fly UCP, establishing Drosophila as an alternative model for *in vivo* UCP studies (FRIDELL *et al.* 2004). We used flies lacking UCP5 activity to determine the biological significance of UCP5 in fly metabolism. Our findings suggest an important role for UCP5 in maintaining metabolic homeostasis. We

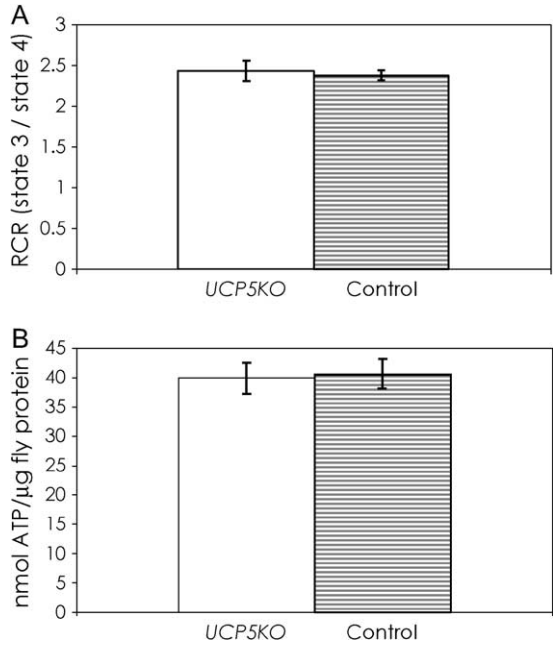

FIGURE 7.—Comparative mitochondrial respiratory control ratios and steady-state ATP levels measured from *UCP5KO* (*O-10*) and control (*W-10*) female flies. (A) Respiratory control ratios for *UCP5KO* (2.43 ± 0.12) and control flies (2.38 ± 0.07) are very similar. (B) Steady-state ATP levels for *UCP5KO* (39.93 ± 2.68 nmol ATP/µg fly protein) and control flies (40.62 ± 2.56 nmol ATP/µg fly protein) are very similar. The bars represent the average ±SEM of three independent experiments.

hypothesize that UCP5 influences hormonal control of metabolism.

Flies lacking UCP5 expression display an unexpected phenotype: Since UCPs diminish the amount of ATP that can be generated from oxidative metabolism, we predicted that flies that lack UCP5 expression would perform better than controls when assayed in tests that reflect energy production. Therefore, we expected flies lacking UCP5 to be more resistant than controls to starvation stress conditions, to gain more weight than controls, and to increase their fertility over controls. However, when we compared the performance of flies that lacked UCP5 expression with genetically matched controls, we observed that *UCP5KO* flies died much faster when subjected to food deprivation conditions, gained less weight on high-calorie diets, and had a diminished fertility status. Previous reports have shown that the expression level of some UCPs are upregulated upon starvation (Dulloo *et al.* 2001; Carroll and Porter 2004). This suggests that uncoupling activity might be an important element in the normal starvation response. However, when we starved flies, we did not observe any significant changes in the level of *ucp5* transcription (data not shown), although we cannot rule out an increase in the translation of UCP5 protein.

Lack of UCP5 expression and metabolic homeostasis alteration: One possibility to explain the unexpected phenotypes observed in *UCP5KO* flies is that they become sickly because they lack UCP5 function. Therefore, the performance of sickly *UCP5KO* flies on any assay that involves energy production and use would be poorer than that of controls. However, when we examined the life span of flies that lacked UCP5 expression, we noted that *UCP5KO* flies lived considerably longer than controls under low-calorie conditions, demonstrating that *UCP5KO* flies may not be merely sickly flies. We also showed that ectopic neuronal expression of the

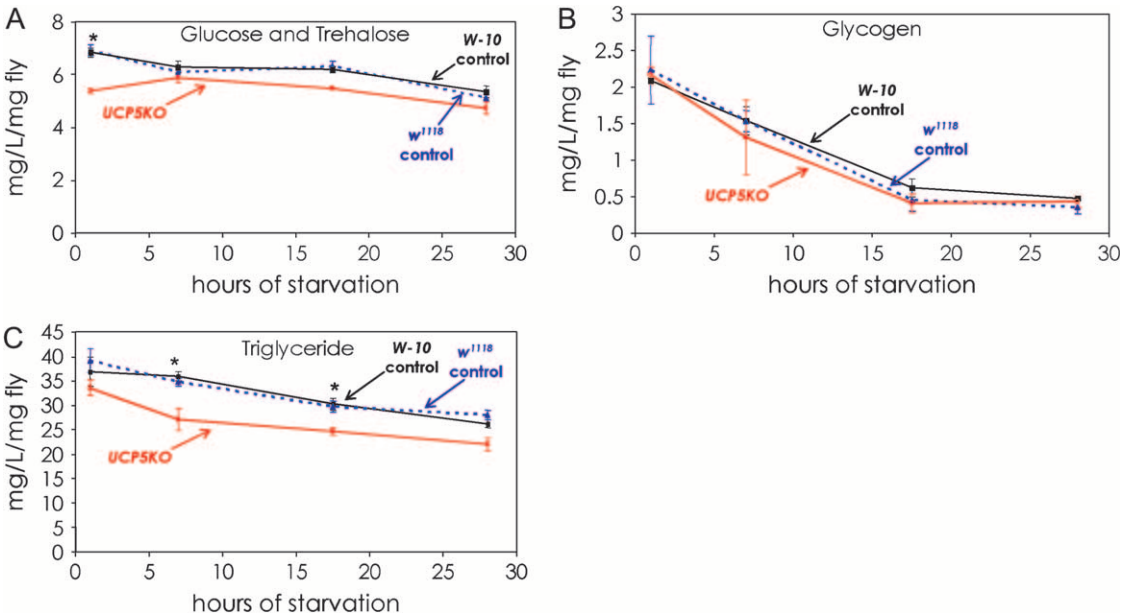

FIGURE 8.—Body composition of *UCP5KO* (*O-10*) and control (*W-10*) fed and starving female flies for (A) glucose and trehalose, (B) glycogen, and (C) triglyceride. Each point represents the average of 30 flies in milligrams/liter/milligrams fly of each metabolite ±SEM for three separate experiments (*$P < 0.05$, Student's *t*-test, $n = 3$).

ucp5 transgene was sufficient to rescue the starvation sensitivity phenotype of flies that lack normal UCP5 expression, suggesting the importance of UCP5 function in the nervous system. Since UCP5 function appears important in the nervous system, it is possible that UCP5 may play a regulatory role in the nervous system of the fly and that lack of UCP5 function leads to flies with altered metabolic homeostasis that are not able to respond to nutritional metabolic challenges.

Involvement of UCP5 in metabolic homeostasis: As mentioned above, expression of UCP5 in the nervous system is sufficient to restore normal levels of starvation resistance, which supports the hypothesis that UCP5 function in the nervous system contributes to the regulation of metabolic homeostasis. Interestingly, the fly nervous system has specific subsets of neurosecretory cells that regulate metabolic balance. These neurosecretory cells have been compared to a "fly pancreas" in that they regulate the release of insulin-like peptides (ILPs) and adipokinetic hormone (AKH), the insect equivalent of glucagon (Rulifson *et al.* 2002; Kim and Rulifson 2004). Since flies without UCP5 have lower-than-normal levels of body sugars, it is possible that UCP5 may affect ILP and/or AKH neurosecretory cells, altering the normal metabolic balance of Drosophila. Effects on insulin levels have been observed in mice in which UCP2 activity has been shown to influence pancreatic β-cell glucose-stimulated insulin secretion by affecting ATP/ADP ratios (Zhang *et al.* 2001). Increased UCP2 activities decrease the ATP/ADP ratio in β-cells and negatively influence insulin secretion by impeding the closure of K^+_{ATP}-dependent channels. Moreover, similar to our results, UCP2 −/− mice displayed lower blood glucose levels and gained less weight than controls when fed high-fat diets (Joseph *et al.* 2002). Recent studies in flies showed that AKH-producing cells express the subunits that form the K^+_{ATP}-dependent channel homolog, making these cells functionally similar to mammalian islet cells in the sensing and regulation of glucose homeostasis (Kim and Rulifson 2004). We hypothesize that the loss of UCP5 activity in fly ILP and/or AKH neurosecretory cells creates a change in the ATP/ADP ratio, which is responsible for an unusual hormonal response that leads to flies with altered metabolic homeostasis. Because changes in UCP5 expression lead to lower sugar levels and altered metabolic homeostasis in the fly, further investigation on UCP5 function may provide new insights into the molecular causes underlying diabetes and other metabolic syndromes.

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