References: Bonilla-Ramirez, L., M. Jimenez-Del-Rio, and C. Velez-Pardo 2011, Biometals 24: 1045-1057; Bonilla-Ramirez, L., M. Jimenez-Del-Rio, and C. Velez-Pardo 2013, Gene 512: 355-363; Feany, M.B., and W.W. Bender 2000, Nature 404: 394-398; Guo, M., 2012, Cold Spring Harbor Perspectives in Medicine 2: a009944; Jimenez-Del-Rio, M., C. Guzman-Martinez, and C. Velez-Pardo 2010, Neurochemical Research 35: 227-238; Liu, Z., S. Hamamichi, B. Lee, D.L. Yang, A. Ray, A.,... and V.L. Dawson 2011, Human Molecular Genetics 20: 3933–3942; Michael J. Fox Foundation 2012, http://www.michaeljfox.org/; Ng, C.H., M.S.H. Guan, C. Koh, X. Ouyang, F. Yu, ... and E.K. Tan 2012, Journal of Neuroscience 32: 14311-14317; Ortega-Arellano, H.F., M. Jimenez-Del-Rio, and C. Velez-Pardo 2011, Neurochemical Research 36: 1073-1086; Smith, W.W., Z. Pei, H. Jiang, D.J. Moore, Y. Liang, ... and C.A. Ross 2005, Proc. Natl. Acad. Sci 102: 18676-18681; Tsao, R., 2010, Nutrients 2: 1231-1246; Vanzour, D., 2012, Oxidative Medicine and Cellular Longevity. 2012: Article ID 914273; Yang, D., T. Li, Z. Liu, N. Arbez, J. Yan, ... and W. Smith 2012, Neurobiology of Disease 47: 385-392; Yue, Z., and L. Lachenmayer 2011, Movement Disorders 26: 1386-1397.

RNAi effects on the alpha glycerophosphate dehydrogenase, the alpha glycerophosphate oxidase and the arginine kinase paralogs of *Drosophila melanogaster.*

MacIntyre, Ross, and Glen Collier. Dept. of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853 and Department of Biology, Tulsa University, Tulsa OK 74101.

Introduction

Alpha glycerophosphate dehydrogenase (GPDH) and alpha glycerophosphate oxidase (GPO) cooperate in the adult thoracic flight muscles to drive the alpha glycerophosphate cycle producing the ATP necessary for continual flight (Sacktor, 1965; and see Figure 1 in Davis and MacIntyre, 1988). Null mutants in either enzyme, as homozygotes or hemizygotes, cannot fly. The structural genes for the flight muscle variants for the two enzymes were mapped in the 1970's and 1980's (Grell, 1967; O'Brien and MacIntyre, 1972; O'Brien and Gethman, 1973; Davis and MacIntyre, 1988). When the genome of *Drosophila melanogaster* was sequenced, two additional paralogs were discovered. Carmon and MacIntyre (2010) compared the sequences and the exon/intron structures of the three forms of GPDH (GPDH-1, 2, and 3) and GPO (GPO-1, 2, and 3). GPDH-1 and GPO-1 encode the flight muscle specific forms mentioned above, whereas GPDH-2 and 3 and GPO-2 and 3 are expressed only in the testis.

Arginine kinase (AK), like GPDH and GPO, is particularly abundant in indirect flight muscle (Lang *et al*., 1980), although low levels are also present in other tissues (James and Collier, 1988). The structural gene for arginine kinase is located 66F (Fu and Collier, 1983; Munneke and Collier, 1988) and is responsible for four alternative protein products, all of which share a common catalytic domain. The single EMS-induced null is due to an amino acid substitution (L182Q) in the common domain. It is an embryonic lethal which precludes assessing its role in flight muscle energetics. There are also two additional paralogs of AK that are only expressed in the testis.

The relevant information on each of the nine paralogs is shown in Table 1. All forms of each enzyme are evolving under purifying selection indicating they are functionally important in the fly.

To further assess the functional roles of the paralogs for GPDH, GPO, and AK, we have inactivated them with RNAi's from the Vienna collection. These RNA lines are also listed in Table 1. To drive the expression of the RNAi's, we have used two Gal4 constructs, one with a tubulin promoter (tub-gal4) and one with a promoter from the *bag of marbles* gene (bam-gal4) (M^{c}Kearin and Spradling, 1990). The former should drive the RNAi's in most if not all cells, whereas the latter should do so in the male germ line. We have assessed three different phenotypes reflecting the actual and possible roles of GPDH and GPO during

development. They are: viability, flight ability, and male fertility and examined in the progeny of Gal4 and RNAi lines.

Table 1. Information on the paralogs of GPDH, GPO and AK.

Paralog	Genome Number	Map Location	RNAi*
GPDH-1	CG 9042	26A	KK105359
GPDH-2	CG 3215	59C	GD4707
GPDH-3	CG 31169	94A	KK101299
GPO-1	CG 8256	52C	KK110608
GPO-2	CG 2137	43D	GD4813
GPO-3	CG 7311	34D	GD41235
AK-1	CG 32031	66F3-4	GD34036
AK-2	CG 5144	66F3	GD 6047
AK-3	CG 4546	89A5	GD 11344

*VDRC stock number

Materials and Methods

Viability is scored as "+" in the tables below if the expected numbers of Gal4;RNAi progeny survive to adulthood. If they do not survive to the adult stage, they are scored as "lethal" in the viability column. To assess flight ability 10-20 adults are placed in a vial which is stored at room temperature overnight. The next day the vial is rotated on a platform shaker for 5 minutes (the "exercise period") and the flies are dumped onto a sheet of white paper. The number of flies that have flown away in a 20 second interval is then recorded over the number of flies originally in the vial. To determine male fertility, five males with the Gal4-RNAi genotype are placed in a vial with virgin females from a dpy^{ov1} stock. Three to five replicates are set up and scored for the eventual emergence of *dpy* plus progeny after 14 days. The number of vials producing progeny is then recorded.

Table 2. RNAi inactivation of single paralogs.

RNAi-Gal4	Viability	Flight Ability	Male Fertility
GPDH-1-tub	lethal	n.d.*	n.d.*
GPDH-1-bam	+	10/10	3/3
GPDH-2-tub	+	10/10	0/3
GPDH-2-bam	+	10/10	0/3
GPDH-3-tub	+	9/10	3/3
GPDH-3-bam	+	10/10	3/3
GPO-1-tub	+	10/10	3/3
GPO-1-bam	+	10/10	3/3
GPO-2-tub	+	10/10	3/3
GPO-2-bam	+	10/10	3/3
GPO-3-tub	+	10/10	3/3
GPO-3-bam	+	9/10	3/3
AK-1-tub	lethal	n.d.*	n.d.*
AK-1-bam	+	10/10	3/3
AK-2-tub	+	10/10	3/3
AK-2-ban	+	10/10	3/3
AK-3-tub	+	10/10	0/3
AK-3-bam	+	10/10	0/3

*Can't be done

Results and Discussion

I: RNAi inactivation of single paralogs

Each of the three forms of the three enzymes was subjected to its RNAi driven by either the tubulin- Gal4 or the bag of marbles-Gal4. The results are shown in Table 2.

Inactivating GPDH-1 with its RNAi driven by the ubiquitous tubulin promoter results in pre-adult lethality in keeping with its pivotal role in intermediary metabolism. Similarly, inactivating AK-1 with its RNAi driven by the tubulin promotor results in embryonic lethality. The only other specific effect is male sterility when either GPDH-2 or AK-3 are inactivated by their respective RNAi driven by either tubulin-Gal4 or by Gal4 driven by the bag of marbles promoter active in the testis. No effect on flight ability was detected when a GPDH, GPO, or AK paralog was inactivated.

II: RNAi inactivation of pairs of paralogs for each enzyme

In order to determine if paralogs of the same enzyme will compensate in any of the three phenotypes, we drove the RNAi's for pairs of the paralogs with either tubulin-Gal4 or the bag of

marbles-Gal4. Double mutant stocks of RNAi; tub-Gal4/ In(3LR)TM3,Sb and RNAi; bam-Gal4 were constructed when the RNAi and tub-Gal4 combinations are viable and fertile. Flies from these stocks were then crossed to flies from the second RNAi stock and the appropriate F1's were scored for the three phenotypes. The results are presented in Table 3.

The expectation here is if the paralogs can compensate for one another, flies carrying both RNAi's activated by Gal4 should show a more extreme phenotype than flies with either RNAi alone. This does not seem to be the case when the double RNAi combinations are viable and fertile. Hence it is clear that the paralogs are affecting distinct and separable functions from one another.

Table 3. RNAi inactivation of paralog pairs-same enzyme.

RNAi's-Gal4	Viability	Flight Ability	Male Fertility
GPDH-1&2-tub	lethal	n.d.*	n.d.*
GPDH-1&2-bam	+	9/10	0/3
GPDH-1&3-tub	lethal	n.d.*	n.d.*
GPDH-1&3-bam	+	10/10	3/3
GPDH-2&3-tub	+	10/10	0/3
GPDH-2&3-bam	+	9/10	0/3
GPO-1&2-tub	+	20/20	3/3
GPO-1&2-bam	+	10/10	3/3
GPO-1&3-tub	+	8/10	3/3
GPO-1&3-bam	+	10/10	3/3
GPO-2&3-tub	+	10/10	3/3
GPO-2&3-bam	+	10/10	3/3

*Can't be done

III: RNAi inactivations of paralogs for different enzymes

Davis and MacIntyre (1988) reported that a combination of a particular GPDH-1 and a GPO-1 null mutant resulted in synthetic lethal phenotype, emphasizing the importance of the alpha glycerophosphate cycle, not only in the operation of the flight muscles but also during pre-adult development. Proceeding from that observation, we wondered if any combination of RNAi's against paralogs of GPDH and GPO would produce synthetic lethals or more extreme phenotypes should they be participating in the alpha glycerophosphate cycle during development. Thus, we produced several such combinations driving their expression once again with tubulin-Gal4 or bag of marbles-Gal4. The data we obtained are presented in Table 4.

Double RNAi's where one is GPDH-1 driven by tubulin-Gal4 are invariably lethal and thus are not shown in Table 4. Also, the male sterility effected by the RNAi against GPDH-2 when driven by either tubulin-Gal4 or bag of marbles-Gal4 was observed and apparently not affected by inactivation of any of the GPO paralogs. In summary we observed no instance of either suppression or enhancement of viability, flight ability, or male fertility in any other combination of paralogs of GPDH or GPO inactivated by their corresponding RNAi's. This again indicates the paralogs of the two enzymes are acting independently of one another during development.

IV: Effect of RNAi inactivation of AK3 on sperm development

When either tubGAL4 or bamGAL4 drivers are crossed to an AK3-RNAi stock, male offspring with both GAL4 and RNAi elements are completely sterile. RT-PCR of RNA extracted from testes of these males using primers specific to the AK3 transcript reveal an absence AK3 transcripts in both qualitative (Figure 1a) and quantitative (Figure 1b) assays. The fold change for AK3 transcripts from testes of normal (Sb/AK3 RNAi) versus knockdown (bamGAL4/AK3 RNAi) siblings shows that the transcript is reduced to extremely low levels in the knockdown. The specificity is also shown by the fact that transcript levels of AK2 are essentially the same between the two genotypes. Further, phase contrast images of testes from these males reveal early stages of spermatogenesis are normal, although no normal mature sperm accumulate in the seminal vesicle (Figure 1d). Closer examination reveals that the process of individualization of the sperm is never completed (Figure 1c). There appears to be a failure of the individualization complex (Fabrizio *et al*., 1973) to complete the process of converting the sixty four spermatocytes contained within the cyst to sixty four individual spermatids. In this case, the testis-specific paralog AK3 does play a critical and specific role in sperm development. Furthermore, the effectiveness of the bamGAL4 driver suggests that transcription

occurred pre-meiotically, while the effects upon sperm function occurred post-meiotically. This is a situation similar to that seen for the testis-specific paralog of another gene, Gld2 (Sartain *et al*., 2011).

Table 4. RNAi inactivation of pairs of paralogs-different enzymes.

RNAi's-Gal4	Viability	Flight Ability	Male Fertility
GPDH-1&GPO-1-bam	+	20/20	3/3
GPDH-1&GPO-2-bam	+	9/10	3/3
GPDH-1&GPO-3-bam	+	20/20	3/3
GPDH-2&GPO-1-tub	+	20/20	0/3
GPDH-2&GPO-1-bam	+	10/10	0/3
GPDH-2&GPO-2-tub	+	18/20	0/3
GPDH-2&GPO-2-bam	+	18/20	0/3
GPDH-2&GPO-3-tub	+	20/20	0/3
GPDH-2&GPO-3-bam	+	17/20	0/3
GPDH-3&GPO-1-tub	+	10/10	3/3
GPDH-3&GPO-1-bam	+	20/20	5/6
GPDH-3&GPO-2-tub	+	20/20	3/3
GPDH-3&GPO-2-bam	+	19/20	3/3
GPDH-3&GPO-3-tub	+	10/10	3/3
GPDH-3&GPO-3-bam	+	10/10	3/3

There is, however, the issue of the RNAi's against GPDH-3, all three GPO paralogs, and AK-2. Since we did not observe any phenotype associated with them when Gal4 is driven by the tubulin and bag of marbles promoters, we cannot be sure they are inactivating their respective messenger RNA's. We have no reason, *a priori*, to suspect them, especially given the dramatic mutant phenotypes associated with the GPDH-1, GPDH-2, AK-1, and AK-3 RNAi's. Also, when Gal4 is driven by the muscle specific promoter actin-88F, all of the GPDH RNAi's, including GPDH-3 and all three GPO RNAi's, produce offspring that are unable to fly. Similarly, the GPO-1 RNAi and AK-1 RNAi, when Gal4 is driven by another muscle specific promoter, mef-2, induced flight-lessness in the Gal4;RNAi containing off-spring. However, in the cases of GPDH-3 and the GPO RNAi's, independent assessment of their knockdown effects on their mRNA's, as was done with AK-3, should be made.

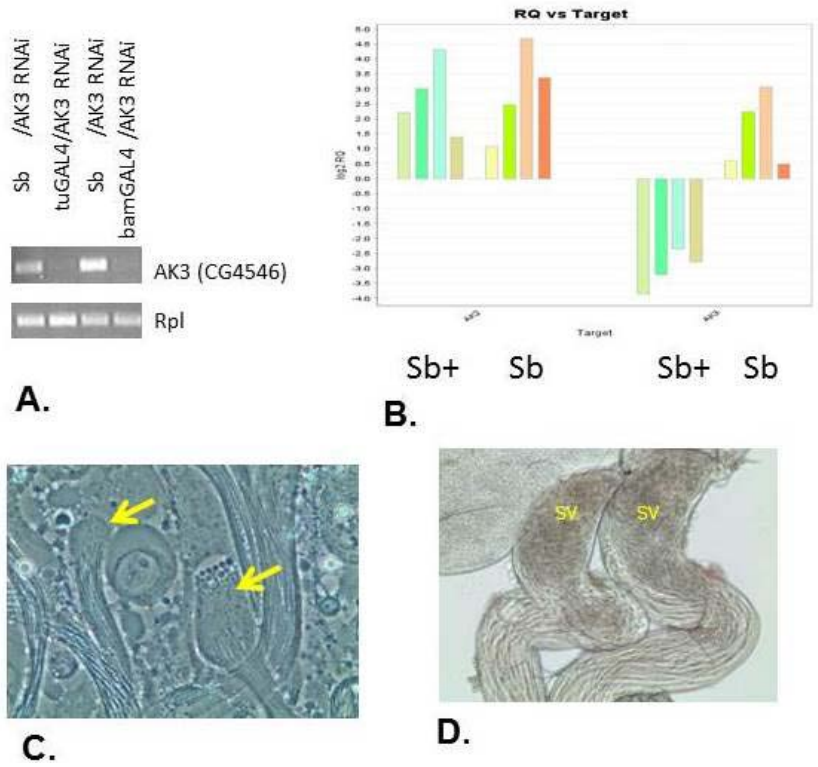

Figure 1. RNAi knockdown of AK3. **A,** Qualitative RT-PCR of AK3 transcripts. RNA from testes of Sb/AK3 RNAi, tuGAL4/AK3 RNAi siblings and Sb/bamGAL4, bamGAL4/AK3 RNAi siblings was used to generate cDNA, which was amplified with primers specific to AK3 (upper panel) or primers specific to Rpl 32.(lower panel). **B,** Quantitative RT-PCR of AK3 transcripts. RNA from testes from four individual Sb/bamGAL4 and four individual bamGAL4/AK3 RNAi siblings was used for RT-PCR using primers specific to AK2, AK3, and RpL32. Reactions were performed on an ABI StepOne Plus platform and comparative CT analysis done with the StepOne software. **C,** Phase contrast images of testis from bamGAL4/AK3 RNAi males. Arrows indicate individualization complexes on cysts of 64 spermatids. **D,** Seminal vesicles of bamGAL4/AK3 RNAi males. No mature sperm are present.

References: Carmon, A., and R. MacIntyre 2010, Journal of Heredity 101: 225-234; Davis, M.B., and R. MacIntyre 1988, Genetics 120: 755-766; Fabrizio, J.J. *et al*., 1998, Development 125: 1833-1843; Fu, L.J., and G.E. Collier 1983, Bull. Inst. Zool. Acad. Sinica 22: 25; Grell, E., 1967, Science 158: 1319-1320; James, J.E., and G.E. Collier 1988, J. Exp. Zool. 248: 185-191; Lang, A.B. *et al*., 1980, J. Muscle Res. Cell Motil. 1: 147-161; McKearin, D., and A. Spradling 1990, Genes and Development 4: 2242-2251; Munneke, L.R., and G.E. Collier 1988, Biochem. Genet. 26: 131-141; O'Brien, S.J., and R. MacIntyre 1972, Genetics 71: 127-138; O'Brien, S.J., and R. Gethmann 1973, Genetics 75: 155-167; Sacktor, B., 1965, *Physiology of Insecta*. (Rockstein, M., ed.). 2: 483-580; Sartain, C.V. *et al*., 2011, Development 138: 1619-1629.

Effect of temperature on the development time and pupation height of *Drosophila*.

Singh, Manish. National Cancer Institute, Frederick, MD 21702; E-mail: manishsingh7237@gmail.com

Temperature is important for the development of insects and other animals. Since insects are cold-blooded organisms, temperature is the most important environmental factor that can influence insect behavior, distribution, development, survival, aging, and reproduction (Mikasa and Narise, 1980; Gillooly *et al.*, 2002; Régnière *et al.*, 2012; Kelly *et al.*, 2013; Danjuma *et al.*, 2014). I chose *Drosophila* (fruit flies) to study the effect of temperature on development time, because they are easy to handle, well understood, possess a short life cycle of just two weeks, and are easy to keep in large numbers. They show complete metamorphosis. There are ~2000 species of *Drosophila* in the world. The average lifespan of *Drosophila* is about 50 days. A female can lay hundreds of fertilized eggs during her brief lifespan. *Drosophila* is also an excellent model organism to study human diseases.

Since temperature affects the development and survival of *Drosophila*, I chose a few laboratory populations to test the developmental time, pupation height, and number of flies emerged.

To perform the experiment, I used the following materials and equipment: 10-15 flies of Oregon-R and Canton-S (3-5 day old individually laboratory-mated female *Drosophila* for each temperature condition); fly food (containing yeast, sugar, agar, cornmeal, water, methylparaben, and ethanol); incubators to keep the flies for testing the development time [18°C, 25°C, and 29°C]; CO_2 chamber used when sorting; and microscope to examine flies.

I used 10-15 flies (males and females) from the main stock and individually transferred them into a fresh vial, while they were sleeping. I prepared three sets (replicates) of vials for each temperature setting (9 total vials with 10-15 flies each). I put these vials into incubators with temperatures set at 18°C, 25°C, and 29°C for 2 days. After 2 days, the flies were taken out from the vials and were observed throughout the developmental stages from egg/embryo until adult. I also observed the pupation height, which was based on the number of pupae resting on the side of the vials. In addition, I also counted the number of flies emerged. Data was analyzed and a graph was made on Microsoft Excel.

I tested if different temperature regimes affect the development time of *Drosophila* and found that at 18°C, flies grow much slower (development time ~20 days) as compared to 25°C (development time ~9 days) and 29°C (~7 days) (Figure 1). I performed these experiments in triplicate and found the same results. I also observed that at lower temperatures the size of the flies is much larger compared to higher temperatures. In addition, I also observed the lowest pupation height at lowest temperature (18°C; Figure 2). Furthermore, I noticed that at lower temperatures flies show less lethality compared to higher temperature.

The above-mentioned results suggest that at lower temperatures *Drosophila* develop slowly. These results also show that at a favorable temperature insects will grow much faster. It is possible that lower temperatures may decrease the rates of depletion of energy substrates (Koštál *et al*., 2016). More lethality observed at high temperatures in our experiments could be due to the accumulation of metabolic waste products or a higher rate of water loss, which is usually greater at later stages of development (Davidson,