Research

Doxycycline-induced expression of sense and inverted-repeat constructs modulates *phosphogluconate mutase* (*Pgm*) gene expression in adult *Drosophila melanogaster*

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Abstract

Background: A tetracycline-regulated (conditional) system for RNA interference (RNAi) would have many practical applications. Such a strategy was developed using RNAi of the gene for *phosphogluconate mutase* (*Pgm*). *Pgm* is a candidate lifespan regulator: Pgm^S allele frequency is increased by selection for increased lifespan, whereas Pgm^M and Pgm^F allele frequencies are decreased.

Results: The *Pgm* alleles were cloned and sequenced and were found to differ by amino-acid substitutions consistent with the relative electrophoretic mobilities of the proteins. The 'tet-on' doxycycline-regulated promoter system was used to overexpress Pgm^S in a wild-type (Pgm^M) background. Enzyme activity increases of two- to five-fold were observed in five independent transgenic lines. Tet-on was also used to drive expression of an inverted-repeat fragment of *Pgm* coding region. The inverted-repeat transcript was expected to form a dsRNA hairpin, induce RNAi, and thereby reduce endogenous *Pgm* gene expression at the RNA level. Endogenous *Pgm* RNA levels in adult flies were found to be reduced or eliminated by doxycycline treatment in five independent inverted-repeat transgenic lines. Our results show that doxycycline-regulated expression of inverted-repeat constructs can cause a conditional reduction in specific gene expression. The effect of sense and inverted-repeat construct expression on lifespan was assayed in multiple transgenic lines. Under the conditions tested, altered *Pgm* gene expression had no detectable effect on adult *Drosophila* lifespan.

Conclusions: A system for conditional RNAi in *Drosophila* adults shows promise for assay of gene functions during aging. Our results indicate that *Pgm* does not have a simple strong effect on longevity.

Background

Conditional gene expression systems such as promoters regulated by tetracycline or doxycycline (DOX) have several advantages for testing the effects of genes on aging and lifespan [1-3]. In the 'tet-on' system, feeding DOX to *Drosophila melanogaster* causes high levels of transgene expression in all tissues. By waiting until the young adult stage to administer DOX, all of pre-adult development is identical between control and experimental groups, and any difference in lifespan must be due to changes in the adult. Subtle differences in the genetic background of *Drosophila* strains can have significant effects on lifespan

[4-6]. With the tet-on system, control and experimental animals have identical genetic backgrounds, and therefore any differences in lifespan must be due to DOX administration and transgene expression. DOX itself, and overexpression of control genes such as *Escherichia coli lacZ*, have no detectable effects on lifespan [1]. In contrast, several genes have been identified for which DOX-regulated overexpression has negative effects on lifespan, and overexpression of the *dGMII* gene, encoding α-mannosidase II, was associated with slightly increased lifespan [7].

Expression of antisense RNA has long been known to be able to inhibit gene expression in *Drosophila* and other organisms [8-12]. Double-strand RNA (dsRNA) formed by hybridization of sense and antisense sequences is thought to initiate a pathway in which homologous RNA sequences are destroyed. This phenomenon has been referred to as post transcriptional gene silencing (PTGS) or RNA interference (RNAi). Expression of inverted-repeat constructs, where the transcript is expected to fold into a dsRNA hairpin, has been shown to be an efficient initiator of RNAi [13-15]. In experiments reported here, the tet-on system was used to drive expression of inverted-repeat constructs in *Drosophila* to determine if a conditional system for RNAi could be created.

In natural populations, lifespan and reproductive period are thought to co-evolve [16]. The duration of the reproductive period is subject to natural selection, and lifespan is altered as a consequence. Significant experimental support for this model has come from laboratory selection experiments with *D. melanogaster* [17-21]. Genetically heterogeneous *Drosophila* populations have been selected over hundreds of generations for late-life reproduction. Such selection results in strains with increased lifespan relative to controls. Selection is thought to function by altering the frequency of gene alleles present in the starting population. Selected strains exhibit a number of correlated phenotypes. The correlated phenotypes can vary somewhat depending on the starting strains used; however, increased stress-resistance appears to be common to all or most long-lived strains [22-26]. In one well studied set of five replicate control (B) and long-lived (O) strains, lifespan was doubled and was correlated with increased stress-resistance and increased glycogen and lipid stores [27,28].

Starch-gel electrophoresis has been used to assay for changes in the frequency of enzyme electrophoretic alleles in the O and B lines. A change in the frequency of Cu/Zn-superoxide dismutase (Cu/ZnSOD) alleles was observed, with the more active allele enriched in the long-lived O strains [29]. Consistent with this observation, overexpression of Cu/ZnSOD in transgenic *Drosophila* has been shown to be sufficient to cause significant increases in lifespan [30,31]. The most dramatic change in allele frequency in the O and B lines was observed for the gene for *phosphogluconate mutase* (*Pgm*) [32]. *Pgm* exists in three electrophoretic forms: fast (Pgm^F), medium (Pgm^M) and slow (Pgm^S). Pgm^M predominates in the B strains, whereas Pgm^S allele frequency is on average tenfold higher in the long-lived O strains relative to the B controls. When O strains were taken off selection for several generations (back-selection), lifespan decreased and Pgm^S allele frequency was reduced to levels more like those in B strains. These results suggest that *Pgm* or a closely linked gene is responding to selection, and make Pgm^S a candidate lifespan regulator.

Pgm encodes the enzyme phosphoglucomutase, which interconverts glucose 1-phosphate and glucose 6-phosphate. Its activity is therefore important for both glycolysis and glycogen synthesis, and the altered *Pgm* allele frequency might therefore be relevant to the increased glycogen and lipid stores of the O strains. If the increased Pgm^S allele frequency is contributing to the unique phenotypes of the long-lived O strains, this might be because Pgm^S has increased or decreased enzyme activity, or enzyme activity has been altered in some way (perhaps by a change in its regulation). One way to begin to test these models experimentally is to engineer transgenic flies with increased or decreased *Pgm* expression and assay for effects on lifespan.

Results

Multiple strains were generated that were homozygous for each of the F, M and S electrophoretic alleles of *Pgm*, by appropriate crosses to a third chromosome balancer stock. *Pgm* enzyme activity and lifespan varied greatly across the strains with no correlation with *Pgm* allele (data not shown). This result was expected, as differences in genetic background between such purified chromosome strains has profound effects on lifespan and the activities of various enzymes [5,6,33]. The *Pgm* coding-region sequences were cloned and sequenced from the Pgm^F, Pgm^M and Pgm^S homozygous strains and from the Canton-S wild-type strain. Amino-acid substitutions were identified that predicted pI values for the alleles that correlated with their mobility on starch gels (Figure 1). The substitution of T for A in Pgm^S creates the amino-acid sequence TTK (in the single-letter amino-acid code) which is a potential phosphorylation site for protein kinase C (PKC) that is absent in Pgm^M and Pgm^F.

If Pgm^S contributes to the increased lifespan of the O lines, this might be due to increased enzyme activity, decreased enzyme activity, or alteration in enzyme activity in some other way such as in its regulation or subcellular localization. The tet-on system was used to test whether simply increasing or decreasing *Pgm* expression would be sufficient to alter lifespan. A P-element transformation vector called USC1.0 was generated, which had unique *Eco*RI and *Pst*I sites downstream of the tet-on doxycycline-regulated promoter (Figure 2a). USC1.0 also contains the mini-*white*$^+$

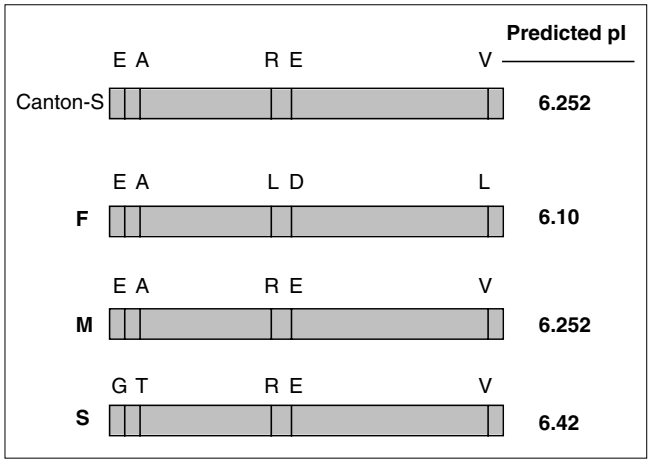

Figure 1
Sequence alterations in various *Pgm* electrophoretic alleles. The amino acids that differ between the Pgm^F, Pgm^M, Pgm^S and wild-type Canton-S alleles are indicated in single-letter code, with the predicted pI values at the right. The GenBank accession numbers for the sequences are AF416982, AF416981, AF416983 and AF416984, respectively.

transformation marker gene. The Pgm^S cDNA sequences were cloned into USC1.0 to create the PGMsense construct (Figure 2b), which should allow overexpression of the PGM^S enzyme. A fragment of Pgm^S coding region of approximately 1 kilobase (kb) (from +711 to +1,794) was cloned into USC1.0 in an inverted-repeat orientation to create construct PGMinvrpt (Figure 2c), which should potentially cause conditional RNAi. For each PGM construct five independent transgenic lines were generated using standard methods. As a control, two trangenic lines were generated containing the USC1.0 vector in which no transgene is expressed.

Expression of the transgenic constructs and the endogenous *Pgm* gene was assayed by northern blot, with or without DOX (Figure 3a). The *Pgm* inverted-repeat sequences were used as probes and should hybridize to both transgenes and the endogenous *Pgm* transcript. In the sense-construct lines, feeding DOX resulted in efficient expression of the transgene at levels five- to ten-fold greater than the endogenous transcript. DOX-induced expression of the inverted-repeat construct had two effects detected by northern blot. First, DOX produced a smear of hybridization resulting from the inverted-repeat transcript (Figure 3a). The smear pattern may have resulted from incomplete denaturation of the hairpin structure and/or instability of the inverted-repeat transcript. Second, the level of the endogenous *Pgm* transcript was found to be reduced by DOX feeding.

The northern blots were also hybridized with a *Pgm*-specific probe corresponding to *Pgm* coding-region sequences (+1 to +700) located outside the region used to create the inverted repeat. This probe is therefore specific for the endogenous *Pgm* transcript. Using this probe the smear of hybridization derived from the inverted-repeat construct was no longer detected, and the endogenous *Pgm* RNA levels were more readily observed and quantitated (Figure 3b). DOX induced expression of the inverted-repeat construct was found to cause a reduction of endogenous *Pgm* RNA levels. The decrease varied across the five transgenic lines, with reductions ranging from 1.3- to 24-fold. The inverted-repeat construct therefore appears to function as expected to induce RNAi and cause decreased expression of the endogenous *Pgm* gene. In the control strains where no transgene is expressed, DOX administration was found to have no effect on *Pgm* transcript levels (Figure 3c).

The indirect PGM activity assay was used to assay for changes in *Pgm* gene expression at the protein level. The sense construct yielded conditional overexpression of PGM activity in each transgenic line, with increases ranging from two- to five-fold (Table 1). Potential reductions in PGM activity were more difficult to assay because of the inherent limitations of the assay. The PGM activity assay is indirect and is coupled to the conversion of $NADP^+$ to NADPH. The extract will contain other activities capable of converting $NADP^+$ to NADPH, thereby creating a significant background activity. Decreases in PGM activity could not be reliably measured because of this unavoidable background. The PGMinvrpt construct yielded decreases in PGM activity of as much as 50%; however, the effect was quite variable from experiment to experiment (Table 1). In the control strains where no transgene is expressed, DOX administration was found to have no consistent effect on PGM levels (Table 1).

To determine whether altered *Pgm* gene expression could affect lifespan, mean lifespan was assayed in multiple PGMsense and PGMinvrpt transgenic lines with or without DOX treatment. The percentage change in mean lifespan caused by DOX and transgene expression is presented for each line. Lifespan was observed to vary across transgenic lines and replicate experiments with changes ranging from -7% to +6%, with one outlier at +16% (Table 2). However, no change in lifespan was observed that was consistent across the sense or inverted-repeat lines or in multiple experiments. The lifespan assays were repeated at 29°C, and again no consistent changes were observed (Table 3). We conclude that under these conditions, altered *Pgm* expression does not have a detectable effect on adult *Drosophila* lifespan.

Discussion

The phenomenon of RNAi (or PTGS) is of great interest for two reasons [9-12]. First, it represents an evolutionarily conserved pathway that probably has important functions in the regulation of gene expression and the control of transposable elements. Second, it provides a means for researchers to test gene functions by experimentally downregulating

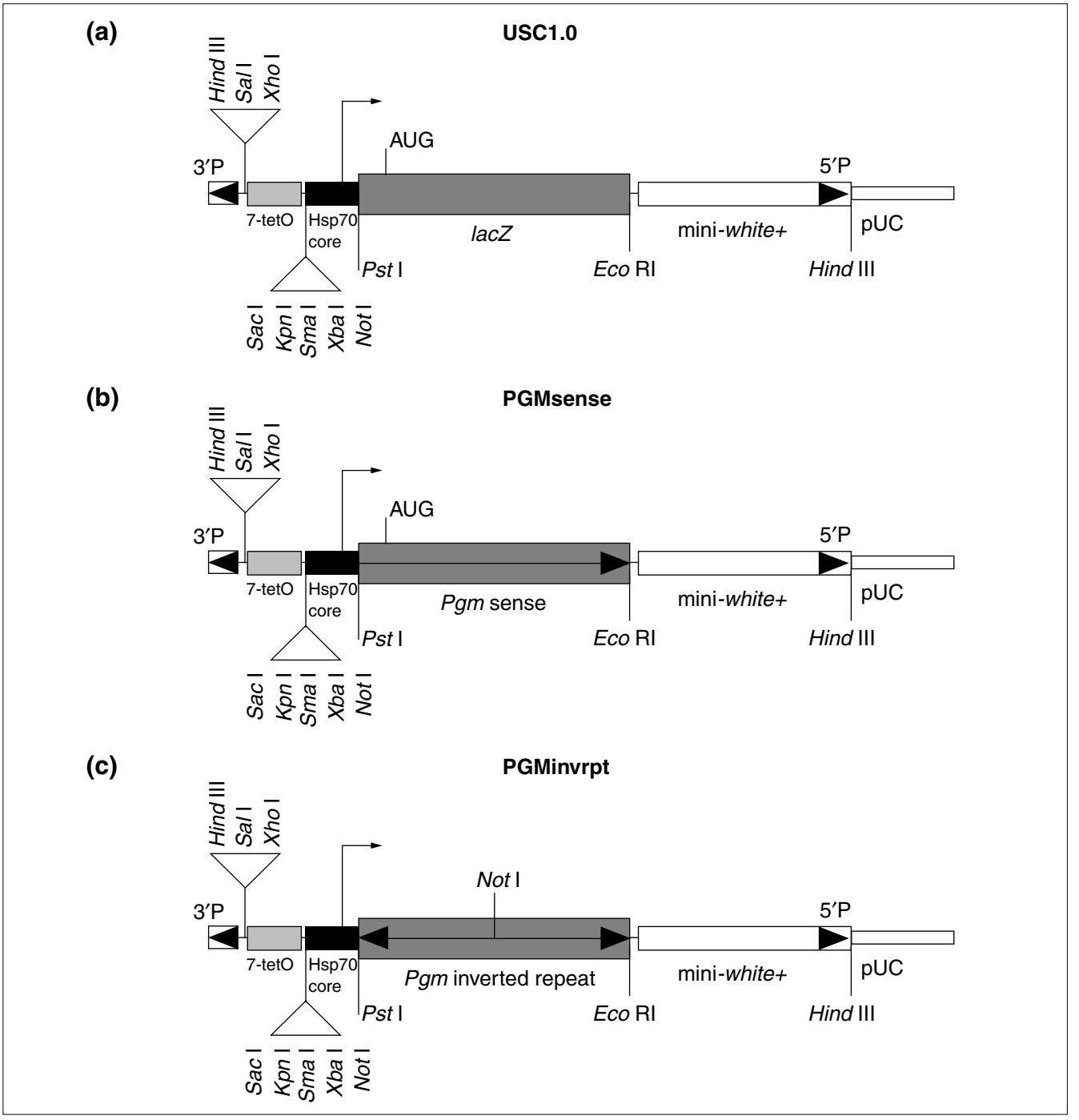

Figure 2
P-element transformation constructs. **(a)** The USC1.0 vector for the tet-on system. Unique *Pst*I and *Eco*RI sites are located downstream of the tet-on promoter, enabling cloning of cDNAs. cDNAs should contain their own ATG start codons and polyadenylation signal sequences. **(b)** The PGMsense construct for conditional overexpression of Pgm^S. **(c)** The PGMinvrpt construct, containing an approximately 1 kb inverted repeat of Pgm^M coding region, as indicated by inverted arrows.

expression of specific genes under well controlled conditions. In *Drosophila*, RNAi can be initiated by injection of dsRNA into embryos, and this has allowed identification of novel phenotypes for genes during development [34]. However, injection has limited potential application in the adult, as it is unlikely that the RNAi could be targeted to all tissues, or to specific tissues, and the trauma of injection is likely to have negative effects on phenotypes such as lifespan.

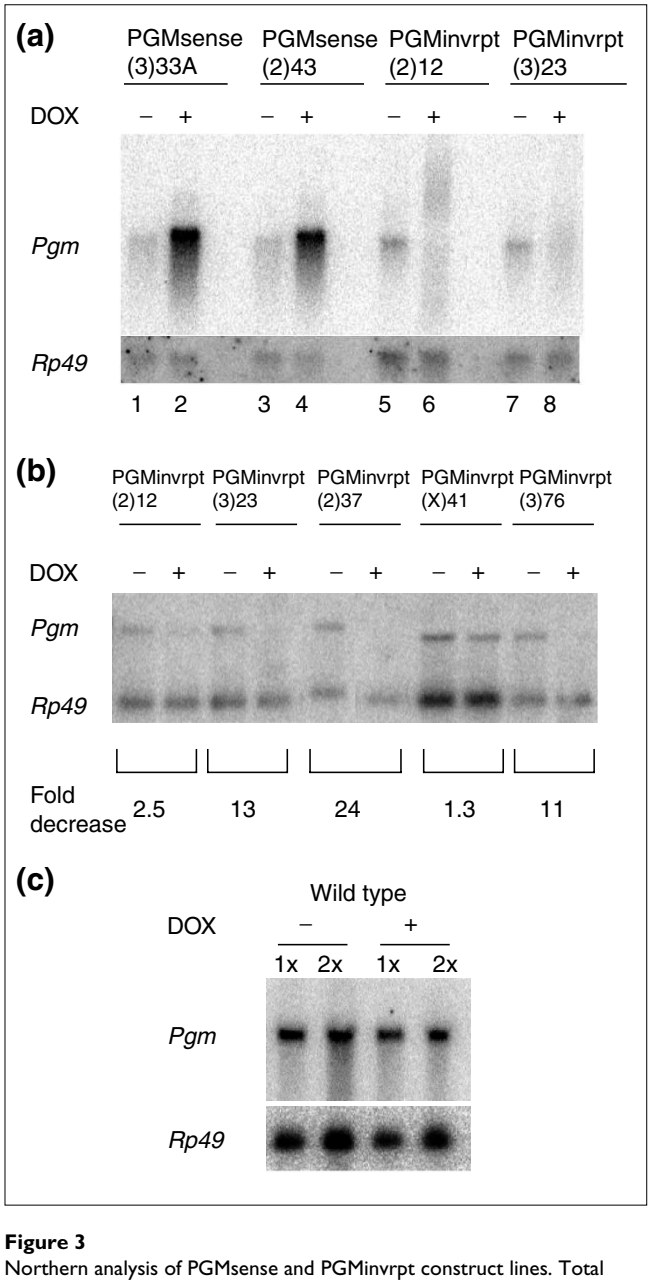

Figure 3
Northern analysis of PGMsense and PGMinvrpt construct lines. Total RNA was isolated from adult males of the indicated PGMsense lines, PGMinvrpt lines, and control line, with or without DOX treatment. **(a)** Northern blot hybridized with a probe that will recognize both endogenous and transgenic *Pgm* transcripts and with a probe specific for *Rp49* as a loading control. **(b)** Northern blot hybridized with a probe that will recognize only the endogenous *Pgm* transcript and with a probe specific for *Rp49* as a loading control. The fold decrease in *Pgm* transcript abundance is indicated below the lanes. **(c)** Northern blot of control strain hybridized with a probe that will recognize the endogenous *Pgm* transcript and with a probe specific for *Rp49* as a loading control.

Transgenic constructs have been used to cause RNAi in *Drosophila*, by using various promoters to drive expression of inverted-repeat fragments of gene coding regions [14,15]. Using the conditional tet-on promoter system to drive expression of an inverted-repeat provided conditional RNAi of the *Pgm* gene. This conditional system should have many applications, both in the study of the RNAi pathway itself and in the design of experiments where RNAi is used as a research tool. Conditional RNAi should be particularly useful to study phenotypes in the adult such as aging and lifespan. Conditional gene-expression systems have previously been used to identify positive regulators of lifespan, such as *Cu/ZnSOD* and *dGMII* [7,31]. In addition, mutations have been used to identify several genes in *Drosophila* that act as negative regulators of lifespan, such as *mth*, *INDY*, *InR* and *chico* [35-38]. For *mth* and *InR* the null phenotype of the genes is lethality. Therefore it is only rare hypomorphic alleles or allele combinations that allow the animals to survive to adult stage where increased lifespan can be observed. Conditional RNAi should provide a powerful means to study these genes and other negative regulators of aging and lifespan. Inactivation or downregulation of the genes specifically in the adult using tet-on RNAi may allow increased lifespan without confounding effects on development. Lam and Thummel have recently reported the use of a heat-shock gene promoter to drive expression of dsRNA, and the efficient conditional inhibition of gene expression during larval and pupal stages of *Drosophila* development [39].

Genetic selection of *Drosophila* populations for late-life reproduction caused the correlated phenotype of increased lifespan and increased the frequency of the slow electrophoretic allele, Pgm^S, of the *Pgm* gene. If Pgm^S contributes to the increased lifespan phenotype of the selected strains, it might be because Pgm^S has increased activity, decreased activity or activity that is altered in some way such as through its regulation or subcellular localization. The *Pgm* alleles were cloned and sequenced and were found to differ by amino-acid substitutions consistent with the relative electrophoretic mobilities of the encoded enzymes.

A recent study reported the sequencing of several fast, medium and slow electrophoretic alleles of *Pgm* from a wild population of *D. melanogaster* [40]. The sequences reported in that study (Verrelli alleles) are comparable to those reported here (Ives alleles) as follows: the medium Verrelli allele used for alignment is identical in amino-acid sequence to the Ives medium allele. The Verrelli fast alleles are identical to the Ives fast allele in each of the three amino-acid positions at which the Ives fast differs from the Ives medium (wild-type) allele. There are a number of differences between the Verrelli slow alleles and the Ives slow allele. The Verrelli slow alleles are not all identical to each other. The Ives slow allele differs from the Ives medium (wild-type) amino-acid sequence at two positions (amino-acid positions 6 (E → G) and 9 (A → T)). The amino-acid change in the Ives slow allele at position 6 (E → G) is not reflected in any of the Verrelli slow alleles. The second amino-acid change in the Ives slow allele (at position 9) is shared by two of the Verrelli slow alleles. In addition, the Verrelli slow alleles have

Table 1

PGM enzyme activity

		Enzyme activity (ΔOD/μg/min)		
Line name	Sex	-DOX	+DOX	Percentage change (*p*-value)
PGMsense(3)33A1	Male	0.1105 ± 0.014	0.5926 ± 0.089	+436% (0.0008)
	Male	0.1035 ± 0.004	0.5901 ± 0.070	+470% (0.0003)
PGMsense(3)33B1	Male	0.1018 ± 0.013	0.4585 ± 0.010	+350% (3.0E-06)
	Male	0.1138 ± 0.014	0.5829 ± 0.018	+412% (3.6E-06)
PGMsense(2)43A2	Male	0.0893 ± 0.010	0.4825 ± 0.014	+440% (2.3E-06)
	Male	0.1100 ± 0.020	0.6706 ± 0.085	+510% (3.6E-04)
PGMsense(2)52A1	Male	0.1131 ± 0.007	0.4939 ± 0.048	+337% (0.0002)
	Male	0.0925 ± 0.012	0.2608 ± 0.060	+182% (0.0088)
PGMsense(X)27A1	Female	0.1301 ± 0.003	0.3093 ± 0.047	+138% (0.0028)
PGMinvrpt(3)76	Male	0.1179 ± 0.0121	0.1162 ± 0.0069	-1.44% (0.83)
	Male	0.0966 ± 0.0076	0.0625 ± 0.0049	-35.3% (0.0028)
PGMinvrpt(3)23B1	Male	0.1198 ± 0.0117	0.0810 ± 0.0070	-32.4% (0.0078)
	Male	0.0789 ± 0.0081	0.0852 ± 0.0047	+7.98% (0.31)
PGMinvrpt(2)12A1	Male	0.1569 ± 0.0118	0.0723 ± 0.0077	-53.9% (0.0005)
	Male	0.0729 ± 0.0075	0.0736 ± 0.0041	+0.96% (0.89
PGMinvrpt(2)37A1	Male	0.2038 ± 0.0134	0.1241 ± 0.0077	-39.1% (0.0009)
	Male	0.1631 ± 0.0089	0.1179 ± 0.0054	-27.7% (0.0017)
PGMinvrpt(X)41A2	Female	0.2853 ± 0.014	0.1757 ± 0.002	-38.4% (0.0002)
Control 1	Male	0.1014 ± 0.0029	0.1019 ± 0.0093	+0.5% (0.932)
Control 2	Male	0.0997 ± 0.0038	0.1118 ± 0.0038	+12.0% (0.017)

amino-acid substitutions that are not shared by the Ives slow allele. The data are consistent with the conclusion that *Pgm* is highly polymorphic and subject to selection in natural populations [40,41]. Consistent with this idea, *Pgm* haplotypes and glycogen content have been found to vary among flies at different latitudes [42].

The tet-on promoter system allowed a test of the hypothesis that increasing or decreasing *Pgm* gene expression would affect lifespan. PGM enzyme levels were increased two- to fivefold using a sense transgenic construct, and *Pgm* RNA levels were decreased 1.3- to 24-fold by driving expression of an inverted-repeat construct. No changes in lifespan were detected that were consistent across multiple transgenic lines or replicate experiments. The results indicate that simply increasing or decreasing *Pgm* gene expression does not significantly affect the lifespan of adult *Drosophila*, at least under the conditions tested.

The Pgm^S allele used in these experiments was cloned from the Ives strain, which is the progenitor of the long-lived selected strains and their controls. The high degree of polymorphism of *Pgm* makes it possible that the slow electrophoretic allele in the Ives strain could consist of multiple DNA sequence forms. The failure to observe effects on lifespan could therefore conceivably have resulted from overexpression of the wrong DNA sequence form of Pgm^S. However, Pgm^S was cloned and sequenced from four independent lines derived from the Ives strain and the clones had the same DNA sequence. The data therefore suggest that most, if not all, of the Pgm^S alleles in these strains have the same sequence, making sequence heterogeneity an unlikely explanation of the present results.

The results suggest several possibilities for the relationship of *Pgm* allele frequency to the increased lifespan of the selected strains. The first is that Pgm^S might in fact contribute to the increased lifespan of the selected strains as a result of an increased or decreased activity, but that the effect on lifespan is too small to be detected in these assays. The lifespan assay appears to have a variability with control strains of approximately -5% to +5%, as seen in the data presented here and elsewhere (G.L., D. Bhole and J.T., unpublished results). It is therefore unlikely that an effect much smaller than 10% could be identified. The second possibility is that *Pgm* allele frequencies are altered because of selection for an unknown linked gene, and this possibility cannot be ruled out at this time. The experiments presented here

Table 2

Mean lifespan at 25°C

		Mean lifespan (25°C)		
Line name	Sex	-DOX	+DOX	Percentage change (*p*-value)
PGMsense(3)33A1	Male	56.723 ± 0.763	56.048 ± 0.743	-1.19% (0.53)
	Male	66.921 ± 0.746	64.955 ± 0.731	-2.94% (0.060)
PGMsense(3)33B1	Male	58.167 ± 0.777	54.140 ± 0.817	-6.92% (0.0004)
	Male	68.169 ± 0.686	67.966 ± 0.614	-0.30% (0.83)
PGMsense(2)43A2	Male	68.256 ± 0.845	65.236 ± 0.870	-4.42% (0.013)
	Male	71.202 ± 0.730	67.268 ± 0.860	-5.53% (0.0005)
PGMsense(2)52A1	Male	63.720 ± 0.844	67.568 ± 0.939	+6.04% (0.0024)
	Male	70.610 ± 0.607	72.508 ± 0.803	+2.69% (0.060)
PGMsense(X)27A1	Female	89.660 ± 0.992	84.275 ± 0.806	-6.01% (<0.0001)
Control	Male	58.568 ± 0.939	58.930 ± 0.938	+0.62% (0.79)
PGMinvrpt(3)76	Male	59.957 ± 0.825	69.817 ± 0.547	+16.45% (<0.0001)
	Male	66.428 ± 0.561	66.801 ± 0.640	+0.56% (0.66)
PGMinvrpt(3)23B1	Male	55.415 ± 0.965	56.486 ± 1.014	+1.93% (0.45)
	Male	69.061 ± 1.302	71.339 ± 1.253	+3.30% (0.21)
PGMinvrpt(2)12A1	Male	71.111 ± 0.979	75.099 ± 1.100	+5.61% (0.0071)
	Male	75.958 ± 0.877	72.464 ± 0.917	-4.60% (0.0060)
PGMinvrpt(2)37A1	Male	59.855 ± 0.647	63.297 ± 0.656	+5.75% (0.0002)
PGMinvrpt(X)41A2	Male	83.443 ± 0.727	82.672 ± 0.948	-0.92% (0.52)
Control	Male	74.312 ± 0.941	70.947 ± 0.810	-4.53% (0.0066)

suggest that tet-on regulated overexpression and RNAi of genes near *Pgm* may be a promising approach.

Third, an interesting model is that Pgm^S contributes to increased lifespan of the selected strains because of an alteration in its regulation and/or subcellular localization. The sequence analysis of the *Pgm* alleles reveals a novel potential site in Pgm^S for phosphorylation by PKC; however, the potential significance of this change is unknown. The Pgm^S allele was used for the overexpression experiments presented here, and presumably the overexpressed enzyme should be subject to any regulation or localization characteristic of Pgm^S. However, the Pgm^S enzyme was overexpressed in a 'wild-type' Pgm^M background, and it may be that any phenotypic consequences of Pgm^S allele function are not apparent when Pgm^M is also present. Finally, another possible explanation for the absence of an effect on longevity is the method of assay. The selected O stocks were handled and assayed with males and females together, repeatedly mating during adult life. The present assay used segregated sexes, which is a different environment. This was done because the segregated-sexes assay is sensitive to even small changes in lifespan, and has been used successfully in the past to detect the effects of other genes on lifespan. It is possible that an effect of PGM on lifespan might be identified with different lifespan assay conditions. However, even if this explanation were to be true, the data still indicate that PGM does not have a simple strong effect on longevity. Further experiments will be required to distinguish between these interesting possibilities for the link between *Pgm* allele frequency and lifespan.

Materials and methods

Drosophila stocks and culture

All transgenic *Drosophila* stocks were generated by P-element germline transformation [43], using a modified microinjection technique [44]. Transgenic lines are named as the construct, followed by the chromosome of insertion in parentheses, followed by a unique line designation. For example, PGMinvrpt(3)76 is PGMinvrpt construct inserted on chromosome 3, independent transgenic line 76. Cytological sites of insertion were not determined. Multiple strains homozygous for each of the Pgm^F, Pgm^M and Pgm^S alleles were generated by purifying third chromosomes from the Ives stock, which is the precursor to the O and B selection stocks [19]. Homozygosity for a particular *Pgm* allele was confirmed by starch-gel electrophoresis assay, as previously described ([32] and data not shown). Other *Drosophila* strains are as described [45]. All stocks were grown on standard cornmeal-agar medium [46] and were cultured at 25°C.

Table 3

Mean lifespan at 29°C

		Mean lifespan (29°C)		
Line name	Sex	-DOX	+DOX	Percentage change (*p*-value)
PGMsense(3)33A	Male	40.902 ± 0.834	43.910 ± 0.782	+7.35% (0.0092)
PGMsense(3)33B	Male	45.693 ± 0.581	44.663 ± 0.618	-2.25% (0.23)
PGMsense(2)43	Male	51.298 ± 0.626	53.989 ± 0.719	+5.25% (0.0050)
PGMsense(2)52	Male	49.874 ± 0.541	48.681 ± 0.647	-2.39% (0.16)
PGMsense(X)27A	Male	35.010 ± 0.470	36.030 ± 0.619	-2.91% (0.19)
PGMinvrpt(3)76	Male	48.827 ± 0.404	48.568 ± 0.621	-0.53% (0.73)
PGMinvrpt(3)23	Male	45.358 ± 0.606	47.726 ± 0.566	+5.25% (0.0046)
PGMinvrpt(2)12	Male	48.759 ± 0.647	51.646 ± 0.598	+5.83% (0.0011)
PGMinvrpt(2)37	Male	49.863 ± 0.583	51.826 ± 0.679	+3.93% (0.029)
PGMinvrpt(X)41	Male	41.747 ± 0.499	39.539 ± 0.981	-5.28% (0.046)

Lifespan assays

Strains homozygous for the transgenic constructs were crossed to rtTA(3)E2 strain, homozygous for the rtTA transactivator construct, to generate progeny heterozygous for both constructs [1]. Control flies were rtTA(3)E2/+, that is, lacking a target construct. To obtain adult flies of defined age, the crosses were cultured at 25°C in urine specimen bottles. Before eclosion of the majority of pupae, bottles were cleared of adults and newly eclosed flies were allowed to emerge over the next 48 h. Most of the males will have mated during this time. The males only were then removed and were designated one day old, and were maintained at 25°C at 40 per vial in culture vials with food, and passaged to new vials every 48 h. For certain experiments the adult males were maintained at 29°C, as indicated. For certain experiments, female flies were used, as indicated. Those flies being fed DOX were kept on food vials supplemented with 250 μg/ml DOX.

DNA sequencing

All DNA sequencing reactions were done using the dideoxy chain-termination protocol and T7 Sequenase v2.0 (Amersham). Multiple clones were sequenced for each of the Pgm^F, Pgm^M and Pgm^S alleles. The clones were obtained by polymerase chain reaction (PCR) amplification of each allele using genomic DNA template isolated from strains homozygous for each allele. The primers used in each amplification are as follows. The primer locations were assigned on the basis of their relative distance from the A in the ATG start codon, which was defined as position +1.

Pgm 5′-end-2 (-27 to -4): 5′-AGCCAGCAGCCGGAAAACTCCAGT-3′; *Pgm* 3′-end (+1706 to +1727): 5′-GGATGGGTTGGTAATCTCAGTG-3′. Each PCR product was gel-purified and cloned into the *Eco*RV site of pBluescript KS+ (Stratagene). Predicted protein motifs were identified using Prosite software [47].

Phosphogluconate mutase enzyme activity assays

The activity assay was carried out essentially as described [48]. Briefly, ten male flies were anesthetized with CO_2 and placed in 200 μl ice-cold 0.1 M Tris buffer solution (pH 7.6). The flies were homogenized using a motorized mortar and pestle. An additional 200 μl Tris buffer was added for a total volume of 400 μl. The sample was centrifuged for 20 min at 12,000*g* at 4°C. Approximately 200-300 μl supernatant was removed, taking care to avoid a top layer of oil and debris. For the assay, 30-40 μl of this supernatant was used in a 3.1 ml assay mixture that contained: 1.5 mM $MgCl_2$, 26 mM histidine solution (pH 7.6), 8 μM glucose-1,6-diphosphate, 0.45 U/ml glucose-6-phosphate dehydrogenase, and 0.2 mM NADP. This solution was mixed at room temperature and then 5 mM glucose 1-phosphate was added. The solution was mixed again and placed in a spectrophotometer. To insure that readings were being taken in the linear range of the enzyme activity, standard curves were generated with respect to both time and amount of protein added. Readings were taken at t = 6 min at OD_{340} and were expressed as ΔOD/μg/min. Protein concentrations were determined using the BioRad protein concentration solution using BSA as standard. All extracts were assayed in triplicate, and the averages and standard deviations are presented. Means were compared using unpaired, two-side *t*-tests and *p*-values are presented. The enzyme assay measures the conversion of $NADP^+$ to NADPH rather than a direct measure of phosphoglucomutase itself. In other words, it is measuring a secondary, coupled reaction.

DNA constructs

All inserts for microinjection were cloned into a derivative of the 7T40 construct [1] called USC1.0. USC1.0 was generated

as follows: First, 7T40 was digested with *Eco*RI, thus liberating the Hsp70 polyadenylation signal sequence. The construct was then religated and transformed. Next, the *Pst*I site in polylinker 1 was destroyed by partial *Pst*I digestion followed by T4 DNA polymerase fill-in and ligation. Clones were screened for destruction of the correct *Pst*I site to generate USC1.0 (Figure 2a). All inserts were cloned into the unique *Pst*I to *Eco*RI sites of USC1.0.

The *Pgm* sense construct was made as follows. The reverse transcription was carried out on total RNA isolated from a strain of flies homozygous for the Pgm^S allele, using the following primer: dTB_IB_O: 5′-TAACCCGGGTCTACAAAGTGATACTGCGTAACTGACTATATTTTTTTTTTTTTTTT-3′. A nested PCR strategy was then used to amplify the Pgm^S cDNA using the following primers: First primer set: B_I: 5′-TAACCCGGGTCTACAAAGTG-3′. *Pgm* 5′-end-2 (-27 to -4): 5′-AGCCAGCAGCCGGAAAACTCCAGT-3′. Second (nested) primer set: B_O: 5′-ATACTGCGTAACTGACTATA-3′. 5B6 (+1,520 to +1,534): 5′-CTGGAAGCTCGGGAG-3′. The resulting PCR product was cloned into the *Eco*RV site of pBluescript KS+ and clones were screened for the correct orientation. The Pgm^S cDNA was then liberated by *Eco*RI complete digest followed by *Pst*I partial digest and cloned into the *Eco*RI to *Pst*I sites of USC1.0 to generate construct PGMsense (Figure 2b).

The PGMinvrpt construct was made as follows: because the final insert would be a large inverted repeat, Pgm^S was PCR-amplified in two pieces, in opposite orientations. The two sets of primers used for the amplifications were: First set: *Pgm*-sense (3′) (+1,773 to +1,794): 5′-AGCTGAATTCCACAAACTTTAATAAATCCGAAAC-3′. *Pgm*-anti Not (+711 to +731): 5′-AGCTGCGGCCGCCCTGAACCGGTTGGGTGCCAC-3′. Second set: *Pgm*-anti Not (+711 to +731): 5′-AGCTGCGGCCGCCCTGAACCGGTTGGGTGCCAC-3′. *Pgm*-anti Pst (+1,773 to +1,794): 5′-AGCTCTGCAGCACAAACTTTAATAAATCCGAAAC-3′. Each of these PCR products was cloned individually into the *Eco*RV site of pBluescript KS+, and then restriction digested with *Eco*RI and *Not*I (first-set fragment) or *Not*I and *Pst*I (second-set fragment). Each of the liberated fragments was gel purified and triple-ligated into the *Pst*I to *Eco*RI sites of USC1.0 to generate construct PGMinvrpt (Figure 2c).

Northern analyses

RNA was extracted using Trizol reagent (Gibco BRL) and the amount of RNA was quantitated by UV spectrophotometry. Approximately 5-10 μg RNA per lane was resolved on a 1.0% agarose gel. RNA markers (Gibco BRL) were used to determine the sizes of bands. After blotting, the RNA was fixed to Genescreen nylon membrane (Dupont) using a UV crosslinker (Stratagene). Probes were ^{32}P-labeled using the Prime-It II kit (Stratagene). To determine fold increases/decreases, northern blots were exposed onto a phosphor screen and results were analyzed using a phosphorimager (ImageQuaNT, Molecular Dynamics). Ribosomal protein 49 gene (*Rp49*) [49] was used as a loading control, and all *Pgm* northern data is normalized to *Rp49* RNA levels.

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