AMERICAN JOURNAL OF MEDICAL AND CLINICAL SCIENCES, 2022
VOL 7, NO. 2, PAGE 1 – 5

RESEARCH ARTICLE

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CPT1 and PyK Gene Expression Changes with Different Physiological Conditions between 2 Resistant to Starvation Fly Lines: A Basic Science Experiment

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ABSTRACT

Background: Many organisms adapt to different physiological conditions by changing their gene expression to increase their chances of survival. *Drosophila melanogaster* fly line 765 show low resistance to starvation and line 362 show high resistance to starvation and were selected to examine how their gene expression would change based on a starved and re-fed environment. The genes selected were both metabolic genes CPT1 and PyK. CPT1 is responsible for fat breaking and PyK is responsible for the catalysis of the final stage in glycolysis.

Methods: Specific *Drosophila melanogaster* fly lines were selected that showed either a high or low resistance to starvation. These lines were selected to examine how their gene expression would change based on a starved and re-fed environment. RNA was isolated from both sets of flies so that reverse transcriptase reactions could be performed using RT-PCR to generate complementary DNA using reverse transcriptase. The complementary DNA served as a template for amplification using endpoint and quantitative PCR to determine if gene expression would change between the two fly lines. A survival curve was also generated to illustrate the graphical differences among the various fly lines.

Results: Quantitative PCR and gene expression data concluded that there was a difference between gene expression of CPT1 and PyK between the two lines. CPT1 quantitative PCR data did not match gene expression predictions, whereas, PyK did in both line 765 and 362.

Conclusions: The finding that CPT1 was able to generate C_t values but was unable to adhere to the expected predictions of gene expression is indicative of line 765 having a problem sensing the nutrients and unlikely to be a problem in transcription. Future directions include to characterize both glycogen and triglyceride levels at time t=0 hrs, starved, and re-fed conditions and to investigate if there is a connection between enzyme efficiency and condition type between the two lines.

ARTICLE HISTORY

Received 30 May 2022
Accepted 27 Jun 2022
Published 02 Jul 2022

KEYWORDS

Biochemistry, CPT1, Energy, Metabolism, Obesity, Physiology.

Introduction

The objective of this experiment was to examine if there was any significant difference in gene expression between fly line 765 low and 362 high that accounts for their starvation resistance difference. The two fly lines, 765 and 362, exhibited drastically different starvation resistance capabilities that should be reflected by their varying gene expression of specific genes. Because there are hundreds of various metabolic genes, this analysis will focus on two specific antagonistic enzymes carnitine palmitoyltransferase I (CPT1) and pyruvate kinase (PyK) [1,2]. Due to their antagonistic nature, spotting any gene expression differences between them will be relatively easy to detect.

Throughout an organism's life, it will encounter multiple different environments both externally and internally through its physiology. The key to organismal survivability is how adeptly it can acclimate to these different changes. On a molecular level, there are different genes that are activated when an organism is exposed to a starvation environment than when that same organism is exposed to an environment with an abundance of food. Biochemically, this disparity between the activation or inactivation of certain genes based on physiological needs is called gene expression [3].

If the environment that the organism is in is considered the input, then the output would be how the organism decides to either activate or inactive various genes to assist in its survival. The way that researchers can measure to what extent an organism's gene expression changed depending on its environment is through an analytical method called quantitative polymerase chain reaction (qPCR). qPCR uses fluorometry and

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low limit of detection (LOD) values to amplify molecules of DNA that can even start from a single molecule of DNA [4]. A very simple method requires a test tube, simple reagents, and a source of heat to properly run the reaction. Normally, heat would denature the enzyme responsible for copying the DNA, polymerase, but PCR methods utilize a special polymerase called Taq polymerase or *Thermus aquaticus* [5]. Taq polymerase is a special kind of enzyme because it is a thermophile and lives in areas of very hot temperatures such as hot springs or the hydrothermal vents of volcanos. This evolutionary adaptation allows subjecting Taq polymerase to high temperatures without the enzyme being denatured or a reduction in functionality.

There are three main stages of any PCR reaction, which are denaturing, annealing, and extension [6]. Denaturing is where the DNA is subjected to high temperatures that actually unwinds the double helix formation and makes it accessible to the primers and polymerase enzymes. Annealing is where the primers are introduced that bind to the open DNA with the help of lowering the temperature to around 60 °C. Extension is where the polymerase elongates the strands of the DNA and thereby, amplifying it as the end result. The whole PCR process is summarized in figure 1 below.

Figure 1 below shows a summary of the individual steps of PCR – denaturation, annealment, and elongation. (Wikipedia Commons)

The qPCR analytical method uses a fluorometric detector to measure the intensity of the sample, which is proportionate to sample concentration [7]. Even though qPCR uses a fluorometric detector, not all biological samples absorb light and fluoresce so a fluorescent dye called SYBR green binds to the DNA after each cycle of amplification, as can be seen in figure 2 below. This dye fluoresces and the intensity of the light is directly proportional to the amount of DNA concentration present. A specialized instrument, called a thermocycler, and then generates a C_t value (cycle threshold) which is the cycle number where concentration was detected that was above the background fluorescence levels. The C_t value is inversely proportional to concentration; as high concentration will result in a low C_t value because it took a lower number of cycles to detect sufficient product accumulation over background levels. This C_t value is highly variable depending on the amount of template used and needs to be normalized in order to compare different genes. A reference gene is used for this normalization factor and is usually a housekeeping gene such as rp49 or actin. A housekeeping gene has consistent levels of expression ubiquitously throughout the cell that is unaffected based on changing environmental conditions.

Figure 2 below shows how the addition of dye SYBR green allows the qPCR detector to quantify the fluorescence emitted, which is proportional to concentration. (German Cancer Research Center).

The two genes of interest for this experiment are CPT1 and PyK. CPT1 is involved in the process of β-oxidation of fatty acids [8]. CPT1 brings fatty acid molecules into the mitochondria for breakdown into energy. For its fatty acid breakdown role,

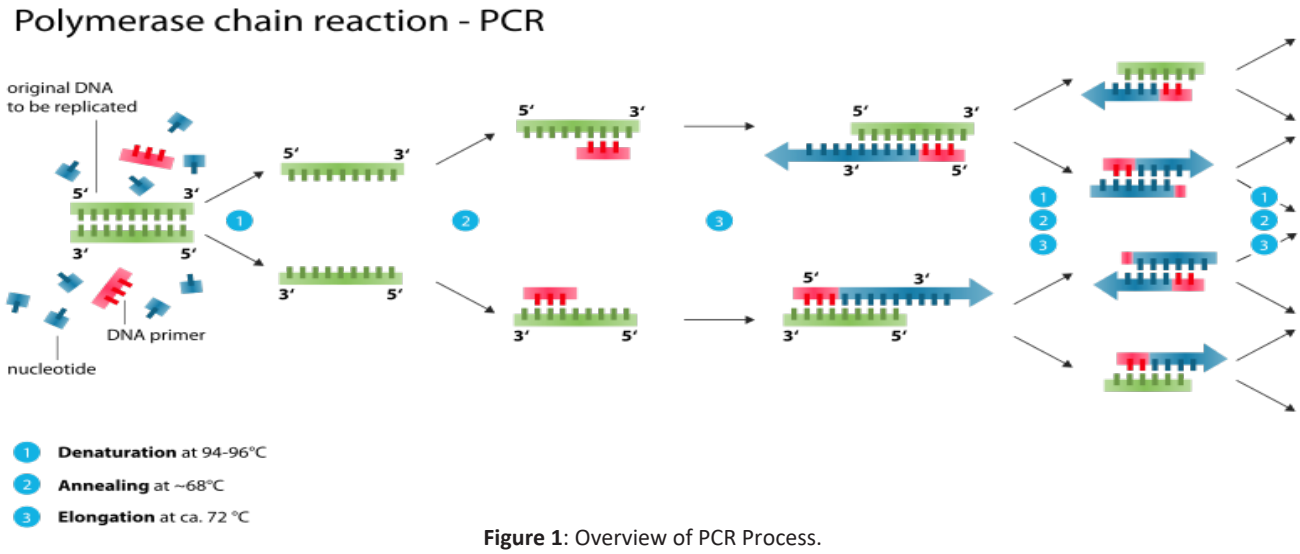

Figure 1: Overview of PCR Process.

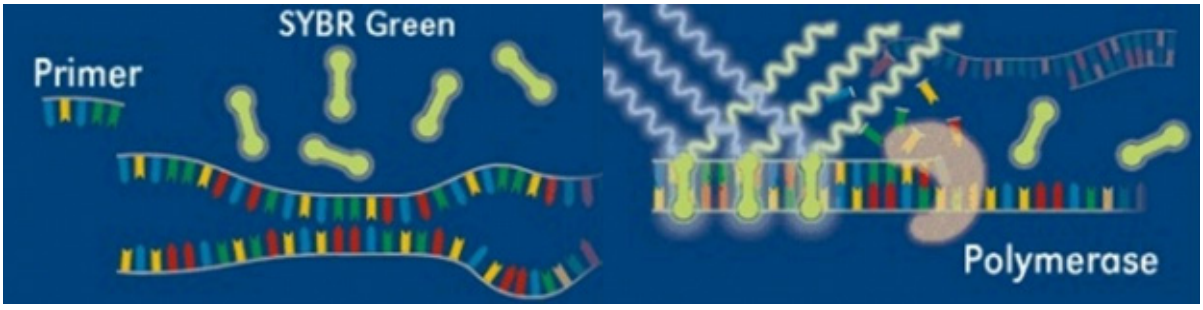

Figure 2: SYBR Dye used for Fluorometrics in qPCR.

CPT1 is implicated with starvation responses and as such, would be expected to have high gene expression during the starved time slot and low gene expression during the re-fed time interval. Conversely, PyK is a major glycolytic enzyme and is responsible for the catalysis of the final reaction in the glycolysis pathway. PyK is responsible for the phosphorylation of phosphoenolpyruvate (PEP) to pyruvate and ATP as a by-product [9]. Since you would not expect an organism to be wasting energy trying to run glycolysis during a starved environment, gene expression would be low for that state, but high for the re-fed condition. All these hypotheses have been tabulated and summarized in table 1 below.

Gene	Expected (starved + re-fed)	
CPT1	↑	↓
PyK	↓	↑

Table 1: Predictions of Gene Expression.

Table 1 above shows the summation of the various gene expression predictions for the two genes – CPT1 and PyK.

Methods

Isolated RNA from adult female flies of both 765 and 362 fly lines using trizol and RNeasy with on-column DNAse digestion protocol. The RNA was then quantitated using Nanodrop and the results tabulated in table 2 below. Reverse transcriptase (RT) reactions were then performed for the generation of cDNA using 2.5 μL of 100 μM oligo dT primers specific for CPT1 and PyK and used 1 μg of RNA template for both CPT1 and PyK per 40 μL reaction tube. The primers for each specific gene were then verified by end PCR. Each gene used 2.5 μL of 10 μM forward and reverse primers for a 25 μL reaction size. The primer sequences for each gene, along with the reference gene rp49, is tabulated below and the PCR program that was used was initial denaturation for 10 min at 94 °C, 30 sec at 95 °C, 1 min at 60 °C, 30 sec at 72 °C, repetition of prior steps for 30 cycles, and hold at 10 °C.

Sample	No. samples	Concentration (ng/μL)	260/280
765 – 0 hrs	29	2135	2.01
765 – 42 hrs starved	32	2460	2.24
765 – re-fed	40	3798	2.15
362 – 0 hrs	52	518	2.18
362 – 65 hrs starved	29	6228	2.21
362 – re-fed	43	2285	2.22

Table 2: Nanodrop Summation.

Table 2 above shows a summary of the Nanodrop data including the concentration, number of samples, and the purity (260/280) ratios of each sample.

Table 3 below shows the specific sequences of the experimental genes CPT1 and PyK, along with the primer sequence for the reference gene rp49.

Sequence of Primers		
Gene	**Forward primer (5' to 3')**	**Reverse primer (5' to 3')**
Pyk	CATCTACGATGAGGCACCGCAG	CGATCAGACCGGTACGGATCTC
CPT1	GCAAGTGCAAATTGAGGAAA	AAGTGCTCCTCACCTTCCAC
rp49	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG

Table 3: Primer sequence of Genes.

qPCR was performed using the same parameters as PCR above. The primer concentration for CPT1 primers and PyK were 500 nM and 400 nM, respectively, for both forward and reverse primer pairs in a 20 μL reaction size. Any reactions based off qPCR data, such as expression values, were calculated using C_t values and by using the $\Delta\Delta$CT and Pfaffl method.

Results

This experiment was designed to investigate the level of gene expression and the mechanism that is activated in a starved state using the fly model. Figure 3 shows the time it took for each fly line to starve to death.

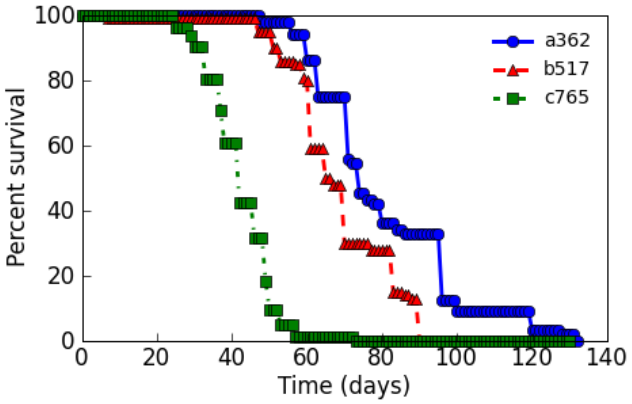

Figure 3: Survival Curve among Three Separate Fly Lines.

Figure 3 above shows the percent survival among the different fly lines.

CPT1 and PyK were selected as the genes of interest due to their antagonistic natures in lipid metabolism. Primer efficiencies were calculated for each fly line and gene to determine how many strands of genetic code was made. Both sets of primers had great efficiency, as they were >70% indicating robust copying and functionality of the primers used. The exact primer efficiencies can be seen in table 4.

Gene	765 Primer Efficiency (%)	362 Primer Efficiency (%)
CPT1	88	153
PyK	81	70

Table 4: Primer Efficiencies for CPT1 and PyK.

Table 4 above shows the breakdown of the primer efficiencies for both CPT1 and PyK.

qPCR was performed on CPT1 and PyK across both sets of fly lines. Rp49 was used as a control, as this is a constitutively active gene and came in at a C_t value of 18-19, which is consistent with the control values. The qPCR shows that CPT1 consistently comes in at lower C_t values when compared to PyK, irrespective of which fly line was tested. PyK took 34 cycles to amplify for

the 765 fly line for both starved and re-fed, but showed a higher expression for the re-fed condition of the 365 line.

Gene	765 (42 hrs starved + re-fed)		362 (65 hrs starved + re-fed)	
CPT1	28.4	30.1	32.6	30.2
PyK	34.3	34.5	36.2	32.2
RP49	19.3		18.5	

Table 5: Individual Gene C_t Values.

Above shows, the cycle number at which enough product accumulated to be detected above background levels.

The main objective of this study was to determine if line 765 had different gene expression than line 362 because the two lines exhibited different resistance to starvation. The qPCR data allows direct comparability between the differing gene expressions due to the C_t value. The results have been tabulated below for summary.

Gene	Method	765 (42 hrs starved + re-fed)		362 (65 hrs starved + re-fed)	
CPT1	$\Delta\Delta_{CT}$	1.23	1.38	0.32	2.3
	Pfaffl	1.59	1.54	0.21	2.3
	AVG	**1.41**	**1.46**	**0.27**	**2.3**
PyK	$\Delta\Delta_{CT}$	0.063	0.19	0.12	2.9
	Pfaffl	0.10	0.26	0.20	2.3
	AVG	**0.082**	**0.32**	**0.22**	**2.6**

Table 6: Expression Values comparing CPT1 and PyK.

Table 6 above compares the expression values between CPT1 and PyK in a starved and re-fed condition breakdown as well as displaying 2 different analytical calculations.

Discussion

Several conclusions can be drawn from the survival curve in figure 3. The c765 line (green) was the line that died out the quickest and was not able to effectively resist the starved environment for prolonged period. Conversely, the a362 line (blue) resisted starvation for the longest period. This data can be used to springboard to more fundamental questions such as how does the level of gene expression compare between the line that was able to resist starvation the longest (a362) vs the line that wasn't as successful (c765). Because of the drastic difference between the two lines and their unique responses to a starved environment, 765 and 362 fly lines will be used to further investigate changes in gene expression.

CPT1 and PyK were the genes selected for this experiment because they should exhibit antagonistic patterns during the starved or re-fed condition. CPT1 is responsible for β-oxidation of fatty acids and is a fat breaker. A such, CPT1 gene expression would be expected to be high during a starved state, for the enzyme would break down fat stores to provide nutrition for the organism. The opposite effect would also be seen in CPT1 gene expression during a re-fed environment, as there would be no physiological need to break down fat reserves when food is abundant. Exactly the opposite trends would be expected for PyK gene expression. PyK is an enzyme responsible for the catalysis of the last stage of glycolysis: the conversion of PEP to pyruvate via a phosphorylation reaction. In a starved state, there would be no physiological advantage of running glycolysis because resources are scarce and energy is valuable. Thus, PyK gene expression values should be low during a starved state. However, during a re-fed environment, PyK gene expression would be expected to be high because of the abundance of glucose within the organism and PyK's role in the glycolytic pathway and breaking it down into energy.

Both primers worked well as can be seen by the high primer efficiencies in table 4. PyK's primer efficiencies ranged from 70-81%, which is a decent efficiency for primer annealment. CPT1's primer efficiencies were better than PyK based on higher percent primer efficiencies. The primer for the 362 line was x > 100% which could be the result of several things. Having such a high primer efficiency could be the result of primer dimers, pipetting errors, or a non-specific interaction by the primers. In addition, the 100% maximum value is relatively arbitrarily set, as a primer efficiency of 100% means that the primers perfectly copied 1 strand giving a total yield of 2; however, if the primer copied more than the one strand, there would be a primer efficiency greater than 100% meaning more than one strand was copied.

qPCR data returned expected results of CPT1 765 line starved condition. The C_t value for that line was 28.4 indicating that there was a lot of product, as lower cycle numbers means more product. The re-fed condition's C_t values was 30.1 meaning that it required more cycles of amplification to occur before product accumulation exceeded background levels. The data is consistent with CPT1's role as a fat breaker for line 765. Conversely, qPCR data for line 362 was not expected because that line displayed low expression for the starved condition and high expression for the re-fed condition. This is counter-intuitive and the opposite of what was expected. Line 765 was the least resistant to starvation, as can be seen on the survival plot in figure 3, but it was the only fly line that had matched experimental gene expression with predicted gene expression.

qPCR data for PyK gene was consistent with predictions only for the starved condition across both fly lines. The starved condition for lines 765 and 362 saw low gene expression, however, saw only saw high expression for the re-fed condition in the 362 line. The 765 line displayed low gene expression values throughout both conditions, which was much unexpected. The 362 line for PyK gene was the only one that matched experimental results with prior predictions, as the starved condition saw low expression and the re-fed condition saw high expression. This is consistent with the glycolytic role of PyK, as in a starved condition, it would be unfavorable to waste energy and precious resources to run glycolysis. The opposite is also true, as in a re-fed condition, it would make sense to have high expression of PyK to break down the excessive glucose concentrations into energy.

The reference gene, rp49, was also measured as a quality assurance control. Rp49 is expected to come in at a low C_t value at around 18-19. qPCR data confirms that rp49 is not only expressed at consistent levels, but also at high levels as for both 765 and 362 lines, the rp49 C_t value came in between the expected 18-19 cycle number. This confirms that the master mix, primers, and PCR protocol are working properly.

The data in table 6 shows that line 765 has inconsistencies because there was no change between the starved and re-fed conditions for CPT1. This does not match the predictions because CPT1 gene expression should be higher in a starved state than in a re-fed state; however, the two expression values are relatively the same. The same line for PyK worked better than it did for CPT1 because it shows low expression for starved and high expression for re-fed. This is more in line to the predictions and what would be expected for a glycolytic enzyme.

Conversely, the 362 line worked well because it responded as expected to the starved and re-fed conditions. Even though it doesn't match the predictions for CPT1, it matches PyK's very nicely. PyK would have low gene expression during a starved condition environment but would drastically see an increase in expression during a re-fed condition environment. The data substantiates this claim and is displayed below. A possible reason that would explain the disparity between the experimentally obtained data and the predictions would be if the 765 line has a problem detecting the nutrients in the condition. The C_t values clearly indicate that it is unlikely to be problem with transcription as there is enough activity to give a C_t value meaning the message is propagated. However, if there was a problem in line 765's ability to sense the nutrient, then it would result in similar results as the ones experimentally obtained.

Conclusion

The main purpose of this experiment was to determine if line 765 and 362's gene expression can account for their drastically different resistance to starvation. Line 765 had a very low resistance to starvation, with 50% of all samples dying off at the end of 41 days; whereas, line 362 exhibited high resistance to starvation, with 50% of all samples dying off at the end of 78 days. The qPCR data strongly suggests that there is a significant difference between the levels of gene expression of CPT1 and PyK in the different fly lines. The PyK gene behaved as it was expected to in both line 765 and 362. PyK functions as the final stage of the glycolytic pathway and would be expected to have low gene expression in the starved condition so as not to waste energy running glycolysis. Conversely, PyK expression levels should be high during the re-fed conditions to allow the breakdown of glucose from the resources ingested. The lab data substantiates this claim as lines 362 and 765 experimental data matches the predictions.

CPT1 data was not as expected for either line 765 or 362, as both lines showed the opposite trends of the predictions. CPT1 is a fat breaking enzyme and is responsible for β-oxidation and transporting fatty acids into the mitochondria for breakdown. Thus, it should have high gene expression during a starved state and low expression during re-fed state. However, neither of these trends could be seen together across both lines. The conclusion that line 765 did not respond at the level of transcription means there is unlikely to be a problem with transcription itself. A possible reason for the disparity between experimental results and predictions is that line 765 may have a problem sensing the nutrients. This would be consistent with the data collected because there is enough gene expression for a C_t value to be registered, which means that a transcriptional message is generated. However, if line 765 has difficulty sensing nutrients then the data obtained would be similar to the data collected in this experiment.

Future directions would be to investigate the glycogen levels and triglyceride (TG) levels. This can be done by performing simple assays like a TG assay to measure if the change in gene expression significantly affects the level of TG found within the organism. Both glycogen and TG levels would be characterized at time t=0 hrs, then at two additional points (starved and re-fed) to determine if one line has more concentration than the other. This experiment will also reveal if the two enzymes CPT1 and PyK have differing levels of efficiency in either a starved or a re-fed environment, which can also help to explain the reason why PyK matched predictions and CPT1 did not.

Gene	Expected (starved + re-fed)		765 low (starved + re-fed)		362 high (starved + re-fed)	
CPT1	↑	↓	↑↑	↑↑	↓↓	↑↑
PyK	↓	↑	↓↓	↓↓	↓↓	↑

Table 7: Summary of Experimental data and Prior Predictions.

Table 7 above shows the summation of the various trends that were either predicted or experimentally determined for CPT1 and PyK gene expression.

Declarations

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article

Authors' contributions

BB conducted the experiments under the supervision of AN, whose research lab was used and who provided all experimental materials and equipment. BB wrote all sections of the manuscript with editing and guidance from AN.

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