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Expression of Adipokinetic Hormone (AKH) - Encoding Gene via Luciferase Reporter in Drosophila under Dietary Stress

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UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

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PROJECT TITLE: Expression of Adipokinetic Hormone (AKH)-Encoding Gene via Luciferase Reporter in Drosophila under Dietary Stress

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

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ABSTRACT

Adipokinetic hormones (AKHs) are the only known metabolic neuropeptides synthesized in intrinsic neurosecretory cells of the corpora cardiaca of many insects. Found in a wide variety of isoforms in several species, AKHs are used to mobilize carbohydrate and lipid substrates from the fat body for use by other parts of the body. Compared to glucagon in mammals, AKH peptides are believed to be responsible for inhibition of protein-, lipid-, and RNA synthesis and contraction of certain muscles. It is also related to an increase in the disaccharide trehalose, which has been linked to resistance to anoxic conditions for up to four hours with complete recovery. To assess whether this tolerance could be extended to dietary stress conditions, UAS-luciferase fruit flies were crossed with those containing UAS-AKH with a *gal4* driver and subjected to salt, paraquat, caffeine, and starvation periods and luminescence measured via a luminometer. No direct correlation between dietary stress and AKH expression could be determined, as results were inconsistent upon repeated trials and in comparison with flies lacking the *gal4* driver.

INTRODUCTION

Regulation of blood sugar levels is a fundamental process in invertebrate animals as well as the more complex mammals such as humans. Disturbance of the homeostatic levels can induce serious ramifications, diabetes for example. In humans, insulin and glucagon counter each other as one signals for glycogen synthesis (insulin) and the other

(glucagon) for glycogen breakdown resulting in the release of glucose and consequently increase in cellular energy.

AKH is also linked to this mode of action through binding to a G protein-coupled receptor, leading to the stimulation of phospholipase C. The second messenger inositol-3-phosphate initiates a signal cascade ending in the release of calcium from intracellular stores and entry of extracellular calcium into the cell. AKHs that go on to activate lipase and increase the concentration of diacylglycerides in the hemolymph or proline (because of fatty acid breakdown in beta-oxidation) cause effects through active adenylate cyclase and cAMP-mediated calcium flux. One further mechanism associated with lipid mobilization is AKH's influence in transport of lipids in the hemolymph via lipophorins. It has been implied that AKH has a role in increasing the capacity of lipids carried by these lipophorins, namely ensuing faster rates to which lipid can be moved from the fat body to the target organs (Gade 2004).

Lipid mobilization during flight, however, is not the only means of AKH influence in organisms. AKH is also believed to be responsible for contraction of specific muscles, esp. those involved in cardiacstimulation and inhibition of synthesis of RNA, fatty acids, and proteins in the fat body (Gade 2004). In fact, the original title given in 1969 to these peptides was hypertrehalosemic hormone (HTH) as it was noticed to have induced increased levels of trehalose, a nonreducing disaccharide the serves as one of the major blood sugar molecules in insects. As *Drosophila* use carbohydrates as their source for flight energy, it was difficult to recognize that later named adipokinetic hormone (AKH) was responsible for both carbohydrate and lipid mobilization (Lee 2004;Isabel 2004).

Flies in which AKH endocrine cells were ablated have been shown to survive longer periods of starvation through hypoactivity. These same mutants had decreased trehalose levels, suggesting that the loss of AKH stimulation is somehow associated with the decrease in trehalose. Organs lacking in the carbohydrate mobilization connected to AKH are unable to receive the necessary energy, thus decreased locomotion. Flies that have slower mobilization of their energy sources are then able to survive longer than wild-type flies as they are preserving the energy contained in their fat body stores (Isabel 2004). Could it be then that lacking in trehalose is actually indicative of longer survival periods in stressed environments?

Contradictorily, Trehalose is also connected with anoxia tolerance in flies with overexpressed trehalose-6-phosphate synthase (*tps1*). Trehalose reduces protein aggregation caused by anoxia and maintains proteins in a partially folded state while under stress, proteins that are later folded correctly by chaperones once stress is alleviated (Chen 2002). Under oxidative stress conditions, intracellular accumulation of trehalose has been correlated with a higher survival rate in *Drosophila* along with a decrease in protein denaturation, thus leading to its uses in cryopreservation of human lymphocytes, red blood cells, and lung slices (Chen 2004;Chen 2002). Furthermore, yeast cells have been shown to be thermotolerant and osmotolerant due to trehalose accumulation (Ribeiro 1999).

Previous work completed in this laboratory demonstrated that AKH stimulates metabolism leading to hypertrehalosemia and hyperlipidemia as well as, hyperactive behavior associated with food scourging. Starved flies responded to their environment with prolonged hyperactivity despite their normal biological rhythms (Lee 2004). Lee

and Park's study created the background for the hypothesis that AKH activity may be linked to survival mechanisms in flies during dietary stress i.e. incubation in salt, paraquat, caffeine, and diluted agarose for simulation of starvation without the potential for dehydration.

MATERIALS AND METHODS

Fly crosses. Prior to this experimentation, transgenic flies containing the UAS-*luc* were created in order to visualize expression of the gene in question, in this case, AKH. Fly lines with a *dAKH-gal4* driver were mated following virgin female selection with UAS-*luc* with the intention that progeny would express luciferase activity in place of the *dAKH* gene for monitoring via a luminometer.

Incubation parameters. Each fly culture contained 12 flies maintained for the respective 12 or 16 hour periods in a humified 24°C incubator. Solutions were made at least 24 hours prior to incubation start time and kept under refrigerated conditions. Solution concentrations are as follows: 5% salt-agarose, 5% paraquat-agarose, 5% caffeine-agarose, and diluted agarose.

Extract preparation. Following the desired incubation, flies were etherized and four chosen at random and placed in Eppendorf tubes. Upon awakening, they were once again knocked down on ice. Homogenates were created using Promega 1X CCLR buffer, each batch made directly prior each extraction, and kept on ice. Two rounds of centrifugation at 13,000K for 8 min in a cold room partitioned with removal of 100µl and then 50µl of clear extract followed (Promega 2000). It is imperative to avoid contamination of

samples with high levels of insoluble fraction and lipid as these can influence the amount of light received by the luminometer during emission. It is also noteworthy that during manipulations, extracts were restricted from light as much as possible.

Data Collection. Five microliters of final fly extract were added to 50µl of Promega luciferase reagent thawed at room temperature within 15 sec on data collection by the luminometer. Severe time constraints were placed on this step of the process to avoid light contamination causing the luciferase reagent to activate prematurely and much of the luminescence generated by the luciferase reporter protein to become lost to the outside atmosphere. Previous calibration of the instrumentation was achieved by running two distilled water blanks and one blank containing 1X CCLR buffer to achieve a baseline measurement.

RESULTS

Luciferase assay following 12 hr incubation under five constructs. Each of the five incubation solutions were selected on the basis of their varying effects on *Drosophila* eating habits and the metabolic results leading to their consumption over long periods of time. Salt was chosent as it is known to induce osmotic and ionic stresses in plant growth (Ueda 2004). Paraquat is a known inducer of oxidative stress (Girardot 2004) while caffeine is known to place flies under stresses involving disturbed biological rhythms preventing sustained sleep state (Ho 2005) and their inability to retain consumed energy as their increased locomotor activity quickly exploits any energy that is readily available. As previously discussed, flies exposed to starvation also display prolonged hyperactivity

associated with food scourging practices (Lee 2004). A level of control was achieved through sucrose only mixtures analogous to flies' preferential cuisine.

The second mode of including a built-in control is comparison of the UAS-*luc* x AKH-*gal4* flies with UAS-*luc* x *yw* subjected under the same conditions. It was hypothesized that UAS-*luc* flies crossed with wild-type *yellow white* exposed to the same dietary stresses would still display luminescence equivalent to baseline with some possible diversion due to the differences in material and some leakage of luciferase expression. However, it was not anticipated that there would be such variability between not only solutions but also the days in which experimentation was performed (Figures 1A-D).

Beginning with the initial trial, awkwardly high levels of luminescence in the constructs other than salt and control indicated a possible misnomer in research methodology (Figure 1A). Subsequent replications, however, did relieve some tension in results as ranges of refractory light units (RLUs) detected stabilized. (Figure 1B. Low,salt=318109, High,parquat=540732; Figure 1C. Low,salt=316766, High,caffeine=483064; Figure 1D. Low,sucrose only=339394, High,salt=403297.) Lacking in a standard construct with the lowest value only adds mildly to the fact that there is yet a clear baseline measurement to which differentiation of luminescence values in UAS-*luc* x AKH-*gal4* can be determined.

To address measurements of luminescence in flies containing the luciferase gene substituted for *dAKH*, variability in data is also a key concern for being able to create support for the hypothesis that AKH is induced during times of dietary stress in order to mobilize carbohydrates and lipids from the fat body for use by other tissues. A trend is

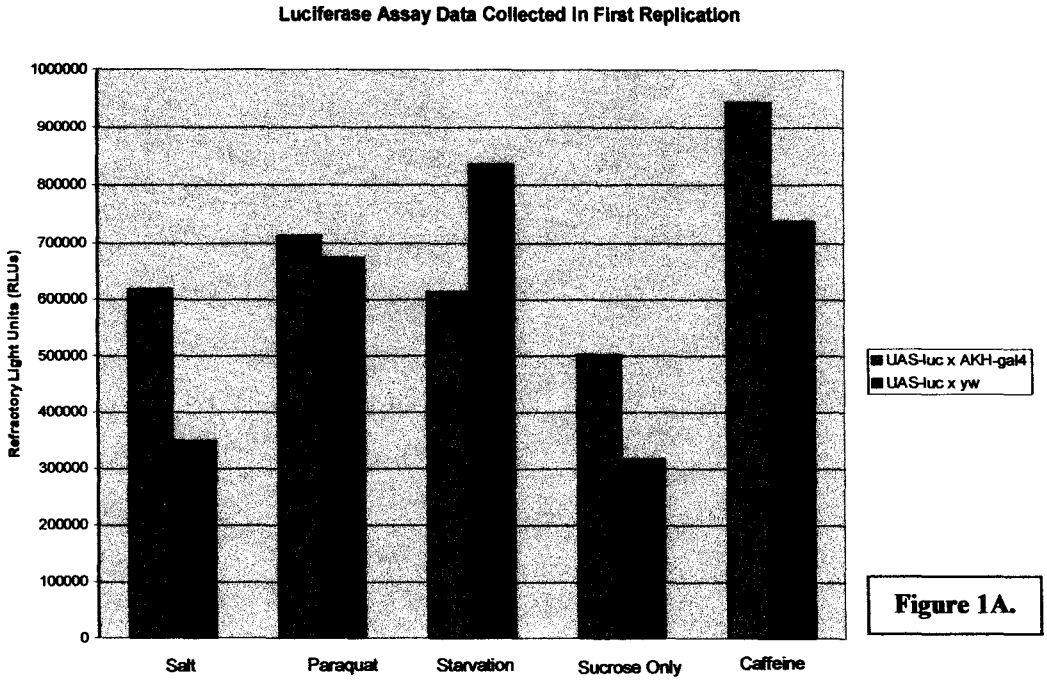
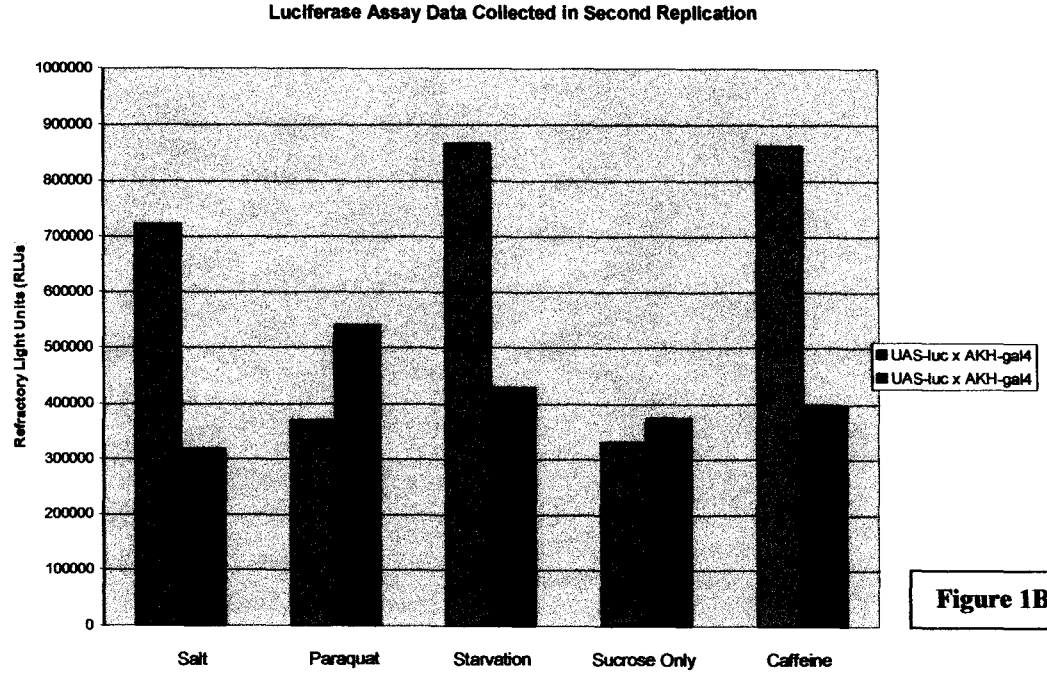


Figure 1A.



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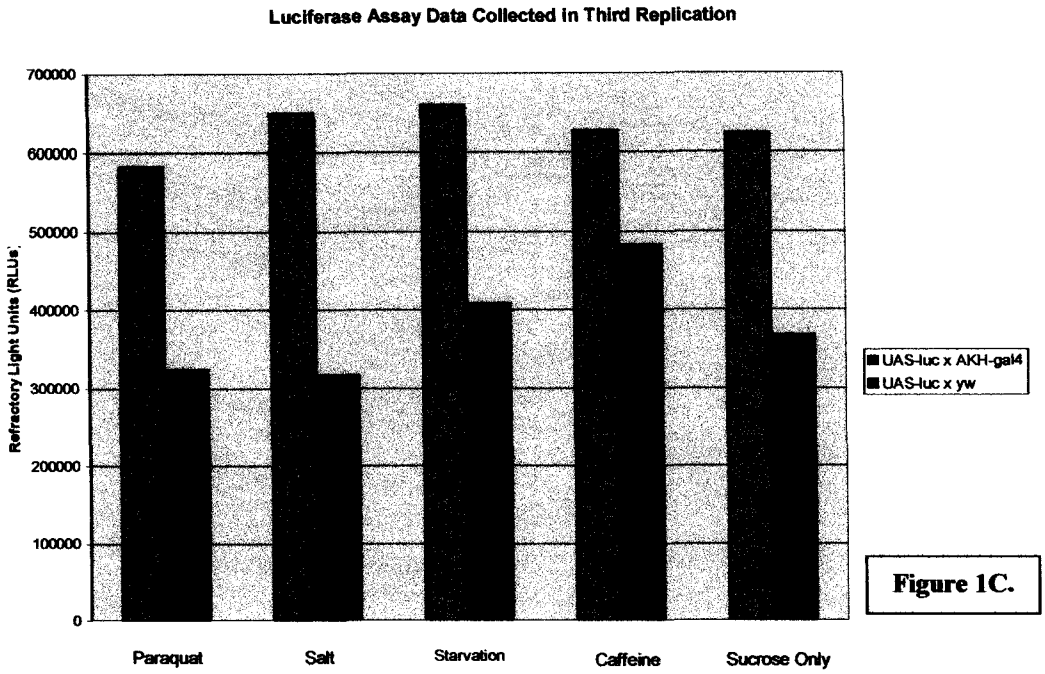

Figure 1C.

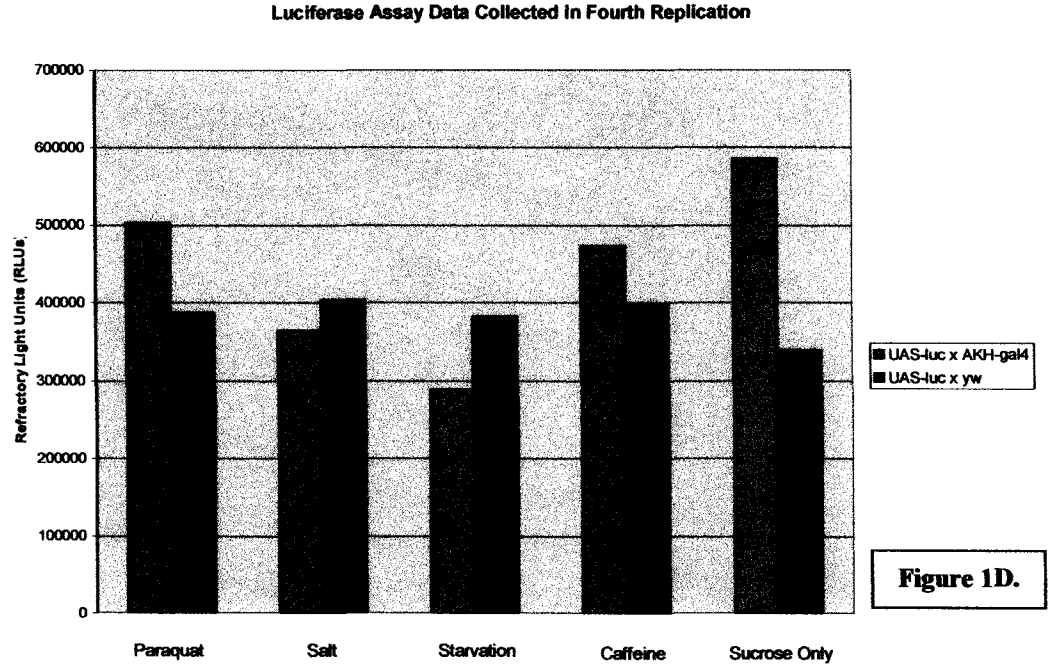

Figure 1D.

Figures 1A-1D. Luciferase Assay following 12 hr incubation under five constructs. Both UAS-*luc* x AKH-*gal4* and UAS-*luc* x *yw* were subjected to the five diets: salt, paraquat, starvation (diluted agarose), sucrose only control, and caffeine for 12 hrs in a humidified 24°C incubator. None of the replications were able to provide a baseline of AKH expression for comparison between the transgenic species.

Figure 1a. Control flies overcome *gal4* driver line in starvation while remaining slightly below in other constructs.

Figure 1b. Control flies luminescence levels remain inconsistent and overcome paraquat and sucrose only trials.

Figure 1c. Better relationship achieved between strains but high luminescence in sucrose only trial in *gal4* strain poses problem when trying to determine if more luminescence is observed in flies under dietary stress.

Figure 1d. Dramatic decrease in overall luminescence levels along with continued prevalence of sucrose only trial in *gal4* strain. Reprisal in control fly groups having greater illumination than their counterparts in salt and starvation constructs.

noted in decreasing luminescence values in following replications. Differences as much as 469909 RLUs (a 50.21% decrease from the initial value), as seen in caffeine measurements, between the first and the last measurements taken are recognized (Figures 1A,D). A second source of concern in considering these evaluations is the dramatic increase in luminescence values in the sucrose only to become equally relevant, and even surpassing, to the luminescence measured in the dietary stress models. For instance, the control construct in the final replication was the highest luminescence measured at 50.9% larger than that of the lowest value, the starvation model, which was the highest measured in the two previous replications (Figure 1D).

Therefore, it is because of the lack in creating a standard of luminescence using UAS-*luc* x *yw* and intense variability between measurements of the different dietary stresses and the replications identical in method that it is yet to be determined using this particular type of assay that a change in nutrition has any effect of AKH expression.

Salt vs. sucrose only control in 16 hr incubation. Due to the variance in the previous experiments, two changes in analysis were made. The first was to extend the incubation period in hopes that more time under stressed conditions would stimulate more AKH expression i.e. more luminescence. The second modification made was to investigate more than one subset of each model and then compare the two transgenic species. Unfortunately, there was still little difference between luminescence recorded in flies subjected to 5% salt solution and those held in sucrose only solution. In fact, in both UAS-*luc* x AKH-*gal4* and UAS-*luc* x *yw*, the average RLUs measured was greater in the control population than in the test population (Figure 2B). As to inter-relating the two

species, the *gal4* driver strain did have consistently higher luminescence measured than the *yw* strain, but still not enough to make any direct conclusions to the relevance of the experimental hypothesis (Figure 2A). Also, the longer incubation period seemed to have no effect on luminescence emitted as values still capped at 334839 RLUs (Sucrose Only 1).

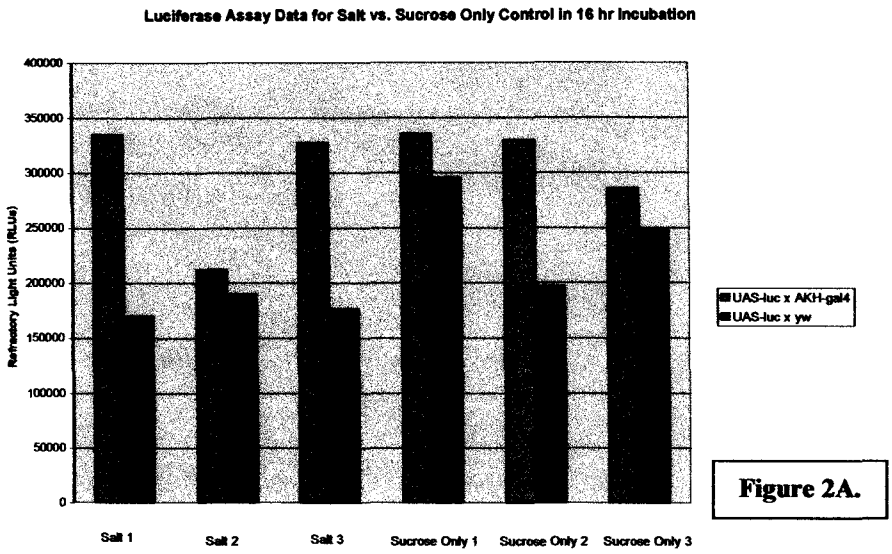

Figure 2A.

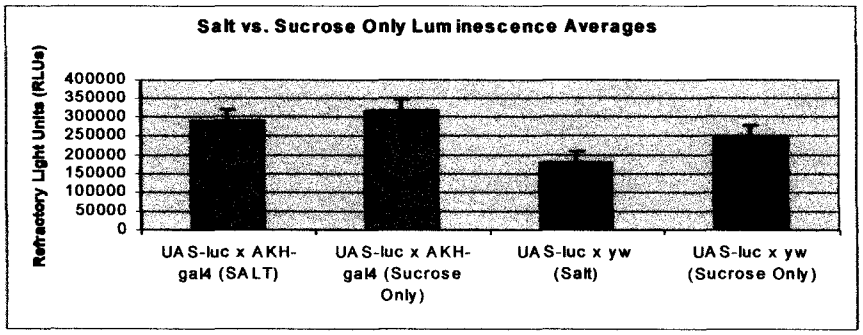

Figure 2B.

Figures 2A-2B. Salt vs. Sucrose Only control in 16 hr incubation. Increase in number of replications and incubation period in hope of collecting more significant data. Figure 2a. *gal4* driver strain has consistently higher luminescence than the control *yw* strain, yet not enough to make any direct conclusions to the relevance of the experimental hypothesis. Figure 2b. Little difference recorded between flies subjected to 5% salt solution and the control though the sucrose only control maintained higher luminescence averages in both strains.

Starvation vs. sucrose only control in 12 hr incubation in UAS-luc x AKH-gal4 strain.

Return to the 12 hr incubation period was based on the determination that a longer period had no discernable effect on the amount of luminescence released. Also, the number of replicates was increased to five of each diet to provide a better representation of each.

Once again, there was no significant evidence, given this method, that there was increased AKH expression in flies subjected to dietary stress conditions. All but one of the starvation replicates remained below the control measurements resulting in a 7692.2 RLU difference between the averages of the starvation and the sucrose only flies. Also, let it be mentioned that these values are exceptionally lower than those begun with in the comparison of all five dietary variants (Figures 3A-B).

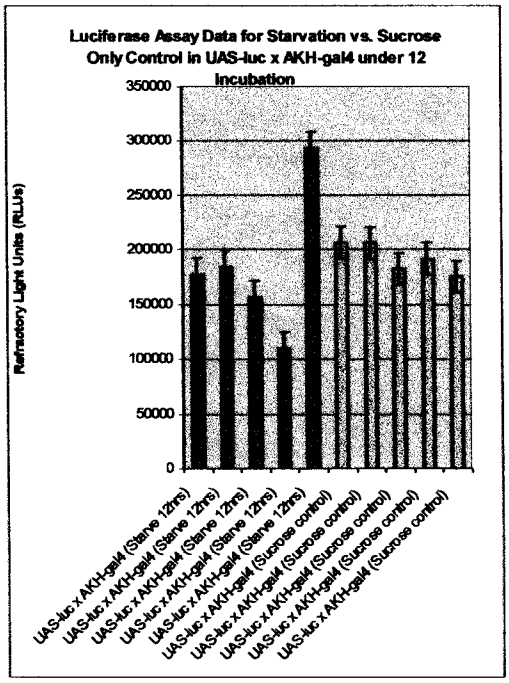

Figure 3A.

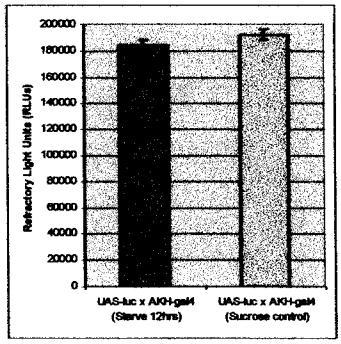

Figure 3B.

Figures 3A-3B. Starvation vs. Sucrose Only Control in 12 hr incubation in UAS-*luc* x AKH-*gal4* strain. Five replicates used for both starvation and sucrose only models in shorter incubation period.
Figure 3a. Only one starvation luminescence measurement exceeded those of control.
Figure 3b. Minute difference between starved and control luminescence averages observed.

DISCUSSION

Adipokinetic hormones (AKHs) have been shown to be responsible for mobilization of carbohydrate and lipid substrates from the fat body of *Drosophila* for use in the classic flight and locomotor scenarios. Furthermore, depletion of *dAKH* gene expression has been linked to reduced trehalose levels, a disaccharide containing two glucose molecules connected via α,α-1,1-glycosidic linkage (Isabel 2004), while trehalose has demonstrated

protective properties in anoxic conditions in flies (Chen 2004) and under osmotic and heat stresses in tropical yeast (Ribeiro 1999). It was thus deduced that there would be a link in AKH expression and survival under these stresses. By changing the diet available for prolonged periods of time, it was hypothesized that the osmotic, oxidative, and locomotor stresses would create an influx of AKH activity as part of the organism's will to survive.

The data collected, however, was inconclusive as to supporting or rejecting the above stated hypothesis. A baseline measurement of expression in sucrose only control constructs and in the fly line lacking the appropriate *gal4* driver was unable to be created, thus making it impossible to positively correlate increased AKH activity with increased luminescence. Focusing on the salt construct with a longer incubation period perpetuated inconsistency among replications as sucrose only luminescence levels were on average were greater than those of flies placed under osmotic stress conditions. The final experimentation completed was focused on starvation –induced AKH expression. Analysis of the results still constitute a higher luminescence average in control flies rather than test populations.

AKH expression is very limited even during bouts of high activity. Hemolymph titer determinations in *S. gregaria* were 3 pmol at maximum after 5 min of flight compared with resting locusts and 0.7 pmol after 30 to 60 min of flight. Much of the peptide remains stored in the corpora cardiaca (Gade 2004). Changes in experimental procedures were made in expectation that longer incubation periods and increased numbers of replicates would provide data suggestive of greater AKH activity in times of stress. Neither of these alterations met the expectations set. It is still unknown as to

whether inconsistent data is the result of the experimentor's methodology, a failed cross, or of some underlying physiology, such as minute expression that cannot be accurately detected. It should be noted that this research is preliminary to further testing of greater magnitude.

Nonetheless, additional changes in future experimentation are proposed. To address the underlying question of responsibility, a new cross could be officiated to rule out any current dispute in the success of the current UAS-*luc* x AKH-*gal4*. Second, the first series of luciferase assay used increasing numbers of flies in each extraction resulting in increasing levels of luminescence (data not shown). It could thus be proposed that doubling the number of flies used for extraction (4 flies used currently) could be one possible solution to the current problems. Third, only multiple replicates of salt and starvation were completed to this point. Continuing experimentation to include multiple replicates of the other two dietary stresses, paraquat and caffeine, would complete the series and possibly provide greater insight as to what specific stresses, should there be any, AKH expression is increased. Fourth, monitoring an increased number of replicates, greater than that of five, the maximum in this research, could simply provide a firmer basis for hypothetical support. Fifth, only males were used in this line of assay. Further questioning could be raised if there are differences between the sexes. Finally, setting up an entirely unique monitoring system could create a trend in increasing AKH expression. Halting incubation in the dietary stressed conditions at an increment of perhaps 4 hr for an extended period of time could elicit an increasing curve in AKH activity as time under stress progresses. Should this occur, results would provide

subsistence to the connection between osmotic, ionic, oxidative, and locomotor activity stresses and induced *dAKH* expression.

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