ANALYSIS OF MFS TRANSPORTERS AND RELATED LIPOPHILIC HORMONES IN ARTHROPOD SPECIES

By

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ABSTRACT

With the development of modern genetic tools and research methods has come a better understanding of how organisms regulate their bodies. One aspect of this regulation includes hormones, which may be characterized in many ways. This includes lipophilic hormones, a group of hormones that may be notably categorized as relatively smaller, hydrophobic hormones present in the development and morphological changes of most organisms. In arthropods, there are not very many studied lipophilic hormones as these hormones cannot be directly studied through commonly used genetic analysis. This leaves the possibility of there being vital lipophilic hormones that have simply not been discovered or studied yet. With the issue of lipophilic hormones being modified by enzymes post-transcription rather than coded directly by genes, another characteristic of hormone regulation must be analyzed: transporters. Major Facilitator Superfamily (MFS) transporters are yet another aspect of hormone regulation that is understudied, but can permit a better analysis of which lipophilic hormones are present throughout development. Through phylogenetic analyses of the genes encoding MFS transporters in insects and other model arthropod species, we aimed to identify hormone transporters that are not yet functionally characterized. By looking for orthologs of ecdysone importers recently identified in fruit flies and mosquitoes, we identified candidate ecdysone importers in the western tarnished plant bug *Lygus hesperus*. A heterologous cell culture system is currently being used to functionally characterize these transporters.

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my faculty mentor and principal investigator, Dr. Naoki Yamanaka, who has always provided his unwavering support and guidance throughout the entirety of my undergraduate career in his lab. Thank you for your unceasing patience, invaluable mentorship, and dedicated willingness to aide in the development of my skills as a researcher and student for the four years you have permitted me to research under your tutelage. I truly appreciate every opportunity you have given me, as I never could imagine myself being where I am now in my academic career without the experiences I gained as a part of your lab.

Thank you additionally to our post-docs Eisuke and Daiki, who taught me specific skills needed to complete luciferase assays. I would also like to thank you both for your continued patience and willingness to help me adjust when my experiments would fail.

Overall, I have gained a significant number of experiences which I have learned a lot from while being in this lab. Despite a lengthy portion of online research, I never stopped learning from my faculty mentor and developed new skills that I could in turn use for this project. Thank you all for your monumental support in what has truly been one of the most enriching involvements of my academic career.

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INTRODUCTION

Throughout all biological systems, hormones play an important role in regulating development and maintaining homeostasis of the living system in which it resides. While the role of hormones within biology are vitally important, some are arguably more difficult to study than others, such as those classified as lipophilic hormones (Berg et al.). This is due to the nature in which lipophilic hormones are created within a living body, which is through diet and the modification of other molecules such as cholesterol. Because of this, the function of these hormones within living systems are not as well studied as other classifications of hormones.

Hormones are responsible for many functions throughout a living system, but can be more commonly divided into a few classifications, such as lipophilic hormones and peptide hormones. In *Drosophila melanogaster*, a widely regarded model organism, two of the most commonly known and studied hormones are the juvenile hormone (JH) and ecdysone (Ec). Within *Drosophila*, the ring gland, which is composed of the corpus allatum, the prothoracic gland, and the corpora cardiaca, is a well-studied organ that is known to produce many hormones including these two lipophilic hormones (Siegmund and Korge). Juvenile hormone is a type of lipophilic hormone that originates from the corpora allata within insects, and is studied to be responsible for the early stages of development of arthropods as well as various aspects of reproduction (Flatt and Kawecki). Similarly, ecdysone is a steroid hormone that originates specifically from the prothoracic gland, and has been found to regulate metamorphosis and other developmental transitions (Yamanaka et al.).

There are also several different types of peptide hormones that regulate development and maintain homeostasis throughout a living organism. Peptide hormones differ from lipophilic hormones in the sense that they can originate directly from gene expression, which involves the

transcription of DNA to RNA and the translation of RNA to the protein (Reiher et al.). Because of this, proteins and peptide hormones are relatively easier to research through fly genetics by utilizing experimental methods such as gene knock-out or knock-down (Yu et al.).

Transporters are very similar to peptide hormones in the sense that they are also composed of proteins, but are instead typically embedded within the cell membranes of various cells (Yan). These proteins regulate the entry and exit of various substrates such as hormones in or out of the cell in which it resides. Much like how hormones are classified based off of their composition and where they originate from, transporters can be classified into many large groups. One large group of transporters is the Major Facilitator Superfamily (MFS), which are generally well studied in terms of their intracellular structure and coupled reactions involving proton exchanges (Bergwitz et al.).

Major Facilitator Superfamily transporters are known to act as a door, which open or close to bring molecules in or out of the cell and across the cell membrane. MFS transporters include many solute carrier transporters, otherwise known as SLC transporters, that mediate the transport of small molecules across cell membranes. In recent studies, ecdysone was found to be transported by MFS-type SLC transporters within *D. melanogaster* as shown in Figure 1 (Okamoto et al.). As a lipophilic steroid hormone, this indicates that functions of many other lipophilic hormones are also mediated by transporters, meaning that their function may be characterized and analyzed through the experimentation of transporter protein encoded genes.

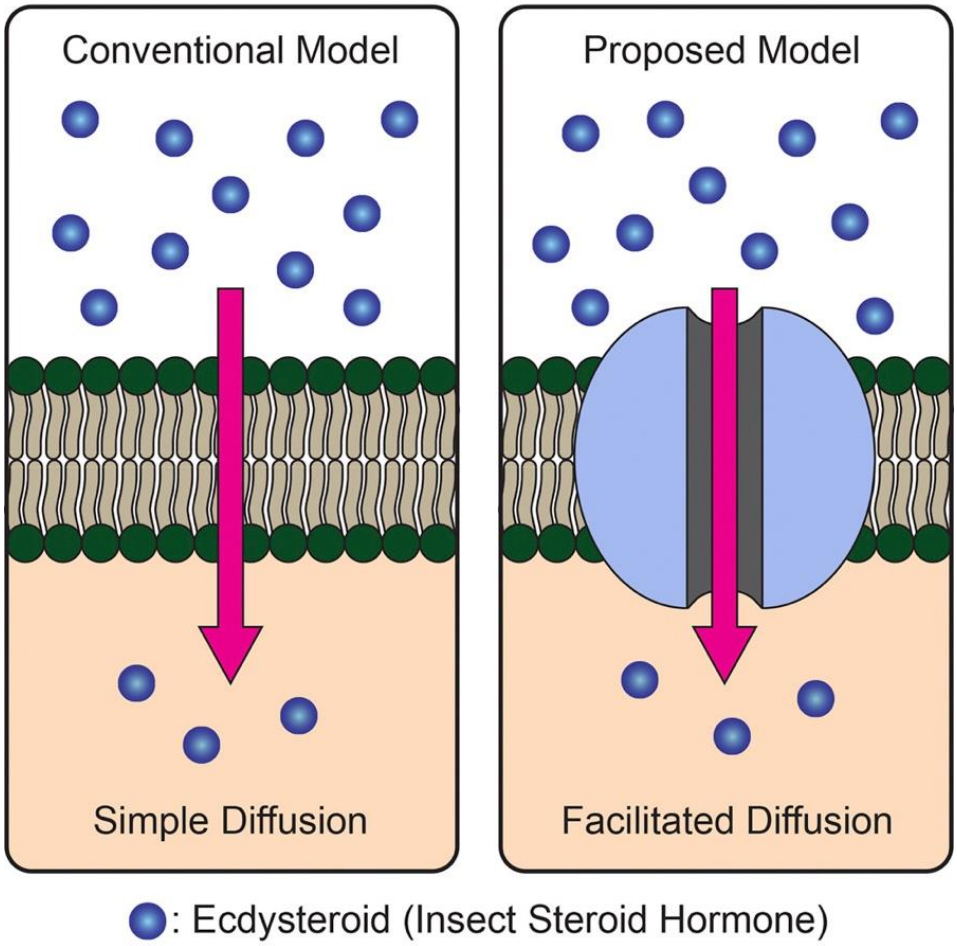

Figure 1 – Proposed model of diffusion of ecdysteroids via transporter mediated diffusion. (Okamoto et al.)

In order to address the underdevelopment of research relating to lipophilic hormone function and the possibility of there being other transporters responsible for lipophilic hormone transport, we analyzed the genes that encode for the MFS transporters in *Drosophila melanogaster* and other arthropod species phylogenetically. These other species include *Apis mellifera*, *Aedes aegypti*, *Lygus hesperus*, *Bombyx mori*, and *Tribolium castaneum.* Using this phylogenetic analysis, genes responsible for certain candidate ecdysone importers may be

experimentally tested via a heterologous cell culture system to observe possible hormone transport interactions and functionally characterize these transporters. For this project, we specifically identified the candidate ecdysone importers in the western tarnished plant bug *Lygus hesperus*, which is an agricultural pest of many cropping systems (Hull et al.).

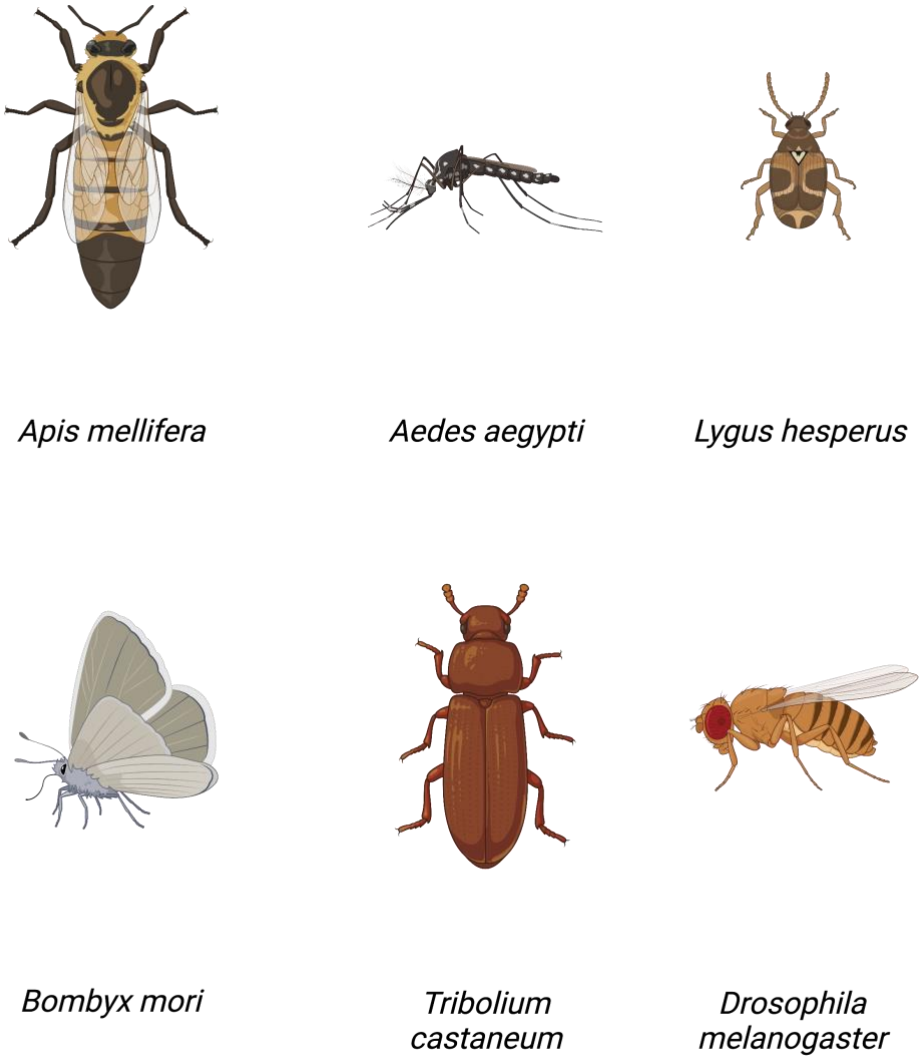

Figure 2 – Included species used for phylogenetic analysis of MFS transporters. Created myself through BioRender.

The intended significance of this project involves many future applications relating to the fields of medicine and biology. Primarily, while there have been other published works that similarly analyze other transporter groups, such as the ABC transporters, this project will organize this information in a way that will make it easily accessible and usable for other *D. melanogaster* research projects (Yamanaka et al.). It will similarly create a baseline of genetic data that will organize this phylogenetic information in a way that has never been done before. Not only that, but the results obtained from the heterologous cell culture system will help develop the understanding of hormone signaling via transporters in many other species, including the common pest *Lygus*. There may also be further applications for this research, such as through the development of pesticides and the use of hormone supplements and therapy in medicine and drug development.

METHODS

Phylogenetic Analysis

For the initial stages of this project, phylogenetic analysis was used to cultivate a series of phylogenetic tress in order to better understand the evolutionary relationship between MFS transporters of the arthropods studied. Primarily, the protein sequences for all MFS transporters of *Drosophila melanogaster* were collected and organized using the online tool FlyBase. Throughout the collection of these sequences, each gene was checked to ensure that if there were multiple variants of the same gene, the variant chosen has already been functionally analyzed if possible and/or has a larger ratio of exon to intron regions. Each gene was similarly analyzed for the expected number of transmembrane domains using Phobius, which in the case of MFS transporters in *D. melanogaster* meant 12 transmembrane domains. Once the most appropriate variant(s) were determined, the protein sequence(s) were collected and sorted by MFS group.

Within *D. melanogaster*, the groups of MFS transporters include SLC2, SLC15, SLC16, SLC17, SLC18, SLC19, SLC22, SLC29, SLC45, SLC46, SLCO, and other ungrouped MFS transporters, coming to a total of 163 genes that are responsible for encoding MFS transporters within *D. melanogaster*. For the other species of arthropods, the NCBI Gene feature was used to collect and organize their MFS protein sequences. These protein sequences were assessed functionally through the NCBI BLASTp feature by analyzing their query cover and e-value by using the genes with a query value greater than 80% and an e-value less than 3×10^{-30}. MegaX, a phylogenetic computer tool, was used to create the phylogenetic trees of each of these species' MFS genes as well as any cross-comparison trees between some or all species.

Luciferase Assays

In the secondary stages of this project, luciferase assays were performed to quantitatively analyze whether or not the MFS transporter proteins were being expressed. HEK293 cells, otherwise known as human embryo kidney cells, were used as the heterologous cell culture system to perform these assays. These assays are reliant on the luciferase reporter plasmid, which is activated by hormone transport across an SLC transporter, where the hormone then travels to form the hormone-receptor complex within the cell that results in the expression of that plasmid. The expressed plasmid then produces an enzyme that once treated with a substrate, results in a quantifiable amount of fluorescence which is measured by a luminometer. The molecular understanding of this assay is visually illustrated in Figure 3.

The maintenance of the HEK293 cells and the production of all required materials were performed weekly. HEK293 cells were incubated at physiological temperature (37°C) in a cell culture flask containing Schneider's *Drosophila* colored media (DMEM) containing L-glutamine and phenol red and treated with 10% Hi-FBS, 1% Antibiotic Penicillin/Streptomycin, and 1%

Non-Essential Amino Acid (NEAA) as shown in Figure 4A. Media was replaced daily or weekly, ranging from 24 hours to no more than four days. All vectors and 20-hydroxyecdysone were reproduced from previous glycerol stocks using QIAprep Spin MiniPrep or MaxiPrep protocol.

The assays themselves were essentially four steps over the course of multiple days. Primarily, transfection was performed to prepare the treatment of the transporter DNA. After the appropriate amount of time needed for the cells to proliferate in the cell culture flask, the cells were split into a 6-well plate, where three of the wells tested the negative control pcDNA empty vector and the other three tested the assumed transporter as shown in Figure 4B. In the initial experiments, Ecdysone Importer (EcI), the SLC importer that was previously mentioned to be found to mediate ecdysone, was used as a positive control to confirm result validity. The vectors were prepared at the following concentrations and amounts before adding 4.5µL of attractene to the total amount of each group and incubating for 48-72 hours at 37°C:

CONTROL	**OVEREXPRESSION**	**VOLUME (µL)/1 well**	**VOLUME (µL)/ 6 wells**
pcDNA-empty (100 ng/µL)	pcDNA-EcI (100 ng/µL)	6	36
pERV3: EcR/RxR (180 ng/µL)	pERV3 (180 ng/µL)	2	12
pEGSH; EcRE-Luc (216 ng/µL)	pEGSH (216 ng/µL)	1	6
pRL-CMV (5.4 ng/µL)	pRL-CMV (5.4 ng/µL)	1	6

After the transfection incubation period, chemical treatment was performed. This involved the preparation of the following concentrations of 20E: 0M, 100nM, 300 nM, 1µM, 3µM, 10µM, 30µM, and 100µM. The concentrated amounts of 20E were diluted with clear growth media, which was DMEM without L-glutamine and phenol red + 1% Antibiotic Penicillin/Streptomycin + 1% NEAA. Once the concentrations of 20E were prepared, the cells from the 6-well plate were transferred equally to half of a clear 96-well plate, consisting of three columns of the transporter DNA and three columns of the negative control DNA as shown in Figure 4C. The cells were then treated with 20E, with each concentration correlating to one row of the 96-well plate. After 20E is added, plate is incubated at 37°C for 16-24 hours.

Lastly, the plate is prepared to be quantified by luminometer. The DNA and 20E treated cells are transferred from a clear 96-well plate to a white 96-well plate to optimize the amount of fluorescence read by the luminometer before being treated with Dual-Glo Luciferase solution. After a 30-minute incubation period where the tray is shaken at room temperature in the dark, the Firefly luciferase activity is measured and recorded. Immediately afterwards, the cells are treated with Dual-Stop&Glo solution to stop the reaction from occurring. Once a 10-minute room temperature incubation period is completed, the Renilla luciferase activity is measured and recorded. The resulting data is consolidated into a linear graph to observe the general luciferase activity across the concentration gradient of 20E.

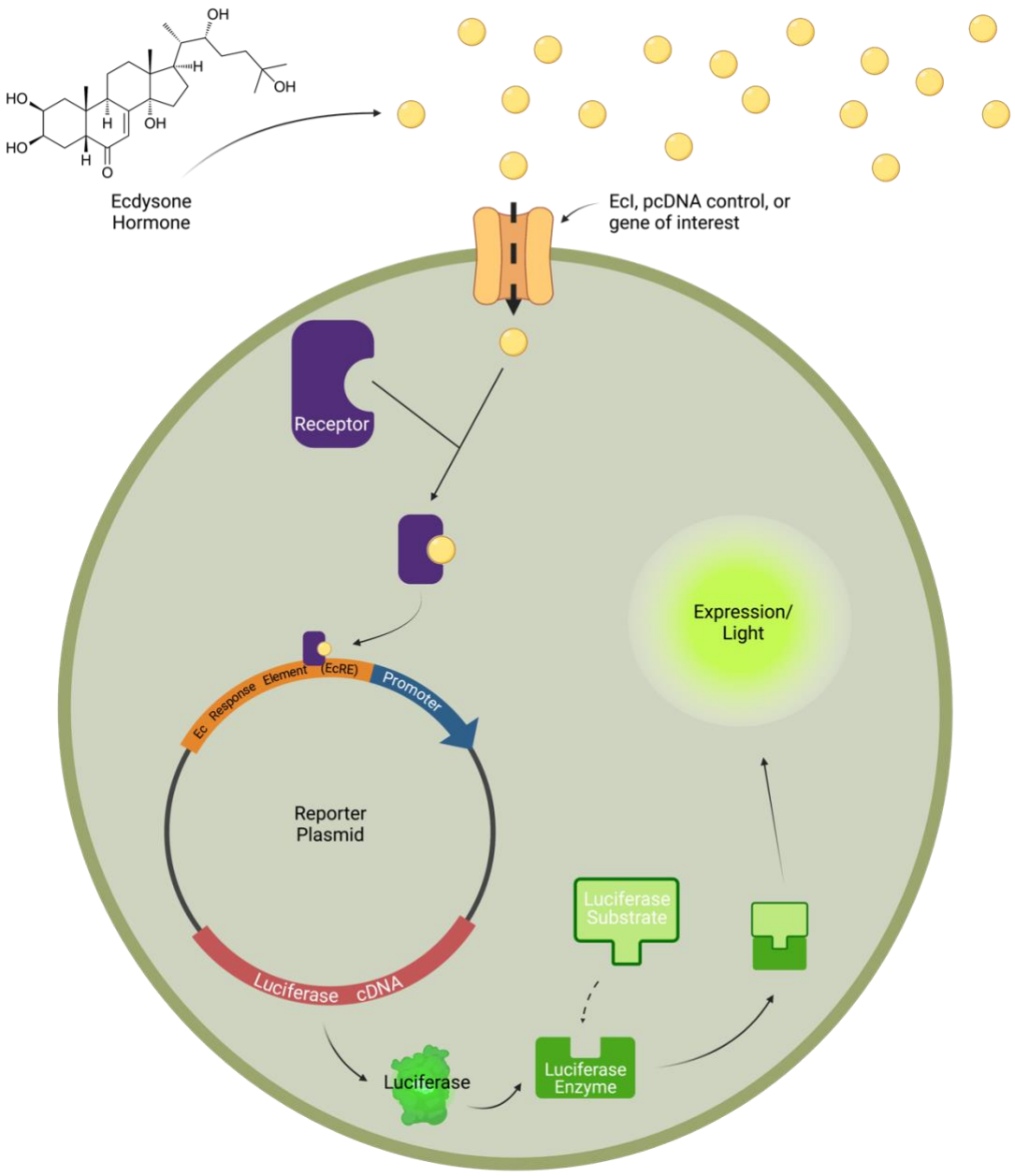

Figure 3 – Molecular function of luciferase assays within a cell. Created myself through BioRender.

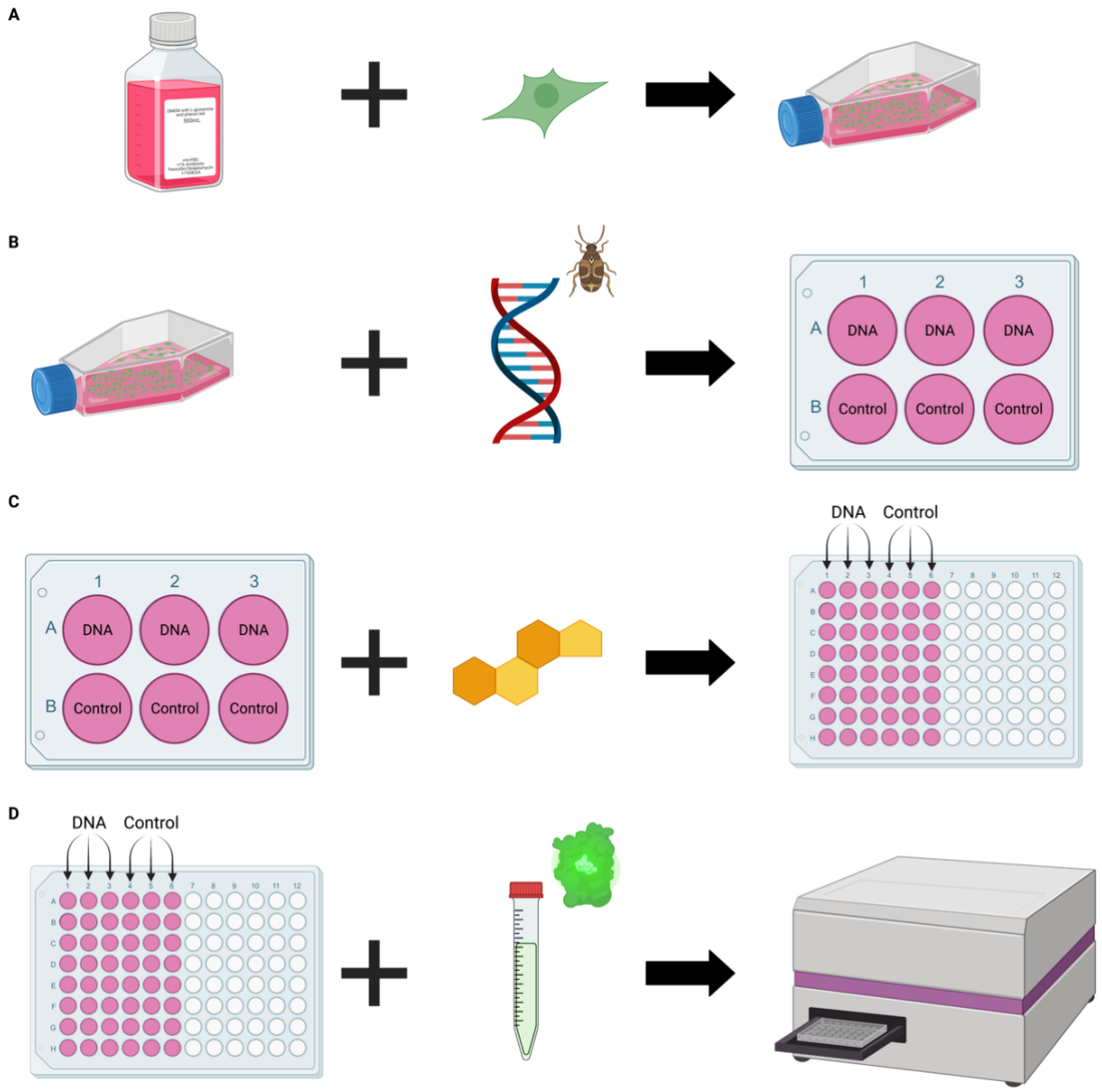

Figure 4 – Process of performing luciferase assays experimentally. A refers to cell maintenance, B refers to transfection, C refers to chemical treatment, and D refers to luciferase assay and luminometer reading. Created myself through BioRender.

RESULTS

Phylogenetic Analysis

Phylogenetic relationships between the protein sequences of each MFS-type SLC transporter groups within *Drosophila melanogaster* were found and distinguished by color as illustrated in Figure 5. Most importantly, the SLCO transporter group, which contains the Oatp74D gene that was discovered to encode an MFS-type SLC transporter that transports the ecdysone hormone, was found to be grouped together and closely related. The SLCO transporter group is colored below in teal with a gold outline.

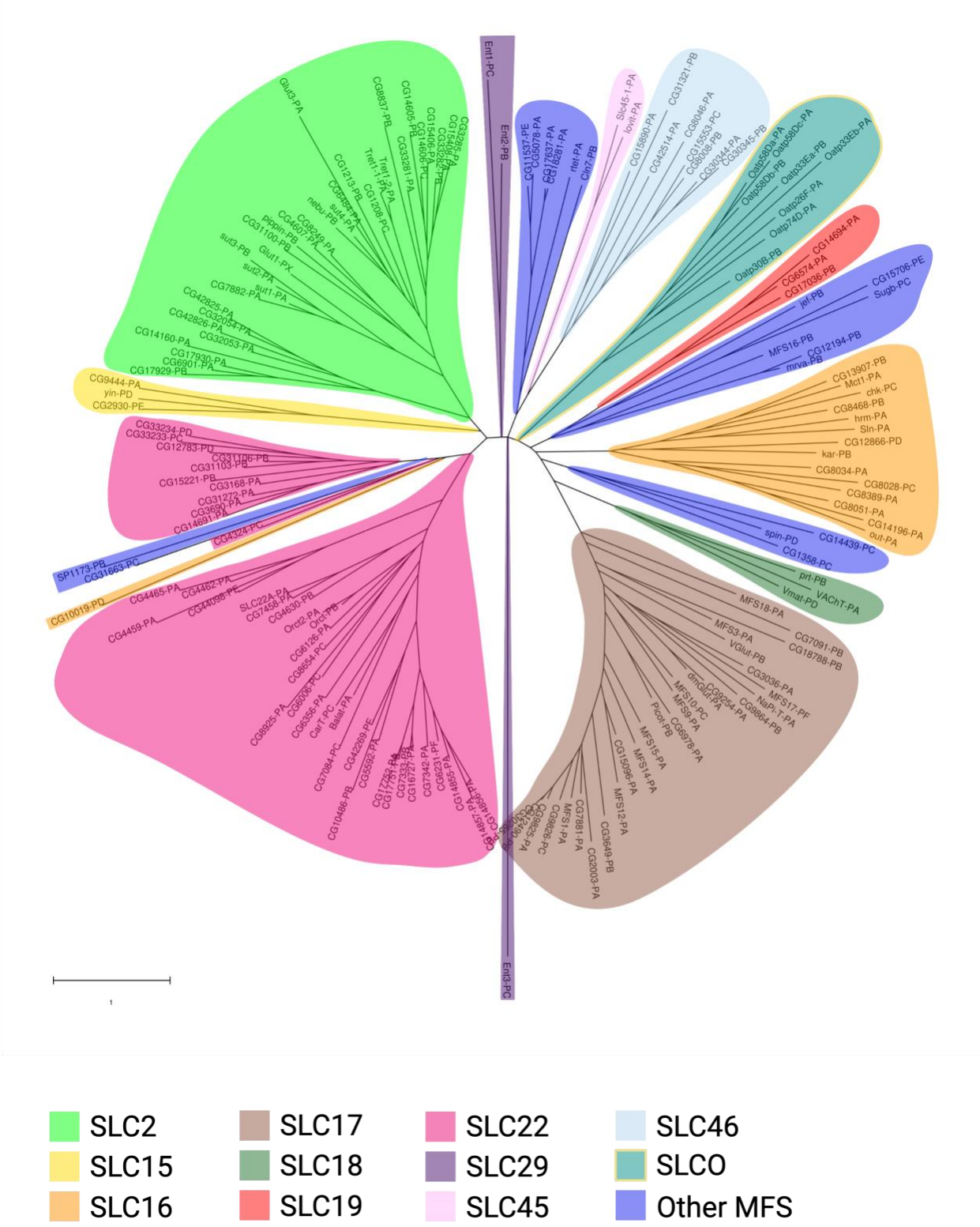

Figure 5 – Phylogenetic tree, color coded by MFS-type SLC transporter group. Created myself through BioRender.

Additionally, phylogenetic relationships between the SLCO transporters of all species, including *D. melanogaster, A. mellifera, A. aegypti, L. hesperus, B. mori,* and *T. castaneum,* were developed. These relationships are displayed and labeled by species in Figure 6B, with the species legend shown in Figure 6A.

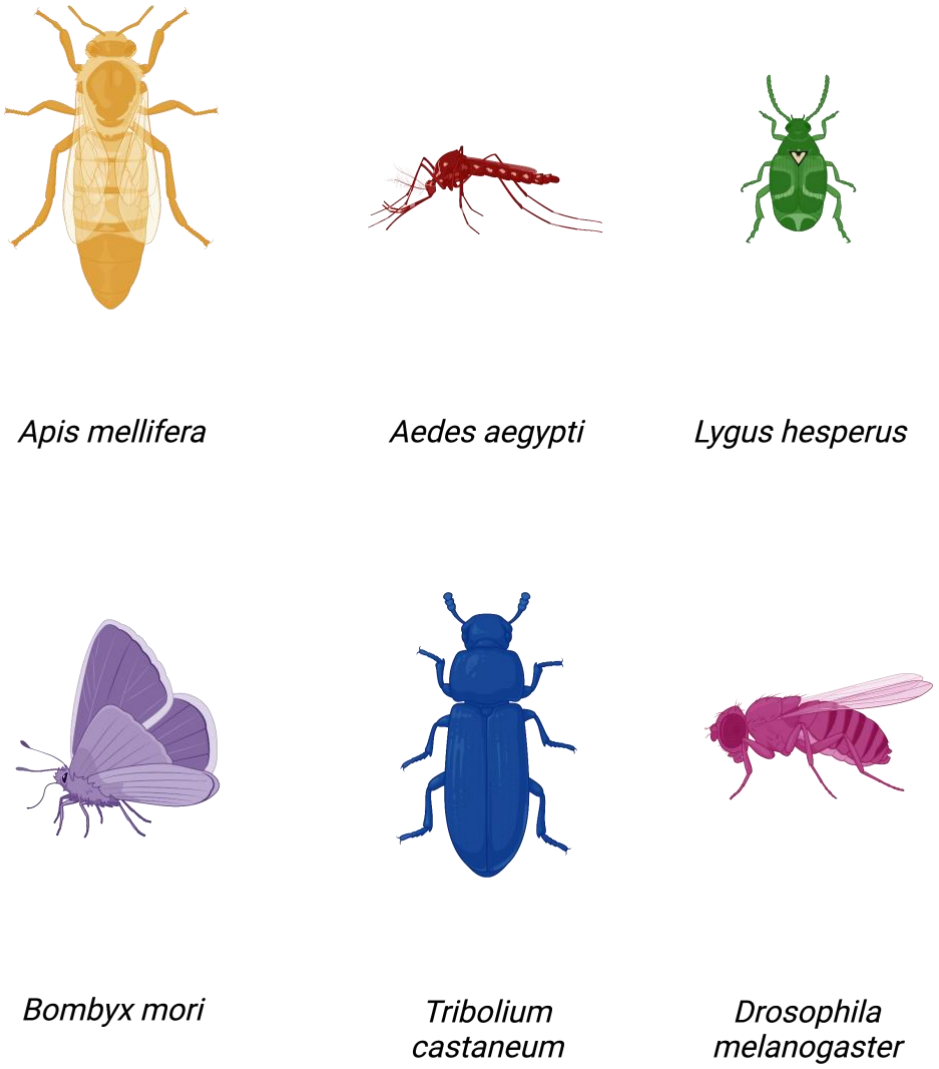

Figure 6A – Phylogenetic tree legend of all species' SLC transporter groups. Created myself on BioRender.

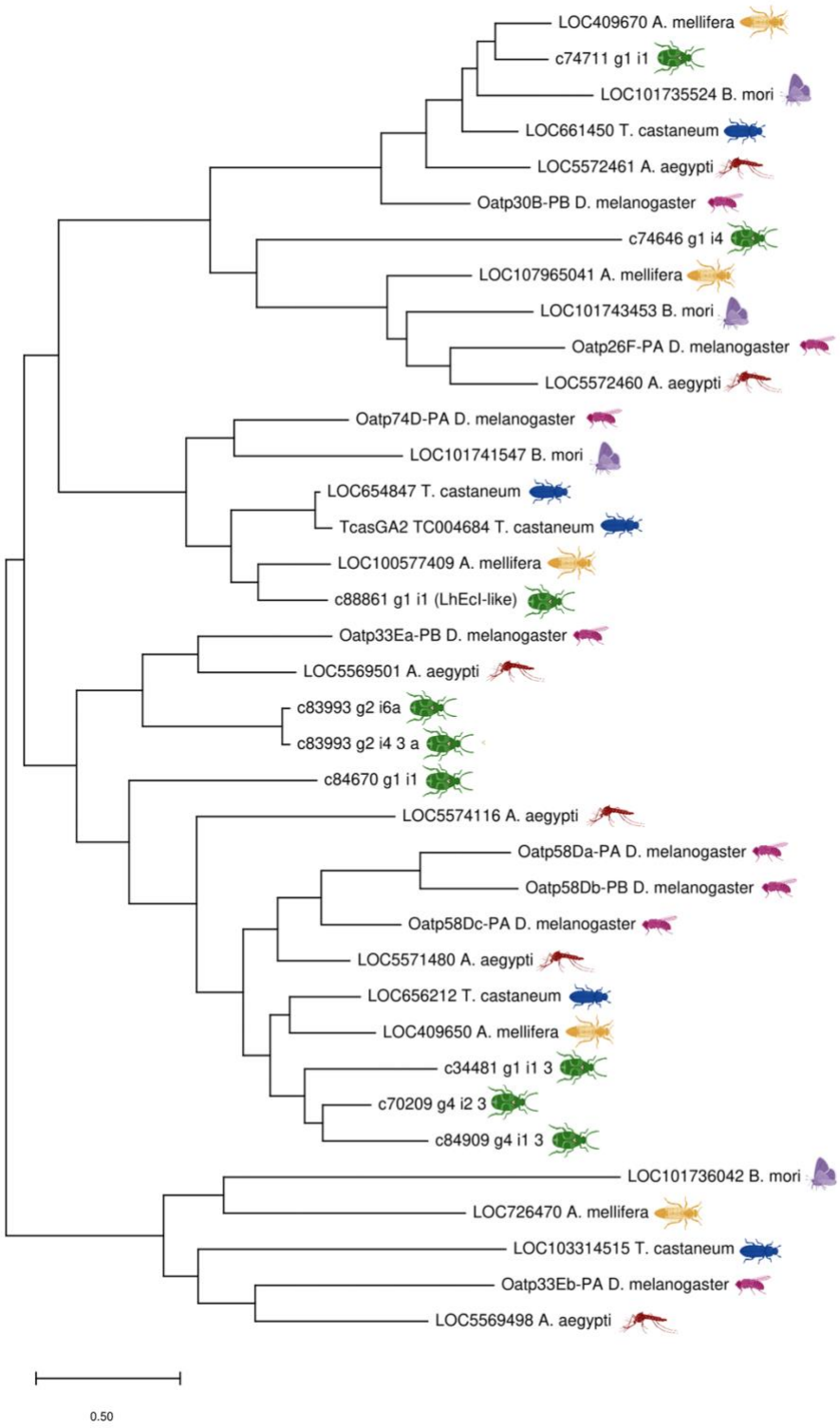

Figure 6B – Phylogenetic tree of all species' SLC transporter groups. Created myself on BioRender.

HEK293 Cells

Many of the primary attempts of maintaining HEK293 were not successful due to preliminary cell death, stress, or contamination. Unhealthy cells were observed as small, clumped together, stacked on top of each other, or free floating in the media. Cells exhibiting stress are shown as highly clumped together, small, and stacked on top of each other as shown within the red circle in Figure 7A. Healthy cells could be observed to have a spaced-out pattern, with all cells attached to the bottom of the culture flask as shown in Figure 7B.

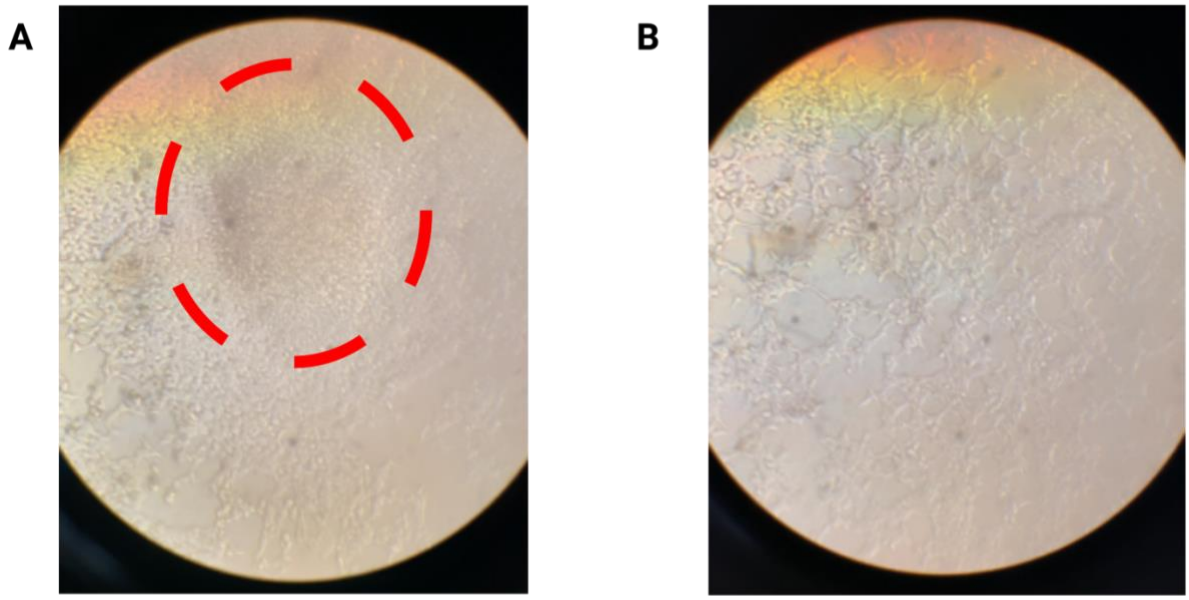

Figure 7 – Unhealthy vs Healthy HEK293 cells. Created myself on BioRender.

Luciferase Assay

Luciferase assays were performed to measure the function of EcI as a positive control and pcDNA empty vector as a negative control. Results confirm the validity of the assay and the function of EcI and are displayed in Figure 8. Expected values of the positive control line of EcI relationship include a maximum relative luciferase activity within 10-20 and a significant positive incline at 10^{-6} M 20E. Expected values of the negative control included consistent relative luciferase activity at 1, with a slight, significant increase starting at 3 x 10^{-5} M 20E. All expected values were met, confirming the function of EcI as an ecdysone importer and the pcDNA empty vector as a negative control.

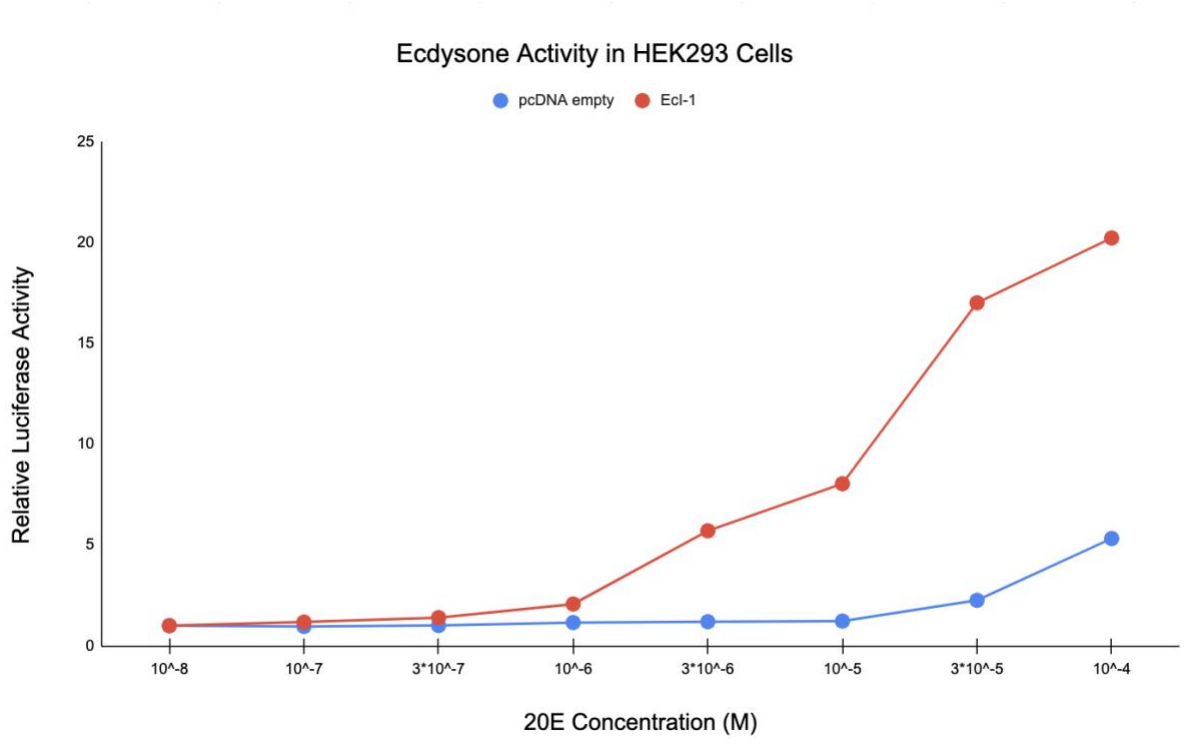

Figure 8 – Relative luciferase activity in HEK293 cells based on transport of various concentrations of 20E by EcI or pcDNA empty vector.

DISCUSSION

The data collected throughout this project displays promising future work within the studies of molecular biology and other scientific advancements, including pesticide and drug development. With our current understanding of MFS-type SLC transporters and their functions throughout living systems, it is vital to continue this research in order to confirm the function of ecdysone transporters through the SLCO genes confirmed through the phylogenetic analysis of the protein sequences in the previously specified arthropod species.

This project will continue with the luciferase assay-based analysis of SLCO transporters in *Lygus hesperus.* As a notoriously harmful pest that has historically caused severe economic and agricultural damage especially within the United States, it is crucial to study the effect of these transporters within the *L. hesperus* system in order to develop a better understanding of how to combat their impact on agriculture (George et al.). Using the SLCO genes of *L. hesperus*, we look to discover whether or not ecdysone and other steroid hormones are mediated by the transporters that these SLCO genes are responsible for. An expected outcome would be that one or more transporter genes are responsible for ecdysone transport and show a dose-response relationship to 20E as observed for EcI in Figure 8.

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Other online tools used:

- BioRender, used for development of some figures: https://biorender.com/
- Phobius, transmembrane domain analytic tool: https://phobius.sbc.su.se/
- FlyBase, fruit fly genetic database system: https://flybase.org/
- NCBI
    - Gene, genome sequence database: https://www.ncbi.nlm.nih.gov/gene
    - BLASTp, protein analysis tool:

      https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins