Identification and characterization of the putative *Sciara coprophila* cold tolerance gene, *Frost* (*ScFst*)

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Background

Insects in temperate climates have adapted ways to survive cold stress, such as when temperatures decline overnight. This adaptation is specifically important when insects prepare for survival during the fall when the temperature decreases at night but is survivable during the day.

The fruit fly, *Drosophila melanogaster,* is a very well-studied species of fly due to the genetic mutations that create easily recognizable phenotypes. Studies have led to research in other organisms like the fungus gnat, *Sciara coprophila* (this species was recently named *Bradysia coprophila* due to new phylogenic studies, but it will be referred to as *S. coprophila* due to the extent of research done using the *Sciara* genus name).

The gene *Frost* (*Fst*) is known to help fly species recover from exposure to cold. The protein is expressed during periods of recovery from cold stress, not during the period of cold stress (Sinclair et al., 2007; Newman et al., 2017). Through protein studies, *Frost* is definitively expressed in *D. melanogaster* during recovery after experiencing cold temperatures. The purpose of this research was to identify and characterize a putative *Frost* ortholog in *S. coprophila*.

The exact function of *Frost* is unknown due to ongoing research; it is, however, known that it does not function as a cryoprotein or antifreeze protein (Newman et al., 2017).

There is evidence that *Frost* is expressed in as little as three hours of cold stress at increasing intervals of recovery (e.g., one-, two-, and three-hour recovery periods) (Bing et. al., 2012).

Recently the genome of *S. coprophila* has been sequenced and assembled and a high-quality transcriptome identified. A putative *S. coprophila Frost* ortholog was identified by Blasting the *D. melanogaster* sequence against the *S. coprophila* transcriptome. Several hits were identified, and a single gene was identified by performing a reciprocal Blast search. In this poster, we report the sequence of the putative *Frost* gene ortholog, *ScFst*. We characterize the expression of this gene following the exposure to cold temperatures using Quantitative PCR (qPCR) and propose a strategy for cloning the *ScFst* cDNA.

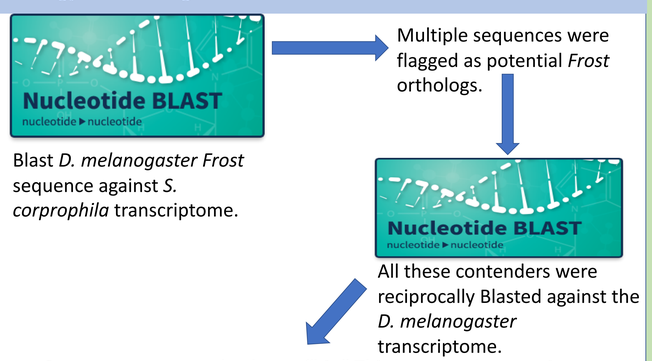

Figure 1. Blast and reciprocal Blast process to identify putative *ScFst*.

Results

Figure 1 identifies our process of identifying the putative *S. coprophila Frost* gene. We gathered the *D. melanogaster Frost* sequence and Blasted it against the *S. coprophila* genome. Several hits were found. These hits were reciprocally Blasted against the total *D. melanogaster* genome, and one sequence resulted as a hit. This sequence became the putative *S. coprhopila Frost* gene *(ScFst)*. The *ScFst c*DNA sequence was gathered using the transcriptome published on NCBI (Figure 2). The protein sequence was compared to the *D. melanogaster Frost* protein sequence using a pairwise alignment. This alignment showed a 39.7% similarity and 22.9% identity (Figure 3). After completing the qPCR for the first adult female experiment, the results showed the desired product was successfully amplified, and no other contaminants were amplified for both the 18S and the *ScFst* cDNA. In this experiment, one peak on the dissociation curve was noted for both sets of data, indicating that the product that was amplified was either the 18S cDNA or the *ScFst* cDNA.

The results of the qPCR experiments were analyzed using the ΔΔCt method to calculate the fold change and expression of *ScFst* relative to the control (Figure 4). This analysis revealed increasing levels of *ScFst* expression over the time course of recovery. This data supports the hypothesis that *ScFst* increases in expression at increasing intervals after recovering from a period of cold stress (Bing et al., 2012, Udaka et al., 2010). We then repeated this analysis using adult females again and included a 0-hour recovery time. This shows an increase in expression at the 1-hour time interval but not at any subsequent time interval (Figure 5). Experiments are ongoing to replicate the original results using adult female flies. A similar experiment was conducted with fourth instar larvae (Figure 6). Interestingly, no increase of *ScFst* was observed relative to the 0-hour control interval.

To start the cloning process, primers designed to amplify the full-length cDNA for *ScFst* were identified and ordered (Figure 7). Preliminary attempts to amplify the *ScFst* cDNA were unsuccessful using Taq PCR. However, research is ongoing (see *Further Directions)*. Once a fragment is produced, it will be cloned into the topo-TA cloning vector, pCR2.1 (Figure 8).

>*ScFst* cDNA Sequence

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ATGGATAATAAAGGCGTTCAACGTAAGAGCGAACGCTCAAACAAAGGAGTACCTCCATCACGTTTTGGATTTCCGGATATTCGAATTGAAAATATCGGATCTGAAGATGG      110
TGCAATTGGAGGCAACAGTACCGTGGAAAACCAACCTAATGAAGGTGCAATTGGGATCGGGGATCAAACAACCATTGAAAAGACTGTGATCCAGTCAAAGAAATATGCTA      220
AGTCTGTCTTGTCGAAAAGCGGTTCAAAGATGAGCAGACGTAGTACAAGAGAGATTCAACATATAAAAGAAACCATGGAAATTGAAGCGGAGCTCCATGAACTCGAAATG      330
ATGGAGATAAAAAGGAAGAAAGATTTCCTTTTACGAAACAAGCAGTTGAAAATGGATCTGAAGCAAGAAGAAGACGCGATGTCCAGTATCGACGATGAACTTGAAGAAGC      440
GGATGATCCGATTATGCAGCCACTGGGATATGACCAGCGTAAAAGAAACGTAAGGGAATGGATAAACAATATGGAAGATCCGATTCATTTCGGCAACTGCCGCGATTGGT      550
TCAAGTACGAATTAGATGCACAATTTGCTGGGTTGTCGGTAAAGGACGTTATGAAGAAATCAAGCAAAGCTACAGTCCTGGCTATTGATTCGCTACCAACAAAAAGAGAA      660
AAATGGTGCCATAAATGCAAAGGAGCAGTTGGACACTATTTACAAGATTGCAACGACTTCAAAAAGCTAACAATCGACAAGCGCAGAGATTTCGTCAGGCAAAATAGTAT      770
TTGCTTCATGTGCTTCAGAAAAGGACACCAAGCTAGCGTTTGTCGGAAAAAGGATTCATTGCCGAAATGCTCCAAGTGTAACAAAGATCATGCTGATATATTGCATGCCG      880
ACAACGCCCAAGTCAACATCGTTCAAAATACCGAAAATAACGTCAAAGAAGTCCGCTGCATCCAAACAAGGCACTCTATGCCAGACGTCAATCGATTCTCGAAATACAAT      990
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CGAAACTCTTTGGTACAAAAAAGCTCAGTTCGATTGTTACTCACGAGAGATCCACGATTTGAAAACGAAAAGCGTCGTAAAGAAGTCGAGCTGCCTTTATCCACTCAGTC     1210
CGTACATCAACAGTGAAGGCGTAATCTGCATGCGTGGTCGTATTCCAGGAACGAACCCAATTATATTGCCTCAAAACCACCGATTCACAAAGATGCTCATACAGTGGTAT     1320
CACGAAATCAATTTACATCAAGGAGTCGACACAGGTTGGAGGATGTTGATGTGGAAAACACCGTCGATCAGCATAATCAAAGTGGGCAATTGA      1413
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Figure 2. cDNA sequence of the putative *Frost* gene in *S. coprophila* (*ScFst*).

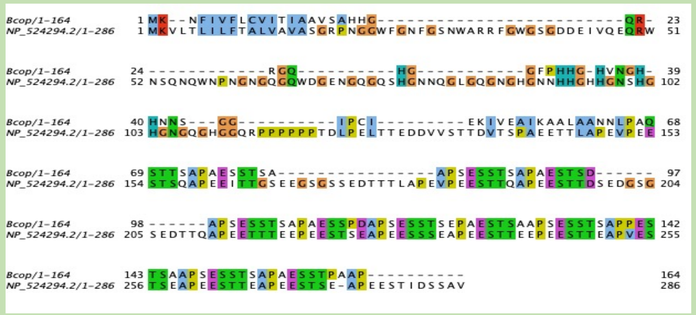

Figure 3. Protein alignment between *ScFst* and *D. melanogaster Frost*.

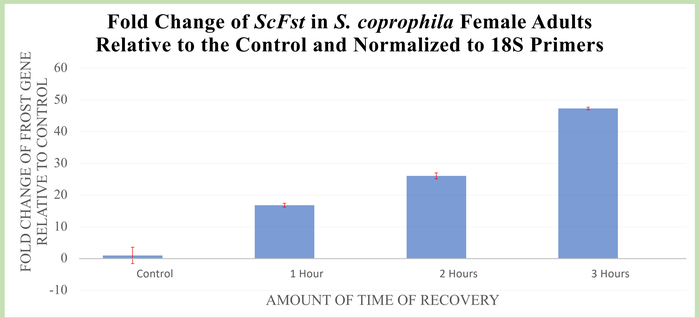

Figure 4. Graph of the first experiment with adult females representing the fold change of *ScFst* relative to the control variable and normalized to the *Sciara* 18S Primers.

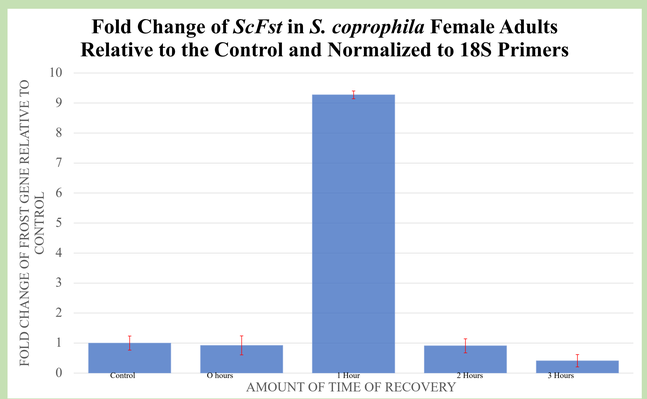

Figure 5. Graph of the second experiment with adult females representing the fold change of *ScFst* relative to the control variable and normalized to the *Sciara* 18S Primers.

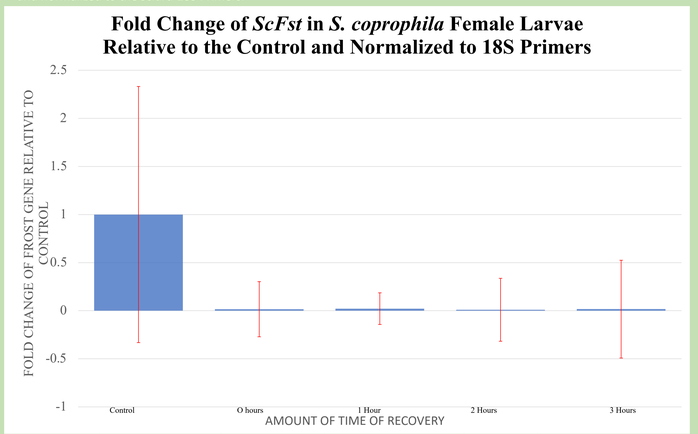

Figure 6. Graph of the experiment with fourth larval instar females representing the fold change of *ScFst* relative to the control variable and normalized to the *Sciara* 18S Primers.

Figure 7. Forward and reverse primers for *ScFst* sequence for cloning into pCR2.1.

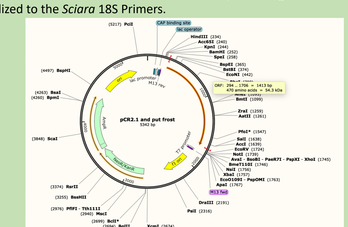

Figure 8. pCR2.1 plasmid with *ScFst*

Materials and Methods

Six adult female flies were left in incubator as a control and eighteen flies were chilled to 4ºC for three hours. The flies recovered at 20º C (Bing et al., 2012, Udaka et al., 2010) for the following times: six flies experienced a one-hour recovery, five flies experienced a two-hour recovery, and five flies experienced a three-hour recovery (one fly in the two-hour recovery period and one fly in the three-hour recovery period were not used due to drowning in condensation in the vial). These groups were separated into 1.5mL Eppendorf tubes and placed into the -80º C freezer after their recovery period to preserve until use.

The flies were then ground using TRIzol reagent in their Eppendorf tubes and total RNA was recovered. Total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (random hexamer primers) from ThermoFisher. DNA is much more stable than RNA, so doing this ensures it won't degrade as quickly and easily. The resulting cDNA was used as template for Real Time PCR using 2X SybrGreen Master Mix (ThermoFisher). Two different reactions were run, one reaction to amplify 18S cDNA and one reaction to amplify *ScFst* cDNA. 18S primers and *ScFst* primers that were ordered from ThermoFisher. These primers specifically copy their complimentary sequences. Real Time PCR reactions were run on Applied Biosystems (ABI) 7300 Real Time PCR System.

For the qPCR reaction, fourteen reactions of 20 µl were made and twelve of the fourteen reactions were used; extra reactions were made to account for pipetting errors. To make each of the twelve reactions, two overall master mixes were made; one master mix contained the *ScFst* forward and reverse primers and the other contained the 18S forward and reverse primers. 15µl of the 18S primer master mix reaction was added to each of twelve wells in the first row of the qPCR well plate and 15µl of the *ScFst* primer master mix reaction was added to each of twelve wells in the second row of the qPCR well plate. Then, the four cDNA template samples were added in triplicate to each of the two rows; the four cDNA template samples consisted of 5µl of the control cDNA, 1-hour recovery cDNA, 2-hour recovery cDNA, and 3-hour recovery cDNA. The resulting fold enrichment over the untreated control was calculated using the ΔΔCt method (Figures 4, 5, and 6).

This protocol was repeated using female larvae as well as more female adults. All variables were kept the same, except a 3-hour cold shock with a 0-hour recovery period variable was added.

The cloning procedure reverse transcriptase was performed as above, substituting the random primers with Poly-dT Oligo to amplify messenger RNA. The DNA was amplified using the *Taq* PCR protocol. The product was run on a 1.2% agarose gel using a 100 base pair ladder for reference. Unfortunately, this experiment has yet to amplify the putative *Frost* gene. Attempts to adjust the annealing temperature using a gradient PCR protocol have also been proven ineffective.

Future Directions

We plan to continue attempting to characterize expression of the putative *ScFst* in *S. coprophila*. These experiments will continue to be performed using the 0-hour recovery step. To further understand expression, we plan to add experimental flies of differing stages, such as embryos, first through fourth larval instar stages, pupae, and adults. This will help to understand the expression of *ScFst* throughout the course of the fly's life cycle. It will also give insight regarding the best stage for *S. coprophila* flies to survive exposure to cold conditions.

Attempts to clone the *ScFst* cDNA will also continue. We plan to test various magnesium concentrations in the *Taq* PCR protocol in order to enhance the likelihood of amplification. Once this fragment is isolated and amplified, it can be cloned into the pCR2.1 plasmid and then subcloned in frame with Green Fluorescent Protein (GFP) in order for it to be visualized in the cells.

Literature Cited

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