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Uncoupling Protein 1 in Bornean Treeshrews

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UNCOUPLING PROTEIN 1 IN BORNEAN TREESHREWS

by

Emily Gagne

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Biology)

The Honors College

University of Maine

May 2019

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ABSTRACT

Many thermoregulatory functions of mammals are related to the fact that they are endotherms. There are several morphological and physiological adaptations that mammals have developed over time to allow them to maintain internal heat, even in cold climates. Many mammals use UCP1, a protein that facilitates non-shivering thermogenesis, to generate heat. Recent work has shown that despite UCP1’s importance for non-shivering thermogenesis, inactivating mutations have occurred in at least 8 of 18 placental orders (Gaudry et al. 2017). My study focused on three species of Bornean treeshrews: *Tupaia tana*, *Tupaia montana*, and *Tupaia minor* (Order: Scandentia), and aimed to confirm whether or not the three tropical treeshrews show evidence of UCP1. I used Polymerase chain reaction (PCR) and designed primer sets to analyze the tissues of *Tupaia montana* and *Tupaia minor* to determine if either show evidence of UCP1. I searched the genome of *Tupaia tana* using the computer software, BLAST, and generated a potential protein sequence for UCP1. I designed primer sets based on areas of conservation in UCP1 for *Tupaia tana*, *Tupaia belangeri*, and mouse, though the bands produced in gel electrophoresis were not significant. My results present preliminary data and methodology to determine if tropical treeshrew species have functional UCP1. My study progresses our understanding of thermoregulation by addressing how mammalian taxa adapt to their unique thermal niches and to differing environmental factors.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Danielle Levesque for her continuous support and teaching me so much on this journey. I became a member of her lab when I started attending lab meetings at the end of my sophomore year, and ever since then, Dr. Levesque has provided nothing but a positive and encouraging learning environment. I cannot imagine a better advisor and mentor to guide me through this thesis process. She has helped me grow as a critical thinker, writer, and scientist.

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CHAPTER 1

LITERATURE REVIEW

Background on endothermy and ways to maintain heat

Endothermy is a trait typical of birds and mammals, in which body heat is generated primarily via a high resting rate (Bennett & Ruben 1979). Because endothermy of birds and mammals evolved separately from each other, endothermy has evolved at least twice (Ruben 1995). There are many clear benefits of endothermy, including the ability to exist in a wide range of ambient temperatures without a significant change in performance. This capacity for high activity level was originally thought to be the main benefit of endothermy (Bennett & Ruben 1979). More recent work has suggested that the evolution of endothermy was driven by a mixture of benefits, including its benefit with parental care, specifically, during incubation of the offspring (Kept 2006, Farmer 2000, Grigg et al. 2004).

Two means of thermoregulation in endotherms include shivering thermogenesis and non-shivering thermogenesis. When ambient temperature drops, the most common responses for endotherms are vasoconstriction to decrease heat loss, followed by activation of either shivering or non-shivering thermogenesis (Haman & Blondin 2017). As the name implies, shivering thermogenesis involves shivering, the process in which muscles involuntarily contract to produce heat (Haman 2006). Shivering thermogenesis can either be activated as an immediate response or after long-term cold exposure. When the skin temperature of an endotherm drops, the spinal dorsal root ganglia neurons are
stimulated (Haman 2017). The stimulation begins a feedback loop with the hypothalamus and the motoneurons, ultimately causing the contraction of the skeletal muscles (Morrison 2016).

In many cases, eutherian mammals use non-shivering thermogenesis (NST), a process that produces heat without spending energy to contract the skeletal muscles (Himms-Hagen 1984). Because shivering is costly and cannot be used for extended periods of time, NST most commonly occurs in newborn mammals and mammals that live in cold ambient temperatures for extended amounts of time (Horvath et al. 1956). NST is most productive for animals with smaller body size and less productive for animals with larger body size (Oelkrug et al. 2015). For all mammals, NST mainly occurs in skeletal muscles and in fat cells known as brown adipose tissue (Janksy 1973). Brown adipose tissue (BAT) is home to a high density of mitochondria and functions to convert energy stores to heat (Lichtenbelt 2012).

**Uncoupling protein 1 - structure and function**

Mitochondria, commonly referred to as the “power house of the cell,” are essential to metabolism. The organelle produces usable energy for the cell, primarily ATP. The mitochondria are also home to protein complexes that facilitate NST. NST is stimulated by the sympathetic nervous system and the thyroid hormone when a mammal is exposed to cold temperatures. During the stimulation of NST, an uncoupling protein known as uncoupling protein 1 (UCP1) causes the release of heat along the inner membrane of the mitochondria (van Marken Lichtenbelt 2012). Mitochondria are bound by both inner and outer membranes with areas of space between them. The outer
membrane of the mitochondria is permeable to most molecules, whereas the inner membrane has tighter regulations that allow for the electrochemical gradient to occur (Rousset et al. 2004). Along the inner membrane of the mitochondria are several complexes that facilitate cellular respiration to produce ATP in the cell.

Typically, there are four main steps of cellular respiration - glycolysis, pyruvate oxidation, citric acid cycle, and oxidative phosphorylation (Mitchell 1978). The citric acid cycle uses the acetyl coA produced during pyruvate oxidation to generate carbon dioxide and other electron carriers including NADH and FADH$_2$. These electron carriers - along with the NADH produced during pyruvate oxidation and glycolysis - deposit their electrons during the process of oxidative phosphorylation (Bear et al. 2016). Energy is released when NADH and FADH$_2$ deposit their electrons in what is known as the electron transport chain. The energy is used to transport protons against the concentration gradient into the intermembrane space of the mitochondria. The protons then flow back across the inner membrane via passive transport through a protein complex (Bear et al. 2016). In many cases, the protons flow through ATP synthase, which results in the production of ATP. In other cases, there is a protein “leak” across the inner membrane in which the protons flow through an uncoupling protein (Rousset et al. 2004). One example of an uncoupling protein is called uncoupling protein 1, or UCP1.

The activation of UCP1 is unique from the other uncoupling proteins because when protons flow through the complex instead of ATP synthase, energy is released as heat (Figure 1, Rousset et al. 2004). The process works via the activation of long-chain fatty acids (LCFAs) that facilitate the shunting of the protons across the inner membrane during non-shivering thermogenesis (Cannon & Nedergaard 2004). The long chain fatty acids...
acids have anions that have hydrophobic tails to allow the LCFAs to stay anchored to UCP1 (Fedorenko et al. 2012). Protons are shunted across UCP1 by attaching to the LCFA anion in the inner membrane space. The proton and the anion are symported across UCP1 and heat is produced as a byproduct (Ricquier & Bouillaud 2000). The production of heat is unique to UCP1, and therefore, other uncoupling proteins cannot compensate for an absence or inactivation of UCP1 (Rousset et al. 2004).

**Figure 1.** The structure of the mitochondria depicting the uncoupling process, in which oxidative phosphorylation is uncoupled from ATP synthase, and the protons are leaked across UCP1. Adapted from Fedorenko et al. 2012.

**History of UCP1 in Eutheria – functional loss through time**

UCP1 is known to be an ancient mammalian protein (Oelkrug et al. 2014). It is hypothesized that UCP1 has been conserved due to its thermoregulatory function,
especially for small and hibernating species (Gaudry & Campbell 2017). UCP1 is not conserved across all mammalian taxa, however, as neither marsupials or monotremes possess a functional UCP1 (Jastroch et al. 2015). To understand functional loss of UCP1, phylogenetic analyses. Recent work has shown that although several Eutherian mammalian species possess the gene for UCP1, pigs do not have a detectable gene (Saito, Saito, & Shingai 2007), introducing the idea that some Eutherian mammalian lineages have lost a functional UCP1.

A recent study used genomic databases to determine that UCP1 inactivation has occurred in 8 of the current 18 eutherian orders of mammals (Gaudry et al. 2017). Although inactivation of UCP1 was recorded to have occurred sometime prior, the largest period to see a trend of UCP1 inactivation was the Early Eocene when the Earth was cooling, and mammalian body size was increasing (Gaudry et al. 2017). Because of this, it is thought that UCP1 was recruited in small-bodied mammals to maintain body temperature during Earth’s cooling (Saito et al. 2007).

**UCP1 and Scandentia**

The extent of inactivation of UCP1 in eutherian mammals is not completely understood; many species either do not have a fully sequenced genome or have not been analyzed for UCP1 orthologs. It is currently unknown whether a lot of tropical species possess a functional UCP1 complex. An example of a poorly understood mammalian order from the tropics includes Scandentia, specifically the genus *Tupaia*. This group of animals, known as treeshrews, are insectivores local to Southeast Asia (Emmons 2000). Scandentia have general characteristics similar to the earliest mammals from the
Cretaceous period: short hands and feet, long muzzles, curved claws, long tails and insectivorous teeth (Emmons 2000). Many Scandentia species still possess these qualities because of recent divergence dates that were estimated via DNA sequencing of mitochondrial ribosomal genes. The Scandentia split from Dermoptera about 80 million years ago. The genus *Tupaia* diverged about 20 million years ago (Fig 3, Roberts et al. 2011).

Treeshrews are often considered scientific enigmas because very few studies have focused on them. There has been recent work, however, that focuses on the thermogenesis of the northern treeshrew, *Tupaia belangeri* (Zhang et al. 2012). The northern treeshrew has a wide geographic distribution and lives in parts of Southeast Asia that reach a colder climate (Zhang et al. 2012). Not only does *T. belangeri* show UCP1 orthologs in the genome (Gaudry et al. 2017), laboratory studies have proven its functionality (Zhang et al. 2012). NST in *T. belangeri* increased via UCP1 when the treeshrew was cold-acclimated, concluding that ambient temperature cues NST (Zhang et al. 2012).

Recent work in UCP1 functional gene loss inspired me investigate whether or not *Tupaia* species local to the tropical regions of Southeast Asia still possess a functional UCP1 (Fig 2). As the majority of Scandentia species are restricted to tropical regions, we chose to focus on three species local to the tropical island of Borneo: *Tupaia montana*, *Tupaia tana*, and *Tupaia minor*. The estimated divergence dates suggest that *Tupaia montana* split most recently from the pen-tailed treeshrew (*Ptilocercus lowii*). *Tupaia montana* diverged 13 million years ago, *Tupaia tana* diverged 8 million years ago, *Tupaia belangeri*, the species with confirmed UCP1, diverged 7 million years ago, and
lastly, *Tupaia montana* diverged 6 million years ago (Roberts et al. 2011). I hypothesize that *Tupaia minor*, *Tupaia montana*, and *Tupaia tana* all possess functional UCP1 because of the close divergence dates between the species observed here and *Tupaia belangeri*. To investigate whether or not the *Tupaia* species have functional UCP1, we used genomic alignment and PCR techniques.

![Figure 2. The current geographic ranges of *Tupaia belangeri*, *Tupaia tana*, *Tupaia montana*, and *Tupaia minor* in Southeast Asia. Geographic ranges from IUCN.](image-url)
Figure 3. The phylogeny of *Tupaia* species and closely-related species. The phylogeny includes the pen-tailed tree shrew (*Ptilocercus lowii*), the northern tree shrew (*Tupaia belangeri*), the pygmy tree shrew (*Tupaia minor*), the large tree shrew (*Tupaia tana*), the mountain tree shrew (*Tupaia montana*), the Sunda flying lemur (*Galeopterus variegatus*), the aye-aye (*Daubentonia madagascariensis*), humans (*Homo sapiens*), and mouse (*Mus musculus*). The branch lengths are to scale with estimated divergence dates. Modified from Hedges et al. 2015.
CHAPTER 2

METHODS AND STUDY DESIGN

Overview

To assess whether *Tupaia montana*, *Tupaia minor*, and *Tupaia tana* possess the genetic code for UCP1, a variety of genomic tools were used. Tissue from both *Tupaia montana* and *Tupaia minor* species were analyzed using PCR, whereas *Tupaia tana* was analyzed using an unpublished genome assembly provided by Dr. Diane Genereux from the Broad Institute (Cambridge, Massachusetts, US).

Acquiring the Scandentia Samples

Both the *Tupaia montana* and *Tupaia minor* samples were obtained from the Genetic Resources Collection from the Museum of Texas Tech University via a Destructive Loan Request. *Tupaia montana*, catalogued by the number TK152252, was a frozen lung sample, while the other, *Tupaia minor*, catalogued by the number TK168468, was tissue within a lysis buffer. Both of samples were collected in Sarawak, Malaysia, though the collecting methods and the date of collection(s) are unknown. Both samples arrived at the University of Maine on dry ice, and *Tupaia montana*, the frozen lung tissue, was stored at -80°C, whereas *Tupaia minor*, the sample within the lysis buffer, was stored at 4°C, as suggested by the Texas Tech Curator of Genetics.
DNA ISOLATION

DNA was isolated from both the *Tupaia minor* and *Tupaia montana* using a *Quick*-DNA took kit provided by Zymo Research. The first tissue sample, *Tupaia montana*, was cut to about 25 mg. A sample of the same size was also cut from the brown adipose fat from the mouse as a positive control. I followed the solid tissue protocol for both *Tupaia montana* and the mouse BAT provided by Zymo Research.

The solid tissue protocol began by placing the mouse BAT sample and *T. montana* lung sample into two separate microcentrifuge tubes that each contained 95 µL of water, 95 µL of *Quick*-DNA took kit solid tissue buffer and 10 µL of proteinase K that had been mixed with storage buffer. Both sample solutions were incubated at about 56°C for 100 minutes before being vortexed and then centrifuged for 1 minute at 12,000 x g. The supernatant from the top of each microcentrifuge was collected. It was estimated that 160 µL of *Tupaia montana* supernatant was collected and 145 µL of mouse supernatant was collected. The supernatants of both samples were transferred to separate clean microcentrifuge tubes and labeled accordingly, before 320 µL of genomic binding buffer was added to the *Tupaia montana* supernatant and 290 µL of genomic binding buffer was added to the mouse supernatant. Both mixtures were transferred to spin columns provided by the *Quick*-DNA took kit sitting within collection tubes and centrifuged for 1 minute at 12,000 x g. Both spin columns were transferred to new collection tubes and each washed using 400 µL of DNA pre-wash. These spin columns were centrifuged again at 12,000 x g for 1 minute. The flow-through from both of the collection tubes was discarded, and then 700 µL of g-DNA wash buffer was used to wash both collection tubes before being
centrifuged for 1 minute at 12,000 x g. The flow-through was discarded, and 200 µL of g-
DNA wash buffer was added to each spin column and then centrifuged at 12,000 x g for 1
minute. Lastly, 30 µL of water was washed through each spin column to elute the DNA.
Each spin column was centrifuged at 20,000 x g for 1 minute. The flow-through was
washed through each spin column again, and then both spin columns incubated at 23°C
for 6 minutes. The spin columns were each centrifuged again at 20,000 x g for 1 minute.
The spin columns were then discarded, and the collection tubes were properly labeled as
“mouse DNA” and “montana DNA.” Each sample was NanoDropped to quantify the
yield of the mouse and Tupaia montana DNA.

_Tupaia minor_ DNA was isolated using a mixture of the solid tissue and biological
fluid protocols provided by Zymo Research Quick-DNA took kit. The Museum of Texas
Tech University general lysis buffer recipe included 50 ml of 2 M Tris-HCl, 200 mL of
0.5 M EDTA, 2 ml of 5 M NaCl, under 975 mL of double-distilled water, and 25 mL of
20% SDS (Longmire et al. 1997). Despite listing this recipe as standard, colleagues at
Texas Tech could not guarantee that the wild-caught _Tupaia minor_ sample was collected
and stored in the same manner. I contacted a specialist at Zy
mo Research to see if the
Quick-DNA Universal Kit would be compatible with the lysis buffer, assuming that its
ture unknown recipe was similar to the one provided in the Longmire et al. (1997)
protocol. The specialist confirmed compatibility and suggested that the _Tupaia minor_
sample follow the biological fluid protocol provided by Zymo Research.

DNA isolation of _Tupaia minor_ began by combining 200 µL of the sample with
200 µL of biofluid & cell buffer and 20 µL of proteinase K that had been mixed with
storage buffer. The sample was vortexed and incubated at 55°C for 45 minutes and then
centrifuged at 12,000 x g for 1 minute. These steps were repeated an additional 3 times in order to use all of the 800 µL of sample that was provided by the Texas Tech Institute. All of supernatant from all four of the collection tubes were then transferred to new collection tubes. A total of 200 µL of genomic binding buffer was added to each of the four new collection tubes and all were vortexed. The solutions were all transferred to four spin columns within collection tubes and centrifuged at 12,000 x g for 1 minute. The collection tubes were all replaced. Then, 400 µL of DNA pre-wash was added to each spin column and all were centrifuged for 1 minute at 12,000 x g before discarding the flow-through. Another wash and centrifuge procedure was done using 700 µL of g-DNA wash buffer for each spin column. The flow-through was discarded, and finally, 200 µL of g-DNA wash buffer was washed through each spin column and centrifuged. Lastly, 30 µL of nuclease-free water was washed through each column, centrifuged at 20,000 x g for 1 minute, and left to incubate for 5 minutes at 23°C. The flow-through of each collection tube was re-run through the spin tube and centrifuged again for 1 minute at 20,000 x g. All of the flow-through, which contained the DNA at this point, was combined. The DNA was labeled “Tupaia minor” and then 1 µL was NanoDropped to quantify the yield of DNA.

GENOMIC ALIGNMENT AND PROTEIN SEQUENCE PREDICTION

Genomic alignment was used in order to understand the UCP1 genomic relatedness between Tupaia belangeri, Tupaia tana, and mouse. Ensembl (Zerbino et. al 2018) was used to search for the coding sequence of UCP1 in Tupaia belangeri, the treeshrew species that lives in colder climate and has functional UCP1. Despite
predicting the transcript of the exons of UCP1, Ensembl did not show the locations of the 5’ and 3’ untranslated regions, so we used the mouse genome to locate the UTRs. We aligned protein coding sequence of UCP1 in *Tupaia belangeri* with the protein-coding sequence of UCP1 in mouse using BLAST, though the 3’ untranslated region was not detected. To detect the translated regions, we aligned the protein-coding UCP1 sequence of the closely-related colugo, *Cynocephalus volans*, with that of *Tupaia belangeri* using BLAST. We identified the untranslated regions in *Tupaia belangeri* and used the information to annotate the UCP1 protein-coding sequence.

We searched the *Tupaia tana* genome assembly for the UCP1-coding sequence using BLAST. We aligned this transcript sequence, along with the transcript sequences from mouse, and *Tupaia belangeri* via a CLUSTAL multiple sequence alignment (Zerbino et al. 2018) for all six exons of UCP1 to show the regions of conservation across the exons (Fig 5, Fig 6).

**PRIMER DESIGN**

Consensus primers for PCR were designed using the conserved regions of the alignments (Fig 5, Fig 6). The sequences for all UCP1 exons in mouse were separately run through the programs called Primer3web and PrimerQuest using standard parameters. For each exon, the suggested forward and reverse primers were compared to the multiple species’ alignment between *Tupaia belangeri*, *Tupaia tana*, and mouse. The mouse genome was searched manually to locate where the forward and reverse primers would bind. The forward primer had the same order of base pairs as the genome, and the reverse primer was the reverse complement of the genome. The primer sets that were located
closest or within the conserved regions were selected. Because no primers fell entirely within the conserved regions, certain base pairs were altered using mixed bases for areas that were not conserved across the three species.

**PCR and Gel Electrophoresis**

PCR and gel electrophoresis and imaging were performed using the designed primer sets. To understand these techniques prior to this project, samples from Maine’s flying squirrels (*Glaucomys sabrinus* and *Glaucomys volans*) were genotyped (Appendix I). All of the primer sets designed for this thesis project arrived from IDT and were reconstituted prior to the PCR procedure. All of the primer tubes were centrifuged before water was added in the correct amount, as designated by the spec sheets provided by IDT.

The first step of PCR was creating a master mix for each of the three primer sets, named Primer Set 1, Primer Set 2, and Primer Set 3. A combination of 60 µL of GoTaq Green Master Mix, 0.72 µL forward primer, 0.72 µL reverse primer, and 52.56 µL of water was vortexed in a nuclease-free centrifuge tube labeled for Primer Set. The formula was repeated in two other centrifuge tubes for Primer Set 2 and Primer Set 3. Strip PCR tubes were labeled 1-12 and 9.5 µL of the correct master mix was added to each tube, followed by 0.5 µL of either DNA or water (Table 1).
Table 1. PCR strip tube labeling: The tubes labeled 1-9 each contained either Mouse BAT, *Tupaia montana*, or *Tupaia minor* DNA. Tubes 1-3 used the master mix with Primer Set 1, Tubes 4-6 used the master mix with Primer Set 2, and Tubes 7-9 used the master mix with Primer Set 3. Tubes 10-12 contained water instead of DNA and were combined with master mix with Primer Set 1, 2, or 3. Tubes 10-12 acted as the negative controls to assure no contamination occurred.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>DNA Sample</th>
<th>Primer Set</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mouse BAT</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td><em>Tupaia montana</em></td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td><em>Tupaia minor</em></td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Mouse BAT</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td><em>Tupaia montana</em></td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td><em>Tupaia minor</em></td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>Mouse BAT</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td><em>Tupaia montana</em></td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td><em>Tupaia minor</em></td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>No DNA (water)</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>No DNA (water)</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>No DNA (water)</td>
<td>3</td>
</tr>
</tbody>
</table>

The PCR tubes were vortexed and run in the thermal cycler (Cell Culture Digital Thermal Cycler) using the standard program provided by BIO RAD. A medium 3% agarose gel was made by combining 180 mL of TAE buffer and 5.367 grams of agarose. The mixture was microwaved until the agarose was fully dissolved and then consistently stirred while it cooled. Then, 100 µL of ethidium bromide was added to the solution and mixed thoroughly before the solution was poured into the gel caster. Two combs were
added, one the end and one in the middle. The gel solidified and then was covered in TAE buffer. The combs were removed and 7 μL of 100 bp ladder was loaded into the first lane, 7 μL of 50 bp ladder was loaded into the second lane, 10 μL of each sample from Tubes 1-12 were added to separate lanes, followed by two more lanes of 100 bp and 50 bp ladders, respectively. The gel was run at 200V for about 25 minutes and then imaged using GenSys software.
CHAPTER 3
RESULTS

Genomic alignment and primer design

Using Ensembl and BLAST, we found the six exons of UCP1 in mouse. In order to make *Tupaia tana* searchable on BLAST to look at the UCP1 exons, the annotated genome of *Tupaia belangeri* was aligned against *Tupaia tana*. BLAST then located the six exons of *Tupaia tana* to give both the genomic transcript and the predicted protein sequence for UCP1. The predicted protein sequence of UCP1 in *Tupaia tana* is as follows within the multiple species alignment:

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Exon 1</th>
<th>Exon 2</th>
<th>Exon 3</th>
<th>Exon 4</th>
<th>Exon 5</th>
<th>Exon 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCPI_tree_shrew</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCPI_Tupaia_tana</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCP1_tana</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

> Mouse, UCPI_tree_shrew, UCPI_Tupaia_tana, UCP1_tana

**Figure 4.** The predicted protein sequence of UCP1 in *Tupaia tana* compared to mouse and *Tupaia belangeri* (labeled UCPI_tree_shrew in the figure). We predicted the protein sequence using the six exons of UCP1 in Blast. Figure provided by B. King 2019.
CLUSTAL multiple sequence alignment showed the areas of conservation between the genomes of the mouse, *Tupaia belangeri*, and *Tupaia tana* for all six exons of UCP1. Some exons were more conserved across all the species than others, including exons 3, 4, and 6. Conservation is indicated by asterisks in Figures 5 and 6. The asterisks show that all three species have the same base pair at that location, and therefore, the base pair is considered conserved across the species.
Figure 5. The genomic alignment of UCP1 exons across *Mus musculus*, *Tupaia belangeri*, and *Tupaia tana*. The asterisks show the regions of conservation, where all three species have the same base pair. The blue region represents exon 3, the green region represents exon 4, the yellow region represents exon 5 and the pink region represents the start of exon 6, which is continued in Figure 6. This figure excludes exons 1 and 2 as well as the introns, or noncoding regions, between the exons shown. Figure provided by B. King 2019.
Figure 6. The genomic alignment of UCP1 exon 6 between *Mus musculus*, *Tupaia belangeri*, and *Tupaia tana*. The asterisks show base pairs that are conserved across all three species and the hyphens indicate the areas of the exon that are unknown. Figure provided by B. King 2019.
The areas of conservation are indicated by regions with several asterisks in a row. Some of the longest conserved regions are in exons 3 and 4. Although the goal was to design a consensus primer set for all three species within these regions, PrimerQuest did not suggest many primer sets within exon 4, and no suggestions were in the conserved regions. The aim of the primer sets was to use as few mixed bases as possible to ensure binding sites. Unfortunately, no suggested primer set fell within completely conserved regions. Because of this, all of the primer sets that were ordered have a variety of mixed bases (Table 2).

Figures 7 and 8 show exons 3 and 6 of UCP1 in mouse, as PrimerQuest and Primer3web suggested primer sets for these regions that would require the least number of mixed bases for both forward and reverse primers. The highlighted bases, like the asterisks in Figures 5 and 6, indicate that that position in mouse has the same base pair in the same location for both Tupaia tana and Tupaia belangeri. The underlined regions indicate where the forward and reverse primers would bind for each primer set.

| CACCTGCCCTCTCTCGGAAACAAGATCTCAGCCGGCTTTAATGACTG | AAGCTTGTTCAACACTTTGGAAAG |
| CACCTGCCCTCTCGGAAACAAGATCTCAGCCGGCTTTAATGACTG | AAGCTTGTTCAACACTTTGGAAAG |

**Figure 7.** Exon 3 of mouse. Highlighted base pairs indicate that the base pair is the same at that location for mouse, Tupaia tana, and Tupaia belangeri. The base pairs that are underlined in black indicate where the forward primer for exon 3 would bind. The base pairs that are underlined in red text indicate where the reverse primer would bind. The top box indicates Primer Set 1, and the bottom box indicates Primer Set 2.
Figure 8. Exon 6 of mouse. Highlighted base pairs indicate that the base pair is the same at that location for mouse, *Tupaia tana*, and *Tupaia belangeri*. The base pairs that are underlined in black indicate where the former primer would bind on exon 6, and base pairs underlined in red text indicate where reverse primers would bind. This primer set is named Primer Set 3.

The forward and reverse primers accounted for base pairs not conserved across mouse, *Tupaia tana*, and *Tupaia belangeri* by using the mixed base “wobbles” provided by Integrated DNA Technologies.

Table 2. The UCP1 primer sets for exons 3 and 6 that include the mixed bases in the oligonucleotides. The letters other than C, T, A or G indicate base pairs that are not conserved across *Tupaia tana*, *Tupaia belangeri*, and mouse.

<table>
<thead>
<tr>
<th>Set</th>
<th>Exon</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>MGGAARCAAGATCTCAGCYG</td>
<td>YRACARGCYTTCTGYGTKGTKR</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>RCAAGATCTCAGCYYGGCTTA</td>
<td>TCGYKTGGKRTCTAAYTCTGT</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>CTGATGAAAGTCMMGRCA GCHR</td>
<td>TCYTTTYCAYYAGTTTGAGY</td>
</tr>
</tbody>
</table>
DNA Isolation

The NanoDrop instrument measured the concentration of DNA from the mouse BAT tissue, the *Tupaia montana* lysis buffer sample, and the *Tupaia minor* frozen lung sample.

Table 3. The concentrations of DNA yielded from the *Mus musculus*, *Tupaia minor*, and *Tupaia montana* samples

<table>
<thead>
<tr>
<th></th>
<th><em>Tupaia montana</em></th>
<th><em>Tupaia minor</em></th>
<th><em>Mus musculus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>[DNA] in ng/µL</td>
<td>439.1</td>
<td>414.58</td>
<td>439.06</td>
</tr>
</tbody>
</table>

PCR and gel electrophoresis

I aliquoted the PCR products into the lanes in the 3% agarose gel, starting with 100 bp ladder, followed by 50 bp ladder, followed by PCR products from tubes 1-12 respectively (Table 1). I imaged the gel using GenSys and then labeled each lane in correspondence with the PCR product tube (Figure 9).
Figure 9. The imaged gel of *Tupaia montana*, *Tupaia minor*, and mouse. The labels reflect those listed in Table 1. The 100 bp ladder is furthest to the left, followed by 50 bp ladder, followed by PCR products from tubes 1-12, followed more 100 bp ladder and 50 bp ladder, respectively.
CHAPTER FOUR

DISCUSSION

Ensembl and BLAST were used to show that *Mus musculus, Tupaia belangeri,* and *Tupaia tana* have several areas of conservation across the six exons of UCP1. Additionally, we located six exons of *Tupaia tana* using BLAST and found both the genomic transcript and the predicted protein sequence for UCP1. The predicted protein sequence suggests that *Tupaia tana* has UCP1, thus indicating that the species has conserved the Eutherian trait, despite living in a tropical region that does not get cold enough to generate non-shivering thermogenesis.

I used PCR and gel electrophoresis to show the presence of 2 bands with the use of Primer Set 2 for PCR tubes 4 and 6, which were mouse BAT and *Tupaia minor,* respectively (Table 1, Figure 9). The bands appeared in the regions predicted according to the amplicon size, though the mouse BAT sample would have been a better positive control had additional primers been ordered with no mixed bases (i.e. the original primers suggested by PrimerQuest through IDT). Regardless, the bands were deemed insignificant and were not extracted for sequencing, as they could not be seen when put on top of the UV light. The PCR and gel electrophoresis/imaging data are preliminary results, however, in that Primer Set 2 has the potential to bind to *Tupaia minor, Tupaia montana,* and mouse, despite the use of so many mixed bases. Prior to graduation, I will be running the PCR and gel electrophoresis again using Primer Set 2, though to make the bands appear cleaner, I will be using 2 µL of DNA instead of the 0.5 µL of DNA that was
used in this study. If the bands are clean enough, they will be extracted and then sent to sequencing for further analysis.

It is important to keep in mind, however, that there are mutations that can affect genomic areas both within and outside of the coding sequences that can alter the gene expression (Friedberg 2003). Examples of loss-of-function mutations include those that reduce the protein’s ability to work (Loeb et al. 2003). Further work would need to be performed to understand possible mutations within transcription factor binding sites, as these binding sites are often where inactivating mutations arise in UCP1 (Gaudry et al. 2017).

My thesis offers preliminary results towards understanding the thermogenesis of Bornean treeshrews. The hypotheses that Tupaia minor, Tupaia montana, and Tupaia tana all possess UCP1 can be neither supported or rejected by these data because of the inconclusive results. Future work should focus on the protein-level to assess functionality of UCP1. One method to check for UCP1 protein levels would be with a Western Blot, a process that allows the target protein to be marked and visualized using both primary and secondary antibodies (Mahmood 2012). The Western Blot procedure would require more specimens, however, as this process would not be possible only using the tissue smaples provided from Texas Tech. The functionality of the protein can also be tested in vivo by measuring NST when the treeshrews are acclimated to long-term cold exposure. One group of treeshrews must act a control while the other is a group of treeshrews would be treated with norepinephrine. The control group would remain in warm temperature while the treated group would remain in the cold. NST could be measured and compared across the two groups (Zhu et al. 2010).
Although the results of my study are currently inconclusive, the methodology I developed can be used in further work to understand the thermogenics of tropical treeshrews and assess whether or not they possess functional UCP1. The results of future research can be used to predict the methods of thermogenesis for each Bornean treeshrew species and draw comparisons across other mammalian taxa. Additionally, my work touches on the importance of understanding the evolution of thermophysiology in little-studied mammalian taxa. Knowing the timescale of inactivating mutations allows for researchers to predict the impacts of environmental factors, including climate change.
REFERENCES


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Ricquier D, Bouillaud F (2000) The uncoupling protein homologues: UCP1, UCP2, UCP3, StUCP and AtUCP. *Biochemical Journal.* 10.1042/bj3450161


APPENDIX I

GENOTYPING FLYING SQUIRRELS

Background – Genotyping flying squirrels in Maine

In order to learn the PCR and gel electrophoresis techniques that I would need for this thesis, I worked on genotyping flying squirrels that were trapped in Maine as a supplement to the capstone project that Tashawna Spellen wrote in May 2018 while working with Dr. Danielle Levesque, Dr. Han Tan and Vanessa Hensley. The project was entitled, “The possibility of hybridization in Maine flying squirrels” and aimed to understand the ranges of northern flying squirrels, *Glaucomys sabrinus*, and southern flying squirrels, *Glaucomys volans* and investigate whether or not there was hybridization between the two species (Spellen et al. 2018). The flying squirrels were trapped from the University of Maine: the Holt Research Forest in Arrowsic, the DeMerritt Forest in Orono, and University Forest at the University of Maine in Orono. Spellen then underwent PCR protocol using the following primers: PvolE6, Pvol74, and Pvol41. These primers did not show results for several samples when gel electrophoresis was performed, so I used the DNA left over from all of the flying squirrel samples and reran the PCR and gel electrophoresis using the forward, reverse, and species-specific primers developed by Rogic et al. (2016). Table 3 shows the numbering system used to label each sample.
Table 4. Numbering system for the flying squirrel samples to be genotyped using PCR and gel electrophoresis. Each sample corresponds to the trapping site that the squirrels were trapped at during the summer of 2017.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>Trapping Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Northern Maine (northern control sample)</td>
</tr>
<tr>
<td>2</td>
<td>N/A – artificial hybrid</td>
</tr>
<tr>
<td>3</td>
<td>DeMerrit Forest (southern control)</td>
</tr>
<tr>
<td>4</td>
<td>DeMerrit Forest</td>
</tr>
<tr>
<td>5</td>
<td>DeMerrit Forest</td>
</tr>
<tr>
<td>6</td>
<td>DeMerrit Forest</td>
</tr>
<tr>
<td>7</td>
<td>DeMerrit Forest</td>
</tr>
<tr>
<td>8</td>
<td>DeMerrit Forest</td>
</tr>
<tr>
<td>9</td>
<td>UM campus</td>
</tr>
<tr>
<td>10</td>
<td>UM campus</td>
</tr>
<tr>
<td>11</td>
<td>UM campus</td>
</tr>
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<td>12</td>
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<td>UM campus</td>
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<tr>
<td>14</td>
<td>UM campus</td>
</tr>
<tr>
<td>15</td>
<td>UM campus</td>
</tr>
<tr>
<td>16</td>
<td>Holt Forest</td>
</tr>
<tr>
<td>17</td>
<td>Unknown</td>
</tr>
<tr>
<td>18</td>
<td>Unknown</td>
</tr>
<tr>
<td>19</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Methods

The PCR primers were prepared in accordance with the methods used in the Rogic et al. (2016) study, using their designed species-specific primers for *G. sabrinus* and *G. volans*, as well as forward and reverse primers to detect cytochrome B (Cytb) mitochondrial gene and the cannabinoid receptor type 1 (CNR1) nuclear gene. Because of this, two primer set mixtures were used with three primers each (Table 5).
Table 5. Primers designed by Rogic et al. (2016) to genotype *Glaucomys sabrinus* and *Glaucomys volans* using PCR and gel electrophoresis. The listed primer sets show the inclusion of the forward primer, the reverse primer, and the species-specific primer designed for each species.

<table>
<thead>
<tr>
<th>Primer Set 1 – Cytb Primer Mix</th>
<th>Primer Set 2 – CNR1 Primer Mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytb L14724</td>
<td>CNR1-5R</td>
</tr>
<tr>
<td>5’ –</td>
<td>5’–</td>
</tr>
<tr>
<td>CGAAGCTTGATATGAAAAACCAT</td>
<td>CAGACTGMAGCTTTCTTGCAGTTCC-</td>
</tr>
<tr>
<td>CGTTG – 3’</td>
<td>3’</td>
</tr>
<tr>
<td>Cytb H15149</td>
<td>CNR1-11F</td>
</tr>
<tr>
<td>5’ – AAACTGCGAGCCCCTCAGA</td>
<td>5’ – AGTGTGGGGAAGAACTTC</td>
</tr>
<tr>
<td>ATGATATTTGTCTCTCA – 3’</td>
<td>ATGGACAT – 3’</td>
</tr>
<tr>
<td>Cytb-sabrinus-2F</td>
<td>CNR1-sabrinus-R</td>
</tr>
<tr>
<td>5’ – NNCGCAAATGGTGCT – 3’</td>
<td>5’ – GCTCCCCAGAAGGTCTGCC – 3’</td>
</tr>
</tbody>
</table>

Before each primer mix was put together, 239 µL of TE buffer was added to Cytb-sabrinus-2F, 339 µL of TE buffer was added to L14724, and 253 µL of TE buffer was added to H15149. Then, 10 µL of this mix were combined with 70 µL of water in a separate centrifuge tube to create primer set 1 (Cytb mix). Then, 330 µL of TE buffer was added CNR1-sabrinus-R, 258 µL of TE buffer was added to CNR1-11F, and 292 µL of TE buffer was added to CNR1-5R. Then, 10 µL of this mix and 70 µL of water were mixed together in a separate centrifuge tube to create primer set 2 (CNR1 mix). Both centrifuge tubes were centrifuged for 20 seconds.

To have a control for a northern sample, a clip of ear tissue from *Glaucomys sabrinus* provided by Dr. Danielle Levesque was combined with 500 µL of NaOH and then boiled for 15 minutes. After cooling, 50 µL of HCl was added. This isolated the
DNA for a positive control. To create an artificial hybrid control, 10 µL of the
_Glaucomyx sabrinus_ was combined with 10 µL of eluted DNA from a _Glaucomyx volans_ sample that was provided by Tashawna Spellen.

To prepare for PCR, all 18 samples plus the hybrid were thawed in the incubator, mixed, and then centrifuged for 20 seconds each. A “master mix” was created for both the Cytb and CNR1 genes. The Cytb mixture combined 150 µL of 2xMM, 20 µL of the Cytb Primer Mix, and 110 µL of nuclease-free water. The CNR1 mixture combined 150 µL of 2xMM, 20 µL of the CNR1 Primer Mix, and 110 µL of nuclease-free water. The Cytb master mix was allotted into 19 centrifuge tubes (14 µL each tube), and then 1 µL of DNA was added separately to one of the centrifuge tubes. The first centrifuge tube had the Northern sample for control, and the second centrifuge tube had the hybrid mix. Then, the CNR1 master mix was allotted into 19 centrifuge tubes (again, 14 µL each tube), and then 1 µL of DNA was added to the centrifuge tubes in the same order as with Cytb.

The samples went through thermal cycling using the parameters as written in Rogenic et al. (2016). Then, two 1% agarose gels were prepared by combined 100 mL of 1.0% SeaKem TAE, 1.0 gram of agarose, and 2 µL of ethidium bromide. A large comb was used placed into the gel to create the slots. A total of 3 µL of DNA ladder was put into the first slot and then 12 µL of PCR prepared for the Cytb gene were put into the remaining 19 slots, with the northern sample first, followed by the artificial hybrid, followed by each sample from Spellen’s capstone project. This was repeated for CNR1. The gel ran at 150V, and then both gels were imaged using the program GeneSys.
Results and Analysis

The imaged gels were used to identify the flying squirrel samples as *Glaucomys volans*, *Glaucomys sabrinus*, or a hybrid of the two. As shown in Table 3, the northern sample was sample 1, the artificial hybrid sample was sample 2, and the southern sample control was sample 3. The samples are labeled below, with sample 1 in the slot furthest to the left next to the ladder, and samples 2 through 19 in order after that.

![Imaged gel for Cytb gel electrophoresis](image)

**Figure 10**: Imaged gel for Cytb gel electrophoresis, with sample 1 as the *Glaucomys sabrinus* control, sample 2 as the artificial hybrid, and sample 3 as the *Glaucomys volans* control.
Figure 11: Imaged gel for CNR1 gel electrophoresis, with sample 1 as *Glaucomys sabrinus*, sample 2 as the artificial hybrid, and sample 3 as the *Glaucomys volans* control.

According to the gel electrophoresis of both the CNR1 and Cytb gel images, all of the flying squirrels belong to the species *Glaucomys volans*, as all of the bands for the other samples match the *Glaucomys volans* control that is shown by slot number 3. The primer mixtures were successful and able to yield bands for all samples for both the CNR1 and Cytb genes.
AUTHOR’S BIOGRAPHY

Emily Gagne will be graduating from the University of Maine in May 2019 with a B.S. in Biology with a minor in Dance. At UMaine, she is the president of the Tap Dance Club, VP of the Hip Hop Club, a University of Maine student choreographer, and the 2019 choreographer for the American Collegiate Dance Association conference. Emily finds so much peace and happiness when dancing in the studio in the Class of 1944 Hall on campus.

Emily is graduating with the highest GPA within the biology major, an accomplishment that has only been made possible by my wonderful professors. Emily has been moved to love science and seek investigation through this program, so much so that it will be bittersweet to graduate. Emily also has appreciated all of the teaching opportunities that the University of Maine has provided me with. Emily has been a Maine Learning Assistant for seven consecutive semesters of my undergraduate career, for courses including Organic Chemistry I, Basic Biology, Biology of Organisms, General Chemistry II, and Elementary Algebraic Models. Emily has also been a one-on-one tutor for these courses throughout college.

Starting in the fall of 2019, Emily will be attending Penn State University to pursue my PhD in Ecology. Emily will be studying how human-induced climate change affects evolutionary genomics. Emily plans on continuing my dance career at Penn State, as well.