Developing a Novel Place Preference Assay to Compare Drosophila Species Over Time

Martha M. Brinson

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DEVELOPING A NOVEL PLACE PREFERENCE ASSAY TO COMPARE DROSOPHILA SPECIES OVER TIME

By
Martha M. Brinson

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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ABSTRACT
MARTHA M. BRINSON: DEVELOPING A NEW PLACE PREFERENCE ASSAY TO COMPAR DROSOPHILA SPECIES OVER CIRCADIAN RHYTHMS

Across phylogeny, integration of external factors, memory, and internal states of the organism dictate organismal behavior and mechanisms. The underlying genetic components can affect these behaviors such as in genomic changes arising from speciation. In this thesis, a new place preference assay was evaluated in the analysis and investigation of two species of Drosophila flies (D. melanogaster and D. simulans) to measure similarites and differences and their attraction to two different food substrates. Sleep and circadian measurements were also recorded during experimentation. The Drosophila Activity Monitor 5M (DAM5M) System and Sleep Circadian Analysis MATLAB Program (SCAMP) analysis were used in experimentation. Two-way ANOVA was carried to determine statistical significance between effects of species and time of day, and differences in activity, sleep, and place preference.
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<td>ACT</td>
<td>Antennal cerebral tract</td>
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<tr>
<td>AL</td>
<td>Antennal lobe</td>
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<td>CAFÉ</td>
<td>Capillary feeder</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CRY</td>
<td>Cryptochrome</td>
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<td>DN</td>
<td>Dorsal neurons</td>
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<td>DAM5M</td>
<td><em>Drosophila</em> Activity Monitor 5M System</td>
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<td>DD</td>
<td>Constant dark</td>
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<td>DP</td>
<td>Dark period</td>
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<td>DPM</td>
<td>Dorsal paired medial neurons</td>
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<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
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<tr>
<td>IR</td>
<td>Infrared</td>
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<td>KC</td>
<td>Kenyon cell</td>
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<td>LD</td>
<td>Light-dark cycles</td>
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<td>LN</td>
<td>Lateral neurons</td>
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<td>MB</td>
<td>Mushroom body</td>
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<td>Per</td>
<td><em>Period</em> clock gene</td>
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<tr>
<td>Pdf</td>
<td>Pigment-Dispersing Factor</td>
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<td>PN</td>
<td>Projection neuron</td>
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<td>POT</td>
<td>Posterior optic tract</td>
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<td>Rut</td>
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<td>SCAMP</td>
<td>Sleep and Circadian Analysis MATLAB Program</td>
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<td><em>Timeless</em> clock gene</td>
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<td>ZT</td>
<td>Zeitgeber time</td>
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Introduction

*Ecological Generalists and Specialists differ in their behaviors.*

Generalist species can survive and thrive in many, different habitats and with a wide range of food sources, while specialist species occupy a narrower niche and have specific environments and food sources (Michálek et al., 2017). Stimulus novelty is of special interest in studying generalist and specialist species. Processes of neophilia and neophobia, the attraction or aversion to novel stimuli, drive and separate reactions to novelty (Corey, 1977). These traits of neophilia and neophobia can be differentially seen in generalists and specialists. Generalist species typically express greater neophilic tendencies, while specialist species typically express greater neophobic tendencies (Hughes, 1997). This difference could help explain the variation in generalist and specialist species behavior. Generalist species display greater behavioral flexibility and having neophilia drives exploration of habitat and its resources, which is important in generalist species and in a role as invasive species (Greenberg, 2003). Invasive species, exacerbated by human involvement and climate change, decrease biodiversity through outcompetition and species extinction, destruction of habitat, and carrying of disease (Fei et al., 2014). In humans, neophilic attributes are associated with increased drug abuse and other high-risk behaviors (Bardo et al., 1996).

Neophobia reduces the negative outcomes associated with exploratory behavior, including increased exposure to predation and other risk factors (Crane & Ferrari, 2017). For specialists, the benefits of neophobia outweigh the benefits of neophilia and allow the species to develop a niche with an abundant resource and decreased competition (Greenberg, 2003). Specialist species are also of importance in climate change. With their dependency on their
specific niche, they are more sensitive to ecological change and destruction of habitat (Colles et al., 2009).

Much is still unknown about the mechanisms underlying neophilic and neophobic behaviors. Studying these differences and changes in food specialization in closely related species provides a broader understanding of how and why these differences exist and is also of increasing importance and interest with its relationship with climate change.

*Specialization in the Drosophila melanogaster group*

*D. sechellia* is a specialist species that evolved to feed on the fruit of a single plant species, *Morinda citrifolia* (Tsacas & Bächli, 1981). *D. sechellia* is endemic to the Seychelles Islands off the coast of East Africa (Tsacas & Bächli, 1981). The fruit of *M. citrifolia*, also known as noni, contains the chemical octanoic acid, which is toxic to many species of *Drosophila* (Legal et al., 1994). Therefore, these species of *Drosophila* will avoid octanoic acid (Amlou et al., 2004). Octanoic acid in *M. citrifolia* is heaviest in its concentration at peak ripening of the fruit and decreases in concentration as further ripening occurs (Amlou et al., 2004). *Drosophila* are known for their feeding behaviors on overripen fruit, furthering intrigue to this unique behavior in the genus. *D. sechellia* has tolerance for relatively high concentrations of octanoic acid; *D. melanogaster* and *D. simulans* both show high sensitivity to octanoic acid (Legal et al., 1992).

*D. sechellia* use the fruit of *M. citrifolia* to both feed and reproduce (Louis & David, 1986). Octanoic acid is essential for the reproduction of *D. sechellia* as it induces oviposition in the flies but has also been observed to inhibit oviposition in the generalist species (Lanno et al.,
2019). *D. sechellia* have mutations that account for the octanoic resistance, but also allowing for low levels of L-DOPA (Lanno et al., 2019). L-DOPA is the precursor to dopamine and in addition to its many hormonal effects, its decreased levels affect fertility by reducing egg size and amount of egg production (Budnik & White, 1987). *Morinda citrifolia* is found to contain very high levels of L-DOPA, leading to *D. sechellia* specialization and reproduction (Lavista-Llanos et al., 2014).

*D. simulans* is more closely related to *D. sechellia* than *D. melanogaster* (Sturtevant, 1920). *D. simulans* is marked by its differences in recombination and behavioral aspects of mating. *D. simulans* has a 30% increase in recombination frequency than *D. melanogaster* (True et al., 1996). It also has light-dependent mating behaviorisms as compared to *D. melanogaster* being a light-independent species. Studies have shown around a 59% decrease in mating when held in constant dark (DD) for two hours (Shahandeh et al., 2020). This light dependency raises questions to the implication of anatomy and physiology in *D. simulans* and its effects of circadian rhythms.

*Circadian Rhythms: Anatomy, Physiology, and importance of monitoring feeding and sleep behaviors.*

Circadian rhythms are the regular behavioral and biological patterns in all organisms examined (Gerstner & Yin, 2010). They are cyclical and last around a 24-hour period, regulated by exogenous cues, such as light-dark cycles (LD), leading to inherent time-of-day in an organism (Peschel & Helfrich-Forster, 2011). The components of circadian rhythm include the endogenous clock and input and output pathways. The endogenous clock functions without
external cues to impact circadian oscillators (Gerstner et al., 2009). Shorter-term factors like the seasonality of environmental cues can impact, entrain, and stabilize this 24-hour cycle with increased or decreased daylight hours, weather patterns, and temperature (Dubruille & Emery, 2008). The input pathways translate and provide information regarding external cues to the endogenous clock to better regulate its timekeeping. Output pathways convey this circadian and time-of-day information to influence other physiological systems in the organism (King & Sehgal, 2020). Circadian rhythms influence organism physiological functions like the sleep cycle, metabolic status and patterns, body temperature, feeding, and bodily and cellular growth and maintenance (Gerstner & Yin, 2010). Circadian rhythms also help establish better efficiency in organisms’ resource usage, such as slowing the metabolic rate at times of rest, due to the macro effects and applications of circadian rhythms and the intersectionality of bodily systems (Marcheva et al., 2013).

*Drosophila* are excellent model organisms to study genetics and behavior due to its ease of breeding, anesthetizing, sexual dimorphism and other distinctive physical qualities of age and virginity, generation time, care, and ethical concern (Markow, 2015). With sexual and age dimorphism, virgin males are noted by their larger shape, tarsal sex combs on the first pair of legs, lighter coloring, rounder shape, darker abdomen, and presence of meconium from larval feeding than their mature or female counterparts (Kopp et al., 2000). In addition to its physical qualities, is the vast nature of study and knowledge of *Drosophila*; its genome, anatomy, the physiological components of molecular processes, memory, and circadian rhythms, as well as the learning and training paradigms that impact these systems, are well studied (Allada & Chung, 2010).
Established in earlier *Drosophila* research, eclosion rhythm, the typically early morning emergence of adult fly from its pupa case, was shown to differ by species, confirming a genetic aspect of this rhythm to be governed by circadian rhythms (Gerstner & Yin, 2010). Clock genes *period (per)* and *timeless (tim)* were established by forward mutagenesis screen in their role to regulate circadian rhythms (Konopka & Benzer, 1971). CLOCK-CYCLE, the co-activator complex, regulates high-level expression of *per* and *tim* through activation of the genes’ transcription in binding to their promoter E-box aspects (King & Sehgal, 2020). *Per* and *tim* form a binding heterodimer that blocks CLOCK-CYCLE transcription (Lee et al., 1999). This results in negative feedback looping lasting around 24 hours per cycle. In addition to difference in eclosion rhythms, *per* and *tim* mutations have also been shown to result in difference in locomotor circadian rhythms (Allada & Chung, 2010).

*Drosophila* circadian pathway receives photic information to entrain through three pathways of light-receiving cells: H-B eyelets, the eyes, and cryptochrome (CRY), a blue light-dependent photopigment (Gerstner & Yin, 2010). The photic information is projected along the posterior optic tract (POT) to the lateral neurons (LN) (Malpel et al., 2002). LN function as clock pacemaker neurons and consist of dorsal LN (LNds), ventral LN, both large and small (l-LNvs, s-LNvs), and lateral posterior neurons (LPNs). s-LNvs and I-LNvs both express Pigment-Dispersing Factor (Pdf), which helps regulate rest and rhythms of activity (Helfrich-Förster, 1995). I-LNvs are connected by POT to the optic lobe. S-LNvs synapse with the accessory medulla, which receives photic information from H-B eyelets (Helfrich-Förster, 1995). Central to *Drosophila* under LD cycle is its locomotive peaks of activity in the morning and evening (Dubowy & Sehgal, 2017). The morning peak is regulated by this relationship between Pdf and LNvs. The evening peak is regulated by the relationship between 5th s-LNvs and LNds (Grima et
al., 2004). In addition to LNs that comprise the around 150 clock neurons that express *per* and *tim*, are dorsal neurons (DN), which has subtypes of DN1s, DN2s, and DN3s. DN1 can be further subdivided into anterior (DN1a) and posterior (DN1p) (King & Sehgal, 2020). Posterior affects locomotive rhythms though the integration of temperature and light cues (Dubowy & Sehgal, 2017). DN2s also integrate temperature cues and can affect temperature preference in *Drosophila* (Dubowy & Sehgal, 2017).

The mushroom body (MB) is the primary site of associative learning, and its pathways allow for entrainment in *Drosophila* (Aso et al., 2014). Kenyon cells (KCs) are the intrinsic neurons that make up the MB. KC dendrites form the MB calyx (Aso et al., 2014). The calyx’s axon fibers form the subsequent lobes of MB (Aso et al., 2014). The MB is separated into α and β lobes, which can be further divided into posterior, core, and surface groups (Crittenden, 1998). The MB and lateral horn are two brain regions in *Drosophila* that receive sensory information via ~150 projection neurons (PNs) from the antennal lobes (ALs) (Løfaldli et al., 2010). Sensory receptors converge on one of the 43 associated glomeruli on the AL, where cholinergic synapses transmit this information (Løfaldli et al., 2010). ~6 PNs are required to input information in a KC to induce activity (Abdelrahman et al., 2021). PNs form the antennal cerebral tract (ACT) that connects to the MB and lateral horn. The calyx is where PNs synapse on a portion of the ACT (Davis, 2005). These pathways are essential for associative learning and memory formation (Aso et al., 2014).

Circadian rhythms also regulate metabolism and metabolic function in organisms (Marcheva 2013). While light is a primary cue in rhythm regulation, changes in food intake or timing strongly impact the endogenous clock (Pickel & Sung, 2020). Food as a natural reward can serve as entrainment with high dopamine release and impact locomotive activity as
anticipatory activity precedes feeding (Opiol et al., 2017). Pars intercerebralis (PI) is a region of the *Drosophila* brain anterior to the MB that innervates the neuroendocrine system (de Velasco et al., 2007). The PI does not have an autonomous circadian clock and relies on the central circadian clock information from LNvs via DN1 neurons (Barber et al., 2016). Insulin-producing cells are located within the PI, playing a large role in feeding behavior by coordinating metabolic rhythms and release of hormones and enzymes (Barber et al., 2016). Research into these intertwining mechanisms is important because of the impacts of dopamine release reinforcement, hedonic behavior, and ties to neophilia creating misalignment in circadian rhythms and can point to a link of genetics and circadian rhythm to addiction and drug abuse behaviors in humans.

*Introduction on Feeding Assays*

Feeding assays allow for the measurement and analysis of food preference and other behavioral mechanisms underlying feeding. Across phylogeny, choice of food and how much of it to consume is shaped by many underlying and interconnected factors such as nutritional density and requirements, sex, reproductive status, development and growth status, and composition of food, including the presence of toxic components (Carvalho & Mirth, 2017). This integration of external and internal sensory information in organisms defines feeding behavior (Mahishi & Huetteroth, 2019). Analysis of feeding behaviors can be viewed by its quality as a currency, with gains of calories and nutrients and losses of energy from foraging or toxicity of food, allowing for organismal risk-benefit analysis and survival (Emelen, 1966). The mushroom body in *Drosophila* integrates this information and is involved in naïve feeding behaviors (Tsao et al., 2018). Dopamine release from feeding serves as a natural reward; these dopaminergic circuits integrate in the MB to form long-lasting feeding memory from naïve feeding for future
survival (Ichinose et al., 2015). Dopamine pathways are also seen in behaviors involving drugs and abuse (Volkow et al., 2004). Information ascertained from feeding assay allow for study of differing preferences of hedonism, novelty, caloric density, plasticity, and sensory preference among closely related species (Stafford et al., 2012).

CAFÉ, FRAPPÉ, and BARCODE are a few of the current feeding assays in use in Drosophila research (Mahishi & Huetteroth, 2019). Appropriate assay choice is dependent on the experiment and provided food characteristics and feeding conditions (Diegelmann et al., 2017). Capillary Feeder (CAFÉ) directly measures food intake by measurements of the meniscus of liquid food in glass capillary vials over time. CAFÉ is noted by its strengths of simplicity, ability to monitor both short-term and long-term, use on individual or groups of flies, and its ability to be used in studies of drug effects (Ja et al., 2007). However, use of liquid food perhaps could make a difference in comparison to solid food being more easily accessible and ability to loiter, important for Drosophila behaviors (Saleem, 2014). CAFÉ also is unable to allow for high-throughput screens with the time required for each genotype. FRAPPÉ, fluorometric reading assay of preference primed by alcohol, is a rendition of CAFÉ to improve its slowness by allowing for high-throughput screens particularly in testing of ethanol exposure with its two-choice capillaries and addition of fluorophore dye and labeling to measure individual fly consumption preferences in group screenings (Peru Y Colón de Portugal et al., 2014). The addition of dye in FRAPPÉ, however, could influence food choice. FRAPPÉ also requires the collection of flies to measure food consumption, making it more labor intensive and lengthy. In CAFÉ and FRAPPÉ assays, ethanol testing can induce starvation in Drosophila and creates difficulties in ascertaining if ethanol is preferred for its chemical abilities or its caloric density (Park et al., 2018). BARCODE strives to fix upon that ethanol testing deficit by being starvation-
independent and using trace amounts of DNA oligonucleotide tags, that are not within human or fly genome, within solid food. These tags are then analyzed post-mortem by qPCR (Park et al., 2018). BARCODE, however, can be labor-intensive and slow and post-mortem analysis does not allow for easy longer-term monitoring.

Place Preference and Spatial Learning Assays.

Place preference of organisms is driven from various environmental factors and memory, such as light, temperature, humidity, and location of food or other rewards or punishments. Positional preference of an animal can be ascertained from their location at different times of the day. When two different food substrates at each end of a tube are introduced, food preference can be inferred at different times of the day (Kim et al., 2017). Drosophila species vary in ecology and behavior and D. melanogaster has been used as a robust model; however, more information such as the evolution of behavior can be gathered when comparing species in the genus. Within the Drosophila genus, both generalist, D. melanogaster and D. simulans, and specialist species, D. sechellia, are found, allowing for study of genetics, food shift and diversity of food intake source, and shaping of behavior (Markow, 2015). This study evaluates a new place preference assay to measure Drosophila species attraction to different food substrates over circadian periods.
A new place preference assay that can incorporate measures of a circadian period.

In this thesis, we examine the use of the *Drosophila* Activity Monitor 5M (DAM5M) System, (TriKinetics Inc., Waltham, MA), for use in a place preference assay. DAM5M is an apparatus that allows for recording and monitoring of locomotive activity in *Drosophila* (Pfeiffenberger et al., 2010). *Drosophila* are crepuscular, with locomotive peaks of activity in the morning and evening in LD cycles. Environmental light cues allow for an estimation of phase and their anticipation of phase change (lights-ON and lights-OFF) mark these peaks (Im et al., 2011). Locomotive activity also allows for the analysis of positional preference of organisms (Chiu et al., 2010). These activities and preferences can be analyzed to provide behavioral insights such as circadian activity and sleep.

DAM5M consists of 32 glass tube channels that *Drosophila* are placed in. Infrared (IR) light beams are used to monitor locomotive information by ascertaining where *Drosophila* are in the channel with breaks in the beams (Pfeiffenberger et al., 2010). DAM5M is different from previous monitor versions in which these multiple IR beams not only allow for measurement of *Drosophila* activity, but also positioning within these tubes. Sleep and Circadian Analysis MATLAB Programming (SCAMP) is then used to analyze the DAM information through activity and sleep analysis and visualization, identification of peaks of activity, and analysis of behavioral anticipation of phase change (Persons et al., 2021). In the analysis and exploration of DAM5M, a two-dimensional place feeding preference assay can be developed to better monitor behavioral activity and food and locomotive preference over circadian periods.
Methods

Drosophila Species and Husbandry

Drosophila melanogaster, and D. simulans flies were acquired from the San Diego Drosophila Species Stock Center and maintained throughout this experiment. The flies were raised on a preparation of cornmeal, sucrose, and yeast extract agar (Lyons & Roman, 2009). All flies were kept in environmental conditions at 25 °C and ~60% relative humidity. Virgin males were acquired following eclosion for use in this experiment. Female Drosophila are unable to be used because of the hindrance of IR beam readings by eggs laid in the tubes. Younger males were chosen to prevent a confounding factor of aging and natural death. Prior to testing, flies were kept in LD cycles for 2-3 days before testing to allow for proper entrainment. This entails 12 hours of light conditions followed by 12 hours of dark conditions. Zeitgeber time (ZT) is used to describe the LD cycle and represents the natural ~24-hour circadian cycle of light. Zeitgebers refer to the exogenous cues used to entrain and regulate this cycle (Gerstner & Yin, 2010). Lights-ON represents dawn and begins the ZT cycle at ZT 0. At ZT 12, 12 hours later, is lights-OFF or dusk (Lyons & Roman, 2009). The experimental food consisted of two choices, a 2% sucrose substrate and a 50% noni substrate. Two food preparations were made for each side cap of the DAM glass tubes. Noni food substrate was created with a 50% noni solution and 1.5% agar. This was created from dilution of pure noni juice with equal parts of deionized water, which was then used to dissolve the agar. Control food substrate was made with solution of 2% sucrose and deionized water and 1.5% agar. The food was then poured into the glass tube caps and allowed time to set.
**Drosophila Activity Monitor (DAM) Set up and Data Collection**

A special setup was required to run the positional preference assay. Modifications to the black tube caps (CAP5 Black, TriKinetics Inc., Waltham, MA) were needed to allow for better air flow through the tubes. Flies cannot survive past two days in tubes with food that has been waxed closed on one side and a cap on the other side or both sides capped. Modified capillary tubes measuring 1.5 cm in length (Sigma-Aldrich Z114952-200EA) were placed in the caps prior to addition of the food to allow for flow of air. The flies were then loaded under carbon dioxide anesthesia into individual glass tubes (PGT5x80 Pyrex Glass, TriKinetics Inc., Waltham, MA), and were closed in with the modified caps. The food substrate caps ran in an alternating placement pattern in each channel up the DAM to correct that the beams for the odd-numbered rows are displaced to the right and to also prevent situational place preference bias. This pattern started with noni food substrate on the left side of channel 32 and followed upward the monitor as seen in *figure 1*. The three species were contained to their respective monitors and received experimental procedures simultaneously. Flies were subjected to three days of LD cycle conditions, followed by five days in constant dark (DD) conditions. The first day was removed from analysis to allow the flies to adjust to experimental settings after being anesthetized and loaded into the tubes. Four IR beams are used to mark the four compartments of the tube, seen in *figure 1*. Occlusions in the beam are recorded to mark locomotive activity and positioning of the flies during experimentation. Sleep is marked and defined as a five-minute interval without beam occlusion (Hendricks et al., 2000).
Figure 1. *Drosophila Activity Monitor 5M (DAM5M) System loaded with activity tubes and modified black caps and food pattern.* (A) food substrate glass cap. (B) breathing glass capillaries. (C) IR beam location. All even numbered channels contained modified black caps as shown with channel 32, containing the noni food substrate nearer to compartment 1 and the control food substrate nearer to compartment 4. All odd numbered channels contained modified black caps as shown with channel 31, containing the control food substrate nearer to compartment 4 and the noni food substrate nearer compartment 1. There were four infrared beams that bisect each tube to record when and where a fly moved.
Data Analysis

Beam-cross data collected from the DAM boards were formatted using DAMFileScan113X (TriKinetics Inc., Waltham, MA) into 1- and 30-min bins. The Sleep and Circadian Analysis MATLAB Program (SCAMP) developed within the Griffith lab was used in aggregation and analysis of DAM experimental data which was originally published in Donelson et al. 2012. SCAMP has been previously used to average experimental days and analyze total sleep, latency to sleep onset, mean sleep episode duration, number of sleep episodes, and how active the flies were when they were awake (Donelson et al., 2012). SCAMP has been modified to include “DAM5M” analysis to analyze positional preference at different times of the day.

GraphPad, statistical software program, along with R, were used. ANOVA was then used to determine significant results with a $p$-value greater than 0.05. SCAMP allows for reversal and alignment of the food preference pattern and selection of specific day ranges. Results were then displayed with actograms, sleep parameter graphs, and dwell box plots.
Results

In examining the use of the *Drosophila* Activity Monitor 5M (DAM5M) System in a place preference assay, SCAMP was used to aggregate and analyze DAM5M experimental data. *Figure 2* was generated from SCAMP and shows activity patterns for *Drosophila* in LD experimental conditions. As seen in previous studies, circadian activity patterns were observed with peaks in activity in anticipation of phase change at ZT-0 and ZT-12. *D. simulans* had dampened activity patterns compared to *D. melanogaster*. Sleep parameter graphs were also generated from SCAMP (figure 3). Sleep is defined as five minutes without activity. Across the species, spikes in amount of sleep were observed at ZT-12. The time of sleep episodes were also observed to increase in dark conditions. *Figure 3* also shows similarities in *D. simulans* and *D. melanogaster*. *Figures 4* and *6* observe dwell and positional preference in *Drosophila* species in LD and DD conditions, respectively. Dwell is defined as percent of time spent in compartments 1 and 2 during experimentation. Compartments 1 and 2 are closer to noni experimental food substrate than the control food substrate. In both *figures 4* and *6*, flies across all species were observed to spend less time in the middle compartments (2 and 3) than in compartments closer to food substrate (1 and 4). In LD conditions (*figure 5*), a two-way ANOVA was used to determine significance of dwell in compartments 1 and 2. There was no statistically significant difference in dwell position between *D. melanogaster* and *D. simulans* \[F (1, 84) = 0.766, p = 0.384\]. There was also no statistically significant difference in dwell position between day and night \[F (1, 84) = 0.472, p = 0.494\].

In *Figure 7*, a two-way ANOVA was also conducted to determine significance of dwell in DD conditions. A two-way ANOVA was carried out on dwell 1+2 (dwell position) by species and time of day. There was no statistically significant difference in dwell position between *D.
melanogaster and D. simulans \( F (1, 74) = 1.175, p = 0.282 \). There was also no statistically significant difference in dwell position between day and night \( F (1, 74) = 0.733, p = 0.395 \).
Figure 2. SCAMP-generated actograms and average activity for different species of *Drosophila* over three days of LD. i) Activity patterns for *Drosophila* in LD conditions over the time period is shown by the actograms over three days. Activity is defined as number of beam breaks in a set time. ii) Mean activity is calculated over the three days shown in minutes. Mean activity for *D. melanogaster* in LD is 96.93 minutes, mean activity for *D. simulans* is 33.19 minutes and mean activity for *D. sechellia* is 33.78 minutes. *D. simulans* and *D. sechellia* show similar dampened activity patterns during the dark period compared to *D. melanogaster*. Morning anticipatory activity can be seen in the times before light-ON (ZT-0) and lights-OFF (ZT-12).
Figure 3. SCAMP-generated sleep parameter graphs for different species of *Drosophila* over three days of LD. Group averages over all three days in LD are plotted for sleep parameters: sleep/30-min bin and mean sleep episode duration. Mean sleep episode duration was observed over light period (LP), dark period (DP), and the 24-hour cycle. An increase in sleep can be observed at lights-OFF (ZT 12) across species. However, mean sleep episode increases are greater for *D. simulans* during dark period.
Figure 4. SCAMP-generated output graphs from DAM5M data analysis for two species of *Drosophila for three days in LD*. Top Panel: The daily line graph output for “Dwell 1+2”, representing how much time the flies spent on the left side of the tube, closer to beam 1 (noni food substrate). Bottom Panel: Heatmap plots of time spent in each compartment for each group (rows) and day (columns). Compartment 1 is the side closer to beam 1 while compartment 4 is closer to beam 4. In this experiment, 0% indicates more time spent near the control food substrate while 100% more time spent near the experimental food substrate.
Figure 5. Dwell position for species of *Drosophila* during three days in LD. A two-way ANOVA was carried out on dwell 1+2 (dwell position) by species and subjective time of day.

There was no statistically significant difference in dwell position between *D. melanogaster* and *D. simulans* \[ F(1, 84) = 0.766, p = 0.384 \]. There was also no statistically significant difference in dwell position between subjective day and night \[ F(1, 84) = 0.472, p = 0.494 \].

*D. melanogaster*, n = 18

*D. simulans*, n = 26
Figure 6. SCAMP-generated output graphs from DAM5M data analysis for two species of *Drosophila* for five days in DD. Top Panel: The daily line graph output for “Dwell 1+2”, representing how much time the flies spent on the left side of the tube, closer to beam 1 (noni food substrate). Bottom Panel: Heatmap plots of time spent in each compartment for each group (rows) and day (columns). Compartment 1 is the side closer to beam 1 while compartment 4 is closer to beam 4. In this experiment, 0% indicates more time spent near the control food substrate while 100% more time spent near the experimental food substrate.
Figure 7. Dwell position for species of Drosophila during five days in DD. A two-way ANOVA was carried out on dwell 1+2 (dwell position) by species and time of day. There was no statistically significant difference in dwell position between D. melanogaster and D. simulans \( F(1, 74) = 1.175, p = 0.282 \). There was also no statistically significant difference in dwell position between day and night \( F(1, 74) = 0.733, p = 0.395 \).

D. melanogaster, n = 14

D. simulans, n = 25

\[
\begin{array}{cc}
\text{Group} & \text{Time} & \text{Group:Time} & \text{Residuals} \\
1 & 926 & 925.6 & 1.175 & 0.282 \\
1 & 577 & 577.1 & 0.733 & 0.395 \\
1 & 120 & 120.1 & 0.152 & 0.697 \\
74 & 58283 & 787.6 & \\
\end{array}
\]
Discussion

Integration of external cues of environment factors (temperature, humidity, and light) and internal states of an organism (hunger, sleep, and growth) dictate behavior and mechanisms. The integration of these factors along with memory can also impact decisions and behaviors, such as previous locations of food, punishments or rewards, or changes in light. This integration drives place preference in organisms. Analysis of place preference and locomotive activity allows for further study of behavior and the influence of underlying genetics and physiological mechanisms. This study was intended to evaluate a new place preference assay to measure similarities and differences in two species of Drosophila (D. melanogaster and D. simulans) and to measure the attraction to two different food substrates while also recording sleep and circadian measurements.

Mean activity was generated for the two species over three days of LD conditions. D. melanogaster had the highest mean activity with 96.93 minutes. Mean activity for D. simulans was 33.78 minutes. Both species had similar activity trends through the circadian period. However, D. simulans showed dampened activity during dark periods as compared to D. melanogaster. As expected, both species show spikes in activity with anticipation of phase change prior to lights-ON (ZT-0) and lights-off (ZT-12). However, D. simulans showed a slight delay in peak average activity observed after lights-ON than D. melanogaster.

Sleep in Drosophila is defined as 5 minutes without activity. In analysis of sleep data of the two species, both species followed similar rhythm trends in sleep during the circadian periods. Also, across species, increases in sleep were observed at lights-OFF, which is expected. However, during dark period, mean sleep episode for D. simulans increased more drastically than D. melanogaster.
Dwell time in compartments 1 and 2 was also measured as a percentage of total time in experimentation. Compartments 1 and 2 are closer to experimental noni food substrate. *D. sechellia* as a specialist dependent on noni is expected to have higher dwell in 1 and 2 as compared to *D. melanogaster* or *D. simulans*. The first section of experimentation entails 3 days of LD conditions: 12 hours of light and 12 hours of darkness. A daily line graph was generated by SCAMP of DAM5M data dwell time plotted for each species. All species were observed to have a stabilizing pattern in anticipation for lights-OFF. Overall, trends were similarly observed in both species, but after lights-OFF, species varied more dramatically than during light conditions. Heatmap plots were also generated for each species of time spent in each compartment. Yellow represents higher activity and blue with lower activity. Across both species, less time was spent in the middle compartments (2 and 3), than compartments closer to food substrate (1 and 4). For *D. melanogaster* and *D. simulans*, time was split relatively equal between compartment 1 and 4 with no observable trends with circadian rhythms. A two-way ANOVA was then carried out on dwell position for species and time of day in LD experimental conditions. There was no statistically significant difference in dwell position between *D. melanogaster* and *D. simulans* \[F (1, 84) = 0.766, p = 0.384\]. There was also no statistically significant difference in dwell position between day and night \[F (1, 84) = 0.472, p = 0.494\].

The second part of experimentation entailed 5 days in constant dark conditions (DD). Species trends in the daily line graph of DD was observed to follow more closely to trends in dark conditions of LD. Also observed was that the phase change activity trend persisted in DD as phase changed from subjective day to subjective night. Both species were observed to have spent less time in middle compartments in DD compared to LD. *D. simulans* spent a larger amount of
time in compartment 1 and a location increase in compartment 1 at phase change. *D. melanogaster* was observed to spend most of the time in compartment 4 throughout DD conditions. There were some location increases observed of *D. melanogaster* in compartment 1 a few hours after phase change.

Two-way ANOVA also determined no statistically significant difference in dwell position between *D. melanogaster* and *D. simulans* \([F (1, 74) = 1.175, p = 0.282]\). There was also no statistically significant difference in dwell position between subjective day and night \([F (1, 74) = 0.733, p = 0.395]\).

In the evolution of the *Drosophila* species, generalist species *D. simulans* diverged from *D. melanogaster* 5.4 million years ago (Tamura et al., 2003). *D. sechellia* diverged from *D. simulans* 0.5 million years ago to become a specialist in the Seychelles (Tamura et al., 2003). All three species share a mostly homosequential set of 4 chromosomes; however, *D. simulans* and *D. sechellia* are distinguished from *D. melanogaster* with an almost identical rearrangement (Podemski et al., 2001). *D. simulans* and *D. melanogaster* are similar in their identities as a generalist, while *D. sechellia* is a specialist. Our results echo these differences in relation and genetics. This could point to a role of underlying genes that make a species generalist or specialist. In experimentation, SCAMP was modified to include “DAM5M” analysis to analyze positional preference at different times of the day. While this is a new program, it shows similar trends to past studies of assays. The development and addition of better and newer assays helps us have increased understanding of the behavioral components of organisms and give more or more accurate validation of other results and suggestions.
Unanswered questions and future studies

Many questions and unknown information exist surrounding *Drosophila* feeding, locomotive, and sleep behavior and the underlying genetics and circadian rhythms that guide and shape these behaviors. This thesis presents results from ongoing research and further testing could affect conclusions, but our results have been statistically proven and show similar trends to other past studies.

One question that arises from this study is the question of whether locomotive or activity behavior of the species of *Drosophila* result from the definitive presence of food substrate and resulting decision-making, or if it is simply a positional preference. We did try to compound this interaction with the alternating pattern of DAM5M loading, but further trials will need to be done better validate this information. Future studies will also look to identify the specific gene markers that our data suggest marks differences in species and identity.

Conclusion

Data obtained observed differences in activity, sleep, and place preference in the three *Drosophila* species. This study was conducted to better understand the genetic components that separate species and their ecological roles, and the better development of assays used to study these behavioral and mechanistic differences. In addition, this research could be used as a further assay to demonstrate key differences and possibly correlate the underlying genetics that differ specialists from generalists. This knowledge is essential particularly in better understanding genetic roles in circadian rhythms and species traits; especially in how it pertains to neophilic and neophobic attributes and their translation in risk attribution in humans and addiction.
behavior, and in invasive species, whose roles and damage are increasingly pertinent with climate change.
List of References


https://doi.org/10.7554/eLife.03785


https://doi.org/10.1111/adb.12105


https://doi.org/10.1371/journal.pone.0096639

https://doi.org/10.7717/peerj.9499


Volkow, N.D., Fowler, J.S., Wang, G.J., & Swanson, J.M. (2004). Dopamine in drug abuse and addiction: results from imaging studies and treatment implications. *Molecular Psychiatry, 9*(6), 557–569. [https://doi.org/10.1038/sj.mp.4001507](https://doi.org/10.1038/sj.mp.4001507)