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Thermal Preference of Immature House Flies (*Musca domestica*) (Diptera: Muscidae)

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Dedication

To the entomology girls, Emilia, Greety, Karla, and Mona. Without you none of this would have happened. And to Molly. For everything.

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Introduction

I. Importance of house flies

House flies (*Musca domestica* L.) (Diptera: Muscidae) are a common nuisance pest that also represent a major health risk to humans and animals due to their close association with microbes, their intimate relationship with filth, and their attraction to humans, animals, and our food (Nayduch & Burrus 2017, Geden et al. 2021). They are most notably problematic in places with poor sanitation or with a close association to confined animal production (Geden et al. 2021).

House flies can carry as many as 130 pathogens (Greenberg 1971, 1973, Nayduch & Burrus 2017, Khamesipour et al. 2018, Nayduch et al. 2023) and have been associated with protozoan parasites, bacteria, viruses, and even some nematodes (Greenberg 1971, 1973, Graczyk et al. 2001, Geden et al. 2021). These pathogens are often acquired by fly contact with or feeding on contaminated surfaces (e.g., animal feces) with some pathogens reported to be transmitted mechanically by adult flies to human or animal hosts (Kobayashi et al. 1999, Collinet-Adler et al. 2015, Nayduch & Burrus 2017, Geden et al. 2021). Additionally, house flies associated with commercial animal production facilities and hospitals can potentially spread antibiotic resistant strains of bacteria (Graczyk et al. 2001, Rahuma et al. 2005, Macovei et al. 2008, Chakrabarti et al. 2010, Doud et al. 2014, Zurek & Ghosh 2014, Schaumburg et al. 2016, Xu et al. 2018, Geden et al. 2021) and even provide a mechanism for horizontal transfer of antibiotic resistance among bacteria co-infecting individual flies (Doud et al. 2014). Control of house flies through improved sanitary measures to reduce immature development sites has resulted

in reduction of human diseases transmitted by these flies including typhoid fever (Cirillo 2006), *Shigella* spp. (McCabe & Haines 1957), and other diarrheal diseases (McCabe & Haines 1957, Chavasse et al. 1999).

House flies can be a severe nuisance to people who live or work in areas where fly numbers are high (Gerry 2015, Geden et al. 2021) and may provide an aesthetic nuisance in the fecal spots they leave behind on surfaces (Geden et al. 2021). The burden is not limited to homes immediately in the vicinity of fly production sites, as nuisance can occur over 3 km away from a major breeding site (Winpisinger et al. 2005) with an increase in the house fly population reported over 6 km away (Winpisinger et al. 2005).

In addition to costs due to nuisance and pathogen transmission, animal production facilities can face additional economic losses due to decreased animal productivity (Geden et al. 2021), decreased marketability of products within an outbreak zone (Geden et al. 2021), and increased cost of treatment (Dhillon et al. 2004, Geden et al. 2021). Fly contamination of human food crops can even distribute pathogen risk well beyond the boundaries of major fly development sites (Alam & Zurek 2004, Talley et al. 2009, Wasala et al. 2013).

II. House fly life cycle

House flies are holometabolous, with distinct egg, larval, pupal, and adult stages that occupy different niches (West 1951, Gerry 2015, Geden et al. 2021). House flies can complete their life cycle (egg-adult) in as little as 6-7 days (West 1951, Geden et al. 2021). The adult stage oviposit in moist, decaying organic matter (West 1951, Bryant & Hall 1975, Geden et al. 2021). Egg stages are small, cylindrical and whitish, typically

about 1 mm in length, and laid in clusters of ~100-150 (West 1951, Bryant & Hall 1975, Gerry 2015, Geden et al. 2021). Emergence from the egg typically occurs between 8-24 hours, though this is largely dependent on temperature (West 1951).

Larval stages are whitish-yellow and tubular shaped ("maggots"), with no apparent appendages aside from sclerotized mouth hooks at the tapered anterior end (West 1951, Geden et al. 2021). Posteriorly their spiracles lay along a flattened end, with spiracular plates shaped like an uppercase "D" (West 1951, Geden et al. 2021). Association with filth habitats such as refuse, feces, and manure is largely due to their larval diet of bacteria and other microbes (West 1951, Nayduch & Burrus 2017, Geden et al. 2021). There are three larval stages and all bear a strong resemblance, with different instars usually differentiated most easily by size (West 1951, Geden et al. 2021). The first instar larvae (L1) will hatch from the egg at about 1 mm long, later undergoing molts to the second instar (L2) and finally third instar (L3), with each instar increasing substantially in size and mass up to ~8mm. The L3 instar then forms a puparium using the third instar cuticle within which each fly develops through the pupal stage to reach the adult stage (West 1951, Geden et al. 2021).

Larvae require a habitat with ~50-80% moisture (Stafford & Bay 1987, Fatchurochim et al. 1989) and a pH ~7-9 (Calvo et al. 2010). Larval density, regulated in part by oviposition behaviors (Bryant & Hall 1975), contributes to total developmental time, survival and adult size (Bryant & Sokal 1967, Barnard & Geden 1993, Chapman & Goulson 2000), with a density of 1 larva per g of substrate being ideal for immature survival (Barnard & Geden 1993). An acceptable temperature range for house fly

development is fairly broad, with a minimum developmental threshold of 12°C (Larsen & Thomsen 1940, West 1951, Wang et al. 2018) and a maximum developmental temperature of up to 45°C (Larsen & Thomsen 1940, West 1951), but survival is highest at 23-28°C (Stafford & Bay 1987, Barnard & Geden 1993, Chapman & Goulson 2000) and immature development is fastest at 32-34°C (Larsen & Thomsen 1940, Barnard & Geden 1993, Wang et al. 2018). There is significant variation in preferred temperature among different life stages (developmental ages) of the house fly with younger larvae (1st and 2nd instars) preferring a much higher temperature (30-37°C) compared to early 3rd instar larvae (28.5-35.3°C) and especially compared to late 3rd instar larvae nearing pupation (0-15.6°C) (Thomsen & Thomsen 1937). Late 3rd instar larvae often leave their developmental environment, seeking a cool and dry place to pupate (West 1951, Geden et al. 2021).

The adult house fly is typically 7 mm or less, with a grey-colored body and four dark longitudinal stripes visible on the top of its thorax between the wings (West 1951, Gerry 2015, Geden et al. 2021). Upon emergence, most adults tend to stay close to their development site (Schoof et al. 1952, Quarterman et al. 1954, Schoof & Siverly 1954**,** Geden et al. 2021), where they mate and lay subsequent generations of eggs (West 1951, Bryant & Hall 1975). Nevertheless, fly dispersal over considerable distances is not uncommon, with dispersal reported >12km from emergence sites (Schoof et al. 1952, Quarterman et al. 1954, Schoof & Siverly 1954**,** Geden et al. 2021). Fly dispersal does not appear to be directed but rather direction of flight seems random (Schoof & Siverly 1954).

III. Management of house flies

Chemical control of house flies in both adult and immature stages, despite having initial success, has resulted in widespread insecticide resistance and control failure (Geden et al. 2021). While most control efforts have targeted the adult stage of the house fly as the pest and pathogen transmitting stage, a change in focus to control of the immature stages would be especially valuable given the rising resistance to pesticides applied against adult house flies (Geden et al. 2021). Integration of novel larval control could provide long-term reduction in adult numbers and reduce the burden of adult fly control.

Current larval house fly management largely focuses on sanitary measures such as proper disposal of animal and food waste (Geden et al. 2021). Among these strategies include rapid drying of animal feces or other potential fly breeding materials to make them unsuitable due to lack of moisture (Fatchurochim et al. 1989, Geden et al. 2021). Use of additives to alter manure pH can alter the substrate microbial community to reduce immature house fly survival while also making the media less attractive to ovipositing adults (Calvo et al. 2010). Sodium bisulfate (Sweeney et al. 2000, Calvo et al. 2010), acetic acid (Lachance et al. 2017), and boric acid (Lachance et al. 2017) are the most promising options for pH alteration and represent little danger to livestock. Composting is another cultural control method, with the resulting very high temperature of the developmental habitat being lethal to house fly larvae (Moon et al. 2001, Abu-Rayyan et al. 2010).

Beyond manure management, some biocontrol measures have been studied (Geden et al. 2021). Fungal pathogens such as *Beauveria bassiana* and *Metarhizium brunneum* have been used to control both adults and immature stages (Machtinger et al. 2016, Geden et al. 2021) with mixed success according to environmental factors and fungal strain. Entomopathogenic bacteria such as *Bacillus thuringiensis* (*Bt*) have been implemented occasionally but require more study before achieving widespread use (Geden et al. 2021). Additional control can be achieved with predators including mites, beetles, and other flies, as well as with several species of parasitoid wasps (Geden et al. 2021).

Manipulation of larval habitat may provide another means for control, though this area is understudied. Larval development sites are generally localized and perhaps amenable to alteration either through manipulation of conditions (temperature, humidity, acidity) or through addition of larval-repelling compounds (e.g., repellents, pheromones, kairomones) that ultimately could drive larvae from a development site or cause larvae to aggregate in less suitable developmental locations.

This thesis evaluates the temperature preference of immature house flies by placing 3rd instar larval flies into developmental media across a thermal gradient and allowing flies to move freely through the media in response to the temperature gradient. Control trials similarly measured larval movement through the channel but in the absence of a thermal gradient applied. Immature flies exposed to the temperature gradient aggregated within a distinct temperature range while flies placed in media without the temperature gradient did not aggregate.

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Chapter 1: Thermal preference of immature house fly (*Musca domestica*) (Diptera: Muscidae)

Abstract

House flies are a widespread, pestiferous insect responsible for causing human nuisance as well as pathogen transmission to humans and animals. While adult fly behavior has been relatively well-studied, little is known about larval behavior. Manipulation of larval behavior may be useful to manage fly populations, reducing adult fly numbers without the need for chemical insecticides. In this study, we determined larval temperature preference by applying a temperature gradient to developmental media containing third instar fly larvae that were initially distributed evenly throughout the media. Following a 4 to 6-h assay period during which larvae could move freely within the media, larvae were found to aggregate within a relatively narrow temperature range similar to previously reported studies.

Introduction

House flies (*Musca domestica* L.) are a widely distributed synanthropic (human-associated) pest common in both domestic and rural landscapes where they develop within a variety of moist organic materials (West 1951). House flies are often associated with animal feces or other filth where they can acquire human and animal pathogens which they are known to mechanically transmit as they move about their environment (Nayduch & Burrus 2017, Geden et al. 2021). This species has been associated with at least 130 human pathogens (Greenberg 1971, 1973, Nayduch & Burrus 2017, Khamesipour et al. 2018, Nayduch et al. 2023).

While immature house flies develop in a multitude of moist organic habitats, there are commonalities among these habitats including an abundant supply of microbes on which the larvae feed (Greenberg 1971, Nayduch & Burrus 2017, Geden et al. 2021), a moisture level between 50-80% (Stafford & Bay 1987, Fatchurochim et al. 1989), and a temperature between 12°C and 45°C (Larsen & Thomsen 1940, West 1951, Lysyk & Axtell 1987, Stafford & Bay 1987, Barnard & Geden 1993, Chapman & Goulson 2000, Wang et al. 2018). Other factors that support successful immature development are a neutral to slightly basic pH of 7-9 of the organic media (Calvo et al. 2010) and suitable larval density (Bryant & Hall 1975, Barnard & Geden 1993). Larval density is largely controlled by adult oviposition behavior (Bryant & Hall 1975) but does not eliminate overcrowding in larval environments which negatively impacts larval development rate, survival, and pupal mass, especially when associated with a higher temperature (Barnard & Geden 1993). Immature survival rates generally increase with lower larval densities, with the highest survival reported at a density of 1 larva per g of manure (Barnard & Geden 1993). In the absence of overcrowding, temperature is the major regulator of larval development rate (Barnard & Geden 1993, Chapman & Goulson 2000). Immature house flies can develop at temperatures between 12°C and 45°C with temperatures above this range resulting in significant mortality (Larsen & Thomsen 1940, West 1951, Wang et al. 2018). Immature house fly survival is greatest at 23-28°C (Stafford & Bay 1987, Barnard & Geden 1993, Chapman & Goulson 2000) while immature development is highest at 32-34°C (Larsen & Thomsen 1946, Barnard & Geden 1993, Wang et al. 2018) with both survival and development rate declining above 35°C (Lysyk & Axtell 1987).

Based on these studies, where temperature variation occurs within a house fly developmental site, house flies might be expected to exhibit a thermal preference of 28-34°C to achieve both high survival and a rapid development rate.

Current management of house flies tends to focus on the adult life stage and is largely dependent on pesticides (Geden et al. 2021). Control measures for immature house flies largely focus on elimination of house fly development sites and use of natural enemies for biological control (biocontrol) (Geden et al. 2021). Biocontrol agents including entomopathogenic fungi, predatory mites, parasitoids, and parasitic nematodes vary widely in effectiveness for control of immature house flies (Geden et al. 2021). Larval habitat, which in commercial animal production facilities largely consists of animal feces, food silage, or moist animal bedding, can be reduced through physical management such as composting (Moon et al. 2001, Abu-Rayyan et al. 2010), drying (Fatchurochim et al. 1989, Geden et al. 2021), or additives to change the substrate pH (Sweeney et al. 2000, Calvo et al. 2010, Lachance et al. 2017).

House fly females oviposit a large batch of eggs (~100-150) during each ovipositional event (West 1951, Bryant & Hall 1975, Geden et al. 2021) and prefer media where young immature house flies are already present (Bryant & Hall 1975), resulting in natural aggregation of immature house flies in field sites. However, even in laboratory colonies of house flies where egg density in larval rearing containers is controlled, immature house flies still tend to aggregate within the container especially in late third instar and pupal stages (personal observation).

The following experiment seeks to connect larval temperature preference with larval aggregation to determine whether immature aggregation is associated with variation in temperature within the immature development site.

Materials and Methods

I. House Flies

House flies were collected from a commercial dairy in southern California (Riverside County) in the fall of 2022. Mixed-sex adult house flies (~500 individuals) were captured via sweep net from several locations at the dairy to ensure a representative gene pool. Captured flies were transferred to a mesh cage and provided water and food (1:1 dry milk powder and sugar) *ad libitum.* After approximately 1 wk, flies were provided with oviposition media consisting of wheat bran, yeast, dry milk, alfalfa pellets, and water (Zahn & Gerry 2018) to obtain eggs. Eggs were transferred to larval rearing media with the same components as the oviposition media (Zahn & Gerry 2018) with flies subsequently maintained in the laboratory at ~27°C, ~38% relative humidity, and 14:10 light:dark period according to previously published procedures (Zahn & Gerry 2018). Third instar larvae (~96 h-old following oviposition or ~2,600 accumulated degree hours (ADH)) from this colony were removed from the standard rearing media using soft forceps immediately before use in a thermal preference assay described below.

II. Thermal Preference Assay – Low Temperature Range

To assess the thermal preference of larvae, four metal U-shaped channels 61 cm long x 4.3 cm wide x 2 cm high and made of 0.32 cm thick hot rolled steel (HRBC11218: KH Metals and Supply, Riverside, CA) were used (Fig.1). The four channels were individually labeled (letter codes A-D) to identify potential channel effects on larval behavior. Channel ends were closed with tape before adding ~350 ml of modified house fly rearing media (2L wheat bran, 113 g dry milk, 8.5 g of yeast, 1L of DI water) to cover a channel depth of 1.5 cm. Alfalfa pellets typically included with the rearing media (Zahn & Gerry 2018) result in substantial clumping of the rearing media and were excluded to ensure a more homogenous media to spread within the metal channels.

Two channels (A and B) were designated as temperature gradient channels, while the remaining two channels (C and D) were designated as the control (non-gradient) channels. During a thermal preference assay, gradient channels containing media were positioned so that one 8 cm end of the channel rested on a chill table (Industrial Inventions Inc. model 1012, Lawrenceville, NJ) while the opposite 8 cm end of the channel rested on a hot plate (Corning PC-400D, Corning, NY) with the remainder of the channel suspended between these two points (Fig.1). Between the chill table and the hot plate, the channel was marked into 3 cm sections labeled sequentially #1-15 starting from the edge of the chill table and ending at the edge of the hot plate. The chill table was set to a surface temperature of ~11°C and the hot plate was set to a surface temperature of ~58°C. Control channels were placed on the laboratory bench near the gradient channels and in the same orientation as the gradient channels but were exposed only to laboratory

room temperature. All channels were placed under LED lamps throughout the experiment to take advantage of the strong negative phototaxis response of house fly larvae (West 1951) to keep the larvae beneath the surface of the rearing media as they moved freely through the channel.

Gradient and control channels were initially set up without house fly larvae to complete a 2-h temperature equilibration period allowing the media within the gradient channels to reach a stable temperature gradient from ~16°C above the chill table to ~41°C above the hot plate while media within the control channels had a mean temperature±standard deviation of 20.8(±0.7) °C across the full length of each control channel since they were exposed only to laboratory room temperature. This 2-h equilibration period was determined in pilot experiments that demonstrated a relatively stable temperature gradient was achieved within this time.

Following the temperature equilibration period, a small indentation was made in the media at the center of each of the 15 channel sections to encourage larvae placed at this location to burrow immediately into the media. After indentations were made in each channel, soft forceps were used to quickly place two 3rd instar larvae into each channel section (total larvae = 30) to start the thermal preference assay period. The total number of larvae utilized per channel was determined in pilot studies to provide robust data without overcrowding the channel. Larvae were subsequently allowed to move freely through the media within each channel for 6-h, allowing larvae sufficient time to traverse the full length of the channel. Pilot studies demonstrated that these larvae could travel the entire channel within 4-h.

At the end of the 6-h assay period, the temperature at the center of each channel section was recorded using a temperature probe (Extech EA15 Easy View Temperature Datalogger; Industrial Electronics, Knoxville, TN) placed into the rearing media at mid-depth (0.75 cm from the surface). The media in each channel section was subsequently removed using a channel-width razor to separate media within each channel section from the adjacent channel section. Media from each channel section was placed into a separate labeled petri dish and sifted to separate and count the larvae (data available in A1 and A2). Media within the metal channel above the chill table or hot plate did not experience a temperature gradient and thus media within these sections were excluded from analyses.

III. Thermal Preference Assay – High Temperature Range

While the thermal preference assay above demonstrated a preferred thermal minimum for immature house flies, the assay did not demonstrate a clear maximum temperature since larval house flies were often found in gradient channel sections adjacent to the hot plate. The thermal preference assay described above was therefore repeated with the same number of maggots/channel but with the hot plate set to a much higher surface temperature of ~117°C resulting in a stable temperature gradient within the media across the gradient channel of ~16.6°C to ~62.5°C. Temperature of the media in the control channels exposed only to laboratory room temperature was a mean of 21.1(±0.3) °C during these trials.

Due to the higher rate of moisture loss from the media in this high temperature range assay, the assay began without the initial 2-h temperature equilibration period and the full assay ran for only 4-h (data available in A3 and A4).

IV. Statistical Analysis

All statistical analyses were conducted with R 4.3.1 (R Core Team, 2023). For each thermal preference assay (low temperature range or high temperature range), the association of larval fly count with channel section or temperature of the media in each channel section was examined using generalized linear mixed effects models (GLMM) using package "lme4" (Bates et al. 2015) with a negative binomial error distribution from package "MASS" (Venables & Ripley 2002). Treatment (control, gradient) was included as a binary variable in the initial model of larval distribution by channel section, but treatment was excluded in subsequent models which were performed separately for each treatment group due to a significant interaction effect of treatment with channel section. Treatment was also excluded in models of larval distribution by temperature which included only data from the gradient channel assays. Assay date and channel code (A-D) were included in the model as random variables, with channel code nested within treatment for models including treatment because channel assignment to treatment remained constant across all replicates. Due to collinearity of treatment and channel section when both variables were included in a model, sequential regression was applied to the larval count by channel section (Graham 2003, Dormann et al. 2013) and used the residuals for each channel section ($section_{res}$) as the explanatory variable in the resulting models. Variable interactions were also included in each model. The relative effect of

each factor was assessed using type II Wald chi-square analysis of deviance conducted with the "car" package (version 3.1-2). Full models are reported in the Appendix. Descriptive statistics including means, interquartile ranges, and standard deviations were also determined with R software.

Results

I. Distribution by Channel Section – Low Temperature Range

The assay was repeated for a total of 14 replicates for the temperature gradient treatment and 14 replicates for the control treatment. At the end of the assay period, 87-97% of the larvae placed in each gradient channel were recovered (total of 380 of 420 larvae) while 73-97% of larvae were recovered from control channels (362 of 420 larvae). Larval recovery rates between gradient and control channels were not significantly different by Welch's two sample t-test ($p = 0.08721$).

Larval distribution across the channels in the low temperature range assay varied by channel section as indicated by a significant difference in the channel section residual ($\chi^2_1 = 8.96$, $p = 0.0028$) (A5), but this was confounded by a significant interaction with treatment ($\chi^2_1 = 14.13$, $p < 0.001$) (A5), indicating that distribution was different for the two treatments. When control and gradient channel data were analyzed separately, larval count did not vary by channel section in control channels ($\chi^2_{14} = 17.09$, $p = 0.25$) (A6) but did vary by channel section in gradient channels ($\chi^2_{14} = 68.50$, $p < 0.001$) (A6). In the control channel, larvae remained relatively evenly distributed across the channel (Fig. 2, Fig. 3A) while in the gradient channel larvae aggregated within channel sections 10-13 which were nearer to the hot plate (Fig. 2, Fig. 3B).

II. Thermal Preference – Low Temperature Range

Larval distribution within temperature gradient channels in the low temperature range assay was related to media temperature ($\chi^2_1 = 13.395$, $p < 0.001$) (A7). Larvae were recovered from media at temperatures from 17.1-38.1°C with a mean(± SD) of 25.6(± 4.5)°C. However, a small number of larvae were also always found in the channel sections immediately adjacent to the hot plate (Fig. 4A) and even occasionally in the media above the hot plate.

III. Distribution by Channel Section – High Temperature Range

This assay was repeated for a total of 12 replicates for the temperature gradient treatment and 5 replicates for the control treatment. Across replicates, 77-100% of the 30 larvae placed into each channel were recovered from gradient channels (total larvae recovered = 314 of 420) while 60-73% of larvae were recovered from control channels (total larvae recovered = 102 of 150). Unlike the low temperature range assay, larval recovery rates between gradient and control channels in the high temperature range assay were significantly different ($p < 0.001$).

Larval distribution across the channels in the high temperature range assays varied by channel section as indicated by a significant difference in channel section residual ($\chi^2_1 = 18.50$, $p < 0.001$) (A8), but this was confounded by a significant interaction with treatment ($\chi^2_1 = 6.45$, $p = 0.011$) (A8), indicating that distribution was different for the two treatments. When control and gradient channel data were analyzed separately, larval count did not vary by channel section in control channels ($\chi^2_{14} = 10.55$, $p = 0.72$) (A9) but did vary by channel section in gradient channels ($\chi^2_{13} = 42.25$, $p < 0.001$) (A9). In the

control channel, larvae remained relatively evenly distributed across the channel (Fig. 5, Fig. 7A) while in the gradient channel larvae aggregated in channel sections 10-13 which were nearer to the hot plate (Fig. 5, Fig. 7B).

IV. Thermal Preference – High Temperature Range

Larval distribution within temperature gradient channels in high temperature range assays was related to media temperature ($\chi^2_1 = 14.048$, $p = 0.002$) (A10). Larvae were recovered from media at temperatures from 18.6-51.1°C with a mean (± SD) of 32.0 (± 5.9)°C, with larvae seldom found in the channel immediately adjacent to or above the hot plate (Fig. 4B).

Discussion

Late-stage house fly larvae and pupae are frequently observed to aggregate in their development media (personal observation). Currently, there is no current clear understanding as to why house fly larvae aggregate, though it is speculated it may be associated with varying environmental conditions such as temperature within the media (Thomsen & Thomsen 1937). In other muscid flies, larval aggregation can increase heat and decrease development times (Charabidze et al. 2011).

In this study, we found that 3rd instar larvae aggregated within a preferred temperature range within temperature gradient channels in both the low and high temperature assays suggesting larvae moved through the media in response to the thermal gradient until they reached a preferred temperature range. The larvae in the low temperature thermal assay aggregated at a lower temperature range than larvae in the high temperature thermal assay, which we originally attributed to a potential drying of the

media near the hot plate in the low temperature assay over the longer time period assayed. Immature house flies are known to develop in media with a >50% moisture concentration (Stafford & Bay 1987, Fatchurochim et al. 1989) and may avoid dry conditions, even if this means moving to a less favorable temperature. However, media subsequently taken from the first and last section of the gradient channel following one 6-h assay showed little difference in moisture content across the length of the gradient channel.

In contrast to the temperature gradient channels, larvae within the control channel remained relatively evenly spread across the entire channel. Either these larvae did not move within the channel since they were even distributed at the start of the assay or larvae moved through the media without direction thereby maintaining an even distribution. It was interesting that fewer larvae were recovered from control channels relative to temperature gradient channels, although this difference was significant only for the high temperature thermal assay. The media within control channels was generally 20-21°C across the entire channel. This temperature is below or near the lower end of the thermal preference range for immature flies, as determined from both the low and high thermal preference assays. In the absence of a thermal gradient allowing for movement to a preferred temperature, larvae within the control channel may have more frequently moved to the media surface and subsequently escaped the channel even though this required exposure to light in conflict with their typical negative phototaxis behavior.

The temperature preference of immature house flies varies with their age (Thomsen & Thomsen 1937, West 1951, Strong-Gunderson & Leopold 1989), with younger larvae (1st and 2nd instars) preferring higher temperatures (30-37°C) relative to early 3rd instar larvae (28.5-35.3°C) and especially to late 3rd instar larvae nearing pupation (0-15.6°C) (Thomsen & Thomsen 1937). The larvae used in this study were ~96-h, old making them early 3rd instar larvae and thus indicating that they would likely find suitable a wide range of temperatures within the gradient provided in these assays. The thermal preference for larval flies in the high temperature assay in these studies (26-38°C) is in line with the thermal preference reported by Thomsen & Thomsen (1937), although the temperature range is slightly broader than that reported by Thomsen & Thomsen perhaps due to the shorter assay time offed flies in the current study (4-6 h) compared to Thomsen & Thomsen (several days for 3rd instar larvae).

While we attempted to only include larvae that were 96-h old (~2,600 accumulated degree hours (ADH)), it is possible that developmental age differed slightly among the two assays (low vs high temperature). Flies were reared for these studies in an insectary room under a constant temperature setting, but variation in temperature and relative humidity within the room is possible particularly among months when environmental conditions outside the insectary building may differ. If larval flies experienced slightly different temperatures before selection for these studies, larval age could vary somewhat among the low and high temperature assays and thus larval temperature preference might also be expected to vary. If larvae used in the high temperature assay were younger, temperature preference would be expected to skew

higher (Thomsen & Thomsen 1937). While a difference in rearing temperature for larvae used in the low and high temperature assays is possible, we believe it to be unlikely or at least very small. Nevertheless, future work should be conducted under carefully monitored conditions to minimize the impact of temperature and more accurately estimate developmental age.

Additional differences between the low and high temperature trials that may have affected the apparent preferred temperature range include the differing assay periods, 6-h for low temperature range assays and 4-h for high temperature range assays, as well as the inclusion or absence of the equilibration period prior to the start of the assay period. House fly larvae may acclimate to a low temperature range over a short period of time (Strong-Gunderson & Leopold 1989) perhaps skewing results to a lower temperature preference in the low temperature assay in which a temperature gradient was already present when larval flies were added to the media within the channel.

In these trials, the small number of larvae that were not recovered from each channel were presumed to have escaped the channel. The top of the channel was exposed with media filling the channel nearly to the top. While immature flies are negatively phototactic (avoid light) and lights were positioned above the channels during the assay period, it is possible that some larvae nevertheless emerged from the top of the media and escaped over the edge of the channel. There was some uncertainty in the high temperature thermal preference assay due to significantly different recovery rates between treatments.

Knowledge of larval temperature preference may be used in developing control measures against house fly. For example, chemical or biological applications to kill immature flies could be targeted to development sites that fall within the house fly temperature preference range for young 3rd instar larvae. Similarly, if habitat temperature can be controlled, larvae could be concentrated within limited areas for more targeted applications of insecticides or biological control agents. Finally, altering temperature conditions of larval habitat to fall outside of the larval temperature preference could exclude larvae from otherwise suitable developmental habitat.

Future work should use a uniform assay period to determine the effect of varying the temperature range across the gradient channel. Additionally, experiments could be run with and without the temperature equilibration period to determine if this change from the low temperature assay to the high temperature assay influenced larval temperature preference. This may provide even greater insight, as there is some evidence that larvae exposed to a slowly decreasing temperature may have higher tolerance to low temperatures (Strong-Gunderson & Leopold 1989). Additional measures to ensure accurate measures of age, such as constant temperature monitoring of rearing rooms, and measures of media moisture will likely provide further insights.

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Figures and Tables

Figure 1: Thermal preference assay set-up. House fly rearing media was placed into metal U-channels marked into 3 cm sections numbered 1-15 starting at the chill table (left) and extending to the hot plate (right). An 8 cm section of the gradient channel on either end rested on the chill table or the hot plate. Wooden supports were placed beneath the chill table and hot plate to level the gradient channels. Control channels were identical metal channels except they were placed on the countertop nearby and held at room temperature across the full length of the channel.

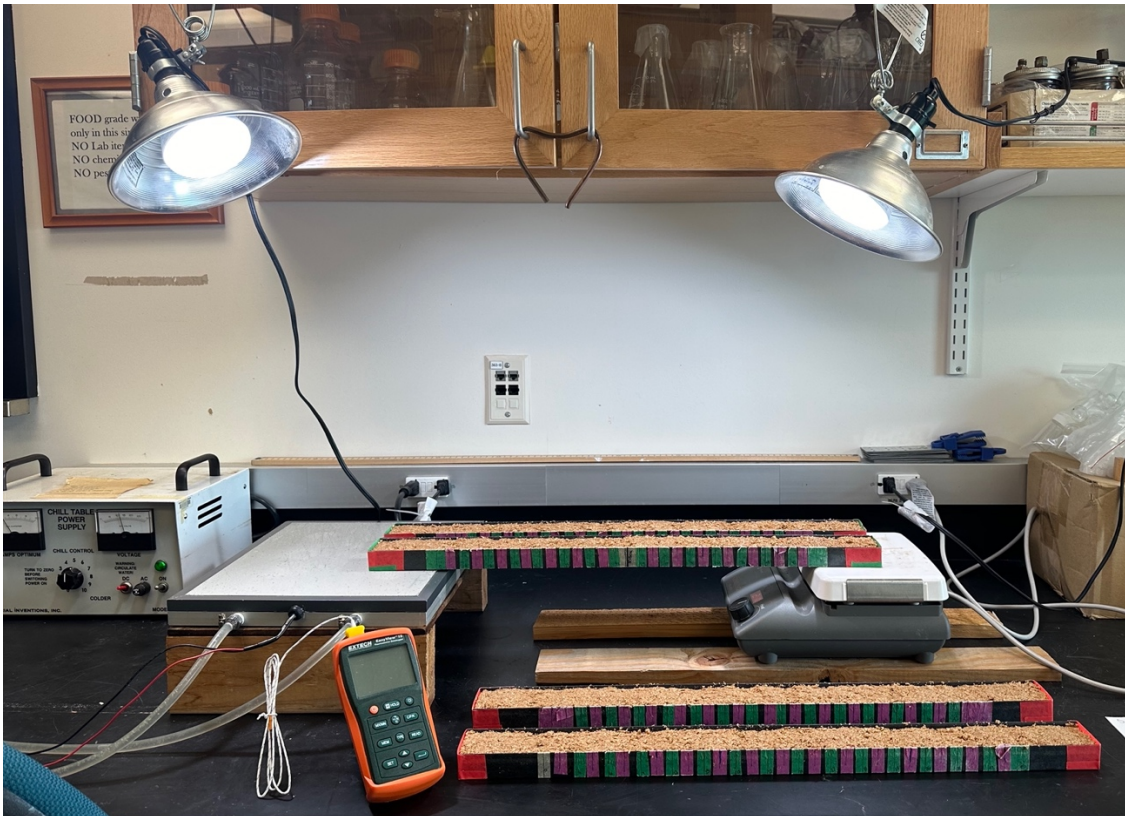

Figure 2: Box and whisker plot of larval house fly distribution by channel section for low temperature range assay. Larval distribution varied among channel sections for the temperature gradient channels ($p < 0.001$) but not for the control channels that lacked a temperature gradient ($p = 0.25$). Plot illustrates the median and IQR of 3rd instar house fly larvae by channel section following the 6-h low temperature thermal preference assay period (total number of larvae recovered from control = 224/420, total number of larvae recovered from gradient = 346/420).

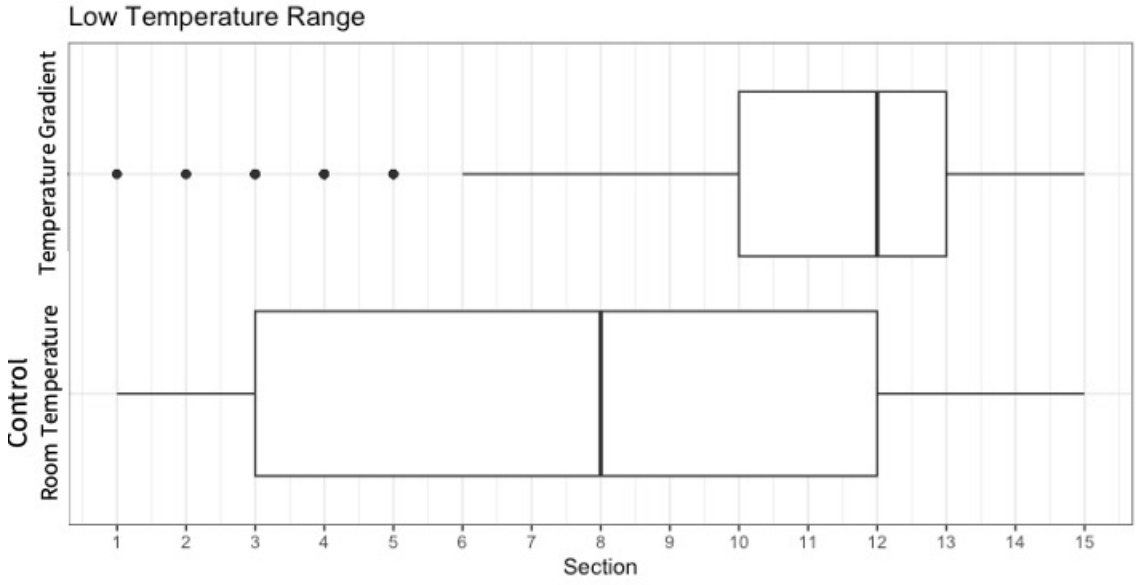

Figure 3: Distribution histograms showing number of 3rd instar house fly larvae by channel section following a 6-h assay period with the channel exposed to room temperature only (3A) or to a temperature gradient (3B) with mean temperature ranging from 17.1 (section 1) to 38.1 °C (section 15). Columns show the sum of larval counts from 14 replicates (30 flies per replicate) (total number of larvae recovered from control = 224, total number of larvae recovered from gradient = 346).

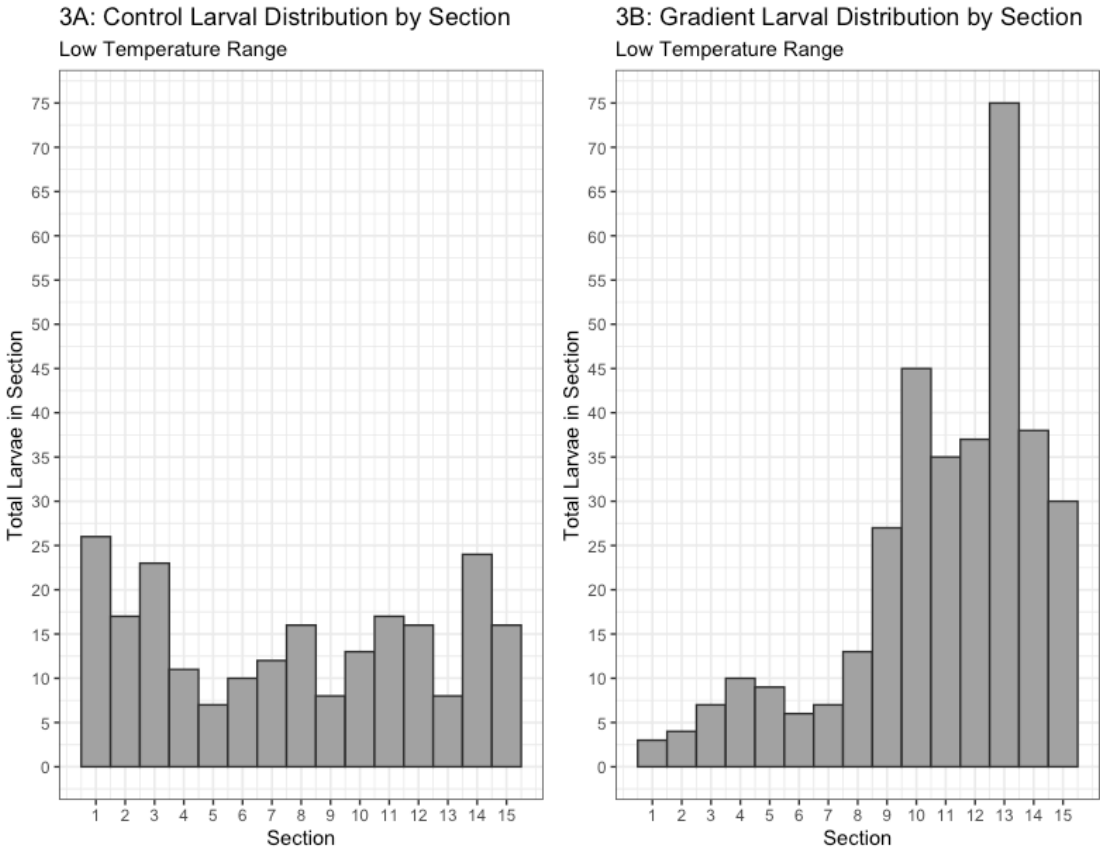

Figure 4: Distribution histograms showing number of larval house flies recovered within media temperature bins (4°C) when exposed to a temperature gradient in either the low temperature range assay (4A: 17.1-38.1 °C) or the high temperature range assay (4B: 18.6-51.8 °C). Columns show the sum of larval counts from 14 replicates (4A) or 12 replicates (4B) (total number of larvae recovered from gradient = 346 (4A) or 314 (4B)).

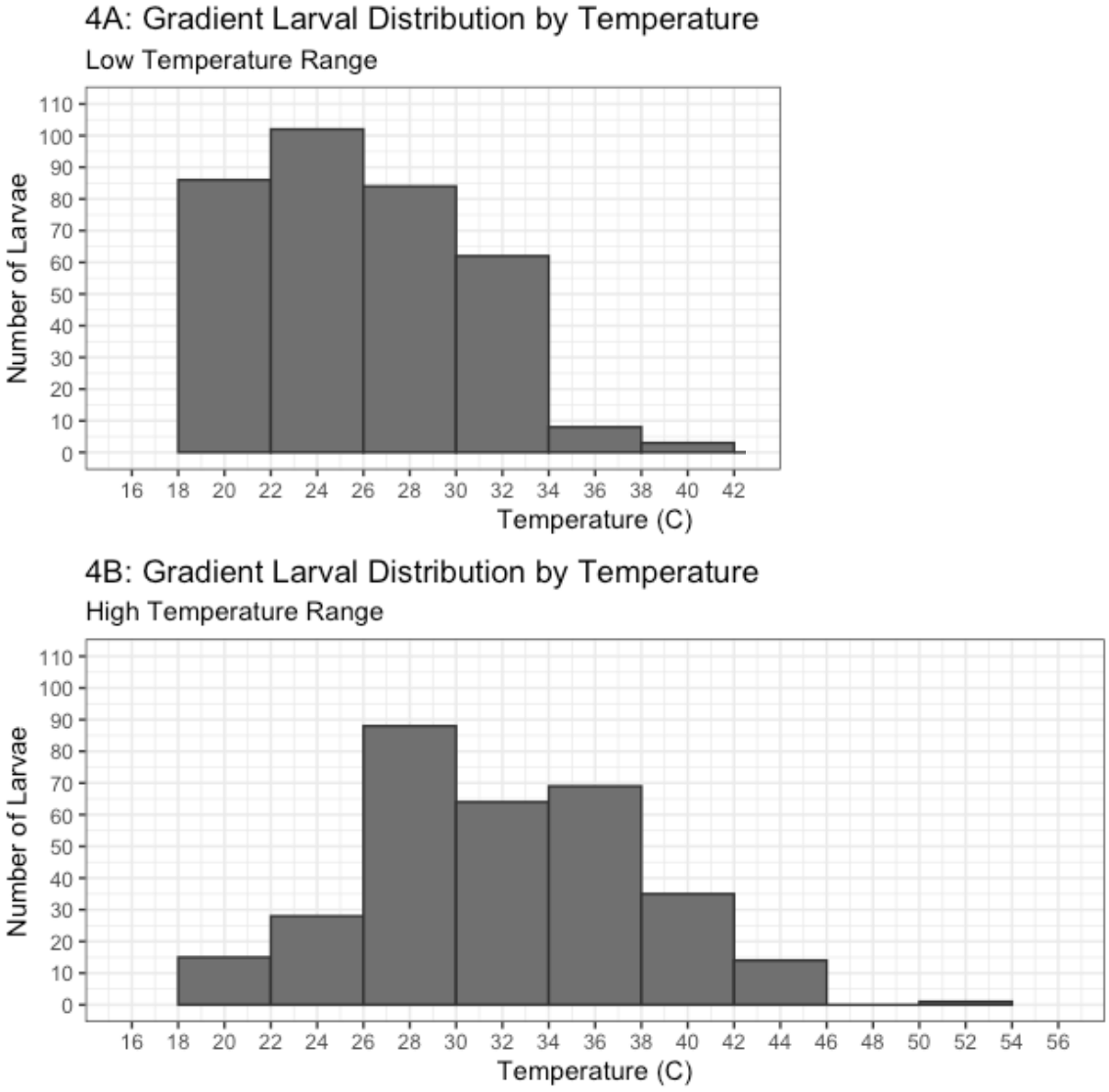

Figure 5: Box and whisker plot of larval house fly distribution by channel section for high temperature range assays. Larval distribution varied among channel sections for the temperature gradient channels ($p < 0.001$) but not for the control channels that lacked a temperature gradient ($p = 0.72$). Plot illustrates the median and IQR of 3rd instar house fly larvae by channel section following the 6-h low temperature range thermal preference assay period (total number of larvae recovered from control = 76/150, total number of larvae recovered from gradient = 314/360).

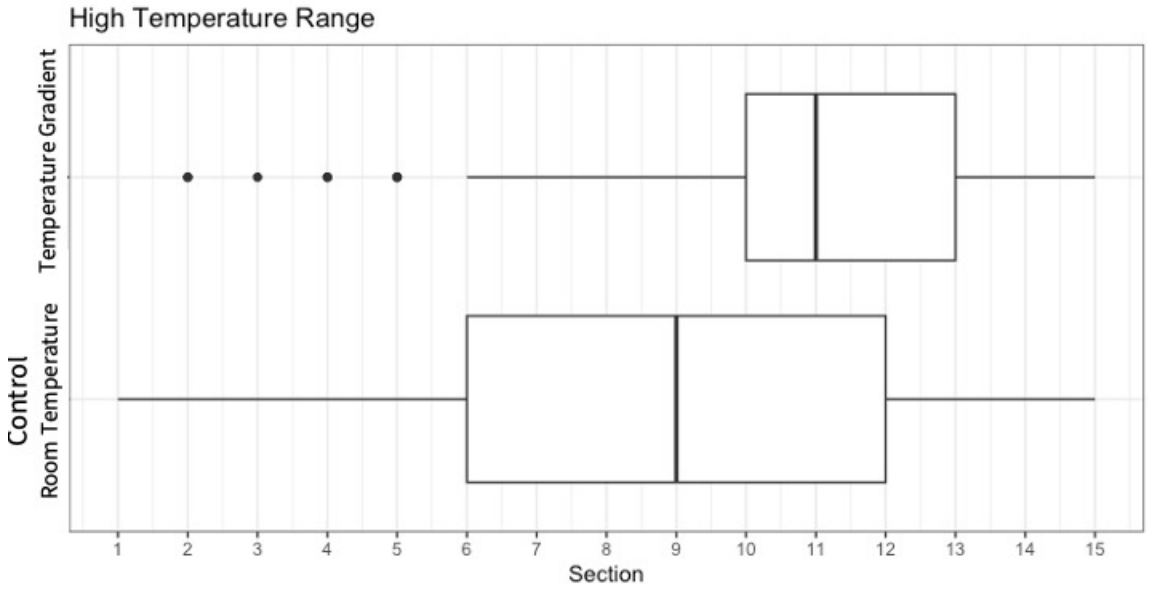

Figure 6: Distribution histograms showing number of 3rd instar house fly larvae by channel section following a 4-h assay period with the channel exposed to room temperature only (6A) or to a temperature gradient (6B) with mean temperature ranging from 18.6 (section 1) to 51.8 °C (section 15). Columns show the sum of larval counts from 5 replicates for the control channels (6A) or 12 replicates for the gradient channels (6B) (30 flies per replicate).

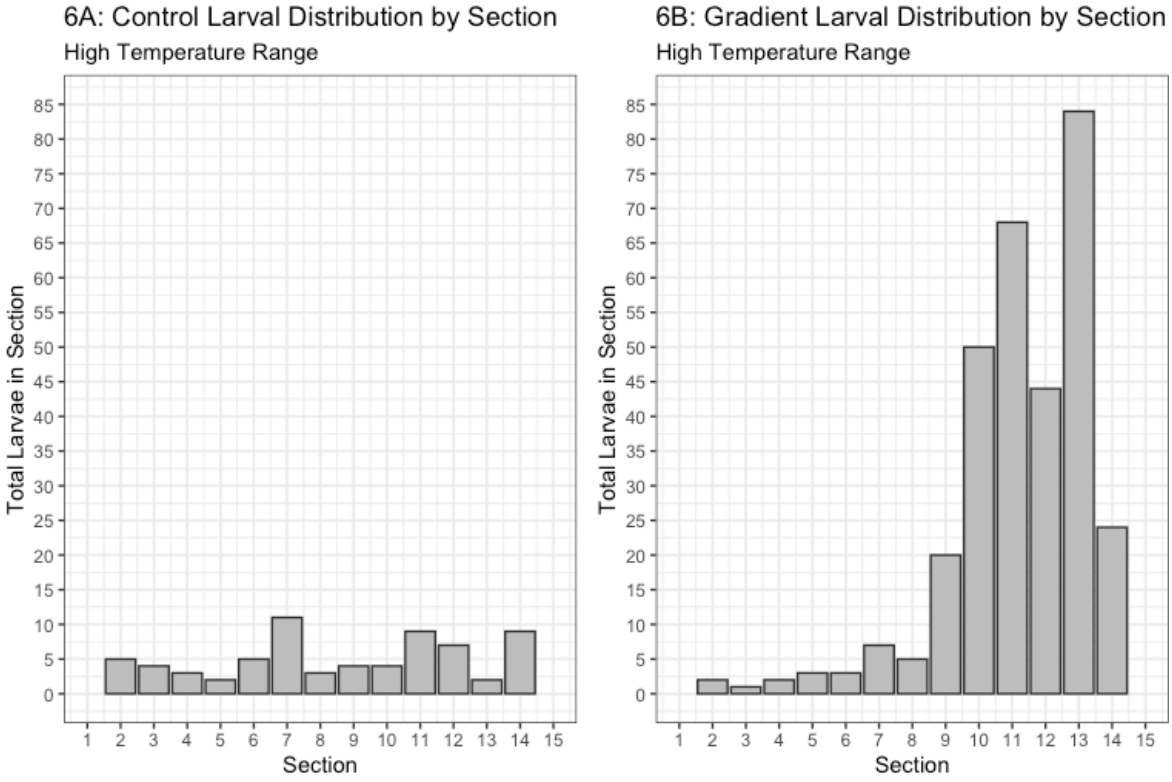

Table A1: Raw data of low temperature range assay larval abundance by section in control channels. Channel sections 0 and 16, which rest on the chill table and hot plate in temperature gradient trials, are

Treatment	Repetition	Counts/ Section																	
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Total**
Control	1C	4	0	0	2	2	2	1	0	1	1	0	0	1	1	1	3	10	29
	1D	2	5	3	0	3	1	0	1	2	1	2	2	0	0	0	0	5	27
	2C	2	0	0	11	1	0	0	1	0	0	5	6	0	0	0	0	0	26
	2D	16	0	2	0	0	0	0	1	1	0	0	0	0	1	0	0	6	27
	3C	10	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	8	23
	3D	10	1	3	0	0	0	0	0	2	2	1	0	1	1	2	1	3	27
	4C	5	0	1	1	0	0	6	1	2	0	0	1	2	0	2	1	3	25
	4D	12	7	1	1	0	0	0	0	1	0	1	0	1	0	0	1	1	26
	5C	1	0	1	4	1	1	0	1	1	1	1	0	1	0	12	0	1	26
	5D	0	0	0	0	1	2	2	0	3	2	0	4	10	0	1	1	3	29
	6C	4	1	1	0	1	1	0	0	0	1	2	2	0	0	3	6	0	22
	6D	10	3	0	0	2	0	0	0	0	0	0	2	0	1	1	3	3	25
	7C	13	3	4	2	0	0	0	3	1	0	0	0	0	0	0	0	0	26
	7D	6	5	1	2	0	0	0	3	1	0	1	0	0	4	1	0	0	24
Total	14	95	26	17	23	11	7	10	12	16	8	13	17	16	8	24	16	43	**362**

Table A2: Raw data of low temperature range assay larval abundance by section in temperature gradient channels. Channel sections 0, which rests on the chill table and corresponds to ~16 °C, and 16, which rests on the hot plate and corresponds to ~41 °C, are excluded from analyses.

Treatment	Repetition	Counts/ Section																	
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Total**
Gradient	1A	0	0	0	0	0	1	0	1	1	3	1	1	4	10	4	2	1	29
	1B	0	0	0	0	0	0	1	1	1	5	4	5	4	3	2	0	0	26
	2A	0	0	0	0	0	2	0	1	0	5	1	2	5	9	0	2	1	28
	2B	0	0	0	1	1	2	0	2	1	1	1	1	3	9	0	4	1	27
	3A	0	1	0	0	5	1	1	0	5	3	2	3	3	0	2	1	1	28
	3B	0	0	0	0	0	0	0	1	0	2	4	3	1	12	1	1	1	26
	4A	0	0	0	1	2	2	3	1	2	2	3	2	4	0	0	3	3	28
	4B	0	0	0	0	0	0	0	0	0	0	8	1	3	3	4	4	3	26
	5A	0	2	2	3	0	0	1	0	2	0	7	4	0	2	1	1	2	27
	5B	0	0	0	1	0	0	0	0	0	2	2	2	3	7	2	0	7	26
	6A	0	0	1	1	0	0	0	0	0	0	5	7	0	7	3	3	1	28
	6B	0	0	1	0	0	0	0	0	1	2	3	1	0	3	8	4	4	27
	7A	0	0	0	0	1	1	0	0	0	1	1	2	7	7	3	3	1	27
	7B	0	0	0	0	1	0	0	0	0	1	3	1	0	3	8	2	8	27
Total	14	0	3	4	7	10	9	6	7	13	27	45	35	37	75	38	30	34	**380**

Table A3: Raw data of high temperature range assay larval abundance by section in control channels. Channel sections 0 and 16, which rest on the chill table and hot plate in temperature gradient trials, are excluded from

Treatment	Repetition	Counts/ Section																	
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Total**
Control	1C	6	1	0	1	1	0	1	0	0	0	1	3	0	0	3	0	2	19
	1D	7	0	0	1	0	0	1	2	3	0	1	1	0	2	3	1	0	22
	2C	0	2	3	0	1	1	0	7	0	0	0	1	0	0	0	3	0	18
	2D	2	0	1	2	1	1	1	1	0	3	0	0	2	0	2	1	5	22
	3C	0	0	1	0	0	0	2	1	0	1	2	4	5	0	1	0	4	21
Total	5	15	4	7	7	3	2	5	11	3	4	4	9	7	2	9	5	11	**102**

Table A4: Raw data of high temperature range assay larval abundance by section in temperature gradient channels. Channel sections 0, which rests on the chill table and corresponds to ~17 °C, and 16, which rests on the hot plate and corresponds to ~63 °C, are excluded from analyses.

Treatment	Repetition	Counts/ Section																	
		0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**Total**
Gradient	1A	0	0	0	0	0	0	0	0	0	2	20	4	1	3	0	0	0	30
	1B	0	0	0	0	0	0	0	1	0	1	1	2	3	15	1	1	0	25
	2A	0	0	0	0	0	0	1	2	0	2	2	13	1	5	0	0	0	26
	2B	0	0	0	0	1	1	2	0	0	0	7	8	8	1	0	0	0	28
	3A	0	0	0	0	0	0	0	1	0	8	9	1	4	5	0	0	0	28
	3B	0	0	1	0	0	0	0	1	1	1	2	5	10	3	4	0	0	28
	4A	0	0	0	0	0	1	0	1	0	1	5	5	6	3	3	0	0	25
	4B	0	0	0	1	1	0	0	0	3	4	0	2	3	4	5	0	0	23
	5A	0	0	0	0	0	0	0	1	0	1	2	3	2	17	0	0	0	26
	5B	0	0	0	0	0	1	0	0	0	0	0	14	1	7	1	0	0	24
	6A	0	0	1	0	0	0	0	0	1	0	2	5	4	10	1	0	0	24
	6B	0	0	0	0	0	0	0	0	0	0	0	6	1	11	9	0	0	27
Total	12	0	0	2	1	2	3	3	7	5	20	50	68	44	84	24	1	0	**314**

Table A5: Larval frequency distribution by section for low temperature range assay. Analysis of effects of treatment, channel section, and their interaction on larval count. Factors were tested using type II Wald chi-square test. The section number was included as residuals (res) from the regression against the treatment (see Methods).

Variable	Chi-squared Statistic	Degrees of freedom	P-value
Treatment	6.7788	1	0.0092247
$Section_{res}$	8.9623	1	0.0027560
Treatment * $Section_{res}$	14.1294	1	0.0001707

Table A6: Larval frequency distribution by section for each treatment in the low temperature range assay. Analysis of effects of larval count on section by treatment type. Factors were tested using type II Wald chi-square test.

Variable	Chi-squared Statistic	Degrees of freedom	P-value
Control	17.086	14	0.2516
Gradient	68.504	14	$3.597x10^{-9}$

Table A7: Larval frequency distribution by temperature for gradient channels in the low temperature range assay. Analysis of effects of temperature on larval count. Factors were tested using type II Wald chi-square test.

Variable	Chi-squared Statistic	Degrees of freedom	P-value
Temperature	13.395	1	0.0002523

Table A8: Larval frequency distribution by section for high temperature range assay. Analysis of effects of treatment, channel section, and their interaction on larval frequency distribution. Factors were tested using type II Wald chi-square test. The section number was included as residuals (res) from the regression against the treatment (see Methods).

Variable	Chi-squared Statistic	Degrees of freedom	P-value
Treatment	18.5037	1	1.696×10^{-5}
$Section_{res}$	16.6883	1	4.405×10^{-5}
Treatment * $Section_{res}$	6.4503	1	0.01109

Table A9: Larval frequency distribution by section for each treatment in the high temperature range assay. Analysis of effects of larval count on section by treatment type. Factors were tested using type II Wald chi-square test.

Variable	Chi-squared Statistic	Degrees of freedom	P-value
Control	10.545	14	0.7214
Gradient	42.245	13	5.973×10^{-5}

Table A10: Larval frequency distribution by temperature for gradient channels in the high temperature range assay. Analysis of effects of temperature on larval count. Factors were tested using type II Wald chi-square test.

Variable	Chi-squared Statistic	Degrees of freedom	P-value
Temperature	14.048	1	0.0001782

Conclusion

Larval biology and behavior have been largely neglected in studies related to the management of house flies. While there is potential for microbe manipulation (Nayduch & Burrus 2017) and biological control (Geden et al. 2021) to manage immature house flies, further investigation of larval behavior is necessary to better understand how larvae respond to these manipulations. To that end, studies described in this thesis were designed to better understand how immature house flies would respond to variation in temperature within their developmental media.

Numerous assay designs and conditions were tested during the development phase of these studies. The essential set up of the thermal preference assay, including use of a thermal gradient applied to a metal 'U' channel, remained constant throughout all assay iterations. In initial assays, larvae were added directly to covered (dark) metal channels without media. It was expected that larvae would move until reaching a portion of the metal channel that was at a preferred temperature where they would aggregate. Unfortunately, larvae moved continuously and without clear orientation over the length of the gradient channel, until they finally wandered to the section of the metal channel above the chill table where they died from freezing. With the failure of these no-media trials, a dry media (vermiculite) was provided as a substrate through which the larvae could move, and which might protect them from direct contact with the metal channel. Dry media trials provided improvement in survival, but larvae were broadly distributed across the entirety of the channel and did not appear to respond to the temperature gradient applied. Additionally, dry media does not represent a typical larval environment,

especially with regard to moisture content needed for development, which likely contributed to the observed results. To create a more "natural" test system, the metal channels were filled with developmental media and house fly eggs were introduced. The eggs hatched and larvae freely dispersed throughout the media in the absence of a temperature gradient until they reached the late L2 or early L3 life stage. Media moisture was maintained by the addition of ~100 mL of DI water distributed daily across the entire surface of the media until the channels were placed on a temperature gradient for 24-h. These assays suffered from a lack of control of larval numbers within each channel (# eggs was difficult to estimate) and a key challenge for all subsequent experiments: media drying near the hot plate. To address media drying, later assay designs (reported in this thesis) utilized an assay period that was long enough to allow larval flies to move the full length of the gradient channel (4-h) but short enough to prevent excessive drying of media adjacent to the hot plate (>8-h).

As in our studies, loss of moisture in media was evident in other foundational experiments (Thomsen & Thomsen 1937), where initial studies failed due to excessive drying of media. Thomsen & Thomsen (1937) addressed media drying by designing a temperature gradient apparatus that included a 'V-shaped' glass cover to capture and return evaporating moisture from the heated media. This design allowed the authors to hold immature house flies in the media over many days while they developed from the egg stage through the pupal stage. In our studies, metal channels with media were held on the temperature gradient for only 8-h in the low temperature assay (2-h equilibration + 6-h assay) or 4-h in the high temperature assay (no equilibration period). These short assay

periods were intended to reduce the impact of drying on heated media within the gradient while still providing larvae sufficient time to move throughout the media within the gradient channel. However, even with these short assay periods, some drying of the media was noted in the gradient channel sections nearest to the hot plate.

It was determined in our studies that early 3rd instar larvae displayed a preference for temperatures of 26-38°C in the high temperature thermal preference assay, a finding that was in line with a much older study (Thomsen & Thomsen 1937) which showed early 3rd instar larvae preferred 28.5-35.3°C. In contrast, the low temperature thermal preference assay indicated that these larvae preferred a much lower temperature range of 21.1-30.1°C. Part of this discrepancy in outcome is due to the availability of higher temperatures in the high temperature range assays, but this does not fully explain the lower minimum. A potential explanation for this could be the increased drying in the low temperature range assay, which endured an additional four total hours of time exposed to the gradient. However, initial investigations into the moisture content of both assays indicated there was no notable difference between them. It is also possible the larvae may have been older, as older larvae exhibit a much lower temperature preference (Thomsen & Thomsen 1937). Another possibility is that the construction of the low temperature range assay, which limited larval movement towards the hot plate, yielded a lower mean. With the high temperature range assay, there was no limit placed on the high or low end, resulting in a mean that much better approximated previous findings.

These findings aim to improve the knowledge of larval house fly behavior and may be implemented in future management practices. If the temperature of developmental sites could be manipulated, either above or below the preferred range, larvae may be driven out of these sites completely and therefore mitigate increasing adult populations. Alternatively, if the media reaches the preferred temperature range, control efforts could be focused on these areas, ensuring the majority of larvae would be in the treatment area. Beyond improvement of control, there has been a recent interest in utilizing house flies as animal food (van Zanten et al. 2015), which would benefit from improved mass rearing methods.

Further studies could take into account the differences in larval preference range by developmental age (Thomsen & Thomsen 1937), using 2nd instar or late 3rd instar larvae for updated temperature ranges of these life stages. Manipulations of other habitat conditions such as moisture, which may be more difficult to achieve along a gradient, could also provide additional insight to larval aggregation behaviors. It may even be useful to conduct similar studies with different densities of larvae, where the limits of this aggregation may be tested.

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