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The Genetics Of Mosquito Heat-Seeking Behavior

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THE GENETICS OF MOSQUITO HEAT-SEEKING BEHAVIOR

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Temperature is a highly dynamic feature of the world, and one that deeply affects living things. Organisms have evolved sophisticated sensory-motor systems to detect and avoid excessive heat or cold—a behavior termed thermotaxis. In rare cases, however, animals use thermosensation not only to regulate their body temperature, but also to locate food sources in their environment. One example of such an adaptation is found in the female *Aedes aegypti* mosquito, which becomes attracted to the body heat of endothermic ("warm-blooded") hosts when in pursuit of a blood meal. Mosquitoes are remarkably adept at finding hosts in their environment and have become major vectors of human disease, but much remains to be understood about the ethology and sensory neurogenetics of this notorious insect. In this thesis, we used high-throughput quantitative behavioral assays and genome-editing techniques to investigate the behavioral rules and molecular basis of mosquito thermotaxis.

We have found that female *Aedes aegypti* are exquisitely sensitive to thermal contrast, and are capable of heat-seeking in diverse ambient environmental temperatures. By seeking relative warmth and avoiding relative cool, mosquitoes can thermotax towards heated targets. However, mosquitoes also avoid stimuli exceeding the body temperature of their hosts. In this manner,

Ae. aegypti are maximally attracted to thermal stimuli approximating endothermic hosts such as humans. We have discovered that the insect thermosensor *TRPA1*, in addition to playing conserved roles in thermoregulation and chemosensation, is important for thermotactic tuning of heat-seeking. *AaegTRPA1*$^{-/-}$ mutant mosquitoes fail to avoid high-temperature stimuli, and do not distinguish between thermal targets that resemble hosts and those that are inappropriately hot. This *AaegTRPA1*-dependent tuning of thermotaxis may be critical for mosquitoes host-seeking in a complex thermal environment in which hosts are warmer than ambient air, but cooler than surrounding sun-warmed surfaces.

These results demonstrate that evolutionarily conserved thermosensors, conventionally used for maintaining thermoregulatory homeostasis, can be repurposed by blood-feeding arthropods to help locate and recognize the thermal signatures of their hosts. Our characterization of the behavioral strategies underlying heat-seeking also helps to establish mosquitoes as a promising model system for the study of thermosensation and thermotaxis. These efforts may inform the design of next-generation repellents and traps for the control mosquito-borne diseases.

For animals.

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It will be hard to leave the cozy home of Rockefeller University. Part of me wants to keep on roaming the tunnels and eating at Weiss cafeteria just a little bit longer, or watch a few more strange vessels parade under the Queensboro bridge. How about one final beer by the ducks? I am excited for what's to come, but before I leave I want to thank the people who made my PhD a real thrill.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

Introduction

1.1 Biology is deeply influenced by temperature

Since ancient times, humans have observed the phenomenon known as temperature, taking great care to avoid the cold of ice and the heat of fire. But it wasn't until around 240 BCE that a particular human known as Philo of Byzantium made the first documented thermometer and began understanding the nature of "hot" and "cold" (McGee, 1988) (**Fig 1.1**). Philo was able to record the thermal expansion and contraction of air inside a hollow sphere. To do this, he connected the sphere to a jug of water using a tube. When he put the device in the sun, the air in the sphere expanded, forming bubbles in the water. When he cooled the device in the shade, the air contracted and the water level rose within the tube. Philo's crucial insight was that temperature can alter the properties of substances such as water and air. This relationship between temperature and matter is not only convenient for designing thermometers, but it is also the essence of why living things are influenced by the thermal environment.

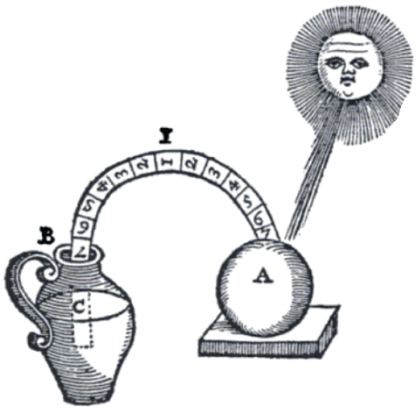

Figure 1.1 The world's first thermometer, made by Philo of Byzantine circa 240 BCE. Radiant heat from the sun warms the air inside a hollow sphere attached to a tube. The thermal expansion of the air results in bubbles. Upon cooling, the water level in the tube rises. Drawn by Robert Fludd in 1574, reprinted from (McGee, 1988).

It was not until the modern era that a deeper understanding of temperature emerged. In 1827, the botanist Robert Brown was looking through his microscope, examining grains of pollen suspended in water, when he noticed that the particles in the preparation were undergoing continuous motion in random directions (Brown, 1828). The origins of this “Brownian” motion were unclear until Albert Einstein derived a mathematical description of this phenomenon in one of his three revolutionary papers of 1905 (Einstein, 1905). Einstein’s analysis, which decisively confirmed the existence of atoms, explained that the matter in Brown’s specimen is comprised of molecules bombarding one another in constant random motion. It is this atomic motion that temperature describes. In other words, temperature is a measurement of the kinetic energy in matter. At high temperatures, molecules tend to undergo more motion, and vice versa. So, for instance, when a human walks from the shade into the sun, her molecules will warm up and speed up—leading to an increase in temperature.

Since the time of Philo, temperature has become one of the most measured parameters in science, and humans have developed complex technology to quantify and control it. In the laboratory, substances have been cooled to nearly absolute zero and scientists have used particle accelerators to generate the highest temperatures in the known universe. In the realm of biology, researchers have also found living things capable of surviving in astounding thermal environments. Such organisms can withstand nearly absolute zero, in the case of the water bear (*Tardigrada*) (Jönsson and Bertolani, 2001), or 120°C

in the case of some Archaea (Kashefi and Lovley, 2003). But these extremophiles are rare exceptions. Most living things can only survive within a narrow range of moderate temperatures. The average optimal body temperature of most living organisms that have been investigated is between 20-30°C (Dell et al., 2011). This is because the molecular processes underlying biology are delicate phenomena, deeply affected by temperature.

When ancient humans declared things “too cold” or “too hot”, they were articulating the fundamental relationship between biology and temperature. For living things, only a particular range of temperatures permits survival, and extreme temperatures are dangerous. That is why most multicellular animals have nervous systems that can: 1) perform thermosensation—the detection of thermal stimuli; and 2) execute thermotaxis—temperature-dependent navigation. For the vast majority of animals, thermosensation and thermotaxis are used to avoid environments and objects that are excessively hot or cold, promoting the outcome of dwelling at optimal physiological temperatures. However, there are rare cases in which animals use thermal cues to locate important resources in their environment. This thesis concerns one such behavioral adaptation in the heat-seeking female *Aedes aegypti* mosquito.

1.1.1 Thermal energy is a dynamic feature of a terrestrial environment

In a terrestrial environment, the atoms of the air, water, objects, and organisms are regularly losing and gaining thermal energy (Cossins and Bowler, 1987). The net flux of heat is from areas of high temperature to areas of low

temperature, and this thermal energy is transferred in four distinct manners: conduction, convection, radiation, and evaporation (Cossins and Bowler, 1987).

Conduction of heat occurs within a material substance in a manner similar to solute diffusion, and is transmitted across macroscopic distances via atomic interactions. Convection is the actual macroscopic translocation of atoms with high kinetic energy from a warmer area to a cooler area. This phenomenon occurs in fluids, such as air and water, often as a result of buoyancy. In a terrestrial environment, convection is constantly occurring throughout the air (Geiger et al., 2009). Radiation is the transfer of heat through electromagnetic energy, such as the sun's radiant heating of earth. In this case, individual atomic reactions in the sun are shedding thermal energy that travels essentially unimpeded through ~93 million miles of space and atmosphere to warm the earth's surface. All objects emit thermal radiation to varying extents, because this is a feature of atoms, and radiation is one of the major forms of heat-loss for organisms (Cossins and Bowler, 1987). Lastly, evaporative heat transfer is fundamentally distinct from the other forms discussed above. This phenomenon occurs when a liquid, such as water, becomes a gas. Because water absorbs a great deal of thermal energy during vaporization, it tends to cool the surface from which it evaporated. This is the physical mechanism by which sweating or panting can cool an animal (Hill et al., 2004).

Animals are profoundly affected by these thermodynamic fluxes. Terrestrial environments often exhibit extremely complex thermal landscapes in which convective air flow, solar radiation, and variable surface temperatures

produce unpredictable and severe temperature fluctuations (Cossins and Bowler, 1987; Geiger et al., 2009). For this reason, most animals must actively regulate their temperature either physiologically or behaviorally.

1.1.2 Temperature is a strong evolutionary force

One of the astounding feats of human evolution and history has been our ability to inhabit almost every climate on earth. This is quite rare, as most organisms reside in more restricted thermal ranges. Terrestrial temperature is highly correlated with latitude on our planet (Angilletta, 2009), and you can find humans living everywhere from freezing polar regions to scorching equatorial deserts. The ubiquity of *Homo sapiens* is perhaps mostly due to cognitive and cultural evolution—more sophisticated brains and the associated ability to generate tools and transmit them across generations. But other species have been inhabiting these environments for far longer timescales without our social adaptations, and have evolved morphologies and physiologies to permit survival at the harsh limits of thermal tolerance.

At the poles, where both terrestrial and aquatic temperatures are routinely subzero, animals have evolved powerful strategies to retain warmth and avoid freezing. For example, one common approach is to insulate the body from heat loss (Cossins and Bowler, 1987). Whales, seals, and polar bears, for instance, use a specialized layer of fat called blubber, and some birds have evolved plumage on their legs. Some animals use a very different strategy, escaping the demands of staying warm by hibernating in a remarkable state of low metabolic

activity (Hill et al., 2004). Other adaptations associated with living in a frigid environment are at the molecular level (Bagriantsev and Gracheva, 2015; Gracheva and Bagriantsev, 2015). For instance, the hibernating thirteen-lined ground squirrel (*Ictidomys tridecmlineatus*) expresses thermogenic (heat-producing) proteins in its brain tissue (Laursen et al., 2015). Many polar fish synthesize antifreeze compounds during winter months to bind ice crystals in the blood plasma and stem the spread of freezing (Lee and Costanzo, 1998). Still other animals tolerate freezing, allowing them to overwinter and thaw out the following spring, such as the wood frog, *Rana sylvatica* (Schmid, 1982).

In equatorial deserts, where both air and ground temperatures soar during the day, animals must contend with serious risk of overheating and desiccating. Some animals have solved this problem by evolving exotic morphologies. For instance, the large, highly vascularized ears of the black-tailed jackrabbit, *Lepus californicus*, radiate excess body heat to the environment (Hill et al., 2004). Among the organs of the body, the brain is especially sensitive to overheating, and many animals selectively cool this organ using countercurrent heat exchange in their circulatory system; cooled venal blood from the respiratory passages is used to lower the temperature of arterial blood supplying the brain (Hill et al., 2004). Dromedary camels allow their body temperature to cycle dramatically, heating up during the hot daytime hours, and cooling down during the cold night. By undergoing these large amplitude temperature cycles, camels avoid the dehydration associated with common thermoregulatory strategies such as sweating or panting (Hill et al., 2004).

These striking adaptations are evidence of the strong evolutionary influence of temperature. These lineages have specialized to flourish in extreme thermal environments, and in many cases cannot survive in more moderate zones. For instance, water temperatures of 4-6°C will elicit lethal *heat* stress in some Antarctic fish and cause lethal *cold* stress in some tropical fish (Hill et al., 2004). But thermal sensitivity does not only apply to polar bears and camels—temperature is one of the most important environmental factors for all living things. Even in moderate thermal environments, animals must closely regulate their internal temperature. In fact, this deep connection between temperature and biology is one of the major concerns regarding anthropogenic climate change, which is altering thermal environments and causing widespread disruptions in ecology (Angilletta, 2009; Hill et al., 2004).

1.1.3 Animal fitness tends to be optimized at intermediate temperatures

In 1919, having recently made headlines by successfully measuring the dimensions of the Milky Way galaxy, the legendary astronomer Harlow Shapley took some time to watch ants. By carefully measuring the velocity of thousands of individual *Liometopum apiculatum* ants as they crawled on the grounds of the Mount Wilson observatory, he discovered that their locomotive speed was strongly correlated with ambient air temperature (Shapley, 1920). On hot days (~40°C) ants tended to walk at a rate of ~6 cm/s, while on cold days (~15°C), ants tended to walk only at ~1 cm/s. This relationship between speed and

temperature was so consistent, that Shapley could predict air temperature within 1°C just by watching ants (Huey and Kingsolver, 2011)!

Shapley's "ant-thermometer" is fundamentally similar to the ancient device created by Philo of Byzantium. In Philo's thermometer, the thermal expansion of air results from changes in the microscopic behavior of its atomic components. Biological life, too, is rooted in molecular processes that are exquisitely sensitive to temperature. The warmer the environment, the faster biological processes unfold. Because all chemical reactions require an initial activation energy (E_a), the rate of a chemical reaction (k) will be related to the amount of kinetic energy in the molecules (temperature, T). The Arrhenius equation, $k = A*e\text{^}E_a/RT$, provides a mathematical description of this empirical relationship, where A is the frequency factor (which includes parameters such as frequency and orientation of molecular collisions) and R is the universal gas constant (Cossins and Bowler, 1987). According to this equation, the rate of a given reaction will increase as temperature increases. The unitless quantity Q_{10} provides a succinct expression of this relationship by describing the rate of change in a biological or chemical process as a consequence of increasing the thermal environment by 10°C (Cossins and Bowler, 1987).

The enzymatic reactions underlying metabolism generally follow an Arrhenius relationship. For instance, in warmer environments, cells tend to have elevated energy states, resulting in more rapid biochemical activity (Cossins and Bowler, 1987). This phenomenon can be observed by measuring oxygen consumption as a proxy for overall metabolism. In one study, the oxygen

consumption of tiger moth caterpillars increased exponentially with temperature, demonstrating the immense effect thermal environment can have on basic biological processes (Hill et al., 2004). The nervous system can also be extremely sensitive to thermal environment, for it too is rooted in physio-chemical processes. For instance, in the sensory receptors of the inner ear, the kinetics of ion channel conductance increase monotonically with temperature and exhibit an Arrhenius relationship (Corey and Hudspeth, 1983). The importance of this effect is underscored by the evolution of compensatory mechanisms; some animals such as the grasshopper *Locusta migratoria* display cell-intrinsic mechanisms to mitigate the effects of thermal fluctuations on auditory neuron ion currents (Roemschied et al., 2014). Even animal behaviors can be described by an Arrhenius relationship, as Shapley found when he further analyzed in his ant data in a subsequent paper (Shapley, 1924).

On the other hand, while enzymes and organisms tend to work more effectively at warm temperatures, they eventually deteriorate when overheated. At a molecular level this can be due to protein denaturation. The biochemical properties of enzymes depend on their tertiary and quaternary structures, which arise from weak atomic interactions that are susceptible to thermal disturbances (Cossins and Bowler, 1987). Therefore, biochemical processes and animal fitness are usually maximized at an intermediate optimal temperature (**Fig 1.2**). These types of performance-temperature curves can be seen in many organismal processes, such as the development of damselfly larvae, growth of *Daphnia magna*, fecundity of butterflies, insect survival, and lizard locomotion

speeds (Angilletta, 2009). Indeed, most living things investigated—from microbes, to plants, to fungi, to vertebrates—have an optimal body temperature averaging around 20-30°C (Dell et al., 2011).

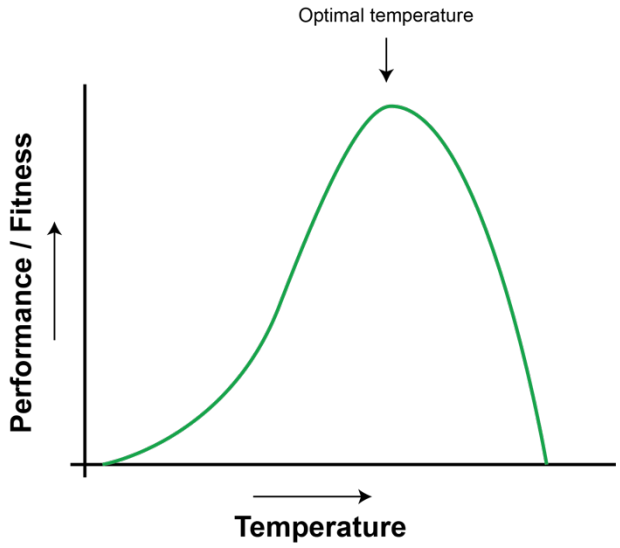

Figure 1.2 A hypothetical curve illustrating the relationship between biological performance/fitness and temperature. Biological processes and organismal fitness are optimized at an intermediate temperature. Adapted from (Huey and Stevenson, 1979).

1.1.4 Ectotherms undergo behavioral thermoregulation

How do animals maintain an optimal, or at least suitable, body temperature in a fluctuating thermal environment? Human body temperature is remarkably steady, holding a nearly constant ~37°C. Whether we are swimming in cold water, or hiking through the desert, our core body temperature barely deviates from its optimum (Hill et al., 2004). Even a 2-3°C increase or decrease in body temperature results in serious physiological distress. How are we able to maintain such a consistent thermal set-point?

All mammals and birds are endotherms, which actively produce body heat through metabolism. When the environment gets too cold, endotherms burn

calories to warm their tissues (e.g. shivering). If the environment gets too warm, endotherms expend energy to sweat or pant to lower body temperature via evaporative cooling. While these homeostatic mechanisms afford animals a great deal of freedom to dwell in diverse thermal environments, it comes at a great energy cost. To supply sufficient calories to support thermoregulation, endotherms must ingest far more food than comparably-sized fish and lizards (Hill et al., 2004).

The vast majority of animals are ectotherms, which do not produce body heat through metabolism. Instead, ectotherm body temperature is determined by environmental factors such as air temperature, surface temperature, and solar radiation (Angilletta, 2009; Stevenson, 1985). For instance, a snail crawling through the woods might be warmed by the summer air, heated by the sun, cooled by the breeze, or chilled by the damp shady earth under a log. In a complex terrestrial setting such as this, an ectotherm has the option to behaviorally thermoregulate by navigating to a suitable thermal environment.

Because behavioral thermoregulation is such a powerful strategy to avoid excessive heat and cold, it is prevalent in the animal kingdom. Humans, for instance, often seek shade and a swimming hole on a hot day, or sunshine and shelter on a cold day. Indeed, many endotherms undergo this form of active thermoregulation. Cats warm in the sun, and grazing sheep cool off in the shade of a tree. Some mammals and birds migrate great distances to seek suitable temperatures, such as Reindeer (*Rangifer tarandus*), which travel 5000 km each year during seasonal changes (Hill et al., 2004). Other endotherms behaviorally

thermoregulate by huddling together for warmth, such as male emperor penguins, *Aptenodytes forsteri*, who incubate their eggs through the harsh Antarctic winter (Gilbert et al., 2008). Even newborn rat pups, *Rattus norvegicus*, navigate to seek the comfortable warmth of their siblings (Alberts, 1978). This type of navigation based on temperature is called thermotaxis.

While thermotaxis is a helpful supplementary thermoregulatory strategy for endotherms, it is a critical one for ectotherms (Hill et al., 2004). In a classic set of experiments, blue-tongued lizards (*Tiliqua scincoides*) were observed to shuttle between a hot (45°C) and a cold (15°C) environment to thermoregulate (Hammel et al., 1967). Thermometers implanted in the animals showed that by thermotaxing between these conditions, they were able to maintain moderate colonic and brain temperatures 30-37°C. Furthermore, the experimenters could influence the behavioral decisions of these lizards by artificially elevating or reducing their internal temperature.

Invertebrates, which are typically ectotherms, also use thermotaxis as an essential means of regulating their temperature. The slime mold (*Dictyostelium discoideum*) is known to migrate across a thermal gradient, avoiding cool and seeking warmth (Poff and Skokut, 1977). Thermotaxis has been studied extensively in the nematode, *C. elegans* (Bargmann and Mori, 1997; Kimata et al., 2012). These animals also migrate on a thermal gradient. Interestingly, their preferred temperature is determined by the temperature at which they were cultivated (Hedgecock and Russell, 1975). Once they reach an area of optimal temperature, *C. elegans* continue moving, but they are careful not to abandon

the area. This isothermal tracking requires detection of thermal gradients <0.1°C (Bargmann and Mori, 1997). Models of *C. elegans* thermotaxis indicate that these behavioral mechanisms form a robust thermoregulatory strategy when deployed in a natural setting with fluctuating temperatures (Ramot et al., 2008b).

Due to their low heat-capacity, small terrestrial ectothermic insects such as flies and mosquitoes rapidly equilibrate to the temperature of their surroundings (Garrity et al., 2010; Heinrich, 1993; Stevenson, 1985). For instance, it is estimated that the body temperature of a 10 mg fly will increase by 10°C within 10 seconds of exposure to direct sunlight (Heinrich, 1993). Thus, for these animals, behavioral strategies such as moving between warmer and cooler environments are vital components of thermoregulation (Heinrich, 1993; Stevenson, 1985). Using thermotaxis, these animals can optimize fitness and avoid damage associated with extreme temperatures. Many studies have investigated thermal sensitivity and thermotaxis in the vinegar fly, *Drosophila melanogaster* (Dillon et al., 2009). This species is able to survive and reproduce only in environments ranging 15-28°C, beyond which incapacitation and mortality typically occur (Hoffmann, 2010). Furthermore, within this permissive thermal range, *D. melanogaster* fitness is strongly affected by temperature (Siddiqui and Barlow, 1972).

Both larval and adult *D. melanogaster* exhibit robust thermotaxis to optimize their internal temperature (Garrity et al., 2010). When crawling freely on a thermal gradient, first instar larvae avoid extremes of heat and cold, accumulating in zones with moderate temperatures (Liu et al., 2003; Rosenzweig

et al., 2005). When adult *D. melanogaster* are allowed to distribute in a thermal gradient, they show a strong preference for air temperatures of ~24-26°C (Hamada et al., 2008; Sayeed and Benzer, 1996). These temperature preferences coincide with the optimal thermal environment that provides maximal fitness for this species (Garrity et al., 2010).

Thermotaxis in a thermal gradient takes place over many minutes, but flies can also make decisions about thermal preference at more rapid timescales. In a two-choice assay, confined adult flies were suddenly given access to two larger chambers, each boasting a different air temperature (Sayeed and Benzer, 1996). When given a choice between high (~30-32°C) and moderate (~22-25°C) air-temperatures, *D. melanogaster* showed strong avoidance of heat, accumulating in the chamber with moderate temperature (Ni et al., 2013; Sayeed and Benzer, 1996). Another study showed that flies can rapidly learn to associate visual cues with thermal environments (Ofstad et al., 2011). In this paradigm, adult flies were confined to an arena with unsuitably high surface temperature (36°C). Over the course of many trials, flies were presented with a single “island” of moderate surface temperature (~25°C), which appeared at different locations in the arena. When the position of the “island” was correlated with arbitrary visual cues surrounding the arena, flies were quickly able to learn to locate the safe spot. This is a clear demonstration of the importance of thermotaxis and behavioral thermoregulation in terrestrial ectothermic insects.

1.2 Some animals use thermosensation to hunt for warm-blooded food sources

"… scorched by the burning peat, and half choked by the blinding smoke, we added quite a novel episode to our experiences in collecting beetles, for on the ground on which it was too hot to place one's hand, many Melanophila were running. They settled… on pine stumps actually glowing, or flying under a blazing August sun through drifts of acrid peat smoke…" (Linsley, 1943)

Many entomologists and fire fighters have remarked upon the strange pyrophilous ("fire-loving") behavior of the *Melanophila acuminata* beetle. Plentiful are the tales of enormous swarms of these insects traveling over kilometers to converge upon forest fires, massive oil-tank infernos, industrial kilns, or even cigarette-smoking stadium audiences (Linsley, 1943; Schmitz and Bousack, 2012). The reason for this peculiar fiery passion is that these beetles have specialized to lay their eggs in the bark of freshly burnt conifers (Campbell et al., 2002). As a result, larvae then hatch among an abundance of nutritive wood without suffering the dangerous defensive toxins produced by live trees (Campbell et al., 2002). This remarkable fire-seeking behavior has evolved multiple times among beetles specialized in this niche, all of which boast sensitive organs capable of detecting infrared heat radiation (Schmitz and Trenner, 2003).

Thermosensation appears to be an evolutionarily conserved feature of animal nervous systems. The ability to respond to temperature fluctuations is highly advantageous, in that it can optimize physiology and prevent tissue damage. However, as in pyrophilous beetles, thermosensation can directly serve other immediate drives besides thermoregulation. For example, while air and

surface temperatures are constantly fluctuating in a terrestrial environment, endotherms, such as mammals and birds, maintain a very consistent warm body temperature. Thus, these creatures provide a highly salient and dependable thermal signature for animals seeking “warm-blooded” prey or hosts (**Fig 1.3**). In these instances, thermotaxis can be a valuable strategy for finding sources of nutrition.

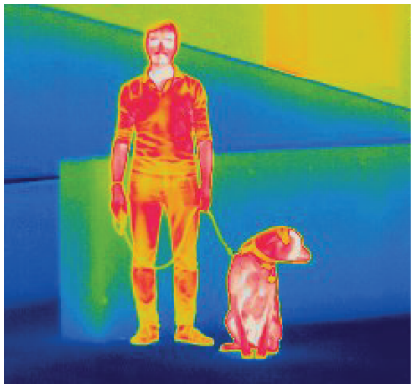

Figure 1.3 “Warm-blooded” animals present salient thermal signatures. A thermal image of a human (*Homo sapiens*) and a dog (*Canis lupus familiaris*) in an outdoor environment.

1.2.1 Snakes and bats use heat-sensation to feed on warm-blooded prey

California ground squirrels (*Spermophilus beecheyi*) must defend themselves against predation by snakes. One confrontational defense strategy is that of “tail-flagging”—waving the tail back and forth—probably as an advertisement of vigilance and readiness (Barbour and Clark, 2012). Squirrels will perform tail-flagging to a variety of snake species, but when confronted with certain types of snakes, they will add a thermal component to this display by increasing the temperature of their tails (Rundus et al., 2007). Why might this be adaptive? It turns out that this strategy is selectively deployed when confronting snakes that use thermosensation to facilitate hunting of warm-blooded prey (Rundus et al., 2007).

Some snakes have evolved specialized organs on their face that are exquisitely sensitive to infrared radiation in their surroundings (Campbell et al., 2002; Gracheva et al., 2010). By providing thermal imagery, these sensors support the tracking and capture of endothermic prey items, such that many of these snakes can successfully hunt even when blindfolded (Buning, 1983). This sensory modality is also probably utilized for thermoregulatory purposes, by remotely locating and assessing warm surfaces suitable for basking (Krochmal and Bakken, 2003). Infrared-sensitive pit organs appear to have evolved twice independently—once among ancient snakes such as boas and pythons, and once again in modern snakes such as pit vipers (Gracheva et al., 2010).

Similar thermosensation capabilities have evolved in the vampire bat, *Desmodus rotondus* (Gracheva et al., 2011). Unlike fruit-eating or insectivorous bats, vampire bats feed exclusively on blood, which they acquire from endothermic prey such as ungulates (McCracken, 2006). This specialization has resulted in multiple dramatic adaptations, such as agile quadrupedal locomotion, altered face morphology, and infrared-sensitive organs (Gracheva et al., 2011; McCracken, 2006). Vampire bats are thought to be capable of detecting warm-blooded prey from many centimeters away, and can use thermosensation to locate suitable feeding spots on their targets (Kürten and Schmidt, 1982).

1.2.2 Many hematophagous arthropods heat-seek

Attraction to warmth is found among many hematophagous (blood-feeding) arthropods. Such animals have evolved to find vertebrate hosts, break

through the skin and consume their protein- and lipid-rich blood, often for the purpose of nourishing developing eggs. This sanguinary life-style has evolved independently at least 20 times in arthropods, including insects, lice, fleas, moths, true bugs, and mites (Mans, 2011). Many of these lineages have experienced convergent evolution, resulting in numerous physiological and morphological adaptations that are common among hematophagous arthropods. These include specialized mouthparts for piercing skin and salivary components to combat vertebrate hemostatic and immune systems (Lehane, 2005; Mans, 2011). They have also converged on behavioral strategies for finding hosts, such as thermotaxis toward “warm-blooded” vertebrates.

Some hematophagous arthropods are permanent ectoparasites (e.g. lice), typically spending their entire lives on the surface of a single host. For these species, acquiring a blood meal is simply a matter of piercing skin and feasting on vasculature. In contrast, blood-feeding is a more sophisticated behavioral challenge for non-ectoparasitic hematophages—those that do not live on the surface of hosts. These species, such as tsetse flies and mosquitoes, must do the hard work of locating hosts in their environment. As a result, they have evolved powerful sensory-motor systems to guide them towards hosts. And because vertebrate hosts such as birds and mammals share many physical characteristics, sensory and behavioral biology in hematophagous arthropods has undergone significant convergent evolution (Lehane, 2005). Many of these species display strong sensitivity and responses to host cues such as body odors, carbon dioxide (CO_2), and visual contrast (Lehane, 2005).

Thermotaxis towards body heat is a potent component of host-seeking for many hematophagous arthropods (Lazzari, 2009). Orientation towards thermal stimuli approximating host temperatures has long been noted in bedbugs (Reinhardt and Siva-Jothy, 2007; Rivnai, 1931), ticks (Lees, 1948), and other blood-sucking arthropods (Lehane, 2005). This behavior has been well studied in the reduviid bugs *Triatoma infestans* and *Rhodnius prolixus*, which are major vectors of Chagas disease (Lazzari, 2009). These insects can home in on warmed objects from a distance of many centimeters (Fresquet and Lazzari, 2011) by sensing radiant heat (Lazzari and Núñez, 1989). It is thought that their thermotaxis is driven by evaluating differences in heat-detection between both antennae. Unilateral antennal ablations result in systematic navigation deviations biased towards the side of the intact antenna. (Flores and Lazzari, 1996). Using their antennae, *R. prolixus* can detect minute amounts of thermal contrast, allowing them to target their biting precisely to a linear heat source mimicking a blood vessel (Ferreira et al., 2007).

1.3 Heat is an important cue for host-seeking female *Aedes aegypti* mosquitoes

As is the case for all small ectothermic arthropods, mosquitoes must be capable of sensing temperature to avoid areas of excessive heat or cold. For instance, the yellow fever mosquito, *Aedes aegypti*, has evolved to survive at tropical and subtropical latitudes (Eisen et al., 2014). Developmental rates of *Ae. aegypti* eggs, larvae and pupae are all maximized between ~15 and ~30°C, and

adults can only survive within thermal environments ~15-38°C (Eisen et al., 2014). Flight performance in this species is dramatically impaired below 15°C and above 32°C (Rowley and Graham, 1968). A crude thermal gradient based on an assay used for *D. melanogaster* (Sayeed and Benzer, 1996), showed that adult *Anopheles stephensi* mosquitoes avoid cold and hot, accumulating at air temperatures of ~24-30°C (Blanford et al., 2009).

However, like other hematophagous arthropods, mosquitoes display an alternate mode of thermotaxis during pursuit of a blood meal. For many mosquito species such as *Ae. aegypti*, obtaining a vertebrate blood meal is required by females for egg production (Clements, 1999). One important component of host-seeking in female mosquitoes is attraction to the body heat of hosts (Clements, 1999).

1.3.1 The body and skin temperatures of *Aedes aegypti* hosts

Ae. aegypti are opportunistic blood-feeders that can feed from a variety of vertebrate hosts (Clements, 1999). Serological or DNA analysis of blood-meal content can be used to determine the origin of blood meals in wild-caught mosquitoes. Studies from geographically distinct populations of *Ae. aegypti* showed that this species feeds on a diverse set of hosts including humans, dogs, and chickens (Barrera et al., 2012; Ponlawat and Harrington, 2005; Tandon and Ray, 2000). In a two choice-olfactometer, even human-preferring strains of *Ae. aegypti* showed appreciable levels of attraction to a chicken, when offered a choice between the bird and a human (Gouck, 1972).

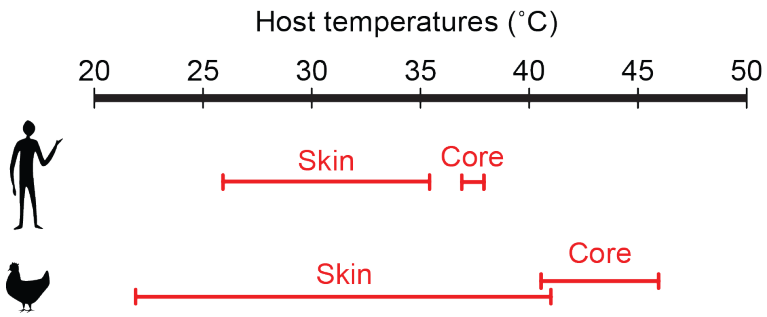

Figure 1.4 Body temperature of *Ae. aegypti* hosts. Typical skin and core temperature ranges of two examples of hosts: humans and chickens (Richards, 1971; Yao et al., 2008).

Ae. aegypti hosts present a diversity of temperatures ranging between ~22-46°C (**Fig 1.4**). Humans, for instance, have core body temperatures of ~37°C, while chickens have core body temperatures ranging between ~41-43°C (Richards, 1971). Birds have even higher body temperatures when active in a warm environment, commonly reaching 43-46°C (Hill et al., 2004). Amazingly, one study noted a body temperature of 47.7°C in the white-crowned sparrow, *Zonotrichia leucophrys* (Prinzinger et al., 1991). The core body temperatures of birds are presumably the highest temperature stimulus that a mosquito would encounter during its interaction with a host. Once feeding, mosquitoes can expect blood temperatures to approximate the body temperature of hosts. Indeed, *Ae. aegypti* feed more quickly from blood warmed to 36-40°C than from cooler blood (Cosgrove and Wood, 1995). Prior to blood-feeding, mosquitoes will experience a wide range of temperatures during approach towards a host, and skin surface temperatures vary widely between hosts and between body parts (Hill et al., 2004). Maximal skin temperature of humans is ~35.5°C (Yao et al., 2008), but birds can present skin temperatures of 41°C (Richards, 1971). At low ambient temperatures (~20°C), skin temperatures at bodily extremities can be as low as ~26°C in humans (Yao et al., 2008), and ~22°C in birds (Richards, 1971).

1.3.2 Heat synergizes with other cues during mosquito host-seeking

"On more than one occasion when mosquitoes have been troublesome at tea-time I have noticed that they seemed to be fond of hovering in the neighbourhood of the tea-pot, being attracted apparently by the heat…" (Howlett, 1910)

The excerpt above come from the earliest account of mosquito attraction to warmth, written by a British entomologist working in India (Howlett, 1910). Howletts's anecdotal observation led him to conduct a simple experiment. When he presented female *Stegomyia scutellaris* or *Culex fatigans* mosquitoes with a test-tube filled with warm water, he noted a "most interesting" effect:

"As soon as the hot air from the tube reached the insects they became restless and began to congregate on the part of the net nearest the tube; as this was brought nearer they became more excited, stabbing with their proboscides through the meshes of the net and displaying the utmost eagerness in their fruitless efforts to puncture the glass... but no reaction at all was obtained when the males were tried with the hot tube…" (Howlett, 1910)

A similar anecdotal account of thermotropism was soon put forth for *Anopheles punctipennis* (Marchand, 1918). These initial reports gave way to more rigorous experimentation to examine the role of thermotaxis in mosquito host-seeking. Early field studies found that multiple mosquito species were attracted to warmed mannequins placed in the Canadian forest (Brown, 1951). This natural thermotropism informs the design of many modern commercial

mosquito traps, because capture rates can increase substantially when heat stimuli are incorporated into trap devices (Kline and Lemire, 1995).

When researchers began modeling host-seeking in the laboratory, it became clear that many species of mosquitoes are attracted to heat (Clements, 1999). Moreover, many early experiments suggested that an increase in ambient CO_2 served to activate mosquito thermotropism in *Ae. aegypti* mosquitoes (Burgess, 1959; Gillies, 1980; Kellogg, 1962; Khan et al., 1966). Other studies have gone on to shown that this is true for the malaria mosquitoes *Anopheles gambiae* and *Anopheles stephensi*, which showed robust heat-seeking to 34-35°C targets only when thermal stimulation was coupled with a pulse of CO_2 (Kröber et al., 2010; Maekawa et al., 2011). A recent publication showed that CO_2 enhanced *Ae. aegypti* landings on a 37°C stimulus in the context of a wind-tunnel (van Breugel et al., 2015).

Mosquito host-seeking is a multimodal behavior—it involves integration of diverse sensory information (Cardé, 2015). Just as elevated ambient CO_2 (Majeed et al., 2014) activated attraction to warmth, it also enhanced attraction to odor (Dekker et al., 2005) and visual targets (van Breugel et al., 2015). Interestingly, thermal cues are dispensable if sufficient alternate cues are present. Host odor and CO_2 could drive feeding from room-temperature blood in an artificial membrane (McMeniman et al., 2014). On the other hand, host odor in the absence of CO_2 elicited feeding from an artificial membrane if the blood is warmed (McMeniman et al., 2014). These results suggest a model in which mosquitoes utilize an array of sensory cues to locate hosts, and that integration

of multiple modalities improves host-seeking. For example, the presence of heat cues enhanced attraction to both visual and olfactory stimuli in wind-tunnels (Spitzen et al., 2013; van Breugel et al., 2015).

1.4 Thermosensitive sensilla of *Aedes aegypti* and other arthropods

Terrestrial arthropod bodies are covered in hard cuticle that protects their internal organs from damage or desiccation by the environment. However, this outer shell can obstruct communication between the organism and its surroundings. To sense external stimuli, arthropod cuticle has specialized external sensory organs called sensilla (Hallberg and Hansson, 1999). Sensilla come in a variety of morphological types, and each houses one or more neurons tuned to detect distinct stimuli. A typical arthropod will have chemosensory, mechanosensory, hygrosensory (humidity-sensing), and thermosensory sensilla.

Thermosensitive sensilla have been documented in numerous arthropods, including mosquitoes, butterflies, cockroaches, stick insects, locusts, moths, ticks, and spiders (Gingl and Tichy, 2001; Zeiner and Tichy, 2000). Study of these organs is challenging because there are usually far fewer thermosensory sensilla than chemosensory sensilla (Altner and Loftus, 1985). Furthermore, thermosensory neurons can be found in a variety of sensilla types, often in combination with hygrosensitive or chemosensitive neurons, making it very difficult to identify these cells using morphology (Altner and Loftus, 1985). Arthropod thermosensation can also take place in tissues other than sensilla,

such as the thermosensory cells found in the brain and aristae of *D. melanogaster* (Gallio et al., 2011; Hamada et al., 2008).

Most of the known arthropod thermoreceptors feature “cold cells”—neurons whose activity increases with cooling and decreases with warming (Zopf et al., 2014a). However, *Ae. aegypti* mosquitoes have sensilla that house a cold cell and a warm cell with complementary receptive-fields (Davis and Sokolove, 1975). Integration of the antagonistic activity of these cells may provide the nervous system with more dependable detection of thermal fluctuations (Liu et al., 2015). Similar sensillar organizations exhibiting antagonistic thermoreceptors have been identified in the antennae of the cave beetle *Speophyes lucidulus* (Loftus and Corbière-Tichané, 1981) and cockroaches (Zeiner and Tichy, 2000), and the forelegs of the tick, *Amblyomma variegatum* (Hess and Loftus, 1984).

In *Ae. aegypti*, such thermoreceptors are housed in a pair of peg-in-pit sensilla located at the distal tip of the antennae (McIver, 1973) (Davis and Sokolove, 1975). The activity of these neurons correlates not only with instantaneous absolute temperature, but also with fluctuations in temperature (ie. warming or cooling) (Gingl et al., 2005). Heat-sensitive thermoreceptors have also been found in homologous antennal sensilla of the malaria mosquito *An. gambiae* (Wang et al., 2009), and morphologically similar sensilla have been identified at the antennal tips of *Culex pipiens* mosquitoes (McIver, 1973). The complete set of thermoreceptors in the mosquito has not been characterized, but morphological evidence suggests that there are additional thermoreceptors near

both the tip and base of the antennae in *Ae. aegypti* and *An. gambiae* (Ismail, 1964; McIver, 1973; Pitts and Zwiebel, 2006).

Some insects have evolved organs that can sense infrared radiation (Campbell et al., 2002), but most thermoreceptive sensilla studied in arthropods respond primarily to convective heat exchange. The thermosensory neurons in these organs are typically separated by <5 μm from the external environment, and thus rapidly equilibrate to the temperature of the surrounding ambient air (Gingl and Tichy, 2001). Arthropods capable of sensing infrared radiation, such as the aforementioned forest fire-seeking beetle *M. acuminate*, have evolved pit organs similar to those found in heat-seeking snakes (Campbell et al., 2002). However, hematophagous insects such as *R. prolixus* sense and thermotax toward radiant heat apparently without the use of specialized infrared detecting organs (Zopf et al., 2014b). The thermoreceptors characterized in *Ae. aegypti* are sensitive to radiant heat, but do not respond to power spectra corresponding to nearby hosts (Gingl et al., 2005). This supports the conclusion that mosquitoes are primarily evaluating convective heat transfer during host-seeking.

1.5 Candidate molecular thermosensors in mosquitoes

To sense temperature in the environment, animals must transduce the energy of thermal fluctuations into neural activity. As we have discussed, cellular molecules are exquisitely sensitive to temperature. The properties of a cell's plasma membrane, the conformational shape of proteins and nucleic acids, and the concentration or activity of signaling molecules can all be greatly affected by

temperature (Digel, 2011). While this may present a problem for most molecules in an organism, which must retain functionality despite temperature fluctuations, it is the operational basis of a molecular thermosensor. In this manner, a change in temperature can drive a molecular process that has evolved to encode a thermosensory impulse in the nervous system.

The inherent thermosensitive nature of biochemistry has resulted in the evolution of a diversity of thermosensation mechanisms. For example, bacterial thermosensors can be formed from a variety of biomolecules, including DNA, RNA, proteins, and lipids (Klinkert and Narberhaus, 2009). In animals, sensory transduction of temperature often relies on thermosensitive ion channels, but other mechanisms have evolved as well. For instance, the thermosensitive AFD neurons of *C. elegans* rely on a molecular signaling network to transduce thermal stimuli (Ramot et al., 2008a). In this scheme, temperature-induced changes in the concentration of cyclic guanosine monophosphate regulate the ion current of channels in the cell (Ramot et al., 2008a).

Because temperature is so intertwined with basic biochemistry, it is possibly one of the most ancient sensory modalities, and many of the genes involved in thermosensation are highly conserved in the animal kingdom. For example, the transient receptor potential (TRP) superfamily of ion channels is associated with thermosensation in divergent lineages including nematodes, insects, fish, birds, reptiles, amphibians, and mammals (Gracheva and Bagriantsev, 2015; Venkatachalam et al., 2014). The genes underlying the specialized host-seeking thermotaxis of the mosquito are unknown, but it is

possible that these animals use evolutionarily conserved thermosensors to accomplish this derived behavior.

1.5.1 *AaegTRPA1* is a candidate mosquito heat-seeking thermosensor

Numerous genes have been implicated in insect thermosensation, most of which are in the TRP channel family (Venkatachalam et al., 2014). The most well-studied insect thermosensor is the TRP ankryin 1 (TRPA1) channel. While this ion channel has been proposed to be a cold-sensor in mammals (Palkar et al., 2015) and in *C. elegans* (Chatzigeorgiou et al., 2010), it is a heat-sensor in *D. melanogaster*, with an activation threshold of ~25-29°C (Viswanath et al., 2003). Since then, it has been found to be a heat sensor in multiple organisms, including other insects, birds, reptiles, and amphibians (Laursen et al., 2014). *TRPA1* is expressed in internal thermosensory cells of the adult *D. melanogaster* brain (Hamada et al., 2008), and is required in both larvae and adult flies for avoidance of high temperatures in thermal gradient assays (Hamada et al., 2008; Rosenzweig et al., 2005). TRPA1 is also implicated in thermoregulation behavior of the red flour beetle, *Tribolium castaneum* (Kim et al., 2015). Additionally, TRPA1 has been shown to be heat-sensitive in the silkworm *Bombix mori* (Sato et al., 2014) and the moth *Helicoverpa armigera* (Wei et al., 2015). Remarkably, heat-sensitive TRPA1 orthologs are also highly expressed in the infrared radiation-detecting organs of the pit-bearing snakes discussed earlier (Gracheva et al., 2010).

TRPA1 encodes a nonselective cation channel with six transmembrane domains, a cytoplasmic C terminus, and a long cytoplasmic N terminus (Laursen et al., 2014). This N-terminal domain includes a distinctively large number of ankyrin repeats, which are involved in protein-protein interactions. There exist at least four isoforms of *DmelTRPA1*, which differ in their thermal sensitivity (Kang et al., 2012; Zhong et al., 2012). It should be noted that the nomenclature of these isoforms is not standardized, and this discussion is based on nomenclature in (Zhong et al., 2012). Isoform TRPA1-A, which is specifically expressed in thermosensory neurons, is highly thermosensitive with a Q_{10} of ~116 (Kang et al., 2012). Isoform TRPA1-D is moderately thermosensitive, with a Q_{10} of ~9 (Kang et al., 2012), while isoforms TRPA1-B and TRPA1-C showed no significant thermosensitivity in heterologous expression assays (Zhong et al., 2012). Surprisingly however, expression of TRPA1-C was able to restore thermosensitivity in *TRPA1* null-mutant flies, though the mechanism underlying this result is unknown (Zhong et al., 2012).

The thermosensitivity of these *DmelTRPA1* isoforms depends on at least two structural features: the N-terminus (Kang et al., 2012) and a 37 amino acid domain bridging the ankyrin repeats and the transmembrane domains (Zhong et al., 2012). The ankyrin repeats themselves also regulate the thermosensitivity of TRPA1. Chimeric transposition of ankyrin repeat domains from fly or rattlesnake TRPA1 conferred heat-sensitivity to human TRPA1 (Cordero-Morales et al., 2011). Additionally, the putative pore region of *DmelTRPA1* has been implicated in determining heat-sensitivity of the channel (Wang et al., 2012). Together,

these results support a model in which specific structural modules adjust the thermosensitivity of the channel. This principle is also seen in the heat-sensitive TRPV1 channel of bats (Gracheva et al., 2011). The heat-sensitive pit organs of vampire bats express a novel isoform of TRPV1 with a truncated c-terminal domain (Gracheva et al., 2011). This structural alteration lowers the activation threshold of the channel from ~40 to ~30°C (Gracheva et al., 2011).

TRPA1 is also known to be a chemosensor of reactive electrophiles such as allyl isothiocyanate, which is the pungent compound found in wasabi (Jordt et al., 2004). This chemosensory function of TRPA1 evolved ~500 million years ago, and is present among mammals and multiple insects such as *D. melanogaster*, the malaria mosquito *An. gambiae*, and the moth *H. armigera* (Kang et al., 2010; Wei et al., 2015). In *D.* melanogaster, expression of *TRPA1* in gustatory chemosensors is required for avoiding ingestion of these noxious electrophiles (Kang et al., 2010). Other organism, such as zebrafish and nematodes, have lost TRPA1 electrophile-sensitivity (Kang et al., 2010). Much like its thermosensitivity, the chemosensitivity of TRPA1 depends on its structure. Particularly important is a set of cysteine residues located in the N-terminal region of the channel (Kang et al., 2010; Macpherson et al., 2007).

The diverse evolutionary trajectories of TRP channels via changes in modular structural features suggest that these genes can take on new roles as animals adapt to novel environments and behavioral niches (Gracheva and Bagriantsev, 2015; Peng et al., 2015). For instance, the honeybee, *Apis mellifera*, appears to have lost *TRPA1* during evolution, but has evolved a

Hymenoptera-specific TRPA channel (*AmHsTRPA*) which is heat-activated (Kohno et al., 2010). Perhaps this newly derived thermosensor arose to accommodate honey bee foraging thermotaxis (Hammer et al., 2009) or to support eusociality, which depends on maintaining brood nest temperatures at ~35°C (Szopek et al., 2013; Tautz et al., 2003). This finding points to the malleability of TRP channels during evolution of novel thermal behaviors.

Could mosquitoes be using TRP channels to facilitate thermotaxis towards warm-blooded prey, much like rattlesnakes and vampire bats (Gracheva et al., 2010; 2011)? It is known that TRPA1 in the malaria mosquito, *An. gambiae*, activates in response to warming to ~40°C (Hamada et al., 2008; Wang et al., 2009). Furthermore, this gene is expressed in neurons associated with thermosensitive sensilla of *An. gambiae* (Wang et al., 2009). The *Ae. aegypti* ortholog of TRPA1 RNA is expressed in numerous tissues, including the antennae, brain, proboscis, and legs (Matthews et al., 2015). Together, these data suggest that *AaegTRPA1* may play an important role in *Ae. aegypti* heat-seeking behavior.

1.5.2 *AaegGr19* is candidate mosquito heat-seeking thermosensor

A second candidate insect thermosensor that may be used in mosquito heat-seeking behavior is the *D. melanogaster* gustatory receptor paralog *DmelGr28b.* While *DmelTRPA1* is known to be an internal thermosensor required for avoidance of heat on the timescale of ~30 minutes, (Hamada et al., 2008), *DmelGr28b* is expressed in peripheral neurons, and is required for rapid

avoidance of heat in adult flies (Ni et al., 2013). The (D) isoform of *DmelGr28b* is expressed in three warmth-activated cells of the fly arista—a feathery bristle protruding from the antenna (Ni et al., 2013; Thorne and Amrein, 2008). Ectopic expression of this transcript confers heat-sensitivity to taste neurons and motor neurons in the fly, suggesting that this gene may encode a primary thermosensor (Ni et al., 2013).

Gustatory receptors (GRs) are conventionally involved in chemosensation, but *DmelGr28b* and its orthologs are associated with atypical functions. For instance, the *C. elegans* homolog of *DmelGr28b, LITE-1*, is required for phototransduction in the ASJ neuron, which mediates avoidance of UV-A light in this organism (Liu et al., 2010). In *D. melanogaster* larvae, this gene is similarly required for phototransduction in the class IV dendritic arborization neurons that tile the body wall, and these neurons are required for avoidance of high intensity light stimuli (Xiang et al., 2010). Thus, while this gene is related to a family of chemosensors, it appears to have properties that allow it to mediate sensation of physical stimuli such as light and heat.

Examination of chemoreceptor genes among *Drosophila* species reveals that *Gr28b* is extremely highly conserved (McBride et al., 2007). The signature of positive selection at this gene is comparable to genes encoding sweet and CO_2 receptors, suggesting that *Gr28b* and its orthologs serve important biological functions. Furthermore, this gene presents an expression pattern strikingly atypical of most gustatory receptors in *D. melanogaster* (Thorne and Amrein, 2008). *AaegGr19* is the clear ortholog of *DmelGr28b* (Ni et al., 2013), and is

expressed in numerous tissues including antennae, brain, proboscis, ovipositor, palps, and legs (Matthews et al., 2015). Together, these data indicate that *AaegGr19* may serve as a mosquito thermoreceptor important for host-seeking thermotaxis.

1.6 Investigating the thermotactic and molecular basis of mosquito heat-seeking

The host-seeking of *Ae. aegypti* and other mosquito species results in the transmission of devastating human diseases (Bhatt et al., 2013), but the molecules and sensory systems underlying these behaviors are only now beginning to be understood (Cardé, 2015). Functional neurogenetics has proven to be a powerful approach for unraveling the mechanisms of behavior in model organisms such as *D. melanogaster* (Benzer, 1971; Vosshall, 2007). Recent advances in genome-editing technologies provide exciting opportunities to identify the molecular underpinnings of complex behaviors in organisms traditionally considered to be genetically intractable, such as mosquitoes (Barrangou, 2014).

This thesis investigates the behavioral strategies and molecular basis of the remarkable heat-seeking activity of female *Ae. aegypti* mosquitoes. We began by developing a high-throughput quantitative assay to model the phenomenon of mosquito heat-seeking in the lab (**Chapter 2**). Using this assay, we were able to elucidate the fundamental behavioral rules of mosquito host-seeking thermotaxis (**Chapter 3**). Next, we generated *AaegTRPA1*$^{-/-}$ mutant

mosquitoes, and characterized conserved roles for this gene in thermosensation and chemosensation (**Chapter 4**). Finally, we characterized the heat-seeking behavior of *AaegTRPA1*$^{-/-}$ mutants, and showed that this gene, and not *AaegGr19*, is required for tuning mosquito thermotaxis away from stimuli that exceed host body temperatures (**Chapter 5**). We conclude with a discussion of our findings and future questions regarding the neurogenetic and thermotactic mechanisms of mosquito heat-seeking (**Chapter 6**).

CHAPTER 2

A laboratory model of mosquito heat-seeking behavior

To understand the behavioral and molecular mechanisms of mosquito heat-seeking, we established an assay to model this behavior in the laboratory. Heat-seeking is a robust innate behavior of female *Ae. aegypti*, and can be easily observed in the laboratory using a rudimentary demonstration. By simply exhaling into a cage containing female mosquitoes, a large proportion of the mosquitoes will be activated, resulting in a frenzy of flight. If at this point a warm bottle of water is placed adjacent to the cage, female mosquitoes will gather at the area nearest the stimulus within moments and probe eagerly through the mesh of the cage.

Our objective was to create a reproducible, robust, and quantitative experimental assay to measure this heat-seeking behavior. We set out to design an experimental arena in which: 1) a group of mosquitoes can be activated by CO_2 to enter a host-seeking state; 2) diverse thermal stimuli can be presented with temporal and spatial precision; and 3) mosquito landings on thermal stimuli can be monitored and quantified.

2.1 A high-throughput quantitative heat-seeking assay

The heat-seeking assay (**Figure 2.1A**) consists of a cubic arena (30 x 30 x 30 cm) in which a group of mosquitoes can be exposed to pulses of CO_2 and presented with thermal stimuli. A Peltier device embedded in one wall of the arena can be set to a wide range of temperatures, and is used to present thermal

stimuli in a temporally precise manner. A camera mounted outside the arena can image the Peltier surface and the surrounding region of the arena with sufficient resolution to distinguish individual mosquitoes (**Figure 2.1B**).

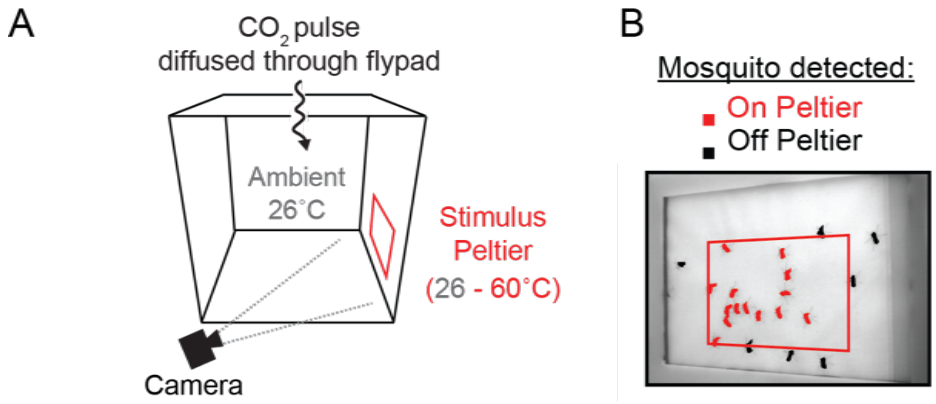

Figure 2.1 A heat-seeking assay to measure mosquito thermotaxis behavior in the laboratory. (A) Schematic of the heat-seeking assay (30 x 30 x 30 cm). Mosquitoes are introduced into a cubic arena where they can be exposed to CO_2 pulses and thermal stimuli. A Peltier device embedded in one wall of the arena can be driven to a wide range of temperatures above ambient (26˚C in most experiments). A camera mounted outside the arena monitors mosquito occupancy in the Peltier region. **(B)** Representative experimental image. Individual mosquitoes detected by a custom MATLAB script are shown in black if they are located in the general Peltier region, and in red if they are detected in the Peltier area (red box).

All aspects of the assay are controlled via an automated computerized system. Once mosquitoes are manually introduced into the assay using a puff of air, a custom MATLAB script is run, executing a pre-programed sequence of stimuli during the course of an experimental trial. This automated system triggers CO_2 pulses, commands Peltier temperature, records Peltier temperature, and triggers image acquisition from the camera monitoring mosquito behavior. The dataset generated by the heat-seeking assay is a series of images acquired at 1 Hz throughout an experimental trial. These images show the Peltier and surrounding region. Using a custom MATLAB script, each image can then be

analyzed to detect individual mosquitoes and record their location within the Peltier region (**Figure 2.1A**).

2.2 Female *Ae. aegypti* heat-seek when activated by CO_2

Our first experiment was to model and quantify the basic phenomenon of mosquito heat-seeking. **Figure 2.2A** shows that female *Ae. aegypti* accumulated on a warmed Peltier stimulus when activated by CO_2. In this experiment, 25 female mosquitoes were introduced into the arena and allowed to acclimate for 5 minutes before stimulus presentation. During this acclimation period, few mosquitoes were detected in the Peltier region. After acclimation, mosquitoes were exposed to a 20-second CO_2 pulse and the Peltier was warmed to 37°C for 5 minutes. We observed that female mosquitoes rapidly accumulated in the Peltier region and that mosquito occupancy remained elevated and stable throughout the stimulus period. We also noted that the animals quickly left the Peltier region when the Peltier was cooled back to ambient temperature (26°C) at

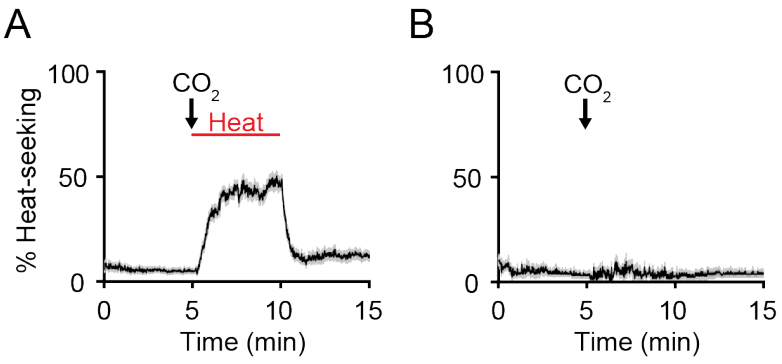

Figure 2.2 Female *Ae. aegypti* heat-seek when activated by CO_2. (A) Percent animals in the Peltier region during a 15-minute heat-seeking trial. n = 15 trials. Black arrow indicates onset of a 20 second pulse of CO_2. The Peltier is kept at ambient temperature (26°C) except during the 5-minute stimulus period (red bar), when it is driven to 37°C. **(B)** Same as in **(A)**, but in these trials the Peltier is kept at ambient temperature and no heat stimulus is presented. n = 6 trials with 20-25 mosquitoes.

the conclusion of the stimulus period. In trials where mosquitoes were exposed to a CO_2 pulse, but no heat stimulus was provided, we saw no accumulation of animals in the Peltier region (**Fig 2.2B, Fig 2.5A**). Despite the lack of heat-seeking behavior, we did observe activation and dramatically increased flight behavior in response to the CO_2 pulse (data not shown), as is seen in other assays (McMeniman et al., 2014). This confirms that the thermal stimulus, not any possible visual cues, attracts mosquitoes to the Peltier region.

To test the importance of CO_2 activation in heat-seeking behavior, we conducted trials without a CO_2 pulse (**Fig 2.3A, Fig 2.5A**). Despite the presence of a heat-stimulus, mosquitoes did not accumulate in the Peltier region when the CO_2 pulse was omitted. We also tested *Ae. aegypti* mutants lacking functional *AaegGr3*, a gustatory receptor required for CO_2 detection (McMeniman et al., 2014) (**Fig 2.3B,C, Fig 2.5B**). These mutants lack normal electrophysiological and behavioral responses to increases in ambient CO_2 (McMeniman et al., 2014).

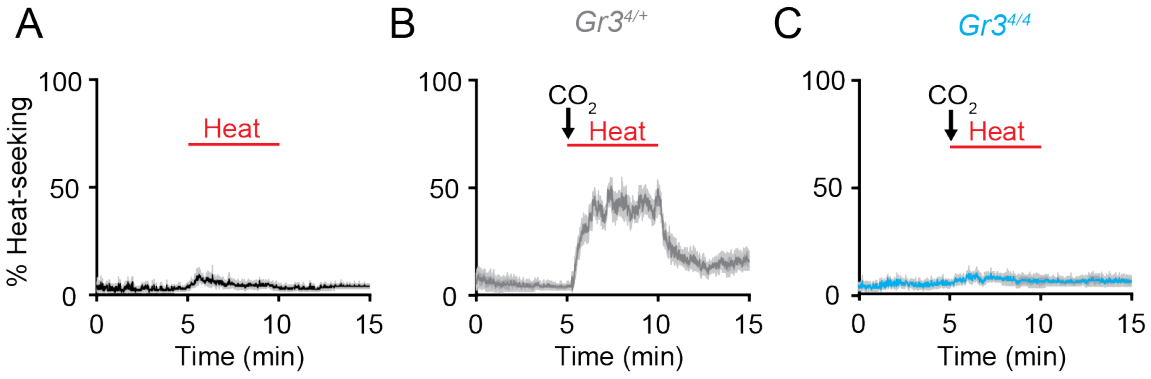

Figure 2.3 Detection of increased ambient CO_2 activates female *Ae. aegypti* to heat-seek. (A) Same as in (**Fig 2A**), but in these trials there is no CO_2 pulse. n = 6 trials. **(B, C)** Heat-seeking behavior of heterozygous **(B)** and homozygous **(C)** *AaegGr3* mutants. n = 6 trials with 20-25 mosquitoes.

While heterozygous *AaegGr3* mutants showed normal heat-seeking when exposed to a CO_2 pulse and a heat stimulus, homozygous *AaegGr3* mutants did not accumulate in the Peltier region in response to these stimuli. This confirms that CO_2 activates mosquito heat-seeking in these experiments, and excludes any possible artifacts due to airflow changes.

2.3 Male *Ae. aegypti* do not heat-seek

Male *Ae. aegypti* do not blood-feed, and one may expect them to lack any host-seeking behaviors. However, male mosquitoes show robust activation and increased flight behavior in response to CO_2 (Matthews et al., 2015). Male *Ae. aegypti* also appear to be attracted to human hosts (Pilitt, 1973). The host-seeking behavior of male mosquitoes may be adaptive as a strategy for finding females, which are presumably at higher densities near hosts. We examined male mosquito responses to thermal stimuli in our assay. While we observed activation and dramatically increased flight behavior in response to the CO_2 pulse (data not shown), males did not accumulate in the Peltier region when presented with a heat stimulus (**Fig 2.4, Fig 2.5A**). This clearly shows that heat-seeking in *Ae. aegypti* is a sexually dimorphic behavior.

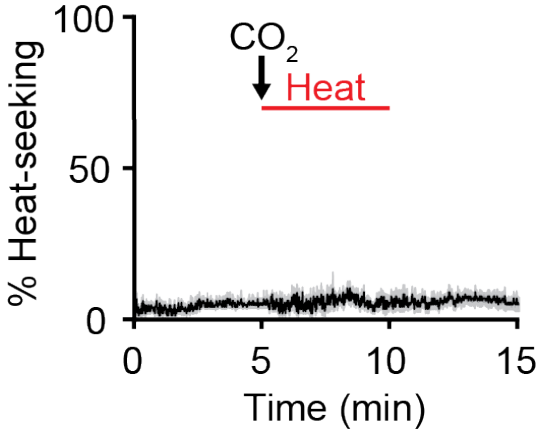

Figure 2.4 Male *Ae. aegypti* do not heat-seek. Heat-seeking behavior of male *Ae. aegypti.* n = 6 trials with 20-25 mosquitoes.

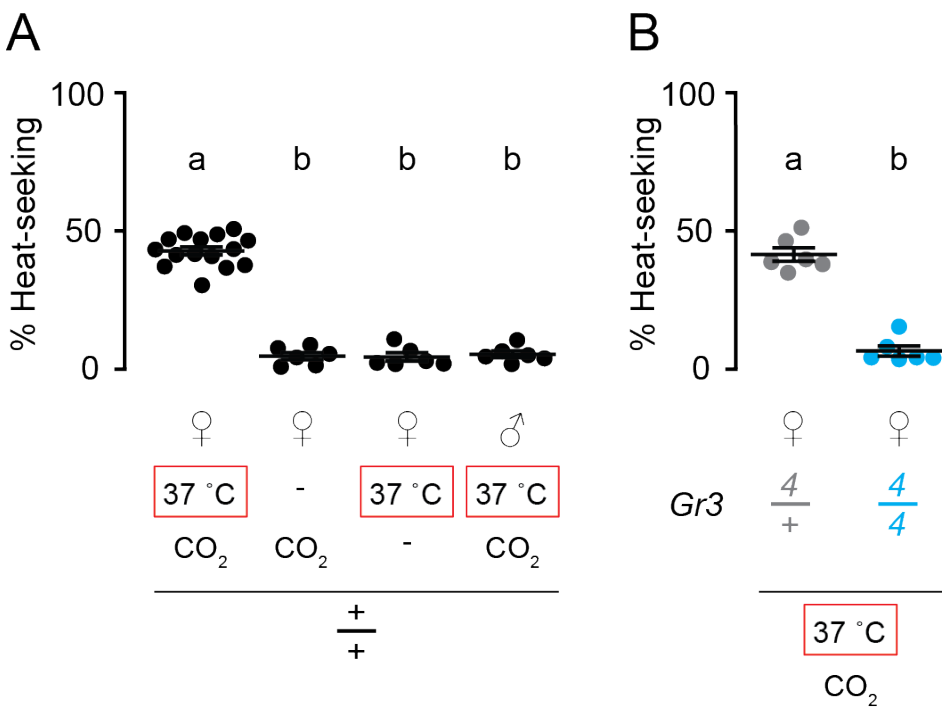

Figure 2.5 Quantification of heat-seeking behavior. Quantification of heat-seeking during minutes 7-10 of assay for different stimulus regimes **(A)** and different mosquito genotypes **(B)**. n = 6-15 trials with 20-25 mosquitoes. Each replicate is indicated by a dot, and mean ± SEM as bars. Variation among both stimulus regimes and mosquito genotypes was significant (one-way ANOVA, $p < 0.0001$ for both **(A)** and **(B)**. Data labeled with different letters are significantly different (Tukey's HSD test comparing all pairs of means [$p < 0.001$]).

2.4 Female *Ae. aegypti* repeatedly heat-seek to 40˚C stimuli

Using the heat-seeking assay, we were able to model robust host-seeking thermotaxis by mosquitoes. It is remarkable that female mosquitoes will accumulate persistently for minutes at a time at a heated stimulus, despite never being rewarded with their final objective of attaining a blood meal. Our next experiment investigated the dynamics of *Ae. aegypti* heat-seeking over longer periods of time. In these trials, 45-50 mosquitoes were introduced into the heat-

seeking assay and exposed to 12 repeated 40°C stimuli, each separated by a 9-minute inter-stimulus period (**Fig 2.6A**).

We found that mosquitoes will repeatedly accumulate on a thermal stimulus over the course of more than 2.5 hours, with no evidence of habituation (**Fig 2.6B, C**). In analyzing these data, we only scored mosquitoes occupying the Peltier area (**Fig 2.1B**, red box). This was done to exclude the effect of any mosquitoes remaining in the vicinity of the Peltier due to prior stimuli.

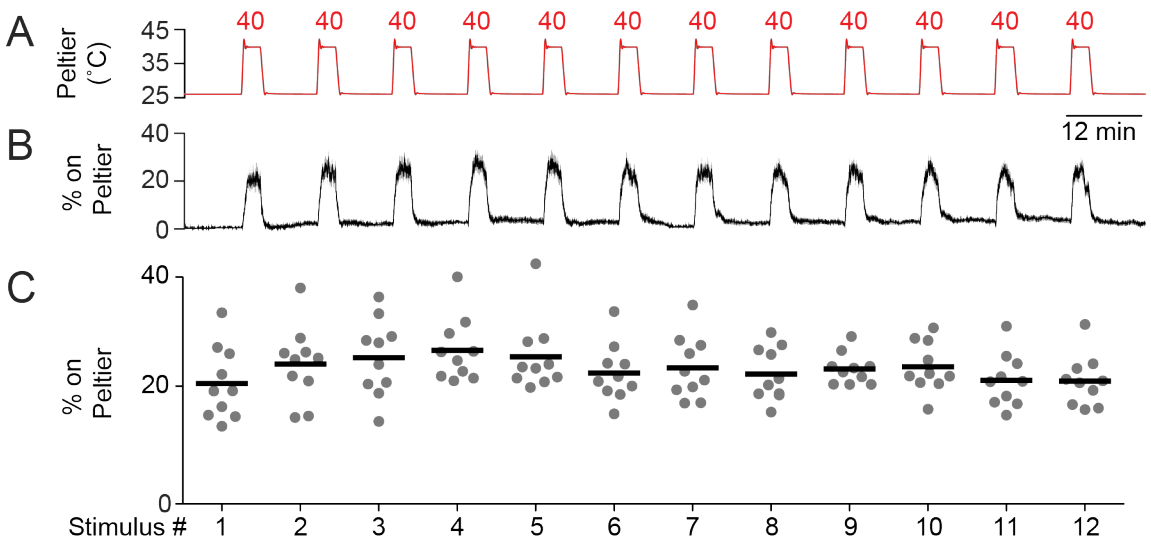

Figure 2.6 Female *Ae. aegypti* robustly heat-seek to repeated 40˚C stimuli. (A) Peltier temperature measured by thermocouple (mean in red, SEM. in gray). **(B)** Percent of mosquitoes on Peltier (mean in black, SEM in gray). n = 10 trials with 45-50 mosquitoes. Note that variance in traces in **(A)** and **(B)** is low, making SEM difficult to see. **C**, Percent mosquitoes on Peltier during seconds 90-180 of each stimulus period in (**A,B**). Each replicate is indicated by a dot, and the mean by a line. There is no significant difference ($p > 0.05$) in Peltier occupancy between the first and last stimulus (repeated measures one-way ANOVA with Bonferroni correction).

CHAPTER 3

The thermotactic strategies of host-seeking mosquitoes

Attraction to warmth has been documented in multiple mosquito species, but the thermotactic strategies underlying this behavior are largely unknown. Body heat from warm-blooded animals can serve as an effective signal for host-seeking mosquitoes, but how exactly does a female mosquito locate a warm host? A challenging aspect of this behavioral task is that mosquitoes have diverse hosts, with a wide range of core body temperatures. An opportunistic mosquito such as *Ae. aegypti* should be flexible in selecting thermotactic targets. On the other hand, as she explores her environment, a mosquito will likely encounter abiotic thermal stimuli that may act as strong distractors. Therefore, discrimination between thermal stimuli may be crucial for optimizing her search for a host. Additionally, variation in ambient air temperature can add further complexity to this task by modifying the thermal contrast between a host and the background temperature.

We set out to use our heat-seeking assay to test mosquito responses to diverse thermal stimuli. The Peltier device allows us to present a wide range of targets for heat-seeking mosquitoes. We can also alter the background temperature of the assay to test the importance of thermal contrast between stimulus and ambient temperature.

3.1 *Ae. aegypti* are maximally attracted to thermal stimuli resembling host body temperatures

Ae. aegypti hosts have core body temperatures up to ~46°C (Richards, 1971) in the case of chickens. We tested mosquito heat-seeking to stimuli ranging from 26°C (ambient) to 60°C (greater than possible host temperature) (**Fig 3.1A**). We found that mosquito heat-seeking depended on the temperature of the stimulus (**Fig 3.1B, C**). As in earlier experiments, mosquitoes were not attracted to the Peltier when it was kept at ambient temperature, but accumulated

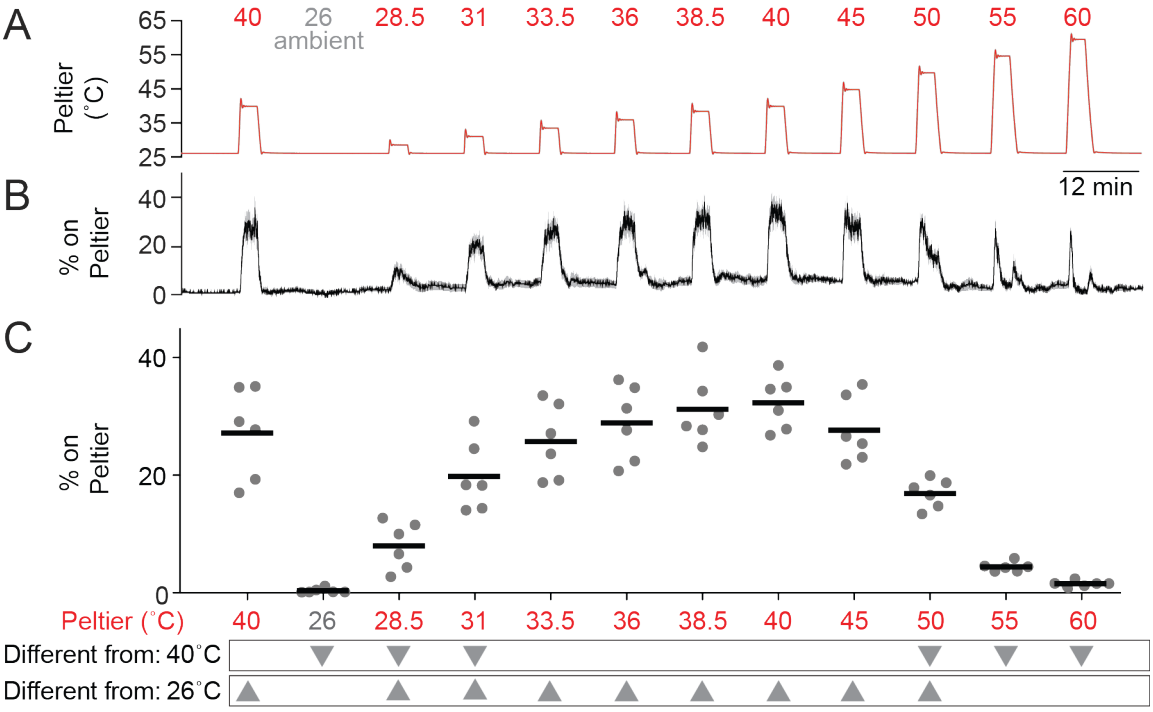

Figure 3.1 Mosquitoes thermotax to stimuli approximating host body temperature. **(A)** Peltier temperature measured by thermocouple (mean in red, SEM. in gray). **(B)** Percent of mosquitoes on Peltier (mean in black, SEM in gray). n = 6 trials with 45-50 mosquitoes. Note that variance in traces in **(A)** and **(B)** is low, making SEM difficult to see. **(C)** Percent mosquitoes on Peltier during seconds 90-180 of each stimulus period in **(A,B)**. Each replicate is indicated by a dot, and the mean by a line. Arrowheads indicate significant differences ($p < 0.05$) from the second presentation of the 40°C stimulus or from 26°C (repeated measures one-way ANOVA with Bonferroni correction).

at the Peltier when it was warmed. Heat-seeking generally increased with thermal contrast, defined as the differential between the stimulus and the ambient temperatures. However, we found reduced Peltier occupancy for high-temperature stimuli, 50-60°C. In fact, Peltier occupancy for 55°C and 60°C stimuli was indistinguishable from an ambient-temperature stimulus.

We next performed a spatial analysis of these data to examine the distribution of mosquitoes in the Peltier region during these experiments. For stimuli 28.5-36˚C, occupancy on the Peltier clearly increased with temperature, and mosquitoes appeared to target the Peltier surface during these stimulus presentations (**Fig 3.2**). However, spatial analysis of responses to stimuli 40-60°C, showed a reduction in Peltier occupancy for 50˚C stimuli, and a strong avoidance of the Peltier area for stimuli 55-60°C (**Fig 3.3**).

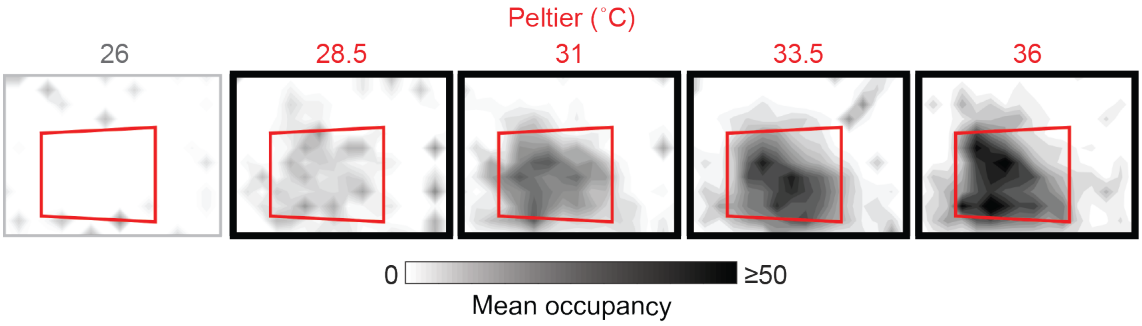

Figure 3.2 Peltier occupancy increases with stimulus temperature for stimuli 26-36°C. Heat maps showing mean mosquito occupancy on the Peltier (red square) and surrounding area, during seconds 90-180 of the indicated stimulus periods in (**Fig 3.1**). Bold borders indicate stimuli with responses significantly different from a 26°C stimulus in (**Fig 3.1**) ($p < 0.05$; repeated-measures ANOVA with Bonferroni correction).

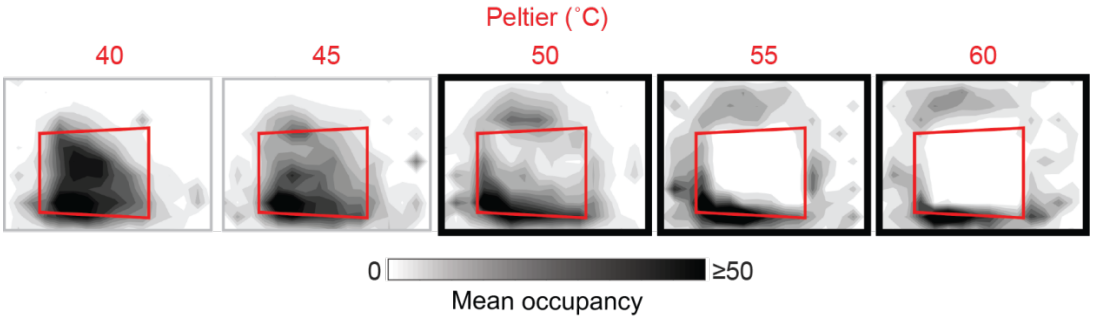

Figure 3.3 Peltier occupancy is reduced for stimuli ≥50°C. Heat maps showing mean mosquito occupancy on the Peltier (red square) and surrounding area, during seconds 90-180 of the indicated stimulus periods in (**Fig 3.1**). Bold borders indicate stimuli with responses significantly different from a 40°C stimulus in (**Fig 3.1**) ($p < 0.05$; repeated-measures ANOVA with Bonferroni correction).

3.2 *Ae. aegypti* heat-seek to host temperatures in a wide range of ambient temperatures

Female mosquitoes searching for a warm-blooded host may be responding to the absolute temperature of a stimulus or may instead be evaluating relative warmth, defined as the differential between a stimulus and background ambient temperature. For instance, is a 36°C stimulus equally attractive at any ambient temperature? Or are mosquitoes somehow comparing stimulus temperature to the surrounding environmental air temperature? To investigate the thermotaxis strategies underlying mosquito heat-seeking behavior, we conducted experiments at three ambient temperatures: 21, 26, and 31°C.

For each background temperature, we first tested stimuli ranging from ambient to 10°C above ambient (**Fig 3.4**). At all background temperatures, there was no heat-seeking for ambient temperature stimuli, demonstrating that thermal contrast is required for heat-seeking in these environments. Peltier occupancy for

stimuli 21- 40°C depended primarily on the differential between the Peltier and ambient temperature (**Fig 3.4B**), rather than the absolute temperature of the Peltier (**Fig 3.4A**). At all ambient temperatures tested, a stimulus 5°C above ambient was sufficient to elicit significant heat-seeking, and elicited approximately half as much Peltier occupancy as a stimulus 10°C above ambient. A spatial analysis of these experiments showed that relative warmth drives heat-seeking to the Peltier area (**Fig 3.5**).

We next tested higher-temperature stimuli, 40-60°C, at each of these background temperatures. Interestingly, heat-seeking to targets 50-60°C was inhibited at all ambient temperatures tested (**Fig 3.6A**), despite the fact that the temperature-differential varied widely in these situations (**Fig 3.6B**). A spatial analysis of these experiments showed that mosquitoes avoid the Peltier area for stimuli 50-60˚C, and that absolute temperature of the stimulus was the primary factor driving this behavior (**Fig 3.7**). Together, these results show that *Ae. aegypti* thermotaxis is driven by seeking relative warmth, but restricted by an absolute upper threshold of ~50-55°C.

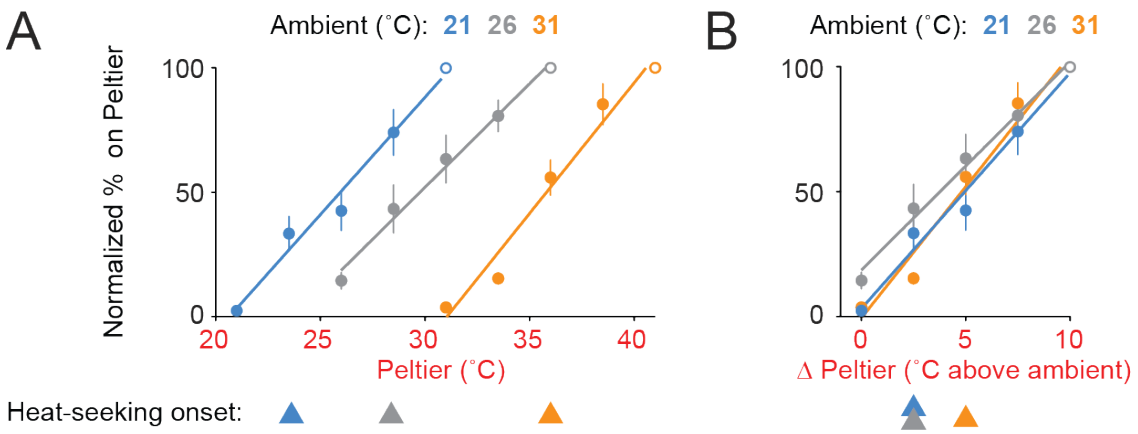

Figure 3.4 Mosquitoes seek relative warmth. (A, B) Heat-seeking at different ambient temperatures (n = 5-6 trials per condition with 45-50 mosquitoes): 21°C (blue), 26°C (gray), 31°C (orange). Data are plotted as mean ± s.e.m. **(A)** Percent of mosquitoes on Peltier during seconds 90-180 of stimuli of indicated temperature, normalized to stimulus 10°C above ambient (open circle) **(B)** Same data as in **(A)**, plotted using differential between ambient and Peltier temperature. For each ambient temperature, arrowheads indicate the lowest temperature stimulus found to elicit a significant increase in heat-seeking compared to an ambient temperature stimulus ($p < 0.05$; repeated-measures ANOVA with Bonferroni correction). For each ambient temperature, linear regressions are plotted (21°C: 10.6 /°C, $R^2 = 0.98$, 26°C: 12 /°C, $R^2 = 0.99$, 31°C :9.5 /°C, $R^2 = 0.97$).

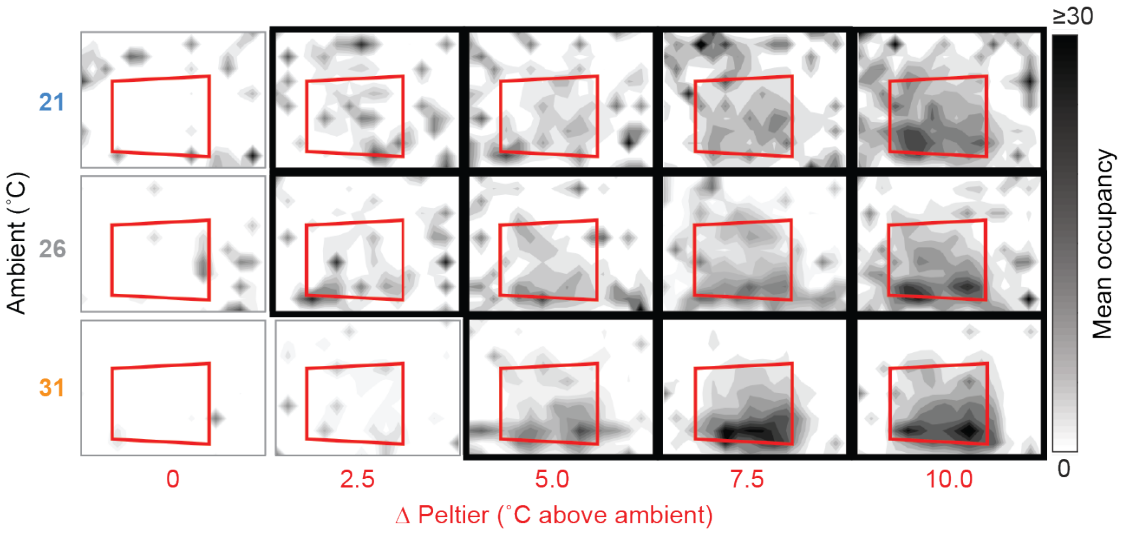

Figure 3.5 Peltier occupancy increases with thermal contrast. Heat maps showing mean mosquito occupancy on the Peltier (red square) and surrounding area, during seconds 90-180 of the indicated stimulus periods. Bold borders indicate stimuli with responses significantly different from an ambient-temperature stimulus in (**Fig 3.4**) ($p < 0.05$; repeated-measures ANOVA with Bonferroni correction).

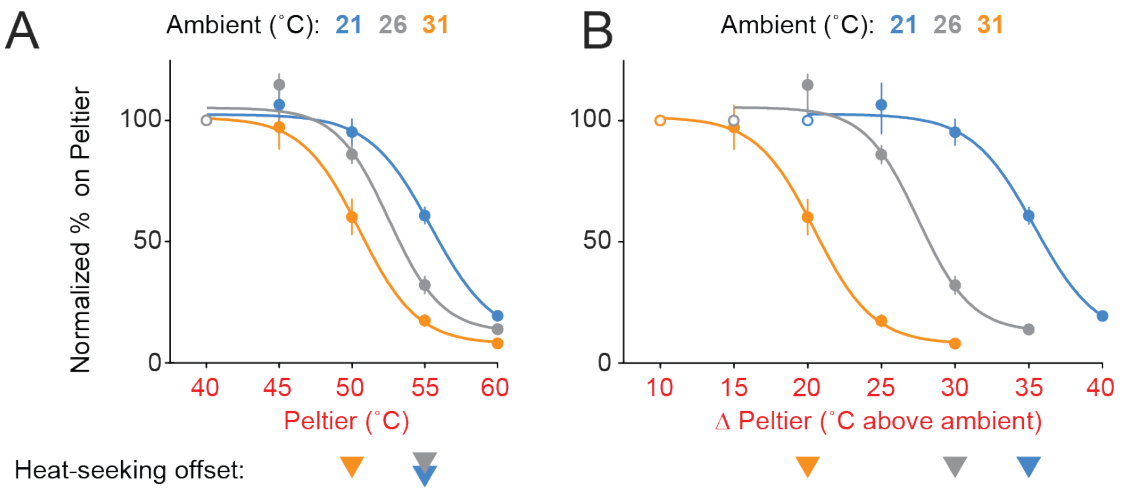

Figure 3.6 Mosquitoes avoid absolute stimulus temperatures above ~50-55°C. (A, B) Heat-seeking at different ambient temperatures (n = 5-6 trials per condition): 21°C (blue), 26°C (gray), 31°C (orange). Data are plotted as mean ± s.e.m. **(A)** Percent of mosquitoes on Peltier during seconds 90-180 of stimuli of indicated temperature, normalized to a 40°C stimulus (open circle) **(B)** Same data as in **(A)**, plotted using differential between ambient and Peltier temperature. For each ambient temperature, arrowheads indicate the lowest temperature stimulus found to elicit a significant reduction in heat-seeking compared to a 40°C stimulus ($p < 0.05$; repeated-measures ANOVA with Bonferroni correction). For each ambient temperature, variable slope sigmoidal dose-response curves are plotted (21°C: IC_{50} = 55.4°C, R^2 = 0.87, 26°C: IC_{50} = 52.5°C, R^2 = 0.92, 31°C: IC_{50} = 50.5°C, R^2 = 0.91).

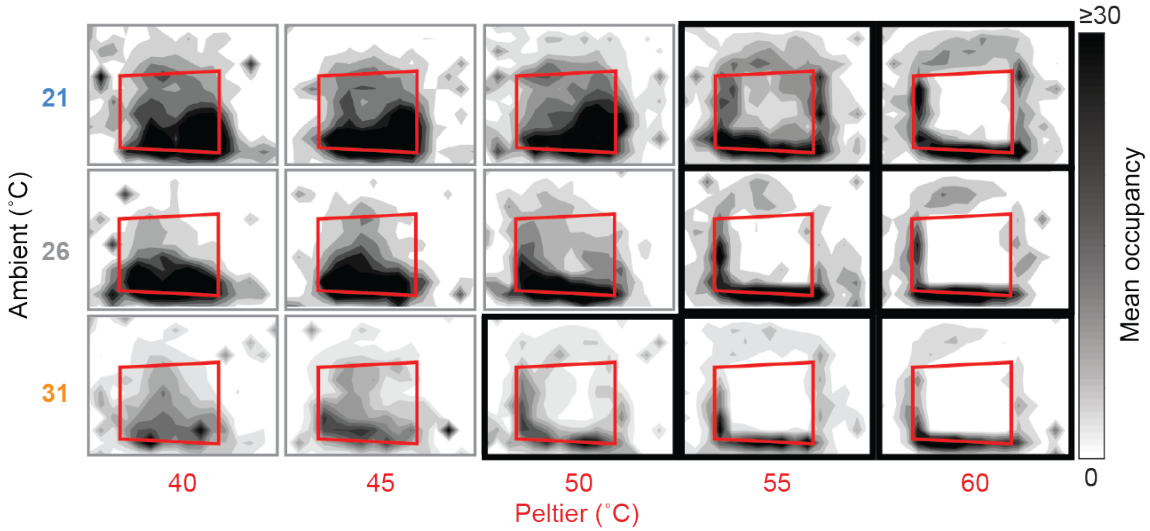

Figure 3.7 Mosquitoes avoid the Peltier area at absolute temperature above ~50-55°C. Heat maps showing mean mosquito occupancy on the Peltier (red square) and surrounding area, during seconds 90-180 of the indicated stimulus periods. Bold borders indicate stimuli with responses significantly different a 40°C stimulus in (**Fig 3.6**) ($p < 0.05$; repeated-measures ANOVA with Bonferroni correction).

3.3 *Ae. aegypti* females avoid relative cool during heat-seeking

Because female mosquitoes were attracted to relative warmth, we hypothesized that they may also avoid relative cool. This complementary behavior would serve to improve host-seeking thermotaxis. We examined mosquito responses to cooling by analyzing the rate at which animals left the Peltier when it was cooled at the conclusion of a stimulus period (**Fig 3.8**). We found that mosquitoes left the Peltier at similar rates regardless of the absolute temperature of the stimulus, demonstrating that mosquitoes avoid relative cool during heat-seeking.

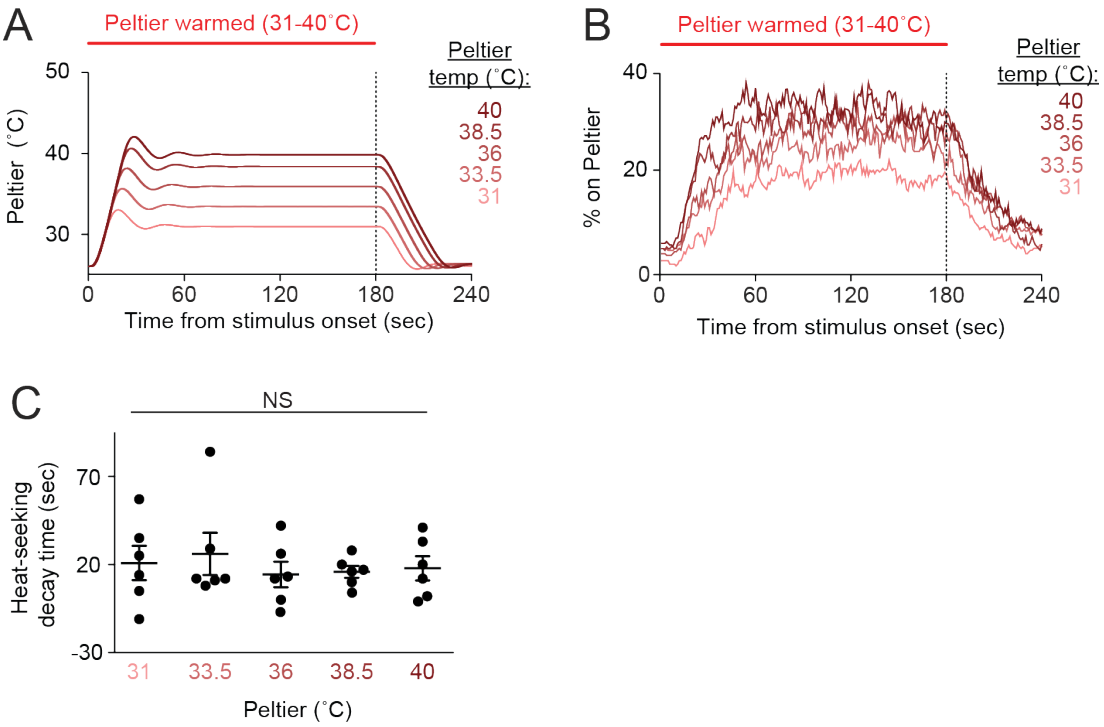

Figure 3.8 Mosquitoes avoid cooling. Analysis of mosquito responses to cooling from data in (**Fig 3.1**). **(A)** Mean Peltier temperature measured by thermocouple during presentation of thermal stimuli (31-40°C). Dashed line indicates the end of the stimulus period. **(B)** Mean percent of mosquitoes on Peltier during thermal stimuli 31-40°C. Dashed line indicates the end of the stimulus period. **(C)** Post-stimulus time at which the percent of mosquitoes on Peltier has decayed to one half of the mean during seconds 90-180 of the stimulus period from (**Fig 3.1**). Each replicate is indicated by a dot, mean ± s.e.m. by lines (NS, not significant; one-way ANOVA with Bonferroni correction).

3.4 *Anopheles gambiae* heat-seek to host-temperatures

We have found that *Ae. aegypti* females are maximally attracted to thermal stimuli resembling warm-blooded hosts. This is achieved by seeking relative warmth, avoiding relative cooling, and avoiding stimuli exceeding host temperatures. We hypothesized that other mosquito species may use similar strategies to locate hosts in their environment.

Anopheles gambiae is the primary vector of human malaria, which claims hundreds of thousands of lives annually (White et al., 2012). As in *Ae. aegypti*, females of this species require a blood meal to produce eggs and use a variety of physical and chemical cues to locate suitable hosts (Clements, 1999). *An. gambiae* show robust heat-seeking behavior in response to CO_2 activation (Kröber et al., 2010), but their responses to different thermal stimuli have never been investigated. Much like *Ae. aegypti*, this species can feed opportunistically on a variety of hosts, including birds (Githeko et al., 1994). Therefore, we hypothesized that *An. gambiae* would display thermotactic tuning resembling that of *Ae. aegypti*.

We used the heat-seeking assay to present diverse thermal stimuli to *An. gambiae* females (**Fig 3.9**). We found that these animals could heat-seek to a 31°C stimulus in a 26°C ambient environment. These mosquitoes may be able to detect stimuli of even smaller thermal contrast, but we did not test responses to stimuli <31°C. *An. gambiae* were maximally attracted to 40°C stimuli, and Peltier

occupancy was reduced for stimuli ≥45°C. Stimuli of 50 and 55°C resulted in Peltier occupancy rates indistinguishable from zero.

This thermotactic tuning in *An. gambiae* is strikingly similar to that of *Ae. aegypti*. Both species are maximally attracted to 40°C stimuli, and avoid stimuli exceeding host temperatures. The fact that these two distinct mosquito species show common heat-seeking strategies suggests that these fundamental components of mosquito thermotaxis may be adaptive, though more species would have to be examined to strengthen this claim.

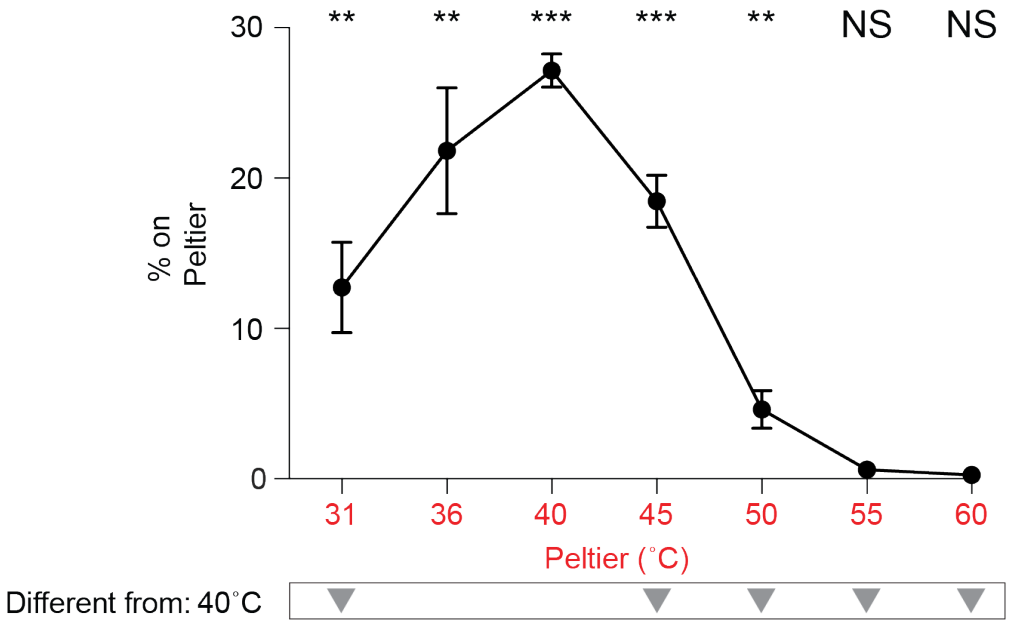

Figure 3.9 *An. gambiae* females are maximally attracted to stimuli approximating host body temperature. Percent mosquitoes on Peltier during seconds 90-180 of the indicated temperature stimulus period. n = 4 trials with 20-25 mosquitoes. Data are shown as mean ± s.e.m.; NS, not significant; **p < 0.01; ***p < 0.001; one sample t-test versus zero preference). Arrowheads indicate significant differences (p < 0.05) from 40°C stimulus (repeated measures one-way ANOVA with Bonferroni correction).

CHAPTER 4

AaegTRPA1 is a mosquito chemosensor and thermosensor

TRPA1 has highly conserved functions as a chemosensor and thermosensor among insects (Venkatachalam and Montell, 2007). We hypothesized that this cation channel may be used by mosquitoes to regulate heat-seeking behavior, but we first sought to establish whether *AaegTRPA1* is important for basic homeostatic behaviors, such as avoidance of noxious irritants or nociceptive air temperatures. If mosquitoes have adapted the function of TRPA1 for host-seeking behaviors, it is possible that this gene may have lost its function in conserved behaviors.

4.1 Targeted mutagenesis of *AaegTRPA1*

We used the Basic Local Alignment Search Tool (BLAST) to identify the *Ae. aegypti* ortholog(s) of *DmelTRPA1*. We found a clear orthologous transcript in the forward strand of supercontig 1.395 (VectorBase accession number AAEL009419). This transcript has 62% identity to *DmelTRPA1* at the cDNA level, and 68% identity at the protein level. This orthology has since been noted by others (Bohbot et al., 2014; Kwon et al., 2010a).

To examine the role of *AaegTRPA1*, we used genome-editing technology to generate animals with null-mutant alleles of this gene. Zinc-finger nucleases are synthetic restriction enzymes that can be designed to target mutations to specific DNA sequences (Carroll, 2011; Cathomen and Joung, 2008). This reagent consists of a zinc finger DNA-binding domain (which recognizes a

specific sequence of base pairs) fused to a Fok1 restriction endonuclease (which when dimerized generate a double-strand break in DNA). By engineering the zinc finger array, one can customize a ZFN to target a particular genomic sequence. The application of two ZFNs targeting unique adjacent sequences can result in Fok1 dimerization and catalytic cleavage of a specific genomic locus.

Cell-endogenous repair machinery can correct DNA breakage in two fundamental ways, each of which can result in mutations at the site of cleavage. One method of repair is non-homologous end-joining (NHEJ), in which the ends of double-stranded DNA breaks are ligated directly without a homologous template. This error-prone process can introduce deletion and insertion events at the cut-site, often resulting in frameshift mutations. Alternatively, cells can respond to DNA breaks using homology directed repair (HDR), which typically uses homologous recombination to restore the damaged locus. If ZFNs are delivered together with a custom donor plasmid containing sequence homologous to the genomic region surrounding the cut-site, the target sequence can be replaced by the donor sequence via HDR. Any sequence in the donor plasmid that is flanked by regions homologous to the target region, will be integrated into the locus during HDR. This process can be used to insert foreign DNA elements into the target region, such as fluorescent markers.

In collaboration with Sigma-Aldrich (St. Louis, MO), we designed ZFNs to target *AaegTRPA1* (**Fig 4.1A**). We established lines of *AaegTRPA1* null-mutant mosquitoes, marked with ubiquitous expression of a fluorescent protein (**Fig 4.1B**). Directed insertion of the fluorescence cassette was confirmed in *AaegTRPA1*$^{-/-}$ mutants by PCR, cloning and sequencing.

Figure 4.1 Genomic organization and targeted mutagenesis of *AaegTRPA1*. (A) Genomic organization of *AaegTRPA1*. Non-coding (white) and coding (black) exons are shown to scale (vectorbase.org). Introns are denoted by connecting lines (not to scale). The *AaegTRPA1* ZFN targets exon 12 (purple arrow). An insertion cassette (shown to scale), including the *Ae. aegypti* polyubiquitin (*polyUB*) promoter driving expression of enhanced cyan fluorescent protein (*ECFP*) and an *SV40* polyadenylation signal, is integrated via homology-directed repair. **(B)** Representative bright field (left) and fluorescence (right) images of wild-type and *AaegTRPA1*$^{-/-}$ female pupae marked by ubiquitous expression of enhanced cyan fluorescent protein (ECFP). Scale bars: 0.5 mm.

4.2 *AaegTRPA1* is required for chemosensation of an electrophile TRPA1 agonist

We hypothesized that *AaegTRPA1*$^{-/-}$ mutant mosquitoes would be unable to sense TRPA1 agonists. To investigate mosquito responses to chemicals, we adapted the *D. melanogaster* capillary feeder (CAFE) feeding assay (Ja et al., 2007) for use in mosquitoes (**Fig 4.2**). In this assay, 5 mosquitoes are introduced into a vial where they have access to two capillaries containing 10% sucrose solution. The amount of liquid consumed from a capillary can be measured by monitoring the height of the meniscus. By adding a chemical into one of the two capillaries and comparing the amount consumed from each capillary, we can assess whether mosquitoes avoid or prefer feeding on the tested chemical.

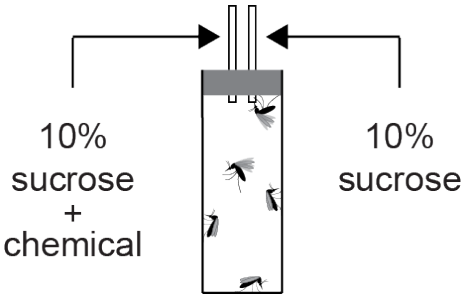

Figure 4.2 A modified capillary feeding (CAFE) assay to test mosquito chemosensation. Schematic of capillary feeding (CAFE) assay. Five mosquitoes are placed in a vial with access to two capillaries containing 10% sucrose. A chemical can be added to one capillary and consumption levels can be observed to measure feeding preference.

N-methylmaleimide is a potent agonist of *D. melanogaster*, and *An. gambiae* mosquito TRPA1 (Kang et al., 2010). Consumption of N-methylmaleimide mixed with sucrose solution is avoided by *D. melanogaster* (Kang et al., 2010). We used our modified CAFE assay to test mosquito avoidance of N-methylmaleimide consumption. When both capillaries contained 10% sucrose, mosquitoes fed equally from both sources (**Fig 4.3**). However, wild-type and heterozygous *AaegTRPA1* mutant mosquitoes strongly avoided

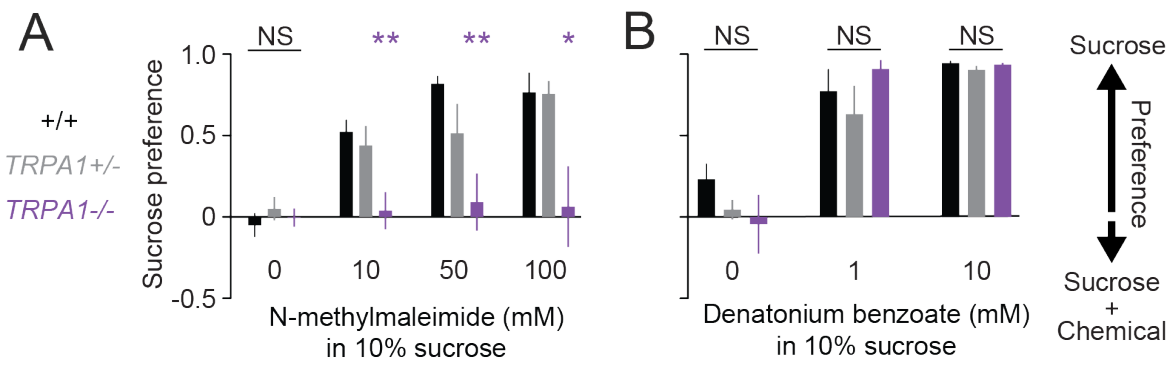

Figure 4.3 *AaegTRPA1*$^{-/-}$ mutants can detect the bitter compound, denatonium benzoate, but not the TRPA1 agonist, N-methylmaleimide. Sucrose preference over sucrose containing the indicated concentration of N-methylmaleimide **(A,** n = 10-12 trials per condition with 5 mosquitoes**)** or denatonium benzoate **(B,** n = 7 trials per condition with 5 mosquitoes**)** for mosquitoes of the indicated genotypes. (NS, not significant; *p < 0.05, **p < 0.01; one-way ANOVA with Bonferroni correction compared to wild-type).

consuming from capillaries containing 10, 50, or 100 mM N-methylmaleimide in 10% sucrose (**Fig 4.3A**). In contrast, *AaegTRPA1*$^{-/-}$ mutant mosquitoes showed no avoidance of N-methylmaleimide consumption at any of the concentrations tested. We interpret this result as a loss of N-methylmaleimide detection in *AaegTRPA1*$^{-/-}$ mutants, leading to no preference between sucrose and sucrose containing N-methylmaleimide (**Fig 4.3A**). This demonstrates that *AaegTRPA1* has a conserved chemosensory role in *Ae. aegypti*.

To examine whether chemosensation defects in *AaegTRPA1*$^{-/-}$ mutants are specific to TRPA1 agonists, we used the modified CAFE assay to also test avoidance of a compound that is not sensed by TRP channels. *D. melanogaster* flies avoid consumption of denatonium benzoate, an extremely bitter compound (Weiss et al., 2011). In our assay, mosquitoes strongly avoided consumption of 1 and 10 mM denatonium benzoate in 10% sucrose (**Fig 4.3B**). *AaegTRPA1*$^{-/-}$

mutants showed normal avoidance of this bitter compound, indicating that *AaegTRPA1* is not broadly required for all forms of chemosensation (**Fig 4.3B**).

4.3 *AaegTRPA1*$^{-/-}$ mutants fail to avoid high air temperatures in a thermal gradient

We asked whether *AaegTRPA1* is required for homeostatic thermoregulation behaviors characteristic of ectotherms. To examine thermal preference, one can allow animals to distribute freely in a thermal gradient. For example, *D. melanogaster* flies avoid both high and low air temperatures, and show a preference for ~24°C air (Sayeed and Benzer, 1996). We tested mosquito thermal preference in a modified thermal gradient assay (**Fig 4.4**). Two Peltier devices are used to drive a thermal gradient across an enclosure 50 cm in length. The air temperature gradient in the assay ranges from ~19 to ~36°C. The enclosure is 6 mm tall, confining mosquitoes to the thermal gradient surface, and

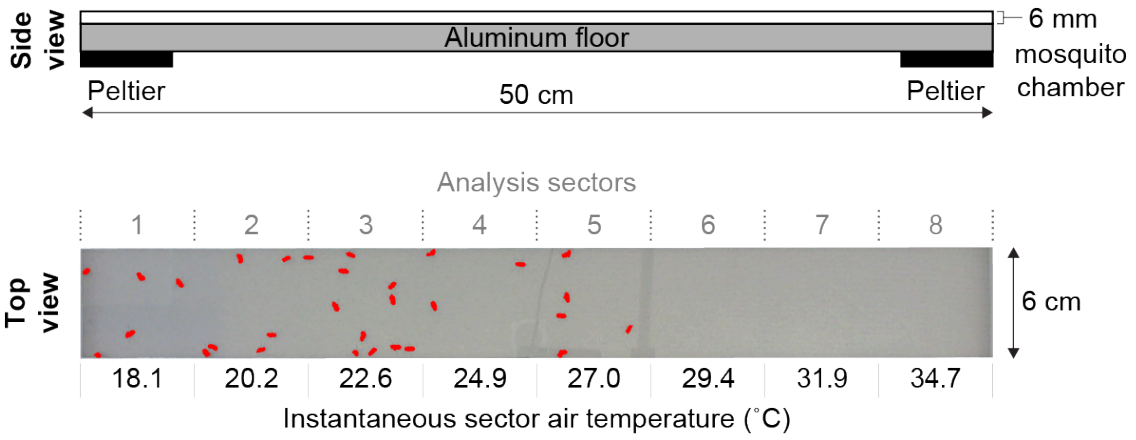

Figure 4.4 A thermal gradient to measure thermal preference of mosquitoes. Schematic of the thermal gradient assay (top, side view). Representative experimental image (bottom, top view) showing mosquitoes outlined in red detected across one lane of the thermal gradient assay, with instantaneous air temperature reported for each of 8 analysis sectors.

is divided lengthwise into 4 lanes, each 6 cm wide. For each trial, groups of mosquitoes are introduced into 3 of the lanes. The fourth lane is used to measure air temperature at 8 evenly spaced intervals spanning the length of the assay. An overhead camera acquires images of the assay once per minute, and computer vision is subsequently used to count mosquitoes in each of the 8 analysis sectors spanning the assay.

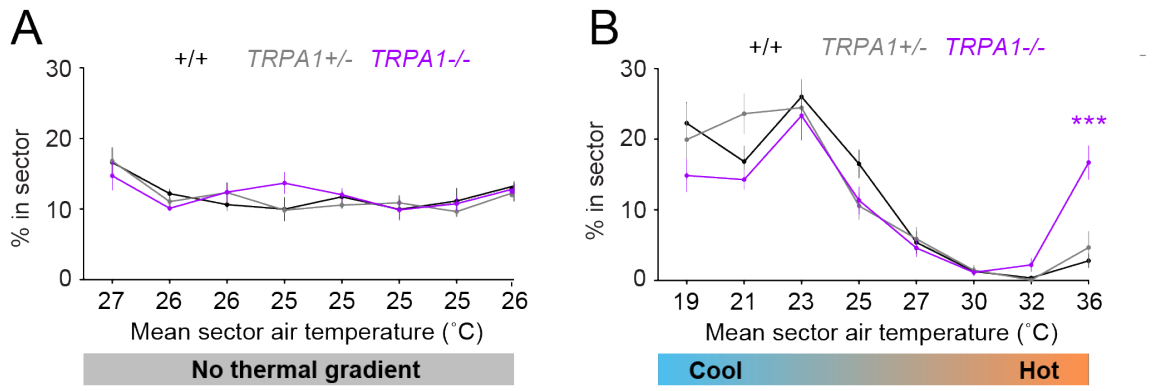

Figure 4.5 *AaegTRPA1*$^{-/-}$ mutants fail to avoid high air temperatures in a thermal gradient. Percent of mosquitoes of indicated genotypes detected in each sector and mean sector air temperature (rounded to nearest °C) in the absence **(A)** or presence **(B)** of a thermal gradient (n = 6 trials per genotype with 25-30 mosquitoes). Data are plotted as mean ± s.e.m. (***p < 0.001; two-way ANOVA with Bonferroni correction compared to wild-type).

In the absence of a thermal gradient, air temperature throughout the assay was ~26°C and mosquitoes distributed evenly throughout the sectors (**Fig 4.5A**). In the presence of a thermal gradient, wild-type and heterozygous *AaegTRPA1* mutant mosquitoes strongly avoided air temperatures above ~25°C, instead preferring sectors with air temperatures of 19-25°C (**Fig 4.5B**). Strikingly, *AaegTRPA1*$^{-/-}$ mutants failed to avoid sector 8, which had the highest air

temperature (~36°C) (**Fig 4.5B**). This disrupted thermal preference resulted in significantly elevated levels of mortality, with an average of ~30% of *AaegTRPA1*$^{-/-}$ mutants dying in sectors 6-8 during the assay (**Fig 4.6**). A more high-resolution temporal analysis (**Fig 4.7**) revealed that *AaegTRPA1*$^{-/-}$ mutants showed normal avoidance of high air temperatures during the first ~15 minutes of the experiment, during which the thermal gradient is being established. It was after this time that *AaegTRPA1*$^{-/-}$ mutants begin to accumulate in sector 8. Unlike wild-type and heterozygous animals, *AaegTRPA1*$^{-/-}$ mutants began to leave the coolest sectors and gathered at the hottest sectors (**Fig 4.8**). We interpret this disruption in *AaegTRPA1*$^{-/-}$ mutant thermoregulation as a defect in heat-sensation, indicating that *AaegTRPA1* is a conserved thermosensor.

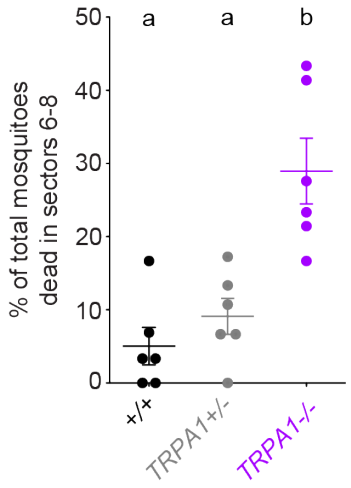

Figure 4.6 *AaegTRPA1*$^{-/-}$ mutants have elevated levels of mortality in a thermal gradient. Percent of total mosquitoes of the indicated genotypes found dead in sectors 6-8 at the conclusion of the experiment. Each replicate is indicated by a dot, and mean ± s.e.m. by lines. Genotypes with different letters are significantly different ($p < 0.01$, one-way ANOVA with Bonferroni correction).

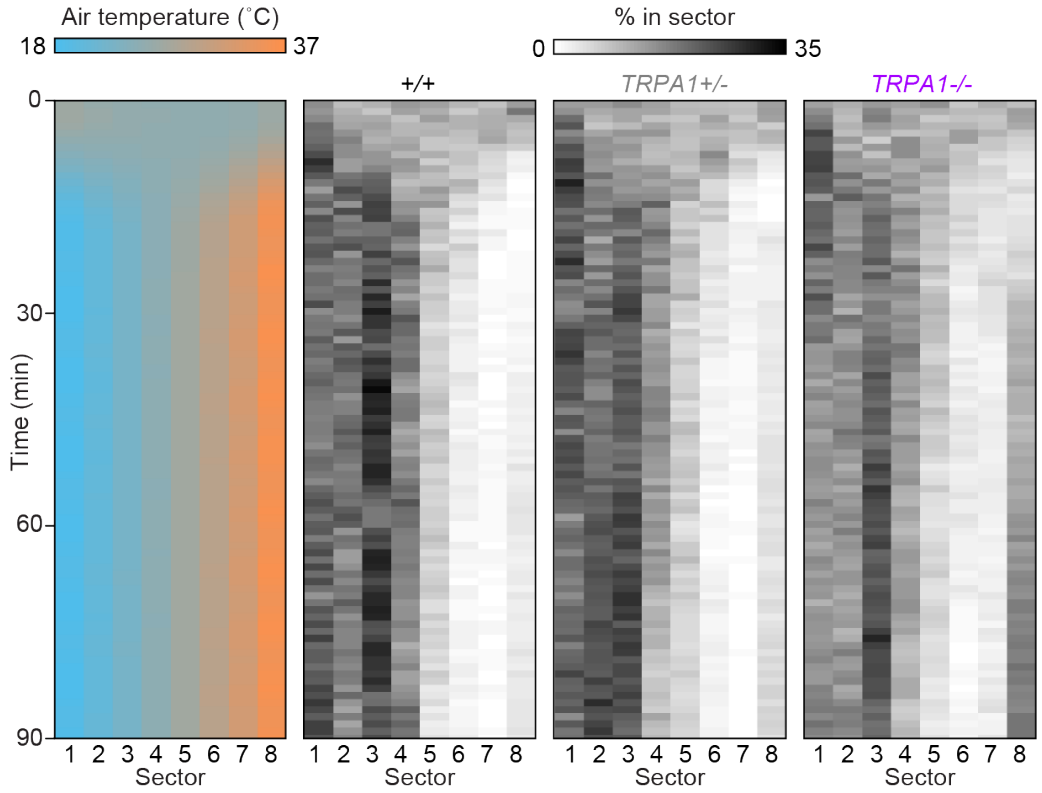

Figure 4.7 *AaegTRPA1*$^{-/-}$ mutants gradually accumulate at the highest-temperature sector. Heat maps showing mean air temperature (left) and percent of mosquitoes of the indicated genotypes detected (right) in each sector over 90 minutes from the onset of a thermal gradient.

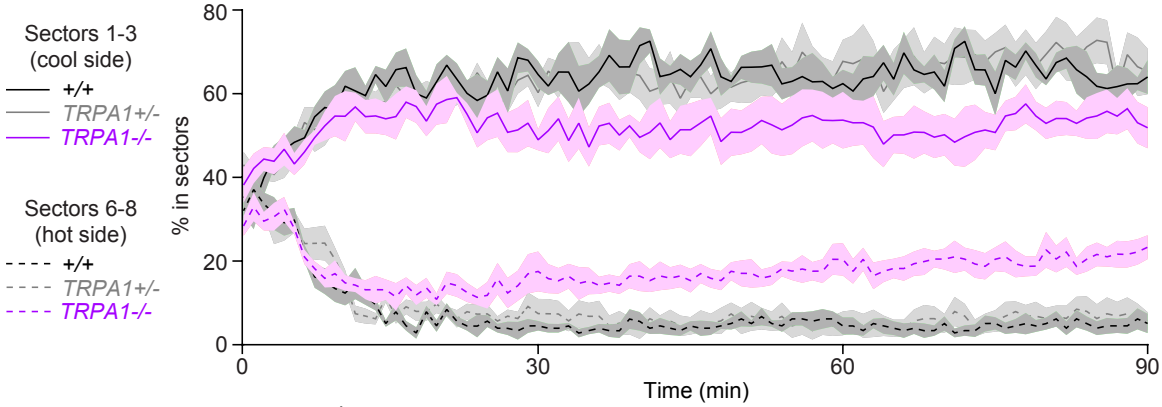

Figure 4.8 *AaegTRPA1*$^{-/-}$ mutants leave cooler sectors and gather at hotter sectors of a thermal gradient. Total percent of mosquitoes of the indicated genotypes detected in sectors 1-3 (cold side, solid line, mean; s.e.m., shading) and sectors 6-8 (hot side, dotted line, mean; s.e.m., shading) over 90 minutes from the onset of a thermal gradient.

CHAPTER 5

AaegTRPA1 tunes mosquito thermotaxis to host temperatures

5.1 *AaegTRPA1*$^{-/-}$ mutants do not avoid thermal stimuli exceeding host temperatures

Having determined that *AaegTRPA1* plays a conserved role in mosquito chemosensation and thermoregulation, we examined whether this gene has evolved a specialized role in mosquito host-seeking thermotaxis. To this end, we examined *AaegTRPA1*$^{-/-}$ mutant responses to diverse thermal stimuli in our heat-seeking assay (**Fig 5.1**).

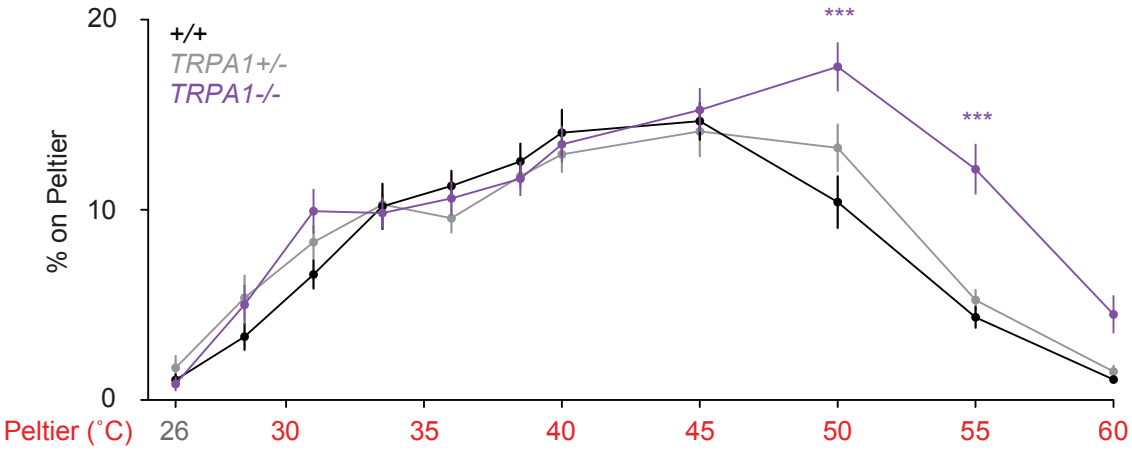

Figure 5.1 *AaegTRPA1*$^{-/-}$ mutants do not avoid thermal stimuli exceeding host temperature. Percent of mosquitoes of indicated genotypes on Peltier during seconds 90-180 of stimuli of indicated temperature (mean ± s.e.m., n = 6-9 trials per genotype with 45-50 mosquitoes; ***p < 0.001; repeated measures one-way ANOVA with Bonferroni correction).

AaegTRPA1$^{-/-}$ mutants showed normal attraction to thermal stimuli 26 to 45°C, with Peltier occupancy indistinguishable from wild-type and heterozygous

mutants. However, *AaegTRPA1*$^{-/-}$ mutants did not show normal avoidance of high-temperature stimuli. While heterozygous mutant responses were indistinguishable from those of wild-type, *AaegTRPA1*$^{-/-}$ mutants showed elevated Peltier occupancy at 50 and 55°C stimuli. This striking disruption in thermotactic tuning was evident in a spatial analysis of mosquito density (**Fig 5.2**). Unlike wild-type, *AaegTRPA1*$^{-/-}$ mutants showed increased Peltier occupancy for 50°C stimuli compared to 45°C stimuli, suggesting a loss of inhibition for landing on high-temperature stimuli. Wild-type and heterozygous mutants clearly avoided the Peltier area for 55°C stimuli, while *AaegTRPA1*$^{-/-}$ mutants showed substantial Peltier occupancy.

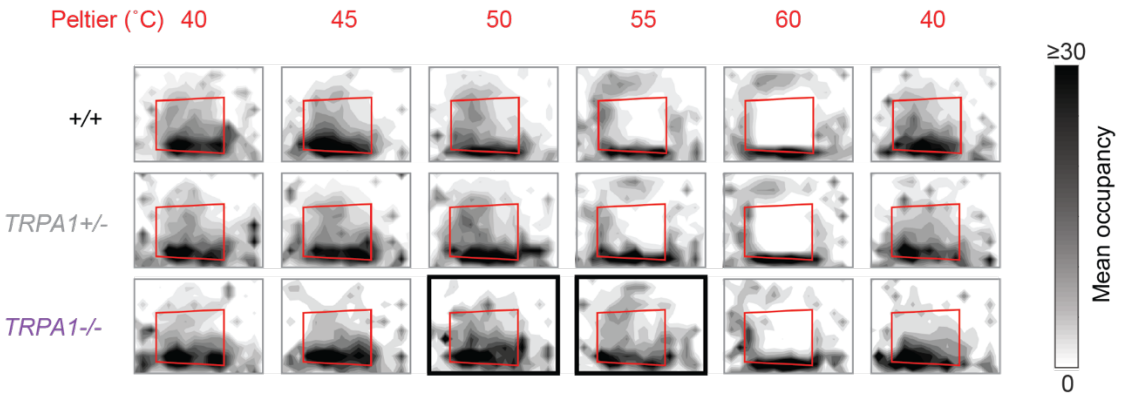

Figure 5.2 *AaegTRPA1*$^{-/-}$ mutants do not avoid the Peltier area for high-temperature stimuli. Heat maps showing mean mosquito occupancy for the indicated genotypes on the Peltier (red square) and surrounding area, during seconds 90-180 of each stimulus period. Bold borders indicate stimuli with responses significantly different from wild-type in (**Fig 5.1**) ($p < 0.05$; repeated-measures ANOVA with Bonferroni correction).

We next examined the temporal dynamics of Peltier occupancy during thermal stimulus periods (**Fig 5.3**). Stimuli 40-45°C elicited rapid accumulation of mosquitoes onto the Peltier, which persisted until the end of the stimulus period. For 50°C stimuli, wild-type and heterozygous animals initially accumulated onto the Peltier, but Peltier occupancy appeared to slowly decrease during the stimulus period. However, *AaegTRPA1*$^{-/-}$ mutants persisted on 50°C stimuli throughout the entire stimulus period, and Peltier occupancy even appeared to increase during this time. A similar and more dramatic pattern was seen for 55°C stimuli. In these stimulus periods, wild-type and heterozygous animals quickly left the Peltier area after initial attraction while *AaegTRPA1*$^{-/-}$ mutants persisted on the Peltier. Although all genotypes avoided 60°C stimuli, *AaegTRPA1*$^{-/-}$ mutants persisted for a longer time on the Peltier. Together, these data show that *AaegTRPA1* is required for normal avoidance of high-temperature stimuli 50-60°C which exceed host temperatures, and that other mechanisms allow for avoidance of more extreme high-temperature stimuli.

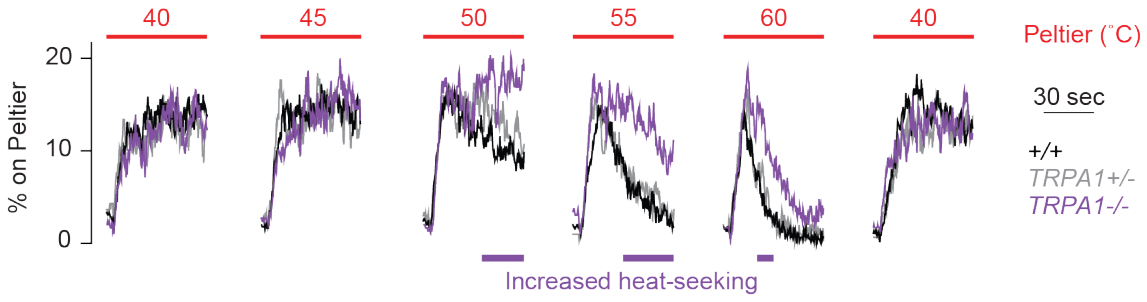

Figure 5.3 *AaegTRPA1*$^{-/-}$ mutants persist on the Peltier during high-temperature stimuli. Mean percent of mosquitoes of indicated genotypes on Peltier during thermal stimuli 40-60°C and during subsequent re-presentation of 40°C. Timespans with statistically significant increases in *AaegTRPA1*$^{-/-}$ mutant Peltier occupancy compared to wild-type are indicated by purple lines (calculated from 15 second bins; $p < 0.05$; one-way ANOVA with Bonferroni correction).

5.2 *AaegTRPA1*$^{-/-}$ mutants do not distinguish between a host-temperature and a high-temperature stimulus

AaegTRPA1$^{-/-}$ mutants failed to avoid thermal stimuli exceeding host temperatures, but they may still be able to preferentially thermotax to host-temperature stimuli when provided with a choice. Using a heat-seeking choice assay with two independently controlled Peltiers, we examined the importance of *AaegTRPA1* in guiding mosquito thermotaxis in a more complex thermal landscape (**Fig 5.4**). In this assay, mosquitoes are simultaneously presented with two thermal stimuli, presented on two adjacent Peltier devices separated by 6.5 cm. A camera is used to monitor mosquito occupancy on both Peltiers.

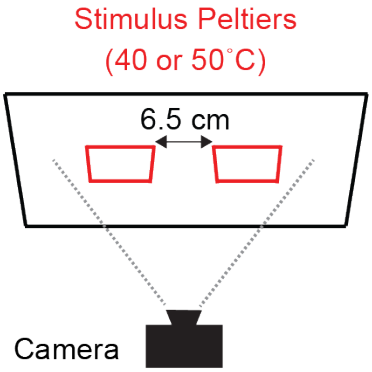

Figure 5.4 A choice heat-seeking assay to measure preference during mosquito thermotaxis. Schematic of heat-seeking choice assay. Two independently-controlled Peltiers are used to present mosquitoes with a choice between thermal stimuli.

When mosquitoes were presented with two 40°C stimuli, we observed strong attraction to both Peltiers, and mosquitoes showed no preference for either stimulus (**Fig 5.5**). We next presented mosquitoes with a choice between a 40°C Peltier and a 50°C Peltier (**Fig 5.6**). Wild-type and heterozygous mosquitoes strongly avoided the 50°C Peltier, and showed a strong preference for the 40°C stimulus. In contrast, Peltier occupancy of *AaegTRPA1*$^{-/-}$ mutants was high for both stimuli, and these animals distributed equally between the

Peltiers. This shows that *AaegTRPA1* is required for distinguishing between thermal stimuli that resemble hosts and those that exceed host temperature.

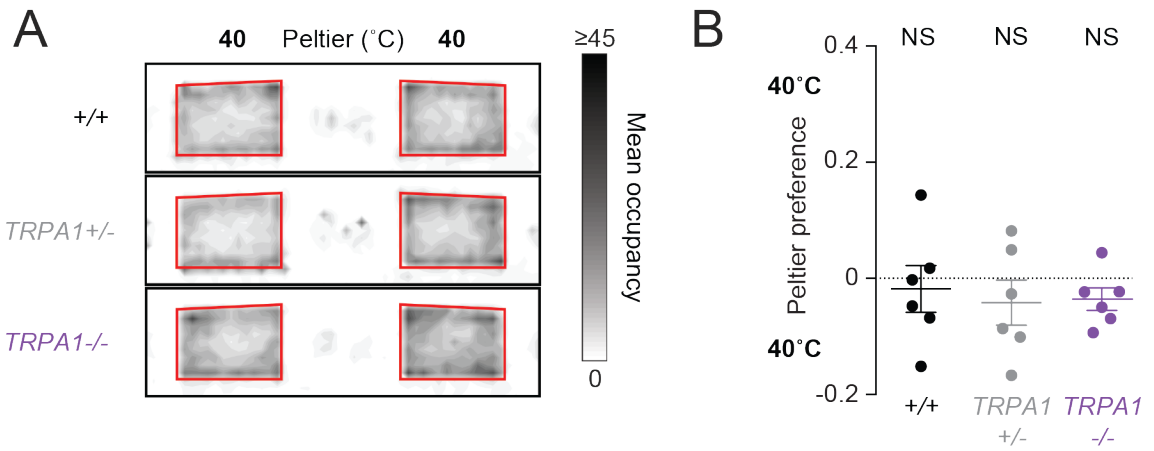

Figure 5.5 Mosquitoes distribute evenly between two 40°C stimuli. (A) Heat maps showing mean mosquito occupancy for the indicated genotypes on two 40°C Peltiers (red squares) and surrounding area, during seconds 60-240 of each stimulus period. n=6 trials per genotype. **(B)** Preference for 40°C versus 40°C Peltiers for indicated genotypes (n = 6 trials per genotype with 45-50 mosquitoes; mean ± s.e.m., with each replicate indicated by a dot; NS, not significant; ***p < 0.001; one sample t-test versus zero preference).

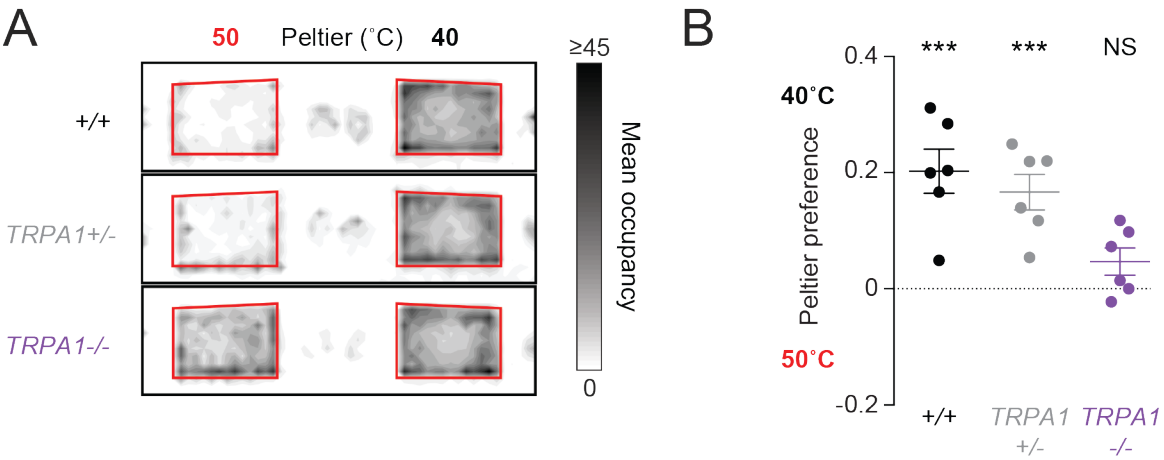

Figure 5.6 *AaegTRPA1*$^{-/-}$ mutants do not distinguish between a 50°C and a 40°C stimulus. (A) Heat maps showing mean mosquito occupancy for the indicated genotypes on two Peltiers (red squares) of the indicated temperatures and surrounding area, during seconds 60-240 of each stimulus period. n=6 trials per genotype with 45-50 mosquitoes. **(B)** Preference for 50°C versus 40°C Peltiers for indicated genotypes (n = 6 trials per genotype; mean ± s.e.m., with each replicate indicated by a dot; NS, not significant; ***p < 0.001; one sample t-test versus zero preference). *AaegTRPA1*$^{-/-}$ mutants are significantly different from wild-type and heterozygous mutants (p < 0.05, one-way ANOVA with Bonferroni correction).

5.3 *AaegGr19*$^{-/-}$ mutants show normal heat-seeking behavior.

Because *AaegTRPA1*$^{-/-}$ mutants retain normal attraction to warmth, this aspect of heat-seeking must rely on other thermoreceptors, still to be identified. We hypothesized that other insect thermoreceptors ordinarily important for ectothermic thermoregulation behaviors may have evolved a function in mosquito heat-seeking. One such candidate gene is the heat-sensor *DmelGr28b*, which has been shown to be important in *D. melanogaster* rapid avoidance of nociceptive high air-temperatures.

We used genome-editing technology to examine the role of *AaegGr19*, the ortholog of *DmelGr28b* (Kent et al., 2007)**.** In collaboration with Sigma-Aldrich (St. Louis, MO), we designed ZFNs to target *AaegGr19*, and we established lines of *AaegGr19* null-mutant mosquitoes, marked with ubiquitous expression of a fluorescent protein (**Fig 5.7**). Directed insertion of the fluorescence cassette was confirmed in *AaegGr19*$^{-/-}$ mutants by PCR, cloning and sequencing.

We tested *AaegGr19*$^{-/-}$ mutant responses to diverse thermal stimuli in our heat-seeking assay. Interestingly, *AaegGr19*$^{-/-}$ mutant mosquitoes showed no thermotaxis defects (**Fig 5.8**). These mutants were indistinguishable from wild-type animals in their responses to stimuli 26 to 60°C, indicating that *AaegGr19* is not required for thermotactic tuning of mosquito heat-seeking.

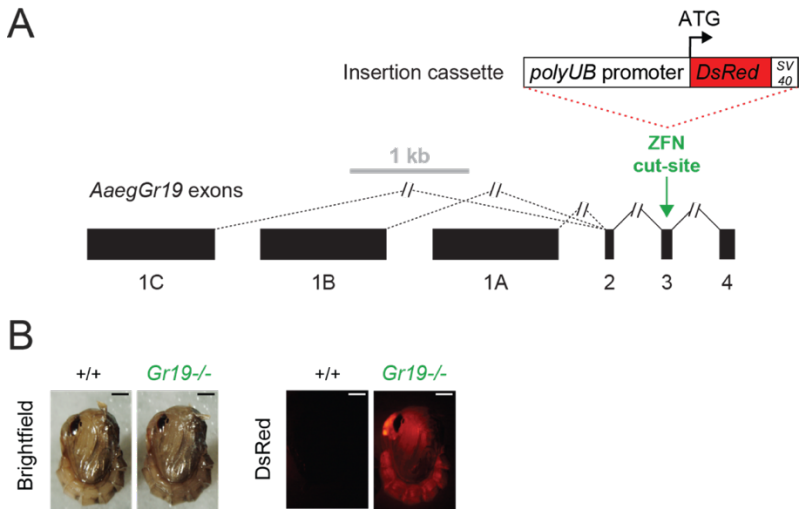

Figure 5.7 Genomic organization and targeted mutagenesis of *AaegGr19*. (A) Genomic organization of *AaegGr19*. Coding (black) exons are shown to scale, introns are denoted by connecting lines (not to scale), and alternative splicing is denoted by dashed lines (vectorbase.org). The *AaegGr19* ZFN targets exon 3 (green arrow). An insertion cassette (shown to scale), including the *Ae. aegypti* polyubiquitin (*polyUB)* promoter driving expression of *Discosoma sp.* red fluorescent protein (*DsRed*) and an *SV40* polyadenylation signal, is integrated via homology-directed repair. **(B)** Representative bright field (left) and fluorescence (right) images of wild-type and *AaegGr19*$^{-/-}$ female pupae marked with ubiquitous expression of *DsRed* Scale bars: 0.5 mm. The wild-type bright-field image is duplicated from (**Figure 4.1**).

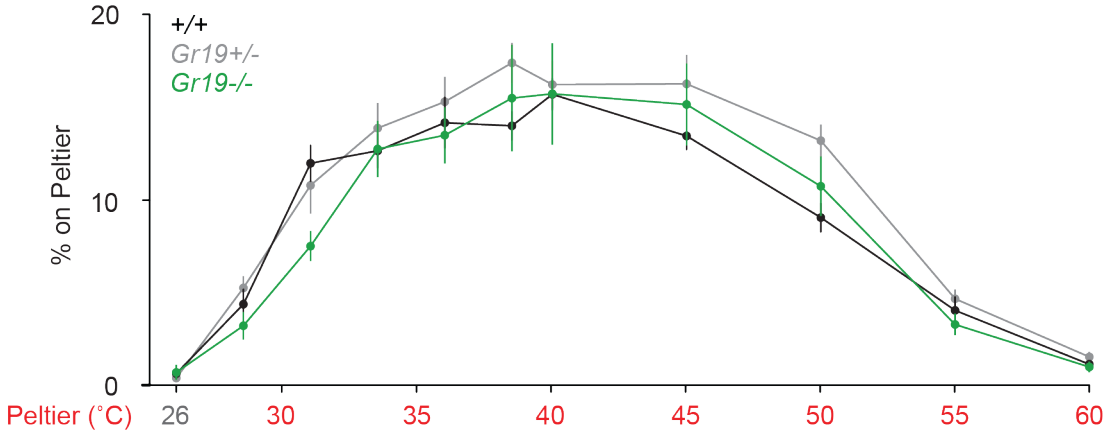

Figure 5.8 *AaegGr19*$^{-/-}$ mutants show normal thermotaxis. Percent of mosquitoes of indicated genotypes on Peltier during seconds 90-180 of stimuli of indicated temperature (mean ± s.e.m., n = 6-9 trials per genotype with 45-50 mosquitoes). Neither *AaegGr19*$^{-/-}$ nor *AaegGr19*$^{+/-}$ were significantly different from wild-type at any stimulus temperature (repeated measures one-way ANOVA with Bonferroni correction).

CHAPTER 6

Discussion

6.1 *AaegTRPA1* is required for an important component of host-seeking

We have found that female *Ae. aegypti* mosquitoes are maximally attracted to thermal stimuli approximating the body temperatures of endothermic hosts such as humans and birds (**Fig 6.1**). By seeking relative warmth and avoiding relative cool, mosquitoes are able to thermotax towards heated targets in diverse ambient background temperatures. Interestingly, heat-seeking mosquitoes avoid thermal stimuli that exceed the body temperatures of hosts. We discovered that the insect heat-sensor *TRPA1* is required for avoidance of high-temperature stimuli in mosquito host-seeking. Without this gene, mosquitoes do not discriminate between thermal targets that resemble hosts, and those that are inappropriately hot.

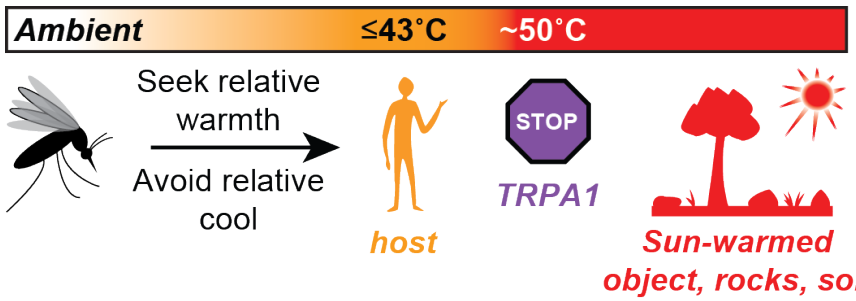

Figure 6.1 A genetic and behavioral model of *Ae. aegypti* host-seeking thermotaxis. Female mosquitoes thermotax towards thermal stimuli resembling hosts. Approach is mediated by seeking relative warmth and avoiding relative cool. Avoidance of objects exceeding host temperatures requires *AaegTRPA1*.

6.1.1 *AaegTRPA1* may be important for focusing thermotaxis toward live hosts

Why might avoidance of high-temperature stimuli be important for a host-seeking mosquito in the wild? One reason this behavior may be adaptive is that natural settings can exhibit objects much warmer than endothermic hosts (Angilletta, 2009; Cossins and Bowler, 1987; Geiger et al., 2009). In an outdoor environment, sun-warmed soil, rocks, trees and human-made objects often have temperatures >45°C, and can thus act as thermal distractors for host-seeking mosquitoes (**Fig 6.2**). Consider a human sitting bare-legged on the sun-lit ground. A mosquito targeting this host will be poorly served by merely thermotaxing to the hottest surface available. Instead, a more optimal strategy for diurnal mosquitoes such as *Ae. aegypti* is to search specifically for biologically relevant thermal stimuli, and to avoid objects exceeding host temperature. Indeed, this is the thermotaxis strategy we have observed in our laboratory models of heat-seeking. We speculate that the tuning of mosquito heat-seeking by *AaegTRPA1* is an important component of host-seeking in a complex natural thermal landscape.

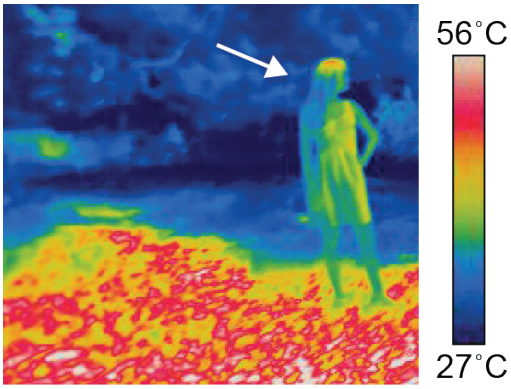

Figure 6.2 Natural environments present thermal stimuli exceeding host temperatures. A thermal image of a human (arrow) standing in a sunlit patch of grass in New York City's Central Park.

A thermal distractor assay could model this scenario in the lab (**Fig 6.3**). Essentially, this experiment would give mosquitoes the choice between two attractive stimuli: 1) a live host; and 2) a heated surface. The assay consists of a human arm situated on a large temperature-controlled surface. If the surface is not heated, then mosquitoes would likely easily locate the live host (**Fig 6.3A**). But what would mosquitoes do if the surface is heated and becomes a thermal distractor (i.e. mimicking sun-lit soil)? Given the strong attraction of mosquitoes to warm objects, the animals may be impaired in finding the human arm, instead busying themselves in futile attempts at biting the thermal distractor (**Fig 6.3B**). If the temperature of the thermal distractor is elevated to 50°C, this may alleviate the impairment due to distraction, as we have found that mosquitoes will not persist on high-temperature stimuli (**Fig 6.3C**). However, we would predict that *AaegTRPA1*$^{-/-}$ mutants would continue to be attracted by the distractor, and thus exhibit greater impairment than their wild-type counterparts (**Fig 6.3D**). Multiple genotypes could be tested simultaneously in this assay, and blood-fed versus non-blood-fed mosquitoes could subsequently be genotyped by fluorescence or PCR.

It is possible that thermal distractors would have no effect in this assay. This could be due to the inadequacy of this model of host-seeking does in recapitulating the complexity of a natural thermal landscape. Alternatively, the adaptive nature of high-temperature avoidance behavior may only be significant over large timescales and populations. Finally, it may also be that other host cues are sufficient to drive preference for a live host versus a heated surface.

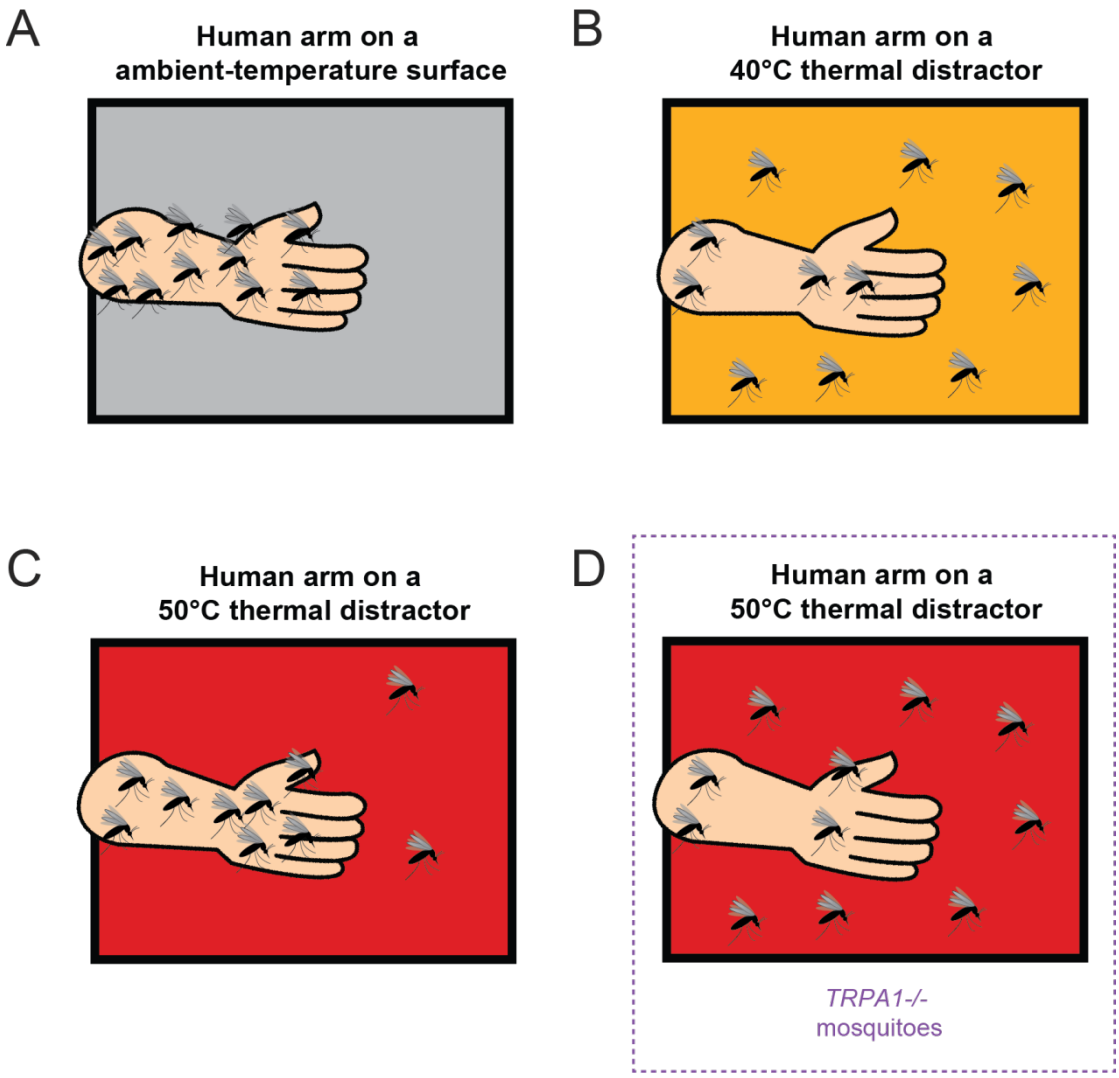

Figure 6.3 A potential thermal distraction assay to assess the importance of high-temperature avoidance in optimizing host-seeking. (A-D) Schematics (top-view) of hypothetical results using different thermal distractor temperatures with wild-type and *AaegTRPA1*$^{-/-}$ mutants. Cartoon mosquitoes show hypothesized distributions of attraction to a human arm and the underlying temperature-controlled surface.

6.1.2 *AaegTRPA1* may be important for protecting mosquitoes from overheating during host-seeking

The *AaegTRPA1*-dependent avoidance of high-temperature stimuli during host-seeking may also be a thermoregulatory or nociceptive response. As we have shown in our thermal gradient assay, *Ae. aegypti* avoid high temperatures outside of the host-seeking context. This is consistent with the thermoregulatory behavior of many other animals. However, because female mosquitoes have evolved a specialized mode of thermotaxis in which they are attracted to heat, this may present a conflict between two drives: heat-seeking and thermoregulation. By heat-seeking, mosquitoes in pursuit of a blood meal may be putting themselves at risk of dangerously elevating their body temperature. For instance, once a mosquito begins blood-feeding, ingestion of this hot meal substantially elevates the body temperature of the animal (Lahondère and Lazzari, 2012). This thermal stress is mitigated by synthesis of heat-shock proteins (Benoit et al., 2011) or by actively generating evaporative cooling through excretion of a liquid droplet during blood-feeding (Lahondère and Lazzari, 2012). These insights suggest that there is a conflict of interest between host-seeking and thermoregulation. Thermophilic behaviors must be accompanied by complementary avoidance behaviors that prevent overheating. Thus, the role of *AaegTRPA1* in regulating thermotaxis may not only be adaptive in its optimization of host-seeking, but may also be important to contend with the thermoregulatory risks associated with this derived behavior.

To assess the role of *AaegTRPA1* outside of the context of host-seeking, a thermal response assay that does not rely on CO_2 activation could be used (**Fig 6.4**). This assay traps mosquitoes onto the surface of a Peltier, at which point they can be exposed to heating or cooling and their behavior observed. A prototype of this assay indicates that this can be done with relative ease. The assay enclosure consists of a long prismatic chamber with a Peltier at one end and a camera at the other. Using a puff of air, mosquitoes are blown into the chamber and onto the Peltier surface, at which point a sliding door forces them to remain there throughout an acclimation period. After acclimation, the Peltier can be heated or cooled, and mosquito occupancy on the Peltier can be monitored by the camera. Mosquito behavior, such as probing, can be monitored by an additional camera mounted near the Peltier.

We hypothesize that mosquitoes will leave the Peltier once it is heated to a particular threshold. If this leaving threshold is ~50°C, then this is evidence that the avoidance of high temperature stimuli during host-seeking may be a feature of general thermoregulation or nociception in the mosquito; especially if this holds true at multiple ambient temperatures. However, if the leaving threshold is <50°C, then this indicates that mosquitoes are violating thermoregulatory optima during host-seeking thermotaxis.

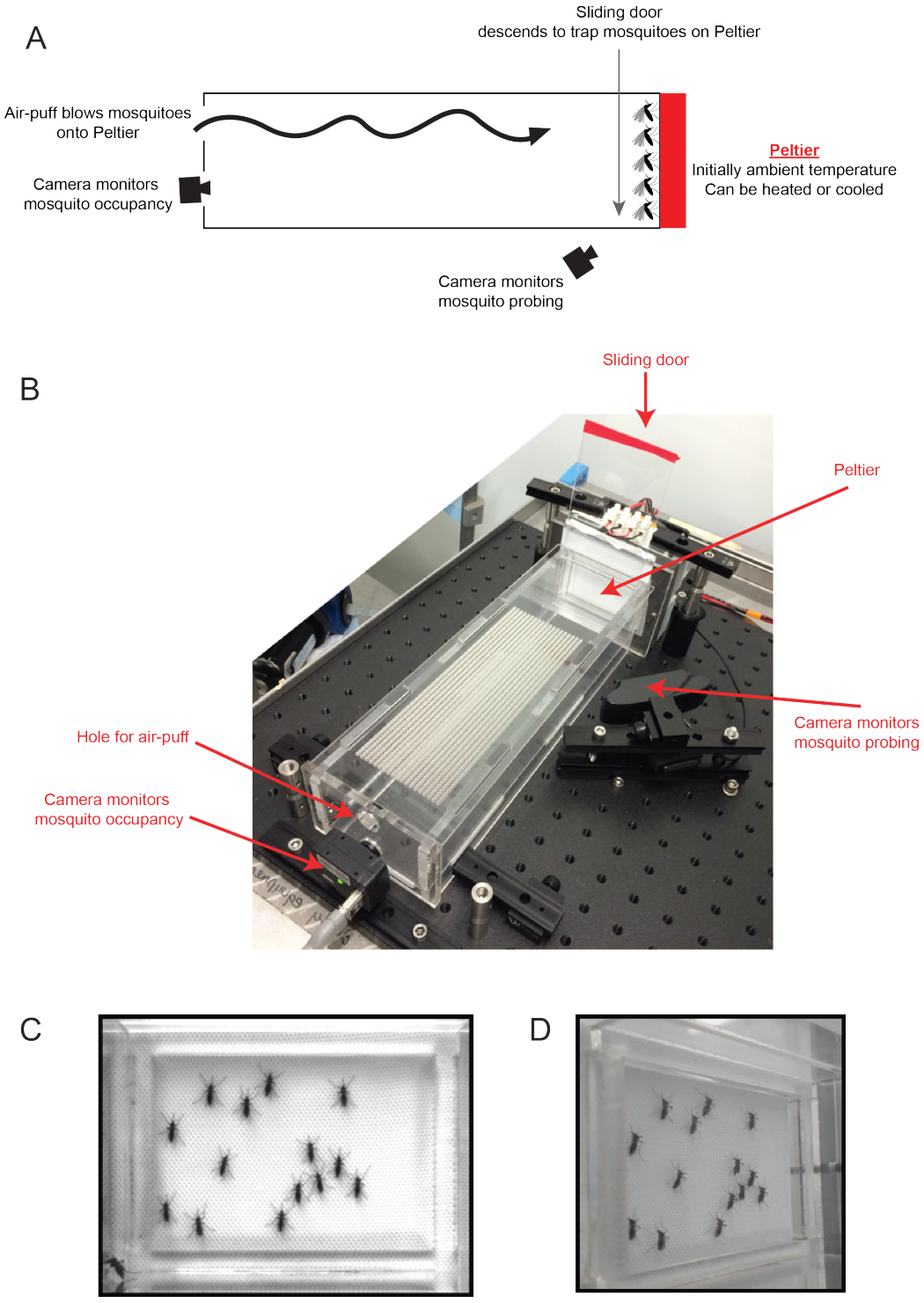

Figure 6.4 A thermal response assay to assess mosquito responses to temperature fluctuations in the absence of CO_2 activation. Side-view schematic **(A)** and photograph **(B)** of thermal response assay. Representative images from the camera monitoring Peltier occupancy **(C)** and the camera monitoring probing behavior **(D)**.

6.1.3 Examining other potential roles of *AaegTRPA1*

The importance of *AaegTRPA1* in larval mosquito thermotaxis has not been investigated, but *DmelTRPA1* is known to be required for larvae to avoid heat in a thermal gradient (Rosenzweig et al., 2008). *Ae. aegypti* larvae are aquatic, and may avoid warm water temperatures that are dangerous or suboptimal. This behavior could be modeled in an aquatic thermal gradient, but water is highly conductive to temperature and achieving a thermal gradient in a small-scale assay may be challenging. Alternatively, larval agitation or escape behaviors could be monitored during aquatic warming. Thermotaxis in mosquito larvae may be an important behavior for maximizing fitness in a natural environment.

Another potentially important function of *AaegTRPA1* is in the detection of the mosquito repellents. Botanical compounds such as camphor, citronella and citronellal have long been used in numerous insect repellent products (Katz et al., 2008). *An. gambiae* and *Cu. pipiens pallens* mosquitoes are strongly repelled by these types of compounds (Kim et al., 2005; Kwon et al., 2010a). *D. melanogaster* flies are also repelled by citronellal, and this behavior requires *TRPA1* as a downstream target of a signaling cascade (Kwon et al., 2010a). Intriguingly, *AgamTRPA1* has been found to be directly and potently activated by citronellal (Kwon et al., 2010a). Camphor, too, acts on TRPA1 by inhibiting the channel (Xu et al., 2005), and activates honeybee *AmHsTRPA*. Testing *AaegTRPA1*$^{-/-}$ mutants in repellency assays would functionally test whether TRPA1 is the target of these repellents in mosquitoes.

Lastly, in addition to its roles in thermosensation and chemosensation, *TRPA1* has been implicated in other sensory processes of insects. Recent work suggests that the TRPA1-D isoform of *D. melanogaster* confers ultraviolet (UV) light sensitivity to a group of neuroendocrine cells in larvae (Guntur et al., 2015). In this process, the channel is being chemically activated by H_2O_2, which is photochemically produced by UV radiation (Guntur et al., 2015). Avoidance of UV light may also be important for mosquito larvae living in natural bodies of water exposed to sunlight.

6.2 Can heat alone drive *Ae. aegypti* blood-feeding?

We and others have found that CO_2 is a potent activator of heat-seeking in mosquitoes. But is CO_2 activation required for mosquitoes to respond to thermal host cues? The apparent CO_2-dependence of heat-seeking could be attributed to multiple plausible mechanisms. It may be that CO_2 modulates thermosensation or the valence of heat-cues in the mosquito nervous system. This would be an instance of multi-modal integration. Alternatively, mosquitoes may simply be more likely to encounter convective heat plumes due to the elevated flight activity induced by CO_2 activation (McMeniman et al., 2014).

Using the thermal response assay described earlier (**Fig 6.4**), we can monitor mosquito responses to thermal cues in the absence of a CO_2 pulse. In pilot experiments, we clearly observed that females sitting on a Peltier will probe at the surface upon warming, with no CO_2 pulse (data not shown). This suggests that elevated ambient CO_2 is not required for responses to thermal stimuli, and is

instead somehow enhancing mosquito landings onto thermal stimuli. Therefore, it is possible that mosquitoes will feed on warm blood in the absence of other host cues. It has been shown that mosquitoes will feed from a warmed artificial membrane containing blood, if they are activated by CO_2, but in this assay, mosquitoes are not necessarily in close proximity to the membrane feeder when it is warmed (McMeniman et al., 2014). It may be that CO_2-elicited flight activity is the key to this multi-modal synergy by making mosquitoes more likely to encounter the warmed membrane feeder. We have begun developing a modified version of this assay, which we have called the mini-membrane feeder (**Fig 6.5**). Here, mosquitoes are confined to a very small chamber and acutely exposed to a warmed membrane feeder. In pilot experiments, we have observed robust blood-feeding in this assay only when the blood is warmed, indicating that heat alone can drive blood-feeding when presented in close proximity (data not shown).

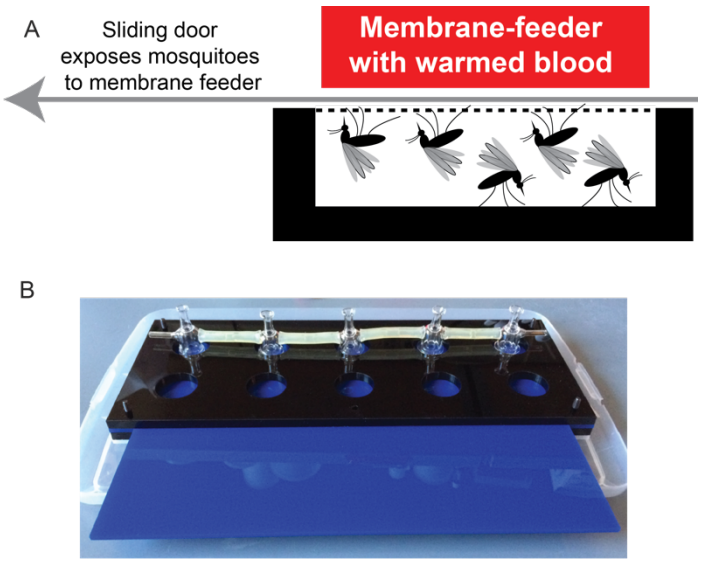

Figure 6.5 Mini-membrane feeder assay to test if blood-feeding can be driven by heat alone. (A) Side-view schematic of a single chamber of the mini-membrane feeder assay. Dashed line represents a mesh barrier. **(B)** Photograph of mini-membrane feeder showing 10 replicate chambers. Membrane feeders sit atop the back row of chambers, and the sliding door (blue) is shut.

6.3 Characterizing the thermal exposure of mosquitoes during heat-seeking

During interactions with a warm-blooded host or a 50°C Peltier, mosquitoes are likely experiencing a wide range of thermal fluctuations. Small ectothermic insects are dramatically and rapidly influenced by the temperature of the air and objects around them. As described earlier, it is estimated that the body temperature of a 10 mg fly will increase by 10°C within 10 seconds of exposure to direct sunlight (Heinrich, 1993). A heat-seeking mosquito can encounter a 10°C thermal gradient in the air within 5 mm of a 37°C stimulus or human arm (van Breugel et al., 2015). This suggests that for a mosquito standing on a warmed Peltier, the antennae, brain, thorax, forelegs, and proboscis may all be experiencing different temperatures. The temperature of mosquito tissues will also be greatly influenced by their material and geometric properties, as well as thermal conduction due to contact with the Peltier. Furthermore, convective plumes forming in the air near a vertical heated plate can be highly dynamic and turbulent in their structure, with thermal air gradients differing considerably at the bottom and top of the plate (Bejan, 2013). These features of thermal stimuli may explain why mosquitoes in our assay often appear to be differentially attracted to the bottom and top of the Peltier, as well as the differences in the behavioral responses of animals in heat-seeking assays versus thermoregulation assays. Future studies characterizing this complex thermal microenvironment, and identifying the relevant thermosensory neurons and receptors will be required to define the thermal fluctuations experienced by mosquitoes during heat-seeking.

Another question is whether mosquitoes are sensing radiant or convective heat during host-seeking. This issue has been addressed in multiple studies, but the contribution of these distinct modes of heat-transfer has not been fully determined (Clements, 1999). Many organisms have been shown to sense infrared radiation (IR), including snakes, bats, and forest-fire seeking beetles (Campbell et al., 2002). These organisms all boast specialized pit organs that transduce radiant heat into neural activity (Campbell et al., 2002), but such organs are not found in blood-feeding arthropods. However, the blood-sucking insects *T. infestans* and *R. prolixus* are be capable of responding to radiant heat without the use of pit organs (Lazzari and Núñez, 1989; Schmitz et al., 2000). This is thought to be accomplished by comparing the activity of two conventional thermoreceptors, one of which is more sensitive to IR and the other which is more sensitive to convective heat (Tichy and Zopf, 2015; Zopf et al., 2014a; 2014b).

The hypothesis that mosquitoes are responding specifically to convective heat cues has been favored since the first description of mosquito heat-seeking (Howlett, 1910). However, experiments investigating mosquito attraction to IR have typically used radiation in frequency ranges that do not match the emission spectra of mosquito hosts (Clements, 1999; Mangum and Callahan, 1968; Peterson and Brown, 1951). Human skin, for instance, emits IR with wavelengths ~8-14 μm and a peak of 10.6 μm (Villaseñor-Mora and Sanchez-Marin, 2009), so experiments testing the role of radiant heat in mosquito host-seeking must present stimuli in this biologically relevant range.

One approach to testing the importance of IR in mosquito thermotaxis is to present IR cues in the absence of convective cues. This can be accomplished by using an IR-transmitting window transparent in the wavelengths of host emission spectra (Lazzari and Núñez, 1989; Schmitz et al., 2000) (**Fig 6.6**). A thermal stimulus can be placed behind this window, and responses of mosquitoes can be observed. By making this window air-tight and actively cooling the surface from behind, conductive and convective heat cues are eliminated. A complementary approach to this problem is to reduce radiant heat cues and observe mosquito responses to only convective cues. For instance, simply applying a layer of aluminum foil to the surface of a thermal stimulus will drastically reduce the radiant emissivity of the target. If mosquito attraction to a Peltier is reduced when the surface is covered in aluminum foil, it would suggest that radiant heat is important for heat-seeking behavior.

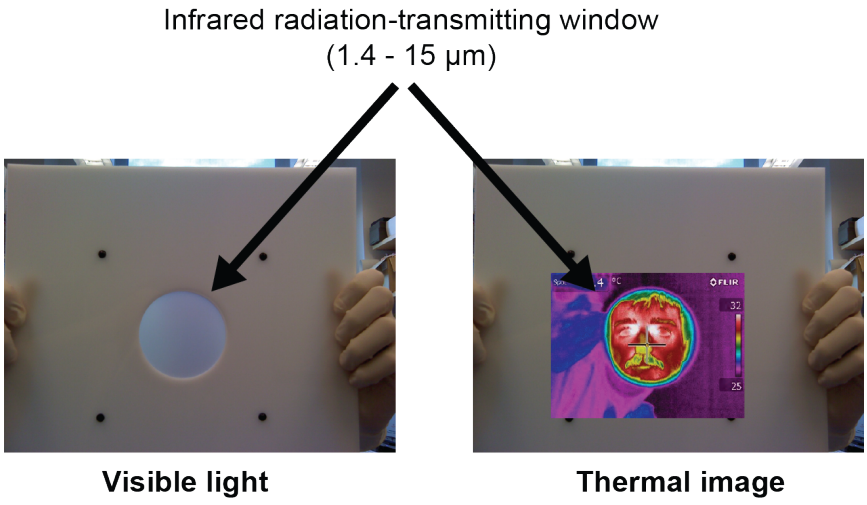

Figure 6.6 An infrared radiation (IR)-transmitting window for testing the role of radiant heat in mosquito thermotaxis. An opaque polymer window (central circle) transparent to 1.4-15 µm infrared emission is inset in a plastic frame. The window is airtight and translucent in visible light (left), but transparent to thermal radiation from a human (right). Note that conventional plastic used for the surrounding frame does not transmit IR, and instead reflects IR in this spectrum, as evidenced by the reflection of the photographer in left portion of the thermal image (right).

6.4 Designing stimuli for next-generation mosquito traps

Our characterization of the behavioral strategies underlying mosquito host-seeking thermotaxis can inform the design of next-generation mosquito traps. We have shown that mosquitoes seek thermal contrast in a variety of ambient temperature backgrounds. While mosquitoes avoid landing on thermal stimuli above ~50-55°C, they remain attracted to the area surrounding these high-temperature targets. In our data, attraction can be seen even for the highest temperature tested, 60°C, and we have seen attraction towards the vicinity of stimuli up to 80°C (data not shown). Fieldwork has shown that inclusion of thermal stimuli enhance mosquito capture rates in outdoor traps (Kline and Lemire, 1995). The heat stimulus used in this field work presented a temperature of 46°C (Kline and Lemire, 1995), but our results indicate that much hotter targets can be used to elicit mosquito attraction, as long as mosquitoes are trapped by the time they reach the stimulus. Higher temperature stimuli will presumably provide thermal cues at greater distances, perhaps enhancing capture rates further.

We also note that we have shown that the electrophile N-methylmaleimde and the bitter compound denatonium benzoate are potent anti-feedants for *Ae. aegypti.* Our modified CAFE assay can be used to identify additional anti-feedant compounds for mosquitoes of any species. Such compounds may be useful for development of future repellents or vector control strategies.

6.5 Approaches to identifying additional mosquito thermosensors

We have identified a role for *AaegTRPA1* in tuning mosquito heat-seeking away from stimuli exceeding host temperatures, but additional thermosensors must be involved in orchestrating this behavior. Firstly, the thermosensors required for initial attraction towards thermal contrast remain unknown. Our results indicate that this attraction must be mediated either by a single thermosensor that adapts to background temperature, or multiple thermosensors each tuned to a distinct absolute threshold. These thermoreceptors can in theory be activated by either cooling or warming to allow for host-seeking thermotaxis. Additionally, because *AaegTRPA1*$^{-/-}$ mutants remain inhibited from landing on targets above ~60°C, other mechanisms must exist to detect these extremely high temperature stimuli.

6.5.1 Investigating the role of *AaegGr19*

No phenotypes were discovered for *AaegGr19* in our experiments, but the high degree of conservation and wide-ranging expression pattern of this gene suggest that it may be biologically important for the mosquito (Matthews et al., 2015; McBride et al., 2007). Though preliminary data (not shown) indicates that *AaegGr19*$^{-/-}$ mutant mosquitoes show normal thermal preference in the gradient assay, it is possible that this gene contributes to mosquito thermoregulation. It is also conceivable that redundancy in the functions of *AaegTRPA1* and *AaegGr19* has masked phenotypes in *AaegGr19*$^{-/-}$ mutant mosquitoes. To this end, double-mutants of *AaegTRPA1* and *AaegGr19* should be tested in mosquito thermotaxis

assays. Additionally, given the importance of *AaegGr19* orthologs in phototransduction in nematodes and fly larvae, light detection may be impaired in *AaegGr19*$^{-/-}$ mutant larvae (Liu et al., 2010; Xiang et al., 2010), but this has not been tested to date. We must also consider that this protein may not be a direct sensor of light or heat, but rather a detector of free radicals associated with these stimuli.

6.5.2 Other known insect thermosensors may be involved in mosquito heat-seeking

Numerous genes have been implicated in insect thermosensation, aside from *TRPA1* and *Gr19* (**Fig 6.7**). This list includes many additional TRP channels. Genome editing techniques could be used to test functional roles for these genes in mosquitoes. While *DmelTRPA1* is activated above ~25-30°C and is important for larval avoidance of warmth (Rosenzweig et al., 2005), the TRPA channels *painless* and *pyrexia* are required for *D. melanogaster* to respond to more extreme heat. *painless* is expressed in the multiple dendritic neurons of the larval body, and is activated above ~43°C (Sokabe and Tominaga, 2009; Tracey et al., 2003). When exposed to a heat probe, wild-type *D. melanogaster* larvae display a stereotyped writhing behavior, but this response is abolished in *painless* mutants (Tracey et al., 2003). *pyrexia* is activated above ~40°C and helps larvae cope with heat stress that results in paralysis (Lee et al., 2005). Non-canonical thermosensors may also play a role in insect heat sensation. For instance, the visual pigment rhodopsin has been implicated in the temperature

discrimination behavior of *D. melanogaster* larvae (Shen et al., 2011), though more work needs to be done to clarify this finding (Minke and Peters, 2011).

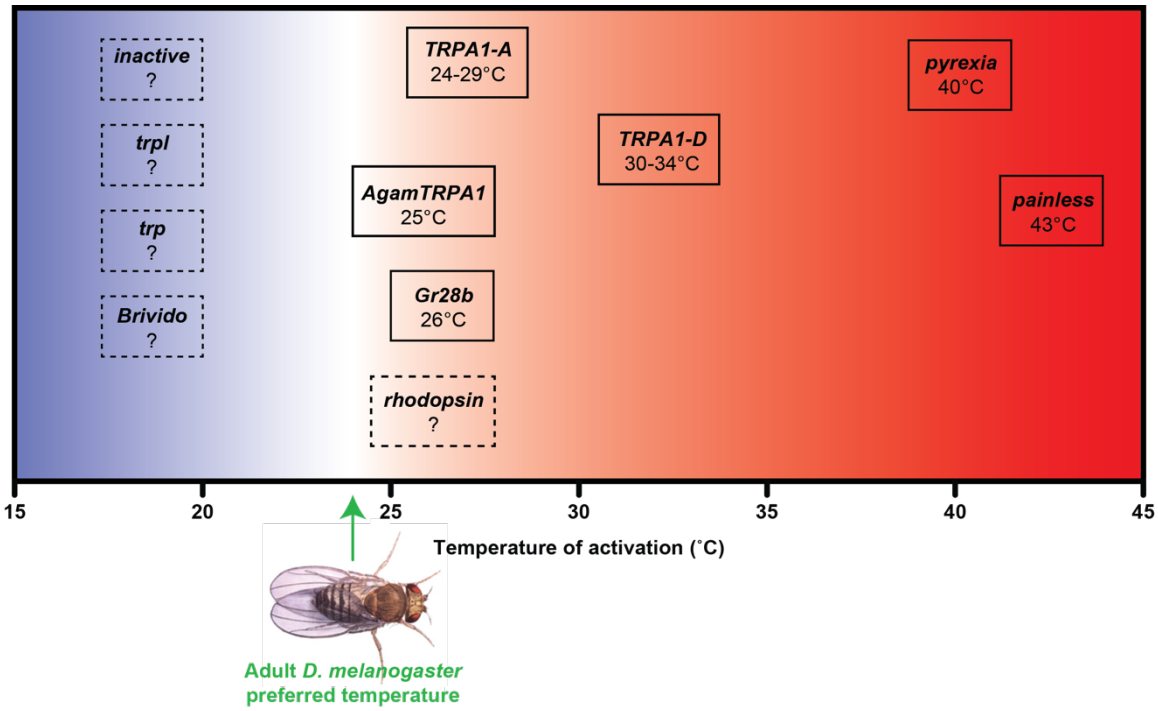

Figure 6.7 Genetics of insect thermosensation. A schematic of known insect thermosensors and their associated temperature of activation in heterologous systems or ectopic expression (solid boxes). Genes without determined activation thresholds (dashed boxes) are situated based on behavioral phenotypes in *D. melanogaster* larvae. All genes refer to *D. melanogaster* except *AgamTRPA1* from the *An. gambiae* mosquito. The approximate preferred temperature of adult *D. melanogaster* is indicated (Sayeed and Benzer, 1996). Fly image from flybase.org. Data from (Gallio et al., 2011; Hamada et al., 2008; Kwon et al., 2010b; Lee et al., 2005; Ni et al., 2013; Rosenzweig et al., 2005; 2008; Shen et al., 2011; Sokabe and Tominaga, 2009; Tracey et al., 2003).

Interestingly, recent work has shown that the warmth-seeking behavior of mammalian spermatozoa involves opsins (Pérez-Cerezales et al., 2015).

Other insect TRP channels are associated with the detection of cold temperatures, though their role as primary molecular thermosensors is unknown. In thermal gradient experiments, *D. melanogaster* larvae avoid temperatures below ~18°C and this behavior depends on the TRP channels *trp*, *trpl*, and the

TRPV channel *inactive* (Kwon et al., 2010b; Rosenzweig et al., 2008). In adult flies, detection and behavioral avoidance of cool is mediated by cool-sensitive neurons of the aristae (Gallio et al., 2011). Expression of the *Brivido* TRP channels *Brv1*, *Brv2*, and *Brv3* in these neurons is required for cool thermosensation (Gallio et al., 2011). By providing antagonistic and complementary thermosensory information, cool detection may be important in mosquito heat-seeking.

6.5.3 Tissue-specific sequencing to identify mosquito thermosensors

It is possible that mosquitoes have evolved novel molecular thermosensors, derived from genes not currently implicated in thermosensation. To use functional genetics to test the role of such genes, a list of candidates must first be generated. One approach is to identify and isolate thermosensitive tissues and to use RNA sequencing to find enriched transcripts.

The thermosensitivity of mosquito sensilla or neurons can be determined by directly testing their physiological responses to temperature. Electrophysiological single-sensillum recordings have located two thermoreceptive peg-in-pit sensilla at the distal tips of female *Ae. aegypti* antennae (Davis and Sokolove, 1975; Gingl et al., 2005). Others have noted additional *Ae. aegypti* antennal sensilla with similar morphology, indicating that they too may be thermoreceptive (Ismail, 1964; McIver, 1973). However, because thermoreceptors are found in a variety of sensillum-types, it is possible that there are even more to be identified. A comparison of female versus male

morphology may help in identifying thermosensitive tissues, since male *Ae. aegypti* do not heat-seek—perhaps this sexually dimorphic behavior can be attributed to differences in sensillar organization. For future studies, the development of new genetic tools in mosquitoes may soon allow for functional imaging to be used to identify thermosensitive sensilla. Once available, a transgenic mosquito strain neuronally expressing fluorescent calcium indicators may allow for thermoreceptors to be located on the basis of neural activity in response to temperature fluctuations.

Another approach to finding thermosensitive sensilla is to compare between mosquito species with varying thermal behaviors. For instance, the *Culex territans*, mosquito is well known to strongly prefer ectothermic hosts such as amphibians and reptiles (Crans, 1970). This dramatically different host-preference may have resulted in the absence of heat-seeking behavior, and possibly the absence of relevant thermosensors. With this in mind, transcriptomic comparison of *Ae. aegypti* and *Cu. territans* sensory tissues may yield candidate mosquito thermoreceptors. To this end, we were able to obtain wild-caught *Cu. territans* larvae from Dr. Brian Byrd (Western Carolina University) and rear them to adulthood in the laboratory. Unfortunately, we found that this species is not activated by CO_2 in the laboratory, making characterization of heat-seeking behavior impossible in our assays. Interestingly, we were able to get *Cu. territans* mosquitoes to feed on bullfrogs, provided by Dr. Jim Hudspeth (Rockefeller University), suggesting that these animals are capable of host-seeking in the

laboratory. However, we were unable to establish a laboratory colony and pursue these experiments further.

6.6 Mosquito heat-seeking as a model-system for the study of thermotaxis

Most of the molecular and neurobiological work on thermosensation has been done in organisms without specialized thermal behaviors. Much remains to be discovered about the transduction, encoding, and integration of temperature stimuli in the animal nervous system, and mosquito heat-seeking offers an excellent opportunity for furthering this field. This specialized behavior is an extraordinarily robust and sophisticated thermosensory phenomenon, which may serve as an excellent model for the study of thermotaxis.

Recent studies have begun to elucidate the coding of temperature information in the *D. melanogaster* nervous system. The aristae of the fly have two distinct groups of thermosensory cells with complementary receptive fields (Gallio et al., 2011). Warm cells are excited by increasing temperature and inhibited by decreasing temperature, and cold cells exhibit the opposite behavior (Gallio et al., 2011). These cells all project to an area of the fly brain called the posterior antennal lobe (PAL), where their pattern of innervation generates a thermotopic map in which warm cells and cold cells project to distinct adjacent glomeruli (Gallio et al., 2011). A set of second-order thermosensory projection neurons (tPNs) have dendrites in the PAL and mainly innervate three higher brain centers: the mushroom body, the lateral horn, and the posterior lateral protocerebrum (PLP) (Frank et al., 2015). Some tPNs respond specifically to

warmth, and others respond only to cool (Frank et al., 2015; Liu et al., 2015). The warmth-activated tPNs receive crossover inhibition from cold-activated tPNs, which can allow for more robust and sensitive detection of temperature fluctuation (Liu et al., 2015). Surprisingly, a certain set of tPNs respond to both warming and cooling, and are important in mediating behavioral avoidance of both hot and cold (Frank et al., 2015). Among tPNs, some show fast-adapting responses to thermal stimuli, which serve to temporally track rapid thermal fluctuations, while other tPNs are slow-adapting, and are thought to encode the absolute magnitude of thermal fluctuations (Frank et al., 2015). Together, these data begin to describe the neural circuitry underlying thermosensation in an insect.

It will be interesting to discover whether mosquito thermosensation displays a similar organizational scheme as that seen in flies, and whether this temperature information is integrated with other host-cues, such as CO_2, odor, or visual information. Integration of thermal and olfactory information is known to occur in insects. For instance, the cockroach *Periplaneta americana* has thermosensory neurons in the antennal lobe which receive input from peripheral cold receptors as well as olfactory sensory cells (Zeiner and Tichy, 2000). In these cells, simultaneous presentation of thermal and olfactory stimuli generate increased neural responses, forming the basis of multimodal sensory integration (Zeiner and Tichy, 2000). It is conceivable that a similar system exists in mosquitoes, by which the co-occurrence of thermal and non-thermal host cues results in enhanced neural responses to stimuli and a robust behavioral output.

MATERIALS AND METHODS

Mosquito Rearing and Maintenance. *Ae. aegypti* wild-type (*Orlando*), *AaegGr19*, and *AaegTRPA1* mutant strains were maintained and reared at 25-28°C and 70-80% relative humidity with a photoperiod of 14 hr light:10 hr dark (lights on at 8 a.m.) as previously described (DeGennaro et al., 2013). Adult mosquitoes were provided constant access to 10% sucrose solution for feeding, and females were provided with a blood source for egg production. Before behavioral assays, mosquitoes were sexed and sorted under cold anesthesia (4°C) and fasted for 15-24 hr in the presence of a water source. All laboratory blood-feeding procedures were approved and monitored by The Rockefeller University Institutional Animal Care and Use Committee protocols 14756 and 11487 and Institutional Review Board protocol LV-0652.

ZFN-mediated targeted Mutagenesis. PCR was carried out using Novagen KOD polymerase (EMD Millipore), products were cloned using pCR4-TOPO (Invitrogen), and Sanger sequenced by Genewiz.

ZFN Design: ZFNs targeting *AaegTRPA1* or *AaegGr19* (VectorBase Accession numbers AAEL009419 and AAEL011073, respectively) were designed and produced by the CompoZr Custom ZFN Service (Sigma-Aldrich). The nucleotide sequences of the ZFN-binding sites (upper case) and nonspecific cut site for wild-type heterodimeric Fok1 endonuclease (lower case) are:

AaegTRPA1: 5’- GTCGTTTTCGTCCATACCgatgtcGTTGCTTAGGACGTT-3’.

AaegGr19: 5’-ACCAACCTTTCACTGCaaatgacCCACCGGAAAGTGGCA-3’.

Homologous Recombination Design: Donor plasmids were generated as previously described (McMeniman et al., 2014). All donor plasmids included homologous arms cloned from wild-type genomic DNA, and consisting of sequence flanking or partially overlapping the ZFN recognition sites.

AaegTRPA1 was targeted using *pSL1180-HR-PUbECFP-TRPA1*. The left homologous arm (1608 bp, primers: forward, 5’-GCATGCATGGGTAACAAGAAGGGTTGT-3’ and reverse, 5’-CGACAAGTGGTTTACTTGTGTGTCAATC-3’) and right homologous arm (3037 bp, primers: forward, 5’-CGTTTTCCATGATGCTCGGC-3’ and reverse, 5’-CGAAGACCAACGCGATGTAGTTCCA-3’) were cloned into the EcoRI and NotI sites of *pSL1180-HR-PUbECFP* (Addgene #47917), respectively.

AaegGr19 was targeted using *pSL1180-HR-PUBdsRED-Gr19*. The left homologous arm (1257 bp, primers: forward, 5’-AGGTATGCCTGGATTGGACGTAAGAAA-3’ and reverse, 5’-GCAGTGAAAGGTTGGTTAACTG-3’) and right homologous arm (1203 bp, primers: forward, 5’-CCACCGGAAAGTGGCATTTACCGC-3’ and reverse, 5’-GCGACGGTCCCTTGCGATGTCGTTAT-3’) were cloned into the XmaI and NotI sites of *pSL1180-HR-PUBdsRED* (Addgene #49327), respectively.

Generation of Mutant Lines: To generate *AaegTRPA1* homologous recombination mutant alleles, 2000 pre-blastoderm stage wild-type embryos were microinjected (Genetic Services Inc.) with *AaegTRPA1* ZFN mRNA (200 ng/µl) and *pSL1180-HR-PUbECFP-TRPA1* (750 ng/µl). To generate *AaegGr19* homologous recombination mutant alleles, 1000 pre-blastoderm stage wild-type embryos were microinjected (Insect Transformation Facility at the University of Maryland) with *AaegGr19* ZFN mRNA (200 ng/µl) and *pSL1180-HR-PUBdsRED-Gr19* (700 ng/µl). Injected G0 animals were crossed to wild-type in multiple batches to generate G1 lines with independent ZFN mutagenesis events. G1 homologous recombination mutant individuals were recovered via fluorescence as previously described (McMeniman et al., 2014), and outcrossed separately to wild-type for 5 generations before establishing four independent homozygous lines: $AaegTRPA1^{ECFP\text{-}1}$, $AaegTRPA1^{ECFP\text{-}2}$, $AaegGr19^{dsRED\text{-}1}$, and $AaegGr19^{dsRED\text{-}2}$.

To confirm directed insertion of the *PUbECFPnls-SV40* cassette into the *AaegTRPA1* locus, a diagnostic PCR product (no wild-type band, 2075 bp mutant band) was amplified using a forward primer anchored outside the boundary of the left homologous arm (5'-CATGGGACAATTTGGCGTAGGCAGTAT-3') and an ECFP reverse primer anchored inside the inserted cassette (5'-AGATCTCGACCCAAGAAAAAGCGGAAG-3'). To establish homozygous $AaegTRPA1^{-/-}$ lines, a diagnostic PCR product (679 bp wild-type band, 3305 bp mutant band) spanning the ZFN cut-site was amplified using primers: forward, 5-

GGTTTCAAGGATGATTGACACACAAG-3', and reverse, 5'-GCAGAGCTGATTTCTCGTAGTTTTCG-3'.

To confirm directed insertion of the *PUBdsRED-SV40* cassette into the *AaegGr19* locus, a diagnostic PCR product (6362 bp wild-type band, 8787 bp mutant band) was amplified using primers anchored outside the boundaries of both homologous arms: forward, 5'-AGCTGATCAACGTTAACAACTACGATG-3', and reverse, 5'-AGAGCATGGTGTAAACTTGACAGCTCAA-3'.

The following lines were used for all experiments: *AaegTRPA1*$^{ECFP\text{-}1/ECFP\text{-}2}$, *AaegTRPA1*$^{ECFP\text{-}1/+}$, *AaegGr19*$^{dsRED\text{-}1/dsRED\text{-}2}$, *AaegGr19*$^{dsRED\text{-}1/+}$. Heteroallelic mutants were used to minimize fitness effects that might be present in homozygous mutants.

Behavioral Assays. All behavioral assays were carried out during the hours of ZT2-ZT12 at 26°C and 70-80% relative humidity unless stated otherwise. Whenever possible, time of day was randomized across conditions. All mosquitoes used were 10-21 day-old females, age-matched across conditions and genotypes.

Heat-seeking Assay: For each experiment with single stimulus periods, each 15-min trial began with the introduction of 20-25 mosquitoes into a custom-made Plexiglass box (30 x 30 x 30 cm). Mosquitoes were given 5 minutes to acclimate. Two Peltier elements (6 x 9 cm surface area; Tellurex) located on opposite walls of the enclosure, and covered with white paper and white plastic mesh, were

used to present heat stimuli. During acclimation, both Peltiers were kept at ambient temperature (26˚C) via controllers (Oven Industries) commanded by a custom MATLAB script. Throughout the trial, carbon-filtered air was pumped into the box via a diffusion pad (59-144, Flystuff.com) installed on the ceiling of the enclosure. After acclimation, 10% CO_2 was added to the air stream for 20 sec via a solenoid valve (Parker-Hannifin) to increase CO_2 concentration 1000 ppm above background levels. At this time, one Peltier was warmed to 37˚C for 5 min and subsequently cooled to ambient for the remaining 5 min of the trial. The side of the heated Peltier was randomized across trials. Mosquito landings at the Peltier were monitored by fixed cameras (FFMV-03M2M-CS, Point Grey Research) acquiring images at 1Hz. Images were analyzed using custom MATLAB scripts to count heat-seeking mosquitoes within a fixed target region comprising a 12 cm x 16 cm area around the center of the Peltier.

For experiments with multiple stimulus periods, assays were performed as described above, with the following changes. All stimulus periods lasted 3 minutes, and were presented on a single Peltier element (6 x 9 cm) covered with a piece of standard white letter size printer paper (extra bright, Navigator) cut to 15 x 17 cm and held taut by a magnetic frame. CO_2 pulses (20 sec) accompanied all stimulus period onsets. A second identical control Peltier element was situated on the wall opposite to the stimulus Peltier, and was set to ambient temperature during all experiments. Peltier temperature set-point is reported in all experiments. When instantaneous temperature of the Peltier is reported, these measurements were taken with a thermocouple embedded in the

Peltier element. For each trial, 45-50 mosquitoes were introduced into the assay, and only mosquitoes directly on the Peltier area during seconds 90-180 of stimulus periods were scored. Heat maps are smoothed 2D histograms of mean mosquito occupancy during seconds 90-180 of stimulus periods, sampled at 1 Hz and binned into 12 x16 image sectors.

Heat-seeking Choice Assay: The heat-seeking choice assay was modified from the heat-seeking assay described above. For each trial, 45-50 mosquitoes were introduced into a custom-made Plexiglas box (38.5 x 28 x 12.5 cm) with two Peltier elements (4.6 x 6.5 cm surface area, ATP-O40-12, Custom Thermoelectric) each covered with a taut piece of standard white letter size printer paper (extra bright, Navigator) cut to 9 x 11cm. The Peltier elements were mounted on a single wall, 3.5 cm above the floor, 8.5 cm from either edge of the enclosure, and adjacent to one another separated by 6.5 cm. Mosquitoes were allowed to acclimate for 10 minutes, at which point the Peltier elements were both warmed to 40°C. After fully warming the Peltier elements for 60 seconds, a CO_2 pulse (20 sec) was added to the airstream and the Peltier elements were kept at 40°C for a 4-minute stimulus period at which point they were returned to ambient temperature (26°C). After a 12-minute interstimulus period, the stimulus was repeated, this time with the left Peltier element warmed to 50°C while the right Peltier element was warmed to 40°C. A camera (Point Grey Research) was used to measure mosquito landings, and only mosquitoes directly on either Peltier area during seconds 60-240 of stimulus periods were scored. Preference

was calculated by subtracting the proportion of total mosquitoes on the left Peltier (40 or 50°C) from the proportion of total mosquitoes on the right Peltier (40°C). Heat maps are smoothed 2D histograms of mean mosquito occupancy during seconds 60-240 of stimulus periods, sampled at 1 Hz and binned into 15 x 52 image sectors.

Capillary Feeder (CAFE) Assay: The mosquito capillary feeder (CAFE) assay was adapted from a similar assay used for *Drosophila* (Ja et al., 2007). For each trial, 5 mosquitoes were fasted with access to water for 24 hours, and placed in a polypropylene vial (#89092-742, VWR) with access to two 5 µl calibrated glass capillaries (#53432-706, VWR) containing 10 % (w : v) sucrose with 5 % (v : v) green food dye (McCormick). Capillaries spaced ~0.5 cm apart traversed the cotton plug (#49-101, Genesee Scientific) of the vial and protruded into the vial < 1 mm to provide a flush surface for mosquitoes to access the liquid while resting on the plug surface. The control capillary contained only green sucrose solution, and the experimental capillary contained green sucrose solution supplemented with 0, 1, or 10 mM of denatonium benzoate (Sigma-Aldrich) or 0, 10, 50, or 100 mM of N-methylmaleimide (Sigma-Aldrich). The experimental capillary with 0 mM chemical was identical to the control capillary, and served as a zero-choice to test for side-bias in the assay. Because a small amount of liquid evaporated during preparation of the capillaries, all choice conditions and genotypes were prepared in a time-staggered format so that any measurement error due to evaporation was spread across the conditions. The levels of remaining liquid in

both capillaries were measured after 18-20 hours and were compared to the known initial liquid level. Experiments started at ZT 8-10 and ended at ZT 4-6 the following day. Consumption values were compared to control capillaries in vials without mosquitoes to account for evaporation. We note that in cases where mosquitoes did not feed from a capillary and all liquid loss was due to evaporation, consumption values could be calculated to be negative due to very small variation in evaporation rates between experimental and control capillaries. Any negative consumption values were rounded to zero. Sucrose preference was calculated by dividing the amount consumed from the control capillary (not containing denatonium benzoate or N-methylmaleimide) by the total amount consumed from both capillaries. In experiments with 0 mM denatonium benzoate or 0 mM N-methylmaleimide, one capillary was arbitrarily chosen as "sucrose only."

Thermal Gradient Assay: The thermal gradient assay was adapted from a similar assay used for *Drosophila* (Hamada et al., 2008; Sayeed and Benzer, 1996). A custom-built enclosure (6 mm tall) was affixed to an aluminum thermal gradient bar (50 x 30.5 cm, TGB-5030, ThermoElectric Cooling America Corp.) driven by two Peltier Elements (AHP-1200CPV, ThermoElectric Cooling America Corp.). The enclosure was separated lengthwise into 4 lanes (each 50 x 6 cm) that were visually isolated from one another. Three lanes were for testing mosquito thermal preference, while the fourth lane was dedicated to measuring air temperature via an array of 8 digital temperature sensors (DS18B20, Maxim, connected to an

Arduino Uno) mounted to the top of the enclosure and distributed evenly across the length of the lane and centered in each analysis sector. An overhead camera (C910, Logitech) monitored mosquito position through the transparent lid of the enclosure. Images were acquired once per minute and analyzed using custom MATLAB scripts to count mosquitoes across 8 analysis sectors of the lane. The assay was conducted in a room maintained at 80-90% relative humidity and 14°C in order to achieve low air temperatures within the gradient enclosure. At the beginning of each 3-hour trial, the air temperature throughout the enclosure was stabilized at ~26°C, and 25-30 mosquitoes were introduced into each lane of the assay. After 90 minutes, a thermal gradient was established (air temperatures: ~19°C to ~36°C) by heating the right Peltier element and cooling the left Peltier element. Mosquitoes were monitored for an additional 90 minutes while exposed to this thermal gradient. Mosquito distributions during minutes 60-90 were monitored in both the “no thermal gradient” and “thermal gradient” conditions. Dead mosquitoes were visually identified at the conclusion of each trial. All genotypes were tested in parallel, and their lane positions were randomized across trials.

Thermal Images. All thermal images were acquired with an infrared camera (E60, FLIR Systems).

Statistical analysis. All statistical analyses were performed using Prism 5 software (GraphPad).

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