Characterising Behavioural Thermoregulation in the Bearded Dragon: The Role of TRPM8

by

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Abstract

Temperature regulation is a necessary part of maintaining life, as most biological processes are influenced by temperature. ThermoTRP channels are considered the primary thermosensors in endotherms, but little is known regarding their function in ectotherms. The goal of this study is to establish TRPM8, a cold sensing channel, as a participant in normal thermoregulation of the bearded dragon (*Pogona vitticeps*), an ectotherm. Animals were placed inside a ramping temperature shuttle box to assess the common behavioural thermoregulatory strategy of shuttling. Shuttling involves the periodic movement between cold and warm environments to maintain body temperature at moderate levels. The temperatures for cold and warm escapes represent sensory thresholds for inducing the shuttling thermoeffector. Animals were administered with: 1) an injection of the TRM8 antagonist capsazepine, 2) an injection of the TRPM8 agonist menthol, and 3) menthol applied topically. No effect was observed with injected drugs, but topical menthol resulted in a 2-3ºC rise in the ambient temperature threshold and 1-2ºC rise in skin temperature threshold for escape from the cold compartment. In an additional experiment, gaping behaviour, a warm temperature thermoregulatory strategy, was assessed. No effect was observed in this behaviour when the same dose of menthol was applied topically. These results point to a role for TRPM8 only in thermoregulation as it relates to cold temperature sensation in lizards, since it does not participate in regulating warm temperature behaviours such as gaping.

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Chapter 1: Introduction

Thermal Optimality

Temperature is critical for life. The kinetic energy of a system changes in tandem with temperature changes, affecting the rates of essential biological processes. Additionally, hot or cold temperature extremes can have immediate negative consequences (*i.e.* freezing or protein denaturation) on an organism's ability to survive. Biological processes generally have an optimal temperature (T_{opt}), at which performance is maximal (P_{max}), and critical thermal maximum (CT_{max}) and minimum (CT_{min}) temperatures, above and below which the process does not function at all (Figure 1; Angilletta, 2009). Performance breadth is another aspect of these criteria, and is quantified as the temperature range across which a certain percentage of maximal performance occurs (conventionally 95% or 80%, depending on the study; Angilletta, 2009).

Thermal optimality curves exist as a result of the physiological limitations brought on by increasing or decreasing temperature. Lowering the temperature limits the energy available for chemical reactions to occur, below the required threshold, the activation energy. At a certain level, known as the Arrhenius breaking point, a reaction is unable to proceed regardless of the amount of energy available because an insufficient number of molecules have achieved activation energy (Tattersall et al., 2012). The energy limitation that occurs at low temperatures results in the CT_{min} of the performance curve (Tattersall et al., 2012). From the CT_{max} perspective, at a certain threshold, adding energy to a reaction will not increase the rate of that reaction. As temperatures continue to increase, various contributing factors begin to inhibit a biological process, including protein denaturation (Somero, 1995) and changes in membrane viscosity outside of a functional state (Angilletta, 2009).

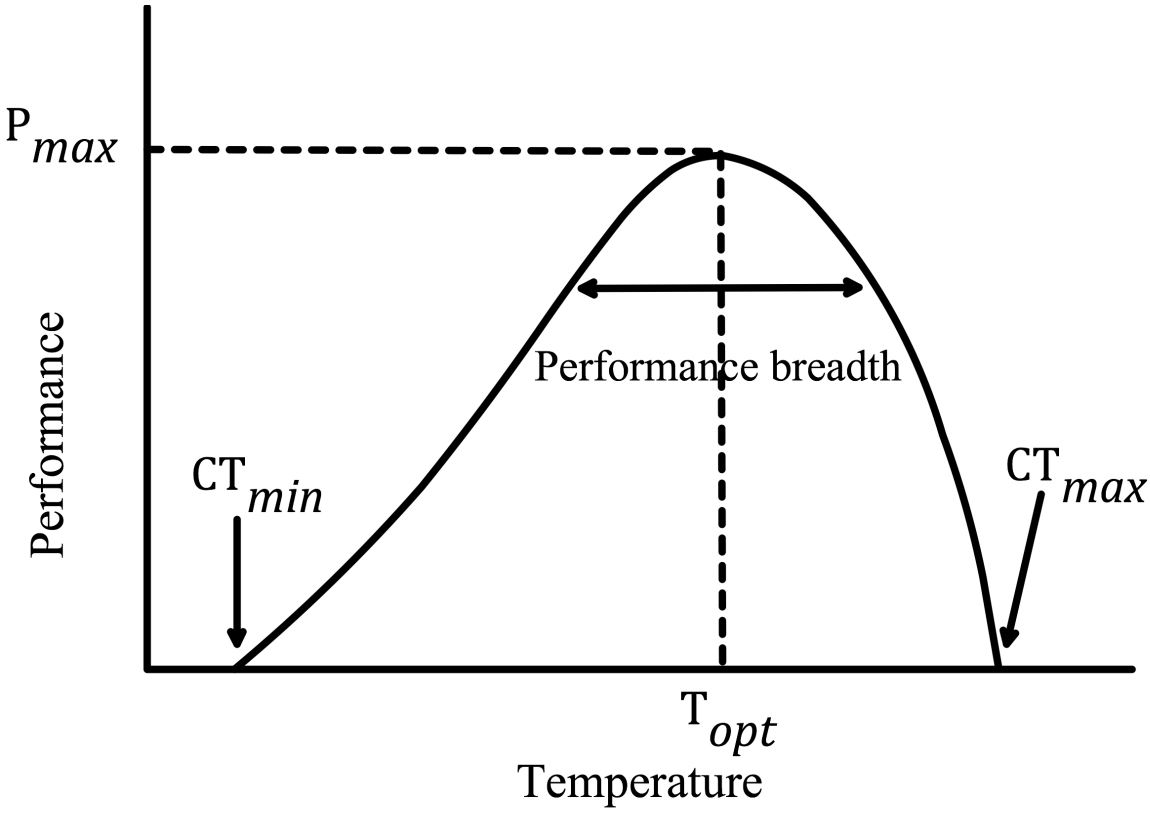

Figure 1: Thermal Optimality Curve

Generalized optimality curve that can be used to describe any temperature sensitive biological process. T_{opt} (optimal temperature) is the temperature at which P_{max} (maximum performance) occurs. Performance breadth is the temperature range across which a certain percentage of maximal performance occurs. Performance breadth can be defined as anywhere from 80-95% of maximal performance depending on the study. CT_{max} (critical thermal maximum) is the maximum temperature at which any performance occurs. CT_{min} (critical thermal minimum) is the minimum temperature at which any performance occurs. No performance occurs above and below the CT_{max} and CT_{min}, respectively. Adapted from Angilletta (2009).

It is important for animals to possess the physiological capacity to maintain temperature within an acceptable performance breadth for survival. Optimal temperature can be quantified for processes at the cellular (e.g. enzyme reaction speed, membrane viscosity), systemic (e.g. digestion, reproduction, sprint speed), or whole animal (e.g. survivorship, growth rate, fecundity) levels (Angilletta, 2009). Performance breadth can be relatively wide, with the trade-off being a relatively low maximum performance (thermal generalist; Figure 2). Alternatively, performance breadth can be narrow, with a relatively high maximum performance (thermal specialist; Figure 2).

The breadth of temperature environments over which life exists has led to the evolutionary adaptation of different optimal temperatures for essential life processes in different species. The large variation in optimal temperatures and thermal habitats makes it impossible to apply a single model across all species, however a trend can be identified whereby the characteristics of thermal optimality correspond to an organism's thermal environment (Angilletta, 2009). For example, the level of performance in animals living at low or high temperatures is maximal in those habitats, but lower at moderate temperatures (Van Berkum, 1988; Franklin, 1998; Wilson et al., 2001). Likewise, many studies have shown a trend in survivorship between populations of the same species living at different latitudes or altitudes, whereby the temperature of an animal's habitat is also its optimal temperature environment for maximal survivorship (Angilletta et al., 2004). Other studies are more conflicted. A pair of studies by Merila et al. (2000) and Stahlberg et al. (2001) on the frog *Rana temporaria* showed opposing results. The latter found that frogs from cold habitats survived better at lower temperatures compared to higher temperatures; the former found that frogs from cold habitats survived better at higher temperatures compared to lower temperatures.

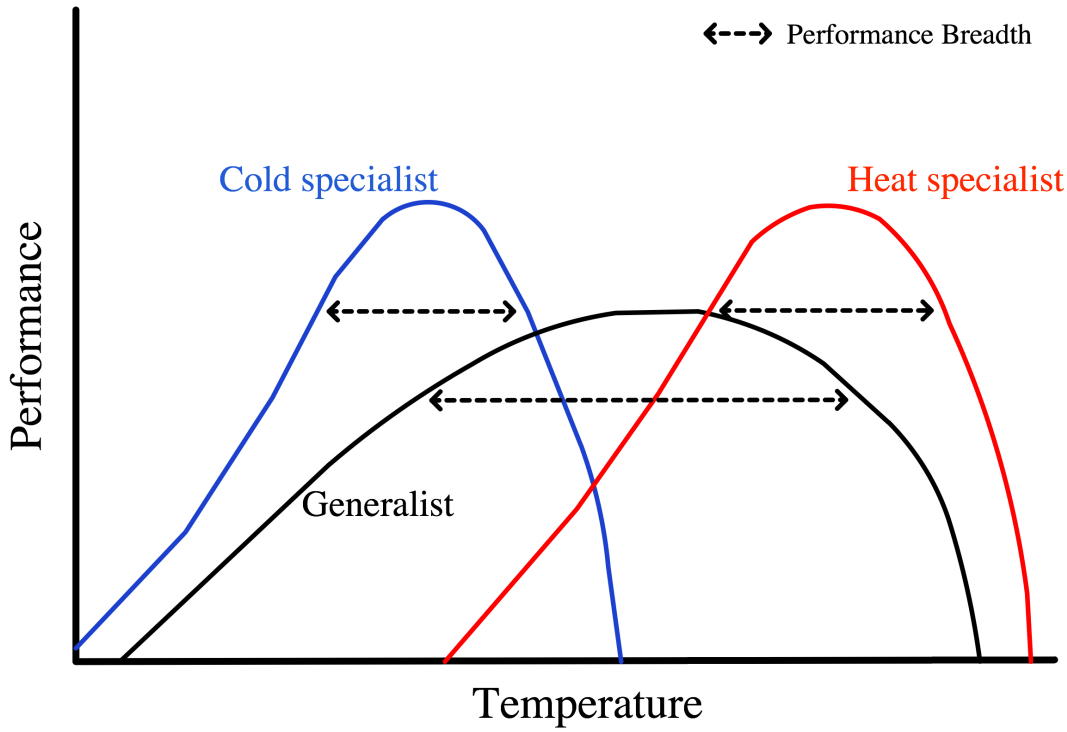

Figure 2: Thermal Optimality Profiles

Optimality curves can be generally categorized into two groups, thermal generalist and thermal specialist. A process with a thermal generalist profile is characterized by a wide performance breadth, with the trade-off being relatively low maximal performance. A process with a thermal specialist profile is characterized by a relatively small performance breadth, but very high maximal performance. Adapted from Angilletta (2009).

Survivorship is, however, only one parameter over which temperature shows influence. In contrast to survivorship, thermal optima of sprint speeds, specifically in lizards, corresponds directly with average body temperature in the field. Additionally, CT_{max} changes proportionately with optimal temperature in lizards, but CT_{min} remains relatively constant across species (Huey and Kingsolver, 1993). This correlation suggests the lower threshold for sprinting is limited by the energy required to perform the respective physiological processes associated with this system. In contrast, the optimal temperature and upper threshold for sprinting have room for evolutionary modification or specific acclimation of a species. Interestingly this does not apply to locomotion of amphibians (*i.e.* swim stroke speed and jumping), which display a specialist-generalist trade-off (Wilson, 2001; Navas, 2006). Species that demonstrate lower CT_{min}, resulting in a wider performance breadth, also have a relatively lower maximal stroke speed (i.e. P_{max}) while swimming.

At the level of cellular processes, a number of ubiquitously expressed enzymes have different isoforms which function optimally at different temperatures, allowing animals in different thermal habitats to perform equally well. For example, lactate dehydrogenase (LDH), which contributes to swimming performance of the mangrove killifish (*Fundulus heteroclitus*), has one isoform that performs optimally above 25ºC and another that performs most efficiently below 25ºC (Powers and Schulte, 1998). Expression patterns of these isoforms correspond to the species' wide distribution along the eastern North American coast (from Newfoundland, Canada to Florida, USA), across which exists large thermal variability. Fish occupying colder habitats primarily express the cold-optimized isoform, while fish living in warmer habitats express primarily the warm-optimized isoform (Powers and Schulte, 1998). Overall, patterns of thermal optimality vary greatly across species, making it very difficult to apply generalized models for

the evolution of thermal optimality curves. There are only a few instances where such models can be applied (e.g. thermal optimality increasing or decreasing proportionately with thermal habitat) and extensive research is still required to determine the appropriateness of available models. Ultimately, organisms function best over a specific range of temperatures and are therefore required to maintain themselves within those temperatures to ensure proper function.

Thermoregulation is the process whereby an organism maintains temperature homeostasis (Angilletta, 2009). As discussed above, an organism must maintain its body within a restricted temperature range for its biological process to function optimally, and it is through thermoregulation that body temperature is maintained within this required range. Thermoregulation can occur via physiological or behavioural mechanisms. Animals possessing the capacity to generate their own heat are deemed endothermic, while animals that cannot, and obtain heat by exploiting environmental temperature, are deemed ectothermic. Generally speaking, endothermic animals thermoregulate primarily via physiological mechanisms, while ectotherms use primarily behavioural mechanisms. Physiological mechanisms of thermoregulation include sweating, shivering thermogenesis (heat production through muscle contraction), non-shivering thermogenesis (brown adipose tissue [BAT]), and vaso-dilation/constriction (increase or decrease heat exchange with the environment; Romanovsky, 2007). Behavioural mechanisms include changes in posture (exploit radiative heat), regulation of activity time (diurnal versus nocturnal activity), and shade/sun seeking behaviour (Bicego et al., 2007). Physiological mechanisms are beneficial because they can be recruited regardless of the external environment, however the trade-off is the relatively high metabolic cost required to maintain heat production.

Behavioural thermoregulation has lower metabolic cost than metabolic heat production (Bennet and Ruben, 1979), but is constrained by an animal's need to perform other tasks throughout the day (e.g. feeding, predator avoidance), which limits their ability to focus solely on thermoregulation in choosing their environment (Huey and Slatkin, 1976). Endotherms often rely on behavioural thermoregulation as a result of these trade-offs, as it reduces metabolic requirements, is faster, and more powerful in many cases than modifying metabolic heat production (Bennet and Ruben, 1979). Regardless of the mechanisms, all organisms rely on thermosensors to monitor internal and peripheral temperature and use the thermal information to recruit the appropriate thermoeffector response (Bicego et al., 2007). The ability to monitor and distinguish temperature is essential in order to carry out an appropriate thermoregulatory response. Organisms use thermosensation to assess their environment and seek out favourable environmental temperatures while avoiding unfavourable ones (Angilletta, 2009). The interplay between optimal temperature, thermoregulation, and thermosensation suggests a shared co-evolution whereby the sensitivity of an organism's thermosensors correspond to their temperature requirements to achieve optimal performance and excel in their environment.

Interplay of Thermosensors and Thermoeffectors

All biological processes are temperature sensitive, and neural signalling of sensory organs is no exception. To produce an action potential through a neuron, ion channels must undergo a physical change to open and allow a flow of ions to pass across the cell membrane. This change is essentially a chemical reaction, and the laws of thermodynamics are such that the rate of a chemical reaction is dependent on temperature (Martinac, 2008). As such, firing rates of all

sensory neurons have varying degrees of temperature sensitivity. Additionally, temperature influences the membrane potential of a neuron as outlined by the Nernst equation:

$$E = \left(\frac{RT}{zF}\right) log \left(\frac{[ion]_o}{[ion]_i}\right)$$

Where E is the equilibrium potential of a given ion, R is the ideal gas constant, T is temperature (Kelvin), z is the charge of the ion, F is Faraday's constant, $[ion]_o$ is the ion concentration outside the neuron, and $[ion]_i$ is the ion concentration inside the neuron. Based on this equation, temperature alters the resting membrane potential of a cell, as a function of changing diffusion rate (proportional to 'RT'). Although this effect is very small, a change in membrane potential can influence the firing rate of a neuron.

Since all neuronal signalling is temperature sensitive, we must differentiate between a change in firing rate due to thermodynamics, and a change in firing rate as the result of thermosensation (*i.e.* a thermosensor). The opening and closing of most ion channels is characterized by a Q_{10} of 2-4, but the gating of thermoTRP (transient receptor potential) ion channels, the molecular drivers of thermosensation (Caterina, 2007), have been demonstrated to be greater than 20 for warm sensitive neurons (Liedtke and Heller, 2007; Martinac, 2008) and less than 0.5 for cold sensitive neurons (Boulant and Dean, 1986), depending on experimental conditions. Thermosensory neurons are thus defined by the large increase in the rate of action potential firing that occurs with changes in temperature, which far exceeds the effect of temperature on other sensory neurons.

Thermosensation relies on temperature sensitive neurons in the periphery and core to detect temperature and relay the information to sites of thermal integration in the brain (primarily the

hypothalamus) so that necessary thermoregulatory action can be taken. Peripheral sensors are those distributed across the skin surface, while core sensors include parts of the brain, spinal cord, and viscera (Bicego et al., 2006; Morrison and Nakamura, 2011). The DRG and TG (dorsal root ganglion and trigeminal ganglion) are paired bundles of neuron cell bodies situated down the length of the spinal cord (many pairs) and the brain (single pair), respectively (Smith, 2000). The axons of some neurons in these ganglion innervate the skin of the periphery (among other tissues); the body in the case of the DRG and the head in the case of the TG (Figure 3). In the periphery, cool and warm temperatures increase the firing rate of free nerve endings of the DRG or TG, which are deemed cool and warm-sensitive fibers (Hensel and Zotterman, 1951a; Boulant and Hardy, 1974; Zhang et al., 1995). Cool temperature stimulus is relayed from the periphery by lightly myelinated Aδ-fibers, while slower signaling, unmyelinated C-fibers relay warm stimulus (Smith, 2000). These two fibers are classified as innocuous sensors as they relay thermal information in the moderate temperature range. A different group of fibers are nociceptive sensors, which respond to noxious stimuli and are responsible for the sensation of thermal pain (extreme hot or cold) (Patapoutian, 2009). All sensory information is subsequently relayed to parts of the central nervous system. Depending on their point of origin, signals travel directly to parts of the brain, such as the hypothalamus, or are first relayed through the spinal cord. Extensive research has shown that the pre-optic area of the hypothalamus (POA) in the brain is itself thermosensitive (Romanovsky, 2007; Morrison and Nakamura, 2011). Evidence suggests that contrary to the periphery, warm sensors are the main driver within the POA, with neurons exhibiting a resting firing rate that increases with warm temperature and decreases with cold temperature (Boulant and Hardy, 1974; Zhang et al., 1995; Morrison and Nakamura, 2011). Additionally, the POA is a central location for integrating thermosensory input coming directly

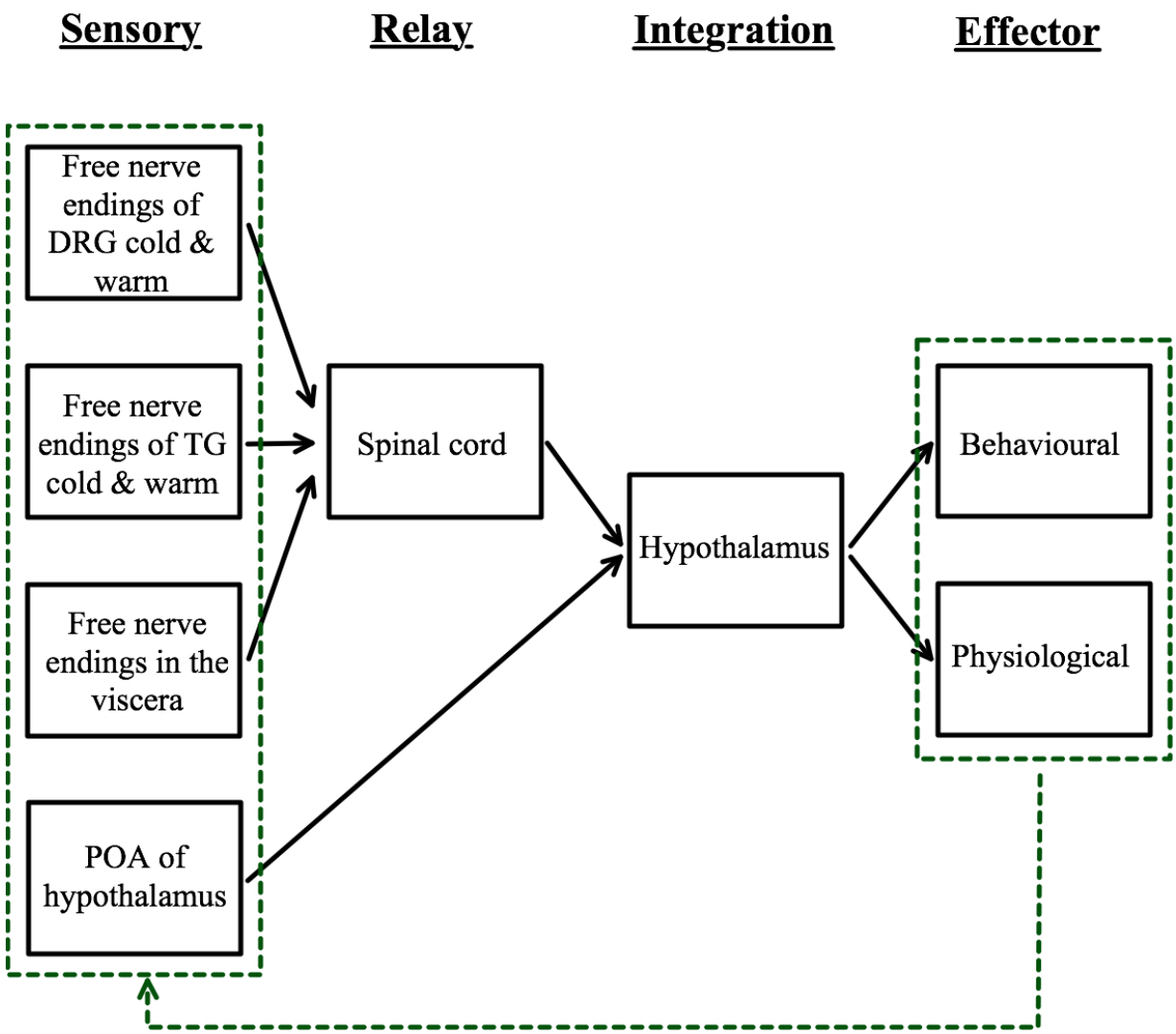

Figure 3: Schematic of Structures Involved in Thermosensation

Thermosensory information is first detected by temperature sensitive nerve endings in the periphery, core, or brain. Depending on the location of signals, information is relayed in the feed forward manner to the spinal cord and subsequently to integration sites in the hypothalamus. Thermoeffectors are recruited by signals from the hypothalamus. The type of thermoeffector recruited is determined by location of incoming thermal information, magnitude, and physiological state of the animal. Behaviour and physiological thermoregulation result in a change of environment or internal temperature, thus acting as a feedback mechanism, which changes the temperature environment of thermosensors.

from thermosensitive areas of the body or transmitted through the spinal cord via the DRG and TG (Romanovsky, 2007). Thermal information is subsequently relayed to thermoeffectors (behavioural and/or physiological), which elicit thermoregulatory action (Figure 3; Griffin, 2004).

Thermoeffectors can be divided into those that are physiological processes and those that are behavioural responses (Romanovsky, 2007). Extensive research has explored which thermosensory input induces the recruitment of specific thermoeffector responses. For endotherms, physiological means of thermoregulation can be recruited without any core thermosensory input. Nakamura and Morrison (2007) showed that acute skin temperature cooling in rats elicits a rapid physiological response in the form of brown adipose tissue (BAT) recruitment and tachycardia (heart rate increase). These responses occur without any changes to core or brain temperature via efferent signals from the hypothalamus. It must be noted that these animals were anesthetized, limiting the availability of all thermoregulatory mechanisms, however the results still highlight the importance that the periphery plays in thermosensation. There exists an ongoing debate over whether peripheral or core thermosensory input is more important in terms of an animal's ability to thermoregulate (Griffin, 2004; Bratincsak and Palkovits, 2005; Bicego et al., 2006; Morrison and Nakamura, 2011). Evidence from unanesthetized rats shows that low ambient temperatures elicit a slight rise or no effect on core and brain temperatures, strengthening the argument for the importance of peripheral sensation (Bratincsak and Palkovits, 2005; Morrison and Nakamura, 2011). These findings do not discount the existence or importance of core and brain thermosensation. Direct warming of the hypothalamus elicits sweating, cutaneous vasodilation, and heat retention behaviours, while direct cooling elicits shivering, cutaneous vasoconstriction, and heat loss behaviours (Boulant,

2000; Griffin, 2004; Bicego et al., 2006). Localized thermal stimulation of the brain does not, however, represent an animal's normal state, in which the periphery is exposed to supra or sub-optimal temperatures before they can influence core and brain temperature.

Behavioural thermoregulation is subject to environmental temperatures, which by extension relies on peripheral sensors to detect environmental conditions. In addition, behavioural mechanisms can be immediate and not limited by metabolic constraints (*i.e.* seeking shade or sun is a more efficient and immediate defence against temperature change than physiological mechanisms). This increased efficiency and immediacy results in situations where peripheral sensation, coupled with behaviour, can counteract any changes in core and brain temperature before they occur (Flouris, 2010). In practice, mammals use a combination of behaviour and physiology to maintain body temperature in a narrow range. In addition, the use of physiology allows for a thermoneutral zone (TNZ), a range of environmental temperatures within which an endotherm does not need to increase metabolism in order to maintain their desired body temperature. In contrast, the core temperature (brain and body) of reptiles, such as lizards, traces the pattern of environmental temperature change more closely than endotherms, despite strong and often precise behavioural thermoregulatory mechanisms (Berk and Heath, 1975; Cadena and Tattersall, 2009). Mammals and reptiles display a difference in the recruitment of thermoregulatory mechanisms. The larger temperature ranges experienced by reptiles compared to mammals, may necessitate differences in the integration of thermal information.

Thermosensation in mammals has been more extensively researched than in reptiles. Much like in mammals, however, the hypothalamus in reptiles plays an important role in directing thermoregulatory behaviour from thermosensory information and is itself thermally

sensitive. Lesioning of the hypothalamus and adjacent areas compromises normal thermoregulatory behaviour in the desert iguana, *Dipsosaurus dorsalis* (Berk and Heath, 1976). Depending on the area lesioned, animals displayed varying magnitudes of change in the following behavioural thermoregulatory characteristics: decreased propensity to thermoregulate, higher maximum temperature and lower minimum temperature experienced, greater variability in upper and lower thresholds for thermoregulation, and in some cases no change. Cabanac et al. (1967) showed that the hypothalamus of the blue-tongued skink lizard (*Tiliqua scincoides*) contains both warm and cold sensitive neurons, which dramatically increase their firing rate in response to temperatures of greater than and less than 25ºC, respectively. Additional work with the blue-tongued skink showed that heating or cooling the hypothalamus induces changes in escape temperature from warm environments (Hammel et al., 1967). Under normal conditions, lizards will shuttle between a cold (15ºC) and hot (45ºC) environment to maintain colonic (defined as body) temperature between approximately 30-37ºC. When the brain was heated, lizards exited the hot side at significantly lower colonic and skin temperatures, and when cooled, they exited at higher temperatures (Hammel et al., 1967). These studies show that the hypothalamus of lizards is temperature sensitive and capable of overriding other thermal input (*i.e.* body and skin) to some degree. However, in the blue-tongued skink at least, the hypothalamus is not all controlling, as direct heating and cooling does not immediately result in a behavioural response (Hammel et al., 1967). Temperature changes in the hypothalamus had the effect of shifting the skin and colonic temperature thresholds which elicit shuttling behaviour, suggesting integration of thermal stimuli is more important than signals from thermosensitive units in the hypothalamus alone (Hammel et al., 1967). A major difference between lizards and the studies performed in mammals is that brain temperature changes by as much as 5ºC

throughout normal shuttling in the lizard while such large variations are rarely observed in mammals. Such a phenomenon may require different levels of thermosensitivity between reptiles and mammals to account for differences in the thermal environment of the brain. Whether peripheral thermosensation operates under similar conditions to the hypothalamus (*i.e.* shifting thresholds for shuttling or working unilaterally) is not known, as very little research has been done on this topic (Bicego et al., 2006).

Thermoregulation in Reptiles

Thermoregulation in reptiles is of great interest from both an ecological perspective with respect to predicting thermophysiological responses to climate change, as well as from an evolutionary perspective in understanding how endothermy/ectothermy may have evolved in vertebrates. Reptiles have the ability to regulate a relatively constant body temperature, typically higher than their surrounding environment. Thermoregulation in reptiles is primarily achieved through careful exploitation of temporal and spatial gradients in temperature in the environment (Bogert, 1948; Seebacher and Franklin, 2005). Selection of a proper habitat (living in a thermally favourable environment) is an important first step for maintaining optimal temperature. Other mechanisms, which also appear in many ectotherm groups, include posture and regulation of activity times. Posture allows animals to alter the amount of heat absorbed from the sun or substrate, while regulating activity times allows them to be active only at certain times of day when they are more likely to encounter optimal temperatures in the environment.

Ectothermy, in general, means that an animal must rely on a repertoire of behavioural strategies to maintain an optimal body temperature, with limited capacity for physiological

mechanisms (Seebacher and Franklin, 2005). Physiology still plays a role, with adjustments in heart rate and peripheral circulation often having a profound impact on the rate of body temperature change, however its effectiveness is highly reduced compared to that of endothermic animals (Seebacher and Franklin, 2005). Furthermore, in the absence of any capacity to produce endogenous heat, ectotherms generally equilibrate with their surrounding environmental temperatures, although body size does influence the rate at which this equilibration occurs. As discussed earlier, physiological, behavioural, and ecological processes are all influenced by temperature, with many displaying a narrow range of temperatures in which they function optimally (Hutchison and Dupré, 1992; Peterson et al., 1993). In reptiles, selection has acted on thermoregulatory effectors (mainly behavioural), resulting in a method to maintain optimal temperature in their environments.

The most effective behavioural strategy employed by reptiles is that of shuttling, defined as movement between hot and cold environments (Cowles and Bogert, 1944; Bogert, 1949). Under normal conditions, optimal temperature is unlikely to be found in the environment, and so animals will move between suboptimal areas (e.g. sun and shade) to maintain body temperature in a moderate range (Cowles and Bogert, 1944; Bogert, 1949). In controlled laboratory experiments using lizards, shuttling behaviour is very defined, with animals moving back and forth regularly between areas of high and low temperature to maintain body temperature at a moderate value between both extremes (Berk and Heath, 1975; Barber and Crawford, 1979; Cadena and Tattersall, 2009). There also exists a relatively high degree of predictability within species in terms of threshold temperatures (ambient, body, skin, brain) that elicit movement from one compartment to the other. Quantifying shuttling behaviour in the field is more problematic, as it is difficult to differentiate between thermoregulatory behaviour and other behaviours (*e.g.*

predator avoidance, hunting, and others; Heath, 1964; Ribeiro et al., 2007). It is these other behaviours that make it impractical for animals to behave as they do in the lab. Animals must perform many other functions to survive day to day, which precludes being able to thermoregulate continuously. Additionally, seasonal variation and general weather trends preclude the occurrence of constant temperature environments such as those used in laboratory studies. Nevertheless, lizards in the wild do demonstrate similar behaviours as those in the lab, such as basking followed by shade seeking leading to maintenance of body temperature in a relatively narrow range (Cowles and Bogert 1944; Bogert, 1949; Heath, 1964; McGinnis and Dickson, 1967; Ribeiro et al., 2007).

In addition to shuttling, some reptiles display a behavioural ventilatory response similar to panting known as gaping. Gaping for the purpose of thermoregulation is most prevalent in lizards, but also displayed by crocodilians (Tattersall et al., 2006). By opening the mouth in hot temperatures, evaporative heat loss leads to cooling of the head, potentially preventing the brain from reaching lethal temperatures (Spotila et al., 1977; Tattersall et al., 2006). The added cooling allows lizards in particular to spend longer periods basking before needing to move to shaded areas. Tattersall and Gerlach (2005) showed that the bearded dragon lizard increased gaping and decreased head temperature compared to body temperature, at high ambient temperatures. Furthermore, lizards generally exhibit cooler head temperature at high ambient temperatures (Tattersall et al., 2006), supporting a role for ventilatory evaporation as a thermoregulatory process.

Physiological mechanisms of thermoregulation available to reptiles are typically limited to those involving circulatory and heart rate adjustments (Bartholomew and Tucker, 1963; Seebacher and Franklin, 2005). Much like endotherms, many reptiles undergo vasoconstriction

or vasodilatation of peripheral blood vessels in response to temperature stress (Grigg et al., 1979; Seebacher and Franklin, 2005). In reptiles, these changes are followed by increasing and decreasing heart rate, known as heart rate hysteresis (Seebacher and Franklin, 2005). Change in heart rate is often incorrectly identified as an initial response to temperature change (Seebacher and Franklin, 2005), when in reality it is a result of circulatory adjustments. The discovery that reptiles undergo heart rate changes preceded the characterising of circulatory adjustments, which potentially led to the widespread mis-association of cause and effect (Bartholomew and Tucker, 1963; Grigg et al., 1979). Vasodilation allows for greater heat dissipation with the environment, while vasoconstriction allows heat retention (Romanovsky et al, 2002). Experimental evidence suggests these processes contribute directly to the rate of heating and cooling in lizards (Bartholomew and Tucker, 1963; Grigg et al., 1979; Seebacher, 2000; Seebacher and Franklin, 2005). Heat retention is especially important in the absence of any capacity for producing heat endogenously (Seebacher and Franklin, 2005). The thermal inertia observed in species of basking lizards can be traced to the capacity for the animals to retain body heat during cooling (Bartholomew and Tucker, 1963; Seebacher, 2000). Although these capabilities are ultimately very limited, they help by ensuring that more energetically costly behavioural mechanisms need not be recruited for fine-tuned temperature control (Seebacher and Franklin, 2005). These circulatory adjustments may also be used in conjunction with behaviours such as shuttling, to finely tune temperature regulation.

Characterizing Lizard Behavioural Thermoregulation

Two primary control models have been proposed to describe behavioural thermoregulatory mechanisms in lizards: proportional control and on-off control (Heath, 1970). Proportional control applies to behaviours whose intensity is determined by the level of thermal strain an individual can endure. Examples include gaping behaviour and postural changes (Heath, 1970; Gerlach and Tattersall, 2005). In terms of gaping, as ambient temperature increases so does the amount of time a lizard will spend gaping. Likewise, the size of the animal's gape is proportional to the ambient temperature above a threshold response temperature (Gerlach and Tattersall, 2005), suggesting that the effort involved in gaping produces a proportional degree of evaporative cooling to offset the higher heat load at elevated temperatures.

Shuttling behaviour typifies the on-off control model (Heath, 1970). Lizards will bask in direct sunlight until temperature (either peripheral or core) reaches a certain threshold, at which point they will move to the shade or water. This effectively turns off the warming behaviour and turns on cooling behaviour. Once temperatures reach a lower threshold during cooling, behavioural thermoeffectors for warm seeking behaviour are triggered and the animal returns to basking.

In addition to being an example of the on-off model, further studies have determined that shuttling behaviour is indicative of a dual threshold thermoregulatory system (Barber and Crawford, 1977). Such a system has been described in a number of lizard species (Barber and Crawford, 1979; Van Berkum et al., 1986; Cadena and Tattersall, 2009). Shuttling is characterised by an upper and lower temperature threshold, above and below which thermosensory information signals thermoeffectors to elicit thermoregulatory behaviour in the

opposing direction (Barber and Crawford, 1977). These two thresholds can be linked to the relevant cold and warm sensor in the periphery and core, which are responsible for detecting the lower and upper threshold, respectively. Since lizards do not have the ability to produce endogenous heat, and temperature is dependent on environmental conditions, these two thresholds can be described for skin, body or brain temperature (Berk and Heath, 1975; Barber and Crawford, 1979). While not identical to one another (*i.e.* thermal inertia of core temperature), these temperature categories are generally proportional and under the direct influence of ambient temperature. In lizards, the dual-threshold system results in regulation of body temperature in a range of several degrees. Within this range, between the two thresholds, very little energy is expended for thermoregulatory behaviour. This is known as the refractory zone (Heath, 1970). The existence of this zone allows animals to perform vital life functions (feeding, reproduction, predator avoidance) without the need to thermoregulate constantly. It is expected that this refractory zone would correspond to the optimal temperature ranges described by Angilletta (2009), although this has not been directly established in reptiles.

A model proposed by Mitchell et al. (1970) integrates well with the existence of a dual threshold system and current thermosensory research. This model suggests a process whereby the balance of incoming warm and cold sensory signals determines the appropriate thermoregulatory action for an animal to take. Research in lizards has already shown that artificial manipulation of one group of thermosensors (*i.e.*, warming or cooling the hypothalamus) does not have an immediate behavioural effect but rather shifts the threshold of other sensors that do induce thermoregulatory behaviours (Hammel et al., 1967). This model also serves as an alternative to the concept of set-points, which dominated the field for many years. In the set-point model, a deviation in body temperature away from an animal's 'set-point'

elicits thermoregulatory response in the opposite direction. However, the concept of a set-point has become somewhat of an analogy, especially in reptiles where temperatures fluctuate over a wide range (Barber and Crawford, 1977).

ThermoTRP Channels

Our understanding of thermosensory mechanisms was recently changed in a fundamental way by the discovery of thermoTRP (transient receptor potential) channels (Caterina, 2007). The opening and closing rate of many ion channels are influenced by temperature, but the thermoTRPs are unique in that they open in direct response to temperature changes (Patapoutian et al., 2003). ThermoTRP channels are members of the large TRP group of primarily non-selective cation channels. Each TRP protein possesses six transmembrane domains with an extracellular loop connecting the fifth and sixth domain. A functioning channel is composed of four proteins connected at the extracellular loop to produce a pore through which cations travel (Flockerzi and Nilius, 2007). The most widely accepted model for channel gating is one involving temperature-dependent voltage gating, which involves a shift of the voltage activation curve towards physiological voltage in response to temperature; a specific temperature range changes a channel's voltage-activation to the voltage of the cell (thus opening), and outside of these temperatures the voltage-activation is different from that of the cell (thus remaining closed; Liedtke and Heller, 2007; Martinac, 2008). There remains significant debate over whether this model applies in all cases or if other mechanisms are involved (Yao et al., 2010).

The thermoTRP channels have been established as the molecular drivers that impart temperature sensitivity to temperature-sensitive neurons (Caterina, 2007). Previously, neurons

were deemed as being either only hot or cold sensitive (Boulant and Hardy, 1974; Zhang, et al., 1995), but with the discovery of thermoTRPs, a new component of thermosensory research was possible. The thermoTRP channels are sensitive to a range of both noxious (extreme) and innocuous (moderate) temperatures. The role of noxious temperature sensitive thermoTRP channels in thermoregulation remains unclear (Caterina, 2007; Gavva, 2008; Romanovsky et al., 2009). In contrast, the role of innocuous temperature sensitive thermoTRP channels in thermoregulation has been well established, although not to the exclusion of other sensory processes (Patapoutian et al., 2003; Caterina, 2007).

Six vertebrate thermoTRPs have been implicated in thermosensation of some form and can be categorized as warm activated (TRPV1-4) and cold activated (TRPM8 and TRPA1), or as noxious (TRPV1-2, TRPA1) and innocuous (TRPV3-4, TRPM8). In addition, three other channels (TRPM2, 4, and 5) are implicated in temperature sensitivity of non-thermoregulatory processes (e.g. sweet taste sensation; Figure 4). These channels are ubiquitously expressed across all vertebrate groups and many invertebrates, however the categories as described above only apply definitively to mammals, with some groups showing specific alterations in the properties of their thermoTRP channels.

TRPV1 was the first thermoTRP to be described. It was long known that humans are sensitive to the chemical capsaicin, found in hot chili peppers, but the method whereby it causes hot, burning pain remained unknown (Toh et al., 1955; Caterina et al., 1997) More recently, it was discovered that a specific capsaicin receptor ion channel exists, dubbed TRPV1 (Caterina et al., 1997). In addition to its chemical sensitivity, it is also activated by temperatures of greater than 42ºC and is therefore deemed a noxious heat receptor (Caterina et al., 1997; Caterina, 2007).

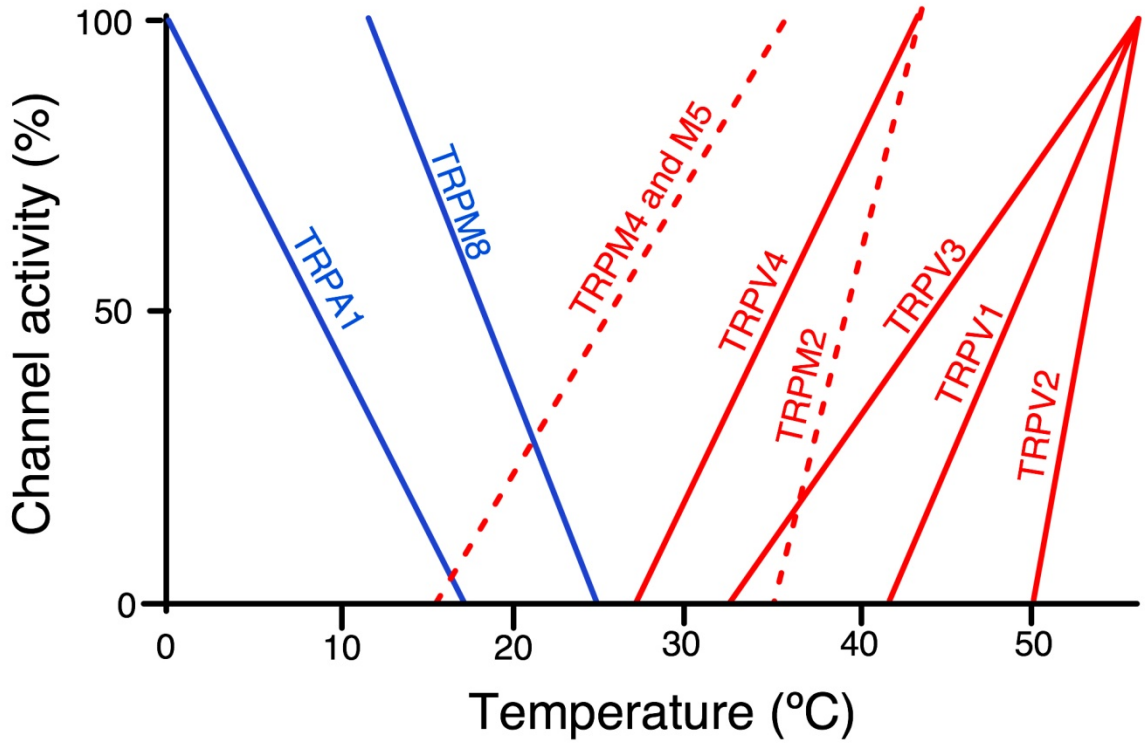

Figure 4: Activation Profile of the ThermoTRP Channels

Values are means from several studies, expressing channels in heterologous systems. Dashed lines represent the TRPM channels that contribute to the temperature sensitivity of taste. These profiles represent very specific conditions for mammalian channels only. They do not necessarily represent the channels' behaviour *in vivo*. Additionally, although significant research exists to support these activations, there is also emerging evidence to the contrary, especially as it relates to TRPV3 and TRPV4 (Huang et al., 2011). Adapted from Romanovsky (2007).

It is expressed primarily in free nerve endings of the DRG and TG, and to a lesser degree, in and around the hypothalamus (Cavanaugh et al., 2011). Owing to its high activation temperature, this channel has generally been disregarded as contributing to thermoregulation, as its activation is outside of normal body temperature ranges and specific to painful temperature stimuli (Romanovsky et al., 2009). In spite of this, many studies have found that activation and blocking of this channel induces hypothermia (drop in body temperature below normal) and hyperthermia (rise in body temperature above normal), respectively, leaving the exact classification of TRPV1 under some debate (Gavva, 2008).

Recent research into TRPV3 and TRPV4, two innocuous receptors (with activation thresholds of greater than 33ºC and 27ºC, respectively), has been more conclusive in determining that these channels play a role in peripheral thermosensation (Guler et al., 2002; Dhaka et al., 2006; Wechselberger et al., 2006; Caterina, 2007). These channels are expressed in the hypothalamus, sensory neurons, and uniquely in skin keratinocytes of rats and mice (Guler et al., 2002; Peier et al., 2002; Xu et al., 2002; Chung et al., 2004). Moqrich et al. (2005) demonstrated that TRPV3 knock out (KO) mice display an impaired ability to sense noxious and innocuous heat. Wild-type mice show a preference for 35ºC versus 22ºC in a two temperature choice box, whereas TRPV3KO mice show no preference for either temperature (Figure 5A). Similarly, when placed in a thermal gradient, wild-type mice will spend the majority of time within a range of 32-36ºC over an hour, whereas TRPV3KO mice show no preference for any particular floor temperature across the gradient over the same time period (Figure 5B). The onset of nociceptive behaviours was somewhat delayed in these 2KO mice at temperatures greater than 50ºC. These results point to a thermoregulatory function for TRPV3 in allowing mammals to select proper

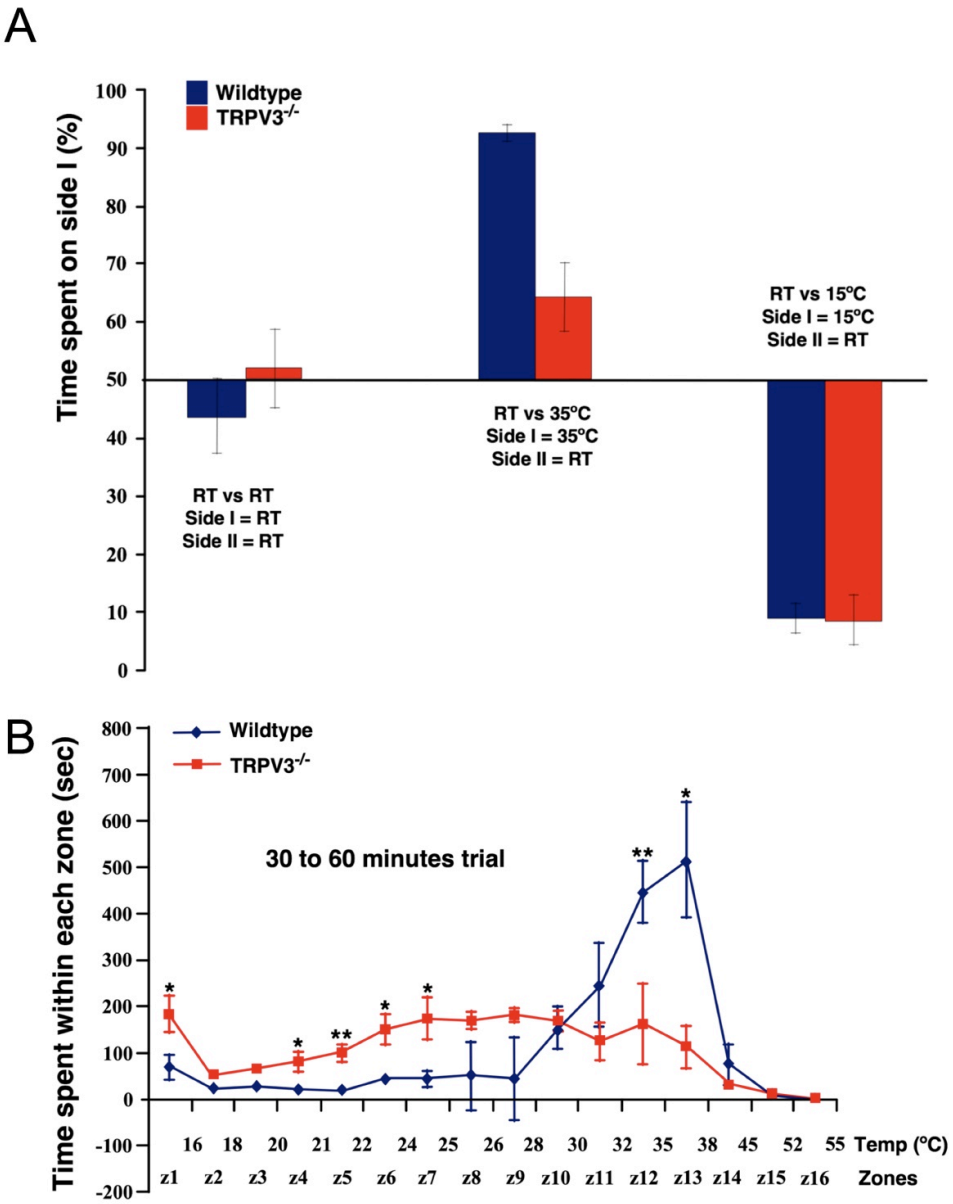

Figure 5: Behavioural Thermoregulation in TRPV3KO Mice

A) Mice were placed in a two temperature choice compartment for 10 minutes. They were free to move between both sides. Three tests were used, room temperature (RT), RT versus 35ºC, and RT versus 15ºC. TRPV3KO mice displayed deficits in sensing the 35ºC compartment. B) Mice were pace in a thermal gradient and allowed to explore freely for two hours. From 30-60 min into the experiment and onward, wild-type mice showed a clear preference of approximately 36ºC, while TRPV3KO mice did not. Adapted from Moqrich et al (2005)

ambient temperature environments, as well as some function in noxious temperature sensation (Moqrich et al., 2005).

A similar experiment by Lee et al (2005) showed TRPV4KO mice modify thermoregulatory behaviour, albeit in a manner distinct from TRPV3KO mice. Wild-type mice spent the majority of time within the 32-36ºC range of a thermal gradient, while TRPV4KO mice settled into a range of 22-32ºC over the same period of time (Lee et al., 2005). In a two-temperature choice situation, Wild-type mice showed no preference between 30ºC and 34ºC, while TRPV4KO's spent significantly more time at the higher temperature. When given a choice between 34ºC and 36ºC however, both groups showed preference for 34ºC of equal magnitude (approximately 80% of time spent), indicating not all warm thermosensory ability is abolished. These results suggest another mechanism is acting as a thermosensor, allowing mice to distinguish temperature greater than 34ºC in the absence of TRPV4 expression. In addition, no difference in physiological thermoregulation was observed between TRPV4KO and wild-type mice. Circadian changes in core temperature proceeded normally in both groups, and body temperature was likewise identical when mice were exposed to a 35ºC or 25ºC ambient environment (Lee et al., 2005). Taken on their own, the results of Lee et al. (2005) show TRPV4 to be a component of thermosensation and behavioural thermoregulation in mice, but with a role distinct from that of TRPV3. Taken together, the TRPV4 and TRPV3 studies highlight a combined role of different thermoTRP channels in normal thermosensation. The TRPV4KO experiment in particular suggests that other peripheral thermal sensors are being recruited to compensate for the lack of TRPV4 action in behavioural thermoregulation.

Lee et al. (2005) did not significantly explore whether abolishing TRPV4 alters thermal sensation of the core or modifies an animal's ability to thermoregulate physiologically. A single

experiment exposed wild-type and TRPV4KO mice to room temperature (25ºC), or 35ºC for two hours followed immediately by 25ºC. No difference was observed between the treatment groups under either condition, providing some evidence that TRPV4 does not contribute to a mouse's ability to detect and maintain core temperature at high temperatures, but rather its role is limited to peripheral sensation. To better explore this concept, it would be beneficial to monitor body temperature throughout the behavioural experiments, to determine if TRPV4KO animals (compared to wild-type) compensate for their altered environmental temperature using non-thermotaxis strategies.

Experiments using TRPV3 and TRPV4 KO mice have been well documented in the literature and used to validate the hypothesis that thermoTRPs contribute to thermal sensation. However, more recent research by Huang et al (2011) revisited a number of earlier KO experiments and their results call into question these conclusions. TRPV3, TRPV4, or both simultaneously, were abolished in mice followed by temperature gradient and two-temperature choice assays. In general, none of the modified mice differed in innocuous temperature sensation or thermal preference compared to their wild-type counterparts. The researchers conclude that other mechanisms may be involved in thermal sensation in the absence of TRPV3 and TRPV4. Furthermore, they highlight the potential effect of methodological differences between studies, as the same mice strains and researchers were involved in the Moqrich et al (2005) work which showed a significant difference in TRPV3KO mice.

As with the warm sensors, the discovery of two cold sensing thermoTRP channels, TRPM8 and TRPA1, have led to a new understanding for how cold sensation occurs. TRPM8 is an innocuous cold receptor activated by temperatures of approximately less than 28ºC and is also known as the menthol receptor, which imparts the sensation of cooling with activation by the

tastant, menthol (Hensel and Zotterman, 1951; McKemy et al., 2002; Peier et al., 2002). TRPA1, in contrast, is considered a noxious cold receptor in mammals with an activation temperature of less than 10ºC and is co-expressed in peripheral nociceptive neurons expressing TRPV1 (Sawada et al., 2007). TRPM8 mRNA is expressed in free nerve endings of the DRG and TG (distinct from those expressing TRPV1 and TRPA1), which, coupled with its innocuous activation range, has led to interest in its participation in peripheral thermosensation (McKemy et al., 2002; Peier et al., 2002; Caterina, 2007). TRPM8KO studies have established an important role for TRPM8 in peripheral thermosensation (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Much like previous TRP channel experiments, knockout models coupled with a thermal gradient and two temperature choice experiments are the predominant means for assessing thermosensation. TRPM8KO mice showed increased time spent at colder temperatures in a thermal gradient and likewise when given a choice between 22ºC and 26ºC (Dhaka et al., 2007). The same was found when choices of 25ºC versus 30ºC, 15ºC versus 30ºC, and 5ºC versus 45ºC (Figure 6) were presented (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). The ability of these mice to differentiate between warm environments was unchanged, as was nociceptive behaviour, which was assessed by exposure to -1ºC (Colburn et al., 2007; Dhaka et al., 2007). These results point to TRPM8 as being the primary means by which peripheral, cold sensory information is relayed to the brain to elicit behavioural thermoregulation.

Further research suggests that the role of TRPM8 extends to the control of physiological thermoregulation as well. Almeida et al. (2012) showed that pharmacological blocking of TRPM8 in rats attenuates physiological cold responses, including shivering, non-shivering thermogenesis, and vasoconstriction. Drugs were administered intravenously, so this does not specifically address whether or not peripheral TRPM8 can elicit these effects. Peripheral

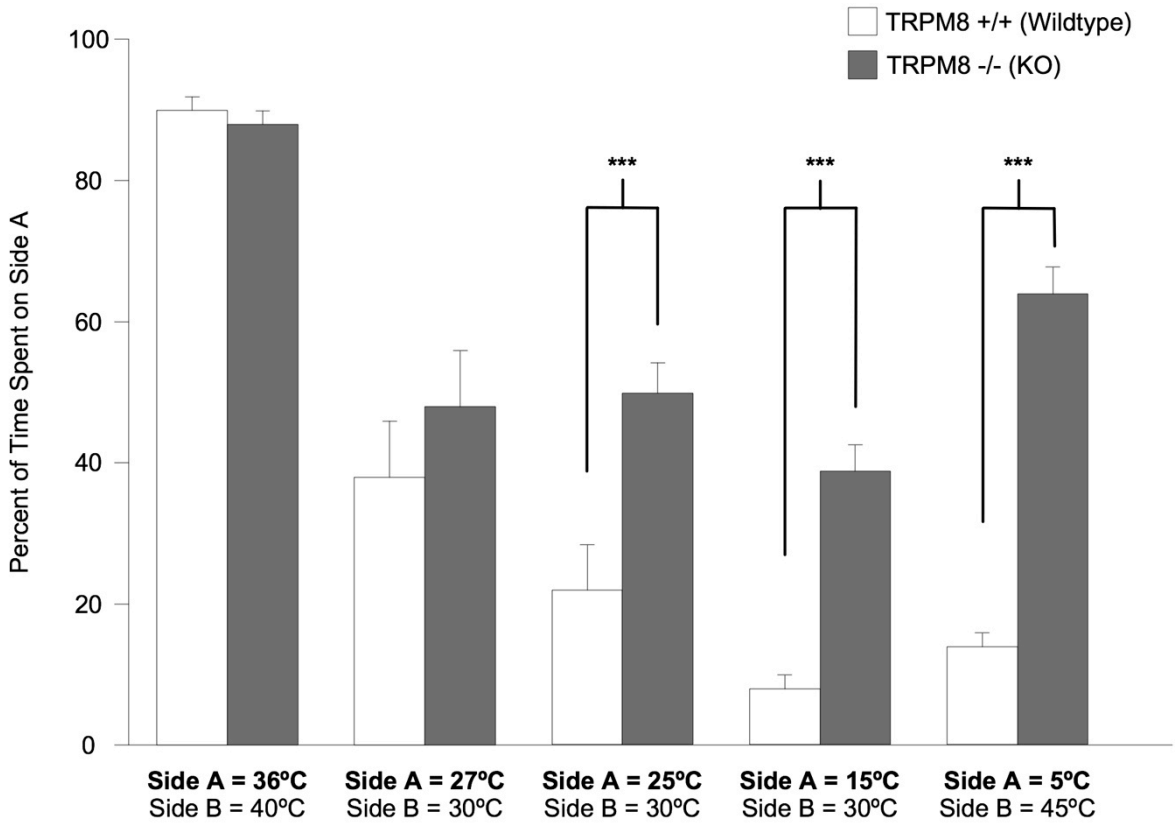

Figure 6: TRPM8 Two-Temperature Choice

Combination of three papers (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007), showing two temperature choice preferences of TRPM8 mice. KO animals and wildtype animals were placed in a two-compartment box, each with a different ambient temperature. TRPM8KO mice show an inability to properly identify and avoid cool temperatures. Avoidance of very cold temperatures (e.g. 5ºC) was not affected. *** indicates significant difference between wildtype and TRPM8KO ($p<0.001$). Leftmost data is from Dhaka et al (2007); middle three data are from Bautista et al (2007); rightmost data is from Colburn et al (2007).

application of the TRPM8 agonist menthol in rats and mice does however elicit a rise in core body temperature (likely through activating heat production responses), in addition to warm-seeking behaviour (Tajino et al., 2007; Klein et al., 2010). This is evidence for the contribution of TRPM8 sensory information in regulating both behavioural and physiological thermoregulation in endotherms.

Research into the thermoregulatory role of thermoTRP channels in vertebrate ectotherms is scarce. What little there is suggests a strongly conserved role for these channels across all vertebrate groups (Saito and Shingai, 2006). Expression of the six thermoTRP channels has been determined in fish (Saito and Shingai, 2006), amphibian (Saito and Shingai, 2006; Meyers et al., 2009; Saito et al., 2011), and reptilian (Seebacher and Murray, 2007; Nagai et al., 2012) species, but few studies have focused on the thermoregulatory function of these channels. The frogs *Xenopus leavis* and *X. tropicalis* express TRPM8, and their respective activation temperatures in the cold range (less than 14ºC) were determined by expression of the channels in frog oocytes (Meyers et al., 2009). Meyers et al. (2009) suggest that much like in mammals, these frogs utilize TRPM8 to detect cold ambient temperature for the purpose of thermoregulation, however this was not established through formal behavioural thermoregulation experiments.

Seebacher and Murray (2007) performed one of the only studies assessing a thermoregulatory role of thermoTRPs in an ectotherm, in this case a crocodile (*Crocodylus porosus*). Crocodiles were injected intraperitoneally with capsazepine, a TRPV1 and TRPM8 channel blocker. Under normal conditions, animals will shuttle back and forth between a warm basking area and cool pool, however, following capsazepine injection, this behaviour ceases almost completely (Figure 7). The authors concluded that this result stems from an inability of the animals to properly sense temperature and respond accordingly when TRPV1 and TRPM8

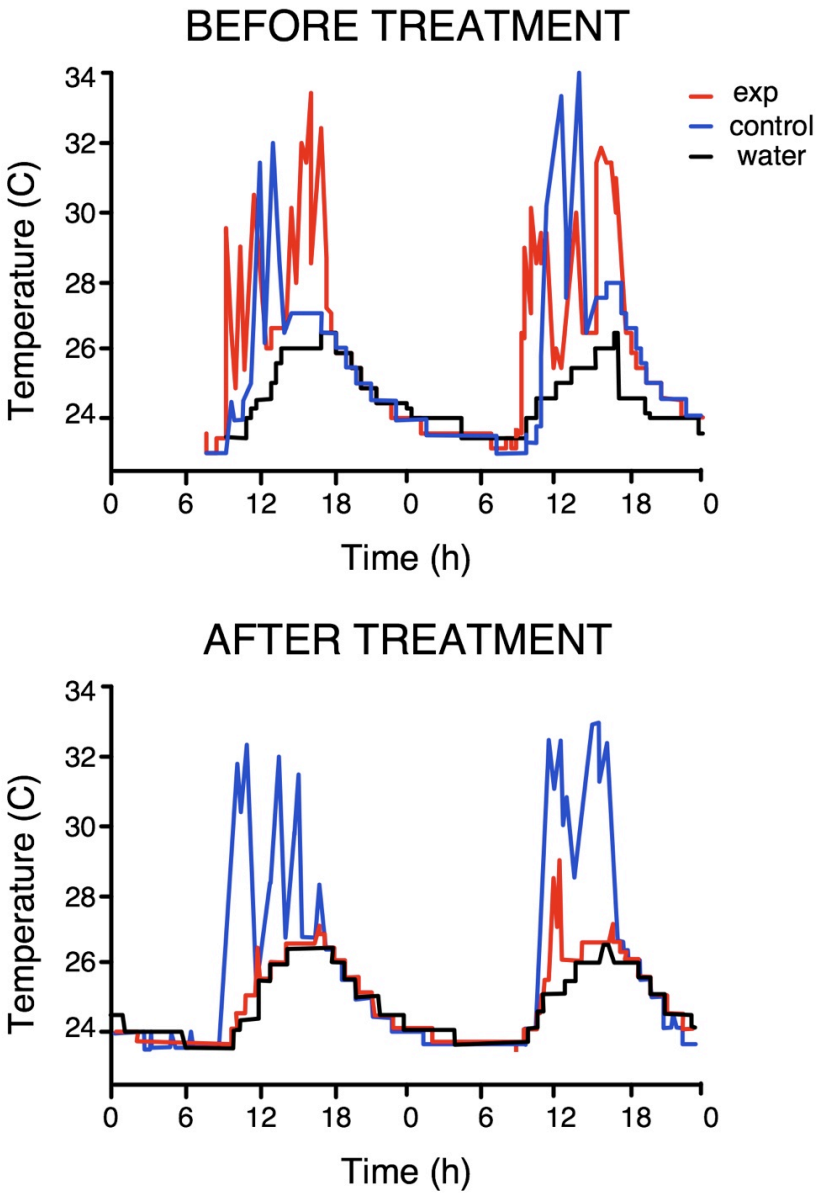

Figure 7: TRPM8 Blocking in a Reptile

Crocodiles (*Crocodylus porosus*) were injected with 16mg/kg capsazepine and allowed to shuttle freely between a warm basking area and cool pool for 48 hours. Control animals were injected with vehicle (DMSO) only. These data represent a single animal in the study. Values are body temperature. Animals treated with capsazepine (red line) ceased normal shuttling behaviour compared to the control injection (blue line). Water temperature fluctuated diurnally (black line). Adapted from Seebacher and Murray (2007).

function are obstructed. One major issue with this study is that it does not address the overwhelming mammalian evidence showing that thermoTRPs within the periphery contribute a large amount of the thermal information that governs thermoregulatory behaviour. The extent to which the drug would enter circulation and reach the periphery was not discussed. Mammalian evidence suggests a very fast clearance time for capsazepine (less than 15 min; Gao et al., 2000; Douat et al., 2011). Additionally, it is debatable what role thermoTRPs in the viscera (where the drug was administered) would have in thermoregulatory behaviour. Ectotherms exhibit a variety of behavioural strategies for thermoregulation, which can influence how thermoTRPs may function as part of the thermoregulatory system. The role of thermoTRPs must be assessed in regards to the behaviours an organism exhibits.

Evolutionary Modification of ThermoTRPs

ThermoTRP channels do not exhibit the same activation thresholds in all species and have been shown to take on unique non-thermoregulatory temperature sensation roles. For example the frogs *X. laevis* and *X. tropicalis* possess TRPM8 with an activation threshold of less than 14ºC (Meyers et al., 2009), far lower than the 28ºC threshold of mouse TRPM8 (Bautista et al., 2007; Dhaka et al., 2007). Although this has only been determined *in vitro*, the lower activation temperature is in accordance with the lower body temperature maintained by the frogs in their natural habitat (Meyers et al., 2009). Another example from *X. tropicalis* is an altered activation temperature for TRPV3. In contrast to the warm sensing mammalian TRPV3, the frog ortholog is activated by temperatures of <16ºC, potentially serving as an additional means of cold sensation in addition to TRPM8 (Saito et al., 2011). This aligns with the normal environmental

temperature to which these animals are exposed, but behavioural studies are required to determine if these activation thresholds translate outside of *in vitro* models. Similarly, the TRPA1 ortholog in the fruit fly *Drosophila melanogaster* is activated by warm temperatures and plays a role in thermotaxis of these animals (Viswanath et al., 2003; Rosenzweig et al., 2007). This is opposite from the proposed role for TRPA1 as a noxious cold sensor in mammals (Viswanath et al., 2003; Sawada et al., 2007). Research is limited in this area, but the emerging hypothesis is that thermoTRP channels have adapted through evolution to provide thermosensory information in a range that is relevant to each individual species (Saito and Shingai, 2006). In general, there is a large degree of conservation between and within thermoTRPs of different vertebrate species (Saito and Shingai, 2006). However, the aforementioned *X. tropicalis* TRPM8 shares 76% amino acid conservation with the mouse ortholog but only 58% conservation in TRPV3.

Two unique examples of thermoTRP modification exist in mammals and reptiles. In both cases it is hypothesized that thermoTRPs expressed in TG nerve fibers have been co-opted for use as infrared detectors in the pit organ located in the head region of bats and some snakes (Gracheva et al., 2010; Gracheva et al., 2011). In rattlesnakes, the pit organ is used in prey detection for hunting of warm-bodied animals by allowing for distance detection of infrared radiation (*i.e.* radiant heat). It was found that TRPA1 is expressed at elevated levels in the TG and has an activation temperature of greater than 28ºC, contrary to the same channels found in mammals (Gracheva et al., 2010).

Vampire bats also possess pit organs innervated by TG nerve fibers, which function in detecting hotspots on warm-bodied prey for blood ingestion. In a similar manner to TRPA1 in rattlesnakes, TRPV1 of vampire bats has been specifically adapted in the TG for increased

expression and an activation temperature in the range of 30ºC (Gracheva et al., 2011), much lower than the greater than 42ºC in other tissues and animals (Patapoutian et al., 2003). In this case, the change was determined to be the result of alternative splicing (Gracheva et al., 2011). Alternative splicing is a means by which multiple mRNA sequences are derived from the same gene through differential splicing of the pre-mRNA sequence. These mRNA are subsequently translated into multiple versions of the same protein (Vazquex and Valverde, 2006). Many factors determine whether these proteins are functional, such as level of expression and which regions are preserved from the pre-mRNA sequence. For example, the *Drosophila* TRPA1 gene produces four unique protein isoforms, only one of which is heat sensitive (Zhong et al., 2012). In the case of the vampire bat, alternative splicing represents a novel means by which thermosensory modification can occur (Gracheva et al., 2011). Research is ongoing into how this applies to the diversity of thermoTRP activation thresholds seen across different species. These examples highlight the diversity of thermoTRP channel function and specialization across vertebrate groups

Thesis Rationale and Objectives

No definitive research exists showing a role for thermoTRP channels in thermoregulatory behaviour of ectotherms, specifically lizards. The role of thermoTRPs in endotherm thermal sensation is well established, but there are significant differences in how these endotherms and ectotherms thermoregulate and integrate thermosensory information. It cannot be assumed that thermoTRPs are the primary thermal sensor for lizards. As such, this research aims to establish a role for the cool sensing channel, TRPM8, in the bearded dragon (*Pogona vitticeps*) lizard. This

work approaches the role of TRPM8 through blocking and activating the ion channels. It is hypothesized that the cold-activated TRPM8 ion channel in the bearded dragon controls cold temperature aspects of shuttling behaviour. Additionally, it is hypothesized that the TRPM8 ion channel does not control gaping, a warm temperature behaviour.

Blocking and Activating TRPM8 via Intraperitoneal Injection

The only known study looking at thermoTRPs as they relate to thermoregulation of ectotherms is the work of Seebacher and Murray (2007) using juvenile crocodiles (*Crocodylus porosus*). The first aim of the present study was to establish if bearded dragons, similarly sized to the juvenile crocodiles (172.9±6.7g), would exhibit the same response to intraperitoneal injections of capsazepine. Several changes were made to the methodology of Seebacher and Murray (2007), as the behavioural response to capsazepine is not definitively established as thermoregulatory in nature based on their methods. Crocodiles were given no incentive to leave the cool pool environment and therefore the choice to remain there may not have been the result of blocking TRPM8. In the event that TRPM8 was blocked, the cool pool area may not have been sufficiently cold (24ºC) to activate the channel under normal conditions (shuttling in the control group may have been under the control of another mechanism). Lastly, blocking of TRPM8 may have influenced another aspect of behaviour, which prevented shuttling regardless of temperature.

To address these shortcomings in the work of Seebacher and Murray (2007), the current study aimed to improve on their methodology by introducing animals to a ramping shuttle box (Cadena and Tattersall, 2009). The difference in this apparatus to that used in Seebacher and

Murray (2007) is that ambient temperature on both sides is constantly changing at a fixed rate (0.7ºC/min), with a 10ºC differential being maintained throughout (Cadena and Tattersall, 2009). If the animal spends too long on the cool side, temperatures will drop to a minimum outside of physiological range (10ºC) and the opposite occurs on the warm side, with temperature rising up to a maximum outside of physiological range (46ºC). It was predicted that capsazepine would block TRPM8, thereby impeding the lizards' ability to sense cool temperatures and leading to a preference for the cool side of the shuttle box. Blocking only TRPM8 would not address whether other cool-sensing channels might be involved in cold-avoidance behaviours in the bearded dragon.

The same ramping shuttle box as used by Cadena and Tattersall (2009) was used in assessing thermoregulatory shuttling behaviour of bearded dragons following intraperitoneal injection of a TRPM8 activator, menthol. The goal was to assess thermoregulatory behaviour of the lizards when TRPM8 was pharmacologically activated, rather than blocked. It was predicted that menthol would activate TRPM8, thereby inducing or augmenting cold sensation, leading to an avoidance of cold temperatures and a preference for the warm side of the shuttle box.

Topical Application of Menthol

In endotherms, thermoTRPs are expressed throughout the body, however it is the peripherally expressed channels (primarily those within peripherally innervated free nerve endings of the DRG) that are thought to be involved in thermal sensation as it relates to behavioural thermoregulation. This relates to the ongoing debate over peripheral versus core thermal sensors. Animals must be able to sense internal and external temperature (or a proxy of

external temperature; *i.e.* skin temperature) to mount an appropriate physiological and/or behavioural response for thermoregulation. A potential concern with the injection methodology is that it is difficult to determine whether the drugs are reaching sites with putative thermal sensors (*i.e.* peripherally expressed TRPM8 within the free nerve endings of the DRG), since rate of diffusion, the overall rates of blood flow, and abundance of capillaries determine the efficacy with which drugs will reach the target sights. In order to better deliver drug to peripheral sites of activation, menthol was applied to the dorsal trunk region of the bearded dragon. Studies on mice have shown topical application of menthol to influence behavioural and physiological thermoregulation, suggesting that with the appropriate vehicle, it can transfer across the skin and to the free nerve endings (Tajino et al., 2007; Klein et al., 2010). It was predicted that peripheral activation of TRPM8 via menthol would induce cool sensation, leading to a compensatory preference for the warm side of the shuttle box.

Upon assessing the thermoregulation of animals in the topical menthol series, it was established that shuttling at warmer temperatures was not altered from control animals. The same topical menthol volume and concentration was used to determine if other thermoregulatory behaviours that are activated at warm temperatures (*i.e.* gaping) were likewise unchanged from the activation of TRPM8 via menthol. From a neurological perspective, the question being addressed was whether 'cold' sensory input can override signals from warm sensors and alter thermoregulatory behaviours. Very little research has explored what areas of thermosensory input (periphery or core) are critical for the regulation of gaping behaviour. It was predicted that topical menthol application would not be sufficient to extinguish or reduce gaping behaviour of bearded dragons in a warm temperature environment. This is based on the notion that gaping occurs for the purpose of brain temperature regulation (Tattersall et al., 2006) and so peripheral

sensory input will be insufficient to override an abundance of warm sensory information from the body and brain to alter an animal's heat loss thermoeffector.

Chapter 2: General Materials and Methods

Experimental Animals

A total of 16 bearded dragons (*Pogona vitticeps*) were used throughout this study, with individual experiments using 8-12 animals. Twelve of the animals were purchased from private breeders, while the remaining four were raised in captivity from eggs. All animals were between one and three years of age at the time of experiments. The lizards were housed in corn cob bedding lined terraria (76 x 76 x 46 cm), each containing a 40W light bulb placed at one end, over a raised stone plate, for basking. A 13W Repti Glow® UV spectrum light provided a substitute for sunlight and cardboard packing materials provided shade, shelter and a climbing surface for extra enrichment. Photoperiod was kept at 12:12 light:dark. Animals were fed a combination of chopped fruits and vegetables three times a week, and supplemented with insects at least once a week. All procedures involving the use of these animals were approved by the Brock University Animal Care and Use Committee (Protocol #10-12-02).

Experimental Set-up

Assessment of Thermoregulatory Shuttling Behaviour

An electronic shuttle box was used to determine thermal preferences of the lizards as they moved from cool to warm temperatures and vice versa (Figure 8). The device consisted of a wooden chamber (119 x 61 x 45 cm) divided into two identical compartments by a Plexiglass® partition. An 11.5 x 14 cm hole at the bottom of the partition connected the two compartments, allowing for shuttling behaviour. A metal bar was fixed 4 cm from the bottom of the hole to

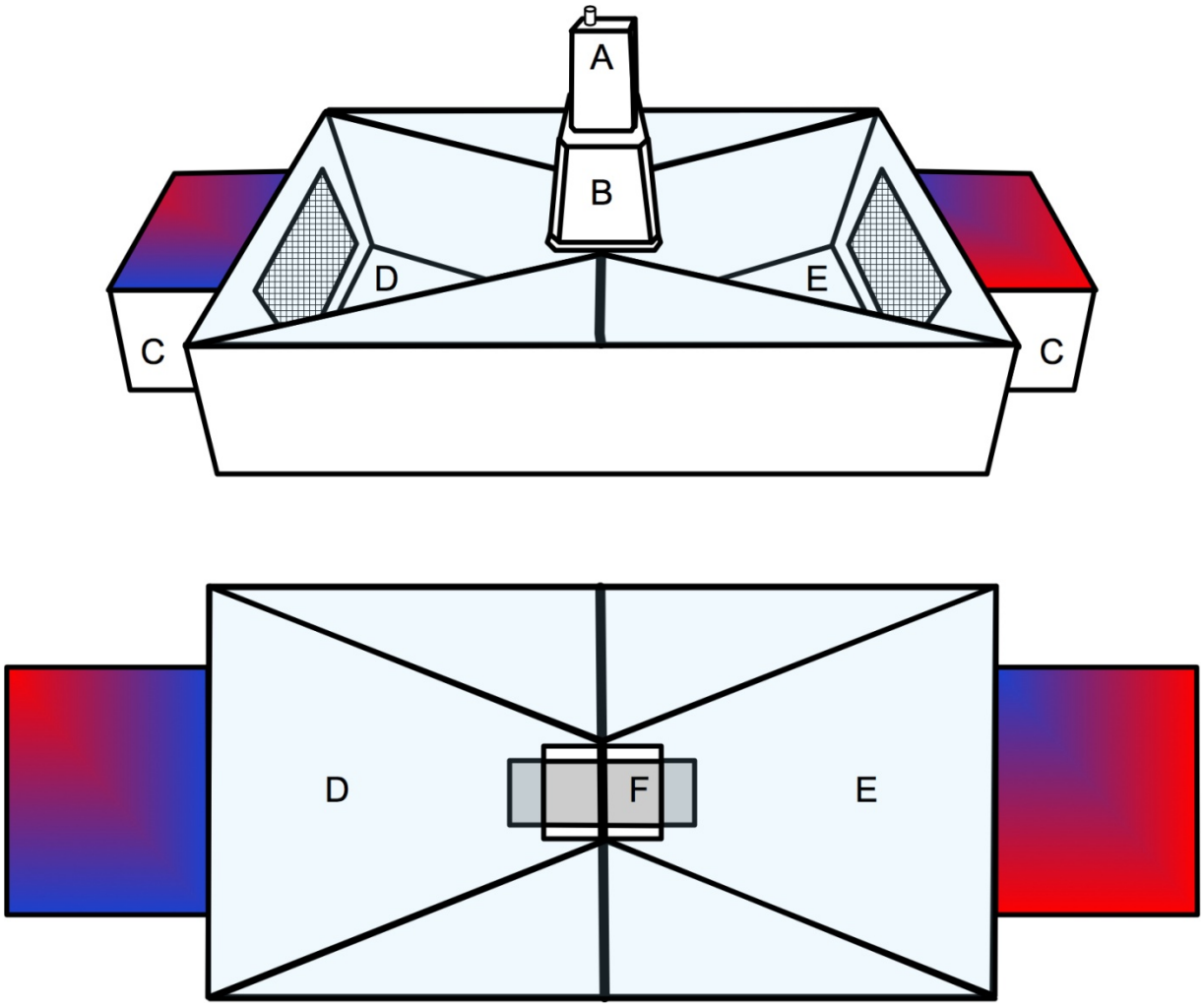

Figure 8: Ramping Shuttle Box

Apparatus for assessment of shuttling behaviour in bearded dragons (*P. vitticeps*). Webcams were mounted above each compartment of the shuttle box (not visible on diagram). Heating and cooling rates were set to 0.7ºC/min. A) Thermal camera B) Bellows to straddle the camera and seal the hole in the plexiglass lid C) Heater + radiator with fan to mix hot/cold air into each compartment D) Cold compartment (CC) of the shuttle box E) Hot compartment (HC) of the shuttle box F) Treadle switch situated between the compartments, which signalled electronic system to indicate the location of the animals and whether heating (HC) or cooling (CC) was required.

prevent animals from readily sitting partially inside each compartment, thereby 'forcing' a behavioural choice of one compartment at a time. Additionally, a thin sheet of clear plastic covered the hole, reducing air mixing between the compartments. Internal wooden walls in each of the compartments ran from each of the corners of the box to the edge of the hole in the partition, creating a "funnel" to guide the lizards towards the switch plate and facilitate shuttling. Uniform lighting was provided by overhead fluorescent bulbs shining through a clear Plexiglass® lid. Cameras mounted above each compartment allowed for continuous monitoring without disturbance from the observer, but were not used for any data collection/analysis.

Heating and cooling of the box was regulated using a custom built electronic system (Brock University, Electronics Shop). A control box monitored ambient temperature via a thermometer in each of the two compartments, allowing it to control the rate of temperature change in the box. Lizards were able to control the temperature inside the box by stepping on a treadle switch located between the two compartments. By activating the treadle switch each time they switched compartments, lizards caused the box to either cool down or warm up at a rate of 0.7ºC/min. This rate was chosen based on the findings of Cadena and Tattersall (2009) that suggested it was a trade-off between minimizing energy expenditure with the need to thermoregulate. The cooling sources consisted of a radiator located at each end of the box through which cold ethanol was circulated from a refrigerated water bath. Heating elements were also located at each end, with a fan positioned behind the heating and cooling elements to blow hot or cool air through the compartment and maintain a constant temperature throughout. When the treadle switch was activated, the heating and cooling systems would activate accordingly.

The two compartments were designated 'hot' and 'cold' based on the 10ºC differential maintained between them at all times. This created a spatial temperature contrast that would guide the lizards to instantly warmer or cooler temperatures. The system was designed to imitate natural conditions, where lizards will move between areas of cold and warmth to maintain body temperature. The cooling and warming occurred to encourage lizards to constantly move (*i.e.* make a choice) between the warm and cool environment, as they would in the wild. Once a lizard was positioned inside the "hot compartment" (HC) the air temperature inside both compartments would automatically rise at a rate of 0.7ºC/min. The temperature continued to climb until the animal moved to the "cold compartment" (CC), stepping on the treadle switch as it moved, activating the cooling system and causing both compartments to cool at a fixed rate of 0.7ºC/min. Maximum and minimum attainable temperatures were set at 46ºC and 10ºC, respectively, as a safety precaution for the animals. These temperatures are not immediately hazardous to the lizards, but long-term exposure can be, creating an incentive for them to leave the box before these limits were reached.

A hole (18 x 14 cm) was cut into the Plexiglass® lid directly above the treadle switch. An infrared (IR) thermal imaging camera (Mikron 7515; Oakland, NJ, USA) was mounted on camera bellows and positioned over this hole. Thermal images were captured at a rate of 2 frames/sec for the duration of the data collection period. During post processing image analysis, an image at the moment immediately prior to the lizards movement from HC to CC was extracted for each shuttle (Figure 9).

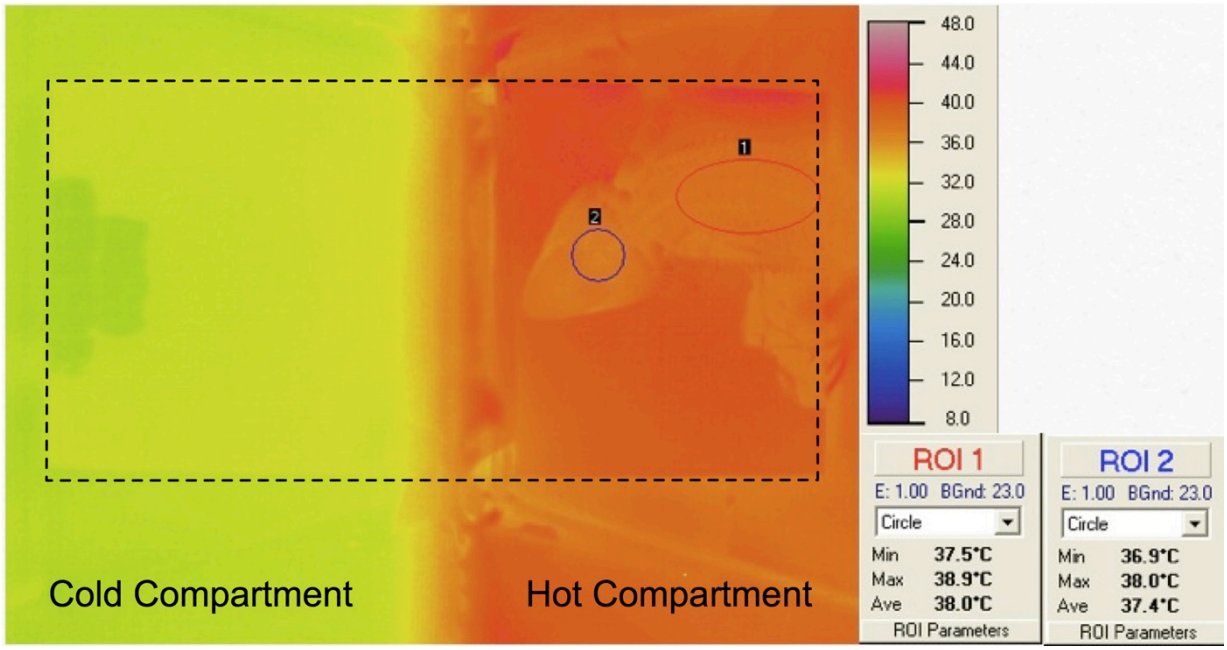

Figure 9: Thermal Image of Dragon in Shuttle Box

Image taken from thermal imaging camera at the moment a bearded dragon switches compartments (hot to cold) in the ramping shuttle box. Circles '1' and '2' are areas drawn using proprietary software and return an average temperature for the area, allowing independent measures of skin temperature at different regions. Dotted line represents the treadle switch, indicating which compartment the animal currently occupies.

Recording Temperatures During Behavioural Thermoregulation

Ambient temperatures (T_a) in the shuttle box were obtained from thermometers suspended in each compartment and automatically recorded using custom-built software (Brock University, Electronics Shop) every 30 s and whenever the lizard switched compartments. The ambient temperatures when animals left the hot compartment were designated UETa (upper escape ambient temperature) and the ambient temperatures when animals left the cold compartment were designated LETa (lower escape ambient temperature). Recordings of lizard's location (HC or CC) were collected simultaneously throughout the duration of experiments.

Thermal images of the lizards were recorded continuously (2 frames/sec, shuttle box; 1 frame/5 min, gaping box), via the IR thermal imaging camera, for the duration of the data collection period. Images were analyzed using custom software from the camera's manufacturer (MikroSpec RT), allowing markers to circle areas and return an average temperature for that area (Figure 9). Using this software, trunk and head temperature were obtained each time an animal switched compartments in the shuttle box (*i.e.* UETts/UEThs [upper escape trunk skin and head skin temperature], and LETts/LEThs [lower escape trunk skin and head skin temperature]) or every 5 min in the fixed temperature gaping box.

Experimental Design to Assess Thermoregulatory Shuttling Behaviour

All experiments were run from 8:00 am to 8:00 pm (except for one group of animals in series III). In the case of experiments using a temperature data logger, the logger was fixed to the animal's back and the probe inserted into the animal's cloaca by 7:45 am. For the purpose of

injections, 1/2 cc tuberculin syringes (27G 1/2) were used to limit 'dead-space', as volumes were relatively small (100-300 µl). Injection/drug application timing followed time courses dependent on the experiment. To begin the experiment, animals were placed inside the "hot compartment". This was done to prevent lethargy from exposure to cold, as the animals had just emerged from their cages during the dark cycle and were already relatively cold (*i.e.* room temperature). This would greatly affect their ability to move and thus their ability to explore and thermoregulate normally. In all experiments, the initial 4.5 hours (8:00 am-12:30 pm) of the trial was deemed exploratory/habituation shuttling, as categorized by previous experiments (Cadena and Tattersall, 2009). The subsequent 7.5 hours (12:30 pm-8:00 pm) was designated data collection, and it is from within this period that all experimental data were obtained and analyzed. In addition to temperature data, the number of times an animal moved between each compartment, for the duration of the data collection period, was assessed. At the end of each experiment, lizards were removed from the box and returned to their housing facilities.

Data Processing and Statistical Analysis

Shuttle Box Temperature Data

The number of shuttles were recorded throughout all experiments and averaged as shuttle/hour across all animals within each treatment. The shuttle box records the location of the lizard throughout the data collection period and samples ambient temperature every 30 seconds, which is used to determine average ambient temperature (T_a) and produce a trace of the ambient temperature environment of a lizard in the box (Figure 10). From this information, values of upper escape ambient temperature (UETa) and lower escape ambient temperature (LETa) can be

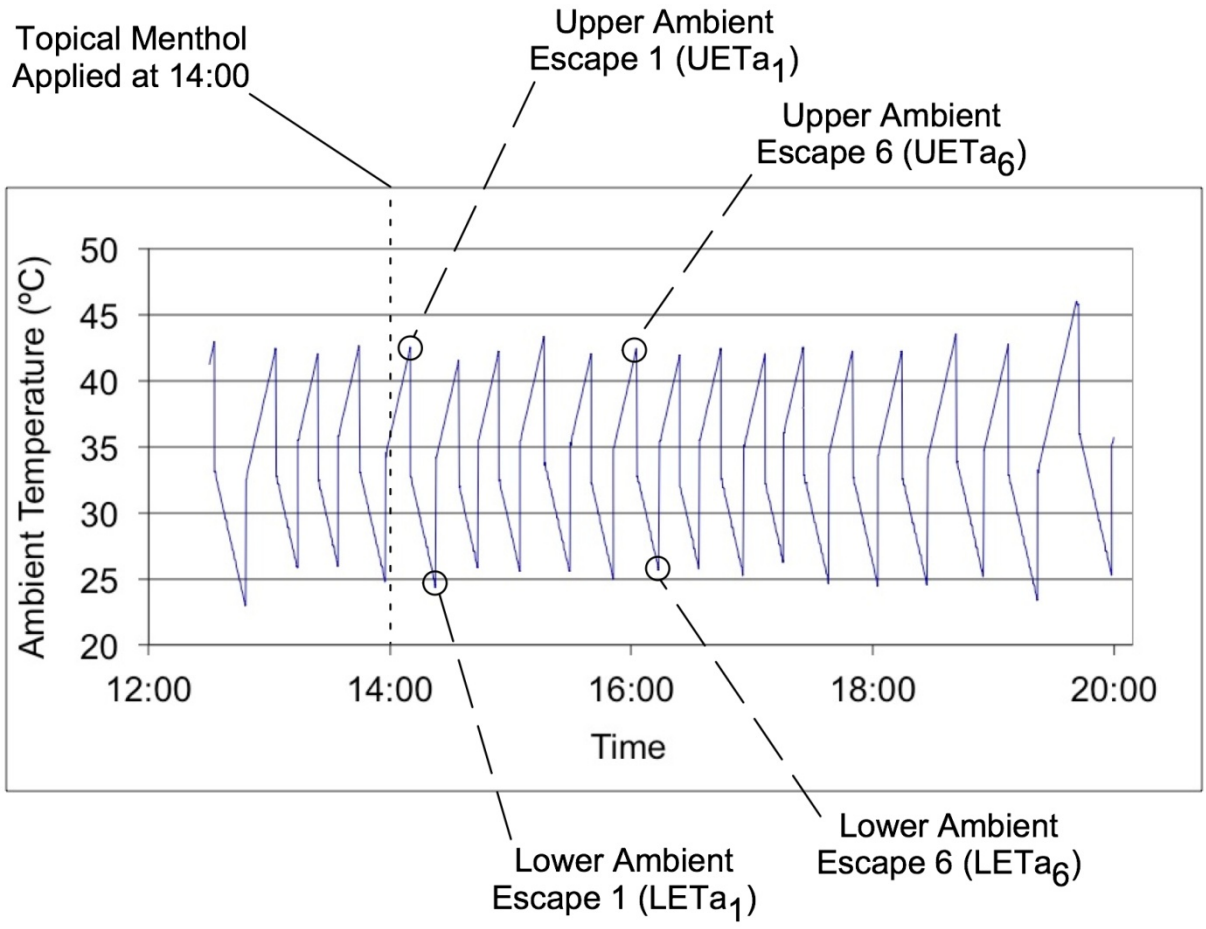

Figure 10: Sample Trace of Ambient Temperature in the Ramping Shuttle Box

Animals shuttle between the warm and cool compartments of the box, causing them to encounter warm and cool environments, respectively. The temperature at which a lizard exits the cold side is denoted LET_i (lower escape temperature), with UET_i (upper escape temperature) being the same metric as it relates to the warm side. This can refer to ambient (as in this figure), skin, or body temperature. 'i'= the shuttle number post-treatment, and is independently assigned to LET_i and UET_i. Events with the same 'i' are averaged for all animals in that treatment.

analyzed. UETa is the temperature at which a lizard exits HC and LETa is the temperature at which a lizard exits the CC. For the purpose of observing changes in escape temperatures over time, the upper escape ambient temperature and lower escape ambient temperature are denoted $UETa_i$ and $LETa_i$, respectively, where 'i' is the shuttle number (e.g. i=1 at the first event following the start of the data collection period and i=6 for the 6^{th} shuttle event; Figure 10).

UET and LET are further categorized by trunk and head skin (T_{ts} and T_{hs}), or body (T_b) temperature. These values are obtained from the thermal camera and data logger, respectively. In the case of skin temperatures, data is limited to UETts, UEThs, LETts, and LEThs values due to the static focal position of the thermal camera over the transition zone between the two compartments. In experiments where body temperature was measured (*i.e.* series I), values were collected every minute, allowing for mean body temperature in addition to UETb and LETb values to be obtained.

Statistical Analysis

A combination of parametric and non-parametric tests were performed, based on the assumptions satisfied by each data set. Sigmaplot 11 was used for all statistical tests, except the Schreirer-Ray-Hare SRH test, which was performed using Sigmastat 4 from the methodology provided by Dyntham (2011). When assumptions of normality and equal variance were met, ANOVAs were performed to compare treatment and control animals. Regular ANOVAs were performed for unpaired data and RM ANOVA were performed for paired data, where appropriate. In the event that a significant interaction effect was found in a two-way ANOVA, a Holm-Sidak *post hoc* test was performed to determine where the significance existed. When

data were not normal, it was determined that residuals were at least normal. In cases where assumptions of the ANOVA were not achieved in any way, a non-parametric Scheirer-Ray-Hare (SRH) test was performed in place of the two-way ANOVA. This test is a more conservative equivalent to the multi-way ANOVA (Dyntham, 2011). No *post hoc* tests are available for the SRH test, so when significance was determined in one of the factors used for the SRH test, targeted Wilcoxon tests were performed to determine which pairs in a factor showed significance. In cases where ANOVA assumptions were not achieved and there were only two groups to test, a Mann-Whitney U or Wilcoxon test was performed for unpaired and paired data, respectively. T-tests were performed to determine significance in number of shuttles and shuttles/hour between different treatment groups within each series.

Partial Sequencing of TRPM8 and TRPV1

Sequences for TRPM8 and TRPV1 proteins in the bearded dragon were obtained by RNA extraction and cDNA sequencing to determine their presence and conservation in this species. Bearded dragons (n=7) were euthanized through a combination of halothane inhalation, followed by an injection of 1mL pentobarbital once all pain reflexes were extinguished. Samples from the liver, heart, dorsal root ganglion (DRG), and skin were taken from the animals within 40-60 min of pentobarbital injection, stored in RNALater (Sigma-Aldrich, Canada), and immediately submerged in liquid nitrogen (long term storage at -80ºC). The Norgen Total RNA Extraction Kit was used to extract total RNA from these tissues. cDNA was made using Bio-Rad cDNA Synthesis kit. Bio-Rad reverse transcriptase was used in conjunction with primers for PCR reaction.

Semi-degenerate primers for TRPV1 were designed by hand from an alignment of rattlesnake, platypus, dog and chicken sequences obtained from GenBank, using the ClustalW2 online alignment tool. The primer sequences were:

F 5' AATGCTGCTGGKGAGTKG

R 5' GSCKGGCCACACCAAARG

Semi-degenerate primers for TRPM8 were designed by hand from an alignment of chicken, frog (*Xenopus laevis*) and predicted *Anolis carolinensis* sequences obtained from GenBank, using ClustalW2. The primer sequences were:

F 5' CTSAAGCCMCGAATGCGY 3'

R 5' CCKCCTCCYTGAGCAAAAC 3'

Resulting products were run on 1% agarose gel. Bands from the gel were cut out and purified using MO BIO UltraClean GelSpin DNA Extraction kit. Sequencing was performed by Genome Quebec at McGill University. Protein alignments to investigate conserved regions for menthol binding were performed by NCBI protein BLAST and ClustalW2.

Chapter 3: Assessing the Role of TRPM8 in Bearded Dragon Thermoregulation by Intraperitoneal Injection

Introduction

The thermoTRP channels are well established as the molecular driver of thermosensation in mammals (Caterina, 2007), but little is understood about their role in ectothermic models, including lizards. The cold sensing channel TRPM8 is ubiquitously expressed across vertebrate groups, typically in DRG and TG neurons, which project into the peripheral sensory nerves and skin (Caterina, 2007). Many studies have explored how removal of a functional TRPM8 gene influences normal behavioural thermoregulation in mammalian models (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Mice lacking TRPM8 display an inability to correctly identify and react to cold temperature environments, while maintaining normal thermoregulation as it relates to warm temperatures. These discoveries have led to the establishment of TRPM8 as a vital thermosensor in directing thermoregulation at cold temperatures (Bautista et al., 2007; Colburn et al., 2007; Dhaka et al., 2007). Further studies have injected TRPM8 antagonists and agonists into mice to explore their effects on thermoregulation. These have included the agonist menthol and a wide variety of antagonists. The general finding is that activation of TRPM8 results in behaviours associated with cold defense, such as warm seeking and heat production (Ruskin et al., 2007). Blocking TRPM8, in contrast, results in reduced heat production and an inability to detect cold temperatures (Knowlton et al., 2011; Almeida et al., 2012; Gavva et al., 2012).

The role thermoTRP channels play in ectotherm thermoregulation has been studied far less extensively than in endotherms. The fact that ectotherms rely heavily on behavioural

mechanisms to thermoregulate suggests differences (Seebacher and Franklin, 2005) in how thermosensory information is collected and integrated. In contrast endotherms can employ extensive autonomic physiological thermoregulation, which generally rely on different neural pathways from behaviours (Romanovsky, 2007).

The ability for lizards to thermoregulate effectively relies on proper detection of external and internal temperatures to mount an appropriate response. This study aims to explore whether, like mammals, the thermoTRP channels serve this role in a lizard species, which employs mainly behavioural thermoregulation. To determine this, bearded dragons (*Pogona vitticeps*) were injected with the TRPM8 blocker capsazepine or the agonist menthol, and then placed in a ramping shuttle box. This apparatus imitates situations in the wild where animals must move between hot and cold environments to maintain body temperature within an optimal range. By assessing the thresholds at which animals move from hot to cold and back, we can determine the effect that blocking and activating TRPM8 has on behavioural thermoregulation. The following experiments aim to establish TRPM8 as a regulator of cold thermosensation in the bearded dragon.

Materials and Methods

Experimental Design

Series I: Intraperitoneal Injection of Capsazepine and Shuttling Behaviour

Capsazepine was used as a TRPM8 antagonist to assess the role TRPM8 plays in thermoregulatory shuttling. Capsazepine was produced at Brock University (Dr. Travis

Dudding, Department of Chemistry). Capsazepine or its vehicle (100% DMSO, dimethyl sulfoxide, Sigma-Aldrich, Canada) was injected at 8:00 am, two days prior to the animal being placed in the shuttle box (Figure 11). This time course was adopted based on a study using capsazepine in crocodiles (Seebacher and Murray, 2007). A concentration of 20 mg/kg was used following a preliminary dose response trial at 1.5, 4.5 and 16 mg/kg that showed no change in shuttling behaviour. This concentration was ultimately chosen based on the limited availability of the drug to run a minimum of 10 trials at a dose exceeding 16mg/kg. Total injected volume was 3 mL/kg. Data loggers were used to record body temperature throughout the 12 h trial, however they were not completely reliable so body temperature was only obtained for 10 of 17 trials. Thermal camera images were obtained for each upper escape and lower escape event during the data collection period. A total of 12 animals were used for Series I (partially paired; n=8 capsazepine, n=9 control). Some animals received both treatments, others only one of the two. Order of treatment and control was randomized for paired experiments, and a minimum of four weeks separated treatments.

Series II: Intraperitoneal Injection of Menthol and Shuttling Behaviour

Menthol was used as TRPM8 agonist to assess the role TRPM8 plays in thermoregulatory shuttling. (-)-Menthol was purchased from Sigma-Aldrich, Canada. This particular isomer is specifically associated with TRPM8 activation in mammals, whereas other forms of menthol exhibit less affinity for mammalian TRPM8 (Behrendt et al., 2004). The effect of menthol occurs relatively faster than that of capsazepine based on studies in mice (Tajino et al., 2007; Klein et al., 2010), so drug or the vehicle (100% DMSO) were injected following 4 h of habituation, at

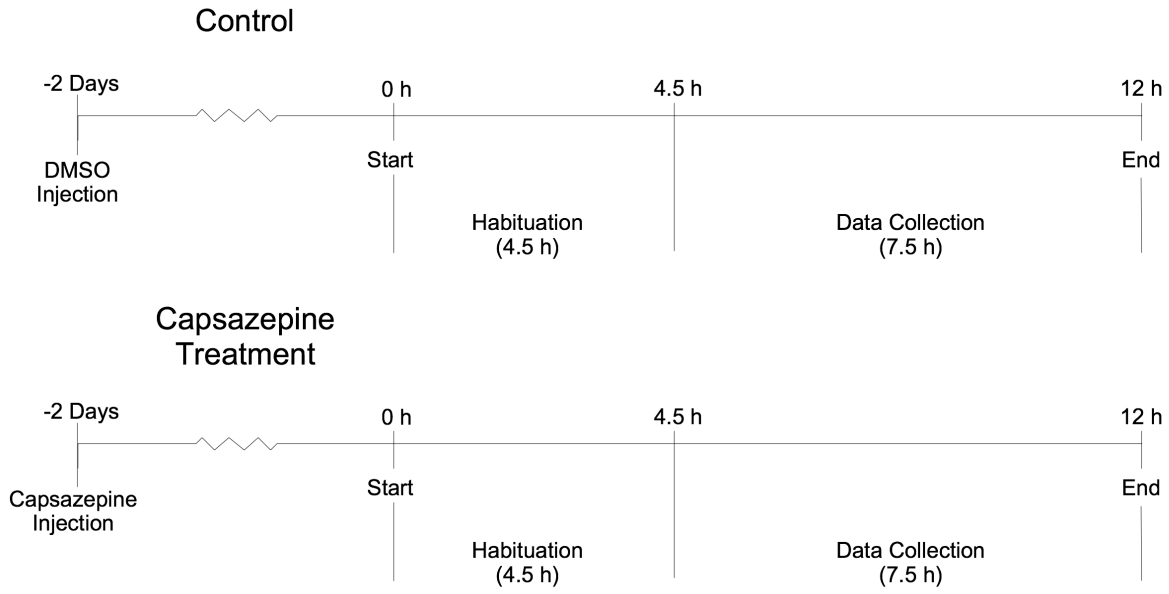

Figure 11: Protocol for Series I: Intraperitoneal Injection of Capsazepine

Time course of capsazepine experiments performed in the ramping shuttle box. Animals were administered 20 mg/kg of drug or vehicle (100% DMSO) two days prior to assessment of behaviour. 4.5 hours of habituation was followed by 7.5 hours of data collection.

12:00 pm (Figure 12). Data collection began at 12:30 pm to give animals a short time to recover from handling and removal from the shuttle box during injection. A concentration of 400 mg/kg was used following no effect observed in drug trials using 100mg/kg and 200mg/kg. 400mg/kg was also deemed the maximum safe dose, as one animal was anesthetised by this dose during preliminary trials, consistent with the anesthetic effects outlined in other studies at elevated doses (Watt et al., 2008). This trial was deemed an outlier and omitted from analysis as only a single animal out of six had such a reaction. A total of 10 animals were used for Series II (unpaired; n=5 menthol, n=5 control). No body temperature or skin temperature data were collected for this series.

Statistical Analysis

Series I: Intraperitoneal Injection of Capsazepine and Shuttling Behaviour

The mean for T_a, UETa, LETa, UETts, and LETts were taken for each individual animal over the 7.5 h data collection period and averaged across replicates. Due to the partially pairing of these trials, data were treated as unpaired. The mean was compared between the treatment (n=8) and control (n=9) group for each metric. A one-way ANOVA was used to test data with normal distribution (T_a, UETa, LETts), while a Mann-Whitney U test was used to compare data that did not meet assumptions for ANOVA (LETa, UETts).

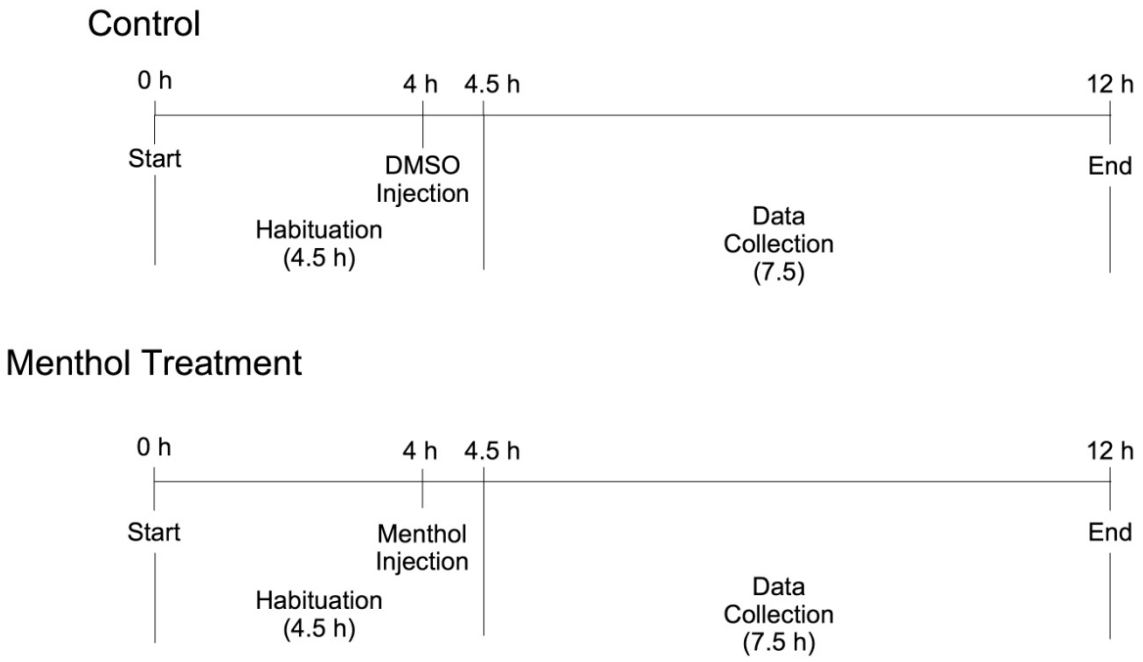

Figure 12: Protocol for Series II: Intraperitoneal Injection of Menthol

Time course of menthol injection experiments performed in the ramping shuttle box. Animals were administered 400 mg/kg of menthol or vehicle (100% DMSO) 30 minutes prior to the beginning of the data collection period.

Series II: Intraperitoneal Injection of Menthol and Shuttling Behaviour

Menthol was expected to have a short time course of effectiveness (Tajino et al., 2007; Klein et al., 2010), so $UETa_2$ to $UETa_{10}$ and $LETa_2$ to $LETa_{10}$ were averaged for each individual shuttle event across experimental animals, to determine the time course of any behavioural changes. Due to the potential for stress associated with handling and injection, the first shuttle ($UETa_1$ and $LETa_1$) was omitted from statistical tests. A two-way repeated measures (RM) ANOVA was used to compare $UETa_{2\text{-}10}$ and $LETa_{2\text{-}10}$. Mean T_a, UETa, and LETa were calculated for all animals and compared between treatment and control groups using a one-way ANOVA

Results

Series I: Intraperitoneal Injection of Capsazepine and Shuttling Behaviour

Capsazepine injection had no observable effect on thermoregulatory shuttling behaviour. Animals in the treatment group did not differ in the amount of shuttling, as categorized by the number of shuttles per hour, during the data collection period (Table 1; U=30.5, p=0.630). Mean T_a between treatment and control groups was not significantly different (Figure 13**;** F_{15}=0.263, p=0.615). There was also no difference in upper or lower ambient or trunk skin escape temperatures (Figure 13; UETa, LETa, UETts, LETts; F_{15}=0.210, p=0.653; U=36.00, p=0.962; U=33.00, p=0.810; F_{15}=1.045, p=0.323).

Table 1: Number of Shuttles

Shuttles per hour (mean±SD) performed in each treatment group of animals throughout their respective data collection periods. A shuttle is defined as a full cycle of movement from the HC to the CC, and back to the HC. Depending on which compartment the animals occupied when data collection began, this can be reversed. No significant difference was found between control(s) and treatment of any series, or between series ($p>0.05$).

Series - Drug	Shuttles/Hour
I – Capsazepine (n=8	2.75±1.2
I - Vehicle (DMSO) (n=9)	3.4±2.2
II – Menthol (n=5)	3.2±0.7
II - Vehicle (DMSO) (n=5)	4.2±1.0
III – Menthol (n=11)	2.3±0.9
III - Vehicle (Ethanol) (n=11)	2.3±1.0
III – DMSO (n=11)	2.9±1.3

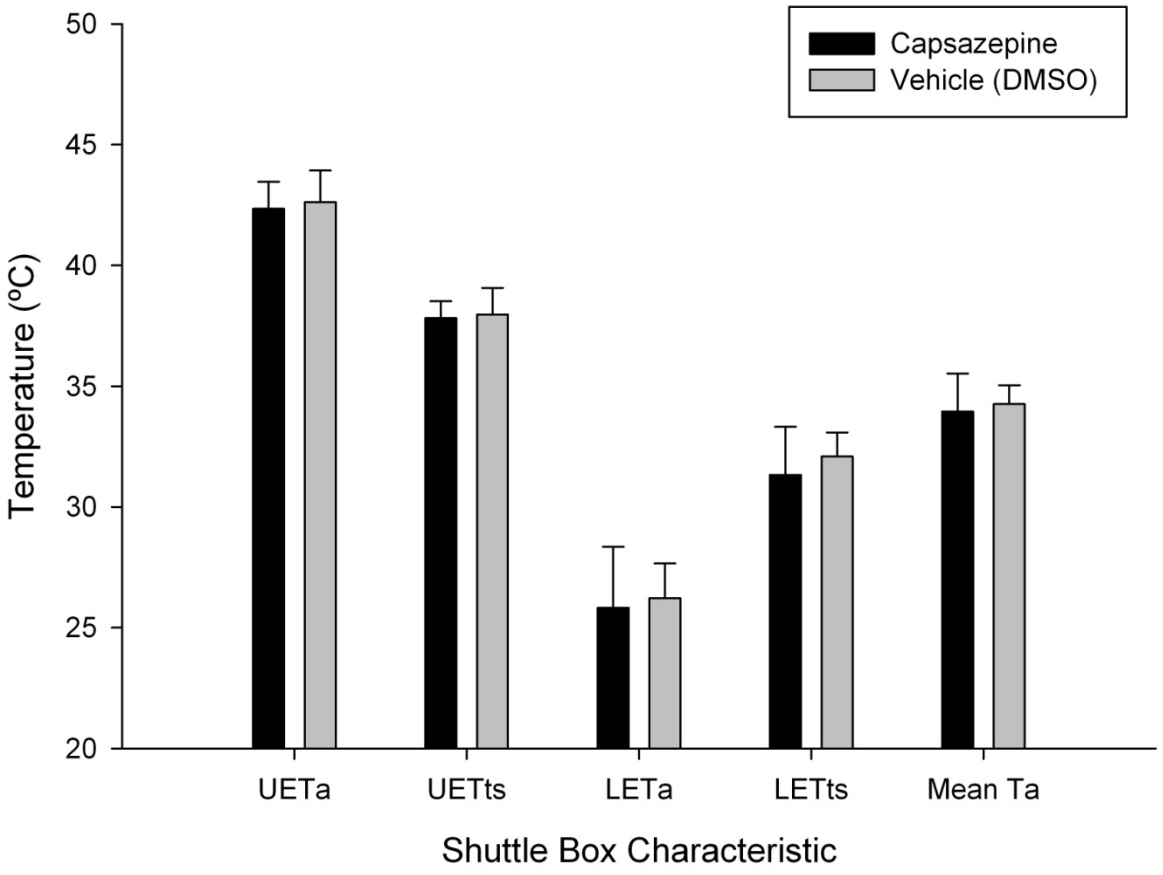

Figure 13: Parameters of Thermoregulation with Injection of Capsazepine or Vehicle (DMSO); Series I

Mean upper and lower escape ambient temperatures (UETa and LETa, respectively), mean upper and lower escape trunk skin temperatures (UETts and LETts, respectively), and mean ambient temperature (T_a) are plotted for bearded dragon lizards (*P. vitticeps*). Animals were injected with a volume of 3mL/kg (20mg/kg capsazepine or 100% DMSO) two days prior to experimentation and tested in an electronic shuttle box for 12 hours. Data collection occurred over the final 7.5 hours. Trials were partially paired and at least one month separated paired trials. Values are mean±SE (n=9, capsazepine; n=8, DMSO).

Series II: Intraperitoneal Injection of Menthol and Shuttling Behaviour

Menthol injection had no observable effect on thermoregulatory shuttling behaviour. Animals in the menthol treatment group did not differ from the control group in the number of shuttles per hour performed during the data collection period (Table 1; t_8=1.785, p=0.112). There was no significant difference in mean T_a, UETa, or LETa over the entire course of the experiment ($F_{1,8}$=0.083, p=0.781; $F_{1,8}$=1.101, p=0.325; $F_{1,8}$=1.226, p=0.300) between menthol and control animals (Figure 14). For $UETa_{2\text{-}10}$ and $LETa_{2\text{-}10}$ temperatures, a two-way RM ANOVA using treatment and shuttle number showed no significant difference in treatment ($F_{8,64}$=0.424, p=0.533; $F_{8,64}$=0.515, p=0.493), shuttle number ($F_{8,64}$=0.524, p=0.834; $F_{8,64}$=1.027, p=0.425), or interaction between the two variables ($F_{8,64}$=1.344, p=0.238; $F_{8,64}$=0.294, p=0.966) post-injection ($UETa_{2\text{-}10}$, $LETa_{2\text{-}10}$; Figure 15).

Sequencing of TRPM8 and TRPV1

Sequencing was undertaken to determine if bearded dragons expressed the relevant TRP channels to this study. A 107aa sequence of TRPV1 was obtained from heart tissue of the bearded dragon (Genbank: HM852065). This section spans from approximately the middle of the sixth transmembrane domain, through the intercellular carboxyl terminus (Tominaga and Tominaga, 2005). This was done primarily for the purpose of learning proper PCR techniques, as TRPV1 is relatively well represented in sequence databases. This confirms that TRPV1 is expressed at least in the heart of these lizards (Figure 16).

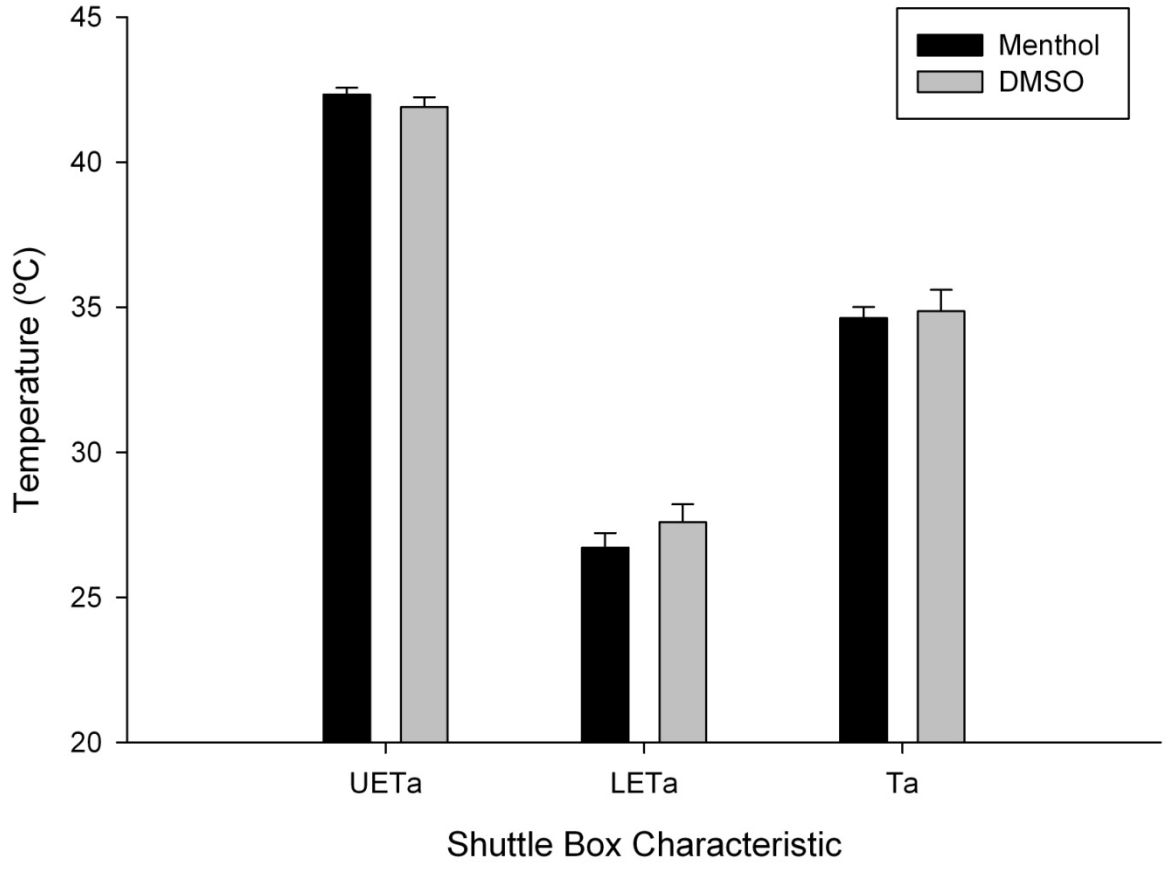

Figure 14: Parameters of Thermoregulation with Injection of Menthol or Vehicle (DMSO); Series II

Mean upper and lower escape ambient temperatures (UETa and LETa, respectively) and mean ambient temperature (T_a) are plotted for bearded dragon lizards (*P. vitticeps*). Animals were injected with a volume of 3mL/kg (400mg/kg menthol or 100% DMSO) following four hours habituation in an electronic shuttle box. Data collection occurred over 7.5 hours, beginning 30 minutes after injection. This series was completely unpaired. Values are mean±SE (n=5, menthol; n=5, DMSO).

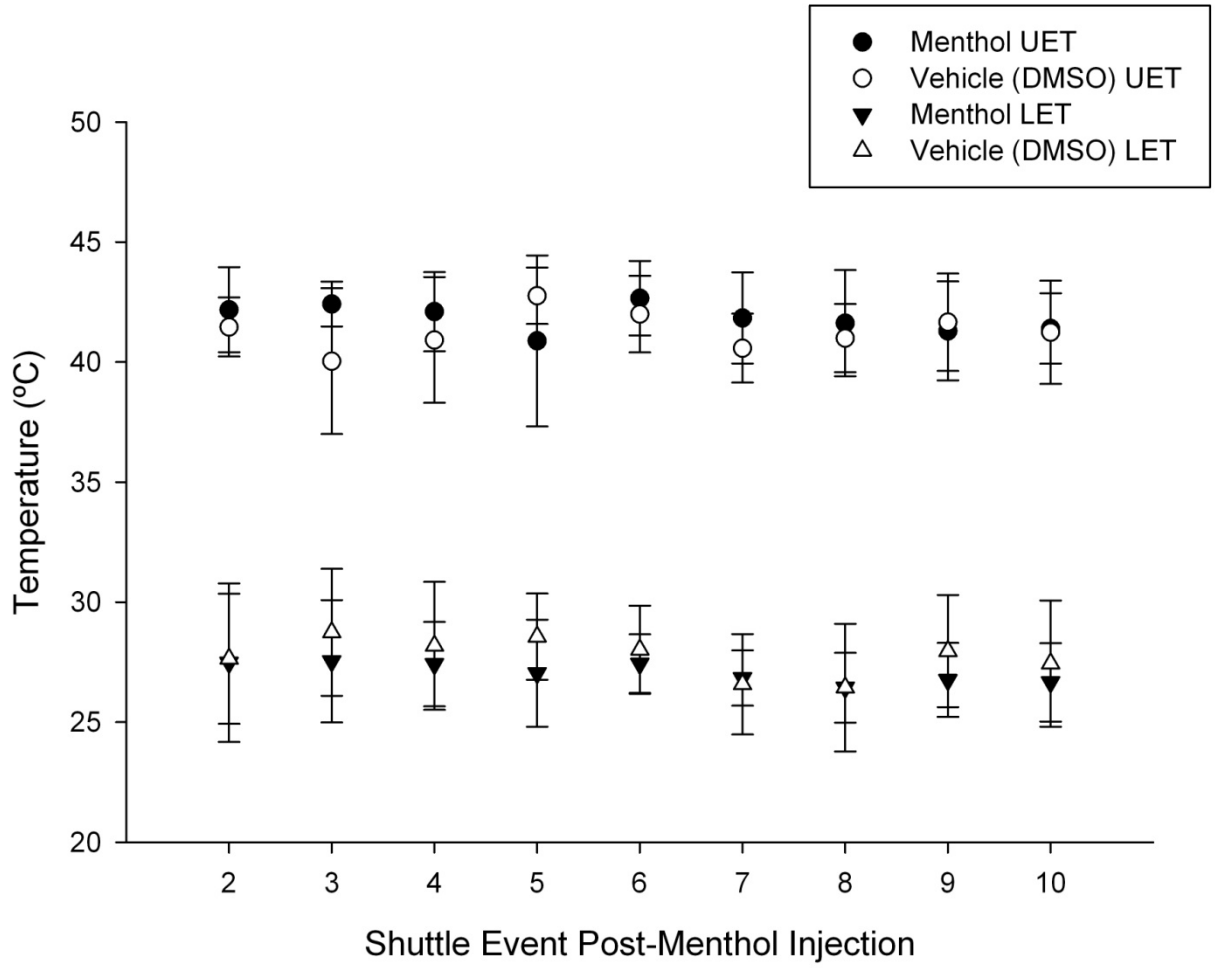

Figure 15: UETa$_{2\text{-}10}$ and LETa$_{2\text{-}10}$ Events Following Injection of Menthol or Vehicle (DMSO); Series II

Upper and lower escape ambient temperature (UETa and LETa, respectively) are plotted as means for each of nine shuttle events performed by bearded dragon lizards (*P. vitticeps*) in an electronic shuttle box. Animals were injected with a volume of 3mL/kg (400mg/kg menthol or 100% DMSO) following four hours habituation in an electronic shuttle box. Data collection occurred over 7.5 hours, beginning 30 minutes after injection. This series is completely unpaired. Values are mean±SD (n=5, menthol; n=5, DMSO).

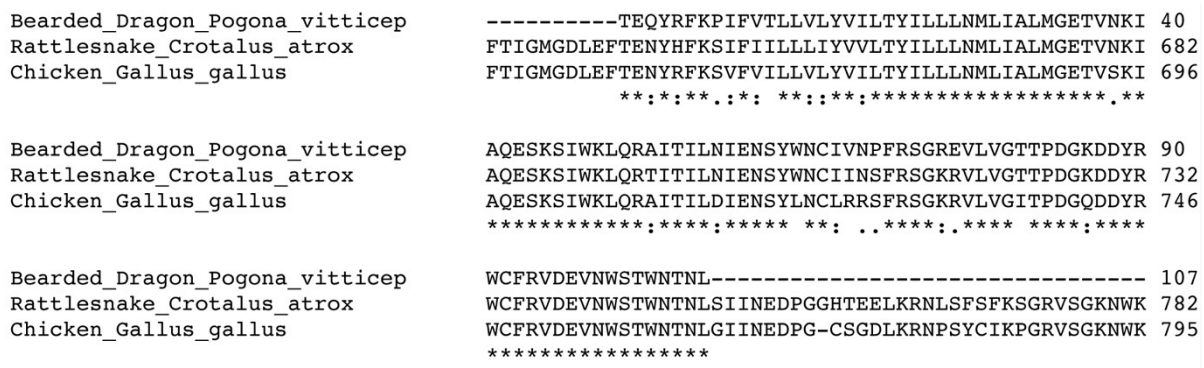

Figure 16: Bearded Dragon TRPV1 Protein Alignment

Portion of TRPV1 protein sequenced from the bearded dragon, aligned with rattlesnake and chicken amino acid sequences. '*' indicates a fully conserved residue, ':' indicates a residue with strongly similar properties, '.' indicates a residue with weakly similar properties. Alignments performed by European Bioinformatics Institute (EBI) online ClustalW2 alignment tool.

A 129aa sequence of TRPM8 was obtained from both bearded dragon liver and DRG (Genbank: JX504010). This section is part of the intercellular amino terminus. This confirms that TRPM8 is expressed in tissues relevant to the peripheral sensory system. An alignment of this partial sequence with the Carolina Anole lizard (*Anolis carolinensis)* shows 93% amino acid identities conserved (Figure 17). Additionally, an alignment of *A. carolinensis* with mouse, chicken, and frog, shows all known residues vital for menthol activation of TRPM8 are conserved in *A. carolinensis* (Figure 18). This includes two transmembrane residues (Y745, R842) in what is believed to be the binding site of menthol, and two TRP domain residues (Y1005, L1009) thought to be important in PIP_2 signalling (Bandell et al., 2006; Malkia et al., 2009; Feng, 2011).

Discussion

Intraperitoneal injections of the TRPM8 antagonist capsazepine and the agonist menthol did not elicit any measurable change in the thresholds for thermoregulatory shuttling behaviour. Many methodological problems may have contributed to this, but also possible are physiological limitations of altering thermoregulation through intraperitoneal injection of drugs. This contrasts with the results of Seebacher and Murray (2007), who concluded capsazepine injections abolished thermoregulatory shuttling behaviour through the blocking of TRPM8. Several endothermic studies also effectively inhibit cold responses with administration of TRPM8 antagonists (Knowlton et al., 2011; Almeida et al., 2012; Gavva et al., 2012), and conclude that the channel has a role in cool-temperature sensation. Whether the intraperitoneal method of administering drugs is efficacious for the purpose of thermoTRP assessment, remains to be seen.

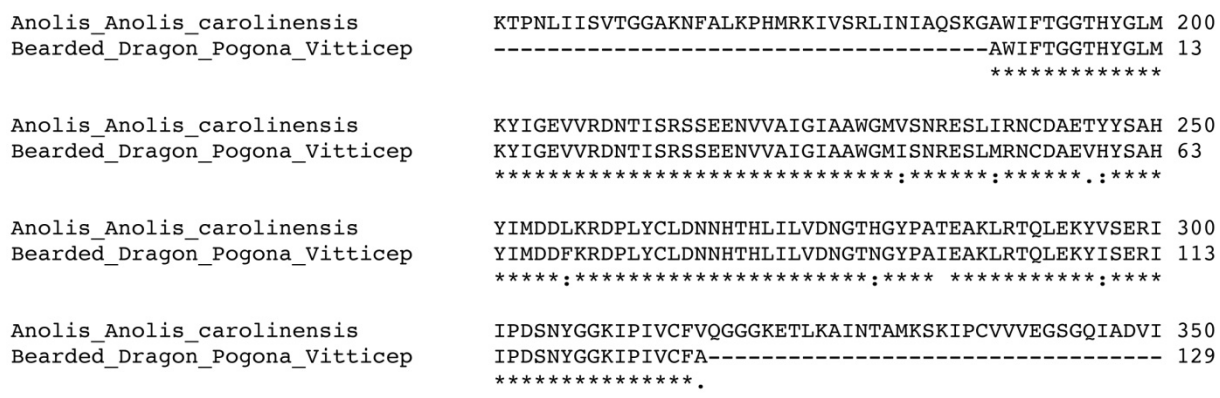

Figure 17: Bearded Dragon TRPM8 Protein Alignment

An alignment of anolis (*Anolis carolinensis*) TRPM8 protein and a portion of bearded dragon (*P. vitticeps*) TRPM8 protein that was sequenced. 93% identities are conserved across this area of the protein. '*' indicates a fully conserved residue, ':' indicates a residue with strongly similar properties, '.' indicates a residue with weakly similar properties. Alignments performed by European Bioinformatics Institute (EBI) online ClustalW2 alignment tool.

Figure 18: TRPM8 Protein Alignment and Menthol Residues

The alignment of the TRPM8 protein sequence from anolis (*A. carolinensis*), mouse (*Mus musculus*), chicken (*Gallus gallus*), and frog (*X. tropicalis*), with the four residues vital for menthol activation of TRPM8 highlighted (Y745, R842, Y1005, L1009; based on mouse sequence). '*' indicates a fully conserved residue, ':' indicates a residue with strongly similar properties, '.' indicates a residue with weakly similar properties. Alignments performed by European Bioinformatics Institute (EBI) online ClustalW2 alignment tool.

Intraperitoneal Injection of TRPM8-Sensitive Compounds Presents Methodological Shortcomings

The finding that bearded dragons did not respond to capsazepine or menthol injection is in contrast to many studies showing that pharmacological blockage and activation of TRPM8 can have a significant effect on thermoregulation. The study by Seebacher and Murray (2007) is the only such *in vivo* research using an ectotherm. While their results are consistent with the prevailing knowledge of thermoTRPs in endotherms, their methodology and conclusions require some analysis. For example, the use of water as a cool environment adds an additional, and potentially confounding variable when compared to the uniformity between environments that is standard in the majority of shuttle box experiments utilizing reptiles (Berk and Heath, 1975; Barber and Crawford, 1979; Cadena and Tattersall, 2009). By using a non-uniform environment, there is no control for the role of TRPM8 in other behaviours; in this case, water-seeking. This is similar to issues that arise when studying shuttling behaviour in the wild. For such an experiment in a lab environment, it is important to control for any other behaviours so that all observed behaviours are more easily classified as thermoregulatory.

These researchers also failed to report the body temperature values that they collected, stating only that body temperature fluctuated between the temperature of the water (22-26ºC) and basking (40-45ºC) environments. This prevents any conclusions from being made in regards to threshold temperatures for shuttling following capsazepine injection and subsequent inactivity of TRPM8. The documented clearance time of capsazepine is another factor which calls into question whether the observed behaviour can be entirely attributed to the drug's action. In mammals, capsazepine has a clearance time of less than 15 minutes (Gao et al., 2000; Douat et al., 2011). Seebacher and Murray (2007) however, document a behavioural change over the

course of 48 hours post-injection. Although reptiles have significantly lower metabolism than mammals, capsazepine may have cleared from the crocodiles, and in the case of this study bearded dragons, a relatively short time into each trial.

Thermoregulatory studies in mammals have been relatively conclusive in establishing a role for TRPM8 using pharmacological based methodology. The literature however is inconsistent in the method of administration for these drugs. Ruskin et al. (2007) injected menthol (TRPM8 agonist) subcutaneously in rats and observed up to a 1ºC increase in body temperature. Gavva et al. (2012) administered a selection of up to five TRPM8 antagonists in rats intravenously and observed approximately a 0.5ºC drop in body temperature. Knowlton et al. (2011) administered PBMC (TRPM8 antagonist) via intraperitoneal injection in wild-type and TRPM8KO mice and observed a short term drop in body temperature greater than 6ºC in the wild-type. Almeida et al. (2012) highlighted this problem with their finding that intravenous administration of a TRPM8 antagonist in rats was more effective at decreasing body temperature than intrathecal or intracerebroventricular administration. They conclude that this is due to a peripheral sensory function for TRPM8 and direct drug action at peripheral sites of channel expression when administered intravenously, but no direct evidence for this is provided. There is a clear consensus that TRPM8 signalling functions as a modulator for thermoregulation in mammals. The disparity in methods of administration however, presents a problem for separating the role of different thermosensory areas (*i.e.* periphery or core). The studies discussed above do not make a definitive case for whether TRPM8 is working as a peripheral or core thermosensor. It would be prudent for future studies of this kind to include assessments of drug dispersal in the body. Such a metric would allow us to determine what areas of TRPM8

expression are being influenced and develop a more accurate model for the intricacies of molecular thermosensation in mammals.

Neither capsazepine nor menthol elicited any changes in behavioural thermoregulation when injected intraperitoneally. Many potential issues may have contributed to this result. Timing may have been a factor for the results of the capsazepine trials. Despite the findings of Seebacher and Murray (2007), it is possible the drug cleared from the body in a relatively short time and the two day period between injection and trial may have been too long. No data exists specifically addressing capsazepine sensitivity of lizard TRPM8, however several studies have noted pharmacological differences in thermoTRPs of other species. For example, chicken TRPV1 is insensitive to the agonist capsaicin (Jordt and Julius, 2002) and frog TRPM8 is insensitive to the synthetic agonist icilin (Meyers et al., 2009). The possibility exists that crocodiles and lizards do not share sensitivity to capsazepine and future experiments would benefit from establishing this through molecular or cellular methods.

It is likely however, based on available evidence, that bearded dragon TRPM8 is sensitive to menthol. The comparison of TRPM8 protein in *A. carolinensis*, a lizard, and other animals is evidence that this lizard at least possesses all known residues required for menthol sensitivity (Figure 28). Coupled with the high degree of conservation in the protein sequence of TRPM8 between species (Figure 29), there is a high likelihood that *P. vitticeps* also possesses the required residues. In terms of why menthol did not elicit a thermoregulatory effect, factors may include the doses used or a similar clearance problem as considered with capsazepine. The anesthetic effects of menthol may preclude the ability to test higher doses in this species. An effort was made to eliminate the potential effect of clearance time by allowing only 30 min to elapse between drug injection and the start of data collection. The extensive handling required to

inject animals may result in a stress response that overlaps with the effect of menthol, negating any behavioural response prior to clearance of the drug.

As previously discussed, it is possible that intraperitoneal injection of these compounds only influences those channels present in the core tissues. It has been established previously in blue-tongued skinks that thermoregulation relies on a combination of sensory input from the core and periphery (Hammel et al., 1967). If the injected compounds blocked or activated only core sensors, it would be expected to have at least a small influence on overall thermoregulation. Additionally, physiological thermoregulation is not completely absent in the bearded dragon lizard (Seebacher and Franklin, 2005) and the recruitment of these mechanisms was not measured during my trials.

Chapter 4: Assessing the Role of TRPM8 in Bearded Dragon Thermoregulation by Topical Application of Menthol to the Trunk

Introduction

In mammals, dorsal root ganglia (DRG) and trigeminal ganglia (TG) neurons extend free nerve endings into the skin, through which sensory information is transmitted to the central nervous system. ThermoTRP channels are expressed in both ganglia, and thus are involved in sensing peripheral temperature (Cavanaugh et al., 2011). Extensive experimentation, using knockout mice, has confirmed that animals lacking TRPV3, V4, or TRPM8 channels are deficient in sensing ambient temperature in the ranges of activation associated with these thermoTRP channels (Guler et al., 2002; Dhaka et al., 2006; Wechselberger et al., 2006; Caterina, 2007; Colburn et al., 2007). Although thermoTRP channels are also expressed in the viscera and the brain (Guler et al., 2002; Xu et al., 2002; Sabnis et al., 2008), these knockout experiments did not observe any behaviours that would indicate impaired core thermosensation (*i.e.* irregular thermoregulation at constant ambient temperature).

The separation between peripheral and core thermosensory control of thermoregulation has been extensively studied and debated in mammals (Griffin, 2004; Bratincsak and Palkovits, 2005; Bicego et al., 2006; Morrison and Nakamura, 2011). Much of this debate originates from experiments that stimulate specific thermosensory areas, resulting in thermoregulatory responses. The periphery is a vital thermosensory structure since it is in direct contact with the environment and the first line of defence in detecting changing ambient temperatures. If these signals do not elicit an appropriate thermoregulatory response, core and brain temperature would eventually be affected (Flouris, 2010). Cooling the peripheral area can elicit immediate thermoregulatory

responses (Jessen, 2000; Nakamura and Morrison, 2007). The hypothalamus also contains highly temperature sensitive areas (Morrison and Nakamura, 2011), which when cooled or warmed can elicit thermoregulation in the opposite direction (Boulant, 2000; Griffin, 2004; Bicego et al., 2006). One of the main reasons this is of such interest, is because it has the potential to provide information on neuronal integration of thermosensory information. The question of what thermosensors activate what thermoeffectors remains poorly understood in endotherms (Romanovsky, 2007; Flouris, 2010), and even less so in ectotherms (Hammel et al., 1967).

Menthol was applied directly to the skin in an effort to activate peripherally expressed TRPM8 and observe the effect on the thermoregulatory behaviours of shuttling and gaping. One of the problems associated with intraperitoneal injection is an inability to determine if the drug is affecting sites of peripheral thermosensation. By directly stimulating the periphery, we can better ensure relevant thermosensors are activated and observe the thermoregulatory effects of stimulating a single thermosensitive area.

Materials and Methods

Experimental Set-up for Examining Thermoregulatory Gaping Behaviour

An environmental chamber (Thermo Forma; Marietta, OH, USA) was used to maintain a constant temperature environment of 37.5-38.5ºC (Figure 19). Lizards were placed inside a clear acrylic box with no lid (24 x 24 x 40 cm). The box was blacked out on three sides to prevent distraction and minimize reflections. The incubator was lit with fluorescent bulbs above and

Figure 19: Gaping Box

Apparatus for assessing gaping behaviour in bearded dragons. Entire apparatus is located inside a Thermo Forma environmental chamber to maintain constant temperature of 37.5-38.5ºC. A) Thermal camera B) Plexiglass box in which animal is placed C) Webcam to record gaping behaviour D) Clock to record timing of behaviour.

below the box containing the animal. An IR thermal imaging camera (Mikron 7515; Oakland, NJ, USA) was hung above the opening in the box, leaving space to allow air circulation to reach the animal. This camera was used to collect skin temperature data from the animal throughout a trial. A webcam was used to monitor the animal and capture gaping behaviour (Figure 20). Gaping is categorized as a sustained opening of the mouth, primarily in response to prolonged exposure to hot temperatures (Gerlach and Tattersall, 2005). Images were captured every 2 s from a computer outside the environmental chamber using Webcam Timershot®. The capture period was not very precise, so a clock was placed in front of the webcam to facilitate assessing the length of time gaping occurred with more accuracy.

Experimental Design

Series III: Topical Application of Menthol and Shuttling Behaviour

Menthol was used topically as a TRPM8 agonist, to assess the role that TRPM8 in free nerve endings of the dorsal trunk skin plays in thermoregulatory shuttling. Menthol or its vehicle (95% ethanol) was applied at 2:00 pm, partway through the data collection period (Figure 21). This allowed the animals to begin exhibiting non-exploratory thermoregulatory behaviour prior to application of the compound. Menthol was applied at a dose of 1200 mg/kg in a total volume of 3 mL/kg. This equated to a 40% menthol solution being applied, coinciding with the maximum surface dose administered to rats in previous experiments (Klein et al., 2010).

Figure 20: Assessing Gaping Behaviour

Sample image from webcam of a bearded dragon in the gaping box exhibiting gaping behaviour. Clock is used to accurately assess the percent of time the animals spend gaping at warm temperatures.

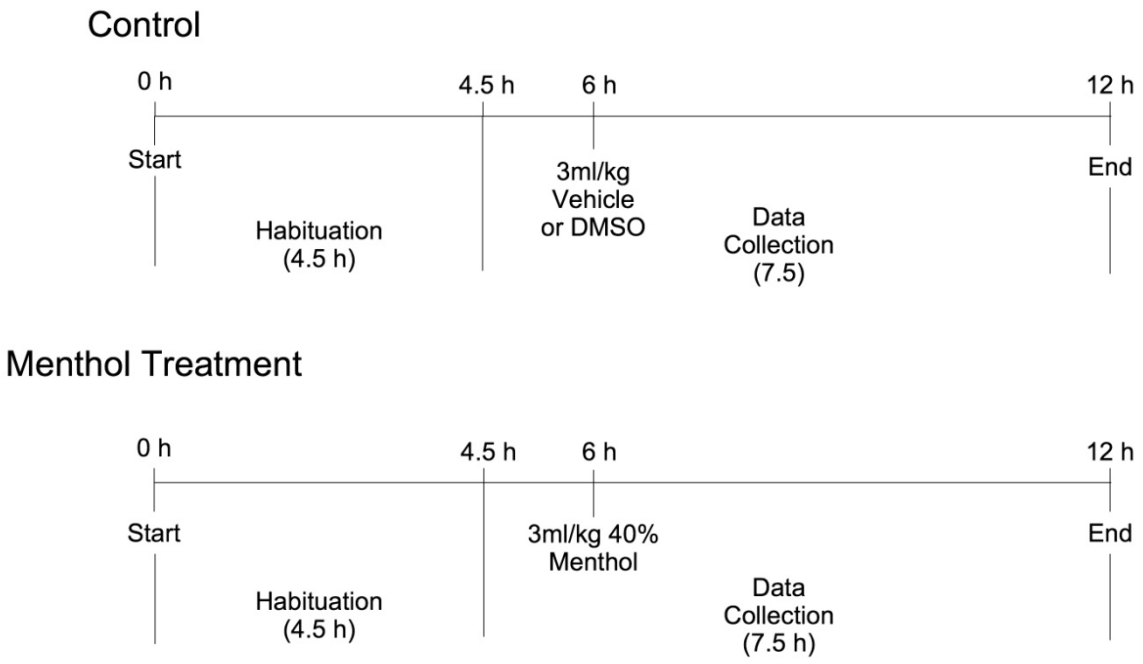

Figure 21: Protocol for Series III: Topical Application of Menthol and Shuttling Behaviour

Time course of topical menthol experiments performed in the ramping shuttle box. Animals were administered 3 mL/kg of 40% menthol, vehicle (ethanol), or DMSO topically. Compounds were applied 1.5 hours into the data collection period to allow animals to begin to exhibit normal periodic shuttling behaviour prior to drug application.

A second control was performed for this series using 100% DMSO, applied at a volume of 3 mL/kg. This was done to account for the possible biological activity of ethanol on thermoTRP channels (Klein et al., 2010). Although DMSO also has evidence of biological activity (Tzabazis et al., 2005), it is a commonly used vehicle that can be absorbed into the lizards' skin, in contrast to water, which simply beads off the lizards' backs. For the DMSO treatment, the data collection period was approximately two hours shorter, as the time of interest was determined to be immediately following application of compound. A total of 11 animals were used for this experiment (fully paired; n=11 menthol, n=11 vehicle [ethanol], n=11 DMSO). No body temperature was recorded for this series. Thermal camera images were obtained only for menthol and ethanol groups at each upper escape and lower escape event during the data collection period. Order of menthol and ethanol trials was randomized, while DMSO trials were done last in all cases. A minimum of four weeks of time elapsed between treatments an individual animal received.

Series IV: Topical Application of Menthol and Gaping Behaviour

In contrast to the shuttle box experiments, gaping experiments were run between 10:00 am and 5:00 pm, with sessions typically lasting 3 h. Previous studies have shown that progressively warmer temperatures increase the frequency and degree of gaping behaviour in bearded dragons (Tattersall and Gerlach, 2005). To assess if chemical activation of 'cool' sensory input can override a warm-induced behaviour, lizards were held at a constant temperature of 38±0.5ºC to induce gaping behaviour, at which point menthol was applied topically. Lizards were first washed in warm water to clear the back of dust and debris and induce cloacal discharge, which

was a typical occurrence in previous experiments (Tattersall and Gerlach, 2005). This had the added benefit of allowing the animals to drink, as hydration status influences the amount of time animals spend gaping (Paramentor and Heatwole, 1975). Lizards were then placed inside an acrylic box blacked out on three sides, inside an environmental chamber (Thermo Forma), for 1-1.5 h to equilibrate to ambient temperature. Menthol or vehicle (95% ethanol) was applied at the same dose as Series III topical experiment (1200 mg/kg, with total volume of 3 mL/kg). Animals were again allowed time to equilibrate following the handling and immediate temperature change to their skin. Frequency of gaping behaviour was observed from 0-60 min post-menthol or vehicle application. A total of 12 animals were used for this experiment (n=12 menthol, n=12 ethanol). Order of treatment and control was randomized and a minimum of two weeks separated treatment and control experiments on the same animal.

Statistical Analysis

Series III: Topical Application of Menthol and Shuttling Behaviour

Previous experiments have shown that the behavioural changes in mice induced by topical application of menthol are relatively short (60-90 minutes; Tajino et al., 2007; Klein et al., 2010). To account for the short time course of the drug, UET and LET events were compared individually. Comparisons between menthol and ethanol group used $UET_{2\text{-}8}$ and $LET_{2\text{-}8}$. Comparisons between all three treatment groups (menthol, ethanol, and DMSO) used $UET_{2\text{-}6}$ and $LET_{2\text{-}6}$. In each case, this was the maximum number of UET's or LET's (ambient and skin) available for all animals and treatment groups. The mean UETa, UETts, LETa, or LETts were calculated for each shuttle event across all animals. Due to the potential for handling induced

disturbances during injection, UET_1 and LET_1 were omitted in all cases from statistical tests. A two-way RM ANOVA was used to test data with normalized distribution ($UETa_{2\text{-}6}$, $LETts_{2\text{-}8}$) and an SRH test was used to test non-normal data ($LETa_{2\text{-}6}$, $UETts_{2\text{-}8}$). Mean T_a, UETa, and LETa were calculated for all animals and compared between treatment and control groups using a one-way RM ANOVA. To assess the time course effects of menthol, LETa values for the first, second, and third hour following menthol application were taken from each individual animal and compared between menthol and ethanol control group using a Wilcoxon test for non-parametric data.

Series IV: Topical Application of Menthol and Gaping Behaviour

Gaping data were collected in 15 min periods over the course of 60 min post-menthol application. A two way RM ANOVA was performed to assess significance between the percentage of time spent gaping with menthol or vehicle over each 15 min period. A Holm-Sidak *post hoc* test was performed to determine where interaction effects occurred between shuttle number and treatment.

Results

Series III: Topical Application of Menthol and Shuttling Behaviour

There was a significant, short-lived increase in lower escape ambient temperature resulting from menthol applied to the trunk region, with no corresponding change in upper escape

temperature. There was no significant difference in the number of shuttles per hour completed by the menthol group compared to the ethanol and DMSO groups (Table 1; $F_{2,20}$=2.939, p=0.077). Mean T_a, UETa, and LETa showed no significant difference over the course of the entire data collection period between the three groups (Figure 22; $F_{2,20}$=0.846, p=0.444; $F_{2,20}$=2.032, p=0.157; $F_{2,20}$=1.000, p=0.385). A Scheirer-Ray-Hare (SRH) test, using shuttle number and all three treatments as factors, showed a significant main effect of treatment for $LETa_2$ through $LETa_6$ (Figure 23; p=0.041), but no significant main effect of shuttle number (p=0.479) or interaction (p=0.118). Subsequent Wilcoxon tests showed that over the course of $LETa_{2\text{-}6}$, the difference occurred between the menthol and ethanol group (Z=2.135, p=0.033), with no significance between menthol and DMSO (Z=-0.581, p=0.564). Although also not significant, the difference between ethanol and DMSO nearly achieved threshold for significance (Z=-1.961, p=0.051).

To determine the magnitude and time-course of the difference between menthol and ethanol control, mean LETa was compared within each of the first three hours following menthol application (Figure 24). The number of shuttles performed during each hour was also recorded (Table 2), with no significant difference found. LETa of menthol was significantly higher in the first hour (Z=-2.578, p=0.007), but not in the second (Z=-0.533, p=0.638) or third (Z=-1.423, p=0.175). This suggests a short effect of topical menthol application, in line with that observed in mammalian studies (Tajino et al., 2007; Klein et al., 2010).

A two way RM ANOVA for treatment and shuttle number showed no significant difference in $UETa_{2\text{-}6}$ (Figure 25), either as a main effect for treatment ($F_{8,80}$=1.251, p=0.308) and shuttle number ($F_{8,80}$=0.325, p=0.86), or as an interaction ($F_{8,80}$=1.324, p=0.244).

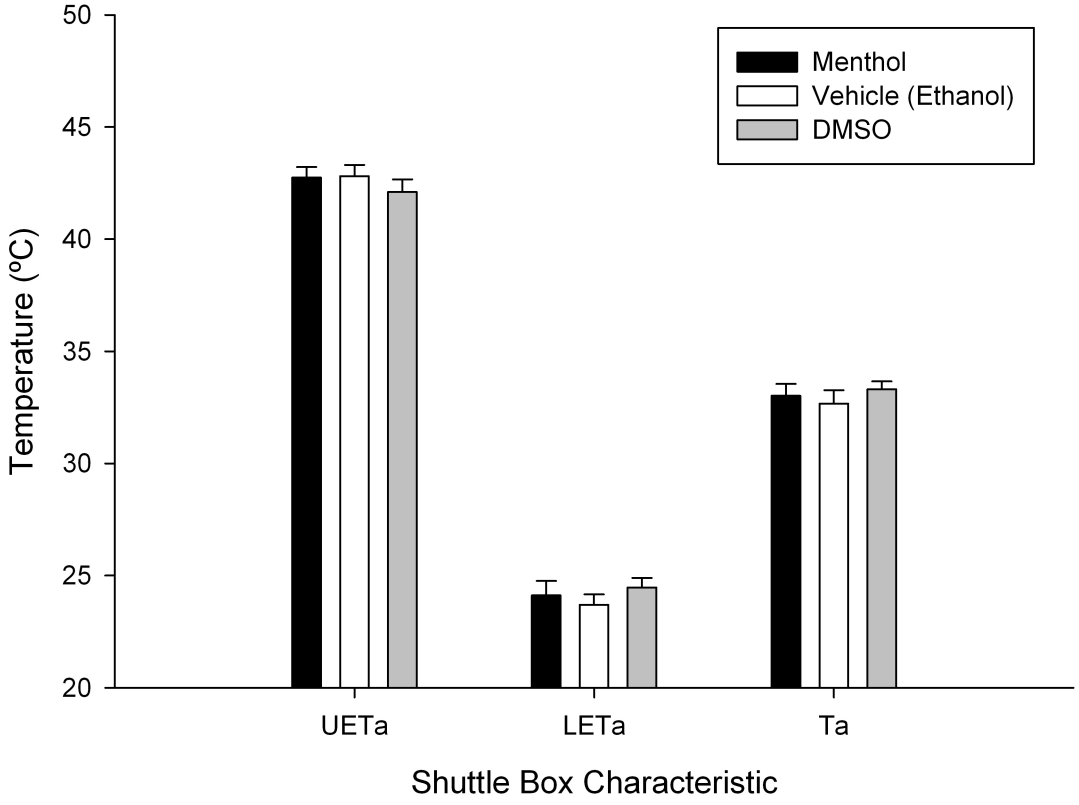

Figure 22: Parameters of Thermoregulation with Topical Application of Menthol, Vehicle (Ethanol), or DMSO; Series III

Mean upper and lower escape ambient temperatures (UETa and LETa, respectively) and mean ambient temperature (T_a) are plotted for bearded dragon lizards (*P. vitticeps*). A volume of 3mL/kg (40% menthol, vehicle [95% ethanol], or 100% DMSO) was applied to the dorsal trunk of the animals following a six hours in an electronic shuttle box. Data collection occurred over the following six hours (except for the DMSO group where data collection occurred over the following four hours). This series was completely paired and at least one month separated trials on the same animal. Values are mean±SE (n=11, menthol; n=11, ethanol; n=11, DMSO).

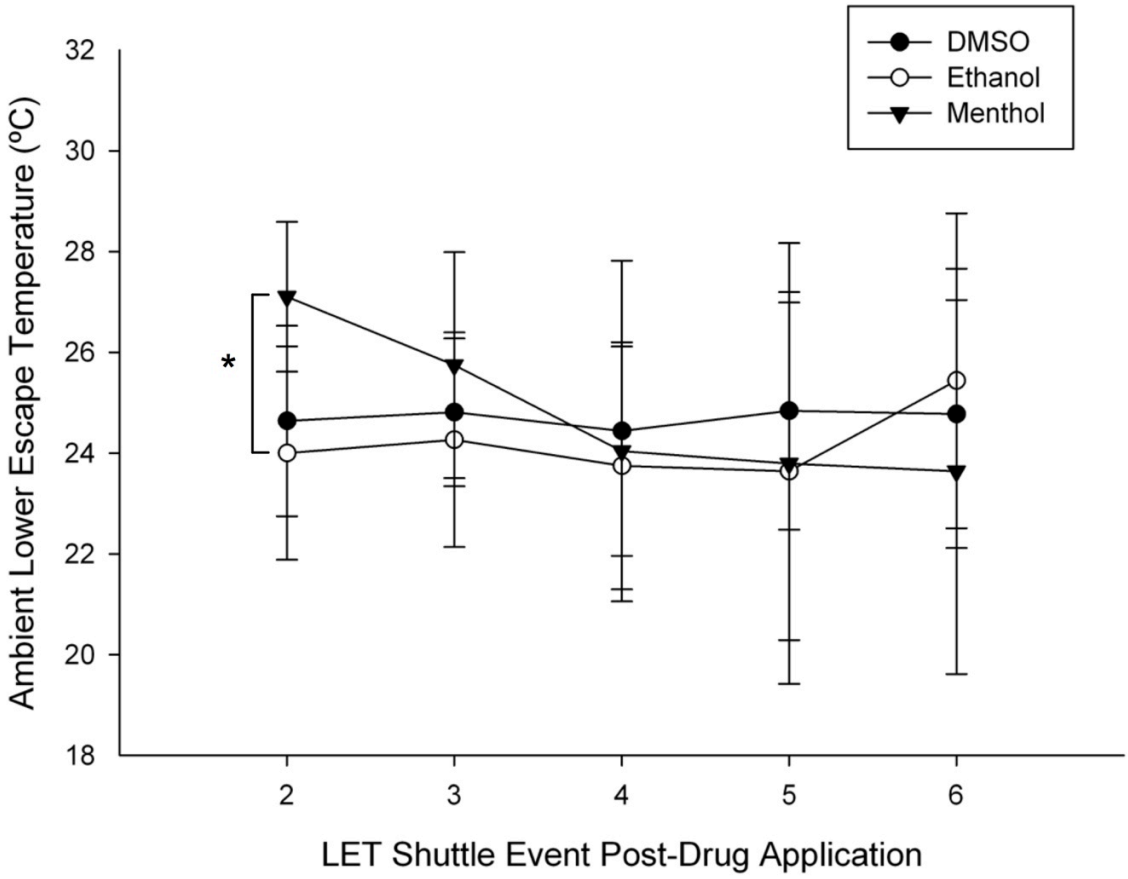

Figure 23: LETa$_{2-6}$ Events Following Topical Application of Menthol, Vehicle (Ethanol), or DMSO to the Trunk; Series III

Mean ambient lower escape temperature (LETa) is plotted for each of shuttle events 2-6 performed by bearded dragon lizards (*P. vitticeps*) in an electronic shuttle box. A volume of 3mL/kg (40% menthol, vehicle [95% ethanol], or 100% DMSO) was applied to the dorsal trunk of the animals following six hours in an electronic shuttle box. '*' denotes significance between menthol and ethanol group, as determined by a targeted Wilcoxon test ($p<0.05$). This statistical test followed the determination that a significant difference existed between treatments, using a Shreir-Ray-Hare ($p<0.05$). This series was completely paired and at least one month separated trials on the same animal. Values are mean±SD (n=11, menthol; n=11, ethanol; n=11, DMSO).

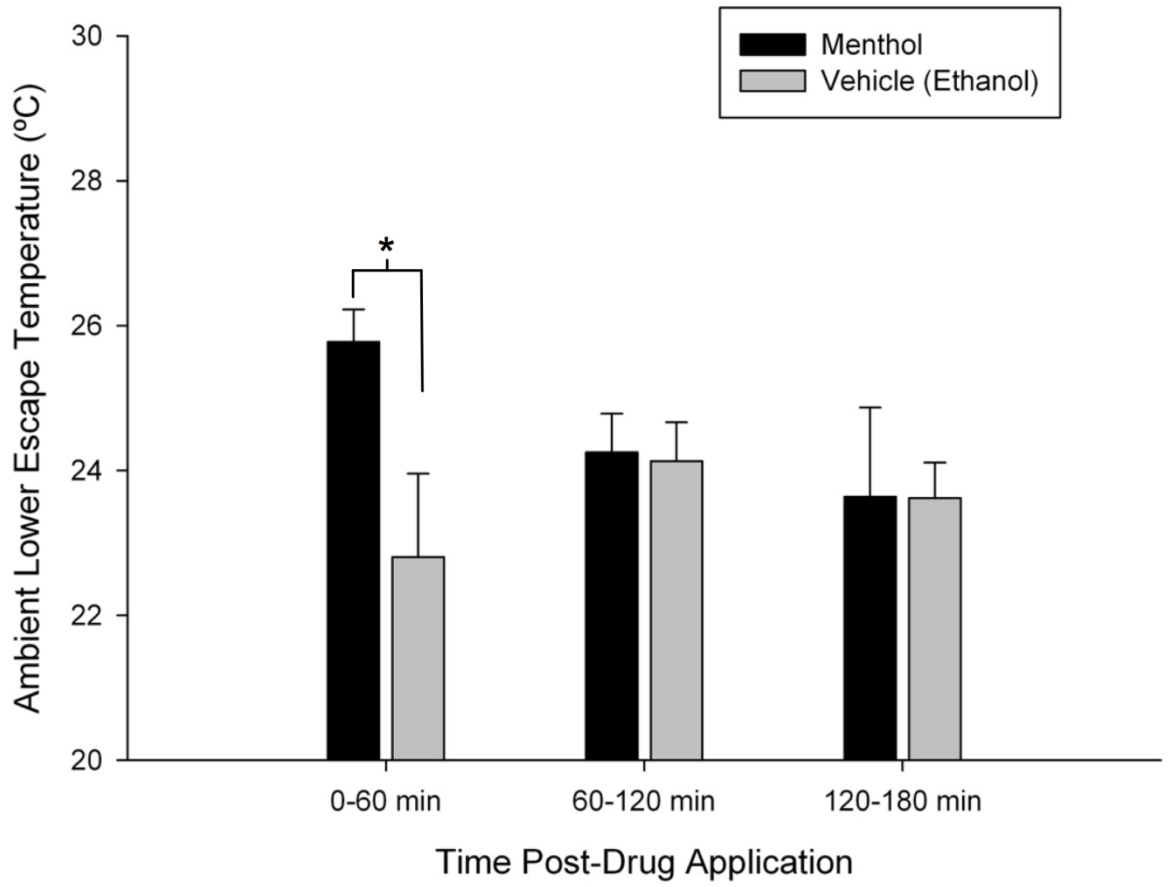

Figure 24: Hourly LETa Means for Topical Menthol or Vehicle (Ethanol); Series III

Mean LETa for each animal was determined for the first three hours following treatment, and averaged for all animals in each group. A volume of 3mL/kg (40% menthol or vehicle [95% ethanol]) was applied to the dorsal trunk of the animals following a six hours in an electronic shuttle box. '*' denotes significant difference exists between menthol and vehicle from 0-60 min ($p<0.05$). This series was completely paired and at least one month separated trials on the same animal. Values are mean±SE (n=11, menthol; n=11, ethanol)

Table 2: Hourly Shuttle Means for Topical Menthol or Vehicle (Ethanol); Series III

Mean number of shuttles performed in each of the first three hours of series III experiments (Mean±SD) for menthol and vehicle (95% ethanol). No significant difference occurred between treatment groups within any time interval ($p>0.05$).

Time (min)	Menthol	Vehicle (Ethanol)
0-60	3.2±1.3	2.1±0.8
60-120	2.7±1.1	2.5±1.6
120-180	2.4±1.4	2.4±1.0

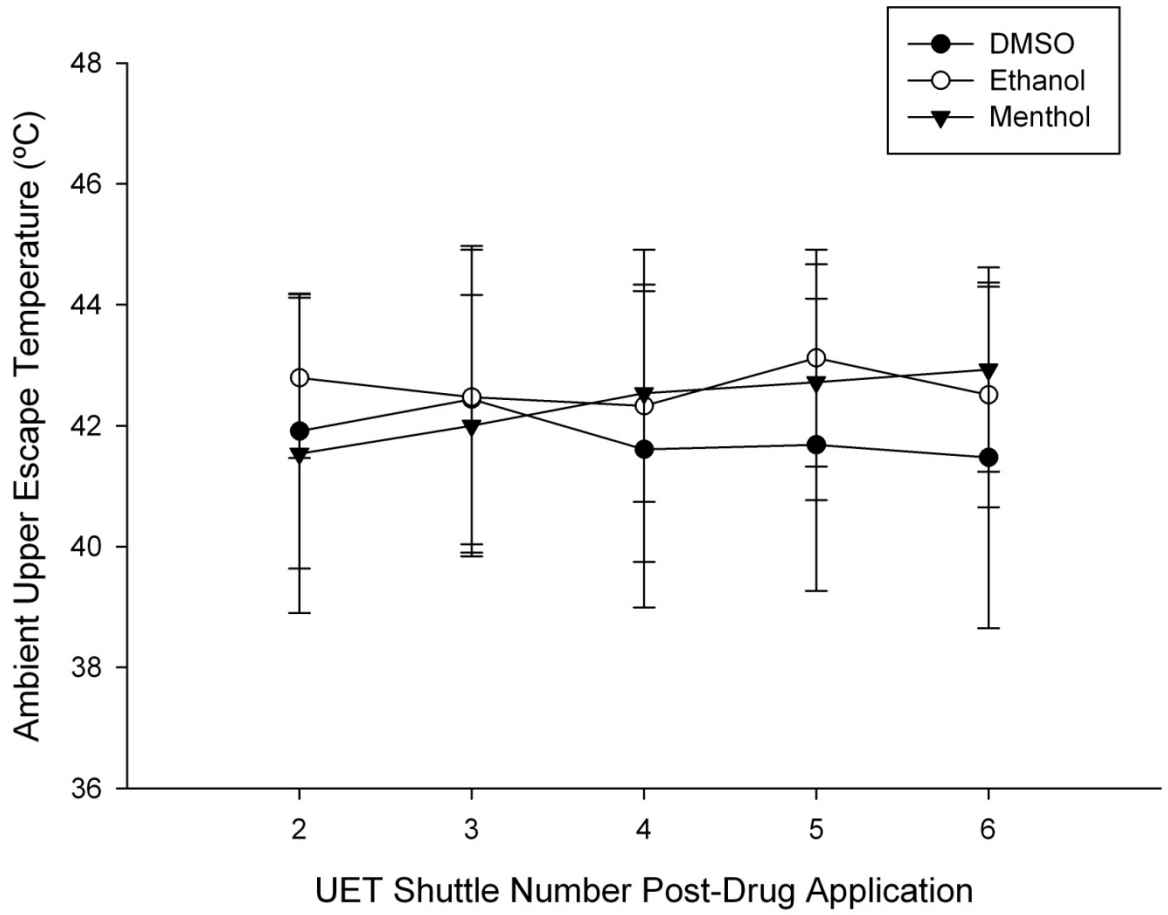

Figure 25: UETa$_{2\text{-}6}$ Events Following Topical Application of Menthol, Vehicle (Ethanol), or DMSO to the Trunk; Series III

Mean ambient upper escape temperature (LETa) is plotted for each of shuttle events 2-6 performed by bearded dragon lizards (*P. vitticeps*) in an electronic shuttle box. A volume of 3mL/kg (40% menthol, vehicle [95% ethanol], or 100% DMSO) was applied to the dorsal trunk of the animals following a six hours in an electronic shuttle box. This series was completely paired and at least one month separated trials on the same animal. Values are mean±SD (n=11, menthol; n=11, ethanol; n=11, DMSO).

LETts and UETts were only available for menthol and ethanol groups. A significant effect was observed in $LETts_2$ between groups (Figure 26). A two way RM ANOVA for treatment and shuttle number showed no main effect of either factor ($F_{4,40}$=4.417, p=0.062; $F_{4,40}$=2.298, p=0.076), but did show a significant interaction effect ($F_{4,40}$=3.943, p=0.009). A Holm-Sidak *post hoc* test indicated that higher skin temperature values occurred at the second shuttle of menthol (32.0±1.3ºC) compared to ethanol (29.5±1.8ºC). No other differences were detected through interaction effects within or between treatment and shuttles. A SRH for treatment and shuttle number of $UETts_{2\text{-}6}$ showed no significant difference in main effect of shuttle number (p=0.941) and treatment (p=0.432), or as an interaction (Figure 26; p=0.896).

Series IV: Topical Application of Menthol and Gaping Behaviour

No significant difference was found in the amount of time spent gaping between treatment and control group within any 15 min period from 0-60 min (Figure 27). There was a significant difference between subsequent 15 min periods within the treatment and control groups, with the percent time spent gaping slowly increasing over each successive 15 min period, over 60 min (Figure 27). A two way RM ANOVA for treatment and time showed no significant main effect of treatment ($F_{3,\ 33}$= 0.048, p=0.831) or interaction effect ($F_{3,\ 33}$=1.078, p=0.372). There was however, a significant difference in the main effect of time ($F_{3,\ 33}$=38.629, p=<0.001). A Holm-Sidak *post hoc* test showed significance between every 15 min period, independent of treatment (Figure 27). Skin temperature remained relatively constant through the entire 60 min period, except for a small drop in the first 15 min caused by application of solution to the back (Table 3).

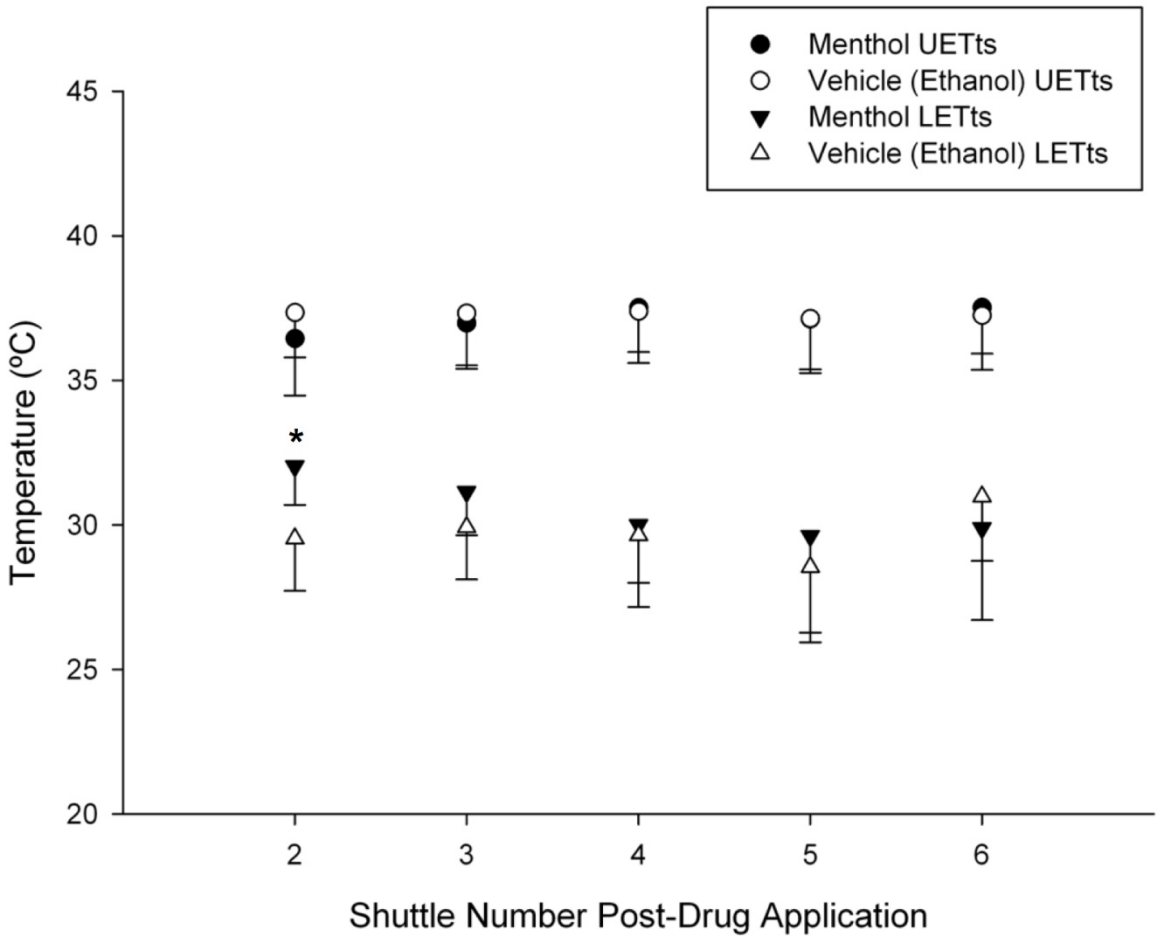

Figure 26: $UETts_{2-6}$ and $LETts_{2-6}$ Events Following Topical Application of Menthol, Vehicle (Ethanol), or DMSO to the Trunk; Series III

Mean upper and lower escape trunk skin temperature (UETts and LETts, respectively) is plotted for each of shuttle events 2-6 performed by bearded dragon lizards (*P. vitticeps*) in an electronic shuttle box. A volume of 3mL/kg (40% menthol or vehicle [95% ethanol]) was applied to the dorsal trunk of the animals following a six hours in an electronic shuttle box. '*' denotes significance between menthol and ethanol group This series was completely paired and at least one month separated trials on the same animal. Values are mean±SD (n=11, menthol; n=11, ethanol).

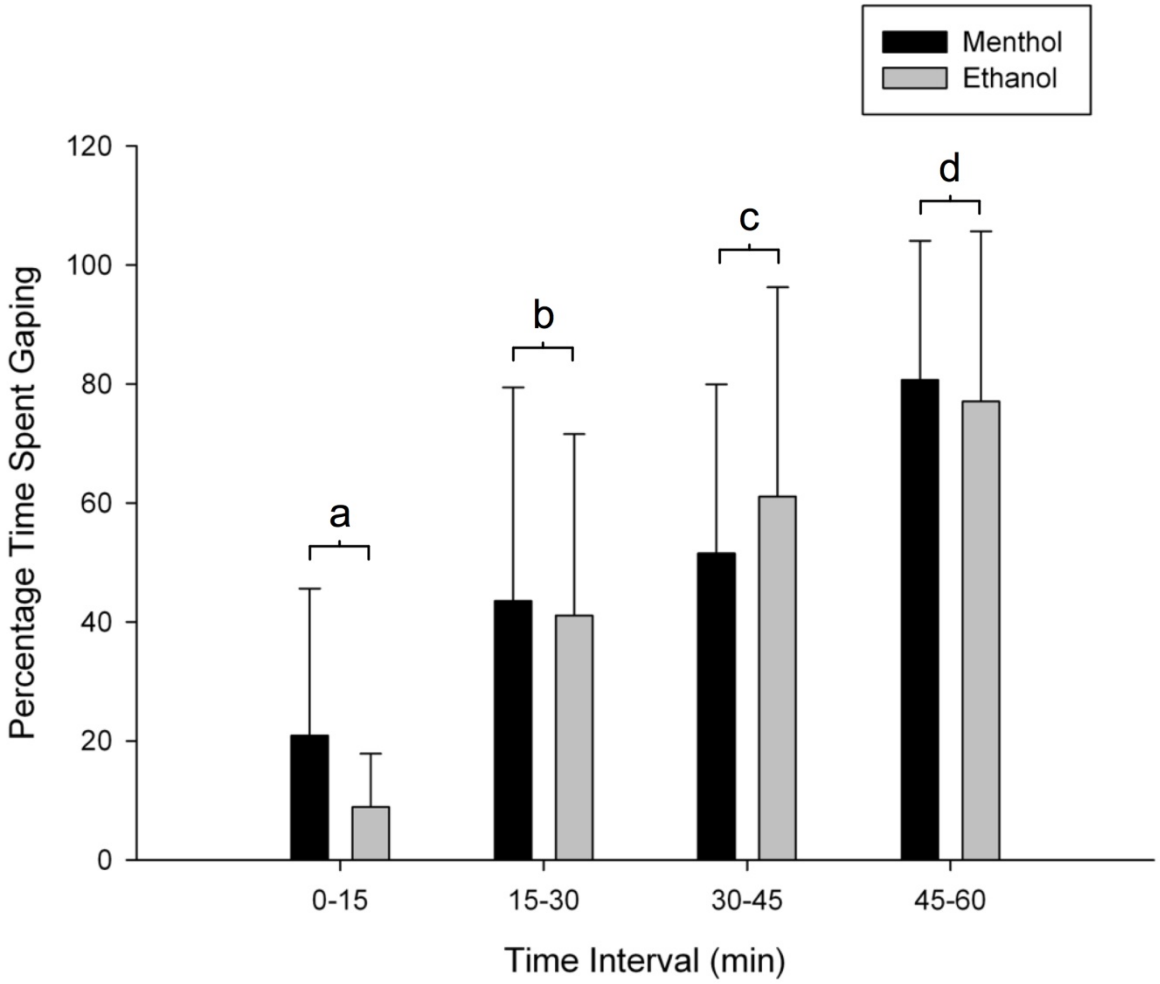

Figure 27: Gaping over 60 Minutes Following Topical Application of Menthol or Vehicle (Ethanol); Series IV

Time 0 represents application of 3mL/kg volume (40% menthol or vehicle [95% ethanol]) to the dorsal trunk of bearded dragon lizards (*P. vitticeps*) following approximately one hour of habituation at 38±0.5 ºC ambient temperature. The percentage of time spent gaping is plotted in 15 minute intervals for 60 minutes. This series was completely paired and at least two weeks separated trials on the same animal. Values are mean±SD (n=11, menthol; n=11, ethanol).

Table 3: Skin Temperature of Animals Following Topical Menthol Application in the Gaping Box

During gaping trials, skin temperature was recorded using an IR thermal imaging camera. No significant difference was found between subsequent 15 minutes time periods, or between treatments. Values are mean±SE.

Time (min)	Menthol (ºC)	Vehicle (Ethanol; ºC)
0-15	36.77±0.1	36.75±0.1
15-30	37.93±0.1	38.12±0.1
30-45	38.13±0.2	38.30±0.1
45-60	38.12±0.2	38.24±0.1

Discussion

Following activation of TRPM8 with topically administered menthol, lizards left the cold compartment of the shuttle box at warmer temperatures compared to vehicle controls. This is indicative of menthol inducing a cooling sensation, which elicits a corrective behavioural response. Bearded dragon shuttling behaviour appears to be at least partially regulated by TRPM8, which fulfils the role of cool temperature detector in the periphery. Tajino et al. (2007) showed similar cold avoidance in mice at a concentration of 10% menthol (compared to 40% in my study), albeit in a two temperature choice assay. Current understanding of thermoTRP channels supports a thermosensory role for TRPM8 in the thermoregulatory response (Dhaka et al., 2007; Almeida et al., 2012) and my results show the potential for a conserved function in the bearded dragon. Furthermore, my results of gaping behaviour show an exclusive role for TRPM8 in behaviours associated with the cold spectrum of thermoregulatory behaviours. TRPM8 activation (i.e. induced cool sensation via menthol) did not reduce or extinguish gaping. This is a logical extension of the mammalian thermoTRP channel profiles (Figure 4); warm sensitive thermoTRP channels regulate warm-associated thermoregulatory behaviours, while cold sensitive thermoTRP channels regulate cold-associated behaviours.

Topically Applied Menthol Modifies the Threshold Temperature for Cold Escape Temperatures

Menthol application resulted in an increase in the threshold for LETa by approximately 2-3ºC for one hour, and to a lesser extent, LETts. The finding here that menthol modifies thermoregulation is novel for reptiles. Menthol has long been known to elicit cooling sensation in mammals (Hensel and Zotterman, 1951) presumably through the singular activation of

TRPM8 (McKemy et al., 2002; Peier et al., 2002). It is probable that the same is true in the bearded dragon. Mice that are administered menthol to the skin show a short term increase in body temperature, indicative of cold sensation inducing the activation of excess heat production (Tajino et al., 2007; Klein et al., 2010). An animal feels cold, and therefore demonstrates behaviour (and/or physiology in the case of endotherms) to raise temperature.

In the bearded dragon, the thermoregulatory response did not occur immediately, but rather required a certain amount of cooling to elicit the behaviour. Animals would actively move to the warm side of the box, return to the cold side once their warm threshold was achieved, and remain on the cold side before returning. This behaviour is similar to experiments where the hypothalamus was warmed and cooled in the blue tongue skink (Hammel et al., 1967). The skinks also required a certain amount of cooling or warming in other areas to elicit a behavioural response (although the threshold for this response rose and fell with warming and cooling of the hypothalamus), rather than immediately responding to changes in hypothalamic temperature. In much the same way, cooling of the back of bearded dragons with menthol potentially resulted in an upward shift in the threshold of other thermally sensitive tissues required to elicit a behavioural response. Such a response suggests that neural information is processed in an additive manner within the lizard brain through thermoTRP signalling pathways.

The delayed thermoregulatory response to menthol in the bearded dragon is in contrast to the more immediate responses observed in mammals. Topical menthol application resulted in an almost immediate increase in metabolic heat production in mice, in the absence of other temperature stimuli (Tajino et al., 2007). There exist many mammalian studies showing that stimulus of a single temperature sensitive tissue (i.e. periphery or core) is enough to elicit a response outside of stimulus to other areas (Jessen, 2000). For example, the conscious goat is a

popular model for these studies. In this model, a goat is placed in a water bath so that skin temperature can be changed, while body temperature is maintained constant. As skin temperature rises, heat production lowers, and vice versa. This demonstrates that in mammals skin temperature signals can modify thermoregulation in the absence of similar signals from other body areas. The same has been shown in human subjects (Jessen, 2000). Contrasted with the findings in lizards discussed above, there may exist a fundamental difference in how thermoTRP signals are processed in the brains of endotherms and ectotherms. This is further supported by the findings of the series IV gaping experiment, discussed below.

Like many thermoTRP sensitive compounds however, a degree of promiscuity has been found to occur with menthol (Macpherson et al., 2006; Voets et al., 2012). In addition to its activation of TRPM8, it has been shown to activate or block TRPV3 and TRPA1, depending on the study and concentration used. Human subjects have reported enhanced feelings of warmth when exposed to solutions of 0.02% menthol (Green, 1985). In a separate study by Hatem et al (2006), four subjects reported a warming sensation following application of 30% menthol to the skin. Further work by Macpherson et al (2006) using *in vitro* preparations identified activation of TRPV3 and inhibition of TRPA1 by menthol, in patch-clamped CHO (chinese hamster ovary) cells. In contrast, Karashima et al (2007) showed a robust activation of TRPA1 by menthol in *in vitro* preparations, including a similar CHO cell patch-clamp, going so far as to conclude that TRPA1 was a necessary element in the whole animal response to menthol. It is difficult to determine which study is correct in their conclusions, however where Macpherson et al (2006) stopped at CHO cell preps, Karashima et al (2007) also performed calcium imaging on wildtype and TRPA1KO trigeminal ganglion (TG). These additional experiments showed that menthol activates approximately 38% of TRPA1 expressing neurons in the TG (as identified by mustard

oil [allyl isothiocyanate] sensitivity). The more extensive experiments lend more weight to the results and conclusions presented by Karashima et al (2007) in regards to TRPA1. Unilateral activation of TRPA1 can be discounted here, as animals did not show any behaviours associated with the immediate onset of pain, as would be expected for a noxious cold receptor. It is possible, however, that some TRPA1 activation occurred in tandem with TRPM8, contributing to the behavioural response observed.

Estimating Thresholds for ThermoTRP Activation

Using lower escape temperatures, an estimate of TRPM8 threshold for activation can be determined. LETa values across all series were approximately 25ºC, while LETts values across all series had a mean of approximately 30ºC. There is a major question of which value better predicts channel activation. It can be argued that for a channel such as TRPM8, which is expressed in free nerve endings of the DRG and TG and embedded in the skin, that skin temperature is a better metric, meaning we can estimate an activation of greater than or equal to 30ºC. This does present problems for species where skin temperature is influenced by changes in circulation. Most mammals can alter their skin temperature in the face of changing ambient temperatures (Jessen, 2000), a process which involves the interplay between ambient temperature, blood temperature, and heat flow in and out of the body. The presence of fur and other insulation further confounds this measurement in mammals (Jessen, 2000). Active fluctuations in skin temperatures presents difficulties in understanding what thermal environment exists around peripheral thermoTRP channels, and what temperature *in vitro* is required for their activation.

For an animal like the bearded dragon, there is only limited capability for changes in peripheral circulation (Seebacher and Franklin, 2005) and nearly zero capacity for metabolic heat production. In spite of these limitations, skin temperature maintained a 5-6ºC differential with ambient temperature (Table 4). This difference may be attributed to heat capacity and thermal conductance of the skin (retaining heat during cooling, and heating slower than the environment). Regardless of the mechanism, it suggests a temperature environment for free nerve endings that is significantly different from ambient. To correctly identify a threshold temperature for TRPM8, more direct measurement of the skin or cellular methodology would compliment the measurements for skin temperature presented here. Dermal implants may be beneficial in establishing any gradient that exists between the outer skin temperature, which I measure, and dermal temperature. It is expected that the temperature of the dermis would fall between the external skin temperature and body temperature (which was higher during cold escapes). With the available data, a threshold of greater than or equal to 30ºC is possible for bearded dragon TRPM8.

Other thermoTRP channels, such as TRPV3 and TRPV4 may detect ambient temperatures more directly. Both channels are expressed in free nerve endings, but these two channels are also uniquely expressed in keratinocytes of mice (Chung et al., 2004). Unlike free nerve endings, which are embedded under the skin, keratinocytes are in direct contact with the ambient environment. This may confer a more direct ability for mammals to sample ambient warmth, compared to cold. Such expression patterns have not been identified in lizards, so it can only be assumed that skin temperature plays a role in determining the temperature environment of these channels in the bearded dragon. The potential for interplay between multiple warm sensitive thermoTRP channels, none of which were tested directly, makes it impossible to make an

informed assumption regarding threshold for activation of warm sensitive thermoTRPs in the bearded dragon.

An activation temperature of 30ºC in the bearded dragon TRPM8 channel is higher than the 28ºC threshold for mammalian TRPM8 channels (Colburn et al., 2007; Dhaka et al., 2007). Many mammalian studies have determined this threshold by recording the activation temperature of TRPM8 *in vitro*. This has been done through the expression of TRPM8 in isolated cells (McKemy et al., 2002; Peier et al., 2002), and by calcium imaging of isolated, TRPM8-expressing TG and DRG neurons (Bautista et al., 2007; Dhaka et al., 2007). None of these experiments address the relatively new discovery that thermoTRPs are voltage-gated channels (Liedtke and Heller, 2007; Martinac, 2008). As such, the aforementioned experiments are unlikely to have taken extra steps to ensure a physiologically relevant environment for their cell preparations, potentially compromising the results. Any difference in membrane potential across the cell membrane would alter the temperature threshold required for channel opening. Controlling for membrane potential in future experiments is of the utmost importance for accurate results, and it would be prudent to re-evaluate the use of widely accepted values for threshold activation temperature. In spite of this, much will likely be gained in future experiments by using *in vitro* preparations to determine activation threshold of bearded dragon TRPM8 and comparing it to lower escape temperature values.

Topical Menthol is Insufficient to Override Warm Sensory Input and Inhibit Associated Behaviours

Gaping is a behaviour brought on by warm temperatures, and as such it can be concluded that warm sensitive neurons contribute to this behaviour. Although not within the scope of this study, from the high level of conservation seen amongst the thermoTRPs (Saito and Shingai, 2006), it is likely that TRPV3, V4 and potentially V1 and V2 are recruited to elicit gaping behaviour. That menthol did not induce a reduction in gaping behaviour, or alter the threshold for upper escape temperature, has implications for how thermal information is integrated.

During periods of warming, the bearded dragon is presumably receiving signals from warm-sensitive neurons, which are integrated in the hypothalamus and activate the gaping thermoeffector. As described above, menthol elicits a cooling sensation when applied to the skin, most likely through the activation of TRPM8. This cooling sensation did not alter gaping behaviour. A few possibilities explain these results. Firstly, with so much warm input being received by the hypothalamus from all areas of the body, cold signalling from just the trunk skin would be insufficient to override warm sensors and shut off the gaping thermoeffector. Second, cold-sensory input from TRPM8 and TRPA1, while integrated in the hypothalamus, may not integrate directly with the gaping thermoeffector neurons in a meaningful way. As such, cold input would not turn off gaping behaviour under any circumstances. Finally, it may be the case that gaping behaviour is not under the control of peripheral thermosensors. In the same way that cold sensory input does not appear to influence gaping, signals from the periphery might not integrate in the hypothalamus in a way that can influence the gaping thermoeffector. Such a case would be similar to how panting appears to function in mammals, with core thermosensory

signals playing a major role (Morrison and Nakamura, 2011), and is somewhat supported by my data.

In mammals, it has been shown that panting requires an increase in core and brain temperatures to occur. Lim and Grodins (1954) demonstrated that peripheral heating does not elicit panting in dogs, direct hypothalamic heating shows only limited onset of panting, and whole body heating most effectively elicits panting. Likewise, Inomoto et al (1983) found that combined core and hypothalamic warming resulted in the greatest amount of panting in rabbits compared to warming of either area independently. It follows logically that a behaviour meant to defend brain and core temperature is under greater control of thermosensors associated with brain and core temperatures, rather than those in the periphery. The finding that whole body heating in mammals is more effective than hypothalamic heating is possibly the result of additive sensory input eliciting a stronger response.

In the bearded dragon, the percent of time spent gaping increased over the course of an hour. During this period, ambient temperature remained at a constant 38±0.5ºC and skin temperature remained relatively constant (Table 3). Although body temperature was not measured directly, it is known that it equilibrates more slowly than skin to ambient temperature (see Chapter 4). Thus we can assume that body temperature continued to equilibrate throughout the 60 minute trial. If core temperature did increase steadily, it suggests that much like in mammals, core temperature increases induce gaping behaviour, with higher temperatures increasing the amount of gaping in the bearded dragon. As the primary source of thermoreception, these results point to a role for centrally located thermoTRP channels (in the viscera or hypothalamus) as the main drivers of gaping behaviour, to the exclusion of peripheral thermosensors.

Dupré and Crawford (1985) studied the thresholds for panting (*i.e.* gaping) in the desert iguana (*Dipsosaurus dorsalis*) and obtained results which contrast those presented here. They exposed iguanas to much higher temperatures (47-65ºC) and found that the onset of gaping occurs at the same skin temperature, independent of ambient temperature. As ambient temperature increased, the temperature of the brain and core, at which gaping began, decreased. They conclude that skin temperature provides the main source of thermosensory information for the on-off control of gaping in this lizard. These experimenters did not determine if gaping is proportionally controlled (as in the bearded dragons) or measure the amount of gaping. They only measured the temperature at which gaping begins, so other factors are potentially at play. The major problem with the experiment by Dupré and Crawford (1985) is the extremely high temperatures used. *Dipsosaurus dorsalis* never exceeds body temperatures of approximately 42ºC in the wild (McGinnis and Dickson, 1967). Animals were exposed to temperatures as high as 65ºC, far exceeding what would be found in their natural habitat, and in such a way that ambient temperatures were reached in a matter of only 30 seconds. The manner in which an animal responds to this type of temperature shock may be very different from their responses to the more gradual temperature changes typical of their natural habitat. The temperature used in my experiment was on the high end, but still within physiological range (mean UETts was approximately 39ºC for the bearded dragon, and skin temperature in the gaping box never exceeded 38.5ºC).

A potential model for gaping behaviour in the bearded dragon involving the thermoTRP channels can be developed. Gaping behaviour functions under a proportional control system whereby the size of the gape increases with increasing temperature (Tattersall and Gerlach, 2005). If we assume that lizards possess a similar repertoire of thermoTRPs as mammals (Figure

4), this behaviour may function through a stepwise activation of progressively higher temperature activated TRP channels. At moderate to high temperatures (30-38ºC), TRPV3 and TRPV4 would be activated to induce moderate gaping. As temperature increases to 40ºC and above, TRPV1 and TRPV2 would be recruited. These higher temperatures were not achieved during my experiment, but Tattersall and Gerlach (2005) showed that animals at 40ºC ambient temperature have the largest gapes and gape almost continuously. It is impossible from the information available to determine which channels exactly contribute to these behaviours, but it is an area worth exploring in the future.

Chapter 5: Characteristics of Shuttling

Introduction

Understanding the intricacies of how an animal thermoregulates can provide extensive information on the thermosensory mechanisms at work. The temperatures that elicit thermoeffector action are useful in identifying the sensory range of an animal. Lizard shuttling behaviour in a laboratory environment has been well described in previous studies. Animals will move between a hot and cold environment at specific ambient, skin, and body temperature ranges (Berk and Heath, 1975). This allows animals to maintain optimal temperature within these upper and lower thresholds, and suggest a thermosensory system adapted to detect and react to these ranges.

Although ectotherms are generally considered to rely solely on behavioural thermoregulation, there is an abundance of evidence to suggest reptiles possess at least a limited capacity for physiological thermoregulation (Seebacher and Franklin, 2005). This has generally been restricted to changes in blood flow through vasoconstriction and vasodilation, as reptiles do not possess any significant capacity for metabolic heat production. In mammals, adjustments in circulation are meant to increase retention of metabolically produced heat, thereby reducing heat loss to the environment and subsequent metabolic costs (Romanovsky, 2007). Several lizard studies have identified this as a viable mechanism, which manifests itself in a reduced rate of cooling compared to heating during shuttling behaviour (Weathers, 1970).

Another interesting aspect of lizard shuttling is a negative skewness in the distribution of body temperatures, which occurs in freely moving animals (Dewitt and Friedman, 1979).

Generally this means that a wider distribution of cold temperature environments are chosen compared to warm temperature environments. From a thermosensory standpoint, this may relate to increased precision in detecting and selecting warm temperatures compared to cold.

This chapter explores how characteristics of bearded dragon thermoregulation integrate with the concept of thermoTRP channels as the primary means of thermosensation. Quantifying the temperatures animals experience through normal shuttling behaviour can be informative in determining which thermoTRPs function in these animals, and how they might contribute to the unique characteristics of lizard behavioural thermoregulation

Materials and Methods

Recording Temperatures During Behavioural Thermoregulation

All data for which body, ambient, and skin temperature was available came from series I experiments. Series III values for ambient and skin temperature were used for comparison. For the shuttle box behavioural experiments where body temperature (T_b) was measured, two custom-built temperature loggers were used (designed and built by Dr. Peter Grey, University of Queensland, Australia). The loggers consisted of a 1.5 x 1.5 cm logic board, a 1.5 x 3 cm battery, and a thin (<1 mm thick) wire at the end of which was a 3 mm long thermistor probe. Over the course of an experiment, a single logger was attached to the animal's back using 3M Impregum® for a total weight of 10 g. The probe was then inserted into the animals cloaca, from which body temperature was recorded every 1 min for the length of the experiment. Data from the logic board was downloaded following the experiment.

The loggers generated temperature data as a five-digit number and had to be calibrated to determine the equivalent temperature scale. Temperature calibration was performed on each logger by placing the probe in a water bath set to 10ºC and increasing temperature by 5ºC every 30 min up to 45ºC. A mean of 10 successive data points (10 five-digit numbers over 10 minutes) was taken for each 5ºC interval and this was designated to the corresponding temperature. These were then fitted to a polynomial equation (R^2=0.99) which was used to convert subsequent data from the loggers into temperatures. The loggers were re-calibrated for temperature after four months to confirm that the values did not change over time. The loggers were also calibrated for time before and after experiments by placing the probe at a non-physiological temperature of 10ºC for 2 min at precisely recorded times. By lining up these spikes to the time, we could ensure that all temperature values corresponded exactly with time. This was done to correct for a slight drift in the loggers internal clock over the course of an experiment.

Data Processing and Statistical Analysis

Correcting for Temperature Logger Time Drift

The data logger was programmed to sample temperature every minute, however it was found to sample at greater than one-minute intervals. Logger data was aligned against the two points of calibration (at the beginning and the end of the experiment). It was then determined, over the course of the 12 h trial, the rate of drift. The number of minutes of drift (7-8 on average) was divided by the total number of sample points over which the drift occurred, to obtain the average drift per sample. This value was applied to each data point using the equation:

$$T_i = t_i + D(i)$$

Where 'i' refers to the sample point number following the first calibration point (with 0 being the first calibration point), T_i is real time, t_i is recorded time, and D is drift/sample point over the course of the experiment.

Shuttle Box Temperature Data

As part of series I, three levels of temperature data were collected in the shuttle box. Ambient temperature was obtained by using the animal's position and the temperature probes placed inside the shuttle box compartments. Skin temperature was obtained from the thermal camera mounted above the treadle switch. Lastly, body temperature (T_b) was obtained using the data logger. In the case of skin temperatures, data is limited to UETts, UEThs, LETts, and LEThs values due to the static nature of the thermal camera. Body temperature however, was collected every minute, allowing for mean body temperature, UETb/LETb values, and a trace for the duration of the data collection period (Figure 28) to be produced. Such a trace is only available for a limited number of trials for which body temperature data is available (n=9), but is meant to highlight how the three temperature variables can be consolidated.

Integration of Ambient, Skin, and Body Temperatures

Body temperature recordings were collected for a total of nine trials during series I (n=4, capsazepine; n=5, control). For these nine trials, ambient and skin temperature were also

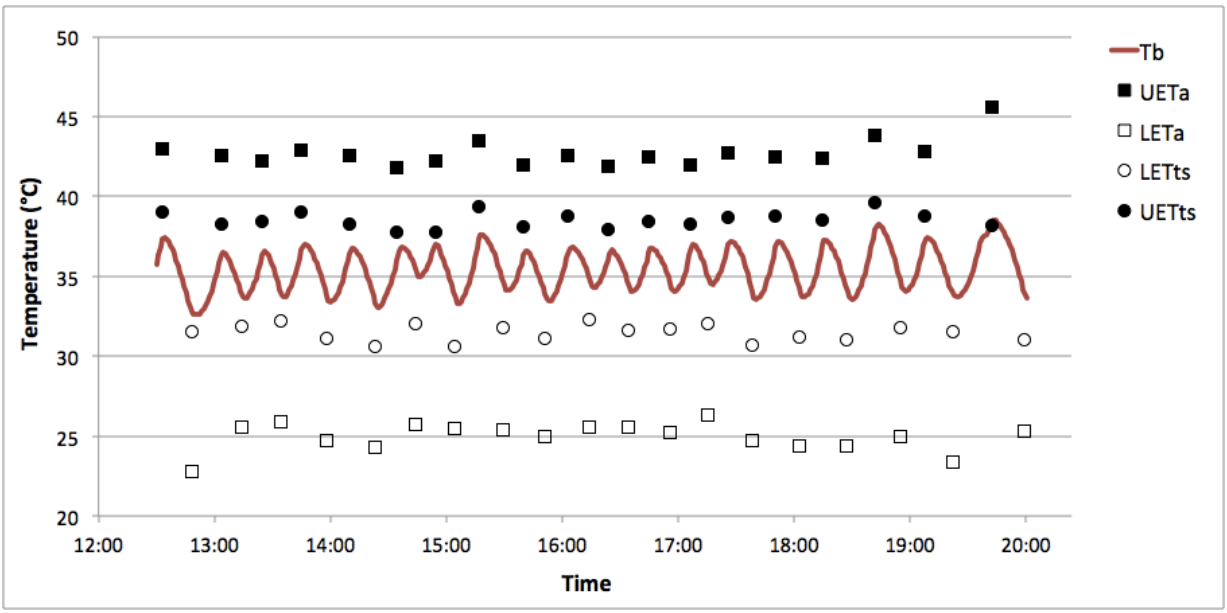

Figure 28: Combined Trace of Ambient (T_a), Skin (T_{ts}), and Body (T_b) Temperature Data

Trace of temperature data produced over the course of a 7.5 hour trial in the ramping shuttle box. T_b is shown as a continuous variable, with values recorded every one minute by the temperature probe in the cloaca. T_a and T_{ts} are both shown as UET and LET values. T_a changes at a constant 0.7ºC between UET and LET values, as determined by the rate of ambient temperature change in the shuttle box. This trace represents data from a single individual bearded dragon administered the vehicle (DMSO) as part of Series I experiments.

available for the duration of the trial period (Figure 28). Since capsazepine had no significant effect on thermoregulatory behaviour, these temperature data from both groups were combined into a single data set (n=9). Series III skin and ambient temperature were the only data sets available for the vehicle (ethanol) and menthol treatments. Both groups were analysed separately as a significant difference in some behaviours were observed, as outlined in chapter 4. All data of series I and series II were treated the same, except for the absence of body temperature data in series III. Absolute difference between body-skin, body-ambient, and skin-ambient temperatures were determined for each UET and LET shuttle event (for the duration of the data collection period). Statistical significance between UET and LET for absolute differences in temperature values was determined using paired t-tests. Paired t-tests were also used to compare UETa, UETts, and UETb to LETa, LETts, and LETb, respectively. While ambient temperatures would be separated by a minimum of 10ºC as a result of the boxes design, it could not be assumed that skin and body temperatures would be significantly different during upper and lower escapes. Coefficient of variation (CV=SD/Mean) was calculated for UET and LET of each temperature category to determine the amount of variation that occurs at the warm and cool sides of the spectrum. CV for UET was compared to LET using paired t-tests, except in the case of non-normal data, where a Wilcoxon test was used.

Results

Quantitative characteristics of bearded dragon shuttling were analysed to describe differences in behaviour and the rate of temperature changes during cooling and warming periods. Mean T_b (body temperature) over the course of the series I data collection period was

35.41±0.19ºC (n=9). UETa, UETts, and UETb temperatures were significantly different from LETa, LETts, and LETb temperatures, respectively ($t=24.565$, $p<0.001$; $t=11.835$, $p<0.001$; $t=6.729$, $p<0.001$; Table 4). A histogram of UETts and LETts shows little overlap between the temperatures at which animals leave the warm and cold sides (Figure 29). A histogram of UETb and LETb shows more overlap, but still distinct ranges over which cold and warm escapes occur (Figure 30). The greater overlap is expected of body temperatures, which change slower with temperature, and over a narrower range than skin temperature, which is in direct content with the ambient environment. The absolute differences between UET values were significantly smaller than those of LET values (ambient-skin, skin-body, body-ambient; $t=-4.707$, $p<0.001$; $t=-9.568$, $p<0.001$; $t=-9.161$, $p<0.001$; Table 4), indicating animals heated up faster than they cooled. In series III, the absolute differences between UET values were significantly smaller than those of LET values for both ethanol ($t=-3.788$, $p<0.01$) and menthol ($t=-5.635$, $p<0.001$) groups (Table 4).

Coefficient of variation (CV) represents a measurement for the amount of variation observed in groups of samples. In this case, CV was calculated to determine the amount of variation in LET and UET values. A higher CV represents a higher amount of variability in the shuttling temperatures, which translates to less consistency and lower precision of shuttles. The LETa value for coefficient of variation (CV) was significantly higher than the UETa value in series I animals for which body temperature data was available ($Z=2.756$, $p=0.003$; Table 5). In series III, this trend extended to a significantly higher CV in LETa compared to UETa in ethanol ($z=2.845$, $p<0.01$) and menthol ($Z=2.934$, $p<0.001$) groups and LETts compared to UETts in both groups (ethanol [$z=2.934$, $p<0.001$]; menthol [$z=2.756$, $p<0.01$]; Table 5).

Table 4: Characteristics of Bearded Dragon Shuttling

Temperature data were collected from trials of series I and III. Difference calculated as absolute value. * indicates significant difference from corresponding value in adjacent column ($p<0.01$). ** ($p<0.001$). Values are mean±SE.

Experiment	Category	UET (ºC)	LET (ºC)
Series I - Animals with Body Temperature Measurements	Ambient	42.66±0.3**	26.49±0.4
	Skin	38.95±0.3**	32.15±0.4
	Body	36.49±0.2**	34.81±0.2
	Difference (Ambient-Skin)	4.71±0.1**	5.67±0.2
	Difference (Skin-Body)	1.47±0.1**	2.65±0.2
	Difference (Body-Ambient)	6.16±0.2**	8.32±0.3
Series III - Menthol	Difference (Ambient-Skin)	5.03±0.2**	6.05±0.2
Series III - Ethanol	Difference (Ambient-Skin)	5.19±0.2*	6.06±0.2

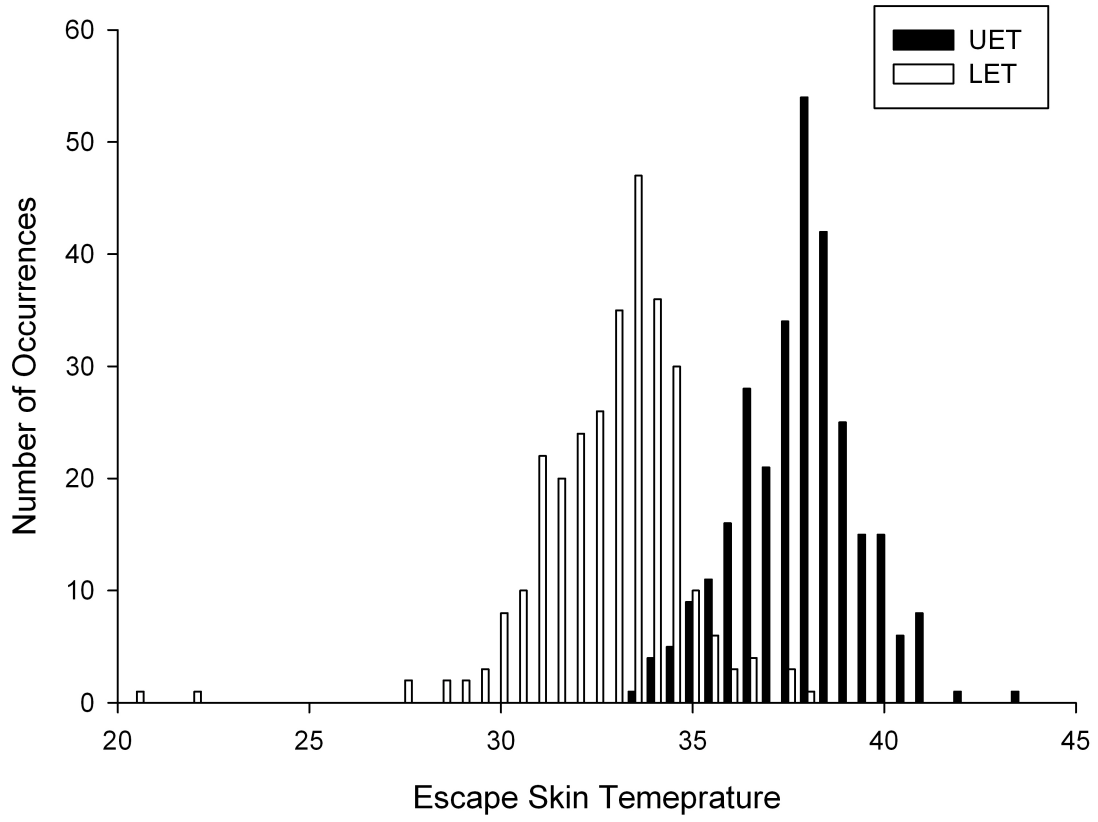

Figure 29: Histogram of Upper and Lower Escape Skin Temperatures, Series I

Histogram showing the number of escapes at each half-degree interval for skin temperature. Animals (n=9) were placed in a ramping shuttle box as part of series I and allowed to move freely between cold and warm sides while temperature decreased or increased, respectively, at a rate of 0.7ºC/min. Skin temperatures were collected using an infrared thermal camera. Upper escape skin temperature (UET) and lower escape skin temperature (LET) are distinct, with very limited overlap.

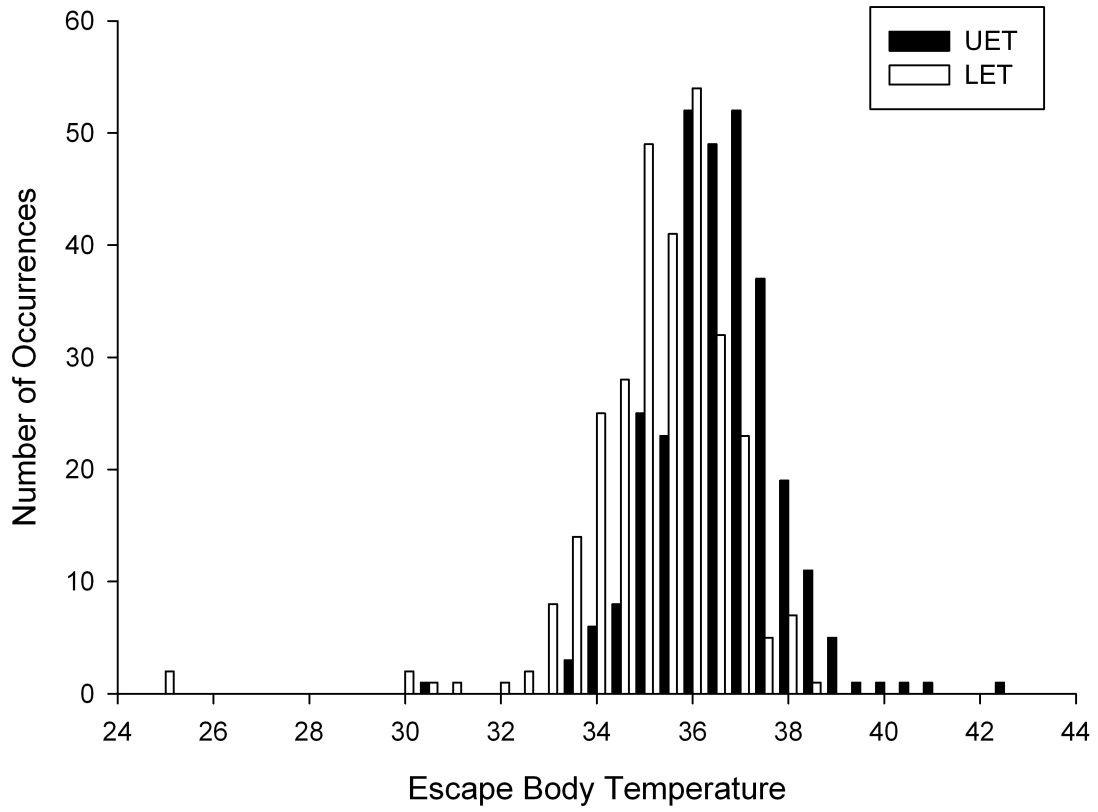

Figure 30: Histogram of Upper and Lower Escape Body Temperatures, Series I

Histogram showing the number of escapes at each half-degree interval for body temperature. Animals (n=9) were placed in a ramping shuttle box as part of series I and allowed to move freely between cold and warm sides while temperature decreased or increased, respectively, at a rate of 0.7ºC/min. Body temperatures were collected using a temperature data logger with probe inserted in the cloaca. Upper escape body temperature (UET) and lower escape body temperature (LET) are somewhat distinct, as determined by the significant difference in means (Table 4), but much less so than for skin temperatures.

Table 5: Coefficient of Variation

Coefficient of variation (CV) was calculated for all temperature categories in series I and III for which data was available. CV was calculated as SD/mean. * indicates significant difference from corresponding value in adjacent column ($p<0.01$). ** ($p<0.001$). Values are mean±SD.

Experiment	Category	UET (%)	LET (%)
Series I - Animals with Body Temperature	Ambient	3.4±1.4*	7.3±3.7
	Skin	2.8±1.4	4.2±2.7
	Body	2.8±1.5	3.8±2.4
Series III - Menthol	Ambient	2.9±1.5**	12.0±10.8
	Skin	2.3±1.1*	7.7±8.0
Series III - Ethanol	Ambient	3.1±1.5*	11.1±9.1
	Skin	2.3±1.0**	7.0±5.2

Discussion

Characteristics of shuttling were evaluated for animals in series I for which body temperature was available (n=9), and all animals in both vehicle (ethanol) and menthol treatment of series III (n=11). Bearded dragon shuttling was characterized by a significant separation between LET and UET in all three categories measured (ambient, skin, and body). This separation is typical of a dual threshold model (see Chapter 1: Characterizing Lizard Behavioural Thermoregulation). Additionally, animals in series I and series III show evidence of a disparity in the rate of heating and cooling. Ambient temperature changed at a constant 0.7ºC/min in both the hot and cold compartment, however the difference between ambient-skin (series I and III), skin-body (series I), and body-ambient (series I) was significantly higher at LET events. This suggests that the rate of cooling was lower than the rate of heating, a phenomenon observed and quantified in numerous species of lizards, and attributed to vasodilation/vasoconstriction to retain or dissipate heat (Weathers, 1970). Another measured characteristic of lizard shuttling is variability in the temperature of UET and LET, as measured by coefficient of variation (CV). CV for LET's was significantly higher at ambient in series I animals, and significantly higher at ambient and trunk skin in series III animals. All three aspects of shuttling behaviour outlined here have been well described in lizards (Weathers, 1970; Barber and Crawford, 1979; Dewitt and Friedman, 1979). With the establishment of thermoTRP channels as a potential thermosensor in the bearded dragon, an opportunity exists to review the role thermoTRP channels may play in defining these characteristics of shuttling behaviour.

Dual Threshold Shuttling in the Bearded Dragon

The bearded dragons in this study displayed dual threshold shuttling, in line with other models of lizard thermoregulation (Barber and Crawford, 1979; Van Berkum et al., 1986) and previous studies with this species (Cadena and Tattersall, 2009). Cadena and Tattersall (2009) recorded values of 34.7±0.22ºC, 25.4±0.82ºC, and 43.0±0.82ºC (n=10; Mean±SEM) for T_b, LETa, and UETa, respectively, using almost identical methodology. The T_b, LETa, and UETa values for the current study were 35.4±0.19ºC, 26.5±0.43ºC and 42.7±0.33ºC (n=9; Mean±SEM), respectively. My values are similar to published values for *P. vitticeps*, and highlight the ease of repeatability in laboratory shuttling experiments. The wide difference between LET and UET are typical of a dual-threshold model, where animals regulate body temperature between a lower and upper threshold to maintain body temperature somewhere in between (Barber and Crawford, 1979). My study also built on this concept by including skin and body temperatures. The data showing that UETts and UETb are statistically significant from LETts and LETb ($p<0.001$), respectively, provides stronger support for the existence of upper and lower thresholds, which are distinct from one another.

With a clear distinction between cold and warm activated channels, the thermoTRPs represent a likely candidate for the maintenance of this system. Without direct knowledge of threshold temperatures for bearded dragon thermoTRPs, the only available option is to apply current knowledge of these channels in mammals to the thermoregulatory behaviours observed in the bearded dragon. On the warm side, TRPV3 and V4 would contribute to warm sensation at moderate temperatures, with TRPV1 and V2 activating at temperatures greater than 42ºC. TRPM8 would act as the innocuous cold sensor, with TRPA1 contributing at very cold temperatures. The distinction between warm and cold-activated channels already present in the

thermoTRPs, is compatible with the concept of dual thresholds. It is unclear how the same channels in mammals translate into a very tightly controlled body temperature. This may be due to both the availability of physiological mechanisms, most importantly metabolic heat production, and differences in how neural information is integrated and relayed to thermoeffectors. It may be the case that dual thresholds do exist in mammals, albeit with much tighter thresholds compared to those in lizards. The sensitivity of core sensors, such as those in the hypothalamus, are a possible factor for mammal's ability to maintain such tight control over their body temperature. Ultimately, these proposals would rely on future experimentation, including more direct comparisons between thermoTRPs in endotherms and ectotherms under similar conditions.

Skewness of Cold Versus Warm-Temperature Thermoregulation

A significant difference in the rate of heating and cooling was seen across all experiments, as defined by a larger disparity between the three temperature categories (ambient, skin, and body) at the moment of LET compared to UET. Lizards cooled slower than they warmed. Other studies have documented this phenomenon, and suggested that it is due to a capacity for physiological thermoregulation (Weathers, 1970). Weathers (1970) compared the rate of heating and cooling in dead and living desert iguanas. Dead lizards heated and cooled at identical rates, while living animals cooled significantly slower than they heated. It was concluded that this change was the result of an ability for lizards to regulate rates of circulation between the core and the periphery. Changes in circulation are well established as one of the few physiological mechanisms for thermoregulation available to reptiles (Seebacher and Franklin, 2005). In the

case of the bearded dragons, it is possible that through the use of vasoconstriction, animals are able to retain heat during cooling, slowing the rate that skin and body temperature lower in the cold compartment. This would be beneficial in the wild for allowing animals to maintain optimal temperature for longer following a period of basking.

In the study by Cadena and Tattersall (2009), a significant difference was not found between CV of ambient and body escape temperatures. A potential reason for this inconsistency is the modification made to the ramping shuttle box for my experiments. The metal bar placed across the crossing point between compartments effectively prevented animals from straddling the hot and cold compartments, something that has occurred in past experiments (Cadena and Tattersall, 2009). My values are therefore indicative of an animal that has made a fully committed choice and the variation presented here is not confounded by animals straddling both sides of the shuttle box. The negative skewness presented by my data has been observed previously as a characteristic of lizard thermoregulation where variation in cold escape temperatures is higher than that of warm escape temperatures (Dewitt and Friedman, 1979). A potential cause for this, outside of those already proposed, is the existence of a greater number and variety of warm sensitive thermoTRP channels (*i.e.* TRPV1-4) than cold sensitive thermoTRP channels (*i.e.* TRPM8, TRPA1). It is possible that this defines the reduced precision in cold escapes compared to warm escapes, leading to a higher degree of variation in cold escapes.

Location of thermoTRP expression may also contribute to the higher precision in warm shuttling. In mammals, TRPV3 and TRPV4 are expressed in keratinocytes, in closer proximity to the ambient environment. This could allow for greater sensitivity to warm temperatures, whereas cold temperature sensation would be situated below the skin within free nerve endings.

Such a distribution in lizards may be contributing the greater precision in warm shuttling compared to cold shuttling.

Chapter 6: Summary and Future Directions

Summary of Results and Conclusions

Bearded dragons express TRPM8 in the DRG, among other tissues. Activation of TRPM8 by menthol applied to the skin resulted in an increase in the threshold for cold escape behaviour. These results suggest TRPM8 functions as a peripheral thermosensor in cool temperature environments. Furthermore, peripheral TRPM8 exerts control over thermoregulatory shuttling behaviour at cold temperatures, pointing to a neural connection between peripheral thermosensation and behavioural thermoeffectors in the bearded dragon. However, the effects were likely reliant on thermosensory input from other areas in the body.

The dual threshold model of thermoregulatory shuttling behavior can be described using current knowledge of mammalian thermoTRP function and activation temperatures. The cold receptors TRPA1 and TRPM8 would detect cool to cold temperatures and produce a cold escape threshold for shuttling. The warm receptors TRPV1-4 would detect warm to hot temperatures, producing a warm escape threshold for shuttling. The increased overlap of multiple warm temperature sensors might contribute to the negative skewness observed in threshold temperatures; lower escape temperatures are more variable and less precise than upper escape temperatures.

The control of gaping behaviour is likewise likely under the control of thermoTRP sensory information. Although not measured directly, the combined sensory input of TRPV1-4 may serve to determine threshold for gaping and amount of gaping. It is clear however, that limited peripheral activation of TRPM8 is insufficient to modify gaping behaviour. Whether this

is the result of insufficient cold sensory input, or a reliance on core temperature sensation to induce gaping behaviour, requires further experimentation.

Although intraperitoneal injection of menthol and the TRPM8 antagonist capsazepine did not elicit any changes in behaviour, many methodological shortcomings prevent any conclusions from being made from these results. Intraperitoneal injections as a delivery system may need to be re-evaluated as to its efficacy in assessing thermoregulation. In the absence of information regarding drug delivery inside the body, it is difficult to make conclusions based on the results of such experiments.

Future Directions

Future experiments would benefit greatly from an expanded understanding of how thermoTRP channels in the bearded dragon function at the molecular and cellular level. While whole animal behavioural experiments have their advantages, mammalian experiments have demonstrated that an understanding of channel activity *in vitro* can be very complimentary. Many mammalian behavioural experiments have included aspects of activation temperatures and sensitivity of channels to relevant compounds (Dhaka et al., 2006). Firstly though, a focus on determining the full sequences for TRPM8 and other channels should be undertaken in the bearded dragon. This would allow for a more thorough analysis of conservation between species, a subject that was only touched here. Following establishment of channel sequences, thermoTRPs can be homogeneously expressed in cell cultures (for example in frog oocytes). Patch clamping or calcium imaging can then be used to determining the temperature threshold for channel activation. The use of physiologically relevant membrane potential would be of

utmost importance, considering the voltage gating of these channels. This would compare favourably with the extensive mammalian data obtained in this way for TRPM8 (McKemy et al., 2002; Peier et al., 2002), TRPV1 (Caterina et al., 1997), TRPV3 (Peier et al., 2002; Xu et al., 2002), and TRPV4 (Guler et al., 2002).

Another well-established tool for thermoTRP research has been knock out (KO) models (Lee et al., 2005; Moqrich et al., 2005; Dhaka et al., 2007). In lizards, use of knock down, or mRNA blocking may serve a similar purpose. The goal is to prevent functional thermoTRPs from operating in animals and test the resulting changes in thermoregulation. Specific KOs for each thermoTRP could better inform the dual threshold model of thermoregulation. For example, in a shuttle box experiment, would TRPM8KO animals show deficits in upper escape shuttling, and would TRPV3KO or V4KO animals show deficits in lower escape threshold? In terms of gaping behaviour, if thermoTRPs are truly the main driver for this behaviour, KO animals could definitively answer questions of how. For example, a TRPV1KO animal would easily determine if the channel plays any role in gaping, which occurs at noxious hot temperatures. Ultimately, thermoTRPs may serve other roles aside from thermosensation, so KO animals for the sake of thermoregulatory assessment can be thought of as a sledgehammer approach, but still provide valuable information.

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