The Pennsylvania State University

The Graduate School

Food Science Department

BIOLOGICAL FACTORS INVOLVED IN SENSORY RESPONSES TO CHEMESTHETIC STIMULI

A Dissertation in

Food Science and Clinical Translational Science

by

Alissa Allen Nolden

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

December 2016

The dissertation of Alissa Nolden was reviewed and approved* by the following:

John E. Hayes
Associate Professor of Food Science
Dissertation Advisor
Chair of Committee

Kathleen Keller
Assistant Professor of Health and Nutritional Sciences and Food Science

Joshua Lambert
Associate Professor of Food Science

Sheila West
Professor of Biobehavioral Health

Robert Roberts
Professor of Food Science
Head of the Department of Food Science

*Signatures are on file in the Graduate School

ABSTRACT

Chemesthetic agents consist of chemical compounds known to activate receptors involved with pain, touch and thermal perception, such as warming, cooling, stinging, pricking, burning and buzzing. Many chemesthetic agents are regularly consumed, including capsaicin, ethanol, menthol, mustard oil, cinnamonaldehyde, and piperine. Psychophysical responses to these compounds have been well investigated in terms of chemesthetic sensations (e.g. the burn of capsaicin); however, little is known regarding their secondary qualities, mainly taste responses.

The first section of this dissertation will explore the chemesthetic and taste sensations from capsaicin and ethanol. Both of these stimuli are consumed regularly, with capsaicin being the active ingredient in chili peppers while ethanol is a key component in alcoholic beverages, both of which are widely used by numerous peoples around the world.

Psychophysical response to these compounds is known to vary greatly across concentrations and individuals. Here, I will establish dose response functions for both ethanol and capsaicin, reporting on both burning and bitterness response to these stimuli. I will then take two different approaches to investigate individual differences in bitterness response. The first approach will explore single nucleotide polymorphisms (SNPs) in bitter taste receptor (*TAS2Rs*) and transient receptor potential vanilloid (*TRPV1*) genes. Multiple bitter compounds are known to vary in the bitterness they evoke due to variability within bitter taste receptor genes (*TAS2Rs*). Whether similar effects are seen for TRPV1 agonists will be tested here.

Second, I will explore the influence of prior experience with and exposure to these stimuli on the sensations they evoke, as differences in response to capsaicin and ethanol have been attributed to intake frequency of chili peppers and alcohol, respectively. Several theories

have been proposed in order to explain reduced responses, including differences in scale usage due to dissimilarities in prior experience, greater familiarity with the compound or, genetic variability in chemosensory receptors. For ethanol, the difference in response will be explored across consumption, but additional research is needed to investigate the mechanism further. In the case of chili pepper intake, reduced responses to capsaicin are widely believed to be due to hypoalgesia or desensitization.

Numerous studies show that perceptual responses to capsaicin are reduced following repeated intermittent exposure to a constant concentration; this phenomenon is commonly known as capsaicin desensitization, but is more accurately called hypoalgesia. Hypoalgesia to capsaicin, and other compounds (e.g. menthol and zingerone) is thought to be unique to chemesthetic agents. Hypoalgesia can be conceptualized as being acute or chronic, depending on the duration of reduced response, with acute hypoalgesia occurring within a test session while chronic hypoalgesia lasts across days. Here, we are interested in the effects of repeated capsaicin exposure, and the development of chronic capsaicin hypoalgesia. There are several purported mechanisms that may result in acute hyperalgesia, including depletion of substance P and calcium ions. However, there is a gap in the literature regarding the mechanism(s) that result in chronic capsaicin hypoalgesia.

To gain insight into the mechanism(s) leading to chronic capsaicin hypoalgesia, I investigate the mRNA expression of *TRPV1*. TRPV1 is activated by both capsaicin and ethanol, along with other exogenous and endogenous compounds. I describe the variability of *TRPV1* expression in human oral tissue as a function of capsaicin exposure, which may provide an explanation for chronic capsaicin hypoalgesia. These results have the potential to translate to human health, as patients with oral cancer and burning mouth syndrome are known to have

increased mRNA expression of *TRPV1* in lingual tissues. Understanding this mechanism of hypoalgesia and TRPV1 regulation may help to inform future treatments to reduce *TRPV1* expression and possibly reduce inflammation and pain.

This dissertation is focused on extending our knowledge of the psychophysical response to chemesthetic stimuli and exploring the effects of repeated exposure on perception. Here, I investigate how individual differences in response to stimuli associate with prior intake along with genetic variability in chemosensory receptors. I evaluate *TRPV1* mRNA expression in humans and explore whether variability in expression is associated with capsaicin response and development of capsaicin hypoalgesia. To do so, the dissertation is broken down into four aims, which are detailed here.

The first aim is to determine the response to capsaicin and ethanol across a wide range of concentrations. Developing a dose response curve for these two stimuli is important in order to determine appropriate concentrations for subsequent research. These stimuli were examined for their variability in burn and bitterness responses, while also exploring whether reported intake of foods containing these compounds is associated with their perception.

The second aim is to explore the relationship between the variability of the reported burn and bitterness of sampled ethanol and individual's genetic differences in *TRPV1* and *TAS2Rs*. My findings complement prior work showing a relationship between alcoholic beverage intake and genetic variability in *TAS2Rs* by providing novel data on the variation of sensations from ethanol with *TAS2R* SNPs. This is also the first report linking differences in ethanol sensations to variability in the *TRPV1* gene.

The third aim is to determine the extent of the effects of repeated capsaicin exposure on the perceptual responses to capsaicin. This will be evaluated i) by measuring self-reported intake free-living individuals and ii) in controlled experiments where participants are systemically and repeated exposed to capsaicin. The development of a robust capsaicin exposure protocol is essential to achieving our fourth aim. This is the first evidence of chronic hypoalgesia after participants rinsed with low concentrations capsaicin for two weeks.

The fourth aim is to determine if capsaicin hypoalgesia, brought on by either dietary chili pepper intake or controlled repeated capsaicin exposure, is associated with a decrease in *TRPV1* expression. The relationship between capsaicin exposure and *TRPV1* expression will be explored in two different studies, the first will use free-living participants and collect self-reported intake of chili peppers, and the second will use a laboratory controlled exposure protocol. These experiments will explore the relationship between *TRPV1* mRNA expression with the perceived burn of sampled capsaicin and the effects of capsaicin exposure. The second study will be designed to measure the change in expression following repeated oral exposure to capsaicin, in order to determine if the development of hypoalgesia is associated with a down-regulation or decrease in *TRPV1* expression. This will be one of the first studies to explore the effects of dietary exposure on expression of chemosensory receptors, and the first related to capsaicin and the TRPV1 receptor.

The findings from this dissertation have the potential to influence multiple areas of inquiry. For example, the dose response curves generated from aim one can influence development and design of sensory studies exploring responses from chemesthetic agents. Aims 3 and 4 have the potential to inform the development of new therapies or treatment options for patients with oral pathologies associated with altered TRPV1 expression, including burning

mouth syndrome or oral cancer. Furthermore, these findings may provoke additional research in other systems and organs that could impact other chronic inflammatory diseases such as irritable bowel syndrome. This work has strong public health relevance, as TRPV1 is involved with the inflammation pathway, inflamed tissues express increased levels of TRPV1, and chronic inflammation is associated with seven of the top ten leading causes of mortality in the United States.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

Thank you John Hayes for your guidance and support throughout my time at Penn State. Your enthusiasm for research was essential in my success, both as a student and researcher. It is with your assistance that I was able to accomplish so much as a graduate student.

Thank you to my committee for their support and guidance. I appreciate your discussions related to both my research and career. Thank you Dr. Pawelczyk and the Clinical Translational Science staff. I am honored to have received the TL1 Fellowship and to be receiving a dual PhD in CTS. This experience has developed my passion for clinical and translational research, and will be a great influence my future research.

There are many students that have passed through while working in the Hayes Lab. The camaraderie significantly enhanced my experience, and I value all the friendships and continuous support from lab members. I am thrilled to be able to join you all on the other side. Thank you to all of the undergraduate students that have assisted me in my research studies. I have enjoyed getting to know each of you and wish you all luck going forward.

Thank you to my parents for always believing in me and always being there for me. I am thankful for your encouragement and guidance. Thank you both to my parents and Erica for the weekend getaways and for visiting me in Happy Valley!

Michael, I am so grateful for all that you have done for me while working towards my PhD. I appreciate all of meals you prepared, making me laugh, and being there to restore balance. I am forever thankful for the sacrifices that you made, in order to be there every day to support me. I enjoyed our time in State College, and I am looking forward to new adventures!

CHAPTER 1

Literature Review

Introduction

Chili peppers, a member of the *Capsicum* genus, are a popular, if not the most popular spice used in cuisines worldwide (Govindarajan and Salzer, 1985; Rozin, 1980). Chili peppers originate from the Americas, and have been consumed since at least 7000 BCE (Smith, 1968). Early European explorers, including Columbus and his crew were the first Europeans to discover chili peppers (see (Govindarajan and Salzer, 1985) for a review). Since their discovery, they have been incorporated into many cuisines in countries around the world, including Southeast Asia and East Africa, where chilies were added to traditionally bland staples, such as grain. For additional information see (Hayes, 2016).

Even though chili peppers have been and continue to remain ubiquitous in diets worldwide, the driving force(s) behind their popularity remains unclear (e.g. (Bègue, Bricout et al., 2015; Byrnes and Hayes, 2013; Dalton and Byrnes, 2016; Rozin, 1978; Rozin P., 1980)). However, liking of chili peppers is likely due to the pungent sensations elicited when consumed, which is attributable to its capsaicinoid content. The most abundant capsaicinoid in chili peppers is capsaicin (8-methyl-*N*-vanillyl-6-nonenamide; 48.6%), followed by dihydrocapsaicin (22%). Capsaicin is known to produce warming, burning, stinging and tingling sensations (Bennett and Hayes, 2012; Green, 1988; Green and Hayes, 2003).

In addition to these potentially desirable sensory properties, capsaicin has long been investigated for its therapeutic properties, such as analgesic and anticarcinogenic properties; see

(Hayman and Kam, 2008; Mason, Moore et al., 2004; Peppin and Pappagallo, 2014; Sharma, Vij et al., 2013; Smith and Brooks, 2014) for comprehensive reviews. While the mechanism(s) regulating these therapeutic effects are complex and not fully understood (e.g. (Holzer, 1991; Vyklicky, Novakova-Tousova et al., 2008; Xu, Zhang et al., 2012; Yang, Xiong et al., 2014), evidence suggests these mechanism(s) involve a receptor, the transient receptor potential cation channel subfamily V member -1 (TRPV1; capsaicin receptor) (Szolcsanyi and Pinter, 2013). Moreover, TRPV1 has been of great research interest, distinct from capsaicin, as it has been associated with a wide variety of human diseases, including chronic inflammation, cancer and neuropathic pain (see (Bishnoi and Premkumar, 2013; Brito, Sheth et al., 2014; Nilius, Owsianik et al., 2007; Premkumar and Bishnoi, 2011) for reviews). Even with this abundance of research on capsaicin and TRPV1 activity, there is a gap in the literature regarding the mechanisms behind oral capsaicin desensitization (or more accurately hypoalgesia; discussed below) and potential effects of repeated capsaicin exposure on TRPV1 expression.

Thus, this review will focus on the oral perception of capsaicin and the effects of repeated exposure. I will discuss and distinguish the differences between psychophysical and pharmacological sensitization and desensitization. I will summarize the nociception pathway and focus on factors involved with regulating TRPV1 activity. As TRPV1 is associated with a wide range of diseases, including chronic inflammation, I will review the literature associating TRPV1 and capsaicin with health outcomes. In summary, this review provides context for the hypothesis that capsaicin exposure may regulate TRPV1 activity by decreasing expression. This will provide insight to the potential mechanism to explain decreased sensory response following repeated capsaicin exposure. Research focused on these biological mechanisms would provide a greater understanding of the pathways through which capsaicin exposure may improve health for those experiencing chronic pain.

Chemesthesis is not a 'taste'

Gustation and somatosensations

Gustatory and somatosensory sensations in the mouth are transmitted via different cranial nerves. Oral somatosensory signals are sent to the brain via the trigeminal nerve (V); whereas gustatory signals utilize three different cranial nerves (VII, IX and X) (see (Simon, de Araujo et al., 2008) for a review).

To perceive a taste (sweet, sour, salty, bitter, or umami), or chemesthetic sensation, a stimulus (e.g. heat) must first activate a chemosensory receptor (either a G-protein coupled receptor or ion channel). Taste receptors are located in taste cells that can be found in some types of lingual papillae on the tongue, as well as on other oral cavity surfaces (Adler, Hoon et al., 2000; Chandrashekar, Hoon et al., 2006). Roughly 50 to 100 taste cells are housed in a single taste bud, located in the epithelial layer of papillae. However, these taste receptors are not exclusive to the oral cavity and are also expressed in other organs (e.g. (Jang, Kokrashvili et al., 2007; Singh, Vrontakis et al., 2011)).

Papillae can be classified into four different types, which are located in different regions of the tongue. Fungiform papillae are located on the anterior tongue, with circumvallate papillae on the posterior and foliate papillae on each posterior side of the tongue. The fourth type, filiform papillae, is the most abundant type, covering much of the tongue, but these do not contain any taste cells. Taste cells form synapses with primary afferent axons, with the various papillae types being innervated by different cranial nerves. This is largely dependent on the location of the papillae on the tongue. The facial (VII) nerve innervates taste buds located in fungiform and

foliate papillae, with the glossopharyngeal (IX) innervating the circumvallate and foliate papillae (Purves, Augustine et al., 2001b; Simon et al., 2008).

Chemesthesis

Chemesthesis is the term given to the broad range of sensations provoked by chemical stimulation of the somatosensory system (i.e. the coolness of menthol and pungency of horseradish) (see (Green, 1996a; Green, 2012)). Unlike tastes, which are perceived only in the oral cavity, chemesthetic qualities may be perceived on all parts of the body. It is important to note that chemesthetic sensations are not considered tastes (i.e. sweet, sour, bitter, salty, and umami) because they are carried by different nerves. Likewise, chemesthetic receptors are not located within taste buds, per se. Nociceptors synapse from the trigeminal nerve and extend to the epithelium of papillae, surrounding taste buds (Roper, 2014). Perception of chemesthetic qualities arises from activation of these nociceptors by a broad range of thermal, mechanical, and chemical stimuli. Nociception as it relates to capsaicin perception will be discussed in more detail below.

Capsaicin is known for its ability to produce the perception of warming and burning sensations, which can vary in intensity from mild to severe. In the oral cavity, capsaicin has been described as eliciting warming, burning, stinging, pricking, and tingling sensations (Bennett and Hayes, 2012; Green, 1989, 1991b), which is often characterized broadly as 'oral irritation' (e.g. (Lawless, 1984; Lawless, Rozin et al., 1985). Repeated exposure to chemesthetic compounds may result in an altered perception (increase or decrease in intensity) of subsequent exposures. This change in perception following capsaicin exposure is of particular interest and has been explored psychophysically in the laboratory. For a more expansive overview of chemesthesis and summary of chemesthetic ingredients commonly found in food see (McDonald, Bolliet et al., 2016).

It is worth mentioning that capsaicin is not the only chemesthetic agent known to elicit secondary sensations. Both capsaicin and ethanol are known to produce bitterness, in addition to sweetness and sourness (Green and Hayes, 2003; Scinska, Koros et al., 2000). The relationship between these secondary responses and the concentration of the compound is largely unexplored. It is important to understand this relationship and quantify the response for primary and secondary sensations. If only the primary quality is measured, participants may combine sensations and rate the one quality higher than if both qualities are measured, a phenomenon known as dumping (Clark and Lawless, 1994). Understanding the dose response function of capsaicin and ethanol, along with other common chemesthetic agents will increase our knowledge of responses to chemesthetic stimuli, secondary taste qualities of compounds and provide opportunity to explore the receptors involved with eliciting this response.

Repeated exposure to capsaicin: changes in psychophysical responses

Psychophysical and pharmacological sensitization and desensitization

Repeated exposure to chemesthetic compounds can result in changes, both increasing and decreasing intensity response for subsequent stimuli, which is not a characteristic of prototypical taste compounds (i.e. sweetness of sucrose or saltiness of sodium chloride). These classic taste solutions do exhibit adaptation under conditions of constant stimulation (Abrahams, Krakauer et al., 1937; McBurney, 1966). Traditionally adaptation to taste stimuli and decreased response to chemesthetic compounds are considered separate phenomenon; however, the underlying mechanisms are not known. Thus, it is possible they are regulated by the same mechanism, but additional research is needed to determine the mechanisms regulating this change in response

following repeated or constant exposure to prototypical tastants. Interested readers can see recent research focusing on changes in expression of bitter taste receptor (TAS2Rs) during periods of constant exposure (Upadhyaya, Chakraborty et al., 2016).

Capsaicin exposure has long been known to exhibit changes in responses (Jancso, 1968). Following initial capsaicin exposure, a subsequent application of the same capsaicin concentration can result (depending on the concentration) in an increased behavioral response; this is referred to as hyperalgesia (or alternatively, allodynia if the stimulus isn't normally perceived as painful). Traditionally, increased sensitivity to capsaicin (or other stimuli, including heat and mechanical stimuli) following repeated capsaicin exposure has been termed sensitization in the human psychophysics literature (Dessirier, Simons et al., 2000; Green, 1989). However, the physiological/pharmacology literature refers to sensitization as the increased responsiveness of nociceptor neurons or the sensitization of a receptor (see (Szallasi and Blumberg, 1999)). While it is expected that hyperalgesia is a result of neuronal sensitization, it is important to differentiate between these two terms as one refers to the psychophysical response and the other refers to the receptor/channel/neuron response. Thus, in order to clearly separate perceptional sensitization from receptor/neuronal sensitization, hyperalgesia and sensitization will be used, respectfully (Purves, Augustine et al., 2001a), for the remainder of the document.

Not only can repeated exposure to chemesthetic compounds result in increased response under some conditions, it can also cause a decrease in psychophysical response and decreased receptor activity in others. Just as there is confusion regarding the terminology used to describe sensitivity and hyperalgesia, the same imprecision exists for terminology used to describe decreased response at the receptor and psychophysical level. For the purpose of this review, hypoalgesia will refer to the decreased psychophysical response to a stimulus, whereas desensitization will be used to refer to decreased receptor/neuron activity.

Capsaicin-induced hyperalgesia

Capsaicin hyperalgesia can occur in the human oral cavity when stimuli are repeatedly applied with a brief break between applications. That is, participants report each subsequent stimulus is more intense (i.e. greater burn) than the previous application when the inter-stimulus interval (ISI) is approximately less than 1 minute, but not more than 2.5 minutes (Green, 1989). Hyperalgesia to capsaicin has been reported in several studies, which varied in application time, number of exposures and time between stimuli (Cliff and Green, 1996; Green, 1989, 1991b), with greatest increases in sensation reported for more frequent applications and shorter delays between applications (Green, 1991b). While the phenomenon of capsaicin hyperalgesia is interesting in terms of understanding receptor and neuron pathologies, it is another phenomenon – capsaicin hypoalgesia – that is of therapeutic interest due its potential use for treating physical and neurological pain (Knotkova, Pappagallo et al., 2008; O'Neill, Brock et al., 2012; Szolcsanyi and Pinter, 2013).

Capsaicin-induced hypoalgesia

Humans have reported hypoalgesia to mechanical and/or temperature stimuli on the skin following topical application of menthol (Andersen, Poulsen et al., 2015; Averbeck, Rucker et al., 2013; Hatem, Attal et al., 2006; Wasner, Schattschneider et al., 2004), mustard oil (Koltzenburg, Torebjork et al., 1994; Olausson, 1998; Schmelz, Schmidt et al., 1996), capsaicin (Kilo, Schmelz et al., 1994; Schaldemose, Horjales-Araujo et al., 2015) and cinnamaldehyde (Averbeck et al., 2013; Namer, Seifert et al., 2005). These studies describe a reduced response to a mechanical or temperature probe following prolonged exposure to the chemesthetic compound. Similar results were reported in the oral cavity for capsaicin, menthol, mustard oil and cinnamaldehyde (Albin,

Carstens et al., 2008). This review will focus on changes in receptor/neuron and behavioral response following repeated exposure of an irritant, rather than the change in response to a mechanical or thermal stimulus following a prolonged exposure to a chemesthetic compound. That is, this review will focus on chemical stimuli, specifically capsaicin and briefly ethanol, which have been extensively investigated by both sensory scientists and pharmacologists.

Hypoalgesia, a decrease in behavioral response to a stimulus following one or more applications can be further classified as acute (lasting minutes) or chronic (lasting across days). Multiple compounds have been shown to induce acute hypoalgesia in the human oral cavity. These include: cinnamaldehyde (Prescott and Swain-Campbell, 2000), menthol (Cliff and Green, 1996; Dessirier, O'Mahony et al., 1997), nicotine (Carstens, Albin et al., 2007; Dessirier, Nguyen et al., 1999), zingerone (Prescott and Stevenson, 1996), mustard oil (Simons, Carstens et al., 2003), eugenol (Klein, Carstens et al., 2013), carvacrol (Klein et al., 2013) and capsaicin (Balaban, McBurney et al., 1999; Carstens et al., 2007; Cliff and Green, 1996; Dessirier et al., 2000; Green, 1989, 1991b; Green and Rentmeister-Bryant, 1998; Hayes, 2000; Karrer and Bartoshuk, 1991; Prescott and Swain-Campbell, 2000; Szolcsanyi, 1977).

It has been hypothesized that the development of hypoalgesia, at least in the oral cavity, is dependent on the time between stimuli (i.e. it requires a hiatus from stimulation) (Green, 1989). For example, data suggests after an initial exposure to capsaicin, a minimum delay of 2.5 to 5 minutes is needed in order for the next applied stimulus to result in a decreased response (Green, 1989, 1991b). These studies, occurring within a single day (i.e. one test session), represent acute capsaicin hypoalgesia, which can occur as a result of a single dose or exposure. Acute capsaicin hypoalgesia has been achieved using concentrations from 2 ppm to 10,000 ppm (Green, 1989; Szolcsanyi, 1977). The occurrence of acute hypoalgesia was previously hypothesized to be due to initial depletion of calcium ions, and/or substance P, following brief capsaicin exposures

(Lembeck and Donnerer, 1981). Capsaicin response is then restored as calcium ions and/or substance P levels are replenished (Vyklicky et al., 2008). However, this hypothesis cannot explain the existence of another phenomenon called stimulus-induced recovery (SIR), which is the temporary reversal of acute hypoalgesia brought about by re-stimulation, specifically rapid stimulation and at concentrations above the 'desensitizing' stimulus (Green and Rentmeister-Bryant, 1998). Even though the existence of SIR appears to contradict the depletion hypothesis, decreased calcium ions and/or neurotransmitters likely play a role in acute hypoalgesia. More research is needed to determine the mechanism regulating acute hypoalgesia and SIR as they relate to repeated capsaicin exposure.

A limitation to prior studies exploring acute hypoalgesia is that they are typically restricted to minutes between exposures within a single test session, rather than exposures that mimic dietary consumption patterns (e.g. exposure during one or more meals over several days/weeks). This type of repeated exposure across several days is thought to result in chronic hypoalgesia. Evidence of chronic capsaicin hypoalgesia (lasting 24hrs or more) has been reported following exposure to concentrations of capsaicin between 2 ppm and 75 ppm (Green and Rentmeister-Bryant, 1998; Hayes, 2000; Karrer and Bartoshuk, 1995; McBurney, Balaban et al., 1997). Karrer and Bartoshuk (Karrer and Bartoshuk, 1991) were the first to investigate the number of days it takes to recover from a single capsaicin stimulus. Participants rated a capsaicin probe of either 10 or 100 ppm capsaicin that was applied with a cotton swab both before and after the participant received a capsaicin treatment, which consisted of painting the tongue using a cotton swab containing the same concentration (10 or 100 ppm capsaicin) and leaving it for 15 minutes. Separate trials were conducted to test recovery time, with follow-up ratings collected 1, 2, 4 or 6 days after the first exposure. Recovery was achieved when a capsaicin stimuli was rated at the same intensity for the previously presented stimuli at the same concentration. Individuals

who had their tongue painted with 10 ppm capsaicin took 2 days to recover, whereas 100 ppm capsaicin required between 2 to 4 days for recovery (Karrer and Bartoshuk, 1991). A second study observed decreased ratings for a 100 ppm filter paper capsaicin stimuli after three days of consuming a 75 ppm capsaicin laced taffy candy daily (McBurney et al., 1997).

In summary, repeated oral exposure to high doses of capsaicin results in decreased burn ratings for subsequent capsaicin stimuli. These controlled laboratory studies are also consistent with the observation that frequent consumers of spicy foods experience report less burn from capsaicin compared to individuals who do not eat spicy foods (Lawless et al., 1985; Ludy and Mattes, 2011, 2012; Rozin, Mark et al., 1981). Chronic hypoalgesia to capsaicin has been achieved with relatively high concentrations of capsaicin (10 and 100 ppm) (Karrer and Bartoshuk, 1991; McBurney et al., 1997), evidence suggests that lower doses of capsaicin may be able to produce chronic capsaicin hypoalgesia. The minimum effective dose of capsaicin required to achieve chronic capsaicin hypoalgesia is not known.

Ethanol and capsaicin elicit burning via the same channel, so it may be possible that the burn from ethanol would decrease following increased exposure to alcohol; however, this association has not been determined. This will not be investigated here; nonetheless, the responses to sampled ethanol will be explored.

Using repeated capsaicin exposure to treat pain

It is through repeated exposure to lower dose (repeated or a single high dose) that capsaicin may result in chronic hypoalgesia for both capsaicin and other painful stimuli. This reduction has led researchers and clinicians to try capsaicin as a treatment for individuals who experience chronic pain. Capsaicin has been injected, applied topically, and provided as an oral

rinse to relieve pain (see (Smith and Brooks, 2014) for a review). In the oral cavity, capsaicin rinses have been used as a treatment to reduce pain in patients with Burning Mouth Syndrome (Scala, Checchi et al., 2003). Patients receiving 200 ppm capsaicin rinse for one week reported reduced oral pain ratings compared to individuals receiving a control rinse (Silvestre, Silvestre-Rangil et al., 2012). Similarly, pain from oral mucositis was significantly reduced in patients after consuming a taffy candy containing 5-9 ppm capsaicin (Berger, Henderson et al., 1995). However, effectiveness varies greatly, mainly due to patients having a difficult time adhering to the treatment due to the initial burning sensation from capsaicin.

The presumed mechanism underlying capsaicin hypoalgesia is complex and regulated via several mechanisms in the nociception pathway. In order to understand these regulatory mechanisms, we will first discuss the capsaicin receptor.

Nociception and the TRPV1 channel

Nociception and capsaicin sensitive neurons

Sensations result from a signal being transduced through network of neurons. A signal is initiated from the peripheral site of activation, before being sent centrally, eventually ending up in the cerebral cortex. The somatosensory system is a part the central and peripheral nervous systems that allows the body to detect and perceive touch, temperature (thermosensation), movement of muscles and joints (proprioception), information from internal organs (interoception) and harmful or noxious stimuli (nociception).

When skin or oral mucosa is exposed to a noxious stimulus, such as capsaicin, it activates physiological channels (nociceptors) on primary afferent sensory neurons, potentially resulting in

the perception of pain. When a noxious stimulus activates peripheral nociceptor(s) expressed on sensory neurons, the neuron must reach sufficient activation potential to cause a signal cascade that is transmitted though the nervous system to the brain. Primary afferent neurons are pseudounipolar, having a cell body in either the dorsal root ganglion (DRG) or trigeminal ganglia (TG), which receive signals from a peripheral axon that innervates the body or face, respectively. These nerves are commonly slow conducting unmyelnated C or thinly myelinated A (beta and delta) axons. Neurological pain and inflammation arises through peptidergic where as non-peptidergic nerves are involved in the development of chronic pain (Nilius et al., 2007). Signals are transduced from the cell body to a central axon and synapse on second-order neurons of the dorsal horn of the spinal cord (or the trigeminal subnucleus caudalis (V)) and then to the brain. The perceived sensation is positively correlated with the stimulus; however, there is a great deal of plasticity throughout all stages of nociception (Millan, 1999). It is because of this plasticity that individuals experience variable pain, and this has lead to a great deal of research surrounding nociception pathways. For a more detailed review of nociception, see (Almeida, Roizenblatt et al., 2004; Dubin and Patapoutian, 2010; Gold and Gebhart, 2010; Julius, 2013).

Discovery and structure of the TRPV1 channel

Capsaicin has long been known to produce analgesic effects, providing individuals with temporary relief from pain (see (Peppin and Pappagallo, 2014; Sharma et al., 2013; Smith and Brooks, 2014) for comprehensive reviews). The mechanisms behind this phenomenon were partially elucidated when Jansco and colleagues found when neurons (*in vitro*) were repeatedly exposed to capsaicin they would become unresponsive (desensitized) (Jancso, 1968; Jancsó and Jancsó, 1949; Jancso, Jancso-Gabor et al., 1967). Furthermore, other compounds with a vanilloid

moiety activated the same capsaicin-sensitive neurons and therefore, the receptor was named the vanilloid receptor. Since its discovery, it has been renamed as the transient receptor potential cation channel subfamily V member 1 (TRPV1), as it resembles the family of transient receptor potential (TRP) channels (Caterina and Julius, 2001; Caterina, Schumacher et al., 1997; Montell, Birnbaumer et al., 2002)

Humans express 27 different TRP channels, with six different subfamilies (-A, -C, -M, -ML, -P, and –V), eight of which are members of the TRPV subfamily (Venkatachalam and Montell, 2007). TRP channels are non-selective cation channels, permeable to calcium and sodium ions, consisting of six transmembrane domains (TM). An intracellular hydrophobic loop lies between TM domains 5 and 6, and together all domains form the ion-conducting pore, assembled in a tetrameric arrangement (Tominaga and Tominaga, 2005). The channel's amino- and carboxy- terminal regions are located on the inner membrane. The N-terminus of TRPV1 has four ankyrin repeats, which has been shown to play a role in regulatory pathways (discussed below) (Lishko, Procko et al., 2007). On the C-terminus, located just below TM6, lies the TRP box (or TRP domain), which is a proline-rich sequence of 25 amino acids, which is conserved across many TRP channels. The vanilloid-binding site is intracellular, located between TM3 and TM4 (Cao, Liao et al., 2013; Liao, Cao et al., 2013), and when bound to a ligand, the protein is stabilized the in an open-pore conformation (Yang, Xiao et al., 2015). For a more detailed review on TRP channels, see (Gaudet, 2010; Huang, Zhang et al., 2006; Julius, 2013; Montell, 2005).

TRPV1 is the most studied of the TRP family of receptors, not only due to it being one of the first TRP channels to be cloned (Caterina et al., 1997), but also due to the channel's involvement with pain (e.g. (Jancso, 1968; Jancso et al., 1967)). Thus, TRPV1 has been suggested as a potential drug target to mediate pain, including acute inflammatory pain and neuropathic pain, such as diabetic neuropathy (e.g. (Rains and Bryson, 1995; Robbins, Staats et

al., 1998). For more detailed reviews, see (Levine and Alessandri-Haber, 2007; Szolcsanyi and Sandor, 2012). It is worth mentioning that TRPV1 is not the only receptor to detect temperature, with the temperature-sensitive receptors (or "thermo-TRP") including six heat-sensitive channels TRPV1, TRPV2, TRPV3, TRPV4, TRPM2, TRPM4, TRPM5 and two cold sensing channels, TRPA1 and TRPM8 (Huang et al., 2006). However, capsaicin is not a ligand for these receptors and much less is known about these TRP receptors, especially with regard to inflammation and disease.

Thermosensation, modulation and modification of the TRPV1 channel

Thermosensation through TRPV1 activation

TRPV1 is directly activated by heat (>43°C) (Caterina et al., 1997; Tominaga, Caterina et al., 1998), resulting in the opening of the channel pore, which allows for an influx of calcium and sodium ions; this leads to the depolarization of the cell, resulting in the release of substance P (SP) and ATP. This depolarization initiates a signal cascade from the nociceptor nerve endings through the CNS and to the brain. Molecules that directly bind to the channel and lower the channel's temperature of activation include protons (pH <6), capsaicin and several other endogenous and exogenous compounds (Tominaga et al., 1998). Sensitizers of the TRPV1 channel found in the food supply include allicin, allyl isothiocyanate, camphor, non-nutritive sweeteners (acesulfame-k, saccharin, aspartame, sodium cyclamate), and ethanol. Other exogenous compounds that activate TRPV1 via this sensitization include resiniferatoxin (RTX) and sulphuric salts (copper, zinc and iron sulfate). See (Nagy, Friston et al., 2014) for an extensive review on the ligands known to sensitize TRPV1.

TRPV1 has four conformations that regulate channel activity, which include closed (inactive), sensitized, open (active), and an intermediate stage (inactive). This fourth, in intermediate state of TRPV1 is where it is unavailable or unresponsive to agonist binding, which is termed desensitization in the pharmacology literature. There are two different stages of desensitization, with each state differing in duration and how it is regulated. The first stage is a short-lived desensitization period, lasting seconds to hours, referred to as acute desensitization, resulting in a decrease in activity during stimulation. This type of inactivity is dependent on intracellular Ca^{2+} levels. During this state, the neuron remains sensitive to other stimuli, with the exception of TRPV1 agonists (Planells-Cases, Valente et al., 2011; Tominaga, 2006).

In contrast, longer-lasting inactivity is due to channel defunctionalization, which refers to decreased response that lasts days to weeks. In this state, the neuron becoming unresponsive to all stimuli, and is likely independent of Ca^{2+} levels (Planells-Cases, Valente et al., 2011; Szolcsányi, 1993; Xu et al., 2012). Both of these stages of desensitization are reversible. However, chronic TRPV1 desensitization brought about by high doses of capsaicin can lead to neuronal toxicity and can eventually cause nerve degeneration. It is the effect of capsaicin induced long-lasting TRPV1 desensitization that results in antinocicieptive effects that is appealing for reducing pain perception.

Determining the various mechanisms regulating TRPV1 activity and desensitization are of great importance, as this could aid in the development of drugs targeting TRPV1 to provide pain relief for individuals experiencing allodynia and hyperalgesia, both of which are symptoms of chronic pain and inflammation. In summary, the ability to inactivate of TRPV1 has substantial therapeutic potential (as reviewed by (Knotkova et al., 2008; Levine and Alessandri-Haber, 2007; Szallasi and Gunthorpe, 2008). Understanding the complex pathways regulating channel sensitivity and expression could potentially offer an explanation for the occurrence and

development of hypo- and hyper- algesia. Even with increased understanding of the TRPV1 receptor, specifically work exploring its desensitization periods, it remains unclear how its activity or inactivity plays a role in hypoalgesia specific to capsaicin. It seems possible that acute desensitization and defunctionaliztion may result in acute and chronic hypoalgesia, respectively.

Modulation of TRPV1 sensitivity

The pathways modulating TRPV1 sensitivity are multifaceted (Planells-Cases et al., 2011) and ultimately result in channel plasticity (Woolf and Salter, 2000). The channel has multiple phosphorylation sites, which regulate the channel's activity. TRPV1 is phosphorylated by protein kinases, including protein kinase C (PKC), protein kinase A (PKA), Src kinase and calmodulin kinase II (CaMKII) (Novakova-Tousova, Vyklicky et al., 2007; Planells-Cases et al., 2011; Qin, 2007; Rohacs, 2015; Rohacs, Thyagarajan et al., 2008; Xu et al., 2012) (see Figure 1-1). Kinases phosphorylate residues on TRPV1, which control the conformation of the channel. Therefore, phosphorylation may result in sensitization or promotion of desensitization and is dependent not only on the residue, but also the kinase.

PKA regulates both sensitization and desensitization of the channel, with phosphorylation of residues Thr144, Thr370 and Ser502 resulting in sensitization and Ser116 and Thr317 involved in desensitization. PKA phosphorylation transforms TRPV1 from a desensitized state to a re-sensitized state. Phosphorylation by PKC sensitizes TRPV1 through several pathways and can directly lower the temperature threshold for channel activation. PKC phosphorylation sites have been located at Ser502 (a PKA phosphorylation site) and Ser800, which are important in sensitizing the channel allowing capsaicin-induced activation (Planells-Cases et al., 2011).

TRPV1 activity is also regulated in part by intracellular calcium ion levels. Following activation, internal calcium levels increase, which bind with calmodulin (CaM), leading to activation of calcineurin phosphatase and CaMKII. This promotes phosphorylation of TRPV1 at Ser502 and Thr704 (Tominaga and Tominaga, 2005). The channel undergoes short-lived (or acute) desensitization when dephosphorylated by calcineruin at these sites, as it competes with binding of ATP. Calmodulin (CaM) binds to the C-terminus and N-terminus of the channel of the channel. However, there are conflicting theories on how this binding regulates TRPV1 activity. Upon removal of CaM from the C-terminus, the channel no longer inactive and is available for further agonist binding (Rosenbaum, Gordon-Shaag et al., 2004).

It was initially suggested that TRPV1 exists in an inactive state when bound by PIP_2 (a phospholipid in the plasma membrane) (Chuang, Prescott et al., 2001). However, recent evidence suggests otherwise. Current literature indicates PIP_2 plays a role in both TRPV1 sensitization and desensitization. TRPV1 is held in an inactive state when in the presence of PIP_2 chelating agents, and upon increasing cytoplasmic PIP_2 and phosphatidylinositol 4-phosphate (PI4P; a PIP_2 precursor), TRPV1 becomes sensitized (Klein, Ufret-Vincenty et al., 2008; Lukacs, Thyagarajan et al., 2007; Stein, Ufret-Vincenty et al., 2006). PIP_2 is cleaved from TRPV1 via phospholipase C (PLC), which is activated by calcium ions and secondary messengers (reviewed in (Rohacs et al., 2008)). Hydrolysis of PIP_2 produces inositol 1,4,5-trisphosphate (IP_3), which triggers the release of calcium from internal stores. Thus, PIP_2 is required for TRPV1 sensitization, whereas depletion from the cytoplasm results in desensitization (Sun and Zakharian, 2015). This suggests that PIP_2 is involved with both positive and negative regulation of TRPV1 (Lukacs et al., 2007; Rohacs, 2015). See Figure 1-1 for summary of the sensitization and desensitization pathways regulating TRPV1.

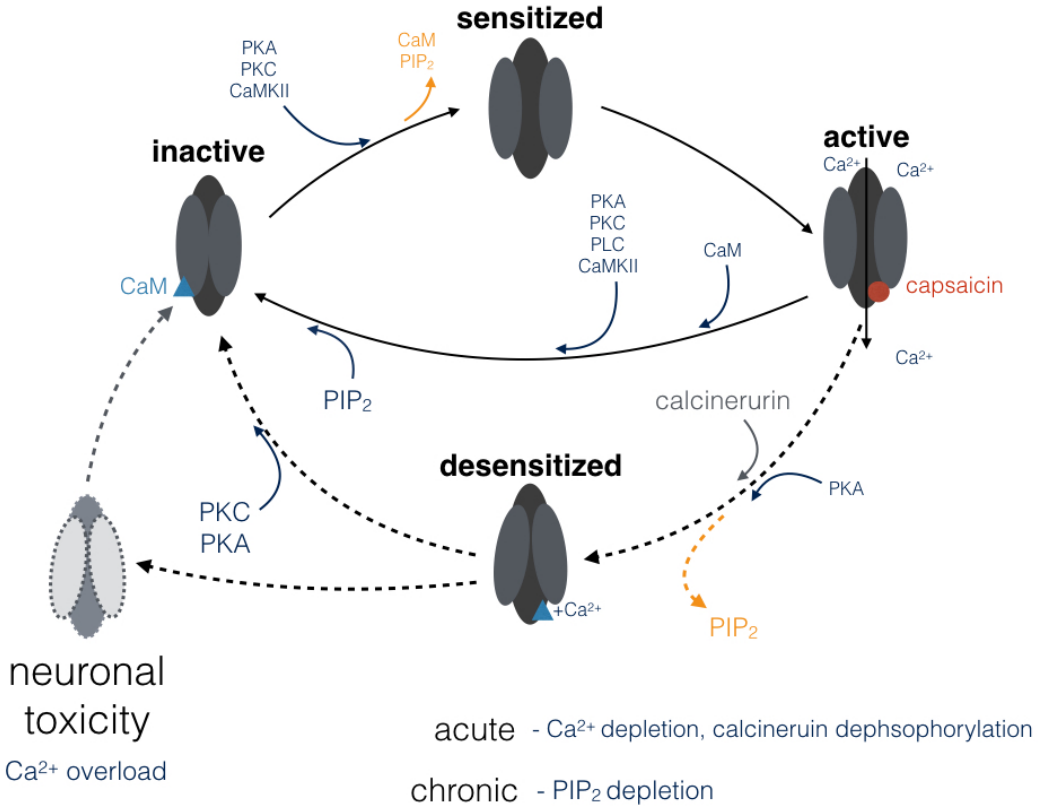

Figure 1-1: A visual representation of the pathways modulating TRPV1 channel activity. The channel can be in the following states: inactive, sensitized, active and desensitized states. Phosphorylation by PKC, PKA, and CaMKII is required to sensitize the channel, making it available for binding. The channel can be further sensitized protons (pH<6) or binding with an agonist, such as capsaicin. This causes a decrease in the activation temperature, resulting in an influx of calcium ions into the cell. This influx of calcium triggers CaMKII mediated binding of CaM. Restoration of intracellular calcium levels resorts TRPV1 activity via CaMKII. However, during recurring or constant agonist exposure the channel can become desensitized, either acute or chronic. Calcium levels and calcinerurin regulate acute desensitized, whereas loss of PIP_2 and other phospholipids leads to chronic desensitization. Calcium overload can lead to mitochondria dysfunction and depleted PIP_2 levels result in neuronal toxicity and neuron degradation. Blue lines represent phosphorylation, orange represents removal and grey represents dephosphorylation. Dashed lines are associated with desensitization pathways.

Modification of TRPV1 channels

Inflammation is an important factor in regulating TRPV1 activity and expression (e.g. (Akbar, Yiangou et al., 2010; Matthews, Aziz et al., 2004). Tissues undergo acidosis following injury; this increase in protons can activate sensory neurons that express TRPV1. During tissue damage, bioactive peptides at the site of injury are also released, including inflammatory mediators. For example, bradykinin stimulates bradykinin receptor 2 (BK_2) resulting in activation of PLC that later lead to the hydrolysis of PIP_2. This further activates PKC and results in the release of Ca^{2+} from intracellular stores. Nerve growth factor (NGF), released during inflammation, promotes thermal hypersensitivity and has short and long-term effects on TRPV1 sensitivity and gene expression. NGF and factors regulating NGF expression have been associated with increased sensitivity and expression of TRPV1 (Zhang, Huang et al., 2005; Zhu, Colak et al., 2011). This includes increased surface expression of TRPV1, mediated via p38 mitogen-activated protein kinase. NGF also activates PLC via the TrkA receptor, promoting TRPV1 sensitization by releasing the TRPV1 channel from PIP_2. Prostaglandin E_2 (PGE_2), an inflammatory cytokine, promotes cyclic AMP activation leading to sensitization of the channel via PKA. In summary, the pathways that modulate TRPV1 activity are multifaceted.

To date, no study has definitively linked this regulation of the receptor with perception. It is generally thought that that acute capsaicin hypoalgesia is a result of acute TRPV1 desensitization, whereas repeated capsaicin exposure resulting in chronic hypoalgesia is a due to chronic TRPV1 channel desensitization or neuronal defunctionalization.

There are several reviews focused on the modulating and modifying pathways of TRPV1 activity (Cortright and Szallasi, 2004; Holzer, 2008; Immke and Gavva, 2006; Nagy et al., 2014; Palazzo, Luongo et al., 2012; Planells-Cases et al., 2011; Suh and Oh, 2005; Tominaga and

Tominaga, 2005; Woolf and Salter, 2000). These regulatory processes are complex and have been extensively studied; however, the mechanisms are not fully understood, suggesting additional research is needed (see (Rohacs, 2015)).

Genetic polymorphisms in TRPV1

Changes in the nucleotide sequence of a gene can arise due to mutations, which can be passed on to offspring. Single nucleotide polymorphisms (SNPs) can occur in the coding or non-coding regions of the gene, and can either result in an altered protein sequence (non-synonymous SNP) or no change the amino acid (synonymous SNP). Non-synonymous SNPs have been shown to alter protein function, transcription, and expression. While functional non-synonymous SNPs within bitter taste receptors (*TAS2R*) genes have been identified and shown to alter function (e.g. (Allen, McGeary et al., 2013; Hayes, Wallace et al., 2011, Nolden, 2016 #1548; Kim, Wooding et al., 2005)), the *TRPV1* gene has received less attention regarding potentially functional SNPs that may relate to perception of noxious sensations. One challenge to studying SNPs in *TRPV1* is the variability in frequencies across ethnic populations (see (Xu, 2007)).

The *TRPV1* gene contains 43,968 base pairs. Based on a search of the NCBI database conducted in June 2016, there are 1046 *TRPV1* SNPs located in exon regions. There are 2465 SNPs in intronic regions with 260 SNPs having a minor allele frequency (MAF) of 0.1 or higher. Looking more closely at these SNPs located in the exon, 32 have a minor allele frequency greater than 0.01, and of those 5 are missense, 23 are in the untranslated regions (3' and 5'), and 4 are synonymous SNPs. Of these the missense SNPs, one variant (Ile585Val) reportedly influences salt perception (Dias, Rousseau et al., 2013) and pain sensitivity (Binder, May et al., 2012; Valdes, De Wilde et al., 2011).

The majority of research on *TRPV1* variants has been investigated *in vitro* using mutagenesis studies (reviewed by (Smutzer and Devassy, 2016). These studies specifically alter amino acids at locations within the gene to determine if the specific location alters structure or activity, allowing conclusions that targeted SNPs would effect ligand binding, activity or expression. However, the mutations that appear to be functional in such studies may not be related to SNPs that are actually observed in the human population. Xu and colleagues (2007) conducted point mutations *in vitro* for 5 SNPs, and concluded that two SNPs (P91S and I315M) may effect expression, with none of the tested SNPs influencing capsaicin response. While many *in vivo* studies have been unsuccessful in identifying SNPs altering response to capsaicin, one *in vivo* gene association study reported two SNPs (in intronic regions) associated with variability in psychophysical measure of perceived burn from sampled ethanol (Allen et al., 2014), a sensitizer of TRPV1 (Trevisani, Smart et al., 2002). More work is needed to determine how SNPs in *TRPV1* alters expression and activity, which may impact pain, effectiveness of TRPV1-mediated treatments, and perception of chemesthetic stimuli.

As mentioned previously, ethanol activates TRPV1, eliciting burning/stinging sensations in addition to bitterness (via TAS2Rs). My research has shown that SNPs within *TRPV1* are associated with differences in the burn from sampled ethanol (Allen et al., 2014). Additional research is needed to determine how SNPs within *TRPV1* associate with burn perception of capsaicin. In addition to genetic variability within *TRPV1* leading to altered responses, it is possible that repeated exposure to alcoholic beverages might change perception by similar mechanisms proposed for dietary capsaicin intake and hypoalgesia.

As detailed above, there are numerous pathways involved in regulating TRPV1 channel activity. Many of the pathways that regulate the modulators and modifiers of TRPV1 activity are up regulated in diseased and damaged tissue, which are regulated by inflammatory responses.

Likewise, TRPV1 expression is also associated with inflammation and diseases (Nilius et al., 2007; Premkumar and Bishnoi, 2011). For this reason, TRPV1 has been suggested as a therapeutic target to regulate pain, inflammation and chronic disease. Additional research is needed to determine if increased TRPV1 expression is an antecedent or consequence of inflammation, and whether this increased expression maintains or promotes an inflammatory state. Regardless of TRPV1 expression and its time course with the onset of inflammation, there is a body of evidence that supports an association between TRPV1 expression and chronic inflammation and disease. These data are discussed below.

TRPV1 expression associated with pain, disease and chronic inflammation

Perception of acute pain is a fundamental warning system to noxious stimuli. In contrast, chronic pain is often a condition for which patients seek treatment. As mentioned above, several pathways modulate and modify TRPV1 sensitivity and expression. Pro-inflammatory cytokines are released as a result of tissue and nerve damage, which mediate TRPV1 regulatory pathways leading to increased TRPV1 sensitivity and expression. Furthermore, depolarization of nociceptive fibers also results in the promotion of neurogenic inflammation, by initiating secondary pathways, leading to vascular leakage, vasodilation, and production of proinflammatory and proalgesic factors. This response has been shown to further sensitize TRPV1 channels. Moreover, TRPV1 expression and sensitivity is increased in patients with pain neuropathy, chronic diseases and inflammation. Thus, TRPV1 has been targeted for drug-based therapies in order to disrupt this inflammatory cycle and aid in the reduction of pain.

Regulation and expression of TRPV1 in patients with inflammation or chronic pain

TRP channels are associated with a range of human disorders. Patients with various chronic diseases have been found to express increased *TRPV1* mRNA compared to their healthy controls (Premkumar and Bishnoi, 2011). Furthermore, expression and immunoreactivity of TRPV1 has been reported to correlate with patients' pain scores (see (Bishnoi and Premkumar, 2013) for a review). For a complete summary of the TRP channels and related diseases, refer to (Nilius et al., 2007; Premkumar and Bishnoi, 2011). However, as this review is focused on perception and irritation in the oral cavity, greater emphasis will be given to TRPV1 expression in diseases involving the oral cavity.

TRPV1 channels are expressed within taste papillae, but not in taste buds themselves (Ishida, Ugawa et al., 2002). Patients with oral neuropathic pain show increased expression of TRPV1 in oral mucosa (Biggs, Yates et al., 2007; Borsani, Majorana et al., 2014; Marincsák, Tóth et al., 2009; Yilmaz, Renton et al., 2007). Borsani and colleagues (2014) reported that tongue punch biopsies from patients with burning mouth syndrome (BMS) had greater TRPV1 immunopositivity (a measure protein quantity and location). Furthermore, greater staining was observed in deeper layers of the epithelium and cytoplasm compared to healthy controls, where TRPV1 was localized to the base layer of epithelium and low amounts of TRPV1 in the cytoplasm (Borsani et al., 2014). This is consistent with previous reports showing greater immunoreactivity area for TRPV1 in oral mucosa from BMS patients compared to controls (Yilmaz et al., 2007). Furthermore, TRPV1 immunoreactivity was significantly associated with perceived pain (Yilmaz et al., 2007). BMS patients experience "unremitting oral burning or similar pain in the absence of detectable oral mucosa changes" (Merskey, 1986). This pain can vary in intensity from moderate to severe (Merskey, 1986).

Oral pain is also a symptom of squamous cell carcinoma (malignant) and leukoplakia (premalignant) of the tongue. Marincsák and colleagues (2009) reported greater immunoreactivity and mRNA expression of *TRPV1* in tongue tissues from patients with both stages of cancer compared to control tissues. These studies and others (Brito et al., 2014; Szolcsanyi and Pinter, 2013) suggest molecules targeting TRPV1 may offer an effective way to reduce oral pain and perhaps treat chronic diseases, including BMS and oral cancer (Borsani et al., 2014; Marincsák et al., 2009; Yilmaz et al., 2007).

Capsaicin as a treatment for pain, and inflammation-induced pain

As mentioned above, capsaicin has therapeutic properties and can treat pain in patients experiencing physiological and neurological pain (Hayman and Kam, 2008; Mason et al., 2004; Oyagbemi, Saba et al., 2010; Smith and Brooks, 2014). This is largely due to its ability to produce hypoalgesia following topical application. Capsaicin has been used in treatment of pain as a result of variety of diseases (see (Hayman and Kam, 2008; O'Neill et al., 2012; Srivastava, 2013)). Other putative health benefits associated with capsaicin include potential influences on energy balance (Ludy, Moore et al., 2011), as well as its antioxidant (Materska and Perucka, 2005) and chemoprotective properties (Surh, Lee et al., 1998); however, these effects are less clear (Surh and Lee, 1996; Surh and Sup Lee, 1995). Capsaicin has been suggested as an effective treatment for orofacial pain, including BMS and oral cancer (e.g. (Berger et al., 1995; Silvestre et al., 2012)).

Capsaicin provides relief for patients with oral pain

The prevalence of orofacial pain is difficult to estimate due to inconsistent diagnosis; however, it is estimated that between 3 to 15% of the population suffers from acute and chronic orofacial pain (Lavigne and Sessle, 2015), with one report as high as 22% in Americans over the age of 18 (Lipton, Ship et al., 1993) and up to 36% in the United Kingdom among those aged 18-65 (Macfarlane, Blinkhorn et al., 2002). The two most common reports of orofacial pain are somatic pain as a result of poor dental hygiene, followed by temporomandibular joint dysfunction (TMJ) (Vickers and Cousins, 2000; Zakrzewska, 2002). Alternatively, a variety of diseases, drugs and surgeries can cause damage to pain pathways that can lead to orofacial neuropathic pain (Christoforou, Balasubramaniam et al., 2015). Oral pain can be constant or intermittent and has been described as aching, burning, tingling, raw and or crawling sensations. Capsaicin has proven to be a useful treatment for many suffering from oral neuropathic pain (Berger et al., 1995; Epstein and Marcoe, 1994; Marino, Torretta et al., 2010; Petruzzi, Lauritano et al., 2004; Silvestre et al., 2012). It is assumed that this treatment of repeated exposure to capsaicin (2– 25 ppm) reduced patients symptoms due to hypoalgesia (Berger et al., 1995; Silvestre et al., 2012). However, in terms of orofacial pain, there is little evidence as to the mechanism in which capsaicin provides therapeutic effects.

Potential mechanisms regulating capsaicin hypoalgesia

There are three potential mechanisms involving TRPV1 regulation that could be working together or separately, to induce capsaicin hypoalgesia. The first possible mechanism I will discuss involves the reduction of TRPV1 channel on the cell surface of the neuron. Second, repeated exposure to capsaicin can result in nerve terminal degeneration, making the TRPV1

channel inaccessible. Third, neurons expressing TRPV1 can become defunctionalized, impairing local nociceptor function. It is likely that all of these can occur following capsaicin exposure, but largely dependent on the dose and exposure duration, with each one associated with greater concentration, respectively.

Capsaicin exposure may down regulate TRPV1 receptor expression

One potential mechanism for capsaicin hypoalgesia is that capsaicin exposure results in down-regulation of the TRPV1 receptor. This decreased receptor expression would explain a reduction in pain perception, as there would be fewer receptors accessible at the cell surface for stimuli to activate and to depolarize the neuron. A relationship between capsaicin exposure and *TRPV1* mRNA expression has been demonstrated in clinical populations of patients with idiopathic rhinitis (IR; inflammation of the nasal membranes; also known as vasomotor rhinitis) (Bernstein, Davis et al., 2011; Blom, Rijsquk et al., 1997; Blom, Severijnen et al., 1998; Ciabatti and D'Ascanio, 2009; Lacroix, Buvelot et al., 1991; Van Gerven, Alpizar et al., 2014; Van Rijswijk, Boeke et al., 2003). Van Gerven and colleagues (2014) were the first to report on the mechanism behind the therapeutic mechanism of capsaicin on IR (idiopathic rhinitis) (see Figure 1-2). Prior to treatment, IR patients had greater mRNA expression of *TRPV1* relative to healthy matched controls. A capsaicin treatment was delivered intranasally and consisted of 5 consecutive doses of a solution containing 30.5 ppm capsaicin, with a total of 8mL administered. IR patients saw a significant reduction in symptoms (nasal obstruction and sneezing) and mRNA expression of *TRPV1* immediately following treatment. Interestingly, at 12 weeks, IR patients continued to show decreased expression and had significantly lower expression than healthy controls (Van Gerven et al., 2014).

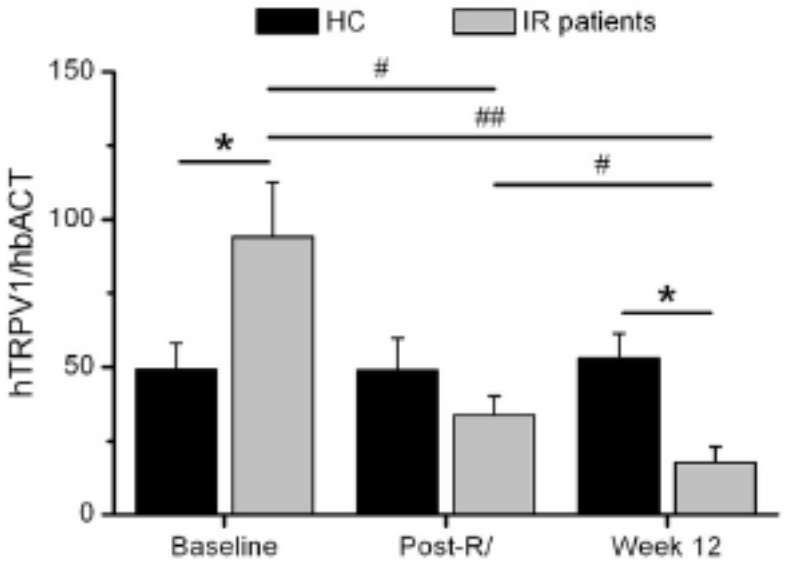

Figure 1-2: TRPV1 expression in human controls (HCs) and patients with idiopathic rhinitis (IR) at different time points in relation to capsaicin treatment. TRPV1 expression (RT-PCR) in homogenates of nasal biopsy specimens in HCs and patients with IR at baseline, immediately after, and 12 weeks after treatment (n=13). (Taken from (Van Gerven et al., 2014).)

In addition to decreased *TRPV1* mRNA expression, there was a reduction of substance P in IR patients, which at baseline was elevated and reached levels similar to that of the healthy control group (Van Gerven et al., 2014). This suggests that a reduction in *TRPV1* expression results in a reduced substance P release and decreased pain response. This study supports the hypothesis that capsaicin exposure may result in a down-regulation of TRPV1 receptors. However, additional research is needed to further elucidate the pathway regulating TRPV1 expression as result of capsaicin activation or other TRPV1 agonists. This decreased mRNA expression does not give proper insight as to potential changes in the location of the channel

expression (i.e. is the channel internalized with in the cell, or are the neurons withdrawing from the cell surface, as discussed below). Further studies relating biology and psychophysics are needed to confirm whether oral capsaicin exposure decreases *TRPV1* mRNA expression, and if this in turn results in decreased capsaicin and pain perception, and to further explore the pathway(s) involved in regulation of TRPV1.

Capsaicin exposure stimulates nerve terminal degeneration

A second mechanism that potentially explains capsaicin hypoalgesia involves degeneration of nerve fibers from the exposed areas. Capsaicin exposure (topical or subcutaneous) in humans can produce pronounced nerve degeneration in the epidermis resulting in decreased response to pain and heat (Kennedy, Vanhove et al., 2010; Malmberg, Mizisin et al., 2004; Nolano, Simone et al., 1999; Polydefkis, Hauer et al., 2004; Simone, Nolano et al., 1998). Simone and colleagues (1998) observed denervation and subsequent reinnervation following an intradermal injection between 0.2ug and 20ug in 20uL (equivalent to 10 to 1,000 ppm). Nerve immunoreactivity for protein gene product 9.5 (PGP 9.5; a neuronal marker) decreases in a dose-dependent manner (Simone et al., 1998). Post capsaicin treatment, participants demonstrated reinnervation of epidermal nerve fibers (Nolano et al., 1999; Simone et al., 1998) with epidermal nerve fiber length only reaching 12 to 29% of pre-treatment length at 4 weeks post-treatment (Simone et al., 1998). Heat and mechanical pain response in participants returned to baseline in the weeks following treatment (4 – 6 weeks), coinciding with reinnervation (Nolano et al., 1999; Simone et al., 1998). Even though denervation of nerve fibers has been observed following capsaicin exposure on the skin, it is unknown if trigeminal innervation in the tongue may be

altered in the same fashion. Furthermore, it has been suggested there are additional mechanisms, in addition to denervation, that give rise to capsaicin hypoalgesia (Anand and Bley, 2011).

Capsaicin exposure induces defunctionalization of local nociceptor function

A potential third mechanism suggests that repeated capsaicin exposure (or a single high dose) leads to capsaicin hypoalgesia and neuronal defunctionalization via two different mechanisms (summarized in (Anand and Bley, 2011)). On one hand, capsaicin inhibits the electron transport chain transport by competitively binding to mitochondrial complex I (Shimomura, Kawada et al., 1989). This activity results in decreased mitochondrial membrane potential (Dedov, Mandadi et al., 2001) and the inhibition of mitochondrial oxidative phosphorylation (Athanasiou, Smith et al., 2007). But on the other hand, prolonged increased intracellular Ca^{2+} (due to opening of the TRPV1 channel and release from internal stores) promotes activation of calcium-dependent enzymes triggering protein synthesis inhibition and depolymerization of cytoskeletal components (Chard, Bleakman et al., 1995; Han, McDonald et al., 2007) and osmotic swelling. Both of these mechanisms – capsaicin-induced inhibition of the electron transport chain and protein synthesis – can result in cell degradation and defunctionaliztion of nerve endings. These mechanisms are thought to explain the phenomenon of hypoalgesia and are termed defunctionalization in the sense that it impairs local nociceptor function for extended periods of time, as a result of capsaicin exposure (Anand and Bley, 2011).

In summary, it is possible that all three mechanisms are involved with capsaicin-induced hypoalgesia. These changes may occur in a progression, with first altering channel expression at the cell surface, followed by nerve defunctionalization and internalization. More research is

needed to determine if oral capsaicin exposure leads to expression changes in TPRV1 channel on the cell surface, and or changes in innervation of TRPV1 containing neurons.

Conclusions

Extensive reviews have been published on the use of TRPV1 agonists, including capsaicin, to mitigate pain (see (Knotkova et al., 2008; Mason et al., 2004; O'Neill et al., 2012). Even with this accumulation of literature supporting capsaicin as a therapeutic agent, the mechanism(s) behind capsaicin induced hypoalgesia and defunctionalization are not fully understood. While some studies have provided insight as to potential mechanisms (see (Anand and Bley, 2011)), there are several reports that conflict with these findings. The proposed mechanisms for the pathways resulting in decreased sensitivity following capsaicin exposure have been demonstrated in human clinical studies, such as nerve degeneration (e.g. (Simone et al., 1998)). However, these studies have almost exclusively as a topical agent on skin. It is unknown if these same effects are tissue specific or are ubiquitous across tissues. There is a lack of studies that combine both psychophysics and biology, which are needed to fully understand the effects of capsaicin exposure. It has yet to be determined if exposure to capsaicin can be used as a preventative treatment to prevent or reduce pain and inflammation. Even though capsaicin is widely available and consumed on a regular basis, very little is known as to the effects of this chronic exposure (Lv, Qi et al., 2015), and how dietary exposure may play a role in mitigating or preventing disease associated with chronic inflammation and pain. Overall, the main hypothesis that repeated exposure of capsaicin results in a decrease in expression of the TRPV1 receptor. Secondary hypotheses are that capsaicin and ethanol elicit variable burn and bitterness response, which is associated with genetic variability in chemosensory receptor genes.

CHAPTER 2

Perceptual qualities of ethanol depend on concentration and variation in these percepts associates with drinking frequency

Adapted from:

Nolden, A.A. and Hayes, J.E.

"Perceptual qualities of ethanol depend on concentration and variation in these percepts associates with drinking frequency."

Chemosensory Perception, 2015, 8(3): 149-157.

Abstract

Ethanol, the pharmaceutically active ingredient in all alcoholic beverages, elicits multiple percepts including sweet, bitter, drying, and burning. However, quality-specific perceptual dose-response functions have not been previously reported. Also, individual differences in ethanol perception may associate with differences in alcoholic beverage use. Here, we describe the chemosensory profile of ethanol across concentrations in a convenience sample of mixed age adults; secondarily, we explore whether individual differences in various qualities from ethanol associate with alcohol use behaviors.

Participants (n = 100, 33 men) aged 21 to 55 (mean: 33 years) tasted ethanol in water (4, 8, 16, 32 and 48% v/v) and rated sweetness, bitterness, drying and burning/tingling on four

general Labeled Magnitude Scales. Demographic question and alcohol use measures (years drinking and reported frequency of drinking occasions) were also collected.

Intensity of most qualities increased as a function of ethanol concentration, although the dominant sensation differed with concentration. The dominant sensation for 8% and 16% ethanol was bitterness (7.4±1.0; 13.5±1.4), whereas for 32% and 48% ethanol, burning/tingling was the dominant sensation (29.7±2.1; 44.7±2.4). Quality-specific intensities of sampled ethanol explained variability in the reported intake frequency for beer, wine, straight spirits and number of drinking occasions. The number of years reported drinking (grand mean 10.5±0.8) was not significantly associated with perceptual ratings for sampled ethanol.

In a convenience sample of mixed aged adults, the sensations from suprathreshold ethanol varied by concentration: bitterness dominated at lower concentrations, while burn dominated at higher concentrations. Exploratory analyses also suggest differences in chemosensory responses across participants may associate with measures of alcohol use.

Introduction

Ethanol elicits both taste and chemesthetic responses, and it is well established that taste intensity (Mattes and DiMeglio, 2001; Scinska et al., 2000) and oral irritation (Green, 1987; Green, 1988) both increase as a function of concentration. However, prior reports fail to consider taste and chemesthetic responses simultaneously. For ethanol in water, the taste detection threshold in humans (Mattes and DiMeglio, 2001) and the preference threshold in rats (Richter, 1956) are both approximately ~1.4% (v/v). In humans, ethanol at and just above threshold has been shown to be predominately bitter (Mattes and DiMeglio, 2001), although burn was not provided as a response option. Other work in humans suggests 3% and 10% (v/v) ethanol are

predominately bitter, although roughly a third of participants endorsed sweetness in addition to bitterness when asked to describe the quality; again however, neither burn nor other chemesthetic qualities were assessed, as participants were only asked about sweet, salty, sour and bitter sensations (Scinska et al., 2000). Moreover, those authors also reported quinine / sucrose mixtures were more qualitatively similar to 10% ethanol than the same quinine concentration in isolation (Scinska et al., 2000); similarly, earlier work shows 8% sucrose in 10% ethanol is isosweet with 10% sucrose in water (Berg, Filipello et al., 1955). Other early work on ethanol chemosensation reported recognition thresholds of 4.2% for sweetness, and 21.2% for burning (Wilson, O'Brien et al., 1973); curiously, while the authors note the quality at lower concentrations may be bitter or 'like an almond', they did not attempt to estimate a recognition threshold for bitterness. Collectively, these reports suggest that in addition to its well-accepted bitterness, ethanol has some inherent sweetness, at least at some concentrations. It also suggests data on chemesthetic qualities are generally lacking, with a few exceptions (e.g., (Green, 1987; Green, 1988; Wilson et al 1973).

Additional information on the sensations from ethanol comes from descriptive analysis on alcoholic beverages or model wines rather than simple ethanol/water mixtures; it is clear from these data that the sensory profile of alcoholic beverages differs by ethanol concentration, at least up to ~15% ethanol. For example, increased ethanol content in alcoholic beverages associates with instrumentally measured changes in physical viscosity and density (Langstaff, Guinard et al., 1991; Nurgel and Pickering, 2005; Pickering, Heatherbell et al., 1998). Using a panel of 12 trained assessors, Nugel and Pickering (2005) observed significant differences in perceived viscosity and density for model wines with increasing ethanol content (0, 3, 7, 10, 12 and 14%) (Nurgel and Pickering, 2005). In model red wines, greater ethanol concentration associates with increased intensity ratings for chemical, woody, spicy, bitterness and burning sensations, and

decreased intensity ratings for fruity, floral and caramel sensations (Villamor, Evans et al., 2013). Contrary to the inherent sweetness discussed above, changes in ethanol concentration did not appear to alter the sweetness of model ice wines containing glucose and fructose, although this may reflect the relatively narrow range of concentrations used (7 to 12% v/v ethanol), as the change in bitterness across concentration also failed to reach significance (Nurgel and Pickering, 2006). The ability to detect ethanol retronasally varies by wine type, and has been reported to range from 1.03 to 1.32% (v/v) (Yu and Pickering, 2008). In summary, the alcoholic beverage literature supports the idea that changes in ethanol concentration influence the sensory quality of these beverages. Nonetheless, there remains a surprising gap in the psychophysical literature regarding the multiple chemosensory qualities evoked by ethanol, as we have been unable to find dose response data on sweetness, bitterness, and irritation of ethanol/water mixtures in a single group of participants. We address this gap here.

Other literature suggests alcoholic beverage liking and intake associates with sensations elicited by ethanol (Intranuovo and Powers, 1998; Lanier et al., 2005). Individual differences in the perception of bitterness and sweetness have been previously associated with intake (Duffy, Davidson, et al., 2004; Duffy, Peterson et al., 2004; Intranuovo and Powers, 1998; Lanier et al., 2005) and alcohol dependence (Kampov-Polevoy, Garbutt et al., 1999; Kampov-Polevoy, Alexey et al., 2004); however, others have failed to replicate this association (Kranzler, Sandstrom et al., 2001; Tremblay, Bona et al., 2009). The sweetness of foods and beverages, including alcohol, associates with increased liking and intake (Lanier et al., 2005), whereas bitterness drives disliking (Hayes et al., 2011; Intranuovo and Powers, 1998; Lanier et al., 2005). Accordingly, studies which explore individual qualities in isolation may be overly reductionist and misleading, as prior work suggests multiple qualities influence liking and intake simultaneously. Lanier and colleagues (2005) found increased sweetness and decreased bitterness elicited from sampled

whiskey and beer associated with greater liking and intake in undergraduate students of legal drinking age. However, those authors did not consider burning sensations in their model. It remains unknown how chemesthetic sensations elicited from ethanol, such as burning, tingling and drying, together with prototypical taste sensations like sweetness and bitterness, may associate with differential intake of alcoholic beverages.

Epidemiological surveys that quantify alcoholic beverage consumption typically use the concept of a standard drink. This unit of measure attempts to take into account the percentage of ethanol in a particular type of beverage (e.g. 12 oz. beer, 1.5 oz. of spirits, or 5 oz. of wine). Critically however, this well accepted metric does not consider the differences in the chemosensory properties of beverages that may arise from different ethanol contents. Accordingly, improving our understanding of the full range of chemosensory responses to ecologically relevant concentrations of ethanol (i.e., at levels found across classes of various alcohol beverages) may provide additional insight into how various sensations from ethanol (e.g., sweetness, bitterness and irritation) potentially influence alcoholic use behaviors.

The cross sectional study described here extends previous work. In a convenience sample of mixed age adults, we report on the chemosensory response to ethanol/water mixtures across a range of concentrations (4% to 48% (v/v)) that are relevant to those typically found in alcoholic beverages (i.e., the range provides ecological validity). These data address a surprising gap in extent literature by illustrating dose response functions for sweetness, bitterness, drying, and burning/tingling sensations across various concentrations within a single group of participants. As a secondary aim, we also explore whether individual differences in the sensations from sampled ethanol may associate with two measures of alcohol related behaviors, including years drinking and drinking occasions in a convenience sample of mixed age adults.

Materials and Methods

Study Overview

A convenience sample of reportedly healthy adults who had previously shown interest in participating in taste and smell experiments in our facility were recruited to participate in a single session study. Interested subjects completed an initial online survey prior to the start of the test in order to determine if they were eligible to participate. Participants were invited to a single test session (~45 minutes), held at the Sensory Evaluation Center at Penn State, where they were asked to rate the intensity of five concentrations of ethanol in water, and answer several demographic questions, including two regarding alcohol use behaviors. Participants gave informed consent and were paid for their time.

Participants

Participants (n = 100; 33 men; mean age = 33.0; age range 21-55 years) were recruited from the Pennsylvania State University campus and surrounding area. Individuals who had previously indicated they were interested in taste and smell research were contacted via email, and asked to complete an online survey to determine if they met study inclusion criteria. These criteria included: not pregnant or breast feeding, non-smoker, no tongue, cheek or lip piercing, no difficulty swallowing or history of choking, no known taste or smell defect, not an undergraduate student, not taking prescription pain medication, no hyperactive thyroid and no history of chronic pain. Participants were also asked if they were willing to taste ethanol in the laboratory to ensure participants had no objection to tasting ethanol, which might result from health, religious, or any other reason. Study procedures were exempted from review by the local Office of Research

Protections staff under the exemption in 45 CFR 46.101(b)(6). All data were collected anonymously (i.e., participant identification was not linked to the sensory, or demographic data, including the alcohol use data).

Stimuli and sampling procedure

Participants rated 4%, 8%, 16%, 32% and 48% (v/v) ethanol in water, concentrations that cover the range of ethanol commonly found in beers, wines and distilled spirits. An additional water (reverse osmosis) sample was included in the sample set as a control. To reduce effects of presentation order, all samples were presented in a counterbalanced Williams Design, which purportedly controls for effects of position, order and carryover effects (Williams, 1949). All samples were presented at room temperature in 10mL aliquots in plastic medicine cups with a three-digit blinding code; they were prepared by diluting neat USP grade ethanol with reverse osmosis water.

Participants were first asked to rinse with room temperature RO water before tasting any stimuli. For each stimulus, participants were instructed to place the entire sample into the mouth and swish it around for 3 seconds, spit it out and wait 5 seconds before making a rating, to account for the delayed onset of chemesthetic sensations. Participants rinsed with room temperature RO water to remove any lingering sensations; a minimum interstimulus interval of 2 minutes was enforced via software, and participants were also instructed to rinse until any remaining sensation from the stimulus was gone.

Psychophysical Scaling

Participants were asked to rate perceived intensity using a general Labeled Magnitude Scale (gLMS). The gLMS ranges from 'no sensation' at 0 to 'the strongest imaginable sensation of any kind' at 100, with empirically spaced adjectives at 1.4, 6, 17, 35 and 51 ('barely detectable', 'weak', 'moderate', 'strong' and 'very strong'; respectfully). Four gLMS scales were presented for each stimulus, and participants used these to rate the intensity of: bitterness, sweetness, drying, and burning/tingling.

Participants were not trained on the individual qualities ahead of time, and burning/tingling were treated as a single percept here to limit 'smearing' bias that can occur when too many scales are provided (see discussion in Bennett and Hayes, 2012). That said, other work suggests these are distinct qualities, and they can be assessed separately under some circumstances (e.g. Bennett and Hayes, 2012; Cliff and Heymann, 1993). Also, it should be noted that while ethanol is frequently described as burning in the psychophysical literature, either explicitly (Allen et al., 2014; Green, 1988) or implicitly (Duffy, Peterson, et al., 2004), chemesthetic aspects of ethanol are often described as 'heat' in the wine literature (e.g., Pickering and Robert, 2006); such nuanced differences may potentially influence how participants perform the task. Finally, for simplicity sake, we also made the assumption naïve participants would consider warming, chemical heat, and mild irritation to be weaker versions of burning/tingling; for a conflicting view with other chemesthetic stimuli besides ethanol, see recent work by Byrnes and colleagues (2015).

Prior to rating any samples, participants were given written instructions on the use of the gLMS and rated 15 remembered or imagined sensations for a brief practice (Hayes, Allen et al., 2013). These items include both food and non-food items to emphasize that the scale should be

used in context to all sensations. Participants' ratings of the orientation items were evaluated to determine whether participants understood the instructions and were using the scale properly. Here, proper scale use was defined as rating items in appropriate order (i.e. dimly lit room < well lit room < brightest light imaginable, and loudness of a whisper < conversation < loudest sound imaginable) and not rating items as 100 (with the exception of 'scalding hot water', 'loudest sound' and 'brightest light'). Under these criteria, four participants failed to use the scale correctly (discussed in more detail below). All psychophysical and demographic data were collected using Compusense *five*, version 5.2 (Guelph, ONT).

Demographics and Measures of alcohol use

After tasting all stimuli, participants answered demographic questions on age and sex, and six questions related to alcohol use (Table 2-1). These questions were adapted from quantity-frequency and food-frequency questionnaires (e.g. Cahalan, Cisin et al., 1969; Glovannucci, Colditz et al., 1991); see (Feunekes, van't Veer et al., 1999) for a review. These questions were used to estimate the frequency at which participants usually consume alcohol, as well as consumption frequency of different types of alcoholic beverage. Specifically, participants were asked: 'How often do you usually drink alcohol?' ('I never drink alcohol', 'less than once a month', 'less than once a week', 'on 1 or 2 days a week', 'on 3 to 4 days a week', 'on 5 to 6 days a week', 'everyday') and 'Over the last month, how often did you drink [TYPE OF ALCOHOL]?', where a separate question was asked for: beer, wine, straight spirits, and spirits with mixers. The response options for these 5 questions were: 'never', 'less than once per month', '1 day per week', '2 days per week', '3 days per week', '4 days per week', '5 days per week', '6

days per week', 'everyday'. Finally, participants were asked 'How long have you been regularly consuming alcohol (in years)?'.

Statistical analysis

Alcoholic use measures were assigned ordinal scores that corresponded with their answers: 0-6 for overall intake, and 0-8 for each beverage specific question (i.e. beer, wine, straight spirits, and spirits with mixers). These values were converted to yearly frequency (e.g. 1-2 times/week = 12, 3-4 times/week = 48, 5-6 times/week = 72, etc.) and then quarter-root transformed to improve normality of the data. Sex was coded as 1 for males and 2 for females. All analyses were conducted using SAS 9.2 (Cary, NC). Pearson correlation (r) and t-tests were conducted to test associations between age and sex respectively, with reported alcohol drinking frequency measures and years drinking. Separate linear regression models were performed using 'proc reg' to test associations between alcoholic beverage use measures and intensity ratings for each sensation across concentrations of sampled ethanol. Stepwise multiple regression analysis was conducted to determine the total amount of variability explained for alcohol use measures explained by all chemosensory ratings across all ethanol concentrations. For multiple regression, multicollinearity was determined for the final stepwise model via the 'vif' (variance inflation factor) option. There was no evidence of multicollinearity here as all VIF's were less than 3 for all significant variables in the final model. Analysis of variance (ANOVA) models were performed via 'proc mixed' to test associations between groups created based on alcohol use measures (drinker group and drinking experience) with quality-specific intensity ratings for sampled ethanol. Percent variance explained (R^2) is reported for both linear and stepwise regression and semi-partial correlation (sr) is reported for stepwise regression.

Results

Evaluation of participants' use of the general Labeled Magnitude Scale

Participants received written instructions describing the gLMS and proceeded to rate 15 remembered or imagined sensations on the gLMS to practice using the scale. Means and standard errors for the scale orientation items are shown in Table 2-2. These ratings were also used to determine participant's ability to follow directions and evaluate their proper use of the scale. Of 100 participants who completed the study, data from four were removed (two participants rated loudest sound or brightest light less than 0.5, one participant only made ratings between 0-2 on a 100 point scale, and one participant rated five items as 100). All other participants (n=96) rated 3 sound and 3 light items in approximately the correct order (i.e., ratings were roughly monotonic, allowing for deviation of up to 5.0 units on a 100 point scale).

Table 2-1: Mean ratings for gLMS orientation items

Orientation question	Mean	±SEM
The brightest light you have ever seen	66.1	2.4
The loudest sound you have ever heard	65.4	2.3
The heat from dipping your hand in scalding hot water	60.3	2.3
The pain from biting your tongue	38.9	1.9
The sourness of a lemon	35.8	1.9
The sweetness of cotton candy	32.6	1.8
The brightness of a well-lit room	27.0	1.5
The bitter taste of black coffee	26.9	1.5
The burn from cinnamon gum	26.6	1.7
The strength of a firm handshake	23.5	1.2
The loudness of a conversation	20.1	1.2
The coolness from a peppermint candy	20.1	1.2
The warmth of a summer breeze on your face	18.8	1.2
The brightness of a dimly lit room	8.6	0.7
The loudness of a whisper	6.1	0.7

SEM standard error of the mean

Sensations from sampled ethanol varied with concentration

The mean intensity ratings for burning/tingling, bitterness and drying increased with increasing concentration, as expected, with differences in the rate at which each sensation increased (Figure 2-1). The functions are shown visually in Figure 2-1, and the power functions for each quality are given below. For 4% ethanol, means for all sensations were similar and fell

between ‘barely detectable’ and ‘weak’. At 8% ethanol, sweetness, drying and burning/tingling were reported as below ‘weak’ with bitterness rated just above ‘weak’. At 16% ethanol, sensations began to differentiate in their intensities, with burning/tingling and bitterness falling below ‘moderate’, whereas drying was rated above ‘weak’ and sweetness just below ‘weak’. At 32% and 48% ethanol, intensity ratings for all sensations continue to diverge with burning/tingling being the predominate sensation, with 32% ethanol being rated above ‘moderate’ and 48% above ‘strong’. Some sweetness for ethanol was reported; however, the ratings fell between ‘barely detectable’ and just above ‘weak’.

Using group means, the estimated quality specific power functions for ethanol in water are as follows:

$$\text{burning/tingling} = 0.86\,[\text{ethanol}]^{1.26},$$

$$\text{bitterness} = 0.92\,[\text{ethanol}]^{0.88},$$

$$\text{drying} = 0.79\,[\text{ethanol}]^{0.98}, \text{ and}$$

$$\text{sweetness} = 0.62\,[\text{ethanol}]^{0.42},$$

where ethanol concentration is given in percent (v/v).

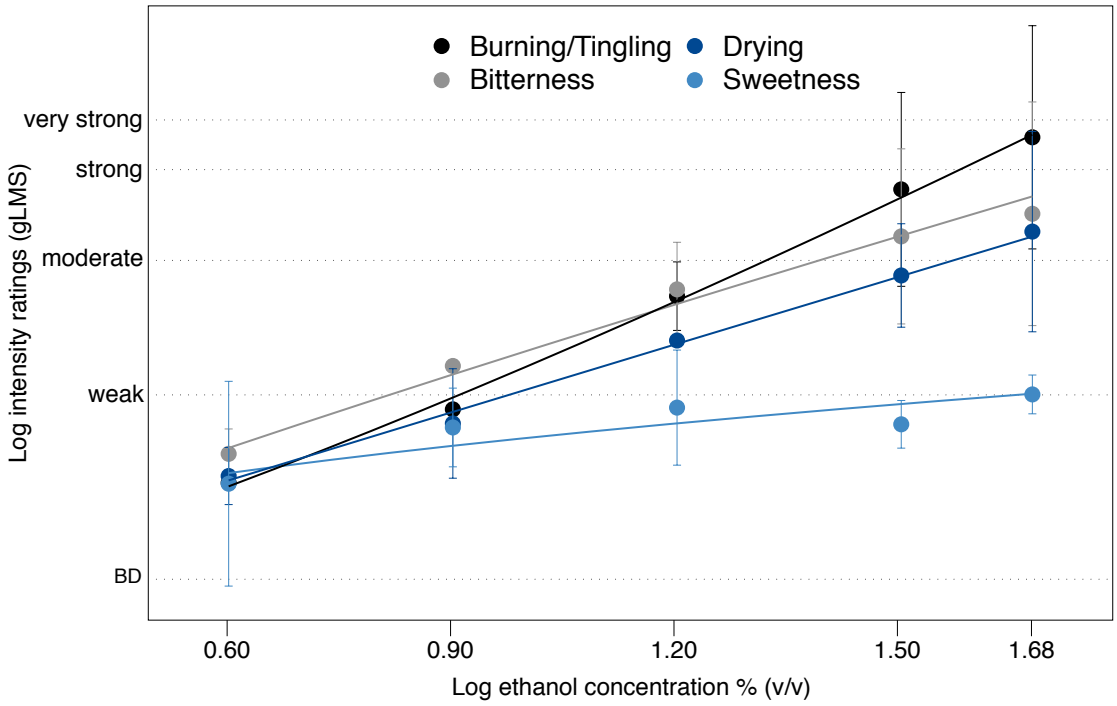

Figure 2-1: Mean log intensity (±S.E.M.) burning/tingling, bitterness, drying and sweetness ratings of sampled ethanol collected on a general Labeled Magnitude Scale (gLMS). At lower ethanol concentrations, bitterness is the dominant sensation; at higher concentrations, burn overtakes bitterness as the dominant sensation. 'BD' is barely detectable.

Alcohol intake was variable across study participants

Individuals reported alcoholic beverage usage including, overall frequency of drinking occasions, and occasions drinking beer, wine, straight spirits and mixed spirits. Most individuals reported consuming alcoholic beverages '1 or 2 days per week', with beer and wine consumed '1 day per week', and straight spirits and mixed spirits were consumed 'less than once per month'. See Table 2-2 for a summary of the characteristics of the 96 study participants. Age was not significantly correlated with frequency of overall alcohol intake (transformed) (r = -0.15, p =

0.14) or frequency of intake for any specific type of alcohol beverage (beer r = -0.10; wine r = -0.13; straight spirits r = -0.04; mixed spirits r = -0.04; all p's > 0.1).

Participants were placed into discrete categories based on the number of days they reported consuming an alcoholic beverage in a typical week (see Table 2-2). Three participants reported never drinking alcohol. Thirty-six participants were categorized as 'light' drinkers (consuming an alcoholic beverage less than once per week), 53 classified as 'moderate' drinkers (consuming at least one alcoholic beverage between 1 to 4 days a week), and 4 were classified as 'heavy' drinkers (consuming at least one alcoholic beverage between 5 days a week to everyday). Based on these data, we estimate the modal frequency of drinking days in our cohort is between 53 – 106 days per year (based on 1-2 drinks / week); this level of intake is comparable to national surveillance data from the 1988 National Health Interview Survey (Russell, Light et al., 2004).

Table 2-2: Characteristics of study participants.

	Total (n=96)	Female (n=64)	Male (n=32)
Mean±SEM			
Age	32.7±0.8	32.6±0.9	33.0±1.5
Years drinking	10.5±0.8	9.8±0.9	11.9±1.7
Drinking frequency (%)[a]			
Never	3.2	1.5	6.2
Light	37.5	42.2	28.1
Moderate	55.2	51.6	62.5
Heavy	4.2	4.7	3.1

Drinking frequency groups were defined as follows: never – reported never consuming an alcoholic beverage, light – reported consuming an alcoholic beverage less than once per week, moderate – reported consuming at least one beverage between 1 and 4 days per week, and heavy – reported consuming at least one alcoholic beverage between 5 days a week to every day.
[a] Percentages are reported for each column.

To test for sex effects on intake, a chi square analysis was performed between drinker group and sex; the three individuals who reported never consuming alcohol (2 male and 1 female) were not included in this analysis. Chi-square analysis between drinker group and sex revealed no relationship between intake frequency group (light, moderate, heavy) and sex (X^2 = 1.69, p = 0.43).

Individuals also reported how long they have been regularly drinking alcohol in years: the grand mean was 10.5 years (±0.84) with a median of 8 years, and a range of 0 to 35 years. As would be expected, age was significantly associated with the number of years participants reported regularly consuming alcohol (r = 0.70; p<0.0001). However, by itself, age was not significantly associated with any sensations across any concentrations of sampled ethanol (all p's

> 0.14). Nor did sex associate with the number of years of regularly consuming alcohol ($t(31) = 1.57$, $p = 0.13$).

Reported frequency of drinking occasions associate with chemosensory perception for sampled ethanol

All reported frequency of drinking occasions were quarter-root transformed for improved normality. Simple linear regressions were performed to determine the amount of variance of frequency of intake explained by reported intensity for each chemosensory sensation. Each sensation was explored separately and separate regressions were conducted for each concentration.

For beer, significant associations were observed for the bitterness of 16%, 32% and 48% ethanol (R^2=5.3, 7.0 and 8.0%; p=0.02, 0.009 and 0.005, respectively), along with burning/tingling for 32% ethanol (R^2 =6.6; p=0.01). No sex differences were observed for beer intake ($t(31)=1.01$; $p=0.95$).

Due to the conceptual and statistical redundancy in the univariate regression models above, stepwise regression was used to determine the amount of variance chemosensory ratings (all sensations across all concentrations) might explain variability in the number of drinking occasions, as well as their relative importance in terms of variance explained; sex and age were also included in the stepwise models a priori. Total R^2 and semi-partial correlation coefficients (sr) are reported for the complete model and individual variables, respectively. For frequency of beer drinking occasions, the final model explained 18.08% of the variance in intake frequency ($p<0.001$) with 4 significant predictors: bitterness of 48% ethanol (sr=-0.22), burning of 32% ethanol (sr=-0.17), drying of 8% ethanol (sr=-0.17) and age (sr=+0.14). In summary, fewer

occasions of beer consumption were associated with greater perceived bitterness and burning/tingling, decreased drying perception, and increased age.

Similarly, a multivariate stepwise model ($p=0.019$) explained 12.1% of the variance in drinking occasions of wine via 4 significant predictors: bitterness of 8% ($sr=-0.04$) and 48% ethanol ($sr=-0.22$), and burning/tingling of 8% ethanol ($sr=-0.27$), along with sweetness of 4% ethanol ($sr=+0.07$). Greater sweetness, and less bitterness and burning/tingling were associated with more frequent occasions of wine intake. In the stepwise model for the frequency of consumption of spirits without mixers, two significant predictors explained 7.7% of the variance ($p=0.02$). The final model included sweetness of 48% ethanol ($sr=-0.20$), and burning/tingling of 8% ethanol ($sr=-0.17$), which were both negatively associated with reported drinking occasions of straight spirits. Conversely, chemosensory responses from ethanol did not predict the frequency of consumption of spirits with mixers. Finally, overall alcoholic beverage drinking occasions resulted in a final model comprised of bitterness of 16% ethanol ($sr=-0.25$), the burning/tingling of 16% ethanol ($sr=+0.17$) and age ($sr=+0.17$), with the model explaining 11.09% of the variance ($p=0.012$) in overall drinking occasions. Frequency of alcoholic beverage consumption occurrences was negatively associated with greater perceived bitterness and age, and was positively associated with burning/tingling of ethanol.

Further analyses were conducted to determine the relationship between ethanol perception across concentrations and the total number of drinking occasions based on drinker groups, as described above (i.e. light, moderate and heavy). Separate mixed model ANOVAs were conducted to determine if there was a significant interaction effect between ethanol concentration and drinker group for each sensation (sweetness, bitterness, burning/tingling, drying). There were no significant interactions between drinker groups by concentration for any rated sensations for sampled ethanol.

Years of alcohol use failed to show any association with chemosensory perception of sampled ethanol

To explore potential associations between sensations and history of alcohol use, participants were segmented into two groups using a median split, with individuals reporting 8 or fewer years in the 'less experienced' group (n=49) and 9 or more years in the 'more experienced group' (n=47). In separate mixed model ANOVAs (group by concentration), there was no evidence of a significant interaction between ethanol concentration and group for burning/tingling ratings [$F(4,376) = 1.69$; $p = 0.15$)] or bitterness ratings [$F(4,376) = 1.00$; $p = 0.40$]. Nor did any of the other sensations from ethanol differ across either the groups based on median splits (not shown).

Discussion

This work confirms previous reports showing the intensity of sampled ethanol increases as concentration increases (Green, 1987; Mattes and DiMeglio, 2001). We extend these findings here by quantifying the differences in intensities of sweetness, bitterness, drying and burning/tingling elicited across a wide range of ethanol concentrations within a single group of individuals. At the concentrations presented here, bitterness was the predominate sensation for 4%, 8% and 16% ethanol. At higher concentrations 32% and 48%, ethanol is primarily burning/tingling, followed by bitterness. Notably, sweetness was perceived across all concentrations, but at a much lower intensity (near 'barely detectable'). These results should be used to guide the selection of the appropriate stimulus (i.e., ethanol concentration) for future studies on chemosensation and ethanol intake.

Regarding the varied qualities from ethanol, our data indicate the power functions on the group means for bitterness, dryness, and sweetness have power exponents less than one, suggesting these psychophysical functions are negatively accelerating. Conversely, burning/tingling has a power exponent above one, suggesting it is a positivity accelerating function. Previously, warmth and pain (electric shock) have both been reported as having power exponents greater than 1 (although thermal pain reportedly has a power exponent near 1). To check our result against prior data on ethanol specifically, we extracted group means from Green (Green, 1987), and calculated power functions. Although not directly comparable due to differences in attribute description (perceived irritation versus burning/tingling), delivery method (regional filter paper disks versus whole mouth sip and spit), and scaling method (magnitude estimation versus gLMS), we find that Green's data also shows a power exponent well above 1, at least on the middle of the tongue.

Our participants were asked to rate burning and tingling sensations on the same gLMS scale. These two qualities were combined here in order to minimize participant confusion of these terms, as we assumed naive participants might find it difficult to discriminate these two chemesthetic qualities, especially at low intensities (e.g. Bennett and Hayes, 2012). Although these two qualities may both be perceived via non-taste mechanisms, they are clearly distinct chemesthetic sensations. Thus, combining them together here under one rating scale limits our ability to explore nuanced differences in chemesthetic sub-qualities (e.g. burn, tingling, stinging, etc.). This is a limitation, as these qualities may potentially influence liking and intake of alcoholic beverages differently.

Years of experience drinking alcohol did not associate with any other perceived taste response from sampled ethanol nor frequency of drinking occasions of alcoholic beverages. Variation in chemosensory responses to sampled ethanol significantly explained variability in

drinking occasions for beer, wine, straight spirits, and overall drinking frequency. In general, greater frequency of drinking occasions was associated with decreased bitterness and burning/tingling. Greater sweetness was associated with increased drinking frequency of wine, but was negatively associated with intake of straight spirits. These findings generally support previous findings showing that greater perceived bitterness is associated with decreased intake (Intranuovo and Powers, 1998; Lanier et al., 2005).

Here, we used ethanol in water mixtures to explore associations between perception and intake. It is critical to consider that ethanol is only one of many sensory active components found in alcoholic beverages. While ethanol contributes to the sweetness, bitterness and burning sensations elicited by alcoholic beverages, other components such as hops and tannins on one hand, versus sucrose and glycerol on the other hand, also have the potential to enhance or suppress these sensations. In terms of external validity, sampling different alcoholic beverages rather than ethanol in water may better mimic real-life situations outside the laboratory. Nonetheless, using ethanol in water as a model system helps tease apart the effects of various constituents, and still reveals significant associations between chemosensation and self-reported alcohol behaviors. This suggest at least some of the effects reported previously are likely due to ethanol itself, and not merely other sensory active components like hops or tannins.

Regarding our participant cohort, of 96 participants, 93 reported consuming at least one alcoholic beverage in the past month. This is higher than national norms: in national surveillance data from 2013, ~64 to 69% of adults aged 21 to 34 and ~59 to 61% of adults aged 35-54 reported consuming alcohol in the last month (SAMSHA, 2014). Given our recruitment criteria, this difference is not entirely unexpected. Critically however, our cohort provides sufficient variability in frequency of alcohol intake to allow us to test the hypotheses explored here.

Additional research among alcohol naïve individuals, as well as heavy and binge drinkers is needed to better understand the potential influence of ethanol sensations on alcohol intake.

Present data suggest future work on chemosensation and alcohol use, misuse and abuse should carefully consider the ethanol concentration used within the context of the specific hypothesis being tested. For example, *TAS2R* bitter receptor gene variants fail to explain differential bitterness of, or liking for, sampled blended whisky (Dotson et al., 2012; Duffy, Davidson, et al., 2004; Hayes et al., 2011); present data suggest this may occur because ethanol in this concentration range found in whiskey (40% v/v; 80 proof) is more burning than bitter. Regarding potential health consequences, previous studies show protective effects of *TAS2R* variants on overall alcohol intake (Duffy et al., 2004a; Dotson et al., 2012). That said, such effects may be less robust in situations or cultures where spirits are the main source of alcohol that is consumed, as present data suggest burning is likely a more salient characteristic than bitterness in high ethanol content beverages. In these situations, *TRPV1* may be a more appropriate candidate gene (Allen et al., 2014) than *TAS2Rs*. To date, no reports have attempted to link *TRPV1* variants to differences in alcohol use, misuse or abuse.

Conclusions

Chemosensory responses from ethanol differ greatly by concentration. Not only do intensities range from 'barely detectable' to 'very strong', but also the predominant sensation is concentration dependent. Here, oral chemosensory sensations from ethanol associated with self-reported measures of alcohol use outside the laboratory. Collectively, these findings suggest ethanol tastes, particularly bitterness and sweetness, along with chemesthetic response, differ

greatly depending on concentration and may provide more nuanced insight into preference and intake of alcoholic beverages.

Acknowledgements

The authors would like to thank Rachel Primrose for assistance with data collection, and all of our participants for their time and involvement in the study.

Funding

This work was supported by a National Institutes of Health grant from the National Institute of Deafness and Communication Disorders [DC010904] to JEH, as well as United States Department of Agriculture Hatch Project [PEN04332] funds, and funds from the Pennsylvania State University. AAN received additional support from the National Institutes of Health via an institutional Clinical and Translational Sciences TL1 Predoctoral Fellowship from the National Center for Advancing Translational Sciences [TR000125], and a Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (F31) from the National Institute of Deafness and Communication Disorders [F31DC01465].

CHAPTER 3

Perceptual and affective responses to sampled capsaicin differ by reported intake

Adapted from:

Nolden, A.A. and Hayes, J.E.

"Perceptual and affective responses to sampled capsaicin differ by reported intake."

Food Quality and Preference (2017) 55: 26-34

Abstract

The present study was conducted to a) generate suprathresold dose-response functions for multiple qualities evoked by capsaicin across a wide range of concentrations, and b) revisit how intensity ratings and liking may differ as a function of self-reported intake. Individuals rated eight samples of capsaicin for perceived burn and bitterness, as well as disliking/liking. Measures of reported preference for chili peppers, chili intake frequency, prior experience and personality measures were also assessed. Here, we confirm prior findings showing that burn in the laboratory differs with reported chili intake, with infrequent consumers reporting more burn. We extend these findings by exploring how capsaicin perception varies by reported liking, and measures of variety seeking. We also address the question of whether differences in burn ratings may potentially be an artifact of differential scale usage across groups due to prior experience, and not chronic desensitization, as is typically assumed. By using generalized scaling methods and recalled sensations, we conclude the differences observed here and elsewhere are not likely due to differences in how participants use rating scales.

Introduction

The chili pepper (*Capsicum ssp.* Solanaceae) is widely used as an ingredient in many cuisines around the world (Lembeck, 1986), with consumption frequencies that may exceed once per day. Surprisingly, the etiology of chili pepper preference is still not well understood, despite several decades of study. Different motives and reasons have been proposed to explain the widespread popularity of chili peppers. Some researchers have speculated their wide use may be due to the biological or pharmacological properties of capsaicin (i.e. anti-bacterial properties, or gustatory sweating) (Abdel-Salam, 2016; Lee, 1954). Other factors that have been identified include culture (Abdel-Salam, 2016), personality traits (Byrnes and Hayes, 2013; Byrnes and Hayes, 2015; Rozin, 1980) and gender (or masochism) (Bègue et al., 2015; Byrnes and Hayes, 2015; Rozin, 1980). While the relative weight of these reasons as drivers of consumption remains unclear, it is well understood that chilies elicit a burning sensation. This burn, in the mouth and elsewhere on the body, is primarily due to capsaicin (PubChem CID: 1548943) and dihydrocapsaicin (PubChem CID: 107982), the two main capsaicinoids found in chili peppers. These compounds are potent agonists of the heat pain receptor TRPV1.

The term chemesthesis was originally coined to describe touch and pain sensations that are initiated by chemical stimuli (Green, 2016). Examples of oral chemesthesis include tingling, buzzing, cooling, and warming. These sensations are clearly distinct from classical taste sensations (i.e. sweet, sour, salty, bitter, and umami) (Green, 1996a). In regard to oral sensation, capsaicin is one of, if not the most, systematically investigated chemesthetic stimulus (e.g., (Green, 1991b; Green and Hayes, 2003; Green and Hayes, 2004; Lawless et al., 1985; Prescott and Stevenson, 1995)).

Despite decades of research investigating the oral burn evoked by capsaicin, response to capsaicin across a wide range of concentrations has not been evaluated in a large group of untrained participants using modern scaling psychophysical methods. Within the psychophysical literature, varied concentrations of capsaicin have been used in many previous studies; unfortunately, it is not possible to extract a single suprathreshold dose response function from these reports due to different delivery systems (liquid solution, cotton swab, filter paper, etc.), type of exposure (sip and spit, sip and swallow, regional application, etc.) and characteristics of the task given to study participants (different scales, or different descriptors such as 'overall sensation', 'irritation', 'pepper heat', 'burn', etc.). To identify appropriate doses for use in subsequent experiments in our laboratory, we desired such a function.

One conventional method for estimating perceived heat from chilies is the Scoville Test, which generates an estimate of perceived intensity in units known as Scoville Heat Units (SHU) (Scoville, 1912). However, due to methodological problems with the classical Scoville Test (see (Gillette, Appel et al., 1984; Govindarajan, Shanthi et al., 1977; Todd, Bensinger et al., 1977), efforts have been made to improve the method of estimating the burn produced by chili peppers and capsaicinoids. Because there is a simple ordered relationship between perceived burn and capsaicinoid concentration, instrumental methods using high performance liquid chromatography-mass spectrometry (HPLC-MS) and gas chromatograph-mass spectrometry (GC-MS) to determine the capsaicinoid content in chili peppers and chili pepper containing foods have been developed (e.g. (Gillette et al., 1984; Othman, Ahmed et al., 2011; Peña-Alvarez, Ramírez-Maya et al., 2009; Todd et al., 1977; Welch, Regalado et al., 2014). These instrumental methods, which have been validated with human sensory data, are often used as a standard method to estimate heat from various foods or ingredients. One example is work by Gillette and colleagues (1984), who used a trained panel (n=10) with fixed references for 'slight', 'moderate',

and ‘approaching strong’ stimuli to estimate a dose response function for n-vanillyl-n-nonamide, a synthetic capsaicin analog, as well as extracts of ground peppers (chilies); however capsaicin itself was not included in their report. Their report later inspired two standard methods from the American Society for Testing Materials (e.g., ASTM E1083-00 and E1396-90), but again, these methods were based on trained panels using fixed intensity references. Additionally, many prior studies in this area have focused solely on burn, irritation or bite; however, capsaicin is known to elicit bitterness in addition to burning in some individuals (e.g. Green and Hayes, 2003; Nolden, McGeary et al., 2016). Accordingly, we chose to address this specific gap in the literature by obtaining intensity estimates for multiple qualities across a wide range of capsaicin concentrations, similar to recent work conducted on ethanol (Nolden and Hayes, 2015).

Greater liking or frequency of chili pepper consumption has been associated with reductions in the reported burn of sampled capsaicin (Cowart, 1987; Lawless et al., 1985; Prescott and Stevenson, 1995; Stevenson and Yeomans, 1993). Based on these data, it widely assumed that regular consumption of chili pepper results in chronic capsaicin desensitization, based on observations that desensitization can occur with exposure in the laboratory and can last over days (reviewed in Hayes, 2016). However, Stevenson and Prescott put forth an alternative explanation that remains untested; namely, observed differences between intake groups may be due to prior experience that influences scale usage, rather than true desensitization (Stevenson and Prescott, 1994). This hypothesis suggests that individuals who frequently consume chili peppers have a larger frame of reference outside of the laboratory regarding chili burn compared to those who do not eat chili peppers regularly; thus, when given the same stimuli in the laboratory, frequent consumers use the scale differently, and rate the stimuli as less intense. It remains untested whether differences in capsaicin responses (i.e., perceived burn) across chili pepper intake groups

are a result of desensitization due to repeated dietary exposure or merely due to prior context that alters use of the rating scale.

The primary aims of the present study were to a) generate a dose-response curve for capsaicin over a wide concentration range using untrained participants without fixed references, and b) reevaluate associations between perceived burn, bitterness and liking of sample capsaicin and chili pepper consumption groups, and investigate whether this relationship is due to diet-induced desensitization or possible context effects. As secondary aims, we also explored the relationship between sampled capsaicin and a trait-based measure of food adventurousness, operationalized via the VARSEEK scale. Here, individuals evaluated eight samples of capsaicin for their bitterness and burning intensity, along with liking/disliking. They also answered questions regarding chili pepper preferences, intake frequency, prior experience, and personality. This study confirms prior work, and extends current knowledge regarding capsaicin perception.

Materials and Methods

Participants

Adults were recruited from The Pennsylvania State University and surrounding community to participate in two 30 minute visits that were scheduled one week apart at the Sensory Evaluation Center at Penn State. Interested individuals completed a brief online questionnaire to see if they met the following study criteria: not pregnant nor breast feeding, non-smoker, no tongue, cheek or lip piercing, no difficulty swallowing or history of choking, no known taste or smell defect, not taking prescription pain medication, no hyperactive thyroid and no history of chronic pain. Individuals meeting these criteria answered additional questions

regarding their liking and intake of foods containing chili peppers. Recruitment was stratified by gender and by liking and intake of chili peppers. These groups included no/low, medium, and high liking, and intake of chili peppers. Participants' self-reported liking of spicy foods, and frequency of intake for a variety of foods containing chili pepper were used to bin participants into groups. At the end of the study, 82 participants (34 men) had completed both sessions, with an average age of 32 (± 0.9) years. A majority of participants reported Caucasian ancestry (n=72), with low representation from Asian (n=7) and Black (n=2) individuals; one individual chose not to disclose ancestry. Procedures were IRB approved, informed consent was obtained, and participants were compensated for their time with a small cash payment.

Stimuli and sampling procedure

Sampled stimuli included 0.11, 0.275, 0.55, 1.1, 2.75, 5.5, 11 and 22 ppm natural capsaicin from Sigma-Aldrich (Sigma #360376). This natural product actually contains a mix of capsaicin and dihydrocapsaicin (~65% / ~35%, respectively, with small variations from lot to lot), but due to their very similar potency, and Sigma's nominal branding as capsaicin, it will be referred to simply as capsaicin for the remainder of the document. In each visit, participants sampled 4 different concentrations of capsaicin, with each participant rating all 8 concentrations across the two visits. Sample sets were counterbalanced across participants, and presented in increasing and alternating order, with two possible orders (0.11, 0.55, 2.75, and 11; and 0.275, 1.1, 5.5 and 22 ppm). This order was chosen both to limit simple carry-over (by presenting lower concentrations first, as is commonly done in threshold testing), and to reduce the potential for sensitization. Prior data (Green, 1991) indicates greater sensitization occurs following higher concentrations (30 ppm) relative to lower concentrations (3 ppm), so the presentation order used

here should minimize sensitization, as the highest stimulus is presented last. Further considerations regarding sensitization are discussed in more detail below.

All stimuli were made from a single stock solution where capsaicin was dissolved in 95% USP grade ethanol. This stock was diluted with reverse osmosis (RO) water to reach the final concentrations, and supplemented with ethanol to standardize all stimuli to equal ethanol concentration of 0.1% (v/v). All stimuli were presented at room temperature in 10mL aliquots in plastic medicine cups labeled with a random three-digit blinding code.

Prior to tasting any stimuli, participants rinsed with room temperature RO water. Participants were instructed to place the entire stimuli in their mouth, swish for 10 seconds, spit it out, and wait 10 seconds before making their ratings. There was a minimum 2.5-minute break between each stimulus, where participants were asked to rinse with water until they no longer perceived any sensation in their mouth; this break was enforced via software. Repeated exposure to capsaicin within a single session can result in sensitization or desensitization depending on the interstimulus interval (ISI) that is used: ISIs of 1.5 minutes or less result in sensitization while ISIs of ~3.5-5.5 min result in desensitization (Green, 1989). To limit this effect a break of 2.5 minutes was selected to be between the times thought to result in sensitization (<1.5 minutes) and desensitization 3.5 to 5.5 minutes {Green, 1989; 1991b; Green and Rentmeister-Bryant, 1998). Here, an initial ISI of 2.5 minutes was used, as prior work with whole mouth stimuli suggests this interval does not result in sensitization or desensitization (Nasrawi and Pangborn 1990). After the 2.5-minute break, participants rated the intensity of any sensation they were still experiencing (i.e., any residual burn). If ratings were greater than 1.4 (‘barely detectable’), then participants waited another minute before continuing on to the next stimuli. Participants were not made aware of the minimum rating required to skip over the additional minute. Therefore, it is very unlikely participants clicked ‘no sensation’ to avoid the additional wait time and finish sooner. This step

was included as a secondary means to limit any carryover effects that might inflate subsequent ratings. However, it should be noted that this choice is also a potential limitation, as changing the ISI might alter the relative occurrence of sensitization versus desensitization. In summary, we cannot know with certainly whether participants might have experienced sensitization or desensitization for subsequent capsaicin stimuli within a session; however, we believe the design tradeoffs made here represent a reasonable attempt to efficiently manage competing concerns about sensitization, desensitization and carry-over effects.

Psychophysical Scaling and Practice/warm-up for scales

Participants rated the burning and bitterness of capsaicin and other non-sample related items on a horizontal general labeled magnitude scale (gLMS). On the left side, the scale is labeled at 0 with ‘’NS’ (no sensation) and on the right labeled ‘the strongest imaginable sensation of any kind’ at 100. Labels were placed at 1.4, 6, 17, 35 and 51 (‘BD’; barely detectable, ‘weak’, ‘moderate’, ‘strong’ and ‘very strong’; respectively). Participants were instructed to not let whether or not they liked or disliked each stimuli influence his or her ratings for intensity. They indicated their affective responses on a generalized bipolar hedonic scale for sampled capsaicin and other non-sampled items (see below). This scale ranges from ‘strongest disliking of any kind’ at -100 on the left to ‘the strongest liking of any kind’ at 100 on the right, with ‘neutral’ at 0 at the center point (Byrnes and Hayes, 2013).

Prior to rating any samples, participants were given written instructions on the use of the gLMS (Snyder, Prescott et al., 2006) and rated 15 remembered or imagined sensations (e.g. Hayes, Allen, et al., 2013). Similarly, participants received instructions for the use of the hedonic scale, followed by a practice session where participants rated 8 items. These items, both for the

gLMS and generalized hedonic scale orientation included food and non-food items in order to emphasize that the scales were to be used in context to all sensations.

Measures of liking and intake of foods containing chili peppers

Affective responses were also collected for 38 items that were not tasted in the laboratory. These items included 15 spicy foods, 14 non-spicy foods and 9 non-food related items. Participants also rated several remembered intensities on a gLMS, including the intensity for the spiciest meal or food they could remember experiencing, and the remembered burn from commercially available hot, medium and mild salsa. They reported intake frequency for a variety of foods, which included questions related to chili peppers and chili pepper containing foods as well as non-spicy foods that were included as controls. Specific wording was as follows: 'How often do you consume … [hot sauce; chili peppers; habanero peppers; red pepper flakes; spice mix containing chilies; fried foods; sweet snacks (candy, chocolate, baked goods); salty snacks (pretzels, potato chips, popcorn); ice cream or frozen yogurt]?' Participants selected either: never, less than once/month, 1-3 times/month, 1-2/week, 3-4/week, 5-6/week, once/day or 2 or more times/day. Stated preference of spicy food was measured by asking participants to report their preferred heat/spice level when ordering food at a restaurant by selecting either: 'I avoid eating spicy foods', 'mild', 'medium', 'spicy' and 'very spicy'. Participants' motives for either consuming or avoiding consuming chili peppers were also obtained (Table 3-1). Lastly, participants were asked to rate 'How much do you like the burn of chili pepper in your food' and 'How much do you like the taste of chili pepper in your food' on a 7-point hedonic scale, ranging from dislike extremely, to like extremely.

Table 3-1: Frequency table for participants responses to both why they do not like or like spicy food.

If you don't like spicy foods, why? \ *If you like spicy foods, why?*	I like the burn/heat	I like the taste	For health reasons	It's what my family eats	I avoid eating spicy foods	**Total**
I like spicy foods	11	33	3	1	0	48
They are too hot	0	11	0	2	10	23
I don't like the taste	0	0	0	1	2	3
I don't feel well when I eat them	0	4	0	1	1	6
Avoid for other reasons	0	1	0	0	1	2
Total	11	49	3	5	14	82

Personality measures

To measure participants desire to seek a variety of foods, they endorsed a subset of statements from the Variety Seeking Tendency Scale (VARSEEK). Participants indicated how much they agreed, or disagreed with each statement (completely disagree, disagree, neither disagree nor agree, agree, or completely agree), and these were coded with values from 0 to 4. Answers were summed to give an overall score of variety seeking, to estimate participants' willingness to try new or unusual foods. A total of 6 statements (of 8) were provided, so potential

scores here ranged from 0 to 24, allowing us to differentiate between non-adventurous and very adventurous consumers.

Statistical analysis

Prior to subsequent analyses, the self-reported food intake was annualized to express estimated consumption frequency on a yearly basis (e.g. less than once per month = 6, one to three times per month = 24, one to two times per week = 62.4, etc.). Sex was coded as 0 for women and 1 for men.

All analyses were conducted using SAS 9.2 (Cary, NC). Analysis of variance (ANOVA) was conducted on annualized intake frequency data to determine if intake significantly differed by sex. A Cochran-Mantel-Haenszel (CMH) test was conducted to determine the relationship between reported preference of spice/heat level (mild, medium, spicy) and sex. Pearson correlation coefficients were conducted via *proc corr* and regression was conducted via *proc reg*. Repeated measures ANOVA were conducted via *proc mixed* to determine associations between groups (e.g. intake and spice preference groups) with bitterness, burning and liking/disliking ratings of sampled capsaicin. Group differences at each concentration were tested using the 'slice' option in SAS. Comparison of slopes from regression models was evaluated between non-normalized and normalized data using *proc glm*.

Results

Variability in liking and intake of spicy foods among participants

Participants were asked a series of questions regarding intake frequency of chili peppers and foods containing chili pepper, in addition to reporting their stated preference of spiciness/hotness level in their food, and how much they like the burn and taste of chili peppers. Intake frequencies were annualized for chili peppers, sweet snacks, salty snacks, fried foods, and ice cream/frozen yogurt; these are summarized in Table 3-2. There was no evidence of a significant difference between men and women for intake frequency for any of the food items (p's>0.05), and the VARSEEK scores (Table 3-2) did not significantly differ between men and women (t=1.17; p=0.6). However, preferred spice level (no heat/avoid, mild, medium, spicy, very spicy) significantly differed by gender (CMH X^2=12.6; p=0.01), with men reporting preferring a higher spice level than women.

Participants were also asked to answer questions regarding why they do, or do not consume spicy foods (Table 3-1). Overall, participants reported they ate spicy foods because they 'liked the way it tasted' (n=49), followed by 'liking the burn' (n=11), for their 'health benefits' (n=3) and because 'it is what their family eats' (n=5). Conversely, of participants reporting not liking spicy foods, reasons given were that they: 'are too hot' (n=23), 'don't feel well when they eat them' (n=6), 'don't like the taste' (n=3), or other reasons (n=2).

In the questionnaire, participants rated how much they liked the burn of chili peppers in their food, followed by how much they liked the taste of chili peppers in their food. Reported liking of both burn and taste were then used in separate regression models to predict annualized intake frequency of chili peppers. Chili pepper intake was estimated by taking the sum of

annualized intake of hot sauce, habanero peppers, chili peppers, red pepper flakes, and spice mix containing chilies. This summed measure of chili pepper intake was significantly correlated with both the reported liking of burn (r=0.37; p=0.0005) and 'taste' (r=0.37; p=0.0005) of chili peppers, consistent with prior work (Byrnes and Hayes 2015, 2016). A stepwise regression with both variables (liking of taste and burn) resulted in 'taste' ceasing to be a significant predictor of intake when burn was included in the model, suggesting these are largely redundant measures. Reported liking of the burn of chili peppers explained 14.3% of the variability in annual intake frequency of chili peppers.

Psychophysical response to sampled capsaicin

Participants rated both burn and bitterness for all concentrations of capsaicin. As expected, intensity ratings for each increased with increasing concentrations. As expected, burn response increased at a faster rate than bitterness (Figure 3-1). As shown in Figure 3-1, separate linear models were fit to model the mean perceived intensity ratings after $\log_{10}$ transforming the capsaicin concentrations:

Burn = 23+21.5 [log ppm] (R^2=0.99) and, bitter =7.9+3.3 5 [log ppm].

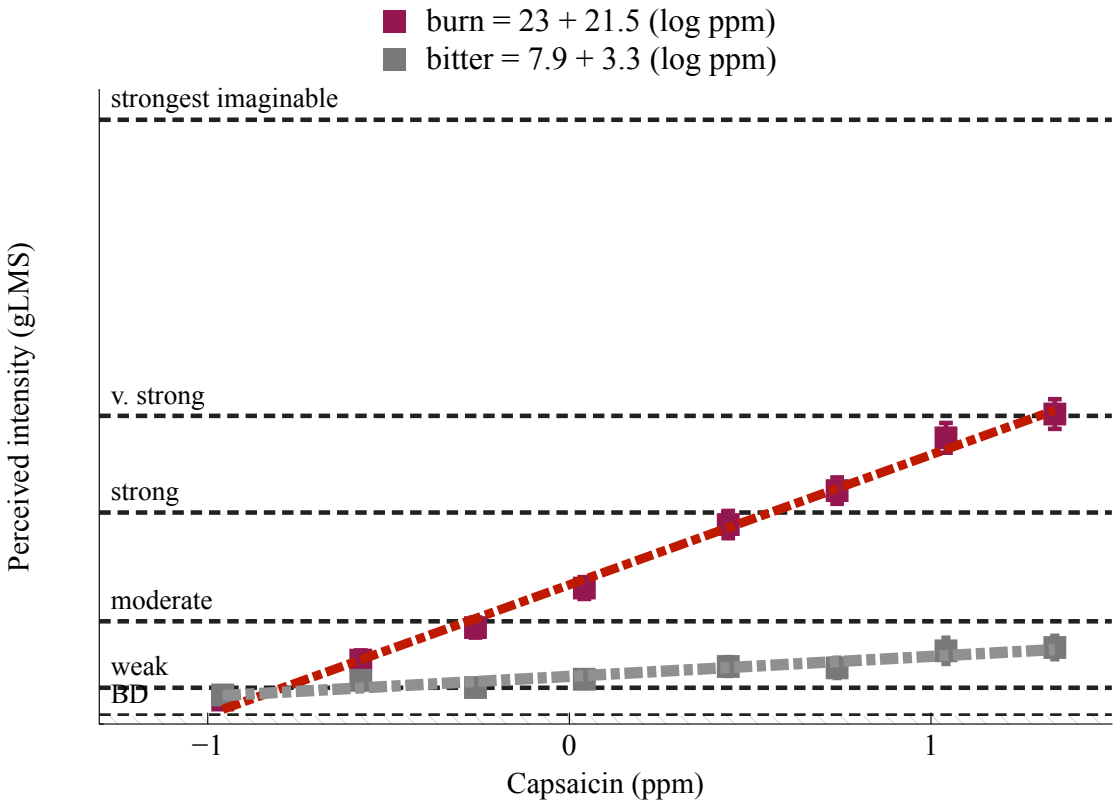

Figure 3-1: Group means and standard errors for bitterness and burning ratings are shown for 8 different concentrations of capsaicin (0.11, 0.275, 0.55, 1.1, 2.75, 5.5, 11 and 22 ppm). The x and y axes are plotted as linear scales, but the capsaicin concentrations (in ppm) were log10 transformed prior to plotting to facilitate fitting a simple linear equation, resulting in a semi-log plot.

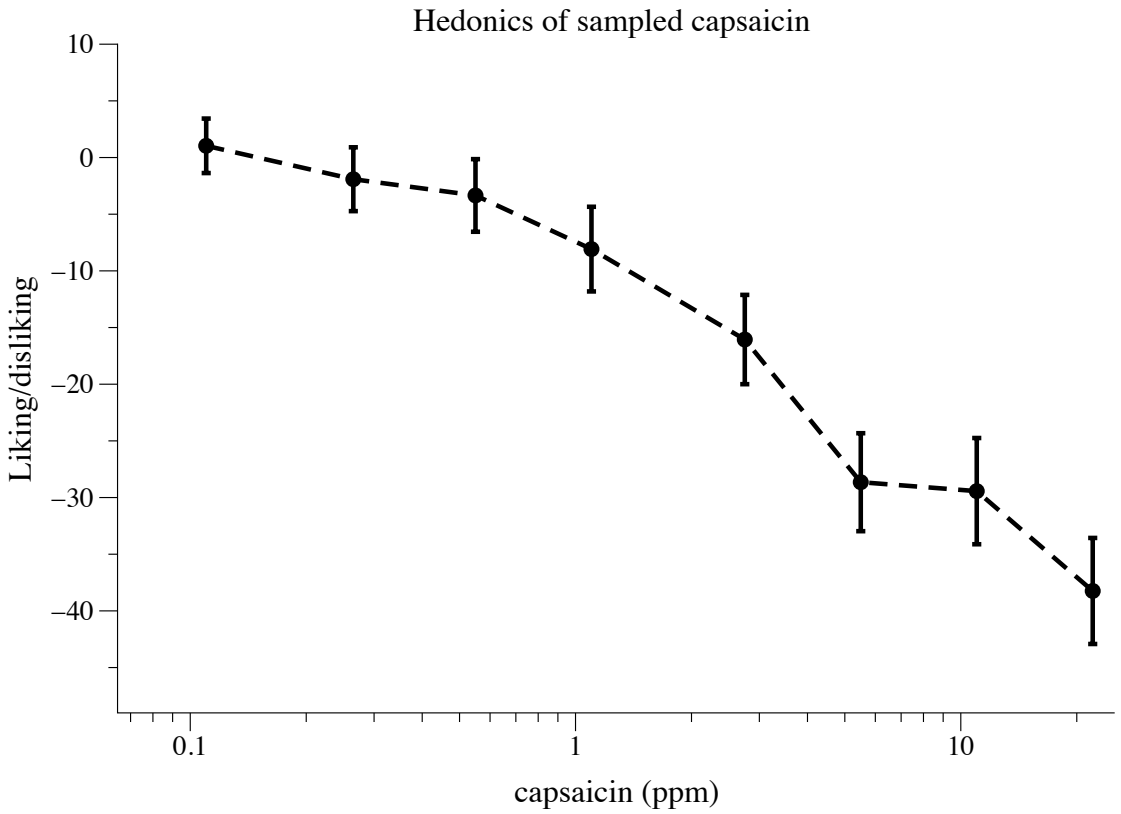

Figure 3-2: Group means and standard errors for liking/disliking ratings are shown for 8 different concentrations of capsaicin (0.11, 0.275, 0.55, 1.1, 2.75, 5.5, 11 and 22 ppm). The y-axis is linear, and the x axis is logarithmic, resulting in a semi-log plot.

As anticipated, hedonic ratings for sampled capsaicin decreased as concentrations increased, as shown in Figure 3-2. At the lowest concentration given, 0.11ppm, capsaicin was rated just above neutral, suggesting some participants found the sample pleasant. In fact, a sizeable fraction of participants rated at least some of the capsaicin concentrations between 'neutral' and 'strongest liking of any kind'. Frequency counts for participants rating above neutral were 27, 29, 42, 39, 24,16,15 and 14, for 0.11, 0.275, 0.55, 1.1, 2.75, 5.5, 11 and 22 ppm capsaicin, respectively. While none of these counts exceed the significance threshold for a

binomial preference test (51 of 82, at alpha = 0.05), this highlights that some participants liked the burn, despite the overall downward trend seen in Figure 3-2.

Notably, the intensity ratings of burn and bitterness did not show a large change in intensity between 11 and 22 ppm, yet mean hedonic ratings for 11 and 22 ppm continued to drop from -29.4±4.6 to -38.6±4.7. To determine if bitterness and burning responses were associated with liking ratings, we explored correlations between each sensation across the different concentrations of capsaicin. Bitterness ratings were significantly associated with liking at the lowest capsaicin concentration ($r = -0.34$; $p=0.0016$), but bitterness was not correlated with liking for any other concentrations ($p's>0.4$). Burning response was significantly correlated with liking for 2.75, 5.5, and 11 ppm ($r = -0.32$, -0.36 and -0.35; $p's<0.003$, respectively). No significant correlations between burn response and liking were observed for any other concentrations ($p's>0.07$).

Table 3-2: Characteristics of study participants.

	All Participants	Females	Males
(n)	82	48	34
Age (years)	32.1±0.9	32.9±1.3	31.0±1.2
Preferred spice level*	2.1±0.1	1.8±0.1	2.4±0.2
VARSEEK score	16.4±0.5	16.4±0.6	16.5±0.7
Frequency of consumption on a yearly basis			
hot sauce	55.5±11.1	44.2±9.7	71.4±23.1
chili peppers	42.0±12.5	34.2±15.0	53.0±21.7
habanero peppers	11.3±3.6	8.2±1.7	15.8±8.3
red pepper flakes	50.9±8.0	39.4±8.1	67.3±15.2
spice mix containing chilies	43.1±9.7	46.8±15.2	38.0±9.4
sweet snacks	185.6±20.0	199.6±28.6	165.8±26.6
salty snacks	195.6±18.9	213.3±27.0	170.5±24.6
fried foods	50.5±5.3	52.2±7.7	48.1±6.9
Ice cream/frozen yogurt	71.2±11.4	68.7±15.6	74.8±16.5

* Indicates significant differences in intake between males and females ($p<0.05$).

Self-reported intake of chili peppers is associated with capsaicin perception

Based on the summed measure of chili pepper intake (using the various annualized intake measures, as described above), participants were segmented into low and high consumers of chili peppers using on a median split of 103.2 times per year. The mean burn ratings of sampled capsaicin in these two groups are shown in Figure 3-3. Separate repeated measures ANOVAs were used to determine whether intake group (high versus low) associated with the perceived burn, bitterness and disliking/liking of the sampled capsaicin.

For burn, concentration [$F(7,560)=178.0$; $p<0.0001$], intake group [$F(1,80)=9.2$;$p=0.003$] and the interaction (concentration by intake group) [$F(7,560)=3.5$; $p=0.001$] were significant. Significant differences between intake groups were observed for the 1.1, 5.5, 11, and 22 ppm capsaicin samples (Figure 3-3). For bitterness, there was a significant main effect of concentration [$F(7,560)=6.35$; $p<0.0001$], but bitterness did not differ by intake group [$F(1,80)=1.68$ $p=0.2$], and there was no evidence of an interaction [$F(7,560)=1.19$; $p=0.3$].

Capsaicin concentration was significantly associated with hedonic ratings of sampled capsaicin [$F(7,560)=24.0$;$p<0.0001$], as shown in Figure 3-4. Also, the main effect of intake group [$F(1,80)=4.9$;$p=0.03$] and the intake group by concentration interaction [$F(7,560)=2.9$;$p=0.005$], were significant for the disliking/liking ratings. Decomposition of significant effects via the slice option in SAS revealed significant differences at 2.75, 5.5, 11, and 22 ppm capsaicin, as shown in Figure 3-4.

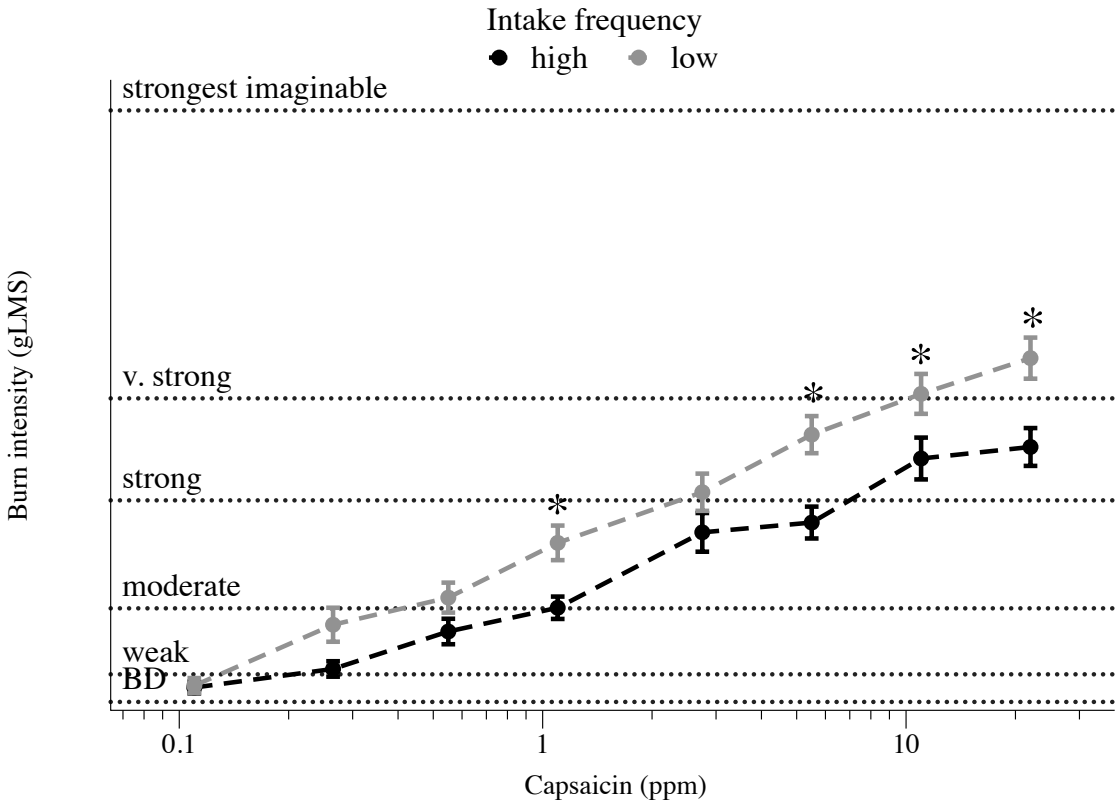

Figure 3-3: Group means and standard errors for burn ratings segmented by frequency of chili pepper consumption. The groups were formed using a median split of the summed annualized frequency for 5 different questions regarding pepper intake (hot sauce, chili peppers, habanero peppers, red pepper flakes, and spice mix containing chilies). * Denotes significant differences ($p<0.05$). See text for p-values.

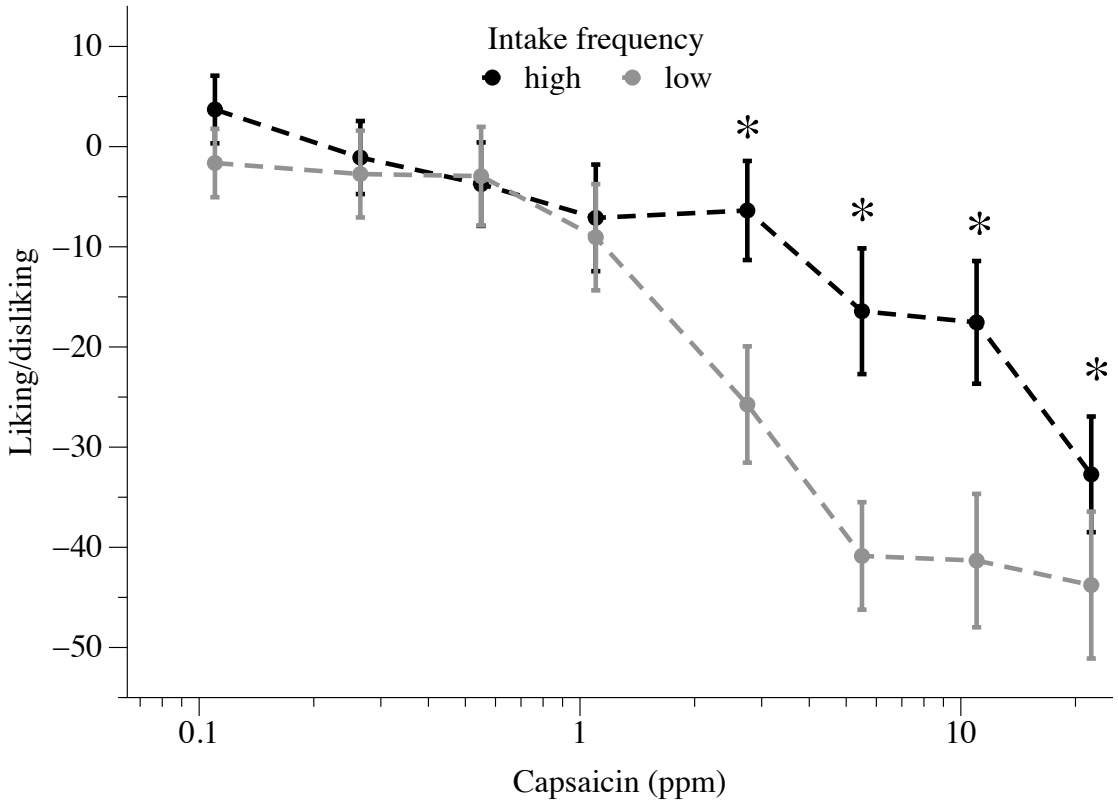

Figure 3-4: Group means and standard errors for hedonic ratings, segmented by intake frequency. See text for details of the ANOVA main effects and interactions. * Indicates significant differences ($p<0.05$) between groups at that concentration.

Self-reported preference for spice level is associated with capsaicin perception

Participants were asked to state their preference for spice/heat in their food using one of five categories: 'no heat/avoid', 'mild', 'medium', 'spicy', 'very spicy'. Given endorsements of 4, 23, 27, 20, and 8, respectively, we collapsed the bottom two categories, and top two categories, resulting in 3 groups of roughly equal size for further analysis (n's of 27, 27, and 28, respectively). Figure 3-5 summarizes the mean burn ratings of sampled capsaicin segmented by

preferred spice level. As above, separate repeated measures ANOVA models were created to test effects of capsaicin concentrations and preferred spice level on burn, bitterness and liking/disliking ratings. In all three models, concentration was significantly associated with liking ($p's<0.0001$), as would be expected. Preferred spice/heat level was significantly associated with burn ratings [$F(2,79)=3.8$; $p=0.02$], with the top group (spicy and very spicy) reporting the lowest burn (Figure 3-5). There was no evidence of an interaction between preference group by concentration [$F(14,553)=1.2$; $p=0.2$], suggesting that the effect of group was uniform over the entire concentration range. Given the significant main effect, we compared differences at each concentration, and significant differences were observed at 1.1, 5.5, 11 and 22 ppm, with participants in the spicy/very spicy group (n=28) reporting lower burn compared to the other two groups. Notably, the no heat/avoid/mild group (n=27) and the medium group (n=27) did not differ from each other. We did not observe any relationship between preferred spicy/heat level and rated bitterness from sampled capsaicin [$F(14,553)$; $p=0.9$).

As would be expected, stated preference for spice/heat was associated with hedonic ratings of sampled capsaicin [$F(2,79)=11.3$; $p<0.0001$], and there was evidence of a preference level by concentration interaction [$F(14,553)=2.2$; $p=0.007$], indicating the magnitude of the differences in liking between the groups changed in size across concentration. This can be seen in Figure 3-6, where the gap between groups increases as concentration increases, and significant differences were observed at the higher capsaicin concentrations.

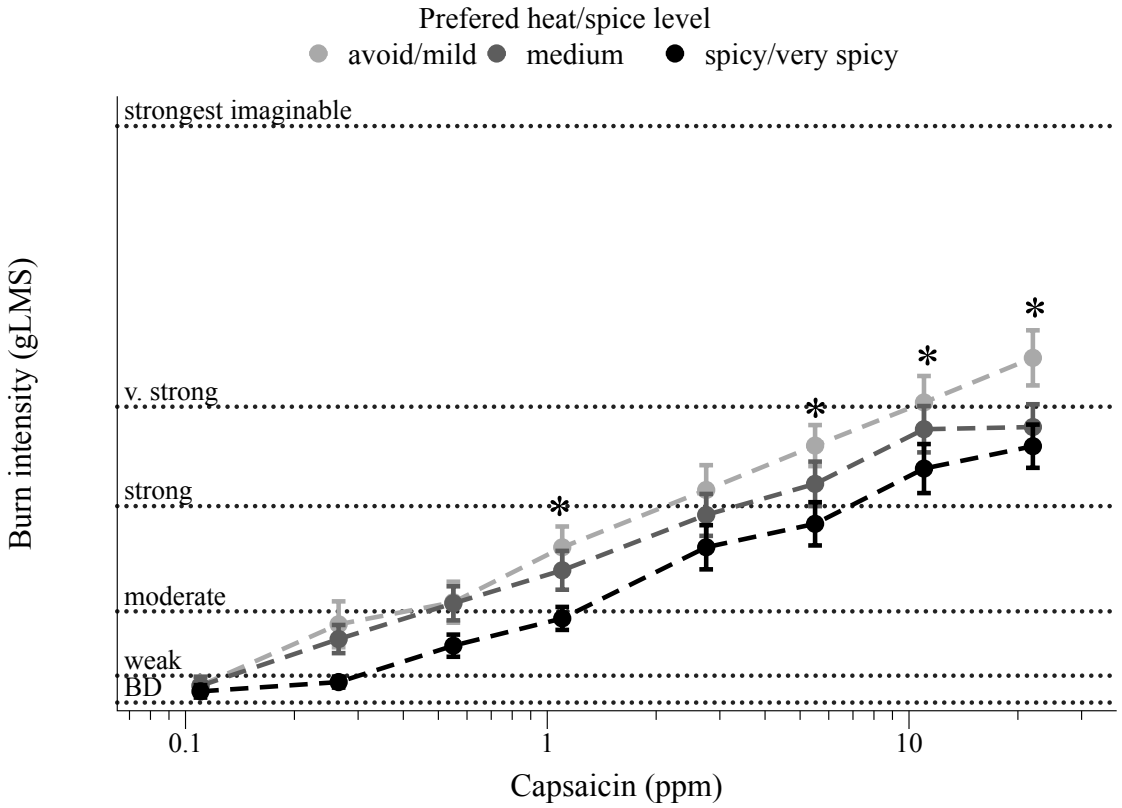

Figure 3-5: Group means and standard errors for burn ratings are segmented by stated (declared) preference for heat/spice level when ordering food at a restaurant: avoid/mild, medium, and spicy/very spicy. * Indicates significant differences ($p<0.05$) between groups at that concentration.

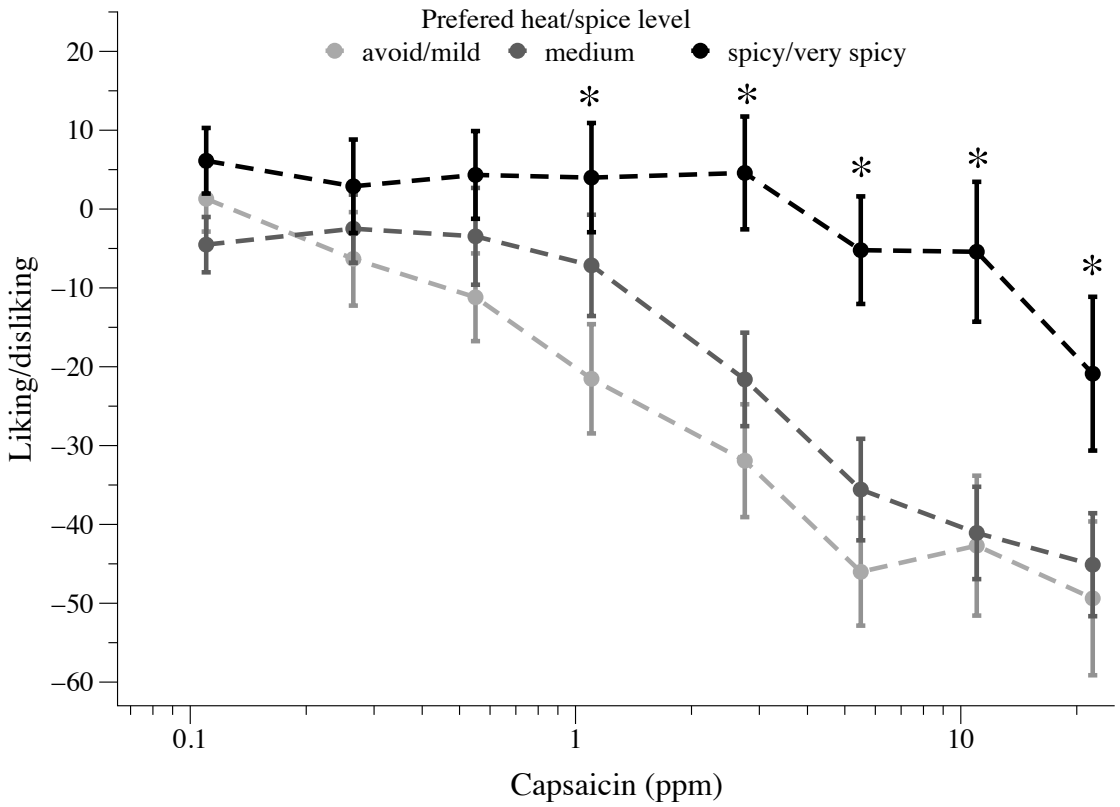

Figure 3-6: Group means and standard errors for hedonic ratings, segmented by stated preference group. See text for details of the ANOVA main effects and interactions. * Indicates significant differences ($p<0.05$) between groups at that concentration.

Variability in variety seeking measures (VARSEEK) is associated with capsaicin perception, liking and intake

Participants indicated their agreement/disagreement with 6 statements from the VARSEEK (see methods). These endorsements were used to generate a score indicating a participant's willingness to try new foods and overall adventurousness in their diet. From these scores, participants were placed into either a non-adventurous or adventurous group using a median split of the overall sum (median=17). Once again, repeated measures ANOVA models were used across all sampled capsaicin concentrations to determine if group (non-adventurous

versus adventurous) associated with burning, bitterness and liking/disliking ratings of sampled capsaicin. Unlike the intake group data above, we did not see any effect of food adventurousness (i.e., personality) on burn [$F(1,80)=0.75$; $p=0.3$]; nor, was there an association between food adventurousness and bitterness [$F(1,80)=0.4$; $p=0.5$]. In contrast, hedonic ratings of sampled capsaicin associated with food adventurousness group [$F(1,80)=3.7$; $p=0.05$], and the group by concentration interaction was significant [$F(7,560)=2.7$; $p=0.007$]. Further analysis revealed the group differences were larger at higher concentrations, as shown in Figure 3-7.

We also tested food adventurousness/variety seeking against reported intake of chili peppers and state preference for spice levels and reported intake of chili peppers. There was a trend of reported frequency of combined chili pepper intake to be associated with individual VARSEEK scores [$F(1,80)=3.2$; $p=0.07$]. Similarly, reported spice preference was significantly associated with variety seeking group ($X^2(2,n=82)=9.9$; $p=0.007$), with greater frequency of adventurous consumers stating a preference for spicy or very spicy heat level in their food.

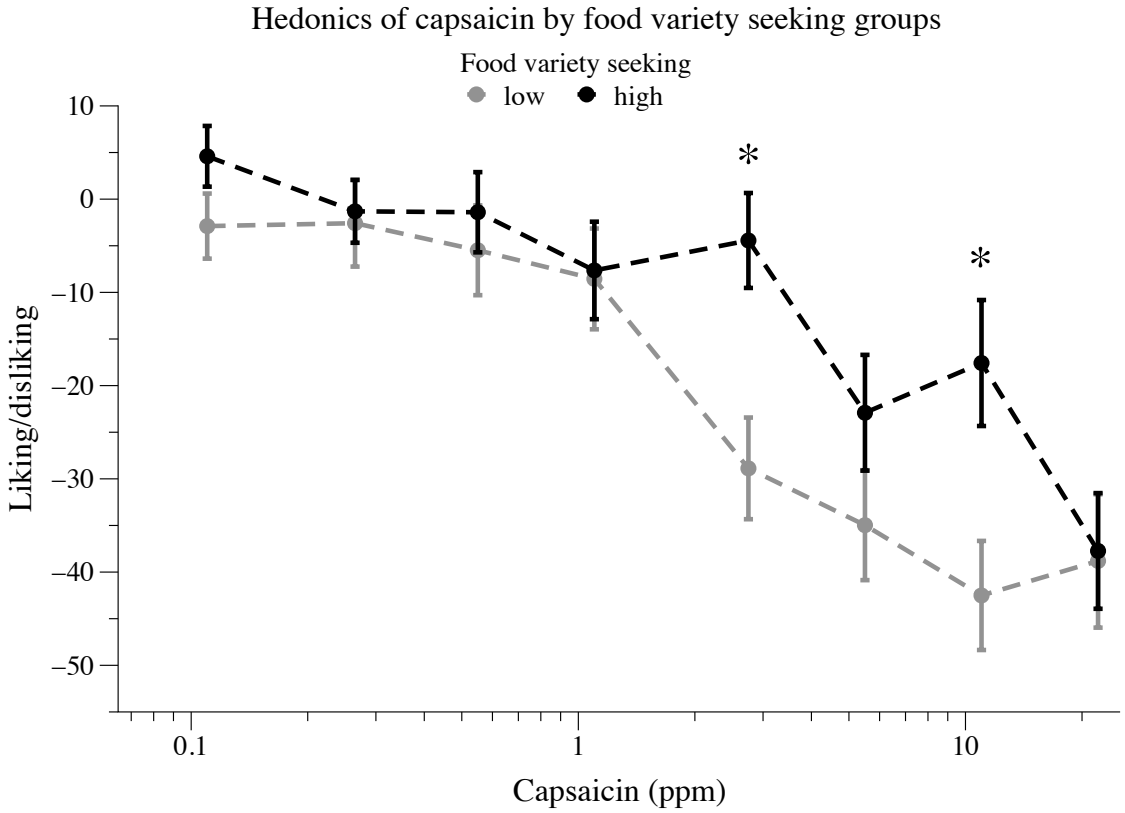

Figure 3-7: Group means and standard errors for hedonic ratings, segmented by VARSEEK scores. Groups were generated via median split of VARSEEK scores, a measure of food variety seeking and food adventurousness. See text for details of the ANOVA main effects and interactions. Stars indicate significant differences ($p<0.05$) between groups at that concentration.

Investigating context effects: exploring associations between capsaicin perception and chili pepper intake with prior experiences

Previously, Stevenson and Prescott speculated that participants who consume chili peppers frequently potentially have a broader context of burn intensity compared to individuals whom choose to avoid chili peppers. In an attempt to determine whether or not participant's prior experience influenced his or her ratings, we also asked participants to rate the remembered intensity for 'commercially available… [mild], [medium] and [hot] salsa', as well as the 'spiciest

meal they have ever eaten' on a gLMS. Mean ratings of remembered intensity of the three salsas and spiciest ever are shown in Figure 3-8, segmented by the same intake groups as used in Figure 3-3. In repeated measures ANOVA on ratings of remembered intensity of mild, medium and hot salsa, the main effect of intake group was significant [$F(1,80)=8.6$; $p=0.004$], with the low intake group reporting more burn (Figure 3-8). Also, the salsa type by intake group interaction was significant [$F(2,160)=3.1$; $p=0.04$]. In individual pairwise comparisons for each salsa level, significant differences were observed for mild and hot salsa ($p=0.02$ and 0.0003, respectively), with medium salsa showing a similar trend ($p=0.054$). Figure 3-8 also shows the group means for 'spiciest meal ever experienced'; these data were analyzed separately via an independent sample t-test, and in sharp contrast to the salsa data, there was no evidence of an effect of group ($t=1.3$; $p=0.3$).

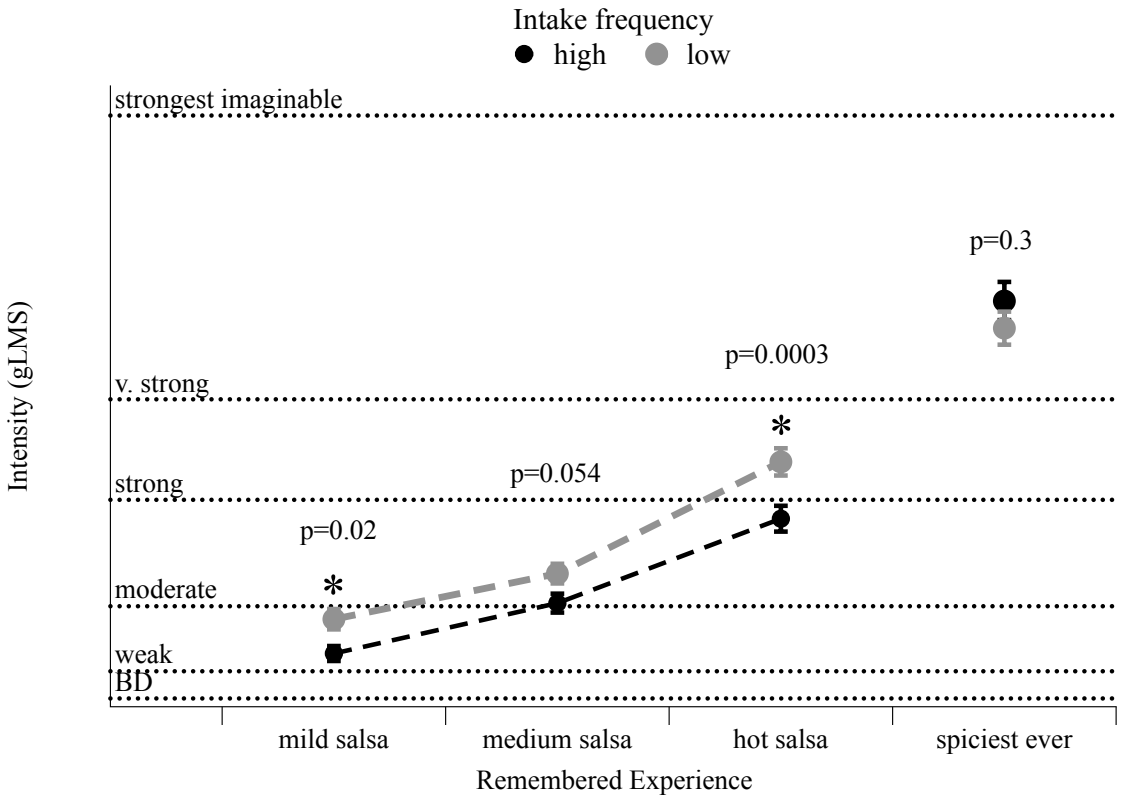

Figure 3-8: Participants rated the remembered intensity of mild, medium, and hot salsa, as well as 'the spiciest meal or food you have ever experienced' on a gLMS. Values are group means and standard errors, segmented by the same low and high intake groups used in Figure 2. For the salsa samples, there was a significant main effect of group, in ANOVA (see text), and the p-values for the individual comparisons are shown. Conversely, the mean intensity ratings for 'spiciest ever' did not differ by group.

To further test if prior experience results in a contextual effect that alters scale usage and thus ratings of burn in a laboratory setting, ratings of spiciest ever were used to normalize the burn ratings of sampled capsaicin. These normalized ratings were calculated by dividing the rating of spiciest ever by the mean rating of spiciest ever across all participants (mean=66.33). This generated a normalization factor for each participant. Each individual's rating for each

capsaicin sample was then multiplied by this factor; thus, their ratings for capsaicin sampled in the laboratory were now expressed relative to the spiciest meal they had ever experienced.

Linear regression (via *proc glm*) was used to test if the slopes for the functions predicting non-normalized and normalized burn ratings of sampled capsaicin were significantly different between intake groups. A significant difference in slopes between normalized and non-normalized (raw) data would suggest prior experience influences ratings of burn collected in a laboratory setting on the gLMS, suggesting greater intake frequency gives individuals a broader context (i.e., a stronger internal referent) against which to compare stimuli given in the laboratory. The burn from sampled capsaicin differed by group even after normalization ($F(1,1280)=43.0$; $p<0.0001$), with the low intake group reporting greater burn, which is wholly consistent with the non-normalized data reported above. However, within the normalized data, the burn by group interaction no longer reached the criteria for significance [$F(7,1280)=1.8$; $p=0.08$]. Nonetheless, this analysis suggests group differences persist even when ratings are adjusted for prior experience.

Discussion

Prior literature exploring capsaicin response lacked details on responses across a broad range of concentrations. Methods used in prior studies have also been inconsistent regarding the specific qualities they measured and used different methods for presenting stimuli, making it difficult to extrapolate across studies. Also, many previous reports focused exclusively on 'overall intensity' or 'irritation'; however, capsaicin can also elicit bitterness in some individuals (Green and Hayes, 2003; Green and Hayes, 2004). Finally, prior studies have used a wide assortment of scaling methods, making it difficult to make comparisons and draw generalized

conclusions about the psychophysical function for capsaicin. Therefore, our first aim here was to generate such quality specific functions for capsaicin over a wide range of concentrations.

Here, dose response functions were generated for both bitter and burn ratings. A priori, we expected to use a power function to do so, given our use of a gLMS to collect these data. However, when a log-log plot was created, it was clearly apparent that a straight line was not a good fit for modeling burn as a function of capsaicin concentration. Thus, a logarithmic function was used instead of a power function, as a straight line fit the data very well in a semi-log plot (see Figure 3-1). The concentration required for a specific desired level of burn can be readily calculated by rearranging the equation from Figure 3-1 as follows:

$$[\log_{10} \text{ppm}] = \frac{2(\mathbf{Burn} - 23)}{43}$$

Thus, to achieve an average burn of moderate (**17** on a gLMS), the logged concentration of capsaicin required would be:

$$\frac{2(\mathbf{17} - 23)}{43} = -0.279 \log_{10} \text{ppm}$$

Taking the antilog of –0.279 gives a final concentration of 0.53 ppm in water needed to have a moderate burn. This approach can be used for any desired intensity.

In terms of perceived quality, capsaicin is predominately perceived as burning, and this increases as a function of concentration. At lower concentrations (<0.275 ppm), bitter and burn intensities are similar (~weak), however; these sensations quickly diverge, with bitterness increasing at a much lower rate, compared to burn. Due to these differences in slope for bitterness and burn response functions, we conclude that participants are able to distinguish between bitterness and burn perceptions, despite a lack of prior training with exemplars for these qualities. We also believe that the bitterness ratings are not merely a matter of 'hedonic dumping' –

wherein the participant rate a stimulus as bitter merely because it is aversive – as we also gave individuals the opportunity to rate their affective responses to the same stimuli. Also, we observed that bitterness intensity was not significantly associated with liking/disliking ratings, with the exception of the lowest capsaicin concentration (0.11ppm). Conversely, burn was significantly associated with hedonic ratings for three concentrations (2.75, 5.5 and 11ppm). This suggests that burn, rather than bitterness, is the primary driver of liking for sampled capsaicin.

A variety of personality measures like sensation seeking and sensitivity to reward have been previously associated with preference and intake of chili peppers (e.g. Byrnes and Hayes, 2013, 2015, 2016; Rozin, 1980). To extend these findings, we included 6 items from the VARSEEK questionnaire to estimate willingness to try new foods, seek a varied diet and food-adventurousness. We found individuals with a greater willingness to try new foods were more likely to state a preference for higher degree of spiciness in their food, and these individuals also reported less disliking for sampled capsaicin. While this specific effect for VARSEEK has not been reported previously, it reinforces a substantial and growing body of evidence that multiple personality traits influence liking and intake of chili containing foods.

Present data confirm previous findings of an association between ratings of sampled capsaicin and frequency of chili pepper intake, and we show here that such effects occur across a very wide range of concentrations (0.11 to 22 ppm). Here, greater intake associated with increased liking (i.e., Byrnes and Hayes, 2015) and less burn from capsaicin (i.e., (Lawless et al., 1985), when comparing frequent consumers to those who consume chili pepper less frequently. We expand on prior findings by showing intake also associates with remembered intensity of commercially available salsas (mild, medium and hot), suggesting effects of intake not only associate with the burn from sampled capsaicin within the laboratory, but also with chili pepper containing products eaten outside of the laboratory. Critical to this conclusion is the prior

observation that judgments of intensity made from memory are similar to those made from sampled stimuli, for both tastants and chemesthestic stimuli (see Stevenson and Prescott, 1997).

Here, stated preference for spice level in food also associated with burn and liking/disliking of sampled capsaicin. Notably, for intensity, the 'avoid/mild' and 'medium' preference groups were more similar to each other, while differing from the 'spicy/very spicy' group. This implies that higher levels of exposure are required to cause in diet-induced capsaicin desensitization, whereas the amount of exposure experienced by the medium spice level group was not efficient to reduce burn relative to the 'avoid/mild' group. Accordingly, the minimal amount of exposure required to induce desensitization warrants further exploration in future work.

To explicitly test Stevenson and Prescott's (1994) hypothesis of prior experience/scale usage, we used two approaches. First, we compared ratings for the spiciest meal ever consumed, and the means did not differ between the groups, suggesting that the groups did not differ in their exposure to very intense stimuli outside the laboratory. However, this interpretation relies on two assumptions that bear further discussion. A gLMS was used here to collect intensity ratings; this scale was developed to make ratings generalized to all possible sensations, and not just burn or oral sensations. While all scales may suffer from context effects, prior work shows the Labeled Magnitude Scale (LMS) anchored to 'oral sensation' exhibits smaller context effects than category scales or the LMS when it is anchored to 'taste' (Lawless, Horne et al., 2000). Although the robustness of the gLMS to context effects has never been formally tested, it does not seem unreasonable to assume that the generalization process should help minimize such effects, especially given the improvements seen for the non-generalized LMS when the top anchor is switched from 'taste' to 'oral sensations'. Also, our conclusion assumes individuals can accurately recall intensities from memory: prior work suggests this is the case (Stevenson and

Prescott, 1997). For additional confirmation, we tested the Stevenson and Prescott (1994) hypothesis using a second method: by normalizing burn ratings for sampled capsaicin obtained in the laboratory against that individual's remembered rating of the spiciest meal ever consumed. When ratings in the laboratory were expressed as fraction of the most intense meal that person had ever consumed, those who eat capsaicin-containing foods more frequently had lower burn ratings for sampled capsaicin. Taken together, these two analyses suggest that prior experience does not associate with chili pepper intake or perceived burn ratings of sampled capsaicin. In turn, this implies that context effects potentially induced by differences in past experiences with chili peppers and spicy foods do not affect laboratory ratings, as speculated previously (Stevenson and Prescott, 1994)

Conclusions

In summary, this study expands our current knowledge of perceptual and affective responses to capsaicin. As expected, reported intake of chili peppers was associated with perceived burn of sampled capsaicin, consistent with previous findings (e.g. Lawless et al., 1985; Prescott and Stevenson, 1995). Specifically, individuals who reported consuming chili peppers more frequently reported lower burn ratings from sampled stimuli compared to individuals who reported consuming chili pepper less frequently. Two theories have been proposed previously to explain the observed difference in burn ratings between intake groups. The predominant explanation given in the literature is capsaicin desensitization induced by dietary exposure. However, an alternative explanation has been raised: specifically, differences in prior experience between intake groups may influence how individuals use the scale in the laboratory (Stevenson and Prescott, 1994). That is, the observed differences may not be perceptual differences per se,

but differential judgment processes in transforming the percept into a response on a scale. By investigating the recalled intensity spiciest meal ever experienced, we have shown – using two different analyses – prior experience does not appear to explain differences in burn ratings of sampled capsaicin between the two intake groups. This suggests that past and present findings are indeed due to capsaicin desensitization as a result of regular chili pepper intake, and not merely a scaling artifact. More pragmatically, present data also provide quality-specific response functions that can be used to guide selection of appropriate capsaicin concentrations in future psychophysical studies.

Funding

This work was supported in part by the National Institutes of Health via an institutional Clinical and Translational Sciences TL1 Predoctoral Fellowship from the National Center for Advancing Translational Sciences [TR000125], and a Ruth L. Kirschstein National Research Service Award (NRSA) F31 Predoctoral Fellowship from the National Institute of Deafness and Communication Disorders [F31DC01465] to AAN. Additional support was provided by United States Department of Agriculture Hatch Project [PEN04565] funds, and discretionary funds from the Pennsylvania State University.

Acknowledgments

The authors would like to thank Cordelia Running, Ryan Elias, Shane McDonald, and David Bolliet for thoughtful discussion of this work, and Gabrielle Lenart for helping to collect these data. We also thank the staff of the Sensory Evaluation Center at Penn State for their assistance in executing the study, as well as our participants for their time and participation.

CHAPTER 4

Differential bitterness in capsaicin, piperine, and ethanol associates with polymorphisms in multiple bitter taste receptor genes

Adapted from:

Nolden, A.A., McGeary, J.E, and Hayes, J.E.

"Differential bitterness in capsaicin, piperine, and ethanol associates with polymorphisms in multiple bitter taste receptor genes."

Physiology & Behavior, 2016, 156: 117-127.

Abstract

To date, the majority of research exploring associations with genetic variability in bitter taste receptors has understandably focused on compounds and foods that are predominantly or solely perceived as bitter. However, other chemosensory stimuli are also known to elicit bitterness as a secondary sensation. Here we investigated whether *TAS2R* variation explains individual differences in bitterness elicited by chemesthetic stimuli, including capsaicin, piperine and ethanol. We confirmed that capsaicin, piperine and ethanol elicit bitterness in addition to burning/stinging sensations. Variability in perceived bitterness of capsaicin and ethanol were significantly associated with *TAS2R38* and *TAS2R3/4/5* diplotypes. For *TAS2R38*, PAV homozygotes perceived greater bitterness from capsaicin and ethanol presented on circumvallate papillae, compared to heterozygotes and AVI homozygotes. For *TAS2R3/4/5*, CCCAGT

homozygotes rated the greatest bitterness, compared to heterozygotes and TTGGAG homozygotes, for both ethanol and capsaicin when presented on circumvallate papillae. Additional work is needed to determine how these and other chemesthetic stimuli differ in bitterness perception across concentrations and presentation methods. Furthermore, it would be beneficial to determine which TAS2R receptors are activated in vitro by chemesthetic compounds.

Introduction

It is generally accepted bitterness is an evolutionarily important mechanism used to protect mammals against ingesting toxins and poisons in foods (Drewnowski and Gomez-Carneros, 2000; Glendinning, 1994). Mammals detect bitterness following the activation of a specific class of G-protein coupled receptors, known as TAS2Rs (Adler et al., 2000; Behrens, Foerster et al., 2007; Chandrashekar, Mueller et al., 2000; Matsunami, Montmayeur et al., 2000; Mueller, Hoon et al., 2005). The specific number of bitter receptor genes varies across species (Go, Satta et al., 2005), with humans having 25 different *TAS2R* genes (Adler et al., 2000; Behrens and Meyerhof, 2006; Chandrashekar et al., 2000) that cluster on chromosomes 5, 7 and 12 (Adler et al., 2000; Matsunami et al., 2000). Similarities between *TAS2R* genes indicate they have diversified over time (Conte, Ebeling et al., 2002; Fischer, Gilad et al., 2005; Go et al., 2005; Shi and Zhang, 2006; Shi and Zhang, 2009; Shi, Zhang et al., 2003; Wooding, 2011), presumably due to environmental pressure such as a changing diet (Leonard, 2002; Li and Zhang, 2013; Shi et al., 2003). As a consequence of this diversification, there are many non-synonymous substitutions in the coding regions of these genes (Behrens and Meyerhof, 2013; Conte et al.,

2002; Fischer et al., 2005; Shi et al., 2003), which in some cases result in functional changes in the receptor (e.g. (Kim, Jorgenson et al., 2003)).

The most widely studied bitter taste receptor gene, *TAS2R38* (located on chromosome 7), has three functional single nucleotide polymorphisms (SNPs). These SNPs (A49P, V262A and I296V) are inherited together (i.e. they form a haploblock), which results in two common haplotypes named for the respective amino acid substitutions: PAV and AVI (Kim et al., 2003; Kim et al., 2005). The *TAS2R38* diplotype is associated with the ability to detect the bitterants phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP), with PAV homozygotes perceiving greater bitterness than heterozygotes, and AVI homozygotes perceiving little to no bitterness (Allen et al., 2013; Boxer and Garneau, 2015; Bufe, Breslin et al., 2005; Calo, Padiglia et al., 2011; Duffy, Davidson, et al., 2004; Garneau, Nuessle et al., 2014; Hayes, Bartoshuk et al., 2008; Kim et al., 2003; Kim et al., 2005; Mennella, Pepino et al., 2011; Wooding, Bufe et al., 2006).

Polymorphisms in other *TAS2Rs* have also been shown to associate with differential bitterness perception of other foods and ingredients. To date, variability in bitterness perception for various bitter foods and beverages have been associated with polymorphisms in *TAS2R3* (Hayes et al., 2011), *TAS2R4* (Hayes et al., 2011; Risso, Morini et al., 2014), *TAS2R5* (Hayes et al., 2011), *TAS2R9* (Allen et al., 2013), *TAS2R14* (Risso et al., 2014), *TAS2R16* (Bufe, Hofmann et al., 2002), *TAS2R19* (Hayes, Feeney et al., 2015; Hayes et al., 2011; Reed, Zhu et al., 2010), *TAS2R31* (Allen et al., 2013; Hayes et al., 2015; Roudnitzky, Bufe et al., 2011), and *TAS2R43* (Pronin, Xu et al., 2007). However, it is critical to note that for many of these putatively functional SNPs identified via gene-phenotype association studies in humans, it is unknown whether these SNPs are truly the causal SNP with respect to mechanism. For instance, in association studies, it is possible that the tested SNP is in linkage disequilibrium with an untested functional SNP (e.g. Hayes et al., 2015; Hayes et al., 2011; Reed et al., 2010)). Although

additional work has also noted associations between gene variants and other behaviors like liking or intake (e.g. Dotson et al., 2012; Duffy, Davidson, et al., 2004; Duffy et al., 2010; Feeney, 2011; Hayes et al., 2011; Hinrichs, Wang et al., 2006; Pirastu, Kooyman et al., 2014; Wang, Hinrichs et al., 2007), here we focus exclusively on associations between genetic variability and bitterness perception, rather than broader influences on hedonics, food choice, and ingestive behavior (see Hayes, Feeney et al., 2013)).

The majority of research on *TAS2R* polymorphisms to date has understandably focused on compounds that are predominantly or solely bitter. However, many other chemosensory stimuli elicit bitterness as a secondary sensation, thus *TAS2R* variation also has the potential to influence perception of these stimuli. For example, *TAS2R31* variants have been associated with differential responses to sulfonyl amide sweeteners (Allen et al., 2013; Roudnitzky et al., 2011) and grapefruit juice (Hayes et al., 2015; Hayes et al., 2011). Likewise, capsaicin, best known for eliciting burning sensations mediated via TRPV1, also provokes bitter sensations in some individuals but not others (Green and Hayes, 2003; Green and Hayes, 2004; Green and Schullery, 2003), although the genetic basis for this is currently unknown. Here, we address this knowledge gap by exploring whether the variability in bitterness of capsaicin, piperine and ethanol associates with select polymorphisms in *TAS2Rs*.

Recently, we reported the bitterness produced by ethanol on the circumvallate papillae was significantly associated with *TAS2R38* diplotype (Allen et al., 2014). Here we expand previously reported data (Allen et al., 2014), and report on the associations between ethanol bitterness and SNPs within *TAS2R3, TAS2R4,* and *TAS2R5*. In a separate group of participants, Hayes and colleagues (2011) previously reported four SNPs on chromosome 7 resulted in a haploblock across *TAS2R3* (rs765007), *TAS2R4* (rs2234001), and *TAS2R5* (rs2234012 and rs2227264). The resulting *TAS2R3/4/5* diplotype was significantly associated with perceived

coffee bitterness, with TGAG homozygotes reporting more bitterness from sampled espresso coffee compared to CCGT homozygotes (Hayes et al., 2011). Accordingly, we also explore the associations between haplotypes across *TAS2R38* and *TAS2R3/4*/5 and the reported bitterness of capsaicin and piperine here.

Materials and Methods

Study overview

Individuals were recruited from the Pennsylvania State University campus and surrounding community (State College, PA) as part of a larger study on the genetics of oral sensation. The overall study consisted of four one-hour one-on-one visits with a researcher in the Sensory Evaluation Center, a custom build sensory testing facility located within the Erickson Food Science Building on the Penn State campus. All testing occurred face to face in a windowless clinical examination room free of distractions. On the first day of the study, researchers provided participants with an oral explanation of the protocol and goals of the study and obtained written consent. This session included collection of anthropometric data (height, weight, body fat %, blood pressure, picture of anterior tongue), collection of salivary DNA, reported liking of foods, beverages, and non-food items and rated intensity of sampled stimuli. These stimuli included potassium chloride (salty/bitter), quinine monohydrochloride dihydrate (bitter), acesulfame-K (sweet/bitter), a monosodium glutamate / inosine monophosphate (MSG/IMP) blend (umami/savory), sucrose (sweet), and capsaicin. Also, participants were specifically instructed 'You may receive stimuli causing more than one quality. Please attend to all sensations on all trials.' Before scheduling visits 2, 3 and 4, the researcher determined if the

circumvallate papillae were visible and could be touched with a wetted cotton swab without triggering a gag reflex. Other than genetic material collected via the salivary DNA sample, data from session one will not be discussed further here, as current hypothesis are focused on data collected in sessions two, three and four. Results from session one have been reported elsewhere (Allen et al., 2013; Byrnes and Hayes, 2013; Hayes et al., 2015).

Briefly, a constant protocol was used across visits two, three and four, with a different chemesthetic stimulus presented on each day (either capsaicin, piperine or ethanol). These chemesthetic stimuli were selected based on other work showing that they were capable of eliciting bitterness, at least in some individuals (Green and Hayes, 2004; Mattes and DiMeglio, 2001; Nolden and Hayes, 2015). Each visit began with a refresher on how to use a general Labeled Magnitude Scale (gLMS) followed by a short practice session. Participants also rated the perceived intensity of five different prototypical tastants presented regionally to each quadrant of the tongue, as reported elsewhere (see Feeney and Hayes, 2014). Participants also rated the perceived intensity of a chemesthetic stimulus (either capsaicin, ethanol or piperine) applied to the left and right circumvallate (CV) papillae, which are located on the posterior tongue, forming a rearward pointing chevron of 8 to 12 dome-shaped structures. Detection thresholds were collected for chemesthetic stimuli using a forced choice method based on ASTM method E-679. As the final stimulus within a session, participants rated the overall intensity of a whole mouth swish-and-spit chemesthetic stimulus.

Participants

All four sessions were completed by 106 participants (40 men) with a mean age of 25.2± 0.63 (SEM). Participants reported ethnicity using the recommended wording from the 1997 OMB

Directive 15 guidelines. The majority of participants reported Caucasian ancestry (n=69), followed by Asian (n=15) and African American (n=1); 11 participants chose not to disclose their ancestry. Participant characteristics are reported in Table 4-1.

Prior to study enrollment, individuals interested in participating completed an online screening questionnaire. Eligibility criteria included: between 18-45 years old, not pregnant nor breastfeeding, had not smoked in the last 30 days, no known defects of smell nor taste, no oral piercings (lip, cheek or tongue), no history of chronic pain, not currently taking any prescription pain medication, no history of choking or difficulty swallowing and no history of thyroid disease. Participants also indicated they were willing to provide a saliva sample to obtain DNA. Written informed consent was obtained from all participants. All procedures were approved by the Pennsylvania State University Institutional Review Board (protocol number #33176). The procedure in this study complies with stipulations outlined in the Declaration of Helsinki.

Table 4-1: Participant characteristics

			Self-reported ethnicity Freq. (row %)			
	Freq.	Mean age (±SEM)	Caucasian	Asian	African American	Unreported
Total	106	25.2 ± 0.63	79 (74%)	15 (14%)	1 (1%)	11 (10%)
Female	66	24.7 ± 0.75	50 (83%)	11 (16%)	1 (1%)	4 (6%)
Male	40	25.8 ± 1.14	29 (72%)	4 (10%)	0 (-)	7 (17%)

Stimuli preparation

Regional application of prototypical tastants

Prototypical tastants were applied regionally, one at a time, to each of the four quadrants of the tongue using a single cotton swab. Swabs were submerged into the tastant solution prior to application, and rolled on the tongue for three seconds. Stimuli included sucrose, sodium chloride, citric acid, quinine, and a monosodium glutamate and inosinate monophosphate (MSG/IMP) mixture. Data for these stimuli were not analyzed here, as they have been described previously (Feeney and Hayes, 2014).

Irritant swabs

Piperine and capsaicin impregnated swabs were prepared prior to the test session. Solutions of either 10,070 ppm piperine and 30.5 ppm capsaicin were prepared using 95% food grade ethanol, resulting in nominal concentrations of approximately 35 mM piperine and 100 uM capsaicin. The swabs were prepared by submerging a cotton swab in the appropriate solution and drawing it across the lip of a plastic medicine cup before placing it on a strip of parafilm and allowing it to air dry. In the test session, capsaicin and piperine impregnated swabs were rewetted with reverse osmosis (RO) water just before the swab was applied participant's tongue (e.g. Green and Hayes, 2004). Ethanol swabs were prepared immediately prior to application by dipping the swab in a 50% (v/v) ethanol solution made with food grade ethanol and RO water, and drawing it across the lip of a plastic medicine cup in the same manner as the other swabs. The concentrations given here should be considered nominal, as it is impossible to know the specific amount of stimulus delivered to the tongue from the swabs. We also assume all of the ethanol evaporated during the drying process. To select the concentrations used here, we consulted prior literature (Green and Hayes, 2004) and refined them via informal piloting by our team to identify concentrations that would produce roughly similar burn intensities.

Detection threshold

Detection threshold estimates were collected using a 3-alternative forced choice (3-AFC) method based on ASTM method E-679. Six concentrations of piperine, capsaicin and ethanol were each prepared from an appropriate stock solution for that stimulus. The same stock solutions of capsaicin and piperine were used for the detection threshold stimuli described here, and the whole mouth sip-and-spit suprathreshold stimuli (next section). The stock solutions consisted of

120 ppm capsaicin and 9120 ppm piperine in ethanol (95%), respectively. Following an initial 100-fold dilution with RO water, followed by additional 2 fold serial dilutions, the resulting detection threshold stimuli for capsaicin were: 0.6 ppm, 0.3 ppm, 0.15 ppm, 0.075 ppm, 0.037 ppm and 0.018 ppm. For piperine, the concentrations were: 4.56 ppm, 2.28 ppm, 1.14 ppm, 0.57 ppm, 0.28 ppm, and 0.14 ppm. Ethanol stimuli were 8%, 4%, 2%, 1%, 0.5%, and 0.25%, which were prepared using 95% food grade ethanol diluted with RO water. These concentrations were chosen to bracket threshold estimates reported previously for capsaicin (Lawless, Hartono et al., 2000; Sizer and Harris, 1985) and ethanol (Mattes and DiMeglio, 2001); we were unable to locate published detection thresholds for piperine, so these levels were selected on the basis of suprathreshold data that suggest piperine is less potent than capsaicin (Green, 1996b; Stevens and Lawless, 1986).

Whole mouth sip-and-spit suprathreshold stimuli

Whole mouth sip-and-spit samples were presented in 15mL aliquots at room temperature. Piperine and capsaicin whole mouth stimuli were prepared via a 100-fold dilution of the same stock solution used to make the detection threshold stimuli (see above). Final concentrations of 9.12 ppm piperine and 1.2 ppm capsaicin, each containing 1% ethanol, were presented to participants (approximately 32uM piperine and 3.9uM capsaicin). Here, we assume the amount of ethanol present (1%) in the capsaicin and piperine samples should not be meaningfully alter the irritancy of these stimuli, as this amount falls below the reported detection threshold of 1.43% (v/v) for ethanol (Mattes and DiMeglio, 2001). The whole mouth ethanol stimulus consisted of 16% (v/v) ethanol in RO water. Whole mouth dose-response functions for capsaicin and piperine using contemporary scaling methods were not available in extant literature when the experiment

was designed, so these concentrations were selected for logistical convenience: they are twice the highest concentration used in the detection threshold concentration series.

Psychophysical scaling

A general labeled magnitude scale (gLMS) was used to collect psychophysical ratings for all stimuli (Hayes, Allen, et al., 2013; Snyder, Fast et al., 2004). This scale ranges from 'no sensation' at 0 to 'strongest imaginable sensation of any kind' at 100, with intermediate descriptors located at 1.4, 6, 17, 35 and 51, which are labeled as 'barely detectable', 'weak', 'moderate', 'strong', and 'very strong', respectively. During each session, the researcher provided an introduction to the scale and the participant practiced rating 15 items, which included food and non-food items. For each sampled stimulus, participants rated sweetness, bitterness, sourness, saltiness, umami and burning/stinging, unless otherwise noted. Participants were asked if they had any questions regarding the sensations and were given verbal examples to ensure their familiarity with each sensation. All psychophysical data were collected using Compusense five, version 5.2 (Guelph ONT, Canada).

Protocol: Sessions 2, 3 and 4

Regional ratings for tastants

Data for regionally applied tastants are not analyzed here, as they have been reported elsewhere (Feeney and Hayes, 2014); we briefly describe the procedure here to better characterize the context within which the participants made their ratings, as this speaks to their ability to perform the task. A timeline depicting the testing sequence for sessions 2, 3 and 4 is provided in

Figure 4-1. Participants were presented with five different prototypical tastants, one at a time, in a randomized order on each of the four quadrants of the tongue (e.g., front right, front left, back right, back left). Participants retracted their tongue into their mouth and were asked to refrain from touching their tongue to the roof of their mouth. Participants rated perceived intensity of sweetness, bitterness, sourness, saltiness, umami and burning/stinging on a gLMS. Mouth temperature RO water, which was maintained in glass bottles in a thermostatically controlled water bath, was provided as rinse water. Participants were instructed to rinse between stimuli, and they waited a minimum of 30 seconds, or longer if a sensation was still present, before the next stimulus was presented. Twenty tastants were presented in total (5 stimuli in 4 regions) with a multi-attribute time intensity (MATI) trial for the irritant presented halfway through the series. Thus, in every visit, participants tasted and rated 10 different prototypical tastants before an irritant was applied to the CV papillae. The participant then tasted and rated 10 more prototypical tastants before the same irritant was applied to the CV papillae on the other side of the tongue. As noted elsewhere (Allen et al., 2014), ratings for these regionally applied prototypical tastants act as a negative control; in a superset of present data (from Feeney and Hayes, 2014), group means for the side tastes for each tastant were extremely low (i.e., mean bitterness for sucrose, citric acid, and sodium chloride were 0.3, 3.0, 2.2, respectively, and mean burning/stinging ratings were 0.35, 1.08, and 0.80). Conversely, means for the expected qualities of each tastant (e.g., sourness for citric acid) were close to "moderate" on a gLMS). Although single time point ratings for regionally applied tastants are somewhat different than the multi-attribute time intensity task described below with regard to the demand characteristics placed on participants, they nonetheless suggest participants were able to successfully distinguish between various qualities during the rating task.

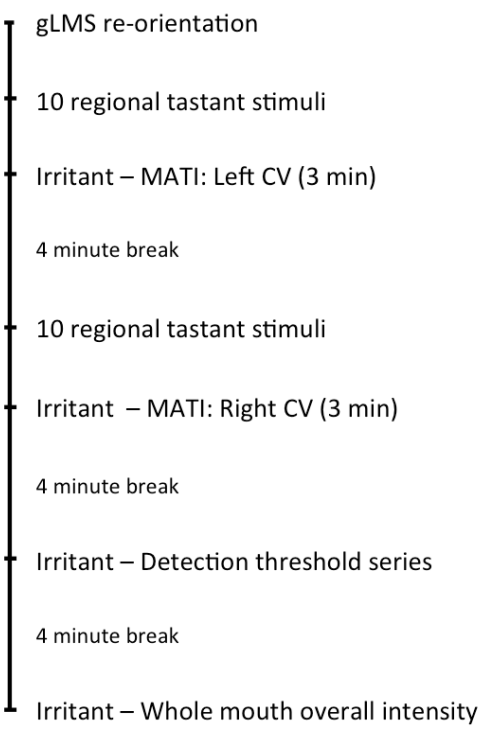

Figure 4-1: Timeline for laboratory visits held on days 2, 3 and 4 of the study. A single irritant (capsaicin, piperine or ethanol) was presented during each visit, with the order of presentation counter-balanced across participants. Abbreviations in the figure are: gLMS, general Labeled Magnitude Scale, and MATI, multiple attribute time intensity.

Multi-attribute time intensity (MATI) for chemesthetic stimuli

Participants were assigned to receive a different chemesthetic stimulus (capsaicin, piperine or ethanol) during each session; presentation order was counter-balanced across participants, so that all participants received all stimuli upon completion of the study. Following the completion of the first 10 regional tastant stimuli, participants received a chemesthetic stimulus on the circumvallate papillae (CV). A researcher applied two cotton swabs taped in tandem onto the left (CV) for 10 seconds in a circular motion. Participants were asked to keep their tongue in their mouth, and lips closed without touching their tongue to the roof of their

mouth. Participants rated sweetness, bitterness, sourness, saltiness, umami and burning/stinging response every thirty seconds for three minutes. Participants were not allowed to rinse with water but were allowed to spit if necessary. After the three minutes of rating, participants were given a four-minute break to rinse with mouth temperature RO water. The participants continued with remaining 10 regional tastant stimuli and then the MATI protocol was replicated for the right CV. Another four-minute break was enforced before continuing on the detection threshold task for the same chemesthetic stimulus. To maintain motivation and attention across the testing session, puzzle books and magazines were provided during the longer 4 minute breaks, and participants were allowed access to their smartphones during these breaks.

Detection thresholds for the chemesthetic stimuli

Participants were presented with three plastic medicine cups, and asked to identify the different sample. Each set of samples contained one spiked sample and two blank samples (see above for sample preparation). All triads were presented in ascending order of concentration. To sample each stimuli, participants were asked to swish the solution for 3 seconds, and spit it out, and wait 5 seconds before sampling the next stimuli, in order from left to right. Participants were not allowed to rinse in between stimuli, but were required to rinse in between sets of stimuli (triads) with mouth temperature RO water (from a glass bottle kept in a temperature controlled water bath). In total, 6 sets of stimuli (triads) were presented; with a 30 second break in between each. All stimuli were presented at mouth temperature in 10mL aliquots. A mandatory break of at least 4 minutes was enforced before moving on to whole mouth sip-and-spit ratings for the same chemesthetic stimulus.

Whole Mouth sip-and-spit

The visit ended with participants rating the intensity of a whole mouth sample of that session's chemesthetic stimulus. The stimulus (either capsaicin, piperine or ethanol) was presented in a plastic cup at mouth temperature. Participants rinsed the entire sample (15mLs) in their mouth for 10 seconds before rating the perceived 'overall intensity' of the stimulus on a gLMS.

Genetic analysis

DNA was collected using Oragene saliva collection kits following manufacturer instructions (Genotek Inc, Ontario, Canada). SNPs (single nucleotide polymorphisms) analyzed here include: rs713598, rs1726866, rs10246939 in *TAS2R38*, as well as SNPs located in *TAS2R3* (rs765007), *TAS2R4* (rs2233998, rs2234001, rs2234002), *TAS2R5* (rs2234012), *TAS2R9* (rs3741845), *TAS2R13* (ra1015443), and *TAS2R31* (rs10772423). Primers were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Genotypes were determined by either MassARRAY technology (Sequenom) or by Taqman assays and independently inspected by two technicians. As a standard procedure, 15% of samples are rerun to ensure reliability. Genotype frequencies of SNPs did not vary from Hardy Weinberg equilibrium (see Table 4-2). Haploview (Barrett, Fry et al., 2005) was used to determine associations between haplotypes and Solid Spline criterion was used to determine haplotype blocks (Figure 4-2). Diplotypes were determined for three SNPs in *TAS2R38* (rs713598, rs1726866, rs10246939) and also for five SNPs located in *TAS2R3* (rs765007), *TAS2R4* (rs2233998, rs2234001, rs2234002) and *TAS2R5* (rs2234012) using PHASE. Individuals with haplotype probabilities less than 0.8 were reported as missing.

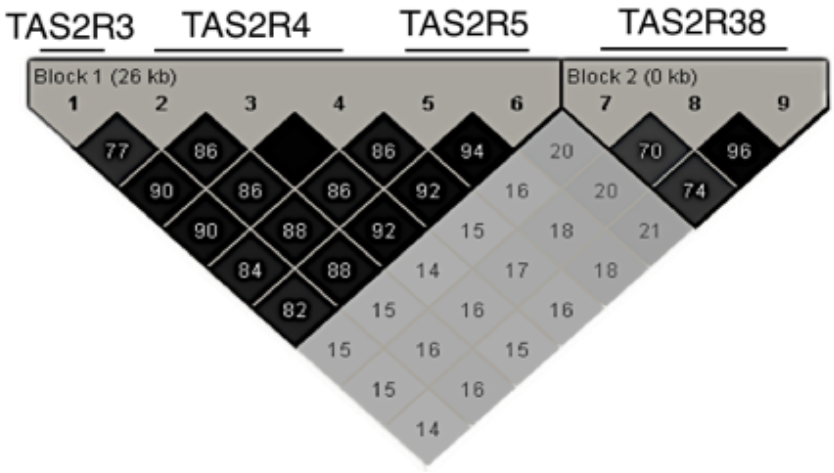

Figure 4-2: Linkage disequilibrium plot for *TAS2R* SNPs on chromosome 7. Numbers in LD plot indicate rounded R^2 values and darker shading indicates higher R^2 values generated via Haploview. For SNP identification, see LD plot # listed in Table 4-2.

Statistical analysis

Data were analyzed using SAS 9.2 (Cary, NC). MATI ratings for the left and right CV were combined and averaged for each participant a priori. Repeated measures analysis of variance (ANOVA) and ANOVA were performed via *proc mixed* to determine the association between genotype and time, and significant effects of gene were determined using the slice option in SAS. Individual detection thresholds were calculated as the geometric mean between the first correct answer followed by all correct answers, and the next lowest concentration. Group detection

thresholds were calculated as the geometric mean of all individual detection thresholds (Lawless and Heymann, 2010).

Table 4-2: Summary for SNPs analyzed

Receptor	LD Plot #	SNP ID	Location	HWE *p*-value
TAS2R3	1	rs765007	5'UTR	0.97
TAS2R4	2	rs2233998	Phe7Ser	0.90
	3	rs2234001	Val96Leu	1.00
	4	rs2234002	Ser171Asn	0.97
TAS2R5	5	rs2234012	5'UTR	1.00
	6	rs2227264	Ser87Asn	1.00
TAS2R38	7	rs713598	Ala49Pro	0.78
	8	rs1726866	Val262Ala	1.00
	9	rs102466939	Ile296Val	0.88

LD Plot #: corresponds to SNP numbers presented in Figure 4-2.
HWE: Hardy-Weinberg equilibrium

Results

Psychophysical response to chemesthetic stimuli

MATI ratings for capsaicin, piperine and ethanol collected separately on the left and right CV were averaged for each participant prior to any analysis. Across all three stimuli, burning/stinging was rated as the dominant sensation during the trial, followed by bitterness (Figure 4- 3). The remaining sensations are not shown in Figure 4-3 to reduce visual clutter: the sourness, sweetness, umami and salty ratings varied in intensity across the stimuli, and for capsaicin and piperine these sensations fell close to or below 'barely detectable'. Ethanol was perceived to have some sourness and sweetness (above 'barely detectable') at time 0 and 30, and these sensations fell below 'barely detectable' by 60 seconds.

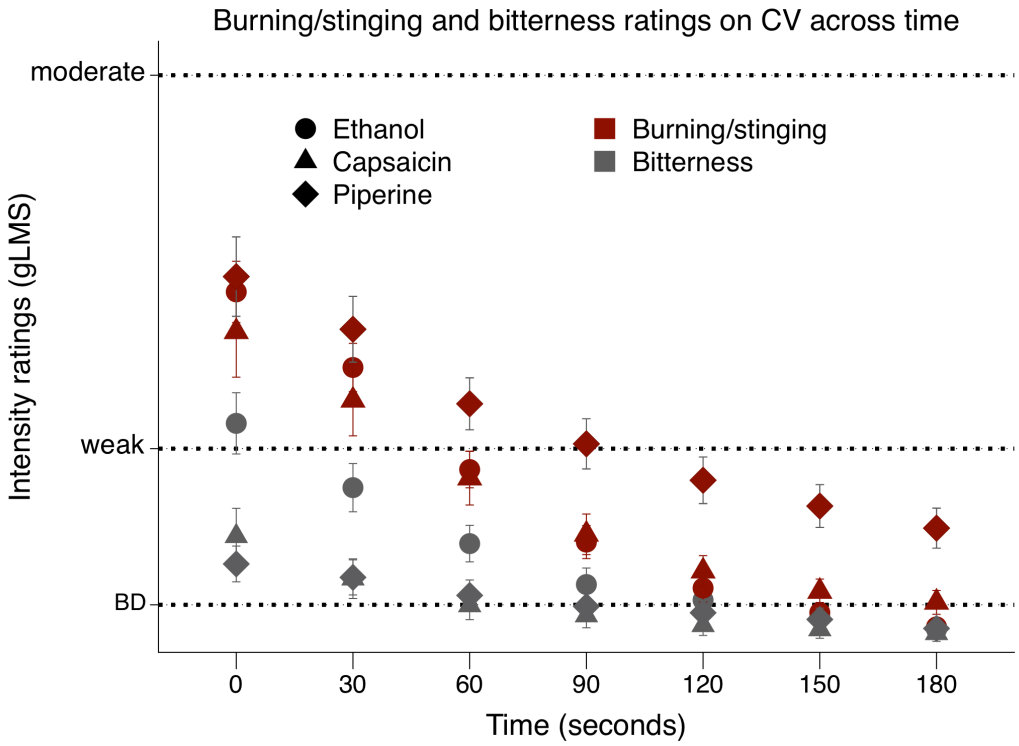

Figure 4-3: Means (±SEM) for burning/stinging and bitterness for 30.5 ppm capsaicin, 50% (v/v) ethanol, and 10,070 ppm piperine presented to the CV via cotton swabs. Ratings were made on a gLMS every 30 seconds over 3 minutes.

TAS2R38 explains variability in bitterness of chemesthetic stimuli presented on the CV

Due to linkage disequilibrium for the three SNPs in *TAS2R38* (see Figure 4-2), diplotypes were determined prior to further analysis. Of the 106 participants who completed all four sessions, 105 were successfully genotyped (diplotype probability > 0.8) for the three SNPs in *TAS2R38* (A49P, V262A, and I296V). There were 25 PAV homozygotes, 46 heterozygotes (PAV/AVI) and 20 AVI homozygotes. The remaining 14 individuals expressed rare diplotypes (6 AVI/AAV, 3 PAV/AAV, 1 PAV/PVI, 1 AVI/PVV, 1 AVI/PVI, 1 PVI/PVV, 1 PVI/PVI).

Repeated measures ANOVA were performed to determine the relationship between common *TAS2R38* diplotypes (PAV/PAV, PAVI/AVI, AVI/AVI) and perceived bitterness over 3 minutes for capsaicin, ethanol and piperine presented on the CV papillae (Figure 4-4). For capsaicin, there was a significant interaction effect between *TAS2R38* diplotype and time for bitterness ([$F(12,528)=2.0$, $p=0.02$]; Figure 4-4a). As would be expected, the main effect of time was also a significant predictor of capsaicin bitterness, [$F(6,528)=10.76$, $p<0.0001$] which decayed over time. The main effect of diplotype was not significant [$F(2,88)=1.69$, $p=0.19$]. Post-hoc analysis of the significant time by diplotype interaction revealed significant differences in the bitterness of capsaicin at 0 and 30 seconds ($p=0.0025$ and $p=0.0344$, respectively).

As reported previously (Allen et al., 2014), perceived bitterness of ethanol was significantly associated with *TAS2R38* polymorphisms. Present ethanol data do not represent an independent replication of our prior report, but are reanalyzed here as haplotypes to allow direct comparison with the other two chemesthetic stimuli. It is important to point out that the previous analysis was conducted in only participants of European ancestry due to ethnic diversity of a different gene of interest (*TRVP1)* (Allen et al., 2014), and that analysis was done SNP by SNP, rather than on the basis of haplotype. For the current analysis, data from all participants was used with haplotypes generated via PHASE. Here, the bitterness of ethanol differed significantly by the *TAS2R38* diplotype [$F(2,88)=3.82$, $p=0.0256$], time [$F(6,528)=36.36$, $p<0.0001$], and the time by diplotype interaction was significant [$F(12,528)=2.25$, $p=0.009$] (Figure 4-4b). Post-hoc analysis via the slice option in SAS revealed ethanol bitterness differed significantly across the diplotypes at 0, 30 and 60 seconds (p's=0.0002, 0.002, 0.01, respectively).

For piperine, bitterness ratings were significantly different across time [$F(6,528)=11.5$, $p<0.0001$] while the main effect of *TAS2R38* diplotype was not significant [$F(2,88)=0.84$, $p=0.43$]; we did observe a significant time by diplotype interaction [$F(12,528)=2.36$, $p=0.0059$].

Despite of this significant interaction, none of the individual time points met the criteria for significance in post-hoc analysis (all p's > 0.08); nonetheless, the pattern in Figure 4-4c is not inconsistent with the results for capsaicin and ethanol (i.e., bitterness appeared to be higher in PAV homozygotes).

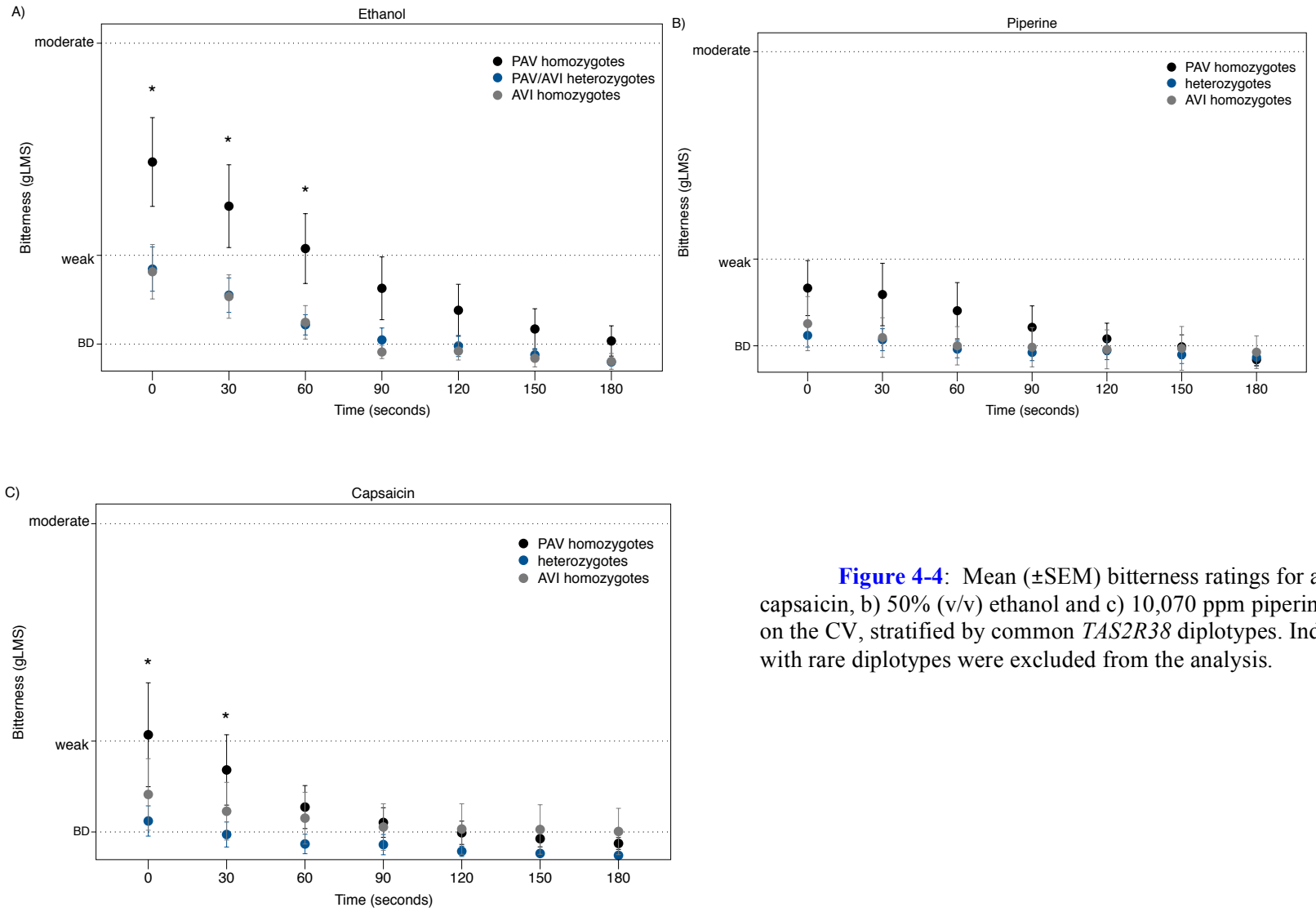

Figure 4-4: Mean (±SEM) bitterness ratings for a) 30.5 ppm capsaicin, b) 50% (v/v) ethanol and c) 10,070 ppm piperine presented on the CV, stratified by common *TAS2R38* diplotypes. Individuals with rare diplotypes were excluded from the analysis.

TAS2R3/4/5 diplotype explains variability in capsaicin, piperine and ethanol bitterness

As expected from prior work, SNPs in *TAS2R3* (rs765007), *TAS2R4* (rs2233998, rs2234001 and rs2234002), and *TAS2R5* (rs2234012 and rs2227264) were in linkage disequilibrium (Figure 4-2), forming a haploblock that was independent from the *TAS2R38* haploblock. Of 106 participants, 102 had a diplotype probability greater than 0.8. In these individuals, we observed two common haplotypes – CCCAGT (frequency = 0.518) and TTGGAG (frequency = 0.413) – which resulted in 3 common diplotypes. There were 28 CCCAGT homozygotes, 46 heterozygotes (CCCAGT/TTGGAG), and 20 TTGGAG homozygotes (see Table 3). Other diplotypes included three individuals expressing CTCAAG/CCCAGT, and one participant for each of the following: CCCAGT/TCCAGT, CCAGT/TCGGAG, CCCAGT/TTGGGT, CTCAGT/CCCAGT, CTGGGG, TTGGAG. Due to low frequencies of the less common diplotypes, analysis was restricted to the three common diplotypes in our cohort.

Table 4-3: Summary of common *TAS2R3/4/5* diplotypes

TAS2R3	**TAS2R4**			**TAS2R5**		
rs765007	rs2233998	rs2234001	rs2234002	rs2234012	rs2227264	n (%)
C	C	C	A	G	T	28 (27%)
Y	Y	S	R	R	D	46 (45%)
T	T	G	G	A	G	20 (20%)

Y = C or T S = G or C R = A or G D = A or G or T

Percentages were based on 102 individuals that had a diplotype probability > 0.8.
See text for frequency of uncommon diplotypes.

Repeated measures ANOVA revealed significant associations between common *TAS2R3/4/5* diplotypes and the bitterness from capsaicin and piperine but not ethanol applied to the CV. For capsaicin, the diplotype by time interaction effect was significant [$F(12,540) = 2.09$, $p = 0.0007$], as was the main effect of time [$F(6,540)=9.86$, $p < 0.0001$]; however, the main effect of diplotype was not significant [$F(2,90) = 1.81$, $p = 0.17$]. Post-hoc analysis via the slice option in SAS revealed significant differences in capsaicin bitterness between the diplotypes occurred at 0 and 30 seconds ($p = 0.0001$ and $p = 0.025$, respectively; Figure 4-4a).

As shown in Figure 4-5b, ethanol bitterness decayed significantly over time [$F(6,540) = 34.23$, $p <0.0001$]. The main effect of *TAS2R3/4/5* diplotype on ethanol bitterness was marginal [$F(2,90) = 2.67$, $p = 0.07$], and there was no evidence of a time by diplotype interaction [$F(12,540) = 1.34$, $p = 0.19$]). Even though the main effect of the *TAS2R3/4/5* diplotype was not significant, a post-hoc analysis was conducted as we might expect differences to occur when bitterness ratings are the highest before bitterness falls below barely detectable as time goes on. In this additional analysis, participants reported significant difference between diplotypes for the bitterness of ethanol at 0 and 30 seconds ($p = 0.017$ and $p = 0.01$, respectively; Figure 4-5b).

For piperine, bitterness decayed over time, as expected [$F(6,540) = 9.61$, $p < 0.0001$], and the *TAS2R3/4/5* diplotype by time interaction was significant [$F(12,540) = 2.14$, $p = 0.01$]; there was no evidence of a main effect of diplotype [$F(2,90) = 0.59$, $p = 0.5$] (Figure 4-5c). As with the *TAS2R38* diplotype data for piperine, there appeared to be a floor effect, as post-hoc analysis of the interaction revealed no significant differences at the individual time points in spite of the significant interaction.

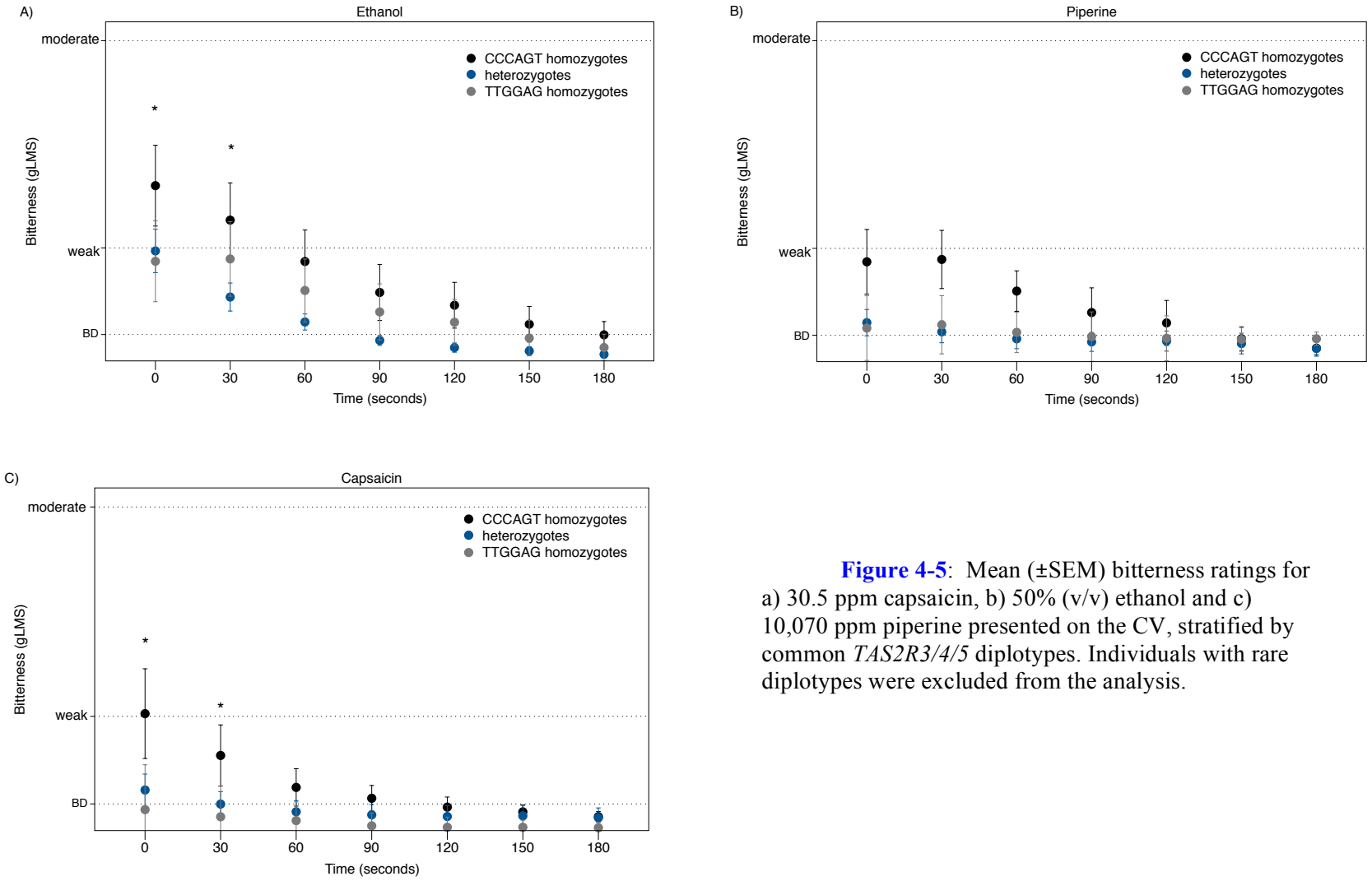

Figure 4-5: Mean (±SEM) bitterness ratings for a) 30.5 ppm capsaicin, b) 50% (v/v) ethanol and c) 10,070 ppm piperine presented on the CV, stratified by common *TAS2R3/4/5* diplotypes. Individuals with rare diplotypes were excluded from the analysis.

Reportedly functional SNPs in TAS2R9, TAS2R13, and TAS2R31 did not associate with variability bitterness of capsaicin, piperine nor ethanol on the CV

In parallel analyses to those described in the two proceeding sections, we also tested putatively functional SNPs in *TAS2R9* (rs3741845), *TAS2R13* (rs1015443) and *TAS2R31* (rs10772423). For capsaicin applied to the CV, there was no evidence of any association with bitterness for either the SNP main effect (all p's > .1) or the SNP by time interactions (all p's > 0.8). Likewise, we failed to observe any effects for piperine (main effect p's > 0.4; interaction p's > 0.4) or ethanol (main effect p's > 0.3; interaction p's > 0.1).

Overall intensity of sip-and-spit whole mouth stimuli

Overall intensity ratings for whole mouth (sip-and-spit) chemesthetic stimuli are shown in Figure 4-6. The average overall intensity rating for piperine was significantly different from capsaicin and ethanol (p's<0.0001). Capsaicin and ethanol did not significantly differ in overall intensity ratings (p=0.13), suggesting our efforts to match their intensity in pilot testing were generally successful.

Individual differences in overall intensity ratings of whole mouth sip-and-spit stimuli were not significantly associated with *TAS2R38 nor TAS2R3/4/5* diplotype (all p's > 0.3). Given the absence of any evidence for an effect with the CV swab, we did not test the SNPs in *TAS2R9*, *TAS2R13,* and *TAS2R31* for the capsaicin and piperine whole mouth stimuli. Also, we did not test for *TAS2R9* and *TAS2R31* effects with whole mouth ethanol; an association between whole mouth ethanol and the rs1015443 SNP in *TAS2R13* was reported previously (Allen et al., 2014).

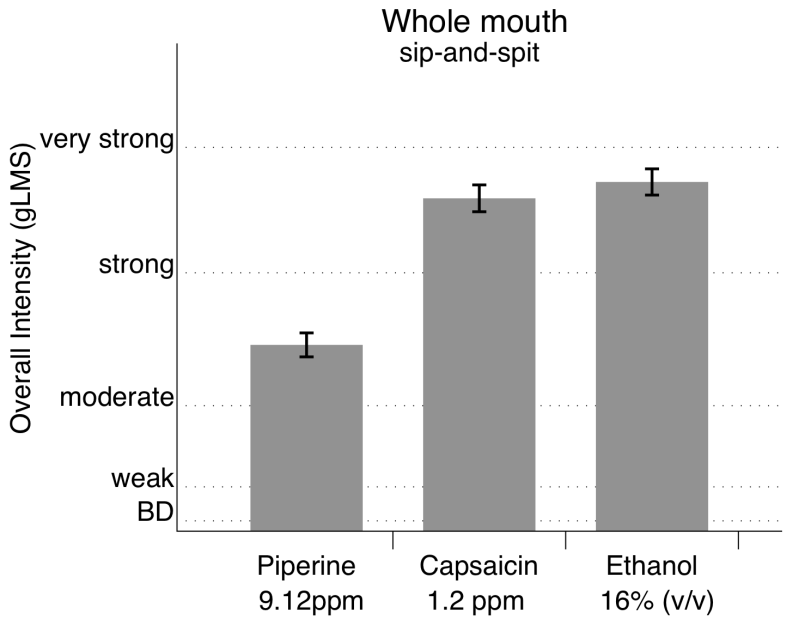

Figure 4-6: Overall intensity ratings (means ± SEM) for whole mouth sip-and-spit stimuli (15 mL).

Detection thresholds for capsaicin, piperine and ethanol

The group geometric mean of individual best estimate thresholds was: 0.52±0.04 ppm for capsaicin, 0.58±0.25 ppm for piperine, and 0.87±0.16% for ethanol (geometric mean± standard error).

TAS2R38 diplotype did not explain variability in individual detection thresholds for any stimuli (capsaicin, piperine or ethanol). However, we did note capsaicin and piperine detection thresholds appeared to trend in the same direction as the suprathreshold data. For capsaicin, AVI

homozygotes and heterozygotes had mean detection thresholds of 0.74±0.1 ppm and 0.72±0.06 ppm, compared to PAV homozygotes 0.52±0.09 ppm, however this difference was not significant (p=0.16). Similarly, piperine detection thresholds for AVI homozgyotes were appeared to be slightly higher (3.12±0.5), compared to heterozygotes (2.45±0.39) and PAV homozygotes (1.53±0.54); however these apparent differences were not significant across the diplotypes (p=0.13). Ethanol detection thresholds did not follow this same trend, with thresholds of 0.94±0.38, 1.06±0.24, and 1.67±0.35, respectively for AVI homozygotes, heterozygotes and PAV homozygotes (p=0.28). *TAS2R3/4/5* diplotype failed to explain variation in individual detection thresholds for capsaicin, piperine nor ethanol (p's > 0.6). The 3 SNPs in *TAS2R9*, *TAS2R13,* and *TAS2R31* were not tested against the detection threshold data.

Discussion

Bitterness of capsaicin, ethanol and piperine

Chemesthetic stimuli are known to elicit both burning and bitter sensations. Present data confirm previous findings that capsaicin, piperine and ethanol presented on the posterior tongue are perceived as mainly burning/stinging, along with bitterness at least in some individuals (Green and Hayes, 2004). Notably, the burning/stinging ratings from capsaicin and ethanol on the CV were similar across time. At time zero, ratings were reported between weak and moderate, and decayed to below weak after 60 seconds and fell to barely detectable by 180 seconds. Conversely, burn ratings for piperine across time decayed more slowly than for capsaicin or ethanol, with means between weak and barely detectable even after 180 seconds. This is consistent with other reports that piperine burn decays more slowly than capsaicin burn (Green

and Hayes, 2004; McDonald, Barrett et al., 2010). Perceived bitterness for ethanol followed a similar time course as burning/stinging, but did not reach the same maximal intensity, suggesting ethanol at this concentration is predominantly burning rather than bitter (Allen et al., 2014; Nolden and Hayes, 2015).

When participants were presented with the chemesthetic swabs, they were asked to rate six qualities, including bitterness and burning/stinging. It is possible that these sensations may have been difficult for untrained participants to discriminate between, as there was no training session with exemplars to familiarize participants with these sensations. However, as mentioned in the methods, separate analysis of the 5 prototypical tastants used in the regional taste test (sucrose, citric acid, NaCl, quinine, and a MSG/IMP blend) showed little to no evidence that participants made inappropriate ratings for qualities that should not be present (e.g., bitterness for sucrose) (Allen et al., 2014). Also, the data described here were collected after participants already had substantial practice in rating diverse, perceptually complex stimuli during their first visit to the laboratory (see Byrnes & Hayes 2013; Allen et al. 2013). Finally, while it remains possible that this task may have been potentially difficult for some participants, the overall burning/stinging and bitterness ratings were not confounded as bitter and burning response curves were distinct for these chemesthetic agents (see Figure 4-3), consistent with other work (Green and Schullery 2003; Green and Hayes 2004). Collectively, this suggests present results were not contaminated by some sort of dumping process or other demand characteristics of the task, in spite of the use of naïve participants who were not previously oriented to these sensations in a training session with specific exemplars.

The capsaicin, piperine and ethanol concentrations applied via a cotton swab to the CV papillae were intended to be intensity matched, to facilitate direct comparisons across the stimuli. This was generally successful, as the burning/stinging ratings were similar at time 0 (as shown in

Figure 4-3). However, it should also be noted that bitterness ratings from ethanol were higher than the bitterness ratings for capsaicin and piperine at these concentrations. It is unknown how bitterness perception may potentially differ when presented at concentrations that are intensity matched for bitterness, rather than burning/stinging. This should be revisited in future work to facilitate comparisons for both bitterness decay rates and associations with bitter taste receptor genetics.

Reported bitterness on CV is explained by TAS2R38 and TAS2R3/4/5 diplotypes

Here, we explored whether *TAS2R38* diplotype can explain variability in bitterness perception of capsaicin and piperine for stimuli presented on the CV and as whole mouth sip-and-spit stimuli. Genetic variability in *TAS2R38* has been previously associated with the bitterness of numerous stimuli, with the most widely reported association occurring with bitterness from 6-n-propythiouracil and phenylthiocarbamide (Allen et al., 2013; Duffy, Davidson, et al., 2004; Kim et al., 2003), but also ethanol (Allen et al., 2014), vegetables (Sandell and Breslin, 2006) and goitrin (Wooding, Gunn et al., 2010). Present data suggests the bitterness of capsaicin, ethanol and possibly piperine, differs by *TAS2R38* diplotype. Here, PAV homozygotes perceived greater bitterness from capsaicin presented on the CV papillae compared to heterozygotes and AVI homozygotes, consistent with the idea that the PAV haplotype codes for the more functional form of the receptor. As expected, the significant differences across diplotype occurred within the first few time points following application, as this is where the greatest intensity was reported. As the burn decays over time, we would not expect the significant differences across diplotype to persist as ratings approach barely detectable on the gLMS; indeed, such a floor effect is apparent in Figure 4-4a. A similar pattern of heightened bitterness among the PAV homozygotes is seen for

piperine, although the use of a concentration that never exceeded weak bitterness (on average) likely obscures the ability to cleanly test for differences across *TAS2R38* diplotype in post-hoc analyses.

Present data also extend upon our prior report that ethanol bitterness on the posterior tongue varies by *TAS2R38* diplotype (Allen et al., 2014). These data are not a true replication of our prior report, and should not be treated as such; however, the analyses shown here are novel in two ways. The present analysis a) is based on diplotype rather than individual SNPs in isolation, and b) uses a mixed race cohort that is a superset of the prior European ancestry only sample. As expected, the PAV homozygotes reported greater bitterness from ethanol, and this difference persisted over the first 90s following application.

SNPs in *TAS2R3*, *TAS2R4*, and *TAS2R5* have been previously reported to be in linkage disequilibrium (Hayes et al., 2011). Here we report that six SNPs (1 from *TAS2R3*, 3 from *TAS2R4* and 2 from *TAS2R5*) are in linkage disequilibrium with each other, but are independent from the well-known haplotype for *TAS2R38*. The resulting *TAS2R3/4/5* diplotype significantly explained variability in bitterness perception of both capsaicin and ethanol that had been applied to the CV papillae. CCCAGT homozygotes rated the greatest bitterness compared to heterozygotes and TTGGAG homozygotes for both ethanol and capsaicin. Unexpectedly, these results are not in agreement with previous findings, which suggested CCGT homozygotes individuals (which are comparable to **C**C**C**A**GT** here) reported lower bitterness response to sampled coffee compared to heterozygotes and TGAG homozygotes (comparable to **T**T**G**G**AG**) individuals (Hayes et al., 2011). The reason for this discrepancy is unclear, and requires an additional study.

Whole mouth ratings and detection threshold

Overall intensity ratings are reported for capsaicin, piperine and ethanol in a whole mouth sip and spit paradigm. In terms of overall intensity, the 1.2 ppm capsaicin and 16% ethanol were not significantly different, while the 9.12 ppm piperine had significantly lower mean ratings. While this suggests the 1.2ppm capsaicin and 16% ethanol were well matched for overall intensity, it is important to note that we do not know the relative contribution of burning/stinging and bitterness to overall intensity, as a whole mouth stimulus. In a separate group of participants, we recently reported that ethanol is more bitter than burning at lower concentrations, with the reverse being true as concentration continues to increase (Nolden and Hayes, 2015). Here, our decision to have participants rate overall intensity rather than individual ratings of multiple attributes is, in hindsight, a clear limitation of the present work. More work is needed to understand how the quality specific profile of capsaicin and piperine may shift with concentration.

The group geometric means of individual best estimate thresholds for capsaicin, piperine and ethanol are 0.52±0.04 ppm, 0.58±0.25 ppm and 0.87±0.16% (±S.E.), respectively. An ascending 3-AFC method was chosen in order to keep test session brief and only included six concentrations; however, it remains possible a more sensitive albeit labor intensive method (e.g. up-down staircase) may result in different associations with *TAS2R38* diplotype. Nonetheless, the present cohort had a capsaicin detection threshold similar to previously results (0.31 ppm) (Lawless, Hartono, et al., 2000), suggesting the method used here was valid. For ethanol, the current cohort reported lower detection threshold 0.87±0.16% (S.E.) than the one reported previously: 1.43±0.11% (S.E.) (Mattes and DiMeglio, 2001). This difference may be due to the different methods used as Mattes and DiMeglio (2001) used a forced choice staircase method in 25 individuals. While their method should to be more sensitive that the rapid method used here, it

is also important to note the difference in sample size. To the best of our knowledge, detection thresholds for piperine have not been previously reported, so we are unable to compare our data to prior values. Finally, the very similar detection thresholds for capsaicin and piperine, in spite of substantial differences in their burn at higher concentrations, serves to recapitulate the observation that threshold values are often a poor predictor of suprathreshold response due to differing slopes of the psychophysical function.

Diplotypes for *TAS2R3/4/5* or *TAS2R38* did not significantly associate with the reported overall intensity of the sip-and-spit stimuli, nor detection thresholds for capsaicin, piperine or ethanol. However, there was a trend for PAV homozygotes to have a lower (more sensitive) detection threshold for capsaicin and piperine compared to AVI homozygotes. This trend was not observed for ethanol. Initially, one might expect that with a more sensitive detection threshold method or with additional participants, that there might be a significant association between *TAS2R38* diplotype and individual detection thresholds for capsaicin and piperine, given the pattern seen in the suprathreshold data. Alternatively however, the primary percept experienced at detection threshold for chemesthetic stimuli like capsaicin and piperine is presumably burning, not bitterness, so accordingly, we should not expect detection threshold to be explained by bitter taste receptor genetics. Thus, determining individual recognition thresholds (rather than detection thresholds), and attempting to associate them with *TAS2R38* diplotype could potentially be more successful in future studies.

Future direction

In vitro methods have been fundamental in identifying bitter compounds that are ligands for TAS2R receptors (Meyerhof, Batram et al., 2010). Furthermore, it can be determined which

amino acid substitutions (either naturally occurring in the population as genetic polymorphisms, or via site directed mutations) influence receptor activation. Changes in receptor activation often explain differences in psychophysical response. However, differences in psychophysical response are frequently associated with genetic polymorphisms in gene association studies in advance of functional expression studies (e.g., Reed et al. 2010, Hayes et al. 2011, Allen et al. 2013). Thus, putatively functional SNPs in such studies may be merely associated with a change in perceptual response without being mechanistically involved if functional expression assays have not been conducted to determine whether the SNP is causal or merely in LD with another nearby SNP that is the functional variant. Presently, it is unknown whether capsaicin, piperine and ethanol activate TAS2R3, -4, -5 or -38. Thus, additional work is needed to determine if these (and other) bitter taste receptors respond to capsaicin, piperine, and ethanol *in vitro*.

Conclusions

Here we explore differential bitterness from capsaicin, ethanol and piperine as a function of *TAS2R* polymorphisms. Measures included suprathreshold ratings from stimuli applied to the circumvallate papillae on the posterior tongue, and as whole-mouth sip-and-spit stimuli; detection thresholds were also collected using a rapid method. Traditionally, chemesthetic compounds are characterized and defined by their ability to elicit irritation: yet, little is known about the perception of secondary sensations (e.g. 'side tastes') from these stimuli. Here we replicate prior reports that capsaicin, piperine and ethanol elicit bitterness in addition to burning/stinging sensations, at least at the concentrations tested. Furthermore, differences in the bitterness from these stimuli associated with genetic polymorphisms in bitter taste receptor genes, specifically in

TAS2R3, -4, -5 as well as *TAS2R38.* Additional work is needed *in vitro* to confirm that these compounds are able to activate bitter taste receptors in functional expression systems.

Funding

This work was supported by a National Institutes of Health grant from the National Institute of Deafness and Communication Disorders [DC010904] to JEH and the National Center for Research Resources to JEM [RR023457], and Shared equipment grants (ShEEP) from the Medical Research Service of the Department of Veteran Affairs to JEM. AAN received additional support from the National Institutes of Health via an institutional Clinical and Translational Sciences TL1 Predoctoral Fellowship from the National Center for Advancing Translational Sciences [TR000125], and a Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (F31) from the National Institute of Deafness and Communication Disorders [F31DC01465]. Additional support was provided by United States Department of Agriculture Hatch Project [PEN04332] funds, and funds from the Pennsylvania State University. The views expressed in this article are those of the authors and do not necessarily reflect the position or policy of the Department of Veterans Affairs.

Acknowledgments

The authors would like to thank Dr. Emma L. Feeney, Dr. Nadia K. Byrnes, and Ms. Meghan Kane for their assistance in collecting psychophysical data, as well as Kayla Beaucage for genotyping our DNA samples. We also thank our study participants for their time and participation.

CHAPTER 5

Polymorphisms in *TRPV1* and *TAS2Rs* associate with sensations from sampled ethanol

Adapted from:

Allen, A.L., McGeary, J.E. and Hayes, J.E.

"Polymorphisms in *TRPV1* and *TAS2Rs* associate with sensations from sampled ethanol."

Alcoholism: Clinical and Experimental Research, 2014, 38(10): 2250-2560.

Abstract

Genetic variation in chemosensory genes can explain variability in individual's perception of and preference for many foods and beverages. To gain insight into variable preference and intake of alcoholic beverages, we explored individual variability in the responses to sampled ethanol. In humans, ethanol elicits sweet, bitter and burning sensations. Here, we explore the relationship between variation in ethanol sensations and polymorphisms in genes encoding bitter taste receptors (TAS2Rs) and a polymodal nociceptor (TRPV1). Caucasian participants (n=93) were genotyped for 16 SNPs in *TRPV1*, 3 SNPs in *TAS2R38* and 1 SNP in *TAS2R13*. Participants rated sampled ethanol on a generalized Labeled Magnitude Scale. Two stimuli were presented: a 16% ethanol whole mouth sip-and-spit solution with a single time-point rating of overall intensity, and a cotton swab saturated with 50% ethanol on the circumvallate papillae (CV) with repeated ratings made over 3 minutes. Area under the curve (AUC) was calculated for the time-intensity data. The ethanol whole mouth solution had overall intensity

ratings near ‘very strong’. Burning/stinging had the highest mean AUC values, followed by bitterness and sweetness. Whole mouth intensity ratings were significantly associated with burning/stinging and bitterness AUC values on the CV. Three TRPV1 SNPs (rs224547, rs4780521, rs161364) were associated with ethanol sensations on the CV, with two (rs224547 and rs4780521) exhibiting strong linkage disequilibrium. Additionally, the *TAS2R38* SNPs rs713598, rs1726866, and rs10246939 formed a haplotype, and were associated with bitterness on the CV. Lastly, overall intensity for whole mouth ethanol associated with the *TAS2R13* SNP rs1015443. These data suggest genetic variations in *TRPV1* and *TAS2Rs* influence sensations from sampled ethanol and may potentially influence how individuals initially respond to alcoholic beverages.

Introduction

Taste strongly influences food intake (Glanz, Basil et al., 1998; IFIC, 2011), including alcohol consumption (Barber and Grichting, 1987; Moore and Weiss, 1995). Ethanol activates olfactory, taste and chemesthetic receptors, and each modality is carried centrally by different nerves; these inputs individually and jointly affect the percept evoked by ethanol. Ethanol reportedly elicits sweet and bitter sensations in humans (Mattes and DiMeglio, 2001) and in mice (Blizard, 2007). Sour and salty sensations have also been reported, but with much lower intensities than bitter or sweet (Mattes and DiMeglio, 2001). Ethanol also activates sweet taste fibers in non-human primates (Hellekant, Danilova et al., 1997) and rodents (Lemon, Brasser et al., 2004). Regarding alcohol behaviors, individual differences in bitterness and sweetness are predictors of alcohol liking and intake in young adults (Lanier et al., 2005).

Multiple studies have linked variation in *TAS2R* bitter receptor genes to alcohol intake. Duffy and colleagues (2004) reported *TAS2R38* haplotypes are associated with alcoholic intake, with AVI homozygotes (who perceive less bitterness from the bitter compound propylthiouracil) consuming significantly more alcoholic beverages than heterozygotes or PAV homozygotes, a finding which was subsequently replicated in a separate cohort (Hayes et al., 2011). In a high-risk familial cohort (COGA), Wang and colleagues (2007) found the same haplotype associated with maximum number of drinks ever consumed within a 24-hour period in African-Americans. More recently, Dotson et al (2012) reported associations between *TAS2R38* and *TAS2R13* polymorphisms and alcohol intakes derived from the Alcohol Use Disorders Identification Test (AUDIT) in head and neck cancer patients. These differences in intake are presumably driven by differences in perceived intensity that lead to lower liking (Duffy, 2007; Duffy, Hayes et al., 2009; Hayes, Feeney, et al., 2013). However, this interpretation is complicated by previous reports where *TAS2R38* haplotypes fail to explain variation in sensations from ethanol (Duffy, Davidson, et al., 2004) or blended whisky (Hayes et al., 2011). Mattes and DiMeglio (2001) observed variable ethanol thresholds and suprathreshold ratings of ethanol across individuals, but these differences did not associate with phenylthiocarbamide detection thresholds (another taste phenotype commonly associated with *TAS2R38* genotype) (Kim et al., 2003). This suggests ethanol may differentially activate bitter receptors beyond TAS2R38, consistent with other data (Dotson et al., 2012; Hinrichs et al., 2006). Of the handful of human *TAS2R*s previously reported to contain functional SNPs, only three (*TAS2R13*, *TAS2R16*, and *TAS2R38*) have been implicated with regard to alcohol intake or dependence in prior literature (Dotson et al., 2012; Hinrichs et al., 2006; Duffy, Davidson, et al., 2004). Due to an extremely low minor allele frequency, the relevant SNP in *TAS2R16* is largely irrelevant in European-Americans, so we confined our analyses here to putatively functional variants in *TAS2R38* and *TAS2R13*.

In addition to bitter and sweet sensations, ethanol also causes irritation commonly described as burning or stinging (Green, 1987; Green, 1988). Burning sensations in the mouth are due, in part, to activation of the transient receptor potential vanilloid receptor 1 (TRPV1). TRPV1 (formerly VR1) is activated by noxious heat, capsaicin (Caterina, Rosen et al., 1999; Tominaga et al., 1998; Tominaga and Tominaga, 2005) and ethanol (Trevisani et al., 2002), even at relatively low concentrations (0.1-3% v/v). TRPV1 is a multimodal nociceptor activated by chemical and thermal stimuli, resulting in a substance P dependent signal cascade that eventually culminates in sensations described as burning. In rodent derived tissue culture, the release of substance P increases with increasing concentrations of ethanol (Trevisani et al., 2002). When the *trpv1* gene is knocked out in mice, knockouts have a higher preference for ethanol and consume more ethanol than wild-type mice (Blednov and Harris, 2009). Collectively, these data suggest the TRPV1 receptor likely plays a role in the perception and acceptability of ethanol.

The objectives of the present study were to determine if polymorphisms in a) *TRPV1* associate with the perception of ethanol, specifically ethanol burn, and polymorphisms in b) *TAS2R38*, and c) *TAS2R13* may explain differences in bitterness of ethanol. Previously, ethanol intensity has been shown to associate with propylthiouracil tasting (Bartoshuk, Conner et al., 1993; Duffy, Davidson, et al., 2004; Prescott and Swain-Campbell, 2000), but due to multiple sensations elicited from ethanol, we anticipated that measuring bitter and burning sensations separately would help elucidate the influence of both bitter taste receptors and heat/pain receptors on alcohol sensations, and potentially intake.

Materials and Methods

Overview

These data presented here are part of a broader study on chemosensory genetics, oral sensation and food preferences (GIANT-CS, phase I). This study involves one test session for all participants, with some participants being invited back for 3 additional sessions on separate days. All sessions (~1 hour each) were scheduled at least one week apart and were completed one-on-one in our laboratory with a member of the research team. During the first session, informed consent was obtained and participants were given a brief explanation of the study aims: to quantify the influence of specific genes on the sensations from capsaicin, piperine and ethanol. Measures of alcohol use, misuse or abuse were not collected, and no special emphasis was given to alcohol related behaviors. Ethanol was not tasted in the first session; the stimuli presented in session 1 have been reported elsewhere (Allen et al., 2013; Byrnes and Hayes, 2013), and are not described here for brevity. Refer to Appendix A for more details regarding session 1 methods. Upon completing session 1, participants were screened to determine if they were eligible to participate in sessions 2, 3 and 4. Eligibility for sessions 2-4 was based on visibility of the individual's circumvallate papillae and the ability to tolerate stimulation with a wetted swab without gagging. Of participants who qualified, 130 individuals returned to complete all four sessions.

Participants

Participants, 18 to 45 years old, were recruited from the Pennsylvania State University campus and surrounding area. Those interested in participating completed an online survey to

determine if they met inclusion criteria. Qualifications include: not pregnant or breastfeeding, non-smoker, no tongue, cheek or lip piercings, no known smell or taste defect, no hyperactive thyroid, no history of chronic pain, and willingness to provide a salivary DNA sample. Of the participants who completed sessions 2-4 (total n=130), the majority reported European ancestry (n=93), with 18 reporting Asian ancestry and 2 reporting African ancestry; 17 individuals declined to provide ancestry. Due to potential differences in allele frequencies across ancestry and the possibility of population stratification, all of the results here are restricted to individuals of European ancestry, resulting in a cohort of 58 women and 35 men with a mean age of 25 (±0.69 SEM) years. Witten informed consent was obtained from all participants. All procedures were approved by the Pennsylvania State University Institutional Review Board (protocol #33186).

Psychophysical Scaling of Test stimuli

A generalized Labeled Magnitude Scale (gLMS) was used to collect psychophysical ratings for stimuli (Hayes, Allen, et al., 2013; Snyder et al., 2004). This scale ranges from 0 to 100 and asks participants to rate the intensity they experience relative to the 'strongest imaginable sensation of any kind' (100). Adjective labels on the scale include: no sensation, barely detectable, weak, moderate, strong, and very strong, located at 0, 1.4, 6, 17, 35, and 51 respectively. This scale is believed to enhance the validity of comparisons across individuals, as compared to visual analog scales (Bartoshuk, Duffy et al., 2003; Bartoshuk, Duffy et al., 2004).

In sessions 2-4, participants were given instructions, identical to those provided during session 1, reorienting them to the scale. This included explanation of the top anchor, 'strongest imaginable sensation of any kind', as well as reminding participants that they should click anywhere along the scale and to not let whether or not they like/dislike the sample to influence

their intensity ratings. Before rating any sampled stimuli, participants completed a warm-up session where they rated 15 remembered sensations using a gLMS (e.g. Hayes, Allen, et al., 2013).

Test Stimuli and Protocol

Following orientation, sessions 2-4 began by presenting 5 stimuli (sucrose, citric acid, NaCl, MSG/IMP, and quinine) on 4 quadrants of the tongue (right and left tip, right and left CV) in a rotating fashion. These test stimuli were not analyzed here, but are included here briefly for completeness. Refer to Appendix A for more detail describing presentation of stimuli. Stimuli were presented in a blocked counterbalanced order, with all 5 stimuli being presented each day for a total of 20 stimuli (each of the 5 tastants in each of the 4 quadrants). After 10 applications, the participant took a break and performed a different task. All five tastants were presented before the same stimulus was presented again.

Participants completed a multiple attribute time intensity (MATI) task for a single irritant after the 10 spatial stimuli described above. Each day consisted of a different irritant, with the irritant remaining constant throughout the session. The irritants presented in this study consisted of ethanol, piperine, and capsaicin; only ethanol results will be discussed here. A 50% v/v ethanol stimulus was presented to the posterior tongue by touching two saturated 'buddy-taped' cotton swab applicators on either their left or right CV for 10 seconds. Intensity ratings were collected every 30 seconds for a total of 3 minutes. Intensity ratings for six qualities were collected (sweetness, bitterness, sourness, burning/stinging, umami/savory and saltiness); the order of the attributes was fixed. Participants were asked to keep their tongue away from the roof of their mouth for the entire 3 minutes and to keep their lips closed to minimize evaporative

cooling. Participants were not allowed to rinse for the 3-minute duration. Following the MATI task, there was a four-minute break where participants were allowed to rinse with mouth temperature reverse osmosis (RO) water.

Following the first MATI task, 10 additional spatial stimuli were applied, and then the second MATI task was completed for the CV papillae on the opposite side. Next, Best Estimated Thresholds (BETs) were collected using the three alternative forced choice (3-AFC) method described in ASTM E-679; these data are not reported here, as detection thresholds do not predict ingestive behavior (Duffy, Peterson, et al., 2004; Lucas, Riddell et al., 2011). These methods are reported in greater detail in Appendix A. The final task within the session involved swishing a 15mL sample of 16% v/v ethanol in the mouth for 5 seconds. Upon spitting out the sample, the participant rated the 'overall intensity' on a gLMS.

The single time point spatial data for the prototypical tastants serve as a negative control here; analysis of a superset of the present data (from (Feeney and Hayes, 2014)) indicate the means for the side tastes/sensations for each tastant were extremely low. For example, mean bitterness for sucrose, citric acid and sodium chloride were 0.3, 3.0, 2.2, respectively. Similarly, mean burning/stinging were 0.35, 1.08, and 0.80. In contrast, means for the expected qualities of each (e.g. sourness for citric acid) were all 13 or higher (just below 'moderate' on a gLMS). While the single point rating is slightly different than the MATI ratings for irritants in terms of participant demand, it suggests participants successfully distinguished between the various qualities in the rating task.

Genetic Analysis

DNA was collected using Oragene salivary collection kits per manufacturer instructions (Genotek Inc, Ontario, Canada). To maximize coverage of *TRPV1* (chr. 17) variation, a tag SNP approach was used with tag SNPs identified in HapMap using the CEU reference population: rs4790521, rs4790522, rs224547, rs4790151, rs161364, rs8065080, rs150908, rs224534, rs222747, rs150846, rs161386, rs222749, rs7217945, rs161381, rs17707155, and rs222741). Additionally, bitter taste receptors SNPs in *TAS2R13* (chr. 12; rs1015443) and *TAS2R38* (chr. 7; rs713598, rs1726866, and rs10246939) were chosen based on previous literature. Genotypes were determined using Sequenom MassARRAY technology (Sequenom, San Diego, CA). Primers were purchased from Integrated DNA Technologies (Coralville, Iowa, USA). Genotypes were automatically assigned via MassARRAY software (Sequenom). Two technicians independently inspected all genotypes and 15% of samples were rerun to ensure reliability.

Statistical Analysis

Data were analyzed using SAS 9.2 (Cary, NC). For MATI data, area-under-the-curve (AUC) was calculated as a summary measure. To test AUC values for individual SNPs, analysis of variance (ANOVA) was performed via *proc mixed,* and posthoc comparisons were made via the Tukey-Kramer method. For SNPs that showed significant associations with AUC ratings, repeated measures ANOVAs were conducted for bitter and burning/stinging ratings using time as a repeated factor via *proc mixed.* If the SNP-by-time interaction was significant, means for the two most extreme groups at each time point were compared using unadjusted t-tests via the LSMEANS option.

Haploview (Barrett et al., 2005) was used to examine the extent of linkage disequilibrium (LD) between each SNP. Haplotype blocks were defined according to Solid Spine of LD criteria (Barrett et al., 2005). LD plots show rounded R-squared values in individual squares.

Results

On the posterior tongue (i.e. on the circumvallate papillae), burning/stinging was the predominant quality for the swab saturated with 50% v/v ethanol, followed by bitterness and sweetness. Means were taken from the ratings for the left and right circumvallate papillae, and the AUC across time was calculated for each participant. Burning/stinging AUCs and bitter AUCs were positively correlated (R^2=11%; p=0.0013). Sweetness and burning/stinging AUCs were significantly correlated (R^2=6.0%; p=0.0183); however sweetness AUCs were not significantly correlated with bitter AUC (p=0.2). For the whole mouth sip and spit procedure using 16% v/v ethanol in water, overall intensity means fell near 'very strong' on the gLMS (47.0±2.1 SEM). Whole mouth ratings of 'overall intensity' for 16% ethanol were also associated with both bitterness and burning/stinging AUCs from 50% ethanol swabs on the CV (R^2= 4.8%; p=0.034 and R^2= 4.5%; p=0.042, respectively).

Figure 5-1 shows the linkage disequilibrium (LD) for the TRPV1 SNPs. R^2 values were reported and haplotypes were generated using Solid Spine of LD criteria (Barrett et al., 2005). Overall, 6 haplotypes were generated showing strong LD between neighboring SNPs, with the exception of rs150908, rs150846, and rs222741, all of which are located within the intronic region. Of the 16 *TRPV1* SNPs included in the genetic analysis (shown in figure 5-1), 3 SNPs exhibited a minor allele frequency (MAF) below 0.25 in our cohort: rs222741, rs161381 and rs222749. As these genotypes had too few participants to perform meaningful statistical analysis

due to low sample number in the minor allele group, they were excluded from further analysis.

The SNPs used in the present analysis are outlined in Table 5-1.

Figure 5-1: Linkage disequilibrium plot (r^2) for 16 TRPV1 SNPs from 93 participants of European ancestry. Darker gray indicates higher r^2 values.

Table 5-1: List of TRPV1 SNPs included in the analysis.

								Reported p-values		
									AUC ratings:	
Receptor	chr.	SNP ID	call rate	HWE p-value	maj/min allele	MAF	SNP location	EtOH WM	Burn	Bitter
TRPV1	17	rs4790521	97.9%	0.71	T/C	0.37	3' UTR	0.35	0.19	0.0033
TRPV1	17	rs4790522	98.9%	0.95	C/A	0.42	3' UTR	0.12	0.79	0.50
TRPV1	17	rs224547	98.9%	0.57	A/G	0.43	Intronic	0.07	0.054	0.0044
TRPV1	17	rs4790151	98.9%	1.0	G/A	0.3	Intronic	0.16	0.84	0.35
TRPV1	17	rs161364	98.9%	0.39	C/T	0.3	Intronic	0.63	0.0021	0.30
TRPV1	17	rs8065080	96.8%	0.96	T/C	0.37	Ile585Val	0.17	0.70	0.43
TRPV1	17	rs150908	95.7%	0.07	G/A	0.35	Intronic	0.80	0.53	0.33
TRPV1	17	rs224534	98.9%	0.56	G/A	0.37	Thr469Ile	0.17	0.94	0.49
TRPV1	17	rs222747	96.8%	0.56	C/G	0.28	Met315Ile	0.48	0.78	0.74
TRPV1	17	rs150846	96.8%	1.0	C/T	0.39	Intronic	0.22	0.86	0.55
TRPV1	17	rs161386	98.9%	0.59	C/T	0.38	Intronic	0.88	0.62	0.23
TRPV1	17	rs7217945	98.9%	0.28	G/A	0.28	Intronic	0.83	0.06	0.27
TRPV1	17	rs17707155	98.9%	0.58	C/T	0.26	Intronic	0.78	0.91	0.39
TAS2R13	12	rs1015443	98.5%	0.28	C/T	0.35	Ser259Asn	0.04	0.98	0.36
TAS2R38	7	rs713598	98.4%	0.37	G/C	0.49	Ala49Pro	0.32	0.87	0.0048
TAS2R38	7	rs1726866	97.7%	0.53	C/T	0.50	Val262Ala	0.86	0.57	0.21
TAS2R38	7	rs10246939	98.5%	0.73	C/T	0.48	Ile296Val	0.87	0.68	0.055

P values are generated via ANOVA. The reported p values are unadjusted.

Exploratory Genotype-Phenotype associations based on AUC scores over time

An intronic *TRPV1* SNP rs224547 (chr. 17) was associated with the summary AUC scores for both bitterness and burning/stinging from 50% v/v ethanol applied to the circumvallate papillae. Burning/stinging AUC scores were associated with rs224547 genotype ($F(89,2)=3.02$; $p=0.0539$). The AA homozygotes (n=32) had the greatest mean AUC with 936.68 (±132.70 SEM) compared to the AG heterozygotes (n=40) who had the smallest mean area of 505.03 (±118.69) with the GG homozygotes (n=20) having a mean area of 772.69 (±167.86). Bitterness AUC scores were also significant for rs224547 ($F(89,2)=5.76$; $p=0.0044$) and in the same direction as burning/stinging values, with the AA homozygotes having the highest mean area. The AA homozygotes reported the greatest mean area, 687.54(±108.92), with the heterozygotes having a mean of 256.22(±97.42). The GG homozygotes had the smallest area for bitterness: 186.75(±137.77).

A second *TRPV1* SNP, rs4790521, was also a significant predictor of bitterness AUC ratings of 50% v/v ethanol on the circumvallate papillae ($F(88,2)=6.09$; $p=0.0033$). This finding is not surprising as rs4790521 is in strong linkage disequilibrium with rs224547, as shown in Figure 5-1. The CC homozygotes (n=14) had the highest mean area for bitterness: 860.09(±164.70). The CT homozygotes (n=41) had a mean area of 419.45(±96.25), with the TT homozygotes (n=36) with the lowest mean area of 185.73(±102.71).

A third *TRPV1* SNP, rs161364, also associated with the AUC burning/stinging ratings for 50% v/v ethanol on the circumvallate papillae ($F(89,2)=6.61$; $p=0.0021$). The TT homozygotes (n=7) had a mean area of 1528.93(±273.59), which was significantly greater ($p=0.001$) than the

CT heterozygotes (n=37), who had a mean area of 476.55(±119.00). The CC (n=48) homozygotes had a mean area of 746.95(±104.48), which was significantly lower than the TT homozygotes (p=0.03); the CC homozygotes did not differ from the CT heterozygotes (746.95 versus 476.55; p=0.15).

TRPV1 SNPs associate with the perception of ethanol

Two SNPs that were significant for the summary AUC estimate across time for the 50% v/v ethanol swab (rs224547 and rs4790521) were analyzed further to explore effects across time; bitterness and burning/stinging at each time point (0,30,60,90,120,150 and 180 seconds) were tested via repeated measures ANOVA. The third significant *TRPV1* SNP, rs161364, was not analyzed further across time due to due to low frequency of the TT homozygotes (n=7).

In repeated measures ANOVA on the bitterness ratings, the time by SNP interaction was significant for the *TRPV1* rs4790521 SNP [$F(12,528)=3.51$, $p<0.001$], as shown in the top panel of Figure 5-2. In the first two minutes after application (i.e. at 0, 30, 60, 90, and 120 seconds), bitterness ratings were significantly different across rs4790521 genotype, with the TT homozygotes giving significantly higher ratings than the CC homozygotes. However, as bitterness decayed after 120 seconds, genotype no longer associated with bitterness, presumably due to floor effects. In repeated measures ANOVA on the burning/stinging ratings, we observed significant main effects for SNP [$F(2,88)=5.36$, $p=0.0064$)], and time [$F(6,528)=25.71$, $p<0.0001$); the time by SNP interaction for the rs4790521 SNP was not significant [$F(12,528)=0.53$; $p=0.89$]. Nonetheless, the pattern was similar to the bitter results as the TT homozygotes tended to report the lowest sensations.

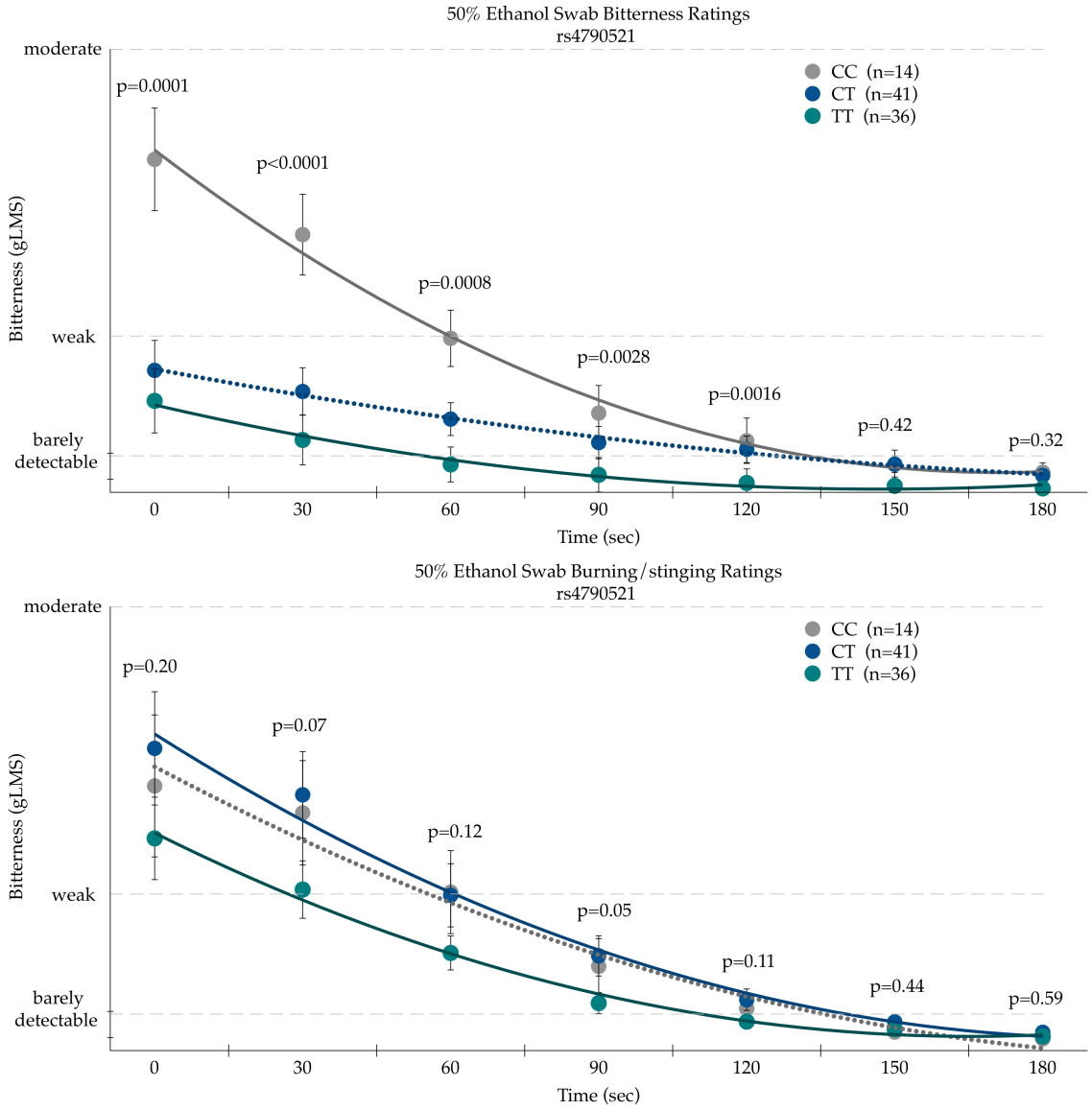

Figure 5-2: Bitterness (top) and burning/stinging (bottom) from 50% ethanol applied to the posterior tongue differs by the TRPV1 SNP rs470521 in repeated measures ANOVA (see text for details). Points are arithmetic means with bars showing the standard error of the mean. *p*-values indicate unadjusted *t*-tests at each time point comparing the two groups of homozygotes to decompose the significant time by SNP interaction.

The second significant SNP in the AUC analysis for bitterness and burn, rs224547, was subsequently analyzed across time. In repeated measures ANOVA for bitterness, there was a main effect of SNP [$F(2,89)=21.40$, $p<0.0001$] and time [$F(6,534)=13.33$; $p<0.0001$], but the influence of the rs224547 SNP did not differ over time [$F(12,534)=0.13$, $p=0.99$]. As shown in Figure 5-3a, the AA homozygotes consistently reported more bitterness than the GG homozygotes. In repeated measures ANOVA on the burning/stinging ratings (Figure 5-3b), there was a main effect of the rs224547 SNP [$F(2,89)=9.10$; $p=0.0003$] and time [$F(6,534)=31.14$; $p<0.0001$], but the influence of this SNP did not differ over time, as the interaction was not significant [$F(12,534)=0.83$, $p=0.62$].

In contrast to the time course data on the posterior tongue, none of the *TRPV1* SNPs tested explained differences in 'overall intensity' ratings of a whole mouth sip and spit solution of 16% v/v ethanol.

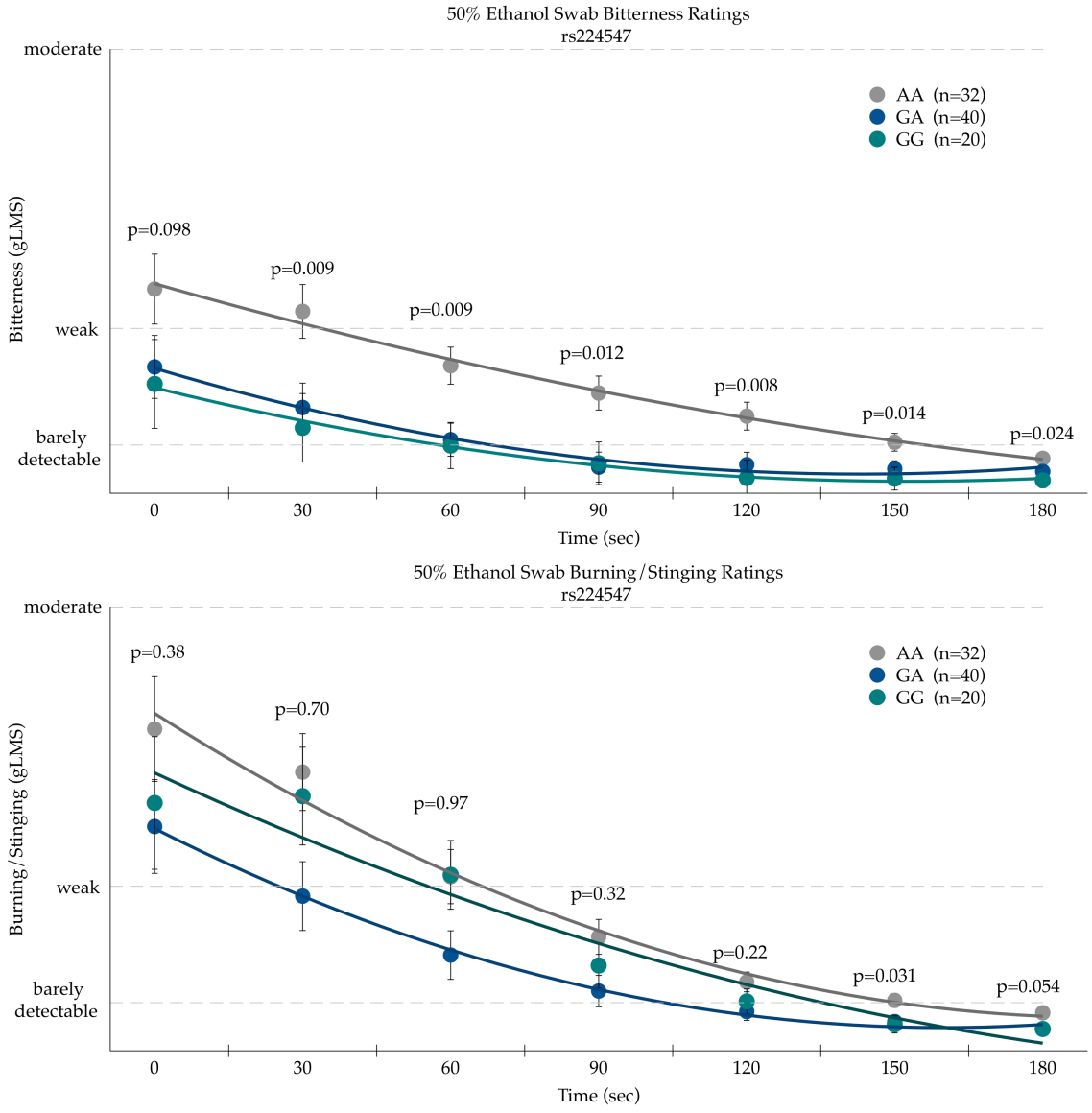

Figure 5-3: Bitterness (top) and burning/stinging (bottom) from 50% ethanol on the posterior tongue differ by the rs224547 SNP in TRPV1 in repeated measures ANOVA (see text for details). Points are arithmetic means with bars showing the standard error of the mean.

TAS2R SNPs associate with the perception of ethanol

Three SNPs in *TAS2R38* (chr. 7, rs713598, rs1726866, and rs10246939, resulting in A49P, V262A, and I296V, respectively) were explored for their association with ethanol bitterness. There was a significant association with A49P (rs713598) with AUC bitterness values for the 50% ethanol swab on the CV [$F(2,89)=3.13$;$p=0.048$]. The Pro49Pro homozygotes (n=18) reported the most bitterness (717.9±149.2); heterozygotes (n=49) and the Ala49Ala homozygotes (n=24) had similar mean areas (240.5±90.4 and 255.2±126.6, respectively). The second SNP Val262Ala (rs1726866) was not associated ($p=0.21$) with bitterness AUC, whereas the third SNP Ile296Val (rs10246939) was associated with bitterness AUC [$F(2,89)=3.00$;$p=0.0549$]. The Val296Val homozygotes (n=19) had the greatest bitterness AUC (696.1±145.4), followed by the heterozygotes (n=50) (345.7±89.6), while the Ile296Ile homozygotes (n=23) reported the least bitterness (238.04±132.2). Accordingly, these three SNPs were chosen for further analysis to explore associations between genotype and mean bitterness on the CV over time (Figure 5-4).

Repeated measures ANOVA indicated the main effects of Ala49Pro genotype [$F(2,89)=13.40$, $p<0.0001$] and time [$F(6,534)=14.84$, $p<0.0001$] were associated with bitterness, although the SNP effect did not vary across time as the time by SNP interaction was not significant [$F(12,534)=1.0$, $p=0.44$]. As shown in Figure 5-4a, the Pro49 homozygotes experienced greater bitterness than Ala49 homozygotes. A similar pattern was observed for Val262Ala (Figure 5-4b), with significant main effects of genotype [$F(2,88)=6.50$, $p=0.0023$] and time ($F(5,528)=14.12$, $p<0.0001$) for bitterness. The time by genotype interaction was not significant for Val262Ala [$F(12,528)=0.76$, $p=0.69$], indicating the effect of genotype did not change over time. As shown in Figure 5-4b, the Ala262 homozygotes reported more bitterness. The Ile296Val SNP in *TAS2R38* showed a similar pattern as the Ala49Pro and Val262Ala SNPs;

the main effects of genotype [F(2,89)=12.96, p<0.0001] and time [F(6,534)=14.07, p<0.0001] were significant for bitterness, and the effect of genotype did not differ over time [F(12,534)=1.13, p=0.34]. As shown in Figure 5-4c, the Val296 homozygotes reported more bitterness than the Ile296 homozygotes. In summary, the consistency across these three SNPs is to be expected due to high linkage disequilibrium (Kim et al., 2003). However, due to the novel association with ethanol sensations described here, we report each separately as it is possible that prior site directed mutagenesis studies which indicate the relative importance of each site for propylthiouracil may not generalize to ethanol.

In contrast to the time course data, these same three SNPs (rs713598, rs1726866, and rs10246939, resulting in A49P, V262A, and I296V) were not associated with the 'overall intensity' ratings of whole mouth 16% ethanol (p=0.32; p=0.86; and p=0.87, respectfully).

Finally, Asn259Ser (rs1015443), a SNP in the bitter taste receptor *TAS2R13* (chr. 12) previously implicated with regard to alcohol intake (Dotson et al., 2012) was significantly associated with 'overall intensity' ratings of whole mouth 16% ethanol (F(2,89)=3.31;p=0.041). The Asn homozygotes (n=12) reported having the highest mean ratings 52.7(±5.8), with the heterozygotes (n=49) rating 50.3(±2.9) and the Ser homozygotes (n=31) reporting the least intensity 39.5(±3.6). Although AUC ratings for bitterness and burning/stinging were not significant (Table 5-1), we performed a repeated measures ANOVA for the MATI data due to significant associations between Asn259Ser and whole mouth intensity ratings. There were significant main effect of genotype [F(2,89)=4.05, p=0.021] and time [F(6,534)=11.16, p<0.0001] for bitterness; however, the interaction of time and SNP was not significant [F(12,534)=0.4, p=0.96] (not shown).

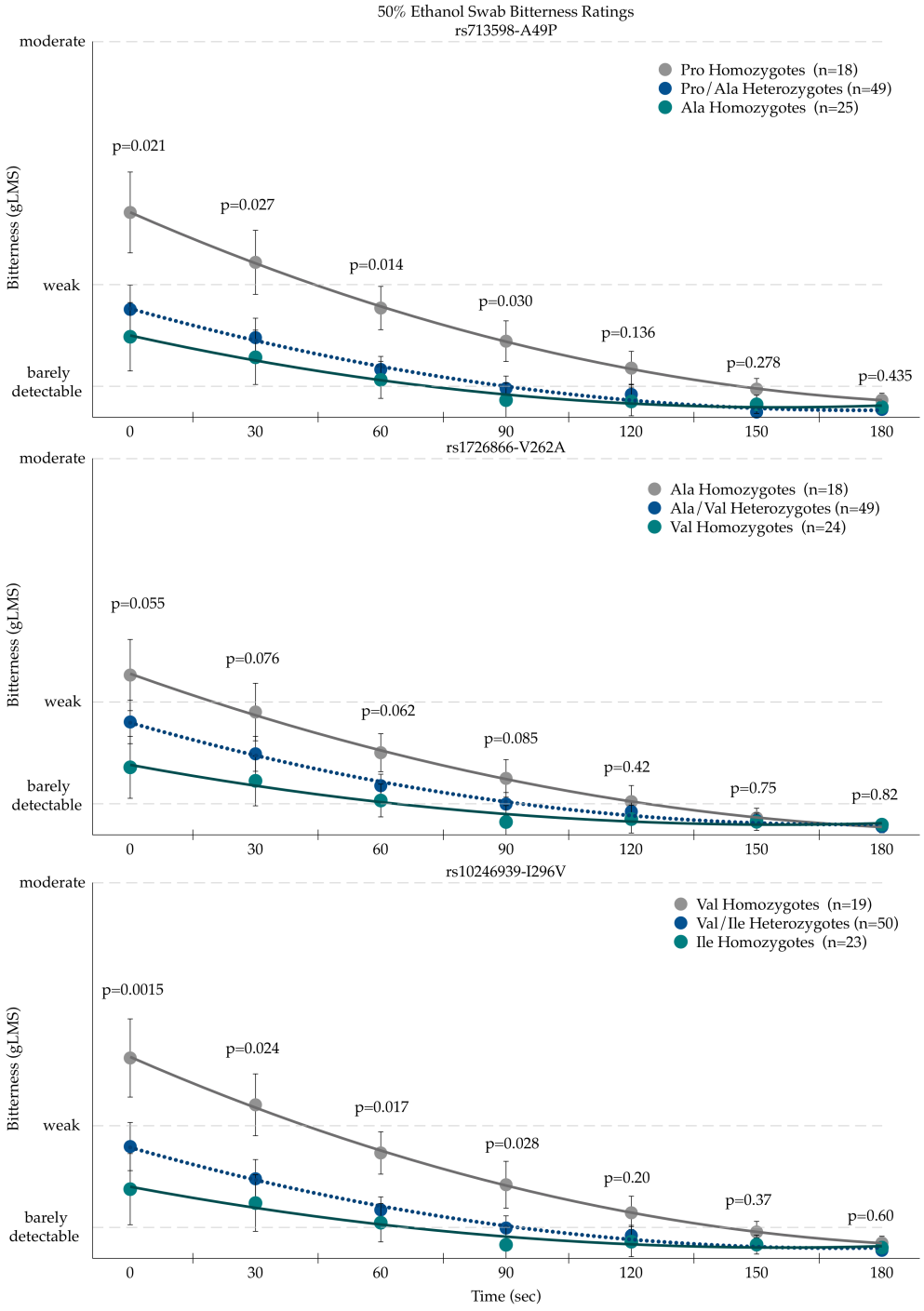

Figure 5-4: Bitterness from 50% ethanol applied to the posterior tongue differs by the TAS2R38 SNPs rs713598 (top), rs1726866 (middle) and rs10246939 (bottom) in repeated measures ANOVAs (see text for details). Points are arithmetic means with bars showing the standard error of the mean.

Discussion

In our cohort, burning/stinging was reported to be the predominate sensation for a 50% ethanol solution applied to the circumvallate papillae, followed by bitterness and sweetness. Summary measures of burning/stinging across time (AUC values) were significantly correlated with summary measures of bitterness across time. Additionally, 'overall intensity' ratings of a whole mouth 16% ethanol solution at a single time point were significantly associated with summary measures of burning/stinging and bitterness over time. Collectively, this suggests those who experience more burn from ethanol also experience more bitterness.

Due to ethanol activating *TRPV1 in vitro* (Trevisani et al., 2002), there is reason to believe that polymorphisms in *TRPV1* might alter the perceived burn from ethanol if the SNPs are functional. The TRP box, a 6-mer region located near the channel gate (C terminus domain) found in all TRP channels, has shown to be key in TRPV1 function (García-Sanz, Valente et al., 2007; Gaudet, 2010; Valente, García-Sanz et al., 2008). This same finding has been shown for TRPM8 for activation from menthol (Bandell, Dubin et al., 2006). Mutations within the TRP box in TRPV1 eliminate response to 1uM capsaicin *in vitro* (Valente et al., 2008). Valente and colleagues (2008) hypothesize reduced activation is due to maintaining the gate in a closed state. *TRPV1* SNPs rs4790521 and rs224547 exhibit strong LD and surround the TRP box. These two SNPs may be in LD with polymorphisms within the TRP box.

Differences in the burning/stinging of ethanol associated with the *TRPV1* SNP rs224547, with AA homozygotes experiencing greater burning/stinging compared to GG homozygotes. However, we did not expect this SNP to associate with bitterness, as was also observed here. Moreover, *the TRPV1* SNP rs4790521 was also found to be associated with bitter AUC ratings, with CC homozygotes reporting significantly more bitterness within the first minute of the CV

being exposed to 50% ethanol. While unexpected, previous reports suggest that bitter and burn sensations are perceptually similar, even though they are thought to be transduced through separate pathways. Lim and Green (2007) reported bitterness from quinine was more similar to the burn from capsaicin than the other prototypical tastes (sour, salty and sweet), suggesting that these two sensations are similar, serving as part of a 'chemofensor complex' (Green, 2012). Other evidence shows the prototypical burning stimulus capsaicin evokes bitterness in some individuals (Green and Hayes, 2004; Green and Schullery, 2003; Lawless and Stevens, 1984). Additionally, non-nutritive sweeteners activate TRPV1 *in vitro* (Riera, 2007; Riera, Vogel et al., 2008) and are often described as bitter by humans (e.g. Allen et al., 2013). Collectively, these data might suggest a simple labeled line receptor-percept hypothesis may be overly reductionist, as burn and bitterness may not be as independent as previously believed. However, this explanation is complicated by the weak phenotypic association observed between burning and stinging, and bitterness in our phenotypic data. Alternatively, we cannot rule out that the genotype-phenotype associations observed here could potentially be an artifact caused by the fixed order of the scales (Bennett, Zhou et al., 2012; Green, Lim et al., 2010), semantic confusion between attributes (Bennett et al., 2012), affective dumping, or some combination thereof. That is, participants always rated sweetness, followed by bitterness and sourness before burning/stinging, which may bias participants toward dumping aversive sensations into the first aversive attribute available to them (bitterness versus burning). Additional work is needed to clarify the relationship between TRPV1 and aversive sensations. Nonetheless, present data suggest that sensations from sampled ethanol vary as a function of genetics, consistent with the idea that variation in chemosensory genes can influence ingestive behavior (Hayes, Feeney, et al., 2013).

These findings suggest that individuals with AA genotype for SNP rs224547 and/or CC genotype for rs4790521 may potentially associate with reduced alcoholic consumption if they

perceive greater bitterness and/or burn from alcohol, which would be expected to deter consumption of alcoholic beverages, at least initially before dependence and reward related associations develop. Present results should be considered provisional until replicated, and additional work testing whether these SNPs associate with alcohol use is warranted.

The *TRPV1* SNP rs161364 was significantly associated with burning AUC ratings. The C allele carriers (CC and CT) did not differ from each other, but the TT homozygotes were different from both groups of C allele carries. This suggests the C allele may be associated with decreased function. However, due to the low frequency of TT individuals, we caution that this finding needs to be replicated to determine the possible functionality of this SNP.

Variations in bitter taste receptor genes have been shown to explain reported bitterness from a wide range of compounds and foods (reviewed by Hayes, Feeney, et al., 2013)). Here, we report the perceived bitterness from 50% ethanol on the posterior tongue was significantly associated with the *TAS2R38* SNPs rs713598 (A49P), rs172866 (Val262A) and rs10246939 (I296V). This is not surprising, as bitter sensations have been reported previously from ethanol (e.g. Mattes and DiMeglio, 2001; Scinska et al., 2000), and alcoholic beverages (e.g. Lanier et al., 2005). Previous work exploring relationships between taste perception and PROP phenotype reported that individuals who perceived propylthiouracil (PROP) as being more bitter also reported greater irritation for ethanol solutions than individuals who reported no bitterness from PROP (Bartoshuk et al., 1993). Since 2003, it has been widely accepted that the perception of PROP is largely explained by three SNPs in *TAS2R38* (Bufe et al., 2005; Duffy, Davidson, et al., 2004; Hayes et al., 2008; Meyerhof et al., 2010) as they exhibit strong linkage disequilibrium (Kim et al., 2003). Polymorphism in *TAS2R38* are associated with food intake via differential sensations (see Duffy et al., 2010; Feeney, 2011; Tepper, 2008) and polymorphisms in other TAS2Rs have also associated with differences in the sensations from foods and beverages. For

example *TAS2R19* variation associates with the bitterness of quinine (Reed et al., 2010), while SNPs in *TAS2R9* and *TAS2R31* associate with the bitterness from acesulfame-K (Allen et al., 2013). Accordingly, it should not be surprising that the bitterness of ethanol associates with variation in *TAS2R* genes that encode bitter taste receptors.

Previously, SNPs in another bitter receptor gene, *TAS2R13,* were found to associate with alcohol intake frequency in a cohort of head and neck cancer patients. Dotson and colleagues (2012) reported that rs1015443 CC homozyogtes reported greater frequency of drinking days, drinks per drinking day, and heavy episodic drinking. Here, we provide evidence the same SNP associates with the overall intensity of whole mouth ethanol, with the CC homozygotes reporting lower ratings than heterozygotes or TT homozygotes.

One must keep in mind the differences between ethanol and alcoholic beverages, as our participants did not sample alcoholic beverages, only ethanol diluted with water. Spirits, beer and wine contain sensory active compounds beyond just ethanol, and these compounds may suppress bitterness (via perceptual masking) or add additional bitterness (e.g. hops). For the whole mouth 16% ethanol solution used here, only 'overall intensity' ratings were collected, so we cannot distinguish between separate percepts like burning and bitterness when regions beyond the circumvallate, like the palate or anterior tongue, are stimulated. Also, this stimulus was delivered via a sip and spit method, so involvement of the pharynx was minimal. Genetic association studies are by definition quasi-experimental (since one cannot randomly assign to genotype); therefore associations may reflect the impact of unmeasured third variables (genetic or otherwise). While limiting the study to Caucasians reduces the threat of population stratification, it does not completely control for this possibility.

Conclusions

Previous work associates SNPs in *TAS2R13* and *TAS2R38* with alcohol intake, and it is assumed differential intake results from differences in sensation. Here, we provide the first evidence ethanol sensations differ with these TAS2R polymorphisms. The novel results reported here should be considered provisional until such a time as they may be replicated in a larger sample. Nonetheless, present data suggest a relationship between sensations from ethanol and genetic variation in *TAS2Rs* and possibly *TRPV1*.

Acknowledgments

The authors thank Dr. Emma Feeney, Dr. Nadia Byrnes, and Meghan Kane, BS for assisting in psychophysical data collection, and Samantha Bennett, MS for assistance with protocol development. We also thank our participants for their time and participation.

Funding

Supported by National Institutes of Health grants from the National Institute National of Deafness and Communication Disorders to JEH [DC01904] and the National Center for Research Resources to JEM [RR023457], and Shared equipment grants (ShEEP) from the Medical Research Service of the Department of Veteran Affairs to JEM. ALA was also supported by the National Center for Advancing Translational Sciences via UL1 [TR000127] and TL1 [TR000125] grants. The views expressed in this article are those of the authors and do not necessarily reflect the position or policy of the Department of Veterans Affairs.

CHAPTER 6

Capsaicin hypoalgesia: the effects rinsing daily with capsaicin

Adapted from:

Nolden, A.A. and Hayes, J.E.

"Capsaicin hypoalgesia: the effect of daily capsaicin exposure"

Submitted to Chemosensory Perception for review.

Abstract

Capsaicin, a bioactive compound naturally found in chili peppers, is a popular ingredient in dishes worldwide. Frequent chili pepper consumption has been associated with decreased capsaicin ratings in the laboratory. The occurrence of this phenomenon, capsaicin hypoalgesia (commonly called capsaicin desensitization) may also have therapeutic value. The present study investigates the development and extent of hypoalgesia following repeated exposure to oral capsaicin at low doses. Participants completed 4 in-person visits, each one week apart, and rated capsaicin (0.2, 1.2 and 5.7 ppm) and control stimuli on a general Labeled Magnitude Scale (gLMS). The first week served as a run-in period, and for the remaining two weeks participants rinsed with either a treatment (1.2 ppm capsaicin) or control (20uM sucrose octaacetate) solution twice per day. Our data suggest that rinsing with 1.2 ppm capsaicin results in a small but significant reduction in perceived burn intensity of sampled capsaicin, compared to rinsing with the control solution. Further work is needed to understand relationships between the dose of capsaicin, along with frequency and duration of exposure that are required to reduce capsaicin

burn, and to investigate the time to recovery. This work provides novel data on the development of hypoalgesia with low dose exposure. Capsaicin has been used for treatment for chronic pain and is thought to have other health benefits. Understanding the development of hypoalgesia and desensitization may be beneficial for future treatment of clinical populations.

Introduction

Capsaicin and dihydrocapsacin are two bioactive and sensory active compounds that belong to a family of pungent compounds known as capsaicinoids. These two compounds are the most abundant capsaicinoids in chili peppers (Sanatombi and Sharma, 2008). They evoke warming, burning and stinging sensations, which are a part of a broader group of sensations that arise via chemical stimulation of the somatosensory system (i.e., chemesthesis) (Green, 1996a; Hayes, 2016). Classical taste stimuli, for example NaCl and sucrose, typically show adaptation (reduced response) following periods of constant exposure (Meiselman, 1968). However, reduced responses psychophysically are not limited to taste stimuli, as they have also been observed for chemesthetic stimuli. While reduced response for taste compounds has been termed adaptation, reduced response to chemesthetic compounds has been termed desensitization; however, little is known about the mechanisms leading to this reduced response. Both adaptation and desensitization refer a reduced response following repeated application; however the mechanisms regulating these responses are not well understood. Additional research is needed to understand the mechanisms leading to decreased response following exposure to both prototypical tastes and chemesthetic stimuli. Furthermore, there is additional confusion regarding this terminology, as desensitization refers to decreased receptor and neuron activity. The more accurate term to

describe a decrease in psychophysical response is hypoalgesia (Purves et al., 2001a). Thus, for consistency, hypoalgesia will be used.

Repeated intermittent capsaicin exposure can result in hypoalgesia (e.g. (Jancso, 1968; Karrer and Bartoshuk, 1991). Acute oral hypoalgesia (within a single test session) in humans has been reported following oral exposure to capsaicin (Dessirier et al., 2000; Green, 1989, 1991b; Jancso, 1968; Karrer and Bartoshuk, 1991), menthol (Cliff and Green, 1996; Dessirier, O'Mahony et al., 2001), piperine (Dessirier et al., 1999), ethanol (Prescott and Swain-Campbell, 2000), eugenol (Klein et al., 2013), carvacrol (Klein et al., 2013), nicotine (Dessirier et al., 1997; Dessirier et al., 2000) and cinnamonaldehyde (Prescott and Swain-Campbell, 2000). Conversely, only a few of these compounds have been shown to produce sensitization, or more accurately hyperalgesia (i.e., increased ratings following exposure), under other exposure conditions (Dessirier et al., 2000; Green, 1989, 1991b; Prescott, 1999). Whether or not an individual experiences acute hypoalgesia or hyperalgesia within a test session has been attributed to the wait time between exposures (i.e., the inter-stimulus interval) (Green, 1991b; Prescott, 1999) and the specific stimulus. Unlike gustatory adaptation, which is transient, hypoalgesia to chemesthetic sensations can also last across days; these are the effects we are interested in here.

Previously, chronic hypoalgesia in humans has generally been investigated in two complementary ways: either, i) by exploring differences in capsaicin responses in adults on ad libitum diets, comparing habitually high and low consumers of chili peppers, or ii) by comparing individuals systematically exposed to known doses of capsaicin in the laboratory. Free-living adults who report consuming chili peppers typically give lower ratings compared to individuals reporting little or no consumption of chili peppers when given the same stimulus. This pattern is widely assumed to result from chronic hypoalgesia due to repeated capsaicin exposure (Cowart, 1987; Lawless et al., 1985; Prescott and Stevenson, 1995; Stevenson and Prescott, 1994;

Stevenson and Yeomans, 1993). Conceivably, this result could also result from a criterion shift in scale usage due to prior experience (Stevenson and Prescott, 1994), but recent work suggests this is not the case (Nolden and Hayes, 2017).

Only a few published studies have used controlled capsaicin exposure to study chronic hypoalgesia across days (Green, 1996b; Green and Rentmeister-Bryant, 1998; McBurney et al., 1997). Others have also explored capsaicin hypoalgesia across days, but they were focused on the recovery time across days following a single dose of capsaicin (Karrer and Bartoshuk, 1991, 1995). These studies have used a wide range of capsaicin concentrations 5 ppm up to 100 ppm that were delivered to the tongue via cotton swabs or filter paper discs placed directly on the tongue. Thus, these exposure protocols, while effective and well controlled, do not directly represent daily consumption of chili peppers. Two studies more closely mimic dietary exposure: McBurney and colleagues (1997) and Green and Rentmeister-Bryant (1998) used capsaicin laced candy (butterscotch or taffy, respectively) as their delivery systems. After consuming a 75 ppm capsaicin butterscotch candy for three days, participants significantly decreased (40%) in their response to a 100ppm capsaicin stimulus applied via a filter paper disk (McBurney et al., 1997). In a different study, participants consumed 3 taffy candies for three days, with each candy containing 5-9 ppm capsaicin. There was significant decrease between ratings of the taffy candy on day one and days two and three within the first two minutes of chewing the candy (Green and Rentmeister-Bryant, 1998). Collectively, these studies leave unanswered questions as to whether lower doses and longer exposure times, such as those more aligned with daily consumption in the typical diet, are able to cause chronic capsaicin hypoalgesia.

Materials and Methods

Overview

To determine the effects of repeated capsaicin exposure on capsaicin perception, participants attend four one-on-one visits with a researcher. These visits were scheduled exactly one week apart. The first visit served as a warm-up, practice session with the scaling method (e.g., Green and Hayes, 2004), with the next visit serving as a baseline visit; following baseline, they were randomized and began the exposure protocol. This included rinsing with a mouthwash consisting of either capsaicin (treatment) or sucrose octaacetate (SOA; control) twice per day for two weeks. There were two follow-up visits, one mid-way through the exposure period, and one at the end. This exposure period was designed to mimic daily exposure to chili peppers and/or capsaicin containing foods among frequent consumers. Intensity ratings for various qualities were collected via a generalized Labeled Magnitude Scale (gLMS) (Snyder et al., 2004) using Compusense Cloud software (Guelph ONT).

Participants

Adults were recruited from The Pennsylvania State University and surrounding community (State College, PA) to participate in this three-week exposure study. Each participant completed 4 visits in a one-on-one setting, each one week apart. Each visit took place in the Penn State Sensory Evaluation Center, a custom built testing facility located in the Erickson Food Science Building on the main Penn State campus. Before enrolling in the study, interested individuals completed an online questionnaire to determine if they met the recruitment criteria. Eligibility criteria included: not pregnant nor breast feeding, non-smoker, no tongue, cheek or lip

piercing, no difficulty swallowing or history of choking, no known taste or smell defect, not taking prescription pain medication, no hyperactive thyroid and no history of chronic pain. Additionally, only infrequent consumers of chili peppers were invited to participate. This included individuals reporting consuming chili peppers (all types, including spicy sauces and condiments) 1-3 times per month or less who also reported a heat/spice level preference of medium or less in their food.

Forty-five participants (18 men) completed all visits. The study population had an average age of 26 (±1.2 std. err.). The majority reported Caucasian ancestry (n=36), with 6 reporting Asian ancestry and 3 reported 'other ancestry not listed'. Procedures were IRB approved and informed consent was obtained. All participants were compensated for their time with a small cash payment.

Test stimuli and sampling procedure

During each visit, participants sampled 6 stimuli in triplicate. These stimuli included three levels (0.2, 1.2 and 5.7 ppm) of natural capsaicin (Sigma-Aldrich; Sigma #360376), each containing 0.002-0.057% Polysorbate-80 (Tween; J.T. Baker; Tween) as an emulsifier. Control stimuli included 0.5M sucrose (Domino), 0.32M NaCl (Macron) and 20uM sucrose octaacetate (Sigma-Aldrich). All control stimuli were made with RO water. The SOA stimulus contained 0.001% Tween for consistency between control and treatment stimuli (see *Exposure Stimuli and Protocol*).

Capsaicin concentrations were selected to be approximately weak, moderate and strong on a gLMS, based on a previously obtained dose response function for capsaicin (Nolden and Hayes, 2017). The natural capsaicin used here is actually a mix of capsaicin and dihydrocapsaicin

(~65% / ~35%, respectively, with variability between lots) but for simplicity will be referred to as capsaicin throughout the manuscript, as the two compounds have similar potency. A single stock solution was made with capsaicin dissolved in 1% Tween. The stock was then diluted with reverse osmosis (RO) water using volumetric glassware to reach the final desired concentrations, with all final capsaicin concentrations containing 0.002, 0.012%, and 0.057% Tween for 0.2, 1.2 and 5.7ppm capsaicin, respectively.

All stimuli were presented at room temperature in 10mL aliquots in plastic medicine cups labeled with a random three-digit blinding code. Before beginning the tasting portion of the study, participants rinsed with room temperature RO water. Each sample was swished in the mouth for 5 seconds then spit out. Participants waited 10 seconds before making their ratings on a gLMS. Samples were presented in a blocked balanced order, with all samples presented once before any duplicates were presented. The highest capsaicin stimuli (5.7 ppm) were presented in the 6^{th}, 12^{th} and 18^{th} position, followed by a longer three-minute break. During this 3-minute break and between all other stimuli (a forced one-minute break) participants were asked to rinse with room temperature RO water until they no longer perceived any sensation in their mouth.

Exposure mouthwash and Protocol

During the second visit, participants were randomly assigned to the treatment or control group. Among completers, there were roughly equal numbers of participants in each group: control (SOA; n=23) or treatment (capsaicin; n=22). The exposure (mouthwash) protocol was described to each participant during the first visit to ensure they understood the protocol fully and were willing to participate. Participants were asked to swish with 15mLs of the mouthwash solution (either capsaicin or SOA) for 30 seconds, twice per day for 14 days. Participants were

asked to refrain from eating or drinking for at least 1 minute after expectorating the solution. Participants were provided a fresh bottle of the mouthwash (210mLs) in sessions 2 and 3 to take home; any missed rinses were reported at the subsequent visit (session 3 and 4).

All mouthwash was made with RO water and stored at room temperature. The treatment mouthwash consisted of 1.2 ppm capsaicin and the control mouthwash consisted of 20uM SOA, each containing Tween (0.0012% and 0.001%, respectively). The same protocol was used to make mouthwash as stimuli (see *Stimuli and sampling procedure above*). Mouthwash was provided in a 250mL plastic (HDPE) light-block bottle (Bel-Art Products).

Orientation and Overview of Psychophysical Scaling

For all stimuli presented in each visit, participants rated the burning, bitterness, saltiness and sweetness intensity for each using a general Labeled Magnitude Scale (gLMS) (Snyder et al., 2004). The scale is labeled at 0 with ‘’NS’ (no sensation) and 100 with ‘the strongest imaginable sensation of any kind’, with additional adjectives placed at 1.4, 6, 17, 35 and 51 (‘barely detectable’ (BD), ‘weak’, ‘moderate’, ‘strong’ and ‘very strong’; respectively).

Prior to rating stimuli, participants were given verbal, followed by written instructions on the use of the scale (Snyder et al., 2004). Following this, participants practiced using the scale by rating 15 remembered or imagined sensations (e.g. Hayes, Allen, et al., 2013; Nolden and Hayes, 2015). This practice was to ensure participants were using the scale correctly and to compare use of the scale across visits. Remembered and imagined sensations included food and non-food and oral and non-oral items in order to emphasize that the scale should be used in the context to all possible sensations.

Survey data

Over the four visits, participants answered a variety of non-sample related questions. These included demographic questions, measures of compliance to the exposure protocol, and frequency of chili pepper intake and liking of spicy foods.

Compliance was measured by having participants report the number of missed rinses for each week (week 1 and 2). To characterize consumption of chili pepper during the study, participants were asked to report the number of meals they consumed that contained capsaicin. We also asked four questions regarding overall liking and intake frequency of chili peppers at the end of the last visit. Participants reported their preferred heat/spice level when ordering food at a restaurant by selecting either: 'I avoid eating spicy foods', 'mild', 'medium', 'spicy' and 'very spicy'. Intake of chili peppers was estimated by having participants report how frequently they consumed chili peppers in the past month and how often they consume foods containing chili peppers that most people would consider medium to very spicy. Participants selected either: never, less than once per month, 1-3 times per month, 1-2 times per week, 3-4 times per week, 5-6 times per week, once per day, or 2 or more times per day. Lastly, participants were asked 'Do you like spicy foods?' by selecting either 'Yes, I like to eat spicy foods', 'No, I try to avoid spicy foods', or 'I don't have a strong preference'.

Statistical analysis

All analyses were conducted using SAS 9.2 (Cary, NC). Sex was coded as 0 for women and 1 for men. Within a visit, all samples were rated in triplicate. These ratings were averaged within each participant a priori, resulting in a single mean rating for each sample for each visit. Change scores were calculated for each participant by subtracting their baseline ratings (collected

at visit 2) by each of their follow-up visits (at week 1 and week 2). T-tests were used to test for significant differences in change scores between exposure groups. All figures and tables report mean values (±SEM).

Results

Summary of study participants

There were 45 participants who completed the study. As noted above, entry criteria for the study included infrequent chili pepper consumption; recruitment was restricted to this low level of consumption level to avoid testing individuals who may already be less sensitive (desensitized/ hypoalgesia) to capsaicin based on habitual exposure in the diet (see Nolden and Hayes, 2017). As a secondary check to ensure all participants actually met these criteria, they were again asked to report frequency of intake and spice preference level at the end of the study. Upon analyzing these questions (see Table 6-1), three participant's responses indicated they did not meet the original recruitment criteria, given that this study was limited to participants that i) reported consuming chili peppers no more than 1-3 times per month and ii) preferred no more than medium spice level in their food. Therefore, these participants were excluded from further analysis.

Table 6-1: Number of participants for each intake frequency of chili peppers and preferred spice/heat level. Participants in ( ) were not included in the analysis.

Intake frequency	Preferred spice/heat level			
	Avoid	Mild	Medium	Spicy
Never	5	11	-	-
< 1/month	0	4	2	-
1-3/month	1	5	8	2
1-2/week	-	4	(2)	-
3-4/week	-	0	(1)	-

Investigating scale usage within participants across visits

To ensure that participants were using the scale correctly, individual's ratings during the second session (i.e., baseline) for remembered and imagined sensations were used. Data were only retained for analysis if the participants rated the three levels of light and sound in appropriate order (i.e. the brightness of: a dimly lit room, a well lit room and the brightest light you have ever seen; the loudness of: a whisper, a conversation, the loudest sound you have ever heard). Based on this, two participants that were removed from further analysis due to incorrect scale usage, resulting in a final sample size of 40 for the study.

In previous studies conducted within our laboratory (unpublished) a learning effect was observed, with changes in ratings across stimuli and remembered sensation, specifically between the first and second visit. Accordingly, the present study included a run-in period and a practice

session prior to the main data collection. To ensure scale ratings stabilized after the baseline visit, non-sample related stimuli were plotted for all participants (n=40) across sessions; these are shown in Figure 6-1.

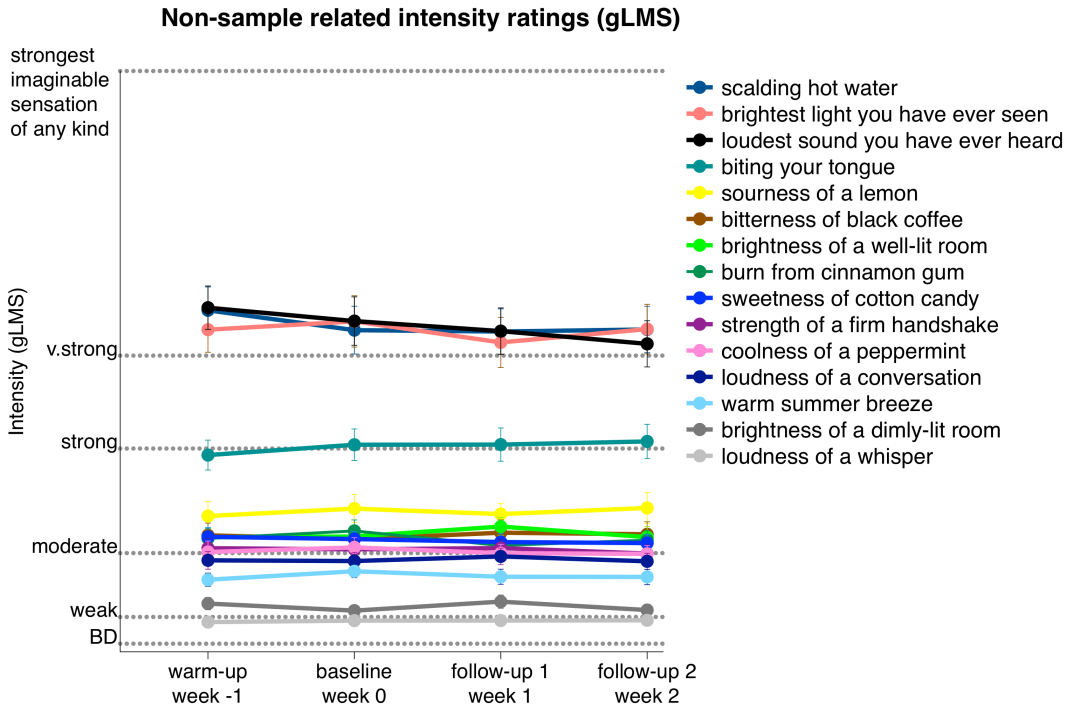

Figure 6-1: Group means (±SEM) for 15 imagined or remembered sensations collected as part of the gLMS orientation / warm-up procedure performed at the beginning of each

Estimating compliance to the exposure protocol

The exposure protocol instructed participants to rinse twice per day for 14 days, totaling 28 total rinses across two weeks. Just over half of the participants successfully rinsed 28 times (57.5%), with several participants missing only one rinse (22.5%) or two rinses (5.0%) and very few missing 3 or more rinses (15%), with no more than 5 missed rinses (of 28 total). Overall, 32

of 40 participants (80%) rinsed at least 27 times of the stipulated 28; accordingly, no participants were removed from analysis for failure to compliance on number of total rinses.

Psychophysical response to sampled capsaicin and control stimuli: capsaicin exposure resulted in reduced burn perception

Participants rated 6 stimuli in triplicate in four different sessions held one week apart, and replicates were averaged within a session a priori. Ratings for capsaicin (0.2, 1.2 and 5.7 ppm) were compared between the treatment and control groups across visits (Figure 6-2). Exposure began after visit 2 (week 0), so comparisons should only be made from this baseline visit. There were two follow-up visits one and two weeks after mouthwash exposure commenced. Allison and colleagues recommend four separate strategies that are appropriate for analysis of the difference between two groups in a longitudinal randomized controlled trial (Allison, Antoine et al., 2016; Allison, Gorman et al., 1993); from their recommended approaches, we chose to create change scores (endpoint measurement minus baseline measurement) and test them with a simple independent sample t-test. Accordingly, to test the effects of capsaicin exposure on capsaicin burn intensity, two change scores, rating at week 1 – rating at baseline (week 0) and rating at week 2 – rating at baseline (week 0), were calculated for all participants and stimuli; these are reported in Table 6-2. These independent sample t-tests test whether the change in ratings for rated stimuli is significantly different between the capsaicin (treatment) and SOA (control) groups. Ratings from visit 2 served as baseline (as visit 1 served as a run-in). This design was chosen a priori to reduce any potential learning effects on the use of the gLMS.

To determine if there was a significant effect of treatment on burn response of capsaicin, t-tests were conducted on the change scores between groups for each concentration. Therefore a bonferroni correction for 6 comparisons would dictate a significance criterion of 0.0083. For

change scores at week 2 (the primary endpoint of the study), there was a significant effect of treatment on perceived burn of 1.2 ppm capsaicin [t(38)= -3.01, p=0.0047]. The capsaicin group (n=21) reporting a mean drop of -3.06±4.4, compared to the control group (1.06±4.2; n=19). Similarly, the same effect was observed for 0.2 ppm [t(38)= -2.14, p=0.039], however this did not meet the bonferroni cut-off and therefore is not significant. The effects were in the expected direction, with the capsaicin group reporting a greater change (-1.0±1.5) compared to the control group (0.01±1.5). Conversely, for 5.7 ppm capsaicin, there was no significant effect of group in perceived burn intensity (t(38)= -1.04, p=0.3) with no differences in burn ratings from the capsaicin group (-2.27±9.7) nor control group (0.77±8.7).

Table 6-2: T-test results: change scores across capsaicin and control after two weeks of exposure

	0.2 ppm capsaicin	1.2 ppm capsaicin	5.7 ppm capsaicin	20uM SOA	0.5 M sucrose	0.32M NaCl
Capsaicin (n=21)	-1.02±1.5	**-3.06±4.4**	-2.27±9.7	-0.32±1.2	0±5.3	-2.35±9.7
Control (SOA) (n=19)	0.01±1.5	**1.06±4.2**	0.77±8.7	-0.10±1.5	1.93±8.1	-0.96±9.1
p-value (t-test)	0.038	**0.0047**	0.30	0.61	0.18	0.79

Group means (±SEM) of change scores week 2 – week 0) for sampled stimuli. Bolded columns indicate where significant differences in the change scores were observed across the two intervention groups. P-values shown are un-adjusted p-values; a bonferroni correction for 6 comparisons would dictate a significance criterion of 0.0083.

Ratings for control stimuli (0.5M sucrose, 0.32M NaCl and 20uM SOA) are shown in Figure 6-3. To test if there was an effect of group on control stimuli change scores were calculated in the same fashion as above (week 2 ratings – week 0 ratings). Change scores for

control stimuli are reported in Table 6-2. T-tests revealed no significant differences for bitterness of SOA (p=0.61), sweetness of sucrose (p=0.18), nor saltiness of NaCl (p=0.79).

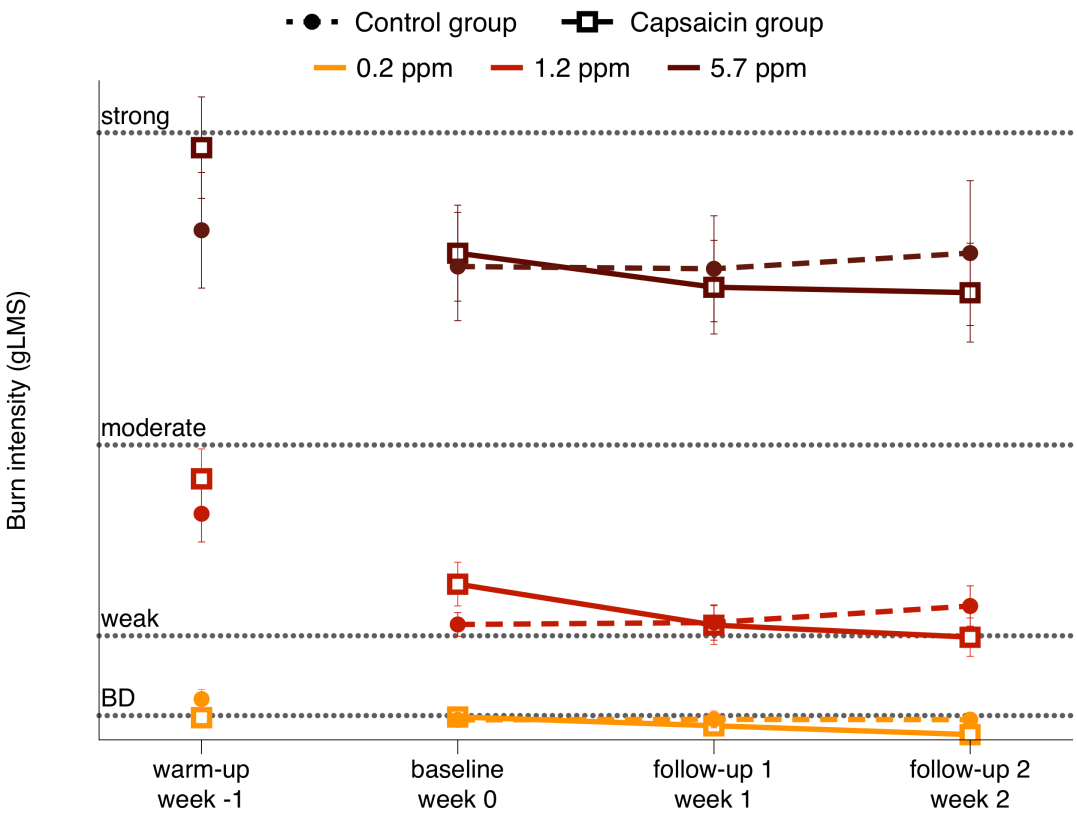

Figure 6-2: Burn ratings for each mouthwash group; individuals were randomized to a group after the baseline visit (week 0). Group means (±SEM) for the SOA control group are shown with solid circles while means for the capsaicin group are shown with open squares. Three capsaicin concentrations (0.2, 1.2 and 5.7 ppm) were used, and data at each concentration were collected in triplicate during each visit and averaged within a participant before any further analysis (see methods). Ratings obtained in the warm-up visit (week -1) were not compared statistically as participants were not assigned to a group and did not receive mouthwash until after their baseline visit. Change scores between the 2nd follow-up (week 2) and baseline (week 0) were different between intervention groups (capsaicin versus SOA mouthwash) for the lower two capsaicin concentrations; at the highest concentration, the apparent drop in the capsaicin group did not reach criterion for significance (see Table 6-2 and text for additional detail).

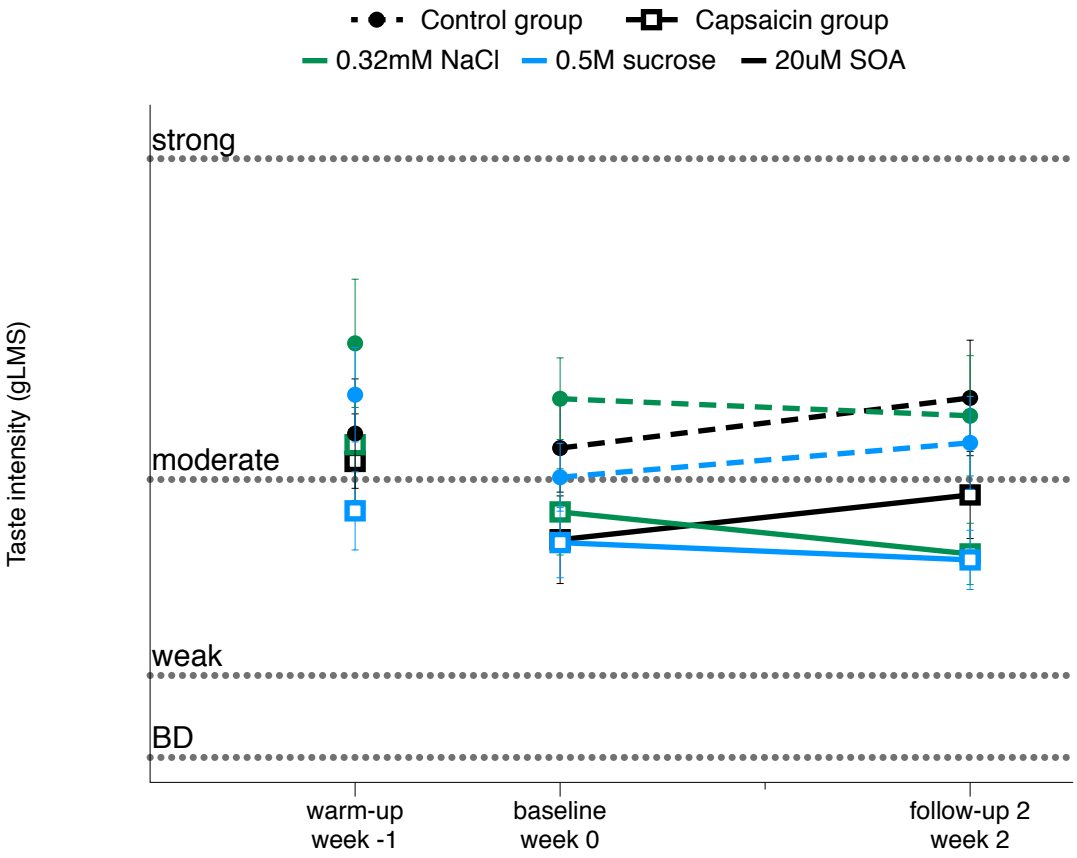

Figure 6-3: Similar to Figure 6-2, except group means (±SEM) for the primary quality for each control stimulus are shown (i.e., bitterness for SOA; saltiness for NaCl; and sweetness for sucrose). Data from the 1st follow-up (week 1) were omitted due to an unexplainable decrease in SOA ratings during week 1, and to reduce visual clutter.

Discussion

The present study was conducted to determine if repeated exposure to a low level of capsaicin (1.2 ppm) over the course of 14 days would result in decreased response to capsaicin (hypoalgesia) at three different concentrations. The study spanned 22 days, with four visits scheduled one week apart (i.e. days 1, 8, 15 and 22). Visit 1 (week -1) served as a warm-up visit, to account for potential learning effects in scale usage. To measure changes in gLMS usage

across visits, mean ratings (±SEM) for 15-remembered or imagined sensations are shown in Figure 6-1; we believe this is the first explicit report on the stability of gLMS ratings across days. As Figure 6-1 shows, ratings of these 15-items, used as part of scale orientation and re-orientation, were generally consistent across visits. The largest change in intensity rating between the first two visits, was observed for 'the burn from scalding hot water' which dropped 3.4 points on the gLMS and stabilized between visits 2 and 4. The second biggest change was for the intensity rating of the 'brightest light you have ever seen', which dropped 1.4 points. Overall, gLMS practice ratings were stable across visits, suggesting there was no learning effect for the participants in the present study. Whether such stability occurs in other studies and populations remains is unknown.

Here, participants were randomized to treatment or control exposure group following visit 2, where participants rinsed with 1.2 ppm capsaicin (treatment) or 20uM SOA (control) for 30 seconds twice per day for two weeks, for 28 exposures total. These solutions were prepared in water and kept at room temperature. When we chew a food, it touches many surfaces of the oral cavity, and not just the anterior tongue, so this oral rinse is more similar to dietary exposure than the application of a filter paper disk to the tongue. Nonetheless, in daily life, we typically don't sample pure compounds in isolation; instead, we consume them within a food matrix, which likely alters not only the ability of the compound to reach its receptor (which is especially relevant for capsaicin, a lipophilic molecule), but also because other sensations evoked by the food matrix may impact the perception of the stimulus (e.g. mixture suppression). However, as there has been little research on repeated whole mouth capsaicin exposure at low doses, the study was simplified to ensure consistent exposure and to promote compliance; thus a simple liquid vehicle (water and Tween) was used to expose participants to either capsaicin or SOA 28 times. Most participants (80%) reporting rinsing at least 27 times, suggesting the protocol was easy for

participants to adhere to, did not induce study dropout, and facilitated the delivery of compounds repeatedly.

Over 4 visits spaced exactly 1 week apart, participants rated three different concentrations of capsaicin stimuli and three control stimuli (sucrose, NaCl, and SOA) in triplicate in a controlled laboratory setting, with two follow-up visits after randomization, allowing us to measure the progression of capsaicin hypoalgesia. When comparing the change in burn intensity for 0.2 and 1.2 ppm capsaicin from week 0 (visit 2) and week 2 (visit 4), there was a significant effect of exposure, with the capsaicin mouthwash group reporting a small but significant reduction in burn relative to the control group who used a mouthwash containing SOA. Conversely, we failed to see a significant effect of group on the burn ratings for 5.7 ppm capsaicin. As the treatment mouthwash consisted of a 1.2 ppm capsaicin solution, we had not expected to see a significant change in the ratings of a higher concentration of capsaicin. To date, there is very limited data exploring the effect of hypoalgesia on concentrations above and below the dose originally used to induce hypoalgesia. Karrer and Bartoshuck (1991) reported that in a single session, ratings for several capsaicin stimuli (1, 10, 100, and 1000 ppm) decreased following exposure to 10 or 100 ppm filter paper disc for 15 minutes. In this case, participants experienced reduced burn from stimuli above the dose used to induce hypoalgesia. In second study, it appeared that participants recovered four days after exposure to 100 ppm for 15-minutes (Karrer and Bartoshuk, 1995). However, both of these studies investigated the effects of a single exposure to potent capsaicin stimulus, rather than repeated exposures to capsaicin.

At the midpoint (i.e., the change scores calculated for one week after starting the mouthwash exposure) there was no effect of group at any concentration. A higher dose, such as 5 – 9 ppm used by Green (1998), may require less time, as after 3 days of 5 – 9 ppm stimuli 3 times per day, participants reported significantly lower burn response for the same stimuli. This

suggests that when the capsaicin dose is relatively low, a period longer than a week is required to cause a decrease in capsaicin response.

There are several limitations related to the results presented here. Present findings are restricted to individuals who report little to no chili pepper consumption (1-3 times per month or less). It is not known how additional capsaicin exposure would affect capsaicin perception in individuals who are already consuming chili peppers regularly and/or in higher doses. Here, capsaicin exposure was achieved by presented in a water/Tween solution that participants rinsed with and spat out. This was done to deliver capsaicin in a way that would roughly mimic dietary capsaicin exposure, versus more controlled methods like cotton swabs or filter paper disks. This increases generalizability and ecological validity at the cost of experimental control, as we were not able to ensure the entire oral surface was perfectly covered in every exposure. Additionally, free-living humans rarely consume isolated compounds as part of their diet. Rather they consume capsaicin in food matrix that may alter its perception and ability of capsaicin to activate its canonical receptor (TRPV1).

Nonetheless, this research has the potential to translate to human health, as exposure to capsaicin has shown to produce analgesic effects. Capsaicin has been used to provide temporary relief from pain (see reviews (Knotkova et al., 2008; Peppin and Pappagallo, 2014; Smith and Brooks, 2014). Oral exposure to capsaicin has been reported to help manage pain in patients with burning mouth syndrome (Silvestre et al., 2012) (see review (Petruzzi et al., 2004). While this study is not intended to investigate the effects of repeated capsaicin exposure in a clinical population, present data may inform the use of capsaicin to treat pain in patients with chronic pain.

Conclusions

A substantial body of evidence indicates reported consumption of chili peppers associates with a decrease in capsaicin burn in the laboratory (Stevenson and Prescott, 1994; Prescott and Stevenson, 1995; Lawless, Rozin et al., 1985; Cowart, 1987; Stevenson and Yeomans, 1993). This phenomenon is generally assumed to reflect as chronic capsaicin hypoalgesia (but also see (Stevenson and Prescott, 1994)). Conversely, there is very little research investigating the required concentration or duration of exposure to induce chronic capsaicin hypoalgesia (or more specifically, a decrease in the psychophysical response to capsaicin across days). Present data confirm limited prior data showing long-term capsaicin hypoalgesia can be experimentally induced with intentional exposure, and extends this finding to dosing levels and exposure conditions that are more in line with dietary exposure. Here, we find that rinsing with 1.2 ppm capsaicin twice per day for 14 days results in a significant reduction in perceived burn intensity of sampled capsaicin, a dose substantially lower than what was been used previously (5 – 9 ppm) in a solid delivery system (Green and Rentmeister-Bryant, 1998). We also found that one week of exposure at low doses was not sufficient to induce hypoalgesia, but that it was achieved after two weeks of exposure. How long someone experiences hypoalgesia to capsaicin after stopping exposure has not been thoroughly investigated. However, recovery has been explored following a single capsaicin exposure, with hypoalgesia lasting upwards of 6 days, with the degree of recovery depending on the exposure concentration (Karrer and Bartoshuk, 1991, 1995). More work is needed to understand relationships between the dose of capsaicin, along with frequency and duration of exposure required to reduce capsaicin burn, and to investigate the time to recovery. Such work may provide insights to potential biological mechanism regulating chronic capsaicin hypoalgesia following repeated dietary exposure to capsaicin, and inform future treatment in clinical populations.

Acknowledgments

The authors thank Gabbrielle Lenart, Lisa Kiem, and Courtney Poorman for assisting in psychophysical data collection and sample preparation. We also thank our participants for their time and participation.

Funding

This work was supported by grants from the National Institutes of Health [F31DC01465 and TR00125] and The Pennsylvania State University.

CHAPTER 7

Psychophysical response to oral capsaicin associates with *TRPV1* mRNA expression in human fungiform papillae

Abstract

Repeated oral exposure to capsaicin across days is known to result in reduced capsaicin response, known as capsaicin desensitization, or more accurately, hypoalgesia. While the effect is robustly observed, the mechanism responsible is not well understood; however, evidence suggests that exposure to capsaicin may alter the biology of the transient receptor potential vanilloid-1 (TRPV1). We hypothesized that chronic repeated capsaicin exposure results in a decrease in the protein expression of TRPV1. The objective of this study was to examine *TRPV1* expression from free-living individuals with diverse (low and high) chili pepper consumption habits, along with their burn ratings for sampled capsaicin. Forty-one individuals sampled and rated the burn of 0.5, 1.0, and 2.0 ppm capsaicin, along with other control stimuli on a general labeled magnitude scale (gLMS). Fungiform papillae were harvested, and mRNA expression of *TRPV1* was determined using qPCR. Greater burn from sampled capsaicin was significantly associated with increased relative *TRPV1* mRNA. There was a significant relationship between burn and self-reported intake of chili peppers; however, there was no relationship between reported intake and *TRPV1* expression. Here, we provide evidence that variability in TRPV1 expression is associated with burn response of capsaicin. Additional research is needed to further explore *TRPV1* expression and how it might be regulated as a result of dietary capsaicin exposure, providing a potential mechanism explaining capsaicin hypoalgesia.

Introduction

Perceptual responses to chemesthetic agents differ from tastants and odorants following repeated exposure: while tastants and odorants typically adapt, chemesthetic agents may show sensitization (hyperalgesia) or desensitization (hypoalgesia), depending on the pattern of exposure. This has been repeatedly shown for capsaicin, and this desensitization (or more precisely, capsaicin induced hypoalgesia) may be acute or chronic (Green, 1989; Green and Hayes, 2003; Karrer and Bartoshuk, 1991). It is well documented that frequent consumers of capsaicin report less burn in the laboratory (e.g., Lawless et al., 1985; Prescott, 1999), and controlled laboratory studies suggest this is not merely the result of a contextual shift (Hayes, 2000; Nolden and Hayes, 2017). Earlier speculation suggested capsaicin hypoalgesia may result from depletion of substance P, but the existence of stimulus-induced recovery (Green 1996, Green and Rentmeister-Bryant 1998) implies that a simple depletion hypothesis may not be sufficient to explain chronic capsaicin-induced hypoalgesia. The mechanism behind chronic hypoalgesia to capsaicin is poorly understood.

Capsaicin is a ligand of the transient receptor potential vanilloid-1 (TRPV1) receptor (Caterina et al., 1997). This ion channel is activated by noxious heat (>43°C), and its activity can be modified directly and indirectly by several stimuli (Caterina et al., 1997; Tominaga et al., 1998), including protons, exogenous ligands (e.g., capsaicin or piperine), and other endogenous metabolites (see (Holzer, 2008; Immke and Gavva, 2006; Planells-Cases et al., 2011) for detailed reviews). During periods of continuous or recurrent capsaicin exposure, the channel enters a desensitized state. These stages of desensitization last seconds to hours and are, at least in part, dependent on Ca^{2+} (Docherty, Yeats et al., 1996; Koplas, Rosenberg et al., 1997; Vyklicky et al., 2008). Presumably, this phase of receptor inactivity is a plausible mechanism that explains the observed decrease in psychophysical response to capsaicin (i.e. acute hypoalgesia) that can be

induced within a single testing session (e.g., Green and Hayes, 2003); however, this cannot explain chronic capsaicin hypoalgesia that lasts across days.

Instead, we hypothesized that chronic capsaicin hypoalgesia is likely the result of changes in the expression TRPV1 and potentially resulting in degradation of TRPV1 sensitive nerve fibers. In skin, exposure to capsaicin (topical or injection) results in a degeneration of nerve fibers in human epidermis (Nolano et al., 1999; Simone et al., 1998). Denervation following capsaicin exposure coincides with a decreased perceptual response, and following discontinuation of capsaicin application, sensitivity to capsaicin returns to baseline along with reinnervation of the epidermis (Nolano et al., 1999; Simone et al., 1998). Whether similar nerve degeneration occurs as a result of capsaicin exposure in oral mucosa at dietarily relevant doses has not been determined empirically, but it could potentially explain chronic capsaicin hypoalgesia.

Alternatively, the other mechanism that may explain chronic hypoalgesia is a decrease in cell surface expression of TRPV1, wherein capsaicin exposure promotes receptor endocytosis and degradation (Sanz-Salvador, Andrés-Borderia et al., 2012). Given a recent report showing that perceived bitterness in humans varies as a function of receptor expression in fungiform papillae (FP) on the surface of the tongue (Lipchock, Reed et al., 2012), we chose to explore whether differential expression of TRPV1 can explain differences in perceived burn. Currently, it is unknown whether repeated oral exposure to capsaicin potentially affects expression of TRPV1 in human FP.

Capsaicin has been widely studied due to its ability to treat persistent and chronic pain (e.g. Yilmaz et al., 2007); for more detailed reviews, see (Knotkova et al., 2008; Mason et al., 2004; Smith and Brooks, 2014). Because capsaicin activates TRPV1, researchers suggest investigating TRPV1 activation and regulation may provide insight as to the mechanisms behind psychophysical capsaicin hypoalgesia.

We hypothesize that TRPV1 expression in FP is down regulated as a response to chronic capsaicin oral exposure, as would occur in a diet with high chili pepper intake, with subsequent impact on the perception of oral burn. The goals of the proposed project include 1) determining if mRNA expression of TRPV1 is associated with threshold sensitivity and suprathreshold intensity of capsaicin in healthy individuals, and 2) testing whether TRPV1 mRNA levels within FP differ among chili users and non-users. If our hypothesis is correct, TRPV1 down regulation would provide a mechanistic explanation for chronic capsaicin desensitization.

Materials and Methods

Overview

This study included two in-person visits on consecutive days. On day 1, participants visited the Penn State Sensory Evaluation Center. In a one-on-one session with a trained experimenter, participants were given an overview of the study and written informed consent was obtained. Following consent, blood pressure was measured and psychophysical data were collected for all stimuli. Participants then reported their liking and intake of chili peppers and other food and non-food items. On day 2, fungiform papillae were harvested at the Clinical Research Center on the Penn State campus.

Participants

Individuals were recruited from The Pennsylvania State University campus and surrounding community (State College, PA). Interested individuals completed a brief online questionnaire to determine if they met the study criteria. These included: Caucasian ethnicity, not

pregnant nor breast feeding, non-smoker, no tongue, cheek or lip piercing, no difficulty swallowing or history of choking, no known taste or smell defect, not taking prescription pain medication, no hyperactive thyroid and no history of chronic pain. Individuals meeting these criteria underwent further screening, answering several questions regarding liking and intake frequency of chili peppers. Recruitment was stratified by gender and by liking and intake of chili peppers. On day 1, participants also had to have a normal blood pressure (101-143/62-91) to proceed with day 2 of the study. All procedures were approved by the Penn State IRB, written informed consent was obtained, and participants were compensated with a cash incentive for their time.

Psychophysical Scaling and Practice/warm-up for scales

Participants rated the burning and bitterness intensity of oral stimuli on a horizontal general labeled magnitude scale (gLMS) (Snyder et al., 2006). On the left hand side, the scale is labeled at 0 with ‘’NS’ (no sensation) and on the right labeled ‘the strongest imaginable sensation of any kind’ at 100. Labels were placed at 1.4, 6, 17, 35 and 51 (‘BD’; barely detectable, ‘weak’, ‘moderate’, ‘strong’ and ‘very strong’; respectively). Participants were instructed to not let whether or not they liked or disliked each stimuli influence their rating of intensity. Prior to rating any samples, participants were given written instructions on the use of the gLMS and rated 15 remembered or imagined sensations (e.g. Hayes, Allen, et al., 2013). The gLMS orientation included food and non-food items in order to emphasize that the scales are to be used in context to all sensations.

Stimuli preparation and sampling protocol

Stimuli were presented in 10mL aliquots at room temperature in duplicate. Sampled stimuli included 0.5, 1 and 2 ppm natural capsaicin (Sigma), 0.5M sucrose (Domino), 0.56mM quinine HCl (Sigma) and 0.32M sodium chloride (NaCl). All samples were prepared with reverse osmosis (RO) water. Three 1000 fold concentrated capsaicin solutions were made with 93% USP grade ethanol and diluted with RO water to reach the final concentrations (each containing 0.1% v/v ethanol). Presentation order was determined using a blocked counterbalanced Williams' design.

Prior to tasting any stimuli, participants rinsed with room temperature RO water. Participants were instructed to swish the entire sample for 5 seconds, spit it out and wait 10 seconds before reporting their response. Participants reported sweetness, bitterness, sourness, saltiness, and burning response on separate gLMS scales, which appeared in different orders (randomized) across participants and samples. Participants rinsed with water between stimuli to remove any lingering sensation and waited a minimum of 1 minute before receiving the next stimuli. A longer 3-minute break enforced after the first set of stimuli. All stimuli were presented in plastic medicine cups labeled with a random three-digit blinding code.

Measures of liking and intake of foods containing chili peppers

After rating all stimuli, participants rated the liking/disliking of remembered sensations, including foods containing chili peppers, and other food and non-food items on a generalized bipolar hedonic scale (e.g. Byrnes and Hayes, 2013). This scale ranges from 'strongest disliking of any kind' at -100 on the left to 'the strongest liking of any kind' at 100 on the right, with

'neutral' at 0 at the center point. Participants rated liking or disliking of 38 items, which included 15 spicy foods, 14 non-spicy foods and 9 non-food related items (Nolden and Hayes, 2017).

Intake frequencies for a variety of foods, including those containing chili peppers were collected via self-report. The questions relating to intake were as follows: 'How often do you consume … [hot sauce, chili peppers, habanero peppers, red pepper flakes, spice mix containing chilies, fried foods, sweet snacks (candy, chocolate, baked goods), salty snacks (pretzels, potato chips, popcorn), ice cream or frozen yogurt]?'. Participants could select one answer for each, including: never, less than once/month, 1-3 times/month, 1-2/week, 3-4/week, 5-6/week, once/day or 2 or more times/day. Liking of spicy food was estimated by having participants select their preferred heat/spice level when ordering food at a restaurant by selecting either 'No heat, I avoid eating spicy foods', 'mild', 'medium', 'spicy' or 'very spicy'. Participants asked rated 'How much they like the burn of chili pepper in your food' and 'How much they like the taste of chili pepper in your food' on a 7-point hedonic scale, ranging from dislike extremely, to like extremely.

Fungiform papillae biopsy

The fungiform papillae biopsies were conducted the following day at the Clinical Research Center located on Penn State campus. Clinical staff collected heart rate and resting blood pressure to ensure participants were in the normal range (to avoid excessive bleeding).

Fungiform papillae were harvested using previously published methods (Lipchock, Mennella et al., 2013; Spielman, Pepino et al., 2010). A licensed clinician, previously trained on this procedure, first checked the oral cavity for inflammation and disease. Using surgical microscissors (McPherson-Vannas; Roboz), 6-10 fungiform papillae were harvested and

transferred to RNAlater (Invitrogen, Life Technologies Corp) using forceps. Tissue samples were stored at -80C. All scissors and forceps were sterilized. During collection, a researcher marked the location for each harvested papillae, consistent with recommendations (Lipchock et al., 2013).

Isolation and quantification of mRNA using real-time RT-PCR

Tissues were thawed on ice and homogenized. RNA was extracted using RNeasy Mini Kit (Qiagen) as described in the manufacturer's protocol. Complementary DNA was synthesized using ABI High Capacity RT Kit. Expression of *PPIA* (peptidylproplyl isomerase A (cyclophilin A); a reference gene) and *TRPV1* was measured by TaqMan quantitative real-time polymerase chain reaction gene expression assays (Hs04194521_s1, and Hs00218912_m1, respectively; Invitrogen, Life Technologies Corp). Individual relative expression of *TRPV1* was determined by subtracting *TRVP1* quantification cycle (C_t) value by *PPIA* C_t (see equation below). Assays were repeated in duplicate where the average for each sample was taken after normalization for each replicate. For analysis the log of normalized relative expression of *TPRV1* to *PPIA* was used.

$$\frac{\text{Expression of } TRPV1}{\text{Expression of } PPIA} = 2^{-((Ct_{TRPV1} - Ct_{PPIA}) - (Avg.Ct_{TRPV1} - Avg.Ct_{PPIA}))}$$

Statistical analysis

All analyses were conducted using SAS 9.2 (Cary, NC). Gender was based on self-report and coded as 0 for women and 1 for men. Repeated measure analysis of variance (ANOVA) was used to determine if variables (i.e. concentration, reported intake, liking of chili peppers and expression) associated with perceived burn from capsaicin stimuli. Linear regression was used to test whether *TRPV1* expression associated with pepper intake. Multiple linear regression was

used to test whether perceived burn of capsaicin was predicted by concentration and *TRPV1* expression. Chi-square was used to determine if gender was associated with self-reported spice/heat preference. T-test and correlation analysis was conducted to determine association of gender and age (respectively) with expression of *TRPV1*.

Results

Characteristics of the study population

Forty-one participants completed both laboratory visits. However, one participant was removed prior to analysis, as their FP tissue sample was undetermined, leaving 40 participants (20 women and 20 men) with an average age of 25.8 (± 1.1) years. In addition to gender, we also stratified recruitment by chili pepper intake: 19 participants reported low intake (1-3/month or less) and 21 reported high chili intake (1-2/week or more). To avoid excessive bleeding during papillae biopsy, all participants were required to have normal blood pressure (between 101-143/62-91 mmHg).

Burn response to sampled capsaicin is associated with chili pepper intake

Mean psychophysical ratings for 0.5, 1, and 2ppm capsaicin, along with control stimuli (0.56mM quinine, 0.5M sucrose and 0.32mM NaCl) are shown in Figure 7-1. The mean burn ratings for capsaicin: 0.5ppm (7.5±1.0), 1ppm (14.9±1.4), and 2ppm (27.0±2.2) are comparable to previous reports (Nolden and Hayes, 2017).

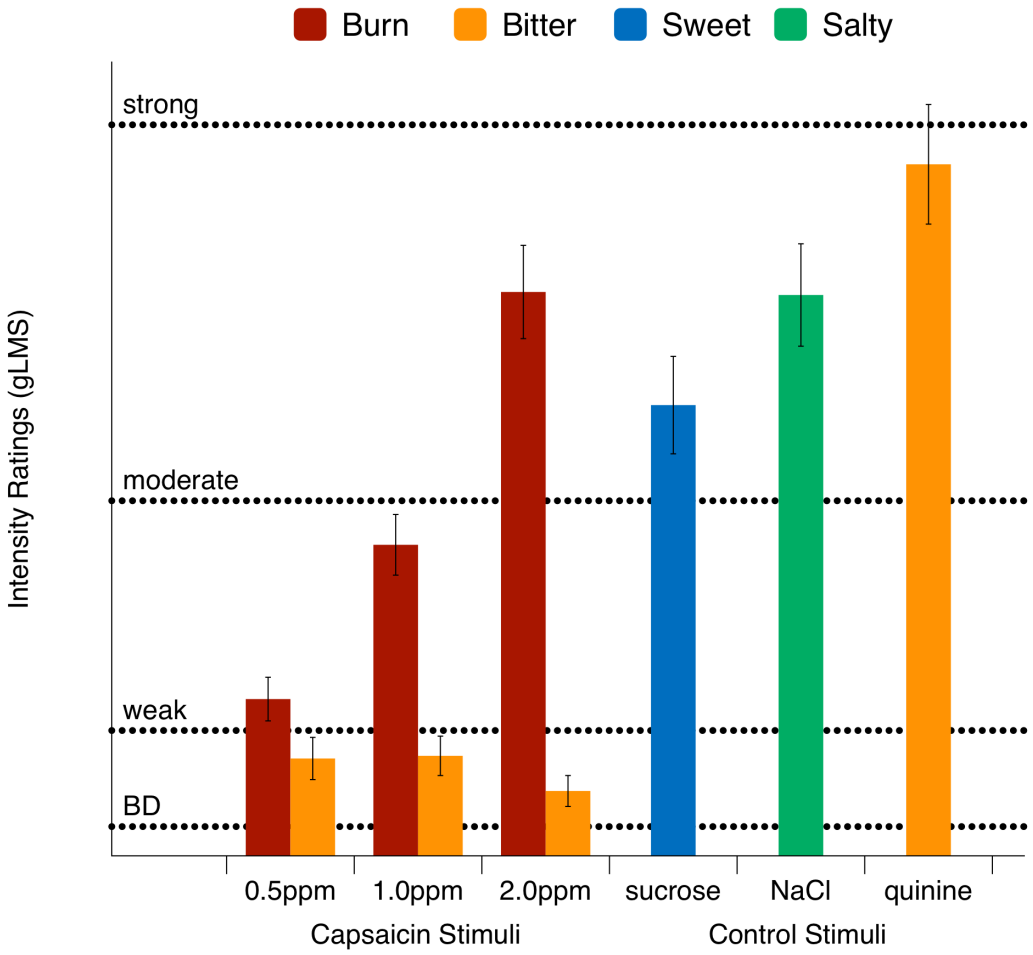

Figure 7-1: Mean ratings (±SEM) for capsaicin and control stimuli. Burn and bitterness response for capsaicin are reported, along with the sweetness of sucrose, saltiness of NaCl and 0.56mM quinine for comparison.

In addition to prior screening, intake of chili peppers was collected during the first day of testing. Participants reported how often they consumed chili peppers every month. To account for different dietary sources of capsaicin, separate questions were asked about hot sauce, chili peppers, habanero peppers, red pepper flakes, and spice mix containing dried chilies using 8 possible response options ranging from never to twice daily. Reported intake for each was annualized (Byrnes & Hayes 2013), and the sum was used as an aggregate measure of chili

pepper intake. Two participants were removed due to their aggregate intake scores were extreme (outliers; both men). The mean aggregate score for the remaining 38 participants was 308.8 (±56.9), with no significant gender differences ($t(36)=1.7$; $p=0.2$). Using a median split (172.8), participants were placed into high intake (n=18) and low intake (n=20) groups, and were compared to the respective group determined during initial recruitment. Generally, individuals were concordant between recruitment and testing, with the exception of 5 individuals. Because the reported intake measures obtained during testing were more detailed, these were used as the primary estimate of chili pepper intake. In addition to intake, each participant also reported his or her preference for spicy food by reporting preferred heat/spice level: avoid (n=11), mild (n=2), medium (n=11), spicy (n=11) or very spicy (n=3). This measure did not associate with (x^2=1.2; p=0.9).

Repeated measure analysis of variance (ANOVA) was used to determine if liking and intake of chili peppers associated with perceived burn across capsaicin stimuli. Concentration was significantly associated with burn ratings [$F(2,72)=86.86$; $p<0.0001$], as expected. Intake group, low versus high, based on a median split of aggregated annualized intake of chili peppers, associated with perceived burn from capsaicin [$F(1,36)=4.84$; $p=0.03$], and the concentration by group interaction was not significant [$F(2,72)=1.8$; $p=0.17$]. Given the main effect of group, post-hoc analysis explored for group differences at each concentration, with the burn response of 2 ppm ($p=0.001$) and 1 ppm ($p=0.02$) significantly different but not the 0.5ppm stimuli ($p=0.9$). Means (±SEM) for both capsaicin and control stimuli by intake group (low and high) are reported in Figure 7-2.

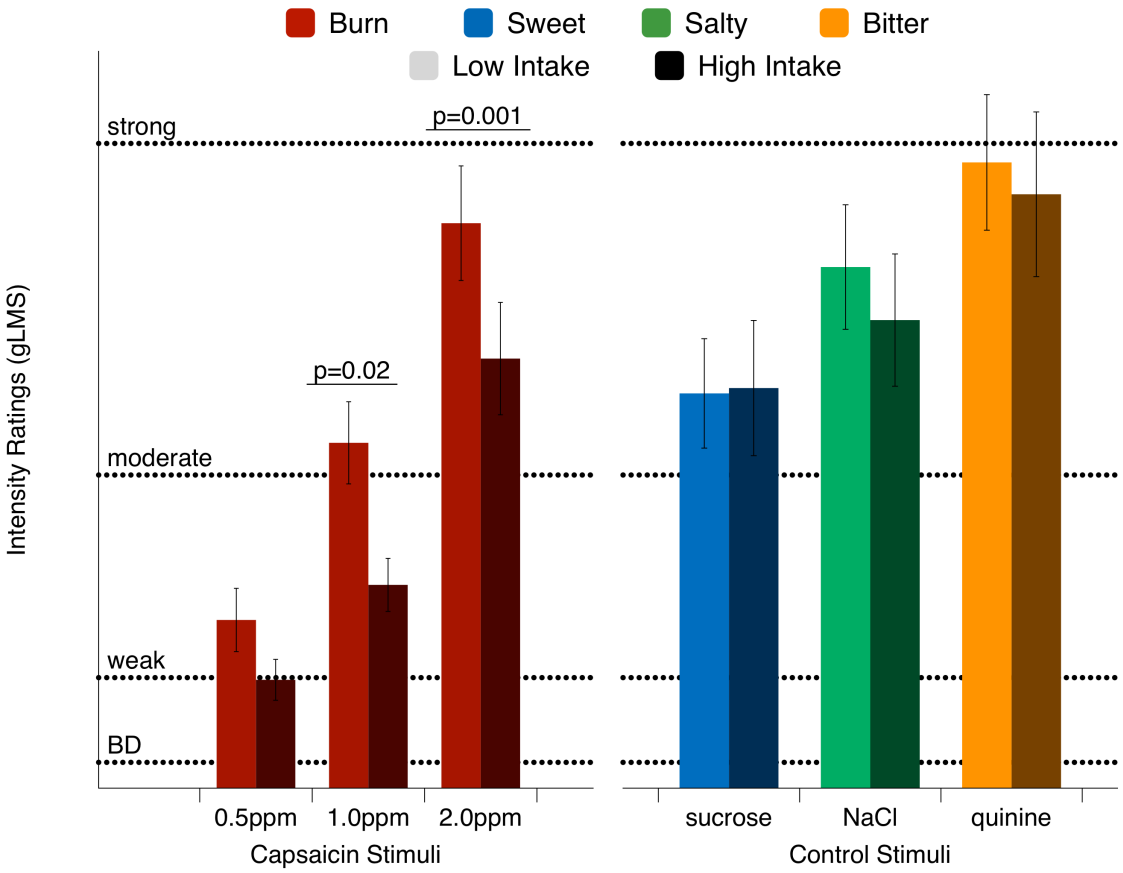

Figure 7-2: Mean ratings (±SEM) for capsaicin and control stimuli by chili pepper intake group (low: lighter color and high: darker color). See text for determination of group. Repeated measures ANOVA was performed to determine if intake group was associated with perceived burn of capsaicin, with concentration as the repeated variable. See text for main and interaction effects, and pvalue for 0.5 ppm capsaicin. Post-hoc analysis was conduct at each concentration with significant differences reported for 1 and 2 ppm (see figure for p-values). There were no significant differences in the sweetness of 0.5M sucrose, saltiness of 0.32mM NaCl and bitterness of 0.56mM quinine between the two intake groups.

For comparison with prior work (Nolden and Hayes, 2017), preferred spice/heat level was segmented into three groups (avoid/mild, medium and spicy/very spicy; n's = 17, 8 and 13, respectively). Preferred spice level was used in a repeated model across capsaicin concentrations (similar to above), to determine if spice preference was associated with burn response. As in the model above, concentration was significant [$F(2,70)=89.58$; $p<0.0001$]; however the main effect

of spice preference was not significant [F(2, 35)=1.53; p=0.23]. The interaction term (concentration by preference group) was significantly associated with burn response [F(4,70)=2.52; p=0.04]. Further analysis revealed significant difference at 2ppm (p=0.03), but not 1 or 0.5 ppm (p's= 0.3 and 0.5).

Multiple linear regression analysis was conducted to determine the amount of variance capsaicin concentration and cumulative chili pepper intake explained for burn ratings from capsaicin. The overall model was significant (F(2,113)=45.04; p<0.0001) and explained 45% of the variance in burn. Both concentration ($sr^2 = 0.36$) and reported intake ($sr^2 = 0.09$) were significant predictors of capsaicin burn.

Relative expression of *TRPV1*

Expression of *TRPV1* mRNA was compared to expression of a reference gene *PPIA* (Peptidylprolyl Isomerase A; Cyclophilin A). Relative expression values were calculated as follows: $2^{((Ct\mathit{TRPV1} - Ct\mathit{PPIA}) - (AvgCt\mathit{TRPV1} - AvgCt\mathit{PPIA}))}$, as done previously (Lipchock et al., 2013) . Non-normalized relative expression varied from 2.897 to 6.046 with a mean of 4.370(±0.58). The log of relative *TRPV1* expression was not associated with gender (t(36)=-0.40; p=0.7) nor age (r=-0.03; p=0.8). The log of relative *TRPV1* expression will be used for remaining analyses.

Gene expression associated with perception of sampled capsaicin

Multiple linear regression was used to determine whether perceived burn of capsaicin was associated with relative *TRPV1* expression across sampled concentrations. The overall model was significant (F(2,111)=35.95; p<0.0001), explaining 39.3% of the variance in burn (Figure 7-

3). Concentration (sr^2=0.36) and *TRPV1* expression (sr^2=0.031) were both significant predictors; with greater concentration and greater *TRPV1* expression each associating with greater burn from sampled capsaicin.

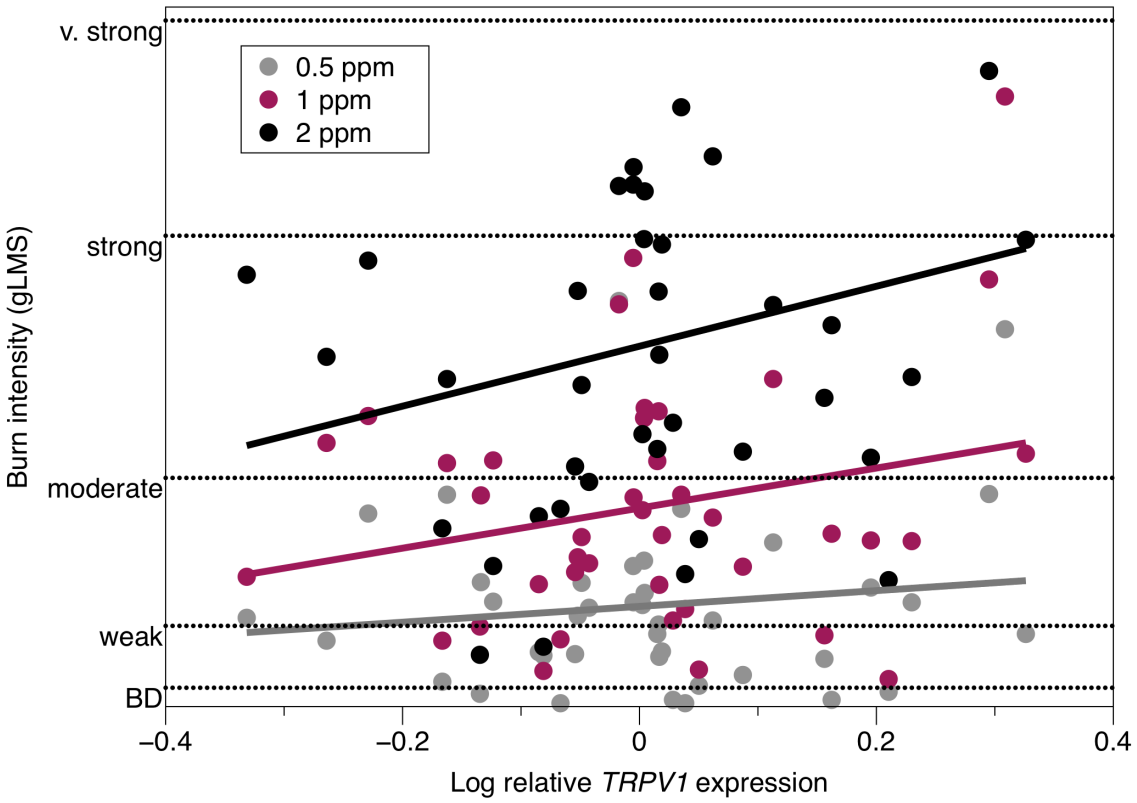

Figure 7-3: Relation between individual's relative expression of TRPV1 and their reported burn response to 0.5, 1.0 and 2.0 ppm capsaicin. Lower relative expression of TRPV1 is associated with reduced burn response, with greater burn response associated with greater relative expression. See text for statistical results.

Gene expression did not associate with reported intake or liking of chili peppers

Linear regression was used to test whether TRPV1 expression is associated with annualized cumulative chili pepper intake. This relationship was not significant ($F(1,36) =0.0$; $p=0.9$). Similarly, no relationship was found when comparing relative expression and groups based on annual intake (low and high) ($t(36)=-0.45$; $p=0.6$)]. Lastly, preferred spice/heat level was not associated with relative expression ($t(2,35)=1.06$; $p=0.3$). These results suggest there is no association between cumulative chili pepper intake or preferred spice level with TRPV1 expression.

Discussion

The present study is the first evidence suggesting *TRPV1* mRNA expression explains variability in capsaicin perception. Here we report that greater *TRPV1* expression is associated with greater burn response to sampled capsaicin. This relationship, while significant, only accounted for a small amount of the variation in burn. This work along with prior work linking greater mRNA expression of functional variant of a bitter taste receptor (*TAS2R38*) with increased bitterness response of propylthiouracil (Lipchock et al., 2013), suggests addition research is warranted to determine how variability in receptor express may influence psychophysical responses to chemosensory stimuli.

In addition to exploring the relationship between *TRPV1* expression and burn response, we investigated whether *TRPV1* mRNA expression is associated with reported intake of capsaicin. This was done to explore whether *TRPV1* expression is reduced in individuals that frequently consume chili pepper, which would provide insight as to the development of capsaicin

hypoalgesia. To explore this hypothesis, we confirmed that participants reporting greater chili pepper intake rated sampled capsaicin stimuli as having less burn compared to low chili pepper consuming participants (consistent with prior reports). In our participants, we failed to observe a relationship between *TRPV1* expression and reported chili pepper intake. This finding may be due to individuals having increased expression due to undetected oral inflammation due to unknown health conditions or oral damage such as biting the tongue or burning the oral cavity. While these events are not known to be associated with a change in *TRPV1* expression, it has been reported that expression is increased in many inflamed tissues, including the tongue (e.g. (Marincsák et al., 2009)).

The associations between burn from sampled capsaicin and the measures of self-reported chili pepper intake used here and elsewhere (Nolden and Hayes, 2017) would seem to suggest that these measures are sensitive enough to determine whether or not they are experiencing capsaicin hypoalgesia. Nonetheless, like any self-report measure, these measures may contain sufficient noise to obscure real associations. Here, participants merely reported frequency of intake for a variety of foods containing chili peppers (hot sauce, a variety of chili pepper types, and spices containing chili pepper). This approach does not provide any information regarding amount consumed, which may be needed to determine how exposure of capsaicin, and ultimately capsaicin hypoalgesia is associated with *TRPV1* expression. An additional potential confound, we did not measure or control for the consumption of other foods and beverages that contain TRPV1 ligands, like black pepper (piperine) and ethanol.

Conclusions

In summary, the data supports the hypothesis that greater *TPRV1* expression is associated with increased burn from sample capsaicin; however, greater expression was not associated with self-reported chili pepper consumption here. We cannot conclude that intake of capsaicin is associated with decreased *TRPV1* expression. Nonetheless, it is still plausible that capsaicin hypoalgesia, a phenomenon brought about by consumption of chili peppers, may be associated with reduced *TRPV1* expression, as we did observe a significant relationship between capsaicin burn response and *TRPV1* expression. Additionally, we recapitulate that chili pepper consumption is significantly associated with variation in capsaicin burn, as consistently reported elsewhere (e.g., Prescott and Stevenson, 1995; Stevenson and Prescott, 1994).

This is the first report on the general variability of *TRPV1* expression in healthy human fungiform papillae. Additional research is required to more accurately measure intake of chili peppers, or control capsaicin exposure, in order to determine whether reduced *TRPV1* expression explains the occurrence and/or the development of capsaicin hypoalgesia. Overall, there is very limited data reporting the variability of chemosensory and taste receptor expression in human fungiform papillae, which may provide a great deal of information about individual variability in perception and potentially influence dietary intake (or vice versa).

Acknowledgements

The authors would like to thank the clinical research staff at the Clinical Research Center at Penn State. We would like to thank both Micaela Hayes MD and Tracey Allen CRNP for collecting participants' fungiform papillae. We also wish to thank Danielle Reed, Corrine Mansfield, Mingyao Sun and Josh Lambert for their guidance in developing study protocol,

Deborah Grove at the Huck Institute at Penn State for assistance with the RT-qPCR data, and Lisa Keim and Gabreille Lenart for helping with psychophysical data collection.

Funding

This work was supported by grants from the National Institutes of Health [F31DC01465 and TR00125] and The Pennsylvania State University.

.

CHAPTER 8

Capsaicin hypoalgesia is inducible in the laboratory, but it does not associate with a decrease in *TRPV1* mRNA expression in human fungiform papillae

Abstract

Capsaicin hypoalgesia is a phenomenon where perceived burn is reduced following repeated exposure. As a result, repeated exposure to capsaicin has been suggested as a potential therapy for patients who experience chronic pain. However the mechanism(s) regulating these hypoalgesic effects are poorly understood. Here, we investigate whether exposure to capsaicin, which leads development of capsaicin hypoalgesia, also results in a reduction in *TRPV1* mRNA expression. This would suggest capsaicin exposure alters capsaicin perception by reducing the availability/accessibility of the TRPV1 channel. Participants (n=51) completed three visits (days 1, 3 and 17) in a 17-day study. During each visit participants sampled several stimuli, including 3, 6 and 9 ppm capsaicin, and rated perceived intensity on a general labeled magnitude scale (gLMS). On days 3 and 17, a clinician harvested fungiform papillae from the anterior tongue. Between days 3 and 17, participants rinsed with either a 6 ppm capsaicin solution or a control (20uM sucrose octaccetate; SOA) twice per day for two weeks. Participants receiving the capsaicin solution reported a significant decrease in their reported burn of capsaicin compared to those receiving the control, indicating we are able to systematically induce hypoalgesia. However, contrary to our hypothesis, *TRPV1* expression was not associated with reported capsaicin burn, nor was capsaicin exposure associated with a decrease in *TRPV1* expression. Additional research is needed to uncover the underlying mechanism for capsaicin hypoalgesia.

Understanding this mechanism may be useful in determining the therapeutic effects of capsaicin for pain management.

Introduction

Capsaicin desensitization, or more accurately hypoalgesia, has been well documented, both orally and extra-orally. However, the mechanism behind this phenomenon is less understood. The hypoalgesia that results following capsaicin exposure has led to the development of drugs and treatments containing capsaicin, which utilize these effects to manage pain symptoms (Knotkova et al., 2008; Mason et al., 2004; Peppin and Pappagallo, 2014). Development of hypoalgesia is also evident in free-living individuals whom frequently consume capsaicin in the diet (Prescott and Stevenson, 1995; Stevenson and Prescott, 1994; Stevenson and Yeomans, 1993), at least in regard to oral exposure. These observations have also been replicated in controlled laboratory trials, showing that capsaicin exposure leads to decreased response to suprathreshold concentrations of capsaicin (Green and Rentmeister-Bryant, 1998; Karrer and Bartoshuk, 1991; Karrer and Bartoshuk, 1995; McBurney et al., 1997) as well as reduced psychophysical responses to other TRPV1 agonists (Green, 1991a; Simons et al., 2003).

While the mechanism(s) regulating these hypoalgesic and therapeutic effects are not fully understood (e.g. (Holzer, 1991; Vyklicky et al., 2008; Xu et al., 2012; Yang et al., 2014), evidence suggests the transient receptor potential cation channel subfamily V member -1 (TRPV1; capsaicin receptor) (Szolcsanyi and Pinter, 2013) is involved. Seminal work by Jansco showed that when neurons are exposed to capsaicin in vitro the neuron becomes unresponsive to subsequent capsaicin challenges, which has been termed desensitization (Jancso, 1968; Jancsó and Jancsó, 1949; Jancso et al., 1967). Acute desensitization of the channel, or short-lasting

desensitization, is thought to be dependent on intracellular calcium ions and reduction of neuropeptides. Alternatively, chronic channel desensitization, or long-lasting desensitization (also known as channel defunctionalization) is independent of calcium ion levels; rather, prolonged desensitization is due to neuronal defunctionalization. This type of desensitization is due to neuropeptide depletion, loss of membrane potential, and inability to transport neurotropic factors. Overtime this can lead to degradation of epidermal nerve fibers (Planells-Cases et al., 2011; Sharma et al., 2013; Szolcsányi, 1993; Xu et al., 2012). Both of these stages of desensitization are reversible; however, in some cases neuronal toxicity has been observed in animal models, which is not reversible, causing permanent nerve damage (Planells-Cases et al., 2011; Ritter and Dinh, 1988; Scadding, 1980). For more information regarding the pharmacological effects of capsaicin see (Sharma et al., 2013). Here, we are interested in the mechanism(s) regulating chronic hypoalgesia, which is likely a result of chronic desensitization of the channel leading to neuronal defunctionalization. We are interested in exploring the development of hypoalgesia following repeated capsaicin exposure, and investing the relationship with *TRPV1* expression.

To date, there are limited studies exploring the biological effects of repeated oral capsaicin exposure in human participants. We hypothesize that *TRPV1* expression in FP is down regulated as a response to chronic capsaicin oral exposure, with subsequent impact on the perception of oral burn. Recently, Lipchock and colleagues (2013) found that participants with greater mRNA expression of a functional haplotype for the bitter taste receptor *TA2R38* in fungiform papillae (FP) reported more bitterness for 6-n-propylthiouracil compared to those with lower expression. Subsequently, in a cross sectional pilot study where individuals differed by reported intake of capsaicin containing foods, we observed a significant relationship between *TRPV1* mRNA expression and perceived burning intensity of sampled capsaicin, with greater expression associated with greater burn response (Nolden and Hayes, unpublished). However, in

our pilot, self-reported chili pepper intake was not associated with *TRPV1* expression, as would have been expected. Because it is unclear whether this may have been due to limitations with measuring frequency of intake (i.e. foods contain chili peppers versus other foods that are spicy, like mustard oil or wasabi) in free living humans, we chose to revisit this question using a longitudinal randomized controlled trial.

Investigating the amount of TRPV1 in fungiform papillae as a result of capsaicin exposure has the potential to translate to human heath, as greater *TRPV1* expression is found in inflamed and cancerous tissues. Marincsak and colleagues (2009) reported that *TRPV1* expression was significantly increased in patients with oral cancer compared to controls. This same association was observed in patients with Burning Mouth Syndrome (BMS), with expression positively correlated with patients' pain ratings (Yilmaz et al., 2007). This same association has been reported in extra-oral tissues (Akbar et al., 2010; Akbar, Yiangou et al., 2008; Guarino, Cheng et al., 2010; Matthews et al., 2004; Sun, Guo et al., 2013; Van Gerven et al., 2014). It has been suggested that because capsaicin exposure can defunctionalize TRPV1, that capsaicin may be helpful in treating or managing pain (Anand and Bley, 2011; Bley, 2012; Jancso, 1968; Peppin and Pappagallo, 2014; Smith and Brooks, 2014).

Here, we investigate if a) capsaicin hypoalgesia results in reduced response to sampled capsaicin and b) whether induction of hypoalgesia by repeated capsaicin exposure results in decreased *TRPV1* mRNA expression in human fungiform papillae (FP). The current study has been designed to systematically and repeatedly expose individuals to capsaicin in a protocol which has previously been shown to be capable of inducing capsaicin hypoalgesia (Nolden and Hayes, unpublished). The primary aim of the present study is to determine if mRNA expression of *TRPV1* decreases in FP within individuals that rinse with capsaicin twice per day for two weeks, compared to the control group. In addition to exploring the effects of repeated capsaicin

exposure on the psychophysical response to capsaicin stimuli, we will also examine whether responses to ethanol decrease. These results will inform our understanding of the mechanism underlying chronic capsaicin hypoalgesia.

Materials and Methods

Overview

This study consisted of three visits over the course of 17 days to the Clinical Research Center at Penn State. During the first visit (day 1) a researcher provided participants with a study overview and written consent was obtained. To ensure the biopsy wasn't contraindicated, blood pressures were obtained and those with 143/91 mmHg or above were removed from the study. Instructions were provided describing the use of the generalized Labeled Magnitude Scale (gLMS), followed by a short practice session. Lastly, participants sampled 10 stimuli and reported their psychophysical response. The second visit (day 3) started with practice gLMS ratings, followed by rating intensities of the same 10 stimuli as day 1. Then, a trained clinician harvested 6-10 fungiform papillae (FP) from the anterior tongue of each participant. At completion of the visit 2, participants were given a rinse solution – either treatment (capsaicin) or control (sucrose octaacetate) depending on random group assignment – and were instructed to rinse with 15mLs of the 'mouthwash' twice per day for two weeks. At the end of two-week exposure period, participants returned for the final visit on day 17; this visit included tasting and rating the same 10 stimuli as before, and collection of additional FP.

Participants

Individuals were recruited from The Pennsylvania State University and surrounding community. Prior to enrollment, interested individuals completed a brief online questionnaire to determine if they met the recruitment criteria. These included being between ages of 18-45, having Caucasian ethnicity, not pregnant nor breast feeding, non-smoker, no tongue, cheek or lip piercings, no difficulty swallowing or history of choking, no known taste or smell defect, not taking prescription pain medication, not taking blood thinners, no known history of oral cancer or disease, no hyperactive thyroid, no history of chronic pain, and willing to taste solutions containing ethanol. Also, recruitment was limited to those that do not frequently consume foods containing chili peppers – this was done to avoid individuals who may already have some baseline desensitization via dietary exposure. To screen for chili pepper use, individuals answered several questions regarding liking and intake of chili peppers. Individuals who reported consuming chili peppers (including hot sauce, red pepper flakes or cayenne pepper, jalapenos, habanero or other hot peppers) less than once per week, and reported a spice preference of medium or less, were invited to participate.

In total, 51 participants (20 men) completed the entire study, with a mean age of 27.3 (±0.7 std. err.) years. Three additional individuals were enrolled, but did not complete the study: two were removed during the first visit due to high blood pressure, and one participant was removed after missing their second visit. Participants were randomized using an unequal allocation ratio of 2 to 3: at study completion, the control group contained 20 participants while the treatment group had 31. Procedures were IRB approved and written consent was obtained. All participants were compensated for their time.

Psychophysical scaling and practice session

Psychophysical ratings for test stimuli were collected on a horizontal gLMS. The scale was labeled with ‘’NS’ (no sensation) at 0 and ‘the strongest imaginable sensation of any kind’ at 100. Labels were located at 1.4, 6, 17, 35 and 51 with the following descriptors: ‘BD’ (barely detectable), ‘weak’, ‘moderate’, ‘strong’ and ‘very strong’, respectively. Participants were given oral and written instructions on the use of the gLMS (Snyder et al., 2006). In these instructions, they were told they should not let whether or not they liked or disliked each stimulus influence their rating of intensity, and that they should to click anywhere along the scale, and not just on the semantic labels. Following the instructions, participants rated 15 remembered or imagined sensations to familiarize them with the task (e.g. Hayes, Allen, et al., 2013). These ratings included food and non-food items in order to ensure participants were making ratings in context to all things, not just chemosensory sensations. This practice / warm-up procedure was completed at each visit, prior to rating sampled stimuli, to ensure participants were using the scale as instructed. These repeated ratings also served as a measure of consistency for scale usage across visits. All data were collected using Compusense Cloud software (Guelph ONT).

Stimuli preparation and sampling protocol

Capsaicin stimuli were presented at 3, 6 and 9ppm. These stimuli were made with natural capsaicin (Sigma) and reverse osmosis (RO) water, with each concentration containing 4% USP ethanol as an emulsifier. The natural capsaicin used here contains a mix of capsaicin and dihydrocapsaicin (~65%/~35%, respectively, with small variations from lot to lot), but due to similar potency, and Sigma’s nominal branding, it will be referred to as capsaicin throughout. Capsaicin stimuli were made from a single concentrated stock made with ethanol. This stock was

diluted with reverse osmosis (RO) water and ethanol to reach the final concentrations (and required 4% ethanol). Control stimuli for tasting and rating also included 0.5M sucrose (Domino) and 20uM sucrose octaacetate (SOA; Sigma-Aldrich), a food grade bitterant with GRAS status. For consistency, the SOA test stimuli (along with mouthwash solutions, discussed below) also contained 4% ethanol. For exploratory analysis, participants also tasted and rated 40% (v/v) ethanol in water.

Of the stimuli listed, four were presented in duplicate (3ppm and 6ppm capsaicin, 20uM SOA, and 0.5M sucrose) and presented in a blocked counterbalanced William's design, except the 6ppm capsaicin stimuli, which was always presented in the 4th and 8th position. The remaining two stimuli, 40% ethanol and 9ppm capsaicin were presented in fixed positions (9th and 10th) to limit potential carry-over effects and potential sensitization or desensitization within a test session. There was a one-minute forced wait time between all stimuli, with an additional minute after the 4th and 8th stimuli (6ppm capsaicin) and after the 9th stimuli (40% ethanol). During this time, participants were instructed to rinse with room temperature RO water until they no longer experienced any sensations. Participants were encouraged to wait more than the forced wait time if they were experiencing any lingering sensation(s). Across visits, the presentation order received by a participant was randomized. All stimuli were at room temperature and presented in 10 mL aliquots in plastic medicine cups labeled with a random 3-digit blinding code.

Prior to tasting any stimuli, participants rinsed with room temperature RO water. Participants were instructed to swish the entire sample for 5 seconds, spit it out and wait 10 to 15 seconds before reporting the intensity. Participants rated sweetness, bitterness, and burning sensations on separate gLMS scales. The order in which the scales appeared on the screen was randomized for every stimuli presented, and the order differed across participants.

Estimating intake for a variety of foods

Although participants were previously screened for liking and intake of chili peppers during recruitment (which restricted participation to individuals that reported consuming chili peppers less than 1-2 times per week and liking medium spice level or below), participants were again asked to report their consumption during the study. Questions used here were taken from intake questions used previously to measure chili pepper intake and liking (Nolden and Hayes, 2017). Question wording was: 'How often do you consume … [foods containing chili peppers; chili pepper containing foods that most people would consider to be medium to very spicy; hot sauce; chili peppers; habanero peppers; red pepper flakes; spice mix containing chilies]?' In a similar fashion, participants reported their intake frequency of: fried foods, sweet snacks (candy, chocolate, baked goods); salty snacks (pretzels, potato chips, popcorn); and ice cream or frozen yogurt. For each question participants could select one of the following frequency categories: never, less than once per month, 1-3 times per month, 1-2 time per week, 3-4 times per week, 5-6 times per week, once per day or 2 or more times per day. Preferred heat/spice level was measured categorically by asking participants whether they prefer: 'No heat, I avoid eating spicy foods', 'mild', 'medium', 'spicy' or 'very spicy', when ordering food from a restaurant. Participants were asked 'How much they like the burn of chili pepper in your food' and 'How much they like the taste of chili pepper in your food' on a 7-point hedonic scale, ranging from dislike extremely, to like extremely. Lastly, participants were asked why they choose to avoid eating spicy foods from a list of the following options: 'they are too hot', 'I don't like the way they taste', 'I don't feel well when I eat spicy foods', 'I like spicy foods, but avoid eating them for other reasons'.

Seven questions were included to estimate frequency of alcohol consumption, as ethanol activates the TRPV1 receptor, which may alter the relationship between capsaicin and TRPV1 expression, or directly influence TRPV1 expression. Using the same frequency responses as

above, participants reported how often they drink: all types of alcohol, straight liquor (without mixers), beer, wine, and mixed drinks. Lastly, participants reported how many drinks they consume on a typical day that they are drinking. Participants selected one from the following options: ‘ I don’t drink alcohol’, ‘One or two drinks’, Three or four drinks’, ‘Five or six drinks’, Seven, eight, or nine drinks’, ’10 or more drinks’.

Generalized liking survey for a variety of food and non-food items

Participants rated their liking/disliking for 37 items, including 15 foods containing chili peppers, 13 non-spicy foods, and 9 non-food items. Ratings were made on a bipolar scale with ‘strongest disliking of any kind’ on the left (-100) and ‘strongest liking of any kind’ on the right (100), with neutral in the middle (0) (e.g., (Byrnes and Hayes, 2013; Nolden and Hayes, 2017)).

Food variety seeking survey

The Variety Seeking Tendency Scale (VARSEEK) was included in the study to estimate an individual’s willingness to try new or unusual foods. Participants responded to each statement by selecting how much they agreed or disagreed (completely disagree, disagree, neither disagree nor agree, agree, or completely agree). Each response was assigned a score (0 to 4) and summed within a participant to give a final score of 0 to 32, reflecting not adventurous to very adventurous in terms of dietary variety and food selection.

Fungiform papillae biopsy

The fungiform papillae (FP) biopsies were conducted at the Clinical Research Center located on Penn State campus. Participants underwent FP biopsies following sampling stimuli on visits 2 and 3 (day 3 and 17). Participants' heart rate and blood pressure were collected prior to each biopsy session.

Collection of FP followed published methods (Lipchock et al., 2013; Spielman et al., 2010). Two health practitioners, who were previously trained on this procedure, harvested FP for this study. Prior to collection, they checked the oral cavity for signs of inflammation and disease, and confirmed that the participants were not taking blood thinners. Surgical microscissors (McPherson-Vannas; Roboz) were used to remove 6 to 10 FP (equal numbers from the left and right side of the tongue). The tissues were transferred to RNAlater (Invitrogen, Life Technologies Corp) using forceps. During collection, a researcher marked the location for each harvested papillae (as done previously (Lipchock et al., 2013)). The tissues were stored at 4°C until processing. All scissors and forceps were cleaned and sterilized in the Clinical Research Center using appropriate methods.

Isolation and quantification of mRNA using real-time RT-PCR

Tissues and RNAlater were brought to room temperature prior to homogenization. RNA was isolated using RNeasy Mini Kit (Qiagen) as per the manufacturer's protocol. ABI High Capacity RT Kit was used to generate complementary DNA. The control gene PPIA (peptidylproplyl isomerase A; Hs04194521_s1) and the target gene TRPV1 (Hs00218912_m1) were acquired from Invitrogen, Life Technologies Corp. Quantity of mRNA was determined

using TaqMan quantitative real-time polymerase chain reaction. Assays were repeated in duplicate.

Individual relative *TRPV1* expression was estimated using the equation below. Three different approaches were used to measure relative expression. First, relative expression for samples harvested during visit 2 (baseline) was normalized to the average of all samples (see below). Second, for samples harvested at the follow-up visit, where participants were assigned to either treatment or control group (i.e. samples from visit 3), individual ΔCt values were subtracted from the average ΔCt of their respective group (treatment and control). Lastly, to measure a change in expression, the follow-up relative expression was subtracted from baseline expression values. However to be consistent with the methods between calculating both baseline and follow-up values, the baseline measurement of relative expression was reanalyzed, following the equation using normalization to their respective groups.

$$\frac{(\text{Expression of } TRPV1)}{(\text{Expression of } PPIA)} = 2^{-(Ct_{TRPV_1} - Ct_{PPIA}) - (\text{Avg.}Ct_{TRPV_1} - \text{Avg.}Ct_{PPIA})}$$

Alternate methods for estimating expression were considered. Individual's ΔCt values were not subtracted from the group average ΔCt for follow-up samples (as done in by Lipchock and colleagues (2013)), as there were two different groups for the second tissue analysis. Similarly, the method used to analyze the follow-up samples was not employed for the first collection, as at the time of collection of the first tissue sample, participants had not yet been randomized into treatment or control groups.

Statistical analysis

All analyses were conducted using SAS 9.2 (Cary, NC). Sex was coded as 0 for women and 1 for men. T-tests were conducted to determine significance between treatment and control group for continuous variables (psychophysical ratings, expression, VARSEEK scores). Similarly, t-tests were conducted to test for significant relationship between sex on age, and VARSEEK scores as well as group effects and VARSEEK scores. A chi-square test was utilized in order to test compliance with mouthwashes with group (treatment and control). Regression analyses were used to explore relationships between continuous variables, specifically VARSEEK scores, and relative *TRPV1* mRNA expression with psychophysical ratings. Multiple regression analysis was used to determine the relationship between relative *TRPV1* expression and burn response across capsaicin concentrations. To determine effects of the repeated mouthwash exposure on psychophysical ratings, a change score was calculated for each participant. After averaging duplicate ratings, visit 3 (follow-up) ratings were subtracted from visit 2 (baseline) ratings to create a change score for each stimulus. This analysis approach was selected from the recommended analysis strategies for determining differences between groups in randomized control trials (Allison et al., 2016; Allison et al., 1993). For all means, the standard error of the mean (SEM) is reported.

Results

Characteristics of the study population

Fifty-one participants completed the study. Participants were randomly assigned to treatment and control groups while stratifying by sex (reported in Table 8-1). During recruitment,

individuals needed to have reported not consuming chili peppers more than 1 to 3 times per month and preferring a spice/heat level of medium or less to participate. In the in-person visits, participants were asked the same questions, which are summarized in Table 8-2. One participant report consuming chili peppers 1 to 2 times per week; however, they were not removed from analysis, as their preferred spice level was mild, suggesting their intake was still likely to be relatively low.

Table 8-1: Participant characteristics between control and treatment groups

	Control (n=20)	Treatment (n=31)
Females/Males	11/9	19/10
Age(±SEM)	28.4±1.3	26.5±0.9
VARSEEK	29.7±1.0	29.0±1.0

.

Table 8-2: Frequency table of self-reported chili pepper intake and preferred spice level.

		Frequency of chili pepper intake			
		Never	< 1 / month	1-3 / month	1-2 / week
Spice level	No heat	4	3	4	0
	Mild	4	13	11	1
	Medium	-	4	7	-

To ensure participants were using the gLMS correctly (see Nolden and Hayes 2015), ratings collected during the gLMS practice in visits 1-3 were examined for each participant. Based on these ratings, no participants were removed based on their performance for the gLMS practice.

The VARSEEK survey was included to estimate each participant's food adventurousness. The overall mean VARSEEK score was 29.3 (±0.75), and no differences were observed for sex ($t(29)=-0.1$; $p=0.8$), age ($F(1,49)=0.2$; $p=0.6$), or treatment group ($t(49)=0.4$; $p=0.6$) (also see Table 8-1).

To estimate compliance for the mouthwash protocol, participants recorded the time of each rinse on a calendar, which was returned to research staff. Overall, 38 individuals (74%) reported not missing any rinses, with 9 missing 1, and few missing 2 and 3 rinses (2 and 1, respectively). There was no differences in compliance between treatment and control groups ($X^2(3)=2.9$; $p=0.3$).

Initial response to sampled capsaicin stimuli (visits 1 and 2)

Participants reported the bitterness, sweetness and burning sensations for 3, 6 and 9ppm capsaicin, along with 20uM SOA, 0.5M sucrose and 40% ethanol. Ratings were made for these stimuli during every visit. Visit 1 served as a warm-up session with visit 2 serving as a baseline measure. For these two visits, the mean burn for 3 ppm capsaicin was 20.3 (±1.5) and 17.3 (±1.3), with some bitterness reported (5.0±1.0 and 5.0±0.07). For the 6ppm capsaicin, the mean burn across the two visits was 28.9 (±2.1) and 27.7 (±2.2), along with some bitterness (6.3±1.4 and 5.9±1.4). Lastly, mean burn for 9ppm capsaicin were similar 28.0 (±2.6) and 28.3 (±2.6) for the two visits, as were the bitterness ratings 5.6 (±1.1) and 5.2 (±1.5). The sweetness of sucrose was 21.1 (±2.1) and 18.9 (±1.5) for the first and second visit. For SOA, the mean bitterness for the

first and second visit was 17.1 (±1.9) and 14.6 (±2.1). Lastly, ethanol was reported as being both burning and bitter for visit 1 (21.6±3.2 and 16.7±2.7) and visit 2 (27.3±3.6 and 16.3±3.0), respectively.

VARSEEK values were used to generate high and low food adventurous groups as done previously (Nolden and Hayes, 2017). There were 30 individuals in the low group with 21 in the high group. Independent t-tests were conducted for each stimuli and sensation to determine if VARSEEK group was associated with psychophysical response. There was no significant association between food adventurousness groups with the burn of capsaicin, sweetness of sucrose, bitterness or burning of ethanol and bitterness of SOA ($p's<0.1$).

Evidence of capsaicin hypoalgesia

Participants were assigned to treatment (n=31) or control (n=20) randomly at the end of their second visit. After rinsing 28 times with their respective rinse over the course of 2 weeks (twice per day), they completed their third visit. Group means for 3, 6, and 9 ppm capsaicin are summarized in Figure 8-1. To test whether there was a significant effect of group, change scores were calculated for each participant for each rated sensation for all stimuli (visit 3 rating – visit 2 rating). Mean change scores by group are reported for each stimulus in Table 3. Independent sample t-tests were used to determine whether there was an effect of treatment on the burn ratings of capsaicin stimuli. There was a significant effect of treatment for 3, 6 and 9 ppm ($t's(49) = 3.28$, 4.15, and 2.43; $p's = 0.0019$, 0.0001, and 0.01), respectively.

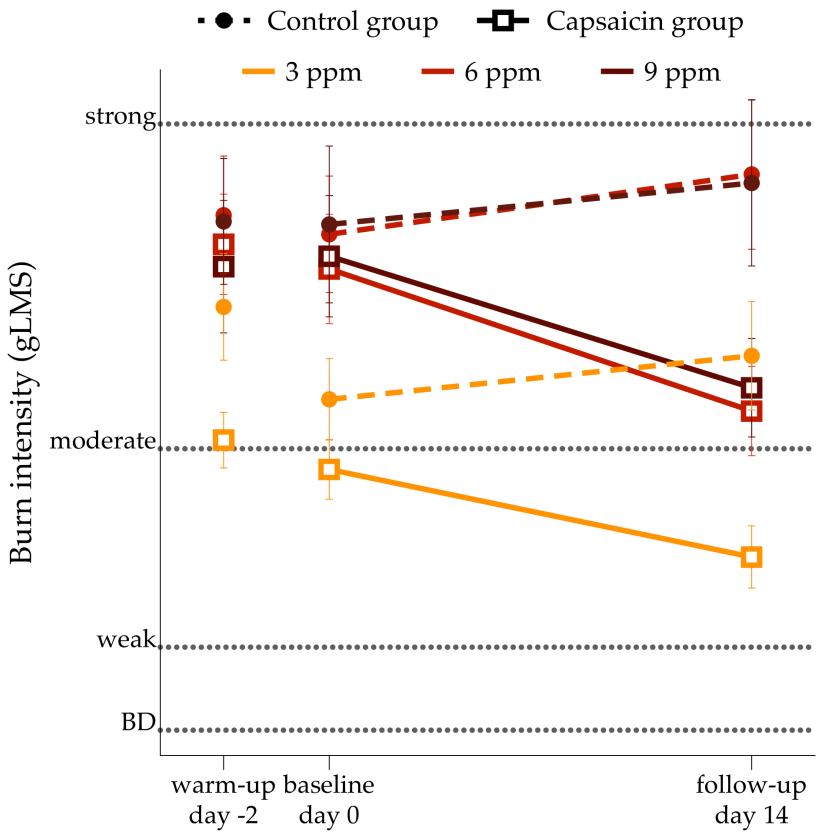

Figure 8-1: Means (±SEM) burn response on gLMS are reported for 3, 6 and 9ppm. Ratings are grouped by mouthwash group for sampled during warm-up (visit 1), baseline (visit 2), and follow-up (visit 3) of the 17-day study. Warm-up ratings are not included in the analysis, as groups had not yet been assigned (lines are not connected to baseline ratings). Control group is represented with filled circles and dashed lines, and the capsaicin group is represented with open squares and solid lines. Significance for effect of group on response was measured using change scores (see text and Table 8-3). 'BD' is barely detectable.

As a control, changes in ratings for the sweetness of sucrose and bitterness of SOA were also examined (Figure 8-2). Change scores for control stimuli (SOA and sucrose) were calculated as above. Independent sample t-tests revealed no significant differences between groups for the

sweetness of sucrose (t(49)=-1.6; p=0.1) or bitterness of SOA (t(49)=-0.8; p=0.3); mean change scores for each group are shown in Table 8-3.

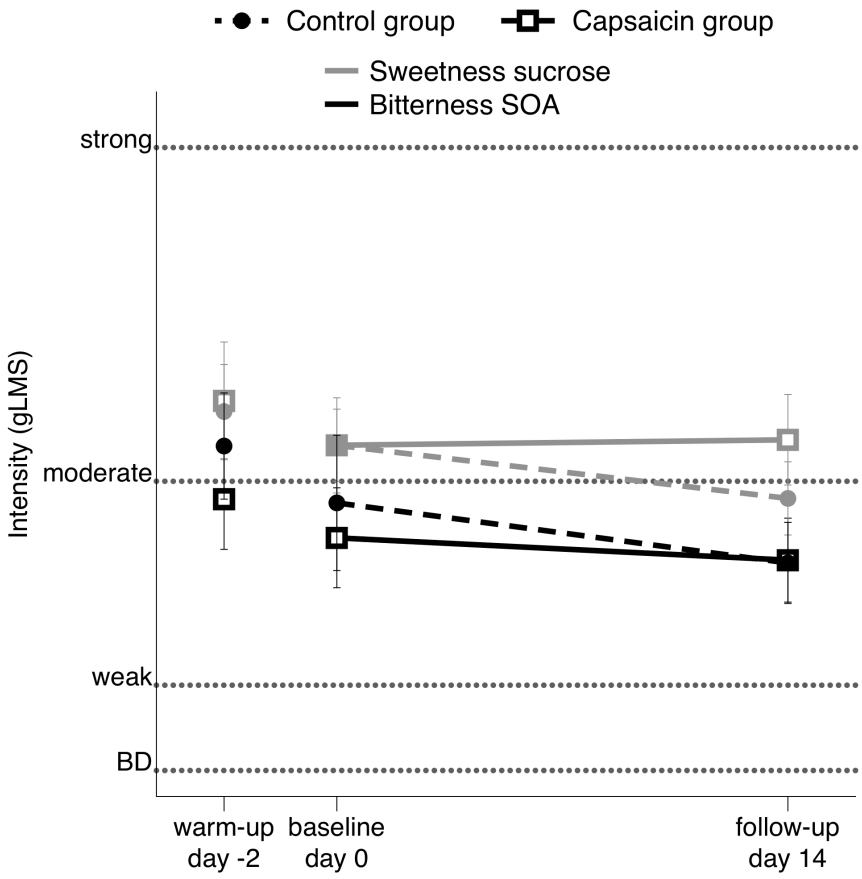

Figure 8-2: Means (±SEM) for the sweetness of sucrose and bitterness of SOA rated on a gLMS. Similar to Figure 1, with ratings collected at warm-up, baseline and follow-up. Ratings are separated by mouthwash group (capsaicin and control). See Table 8-3 for statistics on the relationship between groups and change scores.

Forty percent ethanol (v/v) was also included as an exploratory stimulus to determine if ethanol sensations might be reduced in the capsaicin treatment group due to cross-desensitization. Independent sample t-tests did not indicate a significant effect of group (capsaicin versus SOA) on ethanol bitterness ($t(49)=1.7;p=0.08$) or burn ($t(49)=1.6$; $p=0.09$); the mean change scores for ethanol burn are shown in Table 8-3.

Table 8-3: Independent t-test results: Testing the effects of capsaicin treatment on stimuli ratings (change scores).

	Exposure group		
Stimuli	Control	Treatment	p-value
3 ppm cap	2.41±1.7	-4.85±1.4	0.0019
6 ppm cap	3.32±1.9	-7.87±1.8	0.0001
9 ppm cap	2.29±2.4	-7.29±2.8	0.019
0.5M sucrose	-2.86±1.4	0.29±1.3	0.11
20uM SOA	-3.23±2.4	-1.20±1.0	0.39
40% ethanol	19.1±5.4	7.24±4.4	0.09

Change scores were calculated for all stimuli (visit 2 rating – visit 1 rating). Samples that were in duplicate were averaged prior to calculating change score. Here, change in burn ratings are compared for 3, 6 and 9 ppm capsaicin along with burn of 40% ethanol. Bitterness change scores for SOA and sweetness change scores sucrose are reported.

Relative *TRPV1* expression at baseline is not associated with perceived burn

Fungiform papillae were harvested following visits 2 and 3, with collection at visit 2 serving as a baseline measure of *TRPV1* expression. Two participants were removed from all the expression analysis due to weak reactions, leaving 49 participants total in the analysis, with 20 in the control group and 29 in treatment group. The overall mean logged normalized relative *TRPV1* expression was 0.0155±0.044.

Multiple regression was used to assess the relationship between initial relative expression of *TRPV1* mRNA and burn response of capsaicin across concentrations (burn response = log relative *TRPV1* expression x conc x (log relative *TRPV1* expression x conc; interaction term). The overall model was significant [F(3,143)=5.11; p=0.0022], explaining 9.68% of the variability. Concentration was significant predictors of burn intensity of sampled capsaicin (p=0.0004), while *TRPV1* expression (p=0.68) and the interaction term (conc x expression; p = 0.26) were not significant predictors. Figure 8-3 shows the relationship between *TRPV1* relative expression and burn for 3, 6 and 9 ppm capsaicin.

Linear regression was conducted to determine whether relative *TRPV1* expression is associated with perceived burn of 40% ethanol sampled during visit 1. The resulting model was not significant [Rsq=0.001; p=0.8].

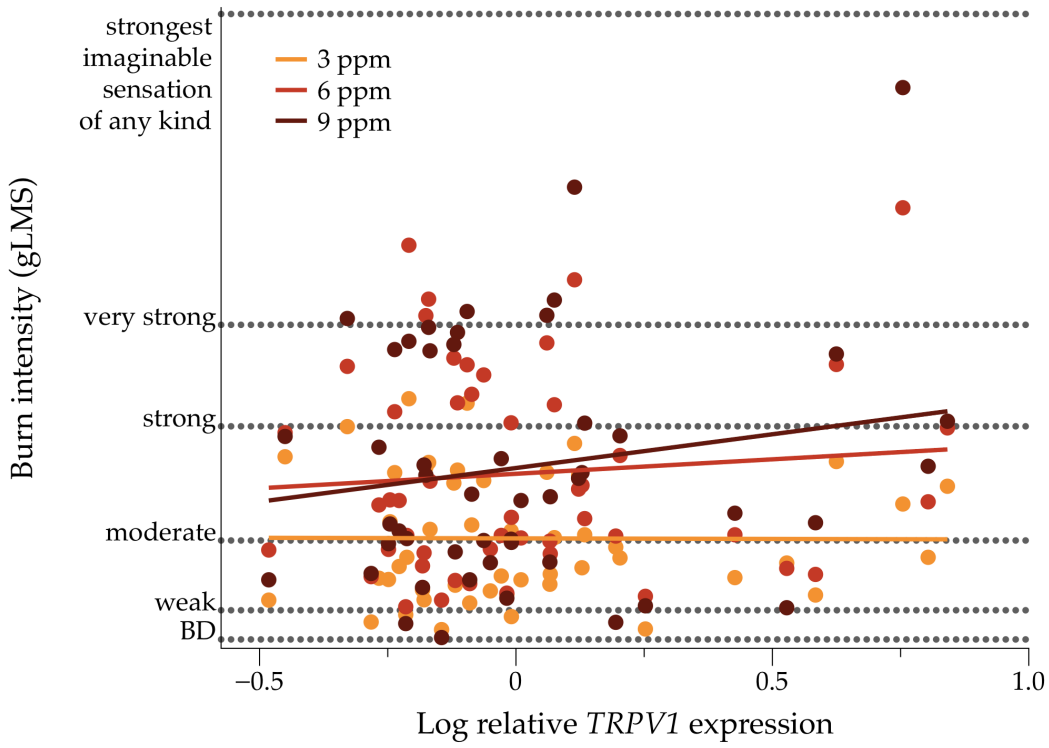

Figure 8-3: Relation between TRPV1 expression and burn ratings of 3, 6 and 9ppm collected on visit 2 (baseline). Burn intensity ratings were collected on a gLMS. See text for p-value.

Relative TRPV1 expression does not decrease as a result of capsaicin exposure

Fungiform papillae biopsies were performed on participants following a 14-day exposure period to either 6ppm capsaicin (n=29) or 20uM SOA mouthwash (n=20). The fold change for relative *TRPV1* expression at follow-up samples is 0.91(±0.11). Figure 8-4 visualizes the change in relative *TRPV1* expression for both the control (left) and treatment (right) groups. To determine if there was an effect of treatment on relative *TRPV1* expression, a t-test was conducted

on relative expression values generated from follow-up samples normalized to individual's group average (either treatment or control). The t-test was not significant [t(47)=0.88; p=0.38], suggesting there was no relationship between mouthwash exposure and TRPV1 expression.

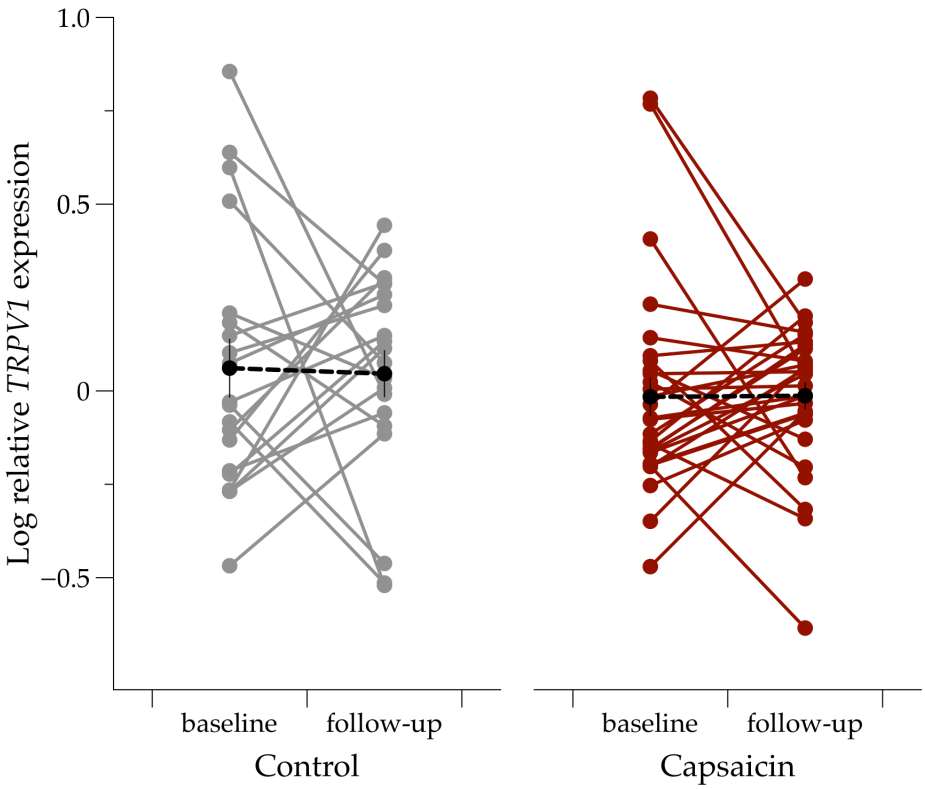

Figure 8-4: Change in TRPV1 expression within participants from visit 2 (baseline; day 3) and visit 3 (follow-up; day 17). Left panel is the change in participant's expression within the control (SOA; grey) group, and the right panel is within the control group (red). Black circles represent the group mean (±SEM) expression, connected by black dashed line.

In a second approach to explore the relationship between capsaicin exposure and *TRPV1* expression, a change score was generated based of subtracting follow-up expression from baseline expression. Unlike the analysis above, this approach takes the individual's baseline *TRPV1* expression into account. Both expression values (baseline and follow-up) for each participant were normalized to their respective group average from each collection (i.e. baseline

and follow-up). The mean change in expression for the control group was -0.014 (±0.10) and treatment group was 0.002(±0.05). A t-test was used to determine if treatment had an effect on the change in mRNA expression values from baseline to follow-up (see above). The test was not significant [t(47)=-0.16; p=0.8], revealing that the variability in change in mRNA expression was not associated with treatment.

Relative TRPV1 expression at follow-up is not associated with burn from capsaicin

Lastly, we explored the relationship between the relative *TRPV1* expression and reported capsaicin burn measured at follow-up (visit 3). In a multiple regression, we tested whether *TRPV1* expression explained variability in capsaicin burn across 3, 6 and 9ppm (Figure 8-5). The two groups (treatment and control) were tested in separate models, as individual relative *TRPV1* expression was normalized to the mean of their respective groups (see methods). The overall model for the SOA control group was not significant [F(2,57)=2.35; p=0.10] (Figure 3, left), whereas the corresponding model was significant for the treatment group [t(2,57)=4.35; p=0.01] (Figure 3, right. This model explained 9.3% of the variability in burn, with concentration being a significant predictor (p=0.01); however, as with the baseline data above, relative *TRPV1* expression was not a significant predictor of burn (p=0.11).

Discussion

Several studies have reported that chronic exposure to capsaicin results in a reduced psychophysical response to capsaicin (i.e., less burn). This phenomenon has been termed as capsaicin desensitization, however the more appropriate term to describe this phenomenon is

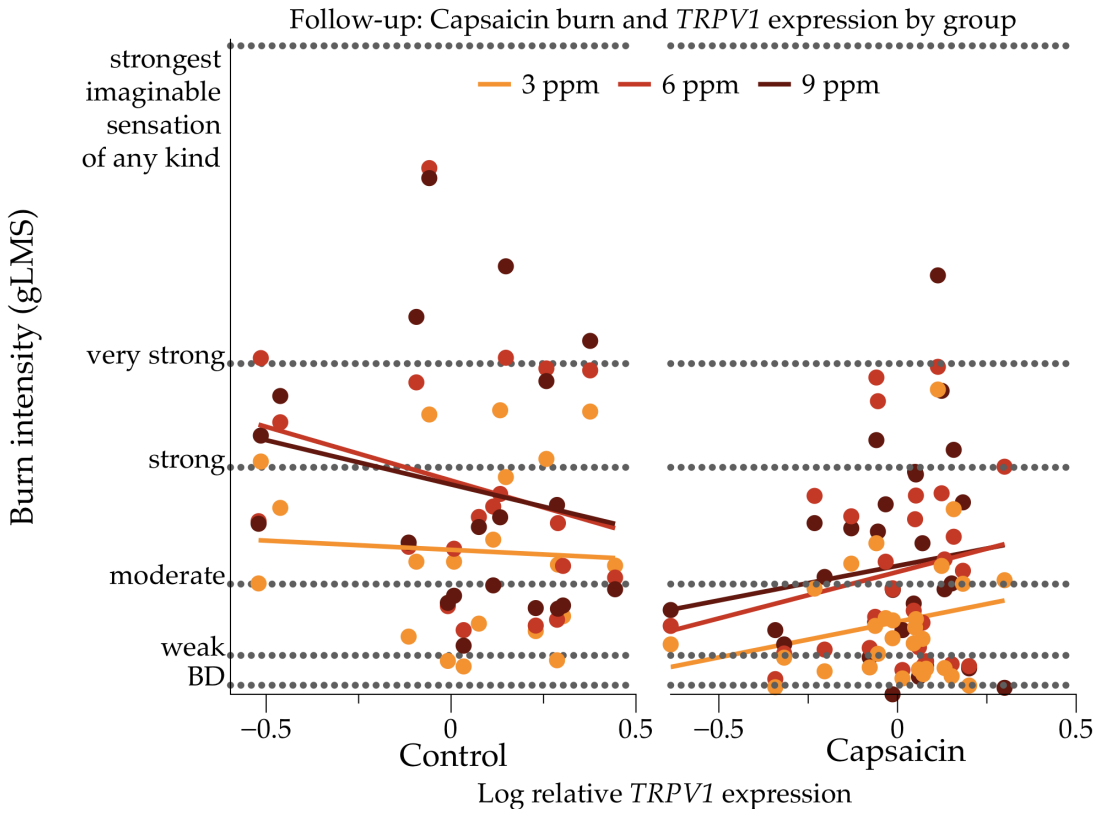

Figure 8-5: Relation between *TRPV1* expression and burn ratings of 3, 6 and 9ppm collected on visit 3 (follow-up; day 17). Left panel is the control group (SOA) and the right panel is the capsaicin group. Relative *TRPV1* expression for an individual is determined by normalizing to the average of the groups' expression (control and capsaicin, respectively). Burn ratings are collected on a gLMS.

hypoalgesia, as strictly speaking, desensitization describes a pharmacological mechanism, and not a psychophysical outcome. The mechanism behind the development of capsaicin hypoalgesia is poorly understood. Here, we explore a straightforward hypothesis; namely, that chronic capsaicin exposure reduces TRPV1 protein at the cell surface, therefore reducing the availability of TRPV1 for binding, which in turn reduces the perceived burn that is elicited by capsaicin.

This hypothesis was based on three different lines of research. First, repeated capsaicin exposure in vitro causes TRPV1 to become desensitized, and can even result in defunctionalization (Planells-Cases et al., 2011; Szolcsányi, 1993). Second, tissues harvested from cancer patients and those experiencing chronic inflammation have shown up-regulation of *TRPV1* (i.e., *TRPV1* mRNA expression) relative to healthy controls (e.g. (Akbar et al., 2010; Akbar et al., 2008; Marincsák et al., 2009)). Finally, there is evidence of neurons withdrawing from the epidermis layer of human skin following repeated exposure of 750ppm capsaicin (e.g. (Nolano et al., 1999; Simone et al., 1998)). Together, these phenomenon suggest that capsaicin hypoalgesia may be a result of TRPV1 defunctionalization and result in TRPV1 internalization.

Here, we explored effects of repeatedly and systematically rinsing with a 6ppm capsaicin mouthwash on both perceived burn from sampled capsaicin and relative *TRPV1* expression. We find that rinsing with 6ppm capsaicin twice per day for 14 days results in a significant decrease in perceived burn of sampled capsaicin (3, 6 and 9ppm), compared to individuals who rinsed with a control solution (20uM SOA). When compared to the control group, individuals rinsing with capsaicin reported a significant decrease in capsaicin ratings between baseline (visit 2) and follow-up (visit 3). Conversely, there were no significant differences between the two groups for changes in ratings for sucrose, SOA or ethanol, suggesting the differences observed are specific to capsaicin. This is the second report of capsaicin hypoalgesia resulting from using a low concentration of capsaicin repeatedly for 14 days. This exposure protocol is presumably much more similar to oral exposure that occurs in the diet, in contrast to prior studies that have used filter paper disks or cotton swabs to deliver the stimulus to a small region of the tongue.

In addition to exploring responses to capsaicin following chronic capsaicin exposure, we also investigated whether responses to ethanol might also decrease. Previously, psychophysical responses for other TRPV1 agonists (e.g., ethanol and cinnamic aldehyde) have been shown to

decrease following short-term exposure to a capsaicin (Green, 1991a); however, the present data suggest chronic capsaicin hypoalgesia does not reduce the burn of 40% ethanol.

After confirming that the capsaicin treatment group developed capsaicin hypoalgesia here, differences in relative *TRPV1* expression were explored. First, we examined whether baseline levels of *TRPV1* mRNA expression was associated with perceived burn from sampled capsaicin collected during visit 2 (baseline). In these participants, baseline *TRPV1* expression was not significantly associated with ratings of sampled capsaicin. While the association was relatively flat for 3 and 6 ppm, there was an apparent trend for greater *TRPV1* expression to be associated with greater perceived burn, but this did not meet our a priori criteria for significance.

Following the exposure period, we tested if treatment altered expression, which would suggest that the capsaicin hypoalgesia observed here was influenced by TRPV1 regulation. However, there was no significant difference between the two group's *TRPV1* expression in FP harvested at the follow-up visit, nor in the change in expression from baseline to follow-up. Similarly, we found no relationship between capsaicin or ethanol burn ratings collected at follow-up and relative *TRPV1* expression within ether the treatment or control group. Thus, additional research is needed to further explore the mechanisms regulating capsaicin hypoalgesia. Even though these data failed to show an effect of capsaicin on *TRPV1* mRNA expression, other methods of quantifying changes in protein regulation may be needed in order to completely disprove the present hypothesis.

One limitation in this study is that mRNA expression measures total protein in the tissue. Even though differences in mRNA have been associated with diseased tissues, a more sensitive measurement may necessary to measure a change in protein expressed on the cell surface. This could be accomplished by conducting immunohistochemistry staining on harvested FP. However, in order to excise enough tissue, and to best measure degradation of neurons in the tongue

epithelium, a tongue punch biopsy may be necessary to conduct this analysis, which is much more invasive than the fungiform papillae biopsy conducted here.

Conclusions

In summary, this study provides direct evidence that repeated capsaicin exposure results in capsaicin hypoalgesia. It has long been thought that the differences in perceived burn from capsaicin in those who frequently consuming capsaicin containing foods, relative to those who do not (e.g. (Lawless et al., 1985; Nolden and Hayes, 2017; Stevenson and Yeomans, 1993)), are a result of capsaicin hypoalgesia. To explore the underlying mechanisms that explain the development of capsaicin hypoalgesia following long-term exposure, we investigated whether mRNA expression of *TRPV1* decreases following repeated capsaicin exposure. In spite of the successful induction of hypoalgesia by rinsing with a 6ppm capsaicin solution twice daily for 14 days, we did not observe a significant decrease in *TRPV1* expression, compared to control participants. This suggests that other methods, such as immunohistochemistry may be needed to observe changes in the location or innervation of TRPV1 as a result of repeated capsaicin exposure. Additionally, *TRPV1* expression was not correlated with burn ratings of sampled capsaicin. More research is needed to determine whether chronic capsaicin exposure results in internalization of the TRPV1 channel and potentially resulting in receding of the nerve endings. These results would provide a mechanism through which capsaicin hypoalgesia is achieved and increase our understanding of the relationship between capsaicin and *TRPV1* expression, which would provide further evidence of the potential therapeutic effects of capsaicin.

Acknowledgements

The authors would like to thank the clinical research staff at the Clinical Research Center at Penn State, and give special thanks to Tracey Banks CRNP and Micaela C. Hayes MD for performing the papillae biopsies. We also thank the Penn State Genomics Core Facility in University Park, PA for assistance with mRNA quantification, and Courtney Poorman for assistance in Compusense Cloud programming and scheduling, sample preparation, and psychophysical data collection. Finally, we sincerely thank our participants for their participation and diligence in completing this study.

Funding

This work was supported by grants from the National Institutes of Health [F31DC01465 and TR00125] and The Pennsylvania State University.

CHAPTER 9

Conclusions and Future Work

An extensive series of experiments were carried out to determine relationships between the variability in capsaicin and ethanol perception with reported intake, genetic differences in *TAS2Rs* and *TRPV1,* and mRNA expression of *TRPV1* in fungiform papillae. The results obtained in these studies expand on our current knowledge of chemosensory response to capsaicin and ethanol, and provide novel information regarding the mechanism(s) regulating capsaicin hypoalgesia. Below are the core findings of these studies:

Dose response (psychophysical) functions were generated for ethanol, quantifying perceived burn and bitter over a range of dietarily relevant concentrations. The relationship between frequency of alcoholic beverage intake and variability in perceptual responses for ethanol were also investigated; these data suggest less intense bitterness from sampled ethanol is associated with greater intake of ethanol outside the laboratory.

Likewise, a perceptual dose response function was generated for capsaicin. Burn was the dominant sensation, with bitterness as a secondary sensation from capsaicin. There was a strong association between the reported burn of capsaicin and frequency of chili pepper intake, with greater intake associated with lower burn. This effect was not due to prior experiences or differences in participants used rating scales, which was consistent with the idea that dietary intake may induce chronic capsaicin hypoalgesia in heavy users.

Two different studies explored the genetic variability in *TAS2Rs* and *TRPV1* in order to explain differences in the bitterness and burn from capsaicin and ethanol. Three SNPs in *TRPV1*

were significantly associated with sensations elicited from ethanol. Interestingly, no *TRPV1* SNPs investigated here associated with differences in capsaicin burn. Three SNPs in *TAS2R38* are in LD and form a haplotype, and six separate SNPs across *TAS2R3, 4* and *5* form another haplotype; each of these are able to explain some differences in bitterness from ethanol and capsaicin. Also, a SNP in *TAS2R13* previously associated with differential intake, was shown to associate with the perception of sampled ethanol.

In two studies, we observed a significant decrease in burn response to capsaicin after rinsing with a capsaicin solution (1.2 and 6ppm) for 2 to 3 weeks, respectively, as compared to participants who rinsed with a control solution. These are the first studies to successfully induce capsaicin hypoalgesia in the oral cavity using experimentally controlled chronic capsaicin exposure with a liquid delivery system.

In one study, reported burn of sampled capsaicin was significantly associated with mRNA expression of *TRPV1* in human FP. Greater expression was associated with greater burn response. However, expression was not associated with intake. In a second study, we failed to observe any relationship between expression and sensation.

We tested the hypothesis that capsaicin hypoalgesia was a result of decreased expression of *TRPV1*. However, repeated exposure to capsaicin solution was not associated with a change in *TPRV1* expression. Here we used qPCR to determine *TRPV1* expression, yet there are other methods to evaluate a change in protein levels. Therefore, this finding does not disprove the proposed hypothesis, and additional research is needed to disprove the stated hypothesis.

Chemesthetic stimuli are known for their ability to produce chemical sensations; however, their taste responses they produce are not well understood. Here we found that two common chemesthetic stimuli, ethanol and capsaicin elicit at least one secondary taste sensation.

It is likely that other chemesthetic compounds may also evoke secondary taste responses (i.e., side tastes), in addition to the primary chemesthetic sensation they are known for. To aid in this research, *in vitro* techniques can be utilized to determine which taste receptors, like TAS2Rs, are activated by a variety of primarily chemesthetic compounds. For example, many TAS2Rs have been investigated for their ability to respond to compounds traditionally thought of as being bitter, with little investigation as to whether other compounds, whose primary quality is not bitter, may activate them as well. These results would be useful for the food and flavor industry when using chemesthetic ingredients in their products, as well as provoking new research questions regarding chemesthetic compounds, taste and chemosensory receptors.

Genetic variability in *TRPV1* was significantly associated with reported burn from ethanol. However, these same SNPs failed to explain variability in the burn response to capsaicin. As the minor allele frequencies for the *TRPV1* SNPs explored here were extremely low, this suggests additional investigation is warranted for confirmation. Conducting a similar study with a larger study population and/or conducting whole gene sequencing may aid in further uncovering potential relationships between genetic variability in *TRPV1* and perceived burn.

These studies described here are the first to explore the effects of experimentally controlled repeated exposure on receptor expression. Previous results found a relationship between coffee consumption and expression of functional *TAS2R38* alleles (Lipchock et al., 2013). However, it is not known is whether coffee consumption led to a change in expression or if expression may play a role in intake and preference. Much more work is needed to explore the influence expression of chemosensory receptors has on both dietary intake and preference of food (e.g., (Hayes, Feeney, et al., 2013)) and also the influence intake may have on subsequent expression. These results could provide new information regarding development of food preferences.

Here, we failed to find evidence that capsaicin hypoalgesia following repeated capsaicin exposure is due to a decrease in *TRPV1* expression. Future work can further develop our understanding of the mechanism(s) resulting in oral hypoalgesia, which could potentially impact research trying to alleviate pain in the oral cavity and elsewhere. Conducting immunohistochemistry staining of the fungiform papillae after exposure could help to determine if there is a change of expression on the cell surface or if there is neuron degradation. This relationship can further be explored in terms of dose of capsaicin and duration of exposure.

Future research investigating the relationship between capsaicin exposure, *TRPV1* expression and health is warranted, as *TRPV1* expression has previously been found to be up-regulated in tissues excised from patients with chronic inflammation and pain. Future studies investigating the effects of repeated capsaicin exposure on patients with oral cancer and burning mouth syndrome would help to reveal if capsaicin would be effective at reducing expression in patients with up-regulated expression. Part of this clinical research should investigate whether these effects would be beneficial in terms of pain, inflammation and health. Due to the pungency of capsaicin, other TRPV1 ligands should be investigated to determine if there are non-chemesthetic compounds that would be just as effective.

The accumulation of work presented here has advanced our understanding of the biological factors that associate with perception, but also the implications of repeated exposure on receptor expression. While these data help to bridge the gap in knowledge regarding chronic capsaicin hypoalgesia, both in terms of scale usage, exposure, and mechanism, many more questions still exist in regards to individual differences in the response to chemesthetic compounds, the impact of genetic variability and the effects of exposure on receptor biology. Future research will help to further expand current findings and investigate new research aims that have been developed as a result of the present finding

REFERENCES

Abdel-Salam, O.M. (2016). Preference for hot pepper: A complex interplay of personal, cultural, and pharmacological effects. *Temperature, 3*(1), 39-40.

Abrahams, H., Krakauer, D., & Dallenbach, K.M. (1937). Gustatory adaptation to salt. *The American Journal of Psychology, 49*(3), 462-469.

Adler, E., Hoon, M.A., Mueller, K.L., Chandrashekar, J., Ryba, N.J.P., & Zuker, C.S. (2000). A novel family of mammalian taste receptors. *Cell, 100*(6), 693-702.

Akbar, A., Yiangou, Y., Facer, P., Brydon, W., Walters, J.R., Anand, P., & Ghosh, S. (2010). Expression of the TRPV1 receptor differs in quiescent inflammatory bowel disease with or without abdominal pain. *Gut, 59*(6), 767-774.

Akbar, A., Yiangou, Y., Facer, P., Walters, J.R., Anand, P., & Ghosh, S. (2008). Increased capsaicin receptor TRPV1-expressing sensory fibres in irritable bowel syndrome and their correlation with abdominal pain. *Gut, 57*(7), 923-929.

Albin, K.C., Carstens, M.I., & Carstens, E. (2008). Modulation of oral heat and cold pain by irritant chemicals. *Chemical senses, 33*(1), 3-15.

Allen, A.L., McGeary, J.E., & Hayes, J.E. (2014). Polymorphisms in TRPV1 and TAS2Rs Associate with Sensations from Sampled Ethanol. *Alcoholism: Clinical and Experimental Research, 38*(10), 2550-2560.

Allen, A.L., McGeary, J.E., Knopik, V.S., & Hayes, J.E. (2013). Bitterness of the Non-nutritive Sweetener Acesulfame Potassium Varies With Polymorphisms in TAS2R9 and TAS2R31. *Chemical senses, 38*(5), 379-389. doi:Doi 10.1093/Chemse/Bjt017

Allison, D.B., Antoine, L.H., & George, B.J. (2016). Incorrect statistical method in parallel-groups RCT led to unsubstantiated conclusions. *Lipids in health and disease, 15*(1), 1.

Allison, D.B., Gorman, B.S., & Primavera, L.H. (1993). Some of the most common questions asked of statistical consultants: Our favorite responses and recommended readings. *Journal of Group Psychotherapy, Psychodrama & Sociometry*.

Almeida, T.F., Roizenblatt, S., & Tufik, S. (2004). Afferent pain pathways: a neuroanatomical review. *Brain research, 1000*(1), 40-56.

Anand, P., & Bley, K. (2011). Topical capsaicin for pain management: therapeutic potential and mechanisms of action of the new high-concentration capsaicin 8% patch. *British journal of anaesthesia, 107*(4), 490-502.

Andersen, H.H., Poulsen, J.N., Uchida, Y., Nikbakht, A., Arendt-Nielsen, L., & Gazerani, P. (2015). Cold and L-menthol-induced sensitization in healthy volunteers—a cold hypersensitivity analogue to the heat/capsaicin model. *Pain, 156*(5), 880-889.

Athanasiou, A., Smith, P.A., Vakilpour, S., Kumaran, N.M., Turner, A.E., Bagiokou, D., Layfield, R., Ray, D.E., Westwell, A.D., & Alexander, S.P. (2007). Vanilloid receptor agonists and antagonists are mitochondrial inhibitors: how vanilloids cause non-vanilloid receptor mediated cell death. *Biochemical and biophysical research communications, 354*(1), 50-55.

Averbeck, B., Rucker, F., Laubender, R., & Carr, R. (2013). Thermal grill-evoked sensations of heat correlate with cold pain threshold and are enhanced by menthol and cinnamaldehyde. *European Journal of Pain, 17*(5), 724-734.

Balaban, C.D., McBurney, D.H., & Stoulis, M. (1999). Time course of burn to repeated applications of capsaicin. *Physiology & behavior, 66*(1), 109-112.

Bandell, M., Dubin, A.E., Petrus, M.J., Orth, A., Mathur, J., Hwang, S.W., & Patapoutian, A. (2006). High-throughput random mutagenesis screen reveals TRPM8 residues specifically required for activation by menthol. *Nature neuroscience, 9*(4), 493-500.

Barber, J.G., & Grichting, W.L. (1987). The assessment of drug attitudes among university students using the short form of the drug attitude scale. *Substance Use & Misuse, 22*(10), 1033-1039.

Barrett, J., Fry, B., Maller, J., & Daly, M. (2005). Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics, 21*(2), 263-265.

Bartoshuk, L.M., Conner, E., Grubin, D., Karrer, T., Kochenbach, K., Palcso, M., Snow, D., Pelchat, M., & Danowski, S. (1993). PROP supertasters and the perception of ethyl alcohol. *Chemical senses, 18*, 526-527.

Bartoshuk, L.M., Duffy, V.B., Fast, K., Green, B.G., Prutkin, J., & Snyder, D.J. (2003). Labeled scales (eg, category, Likert, VAS) and invalid across-group comparisons: what we have learned from genetic variation in taste. *Food Quality and Preference, 14*(2), 125-138.

Bartoshuk, L.M., Duffy, V.B., Green, B.G., Hoffman, H.J., Ko, C.W., Lucchina, L.A., Marks, L.E., Snyder, D.J., & Weiffenbach, J.M. (2004). Valid across- group comparisons with labeled scales: the gLMS versus magnitude matching. *Physiology & behavior, 82*(1), 109-114.

Bègue, L., Bricout, V., Boudesseul, J., Shankland, R., & Duke, A.A. (2015). Some like it hot: Testosterone predicts laboratory eating behavior of spicy food. *Physiology & behavior, 139*, 375-377.

Behrens, M., Foerster, S., Staehler, F., Raguse, J.D., & Meyerhof, W. (2007). Gustatory expression pattern of the human TAS2R bitter receptor gene family reveals a heterogenous population of bitter responsive taste receptor cells. *The Journal of neuroscience, 27*(46), 12630-12640.

Behrens, M., & Meyerhof, W. (2006). Bitter taste receptors and human bitter taste perception. *Cellular and molecular life sciences: CMLS, 63*(13), 1501.

Behrens, M., & Meyerhof, W. (2013). *Bitter taste receptor research comes of age: from characterization to modulation of TAS2Rs.* Paper presented at the Seminars in cell & developmental biology.

Bennett, S.M., & Hayes, J.E. (2012). Differences in the chemesthetic subqualities of capsaicin, ibuprofen, and olive oil. *Chemical senses, 37*(5), 471-478.

Bennett, S.M., Zhou, L., & Hayes, J.E. (2012). Using Milk Fat to Reduce the Irritation and Bitter Taste of Ibuprofen. *Chemosensory perception, 5*(3-4), 231-236.

Berg, H., Filipello, F., Hinreiner, E., & Webb, A. (1955). Evaluation of thresholds and minimum difference concentrations for various constituents of wines. II. Sweetness: the effect of ethyl alcohol, organic acids and tannin. *Food Technol, 9*(6), 138-140.

Berger, A., Henderson, M., Nadoolman, W., Duffy, V., Cooper, D., Saberski, L., & Bartoshuk, L. (1995). Oral capsaicin provides temporary relief for oral mucositis pain secondary to chemotherapy/radiation therapy. *Journal of pain and symptom management, 10*(3), 243-248.

Bernstein, J.A., Davis, B.P., Picard, J.K., Cooper, J.P., Zheng, S., & Levin, L.S. (2011). A randomized, double-blind, parallel trial comparing capsaicin nasal spray with placebo in subjects with a significant component of nonallergic rhinitis. *Annals of Allergy, Asthma & Immunology, 107*(2), 171-178.

Biggs, J.E., Yates, J.M., Loescher, A.R., Clayton, N.M., Boissonade, F.M., & Robinson, P.P. (2007). Changes in vanilloid receptor 1 (TRPV1) expression following lingual nerve injury. *European Journal of Pain, 11*(2), 192-201.

Binder, A., May, D., Baron, R., Maier, C., Tölle, T.R., Treede, R.-d., Berthele, A., Faltraco, F., Flor, H., & Gierthmühlen, J. (2012). *Transient receptor potential channel polymorphisms are associated with the somatosensory function in neuropathic pain patients*: Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU).

Bishnoi, M., & Premkumar, L.S. (2013). Changes in TRP Channels Expression in Painful Conditions. *Open Pain J, 6*, 10-22.

Blednov, Y., & Harris, R. (2009). Deletion of vanilloid receptor (TRPV1) in mice alters behavioral effects of ethanol. *Neuropharmacology, 56*(4), 814-820.

Bley, K. (2012). Effects of topical capsaicin on cutaneous innervation: implications for pain management. *Open Pain J, 6*, 81-94.

Blizard, D.A. (2007). Sweet and bitter taste of ethanol in C57BL/6J and DBA2/J mouse strains. *Behavior genetics, 37*(1), 146-159.

Blom, H., RIJSWUK, J., Garrelds, I., Mulder, P., Timmermans, T., & WIJK, R.G. (1997). Intranasal capsaicin is efficacious in non‐allergic, non‐infectious perennial rhinitis. A placebo‐controlled study. *Clinical & Experimental Allergy, 27*(7), 796-801.

Blom, H., Severijnen, L., Van Rijswijk, J., Mulder, G., Gerth van Wijk, R., & Fokkens, W. (1998). The long-term effects of capsaicin aqueous spray on the nasal mucosa. *Clinical and Experimental Allergy, 28*, 1351-1358.

Borsani, E., Majorana, A., Cocchi, M.A., Conti, G., Bonadeo, S., Padovani, A., Lauria, G., Bardellini, E., Rezzani, R., & Rodella, L.F. (2014). Epithelial expression of vanilloid and cannabinoid receptors: a potential role in burning mouth syndrome pathogenesis. *Histology and Histopathology, 29*(4), 523-533.

Boxer, E.E., & Garneau, N.L. (2015). Rare haplotypes of the gene TAS2R38 confer bitter taste sensitivity in humans. *SpringerPlus, 4*(1), 1-4.

Brito, R., Sheth, S., Mukherjea, D., Rybak, L.P., & Ramkumar, V. (2014). TRPV1: a potential drug target for treating various diseases. *Cells, 3*(2), 517-545.

Bufe, B., Breslin, P.A., Kuhn, C., Reed, D.R., Tharp, C.D., Slack, J.P., Kim, U.K., Drayna, D., & Meyerhof, W. (2005). The molecular basis of individual differences in phenylthiocarbamide and propylthiouracil bitterness perception. *Current Biology, 15*(4), 322-327.

Bufe, B., Hofmann, T., Krautwurst, D., Raguse, J.D., & Meyerhof, W. (2002). The human TAS2R16 receptor mediates bitter taste in response to beta-glucopyranosides. *Nature genetics, 32*(3), 397-401.

Byrnes, N., & Hayes, J. (2016). Behavioral measures of risk tasking, sensation seeking and sensitivity to reward may reflect different motivations for spicy food liking and consumption. *Appetite, In Press*.

Byrnes, N.K., & Hayes, J.E. (2013). Personality factors predict spicy food liking and intake. *Food Quality and Preference, 28*(1), 213-221.

Byrnes, N.K., & Hayes, J.E. (2015). Gender differences in the influence of personality traits on spicy food liking and intake. *Food Quality and Preference, 42*, 12-19.

Cahalan, D., Cisin, I., & Crossley, H. (1969). American drinking practices: A national survey of behaviour and attitudes (Monograph No. 6). *New Brunswick, NJ: Rutgers Center of Alcohol Studies*.

Calo, C., Padiglia, A., Zonza, A., Corrias, L., Contu, P., Tepper, B.J., & Barbarossa, I.T. (2011). Polymorphisms in TAS2R38 and the taste bud trophic factor, gustin gene co-operate in modulating PROP taste phenotype. *Physiology & behavior*.

Cao, E., Liao, M., Cheng, Y., & Julius, D. (2013). TRPV1 structures in distinct conformations reveal activation mechanisms. *Nature, 504*(7478), 113-118.

Carstens, E., Albin, K.C., Simons, C.T., & Carstens, M.I. (2007). Time course of self-desensitization of oral irritation by nicotine and capsaicin. *Chemical senses, 32*(9), 811-816.

Caterina, M.J., & Julius, D. (2001). The vanilloid receptor: a molecular gateway to the pain pathway. *Annual review of neuroscience, 24*(1), 487-517.

Caterina, M.J., Rosen, T.A., Tominaga, M., Brake, A.J., & Julius, D. (1999). A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature, 398*(6726), 436-441.

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D., & Julius, D. (1997). The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature, 389*(6653), 816-824.

Chandrashekar, J., Hoon, M.A., Ryba, N.J.P., & Zuker, C.S. (2006). The receptors and cells for mammalian taste. *Nature, 444*(7117), 288-294.

Chandrashekar, J., Mueller, K.L., Hoon, M.A., Adler, E., Feng, L., Guo, W., Zuker, C.S., & Ryba, N.J.P. (2000). T2Rs function as bitter taste receptors. *Cell, 100*(6), 703-711.

Chard, P., Bleakman, D., Savidge, J., & Miller, R. (1995). Capsaicin-induced neurotoxicity in cultured dorsal root ganglion neurons: involvement of calcium-activated proteases. *Neuroscience, 65*(4), 1099-1108.

Christoforou, J., Balasubramaniam, R., & Klasser, G.D. (2015). Neuropathic Orofacial Pain. *Current Oral Health Reports, 2*(3), 148-157.

Chuang, H.-h., Prescott, E.D., Kong, H., Shields, S., Jordt, S.-E., Basbaum, A.I., Chao, M.V., & Julius, D. (2001). Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns (4, 5) P2-mediated inhibition. *Nature, 411*(6840), 957-962.

Ciabatti, P.G., & D'Ascanio, L. (2009). Intranasal Capsicum spray in idiopathic rhinitis: a randomized prospective application regimen trial. *Acta oto-laryngologica, 129*(4), 367-371.

Clark, C.C., & Lawless, H.T. (1994). Limiting response alternatives in time-intensity scaling: an examination of the halo-dumping effect. *Chemical senses, 19*(6), 583-594.

Cliff, M., & Heymann, H. (1993). Descriptive Analysis of Oral Pungency. *Descriptive Sensory Analysis in Practice*, 641-652.

Cliff, M.A., & Green, B.G. (1996). Sensitization and desensitization to capsaicin and menthol in the oral cavity: interactions and individual differences. *Physiology & behavior, 59*(3), 487-494.

Conte, C., Ebeling, M., Marcuz, A., Nef, P., & Andres-Barquin, P. (2002). Identification and characterization of human taste receptor genes belonging to the TAS2R family. *Cytogenetic and genome research, 98*(1), 45-53.

Cortright, D.N., & Szallasi, A. (2004). Biochemical pharmacology of the vanilloid receptor TRPV1. *European Journal of Biochemistry, 271*(10), 1814-1819.

Cowart, B.J. (1987). Oral chemical irritation: does it reduce perceived taste intensity? *Chemical senses, 12*(3), 467-479.

Dalton, P., & Byrnes, N. (2016). Psychology of chemesthesis–why would anyone want to be in pain? *Chemesthesis: Chemical Touch in Food and Eating*, 8.

Dedov, V., Mandadi, S., Armati, P., & Verkhratsky, A. (2001). Capsaicin-induced depolarisation of mitochondria in dorsal root ganglion neurons is enhanced by vanilloid receptors. *Neuroscience, 103*(1), 219-226.

Dessirier, J.-M., Nguyen, N., Sieffermann, J.-M., Carstens, E., & O'Mahony, M. (1999). Oral irritant properties of piperine and nicotine: psychophysical evidence for asymmetrical desensitization effects. *Chemical senses, 24*(4), 405-413.

Dessirier, J.-M., O'Mahony, M., & Carstens, E. (1997). Oral irritant effects of nicotine: psychophysical evidence for decreased sensation following repeated application and lack of cross-desensitization to capsaicin. *Chemical senses, 22*(5), 483-492.

Dessirier, J.-M., O'Mahony, M., & Carstens, E. (2001). Oral irritant properties of menthol: sensitizing and desensitizing effects of repeated application and cross-desensitization to nicotine. *Physiology & behavior, 73*(1), 25-36.

Dessirier, J.-M., Simons, C., Sudo, M.e., Sudo, S., & Carstens, E. (2000). Sensitization, desensitization and stimulus-induced recovery of trigeminal neuronal responses to oral capsaicin and nicotine. *Journal of neurophysiology, 84*(4), 1851-1862.

Dias, A.G., Rousseau, D., Duizer, L., Cockburn, M., Chiu, W., Nielsen, D., & El-Sohemy, A. (2013). Genetic variation in putative salt taste receptors and salt taste perception in humans. *Chemical senses, 38*(2), 137-145.

Dinehart, M., Hayes, J., Bartoshuk, L., Lanier, S., & Duffy, V. (2006). Bitter taste markers explain variability in vegetable sweetness, bitterness, and intake. *Physiology & behavior, 87*(2), 304-313.

Docherty, R., Yeats, J., Bevan, S., & Boddeke, H. (1996). Inhibition of calcineurin inhibits the desensitization of capsaicin-evoked currents in cultured dorsal root ganglion neurones from adult rats. *Pflügers Archiv, 431*(6), 828-837.

Dotson, C.D., Wallace, M.R., Bartoshuk, L.M., & Logan, H.L. (2012). Variation in the Gene TAS2R13 is Associated with Differences in Alcohol Consumption in Patients with Head and Neck Cancer. *Chemical senses, 37*(8), 737-744.

Drewnowski, A., & Gomez-Carneros, C. (2000). Bitter taste, phytonutrients, and the consumer: a review. *The American journal of clinical nutrition, 72*(6), 1424-1435.

Dubin, A.E., & Patapoutian, A. (2010). Nociceptors: the sensors of the pain pathway. *The Journal of clinical investigation, 120*(11), 3760.

Duffy, V.B. (2007). Variation in oral sensation: implications for diet and health. *Current opinion in gastroenterology, 23*(2), 171-177.

Duffy, V.B., Davidson, A.C., Kidd, J.R., Kidd, K.K., Speed, W.C., Pakstis, A.J., Reed, D.R., Snyder, D.J., & Bartoshuk, L.M. (2004). Bitter Receptor Gene (TAS2R38), 6-n-Propylthiouracil (PROP) Bitterness and Alcohol Intake. *Alcoholism: Clinical and Experimental Research, 28*(11), 1629-1637. doi:10.1097/01.alc.0000145789.55183.d4

Duffy, V.B., Hayes, J.E., Davidson, A.C., Kidd, J.R., Kidd, K.K., & Bartoshuk, L.M. (2010). Vegetable intake in college-aged adults is explained by oral sensory phenotypes and TAS2R38 genotype. *Chemosensory perception*, 1-12.

Duffy, V.B., Hayes, J.E., Sullivan, B.S., & Faghri, P. (2009). Surveying Food and Beverage Liking. *Annals of the New York Academy of Sciences, 1170*(1), 558-568.

Duffy, V.B., Peterson, J.M., & Bartoshuk, L.M. (2004). Associations between taste genetics, oral sensation and alcohol intake. *Physiology & behavior, 82*(2-3), 435-445.

Epstein, J.B., & Marcoe, J.H. (1994). Topical application of capsaicin for treatment of oral neuropathic pain and trigeminal neuralgia. *Oral surgery, oral medicine, oral pathology, 77*(2), 135-140.

Feeney, E. (2011). The impact of bitter perception and genotypic variation of TAS2R38 on food choice. *Nutrition Bulletin, 36*(1), 20-33.

Feeney, E.L., & Hayes, J.E. (2014). Exploring associations between taste perception, oral anatomy and polymorphisms in the carbonic anhydrase (gustin) gene *CA6*. *Physiology & behavior, 128*, 148-154.

Feunekes, G.I., van't Veer, P., van Staveren, W.A., & Kok, F.J. (1999). Alcohol intake assessment: the sober facts. *American journal of epidemiology, 150*(1), 105-112.

Fischer, A., Gilad, Y., Man, O., & Pääbo, S. (2005). Evolution of bitter taste receptors in humans and apes. *Molecular biology and evolution, 22*(3), 432-436.

García-Sanz, N., Valente, P., Gomis, A., Fernández-Carvajal, A., Fernández-Ballester, G., Viana, F., Belmonte, C., & Ferrer-Montiel, A. (2007). A role of the transient receptor potential domain of vanilloid receptor I in channel gating. *The Journal of neuroscience, 27*(43), 11641-11650.

Garneau, N.L., Nuessle, T.M., Sloan, M.M., Santorico, S.A., Coughlin, B.C., & Hayes, J.E. (2014). Crowdsourcing taste research: genetic and phenotypic predictors of bitter taste perception as a model. *Front Integr Neurosci, 8*, 33. doi:10.3389/fnint.2014.00033

Gaudet, R. (2010). Structural Insights into the Function of TRP Channels. In Wolfgang Liedtke (Ed.), *TRP ion channel function in sensory transduction and cellular signaling cascades*: CRC Press.

Gillette, M., Appel, C., & Lego, M. (1984). A new method for sensory evaluation of red pepper heat. *Journal of Food Science, 49*(4), 1028-1033.

Glanz, K., Basil, M., Maibach, E., Goldberg, J., & Snyder, D. (1998). Why Americans Eat What They Do: Taste, Nutrition, Cost, Convenience, and Weight Control Concerns as Influences on Food Consumption. *Journal of the American Dietetic Association, 98*(10), 1118-1126.

Glendinning, J.I. (1994). Is the bitter rejection response always adaptive? *Physiology & behavior, 56*(6), 1217-1227.

Glovannucci, E., Colditz, G., Stampfer, M.J., Rimm, E.B., Litin, L., Sampson, L., & Willett, W.C. (1991). The assessment of alcohol consumption by a simple self-administered questionnaire. *American journal of Epidemiology, 133*(8), 810-817.

Go, Y., Satta, Y., Takenaka, O., & Takahata, N. (2005). Lineage-specific loss of function of bitter taste receptor genes in humans and nonhuman primates. *Genetics, 170*(1), 313-326.

Gold, M.S., & Gebhart, G.F. (2010). Nociceptor sensitization in pain pathogenesis. *Nature medicine, 16*(11), 1248-1257.

Govindarajan, V., & Salzer, U.J. (1985). Capsicum-production, technology, chemistry, and quality part 1: History, botany, cultivation, and primary processing. *Critical Reviews in Food Science & Nutrition, 22*(2), 109-176.

Govindarajan, V., Shanthi, N., & Dhanaraj, S. (1977). Evaluation of spices and oleoresins. II. Pungency of Capsicum by Scoville heat units-a standardized procedure. *Journal of Food Science and Technology, 14*(1), 28-34.

Green, B. (1996a). Chemesthesis: pungency as a component of flavor. *Trends in Food Science & Technology, 7*(12), 415-420.

Green, B.G. (1987). The sensitivity of the tongue to ethanol. *Annals of the New York Academy of Sciences, 510*(1), 315-317.

Green, B.G. (1988). Spatial and temporal factors in the perception of ethanol irritation on the tongue. *Perception & Psychophysics, 44*(2), 108-116.

Green, B.G. (1989). Capsaicin sensitization and desensitization on the tongue produced by brief exposures to a low concentration. *Neuroscience letters, 107*(1), 173-178.

Green, B.G. (1991a). Capsaicin cross-desensitization on the tongue: psychophysical evidence that oral chemical irritation is mediated by more than one sensory pathway. *Chemical senses, 16*(6), 675-689.

Green, B.G. (1991b). Temporal characteristics of capsaicin sensitization and desensitization on the tongue. *Physiology & behavior, 49*(3), 501-505.

Green, B.G. (1996b). Rapid recovery from capsaicin desensitization during recurrent stimulation. *Pain, 68*(2-3), 245-253.

Green, B.G. (2012). Chemesthesis and the chemical senses as components of a "chemofensor complex". *Chemical senses, 37*(3), 201-206.

Green, B.G. (2016). Introduction: what is chemesthesis? In shane T mcdonald, David A. Boilliet, John E. Hayes (Ed.), *Chemesthesis: Chemical Touch in Food and Eating* (pp. 1-5).

Green, B.G., & Hayes, J.E. (2003). Capsaicin as a probe of the relationship between bitter taste and chemesthesis. *Physiology & behavior, 79*(4), 811-821.

Green, B.G., & Hayes, J.E. (2004). Individual Differences in Perception of Bitterness from Capsaicin, Piperine and Zingerone. *Chemical senses, 29*(1), 53-60. doi:10.1093/chemse/bjh005

Green, B.G., Lim, J., Osterhoff, F., Blacher, K., & Nachtigal, D. (2010). Taste mixture interactions: surpression, additivity, and the predominance of sweetnenss. *Physiology & behavior, 101*(5), 731-737.

Green, B.G., & Rentmeister-Bryant, H. (1998). Temporal characteristics of capsaicin desensitization and stimulus-induced recovery in the oral cavity. *Physiology & behavior, 65*(1), 141-149.

Green, B.G., & Schullery, M.T. (2003). Stimulation of bitterness by capsaicin and menthol: differences between lingual areas innervated by the glossopharyngeal and chorda tympani nerves. *Chemical senses, 28*(1), 45.

Guarino, M., Cheng, L., Ma, J., Harnett, K., Biancani, P., Altomare, A., Panzera, F., Behar, J., & Cicala, M. (2010). Increased TRPV1 gene expression in esophageal mucosa of patients with non-erosive and erosive reflux disease. *Neurogastroenterology & Motility, 22*(7), 746-e219.

Han, P., McDonald, H.A., Bianchi, B.R., El Kouhen, R., Vos, M.H., Jarvis, M.F., Faltynek, C.R., & Moreland, R.B. (2007). Capsaicin causes protein synthesis inhibition and microtubule disassembly through TRPV1 activities both on the plasma membrane and intracellular membranes. *Biochemical pharmacology, 73*(10), 1635-1645.

Hatem, S., Attal, N., Willer, J.-C., & Bouhassira, D. (2006). Psychophysical study of the effects of topical application of menthol in healthy volunteers. *Pain, 122*(1), 190-196.

Hayes, J.E. (2000). *Psychophysical and physiological response of humans to the oral irritant capsaicin.* (M.S.), Cornell University.

Hayes, J.E. (2016). Types of chemesthesis I. Pungency and burn: historical perspectives, word usage, and temporal characteristics. *Chemesthesis: Chemical Touch in Food and Eating*, 92.

Hayes, J.E., Allen, A.L., & Bennett, S.M. (2013). Direct comparison of the generalized Visual Analog Scale (gVAS) and general Labeled Magnitude Scale (gLMS). *Food Quality and Preference, 28*(1), 36-44.

Hayes, J.E., Bartoshuk, L.M., Kidd, J.R., & Duffy, V.B. (2008). Supertasting and PROP Bitterness Depends on More Than the TAS2R38 Gene. *Chemical senses, 33*(3), 255-265. doi:10.1093/chemse/bjm084

Hayes, J.E., Feeney, E.L., & Allen, A.L. (2013). Do polymorphisms in chemosensory genes matter for human ingestive behavior? *Food Quality and Preference, 30*(2), 202-216.

Hayes, J.E., Feeney, E.L., Nolden, A.A., & McGeary, J.E. (2015). Quinine Bitterness and Grapefruit Liking Associate with Allelic Variants in TAS2R31. *Chemical senses*, bjv027.

Hayes, J.E., Sullivan, B.S., & Duffy, V.B. (2010). Explaining variability in sodium intake through oral sensory phenotype, salt sensation and liking. *Physiology & behavior, 100*(4), 369-380.

Hayes, J.E., Wallace, M.R., Knopik, V.S., Herbstman, D.M., Bartoshuk, L.M., & Duffy, V.B. (2011). Allelic variation in TAS2R bitter receptor genes associates with variation in sensations from and ingestive behaviors toward common bitter beverages in adults. *Chemical senses, 36*(3), 311-319. doi:10.1093/chemse/bjq132

Hayman, M., & Kam, P.C. (2008). Capsaicin: a review of its pharmacology and clinical applications. *Current Anaesthesia & Critical Care, 19*(5), 338-343.

Hellekant, G., Danilova, V., Roberts, T., & Ninomiya, Y. (1997). The taste of ethanol in a primate model: I. Chorda tympani nerve response in *Macaca mulatta*. *Alcohol, 14*(5), 473-484.

Hinrichs, A.L., Wang, J.C., Bufe, B., Kwon, J.M., Budde, J., Allen, R., Bertelsen, S., Evans, W., Dick, D., & Rice, J. (2006). Functional Variant in a Bitter-Taste Receptor (*hTAS2R16*) Influences Risk of Alcohol Dependence. *The American Journal of Human Genetics, 78*(1), 103-111.

Holzer, P. (1991). Capsaicin: cellular targets, mechanisms of action, and selectivity for thin sensory neurons. *Pharmacol Rev, 43*, 143-201.

Holzer, P. (2008). The pharmacological challenge to tame the transient receptor potential vanilloid-1 (TRPV1) nocisensor. *British journal of pharmacology, 155*(8), 1145-1162.

Huang, J., Zhang, X., & McNaughton, P.A. (2006). *Modulation of temperature-sensitive TRP channels.* Paper presented at the Seminars in cell & developmental biology.

IFIC. (2011). *Consumer Attitudes Toward Food Safety, Nutrition & Health*. Retrieved from

Immke, D.C., & Gavva, N.R. (2006). *The TRPV1 receptor and nociception.* Paper presented at the Seminars in cell & developmental biology.

Intranuovo, L.R., & Powers, A.S. (1998). The Perceived Bitterness of Beer and 6-n-Propylthiouracil (PROP) Taste Sensitivity. *Annals of the New York Academy of Sciences, 855*(1), 813-815.

Ishida, Y., Ugawa, S., Ueda, T., Murakami, S., & Shimada, S. (2002). Vanilloid receptor subtype-1 (VR1) is specifically localized to taste papillae. *Molecular brain research, 107*(1), 17-22.

Jancso, N. (1968). Desensitization with capsaicin and related acylamides as a tool for studying the function of pain receptors. *Pharmacology of pain, 9*, 33-55.

Jancsó, N., & Jancsó, A. (1949). Desensitization of sensory nerve endings. *Kísérletes Orvostudomány, 2*(2).

Jancso, N., Jancso-Gabor, A., & Szolcsanyi, J. (1967). Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *British journal of pharmacology and chemotherapy, 31*(1), 138.

Jang, H.-J., Kokrashvili, Z., Theodorakis, M.J., Carlson, O.D., Kim, B.-J., Zhou, J., Kim, H.H., Xu, X., Chan, S.L., & Juhaszova, M. (2007). Gut-expressed gustducin and taste receptors regulate secretion of glucagon-like peptide-1. *Proceedings of the National Academy of Sciences, 104*(38), 15069-15074.

Julius, D. (2013). TRP channels and pain. *Annual review of cell and developmental biology, 29*, 355-384.

Kampov-Polevoy, A.B., Garbutt, J.C., & Janowsky, D.S. (1999). Association between preference for sweets and excessive alcohol intake: a review of animal and human studies. *Alcohol and Alcoholism, 34*(3), 386.

Kampov-Polevoy, A., Alexey, B., Eick, C., Boland, G., Khalitov, E., & Crews, F.T. (2004). Sweet liking, novelty seeking, and gender predict alcoholic status. *Alcoholism: Clinical and Experimental Research, 28*(9), 1291-1298.

Karrer, T., & Bartoshuk, L. (1991). Capsaicin desensitization and recovery on the human tongue. *Physiology & behavior, 49*(4), 757-764.

Karrer, T., & Bartoshuk, L. (1995). Effects of capsaicin desensitization on taste in humans. *Physiology & behavior, 57*(3), 421-429.

Kennedy, W.R., Vanhove, G.F., Lu, S.-p., Tobias, J., Bley, K.R., Walk, D., Wendelschafer-Crabb, G., Simone, D.A., & Selim, M.M. (2010). A randomized, controlled, open-label study of the long-term effects of NGX-4010, a high-concentration capsaicin patch, on epidermal nerve fiber density and sensory function in healthy volunteers. *The Journal of Pain, 11*(6), 579-587.

Kilo, S., Schmelz, M., Koltzenburg, M., & Handwerker, H. (1994). Different patterns of hyperalgesia induced by experimental inflammation in human skin. *Brain, 117*(2), 385-396.

Kim, U., Jorgenson, E., Coon, H., Leppert, M., Risch, N., & Drayna, D. (2003). Positional cloning of the human quantitative trait locus underlying taste sensitivity to phenylthiocarbamide. *Science's STKE, 299*(5610), 1221.

Kim, U., Wooding, S., Ricci, D., Jorde, L.B., & Drayna, D. (2005). Worldwide haplotype diversity and coding sequence variation at human bitter taste receptor loci. *Human mutation, 26*(3), 199-204.

Klein, A.H., Carstens, M.I., & Carstens, E. (2013). Eugenol and carvacrol induce temporally desensitizing patterns of oral irritation and enhance innocuous warmth and noxious heat sensation on the tongue. *Pain, 154*(10), 2078-2087. doi:10.1016/j.pain.2013.06.025

Klein, R.M., Ufret-Vincenty, C.A., Hua, L., & Gordon, S.E. (2008). Determinants of molecular specificity in phosphoinositide regulation rhosphatidylinositol (4, 5)-bisphosphate (PI (4, 5) P2) is the endogenous lipid regulating TRPV1. *Journal of Biological Chemistry, 283*(38), 26208-26216.

Knotkova, H., Pappagallo, M., & Szallasi, A. (2008). Capsaicin (TRPV1 Agonist) therapy for pain relief: farewell or revival? *The Clinical journal of pain, 24*(2), 142-154.

Koltzenburg, M., Torebjork, H.E., & Wahren, L.K. (1994). Nociceptor modulated central sensitization causes mechanical hyperalgesia in acute chemogenic and chronic neuropathic pain. *Brain, 117*(3), 579-591.

Koplas, P.A., Rosenberg, R.L., & Oxford, G.S. (1997). The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. *The Journal of neuroscience, 17*(10), 3525.

Kranzler, H.R., Sandstrom, K.A., & Van Kirk, J. (2001). Sweet taste preference as a risk factor for alcohol dependence. *American Journal of Psychiatry, 158*(5), 813-815.

Lacroix, J., Buvelot, J., Polla, B., & Lundberg, J. (1991). Improvement of symptoms of non-allergic chronic rhinitis by local treatment with capsaicin. *Clinical & Experimental Allergy, 21*(5), 595-600.

Langstaff, S.A., Guinard, J.X., & Lewis, M. (1991). Instrumental evaluation of the mouthfeel of beer and correlation with sensory evaluation. *Journal of the Institute of Brewing, 97*(6), 427-433.

Lanier, S.A., Hayes, J.E., & Duffy, V.B. (2005). Sweet and bitter tastes of alcoholic beverages mediate alcohol intake in of-age undergraduates. *Physiology & behavior, 83*(5), 821-831.

Lavigne, G.J., & Sessle, B.J. (2015). Canadian Orofacial Pain Team workshop report on the Global Year Against Orofacial Pain. *Pain Research & Management: The Journal of the Canadian Pain Society, 20*(1), 7.

Lawless, H. (1984). Oral chemical irritation: Psychophysical properties. *Chemical senses, 9*(2), 143.

Lawless, H., Rozin, P., & Shenker, J. (1985). Effects of oral capsaicin on gustatory, olfactory and irritant sensations and flavor identification in humans who regularly or rarely consume chili pepper. *Chemical senses, 10*(4), 579-589. doi:10.1093/chemse/10.4.579

Lawless, H., & Stevens, D.A. (1984). Effects of oral chemical irritation on taste. *Physiology & behavior, 32*(6), 995-998.

Lawless, H.T., Hartono, C., & Hernandez, S. (2000). Thresholds and suprathreshold intensity functions for capsaicin in oil and aqueous based carriers. *Journal of sensory studies, 15*(4), 437-477.

Lawless, H.T., & Heymann, H. (2010). *Sensory evaluation of food: principles and practices* (Vol. 5999): Springer Science & Business Media.

Lawless, H.T., Horne, J., & Spiers, W. (2000). Contrast and range effects for category, magnitude and labeled magnitude scales in judgements of sweetness intensity. *Chemical senses, 25*(1), 85-92.

Lee, T. (1954). Physiological gustatory sweating in a warm climate. *The Journal of physiology, 124*(3), 528-542.

Lembeck, F. (1986). Columbus, Capsicum and capsaicin: past, present and future. *Acta Physiologica Hungarica, 69*(3-4), 265-273.

Lembeck, F., & Donnerer, J. (1981). Time course of capsaicin-induced functional impairments in comparison with changes in neuronal substance P content. *Naunyn-Schmiedeberg's archives of pharmacology, 316*(3), 240-243.

Lemon, C.H., Brasser, S.M., & Smith, D.V. (2004). Alcohol activates a sucrose-responsive gustatory neural pathway. *Journal of neurophysiology, 92*(1), 536-544.

Leonard, W.R. (2002). Dietary change was a driving force in human evolution. *Scientific American, 287*(6), 106-116.

Levine, J.D., & Alessandri-Haber, N. (2007). TRP channels: targets for the relief of pain. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1772*(8), 989-1003.

Li, D., & Zhang, J. (2013). Diet shapes the evolution of the vertebrate bitter taste receptor gene repertoire. *Molecular biology and evolution*, mst219.

Liao, M., Cao, E., Julius, D., & Cheng, Y. (2013). Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature, 504*(7478), 107-112.

Lim, J., & Green, B.G. (2007). The psychophysical relationship between bitter taste and burning sensation: evidence of qualitative similarity. *Chemical senses, 32*(1), 31-39.

Lipchock, S.V., Mennella, J.A., Spielman, A.I., & Reed, D.R. (2013). Human bitter perception correlates with bitter receptor messenger RNA expression in taste cells. *American Journal of Clinical Nutrition, 98*(4), 1136-1143. doi:Doi 10.3945/Ajcn.113.066688

Lipchock, S.V., Reed, D.R., & Mennella, J.A. (2012). Relationship between bitter-taste receptor genotype and solid medication formulation usage among young children: a retrospective analysis. *Clinical therapeutics, 34*(3), 728-733.

Lipton, J., Ship, J., & Larach-Robinson, D. (1993). Estimated prevalence and distribution of reported orofacial pain in the United States. *The Journal of the American Dental Association, 124*(10), 115-125.

Lishko, P.V., Procko, E., Jin, X., Phelps, C.B., & Gaudet, R. (2007). The ankyrin repeats of TRPV1 bind multiple ligands and modulate channel sensitivity. *Neuron, 54*(6), 905-918.

Lucas, L., Riddell, L., Liem, G., Whitelock, S., & Keast, R. (2011). The influence of sodium on liking and consumption of salty food. *Journal of food science, 76*(1), S72-S76.

Ludy, M.-J., & Mattes, R.D. (2011). Noxious stimuli sensitivity in regular spicy food users and non-users: Comparison of visual analog and general labeled magnitude scaling. *Chemosensory perception, 4*(4), 123-133.

Ludy, M.-J., & Mattes, R.D. (2012). Comparison of sensory, physiological, personality, and cultural attributes in regular spicy food users and non-users. *Appetite, 58*(1), 19-27.

Ludy, M.-J., Moore, G.E., & Mattes, R.D. (2011). The effects of capsaicin and capsiate on energy balance: critical review and meta-analyses of studies in humans. *Chemical senses*, bjr100.

Lukacs, V., Thyagarajan, B., Varnai, P., Balla, A., Balla, T., & Rohacs, T. (2007). Dual regulation of TRPV1 by phosphoinositides. *The Journal of neuroscience, 27*(26), 7070-7080.

Lv, J., Qi, L., Yu, C., Yang, L., Guo, Y., Chen, Y., Bian, Z., Sun, D., Du, J., & Ge, P. (2015). Consumption of spicy foods and total and cause specific mortality: population based cohort study.

Macfarlane, T.V., Blinkhorn, A.S., Davies, R.M., Kincey, J., & Worthington, H.V. (2002). Oro-facial pain in the community: prevalence and associated impact. *Community dentistry and oral epidemiology, 30*(1), 52-60.

Malmberg, A.B., Mizisin, A.P., Calcutt, N.A., von Stein, T., Robbins, W.R., & Bley, K.R. (2004). Reduced heat sensitivity and epidermal nerve fiber immunostaining following single applications of a high-concentration capsaicin patch. *Pain, 111*(3), 360-367.

Marincsák, R., Tóth, B.I., Czifra, G., Márton, I., Rédl, P., Tar, I., Tóth, L., Kovács, L., & Bíró, T. (2009). Increased expression of TRPV1 in squamous cell carcinoma of the human tongue. *Oral diseases, 15*(5), 328-335.

Marino, R., Torretta, S., Capaccio, P., Pignataro, L., & Spadari, F. (2010). Different therapeutic strategies for burning mouth syndrome: preliminary data. *Journal of Oral Pathology & Medicine, 39*(8), 611-616. doi:10.1111/j.1600-0714.2010.00922.x

Mason, L., Moore, R.A., Derry, S., Edwards, J.E., & McQuay, H.J. (2004). Systematic review of topical capsaicin for the treatment of chronic pain. *Bmj, 328*(7446), 991.

Materska, M., & Perucka, I. (2005). Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (Capsicum annuum L.). *Journal of Agricultural and Food Chemistry, 53*(5), 1750-1756.

Matsunami, H., Montmayeur, J.P., & Buck, L.B. (2000). A family of candidate taste receptors in human and mouse. *Nature, 404*(6778), 601-604.

Mattes, R.D., & DiMeglio, D. (2001). Ethanol perception and ingestion. *Physiology & behavior, 72*(1-2), 217-229.

Matthews, P.J., Aziz, Q., Facer, P., Davis, J.B., Thompson, D.G., & Anand, P. (2004). Increased capsaicin receptor TRPV1 nerve fibres in the inflamed human oesophagus. *European journal of gastroenterology & hepatology, 16*(9), 897-902.

McBurney, D.H. (1966). Magnitude estimation of the taste of sodium chloride after adaptation to sodium chloride. *Journal of Experimental Psychology, 72*(6), 869.

McBurney, D.H., Balaban, C.D., Christopher, D.E., & Harvey, C. (1997). Adaptation to capsaicin within and across days. *Physiology & behavior, 61*(2), 181-190.

McDonald, S., Barrett, P., & Bond, L. (2010). What kind of hot is it? *Perfumer & flavorist, 35*(7), 32-39.

McDonald, S.T., Bolliet, D.A., & Hayes, J.E. (2016). *Chemesthesis: Chemical Touch in Food and Eating*: John Wiley & Sons.

Meiselman, H.L. (1968). Magnitude estimations of the course of gustatory adaptation. *Perception & Psychophysics, 4*(4), 193-196.

Mennella, J.A., Pepino, M.Y., Duke, F.F., & Reed, D.R. (2011). Psychophysical dissection of genotype effects on human bitter perception. *Chemical senses, 36*(2), 161-167.

Merskey, H.E. (1986). Classification of chronic pain: Descriptions of chronic pain syndromes and definitions of pain terms. *Pain*.

Meyerhof, W., Batram, C., Kuhn, C., Brockhoff, A., Chudoba, E., Bufe, B., Appendino, G., & Behrens, M. (2010). The molecular receptive ranges of human TAS2R bitter taste receptors. *Chemical senses, 35*(2), 157-170.

Millan, M.J. (1999). The induction of pain: an integrative review. *Progress in neurobiology, 57*(1), 1-164.

Montell, C. (2005). The TRP superfamily of cation channels. *Science Signaling, 2005*(272), re3-re3.

Montell, C., Birnbaumer, L., & Flockerzi, V. (2002). The TRP channels, a remarkably functional family. *Cell, 108*(5), 595-598.

Moore, M., & Weiss, S. (1995). Reasons for non-drinking among Israeli adolescents of four religions. *Drug and alcohol dependence, 38*(1), 45-50.

Mueller, K.L., Hoon, M.A., Erlenbach, I., Chandrashekar, J., Zuker, C.S., & Ryba, N.J.P. (2005). The receptors and coding logic for bitter taste. *Nature, 434*(7030), 225-229.

Nagy, I., Friston, D., Valente, J.S., Perez, J.V.T., & Andreou, A.P. (2014). Pharmacology of the capsaicin receptor, transient receptor potential vanilloid type-1 ion channel *Capsaicin as a Therapeutic Molecule* (pp. 39-76): Springer.

Namer, B., Seifert, F., Handwerker, H.O., & Maihöfner, C. (2005). TRPA1 and TRPM8 activation in humans: effects of cinnamaldehyde and menthol. *Neuroreport, 16*(9), 955-959.

Nilius, B., Owsianik, G., Voets, T., & Peters, J.A. (2007). Transient receptor potential cation channels in disease. *Physiological reviews, 87*(1), 165-217.

Nolano, M., Simone, D.A., Wendelschafer-Crabb, G., Johnson, T., Hazen, E., & Kennedy, W.R. (1999). Topical capsaicin in humans: parallel loss of epidermal nerve fibers and pain sensation. *Pain, 81*(1), 135-145.

Nolden, A.A., & Hayes, J.E. (2015). Perceptual qualities of ethanol depend on concentration, and variation in these percepts associates with drinking frequency. *Chemosensory perception, 8*(3), 149-157.

Nolden, A.A., & Hayes, J.E. (2017). Perceptual and affective responses to sampled capsaicin differ by reported intake. *Food Quality and Preference, 55*, 26-34.

Nolden, A.A., McGeary, J.E., & Hayes, J.E. (2016). Differential bitterness in capsaicin, piperine, and ethanol associates with polymorphisms in multiple bitter taste receptor genes. *Physiology & behavior, 156*, 117-127.

Novakova-Tousova, K., Vyklicky, L., Susankova, K., Benedikt, J., Samad, A., Teisinger, J., & Vlachova, V. (2007). Functional changes in the vanilloid receptor subtype 1 channel during and after acute desensitization. *Neuroscience, 149*(1), 144-154.

Nurgel, C., & Pickering, G. (2005). Contribution of glycerol, ethanol and sugar to the perception of viscosity and density elicited by model white wines. *Journal of texture studies, 36*(3), 303-323.

Nurgel, C., & Pickering, G. (2006). Modeling of sweet, bitter and irritant sensations and their interactions elicited by model ice wines. *Journal of sensory studies, 21*(5), 505-519.

O'Neill, J., Brock, C., Olesen, A.E., Andresen, T., Nilsson, M., & Dickenson, A.H. (2012). Unravelling the mystery of capsaicin: a tool to understand and treat pain. *Pharmacological reviews, 64*(4), 939-971.

Olausson, B. (1998). Recordings of human polymodal single C-fiber afferents following mechanical and argon-laser heat stimulation of inflamed skin. *Experimental brain research, 122*(1), 55-61.

Othman, Z.A.A., Ahmed, Y.B.H., Habila, M.A., & Ghafar, A.A. (2011). Determination of capsaicin and dihydrocapsaicin in Capsicum fruit samples using high performance liquid chromatography. *Molecules, 16*(10), 8919-8929.

Oyagbemi, A., Saba, A., & Azeez, O. (2010). Capsaicin: a novel chemopreventive molecule and its underlying molecular mechanisms of action. *Indian journal of cancer, 47*(1), 53.

Palazzo, E., Luongo, L., de Novellis, V., Rossi, F., Marabese, I., & Maione, S. (2012). Transient receptor potential vanilloid type 1 and pain development. *Current opinion in pharmacology, 12*(1), 9-17.

Peña-Alvarez, A., Ramírez-Maya, E., & Alvarado-Suárez, L.Á. (2009). Analysis of capsaicin and dihydrocapsaicin in peppers and pepper sauces by solid phase microextraction–gas chromatography–mass spectrometry. *Journal of Chromatography A, 1216*(14), 2843-2847.

Peppin, J.F., & Pappagallo, M. (2014). Capsaicinoids in the treatment of neuropathic pain: a review. *Therapeutic advances in neurological disorders, 7*(1), 22-32.

Petruzzi, M., Lauritano, D., De Benedittis, M., Baldoni, M., & Serpico, R. (2004). Systemic capsaicin for burning mouth syndrome: short-term results of a pilot study. *Journal of oral pathology & medicine, 33*(2), 111-114.

Pickering, G.J., Heatherbell, D., Vanhanen, L., & Barnes, M. (1998). The effect of ethanol concentration on the temporal perception of viscosity and density in white wine. *American journal of enology and viticulture, 49*(3), 306-318.

Pickering, G.J., & Robert, G. (2006). Perception of mouthfeel sensations elicited by red wine are associated with sensnitivity to 6-N-propylthiouracil. . *Journal of Sensory Studies, 21*(3), 249-265.

Pirastu, N., Kooyman, M., Traglia, M., Robino, A., Willems, S.M., Pistis, G., d'Adamo, P., Amin, N., d'Eustacchio, A., & Navarini, L. (2014). Association analysis of bitter receptor genes in five isolated populations identifies a significant correlation between TAS2R43 variants and coffee liking. *PloS one, 9*(3), e92065.

Planells-Cases, R., Valente, P., Ferrer-Montiel, A., Qin, F., & Szallasi, A. (2011). Complex regulation of TRPV1 and related thermo-TRPs: implications for therapeutic intervention *Transient Receptor Potential Channels* (pp. 491-515): Springer.

Polydefkis, M., Hauer, P., Sheth, S., Sirdofsky, M., Griffin, J.W., & McArthur, J.C. (2004). The time course of epidermal nerve fibre regeneration: studies in normal controls and in people with diabetes, with and without neuropathy. *Brain, 127*(7), 1606-1615.

Premkumar, L., & Bishnoi, M. (2011). Disease-related changes in TRPV1 expression and its implications for drug development. *Current topics in medicinal chemistry, 11*(17), 2192-2209.

Prescott, J. (1999). The generalizability of capsaicin sensitization and desensitization. *Physiology & behavior, 66*(5), 741-749.

Prescott, J., & Stevenson, R.J. (1995). Effects of oral chemical irritation on tastes and flavors in frequent and infrequent users of chili. *Physiology & behavior, 58*(6), 1117-1127.

Prescott, J., & Stevenson, R.J. (1996). Desensitization to oral zingerone irritation: effects of stimulus parameters. *Physiology & behavior, 60*(6), 1473-1480.

Prescott, J., & Swain-Campbell, N. (2000). Responses to repeated oral irritation by capsaicin, cinnamaldehyde and ethanol in PROP tasters and non-tasters. *Chemical senses, 25*(3), 239.

Pronin, A.N., Xu, H., Tang, H., Zhang, L., Li, Q., & Li, X. (2007). Specific alleles of bitter receptor genes influence human sensitivity to the bitterness of aloin and saccharin. *Current Biology, 17*(16), 1403-1408.

Purves, D., Augustine, G.J., Fitzpatrick, D., Katz, L.C., LaMantia, A.-S., McNamara, J.O., & Williams, S.M. (2001a). Hyperalgesia and Sensitization.

Purves, D., Augustine, G.J., Fitzpatrick, D., Katz, L.C., LaMantia, A.-S., McNamara, J.O., & Williams, S.M. (2001b). The organization of the taste system.

Qin, F. (2007). Regulation of TRP ion channels by phosphatidylinositol-4, 5-bisphosphate *Transient Receptor Potential (TRP) Channels* (pp. 509-525): Springer.

Rains, C., & Bryson, H.M. (1995). Topical capsaicin. *Drugs & aging, 7*(4), 317-328.

Reed, D.R., Zhu, G., Breslin, P.A.S., Duke, F.F., Henders, A.K., Campbell, M.J., Montgomery, G.W., Medland, S.E., Martin, N.G., & Wright, M.J. (2010). The perception of quinine taste intensity is associated with common genetic variants in a bitter receptor cluster on chromosome 12. *Human molecular genetics, 19*(21), 4278-4285.

Richter, C.P. (1956). Loss of appetite for alcohol and alcoholic beverages produced in rats by treatment with thyroid preparations. *Endocrinology, 59*(4), 472-478.

Riera, C. (2007). Artificial sweeteners and salts producing a metallic taste sensation activate TRPV1 receptors.

Riera, C.E., Vogel, H., Simon, S.A., Damak, S., & le Coutre, J. (2008). The capsaicin receptor participates in artificial sweetener aversion. *Biochemical and Biophysical Research Communications, 376*(4), 653-657. doi:Doi 10.1016/J.Bbrc.2008.09.029

Risso, D., Morini, G., Pagani, L., Quagliariello, A., Giuliani, C., De Fanti, S., Sazzini, M., Luiselli, D., & Tofanelli, S. (2014). Genetic signature of differential sensitivity to stevioside in the Italian population. *Genes & nutrition, 9*(3), 1-9.

Ritter, S., & Dinh, T.T. (1988). Capsaicin-induced neuronal degeneration: Silver impregnation of cell bodies, axons, and terminals in the central nervous system of the adult rat. *Journal of Comparative Neurology, 271*(1), 79-90.

Robbins, W.R., Staats, P.S., Levine, J., Fields, H.L., Allen, R.W., Campbell, J.N., & Pappagallo, M. (1998). Treatment of intractable pain with topical large-dose capsaicin: preliminary report. *Anesthesia & Analgesia, 86*(3), 579-583.

Rohacs, T. (2015). Phosphoinositide regulation of TRPV1 revisited. *Pflügers Archiv-European Journal of Physiology*, 1-19.

Rohacs, T., Thyagarajan, B., & Lukacs, V. (2008). Phospholipase C mediated modulation of TRPV1 channels. *Molecular neurobiology, 37*(2-3), 153-163.

Roper, S.D. (2014). TRPs in Taste and Chemesthesis. In B. Nilius & V. Flockerzi (Eds.), *Mammalian Transient Receptor Potential* (Vol. 223, pp. 827-871).

Rosenbaum, T., Gordon-Shaag, A., Munari, M., & Gordon, S.E. (2004). Ca2+/calmodulin modulates TRPV1 activation by capsaicin. *The Journal of general physiology, 123*(1), 53-62.

Roudnitzky, N., Bufe, B., Thalmann, S., Kuhn, C., Gunn, H.C., Xing, C., Crider, B.P., Behrens, M., Meyerhof, W., & Wooding, S.P. (2011). Genomic, genetic and functional dissection of bitter taste responses to artificial sweeteners. *Human Molecular Genetics, 20*(17), 3437-3449.

Rozin, P. (1978). *The use of characteristic flavorings in human culinary practice.* Paper presented at the Arthur D. Little, inc., Flavor Symposium. Cambridge, Mass.(USA). 1977.

Rozin, P., Mark, M., & Schiller, D. (1981). The role of desensitization to capsaicin in chili pepper ingestion and preference. *Chemical senses, 6*(1), 23-31.

Rozin P., S.D. (1980). The Nature and Acquisition of a Preference for Chili Pepper by Humans.

Russell, M., Light, J.M., & Gruenewald, P.J. (2004). Alcohol consumption and problems: the relevance of drinking patterns. *Alcoholism: Clinical and Experimental Research, 28*(6), 921-930.

SAMSHA. (2014). *Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings, .* Retrieved from Rockville, MD: http://www.samhsa.gov/data/sites/default/files/NSDUHresultsPDFWHTML2013/Web/NSDUHresults2013.pdf

Sanatombi, K., & Sharma, G. (2008). Capsaicin content and pungency of different Capsicum spp. cultivars. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca, 36*(2), 89.

Sandell, M.A., & Breslin, P.A. (2006). Variability in a taste-receptor gene determines whether we taste toxins in food. *Current Biology, 16*(18), 792.

Sanz-Salvador, L., Andrés-Borderia, A., Ferrer-Montiel, A., & Planells-Cases, R. (2012). Agonist-and Ca2+-dependent desensitization of TRPV1 channel targets the receptor to lysosomes for degradation. *Journal of Biological Chemistry, 287*(23), 19462-19471.

Scadding, J. (1980). The permanent anatomical effects of neonatal capsaicin on somatosensory nerves. *Journal of anatomy, 131*(Pt 3), 471.

Scala, A., Checchi, L., Montevecchi, M., Marini, I., & Giamberardino, M.A. (2003). Update on burning mouth syndrome: overview and patient management. *Critical Reviews in Oral Biology & Medicine, 14*(4), 275-291.

Schaldemose, E.L., Horjales-Araujo, E., Svensson, P., & Finnerup, N.B. (2015). Altered thermal grill response and paradoxical heat sensations after topical capsaicin application. *Pain, 156*(6), 1101-1111.

Schmelz, M., Schmidt, R., Ringkamp, M., Forster, C., Handwerker, H., & Torebjörk, H. (1996). Limitation of sensitization to injured parts of receptive fields in human skin C-nociceptors. *Experimental brain research, 109*(1), 141-147.

Scinska, A., Koros, E., Habrat, B., Kukwa, A., Kostowski, W., & Bienkowski, P. (2000). Bitter and sweet components of ethanol taste in humans. *Drug and Alcohol Dependence, 60*(2), 199-206.

Scoville, W.L. (1912). Note on capsicums. *Journal of the American Pharmaceutical Association, 1*(5), 453-454.

Sharma, S.K., Vij, A.S., & Sharma, M. (2013). Mechanisms and clinical uses of capsaicin. *European journal of pharmacology, 720*(1), 55-62.

Shi, P., & Zhang, J. (2006). Contrasting modes of evolution between vertebrate sweet/umami receptor genes and bitter receptor genes. *Molecular biology and evolution, 23*(2), 292-300.

Shi, P., & Zhang, J. (2009). Extraordinary diversity of chemosensory receptor gene repertoires among vertebrates. *Chemosensory Systems in Mammals, Fishes, and Insects*, 57-75.

Shi, P., Zhang, J., Yang, H., & Zhang, Y.-p. (2003). Adaptive diversification of bitter taste receptor genes in mammalian evolution. *Molecular biology and evolution, 20*(5), 805-814.

Shimomura, Y., Kawada, T., & Suzuki, M. (1989). Capsaicin and its analogs inhibit the activity of NADH-coenzyme Q oxidoreductase of the mitochondrial respiratory chain. *Archives of biochemistry and biophysics, 270*(2), 573-577.

Silvestre, F.-J., Silvestre-Rangil, J., Tamarit-Santafé, C., & Bautista, D. (2012). Application of a capsaicin rinse in the treatment of burning mouth syndrome. *Medicina oral, patologia oral y cirugia bucal, 17*(1), e1.

Simon, S., de Araujo, I., Stapleton, J., & Nicolelis, M. (2008). Multisensory processing of gustatory stimuli. *Chemosensory perception, 1*(2), 95-102.

Simone, D.A., Nolano, M., Johnson, T., Wendelschafer-Crabb, G., & Kennedy, W.R. (1998). Intradermal injection of capsaicin in humans produces degeneration and subsequent reinnervation of epidermal nerve fibers: correlation with sensory function. *The Journal of neuroscience, 18*(21), 8947-8959.

Simons, C.T., Carstens, M.I., & Carstens, E. (2003). Oral irritation by mustard oil: self-desensitization and cross-desensitization with capsaicin. *Chemical senses, 28*(6), 459-465.

Singh, N., Vrontakis, M., Parkinson, F., & Chelikani, P. (2011). Functional bitter taste receptors are expressed in brain cells. *Biochemical and biophysical research communications, 406*(1), 146-151.

Sizer, F., & Harris, N. (1985). The influence of common food additives and temperature on threshold perception of capsaicin. *Chemical senses, 10*(3), 279-286.

Smith, C.E. (1968). The New World centers of origin of cultivated plants and the archaeological evidence. *Economic Botany, 22*(3), 253-266.

Smith, H., & Brooks, J.R. (2014). Capsaicin-Based Therapies for Pain Control *Capsaicin as a Therapeutic Molecule* (pp. 129-146): Springer Basel.

Smutzer, G., & Devassy, R.K. (2016). Integrating TRPV1 Receptor Function with Capsaicin Psychophysics. *Advances in Pharmacological Sciences, 2016*.

Snyder, D., Fast, K., & Bartoshuk, L.M. (2004). Valid comparisons of suprathreshold sensations. *Journal of consciousness studies, 11*(7-8), 7-8.

Snyder, D.J., Prescott, J., & Bartoshuk, L. (2006). Modern Psychophysics and the Assesment of Human Oral Sensation. In Hummel T.D. & Welge-Lussen A.B. (Eds.), *Taste and Smell* (Vol. 63, pp. 221-241): S. Karger AG.

Spielman, A.I., Pepino, M.Y., Feldman, R., & Brand, J.G. (2010). Technique to collect fungiform (taste) papillae from human tongue. *Journal of visualized experiments: JoVE*(42).

Srivastava, S.K. (2013). *Role of Capsaicin in Oxidative Stress and Cancer*: Springer.

Stein, A.T., Ufret-Vincenty, C.A., Hua, L., Santana, L.F., & Gordon, S.E. (2006). Phosphoinositide 3-kinase binds to TRPV1 and mediates NGF-stimulated TRPV1 trafficking to the plasma membrane. *The Journal of general physiology, 128*(5), 509-522.

Stevens, D.A., & Lawless, H.T. (1986). Putting out the fire: effects of tastants on oral chemical irritation. *Perception & Psychophysics, 39*(5), 346-350.

Stevenson, R.J., & Prescott, J. (1994). The effects of prior experience with capsaicin on ratings of its burn. *Chemical senses, 19*(6), 651-656.

Stevenson, R.J., & Prescott, J. (1997). Judgments of chemosensory mixtures in memory. *Acta psychologica, 95*(2), 195-214.

Stevenson, R.J., & Yeomans, M.R. (1993). Differences in ratings of intensity and pleasantness for the capsaicin burn between chili likers and non-likers; implications for liking development. *Chemical senses, 18*(5), 471-482.

Suh, Y.-G., & Oh, U. (2005). Activation and activators of TRPV1 and their pharmaceutical implication. *Current pharmaceutical design, 11*(21), 2687-2698.

Sun, F.-J., Guo, W., Zheng, D.-H., Zhang, C.-Q., Li, S., Liu, S.-Y., Yin, Q., Yang, H., & Shu, H.-F. (2013). Increased expression of TRPV1 in the cortex and hippocampus from patients with mesial temporal lobe epilepsy. *Journal of Molecular Neuroscience, 49*(1), 182-193.

Sun, X., & Zakharian, E. (2015). Regulation of the Temperature-dependent Activation of Transient Receptor Potential Vanilloid 1 (TRPV1) by Phospholipids in Planar Lipid Bilayers. *Journal of Biological Chemistry, 290*(8), 4741-4747.

Surh, Y.-J., Lee, E., & Lee, J.M. (1998). Chemoprotective properties of some pungent ingredients present in red pepper and ginger. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 402*(1), 259-267.

Surh, Y.-J., & Lee, S. (1996). Capsaicin in hot chili pepper: carcinogen, co-carcinogen or anticarcinogen? *Food and Chemical Toxicology, 34*(3), 313-316.

Surh, Y.-J., & Sup Lee, S. (1995). Capsaicin, a double-edged sword: toxicity, metabolism, and chemopreventive potential. *Life sciences, 56*(22), 1845-1855.

Szallasi, A., & Blumberg, P.M. (1999). Vanilloid (capsaicin) receptors and mechanisms. *Pharmacological reviews, 51*(2), 159-212.

Szallasi, A., & Gunthorpe, M.J. (2008). Peripheral TRPV1 receptors as targets for drug development: new molecules and mechanisms. *Current pharmaceutical design, 14*(1), 32-41.

Szolcsanyi, J. (1977). A pharmacological approach to elucidation of the role of different nerve fibres and receptor endings in mediation of pain. *Journal de physiologie, 73*(3), 251-259.

Szolcsányi, J. (1993). Actions of capsaicin on sensory receptors. *Capsaicin in the Study of Pain*, 1-26.

Szolcsanyi, J., & Pinter, E. (2013). Transient receptor potential vanilloid 1 as a therapeutic target in analgesia. *Expert Opinion on Therapeutic Targets, 17*(6), 641-657. doi:10.1517/14728222.2013.772580

Szolcsanyi, J., & Sandor, Z. (2012). Multisteric TRPV1 nocisensor: a target for analgesics. *Trends in Pharmacological Sciences, 33*(12), 646-655. doi:10.1016/j.tips.2012.09.002

Tepper, B.J. (2008). Nutritional implications of genetic taste variation: the role of PROP sensitivity and other taste phenotypes. *Annual Review Nutrition, 28*, 367-388.

Todd, P., Bensinger, M., & Biftu, T. (1977). Determination of pungency due to capsicum by gas-liquid chromatography. *Journal of Food Science, 42*(3), 660-665.

Tominaga, M., Caterina, M.J., Malmberg, A.B., Rosen, T.A., Gilbert, H., Skinner, K., Raumann, B.E., Basbaum, A.I., & Julius, D. (1998). The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron, 21*(3), 531-543.

Tominaga, M., & Tominaga, T. (2005). Structure and function of TRPV1. *Pflügers Archiv, 451*(1), 143-150.

Tremblay, K.A., Bona, J.M., & Kranzler, H.R. (2009). Effects of a diagnosis or family history of alcoholism on the taste intensity and hedonic value of sucrose. *The American Journal on Addictions, 18*(6), 494-499.

Trevisani, M., Smart, D., Gunthorpe, M.J., Tognetto, M., Barbieri, M., Campi, B., Amadesi, S., Gray, J., Jerman, J.C., & Brough, S.J. (2002). Ethanol elicits and potentiates nociceptor responses via the vanilloid receptor-1. *Nature neuroscience, 5*(6), 546-551.

Upadhyaya, J.D., Chakraborty, R., Shaik, F.A., Jaggupilli, A., Bhullar, R.P., & Chelikani, P. (2016). The Pharmacochaperone Activity of Quinine on Bitter Taste Receptors. *PloS one, 11*(5), e0156347.

Valdes, A.M., De Wilde, G., Doherty, S.A., Lories, R.J., Vaughn, F.L., Laslett, L.L., Maciewicz, R.A., Soni, A., Hart, D.J., & Zhang, W. (2011). The Ile585Val TRPV1 variant is involved in risk of painful knee osteoarthritis. *Annals of the rheumatic diseases*, annrheumdis148122.

Valente, P., García-Sanz, N., Gomis, A., Fernández-Carvajal, A., Fernández-Ballester, G., Viana, F., Belmonte, C., & Ferrer-Montiel, A. (2008). Identification of molecular determinants of channel gating in the transient receptor potential box of vanilloid receptor I. *The FASEB Journal, 22*(9), 3298-3309.

Van Gerven, L., Alpizar, Y.A., Wouters, M.M., Hox, V., Hauben, E., Jorissen, M., Boeckxstaens, G., Talavera, K., & Hellings, P.W. (2014). Capsaicin treatment reduces nasal hyperreactivity and transient receptor potential cation channel subfamily V, receptor 1 (TRPV1) overexpression in patients with idiopathic rhinitis. *J Allergy Clin Immunol, 133*(5), 1332-1339, 1339.e1331-1333. doi:10.1016/j.jaci.2013.08.026

Van Rijswijk, J., Boeke, E., Keizer, J., Mulder, P., Blom, H., & Fokkens, W. (2003). Intranasal capsaicin reduces nasal hyperreactivity in idiopathic rhinitis: a double-blind randomized application regimen study. *Allergy, 58*(8), 754-761.

Venkatachalam, K., & Montell, C. (2007). TRP channels. *Annual review of biochemistry, 76*, 387.

Vickers, E.R., & Cousins, M.J. (2000). Neuropathic Orofacial Pain Part 1-Prevalence And Pathophysiology. *Australian Endodontic Journal, 26*(1), 19-26.

Villamor, R.R., Evans, M.A., & Ross, C.F. (2013). Ethanol, tannin, and fructose concentration effects on sensory properties of model red wines. *American Journal of Enology and Viticulture*, ajev. 2013.12118.

Vyklicky, L., Novakova-Tousova, K., Benedikt, J., Samad, A., Touska, F., & Vlachová, V. (2008). Calcium-dependent desensitization of vanilloid receptor TRPV1: a mechanism possibly involved in analgesia induced by topical application of capsaicin. *Physiol Res, 57*(Suppl 3), S59-68.

Wang, J.C., Hinrichs, A.L., Bertelsen, S., Stock, H., Budde, J.P., Dick, D.M., Bucholz, K.K., Rice, J., Saccone, N., & Edenberg, H.J. (2007). Functional Variants in TAS2R38 and TAS2R16 Influence Alcohol Consumption in High-Risk Families of African-American Origin. *Alcoholism: Clinical and Experimental Research, 31*(2), 209-215.

Wasner, G., Schattschneider, J., Binder, A., & Baron, R. (2004). Topical menthol—a human model for cold pain by activation and sensitization of C nociceptors. *Brain, 127*(5), 1159-1171.

Welch, C.J., Regalado, E.L., Welch, E.C., Eckert, I.M., & Kraml, C. (2014). Evaluation of capsaicin in chili peppers and hot sauces by MISER HPLC-ESIMS. *Analytical Methods, 6*(3), 857-862.

Williams, E. (1949). Experimental designs balanced for the estimation of residual effects of treatments. *Australian Journal of Chemistry, 2*(2), 149-168.

Wilson, C., O'Brien, C., & MacAirt, J. (1973). The effect of metronidazole on the human taste threshold to alcohol. *British Journal of Addiction to Alcohol & Other Drugs, 68*(2), 99-110.

Wooding, S. (2011). Signatures of natural selection in a primate bitter taste receptor. *Journal of molecular evolution, 73*(5-6), 257-265.

Wooding, S., Bufe, B., Grassi, C., Howard, M.T., Stone, A.C., Vazquez, M., Dunn, D.M., Meyerhof, W., Weiss, R.B., & Bamshad, M.J. (2006). Independent evolution of bitter-taste sensitivity in humans and chimpanzees. *Nature, 440*(7086), 930-934.

Wooding, S., Gunn, H., Ramos, P., Thalmann, S., Xing, C., & Meyerhof, W. (2010). Genetics and bitter taste responses to goitrin, a plant toxin found in vegetables. *Chemical senses, 35*(8), 685-692.

Woolf, C.J., & Salter, M.W. (2000). Neuronal plasticity: increasing the gain in pain. *Science, 288*(5472), 1765-1768.

Xu, H.a.T., Wei and Oyama, Terry and Anderson, Sharon and Cohen, David. (2007). Functional eddects of nonsynonymous polymorphisms in the human TRPV1 gene. *American Journal of Physiology, 293*(6).

Xu, Y.-p., Zhang, J.-w., Li, L., Ye, Z.-y., Zhang, Y., Gao, X., Li, F., Yan, X.-s., Liu, Z.-g., & Liu, L.-j. (2012). Complex regulation of capsaicin on intracellular second messengers by calcium dependent and independent mechanisms in primary sensory neurons. *Neuroscience letters, 517*(1), 30-35.

Yang, F., Xiao, X., Cheng, W., Yang, W., Yu, P., Song, Z., Yarov-Yarovoy, V., & Zheng, J. (2015). Structural mechanism underlying capsaicin binding and activation of the TRPV1 ion channel. *Nature Chemical Biology, 11*(7), 518-524.

Yang, R., Xiong, Z., Liu, C.J., & Liu, L.J. (2014). Inhibitory Effects of Capsaicin on Voltage-Gated Potassium Channels by TRPV1-Independent Pathway. *Cellular and Molecular Neurobiology, 34*(4), 565-576. doi:10.1007/s10571-014-0041-1

Yilmaz, Z., Renton, T., Yiangou, Y., Zakrzewska, J., Chessell, I., Bountra, C., & Anand, P. (2007). Burning mouth syndrome as a trigeminal small fibre neuropathy: increased heat and capsaicin receptor TRPV1 in nerve fibres correlates with pain score. *Journal of Clinical Neuroscience, 14*(9), 864-871.

Yu, P., & Pickering, G.J. (2008). Ethanol difference thresholds in wine and the influence of mode of evaluation and wine style. *American journal of enology and viticulture, 59*(2), 146-152.

Zakrzewska, J.M. (2002). Facial pain: neurological and non-neurological. *Journal of Neurology, Neurosurgery & Psychiatry, 72*(suppl 2), ii27-ii32.

Zhang, X., Huang, J., & McNaughton, P.A. (2005). NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels. *The EMBO journal, 24*(24), 4211-4223.

Zhu, Y., Colak, T., Shenoy, M., Liu, L., Pai, R., Li, C., Mehta, K., & Pasricha, P.J. (2011). Nerve Growth Factor Modulates TRPV1 Expression and Function and Mediates Pain in Chronic Pancreatitis. *Gastroenterology, 141*(1), 370-377. doi:http://dx.doi.org/10.1053/j.gastro.2011.03.046

Appendix A

Supplemental methods for Chapter 5

Overview

These data were collected as part of a broader study on chemosensory genetics, oral sensation and food preferences (GIANT-CS, phase I). The GIANT-CS, phase I study involves one test session for all participants, with some participants being invited back for 3 additional sessions on separate days (details below). Interested participants were screened prior to the first visit to ensure that they were qualified. Eligibility criteria included: between 18-45 years old, not pregnant or breastfeeding, non-smoker (had not smoked in the last 30 days), no known defects of smell or taste, no lip, cheek or tongue piercings, no history of any condition involving chronic pain, not currently taking any prescription pain medication, no reported history of choking or difficulty swallowing and no history of thyroid disease. Participants also needed to be willing to provide a DNA sample via saliva. Written informed consent was obtained from all participants. All procedures were approved by the Pennsylvania State University Institutional Review Board (protocol number #33176).

Session 1

During the first session, informed consent was obtained and participants were given a brief explanation of the aims of the study: "to quantify the influence of specific genes on the sensations from capsaicin, piperine and ethanol." Participants were oriented to a generalized bipolar hedonic scale and subsequently completed a general degree of liking questionnaire that

contained 63 food and non-food items. Next, anthropometric data (height, weight, BMI and blood pressure) were collected for exploratory analysis. A salivary DNA sample was collected using Oragene kits followed by digital microscopy of the anterior tongue. Participants were oriented to the general Labeled Magnitude Scale (gLMS), and also completed an orientation in which participants practiced using the scale (e.g. (Hayes, Allen, et al., 2013)). Participants tasted six perceptually complex tastants and irritants (0.56 M potassium chloride (salty/bitter), 0.41 mM quinine HCl (bitter), 25 mM acesulfame-K (sweet/bitter), 100 mM MSG + 50mM IMP (umami/savory), 0.5 M sucrose (sweet), and 25 uM capsaicin (burning/stinging)). Intensity ratings were collected for multiple qualities (sweetness, bitterness, sourness, burning/stinging, saltiness and umami). The final task in session 1 was to complete a standard propylthiouracil (PROP) phenotyping protocol with PROP, salt and auditory stimuli. PROP and salt solutions each had 5 concentrations and were presented in duplicate with auditory stimuli presented at 5 different tones with 5 replicates (Dinehart, Hayes et al., 2006; Duffy, Peterson, et al., 2004; Hayes, Sullivan et al., 2010)). After leaving the laboratory, participants also completed an online survey that included several different personality measures. Total time in the laboratory for the first session was approximately 1 hour, and all data were collected one-on-one with project staff.

Sessions 2, 3 and 4

Upon completing session 1, some participants were invited to participate in three additional testing sessions. In order to be eligible to participate in sessions 2, 3 and 4, the individual's circumvallate papillae on the posterior tongue had to be visible and accessible with a cotton swab. Of participants who qualified, 130 individuals returned to complete all four sessions.

Participants were reoriented to the gLMS using instructions identical to those provided during session 1. This included explanation of the top anchor, 'strongest imaginable sensation of any kind', as well as reminding participants that they should click anywhere along the scale and to not let whether or not they like/dislike the sample to influence their intensity ratings. Before rating any sampled stimuli, participants completed a warm-up task, identical to the one presented in session one.

Sessions 2, 3, and 4 began by presenting 5 different stimuli (sucrose, citric acid, NaCl, MSG/IMP, and quinine) on four different areas of the tongue (right tip, left tip, right CV and left CV) in a rotating fashion. The samples were presented at room temperature solutions were made with reverse osmosis (RO) water. These stimuli will be referred to as spatial stimuli. A single cotton swab applicator was submerged in the appropriate solution and blotted to remove excess liquid. The swab was rolled across one of the four quadrants of the tongue for 3 seconds. The participant was instructed to keep their tongue away from the roof of their mouth, make a rating for each quality while keeping their lips closed. The qualities were identical to session 1 (sweetness, bitterness, sourness, burning/stinging, saltiness and umami). Samples were presented in a blocked counterbalanced order, with all 5 stimuli being presented each day for a total of 20 samples (5 stimuli in each of the 4 quadrants). After 10 samples, the participant began a different task (see below). All five stimuli were presented in rotating areas, before the same stimulus was presented again.

Participants completed a multiple attribute time intensity (MATI) task for a single irritant after 10 spatial samples. Each day consisted of a different irritant, but remained constant throughout a single session. The three irritants presented in this study consist of capsaicin, piperine and ethanol. Participants received either a 50% v/v ethanol, 100uM capsaicin or 70mM pipierine. The sample was presented by touching two cotton swab applicators on either their left

or right CV for 10 seconds. Intensity ratings were collected every 30 seconds for a total of 3 minutes. Intensity ratings for six qualities were collected (sweetness, bitterness, sourness, burning/stinging, umami/savory and saltiness). Participants were asked to keep their tongue away from the roof of their mouth, while making their ratings and to keep their lips closed to minimize evaporative cooling. Participants were not allowed to rinse for the duration of the three minutes. Only a single irritant was presented within a single session, and the order in which the irritants were presented across sessions was counterbalanced across individuals. There was a four-minute break following the MATI task where participants were allowed to rinse with mouth temperature RO water. Following the first MATI task, the second 10 spatial samples were applied and then completed the second MATI task for the remaining CV.

Threshold data was collected following the second 4-minute break. Estimated detection thresholds were collected using (ASTM-E679) protocol. Briefly, participants were asked to select the different sample using a three alternative forced choice (3-AFC) test. One irritant concentration was paired with two blank samples. For blanks paired with capsaicin samples, 1% of ethanol was added to mimic the ethanol present in the capsaicin test samples. A total of 6 3-AFC pairs were presented.

The final task within a session was to rate the 'overall intensity' on a gLMS for a 15mL of either 16%v/v ethanol, 0.912g/L piperine, or 0.12g/L capsaicin (with 1% ethanol) solution presented at mouth temperature. Participants sipped the sample, swished for 5 seconds to cover all oral surfaces, and then spit it out.

VITA

Alissa Allen Nolden

EDUCATION

2016 PhD., Food Science and Clinical Translational Science
The Pennsylvania State University, University Park, PA
2013 M.S., Food Science
The Pennsylvania State University, University Park, PA
2011 B.S., Food Science with minors in Chemistry and Resource Economics
University of Massachusetts, Amherst, MA

PUBLICATIONS

Nolden AA and Hayes JE. 2017. Perceptual and affective responses to sampled capsaicin differ by reported intake. Food Quality and Preference. 55: 26-34.

Nolden AA and Hayes JE. 2016. Differential bitterness in capsaicin, piperine and ethanol associates with polymorphisms in multiple bitter taste receptor genes. Physiology & behavior. 156: 117-127.

Nolden AA and Hayes JE. 2015. Perceptual qualities of ethanol depend on concentration, and variation in these percepts associates with drinking frequency. Chemosensory Perception. 8(3): 149-157.

Hayes JE, Feeney EF, **Nolden** AA, McGeary JE. 2015. Quinine and grapefruit juice bitterness associate with allelic variants in TAS2R31. Chemical Senses. 40(6): 437-443.

Allen AL, McGeary JE and Hayes JE. 2014. Polymorphisms in *TRPV1* and *TAS2Rs* associate with the burning and bitterness of sampled ethanol. Alcoholism: Clinical and Experimental Research. 38(10): 2550-2560.

Hayes JE, Feeney EF, and **Allen** AL. 2013. Do polymorphisms in chemosensory genes matter for human ingestive behavior? Food Quality and Preference. 30(2): 202-216.

Allen AL, McGeary JE and Hayes JE. Rebaudioside A and Rebaudioside D Bitterness do not Covary with Acesulfame-K Bitterness or Polymorphisms in *TAS2R9* and *TAS2R31*. Chemosensory Perception. 6(3): 109-117.

Allen AL, McGeary JE, Knopik VS, Hayes JE. 2013. Bitterness of the non-nutritive sweetener Acesulfame Potassium varies with polymorphisms in *TAS2R9* and *TAS2R31*. Chemical Senses. 38(5): 379-389.

Hayes JE, **Allen** AL, and Bennett SM. 2013. Direct comparison of the generalized Visual Analog Scale (gVAS) and general Labeled Magnitude Scale (gLMS). Food Quality and Preference. 28(1): 34-44.

FUNDING & AWARDS

1 F31 DC014651-01A1, NIH/NIDCD: Role: PI, Co-sponsors: Drs. Hayes & Lambert
Title: TRPV1 expression, oral burn and capsaicin desensitization in humans, 2015-2017

NIH Clinical and Translational Science Institute TL1 Fellowship, 2013-2014

John and Jane Ziegler Award in Sensory Science, 2015

Robert and Jeanne McCarthy Memorial Graduate Teaching Award & Scholarship, 2015

IFT Sensory and Consumer Science Division Sliver Celebration PhD Degree Scholarship, 2014