Measuring Intake and Behavioral Responses to Phenylthiocarbamide for the Identification of Phenotypic Expression of Bitter Tasting Ability in Sheep

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Abstract

Research on the genetic and phenotypic expression of bitter taste perception in sheep suggests bitter-tolerant sheep may be more inclined to consume less palatable rangeland plants. In the absence of definitive genetic tests, the current work focuses on the development of accurate and precise methods for the classifying of bitter-taste-sensing phenotypes (i.e., bitter taster) in sheep. Existing methods for determining bitter taster status of sheep using phenylthiocarbamide (PTC)-spiked drinking water were lengthy, laborious, and limited on sample size. Our first experiment outlines refined methodology for the classification of nearly three times the number of rams, utilizing only two concentrations of PTC-solution over a 2-day test period. During this trial, behavioral reactions uniquely associated with each PTC-tasting phenotype were also identified. A later attempt to develop methods utilizing an involuntary drench to deliver PTC-solution suggests that the use of behavioral analysis for the identification of bitter tasting phenotypes in sheep may be limited to voluntary preference tests. Although bitter taster status impacts dietary preference in humans, literature regarding the influence of bitter taste perception on the foraging behavior of sheep is limited. Subsequent tests using intake of both internal and topical applications of PTC-treated feed to identify bitter tasting ability were inconclusive. While some mature rams showed reluctance to consume PTC-treated feed, ram lambs consumed PTC pellets to near entirety. Although ram lambs did not demonstrate an aversive feed intake response, behavioral reactions indicative of bitter sensitivity were observed among individual sheep. Factors outside of genetics, such as physiological state or learned behaviors may play an important role in voluntary diet decision making in sheep. Efficient methods for the identification of bitter taster phenotypes are the first step in determining the effects of bitter tasting ability on dietary selection in sheep.

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Dedication

To my family and friends, without whom this path would be unfulfilled. To my parents Clay and Cary, for always encouraging me to reach further and dream bigger. To my husband Brad, for his love and support even when we were a thousand miles apart. And to my daughter Janie Louise, I may not have known it from the start, but this journey was always for you.

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Statement of Contribution

Refined methodology for identification of bitterness aversion in mature rams through quantification of fluid intake and behavioral response to phenylthiocarbamide

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Claire Southerland's responsibilities included: Experimental design. collecting and organizing data, conducting statistical analyses, constructing tables and figures, and writing, formatting, revising, and submitting the manuscript.

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Chapter 1: Literature Review

Shrub encroachment

Shrub encroachment onto rangelands has become an area of increasing interest, having been cited as the cause of both structural (Zarovali et al., 2007; Knapp et al., 2008) and functional (McKinley et al., 2008; Schlesinger et al., 1999; Throop & Archer, 2008) environmental changes. Approximately 330 million hectares of non-forested United States rangelands are undergoing conversion to woody shrubland, primarily in the arid west (Pacala et al., 2001; Estell et al., 2012). Contributing factors to shrub encroachment include climate change, persistent drought, increasing atmospheric CO_2, alteration of wildfire regimes, declines in native browsers, and unrestricted grazing of domestic livestock (Archer et al., 2017). However, the increasing abundance of indigenous woody species has primarily been attributed to decreased fire frequency and the herbivorous removal of fine fuels which consist of lighter weight plant material, such as grasses, leaves, needles and small twigs (Fuhlendorf et al., 2008).

Prominent methods of brush control include mechanical removal, chemical application, and prescribed fire (Wambolt & Payne, 1986). However, each one of these practices comes with a unique set of drawbacks. For example, success of prescribed burning is dependent on fuel loads and may need to be combined with more costly and terrain-limited mechanical options, such as mowing or ground-level chaining (Ansley & Wiedemann, 2008). While chemical methods are equally effective, determining the plant community-level effects of herbicide application may be difficult to predict in species-rich areas (Laufenberg et al., 2005). Alternatively, grazing management—particularly the use of sheep or goats due to their willingness to browse shrubs during certain times of the year (Coblentz, 1977)—may be a more practical solution to shrub encroachment. Suggested grazing strategies include, high-intensity short-duration (Seefeldt, 2005), long-term (Wambolt & Payne, 1986), mixed livestock species (Wilcox et al., 2021), or a combination of grazing with other methods of removal, such as burning (Fuhlendorf et al., 2008; Wilcox et al., 2021) or herbicide application (Magadlela et al., 1995).

Shrub encroachment reduces rangeland herbage production and nutritive value (Zarovali et al., 2007); and thus, can negatively impact sustainable commercial livestock production (Fuhlendorf et al., 2008). Fuhlendorf et al. (2008) evaluated several models which integrated various levels of grazing intensities and fire return intervals for the improvement of both grassland ecosystems and animal productivity. These methods operated under the assumption that the persistence of rangelands

may depend upon the disturbances with which they co-evolved (Parr et al., 2014; Veldman et al., 2015; Wilcox et al., 2021). This work suggested that the type of livestock grazing regime (none, moderate, or heavy) effected the rates and dynamics of the physiognomic transformation to juniper-shrubland (Fuhlendorf et al., 2008). It was determined that moderate grazing combined with a fire return interval of 5-10 years could be used to maintain juniper growth, while in the absence of increased fire return intervals, ungrazed or heavily grazed areas experienced unrestricted conversion to shrubland (Fuhlendorf et al., 2008).

Grazing livestock has been suggested to have applications for the targeted control of shrubs such as juniper (*Juniperus* spp.) (Pritz et al., 1997; Campbell et al., 2007; Fuhlendorf et al., 2008; Waldron et al., 2009), and sagebrush (*Artemisia tridentata* Nutt.) (Bork et al., 1998; Snowder et al., 2001; Seefeldt, 2005; Fraker-Marble et al., 2007). In addition to removing woody plant material in rugged and often inaccessible areas, the grazers are then able to convert the undesirable biomass into saleable products (meat, milk and fiber) (Sharrow et al., 1989; Hart, 2001; Wilcox et al., 2021). Research suggests that the genetic selection of goats and sheep to respectively target juniper (Waldron et al., 2009) and sagebrush (Snowder et al., 2001) maybe possible. While a range of literature exists for juniper consumption by goats (Pritz et al., 1997; Campbell et al., 2007; Utsumi et al., 2009; Dietz et al., 2010; Miller & Scott, 2021), further research is needed to confirm the efficacy of selecting for sheep to target sagebrush as a component in their diet (Seefeldt, 2005).

Notably, some woody rangeland plants produce terpenes, or essential oils, which are elicited upon mastication (Cedarleaf et al., 1983; Johnson et al., 1985; Utsumi et al., 2009). It is widely accepted that bitter tasting ability in general evolved to detect these secondary plant compounds (Garcia-Bailo et al., 2009). When the sensation of bitter taste is paired with negative post-ingestive feedback, livestock may become conditioned to avoid bitter tasting feeds (Burritt & Provenza, 1996; Frederick D. Provenza, 1996). Research on the genetic (Davenport et al., 2021) and phenotypic (Henslee et al., 2019; Southerland et al., 2022) expression of bitter taste perception in rams has suggested that bitter-tolerant sheep may be more inclined to consume less palatable rangeland plants like sagebrush. Yet, formation of taste preferences are highly complex and have been suggested to be driven by several factors, including genetics, age, health, post-ingestive feedback and learned behaviors (Tepper, 2008). Understanding the dynamic relationship between bitter tasting ability and dietary preferences could improve targeted grazing strategies for the effective control of shrub encroachment onto rangelands.

Dietary selection and grazing behavior of domestic ruminants

In recent years, mismanagement of grazing herbivores, which includes are removal of grazing, on rangelands has been linked to decreasing plant (Courchamp et al., 2003) and soil diversity (Williams

et al., 2008), increasing the spread of nonnative plant species (Parker et al., 2006; Oduor et al., 2010), and altering fire regimes (Kimuyu et al., 2014). However, many of these issues appear to resolve with proper grazing management (Bailey et al., 2019; Marchetto et al., 2021). Through careful control of animal density, duration of grazing, and season of use, grazing can be conducted without negatively impacting the landscape (Svejcar et al., 2014; Bailey et al., 2019). One method, known as "targeted" or "prescribed" grazing can even be used to restore degraded rangelands by promoting healthy flora, fauna, soil, water resources, and ecological cycles (Svejcar et al., 2014; Bailey et al., 2019). Most frequently, targeted grazing by domestic livestock is used to decrease the prevalence of a specific unwanted plant species, such as leafy spurge (*Euphorbia esula*) (Olsen, 1978; Walker et al., 1992; Lacey & Sheley, 1996; Rinella & Bellows, 2016), cheatgrass (*Bromus tectorum*)(Diamond et al., 2009; Schmelzer et al., 2014; Perryman et al., 2020), big sagebrush (*Artemisia tridentata* Nutt.) (Petersen et al., 2014), and many other plant species discussed by Marchetto et al., 2021.

Targeted grazing can be used either alternatively or complimentary to mechanical, chemical and pyric methods of vegetation management (Magadlela et al., 1995; Fuhlendorf et al., 2008; Wilcox et al., 2021). Targeted grazing is often thought of as a more "environmentally friendly" method by converting undesirable vegetation into saleable products (Fuhlendorf et al., 2008) in terrain-limited areas, without the risk of chemical residues (Sharrow et al., 1989; Hart, 2001; Bailey et al., 2019; Wilcox et al., 2021). However, in order for targeted grazing to be successful, rangeland managers must select the appropriate livestock species and grazing management strategy to match landscape goals (Frost & Launchbaugh, 2003; Tóth et al., 2018).

Livestock species vary in their dietary preferences and grazing behaviors (Walker, 1994). These differences are primarily driven by morphological and physiological characteristics of an animals' mouth and gut anatomy (Hofmann, 1989) which divide ruminants into three categories: roughage eaters, concentrate selectors, and intermediate feeders (Hofmann, 1989). Comparatively, roughage eaters have more rigid lips, broader muzzles, and a highly keratinized tongue; as well as smaller salivary glands, larger rumens, and smaller livers relative to their body weight (Hofmann, 1989; Van Soest, 1994). Cattle and sheep are considered roughage eaters (Hofmann, 1989), while goats are classified as intermediate feeders. However, there is some contention over the classification of sheep due to their versatility and adaptive diets which closely identifies with that of intermediate feeders (Vallentine, 1991).

These differences make certain livestock species more equipped to selectively graze on various vegetation types. The dietary inclusion of grasses, forbs and browse is 50, 30, and 20 % for sheep; 70, 15 and 15 % for cattle; and 30, 10, 60 % for goats, respectively (Walker, 1994). As such, cattle are most frequently used to target grasses, sheep for forbs, and goats for shrubs (Marchetto et al., 2021).

However, due to goat's high selectivity for browse (leaves, twigs, and buds of woody plants), it has been suggested that applied grazing be followed with targeted mechanical or chemical treatments to allow regeneration of more desirable native shrubs (Rathfon et al., 2021). The dietary composition of goats, and to a lesser extent sheep (Kronberg & Malechek, 1997; Papachristou, 1997), varies seasonally, consuming the most available and nutritive forage at a given time of year (Coblentz, 1977). Although goats possess the ability to detoxify higher amounts of secondary compounds compared to other ruminants (Salem et al., 2006), sheep, too, can be very efficient browsers under seasonal grazing pressures (Valderrábano et al., 1996).

In addition to different plant species, domestic livestock vary in their preference for plant parts. First and foremost, grazers and browsers will select for nutritious green vegetation over dead plant material (Hamilton et al., 1973; Gurung et al., 1994). Goats best utilize their deep and narrow mouths, prehensile lips and tongue to selectively harvest leaves and twigs (Hofmann, 1989; Van Soest, 1994), consuming forage from the top down (Norton et al., 1990). The cleft lip of sheep allows them to single out the highly digestible plant components, utilizing their incisors to remove vegetation close to the ground first (Norton et al., 1990; Rook et al., 2004). However, the rigid lips of cattle do not allow them to be as selective and instead will use their tongue to gather large quantities biomass which consequently is sometimes of lower-quality (Jerrentrup et al., 2015). As such, complimentary use of livestock with different feeding styles could be useful in promoting more even use of rangeland plants (Walker, 1994).

Not only do herbivores seek out plants with high nutrient content, but they also prefer forages with lower chemical and physical defenses, like thorns (Hanley, 1997). To avoid herbivory, many rangeland plants produce secondary compounds, otherwise known as phytotoxins, which are drawn out during the grazing process (Cedarleaf et al., 1983; Bennett & Wallsgrove, 1994). When ingested in enough quantity, these secondary compounds can induce illness and disrupt digestive functions (Launchbaugh et al., 2001). For example, Ngugi et al. (1995) determined that in sheep, for every 1% increase of sagebrush content in the diet, there was a 2.1% decrease in dry matter (DM) digestibility, which may be due to a depression of ruminal microbial activity caused by ingestion of terpenes, a phytotoxin (Nagy et al., 1964).

The toxic anti-microbial effects of terpenes stem from their ability to promote cell rupture and inhibit protein and DNA synthesis (Álvarez-Martínez et al., 2021). These negative digestive consequences are a regulated process that protect plants from overuse by limiting how much herbivores can consume (Coley et al., 1985). However, herbivores may benefit from the antiparasitic effects of secondary plant compounds (Villalba et al., 2017). It has been suggested that regulated self-selection of secondary plant compounds triggered by need may even improve fitness in sick animals

(Singer et al., 2009). This behavior has been noted in parasitized sheep and goats in which selection for feeds that contained condensed-tannins reduced parasite loads (Villalba et al., 2010 Juhnke et al., 2012; Amit et al., 2013), but in the absence of parasitic infection, continued ingestion of condensed-tannins may have negative impacts on animal growth (Burke et al., 2014). Although the literature does not extend to the antiparasitic effects of terpenes on grazing livestock, it is possible that preference for foods containing secondary plant compounds may depend on post-ingestive feedback and the cost versus benefit within individuals (Villalba et al., 2017).

When foraging, ruminants naturally select a variety of feedstuffs in order to limit nutrient deficiencies and minimize metabolic discomfort caused by ingesting secondary plant compounds (Launchbaugh et al., 2001). The concentration of secondary metabolites is known to vary within plant species (Maknickienė & Asakavičiūtė, 2008; Olsoy et al., 2020) and across phenological states (Lee et al., 2007; Maknickienė & Asakavičiūtė, 2008), geographic regions (Owens et al., 1998; Olsoy et al., 2020), and seasons (Cedarleaf et al., 1983; Owens et al., 1998). For example, in juniper, terpene content is generally highest in the winter and spring, but these levels may fluctuate arbitrarily throughout the year (Owens et al., 1998). On the other hand, the terpene content of sagebrush spikes substantially when flowers mature around July, gradually declining until reaching its lowest point in new spring leaves near May/June (Kelsey et al., 1982; Cedarleaf et al., 1983). The fluctuation in terpene content of sagebrush is thought to be a cyclical response to grazing pressures (Cedarleaf et al., 1983) and is strongly associated with phenological stages (Kelsey et al., 1982), suggesting that dietary preference for terpene producing plants may be highly dependent on the seasonality of the target plant species.

Dietary selection for plants that contain secondary plant compounds can also be influenced by genetics. Preference for sagebrush in sheep is moderately (0.28) heritable (Snowder et al., 2001) and nearly double the heritability estimate for juniper in goats (0.13, Waldron et al., 2009). High consumption of these shrubs maybe due to physiological differences within individual animals (Campbell et al., 2007; Fraker-Marble et al., 2007). Fraker-Marble et al. (2007) evaluated the differences in ingestion and digestion of sagebrush by sheep classified by fecal NIRS as either "high" or "low" sagebrush consumers. They determined that the DM digestibility of the sagebrush ration was greater in sheep classified as "high" sagebrush consumers compared to "low" sagebrush consumers, suggesting that those more willing to consume sagebrush receive a greater digestive benefit from this terpene containing shrub. Additional studies have shown that chronic exposure to terpenes allows for the adaptation of rumen microbes (Diziba et al., 2006), increasing the ability of sheep to consume terpenes (Villalba et al., 2002).

Grazers have adapted a variety of mechanisms for avoiding plant toxicity which includes sensory perception (Launchbaugh et al., 2001), enhanced ruminal metabolism (Stegelmeier et al., 1999), and adapted microbial populations (Allison & Cook, 1981). However, some herbivores are more tolerant of plant toxins than others (Launchbaugh et al., 2001). Due to their comparatively large livers, goats can process secondary compounds more efficiently than other livestock species (Hofmann, 1989). It should also be noted that sheep are more capable of detoxifying the alkaloids found in tall larkspur and tansy ragwort than cattle (Olsen, 1978; Stegelmeier et al., 1999). Thus, co-grazing with sheep or goats may benefit both animal and land managers by selecting plants that may be toxic to or otherwise avoided by cattle (Walker, 1994).

A meta-analysis consisting of 70 targeted grazing studies determined that grazing by cattle, sheep, and goats not only reduced prevalence of the targeted species, but also lead to increases in plant richness and biodiversity (Marchetto et al., 2021). Experiments that did not show significant reductions in targeted plants still reported increases of the undesirable species in the control plots that were not observed in grazed areas (Cornett et al., 2006; Lagendijk et al., 2017). While there is much evidence to support the benefits of targeted grazing, the results are directly dependent on selecting the appropriate livestock species for the desired outcomes (Metera et al., 2010; Tóth et al., 2018).

The Senses and Dietary Selection

Animals rely on their sensory organs to collect information about their environment. Ruminants utilize their sense of sight, touch, smell, and taste to assist with dietary selection (Ginane et al., 2011). These stimuli are then used to make inferences about the nutritional quality of feed, which in turn allows them to make appropriate foraging decisions (Forbes, 2007). For example, the sense of sight is used to both locate (Arnold, 1996; Edwards et al., 1997; Hirata et al., 2019) and differentiate among various feeds (Cahn & Harper, 1976; Bazley, 1990; Edwards et al., 1997; Hirata et al., 2019). Ruminants are able distinguish between live and dead forages (Hirata et al., 2019), sward height (Bazley, 1990), plant species (Edwards et al., 1997) and polymorphisms of the same species (Cahn & Harper, 1976). Meanwhile, the sense of touch helps deter ruminants from consuming plants with physical defenses, often avoiding harsh or spiny plant material (Vallentine, 1991).

Olfaction and taste perception exhibit a close, yet complex relationship. Both volatile and soluble compounds interact in the mouth to give feed its unique flavor (Forbes & Mayes, 2002). The sense of smell is often utilized first and allows ruminants to investigate the palatability of a plant without having to take a bite (Ginane et al., 2011). This ability is useful in the detection of aromatic volatile plant oils (Cannas et al., 2009). However, smell is thought to be more important in appetite stimulation than feed selection (Tribe, 1949). Although impairment of any sense can influence plant

preference, the senses of sight, touch, and smell are considered supplementary to taste (Krueger et al., 1974; Tribe, 1949).

Taste is the last sense experienced before food is swallowed; and as such, plays an essential role in determining the acceptability of feed (Vallentine, 1991). Taste receptors across the oral cavity serve similar functions, but they differ in their ability to detect various compounds. In mammals, there are two distinct families of seven transmembrane G protein-coupled taste receptors (reviewed by Shi & Zhang in 2006). Type I taste receptors (TAS1Rs) are responsible for the perception of sweet and umami flavors (Kitagawa et al., 2001; Max et al., 2001; Montmayeur et al., 2001; Nelson et al., 2001, 2002; Sainz et al., 2001; Zhao et al., 2003), while type II taste receptors (TAS2Rs) perceive bitterness (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000; Meyerhof, 2005). Soluble taste stimuli bind to taste receptors cells, which are clustered into tastebuds and located on the gustatory papillae of the tongue (Reece, 2009). This interaction initiates an afferent signal, which is transmitted to the brain via the facial (IV), glossopharyngeal (IX), or vagus (X) nerves (Bachmanov & Beauchamp, 2007; Roper, 2013). Animals process this information to make associations between flavors and feeds that contain vital nutrients (Bach et al., 2012) and those that could be potentially life threatening (Launchbaugh & Provenza, 1994).

The five basic taste modalities are considered to reflect feed characteristics—sweet for carbohydrates, umami for protein content, sodium for mineral presence, sour to indicate spoilage, and bitter to indicate toxicity (Bachmanov & Beauchamp, 2007). In general, ruminants are believed to have the highest preference for umami flavors and the greatest aversion to bitterness (Goatcher & Church, 1970c, 1970d; Ginane et al., 2011), but the sensitivity of ruminants to each flavor varies by species. Cattle demonstrate the highest level of discrimination between flavored and control feeds; and like goats, have the lowest tolerance for saltiness (Goatcher & Church, 1970b, 1970c). Goats exhibit a mild preference for bitterness (Goatcher & Church, 1970d), which may be explained by the dichotomy of browsers versus grazers (Glendinning, 1994). Sheep tend to be the least discriminatory of the domesticated ruminant livestock (Goatcher & Church, 1970b, 1970c). However, while sheep are the most sensitive to sweet flavors (Goatcher & Church, 1970c), they express low tolerance for bitter flavors at high concentrations (Goatcher & Church, 1970d). Further research on the expression of bitter taste perception in sheep has alluded to the potential rangeland management applications of sheep selectively bred based on bitter-taster status (Henslee et al., 2019; Davenport et al., 2021; Southerland et al., 2022) or voluntary consumption of browse (Snowder et al., 2001 Walker et al., 1992), which contain bitter tones.

Perception of Phenylthiocarbamide

Compared to the other taste modalities, there seems to be a great interest in bitter taste perception. Perhaps this is because the ability to identify bitterness is viewed as an evolutionary protection mechanism against toxicosis (Chandrashekar et al., 2000; Dong et al., 2009; Garcia-Bailo et al., 2009). The number of bitter tastants and the intensity in which they are perceived is variable (Kim et al., 2003; Dong et al., 2009), allowing animals to detect harmful compounds they may frequently encounter in their diet (Wong et al., 1996; Dong et al., 2009). This variation has been recorded both between species and amongst individuals of the same species (Fox, 1932; Blakeslee & Salmon, 1935; Goatcher & Church, 1970b, 1970c; Dong et al., 2009).

One of the earliest accounts of variation in bitter taste perception was recorded by Arthur Fox (1932) when he accidentally spilled a bottle of phenylthiocarbamide (PTC) while working in the lab. Although he perceived the PTC dust to be completely flavorless, his colleagues complained of the bitter taste and scent that the airborne particles produced. He went on to observe and classify individuals by PTC tasting ability but found that the dichotomy of "tasters" versus "non-tasters" could not be explained by age, race, nor gender (Fox, 1932).

The "taster" classification was later refined to include a subcategory of "super-tasters" who at very low concentrations reported PTC as having an extremely bitter taste (Bartoshuk et al., 1994). Sensitivity to PTC exhibits a strong positive correlation ($r = 0.84$) with the density of fungiform papillae found on the anterior portion of the tongue (Essick et al., 2003). As such, super-tasters were determined to have greater lingual tactile acuity than intermediate and non-tasters, respectively (Bartoshuk et al., 1994; Tepper & Nurse, 1997; Yackinous & Guinard, 2001; Essick et al., 2003). Sensitivity to PTC is often determined using threshold or suprathreshold methods. Threshold tests measure an individual's ability to discriminate between low concentrations of PTC, but do not account for variation in perception of intensity. Research conducted by Blakeslee and Salmon (1935) observed the range of PTC perception in humans to be linear, arguing that the use of thresholds to classify individuals by PTC-taster status may be arbitrary. While some individuals reported the intensity of bitterness increased incrementally with respect to rising levels of PTC, others sensed extreme bitterness as soon as the compound was detectable (Blakeslee & Salmon, 1935). Suprathreshold methods account for this variation by using a rating scale to determine intensity of perception at high concentrations. However, lack of standardized criteria for sorting individuals into PTC-taster categories can result in inconsistent classifications (Tepper, 2008).

Since Arthur Fox's initial discovery, PTC has been used to evaluate bitter sensitivity in species such as mice (Nelson et al., 2003), monkeys (Suzuki et al., 2010), and sheep (Henslee et al., 2019;

Southerland et al., 2022). Due to the inability of these animals to verbally communicate the concentration and strength in which PTC is perceived, preference tests evaluating voluntary intake are commonly used to interpret acceptance or rejection thresholds. In 2019, Henslee et al. conducted a study on sheep that was designed similarly to the taste response studies with human subjects performed by Goatcher & Church (1970a, 1970b, 1970c, 1970d). The study aimed to determine if sheep could detect PTC, and if so, identify their threshold for PTC avoidance. The preference study evaluated the fluid intake response of individual rams (n = 30) to 8 different concentrations (0.20 – 12.29 mM) of PTC over two 14-d trial periods. They observed that as PTC concentration increased, intake of PTC-solution decreased, suggesting that sheep could discern between PTC-solution and water (Henslee et al., 2019). Furthermore, these rams were sorted into groups of high, medium, and low PTC consumers, similar to the three-group method of classification suggested for humans (Bartoshuk et al., 1994).

Initial studies on humans suggested that inability to taste PTC was a Mendelian simple recessive trait (Blakeslee, 1932). However, this theory was later dispelled (Das, 1958), demonstrating this phenotype to be a trait of much more complicated inheritance. Today it is understood that the number of bitter tastants and the intensity in which they are perceived is dependent on genetic variation (Kim et al., 2003; Dong et al., 2009). The TAS2R responsible for the perception of PTC in humans (hTAS2R38) also detects 6-n-proplthiouracil (PROP), and other compounds of thiourea moiety (N-C=S) (Kim et al., 2003). This includes glucosinolates, a class of anti-thyroid compounds found in brassica vegetables (Fahey et al., 2001; Sandell & Breslin, 2006). As such, PTC "tasters" are more likely to limit their ingestion of naturally occurring goitrogens (Greene, 1974) and other bitter tasting foods (Fischer et al., 1961). Additionally, PTC-tasting ability is also suspected to be associated with decreased vegetable consumption and increased preference for sweet and fatty foods (Garcia-Bailo et al., 2009). The *TAS2R* gene family in of domestic ruminants also contains the *TAS2R38* gene (Henslee et al., 2020), leading researchers to believe that PTC sensitivity may also be linked to feed selection in grazing animals. Thus, the methods of Henslee and colleagues (2019) were later refined for the rapid identification of PTC sensitivity in sheep to develop a method which could be used to classify individual rams for use in future dietary preference studies (Southerland et al., 2022).

The Genetics of Bitter Taste Perception

Type II taste receptors (TAS2R) found on the tongue and within the gastro-intestinal (GI) tract are responsible for perception of bitter flavors (Adler et al., 2000; Chandrashekar et al., 2000; Matsunami et al., 2000; Meyerhof, 2005). The TAS2Rs located throughout the GI tract are thought to be involved in the protective response, inducing behaviors such as vomiting or aversion when a

potentially harmful substance is ingested (Sternini et al., 2008). Some TAS2Rs detect a wide range of bitter tasting compounds, while others are ligand specific (Chandrashekar et al., 2000; Bachmanov & Beauchamp, 2007). Compounds more common in the diet may bind to a TAS2R which has been finely tuned to recognize that specific chemical (Shi et al., 2003; Drayna, 2005), while other TAS2Rs act more broadly, aiding in the detection of more novel compounds (Shi et al., 2003).

The number of functional *TAS2R* genes varies by species and are thought to have closely evolved with feeding ecology (Bachmanov & Beauchamp, 2007; Dong et al., 2009). For example, omnivores such as humans (n=25) and mice (n=34) who have a more diverse diet also express a higher number of functional *TAS2R* genes than herbivores (n=20-23) (Go et al., 2005; Henslee et al., 2020). Gene loss or expansion of the *TAS2R* gene family is believed to reflect the diversity of bitter-tasting chemical compounds encountered in the diet for optimal animal survivability (Glendinning, 1994; Go et al., 2005; Dong et al., 2009; Bachmanov et al., 2014).

The most widely studied bitter gene is *TAS2R38*—often referred to as the PTC gene—which is responsible for the detection of phenylthiocarbamide (PTC), 6-n-propyl-2-thiouracil (PROP), and other compounds containing thiourea moiety (Kim et al., 2003). How well a ligand binds to the TAS2R is dependent on this orientation of the G-protein. Genetic variation caused by single nucleotide polymorphisms (SNPs) within a *TAS2R* gene can biochemically alters the G-protein, which in turn modifies the strength of the "bitter signal" received by the brain (Kim et al., 2003). In humans, three functional SNPs resulting in amino acid changes at codon position 49, 262, and 296 result in either the PTC "taster" PAV (Proline/Alanine/Valine) or "non-taster" AVI (Alanine/Proline/Isoleucine) haplotypes (Kim et al., 2003; Drayna, 2005). While several haplotypes exist, the AVI and PAV haplotypes are responsible for up to 85% of the variation in PTC sensitivity (Kim et al., 2003; Drayna, 2005), and are observed in 96% of European and 100% of East Asian decedents (Kim et al., 2003). However, rare haplotypes are almost exclusively associated with certain ethnic populations, such as PVI and AAI, found only within individuals of sub-Saharan African ancestry (Kim et al., 2003).

In humans, the ability to taste thiourea compounds has been linked to the ability to taste other bitter foods (Fischer et al., 1961). Homozygous PAV individuals reported glucosinolate containing vegetables as 60% more bitter than AVI homozygotes did, whereas PAV/AVI heterozygotes assigned intermediate scores to the same foods (Sandell & Breslin, 2006). Polymorphisms within *TAS2R3*, *TAS2R5*, *TAS2R9* and *TAS2R31* have also been linked to human intake of bitter-tasting brassica vegetables, grapefruits and even the bitter undertones of artificial sweeteners (Allen et al., 2013; Mikołajczyk-Stecyna et al., 2020). However, there are many foods which contain beneficial bitter phytochemicals such as the flavonoids in citrus fruit, polyphenols in green tea and red wine,

glucosinolates in brassica vegetables, and isoflavones in soy products (Drewnowski et al., 2001). It has been theorized that individuals with a heighted sense of bitterness may prefer sweet and fatty foods over antioxidant-rich fruits and vegetables (Garcia-Bailo et al., 2009).

The highly conserved *TAS2R38* gene is also present in domestic ruminants (Henslee et al., 2020). The percent similarity in nucleotide sequence is 95% for cattle and sheep, 95% for cattle and goats, and 98% for sheep and goats (Henslee et al., 2020). Recent studies have attempted to identify which TAS2R genes are associated with PTC-taster status in sheep (Davenport et al., 2021). Blood samples were collected from mature Rambouillet and Targhee rams (n = 26) previously identified as high, medium, or low PTC consumers (Henslee et al., 2019). A total of 19 TAS2R genes were applied and sequenced resulting in the identification of 1,049 SNPs and 26 haplotypes. Of these, 24 SNPs and 11 haplotypes across 9 TAS2R genes were found to be associated with PTC consumption, including *TAS2R38* and *TAS2R16* (Davenport et al., 2021). In humans *TAS2R16* detects β-glucopyranosides (Bufe et al., 2002). Bitter tasting β-glucopyranosides such as salicylic acid are found in willow bark, and therefore may be important for sheep encountering similar substances in their grazing regime. Although further research is needed, this suggests that a similar relationship between bitter tasting ability and dietary preferences may also exist in domestic livestock.

Factors of Dietary Preference and Aversion

Some populations of animals, such as the plateau zokor (*Eospalax baileyi*) are physiologically, biochemically, and genetically adapted to detoxify bitter secondary plant compounds (Tan et al., 2022). This species of subterranean rodent is characterized by its extreme visual impairment (Bradley, 2010); and thus, relies on its sense of taste and smell to intentionally select bitter feeds (Zhao et al., 2016). Zhao et al. (2016) examined underground food caches (1-4) from several burrowing systems of zokor (n = 56) and found that bitter plants comprised 40% of the collected forages. Genetic analysis determined that variation in five zokor TAS2R genes was associated with the selection of bitter plants (zTAS2R115, zTAS2R119, zTAS2R126, zTAS2R134, and zTAS2R136). These results agreed with previous studies suggesting that zokors do not forage randomly, but rather based on taste profile (Xie et al., 2014).

However, the bitter preference observed in the zokor is rare. In 1970, Goatcher and Church performed a series of preference studies on ruminants and found that of the five primary flavors, domestic ruminants are the most sensitive to bitterness (Goatcher & Church, 1970a, 1970b, 1970c, 1970d). One of these studies evaluated the fluid intake response of two groups of sheep (n = 5) to increasing concentrations of quinine, which is a bitter tasting compound similar to PTC. They determined that as the concentration of quinine increased, fluid intake decreased across groups,

suggesting that sheep are more willing to consume bitter-tasting solutions at lower concentrations (Goatcher & Church, 1970b). Since then, further documentation of bitter-taste sensitivity in sheep has been recorded both within controlled environments (Henslee et al., 2019; Southerland et al., 2022) and on the range (Krueger et. al 1974). While Goatcher and Church provided some of the initial evidence of bitter sensitivity in sheep, their study only measured aversion on a group basis. Later studies, which focused on the fluid intake response of individuals, determined that much like humans (Blakeslee, 1932) and mice (Nelson et al., 2003), individual rams exhibited various thresholds of aversion to bitterness (Henslee et al., 2019).

Food preferences are often formed via interactions between taste and either positive or negative post-ingestive feedback, which is determined by an animal's physiological state and the chemical composition of the ingested plant (Provenza, 1996). For example, lambs express preference for feeds containing nutrients in which they are deficient, but this preference declines as nutrient levels improve (Villalba & Provenza, 1996). Bitter-tasting phytotoxins are generally associated with decreased food preference. However, toxins will not prevent ruminant consumption of a plant if it is also rich in vital nutrients (Wang & Provenza, 1996). Consuming a diet high in plant variety is thought to protect ruminants from the toxic effects of secondary plant compounds (Launchbaugh et al., 2001). Ruminants reduce their rate of intake and limit ingestion of both novel and known feeds until post-ingestive consequences can be determined (Provenza et al., 1992; Launchbaugh et al., 2001).

When an animal becomes ill after ingesting either a familiar or novel feed, they usually will avoid consumption of that feed in the future (Burritt & Provenza, 1996). Bitter aversions can develop when a secondary metabolite stimulates the portion of the nervous system that controls nausea and vomiting (Launchbaugh et al., 2001). These taste feedback mechanisms are involuntary responses that unconsciously generate flavor preferences and aversions. Provenza et al. (1994) emphasized this point by anesthetizing a group of sheep and administering intraruminal injections of lithium chloride (LiCl) to induce negative post-ingestive feedback 90-minutes after consuming their morning ration. Sheep that had received LiCl were reported to have depressed feed intake compared with the control group (Provenza et al., 1994). Favreau et al. (2010) later reported that sheep were indifferent to quinine-treated versus non-treated feed until LiCl was added to the bitter diet. The addition of LiCl to the quinine-treated feed resulted in a decrease in intake during subsequent feeding. This indicated that although sheep could taste the bitterness within the feed, the level of quinine alone was not enough to modify intake (Favreau et al., 2010), further demonstrating the important relationship between tolerance for bitter feeds and post-ingestive cues.

In addition to exposure, dietary selection and preferences are also learned through offspring and dam interactions (Mirza & Provenza, 1994; Glasser et al., 2009), as well as other peer interfaces (Lane et al., 1990; Provenza & Burritt, 1991; Thomas et al., 2015). To avoid consumption of toxic plants, ruminants are often reluctant to consume novel forages (Launchbaugh et al., 2001). However, this neophobia can be quickly overcome in lambs by watching their mothers consume or avoid certain feeds (Mirza & Provenza, 1994). Furthermore, the results of a cross-fostering experiment suggested that in goat kids, learned aversion to tannin-rich browse is influenced more so by the dietary preferences exhibited by the maternal model than genetics (Glasser et al., 2009).

A behavioral study conducted by Lane et al. (1990) aimed to determine whether peer interaction would alter dietary preferences in heifers (n = 5) conditioned with LiCl to avoid Barbey larkspur (*Delphinium barbeyi L. Huth*). When isolated from non-averted peers, aversion to larkspur persisted in averted cattle for two consecutive summers. However, the conditioned aversion rapidly declined after only two days co-grazing with non-averted (n = 5) heifers. Similar results were observed when lambs that had been conditioned to avoid alder-leaf mountain mahogany (*Cercocarpus montanus*) increased their intake of the plant when foraging with non-conditioned peers (F. D. Provenza & Burritt, 1991).

Literature suggests that early experiences have a greater, longer lasting influence on an animal's preferences than those that occur later in life (Nolte & Provenza, 1992; Distel et al., 1994; Provenza, 1996). For example, Nolte & Provenza (1992) observed that orphan lambs offered onion flavored milk preferred onion flavored forages well into adulthood. Cattle with experience grazing on the sagebrush-steppe consume significantly more sagebrush than their naïve counterparts (Petersen et al., 2014). Exposing goats to Redberry Juniper (*Juniperus pinchotii Sudw.)* at weaning conditions the animals to consume greater quantities of juniper on pasture (Dietz et al., 2010). Additionally, lambs that are exposed early in life to feed containing terpenes and condensed-tannins will consume notably greater amounts of these secondary plant compounds later in life, even when forages of greater quality are available (Villalba et al., 2004; Shaw et al., 2006). This supports the theory suggested by Launchbaugh et al. (2001) that ruminants may be trained to consume bitter forages if the animal is exposed to a particular plant or flavor earlier in life (Launchbaugh et al., 2001). Further research on juvenile lambs suggested that early dietary experiences may improve the lamb's ability to harvest (Ortega-Reyes & Provenza, 1993), digest (Distel et al., 1994), and detoxify (Distel & Provenza, 1991) plants after reaching maturity.

Although flavor and forage preferences can be learned, individual dietary experiences with post-ingestive feedback are more likely to shape dietary preferences than social interactions (Launchbaugh et al., 2001). This was demonstrated by Provenza et al. (1993) when lambs administered LiCl avoided

consuming elm, despite a clear preference within the ewes. It was determined that the post-ingestive feedback lambs received from the LiCl was more important than the mother as a social model in forming dietary preferences or aversions (Provenza et al., 1993).

Conclusion

There are many factors contributing to the formation of dietary preferences and aversions. Current research has emphasized the dependence of feed selection and flavor aversions on the taste-feedback mechanism (Provenza et al., 1993; Burritt & Provenza, 1996; Provenza, 1996; Villalba & Provenza, 1996; Launchbaugh et al., 2001). However, this conditioned response may also depend on learned behaviors and the genetic predisposition for bitter tasting ability (Kim et al., 2003). The innate dietary preferences and grazing behaviors exhibited by sheep and goats makes them ideal candidates for the targeted control of undesirable woody rangeland plants. While dietary inclusion of juniper and sagebrush is heritable (Snowder et al., 2001; Waldron et al., 2009), it is unclear what role bitter taste perception may play in the voluntary consumption of secondary plant compounds.

The current line of research on phenotypic expression of PTC tasting ability in sheep will provide further insight into this dynamic relationship. Future research should focus on the identification of bitter tasting genotypes and the subsequent effects on dietary selection. But first, streamlined methods for the accurate and precise identification of phenotypic expression of bitter tasting ability in sheep must be developed and is the focus of the current work reported herein. Ideal tests should be short-duration, minimize animal stress, reduce laboriousness, and increase sample size. Understanding how bitter taste perception influences dietary selection and foraging behavior in ruminants could have major implications for both animal production and range management practices. The long-term goal of this line of work is the development of breeding programs based on bitter tasting ability. Although further research is required, lineages of sheep selected for bitter tolerance have the potential to maintain landscapes overrun by less palatable rangeland plants like sagebrush.

Chapter 2: Refined methodology for identification of bitterness aversion in mature rams through quantification of fluid intake and behavioral response to phenylthiocarbamide

Abstract

Sheep have a sensitive threshold for bitterness, which may affect feed selection. Previous research attempted to classify sheep according by bitter sensitivity by measuring fluid intake of the compound phenylthiocarbamide (PTC) at various concentrations over a 14-d period. Although successful, the study was laborious, lengthy, required multiple layers of acclimation and testing, and accommodated a limited number of animals. Accordingly, a more efficient test is needed to classify bitter-taster status of sheep. The objective of this experiment was to develop a more rapid and refined method to assist in identification of PTC-taster status in rams utilizing only two concentrations of PTC-mixture over a 2-day test period. Mature Targhee, Rambouillet, Polypay, and composite-breed rams (n = 44), were equally stratified into three barns and subjected to 4-d acclimation, 2-d sham, and 2-d testing phases. The sham phase was conducted to determine if ethanol (delivery vehicle for PTC) influenced voluntary fluid intake or behavior; no effect ($P \geq 0.13$) was observed. The 2-d test phase was a side-by-side preference study between water and one of two concentrations (0.20 and 2.03 mM) of PTC-mixture delivered on alternate days. Video surveillance was conducted to evaluate the number and duration of approaches to the PTC-mixture and subsequent sniffing, head bobbing, jerking, shaking, and lip movements, smacking, and licking behaviors. Overall, intake of PTC-mixture was less ($P < 0.001$) than water intake. The PTC-taster classifications were determined based on average PTC intake relative to ± 1.0 standard deviation of the study population mean and used to sort rams into super- (n = 9), intermediate (n = 29), or non- (n = 6) PTC-taster classifications. The amount of time spent drinking either PTC-mixture or water was different ($P < 0.001$) amongst PTC-taster classifications and correlated with both water (r = 0.23, $P = 0.028$) and PTC-mixture (r = 0.46, $P < 0.001$) intake. Head bobbing ($P = 0.035$) and lip smacking ($P < 0.001$) were associated with PTC intake across all classes. Super-tasters appeared to utilize sense of smell (sniffing) more frequently to identify PTC-mixture than the intermediate or non-tasters ($P \leq 0.012$). Non-tasters did not exhibit

behavioral differences ($P \geq 0.12$) regardless of solution type. The streamlined methodology outlined in this study will help facilitate future studies aimed at better understanding forage selection and consumption in sheep.

Keywords: behavior, bitterness-avoidance, phenylthiocarbamide, sheep

Introduction

Of the five primary flavors (sour, salty, sweet, umami, and bitter), sheep are the most responsive to bitterness (Goatcher and Church, 1970b). Documentation of bitter-taste sensitivity in sheep has been recorded both within controlled environments and on the range (Goatcher and Church, 1970b; Krueger et. al 1974; Henslee et al., 2019). Furthermore, previous research in humans suggests that genetics influence phenotypic sensitivity to bitter-taste (Kim et al., 2003). In humans, more sensitive individuals (those who perceive bitterness with great intensity) are likely to form an aversion to bitter flavor that ultimately influence their food choices (Garcia-Bailo et al., 2009). The synthetic compound phenylthiocarbamide (PTC) has been used to evaluate bitter sensitivity in various species including humans, mice, and sheep (Blakeslee and Salmon, 1935; Nelson et al., 2003; Henslee et al., 2019). While PTC is not found in nature, the ability to taste this chemical, and other thiourea moiety compounds, has been correlated ($r = 0.48$) with the ability to taste bitterness in foods (Glanville and Kaplan, 1965), such as broccoli and coffee.

Henslee et al. (2019) classified PTC sensitivity in rams based on their aversion to PTC at various concentrations (0.20 – 12.29 mM) in water over a 5-d adaptation and 9-d testing period. While the study was successful, the methodology was laborious, lengthy, required multiple layers of acclimation and testing, and only accommodated a limited number of animals (n = 15) per trial. Interestingly, Henslee et al. (2019) also noted that PTC elicited a behavioral response in some rams (i.e., lip smacking, head shaking, etc.), although the data was not recorded. In addition to fluid intake, flavor aversions have been studied by interpreting behavioral response post-taste (Travers and Norgren, 1986; Grill and Norgren, 1978; Rosenstein and Oster, 1988) and used to evaluate chemical sensory responses and hedonic predispositions (Rosenstein and Oster, 1988). Stereotypical reactions observed in mice, apes, and newborn human infants associated with flavor preference or aversion are mouth movements, lip smacking, rhythmic tongue protrusions, head shaking, gaping, and midface grimacing (Travers and Norgren, 1986; Steiner et al., 2000).

Encroachment of shrubs, such as sagebrush, and other invasive plants onto rangelands is an area of both economic (Wambolt and Payne, 1986) and environmental concern (Johnson et al., 1996). Bitter tasting ability has been theorized to have evolved to detect secondary metabolites (Garcia-Bailo et al., 2009) found in many rangeland shrubs (Cedarleaf et al., 1983; Johnson et al., 1985). Sheep

primarily rely on sense of taste when making decisions regarding feed selection (Krueger et al., 1974; Favreau et al., 2010); therefore, the ability to detect certain flavors may influence foraging choices. Further understanding the mechanisms which influence dietary preferences in sheep could benefit producers through the application of targeted grazing management strategies, particularly on rangelands, where grazing management may be the most feasible tool for managing forage composition.

Because identification of bitter-tasting ability is dependent on assessing the individual, the objective of this study was to refine the laborious and lengthy methodology used by Henslee et al. (2019) to assist with more rapid identification of PTC-taster status in rams; as well as identify different indicators of PTC sensitivity other than intake. We first hypothesized that classification as "super-" or "non-" bitter tasters based on fluid intake of PTC could be achieved using only two PTC concentrations (0.20 and 2.03 mM) over two days. Second, we hypothesized that rams would exhibit a visual behavioral response to PTC-mixtures based on their differing sensitivity to bitterness. The results of this study will facilitate further research, allowing us to better understand the influence PTC tasting ability could have on dietary selection and, ultimately, may lead to better understanding of grazing behavior in sheep.

Materials and Methods

Animals

This experiment was conducted in January 2021 at the USDA, ARS, Range Sheep Production Efficiency Research Unit, U. S. Sheep Experiment Station located near Dubois, ID, USA. All animal procedures were approved by an Institutional Animal Care and Use Committee (USDA, ARS, Dubois, ID, USA) in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Forty-four mature rams ($\geq$ 2 years of age), including Polypay (n = 6; 89.2 $\pm$ 11.6 kg), Rambouillet (n = 16; 94.8 $\pm$ 10.3 kg), Targhee (n = 10; 98.6 $\pm$ 6.6 kg), and a composite breed (Suffolk $\times$ Columbia $\times$ Texel; n = 12; 102.6 $\pm$ 9.1 kg) were used. To ensure adequate breed representation in each of the three barns, rams were blocked by breed and arbitrarily assigned to one of three groups consisting of 14, 14, and 16 rams each to match barn capacity. Each group was randomly assigned to a barn and within a barn, to individual pens. Barns were maintained at approximately 10°C, ventilators set on a 10% hourly cycle, and on a 12-h light-dark cycle. To encourage morning fluid intake, feed and water were withheld from rams from 1700 to 0700 h daily throughout all phases of the study.

Experimental Design

The experimental design was adapted from Henslee et al. (2019) with the procedural modifications that included addition of a sham phase, reduction in days necessary to complete the test phase, and reduction in number of phenylthiocarbamide (PTC) test concentrations from five to two. The trial was divided into three phases: acclimation, sham, and test.

Acclimation phase. A 4-d acclimation phase was conducted to adapt animals to the housing environment and daily routine. At 0700h, rams were offered 1.75 kg of alfalfa pellets at 0700 h for a duration of 30 min. Feed buckets were removed, and any refusals were weighed (data not shown). For the morning water-intake measurement, two identically colored buckets containing 3.0 kg of water each were placed in each pen and secured in a holding rack. All water used in this study was collected via the "cold" tap from the location municipal source just prior to delivery. After 2 h, buckets were removed, and water refusals were weighed. Immediately afterwards, for the afternoon water and feed delivery, rams were given two buckets of water (4.0 kg each) and additional alfalfa pellets to target a daily delivery of 2.8% (as-fed) of body weight. At 1700 h, water and feed were removed and refusals weighed. These feeding, watering, and intake-measurement procedures were maintained through the subsequent phases.

Sham phase. A 2-d sham phase was added to the initial protocol (Henslee et al., 2019) to determine if ethanol (EtOH), which is used to transfer dissolved PTC to water, affected voluntary water intake. Stock solutions of EtOH were prepared the day before delivery by heating 12.5g of EtOH just below boiling point (78°C) and adding it to 500g of cold water. Solutions were stored overnight and allowed to reach room temperature. On each sham day after the morning feeding, rams were offered one bucket of water (3000g) and one bucket with 1part EtOH stock solution diluted in 5 parts water side-by-side (3000g total). Rams were randomly assigned to receive the EtOH-mixture on either the left or right side of the holding rack. The position of the EtOH-solution was alternated on day two.

Test phase. A 2-d test phase was conducted to identify rams that were either sensitive ("tasters") or insensitive ("non-tasters") to PTC delivered in water. Solution types, the PTC-mixture (PTC + EtOH + water) or water, were offered side-by-side to rams as described in Henslee et al. (2019). Final PTC-mixture concentrations of 0.20 mM and 2.03 mM PTC were tested. Based on the findings of Henslee et al. (2019) a 0.20 mM PTC-mixture is believed to be suitable for identification of rams with a high degree of sensitivity (i.e., super-tasters) to PTC, while a concentration of 2.03 mM PTC serves to identify rams that are insensitive to PTC (i.e., non-tasters). Henslee et. al (2019) did not observe any carry over effects of PTC-mixture on subsequent water intake, thus the "rest" day between PTC delivery was excluded. Between the adaptation and test phases, these modifications allowed testing of three times the number of rams in six fewer days compared with Henslee et al. (2019).

Stock solutions of 0.20 and 2.03 mM PTC (Sigma-Aldrich, Inc, St Louis, MO) were prepared the day prior to delivery by adding 92.7 and 927 mg PTC, respectively, to 12.5g EtOH and heated until PTC was dissolved. The PTC-EtOH solutions were then added to 500g of cold water and allowed to reach room temperature. Stock solutions were remade if PTC was observed to precipitate out of the stock solution. On test days, stock solutions were diluted just prior to delivery at a rate of one part stock solution to five parts water to form final PTC- solution test concentrations of 0.20 or 2.03 mM.

Within each barn, rams were randomly assigned to one of two groups to receive either 0.20 or 2.03 mM concentration of PTC-mixture in a side-by-side offering with water on the first test day. The side of the holding rack in which the PTC-mixture was delivered relative to the water was randomized. After the morning feeding, each ram received one bucket of water and one bucket of the assigned concentrations of PTC-mixture. After 2 h, all buckets were removed, and the remaining fluids were weighed and recorded. On the second test day, the location of fluids within the holding rack was reversed and the respective alternate concentration of PTC-mixture was offered.

Video Surveillance

Video surveillance was conducted on the last day of the acclimation and sham phases and both test days. Cameras (LX1081-88, Lorex Technology, Inc., Markham, ON, Canada) were set to record 30 frames-per-second in 1080p from 0730 to 0930 h. Within each barn, cameras were mounted at equal height and distance from the pens to record the behavioral reactions of two rams per camera. Evaluated behaviors include head bobbing, head jerking, head shaking, lip smacking, lip licking, lip movement, sniffing, number of bucket approaches, and drinking duration are described in Table 2.1.

Statistical Analysis

Unless otherwise specified, statistical analyses of fluid intake variables were conducted using the PROC MIXED procedure of SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC). The differential response between fluid intake of water + EtOH-solution as a percent of total fluid intake (TFI) for the sham phase was modeled in a pooled analysis across days. A similar test was used to determine the overall difference between tap water and PTC water intake within the fixed effect of PTC concentration (0.20 and 2.03 mM). The fluid intake response variables evaluated in the experiment included total morning test period fluid intake as a percentage of total fluid offered, PTC intake as percentage of total test fluid intake, water intake as a percentage of total test fluid intake, afternoon water intake as a percentage of water offered, and daily fluid intake as a percentage total fluid offered throughout the day.

Rams were classified based on average PTC intake as a percentage of total test fluid intake across concentrations. The PTC-taster classification categories were determined by ±1.0 standard deviation (SD) of the population mean to sort rams into super- ($\leq$ -1.0 SD), intermediate ($<$ 1.0 to $>$ -1.0 SD), or

non- (≥ 1.0 SD) PTC tasting groups (Fig. 1). The three-group method of classification utilized in this study was initially suggested by Bartoshuk et al. (1994) and has been consistently used to describe the range of bitter taste perception in humans. The fixed effects of concentration and PTC-taster classifications were evaluated for all intake response variables. Morning, afternoon, and daily feed intakes were included as covariates for PTC intake but were determined to be non-significant ($P > 0.05$) and excluded from the final model. Additionally, fixed effects of barn and breed, as well as their interactions with concentration and PTC-taster classifications were initially included but were excluded from the final model due to lack of significance ($P > 0.05$).

To determine if an individual ram's normal fluid intake was an indicator of PTC-taster status, total morning fluid intake (kg) during the last two days of the adaptation and test phase, as well as the difference between the two were analyzed for the main effect of PTC-taster classifications. Additionally, the PROC CORR procedure was conducted to analyze the relationship between fluid intake relative to solution type and duration of drinking. All means are expressed as least squares mean ± standard error. Mean comparisons were made using pair-wise contrasts (PDIFF). Significance was set at $P \leq 0.05$.

Video footage was evaluated to identify behavioral differences between the PTC classifications. The PROC GLIMMIX procedure of SAS was used to evaluate the probability of a behavior being exhibited after consuming solutions (EtOH, PTC, or water). Results from the sham phase were evaluated for differences between solutions. Data from the test phase was analyzed for the main effects of PTC taste classification, PTC concentration and solution type, as well the interactions for PTC classification by solution type and PTC classification by PTC concentration. The model also included a random statement of ram nested in PTC classification by solution type. The number of bucket approaches and drinking duration were weighted by the number of bucket approaches per individual ram. For the head bobbing, head shaking, lip licking, and lip smacking behaviors, binomial data was converted to a percentage relative to number of bucket approaches. Due to the limited number of observations for head jerking and head shaking, these behaviors were analyzed using the PROC MEANS statement and are reported as observational means ± SD.

Results

Fluid Intake

During the sham phase, no difference ($P = 0.16$) was observed between intake of water (54.2 ± 27.6%) and EtOH-solution (45.7 ± 27.6%) with the pooled analysis; although, a day effect ($P = 0.035$) was observed. On day one, rams consumed approximately 21% more water than EtOH-

solution (P = 0.013), whereas, on day two, intake of the EtOH-solution matched the intake of water (P = 0.61, data not shown).

During the test phase, no effect ($P \geq 0.23$) of PTC concentration was observed for any of the fluid intake variables (morning, afternoon, daily; Table 2.2). However, aversion to PTC-mixture was demonstrated by rams within the 0.20 and 2.03 mM PTC concentrations ($P \leq 0.01$, Table 2.2), where intake of water was greater than intake of PTC-mixture (approximately 60:40). Moreover, water intake was inversely correlated (r = -0.49, $P < 0.001$) to PTC-mixture intake as a percentage of each fluid offered (Table 2.2).

Individual rams across the bitter-tasting spectrum were identified based on their average PTC intake across PTC concentrations relative to the test population mean of 38.9 ± 15.1%. Classification by PTC intake resulted in super-tasters (n = 9), intermediate tasters (n = 29), and non-tasters (n = 6) (Figure 2.1). Intake of PTC (% TFI) for rams identified by PTC-taster classifications at each concentration (0.2 and 2.03 mM) can be found in Figure 2.2. In a retrospective analysis, it was determined that the amount of morning fluid (kg) consumed during the adaptation and test phases were not different (P = 0.50) amongst PTC-taster classifications, nor was the difference between the amount of fluid consumed by individual rams during the adaptation and test phases indicative of their PTC-taster status ($P \geq 0.33$, data not shown). However, classification by PTC intake did influence (P = 0.007; Table 2.2) TFI during the morning delivery of test fluids. Rams classified as super-tasters drank numerically less fluid (54%, P = 0.09) during this 2-h window compared with non-tasters (63%) and consumed even less (P = 0.001) than intermediate tasters (66%).

When the test period (morning) TFI was divided into solution types (PTC vs. water), there were differences in the percent intake of PTC-mixture ($P < 0.001$) and water ($P < 0.001$) across PTC-taster classifications (Table 2.2). Percent PTC intake of TFI was different ($P < 0.001$) for the super-, intermediate and non-tasters from 15%, 40%, and 61%, respectively (Table 2.2), which were all different ($P \leq 0.001$) from each other. Moreover, super-tasters consumed the most water during this period (84% of TFI), followed by intermediate (59% of TFI), and non-tasters (38% of TFI), which were different ($P \leq 0.001$) from each other.

There was no difference (P = 0.10) in afternoon water intake between classifications. However, while total morning fluid intake was lowest amongst the super-tasters, they exhibited numerically greater intake of water (50% of TFI) than that of the intermediate (46% of TFI) and non-tasters (40% of TFI, Table 2.2) in the afternoon. This numerical difference is supported by the lower fluid intake of super-taster rams during the morning delivery of fluids which may have driven these specific rams to consume more water in the afternoon, Ultimately, TFI was not different ($P \geq 0.18$) for the

intermediate and non-tasters during either the morning or afternoon periods. There was no effect ($P =$ 0.21) of PTC classification on total daily fluid intake.

Behavior

Throughout the sham phase of the experiment, there were no differences ($P \geq 0.13$) between EtOH-solution and water intake for any of the behavioral traits evaluated (data not shown). During the test phase, the number of times a ram approached a particular bucket was not influenced by any main effects or interactions ($P \geq 0.19$, Tables 3 and 4). However, greater concentration of PTC in water numerically increased ($P = 0.06$) the total number of trips made to the fluid buckets from 19.2 ± 1.9 to 22.2 ± 1.9 when offered the 0.2 or 2.03 mM PTC-mixture, respectively.

The total duration of time that rams spent drinking from both buckets during the morning delivery of test fluids was also not significant for any of the main effects or interactions ($P \geq 0.24$, data not shown). However, when evaluating total time spent at each individual bucket, there was an interaction between PTC-taster classification and solution type ($P < 0.001$, Table 2.3). The amount of time that the super-tasters spent drinking water (247 s) was greater ($P < 0.001$) than the intermediate (132 s) and non-tasters (99 s). Conversely, non-tasters spent more ($P \leq 0.002$) time drinking out of the PTC bucket (172 s) than the super- (37 s) or intermediate tasters (85 s). The intermediate tasters were not different from the non-tasters ($P = 0.13$) in the amount of time spent drinking water. Nor were the intermediate tasters different ($P = 0.36$) from the super-tasters in the total amount of time that they spent drinking PTC-mixture. However, the intermediate tasters did spend nearly a full minute more ($P = 0.021$) drinking from the water rather than the PTC-mixture. The amount of time that the super-taster rams spent drinking water was 6-fold greater ($P < 0.001$) than the PTC-mixture. The non-tasters did not differ ($P = 0.07$) in the amount of time spent drinking PTC-mixture when compared to water. The amount of time spent drinking was positively correlated with both fluid intake for both water (r = 0.23, $P = 0.028$) and PTC-mixture (r = 0.46, $P < 0.001$) intake (Figure 2.3). The average rate of fluid intake across all PTC-taster classes was approximately 15 g/s and was the slowest among the intermediate tasters. Respective intake rates for water and PTC were 11 g/s and 15 g/s for the super-tasters; 19 g/s and 17 kg/s for the intermediate tasters; and 12 g/s and 15 g/s for the non-tasters.

There was also an interaction ($P = 0.008$) between PTC classification and solution type that influenced the drinking duration of a single approach that a ram spent drinking from a particular solution type (Table 2.3). Super-tasters spent more ($P < 0.001$) time drinking water (34 s) than PTC-mixture (12 s). There was no difference ($P = 0.09$) in the amount of time intermediate tasters spent drinking water (18 s) than PTC-mixture (13 s). Likewise, non-tasters spent a similar ($P = 0.79$) amount of time drinking from either bucket, regardless of whether the contents consisted of water (17 s or PTC-mixture (18 s). It should be noted that the drinking duration of a single drinking approach to

the PTC-mixture was not different ($P \geq 0.26$) across PTC classifications. While the duration of a single drinking event for water was not different ($P = 0.27$) between intermediate and non-tasters, super-tasters had a longer ($P \leq 0.015$) duration of a single drinking event for water than intermediate and non-tasters.

For the head bobbing, head shaking, lip licking, and lip smacking behaviors, data is reported as a percentage of the number of observations relative to number of bucket approaches. The main effects of PTC-mixture concentration ($P = 0.008$) and solution type affected ($P = 0.035$) the amount of lip smacking. The frequency at which lip smacking was observed was greater ($P = 0.008$) when rams were offered the 2.03 mM ($11.3 \pm 1.8\%$) compared with 0.20 mM ($6.4 \pm 1.3\%$) PTC-mixture. Regardless of PTC-taster classification or PTC-mixture concentration, lip smacking was 3-fold more likely ($P < 0.001$) to be exhibited after drinking from the bucket containing PTC-mixture ($14.8 \pm 2.5\%$) compared with water ($4.8 \pm 1.2\%$). Solution type also affected ($P = 0.035$) the occurrence of head bobbing, as this behavior was observed more frequently when rams consumed PTC-mixture ($16.1 \pm 2.7\%$) versus water ($9.1 \pm 1.8\%$). There was no PTC classification effect ($P \geq 0.35$, data not shown), PTC-taster classification by PTC-mixture concentration interaction ($P \geq 0.50$, Table 2.4), or solution type effect ($P \geq 0.80$, Table 2.3) for either the lip smacking or head bobbing behaviors.

There was an interaction observed between PTC classification and solution type for sniffing ($P = 0.015$, Table 2.3). The frequency at which the different PTC classifications (super- = 20%; intermediate = 23%; and non-tasters = 28%) sniffed the water bucket were similar ($P \geq 0.34$). However, the frequency in which the super-tasters sniffed the PTC-mixture (52%) was greater ($P \leq 0.012$) than both the intermediate (33%) and non-tasters (22%), which were not different ($P = 0.15$) from one another. Within PTC-taster classifications, the non-tasters did not sniff one solution type more frequently than the other ($P = 0.54$); whereas both the super- and intermediate tasters sniffed the PTC solution more ($P \leq 0.020$) often than the water.

Observance of lip movements within the various PTC classifications interacted with PTC concentration ($P = 0.046$, Table 2.4). After drinking the 0.20 mM PTC-mixture, frequency of lip movements was recorded at 16%, 35% and 26% for the super-, intermediate, and non-tasters, respectively. At the lower PTC concentration, only the super- and intermediate tasters were different ($P = 0.001$) from each other. At the higher concentration of 2.03 mM, the super-tasters moved their lips less (14%) than the intermediate (25%, $P = 0.026$) and the non-tasters (32%, $P = 0.010$). However, the latter two groups were not different ($P = 0.25$). Within their respective PTC classifications, the super-tasters ($P = 0.74$) and non-tasters ($P = 0.32$) showed no difference in lip movements, regardless of PTC concentration. However, lip movements were recorded more frequently ($P < 0.001$) for the intermediate tasters after drinking from the 0.20 mM solution (35%) as

opposed to the 2.03 mM solution (25%). While not significantly different ($P = 0.06$), it should also be noted that for the non-tasters exhibited numerically more lip licking than both the super-tasters (11%) and intermediate tasters (7%).

Out of the 175 observations per behavior, few instances of head jerking and head shaking were recorded during this study; therefore, only numerical differences and values are discussed. Throughout the sham phase, mean percent occurrence for either of these behaviors after drinking from the EtOH or water buckets was < 0.01% (data not shown). However, both behaviors occurred more frequently after rams drank from the PTC bucket as opposed to the water during the test phase. The number of times head jerking after drinking from either the water or PTC bucket was two and three, respectively. Likewise, the number of rams which shook their heads was greater after drinking PTC (n = 10) than water (n = 7, data not shown). Results of any behavioral reactions occurring after drinking water were expected to be less than or equal to that of the reaction to PTC. While this was true for both the super- and non-tasters, it should be noted that occurrence of head shaking was numerically greater in the intermediate tasters after drinking water (1.1 ± 4.7) than PTC (0.3 ± 1.3, data not shown).

The head jerking behavior was not expressed by any ram classified as a non- PTC-taster, but was exhibited more often by the super-tasters ($0.6 \pm 2.6\%$) than the intermediate ($0.2 \pm 1.4\%$) group (data not shown). This behavior appeared to occur slightly more often in super-tasters after rams sampled from the 0.20 mM ($0.8 \pm 3.4\%$) as opposed to the 2.03 mM ($0.3 \pm 1.6\%$) solution, while frequency of head jerking was exhibited within the intermediate tasters $0.1 \pm 1.3\%$ and $0.2 \pm 1.6\%$ for the 0.20 and 2.03 mM solutions, respectively. Head shaking was observed more frequently in the super- ($1.3 \pm 4.0\%$), followed by the non- ($1.3 \pm 5.2\%$) and intermediate tasters ($0.7 \pm 3.6\%$, data not shown). The super-tasters expressed this behavior at similar rates regardless of being treated with the 0.20 mM ($1.4 \pm 4.1\%$) or 2.03 ($1.3 \pm 4.1\%$) mM PTC concentration. The same was true for the intermediate tasters who exhibited head shaking $0.7 \pm 4.4\%$ and $0.7 \pm 2.5\%$ on the 0.20 mM and 2.03 mM PTC concentrations, respectively. However, only after sampling the 2.03 mM solution did any non-tasters shake their head ($2.6 \pm 7.3\%$).

Discussion

Aversion to PTC was previously quantified in rams by measuring fluid intake response to various concentrations of PTC in water (0.20 – 12.29 mM) over a 14-d test (Henslee et al., 2019). The objective of this experiment was to refine the methodology of Henslee and coworkers (2019) and develop a more rapid method for identification of PTC-taster status in rams. This was accomplished by recording individual fluid intake and behavioral response to only two concentrations (0.20 and

2.03 mM) of PTC-mixture over a 4-d acclimation and 2-d test phase. The introduction of PTC-mixture did not modify individual baseline fluid intake of rams (n = 44) between the adaptation and test phases. This would support both the reduction of the adaptation phase from five to four days and the elimination of the "rest day" utilized by Henslee et al. (2019). Moreover, there were no effects of ethanol during the sham phase resulting in the removal of this phase from the testing protocol. The suggested procedural modifications would allow for testing three times the number of rams in less than half of the time than the Henslee et al. (2019) protocol.

Concentration of PTC-mixture did not have a main effect on voluntary TFI within the experimental group. Similarly, Henslee et al. (2019) did not observe any effects of PTC concentration on fluid intake until a 4.39 mM PTC-mixture was offered, at which point suppression of total fluid intake occurred and water became preferred over PTC-mixture across all rams. The range of bitter sensitivity amongst individuals is known to vary considerably (Blakeslee and Salmon 1935; Henslee et al., 2019). Thus, differences within the experimental group attributed to the main effect of PTC concentration were expected to be minimal. This was true for almost all evaluated variables, except for lip smacking which increased across all PTC-taster classes in response to the 2.03 mM concentration. However, the amount of lip smacking observed at the higher concentration decreased numerically across PTC-taster classifications regardless of concentration, with the super-tasters exhibiting this behavior the most, followed by the intermediate then non-tasters.

Throughout the test phase we observed considerable variation in PTC intake between rams, emphasizing the complexity of bitter aversion amongst individuals (Figure 2.1). On average, rams (n = 44) drank more water (61%) than PTC-mixture (39%) suggesting that some rams were sensitive to the PTC-mixtures. Blakeslee and Salmon (1935), as well as Henslee et al. (2019), provided some of the initial evidence that the threshold for PTC avoidance varies amongst individuals in both humans and rams, respectively. In the present study, mean individual PTC intake ranged from 8 - 66% (Figure 2.1). These findings support Henslee et al. (2019), further illustrating that individual rams have differing degrees of sensitivity to PTC. Within this study, day did not affect any fluid intake variables within the experimental group or PTC-taster classifications. However, total fluid intake (% offered) did fluctuate within individuals. This variation may be reduced by incorporating a replicate delivery of both PTC concentrations, increasing both the accuracy and precision of this test.

While sheep have a sensitive threshold for bitterness, some individuals have demonstrated tolerance to bitter-tasting compounds (Goatcher and Church,1970b; Provenza et al., 1992; Launchbaugh et al., 2001). However, the precise amount of variation in bitter-taste perception within sheep is currently unknown. To ensure identification of rams on the furthest ends of the bitter tasting spectrum, rams were classified into three PTC-taster classifications (Bartoshuk et al., 1994) based on

their average PTC intake across both concentrations relative to the population mean. This resulted in one large intermediate and two smaller super- and non-taster groups that were distinctly different from one another in both their PTC and water intake.

The 0.20 mM and 2.03 mM solutions utilized in this study were distinctly aimed at identifying the super- and non- PTC-tasters, respectively (Figure 2.2). Thus, considerable variation was expected within the intermediate class. The range of PTC (%TFI) intake within the intermediate tasters was 24 – 52%. Our classification method was designed to identify those on the extreme ends of the bitter-tasting spectrum; with changes in concentration would primarily act on the intermediate tasters. The one behavior that elicited a significant difference within the intermediate tasters was lip movement, which was exhibited less frequently with intake of the 2.03 mM PTC concentration. Lip movements (referred to in other studies as rhythmic mouth movements) are thought to be either a preferential or a neutral flavor response (Steiner, 2000; Berridge and Pecinña, 1995). This decrease in lip movements exhibited by the intermediate tasters may indicate a loss of indifference or lack of preference towards the higher concentration of PTC.

The combined average PTC (15%) consumed by the super-tasters at both concentrations in this study was similar to the "low" PTC-consuming rams identified by Henslee et al. (2019) at the 0.20 and 2.03 mM concentrations. In addition to exhibiting a preference for water, total morning fluid intake was numerically lower amongst the super-tasters. The lower morning fluid intake exhibited by this class is indicative of their bitter-taste sensitivity, in which their drive to drink was overcome by their aversion to PTC. However, this relatively lower fluid intake in the morning was followed by an increase in afternoon fluid intake, which exceeded the other two classifications by 7.5%. Ultimately, total daily fluid intake was similar for all PTC-taster classifications (8 kg; data not shown).

While the amount of fluid consumed by non- and intermediate tasters during the morning delivery of test fluids was similar, the non-tasters consumed more PTC-mixture than water which without further investigation may suggest a PTC preference. In theory, a non-discriminate animal would consume equally from either bucket (Goatcher and Church, 1970a, b). This is not always the case when dealing with unpredictable behaviors in animals, particularly indiscriminate animals which may consume more PTC-mixture by chance and convenience rather than preference. However, the non-tasters did not approach or spend more time drinking from either the water or PTC-mixture, emphasizing the significance of a combined approach to interpreting PTC sensitivity utilizing both intake and behavioral data.

Aside from genetics, learned experience may also contribute to the range of PTC intake observed in this study. Exposure to flavors can influence the formation of preferences and aversions throughout a sheep's lifetime (Nolte and Provenza, 1992; Launchbaugh et al. 2001). Despite the novelty of the

PTC, the ability to taste this compound has been linked to the ability to taste other bitter-tasting foods (Glanville and Kaplan, 1965), which the mature rams may have been encountered on the range. Both head bobbing and lip smacking were associated with PTC intake irrespective of PTC-taster classification. Indicating that some individuals within each PTC-taster classification recognized the PTC as distinctly different from the water. Measuring these two behavioral responses in combination with fluid intake may be useful in recognizing individuals with the ability to taste PTC but may have developed a tolerance for bitterness.

The number of bucket approaches was independent of PTC-taster classification, PTC concentration and solution-type, demonstrating the difficulty rams had determining which bucket contained PTC-mixture without further investigating the contents (i.e., tasting or sniffing). For example, one super-taster repeatedly approached, sniffed, and sampled from the PTC bucket despite exhibiting aversive behavioral reactions (head bobbing, lip smacking and licking, as well as the more extreme behaviors of head jerking and shaking) after each drinking attempt (data not shown). In general, when the bucket contents were sampled orally, the amount of solution consumed by each type of taster appears to reflect the time spent drinking from each bucket (Figure 2.3). The super-tasters, however, appeared to be the most cautious of the three groups, relying more heavily on their sense of smell to identify the PTC solution. However, while smell has been demonstrated to be important in the selection of forages, for the most part it remains supplementary to taste (Tribe, 1949; Krueger et al., 1974).

The interaction of PTC-taster classification and concentration showed numerical differences in the presence of lip licking across all classes. Generally, lick cluster size and rate will increase with preference and decrease with aversive stimuli (Hsiao and Fan, 1993; Spector and St. John, 1998; Lin et al. 2016). Lick pattern analysis was not conducted during this experiment but could be used in the future to better interpret the resulting increase of tongue protrusions at the higher PTC concentration. Aside from this, non-tasters exhibited the same behaviors regardless of solution type, and PTC concentration.

The infrequent observance of head jerking and shaking behaviors raises questions about their application in identifying bitter aversion. It has been reported that in human infants, head shaking is considered a rare behavior most frequently observed after exposure to the bitter compound quinine (Steiner et al., 2000). In this study, head shaking was not observed within the non-taster group. This supports that while this behavior is considered rare aversive reaction, it may still be useful in determining PTC-taster status.

Conclusion

The methodology outlined in this study facilitates more efficient identification of rams as super- and non-PTC-tasters. The reduction in trial time from 14 to 6 days resulted in three significantly different PTC-taster groups. Confidence in these results is further supported by the lack of ethanol interference during PTC preparation and delivery. Drinking duration, head bobbing, lip smacking, lip movement, and sniffing behaviors can be used to supplement fluid intake data in determining bitter sensitivity. In this capacity, we will be able to design future experiments more efficiently, deepening our understanding of the influence bitter-taste sensitivity may have on feed selection in sheep. While bitter tasting ability modifies intake of PTC-treated water, additional studies are needed to determine the effects on consumption of bitter-tasing feed.

Sheep that are attuned to select forages based on bitter-tasting phenotypes could have targeted grazing applications either for the control or conservation of vegetative species based on plant flavor profiles. Future applications of this research may provide producers the necessary information to select bitter-tolerant sheep that can be used to target consumption of various forages, such as sagebrush, found on rangelands in the Intermountain west.

Acknowledgment

Tables and Figures

Table 2.1: Ethogram of behaviors observed and their corresponding descriptions during the morning delivery of fluid during the adaptation, sham, and test phases.

Behavior	Description
Approach	Head hovering over or inside of bucket
Drinking	Head inside of bucket, consuming fluid
Head bobbing	Repetitive vertical movement of head up and down
Head jerking	Rapid movement of head away from bucket
Head shaking	Rapid repetitive movement of head side to side
Lip licking	Protrusion and passing of tongue over lips
Lip movement	Rhythmic rubbing of lips together, minimal opening of mouth
Lip smacking	Wide repetitive opening and closing of mouth
Sniffing	Head hovering over bucket with nose pointed toward solution

Table 2.2: Least squares means (± standard error) for fluid intake by rams across phenylthiocarbamide (PTC) concentrations and within PTC-taster classifications[1].

Variable	PTC concentration, mM			PTC-taster classifications			
	0.20	2.03	*P-value*	Super-	Intermediate	Non-	*P-value*
Total test period (morning) fluid (PTC water + tap water) intake, % offered	63.6 ± 2.0	63.9 ± 2.0	0.910	54.6 ± 2.5[a]	66.6 ± 1.3[b]	63.1 ± 2.9[ab]	0.007
Water, % of test period fluid intake	60.3 ± 3.2	62.4 ± 3.2	0.681	84.3 ± 3.3[a]	59.3 ± 1.7[b]	38.5 ± 3.9[c]	<0.001
PTC-mixture, % of test period fluid intake	39.6 ± 3.2	37.5 ± 3.2	0.681	15.6 ± 3.3[a]	40.6 ± 1.7[b]	61.4 ± 3.9[c]	<0.001
Afternoon period water intake, % offered	44.8 ± 1.8	48.1 ± 1.8	0.236	50.9 ± 2.3	46.2 ± 1.3	40.8 ± 2.8	0.105
Total (all periods) daily fluid (all forms) intake, % offered	53.0 ± 1.3	54.9 ± 1.3	0.323	52.6 ± 1.7	55.0 ± 0.9	50.3 ± 2.0	0.216

[1] Rams were divided into PTC-taster classifications based on average PTC intake of individual rams administered two concentration levels (0.20 and 2.03 mM): super-tasters ≤ -1.0 SD, intermediate tasters= < 1.0 to > -1.0 SD, and non-tasters= ≥ 1.0 SD. Mean PTC intake: 38.9 ± 15.1%.
[a,b]Means across PTC-taster classifications with different superscripts are different ($P \leq 0.01$)

Table 2.3: Least squares means (± standard error) for exhibited behaviors across test fluid (water vs. PTC) within PTC-taster classifications[1] for the 2-h morning delivery period.

Variable	PTC-taster classifications						P-value
	Super-		Intermediate		Non-		
	Water	PTC	Water	PTC	Water	PTC	
Approaches, count	15.8 ± 3.2	8.3 ± 3.2	13.4 ± 1.7	10.8 ± 1.7	8.9 ± 3.9	13.1 ± 3.9	0.272
Drinking duration, total, s	247.1 ± 26.9^a	37.9 ± 26.9^b	132.7 ± 15.0^c	85.0 ± 15.0^b	$99.7 \pm 33.0^{b,\,c}$	$172.7 \pm 33.0^{a,\,c}$	< 0.001
Drinking duration, single approach, s	34.1 ± 3.7^a	12.2 ± 3.7^b	18.9 ± 2.0^b	13.9 ± 2.0^b	17.2 ± 4.5^b	18.9 ± 4.5^b	0.008
Head bobbing[2], %	13.3 ± 4.0	18.8 ± 5.5	7.9 ± 1.6	16.1 ± 2.7	7.2 ± 3.5	13.7 ± 5.1	0.801
Lip licking[2], %	4.8 ± 2.2	9.0 ± 3.8	8.9 ± 1.9	13.3 ± 2.6	10.1 ± 4.9	14.4 ± 6.0	0.947
Lip movement[2], %	19.9 ± 4.7	11.8 ± 3.8	33.8 ± 3.5	26.4 ± 3.2	28.1 ± 7.5	31.2 ± 7.5	0.533
Lip smacking[2], %	6.4 ± 2.3	17.5 ± 5.2	5.8 ± 1.2	15.4 ± 2.5	3.0 ± 2.0	11.9 ± 4.6	0.902
Sniffing[2], %	20.7 ± 4.2^a	51.9 ± 6.6^b	$23.5 \pm 2.6^{a,\,c}$	33.1 ± 3.2^d	$28.1 \pm 6.8^{a,\,c,\,d}$	$22.8 \pm 5.6^{a,\,c,\,d}$	0.015

[1] Rams were divided into phenylthiocarbamide (PTC) taster classifications based on average PTC intake of individual rams administered two concentration levels (0.20 and 2.03 mM): super-tasters $\leq$ -1.0 SD, intermediate tasters= < 1.0 to > -1.0 SD, and non-tasters= ≥ 1.0 SD. Mean PTC intake: $38.9 \pm 15.1\%$.

[2] Data reported as a percentage of number of times the behavior occurred relative to the number of bucket approaches.

[a,b] Means across PTC-taster classifications with different superscripts are different ($P \leq 0.05$).

Table 2.4: Least squares means (± standard error) for exhibited behaviors as a percentage relative to number of bucket approaches across phenylthiocarbamide (PTC) concentrations within PTC-taster classifications[1].

Variable	Super-		Intermediate		Non-		P-value
	0.2 mM	**2.03 mM**	**0.2 mM**	**2.03 mM**	**0.2 mM**	**2.03 mM**	
Approaches	11.3 ± 2.6	12.7 ± 2.4	10.2 ± 1.4	14.0 ± 1.3	11.9 ± 3.1	10.1 ± 3.2	0.196
Drinking duration, total, s	14.22 ± 24.5	142.8 ± 24.5	100.7 ± 13.6	117.0 ± 13.6	133.0 ± 30.0	139.4 ± 30.0	0.849
Drinking duration, single approach, s	26.4 ± 3.7	19.9 ± 3.7	17.5 ± 2.0	15.2 ± 2.0	18.0 ± 4.5	18.1 ± 4.5	0.694
Head bobbing[2], %	15.1 ± 4.0	16.44 ± 3.8	9.1 ± 1.6	14.2 ± 1.9	11.3 ± 4.0	8.8 ± 3.4	0.502
Lip licking[2], %	7.4 ± 2.6	5.9 ± 2.0	12.4 ± 2.1	9.6 ± 1.6	8.5 ± 3.4	16.9 ± 5.5	0.065
Lip movement[2], %	16.0 ± 3.7[a, c]	14.9 ± 3.3[a]	35.2 ± 3.0[b]	25.3 ± 2.4[c]	26.7 ± 5.8[a, b, c, d]	32.7 ± 6.4[b, c, d]	0.046
Lip smacking[2], %	8.2 ± 2.7	13.8 ± 3.4	7.4 ± 1.4	12.4 ± 1.8	4.4 ± 2.2	8.4 ± 3.4	0.976
Sniffing[2], %	52.6 ± 1.7	51.9 ± 6.6	11.3 ± 2.6	12.7 ± 2.4	11.3 ± 2.6	12.7 ± 2.4	0.650

[1] Rams were divided into phenylthiocarbamide (PTC) taster classifications based on average PTC intake of individual rams administered two concentration levels (0.20 and 2.03 mM): super-tasters ≤ -1.0 SD, intermediate tasters= < 1.0 to > -1.0 SD, and non-tasters= ≥ 1.0 SD. Mean PTC intake: 38.9 ± 15.1%.

[2] Data reported as a percentage of number of times the behavior occurred relative to the number of bucket approaches.

[a,b] Means across PTC-taster classifications with different superscripts are different ($P \leq 0.05$)
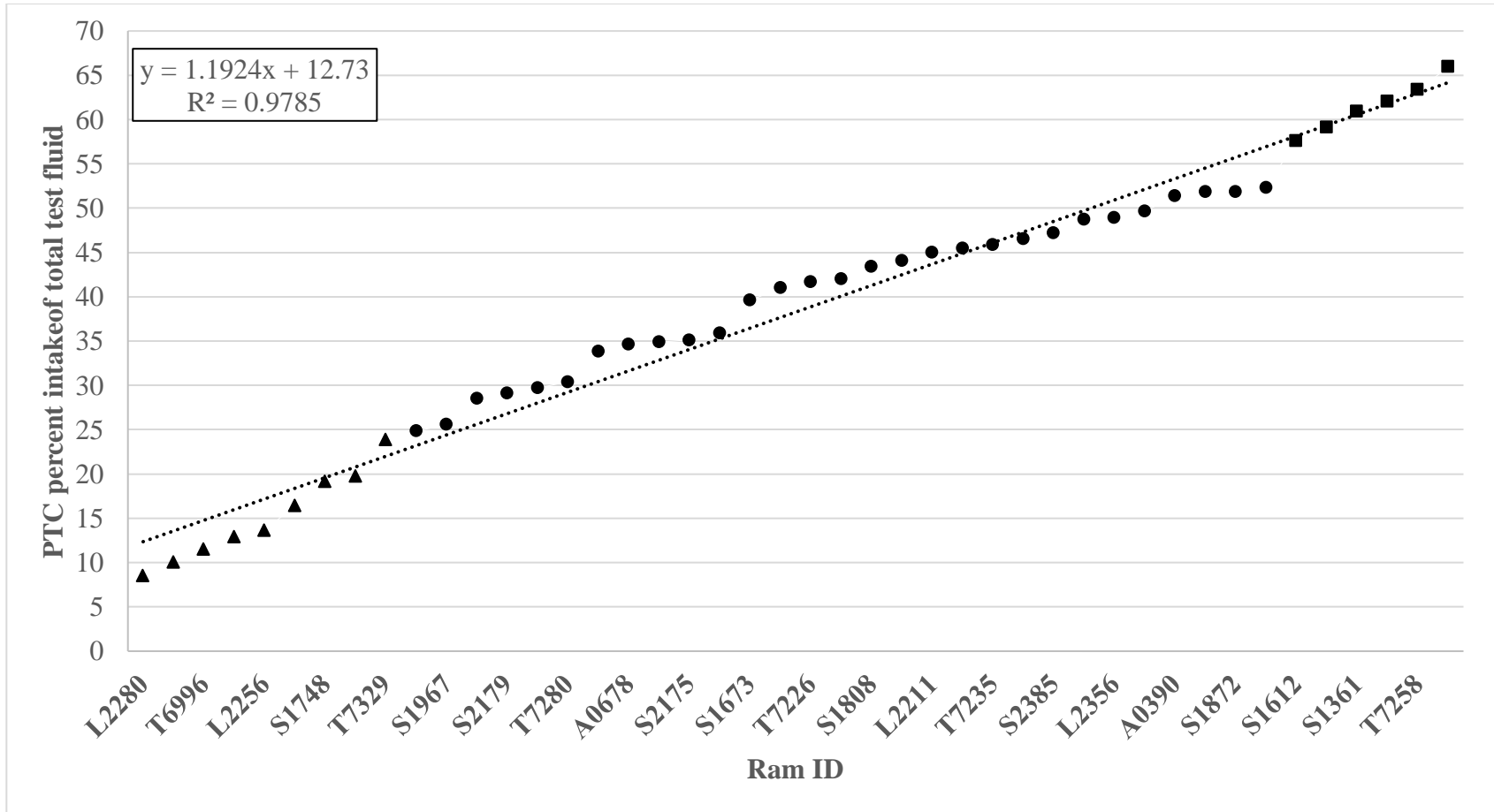
Figure 2.1: Average phenylthiocarbamide (PTC) intake of individual rams administered two concentration levels (0.20 and 2.03 mM) and further classified by taster status: super- (▲ = ≤ -1.0 SD), intermediate (● = < 1.0 to > -1.0 SD) and non- (■ = ≥ 1.0 SD) tasters. Mean PTC intake: 38.9 ± 15.1%.
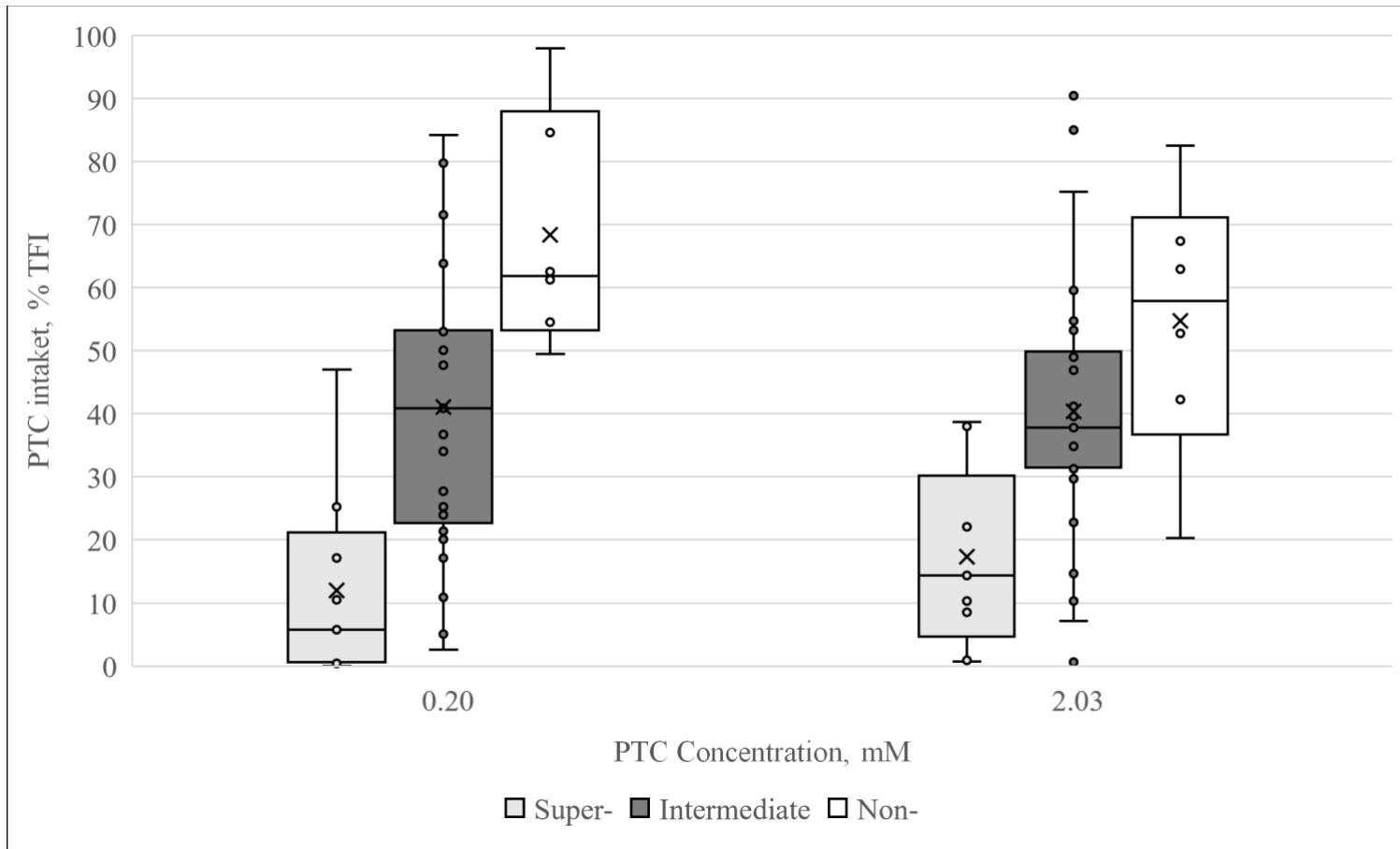
Figure 2.2: Phenylthiocarbamide (PTC) intake as a percentage of total fluid intake for rams at the two concentration levels (0.20 and 2.03 mM) and further classified by taster status: super- (⊘ = ≤ -1.0 SD), intermediate (◉ = < 1.0 to > -1.0 SD) and non- (○ = ≥ 1.0 SD) tasters. Mean PTC intake: $38.9 \pm 15.1\%$. PTC-taster classifications are further divided into quartiles demonstrating the mean (✕), median (—), and range of values for PTC intake.
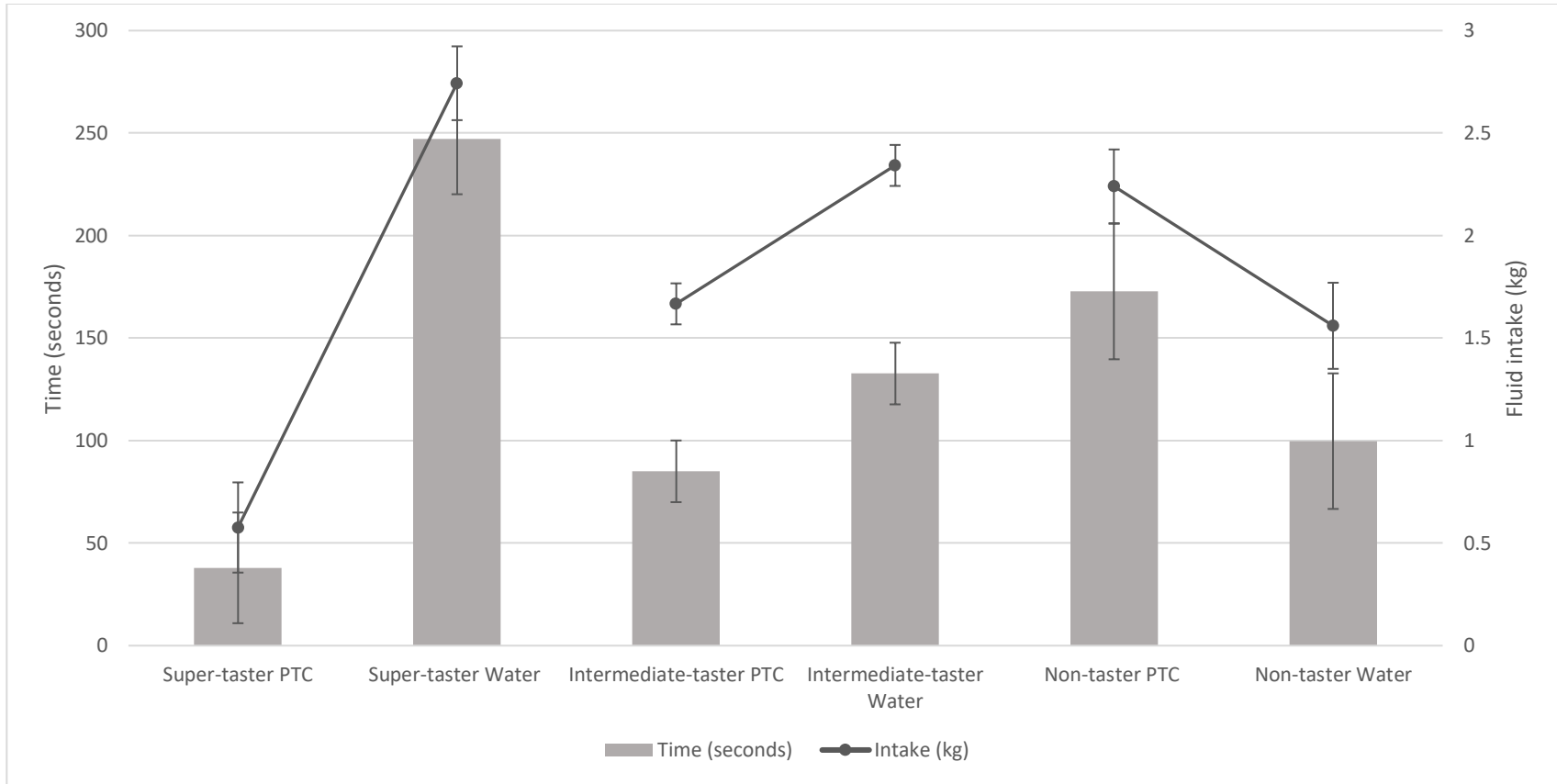
Figure 2.3: Fluid intakes and drinking durations of test solutions (water vs. phenylthiocarbamide, PTC) presented by PTC-taster classifications.

Chapter 3: Feed intake and behavioral responses of sheep provided feed containing phenylthiocarbamide

Abstract

Bitter-taster status in sheep can be identified by measuring fluid intake of phenylthiocarbamide (PTC) spiked water. The objective of this study was to determine the effects of PTC delivered in feed, rather than water, on dietary intake and behavior in PTC-naïve ram lambs (n = 26) and mature rams (n = 12) previously classified as either super- (n = 3), intermediate- (n = 5), or non-PTC tasters (n = 4) using PTC-spiked drinking water. Ground alfalfa (70%), corn (15%), and beet pulp (15%) was mixed with ethanol (5 g/kg, as fed) only (control) or PTC solubilized in ethanol (5 g/kg) and pelleted to create five diets of 110, 220, and 330 mg/kg PTC for mature rams, or 180 and 280 mg/kg PTC for ram lambs. Rams received control and PTC diets (0.87% BW) daily for 30 min in a side-by-side presentation and replicated for each concentration. Feed intake duration, number of bucket approaches and switches, sniffs, drinks, lip smacks and licks, and head bobs, jerks, and shakes were recorded using video surveillance. Consumption of PTC and control diets were similar for all rams ($P \geq 0.22$) when measured as a percentage of total diet (control + PTC) consumed. While mature rams began to exhibit signs of PTC aversion at 220 mg/kg (CV = 34.1%; $P = 0.16$), lambs were indifferent ($P = 0.61$) to concentrations up to 280 mg/kg (CV = 13.9%). Total intake of feed offered to mature rams decreased ($P = 0.01$) between 110 mg/kg ($80.9 \pm 3.2\%$) and 330 mg/kg ($72.3 \pm 3.2\%$), while 220 mg/kg ($78.4 \pm 3.2\%$) was intermediate ($P \geq 0.06$). Total feed intake duration was less ($P \leq 0.01$) when mature rams were offered 330 mg/kg (805.6 ± 440 s) compared with 110 (858.9 ± 44.3 s) and 220 mg/kg (862.9 ± 44.0 s). Although ram lambs consumed total test feed to near entirety, ram lambs took longer ($P < 0.01$) to consume 280 mg/kg PTC (438.4 ± 26.5 s) pellets than control (332.2 ± 27.4 s). Observance of head bobbing and lip smacking increased after mature rams (solution type × replication, $P \leq 0.01$) and ram lambs (solution type, $P \leq 0.01$) consumed PTC pellets. The rejection threshold appears to be different for mature rams versus ram lambs. These results suggest that sheep perceive PTC concentrations between 180 and 280 mg/kg incorporated into feed pellets. More research is needed to understand the influence of PTC tasting ability on dietary selection in sheep.

Introduction

Ruminants rely on their sense of sight, touch, smell, and taste to make appropriate dietary decisions (Forbes, 2007; Ginane et al., 2011). The sense of taste is thought to play a particularly important role in determining feed acceptability since it is the last sensation experienced before food is swallowed (Vallentine, 1991). The five basic taste modalities—sour, salty sweet, umami, and bitter—are considered to reflect certain feed characteristics (Bachmanov & Beauchamp, 2007). Of these, ruminants show the greatest aversion toward bitter flavors (Goatcher & Church, 1970b, 1970d; Ginane et al., 2011), which are commonly associated with secondary metabolites (Garcia-Bailo et al., 2009) found in many rangeland shrubs (Cedarleaf et al., 1983; Johnson et al., 1985).

In the United States, the encroachment of shrubs onto western rangelands results in both structural (Zarovali et al., 2007; Knapp et al., 2008) and functional (McKinley et al., 2008; Schlesinger et al., 1999; Throop & Archer, 2008) environmental changes. Targeted grazing management has been suggested as a means to restore these degraded rangelands (Svejcar et al., 2014). However, to avoid herbivory, many rangeland shrubs produce bitter tasting secondary compounds, which are drawn out during the grazing process (Cedarleaf et al., 1983; Bennett & Wallsgrove, 1994) and may reduce palatability. In sheep, taste-blindness has been shown to increase the acceptance of bitter-tasting forages (Krueger et al., 1974). Thus, further understanding the influence bitter-tasting ability has on the dietary selection of range sheep may improve grazing management strategies designed to maintain shrub canopies.

Bitter taste perception has been studied in various species, including humans (Blakeslee, 1932; Blakeslee & Salmon, 1935; Fox, 1932) and sheep (Henslee et al., 2019; Southerland et al., 2022), using the synthetic compound phenylthiocarbamide (PTC). Similar to humans, individual sheep express different levels of aversion to PTC solutions and (Blakeslee & Salmon, 1935; Henslee et al., 2019; Southerland et al., 2022) can be classified into three categories of PTC tasters—super-, intermediate and non-tasters (Bartoshuk et al., 1994; Southerland et al., 2022). In humans, PTC sensitivity has been linked to the ability to taste and form aversions to bitter tasting foods (Fischer et al., 1961; Garcia-Bailo et al., 2009).

While sheep demonstrate an aversive response to bitter compounds in solution, research suggests that sheep were indifferent to bitter-flavored feed when quinine-HCl, a bitter-tasting compound similar to PTC, was added to the diet at the same concentration used to elicit an aversive response in drinking water (Favreau et al., 2010). Therefore, the objective of this study was to determine the effects of PTC delivered in feed on dietary intake and behavior in sheep. Both PTC-naïve ram lambs and mature rams that had been previously identified as either super-, intermediate, or non-PTC tasters

were tested. We hypothesized that rams previously classified using PTC-spiked drinking water would classify similarly when PTC was added to feed and that ram lambs could be classified by measuring feed intake of two concentrations (180 mg/kg and 280 mg/kg) of PTC incorporated into feed pellets. The results of this study will be used to further understand the influence of PTC tasting ability on dietary selection in sheep.

Materials and methods

Animal Care

This series of experiments was conducted between December 2021 and February 2022 at the USDA, ARS, Range Sheep Production Efficiency Research Unit, U. S. Sheep Experiment Station located near Dubois, ID, USA. All animal procedures were approved by an Institutional Animal Care and Use Committee (Protocol: 22-02; USDA, ARS, Dubois, ID, USA) in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Pellet Preparation

All pellets were prepared within 2 weeks of starting each experiment in 30 kg batches. Alfalfa (70%), corn (15%), and beet pulp (15%) meal were mixed in a ribbon mixer for 10 min. For the control pellets, 150g of ethanol (EtOH) was heated to just below boiling point (78°C) and added to 300 g of cold water. For the PTC pellets, 3.3, 5.4, 6.6, 8.4, or 9.9 g of PTC (Sigma-Aldrich, Inc, St Louis, MO) was solubilized in 150g of EtOH and heated to just below boiling point (78°C) to make pellets consisting of 110, 180, 220, 280, or 330 mg/kg as fed, respectively. The EtOH-water or PTC-EtOH-water solution was then added to the meal and mixed for 10 min before sitting for an additional 10 min. To reduce fines and increase production rate, Super-lube powder (Uniscope, Inc., CO; 60 g) was then added to each batch and allowed to mix for 5 min. The complete mixture was then pelleted through a 6.3 mm die using a CME Mill-10, 10 HP Pellet Mill (Colorado Mill Equipment, CO) and allowed to cool to room temperature before storage.

Experiment 1

Experiment 1 took place in December 2021 and utilized twelve mature rams ($\geq$ 2 years of age, 112.5 $\pm$ 13.3 kg) including Polypay (n = 2), Rambouillet (n = 2), Targhee (n = 5) and a composite breed (Suffolk $\times$ Columbia $\times$ Texel; n = 3) that had been previously classified (Southerland et al., 2022) into PTC-taster categories (super-, n = 3; intermediate, n = 5; and non-tasters, n = 4) using PTC-spiked drinking water. Rams were randomly assigned to individual pens within a barn that was maintained at approximately 10°C with ventilators set on a 10% hourly cycle. Barn lights were turned on and off at 700h and 1700h, respectively. Water was provided for *ad libitum* (metered) intake and refreshed twice daily. The trial was divided into two phases: acclimation and test.

Acclimation phase. A 4-d acclimation phase was conducted to adapt animals to the housing environment and daily routine and collect baseline feed and water intake data. At 0700h, previously offered PM water was removed and weighed (data not shown), followed by a delivery of fresh AM water. For the morning feed intake measurement, two identical buckets of control pellets (0.87% BW each) were secured in a holding rack at 0730h. After 30 min, the feed buckets were removed, and weigh backs recorded. At 1500h, AM water was removed, weigh backs recorded (data not shown), and fresh PM water delivered. For the afternoon feeding at 1530h, rams were again offered two identical buckets of control pellets (0.87% BW each) for 30 min, after which feed buckets were removed and weigh backs recorded. Total daily feed intake was targeted at 3.5% BW.

Test phase. Following the acclimation phase, a 6-day test phase was conducted to identify sensitivity to PTC internally incorporated into feed. The feeding, watering, and intake-measurement procedures outlined in the acclimation phase were maintained through the test phase. Rams were randomly assigned to one of three groups (n = 4) to determine the order in which PTC concentrations were received. On days 1-3, during the morning feeding, rams received PTC pellets containing either 110, 220 or 330 mg/kg of PTC in a side-by-side offering with control pellets. The side of the holding rack in which the PTC pellets were delivered relative to the control was randomized. After 30 min, buckets were removed and weigh backs recorded. On days 4-6, the order of delivery was re-randomized for the various PTC concentrations and the location of the PTC pellets relative to the control was alternated.

Rams were classified based on average PTC intake as a percentage of total morning feed intake across concentrations. The PTC-taster classification categories were determined by ±1.0 standard deviation (SD) of the population mean to sort rams into super- (≤ -1.0 SD), intermediate (< 1.0 to > -1.0 SD), or non- (≥ 1.0 SD) PTC tasting groups and compared the previous classifications as established using PTC spiked water by Southerland et al. (2022).

Experiment 2

Experiment 2 included twenty-eight ram lambs (~11 mo of age) naïve to PTC in February 2022. One ram failed to acclimate to the daily feeding and watering schedule while another ram had health issues; hence, both rams were removed from the trial. The resulting sample size consisted of 13 Rambouillet (74.0 ± 3.4) and 13 Targhee (72.1 ± 5.1) ram lambs. To ensure adequate breed representation in each of the three barns, rams were blocked by breed and arbitrarily assigned to one of two groups (n = 14) to match barn capacity. Barns were maintained at approximately 10°C with ventilators set on a 10% hourly cycle. Lights were turned on and off at 700h and 1700h, respectively. Water was provided *ad libitum* (metered) and refreshed twice daily. The trial was divided into two phases: acclimation and test.

Acclimation phase. A 4-d acclimation phase was conducted to adapt rams to the housing environment and daily routine. The same feeding, watering, and intake-measurement procedures outlined in Experiment 1 were conducted throughout the acclimation and test phases of Experiment 2.

Test phase. A 4-day test phase was conducted to identify sensitivity to PTC internally incorporated into feed. Rams were randomly assigned to one of two groups (n = 14) to determine the order in which PTC concentrations were received. On days 1-2, during the morning feeding, rams received PTC pellets containing either 180 or 280 mg/kg of PTC in a side-by-side offering with control pellets. The side of the holding rack in which the PTC pellets were delivered relative to the control was randomized. After 30 min, the buckets were removed, and weigh backs recorded. On days 3-4 the order of delivery was repeated for each concentration of PTC and the location of the PTC pellets relative to the control was alternated.

Video surveillance

For both experiments, video surveillance was conducted on the last two days of the acclimation and all test days. Cameras (LX1081-88, Lorex Technology, Inc., Markham, ON, Canada) were set to record 30 frames-per-second in 1080p from 0730 to 0800 h. Within each barn, cameras were mounted at equal height and distance from the pens to record the behavioral reactions of two rams per camera. Evaluated behaviors described in Table 4.3.1 included head bobbing, lip smacking, lip licking, sniffing, number of bucket approaches, number of bucket switches, and drinking duration.

Statistical analyses

Feed intake. Statistical analyses were conducted using the PROC GLIMMIX procedure of SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC). The differential response between control and PTC pellet intake as a percent of total feed intake (TFI) was modeled in a pooled analysis to determine the effects of PTC concentration. The feed intake variables evaluated in this experiment include total feed intake during the morning feeding as a percentage of total feed (control + PTC) offered, PTC pellet intake as percentage of total feed intake during the morning feeding, control pellet intake as a percentage of total feed intake during the morning feeding, afternoon feed intake as a percentage of total feed offered, and daily feed intake as a percentage total feed offered throughout the day. Using body weight as a covariate, the fixed effects of concentration, and day within replication were evaluated for all intake response variables. Additionally, fixed effects of barn and breed, as well as any interactions were initially included but were excluded from the final model due to lack of significance ($P > 0.05$). The model also included a random statement of ram nested within replication. All means are expressed as least squares mean ± standard error. Mean comparisons were made using pair-wise contrasts (PDIFF). Significance was set at $P \leq 0.05$.

Behavioral Response. Video footage was evaluated to identify ram feeding behaviors observed at each concentration. The PROC GLIMMIX procedure of SAS was used to evaluate the probability of a behavior being exhibited after consuming control or PTC pellets. Data was analyzed for the fixed effects of feed type (control or PTC), PTC concentration, replication, as well as the interactions for feed type by PTC concentration, and feed type by replication. The model also included a random statement of ram nested within replication. The number of bucket approaches to each feed type, switches, and feed intake duration were weighted by the total number of bucket approaches per individual ram. For the head bobbing, head shaking, lip licking, and lip smacking behaviors, binomial data was converted to a percentage relative to number of bucket approaches.

Results

Experiment 1

Feed Intake. For mature rams, the difference between PTC and control pellet intake was similar for each concentration ($P = 0.22$, Table 3.2). Intake of PTC pellets as a percentage of total intake (control + PTC) during the morning feeding was 52.6 ± 2.8 %, 45.5 ± 2.8 %, and 51.5 ± 2.8 % for the 110, 220, and 330 mg/kg treatments respectively ($P = 0.16$, Table 3.2). When rams were offered 220 mg/kg pellets, PTC intake (45.5 ± 2.8 %) was 8.8% less than the control (54.4 ± 2.8); however, the difference was not significant ($P = 0.16$, Table 3.2). There was considerable variation in individual PTC pellet intakes, which ranged from 1.5 to 89.8% of total intake during the morning feeding as presented in Figure 3.1. The CV for PTC intake of the 110, 220 and 330 mg/kg PTC pellets increased from 22.4 to 34.1 to 36.1%, respectively (Figure 3.1). An effect of day within replication was observed ($P = 0.011$), in which PTC intake on day two of the second replication (62.5 ± 3.9 %) was greater than day one (40.4 ± 3.9 %) and three (49.2 ± 3.9 %) of the first replication, as well as day one (48.8 ± 3.9 %) and three of the second replication (46.6 ± 3.9 %; $P \leq 0.021$).

Total intake during the morning feeding decreased ($P = 0.01$) between 110 mg/kg (80.4 ± 2.6 %) and 330 mg/kg (71.9 ± 2.6 %), while 220 mg/kg (78.0 ± 2.6 %) was intermediate ($P \geq 0.07$, Table 3.2). The decreased feed intake exhibited when rams were offered the 330 mg/kg PTC concentration was the result of numerical decreases ($P \geq 0.06$) in both PTC and control pellet intake (% offered, data not shown) which decreased by 11.4 ± 5.7 % and 5.7 ± 6.1 % from the 110 mg/kg, respectively. The afternoon and total daily feed intakes were similar ($P \geq 0.06$) to each other. Although, total daily feed intake was numerically lower ($P \geq 0.06$) when rams were offered the 330 mg/kg pellets (78.6 ± 2.1 %), compared with the 110 (83.9 ± 2.1) or 220 (82.9 ± 2.1) mg/kg pellets.

Rams were classified by average PTC pellet intake as a percentage of total morning feed intake relative to ± 1.0 SD of the population mean (50.2 ± 7.5 %). Using this method, only one super- ($\leq$

42.7 %), one non- (≥ 57.7 %), and five intermediate tasters (< 57.7 % to > 42.7 %) were categorized similarly to the former classification established by Southerland et al. (2022) when PTC was added to drinking water. The remaining five rams were categorized into the intermediate class.

The smaller CV and indifference to PTC which resulted when mature rams were offered the lowest concentration of PTC pellets suggests that 110 mg/kg applied to feed may be too weak to identify individuals of even the greatest PTC sensitivity (i.e., super-tasters). Meanwhile, the higher CV and depression of feed intake exhibited when mature rams were offered the highest concentration of PTC pellets may indicate that 330 mg/kg is too strong to accurately identify PTC tasting ability. This, along with the variation in PTC pellet intake exhibited by individual rams offered 220 mg/kg indicates that the level of PTC inclusion necessary to sort mature rams into their respective PTC taster classifications is intermediate to the concentrations selected for this experiment. Therefore, PTC concentrations of 180 and 280 mg/kg were selected for Experiment 2.

Behavioral response. The total number times a mature ram approached the feed buckets (65.5 ± 28.8), as well as the number of individual approaches to either the control (34.8 ± 3.0) or PTC (34.2 ± 3.0) buckets were not influenced by any fixed effects or interactions ($P \geq 0.20$). However, the number of times a ram switched from the control to the PTC bucket, or vice versa, decreased ($P \geq 0.015$) from 21.4 ± 2.1 when offered 110 mg/kg PTC pellets to 16.4 ± 2.0, and 18.4 ± 2.0 when offered the 220 and 330 mg/kg PTC pellets, respectively, which were similar to each another ($P = 0.08$). While there were no main effects or interactions ($P \geq 0.10$) for time spent eating either PTC or control pellets, total feed intake duration was less ($P \leq 0.025$) when rams were offered 330 mg/kg (805.6 ± 44.0 s) compared with 110 (858.9 ± 44.3 s) and 220 mg/kg (862.9 ± 44.0 s), which may be due to the reduced feed intake at the highest concentration of PTC.

An interaction of replication by feed type was observed for both the head bobbing ($P \leq 0.001$; Figure 3.2a) and lip smacking ($P = 0.018$; Figure 3.2b) behaviors. During replication one, instances of head bobbing occurred more frequently ($P \leq 0.001$) after rams ate PTC pellets (9.2 ± 1.7 %) than after eating control (4.4 ± 0.9 %). However, during the second replication, occurrence of head bobbing increased ($P = 0.036$) to 8.2 ± 1.5 % after sampling the control feed which was not different ($P = 0.08$) from the PTC pellets (6.4 ± 1.2 %). The frequency in which head bobbing was observed after consuming PTC were not different ($P = 0.08$) between replications. A similar effect was observed for the lip smacking behavior (Figure 3.2B), which occurred more frequently during replication one ($P = 0.005$) after rams ate PTC pellets (3.2 ± 0.9 %) than after eating control (1.5 ± 0.5 %). However, during replication two, these values (2.1 ± 0.6 % for PTC) and (2.3 ± 0.7 % for control) were not different ($P = 0.62$) from one another, nor was the rate of occurrence after consuming each feed type different from replication one ($P \geq 0.33$).

For the sniffing behavior, an interaction of concentration by feed type was observed ($P = 0.003$, Figure 3.3). When mature rams were offered pellets containing 110 mg/kg of PTC side-by-side with control, rams sniffed the control bucket (8.5 ± 1.3 %) less than ($P \leq 0.001$) the PTC bucket (14.6 ± 1.9 %). However, rams sniffed the control bucket (13.9 ± 1.9 %) at a rate similar to ($P = 0.42$) the 220 mg/kg PTC pellets (12.5 ± 1.8 %). The frequency of sniffing was also the same ($P = 0.80$) when the control (12.2 ± 1.7%) was offered side-by-side with 330 mg/kg PTC pellets (12.6 ± 1.7%). Rams sniffed the PTC bucket at a similar rate ($P \geq 0.23$) regardless of PTC concentration. However, this was not true for the control in which bucket sniffing occurred less ($P \leq 0.017$) when paired with the 110 mg/kg PTC pellets, as opposed to the control buckets offered with the 220 and 330 mg/kg PTC pellets, which were not different ($P = 0.32$) from one another. Neither the drinking nor the lip licking behaviors were influenced by any main effects or any interactions thereof ($P \geq 0.18$).

Experiment 2

Feed intake. The population mean for average intake of PTC pellets as a percentage of total intake by ram lambs during the morning feeding was 50.8 ± 2.8%. Intake of PTC was not influenced by concentration ($P = 0.86$) or day within replication ($P = 0.61$, Table 3.2). There was also no difference between the type of feed consumed (PTC vs. control) when offered either the 180 (1.4 ± 2.3 %) or 280 (3.0 ± 2.3%) mg/kg PTC pellets (Table 3.2). While the individual intake of PTC pellets ranged from 16.4 % on the low to 97.4 % on the high PTC concentration, the CV for PTC intake was 16.6 and 13.9 % for the 180 and 280 mg/kg PTC concentrations, respectively.

Ram lambs were then classified in the same manner as the mature rams in Experiment 1, utilizing average PTC pellet intake as a percentage of total morning feed intake relative to ± 1.0 SD of the population mean (Figure 3.4). Only one lamb identified as a non-taster (≥ 53.6 %), while the remaining 25 lambs fell into the intermediate category (< 53.6 % to > 48.0 %). No lambs exhibited a dietary aversion to PTC pellets that would identify them as super-tasters (≤ 48.0 %). Ram lambs consumed 97.3 ± 10.5 % and 99.7 ± 1.2 % of their total feed offer during the morning and afternoon feedings, respectively. As such, there was no effect of PTC concentration or day within replication for total morning, afternoon, and daily feed intake ($P \geq 0.56$; Table 3.2).

Behavioral response. There were no main effects or interactions ($P \geq 0.08$) for the total number of times a lamb approached the feed buckets (60.7 ± 22.3), or the number of individual approaches to either the control (30.4 ± 1.7) or PTC (33.6 ± 1.7) buckets. Unlike the mature rams, the number of times a ram lamb switched between the control to the PTC bucket was similar ($P = 0.30$) regardless of being offered pellets containing 180 (21 ± 1.3) or 280 (20.2 ± 1.3) mg/kg PTC. There was no difference ($P = 0.92$) in the amount of time spent eating out of either bucket when control (380.9 ± 27.4 s) was offered side-by-side by with the 180 mg/kg (377.8 ± 26.0) PTC pellets. When offered

pellets containing 280 mg/kg PTC, the time spent eating out of the PTC bucket numerically increased ($P = 0.07$) to 438.4 ± 26.1 s. While the amount of time eating control pellets did not change ($P = 0.17$; 332.2 ± 27.4 s), time spent eating the control and PTC feed were different from one another ($P = 0.002$, Figure 3.5) when offered the 280 mg/kg PTC concentration. Total time spent eating (control + PTC) was not different ($P = 0.24$) for the 180 (744.0 ± 31.4 s) and 280 (767.4 ± 31.4 s) mg/kg PTC concentrations or by replication ($P = 0.71$, data not shown).

An effect of feed type was observed for both the bobbing and lip smacking behaviors. Head bobbing was observed more frequently ($P < 0.001$) after lambs ate PTC pellets (7.3 ± 1.0 %) than after eating from the bucket containing control pellets (4.4 ± 0.6 %). But the head bobbing behavior was not affected by concentration, replication, or any interaction thereof ($P \geq 0.08$, data not shown). Lip smacking was also observed at a greater rate ($P = 0.008$) after lambs ate from the PTC bucket (2.5 ± 0.4 %) when compared to the bucket containing control pellets (1.5 ± 0.2 %). Much like the head bobbing behavior, aside from feed type, lip smacking was not influenced by any other main effects or interactions ($P \geq 0.08$, data not shown).

Unlike Experiment 1, the sniffing behavior was not influenced by PTC concentration ($P = 0.53$), replication ($P = 0.14$), or any interaction of replication and concentration with feed type ($P \geq 0.91$, data not shown). Interestingly, ram lambs sniffed the control bucket (21.5 ± 1.4 %) numerically more than the bucket containing PTC pellets (19.5 ± 1.3 %); however, this difference was not significant ($P = 0.05$). No main effects or interactions were observed for either the drinking or lip licking behaviors ($P \geq 0.05$, data not shown).

Discussion

Bitter aversion can be quantified in rams by measuring fluid intake of two concentrations of PTC-solution (0.20 mM and 2.03 mM) (Southerland et al., 2022). These two concentrations serve to respectively separate the super- and non-PTC tasting sheep from the intermediate tasters. In humans, those who can detect minute quantities of PTC (i.e. super-tasters) are most likely to form an aversion to bitterness, which can influence their dietary selections (Garcia-Bailo et al., 2009). Research on a small sample size of sheep (n = 26), suggested that the TAS2R38 gene that codes for PTC tasting ability in humans (Kim et al., 2003), could also be linked to PTC tasting ability in sheep (Davenport et al., 2021). Previous studies evaluating PTC-tasting ability, have alluded to the possible selection of bitter-tolerant sheep for the targeted control of less palatable rangeland plants (Henslee et al., 2019; Southerland et al., 2022). However, before such conclusions can be made, we must first determine if bitter tasting ability impacts dietary selection in sheep.

Favreau et al. (2010) also attempted to answer this question by feeding quinine HCl treated grass hay to 5-month-old ewe lambs and determined that unless a post-ingestive consequence (LiCl) was associated with the bitter stimuli, sheep were indifferent to the quinine-treated feed. However, the discrimination threshold of quinine in sheep is lower than the rejection threshold (Goatcher & Church, 1970b). The lack of aversion to the perceived bitterness was therefore attributed to a dose effect (Favreau et al., 2010). Concentrations of PTC-solution previously used to classify tasting ability in mature rams contained 30.9 mg (0.20 mM) and 309 mg (2.03 mM) of PTC per kg of drinking water (Southerland et al., 2022). Thus, the objective of this study was to determine the effects of various concentrations of PTC on the dietary selection of both lambs and mature male sheep.

During Experiment 1, mature rams were exposed to three concentrations (110, 220 and 330 mg/kg) of PTC pellets. Based on behavioral observations and feed intake data, all mature rams were indifferent to the lowest concentration of PTC. Taste perception relies on the fluid component of the external milieu of the taste bud (Matsuo, 2000). For a taste stimulant to be perceived, it must first be dissolved in salivary water for transport and diffusion by taste buds (Matsuo, 2000). Slower flow rates of taste solution correspond with a smaller magnitude of response and longer periods of time to reach max response (Smith and Bealer, 1975; Marowitz and Halpern, 1977). Additionally, taste stimulants delivered in aqueous solution are more immediately dissolved into saliva than those in dry solids (Matsuo, 2000). While 110 mg/kg is a higher concentration than what was necessary to elicit a reaction from super-tasters with water, the pellets formulated for this feed trial contained 88.5% dry matter. Food pellets must be gradually ground into smaller pieces before being dissolved into saliva, which results in a longer period of time for the stimulus to reach the receptor site (Matsuo, 2000). Thus, the perception of PTC internally incorporated into feed pellets would rely heavily upon mastication and saliva production (Matsuo, 2000), which could ultimately influence bitter sensitivity by requiring a greater concentration in order for PTC to be perceived.

While the individual feed intake variation increased when mature rams were offered pellets containing 330 mg/kg PTC, a decrease in overall feed intake (control + PTC, % offer) was also exhibited. This is similar to the reduction in overall fluid intake exhibited by rams offered 4.39 mM PTC-solution (Henslee et al., 2019), and deemed high for identification of PTC tasting ability in rams (Southerland et al., 2022). The increased observance of bucket sniffing, as well as the reduction in bucket switches at the 330 mg/kg PTC concentration, demonstrates a greater level of selectivity and reliance on a secondary sense to make informed decisions regarding which bucket to consume feed from.

Respectively, these results indicate that an inclusion of 110 and 330 mg/kg PTC were likely either too low or too high to accurately quantify PTC aversion in sheep. However, when dosed with 220 mg/kg concentration, not only was the individual variation in PTC pellet intake high (Figure 3.1), but consumption of control pellets exceeded that of PTC by 10 % of total morning feed intake (Table 3.2). These results indicate that some rams within the population were beginning to express aversion toward the 220 mg/kg PTC pellets (Figure 3.1). This is furthered by the increased occurrence of head bobbing and lip smacking after consuming PTC treated feed which were also observed in mature rams after consuming PTC-spiked drinking water (Southerland et al., 2022). As the trial progressed, the effect of feed type on head bobbing and lip smacking became less pronounced, suggesting that rams may become more tolerant of PTC-treated feed over time.

Seven of the twelve rams in this study were classified in the same category as to their former classification, established when PTC was added to drinking water (Southerland et al., 2022). Using this method, two super- and three non-tasters failed to differentiate from the intermediate group. Average PTC intake was 51.5 ± 6.0 % and 48.8 ± 2.7 % for undifferentiated super- and non-tasters, respectively. Concentration of PTC did not have an effect on voluntary feed intake of PTC or any reactive behaviors within the experimental group. These results suggest that the ideal concentrations identification of PTC-tasting ability in sheep maybe intermediate to the concentrations selected for Experiment 1 which would account for the inconsistency in classification within rams treated with both PTC-solution and the PTC-treated pellets.

However, when concentrations of 180 and 280 mg/kg were tested during Experiment 2, inclusion of PTC did not have an adverse effect on pellet intake by ram lambs. Ruminants are known to reduce their intake in order to determine digestive consequences (Provenza et al., 1992; Launchbaugh et al., 2001), but it has been suggested that rate of feed intake may also be important (Launchbaugh et al., 2001). Although PTC and control pellet intakes were similar during Experiment 2, ram lambs took longer to eat the 280 mg/kg PTC pellets, which may have allowed for determination of any post-ingestive feedback. Furthermore, the increased observance of head bobbing and lip smacking post PTC consumption indicates that some of the ram lambs were able to sense the PTC. These results are similar to the findings of Favreau et al. (2010) in that lambs were able to identify which pellets contained PTC, but the perceived bitterness did not alter intake of PTC-treated feed. While mature rams began to exhibit signs of PTC aversion at concentrations of 220 mg/kg, ram lambs were willing to consume pellets containing 280 mg/kg PTC. This suggests that the PTC concentration necessary to elicit an aversive response in lambs is different than the dosage necessary to modify feed intake in mature sheep. One explanation for the variable response to the different PTC concentrations between the two age groups could be the influence of learned experience and formed associations between the

consumption of bitter feed and negative post-ingestive feedback (Favreau et al., 2010; Provenza et al., 1993, 1994).

The formation of food preferences and aversions often occurs when a taste stimuli is paired with either positive or negative post-ingestive feedback (Provenza, 1996). Of the five main flavor profiles, ruminants are often the most averse to bitter flavors (Goatcher & Church, 1970b, 1970d; Ginane et al., 2011) which develop when toxic secondary plant metabolites stimulate the portion of the nervous system that controls digestive upset (Launchbaugh et al., 2001). If an animal becomes ill or experiences digestive discomfort from a particular plant, they tend to avoid consumption of it in the future (Burritt & Provenza, 1996). These taste feedback mechanisms are involuntary responses that unconsciously generate flavor preferences and aversions through associations made with external stimuli—such as the sense of taste (Provenza, 1996). Exposure is a critical component in the formation flavor aversions throughout a sheep's lifetime (Nolte and Provenza, 1992; Launchbaugh et al. 2001). While PTC is not found in nature, PTC-tasting ability has been associated with the ability to taste other bitter foods (Glanville and Kaplan, 1965). Thus, mature rams may be more responsive to lower concentrations of PTC due to previous exposure to bitter flavors and involuntary associations with negative post ingestive feedback.

However, toxic effects of secondary metabolites will not always prevent ruminant consumption of a plant if it is also rich in vital nutrients (Wang & Provenza, 1996). In addition to the chemical composition of the plant, the formation of flavor aversions also depends on the physiological state of the animal (Provenza, 1996). Mature rams have lower nutrient requirements than growing ram lambs, and in theory, could metabolically afford to be more selective with their pellet choices. While all feed amounts were calculated to meet daily nutritional requirements, it is possible that any flavor aversion experienced by ram lambs was confounded by growth stage and experience, causing lambs to consume higher concentrations of PTC pellets more willingly.

Conclusion

Results from this research suggest that when internally incorporated into feed pellets, the perception threshold of PTC ranges between 180 and 280 mg/kg. However, lambs and mature male sheep exhibit different responses to PTC-treated pellets, suggesting that the rejection threshold may vary by physiological state and learned experiences. While feed intake measures appear to provide information regarding the willingness to consume PTC-treated feed, interpreting behavioral responses such as duration of feeding, head bobbing and lip smacking may be more indicative of PTC-taster ability. The ability to taste bitterness may not directly influence dietary intake, regardless, the role of sensation and bitter taste perception are crucial to the formation of learned flavor aversions and their

influence on dietary decisions. Although selective breeding based on bitter-tasting phenotypes may be possible, the influence of this ability on dietary selection may depend on the formation of flavor aversions that commensurate with grazing experience. Further research is needed to understand the dynamic relationship between PTC tasting ability on dietary selection in sheep and their possible application for the targeted control of less palatable range plants.

Acknowledgement

Tables and Figures

Table 3.1. Ethogram of behaviors observed and their corresponding descriptions during the morning feed delivery during the adaptation and test phases during experiments 1 and 2.

Behavior	Description
Approach	Head hovering over or inside of bucket
Feeding	Head inside of bucket, consuming feed
Head bobbing	Repetitive vertical movement of head up and down
Lip licking	Protrusion and passing of tongue over lips
Lip smacking	Wide repetitive opening and closing of mouth
Sniffing	Head hovering over bucket with nose pointed toward feed
Switch	Change of feeding or sniffing from one bucket to another

Table 3.2. Least squares means (± standard error) for feed intake by rams within experiment (1-mature rams and 2-yearling rams) and across phenylthiocarbamide (PTC) concentrations.

| Variable | Experiment 1 | | | | Experiment 2 | | |
| | PTC concentration | | | | PTC concentration | | |
	110 mg/kg	220 mg/kg	330 mg/kg	P-value	180 mg/kg	220 mg/kg	P-value
Total morning feed (control + PTC pellets) intake, % offered	80.4 ± 2.6 [a]	78.0 ± 2.6 [a,b]	71.9 ± 2.6 [b]	0.040	97.7 ± 1.6	97.7 ± 1.6	0.994
Control, % of total morning feed intake	47.3 ± 2.8	54.4 ± 2.8	48.4 ± 2.8	0.168	49.3 ± 1.2	48.5 ± 1.2	0.618
PTC, % of total morning feed intake	52.6 ± 2.8	45.5 ± 2.8	51.5 ± 2.8	0.168	50.6 ± 1.2	51.4 ± 1.2	0.618
Afternoon feed intake, % offered	87.3 ± 2.3	87.7 ± 2.3	85.3 ± 2.	0.647	99.7 ± 2.3	99.8 ± 2.1	0.563
Total daily feed intake, % offered	83.9 ± 2.1	82.9 ± 2.1	78.6 ± 2.1	0.060	98.7 ± 2.1	98.8 ± 2.1	0.914

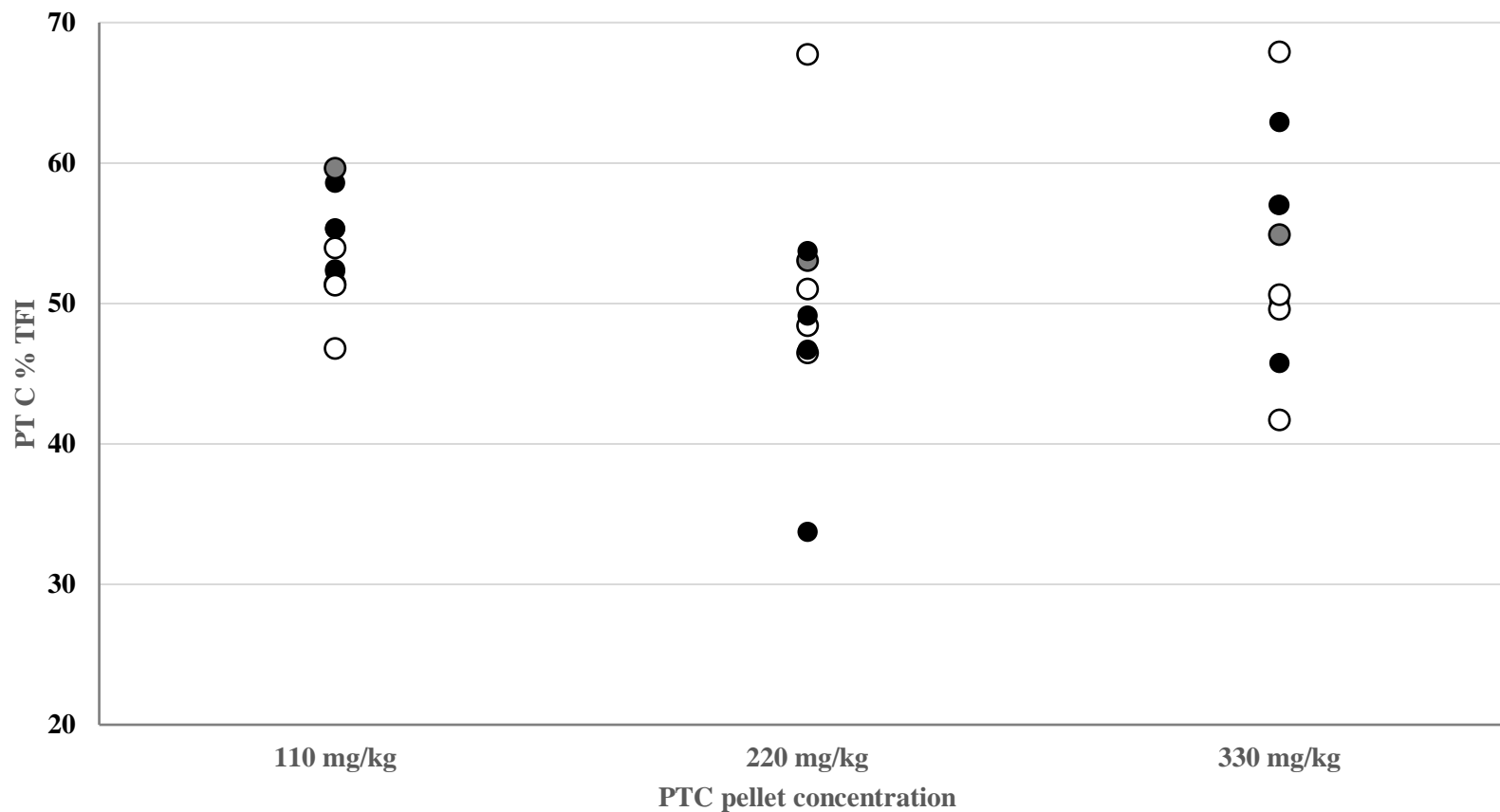[a,b]Means across PTC concentrations with different superscripts are different ($P \leq 0.05$).

Figure 3.1. Least squares means of average PTC intake as a percentage of total feed intake during the morning feeding in experiment 1 for mature rams at each concentration. Classifications using feed intake were devised based on average PTC intake as a percentage of total morning feed intake across concentrations. The PTC-taster classification categories were determined by ±1.0 standard deviation (SD) of the population mean to sort rams into super- ($\leq$ -1.0 SD), intermediate ($<$ 1.0 to $>$ -1.0 SD), or non- ($\geq$ 1.0 SD) PTC tasting groups which are represented by a dotted, solid, or dashed line, respectively. These feed classifications are compared the previous classifications for ⬤ super-, ● intermediate, or ○ non-tasters as established using PTC spiked water by Southerland et al. (2022).
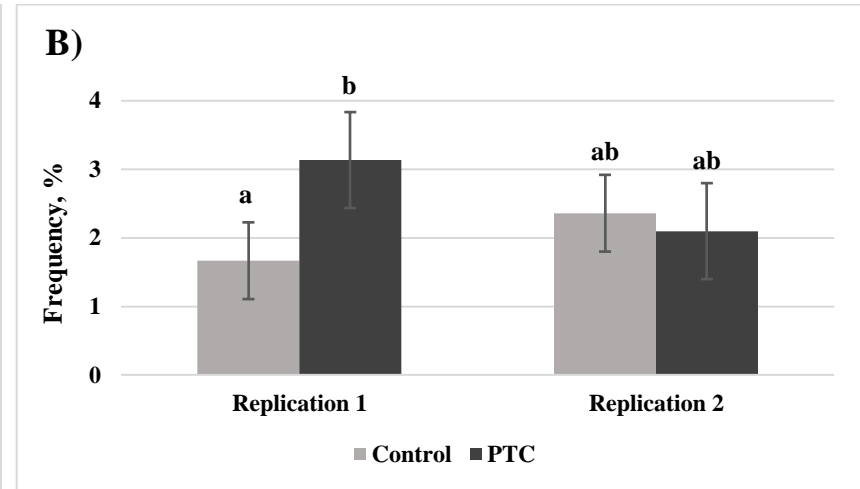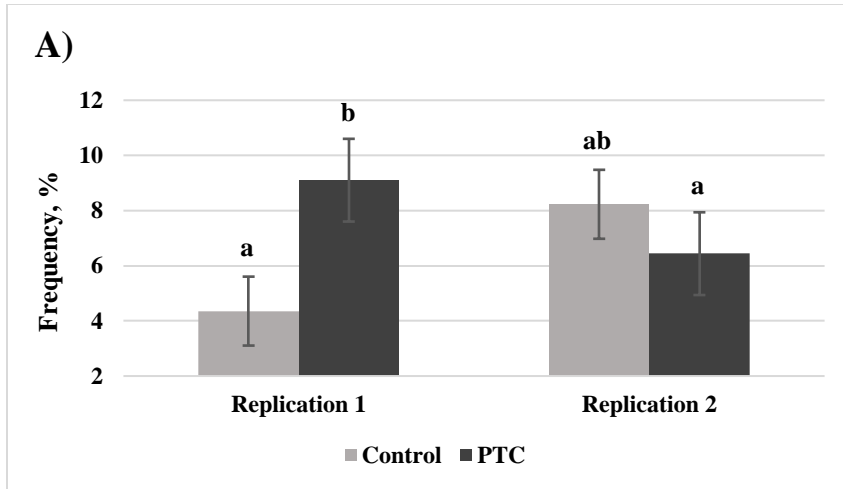
Figure 3.2. Least squares means (± SE) of the frequency in which **A)** head bobbing and **B)** lip smacking behaviors were exhibited by mature rams after eating control or PTC pellets during each replication (solution type × replication) in experiment 1.
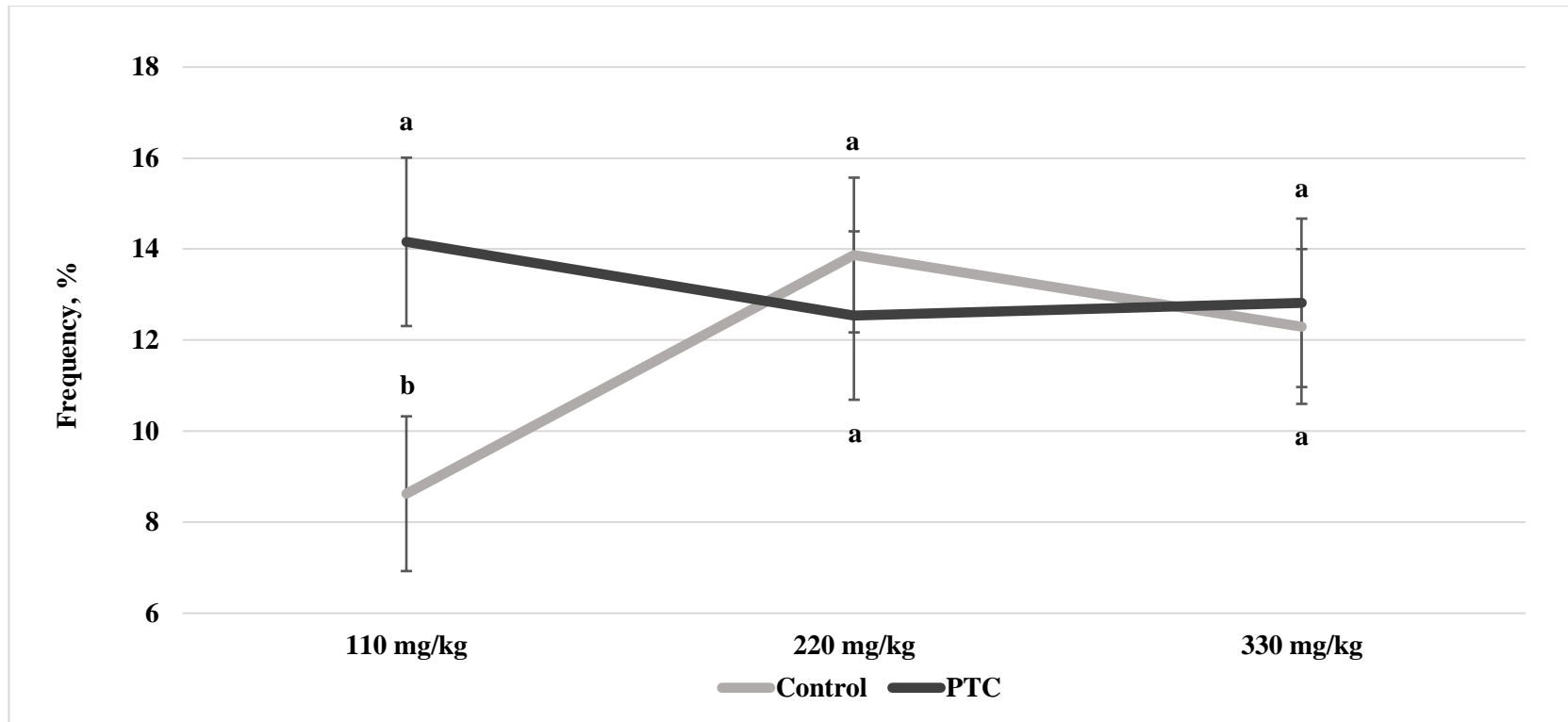[a,b]Means are different ($P \leq 0.05$).

Figure 3.3. Least squares means (± SE) of the frequency in which the sniffing behavior by mature rams occurred for buckets containing either control or PTC pellets at each concentration (110, 220, and, 330 mg/kg) in experiment 1.
[a,b]Means are different ($P \leq 0.001$).

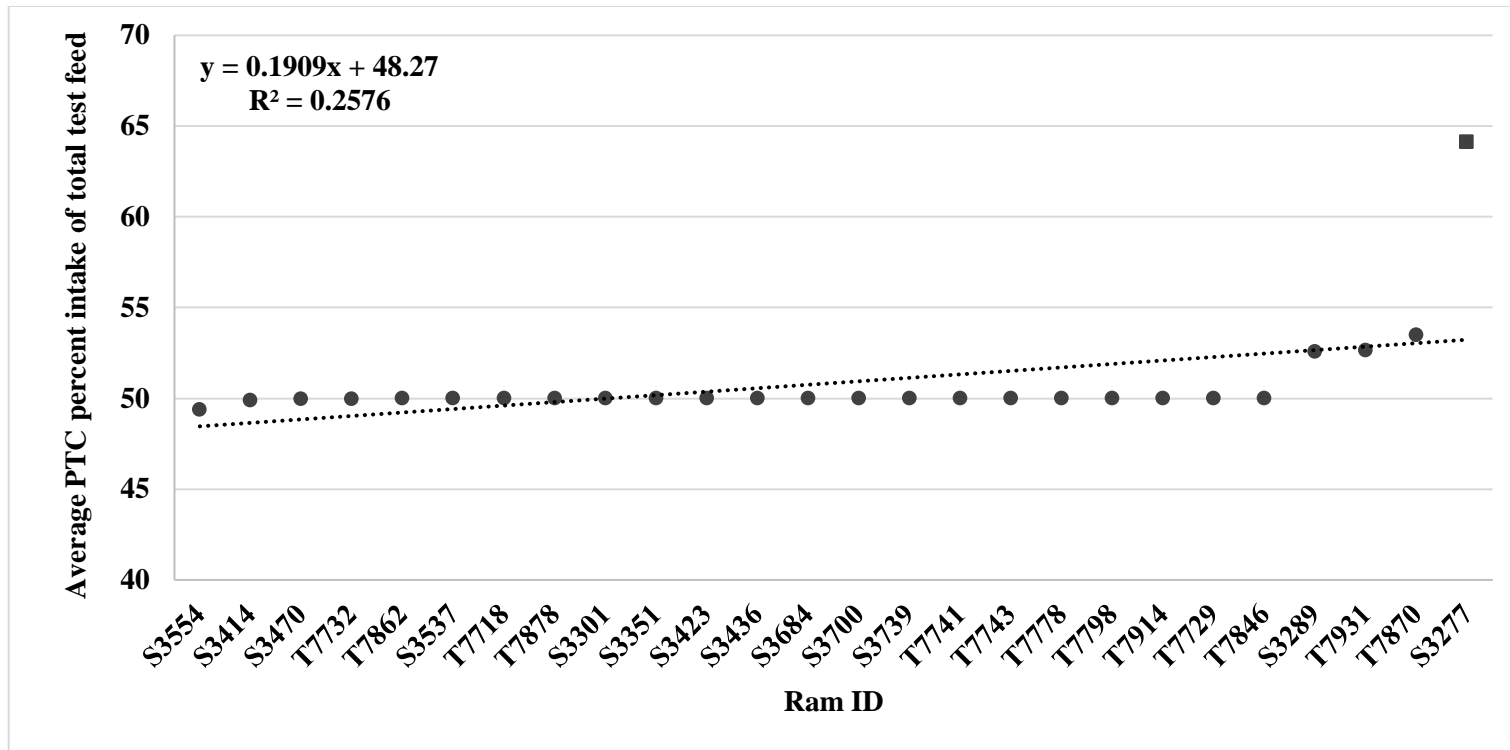$$y = 0.1909x + 48.27$$
$$R^2 = 0.2576$$

Figure 3.4. Average phenylthiocarbamide (PTC) intake of individual ram lambs administered two concentrations (180 and 280 mg/kg) of PTC internally incorporated into feed pellets and further classified by taster status: intermediate ($\bullet$ = < 1.0 to > -1.0 SD) and non- ($\blacksquare$ = ≥ 1.0 SD) tasters in experiment 2. Mean PTC intake: 50.8 ± 2.8%.
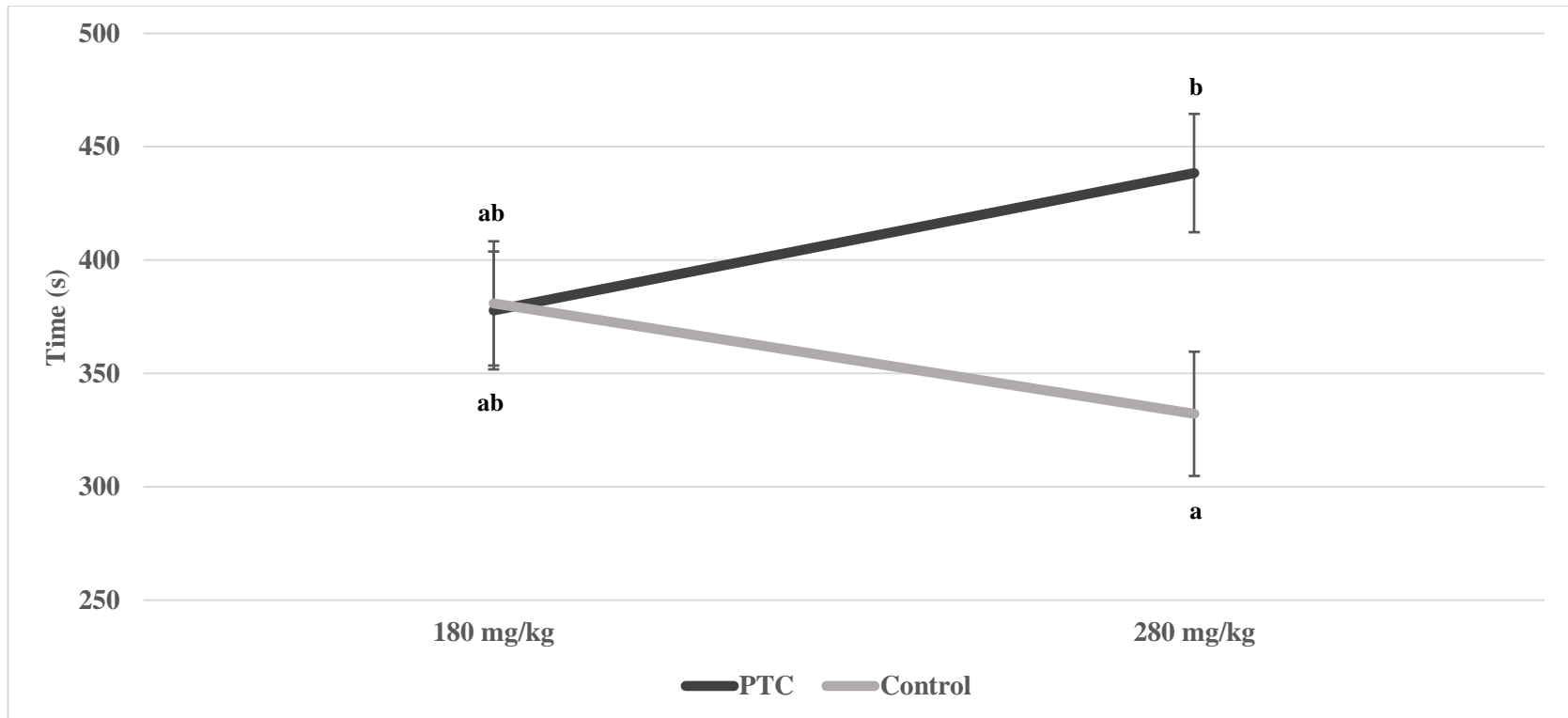
Figure 3.5. Least squares means (± SE) for the amount of time ram lambs spent eating (sec) out of either control or PTC bucket when offered treated pellets containing 180 or 280 mg/kg of PTC (solution type × concentration) in experiment 2.
[a,b]Means are different ($P = 0.002$).

Chapter 4: Intake and behavioral responses of ram lambs provided phenylthiocarbamide topically applied to feed

Abstract

Voluntary fluid intake studies utilizing phenylthiocarbamide (PTC) have demonstrated variation among sheep in their willingness to consume bitter compounds. Bitter sensitivity has been linked to dietary preferences in other species, leading researchers to inquire whether this ability impacts foraging decisions. A series of experiments was conducted on ram lambs to determine whether bitter tasting ability could be identified using an oral drench or measuring intake of alfalfa pellets topically sprayed with PTC-solution. The drench (2.5 mM PTC or water) was applied directly to the tongue and lambs (n = 62) were monitored (30 sec) for any aversive response. None of the observed behaviors were associated with PTC ($P \geq 0.20$). Reactive behaviors to the involuntary drench were observed regardless of treatment (PTC, 4.8 ± 0.3; water, 4.9 ± 0.3; $P = 0.75$). A pilot study was then conducted to determine 1) if group-housed lambs (n = 6) would exhibit an aversive response to topical applications of PTC-treated (LOW: 20 mM; HIGH: 30 mM) feed and 2) the maximum duration of feed intake (981.4 ± 70.6 sec*; $P \leq 0.011$). Lambs exhibited a greater ($P \leq 0.002$) number of feeding events, positional changes around the feed bunk, and increased ($P = 0.002$) reliance on olfaction when offered PTC. During two back-to-back feed trials, 28 lambs (Polypay: n = 10; Rambouillet: n = 10; Targhee: n = 8) were offered alfalfa pellets sprayed with either LOW or HIGH PTC solutions (PTC + EtOH + water) followed by control (EtOH + water) pellets for 15 min each. Feed intake was not affected by PTC ($P = 0.18$). Control pellet intake was greatest ($P \leq 0.001$) after consuming HIGH PTC pellets and was directly correlated ($P <0.001$, r = 0.45) to intake of PTC-treated feed. Lambs exhibited more ($P \leq 0.001$) behavioral reactions to PTC and demonstrated a more continuous feeding pattern when offered control pellets ($P \leq 0.005$). Classifications were devised based on the range of behaviors observed over both concentrations of PTC (low: 0-3, n = 14; moderate: 4-6, n = 30; high: 7-10, n = 11). Highly and moderately reactive lambs consumed the greatest amount of PTC and control pellets ($P \leq 0.006$). Differences were observed between the classes ($P \leq 0.035$) for the sniffing, head shaking and butting, biting, pawing, and fenceline behaviors which may aid in the identification of bitter sensitivity. Further research is needed to determine the effects of bitter tasting ability on the willingness of sheep to consume bitter feeds.

Introduction

Bitter taste perception has long been viewed as an evolutionary protection mechanism against toxicosis (Chandrashekar et al., 2000; Dong et al., 2009; Garcia-Bailo et al., 2009). However, there are also many foods that contain bitter phytochemicals that have beneficial properties such as anti-inflammatory flavonoids and glucosinolates found in citrus fruits and brassica vegetables, respectively (Drewnowski et al., 2001). Varying degrees of bitter sensitivity have been reported both between species and among individuals within a species (Fox, 1932; Blakeslee and Salmon, 1935; Goatcher and Church, 1970b; Goatcher and Church, 1970d; Dong et al., 2009). This variation is thought to be closely linked to feeding ecology (Bachmanov and Beauchamp, 2007; Dong et al., 2009) and may impact dietary selection and decision making (Garcia-Bailo et al., 2009). For example, humans with a heighted sense of bitterness have been found more likely to select against antioxidant-rich fruits and vegetables (Garcia-Bailo et al., 2009).

Bitter tasting ability has also been studied in sheep (Goatcher and Church, 1970b; Henslee et al., 2019; Southerland et al., 2022), and other ruminants (Goatcher and Church, 1970d) using solubilized quinine HCl and phenylthiocarbamide (PTC). In humans, perception of thiourea compounds such as PTC and propylthiouracil (PROP) has been linked to the ability to taste other bitter foods (Fischer et al., 1961). Research has determined that individual sheep show varying degrees of PTC sensitivity (Henslee et al., 2019) and can be categorized as either super-, intermediate, or non-bitter tasters based on voluntary fluid intake of PTC-spiked drinking water (Southerland et al., 2022). These findings have led researchers to question whether bitter taste perception also influences dietary selection in grazing animals; and if so, could this ability be employed in grazing regimes to selectively target plants based on bitter flavor profile. However, the formation of taste preferences and flavor aversions are highly complex and may be driven by several factors, including genetics, age, health, post-ingestive feedback, and learned behaviors (Tepper, 2008).

Streamlined methodology for the identification of PTC tasting phenotypes will allow researchers to conduct future research on the topic more efficiently, deepening our understanding of the effects of bitter taste perception on dietary selection in range sheep. Genetic tests to determine bitter tasting ability in sheep are currently unavailable. Therefore, to better understand how bitter taste perception influences feed intake and diet selection in range sheep, exploratory studies have begun to determine the most efficient methods for identifying bitter tasting phenotypes. Initial preference tests measured voluntary fluid intake of eight different concentrations (0.20 – 12.29 mM) of PTC solubilized in drinking water (Henslee et al., 2019). This method was later refined by Southerland et. al (2022) using only two concentrations (0.20 and 2.03 mM) of PTC-solution to classify mature rams by bitter

sensitivity. During this study, video surveillance was simultaneously conducted to identify any aversive behaviors (i.e. drinking duration, head bobbing, lip smacking, and rhythmic lip movements) associated with the various classes of PTC-tasters (Southerland et al., 2022). While less laborious than the methodology utilized by Henslee et al. (2019), fluid intake studies required a 6-d trial period and sample sizes were limited by testing facility capacity (Southerland et al., 2022). As such, the objectives of this study were to determine if bitter taster status in sheep could be identified using either an oral drench of PTC-spiked drinking water directly applied to the tongue or through feed intake and behavior of sheep provided alfalfa pellets with PTC topically applied to the pellet surface.

Materials and Methods

Animal Care

This series of experiments was conducted between August and October 2022 at the USDA, ARS, Range Sheep Production Efficiency Research Unit, located near Dubois, ID, USA. All animal procedures were approved by an Institutional Animal Care and Use Committee (USDA, ARS, Dubois, ID, USA) in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Experiment 1

Experiment 1 took place in August and September 2022 and utilized 62 ram lambs (approximately 5 mo of age; 42.6 ± 4.3 kg) of Polypay (n = 20), Rambouillet (n = 23), and Targhee (n = 19) breeds. The purpose of this experiment was to determine if bitter sensitivity could be rapidly identified by measuring behavioral reactivity to a one-time oral drench of PTC-solution. Lambs were kept outdoors and divided evenly at random into three groups, irrespective of breed. Each group was then assigned to neighboring pens where they were housed throughout the duration of the trial. Lambs were fed an alfalfa-based total mixed ration twice daily from a feed bunk with *ad libitum* access to water.

Acclimation Phase. An 8-d acclimation phase was conducted to normalize animals to the housing environment and drenching routine. For d 1 and d 2 of the trial, lambs remained in feedlot pens with no further activity. On d 3 and d 4, all three groups of lambs were gathered from their pens at 0800 h and moved through an indoor race and chute single-file without further handling. Once through the chute, each group was held in one of three outdoor holding pens until all lambs had moved through the facility. All three groups were then returned to their home pens. This method was used to move lambs through the facilities for the remainder of the trial.

On d 5 and d 6, once each lamb reached the chute they were orally drenched with water (10 mL) and immediately released to one of the holding pens. The water was administered using a drench gun

inserted into the mouth at the diastema and angled so that water was dispensed directly onto the tongue. This procedure was repeated on d 7 and d 8; however, after receiving the drench, lambs were retained in the chute for 30 sec before being released into the holding pens. Once all sheep had been drenched, the groups were returned to their home pens.

Testing Phase. For the 2-d test phase, lambs were randomly assigned to one of two treatment groups (n = 31) to be drenched with either water or 2.5 mM PTC-solution. The PTC-solution was prepared the day prior to administration by adding 385.5 mg PTC to 12.5 g EtOH and heated until PTC was dissolved. The PTC-EtOH mixture was then added to 1000g of cold water and allowed to reach room temperature. On d 9 lambs were removed from their pens at 0800 h and moved through the facilities, similar to the acclimation phase. Voluntary fluid intake studies show that compared to water only, EtOH-spiked water does not influence sheep behavior (Southerland et al., 2022). Therefore, each lamb received a 10 mL drench of either 2.5 mM PTC or water only and was monitored for behavioral reactivity using video surveillance. After 30 sec, lambs were released into the holding pens until all individuals had been drenched and each group was returned to their home pen. On d 10, the drench solutions were alternated by treatment group and the procedure was repeated, similar to the day before.

Video surveillance. Video surveillance was conducted on the last day of the acclimation phase to establish baseline behaviors, as well as on both testing days. Cameras (LX1081-88, Lorex Technology, Inc., Markham, ON, Canada) were set to record 30 frames-per-second in 1080 p. One camera was mounted at the end of the chute eye-level with the lambs, while a second camera was mounted approximately 8 ft above the chute to record a top-down view. Evaluated behaviors include bleating, head bobbing, head shaking, jumping, lip smacking, lip licking, and attempts to turn around in the chute (Table 4.1).

Experiment 2 - Pilot

The purpose of this pilot experiment was to 1) determine if 20- and 30-mM PTC-solutions topically applied to feed pellets were appropriate to elicit an aversive reaction in rams and 2) confirm the optimal amount of time to capture intake and behavioral measures indicative of bitter taste aversion. In September 2022, six lambs of Rambouillet (n = 3; 48.3 ± 2.3 kg) and Targhee (n = 3; 50.7 ± 6.2 kg) breeds were selected from Experiment 1. The lambs were group housed outdoors in a feedlot style pen and provided *ad libitum* access to water. Each lamb was assigned a number 1 - 6 which was painted on their back for identification.

Pellet Preparation. Feed pellets (Sebs Feed and Supply, Terreton, ID, USA) were prepared on the same day for all treatments, one week prior to the experiment. For the control pellets, EtOH was heated to just below boiling point (78°C) and added to room temperature EtOH in a 1:7 ratio. For the

treated pellets, 1.5 g and 2.2 g of PTC were added to 50 g EtOH and heated until PTC was completely dissolved. Each EtOH-PTC mixture was then added to 350 g of room temperature EtOH to produce a LOW and HIGH concentration of 20- and 30-mM PTC-solutions, respectively. Pellets (2 kg per treatment) were spread out on separate 8 in x 6 ft layer tarps and treatment solutions were sprayed onto the pellets using a 18V battery operated chemical sprayer (Ryobi One +; Ryobi Limited, Andersen, SC, USA) at a continuous rate of 25 ml per kg of feed. Pellets were then air dried, mixed, spread out into a single 8 in x 6 ft layer, and resprayed at the same rate. Spray rate of the 20- and 30-mM PTC concentrations were designed to target applications of 180 and 280 mg/kg of PTC-treated feed. Pellets were allowed to air dry before being stored in sealed containers for later use.

Acclimation Phase. Lambs were acclimated to the new housing environment and daily routine for 2-d prior to receiving PTC-treated feed. Feed bunks were swept clean before and after each feeding. All feed was evenly spread along feed bunk to ensure equal access. Feed amounts were calculated so that total daily feed offered was equal to 4% BW for each lamb. At 0700 h, lambs were provided 3.0 kg of alfalfa hay. After 2 h, hay was removed and weighed back and 3.0 kg of control pellets were delivered at 1100 h for a duration of 30 min. Remaining pellets were then removed and weighed back, feed bunks were cleaned, and an additional 3.0 kg of control pellets was delivered. After 30 min, any remaining pellets were removed and weigh backs recorded. At 1500 h, lambs were provided with an additional 3.0 kg of alfalfa hay. After 3 h the hay was removed from the feed bunks and weighed back.

Test Phase. Immediately following the acclimation, a 2-d test phase was conducted. The feeding schedule and intake-measurement procedures described in the acclimation phase were maintained throughout the test phase. On d 1, at 1100 h, 3.0 kg of control pellets were delivered as outlined in the acclimation phase. After 30 min the control pellets were removed and weighed, the feed bunk was cleaned, and 3.0 kg of the LOW PTC-treated pellets were delivered. After 30 minutes, any remaining pellets were removed and weighed back. On d 2, this procedure was repeated using the HIGH concetration PTC-treated pellets.

Video surveillance. Two cameras (LX1081-88, Lorex Technology, Inc., Markham, ON, Canada) set to record 30 frames-per-second in 1080 p were used to monitor feeding duration and behavioral reactions from 1100 - 1200 h on all four days of the trial. One camera was mounted to a wooden board for stability at end of the feed bunk and angled so that all lambs and feed pellets were visible within the frame. A second camera was mounted to a post on the opposite end approximately 2 ft above the feed bunk to capture a top-down view. Reported behaviors for each lamb included duration of feeding, number of feeding events, positional changes around the feed bunk, head bobbing, head shaking, lip licking, and sniffing (Table 4.1).

Experiment 3

The purpose of this experiment was to quantify intake and behavioral responses associated with bitter-taste perception in individual lambs fed alfalfa pellets topically treated with either a 20- or 30-mM PTC-solution. Pellet concentrations and duration of feed offerings were based on the results of Experiment 2. All pellets were prepared one week prior to the start of the first trial as described in Experiment 2 (see section *2.3.1*).

At the conclusion of the pilot study (Experiment 2), the remaining ram lambs from Experiment 1 (n = 56) that were not used in Experiment 2 were blocked by breed and randomly divided into two groups to be tested sequentially for bitter sensitivity. Each group contained an even number of Polypay (n = 10), Rambouillet (n = 10), and Targhee (n = 8) lambs. Average BW was 51.0 ± 4.3 kg for Group 1 and 54.1 ± 4.6 kg for Group 2. Within each group, lambs were blocked by breed and arbitrarily assigned to one of two indoor testing facilities. Within a testing facility, lambs were randomly assigned to individual pens, however, pens alternated by breed. Testing facilities were maintained at near constant temperature at or less than outdoor ambient temperature, using evaporative coolers as needed, with ventilators set on a 10% hourly cycle. Testing facility lights were turned on at 0700 h and off at 1900 h.

Beginning September of 2022, Group 1 entered the testing facilities and underwent a 5-d acclimation and 4-d test phase. Upon completion of the trial, Group 1 lambs were removed, and testing facilities were cleaned. The following day, Group 2 entered the testing facilities and began the same 5-d acclimation and 4-d test phase which concluded in October 2022. During the acclimation phase, one Polypay lamb in Group 2 was removed from the trial due to change in health status.

Acclimation phase. A 5-d acclimation phase was conducted to familiarize lambs to the housing environment, as well as the feeding and watering routine. At 0730 h, any remaining water was removed, weighed back, and replaced with fresh water for the morning. At 0800 h rams were provided alfalfa hay in an amount equivalent to 1% of their body weight. After 3 h, the hay was removed, and weigh backs were recorded. Throughout the trial, all feed was delivered in identical black 6.5-gal rubber feed buckets that were specific to each ram. At 1215 h, lambs were first offered a single bucket containing control pellets (1% BW). Based on the findings of experiment 2, the optimum amount of time needed to measure bitter taste aversion was determined to be 15 min (see *section 3.2*), at which time the buckets were removed and immediately replaced with a second offering of control pellets (1% BW) for an additional 15 min. Feed buckets containing the second offering of control feed were then removed, and all weigh backs were recorded. At 1330 h, morning waters were removed, weighed back, and replaced with fresh nightly water. Additional alfalfa hay

(1% BW) was delivered at 1400 h so that total feed offered was equivalent to 4% BW. After 3 h, hay was removed, and weigh backs were recorded.

Test phase. Immediately following the acclimation phase, a 4-d test phase was conducted to identify signs of PTC sensitivity in lambs. The feed and fluid delivery schedule outlined in the acclimation phase remained consistent throughout the test phase. At 1215 h on d 1 and d 2 of the test phase, lambs were first offered a single bucket containing pellets treated with LOW PTC. After 15 min, the PTC was removed and immediately replaced with control pellets for a duration of 15 min. Feed buckets containing the control feed were then removed, and all weigh backs were recorded. These procedures were repeated on d 3 and d 4 of the test phase with an offering of HIGH PTC-treated pellets.

Video surveillance. Video surveillance was conducted on the last day of the acclimation phase and on all four test days. Cameras (LX1081-88, Lorex Technology, Inc., Markham, ON, Canada; 30 frames-per-second; 1080 p) were set to record from 1200 h to 1300 h. Within each testing facility, cameras were mounted at equal height and distance from the pens to capture the behavioral reactions of two rams per camera. Evaluated behaviors include head bobbing, head butting, head shaking, lip licking, lip smacking, biting, pawing, sniffing, drinking, feeding events, feeding duration, and attempts to reach neighboring feed through the fence-line (Table 4.1).

 Statistical Analyses

All means are expressed as least squares mean ± standard error, unless otherwise stated. Mean comparisons were made using pair-wise contrasts (Statistical Analysis System, SAS Institute Inc., Cary, NC). Significance was set at $P \leq 0.05$.

Behavioral Response. Video footage was used to evaluate how often reactive behaviors were exhibited in response to PTC using the PROC GLIMMIX procedure of SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC). Data from Experiment 1 was analyzed for the fixed effects of treatment (water or 2.5 mM PTC-solution) and included a random statement of ram. The initial model also included group, as well as the interaction between group and treatment. These effects were ultimately excluded from the model due to lack of significance ($P > 0.05$). Behaviors are reported as the total number of occurrences within the 30 sec retainment period.

Behavioral data from Experiment 2 was analyzed for the fixed effects of PTC concentration (0, LOW or HIGH) and included a random statement of ram. Day, as well as the interaction between day and concentration, were non-significant ($P > 0.05$) and excluded from the final model. Reactive behaviors, as well as duration of a single feeding event were weighted by the total number of feeding events per ram within each 30 min offering. All reactive behaviors are reported as the as the number of occurrences within the treatment period.

For Experiment 3, data was analyzed for the fixed effects of breed, testing facility, group, replication, treatment (PTC vs. control), PTC concentration, as well as the interaction between treatment and concentration. The model also included a random statement of ram nested within replication. All other interactions as well as the fixed effect of day were excluded from the final model due to lack of significance ($P > 0.05$). All reactive behaviors are reported as a frequency of occurrence (number of observations for each specific behavior divided by the total number of feeding events per ram).

Feed and fluid intake. Due to the small sample size and limitations presented by group housing in Experiment 2, feed intake was analyzed using the PROC MEANS statement and is reported as an observational mean ± SD. For Experiment 3, statistical analysis was conducted using the PROC GLIMMIX procedure of SAS. The feed intake variables evaluated in this experiment include morning, afternoon, and daily hay intake; intake of first and second pellets as a percentage of pellets offered, and total daily feed intake (hay + pellets). Using body weight as a covariate, the fixed effects of concentration (control, LOW, HIGH), group, testing facility, and breed were evaluated for all intake response variables. Initial evaluations included the fixed effects of day and replication, as well as day × replication but were excluded from the final model because they were determined to be not significant ($P > 0.05$). The model also included a random statement of ram nested within replication. Morning, afternoon, and daily water intake were evaluated using this same statistical model. The PROC CORR procedure was conducted to analyze the relationship between PTC pellet intake and subsequent consumption of control pellets.

Classification. For Experiment 3, rams were classified based on the total number of reactive behaviors observed at both PTC concentrations. The average number of behaviors observed were then used to create three reactivity classifications. Classes were determined by ± 1.0 standard deviation (SD) of the population mean (4.1 ± 1.9) and rounded to the nearest whole number to sort rams into groups of high (≥ 1.0 SD; 7 - 10 behaviors), moderate (< 1.0 to > -1.0 SD; 4 - 6 behaviors), or low (≤ 1.0, 0 - 3 behaviors) reactivity to PTC (Fig. 4). The fixed effects of PTC-taster classification, treatment, and classification × treatment were evaluated for all behavioral response and feed intake variables. These models also included a random statement of ram nested within replication.

Results

Experiment 1 – PTC Drench

Bitter sensitivity could not be determined by measuring the behavioral response of lambs to an oral drench of 2.5 mM PTC-solution. None of the observed behaviors, including those previously linked to PTC-taster status (Southerland et al., 2022), were associated with PTC (*P ≥ 0.20; Table*

4.2). Rather, all lambs exhibited some form of reactive behavior to the involuntary drench regardless of treatment (PTC, 4.8 ± 0.3; water, 4.9 ± 0.3; *P* = 0.75). Although it was not significant, of these behaviors, lip licking and attempting to turn around in the chute were exhibited the most frequently (*P ≥ 0.20)* after receiving PTC-solution, while lip smacking and head shaking were exhibited the least (*P ≥ 0.72; Table 4.2).*

Experiment 2 – Pilot

During the pilot test for topical applications of PTC treated feed, morning hay intake was 2.5 kg during the acclimation phase, followed by 2.7 and 2.9 kg on days when ram lambs were offered 20- and 30-mM PTC pellets, respectively. During the acclimation mean total (first + second offering) pellet intake was 0.5 kg. For future studies on group housed lambs, additional acclimation days will be necessary to establish a baseline for pellet intake. On the first day of the test phase, lambs consumed 1.5 kg of the LOW PTC-treated feed followed by 1.3 kg of control. Intake of the HIGH PTC-treated pellets on d 2 was 2.9 kg, followed by 0.8 kg of control pellets. Afternoon hay intake remained consistent throughout the trial (2.8 kg). Although as a group these results do not reflect PTC aversion, individual sheep are known to exhibit variable degrees of bitter sensitivity (Henslee et al., 2019). During this pilot, individual analysis of PTC intake was not possible due to limitations associated with group housing.

The total amount of time lambs spent eating control pellets was 396.1 ± 53.8 sec and increased (*P ≤ 0.011) to 713.9 ± 70.5 sec and 981.4 ± 70.6 sec when offered LOW and HIGH PTC pellets, respectively which was less than the 30 min (1,800 sec) allotted feed consumption time. No difference (*P* = 0.32) was observed for the average duration of a single feeding event which was 248 ± 41.8 sec for the control, 167.8 ± 38.2 sec for the LOW, and 177.9 ± 31.9 sec for the HIGH PTC pellets. However, rams exhibited a more continuous feeding pattern when offered control pellets. This was indicated by the number of head lifts which subsequently resulted in differences between the number of feeding events at each concentration. The number of feeding events was 7.1 ± 0.9 and 4.9 ± 0.9 when offered HIGH and LOW PTC pellets, respectively (*P* = 0.13), and decreased (*P ≤ 0.028) to 2.0 ± 0.7 when control pellets were offered at the next feeding event. The number of positional changes or moves around the feed bunk was also lower (*P ≤ 0.029) when lambs were offered control (0.4 ± 0.6) compared to the LOW (2.6 ± 0.6) or HIGH (4.0 ± 0.5) PTC pellets, which were not different (*P* = 0.10) from each other.

While the number of times lambs sniffed the LOW PTC (0.66 ± 0.8) and control pellets (1.6 ± 0.8) were not different (*P* = 0.33), lambs appeared to investigate their feed more closely when offered the HIGH PTC concentration (5.0 ± 0.70) by sniffing their pellets more frequently (*P ≤ 0.002). Although it was not statistically different (*P* = 0.11), the number of head bobs was numerically

greater after lambs consumed both the LOW (5.4 ± 2.8) and HIGH (4.9 ± 2.7) PTC pellets than after the control (1.0 ± 2.9). The number of head shakes, and lip licks were not influenced by concentration ($P \geq 0.08$, Table 4.3). Despite the small sample size (n = 6) of lambs utilized in this experiment, the increased demonstration of aversive behaviors with respect to increasing PTC concentration suggests that topical application of 20 mM (LOW) and 30 mM (HIGH) PTC-solutions may be useful for the identification of bitter tasting ability in lambs during Experiment 3. Moreover, to avoid capturing any behaviors unrelated to bitter tasting ability, the duration of pellet offerings for Experiment 3 was reduced 15 min.

Experiment 3

Intake. Ram lambs did not demonstrate an aversive feed intake response to PTC. During the acclimation, intake of the first offering of pellets (control) was 90.6 ± 1.8 %. This remained consistent ($P = 0.18$) throughout the trial, with lambs consuming 90.8 ± 1.4 % of LOW PTC and 92.9 ± 1.4 % of HIGH PTC-treated pellets. However, it was noted that when lambs were offered HIGH PTC, intake of the subsequent offering of control pellets (64.9 ± 2.4 %) was greater ($P \leq 0.001$) than when offered LOW PTC (57.6 ± 2.4 %) and control pellets during the acclimation phase (53.6 ± 3.1 %; $P = 0.25$). Intake of PTC-treated pellets was directly correlated ($P <0.001$, r = 0.45) to intake of the subsequent offering of control pellets. As a result, total pellet intake was greatest ($P <0.001$) when lambs were offered the HIGH (79.0 ± 1.4 %) as opposed to the LOW (74.2 ± 1.4 %) PTC concentration and control (72.0 ± 1.9 %), which were not different from each other ($P = 0.32$).

Morning hay intake was 94.2 ± 1.4 % during the acclimation phase and 93.0 ± 1.1 % for the HIGH PTC treatment ($P = 0.48$). But on days that lambs were offered the LOW concentration of PTC pellets, morning hay intake was lower ($P \leq 0.022$; 89.9 ± 1.1 %). A similar pattern was observed for the afternoon hay, in which intake was lower ($P \leq 0.033$) on the days that rams were offered LOW (90.0 ± 1.1 %) compared to the HIGH (93.2 ± 1.1 %) concentration of PTC and control (93.9 ± 1.5 %) which were not different from each other ($P = 0.70$). As a result, daily feed intake (hay + pellets) was the greatest ($P \leq 0.050$) when lambs were offered HIGH (86.1 ± 0.9 %), followed by control (83.0 ± 1.2 %) and the LOW concentration of PTC (82.0 ± 0.9 %).

During the acclimation phase, morning water intake was the highest (57.8 ± 1.6 %; $P \leq 0.06$) but remained similar ($P = 0.11$) throughout the testing phase (LOW: 52.7 ± 1.2 %; HIGH: 54.8 ± 1.2 %). As such, water intake on control only days was greater ($P = 0.008$) than when rams received HIGH PTC and numerically different ($P = 0.06$) from when rams received the LOW concentration of PTC pellets. However, in the afternoons, after the lambs had received their daily pellet rations, water intake increased ($P \leq 0.001$) from 55.2 ± 1.7 % during the acclimation phase to 61.5 ± 1.3 % (LOW) and 67.7 ± 1.3 % (HIGH) during the testing phase. This influenced overall daily water intake which

was greatest ($P \leq 0.001$) when lambs were offered HIGH PTC pellets (61.1 ± 0.8 %), compared to the LOW (57.0 ± 0.8 %), and control (56.3 ± 1.1 %) days ($P = 0.16$, Figure 4.1).

A group effect was observed in which lambs from Group 1 consumed more pellets during both the first (95.3 ± 1.9 %, $P = 0.005$) and second (87.6 ± 1.6 %, $P \leq 0.001$) offerings, than Group 2 who respectively consumed 87.6 ± 1.6 % and 47.0 ± 2.8 % of their pellet rations. Total pellet intake for Group 1 was 15 % greater ($P \leq 0.001$) than Group 2, resulting in a 9 % higher ($P \leq 0.001$) daily feed intake. This was accompanied by higher levels of afternoon water intake ($P = 0.030$) for Group 1 (64.1 ± 1.7 %) than Group 2 (58.8 ± 1.4%). Although, morning water intake was similar ($P = 0.11$) for both Group 1 (57.0 ± 1.7 %) and Group 2 (57.3 ± 1.4 %), the difference in afternoon water consumption impacted daily water intake, which was 5 % greater ($P = 0.003$) for Group 1 than Group 2.

A testing facility effect also occurred in which lambs housed in Building 21 consumed more ($P = \leq 0.001$) of the subsequent offering of control pellets (69.2 ± 2.4 %) than rams in Building 93 (48.2 ± 3.3 %). Overall, the lambs in Building 21 consumed 81.3 ± 1.4 % of their total pellet ration, which was greater ($P < 0.001$) than those in Building 93 (68.9 ± 2.0 %). As a result, daily feed intake in Building 21 was 7 % higher ($P < 0.001$) than Building 93. Likewise, afternoon water intake (66.2 ± 1.2%) in Building 21 was greater ($P < 0.001$,) than in Building 93 (56.7 ± 1.7 %), resulting in a daily water intake that was 4 % higher ($P = 0.004$) in Building 21.

Throughout the experiment, Targhee lambs consumed (69.6 ± 3.7 %) more ($P \leq 0.006$) during the subsequent offering of control pellets than the Polypay (55.1 ± 3.5 %) or Rambouillet (51.3 ± 3.4 %) lambs, which were not different ($P = 0.46$) from each other. Total pellet intake for Targhee lambs was 81.5 ± 2.2 % which was greater ($P \leq 0.006$) than both the Polypay (72.9 ± 2.1 %) and Rambouillet (70.9 ± 2.0 %) lambs. As a result, daily feed intake was greater ($P \leq 0.004$) for Targhee lambs (86.7 ± 1.4 %) than either of the other breeds (Polypay: 82.7 ± 1.3 %; Rambouillet: 81.7 ± 1.2 %). No differences ($P \geq 0.53$) in total pellet or daily feed intake were observed between the Rambouillet and Polypay lambs. Additionally, Targhee lambs drank more water ($P = 0.004$, 58.8 ± 1.9 %) in the mornings than Rambouillet (51.4% ± 1.7 %) lambs, with Polypay (55.1 ± 1.9 %) lambs falling intermediate to the two ($P \geq 0.16$). Similarly, afternoon water intake was greater for Targhee (66.1 ± 1.8 %, $P \leq 0.022$) lambs than both the Polypay (59.9 ± 1.8 %) and Rambouillet (58.4 ± 1.8 %) lambs, which were not different from one another ($P = 0.57$). As a result, Targhee lambs drank more ($P \leq 0.005$) water throughout the day, while daily water intake for Polypay (58.4 ± 1.8 %) and Rambouillet (58.4 ± 1.8 %) lambs remained similar ($P = 0.16$).

Behavior. During the test phase, a treatment × concentration interaction occurred which influenced the amount of time that lambs spent eating either PTC or control pellets ($P = 0.010$). When

lambs were offered LOW PTC pellets the total duration of feeding was 586.3 ± 15.9 sec, which decreased ($P = 0.004$) to 532.0 ± 16.0 sec when offered the HIGH PTC concentration. However, when lambs received the subsequent offering of control pellets, the total time spent eating was similar ($P = 0.41$), regardless of PTC concentration offered earlier in the day (LOW: 489.2 ± 16.0 sec; HIGH: 505.11 ± 15.9 sec). Lambs took longer ($P < 0.001$) to eat the LOW concentration of PTC pellets than the control pellets that were offered immediately after, but the duration of feeding on HIGH PTC compared to the subsequent offering of control pellets was not different ($P = 0.15$; Figure 4.2). The duration of a single feeding event (time spent eating without lifting their head from the bucket) was shorter ($P = 0.005$) for PTC (37.2 ± 1.9 sec) than for the control (41.1 ± 2.0 sec), regardless of PTC concentration ($P = 0.51$). Additionally, a treatment effect was observed in which the number of feeding events were fewer ($P < 0.001$) when offered control (15.9 ± 0.8) versus PTC pellets (19.9 ± 0.8).

Ram lambs were more likely ($P \leq 0.001$) to exhibit a behavioral reaction when fed PTC (4.7 ± 0.1) compared to control pellets (3.4 ± 0.1). Regardless of concentration ($P = 0.93$), the average number of aversive reactions exhibited by lambs was four out of the ten recorded reactive behaviors. A concentration effect was observed in which lambs bobbed their head more frequently ($P = 0.008$) after consuming the LOW (20.9 ± 1.5 %) compared to the HIGH (17.6 ± 1.5 %) concentration of PTC treated pellets. Similarly, the rate of lip smacking was 12.1 ± 1.0 % and 9.1 ± 1.0 % for the LOW and HIGH PTC-treated pellets, respectively ($P = 0.003$). The frequency for head bobbing and lip smacking after consuming PTC was 17.7 ± 1.5 % and 9.6 ± 1.0 %, respectively. Yet, lambs exhibited a higher frequency ($P \leq 0.039$) for both head bobbing (20.8 ± 1.5 %) and lip smacking (11.7 ± 1.0 %) during the subsequent offering of control pellets. Lambs were also observed at the water buckets more frequently ($P < 0.001$) after consuming control pellets (7.2 % ± 0.5 %) versus PTC (3.2 ± 0.5 %). On days when LOW PTC was offered, the rate lambs drank from the water buckets was 6.1 ± 0.5 % compared to 4.3 ± 0.5 % on days when HIGH PTC was offered ($P = 0.011$). A testing facility effect was also observed in which lambs housed in Building 21 (6.9 ± 0.5 %) were observed drinking water more often ($P < 0.001$) than those housed in building 93 (3.5 ± 0.5 %).

Lambs demonstrated caution by sniffing the buckets containing PTC treated feed (13.8 ± 0.9 %) more frequently ($P < 0.001$) than the control pellets (8.1 ± 0.9 %). However, a replication effect was observed in which instances of sniffing were fewer ($P = 0.011$) on the second replication (8.9 ± 1.1 %) of each concentration of PTC compared to the first (12.9 ± 1.1 %). Head shaking was observed at a similar rate ($P = 0.82$) regardless of PTC concentration (LOW: 3.4 ± 0.4 %; HIGH: 3.3 ± 0.4 %). But the frequency of this behavior increased ($P < 0.001$) from 2.3 ± 0.4 % when offered control pellets to 4.4 ± 0.4 % when offered PTC treated pellets. Meanwhile, the rate of lip licking was not

affected by concentration (LOW: 2.0 ± 0.3 %; HIGH: 2.6 ± 0.3 %; $P = 0.17$) or treatment (PTC: 2.7 ± 0.3 %; control: 1.8 ± 0.3 %; $P = 0.05$).

Lambs appeared more agitated ($P < 0.001$) when offered PTC by exhibiting behaviors such as biting, head butting, and pawing at the PTC buckets. Bucket biting occurred more frequently (10.3 ± 1.1 %; $P < 0.001$) when presented with PTC compared to control pellets (4.6 ± 1.1 %). The rate of bucket biting increased ($P = 0.045$) from 6.5 ± 1.1 % when lambs were offered LOW PTC to 8.4 ± 1.1 % when offered HIGH PTC pellets. Although head butting was rarely exhibited when lambs were offered control pellets (0.4 ± 0.3 %), this behavior became more prevalent ($P < 0.001$) when presented with PTC treated pellets (2.5 ± 0.3 %). However, no PTC concentration effect was observed ($P = 0.66$) for the head butting behavior regardless of whether lambs had consumed the LOW (1.4 ± 0.3 %) or HIGH (1.6 ± 0.3 %) PTC pellets. Likewise, lambs pawed at their pellet buckets less ($P < 0.001$) when offered control (0.4 ± 0.3 %) versus PTC (2.8 ± 0.3 %) treated feed, but this behavior was exhibited at the same frequency ($P = 0.60$) for both the LOW (1.5 ± 0.3 %) and HIGH (1.7 ± 0.3 %) PTC pellets. In addition, lambs attempted to reach through the fence to access the feed buckets of neighboring lambs more frequently ($P < 0.001$) when offered PTC (5.9 ± 0.5 %) than when offered control pellets (1.3 ± 0.5 %). This behavior was observed approximately 3% of the time, regardless of PTC concentration provided during the first pellet offering ($P = 0.72$). Lambs housed in Building 93 attempted to reach through the fence more often ($P = 0.019$; 4.1 ± 0.6 %) than those housed in Building 21 (2.0 ± 0.6 %).

During this experiment, both group ($P \leq 0.003$) and breed ($P \leq 0.030$) effects were observed. In general, Group 1 (4.6 ± 0.1) displayed more ($P \leq 0.001$) behavioral reactions than Group 2 (3.5 ± 0.1), beginning with the total number of feeding events, which were 20.2 ± 1.0 and 15.6 ± 1.1 for Group 1 and 2, respectively ($P = 0.003$). Additionally, Group 1 relied more heavily on their sense of smell (13.3 ± 1.1 %,), sniffing the feed buckets more often ($P = 0.002$) than Group 2 (8.6 ± 1.1 %). Group 1 also exhibited more ($P \leq 0.014$) of the aggravated behaviors. Biting ($P = 0.002$), head butting ($P = 0.007$), and pawing ($P = 0.014$) at the pellet buckets were exhibited at a respective rate of 10.5 ± 1.4 %, 2.2 ± 0.3 %, and 2.2 ± 0.3% for Group 1 and 4.4 ± 1.4 %, 0.7 ± 0.3 %, and 1.0 ± 0.3%, for Group 2. The lambs in Group 1 were also observed trying to reach through the fence to access neighboring feed buckets twice as often (4.2 ± 0.6 %, $P = 0.010$) as the lambs tested in Group 2 (1.9 ± 0.6 %). Lip smacking was the only behavior to be reported more frequently ($P < 0.001$) for Group 2 than Group 1, which occurred at a rate of 14.1 ± 1.2 % and 7.2 ± 1.2 %, respectively.

Similar to the results of the intake analyses, Targhee lambs stood apart from the other two breeds with respect to some of the observed behavioral reactions. For instance, Targhee lambs exhibited fewer eating events ($P \leq 0.001$, 12.1 ± 1.4) than Polypay (22.5 ± 1.3) or Rambouillet (19.1 ± 1.2)

lambs, which were not different ($P = 0.06$) from one another. Additionally, the average duration of a single feeding event was longer ($P \leq 0.001$) for Targhee lambs (53.4 ± 3.5 sec) than the Rambouillet (35.7 ± 3.0 sec) or Polypay (28.3 ± 3.0 sec) lambs, which were only numerically different ($P = 0.08$) from one another. Lip licking was exhibited more often by Targhee ($P = 0.009$, 3.3 ± 0.5 %) than Rambouillet (1.5 ± 0.4 %) lambs, with Polypay (2.0 ± 0.4 %) falling intermediate to the two ($P \geq 0.07$). Rambouillet lambs pawed at their feed buckets the least ($P \leq 0.043$; 0.4 ± 0.4 %), followed by the Polypay (1.6 ± 0.4 %) and Targhee (2.9 ± 0.4 %) lambs. Interestingly, Polypay lambs were less likely (1.2 ± 0.7 %; $P \leq 0.31$) to try and feed from the neighboring pens than Rambouillet (4.5 ± 0.7 %) or Targhee lambs (3.4 ± 0.7 %) which were similar to each other ($P = 0.002$). Of the three breeds, Polypay lambs exhibited the greatest number ($P \leq 0.021$) of aversive reactions (4.4 ± 0.1), as opposed to Rambouillet (3.9 ± 0.1) or Targhee (3.8 ± 0.1) lambs, which were not different ($P = 0.56$).

Classification. Ram lambs were classified into reactivity categories of high (7 - 10 behaviors; n = 11), moderate (4 - 6 behaviors; n = 30), or low (0 - 3 behaviors; n = 14) behavioral reactivity to both PTC concentrations. Lambs that exhibited a wider range of aversive reactions (high: 97.5 ± 1.4 %; moderate: 95.9 ± 2.4 %; $P = 0.58$), consumed greater ($P \leq 0.001$) amounts of PTC pellets than those in the low reactivity group (82.2 ± 2.1 %; Fig 3). Highly and moderately reactive lambs also consumed the greatest ($P \leq 0.006$) amount of control pellets but, were only numerically different from one another ($P = 0.09$; Fig 3). Control pellet intake was 77.0 ± 5.1 %, 67.0 ± 3.0 % and 51.8 ± 4.5 % for the high, moderate, and low reactivity groups, respectively.

The total amount of time lambs spent consuming PTC pellets was similar (~560 sec; $P \geq 0.67$), regardless of classification by behavioral reactivity. However, during the subsequent offering of control pellets, lambs in the moderate and low reactivity groups decreased ($P \leq 0.016$) their feeding duration by 44 and 166 sec, respectively. Meanwhile, lambs in the high reactivity groups maintained a longer feeding period (573.4 ± 27.9 sec) that was not different ($P = 0.51$) than when offered PTC pellets. Total duration of control pellet consumption was shorter ($P \leq 0.001$) for low reactivity lambs (403.8 ± 24.9 sec) than for lambs in the high (573.4 ± 27.9 sec) and moderate (514.6 ± 16.8 sec) reactivity groups, which were only numerically ($P = 0.07$) different from each other. The average duration of a single feeding event was similar ($P = 0.78$; ~ 38 sec) for all behavioral reactivity groups, irrespective of being offered PTC or control pellets ($P = 0.13$). Additionally, the number of feeding events was less ($P \leq 0.001$; 13.8 ± 1.7) for lambs that expressed low behavioral reactivity to PTC, than the moderately (20.3 ± 1.1) or highly (18.5 ± 1.9) reactive lambs, which were not different from one another ($P = 0.41$).

Throughout the trial, morning hay intake was 94 % for lambs classified in the high and moderate classes and 90 % for the low class ($P = 0.05$). In the afternoon, post-consumption of alfalfa test

pellets, notable differences (P = 0.013) in hay intake were reported. Lambs in the highest reactivity class consumed 97.1 ± 1.7 % of their afternoon hay which was greater ($P \leq 0.028$) than both the moderate and low reactivity groups. Lambs classified as having a moderate reaction to PTC consumed 92.5 ± 1.0 % while the low reactivity group consumed 90.0 ± 1.6 % of their afternoon hay (P = 0.20). This resulted in a total daily feed intake of 78.6 ± 1.7 % for the low reactivity group, which was lower ($P \leq 0.001$) than both the high (91.3 ± 1.9 %) and moderate (88.1 ± 1.2 %) groups, which were similar (P = 0.17) to each another.

Classification by behavioral reactivity to PTC did not influence morning, afternoon, or daily water intake ($P \geq 0.58$), nor the number of trips to the water bucket (P = 0.28, data not shown*).* Additionally, the rate of lip licking was exhibited at a similar rate (~ 2 %) regardless of classification by behavioral reactivity (P = 0.71). However, each of the reactivity groups were different from one another with respect to how often they sniffed either the PTC-treated ($P \leq 0.010$) or control pellets ($P \leq 0.035$). Lambs that exhibited the least number of reactive behaviors (≤ 3) sniffed both the control and PTC pellets approximately 9 % of the time they approached the feed buckets (P = 0.39). However, lambs classified in both high and moderately reactive groups were more cautious of PTC-treated pellets, decreasing ($P \leq 0.040$) their rate of sniffing from 20.3 ± 2.0 % and 14.0 ± 1.2 % to 15.6 ± 2.0 % and 4.6 ± 1.2 %, respectively, when offered control pellets. In general, highly reactive lambs shook their head most frequently (5.2 ± 0.8 %), followed by the moderate (3.0 ± 0.5 %) and less (2.3 ± 0.7 %) reactive lambs ($P \leq 0.002$).

After consuming PTC-treated pellets, lambs exhibited head bobbing at a rate of 20.5 ± 3.3 %, 14.4 ± 2.0 %, and 21.4 ± 3.3 % for the high, moderate, and low reactivity groups, respectively ($P \geq$ *0.05)*. But when offered control pellets, the rate of head bobbing was similar ($P \geq 0.28$) for all three reactivity classes (high: 18.1 ± 3.3 %; moderate: 20.2 ± 2.0 %; low: 23.0 ± 3.0 %). Interestingly, when moderately reactive lambs were offered control pellets, the rate of head bobbing increased ($P <$ 0.001) by 6 % but did not change for either the high or low reactivity groups ($P \geq$ *0.45)*. When offered PTC treated pellets, lip smacking was observed at a rate of 12.7 ± 2.1 % and 7.0 ± 1.4 % and for the low and moderate reactivity groups, respectively, with the highly reactive lambs (11.7 ± 2.3 %) falling intermediate ($P \geq$ *0.08)*. The rate of lip smacking decreased (P = 0.010) by 6 % for lambs in the highest reactivity group but increased ($P < 0.001$) by 4 % for moderately reactive lambs when offered the control pellets. The presence of the lip smacking behavior for the lowest reactivity group was similar (P = 0.29), regardless of consuming PTC or control feed. During the subsequent offering of control pellets, lip smacking was observed less ($P \leq 0.033$) for highly (6.0 ± 2.3 %) reactive lambs than for the low (14.8 ± 2.1 %) or moderately (11.9 ± 1.4 %) reactive lambs, which were not different (P = 0.25) from each other.

When presented with PTC pellets, lambs were observed biting the feed buckets at a rate of 24.1 ± 2.3 %, 8.6 ± 1.4 % and 2.8 ± 2.0 % for the high, moderate or low reactivity groups, respectively ($P \leq$ 0.022). For highly reactive lambs, this value decreased ($P < 0.001$) to 12.8 ± 2.3 % when offered control pellets but, was still greater ($P \leq 0.001$) than either the moderate (2.9 ± 1.4 %) or low (1.7 ± 2.0 %) reactivity groups, which were not different ($P = 0.63$). Rams classified as moderately reactive also exhibited higher rates of bucket biting when presented with PTC-treated pellets as opposed to the control, but rams that exhibited a smaller range of behaviors (≤ 3) did not (2.8 ± 2.0 %; $P = 0.55$). The high (6.2 ± 0.7 %), moderate (2.1 ± 0.4 %) and low (0.4 ± 0.6 %) reactivity groups, expressed sequentially decreasing ($P \leq 0.035$) rates of head butting when buckets contained PTC treated pellets. However, head butting was rarely observed when lambs consumed control feed (high: 1.4 ± 0.7 %; moderate: 0.1 ± 0.4 %; low: 0.5 ± 0.6 %; $P \geq 0.13$). While the rate of head butting decreased ($P \leq 0.001$) for lambs classified as high and moderately reactive when offered control feed, these values were not different ($P = 0.63$) for lambs in the lower reactivity group.

Bucket pawing was rarely observed when lambs were offered control pellets (~ 0.3 %; $P \geq 0.90$). When low reactivity lambs were offered PTC-treated pellets, bucket pawing occurred at a similar rate (0.5 ± 0.6 %; $P = 0.83$) but, increased ($P \leq 0.001$) to 2.4 ± 0.4 % and 6.6 ± 0.6 %, respectively for moderately and highly reactive lambs. When offered PTC, the rate at which bucket pawing was observed was different ($P \leq 0.011$) for each behavioral reactivity class. Lambs classified in the lowest reactivity group rarely attempted to reach through the fence to the neighboring feed buckets, regardless of consuming control (0.5 ± 1.0 %) or PTC-treated pellets (1.6 ± 1.0 %; $P = 0.28$). When lambs were offered PTC treated pellets, the frequency in which lambs attempted to reach through the fence and access neighboring feed was different for each reactivity group ($P \leq 0.013$). Moderately reactive lambs were similar ($P = 0.74$) to the low reactivity lambs in that attempts to reach their head through the fence were minimal (0.9 ± 0.7 %) when offered control pellets; however, observance of this behavior increased ($P \leq 0.001$) to 4.8 ± 0.7 % when offered PTC. For the highest reactivity group, this fenceline behavior was observed 3.5 ± 1.1 % of the time when offered control pellets and increased ($P \leq 0.001$) to 9.2 ± 1.1 % of the time when offered PTC-treated feed.

Discussion

Flavor preferences and aversions can be quantified by measuring both fluid (Henslee et al., 2019; Nelson et al., 2003) and feed intakes (Villalba and Provenza, 1996; Wang and Provenza, 1996; Favreau et al., 2010), as well as interpreting any behavioral response exhibited post-taste (Travers and Norgren, 1986; Grill and Norgren, 1978; Rosenstein and Oster, 1988). When offered the choice between PTC-spiked and plain drinking water, mature rams demonstrated a range of aversive

behavioral reactions indicative of bitter sensitivity such as head bobbing, lip smacking, rhythmic mouth movements, and sniffing (Henslee et al., 2019; Southerland et al., 2022). Thus, we hypothesized that applying PTC-solution directly to the tongue would yield behaviors similar to those observed during voluntary fluid intake studies, allowing researchers to expedite the phenotyping process. However, the ability to discern bitter-taster status was not apparent when the testing methodology replaced a voluntary side-by-side preference test with an involuntary drench of PTC-solution. The high level of aversive behaviors exhibited by ram lambs during Experiment 1 regardless of treatment type (PTC vs. water) suggests that measurements of behavioral reactivity were confounded by the fluid delivery method. Future experiments evaluating behavioral response to flavored solutions may wish to reconsider methods involving minimal handling of animals and voluntary consumption of fluids.

Decreased consumption of treated feed is typically used to determine the presence of a feed aversion. However, during Experiment 3, ram lambs did not demonstrate an aversive feed intake response to topical applications of PTC-treated alfalfa pellets. However, other feed intake and behavioral modifications were observed which may correspond to bitter tasting ability. The range of behavioral reactions exhibited post-voluntary consumption of PTC-treated pellets may be indicative of bitter taster status, with highly reactive individuals representing super-tasters and less reactive individuals as non-tasters. The testing facility and breed effects observed in this study have not been noted in previous experiments involving fluid delivery of PTC that were conducted within the same facilities and using the same breeds of sheep (Henslee et al., 2019; Southerland et al., 2022). Slight variation within the testing facilities (i.e., pen orientation and size) may be responsible for some of these differences in behavioral reactivity. Experimental replications focused on PTC-treated feed and increased sample size may be necessary to further evaluate these effects.

Initial observations of aversive reactions (i.e., head bobbing and lip smacking) to PTC-spiked drinking water were made by Henslee et al. (2019); however, no behavioral data was recorded in that experiment. Subsequently, a list of possible indicators of bitter sensitivity in sheep was developed by Southerland et al. (2022). Of these, head bobbing, lip smacking, sniffing, and duration of intake were hypothesized to be associated with PTC-taster status (Southerland et al., 2022). During this study, head bobbing and lip smacking were two of the most predominantly observed behaviors. However, they could not be linked directly to PTC consumption or classification by behavioral reactivity. While head bobbing and lip smacking were identified to correspond with consumption of PTC-spiked drinking water and bitter sensitivity (Henslee et al., 2019; Southerland et al., 2022), existing literature has not determined a definitive relationship between these behaviors and the various PTC taster

classifications (Southerland et al., 2022). As such, further investigation is required to justify the use of the head bobbing and lip smacking behaviors as indicators of bitter sensitivity.

In one side-by-side preference study, mature rams reportedly utilized their olfactory sense to help differentiate PTC-solution from regular drinking water (Southerland et al., 2022). While olfaction is thought to play a more prominent role in appetite stimulation than dietary selection (Tribe, 1949), ruminants are known to utilize their sense of smell to investigate the palatability of a plant without first having to taste it (Ginane et al., 2011). Although in the current study, PTC-treated and control pellets were offered sequentially and not simultaneously, lambs that were classified as moderately and highly reactive demonstrated greater rates of bucket sniffing before making the decision to consume PTC treated feed compared to untreated control feed. Additionally, aversive behaviors such as head butting, biting, and pawing that have not been previously reported in PTC-sensitive rams were also observed. The occurrence of these aggravated behaviors was greatest when lambs were offered PTC-treated pellets and was directly related to increasing behavioral reactivity. Once PTC pellets were removed and control feed was administered, occurrence of these specific behaviors greatly diminished across all reactivity classes. Suggesting that the head butting, biting, and pawing behaviors may be useful in the identification of PTC taster status in sheep.

Although bitter aversion can be quantified by measuring voluntary fluid intake of PTC-spiked drinking water (Southerland et al., 2022), intake measurements of PTC-treated feed do not appear to be sufficient for classification of lambs by bitter taster status (i.e., super-, intermediate, and non-tasters). In the absence of post-ingestive consequences, Favreau et al. (2010) observed a similar reaction in 5-month-old ewe lambs, reporting an indifference to topical applications of quinine HCl to chopped grass hay unless paired with post-ingestive consequences induced by LiCl. While genetics play a primary role in determining the expression of bitter tasting ability (Davenport et al., 2021; Kim et al., 2003), behaviors learned through maternal (Mirza & Provenza, 1994; Glasser et al., 2009), peer (Lane et al., 1990; Provenza & Burritt, 1991; Thomas et al., 2015), and individual experience with post-ingestive consequence (Favreau et al., 2010; Launchbaugh et al., 2001; Provenza et al., 1994) greatly contribute to formation of dietary preferences and flavor aversions in ruminants. These results, along with that of Favreau et al. (2010), suggest that in the absence of negative post-ingestive feedback, that the demand for nutrients in growing lambs may take precedence over the sense of taste. Although aversion to PTC has been demonstrated in mature rams (≥ 2 years) (Southerland et al., 2022), the identification of this ability may be more complex in younger, less experienced sheep like those utilized in this study and by Favreau et al. (2010).

Bitter flavors are often associated with plant toxicity (Garcia-Bailo et al., 2009). When foraging, ruminants tend to select a variety of feedstuffs to counteract any negative post-ingestive consequences

caused from ingestion of secondary plant compounds (Launchbaugh et al., 2001). As such, the increase in control pellet intake and longer feeding durations that were demonstrated post-PTC consumption may have been an attempt to offset the potential post-ingestive effects associated with the consumption of bitter tasting feed. This is particularly evident in the highly reactive group of lambs, who demonstrated increases in afternoon hay intake as well.

Determination of post-ingestive consequences by ruminants is typically expressed via a reduction in feed intake; however, it has been suggested that the rate of feed intake may also be important (Launchbaugh et al., 2001). Although the amount and total duration of PTC consumption were similar for all reactivity groups, suspected non-tasters with low reactivity scores were more likely to feed uninterrupted, expressing less feeding events (head lifts out of the bucket) than moderately and highly reactive lambs. In order to determine post-ingestive effects, ruminants often exhibit caution when sampling novel feeds (Launchbaugh et al., 2001). As such, the caution exhibited by the more reactive lambs when consuming PTC-treated feed may be indicative of their bitter tasting ability.

Conclusion

During this study, direct measurements of PTC intake were not conclusive indictors of bitter aversion or PTC taster status. In order to observe this modified intake, flavor aversions may need be formed and learned via experience with post-ingestive consequence. As such, measurements of PTC-intake for the identification of bitter tasting ability may be more conclusive in studies utilizing mature sheep.

Other intake measurements, such as increased consumption of control feed post PTC-intake, paired with behavioral reactivity scores may be indicative of bitter taster status in lambs. The range of aversive behaviors exhibited by lambs post-taste appears to be a more appropriate predictor of PTC-tasting ability than measuring the frequency in which individual behaviors are observed. Increased reliance on the olfactory sense, as well as observance of head butting, bucket biting, and pawing behaviors were more prominent in lambs that demonstrated a wider range of aversive behavioral reactions and may aid in the identification of more bitter sensitive sheep. However, measurements of behavioral reactivity may depend on the method of PTC administration and the voluntary consumption of PTC. Before targeted grazing regimes can be implemented, further research is needed to determine the effects of bitter tasting ability on the willingness of sheep to consume bitter tasting feeds.

Acknowledgement

Tables and Figures

Table 4.1. Ethogram of behaviors observed by ram lambs and their corresponding descriptions when evaluated for response to a bitter-tasting compound when administered orally or on feed.

Behavior	Description
Bleating	Vocalizing[1]
Drinking	Head inside of water bucket, consuming fluid[3]
Event	Head hovering over or inside of feed bucket[2,3]
Feeding	Head inside of feed bucket, consuming feed[2,3]
Head bobbing	Repetitive vertical movement of head up and down[1,2,3]
Head butting	Targeted strike of the head directed at the feed bucket[3]
Head shaking	Rapid repetitive movement of head side to side[1,2,3]
Jumping	Pushing off the ground into the air with all four legs in the air[1]
Lip licking	Protrusion and passing of tongue over lips[1,2,3]
Lip smacking	Wide repetitive opening and closing of mouth[1,3]
Pawing	Targeted strike of the hoof directed at the feed bucket[3]
Sniffing	Head hovering over bucket with nose pointed toward feed[2,3]
Stomping	Lifting the leg and forcefully bringing the hoof to the ground[3]
Turning	Rotation of the head, neck, or full body towards the back of the chute[1]

[1]Experiment 1 – evaluation of behavioral response to an oral drench of either 2.5 mM PTC or water
[2]Experiment 2 – evaluation of feed intake and behavioral response to alfalfa pellets treated topically with either control (EtOH only), LOW (20 mM), or HIGH (30 mM) PTC in group housed lambs
[3]Experiment 3 – evaluation of feed intake and behavioral response to alfalfa pellets treated topically with either control, LOW, or HIGH PTC in individually penned lambs

Table 4.2. Least squares means (± the standard error) for the number of times a behavior was observed within the 30 second retainment period after lambs were orally drenched with either water or 2.5 mM PTC-solution (Experiment 1).

	Treatment Observations		
Behavior	**PTC**	**Water**	***P*-value**
Bleating	0.14 ± 0.07	0.22 ± 0.07	0.27
Head bobbing	0.20 ± 0.05	0.14 ± 0.05	0.37
Head shaking	0.11 ± 0.05	0.11 ± 0.05	1.00
Jumping	0.32 ± 0.14	0.30 ± 0.14	0.91
Lip smacking	0.09 ± 0.04	0.08 ± 0.04	0.74
Lip Licking	1.69 ± 0.14	1.56 ± 0.14	0.50
Turning	2.09 ± 0.21	2.33 ± 0.21	0.20

Table 4.3. Least squares means (± the standard error) for either the number of times a behavior was observed or the duration of the behavior within the 30 minute period that lambs were offered alfalfa pellets topically sprayed with either control (water-EtOH), LOW (20 mM) or HIGH (30 mM) PTC-solution (Experiment 2).

| Behavior | Treatments | | | |
	Control	LOW	HIGH	*P*-value
Feeding events, counts	2.0 ± 0.7^a	4.9 ± 0.9^b	7.1 ± 0.9^b	0.002
Feeding duration, total, s	396.1 ± 53.8^a	713.9 ± 70.5^b	981.4 ± 70.6^c	< 0.001
Feeding duration, single event, s	248.8 ± 41.8	167.8 ± 38.2	177.9 ± 31.9	0.325
Moves, counts	0.4 ± 0.6^a	2.6 ± 0.6^b	4.0 ± 0.5^b	0.002
Sniffing, counts	1.6 ± 0.8^a	0.6 ± 0.8^a	5.0 ± 0.7^b	< 0.001
Head bobbing, counts	1.0 ± 2.9	5.4 ± 2.8	4.9 ± 2.7	0.113
Head shaking, counts,	0.0 ± 0.3	0.2 ± 0.3	0.7 ± 0.3	0.088
Lip licking, counts	0.4 ± 0.2	0.0 ± 0.1	0.1 ± 0.1	0.375

[a,b]Means across pellet concentrations with different superscripts are different ($P \leq 0.029$)
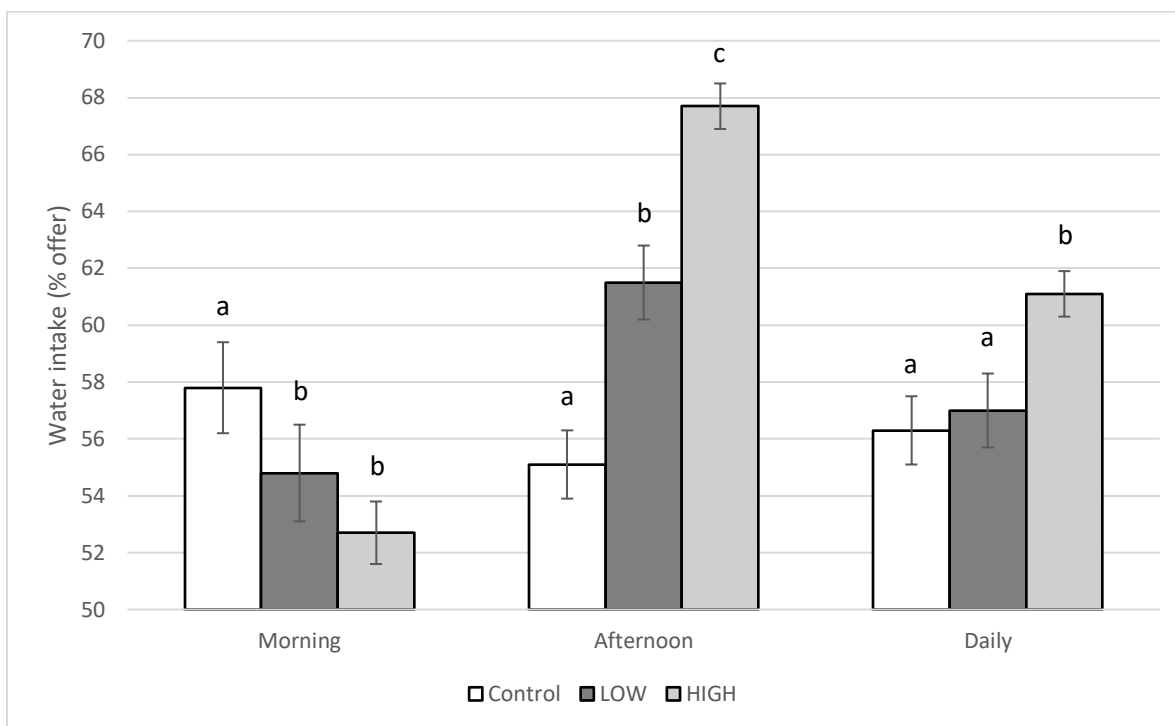
Figure 4.1. Least squares means (± the standard error) for morning, afternoon and daily water intake as a percentage of water offered on days where lambs were presented with either control, LOW (20 mM), or HIGH (30 mM) PTC pellets (Experiment 3).
[a,b]Means within the morning, afternoon, and daily time periods with different superscripts are different ($P \leq 0.009$)
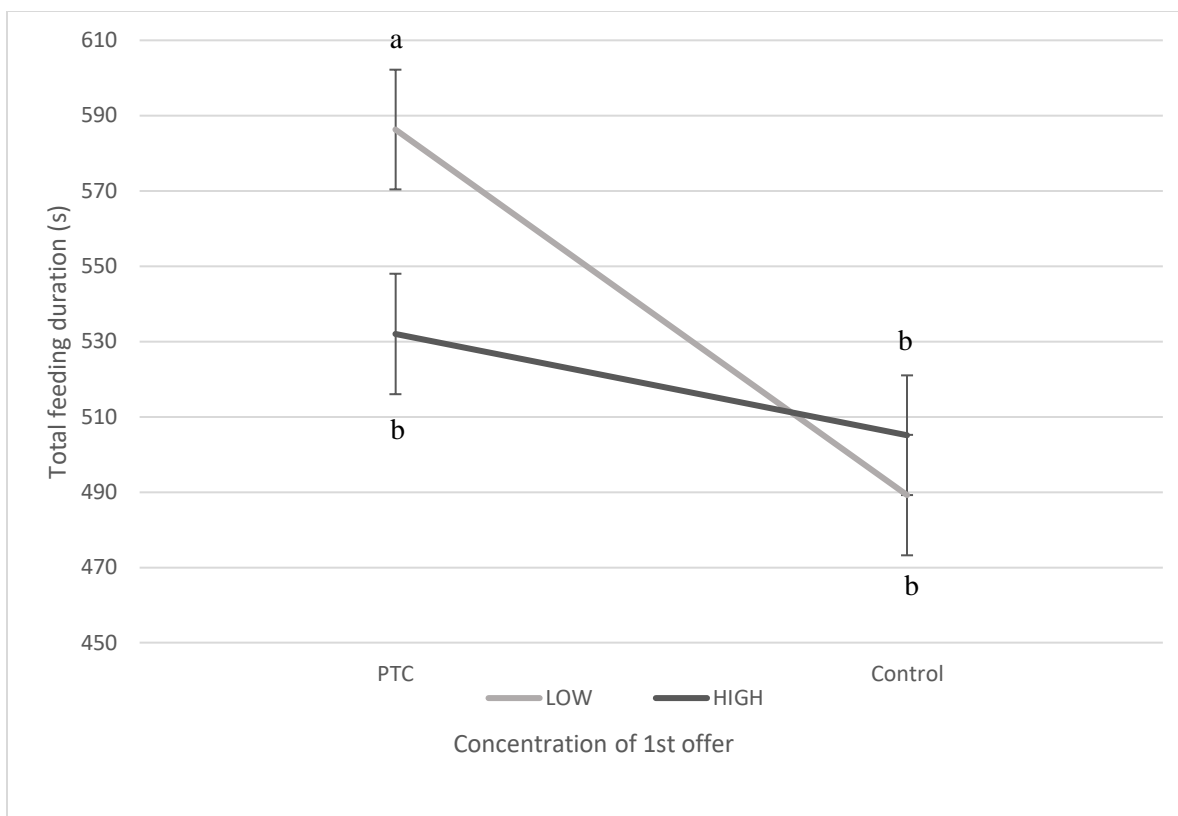
Figure 4.2. Least squares means (± the standard error) total feeding duration (sec) for the PTC or control pellet offerings on days where lambs were presented with either LOW (20 mM) or HIGH (30 mM) PTC pellets (Experiment 3).
[a,b]Means with different superscripts are different ($P \leq 0.030$)

Figure 4.3. Least squares means of PTC and control pellet intake as a percentage of pellets offered for ram lambs classified into high (n = 11), moderate (n = 30), and low (n = 14) behavioral reactivity classes. Classes were determined by ± 1.0 standard deviation (SD) of the population mean (4.1 ± 1.9) and rounded to the nearest whole number to sort rams into groups of high (≥ 1.0 SD; 7-10 behaviors), moderate (< 1.0 to > -1.0 SD; 4-6 behaviors), or low (≤ - 1.0 SD, 0-3 behaviors) behavioral reactivity to PTC (Experiment 3).

Chapter 5: Conclusion

Properly managed grazing can improve soil composition, promote nutrient cycling, support healthy fire regimes, and generate habitat for native plant and animal species (Svejcar et al., 2014; Bailey et al., 2019; Marchetto et al., 2021). The targeted control of overgrown shrubs such as sagebrush is necessary to maintain populations of ecologically important species like sage grouse (Petersen et al., 2014; Bailey et al., 2019). However, these resilient woody plants often contain secondary plant compounds (Cedarleaf et al., 1983; Johnson et al., 1985; Utsumi et al., 2009) that ruminants may associate with negative post-ingestive feedback (Burritt & Provenza, 1996; Frederick D. Provenza, 1996).

The implementation of breeding programs based on bitter sensitivity has been suggested as a tool to assist with seasonal grazing management of less palatable rangeland shrubs. In order to provide more insight on genetic determinants of bitter tasting ability, the development of efficient methods for the accurate and precise identification of phenotypic expression of bitter tasting ability in sheep are needed. However, the formation of flavor preferences and aversions can be influenced by many factors, including genetics, age, health, post-ingestive feedback, and learned behaviors (Tepper, 2008) which may accentuate the difficulty of determining bitter tasting phenotypes in sheep. Taste perception relies heavily on fluid transport of bitter solutes to the taste receptor (Matsuo, 2000) which we suspect plays a role in the successful use of PTC (0.20 mM and 2.03 mM) delivered in drinking water rather than internal or topical applications (180 mg/kg and 280 mg/kg) of PTC to feed to classify rams into three distinct bitter taster groups (Henslee et al., 2019; Southerland et al. 2022). Behaviors such as duration of consumption, head bobbing, lip smacking, rhythmic mouth movements, sniffing, pawing, biting, and headbutting were all found to be associated with perception of phenylthiocarbamide (PTC). However, further analysis suggests that the range of behaviors exhibited post-taste may be more useful in identifying bitter aversion as opposed to measuring the occurrence of specific reactive behaviors. While behavioral data may be used to help identify PTC-tasting ability, measurements of behavioral reactivity may depend on the method of PTC administration and the voluntary consumption of PTC, as opposed to an involuntary oral drench.

Our results indicate that studies which combine the use of voluntary fluid intake measurements with behavioral analysis may be the most appropriate methods for identifying bitter tasting phenotypes in sheep. The long-term goal of this line of work is the development of lineages of sheep for the control of shrubs suspected to have strong bitter profiles. Lower concentrations (0.20 mM) of PTC-spiked drinking water are capable of being used to single out sheep with super-tasting ability, while higher concentrations (2.03 mM) serve to identify bitter tolerant individuals or non-tasters. To

further enhance the testing process, research strictly focused on the identification on non-bitter tasters could eliminate the use of lower PTC concentrations. This select group of sheep could then be used to evaluate the effects of bitter tolerance on the dietary selection of rangeland plants that contain bitter compounds. Although additional work is needed, the methodologies outlined in this study will facilitate further research in understanding forage selection and consumption in sheep.

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