Università degli Studi di Cagliari

DOTTORATO DI RICERCA

Scienze Morfologiche e Funzionali

Ciclo XXVI

Sensitivity to chemical stimuli plays a fundamental role in the food preferences. Examples in the evolutionary scale:

1. Role of the walking leg chemoreceptors in the red swamp crayfish *Procambarus Clarkii*
2. PROP bitter taste sensitivity and its nutritional implications in Humans.

Settore scientifico disciplinare di afferenza: BIO/09

Presentata da: **Dott.ssa Melania Melis**
Coordinatore Dottorato Prof.ssa Valeria Sogos
Tutor/Relatore Dott. Paolo Solari

Esame finale anno accademico 2012 - 2013

Melania Melis gratefully acknowledges Sardinia Regional Government for the financial support of her PhD scholarship (P.O.R. Sardegna F.S.E. Operational Programme of the Autonomous Region of Sardinia, European Social Fund 2007-2013 - Axis IV Human Resources, Objective l.3, Line of Activity l.3.1.)

This work was partly supported by the "Fondazione Banco di Sardegna".

Abstract

In this thesis, we studied two examples of the sensitivity to chemical stimuli and its role in the food preferences in two models of the evolutionary scale.

The red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Crustacea: Decapoda) is an invasive species of freshwater habitats that has spread worldwide. In crayfish, like in other decapod crustaceans, reception of chemical cues occurs by way of peripheral chemoreceptors grouped within sensory hairs and typically located on the cuticle of cephalothoracic appendages. Antennules and pereopods (walking legs), in particular, have been reported to be olfactory organs involved in a number of behavioral responses, such as, sex recognition and localization of food sources in the environment. By way of extracellular nerve recordings coupled with behavioral bioassays, we investigated the sensitivity spectra of the walking leg chemoreceptors in the crayfish *P. clarkii* in response to different compounds of feeding significance and related to its omnivorous habits. Our results confirmed a marked sensitivity of the legs to trehalose, cellobiose, sucrose, maltose, glycine and leucine. Some sensitivity to glucose, fructose, asparagine (all food indicators) and taurocholic acid was also found, the sugar-sensitive chemoreceptor units resulting as broadly tuned to the carbohydrates. Responses were highly phasic to trehalose (hemolymph sugar in the body fluid of many invertebrates), phasic to glycine and leucine and phasic-tonic to the other compounds. This suggests that chemoreceptor phasicity is an additional property for better discrimination of the protein components in the diet from other stimuli. The behavioral bioassays excluded, at least under confined experimental conditions, any involvement of antennules in the detection of food-related compounds, thus emphasizing the role of the crayfish legs as the main short-distance, broad-spectrum sensors for feeding. Such information may be valuable for the identification of key chemicals aimed at the future development of strategies for crayfish population control programs.

Taste sensitivity varies greatly in humans, influencing eating behavior and therefore may play a role in body composition. PROP bitter taste sensitivity is the most studied example of the individual variability of taste sensitivity. Some studies show that PROP bitter taste sensitivity may be correlated with sensitivity to other oral stimuli, food preferences and BMI, while other studies did not confirm this association. It is known that PROP phenotype is associated with variant in bitter taste receptors TAS2R38 and with density of fungiform papillae on tongue surface. Although most of PROP phenotypic variations are explained by the allelic diversity of the bitter receptor TAS2R38, they cannot explain the PROP taster status-related differences above all that in the perception to different oral stimuli. The aim of this study was identify and

characterize other factors that may contribute to differences in the genetic predisposition to taste PROP and identify confounding variables which may explain the controversial data in the literature about the relationship between PROP taste sensitivity and BMI. 1) We investigated the possible relationship between PROP bitter taste responsiveness and salivary proteins by using HPLC-ESI-MS on saliva sample before and after PROP taste stimulation. 2) We evaluated the role of proteins and free amino acids in modulating bitter taste responsiveness. Subjects rated PROP bitterness after supplementation of two salivary proteins (Ps-1 and II-2), and the free form of constituent amino acids of the two proteins sequences (L-Arg and L-Lys) whose interaction with PROP was demonstrated by ^{1}H-NMR spectroscopy. 3) We investigate the role of polymorphism rs2274333 (A/G) in the gene that codify for the salivary trofic factor gustin protein, in PROP sensitivity and fungiform papilla density and morphology and *in vitro* we investigate the effect of this gustin gene polymorphism on cell proliferation and metabolic activity, following treatment with saliva of individuals with and without the gustin gene mutation, and with isolated protein, in the two iso-forms. 4) We investigated whether the endocannabinoid system, which modulates hunger/satiety and energy balance, plays a role in modulating eating behaviour influenced by a sensitivity to PROP which could explain the controversial data in literature. In particular we determined the plasma profile of the endocannabinoids 2-arachidonoylglycerol (2-AG), anandamide (AEA) and congeners in normal-weight PROP super-tasters and non-tasters, also we assessed the cognitive eating behavior disorder by the Three-Factor Eating Questionnaire.

The results showed that: 1) Basal levels of II-2 and Ps-1 proteins, belonging to the basic proline-rich protein (bPRPs) family, were significantly higher in PROP super-taster than in non-taster unstimulated saliva, and PROP stimulation elicited a rapid increase in the levels of these same proteins only in PROP super-taster saliva. 2) Supplementation of Ps-1 protein in individuals lacking it in saliva enhanced their PROP bitter responsiveness. ^{1}H-NMR results showed that the interaction between PROP and L-Arg is stonger than that involving L-Lys, and taste experiments confirmed that oral supplementation with L-Arg increase more PROP bitterness intensity than L-Lys. 3) Gustin and *TAS2R38* genotypes were associated with PROP threshold, while bitterness intensity was mostly determined by *TAS2R38* genotypes. Fungiform papillae densities were associated with both genotypes (with a stronger effect for gustin), but papilla morphology was a function of gustin alone. I*n vitro* experiment, the treatment of isolated cells with saliva from individuals with AA form, and direct application of the active iso-form of gustin protein, increased cell proliferation and metabolic activity. 4) The disinhibition score of non-taster was higher than those of super-tasters. In addition, we found that the concentration

of endocannabinoid AEA (anandamide) and 2-AG (2-arachidonoylglycerol) was lower in the plasma of non taster compared with super-tasters subjects.

In conclusion, among the factors contributing to individual differences of PROP sensitivity, in addition to the *TAS2R38* variants with its different affinity for the stimulus, we found: 1-2) the specific salivary proteins of bPRP family (Ps-1) and L-Arg that could be involved in twist and turn of the PROP molecule, thus facilitating its binding with the receptor. 3) A gustin gene polymorphism that, by modulating the protein activity, controls the growth and maintenance of taste buds and 4) the higher disinhibition behaviour in non-tasters may be compensated in part, in normal-weight subjects, by the decrease of peripheral endocannabinoids to downregulate the hunger-energy intake circuitry.

Keyword: Decapod crustaceans, *Procambarus Clarkii*, walking legs, sugars, amino acids;

PROP taste sensitivity, TAS2R38, trophic factor Gustin (CA6) gene, basic proline-rich proteins, endocannabinoids, controll of eating behavior.

Corresponding author: Melania Melis, Department of Biomedical Sciences, Section of Physiology, University of Cagliari, 09042 Monserrato, Italy. E-mail: melaniamelis@unica.it

Contents

Section 2

This thesis is based on the work contained in the following publications:

- Solari P, **Melis M**, Sollai G, Masala C, Palmas F, Sabatini A, Crnjar R. ***Role of the leg chemoreceptors in the red swamp crayfish Procambarus clarkii: an electrophysiological and behavioral study.*** Submitted.

- Cabras T, **Melis M,** Castagnola M, Padiglia A, Tepper BJ, Messana I, Tomassini Barbarossa I. (2012) ***Responsiveness to 6-n-Propylthiouracil (PROP) is associated with salivary levels of two specific basic Proline-rich proteins in Humans.*** PLOS One 7(2): e30962.

- Tomassini Barbarossa I, Carta G, Murru E, **Melis M**, Zonza A, Vacca C, Muroni P, Di Marzo V, Banni B. ***Taste sensitivity to 6-n-propylthiouracil is associated with endocannabinoid plasma levels in normal-weight individuals.*** Nutrition. 2013 Mar; 29(3):531-536.

- **Melis M**, Atzori E, Cabras S, Zonza A, Calò C, Muroni P, Nieddu M, Padiglia A, Sogos V, Tepper BJ, Tomassini Barbarossa I. (2013) ***The gustin (CA6) gene polymorphism, rs2274333 (A/G), as a mechanistic link between PROP tasting and fungiform taste papilla density and maintenance***. PLoS One 8(9):e74151.

- **Melis M**, Aragoni MC, Arca M, Cabras T, Caltagirone C, Castagnola M, Crnjar R, Messana I, Tepper BJ, Barbarossa IT. (2013) ***Marked increase in PROP taste responsiveness following oral supplementation with selected salivary proteins or their related free amino acids***. PLoS One 8(3):e59810.

Section 1 of thesis

Role of the walking leg chemoreceptors in the red swamp crayfish *Procambarus Clarkii*

General introduction

The red swamp crayfish *Procambarus clarkii* (GIRARD, 1852) (Crustacea: Decapoda), also known as the Lousiana Crayfish or killer Crayfish (Figure 1), is an invasive species that has spread worldwide [1]. It originated in the South-Central United States and, following introduction for aquaculture purpose [2], it now displays a cosmopolitan distribution in all the continents, except for Australia and Arctic and Antarctic areas [3,4]. *P. clarkii* also inhabits a large number of ponds, lakes and streams from different Italian regions [2], where the populations are expanding exponentially. The first population was documented in Piedmont in 1989 [5], but this species is now widespread in Lombardy, Liguria and Emilia Romagna [6], Tuscany [2,7], Umbria [8], Marche [9], Lazio [2], Abruzzo [2] and Sicily [10]. Since 2005, the presence of *P. clarkii* has also been reported in some rivers of the Central-Northern Sardinia and, more recently, also in the South of the island. At present, the red swamp crayfish is considered the most damaging invasive species in Italy and, due to its high dispersive abilities [11,12], it now represents the most common large-size invertebrate species [3]. Because of its huge ecological value, it is now listed among the "100 most invasive" species in the "Delivering Alien Invasive Species in Europe" project (DAISIE, 2010) [13].

In particular, *P. clarkii* represents a serious threat for autoctonous crayfish species such as *Austropotamobius pallipes*, to which it can transmit the oomycete *Aphanomyces astaci* (Schikora 1906), vector of the "crayfish plague" [14-17].

On the whole, *P. clarkii* alters the biodiversity and also influences human activities [18], by damaging crops [3], transmitting toxins, pathogens, pesticides and heavy metals [1,19,20]. Ultimately, in the environmental contexts in which *P. clarkii* was introduced, it usually overpowered the other species and in a short time become, in absence of suitable natural predators, the strongest ring in the ecological chain.

Figure 1 - The red swamp crayfish *Procambarus clarkii*.

Chemical communication in the aquatic environment

The ability to recognize chemical stimuli is a fundamental aspect of the sensory repertoire of all animals. Even in aquatic environments the chemical communication plays a crucial role and stimulating compounds may diffuse over a long distance, by carrying crucial information for animal survival. For instance, many crustaceans make use of complex odors to precisely locate a chemical source of interest, even when this is at a considerable distance. Given the importance of the chemical communication, decapod crustaceans are able to discriminate among countless different stimuli by way of a large endowment of peripheral chemoreceptors grouped within sensory hairs called sensilla, typically distributed over the entire body surface of the animal. Among these, antennules, antennae, pereopods (major claws and walking legs) and mouthparts represent the major chemosensory organs [21-31].

Many of these types of sensilla are bimodal, i.e. chemo-mechanoreceptors, in such a way that the combination of chemical and hydrodynamic inputs can synergistically improve the temporal properties of orientation to the odor source, by decreasing the search time [32-34].

A large number of studies have shown that the antennules are the main sensory organs in the primary olfactory chemoreception in decapod crustaceans. They are biramous organs, with a lateral and a medial flagellum (Figure 2). In particular, the lateral flagellum of *P. clarkii*, a vertically held, curved, conical cylindrical structure, contains several sensillum morphotypes and plays a key role in both food search [35-40]

and mates [41-45] and also mediates social and agonistic behaviors [46,47]. It bears peculiar chemoreceptors grouped within the aesthetasc sensilla or, more simply, the "aesthetascs" (Figure 3), that are hair-like protrusions of the flagellum containing the dendrites of many chemosensory neurons [48,49]. Each aesthetasc is approximately 100 μm in length, 15-20 μm in base diameter, and less than 10 μm in tip diameter, with a ~1.9 μm thick cuticle that shows a change to transparency approximately three-fifths of the way from base to tip [50]. This transparent portion of the aesthetasc is where chemoreception is thought to take place [50,51] (Figure 3B). The aesthetascs are usually paired, spaced approximately 1 aesthetasc diameter apart, with 2-6 aesthetascs per annulus and 40-110 olfactory chemoreceptor neurons (ORNs) per aesthetasc [52].

Axons from aesthetasc ORNs exclusively project to the glomeruli in the ipsilateral olfactory lobe (OL) of the brain, possibly in a 1:1 connectivity pattern [50,53,54]. There is no anatomical or physiological evidence that the aesthetascs are equipped with mechanoreceptor neurons [32]. Antennules of *P. clarkii*, like those of other crayfish species, also contain other non-aesthetasc sensillum morphotypes; among them, two main types are represented by the standing feathered sensilla, highly sensitive to hydrodynamic stimuli [55] and by the beaked sensilla, with possible bimodal chemo-mechanoreceptor features [32]. Antennular flagella also bear at least three additional morphotypes of setae: the procumbent feathered setae, the filamentous setae and the asymmetric setae [32]. In lobster, asymmetric setae are believed to be bimodal chemo-mechanoreceptors sensilla [56]; however, some of them, including those of the medial flagellum, also respond to chemical stimulation [36,57-60]. Due to the lack of aesthetascs, the chemical sensitivity of the lateral flagellum is mediated by the other sensillum morphotypes.

Decapod crustaceans display a peculiar behavior known as antennular "flicking" [52,61,62]. It consists of a rapid down stroke of the antennules, followed by a slower return stroke: during the down stroke, water flows between the aesthetascs, whereas during the slower return stroke and during the stationary pause between flicks, water is trapped between the aesthetascs. During this time, molecular diffusion is thought to transport odorants to the surface of the aesthetascs [35,63], so that the antennular flicking definitely improves the interactions between odorants and chemoreceptors. Even if it has been well established that the aesthetascs play an important role in crayfish intraspecific communication [47], data in the literature are still controversial. In fact, some authors reported that the ablation of the entire lateral flagellum inhibits the perception of food odors [33,36,57] and sex discrimination [41,60,64]. Conversely Ameyaw-Akumfi [65] showed that the ablation of both flagella may not affect the ability of *P. clarkii* to perceive odors of feeding significance. Moreover, Dunham et al. [57] reported that the

medial flagellum could mediate a response to sucrose, whereas Giri and Dunham [36] observed that crayfish with only the medial flagella were unable to locate long-distance food sources. It was also reported that in *P. clarkii* females both flagella were equally successful in detecting odors from conspecific males [60]. In this respect, male crayfish seem to exclusively rely on chemical cues to find reproductive females, while the latter normally make use of a combination of both chemical and visual stimuli for recognition of conspecific males [66-69] .

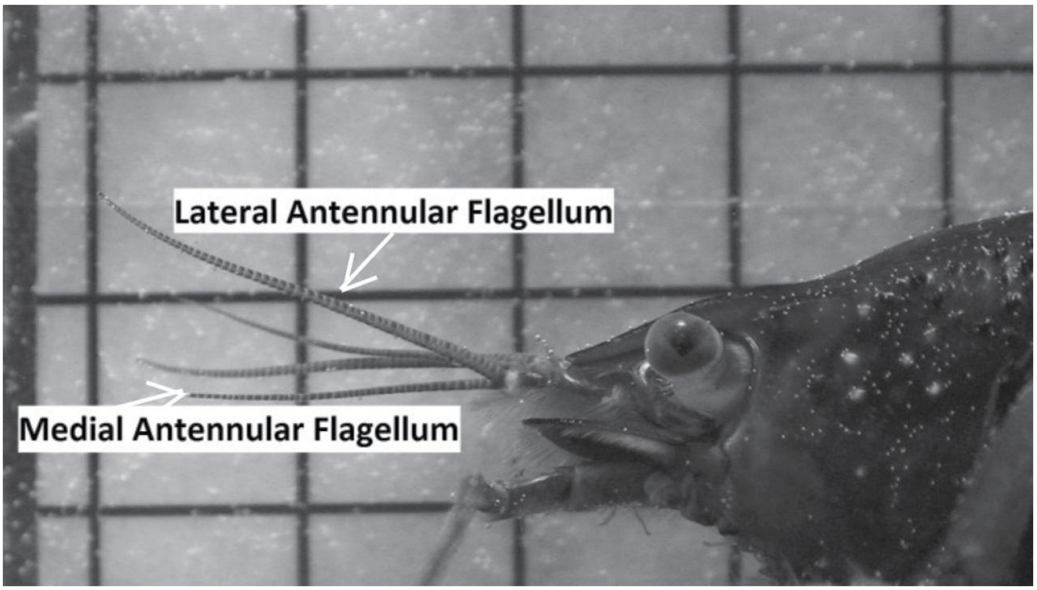

Figure 2 - Lateral and medial antennular flagella of *P. clarkii.*

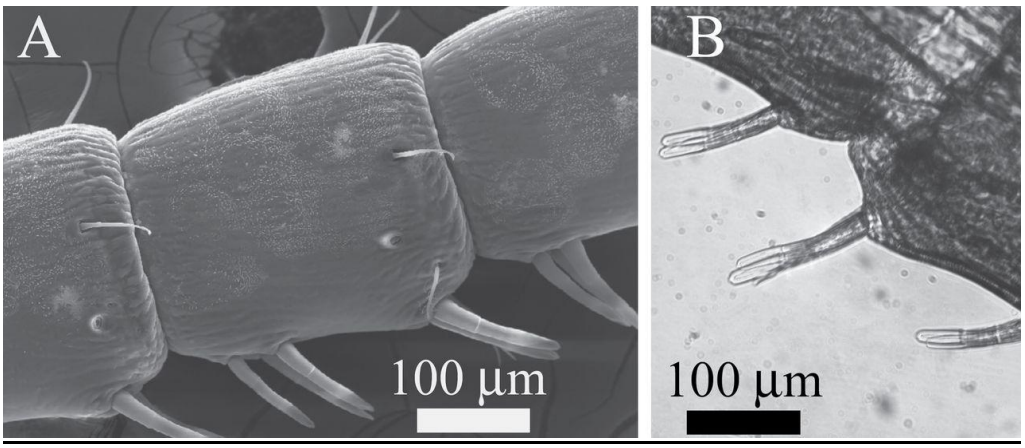

Figure 3 - (**A**) Scanning electron microscope images of a portion of a lateral flagellum showing the placement and spacing of aesthetascs on the flagellar annuli. (**B**) Light micrograph of a living lateral flagellum, and 3 pairs of aesthetascs on its ventral surface. Note the optically transparent distal regions containing the cuticle and the olfactory receptor neurons (ORNs) [52].

Finally, similarly to what observed in other crayfish species, also *P. clarkii* possesses a large array of chemoreceptive organs distributed on the walking legs, especially on the second and the third pairs [24,28,70-72]. Previous electrophysiological and behavioral investigations suggested that these leg chemosensilla act as detectors for food-related compounds, on the basis of their marked sensitivity to a number of sugars and amino acids [24].

Sistematic and anatomy of *Procambarus clarkii*

Domain	*Eukaryota*
Kingdom	*Animalia*
Phylum	*Arthropoda*
Subphylum	*Crustacea*
Class	*Malacostraca*
Order	*Decapoda*
Super-family	*Astacoidea*
Family	*Cambaridae*
Genus	*Procambarus*
Species	*P. clarkii*
	GIRARD, 1852

P. clarkii is a decapod crustacean from the family of *Cambaridae*, which includes only genera of North American freshwater crayfish [73]. The order *Decapoda* is very broad and mostly includes marine crustaceans, although some forms become successful invaders of freshwater ecosystems.

Like other crustaceans, the crayfish body comprises two main regions, the **cephalothorax** and the **abdomen,** covered by a hardened chitinous exoskeleton impregnated with calcium salts, which is periodically replaced to allow for growth of the animal (molt) (Figure 5). Each segment from both the cephalothorax and the abdomen contains a pair of appendages. In particular, the head region houses the following six pairs (Figures 6 and 7):

1) **compound eyes**, that are located on the eyestalk and can be moved in all directions by the oculomotor muscles;
2) **antennae,** that mainly provide the animal with hydrodynamic, mechanical and equilibrium sensory information;
3) **antennules,** that represent the major chemosensory organs and also contain the "aesthetasc" sensilla;
4) **mandibles,** or jaws, heavily calcified and equipped with powerful muscles; they are used for shearing or crushing the food;
5) two pairs of **maxillae,** that hold the solid food, and tear and pass it to the mouth. The second pair of maxillae also helps propelling water currents to the gills;
6) **maxillipeds,** which hold the food during eating and also function as auxiliary, potentially chemosensitive, mouthparts.

The five thoracic segments bear each an appendage pair, also called pereopods, with the function of walking legs or pincers. In this respect, the five pairs of legs are considered as a distinctive feature of decapods, hence the name of the entire order (decapoda = "ten feet"). In particular, the first pair, also called **chelipeds,** contains large claws**,** formed by a movable part, the dactylopodite, and a fixed part, the propodite, articulated on a segment named carpus, and mainly serve for defense and prey capture (Figure 8). Each of the four remaining segments contains a pair of **walking legs** better suited for locomotion, the second and the third pairs being also equipped with small pincers, normally used for food manipulation. It is worth mentioning that the legs also display chemosensory activity.

The first five pairs of abdominal appendages are named pleopods (or **swimmerets**). Pairs 2 to 5 are biramous, narrow and whiplike appendages, not very long. Pleopods of females are better developed than those of males as they are also used to carry and oxygenate eggs. In males, the first pleopod pair is modified and serve as a copulatory organ, in order to transfer the spermatozoa to females and is referred to as gonopods. Small hooked teeth are also present at the base of the third and the fourth pair of the male pereopods; they are exclusive of the family of Cambaridae and help holding the female during mating. The male spermatophores are received by females in appropriate seminal receptacles forming a characteristic structure known as annulum ventralis (or seminal receptacle) (Figure 9) [3]. The sixth abdominal segment contains a modified pair of **uropods.** In the middle of the uropods there is the **telson** containing the anus. Uropods and telson together form the tail fan that crayfish use for backward movements.

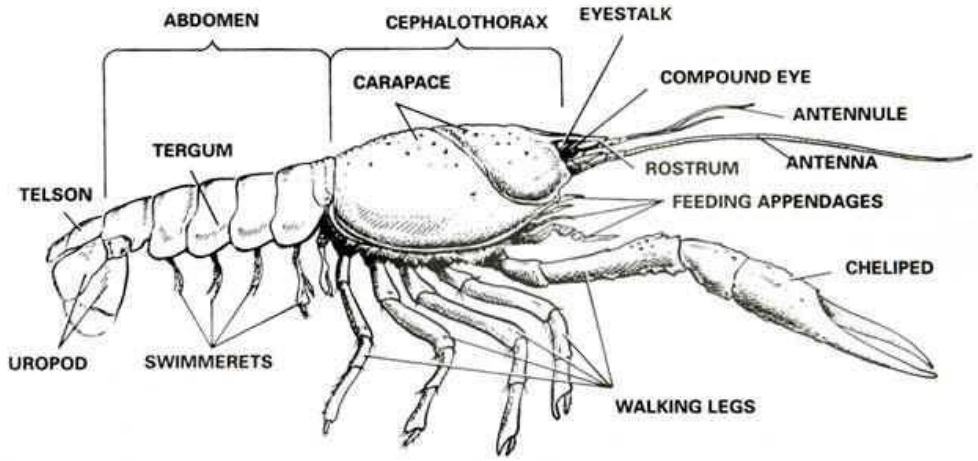

Figure 5 - General morphology of the body of decapod crustaceans.

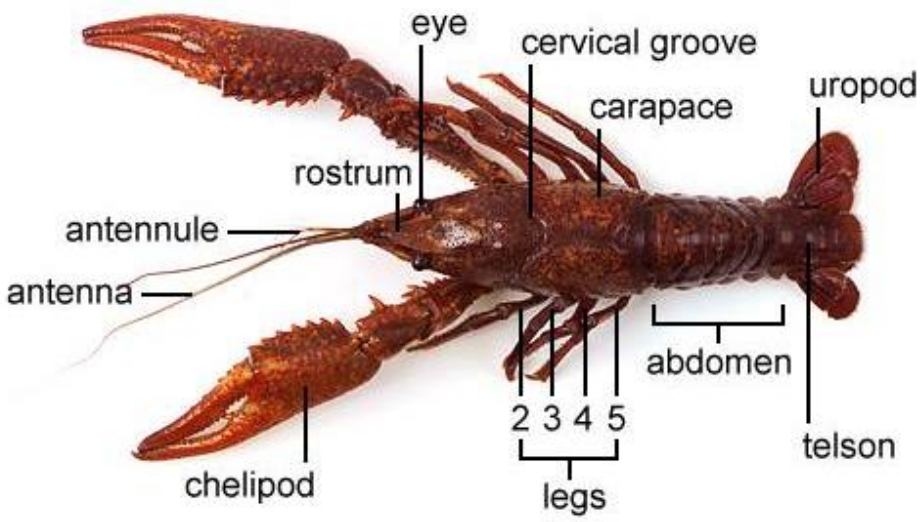

Figure 6 - General morphology of the *P. Clarkii* body (dorsal view).

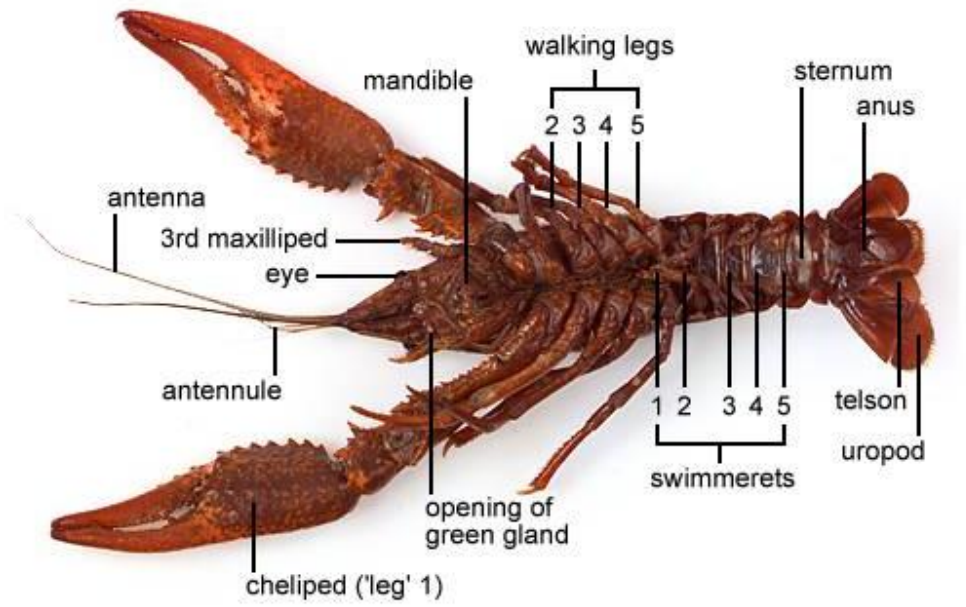

Figure 7 - General morphology of the *P. Clarkii* body (ventral view).

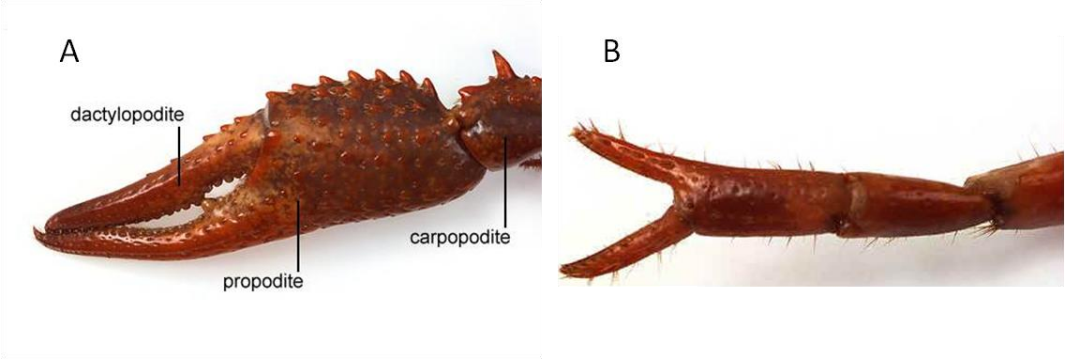

Figure 8 - The largest prominent pair of appendages, the **chelipeds** or claws (1st leg; A) and 2nd walking leg (B) in *P. Clarkii*.

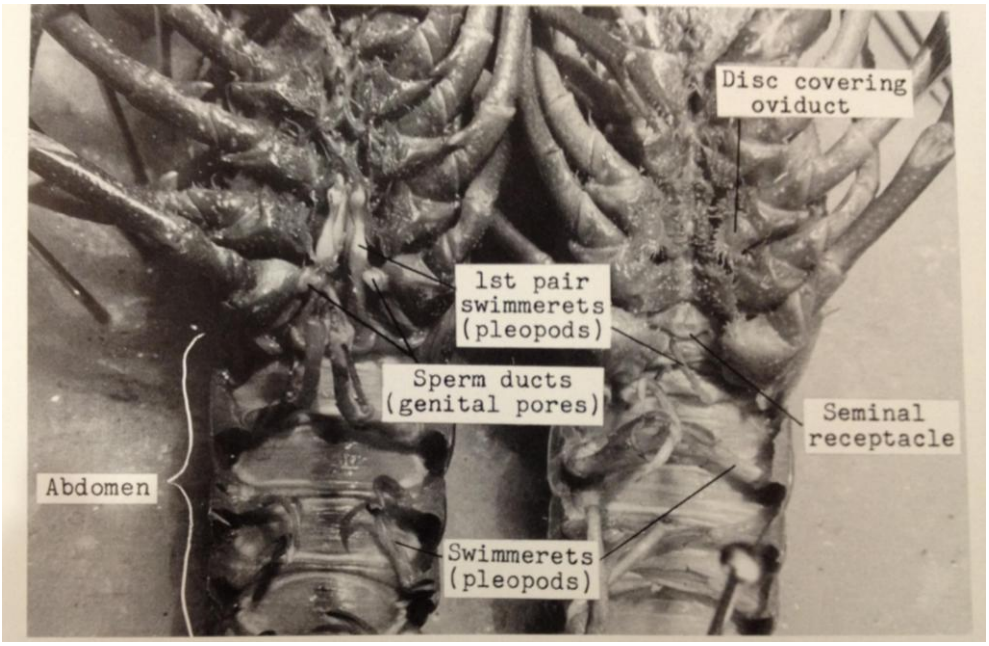

Figure 9 - Ventral side of the abdomen in *P. clarkii* male (left side) and female (right side). In the male the first pairs of swimmerets are greatly enlarged and act as copulatory organs for the transfer of sperm from the sperm duct opening at the base of the last pair of walking legs to the female's seminal receptacle.

Biological cycle and ecology

P. clarkii is a digger crustacean [3], with a very rapid development/growth, living in marshes, swamps, ponds and slow moving rivers and streams, but have also become established in lakes. Its biological cycle, usually of short duration (about 12-18 months), is remarkably plastic [2] and well adapts to the hydrological cycles, temperature changes and seasonal large water level fluctuations [74,75]. Although the optimal temperature range is comprised between 21 and 27 °C, this crayfish species is also able to tolerate extreme temperatures up to 35 °C [2,3,76]. The feeding habits of crayfish may greatly vary, being dependent upon the size and the age of the animal, the availability and the distribution of food resources and the presence of predators in the ecological niche [77,78]. They are usually classified as herbivores, detritivores (consumers of decomposing organic matter [65,79], omnivores [80,81] and, more recently, also as obligate carnivores, which means that they "require" some animal matter in the diet for optimal growth and health [77,82]. The analysis of stomach contents confirms the omnivorous habits, with preferences for herbivory in adults and carnivory or cannibalism in youth [83].

From an ecological point of view, *P. clarkii* is described as a r-selected species, i.e. a typical colonizer of unstable environments. Moreover, it shows high fecundity (300-600 eggs) [84], rapid growth, early sexual maturity, that can be reached after about 3-5 months of age (animal length of 55-125 mm), usually associated to multiple reproductive cycles per year [3]. Once specimens reach the sexual maturity, the alternation of two different forms occurs, indicated as form I (F1) and form II (F2) (Figure 10). The F1 form corresponds to the sexually active crayfish and is maintained throughout the

reproductive period; it entails some important morphological changes that are more evident in males, whose claws, at this stage, lengthen and become more robust. Also the body livery varies, especially in males, from the soft colors typical of the F2 form to the intense red of the F1 form. In females, changes are less dramatic and simply regard an enlargement of the claws. Usually, a few weeks after mating (in females about three weeks after egg hatching), crayfish molt, returning to the sexually inactive F2 form, characterized by shorter claws, less pronounced colors, absence of hooks and little gonopods in males. The F1 form will take place through a molt at the next reproductive cycle, even if adults living in constantly warm waters frequently maintain the F1 form, that is also the most frequent one in specimens of great size [18].

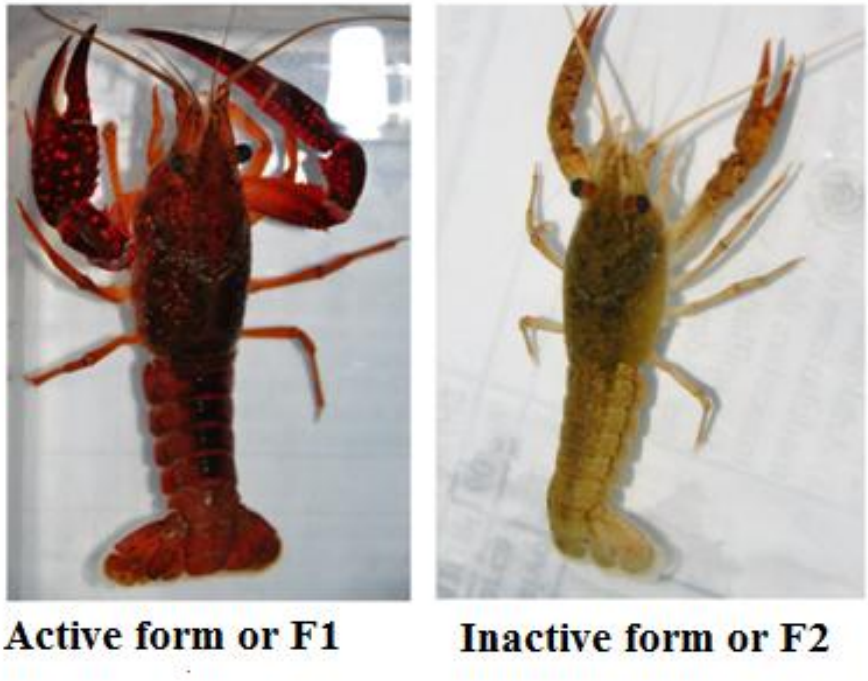

Figure 10 - The two different forms of sexual maturity in *P. clarkii*.

The complex physiological and behavioral changes related to molt are controlled by hormones, among which the most important are the MHs (Molting Hormones, stimulators of the molt) and the MIHs (Molting Inhibiting Hormones) [85]. The MH hormones are represented by a group of steroids known as ecdysone-derivatives (compounds derived from cholesterol), responsible for the initiation of the pre-molt phase, and produced by a gland located in the cuticle at the internal branchial room (Y organ). The MIHs are compounds of peptide nature and inhibit the synthesis of ecdysteroid; they are secreted by a glandular complex localized on the eyestalk (X organ). During the molt the outer exoskeleton breaks down and the new exoskeleton, soft, wrinkled, stretches to accommodate the increased size of the animal. The stages

between molts are called Inter-Molt phases or ages. Like all crustaceans, molt does not stop at sexual maturity, but continues throughout the life time [86]. Immediately after the molt, crayfish are vulnerable, because the exoskeleton is not sufficiently hardened, and the animals assume, therefore, a self-effacing behavior, remaining hidden within the den [87].

According to a recent study [88], the females of this species are able to make a "cryptic" choice of males, adjusting the amount of deutoplasm in the eggs depending on the size of the partner, and abandoning or cannibalizing the clusters of eggs when mating with small or low quality males. This would allow them to save time and energy to devote them to a possible more beneficial coupling [88]. Maturation of eggs lasts from 6 weeks to 8 months, depending on the quality of the site and on water temperature: 2-3 weeks at 22 °C, up to 2-3 months at lower temperatures, while beneath 10 °C egg maturation blocks. During this time, especially in warmer habitats and in presence of low oxygen levels, females periodically expose eggs to air and, also thanks to the constant movements of pleopods to which eggs adhere, they can get adequate oxygen supply. The growth of newborns is very fast and, at 20-30 °C, they can molt every 5-10 days. Females of *P. clarkii* exhibit extreme care of the progeny: the bond established between females and juveniles is mediated by species-specific chemical stimuli, aimed at protection and survival of the progeny [87,89].

After the release of the young specimens, females remain hidden in sheltered places for most part of the time, until resuming a normal active behavior [87]. The innate aggressiveness of these animals leads them to an early establishment of dominance hierarchies among group members, ensuring better access to resources [90]. This competitive behavior seems to be influenced by the levels of neuromodulators, such as biogenic amines (serotonin, dopamine, octopamina, norepinephrine) in the nervous system [91], and is mediated by a massive release of chemical signals during the social interactions [92]. These olfactory signals are released, along with urines, through the nephropores, excretory organs located at the base of the antennules, and are involved in the recognition of the status of dominance between individuals [91,93,94] and in sex identification for mating [95,96]. Like the other crustaceans, this species show a remarkable capacity of regenerating the lost appendages [97].

From a behavioral point of view, *P. clarkii* shows two alternating patterns of activity: a **nomadic phase**, without any daily periodicity, characterized by high locomotion activity, during which the breeding males are able to cover up to 17 Km in four days, and a **static phase** during which, especially by day, crayfish hide in the tunnels of their burrows, which can be more than 2 m long, and only emerge at dusk to feed [3,87]. When the red swamp crayfish feels the urge of migration, it can cover a long

distance (3 km in one night), by walking also outside of water without any apparent trouble, even during the daylight hours.

The high mobility coupled to the ability to adapt to the most diverse, sometimes extreme, environmental conditions has favored the dispersion and the success of this species. In fact, *P. clarkii* can also colonize brackish waters (salinity 20 ‰) that are usually inhospitable for native crayfish, to tolerate draught/desiccation [98], acidity and anoxia, being able to survive at pH values between 5.6 and 10.4 and oxygen concentrations below 1 mg/L. This is also possible for the particular conformation of their gill rooms, that in the presence of minimal amounts of water may efficiently extract oxygen from both air and water, therefore integrating the two breathing modalities [3].

Risks related to the introduction of alien species and population control strategies.

Invasive species are responsible for numerous ecological impacts and are considered as the second cause for the extinction of native species after the loss and destruction of habitats [99], and also inevitably produce long- and short-term economic damage [100,101]. The acronym NICS (Non Indigenous Crayfish Species) groups all crayfish species that are not native to the waters in which they are located but that have been introduced deliberately or accidentally by humans [102]. The prevention of NICS introductions is much cheaper economically, while as well as desirable from an environmental point of view, with respect to the adoption of countermeasures after their stabilization, by making the eradication process or any population control extremely difficult and rather expensive.

Since 1850, many crayfish species have been introduced in Europe from other geographical areas and some of them, because of their biological, ecological and behavioral features, have managed to stabilize in the new environment and to grow in such an uncontrolled way so as to successfully colonize the majority of freshwater habitats [103]. The huge increase in population size also led to the invasion of new environments, thus causing a negative impact at different levels, from genetics to the ecosystem [14]. In Italy, the red swamp *P. clarkii* represents the most common NICS and its rapid diffusion was mainly due to commercial purposes [102]. The same characteristics that facilitated breeding (like resistance to diseases and to extreme environmental conditions, generalist and opportunistic feeding habits, high fecundity, etc.) [1] also made it able to easily colonize a wide variety of habitats, causing severe ecological alterations. In particular, the red swamp crayfish has the potentiality to affect

most of the other species living in the same habitat and therefore results in a drastic reduction of fish, amphibian, mollusk and aquatic plant communities, thus altering the biodiversity at all levels [102]. Moreover, its intense digging activity causes structural damage to riverbanks and lakes [104] and determines the phenomenon of bio-disturbance, such as clouding of waters that leads to the reduction of primary productivity [14,18]. *P. clarkii* may also have a negative impact on human health: its ability to live in contaminated environments and to accumulate pollutants in their tissues increases the risk to transfer them to consumers, including humans [102].

Eradication consists of extirpation of the entire invasive population from a given area by way of a time-limited campaign [105]. In this respect, "time-limited' means that the eradication needs to be achieved within a prefixed deadline, because an eradication campaign in absence of any specified end point should be defined as a continuous control, that is harvesting or killing a proportion of a population on a sustained basis [106]. Obviously this practice is still considered the best (and cheapest) remediation tool. However, eradication programs are viewed with incredulity by many conservation biologists, particularly in Europe [107-109]. The eradication and also continuous control of NICS should be socially and ethically acceptable, efficient, non-polluting, and especially should not damage native flora and fauna, humans, animals, and farm [105]. Although all of these criteria are difficult to be met, genuine attempts should be made to do so [110]. The type of action should be chosen specifically for each individual case, it is important in fact to consider the circumstances for which a given intervention is biologically possible, as well as acceptable under ecological, economic, political, and ethical viewpoints [111].

Currently, different strategies have been proposed, alone or combined, for either eradication or the continuous control of invasive populations of NICS. The methods are distinguished in the following five broad categories [105]: mechanical removal, physical methods, biological control methods, biocides and autocidal methods.

The **mechanical removal** is implemented through the use of various types of traps (traps Swedish, Evo- traps, collapsible traps, nets folding "fyke", etc.; [112,113]) or by "electrofishing" [114,115]. However, trapping should be conducted over an extended period of time so as to get some significant results. All this means considerable costs and extended human resources. Currently, one of the most valid techniques for the control of invasive shrimp is that of "intensive trapping", which consists of massive catches of individuals through the use of lobster pots with baits containing odors [102]. The **physical methods** consist in the drainage of ponds, diversion of rivers and construction of artificial barriers that may be used in the case of confined populations of NICS. The drought may not be effective against burrowing species such as *P. clarkii*, which can

survive out of water for long periods of time. The **biological control**, or biocontrol, includes a range of interventions based on the use of natural enemies of NICS. Theoretically, this is one of the preferable strategies, because it is permanent, nonpolluting, environmentally friend and ethical. The risks are represented by the fact that natural enemies are not always specific to the target species, but they can also attack native species. For this reason, the control agents should be carefully selected and monitored. The traditional enemies of crayfish are relatively large predators, such as birds and fish, or disease-causing organisms and microbes that produce toxins, such as, for example, the bacterium *Bacillus thuringiensis* [105]. Several studies have shown that fish predation may have a certain degree of impact on the NICS populations, particularly for eels, burbots, perchs and pikes [116]. The European eel, *Anguilla anguilla*, seems to be a good candidate to mitigate the damage caused by *P. clarkii* in Italy [117]. The study confirmed that eel can actually preying on *P. clarkii*, although limitations in buccal opening enable it to capture almost exclusively small individuals [118]. However, eels, like other predatory fish, may alter the crayfish behavior, inducing a reduced activity and consequently an increase in the time spent within the shelter [117,119-122]. In any case, this fact may result in a decrease in the crayfish trophic activity followed by an increase in mortality due to starvation and also reduce the impact on the most affected components of the community, such as macrophytes and snails [105]. The **biocide methods** are based on the use of pesticides or related chemicals to control invasive and noxious species and can be used alone or in combination with mechanical or physical methods [105]. Because of their widespread use, there is frequent development of resistance in the target organism. In addition, since biocides are not species-specific, a high risk of bioaccumulation and biomagnification in the food chain may exist [105]. Biocides that have been used to control the NICS include insecticides, organochloride, organophosphate, pyrethroids, extracts from the flowers of *Chrysanthemum cinerariaefolium and C. cineum* (eg Pyblast Agropharm Ltd, UK), rotenone and surfactants [105]. Finally, the **autocidal methods** include the technique of release of sterile males (SMRT) and the use of sex pheromones. Although initially expensive, these techniques do not cause environmental contamination or impacts on non-target organisms, because it is largely species-specific [105]. The SMRT technique is based on the capture of males, their sterilization by ionizing radiation and the subsequent release into the environment. Studies have shown that X-rays did not involve either the survival or the mating ability, but affected the reproductive success by reducing the number of eggs. Overall, the available data suggest that the release of a sufficient number of irradiated males can reduce the size of populations of NICS and that the reduction in fertility may persist for more than 1 year [123].

The release of female sex pheromones in an area can confuse the males and prevents them from finding mates [105]. Alternatively, sex pheromones may be used to bait traps for massive captures and this procedure is environmentally safe because sex attractants are in most cases species-specific; however, an apparent limitation consists in that it can be applied not all year round but during the reproductive season only [105].

Outline of the work

The majority of decapods, including crayfish, lobsters, crabs and shrimps, rely on chemical signals to detect and locate food [36,57,124-127], mates [128,129] and suitable living habitats [130-132], for the choice of shelters [133-135], to escape from potential predators [134] and for conspecific interactions [35,36,43,60,136,137]. The categories of stimulating compounds may differ among the various species, but typically include amino acids, nucleotides and their derivatives, amines and, in some cases, carbohydrates. Compounds that stimulate crustacean chemoreceptor cells are typically those that possess the best signal properties, i.e., they are found at high concentrations within food items, but are present at much lower "background" levels in the environment [24]. As a general standpoint, amino acids represent some of the most important chemosensory stimuli for crustaceans, and sensitivity to amino acids has been demonstrated in a wide range of crustacean species [71,138-142]. In contrast, chemosensory sensitivity to carbohydrates has been reported for just a few crustaceans with herbivorous or omnivorous feeding habits [24,139,140,143-146]. One class of compounds that has not been tested on crustaceans yet is that of the bile acids. They are potent olfactory stimuli for fish [147], which release bile acids in their feces [148], it may also represent a potential signals for social and/or territorial interactions, as reported for freshwater habitats [149].

Based on these considerations, and taking into account the predominantly omnivorous feeding habits of *P. clarkii* [83], aim of this study was to investigate, using a combined approach of behavioral and electrophysiological tests, the sensitivity of the walking leg (pereopods) in this crayfish species. As shown in the manuscript that follows (chapter I), we studied the leg sensitivity to a number of food-related compounds, such as the disaccharides sucrose, maltose, trehalose and cellobiose, the monosaccharides glucose and fructose, the aminoacids leucine, glycine and asparagine and to a representative of bile acids, taurocolic acid. Understanding the relationship between sensory inputs and behavioural outputs can be of great importance for the development of biological control strategies aimed at the reduction and/or the eradication of invasive crayfish populations, possibly based on the use of attractants that may help improving the mass trapping efficiency.

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I

Role of the walking leg chemoreceptors of the red swamp crayfish *Procambarus clarkii*: an electrophysiological and behavioral study.

Paolo Solari
Melania Melis
Giorgia Sollai
Carla Masala
Francesco Palmas
Andrea Sabatini
Roberto Crnjar

Submitted for publication

Abstract

By way of extracellular nerve recordings coupled with behavioral bioassays, we investigated the sensitivity spectra of the walking leg chemoreceptors in the red swamp crayfish *Procambarus clarkii,* an invasive species of freshwater habitats, in response to different compounds of feeding significance and related to its omnivorous habits. Our results confirmed a marked sensitivity of the legs to trehalose, cellobiose, sucrose, maltose, glycine and leucine. Some sensitivity to glucose, fructose, asparagine (all food indicators) and taurocholic acid was also found, the sugar-sensitive chemoreceptor units resulting as broadly tuned to the carbohydrates. Responses were highly phasic to trehalose (hemolymph sugar in the body fluid of many invertebrates), phasic to glycine and leucine and phasic-tonic to the other compounds. This suggests that chemoreceptor phasicity is an additional property for better discrimination of the protein components in the diet from other stimuli. The behavioral bioassays excluded, at least under confined experimental conditions, any involvement of antennules in the detection of food-related compounds, thus emphasizing the role of the crayfish legs as the main short-distance, broad-spectrum sensors for feeding. Such information may be valuable for the identification of key chemicals aimed at the future development of strategies for crayfish population control programs.

Introduction

The red swamp crayfish *Procambarus clarkii* (Girard, 1852) (Crustacea: Decapoda) is considered one of the most hazardous invasive alien species (IAS) of freshwater habitats. Since its use in aquaculture practices, it has spread worldwide, consuming every kind of trophic substrates, becoming a generalist top consumer in the alimentary chain and greatly contributing to the decline of native crayfish (Alcorlo et al. 2004; Gherardi 2006; Gherardi and Acquistapace 2007; Gherardi et al. 2011; Lodge et al. 2012).

Like the other decapod crustaceans, crayfish rely on chemical senses to produce a number of adequate behavioral responses, from orientation to social communication, detection of predators, sex recognition and localization of food resources in their environment (Breithaupt and Eger 2002; Grasso and Basil 2002; Bergman and Moore 2005; Aquiloni et al. 2009; Breithaupt 2011; Schmidt and Mellon 2011). The discrimination of the different stimuli is mediated by peripheral chemoreceptors grouped within sensory hairs called sensilla and typically located on the cuticle of cephalothoracic appendages, including antennae, maxillipeds (mouthparts) and especially antennules and pereiopods (major claws and walking legs) (Schmidt and Mellon 2011). Many of these types of sensilla are bimodal, i.e. chemo-mechanoreceptors, in such a way that the combination of hydrodynamic and chemical inputs can greatly enhance the temporal properties of orientation to odor sources, by decreasing the search time (Wolf et al. 2004; Mellon 2007). The feeding behavior and the related stimulatory compounds may vary, both qualitatively and quantitatively, among the different crayfish species, but also intraspecifically, being greatly dependent upon the size and the age of the animal, the availability and distribution of food resources in the ecological niche, as well as the presence and the distribution of predators (Momot 1995; Lodge et al. 2012). It has been well established that the chemoreceptor neurons (CRNs) of carnivorous crustaceans are highly sensitive to small, nitrogen-containing compounds, like amino acids, amines, nucleotides and peptides, that are indicators of good quality food as usually prevalent in the tissues of their animal prey, while they are relatively insensitive to carbohydrates and sugars (Zimmer-Faust 1993; Schmidt and Mellon 2011). These CRNs usually tend to be narrowly tuned so as to specifically respond to single molecules or to a restricted set of structurally related compounds (Derby et al. 1991; Voigt and Atema 1992; Derby 2000). Conversely, herbivores and omnivores like crayfish (Tierney and Atema 1988; Momot 1995) are often sensitive to sugars common to plants, bacteria and diatoms and possess CRNs that are less strictly tuned, but still retain specificity, also to amino acids, pyrimidines or amines (Bauer and Hatt 1980; Bauer et al. Hatt 1981). Feeding deterrents also play a critical role in food selection by crayfish, even though this topic remains poorly

investigated (Lane and Kubanek 2006; Parker et al. 2007). Previous electrophysiological and behavioral studies also highlighted the role of the walking legs of *P. clarkii* as potential food detectors, on the basis of their marked sensitivity to a number of disaccharides such as trehalose, cellobiose, maltose and to the amino acids leucine and glycine (Corotto and O'Brien 2002; Corotto, et al. 2007).

Based on these considerations, aim of the present study was to investigate, by way of a combined electrophysiological and behavioral approach, the ability of the walking legs in the crayfish *P. clarkii* to detect a set of chemical stimuli possibly related to food. To do this, we selected a number of compounds, such as a few sugars (trehalose, cellobiose, maltose, sucrose, glucose and fructose) and amino acids (glycine, leucine, asparagine, serine and threonine). We also chose a representative of the bile acids, taurocholic acid, that has been reported to occur in feces of freshwater fishes (Zhang et al. 2001) and for its potential role as a semiochemical for social and/or territorial interactions in freshwater habitats (Martinovic-Weigelt et al. 2012). We first tested these stimuli by way of extracellular recordings, in order to examine the response patterns and also the adaptation properties of the leg CRNs. The same compounds were then tested in behavioral bioassays to better assess their stimulating effectiveness on the crayfish.

From an applied point of view, any insight about the crayfish ability to detect potential food-related compounds and the underlying mechanisms may help improving the efficiency of mass trapping strategies aimed at the reduction and/or the eradication of the crayfish populations.

Materials and methods

Animal collection and rearing conditions

All experiments were performed on wild, intermolt adult red swamp crayfish *P. clarkii* from both sexes, 70-100 mm in carapace length, collected using a backpack electrofishing unit at the Santa Lucia of Capoterra river (southern Sardinia, Italy) during the spring 2011-2013. They were kept in aerated and bio-conditioned (Aquasafe, Tetra) tap water (hereafter referred to as tap water), at 22-23 °C, 16 h light/8 h dark photoperiodic regime, and fed with lettuce, squid or commercial pellet food (Shrimps natural, SERA Gmbh) three times a week. Individuals were kept separate to avoid any exposure of males to females and to prevent the risk of reciprocal attacks or cannibalism.

Electrophysiological experiments and stimulus delivery apparatus

Recordings were performed from second and third walking leg pairs, according to the procedure described by Derby (1995) and Corotto and O'Brien (2002). Briefly,

immediately before experiments, the legs were excised, from ice-anaesthetized crayfish, at the middle of the merus, and dissected in a modified van Harreveld's cold saline solution (in mM: 205.0 NaCl, 13.5 $CaCl_2$, 5.4 KCl, 2.6 $MgCl_2$, 2 glucose and 2.4 HEPES, pH 7.5 (Derby 1995)).

Cuticle, muscles and apodemes were carefully removed in order to expose the nerve bundles and the artery as they emerge from the proximal end of the carpus. They were separated from each other by using sharp insect pins and fine forceps and the nerve was further divided into four to six bundles. Legs were then transferred to a custom-made olfactometer, consisting of a small rectangular Plexiglas® container (13 cm long x 3 cm wide x 1.5 cm deep), with a drain for wastewater collection and the open end of a plastic tube (5 mm diameter, 3 cm long), where intact dactylus and propodus could be inserted and continuously perfused with a 20 ml/min main carrier flow of tap water. The proximal cut end of legs with the exposed nerve bundles and the artery was secured on the top of a separate, Sylgard-made, cylindrical support (1 cm diameter x 1 cm high) filled with saline, arising from the bottom of the container, that acted as the recording chamber. The leg artery was then cannulated and perfused with cold, oxygenated saline, at a flow rate of 0.5-1 ml/min. In order to better de-fasciculate axonal bundles, enzymatic digestion was also performed, by exposing the nerve bundles to 10 mg/ml Pronase E for about 2 min. The time from leg ablation to perfusion was 10-15 min, while nerve recordings usually started within the next 15 min.

The extracellular spike activity from nerve fascicles was recorded "en passant", by way of fine-tipped borosilicate glass suction electrodes. Recordings were preamplified and band-pass filtered (0.1-1 KHz) by using an A-M System (Everett, WA, USA) four-channel differential AC amplifier (Model 1700), digitized by means of an Axon Digidata 1440A A/D converter (sampling rate, 10 KHz) and stored on PC for later analyses. Following spike sorting based on amplitude and waveform (Axon Clampfit 10.0), the neural activity in each recording was resolved as responses from 1 to 3 receptor cells.

Axon bundles were considered for their chemosensory component and were therefore tested with all stimuli only when they at first responded to either a sugar such as trehalose or an amino acid such as leucine. These search stimuli were chosen on the basis of their previously reported stimulating effectiveness for the leg chemoreceptors (Corotto and O'Brien 2002). In order to exclude mechanosensory components in the bundle response, stimulations with same aliquots of tap water were also performed.

Behavioral experiments

Animals were individually exposed to test items in Plexiglas® tanks (36 cm long x 25 cm wide x 15 cm deep), containing 5 liters of tap water (22-23 °C) according to the

procedure adopted by Kreider and Watts (1998). A 7.2- x 25-cm stimulus delivery area was separated from the rest of the tank by a rectangular, opaque Plexiglas® divider, perforated by ca. 30 evenly spaced 1-cm-diameter holes. The holes allowed water and test stimuli to move into the crayfish area. At the beginning of each test, crayfish were allowed to acclimate until becoming motionless; before stimulus supply, the response of each animal to a same aliquot of tap water (blank control) was monitored. Stimuli were added to the tank at increasing concentrations via the stimulus delivery area and each crayfish was allowed 3 min to respond. Trials were video-recorded for later analysis by way of a Samsung SMX-F34 color digital camera mounted above the test tank.

The behavioral responses were determined by using a 2-level ranking score partly according to Kreider and Watts (1998): 1) movement of walking legs with dactyl probing (s/3 min), that indicates a food search response and 2) rate of antennular flicking (flicking/3 min). At the end of each stimulation series, the pellet food was tested as a known responsiveness control. On test days the crayfish were not fed for at least 12 hr prior to experimentation.

Stimuli and supply protocol

The following compounds of potential feeding significance, mostly already known to elicit responses from leg CRNs of this and other crayfish species (Tierney and Atema 1988; Corotto and O'Brien 2002; Corotto et al. 2007), were tested as stimuli: the disaccharides trehalose, cellobiose, maltose, and sucrose, the monosaccharides glucose and fructose and the amino acids glycine, leucine, asparagine, serine and threonine. Moreover, also taurocholic acid was tested, a bile acid previously reported as present in feces of freshwater fishes (Zhang et al. 2001) and a potential chemical signal for territoriality and reproduction in freshwater habitats (Martinovic-Weigelt et al. 2012). All chemicals used were obtained from Sigma-Aldrich (Italy).

For electrophysiological experiments, chemicals were preliminarily dissolved in tap water at a 100 mM concentration, except for taurocholic acid (tested at 10 mM). By using an electronic perfusion system (Valve Link 8, Automate Scientific, Inc.) they were supplied for about 15 s, at a flow rate of 2.2 ml/min, directly into the main carrier flow (20 ml/min) bathing the preparation, thus undergoing a predicted 10-fold dilution to 10 mM (1 mM for taurocholic acid) at the leg. Stimuli were presented in a randomized sequence, separated by interstimulus intervals of at least 3 min. In independent experiments the time course of the stimulus was determined by way of colorimetric measurements, using a blue food (E131) dye (Fig. 1) and a video-recording system (Samsung SMX-F34 color digital camera) coupled to the stereomicroscope. Video information was stored on a computer as mpg files, converted into single frames and

analyzed for differences in pixel intensity (Adobe Photoshop CS2 software). Additional experiments with dye solution showed that three rinses with tap water were sufficient to eliminate completely any vestige of a previous stimulus from the perfusion system.

For behavioral trials, stimuli were dissolved in tap water and used at the three different concentrations 10^{-5}, 10^{-3} and 10^{-1} M, except for taurocholic acid (tested at 10^{-6}, 10^{-4} and 10^{-2} M). 10-ml aliquots of each compound were supplied in approx. 1 l of water contained in the stimulus delivery area (ratio 1:100) where complete mixing was achieved by agitation with a magnetic stir plate. Chemicals thus rapidly reached a final concentration of 10^{-7}, 10^{-5} and 10^{-3} M (10^{-8}, 10^{-6} and 10^{-4} M for taurocholic acid) advecting and diffusing throughout the holes into the crayfish area.

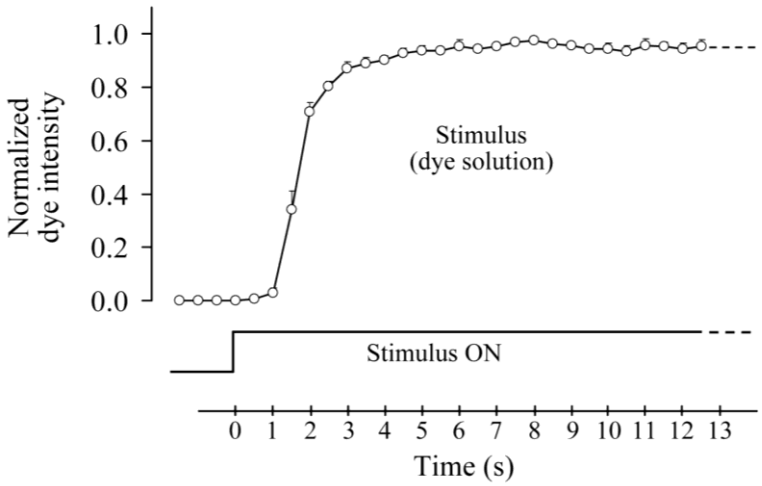

Fig. 1 Time course of stimulus delivery used during the electrophysiological experiments as monitored by colorimetric measurements (blue food dye E131). Normalized mean values in arbitrary units ± SE (vertical bars) from 5 replicates.

Statistical analysis

Results are expressed as the mean ± SE. One-way ANOVA was used to assess significant differences in spike firing frequencies to the tested compounds within the first 500 ms of the leg chemoreceptor responses. The electrophysiological data on the time-course and the adaptation rate of leg CRNs were estimated as follows: based on chemical stimulation and impulse recordings lasting 10 s, the declining sensitivity curves in response to the tested stimuli were plotted by counting the number of spikes generated

every 500 ms vs. the time interval of occurrence. Exponential decay curves were then fitted on data points by using non-linear regression analysis (GraphPad Prism 6.01 software). According to Ozaki and Amakawa (1992), the half-time ($t_{1/2}$), defined as the time it took the impulse frequency to decrease by 50% of the first count, was chosen as the main parameter to establish the speed of the sensitivity decline for each response curve. For a better comparison of the different time-courses evoked by the different stimuli, the two following other parameters were also considered: 1) the discharge plateau, defined as the spike firing frequency value at infinite time, which may be referred as an indicator of the discharge adaptation degree by also considering the steady residual response and 2) the discharge span, considered as the difference between the peak firing frequency and the plateau, and therefore an indicator of the overall magnitude of the response to a given stimulus. One-way ANOVA was used to compare the half-times, the discharge plateaus or the spans within each compound class (sugars or amino acids).

Multi-factorial analysis of variance (3-way ANOVA) was used to analyze the effectiveness of the tested compounds during behavioral trials and specifically to compare differences related to gender, appendage type (antennules vs. legs) and stimulus concentrations. Since no differences related to gender were detected, data were then analyzed by means of a 2-way ANOVA, by considering the appendage type and the different stimulus concentrations. ANOVA analyses were made using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). Post-hoc comparisons were conducted with the Fisher LSD test and P values < 0.05 were considered significant.

Results

General features of the leg chemoreceptor response

Chemically evoked responses were successfully recorded from the nerve fascicles of the sensory neurons located in the walking legs following stimulation with most of the tested stimuli. Responses were mostly of a mononeural type, but in some cases 2-3 units were active as resulted by the spike sorting analysis. In any case, the chemosensory cells did not display any spontaneous activity and appeared virtually silent in absence of stimulation.

One-way ANOVA indicated that within the first 500 ms of stimulation the leg CNRs displayed different responses to the tested compounds ($F_{9,106} = 15.564$, $P < 0.0001$; Fig. 2). Among the sugars, the disaccharide trehalose resulted the most stimulating compound, with a spike firing frequency of 29.06 ± 1.36 spikes/500 ms, followed by cellobiose ($P < 0.001$, Fisher LSD). The other disaccharides sucrose and maltose as well as the monosaccharides glucose and fructose were less effective than cellobiose (P <

0.01, Fisher LSD). The amino acids glycine and leucine evoked a response which was comparable with that to trehalose (P > 0.27, Fisher LSD), while that to asparagine was weaker and not dissimilar from those to sucrose, maltose and glucose (P > 0.44, Fisher LSD). Serine and threonine failed to evoke unambiguous responses from the leg CRNs and were not further considered. Also taurocholic acid showed a stimulating effectiveness, similar to that displayed by cellobiose (P = 0.74, Fisher LSD).

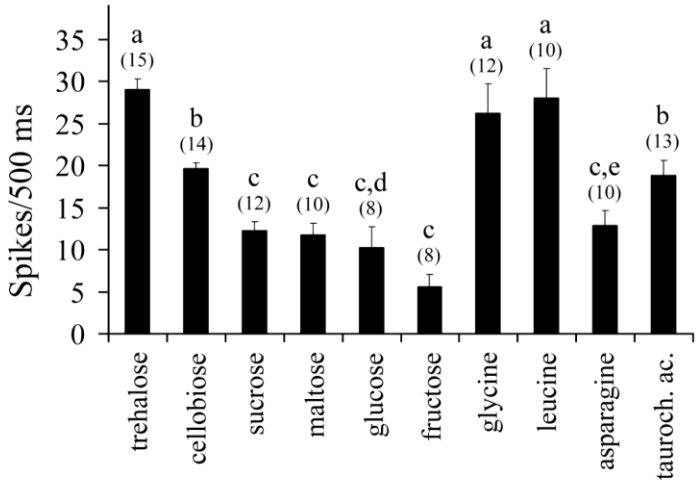

Fig. 2 Spike firing frequencies (spikes/s) recorded from the leg chemoreceptors following stimulation with the different tested compound. Mean values ± SE (vertical bars). Number of individual receptor cells recorded from is given in brackets. Bars followed by different letters are significantly different (P < 0.05; Fisher LSD test subsequent to one-way ANOVA).

Specificity and temporal pattern of the leg chemoreceptor response

The specificity and the time course of adaptation in individual chemosensory cells from the walking legs were investigated, over a 10 s period and at 500 ms intervals. Nonlinear regression analysis showed that the responses to all tested compounds decreased over time by following exponential, one-phase-decay kinetics. As also shown by sample recordings and spike templates of Fig. 3a and b, all coming from one same experiment on the same nervous fiber, di- and monosaccharides specifically activated a "sugar" receptor cell which, in turn, resulted insensitive to the other tested compounds. The tested sugars elicited different time courses of adaptation from the leg chemoreceptors according to the three parameters considered, half-time, discharge plateau and span (Fig. 4). In fact, the stimulation with trehalose produced a very phasic

response profile as compared to the phasic-tonic ones evoked by all the other sugars that never reached full adaptation within the stimulation interval.

In details, one-way ANOVA indicated that the responses to the sugars varied, even though to a different extent, in half-time ($F_{5,65} = 3.2208$, $P = 0.0117$), in discharge plateau ($F_{5,65} = 22.909$;, $P < 0.0001$) and span ($F_{5,65} = 59.695$, $P < 0.0001$). Post-hoc comparisons within half-times showed that the response to trehalose halved faster than those to all other sugars ($P < 0.02$, Fisher LSD), except for fructose, which anyway resulted the least stimulating compound. Instead, cellobiose, maltose, sucrose, glucose and fructose all evoked responses with comparable half-times. As for the discharge plateau, the response to trehalose exhibited the lowest value, while that to cellobiose the highest one with respect to the other sugars ($P < 0.01$, Fisher LSD), that, in turn, did not differ from one another. The response to trehalose also displayed the highest discharge span, while that to fructose the lowest one ($P < 0.01$, Fisher LSD); span for cellobiose was higher than that for glucose ($P < 0.05$, Fisher LSD), but comparable to those of both the other disaccharides maltose and sucrose. The time-courses and the adaptation rates of the three tested amino acids glycine, leucine and asparagine are depicted in Fig. 5. One-way ANOVA showed that the three amino acids differed from one another in both the half-times ($F_{2,29} = 3.5218$, $P < 0.05$) and the discharge span ($F_{2,29} = 8.149$, $P = 0.0015$), but not in plateau ($F_{2,29} = 0.3022$, $P = 0.7414$). Post-hoc comparisons for half-times indicated that the response to leucine halved faster than those to glycine and asparagine ($P < 0.05$, Fisher LSD), while no differences were detected in the response plateau ($P>0.05$, Fisher LSD). Besides, the discharge span to asparagine, the least stimulating among the three amino acids, was significantly lower with respect to both glycine and leucine ($P < 0.05$, Fisher LSD). Finally, also the taurocholic acid evoked a response with a phasic-tonic time course (Fig. 5), marked by an appreciable phasic component, with a half-time of 1.06 ± 0.11 s, a discharge plateau of 3.20 ± 0.49 spikes/500 ms and a span of 15.38 ± 1.76 spikes/500 ms.

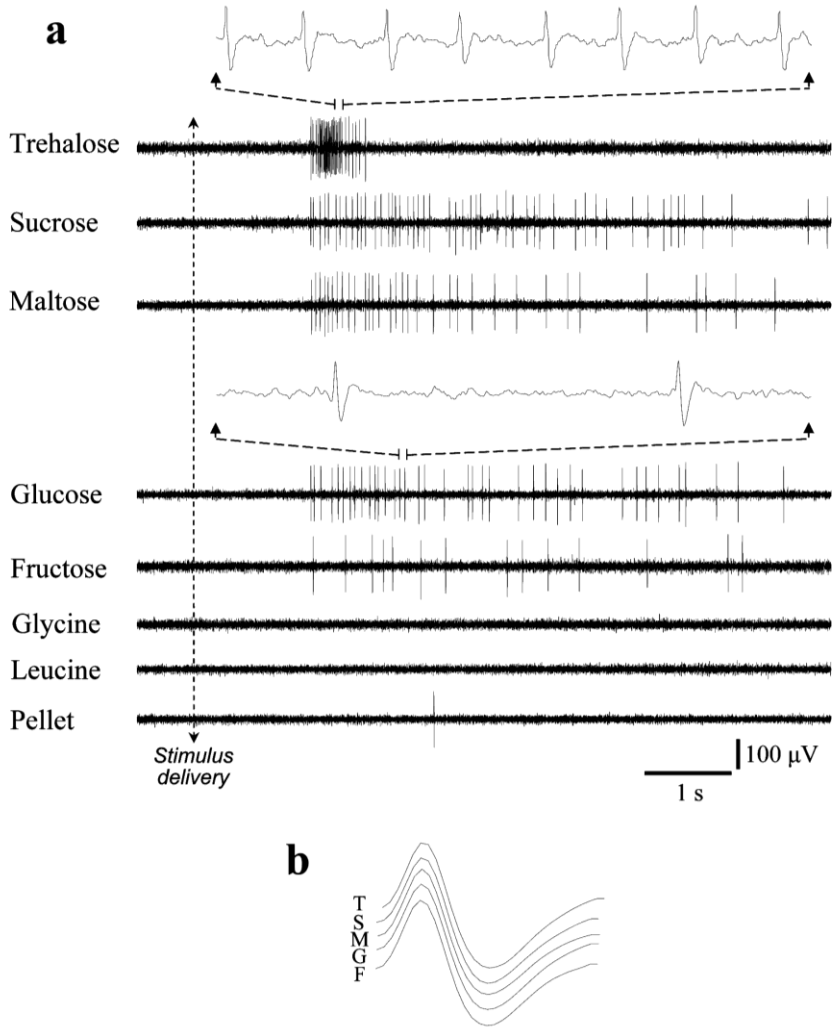

Fig. 3 a Representative samples of spike discharges from one same leg chemoreceptor in response to the different tested sugars, as compared with the response to glycine, leucine and the pellet food (control). **b** Spike templates obtained from the leg chemoreceptor recordings shown in a following stimulation with trehalose (T), sucrose (S), maltose (M), glucose (G) and fructose (F).

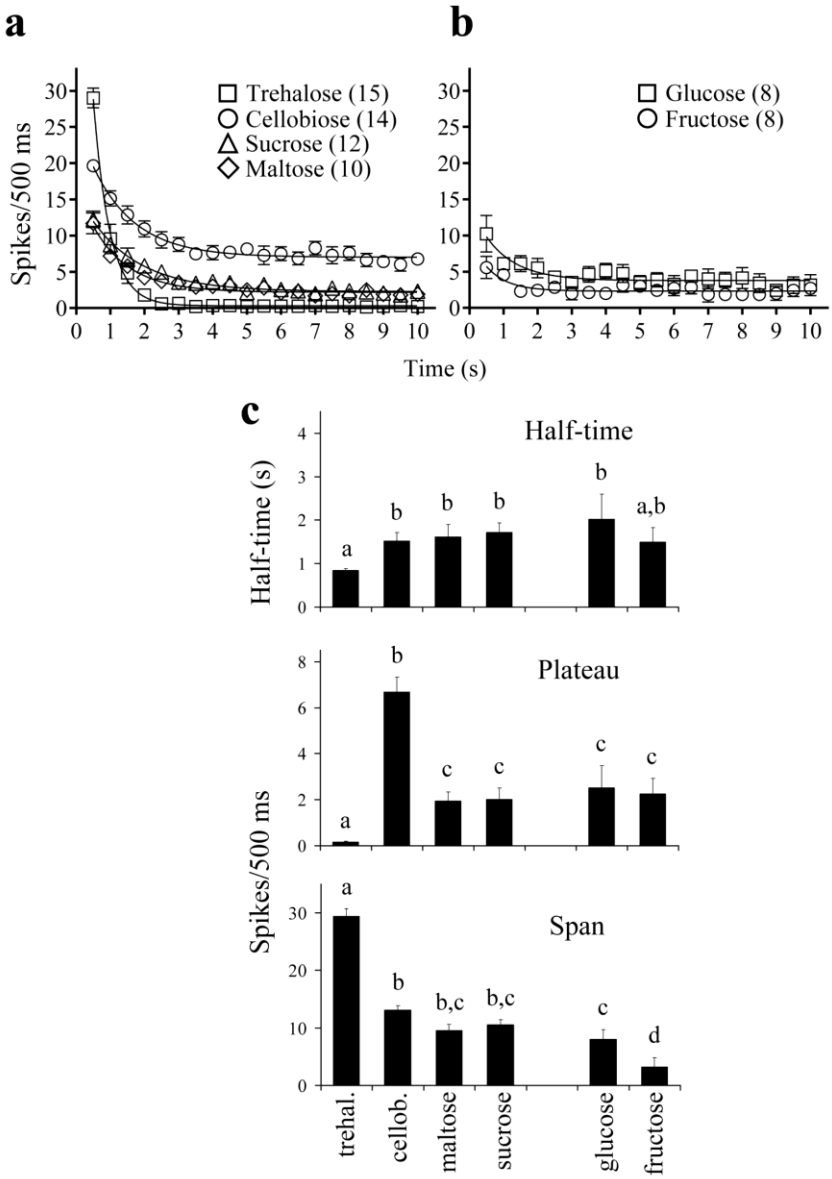

Fig. 4 Declining sensitivity curves of the response of leg CRNs following long-lasting (10 s) stimulation with the tested disaccharides (**a**) and monosaccharides (**b**). **c** Half-time, discharge plateau and span values of the responses to the same chemicals. Mean values ± SE (vertical bars). Number of individual receptor cells recorded from is given in brackets. Bars followed by different letters are significantly different ($P < 0.05$; Fisher LSD test subsequent to one-way ANOVA).

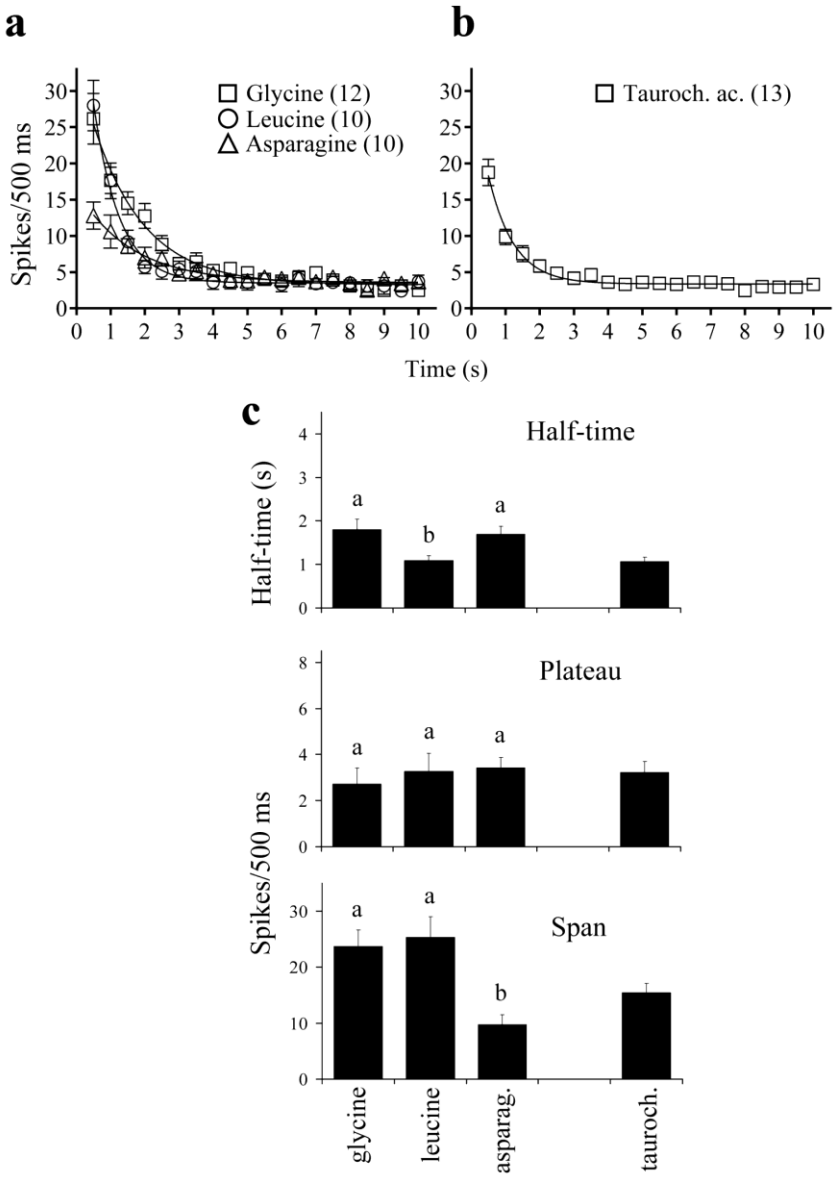

Fig. 5 Declining sensitivity curves of the response of leg CRNs following long-lasting (10 s) stimulation with the tested amino acids (**a**) and taurocholic acid (**b**). **c** Half-time, discharge plateau and span values of the responses to the same chemicals. Mean values ± SE (vertical bars). Number of individual receptor cells recorded from is given in brackets. Bars followed by different letters are significantly different ($P < 0.05$; Fisher LSD test subsequent to one-way ANOVA).

Behavioral experiments

Mean values ± SE of both the walking leg movements and the antennular flicking activity in response to the different compounds are shown in Figs 6 and 7. A preliminary 3-way ANOVA revealed, for each compound, no significant interactions of the appendage type (antennules and legs) and of the stimulus concentration across gender for sugars (maltose: $F_{3,64} = 0.028$, $P = 0.892$; cellobiose: $F_{3,56} = 1.17$, $P = 0.33$; sucrose: $F_{3,56} = 0.16$, $P = 0.92$; fructose: $F_{3,48} = 0.4$, $P = 0.787$; glucose: $F_{3,56} = 0.39$, $P = 0.760$; trehalose: $F_{3,48} = 0.46$, $P = 0.714$), for amino acids (glycine: $F_{3,56} = 0.44$, $P = 0.728$; leucine: $F_{3,72} = 0{,}70$, $P = 0.555$; asparagine: $F_{3,56} = 0.27$, $P = 0.847$) and also for taurocholic acid ($F_{3,64} = 0.85$, $P = 0.472$). Since no sex related differences were found, data from males and females were pooled and analyzed, for each compound, with a 2-way ANOVA, by including in the analysis only the stimulus concentrations and the two appendage types, for which a significant two-way interaction was detected.

In details, as for the disaccharides trehalose ($F_{3,48} = 3.615$, $P = 0.0196$) and cellobiose ($F_{3,56} = 10.14$, $P = 0.0001$), but also the monosaccharides fructose ($F_{3,56} = 5.8$, $P = 0.002$) and glucose ($F_{3,48} = 6.996$, $P = 0.0005$), the post-hoc comparison showed that the leg movements increased, with respect to the blank, regardless of the stimulus concentration ($P < 0.0020$, Fisher LSD; Fig. 6). In the case of glucose, the response to the highest tested concentration resulted significantly higher than those to the other concentrations ($P < 0.03$, Fisher LSD). Conversely, no variation in flicking activity was detected following stimulation with all these sugars. Comparison between the activity of the two appendage types displayed a statistical difference at any tested concentration ($P < 0.007$, Fisher LSD). In the case of maltose ($F_{3,64} = 4.574$, $P = 0.006$) and sucrose ($F_{3,64} = 4.11$, $P = 0.01$), the post-hoc comparison showed that the movements of the walking legs increased, with respect to blank, following stimulation with the concentrations 10^{-5} and 10^{-3} M ($P < 0.01$, Fisher LSD). Also in these cases, the antennules were not responsive to maltose and sucrose, the flicking activity being lower than the leg response at any tested concentration for sucrose and at the two highest ones for maltose ($P < 0.0060$, Fisher LSD). ANOVA revealed a significant two-way interaction between antennular flicking and movement of legs also among the amino acids (glycine: $F_{3,56} = 3.36$, $P = 0.025$; leucine: $F_{3,72} = 2{,}72$, $P = 0.048$; asparagine: $F_{3,56} = 6.86$, $P = 0.001$; Fig. 7). In particular, the post-hoc comparison showed that the leg movements increased at increasing concentration of glycine, the response to blank being lower than those to the two highest concentrations ($P < 0.03$, Fisher LSD), and also a significant increase at the concentration 10^{-3} M was found with respect to 10^{-7} M ($P < 0.02$, Fisher LSD). Similarly to what observed for all tested sugars, the antennules were not responsive to glycine. The activity of legs was higher than that of antennules at 10^{-5} and 10^{-3} M ($P < 0.01$, Fisher

LSD). As for leucine, the post-hoc comparison showed that the leg movements increased, with respect to the blank, regardless of the stimulus concentration ($P < 0.0070$, Fisher LSD). Once again, no variation in flicking activity was detected following stimulation with leucine, the activity of the legs being different from that of the antennules at any tested concentration ($P < 0.0010$, Fisher LSD). The response to asparagine was more complex. In fact, the post-hoc comparison showed that at any concentration the leg activity was higher than that to the blank. In the case of this amino acid, an increase in antennular flicking was also detected, even though only at the highest concentration ($P < 0.01$, Fisher LSD). Differences between legs and antennules were therefore present only at the concentrations 10^{-7} and 10^{-5} M ($P < 0.001$, Fisher LSD). A significant interaction between antennular flicking and leg movements was found also for taurocholic acid ($F_{3,64} = 4.6$, $P = 0.005$). The post-hoc comparison showed that the movements of the legs increased, with respect to blank, at any tested concentration ($P < 0.0008$, Fisher LSD), and also a significant increase of the response was present at the concentration 10^{-3} M with respect to 10^{-7} M ($P < 0.02$, Fisher LSD). No activity was observed in the antennular responses to taurocholic acid, that were invariantly lower than those of the legs ($P < 0.04$, Fisher LSD).

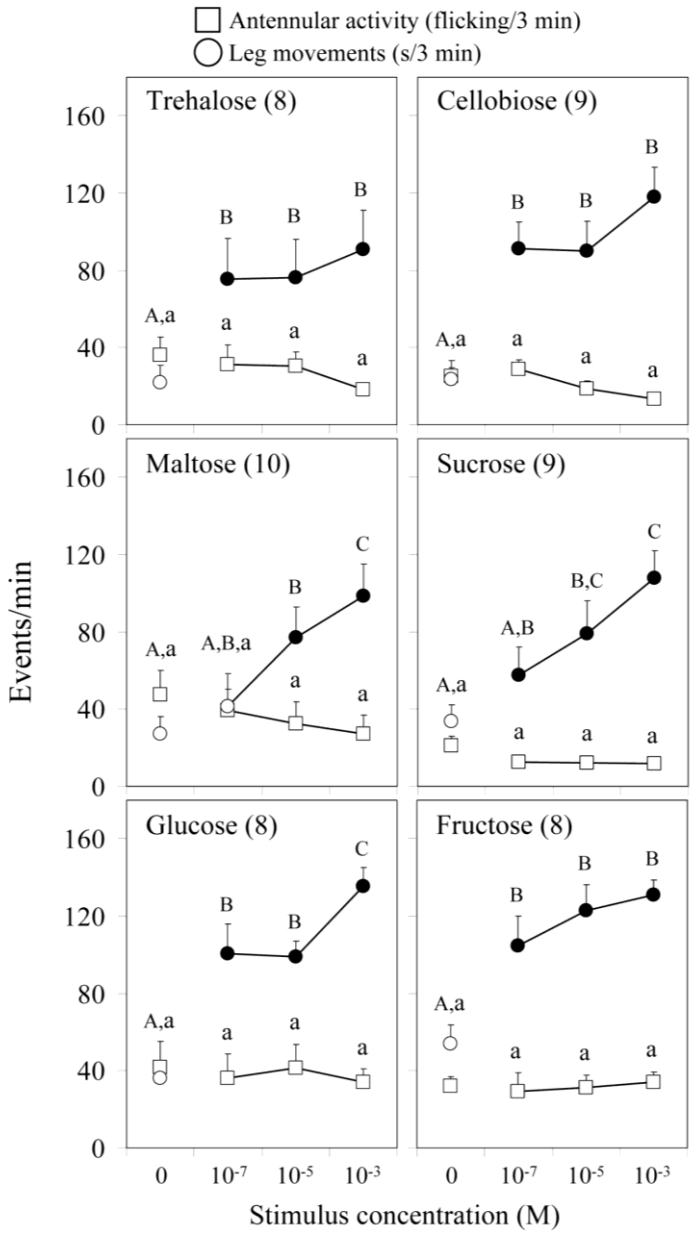

Fig. 6 Frequency of antennular flicking and duration of leg movements determined over a 3-min interval during stimulation with the different sugars in the whole crayfish bioassay. Mean values ± SE (vertical bars); number of replicates for each compound is indicated in brackets. Bars followed by different letters for a given compound (lowercase for antennules, uppercase for legs) are significantly different ($P < 0.05$; Fisher LSD test subsequent to two-way ANOVA). Filled symbols represent the significant differences between leg and antennular responses at a given stimulus concentration ($P < 0.05$; Fisher LSD test subsequent to two-way ANOVA).

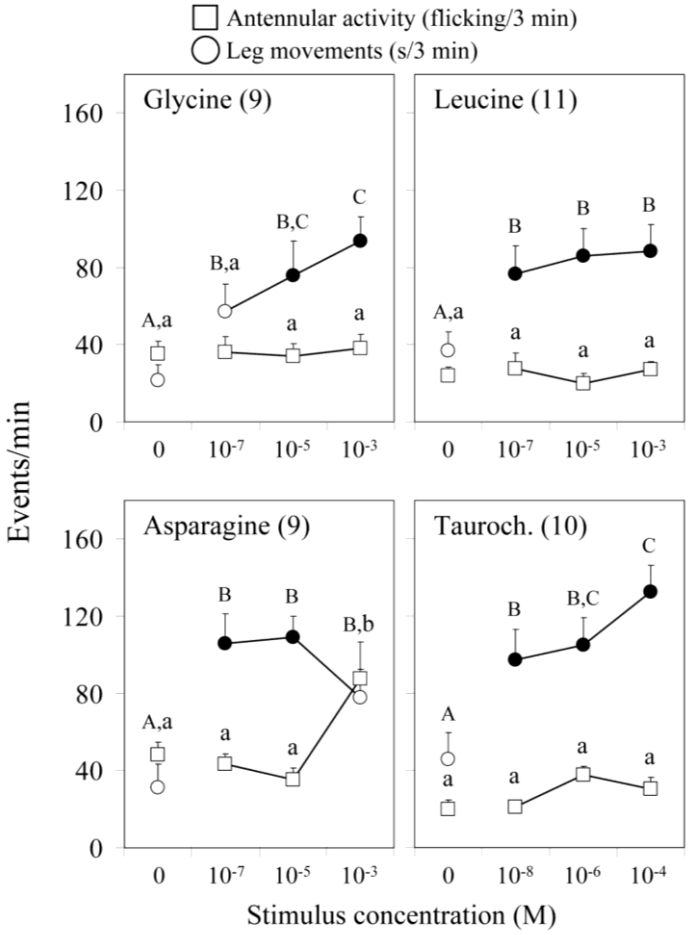

Fig. 7 Frequency of antennular flicking and duration of leg movements determined over a 3-min interval during stimulation with the different amino acids and taurocholic acid in the whole crayfish bioassay. Mean values ± SE (vertical bars); number of replicates for each compound is indicated in brackets. Bars followed by different letters for a given compound (lowercase for antennules, uppercase for legs) are significantly different ($P < 0.05$; Fisher LSD test subsequent to two-way ANOVA). Filled symbols represent the significant differences between leg and antennular responses at a given stimulus concentration ($P < 0.05$; Fisher LSD test subsequent to two-way ANOVA).

Discussion

The present electrophysiological and behavioral investigation extends our knowledge about the chemoreception properties in decapod crustaceans, by adding data on the ability of the crayfish *P. clarkii* to detect a number of food-related compounds by means of the sensory input provided by its walking legs.

First, our electrophysiological results confirm the sensitivity of the legs to a number of sugars like the disaccharides trehalose, cellobiose, sucrose, maltose and to the amino acids glycine and leucine, as previously reported by Corotto and O'Brien (2002). Trehalose, glycine and leucine resulted the most stimulating compounds, at least in the initial, phasic part of the response. In addition, we found a stimulating effectiveness for the amino acid asparagine, that had not been tested on *P. clarkii* yet, and also for the two monosaccharides glucose and fructose and for the taurocholic acid, all compounds that Corotto and O'Brien (2002) found ineffective.

Behavioral results are consistent with the electrophysiological findings, as all compounds found to stimulate the isolated leg CRNs also led to an increase in the movements of the walking legs coupled to dactyl probing. This expands the previous behavioral findings reporting trehalose, maltose, glucose and glycine as stimulants for the legs of *P. clarkii* (Corotto et al. 2007). Clearly, the design of our bioassay does not exclude that this behavior be also mediated, at least in part, by stimulation of other sensory appendages. The apparent discrepancy between our electrophysiological results and those by Corotto and O'Brien (2002) could be explained by considering that we used a 100-fold higher stimulus concentration (10 mM). On the other hand, in a subsequent behavioral study also Corotto et al. (2007) reported a stimulating effect exerted by high glucose concentrations and therefore concluded that receptor cells responsive to glucose were present in the legs of *P. clarkii*. Thus, our data support the hypothesis that CRNs sensitive to glucose, and possibly to several other compounds like fructose and taurocholic acid, may be present in the legs, but only responsive at high stimulus concentrations.

When dealing with large receptor populations, such as those typical of crustaceans and, specifically, of crayfish legs (Sutherland and Nunnemacher 1968), experimental samples on a limited number of cells may be only partially representative of the whole population of sensory neurons, and therefore a lack of response does not necessarily imply that the appendage is insensitive to a given compound. Our results suggest that the legs of *P. clarkii* are sensitive to a broad-spectrum of food-related compounds, but further investigation is needed to discover novel or undetected sensitivity towards a potentially very wide range of stimulants. This is consistent with the omnivorous/scavenger feeding habits of crayfish (Tierney and Atema 1988; Momot

1995) and, in particular, of *P. clarkii*, a generalist consumer of invertebrates in combination, depending on the prey availability, with macrophytes, algae and detritus (Alcorlo et al. 2004). Our electrophysiological data also show, at least in a number of cases concerning the responses to sugars, for which mononeural discharges were recorded, that one same CRN is sensitive to the different sugars tested, both mono- and disaccharides, but not to other compounds like the amino acids glycine and leucine. This fact suggests that the CRNs, or at least subpopulations of CRNs, responsive to sugars in the legs are relatively broadly tuned, so as to respond to a class of related compounds, in our case the carbohydrates, instead to single chemicals. This property for sugar CRNs is in agreement with what previously reported for the crayfish *Orconectes limosus*, where different types of single units were found to be sensitive either to amino acids, amines or pyridines, with the amino acid-responding units that, even if limited in their response range to that particular class of molecules, displayed no or little selectivity within the class (Bauer and Hatt 1980; Bauer et al. 1981). In this respect, crayfish seem to differ from marine crustaceans, where the spectral sensitivity usually tends to be more narrowly tuned. However, this kind of information mainly concerns the sensory appendages as sensors of good quality food indicators in relation to a carnivorous diet, such as amino acids and other nitrogen compounds like taurine, hydroxyproline, glutamate, nucleotides or ammonium, rather than to carbohydrates (Johnson et al. 1985; Tierney et al. 1988; Derby et al. 1991; Voigt and Atema 1992; Zimmer-Faust 1993; Derby 2000; Schmidt and Mellon 2011). In any case, the possibility exists that the evolutionary pressure for food search may have led, in *P. clarkii* and possibly in other crayfish species, to a relatively broadly tuned and less discriminative sensory strategy of the leg CNRs that could be well suited to the omnivorous/scavenger feeding habits of the crayfish.

As far as we know, little attention has been devoted to the study of dynamic properties of crustacean chemosensory cells, so that we mainly focused our interest in the time course and in the adaptation rate of the leg CRNs, by keeping in mind that such information may also account for matching of the cells to fluctuations in stimulus intensity (Atema 1985). It is commonly accepted that cells with short, phasic responses can efficiently encode rapid variations in stimulus concentration, while slowly adapting cells are better suited to monitor the time course of prolonged stimuli; however, these two coding modalities could also fulfil the need to discriminate between stimuli that, at a same concentration, might qualitatively differ in their molecular properties. To do this, and also according to Ozaki and Amakawa (1992), we selected, as the main parameter, the half-time, in combination with both the plateau discharge and the span amplitude. The lower are both the half-time and the discharge plateau value, along with a high span,

the faster is the discharge adaptation rate, i.e. more phasic the response, to a given stimulus. On this basis, we observed that, following long lasting (10 s) sugar supply at high concentrations (10 mM), leg CRNs were found to rapidly and completely adapt, thus displaying a short phasic response, only to the disaccharides trehalose (shortest half-time coupled to plateau values near zero spikes and maximal span; Fig. 4).

Conversely, all other compounds elicited, at that same concentration, phasic-tonic responses, with a decline in sensitivity that, on an arbitrary scale of response phasicity, placed the two amino acids leucine and glycine immediately after threalose.

The phasic nature in the response to both leucine and glycine is in good agreement with what previously reported for the crustacean chemosensory cells sensitive to amino acids, related substances, or purine nucleotides, in both marine decapods and crayfish (Bauer et al. 1981; Derby and Atema 1982a; Hatt 1984; Schmidt and Gnatzy 1989; Garm et al. 2005). Such information on carbohydrates and especially on the phasic response pattern to threalose was still undocumented, also because only a few crustaceans were previously found to display sensitivity to sugars (Tierney and Atema 1988; Rittschof and Buswell 1989; Corotto and O'Brien 2002; Corotto et al. 2007). The possibility exists that also the dynamic properties of the leg CRNs could be somewhat tuned to the feeding habits of *P. clarkii*. For instance, glycine and leucine are commonly present at high concentrations in many invertebrate tissues (Awapara 1962) and therefore represent protein food indicators for the carnivorous provision in the crayfish diet. Instead, carbohydrates are more likely indicators for the vegetarian counterpart of the diet, as sugars usually occurs in the macrophytes and in plant detritus (Ljungdahl and Eriksson 1985; Mohr and Schopfer 1995) that *P. clarkii* consumes as well. In this respect, trehalose represents an anomalous carbohydrate; in fact, although chemically belonging to the class of the sugars, from a feeding point of view for the crayfish it represents an indicator of a protein diet, since it is a hemolymph sugar commonly found in the body fluid of invertebrates (Fairbairn 1958). In spite of the differences found in the dynamic properties of leg CRNs, the behavioral patterns elicited by the crayfish in response to the various compounds were not dissimilar from one another, thus indicating that qualitatively different sensory inputs may lead to a common behavioral stereotype for food search. The lack of a dose-response relationship mostly registered during the behavioral trials, especially at the lowest concentrations, also suggests that the sensitivity of the legs is not so fine as to precisely discriminate among 100-fold step concentration increases of stimuli supplied in a sequence. In this respect, the crayfish chemosensory system may act as a detector of relative rather than absolute stimulus intensity, by re-setting the response threshold to a zero-level in the presence of constant background

chemical noise, similarly to what previously described in lobsters (Borroni and Atema 1988).

As far as we know, this is the first report for a sensitivity to taurocholic acid in crustaceans and, more specifically, in crayfish. Even if at present the significance of this compound for *P. clarkii* remains unknown, we cannot exclude that, rather than being a food indicator (it occurs in feces of freshwater fishes (Zhang et al. 2001)), it may also represent a semiochemical for social and/or territorial interactions, as reported for freshwater habitats (Martinovic-Weigelt et al. 2012). Further investigations in this and other crustacean species are required to elucidate this aspect. It is noteworthy that, unlike what observed for the legs, none of the tested compounds specifically elicited antennular flicking in the crayfish, except for asparagine at the highest concentration. This fact highlights the critical role of the legs in food detection and search, but also strengthens the belief that the antennules, even if reported to also perceive food odors (Giri and Dunham 1999), might primarily serve alternative roles, such as sex discrimination, reproduction and social status assessment (Ameyaw-Akumfi and Hazlett 1975; Dunham and Oh 1992; Giri and Dunham 2000; Horner et al. 2008). The fact that animals with ablated antennules were still successful in locating food sources in a confined arena (unpublished results) supports the idea that they may not be needed for short-distance food sensing, as previously suggested by Ameyaw-Akumfi (1977) and Giri and Dunham (1999), their input being overridden by that of legs or other chemoreceptive organs. As for asparagine, it could represent a key compound for crayfish as it stimulates CRNs from both the legs and the antennules. This amino acid, occurring in plants and also in microorganisms and animal tissues either as a free amino acid or incorporated into proteins (Meister 1965), was reported to weakly stimulate the legs also in the crayfish *Orconectes limosus* (Bauer et al. 1981) and both the antennules and the legs in lobsters (Derby and Atema 1982b; Tierney et al. 1988). Asparagine was also suggested as an essential amino acid for crayfish (Van Marrewijk and Zandee 1975), hence the importance to sense it more finely than other feeding substrates.

In conclusion, the present findings add relevant information on the ability of the crayfish *P. clarkii* to detect food-related compounds by way of the sensory input provided by the legs. While confirming a marked sensitivity of the legs to the disaccharides trehalose, cellobiose, sucrose and maltose and to the amino acids glycine and leucine, we also report novel sensitivity to the monosaccharides glucose and fructose, to the amino acid asparagine (potential food indicators) and to taurocholic acid, the sugar-sensitive CRNs being broadly tuned to this chemical class, instead of to its single components. In terms of dynamic properties, the leg CRNs show highly phasic responses to trehalose (hemolymph sugar in the body fluid of many invertebrates),

phasic to glycine and leucine and phasic-tonic to the other tested compounds, as whether phasicity of chemoreceptor responses were an additional property for better discrimination of the protein components in the diet from other stimuli. Results of our behavioral bioassays also exclude, at least in confined experimental arenas, any involvement of antennules in the detection of food-related compounds, and together with the electrophysiological findings emphasize the role of the legs as the main short-distance, broad-spectrum sensors for food detection, coherently with the omnivorous/scavenger habits of this animal.

This information gains further importance in the light of the invasive attitude of *P. clarkii* throughout the world. In fact, by providing knowledge on the crayfish ability to detect new chemicals, our study may contribute, from an applied point of view, to the discovery of attractive key compounds that could help improving the efficiency of mass trapping strategies aimed at the reduction and/or the eradication of the crayfish populations. In this respect, as a follow-up of the present study we shall evaluate the attractiveness of these stimuli on *P. clarkii* and especially if they may surpass, alone or in blend, the efficacy of the baits traditionally employed in crayfish mass trapping.

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Section 2 of thesis

PROP bitter taste sensitivity and its nutritional implications in Humans

General introduction

The ability to distinguish nutrient-rich food sources from noxious substances is essential for an animal's survival [1]. Although olfaction and vision participate to food identification, taste provides the final checkpoint for food acceptance or rejection. Furthermore, taste perception is one of the most important determinants that strongly influences the food choice, eating behavior and therefore the nutritional status and health of the individual. In fact, taste perception and food preferences have been extensively reported as factors influencing eating behavior and body mass [2,3].

Anatomy and physiology of taste

It is generally assumed that the sense of taste can differentiated five distinct sensory qualities: sweet, umami, sour, salty, and bitter (Fig. 1)

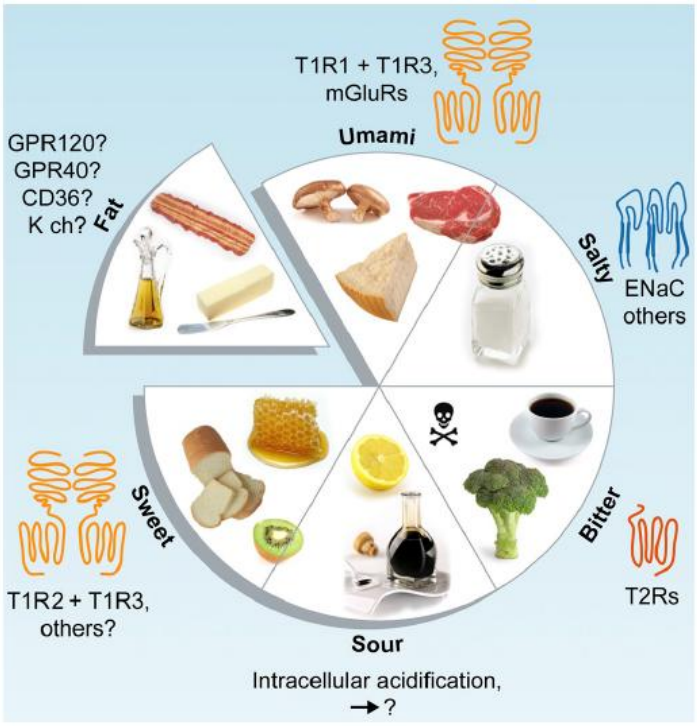


Figure 1 - Taste qualities, the taste receptors that detect them, and examples of natural stimuli. Five recognized taste qualities: sweet, sour, bitter, salty, and umami. They are detected by taste buds. Bitter taste is thought to protect against ingesting poisons, many of which taste bitter. Sweet taste signals sugars and carbohydrates. Umami taste is elicited by l-amino acids and nucleotides. Salty taste is generated mainly by Na+ and sour taste potently by organic acids. Evidence is mounting that fat may also be detected by taste buds via dedicated receptors [4].

There are likely additional qualities such as fatty, metallic, and others that might also be considered basic tastes [4]. Each of these is believed to represent different nutritional or physiological requirements or indicate potential dietary risk so the primary taste categories reflect complementary strategies to obtain essential nutrients and avoid harmful chemicals [5,6]. Sweet, umami, and salty are associated with specific classes of nutrients and they are perceived as good and pleasant at low and moderate concentrations but are avoided at high concentrations [7]. Sweet taste usually indicates the detection of sugars soluble carbohydrates that serve as an energy source. In addition to sugars (*e.g.*, fructose, glucose, and sucrose), other compounds can be perceived as sweet tasting by humans [6] such as D-amino acids, peptides (*e.g.*, aspartame), certain organic anions (*e.g.*, saccharin), and some proteins (*e.g.*, monellin and thaumatin). The taste of umami is associated with the taste of L-glutamate and a few other L-amino acids, reflects a food's protein content [4] and this taste is induced by glutamic acid, inosinic acid, and guanylic acid, which exist in salt form, usually as monosodium glutamate (MSG), disodium inosinate (IMP), or disodium guanylate (GMP) [8]. Umami is often described as "savory" or "meaty", although many foods in addition to meat contain these compounds. Salty taste indicates the presence of sodium (Na+) and other salts as lithium, or potassium [9], essential for maintaining the homeostasis in the body [10,11]. Unlike sweet, umami, and salty, tastes categorized as bitter and sour are associated with compounds that are potentially harmful. In fact the perception of bitter taste is associated with different compounds including alkaloids (*e.g.*, caffeine, strychnine, quinine, and glycosides) and it is also considered innate aversion and is thought to guard against consuming poisons or that can be toxic or inhibit digestion. Sour taste, like bitter taste, is generally considered innate aversion, this quality is important for detection of acid (*i.e.*, free protons or H+ ions) and this can be useful to avoid ingesting excess acids and overloading the mechanisms that maintain acid–base balance for the body [4]. Although, acceptable at low concentrations, sour taste elicits a rejection response at higher concentrations and can be used to detect unripe fruits and spoiled foods [10,12]. Nonetheless, people learn to tolerate and even seek out certain bitter- and sour-tasting compounds such as caffeine and citric acid (e.g., citrus fruits), overcoming innate taste responses. Variations of taste preference may arise from genetic differences in taste receptors and may have important consequences for food selection, nutrition, and health [13-16]. The degree of pleasantness of taste, however, is subjective and can be influenced by experience and nutritional needs. Both animals and humans tend to reject food from which they have been intoxicated in the past. The same food can be perceived as very pleasant and desirable at the beginning of a meal and unpleasant when the subject

reaches its fill. According to gustatory and olfactory information we have the ability to make a choice between different foods and to choose the one that provides the most appropriate nutritional needs for the body.

Taste in humans begins with the activation of taste cells where taste reception and signal transduction mechanisms are located. Taste buds are aggregates in groups of 50-100 polarized neuroepithelial cells [17,18] that form compact, columnar pseudostratified "islands" embedded in the surrounding stratified epithelium of the oral cavity [4]. The human gustatory system includes approximately 5000 taste buds in the oral cavity, situated on the superior surface of the tongue, and also isolated taste buds are scattered on the surface of the palate and throat and on the epiglottis [19]. On the tongue, taste buds are grouped in specialized structures called gustatory papillae of the lingual epithelium of gustatory papillae. Three different morphological structures of taste papillae are topographically arranged on the tongue: Fungiform papillae (mushroom-shaped) are located on the anterior two-thirds of the tongue and are more densely concentrated toward the tip, foliate papillae (leaf-shaped) on the lateral sides, and circumvallate on the posterior two-thirds (Figure 2). There are also filiform papillae located across the entire superior surface, but these do not contain taste buds (*i.e.*, they are non-gustatory). Every taste bud consists of a single apical pore where microvilli of taste receptor cells (TRCs) come into contact with tastants present within the oral cavity (Figure 3).

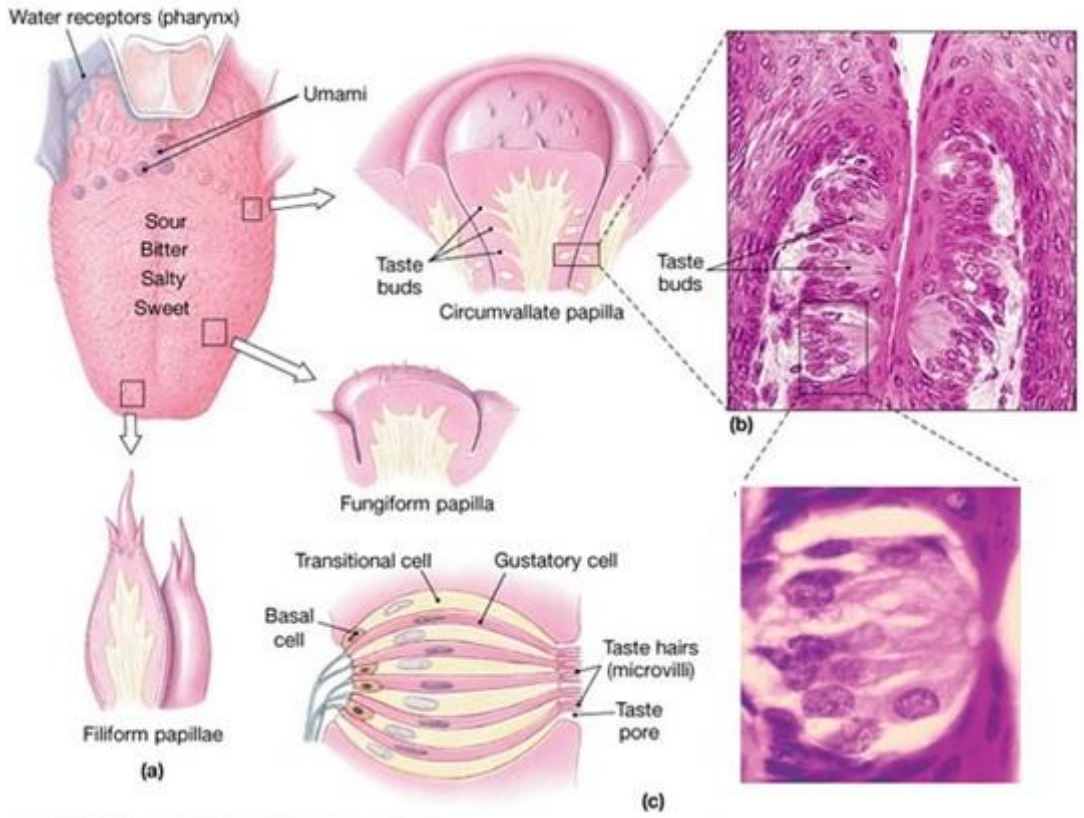


Figure 2 - Lingual gustatory papillae and taste buds (A) The lingual papillae on the superior surface on the tongue, the circumvallate papillae are relatively large and are surrounded by deep epithelial folds; the fungiform papillae have typical mushroom-shaped and filiform papillae provide friction that helps objects around in the mouth but not contain taste buds. (B) Taste buds in circumvallate papillae (C) Diagrammatic view of a taste bud that shows details of gustatory cells and supporting cells and the taste pore.

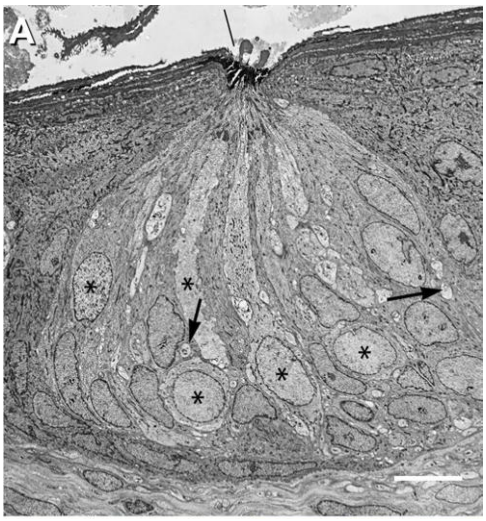
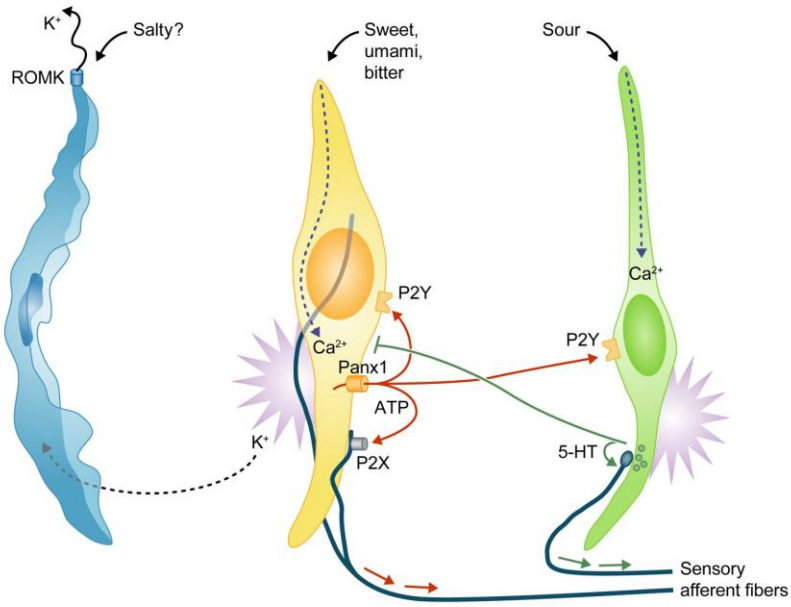


Figure 3 - Electron micrograph of a taste bud showing cells with dark or light cytoplasm, and nerve profiles (arrows). Asterisks mark Type II (receptor) cells [20]. Each taste bud contains four cell types: Type I, Type II, Type III, and Type IV.

Each taste bud contains four cell types: Type I, Type II, Type III, and Type IV. Type I cells (or dark cell) are the most abundant cells in taste buds, with extended cytoplasm lamellae that engulf other cells. This cell are termed ‘‘glial-like’’ because they appear to be involved in synaptic transmission and limiting the spread of transmitters [4]. In fact the type I cells express different neurotransmitter clearance, in particular express GLAST, a transporter for glutamate, indicating that they may be involved in glutamate uptake [21] and also express NTPDase2, a plasma membrane–bound nucleotidase that hydrolyzes extracellular ATP [22] that it serves as a neurotransmitter in taste buds [23]. Type I cells may regulate the ionic milieu [24,25], in effect express ROMK, a K channel that may be involved in K+ homeostasis within the taste bud [25]. During prolonged trains of action potentials elicited by intense taste stimulation, type I cells may serve to eliminate K+ (see blue cell in Fig. 4) that would accumulate in the limited interstitial spaces of the taste bud and lead to diminished excitability of Type II and III cells. Thus, type I cells appear overall to function as glia in taste buds. Lastly, type I cells may exhibit ionic currents implicated in salt taste transduction [26]. The type II (or light cells) are spindle-shaped cells with a large, round, clear nucleus, this cells were also renamed “receptor” cells [17] because this type of cells contain, in the integral plasma membrane, elements necessary for taste transduction, such as the receptors for bitter, sweet, and umami compounds. These cells are considered the primary receptor cells in the taste bud [7,17,27-29]. These type II cell express G protein–coupled receptors (GPCR) with seven trans-membrane domains, specific for only one taste quality, such as sweet or bitter, but not both [30]. Type II cells also express voltage-gated Na and K channels essential for producing action potentials, and secretion of ATP (yellow cell in Fig. 4). In brief, Type II cells are “tuned” to sweet, bitter, or umami taste [31] but this cells do not appear to be directly stimulated by sour or salty stimuli [4].The type III cells share many neuron-like properties, they are characterized by synapses with afferent sensory nerves and are called presynaptic, or synaptic cells (green cell in Fig. 4) [17,32]. These cells express enzymes for the synthesis of at least two neurotransmitters and voltage-gated Ca channels typically associated with neurotransmitter release [17,33] and showing depolarization-dependent Ca^{2+} transients typical of synapses. Like receptor cells, presynaptic cells also are excitable and express a complement of voltage-gated Na and K channels to support action potentials [34-37]. In addition to these neuronal properties, presynaptic cells also respond directly to sour taste stimuli and carbonated solutions and are presumably the cells responsible for signaling these sensations Huang [31,38-40]. The presynaptic cells, in the contrary to receptor cells, are not tuned to specific taste qualities but instead respond generally to sweet, salty, sour, bitter, and umami compounds [31]. Type IV cells are basal and a nonpolarized, presumably undifferentiated or immature taste cells

(progenitor cells) [41]. Basal cells are small round cells at the base of the taste bud that are thought to be stem cells from which other cells are derived during cell turnover [32].



Type I glial-like cell	
Neurotransmitter clearance	
GLAST	Glutamate reuptake
NTPDase2	Ecto-ATPase
NET	Norepinephrine uptake
Ion redistribution and transport	
ROMK	K^+ homeostasis
Other	
OXTR	Oxytocin signaling?

Type II receptor cell	
Taste transduction	
T1Rs, T2Rs	Taste GPCRs
mGluRs	Taste GPCRs
Gα-gus, Gγ13	G protein subunits
PLCβ2	Synthesis of IP3
TRPM5	Depolarizing cation current
Excitation and transmitter release	
$Na_v1.7$, $Na_v1.3$	Action potential generation
Panx1	ATP release channel

Type III presynaptic cell	
Surface glycoproteins, ion channels	
NCAM	Neuronal adhesion
PKD channels	Sour taste?
Neurotransmitter synthesis	
AADC	Biogenic amine synthesis
GAD67	GABA synthesis
5-HT	Neurotransmitter
Chromogranin	Vesicle packaging
Excitation, transmitter release	
$Na_v1.2$	Action potential generation
$Ca_v2.1$, $Ca_v1.2$	Voltage-gated Ca^{2+} current
SNAP25	SNARE protein, exocytosis

Figure 4 - The three major classes of taste cells. The Table below shows the list of some proteins that are expressed in a cell type [4].

The transduction pathways in the gustatory system involve a variety of mechanisms and appear to differ from the transduction mechanisms of the other special senses [12,32,42] and also the five basic tastes are recognized in different pathways. Salty and sour taste sensations are both detected through ion channels. Sweet, bitter, and umami tastes, however, are detected by way of G protein-coupled taste receptors with seven transmembrane domains. In particular this qualities are detected by the two families of taste receptors TAS1R and TAS2R (T1R and T2R) [13] located in the apical microvilli of Type II cells [27,29,30,43-45]. These taste receptors have similar signaling effectors,

as stated above, sweet, umami and bitter compounds are the taste receptor-associated G protein gustducin (GPCRs), each of this quality activate different taste GPCRs that are expressed in discrete sets of receptor cells. Ligands binding at the taste receptors activate second messenger cascades to depolarize the taste cell. During sweet, umami, and bitter transduction, the compounds act as agonists, binding to GPCRs and resulting in the initiation of signal transduction cascades. GPCRs couple to specific intracellular G-proteins. The G-protein subunit α-gustducin (Gα gustducin) participates in bitter and sweet taste transduction [27,30,46-51]. The subunit Gγ13 is also involved in bitter taste transduction [52]. The human TAS1R family contains just 3 genes, *TAS1R1*, *TAS1R2*, and *TAS1R3*. The sugar taste is identified of the receptor cells expressing the heterodimer T1R2/T1R3 it can detect also synthetic sweeteners, and sweet-tasting proteins such as monellin and brazzein [30,53,54]. A second class of receptor cells expresses the heterodimeric GPCR, T1R1/T1R3, which responds to umami stimuli, particularly the combination of l-glutamate and compounds that accumulate in many foods after hydrolysis of proteins [43,44]. The bitter taste is identified of the TAS2R (T2R) family of GPCRs. [55]. There are 25 apparently functional *TAS2R* genes in humans encode members of the T2R family, whose products are responsible for bitter perception [55-57]. These taste receptors exhibit heterogeneous molecular receptive ranges: some are narrowly tuned to 2–4 bitter-tasting compounds, whereas others are promiscuously activated by numerous ligands [58]. More recently, detailed analyses on human taste buds confirm that different bitter responsive taste cells express subsets of 4–11 of the T2Rs in partially overlapping [59]. Bitter sensing taste cells are known to functionally discriminate among bitter compounds [60]. This pattern of T2R expression, along with polymorphisms across the gene family, is thought to allow humans and animals to detect the vast range of potentially toxic bitter compounds found in nature [13]. The mechanism of transduction is implicated the activation of GPCR that are present in the apical surface of the taste cells [10]. For example for sugars, the activation of GPCR depolarize the taste cells due at activation of the intracellular second messenger cyclic adenosine monophosphate (cAMP) lead to transmitter release from taste cells, but how this happens is not known [61-63]. The other intracellular messenger involves phospholipase Cβ2 (PLCβ2) and 1,4,5-triphosphate (IP3) [29,64-67]. The GPCR stimulation activates PLCβ2, which produces IP3. IP3R3 receptors, located in the endoplasmic reticulum, they are then stimulated and release Ca2+ from intracellular stores. Increased intracellular Ca2+ activates TRPM5 channel that is the transient receptor potential (TRP) ion channel. The activation of TRPM5 allow the entry of sodium ions (Na+), influx leading to cell depolarization [12,66-69]. The combination of depolarization resulting from the influx of Na+ and rise in intracellular Ca+ opens

pannexin channels (Panx) in the taste-cell membrane, releasing adenosine triphosohate (ATP) from the cell. The role of ATP is not completely clear. The ATP secreted from receptor cells in turn activates purinergic receptors on the sensory nerve fibers innervating the taste buds, thereby sending a signal to the brain [4] and at the same time Type II cells secrete the paracrine transmitter ATP and excites adjacent presynaptic cells (Type III) and stimulates them to release serotonin and/or norepinephrine [23,70] and elicit afferent nerve output [12,32]. In this last model, given the complementary attributes of these two cells type, Roper [32] has suggested that groups of Type II and Type III cells form a "gustatory processing unit".

Salty and sour taste transductions take place in the microvilli of taste cells and along the basolateral membranes [71]. Sour taste stimuli (acids) are detected by a small subset of cells such as presynaptic cells [31]. The membrane receptor or ion channel that detects acid stimuli remains as yet unidentified. The candidate for sour taste receptors can be identified by non selective cation channels formed by PKD2L1 and PKD1L3 [38,72,73]. This channel is sensitive to extracellular pH instead that a decrease of cytoplasmic pH, which is known to be the direct stimulus for sour taste (Fig. 4 B) [39,74]. There is evidence that the organic acids such as acetic acid, which are not fully dissociated at physiological pH values, can directly permeate through the plasma membrane, of Type III cells, and acidify the cytoplasm and thereby elicit an electrical response. According to this mechanism, intracellular hydrogen ions inhibit or block a proton-sensitive K channel (normally function to hyperpolarize the cell). By a combination of direct intake of hydrogen ions (which itself depolarizes the cell) and the inhibition of the hyperpolarizing channel, sourness causes in the taste cell trigger action potentials and release neurotransmitter. The complete transduction pathways which detect sour taste are still not completely understood [4].

Taste buds detect Na salts by directly permeating Na^+ through apical ion channels. This ion channels are named amiloride-sensitive epithelial Na channel, ENaC (figure 5 C) [10,75-77]. Permeation of Na^+ determines the depolarization of taste. The amiloride-sensitive channel is also permeable to H^+ ions, so the transduction of substances that are perceived as sour is due to an input of these ions through amiloride-sensitive Na^+ channels. Sour and salty, in relation to their concentrations in the saliva, in part interfere with each other at peripheral [78]. Similarly, also the transduction of salts of K^+ may result in the entry of these ions across the apical K^+ channels. The differences are observed in the ability to perceive the taste of different salts of Na^+ could also depend on the different permeability of the respective anions through the tight junctions and the consequent ability to affect other ion channels localized at the level of the basal lateral membranes of taste cells.

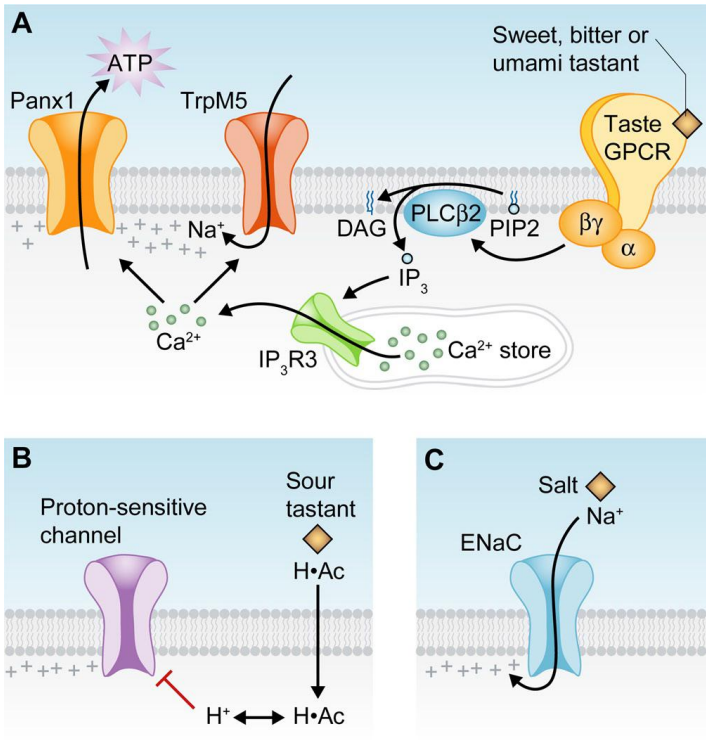


Figure 5 - Mechanisms of transduction of gustatory stimuli of the five taste qualities in taste cells (A) In receptor (Type II) cells, sweet, bitter, and umami ligands bind taste GPCRs, and activate a phosphoinositide pathway that elevates cytoplasmic Ca2+ and depolarizes the membrane via a cation channel, TrpM5. The combined action of elevated Ca2+ and membrane depolarization opens the large pores of gap junction hemichannels, likely composed of Panx1, resulting in ATP release. (B) In presynaptic (Type III) cells, organic acids permeate through the plasma membrane and acidify the cytoplasm where they dissociate to acidify the cytosol. Intracellular H+ is believed to block a proton-sensitive K channel and depolarize the membrane. (C) The salty taste of Na+ is detected by direct permeation of Na+ ions through membrane ion channels, including ENaC, to depolarize the membrane [4].

Most of the transduction mechanisms determines the depolarization of the membrane of the taste cell (receptor potential), which in turn determines increase of the concentration of Ca^{++} for opening of voltage-dependent channels or for mobilization from intracellular stores. The increase in the Ca^{++} causes the exocytosis of chemical mediator and the consequent transmission of the signal (make synapses) to the primary gustatory afferent fibers.

Taste afferent nerve fibers transmit sensory input from taste buds to the brain belong the three cranial nerves. The anterior two-thirds of the tongue and palate are

innervated by facial nerve (cranial nerve VII). The taste buds of fungiform papillae and foliate papillae are innervated by the chorda tympani, a branch of the facial nerve [79-82]. The posterior third are innervated from the lingual branch of branch of glossopharyngeal nerve (cranial nerve IX). The region around the throat, including the glottis, epiglottis and pharynx, receive branches of the vagus nerve (cranial nerve X). In general, each fiber can respond, , two or three or all four gustatory qualities, although with a different intensity . The first synapse within the gustatory system is at the terminals of the sensory afferent fibers and individual synaptic cells [83]. The input from the chorda tympani nerve synapses at the geniculate ganglion and input from the glossopharyngeal nerve synapse at the petrosal ganglion. The central axons of these primary sensory neurons in the respective cranial nerve ganglia project to rostral and lateral regions of the nucleus of the solitary tract in the medulla, which is also known as the gustatory nucleus of the solitary tract complex (Figure 6). From the gustatory nucleus, neurons project to the small group of neurons of the ventral posterior medial nucleus (VPM) of the thalamus. This nucleus projects to several regions of the cortex, including the anterior insula and frontal operculum (gustatory cortex) in the ipsilateral cerebral cortex. The gustatory cortex is responsible for conscious discrimination of gustatory stimuli [83-85]. Destruction of the insula causes ageusia, the total inability to perceive any compounds [86]. Finally, reciprocal projections connect the nucleus of solitary tract via the pons (one of the three components of the brainstem, lying between the midbrain rostrally and the medulla caudally) to the hypothalamus and amygdale. These projections presumably influence appetite, satiety and other homeostatic responses associated with eating. Projections from the gustatory cortex are also managed anteriorly to the dysgranular caudolateral region of the orbitofrontal cortex where they join with those from the visual and olfactory areas. It is here that the convergence of visual, olfactory, and gustatory sensory input allow for an awareness of flavor, which is the combination of taste, olfaction, and somatosensory perception (such as texture and pain) [85,87,88].

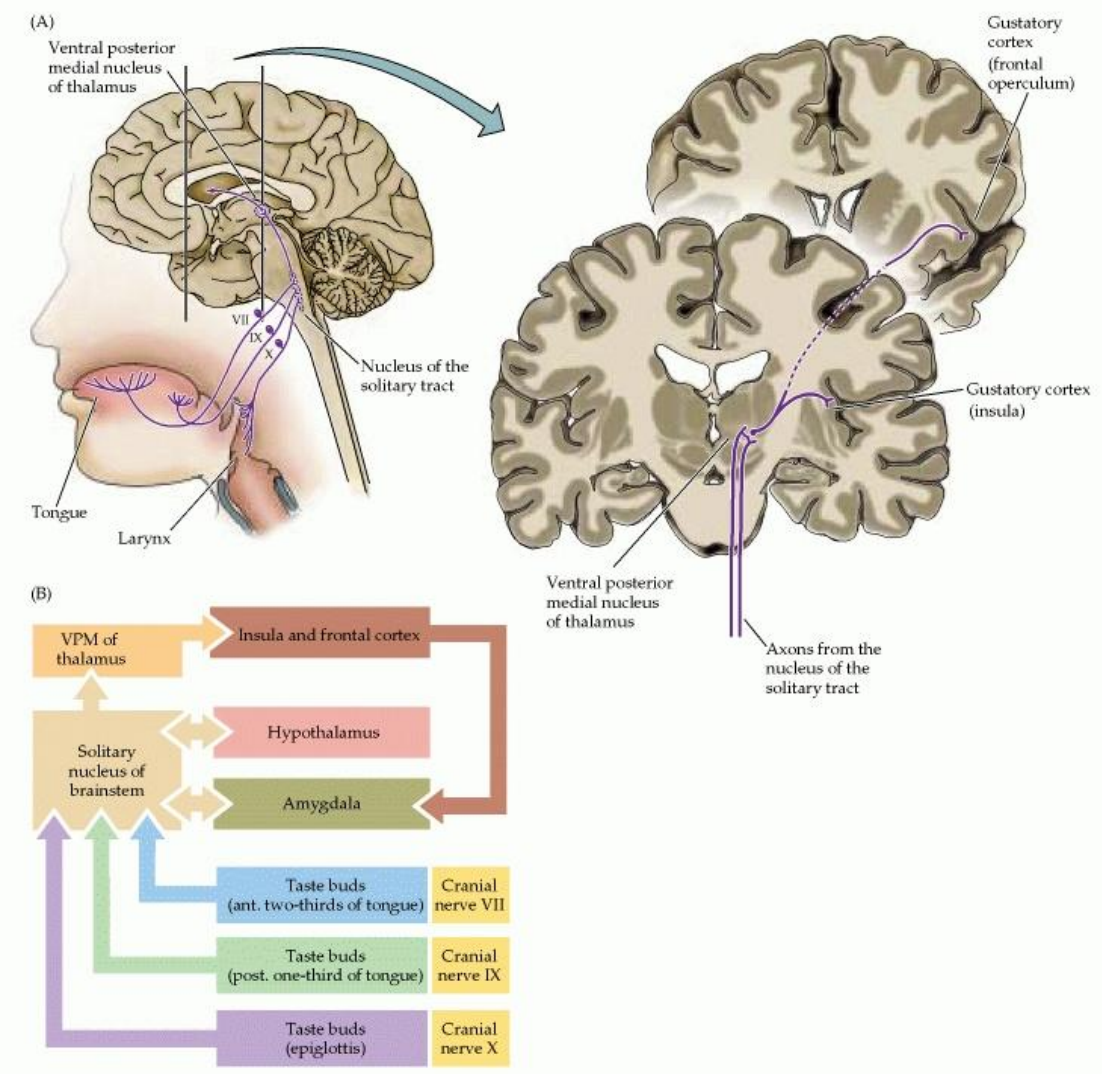
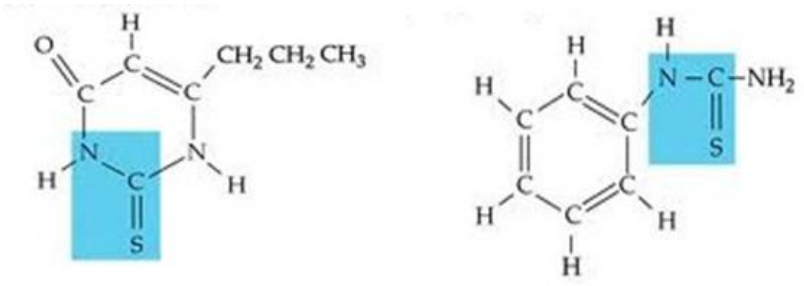


Figure 6 - Organization of the human taste system. (A) Drawing on the left shows the relationship between receptors in the oral cavity and upper alimentary canal, and the nucleus of the solitary tract in the medulla. The section on the right shows the VPM nucleus of the thalamus and its connection with gustatory areas of the cerebral cortex. (B) Diagram of the basic pathways for processing taste information.

PROP bitter taste sensitivity

Taste sensitivity varies greatly in individual and the individuals differences strongly influence food choice and satiety [89]. The physiologic role of taste variability could be related to evolutionary adaptation to specific environments to recognize substances potentially dangerous or necessary for bodily function [90]. For example in the case of bitter taste it is know that it play a dual role in human nutrition as both a warning signal and an attractant. Some plants produce a large diversity of bitter-tasting compounds as protection against predation [91]. These substances include bitter alkaloids such as quinine and brucine, isothiocyanates from cabbage and mustard seeds, as well as certain fatty acids, amino acids and peptides [92,93]. Strong bitter taste is closely associated with the presence of substances toxic, the ability of humans to detect bitterness at low concentrations represents an important evolutionary adaptation for limiting or avoiding the consumption plant foods that could be harmful [89]. However, several classes of bitter polyphenols, such as tannins, catechins and anthocyanins (from grapes, tea, coffee, dark-colored fruit, citrus and chocolate) and isoflavones derived from soy, and glucosinolates from cruciferous vegetables [94] provide positive health benefits by acting as anti bacterials and antioxidants [95].

The ability to taste the bitter thiourea compounds, phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP) is a well-studied human trait [96]. Thiourea compounds contain the thiocyanate moiety (N-C=S) which is responsible of their bitter taste [97,98] (Figure 7). The N-C=S grouping is also characteristic of glucosinolates and goitrin, naturally-occurring substances commonly found in cruciferous vegetables such as broccoli, cabbage, cauliflower and Brussels sprouts (*Brassica oleracea*), Chinese cabbage and turnips (*B. rapa)*, mustard greens (*B. juncea*), and radishes (*Raphanus sativus*) and other plants of the *Brassica* family [99]. Goitrin has potent anti-thyroid properties, and can be toxic when consumed in large quantities by populations at-risk for thyroid deficiency [100]. In areas of low iodine over-ingestion of isothiocyanates is associated with thyroid disease and goiter, while, on the other hand, these vegetables exhibit potent anti-cancer effects [99]. Depending on regional influences (over) ingestion of such vegetables can have positive as well as negative health effects for individuals which might lead to balancing selection for *TAS2R38* gene variants [101]. One interesting explanation for the persistence of this trait in humans is that it served as an evolutionary adaptation to local eating environments [102]. Larger rejection of Brassica plants would provide survival advantages to those who were more sensitive to their bitter taste [103].



Molecular Weight: 170.23 $C_7H_{10}N_2OS$
6-n Propylthiouracil
(PROP)

Molecular Weight: 152.22 $C_7H_8N_2S$
Phenylthiocarbamide
(PTC)

Figure 7 - Chemical structure of 6-n Propyltiouracil (PROP) and Phenylcarbamide (PTC). In Blue are highlight the isothiocyanate chemical group.

Individual variability in sensitivity to the bitter taste of PTC was first recognized by Fox more than eight decade ago [97]. Based on threshold methods, PROP Sensitive and non sensitive individuals are defined as tasters and nontasters, respectively. The frequency of nontasters varies among populations, from as low as 7% to more than 40% [104]. In the Caucasian population, the estimated frequency of nontasters is ~30% [105-109]. Bartoshuk [109,110] first used the term "supertaster" to distinguish individuals who perceived PROP as extremely bitter from those who perceived PROP as moderately bitter. Although numerous studies support the classification of individuals into three phenotypic groups (nontasters, medium tasters and supertasters) [107,111-118], other work suggests that PROP tasting may be a more continuous phenotype [101,111,114,119,120]. The ability to taste PROP is associated with haplotypes of the *TAS2R38* gene defined by three single-nucleotide polymorphisms that result in three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile) [101,114] (Figure 8). There are two common haplotypes, PAV the dominant (sensitive) variant and AVI the recessive (insensitive) one. Nontasters are homozygous for the AVI haplotype, and it was assumed that supertasters were homozygous for the PAV haplotype and medium tasters were heterozygous for the PAV haplotype. However, studies have reported considerable genotypic overlap between the medium and supertaster groups [101,107,121] with substantial numbers of supertasters carrying the PAV/AVI diplotype. Other work suggests that the presence of two PAV alleles (as opposed to one) confers no additional advantage for perceiving more bitterness intensity from PROP, at least in the suprathreshold (above threshold) range [119]. Thus, *TAS2R38* genotypes do not completely explain the oro-sensory differences between medium and supertasters. In fact, *TAS2R38* genotype predicts the majority (55-85%) but not all of the phenotypic

variance in PROP threshold, implying that epigenetic factors may be involved in the expression of the trait [57,89,108,119].

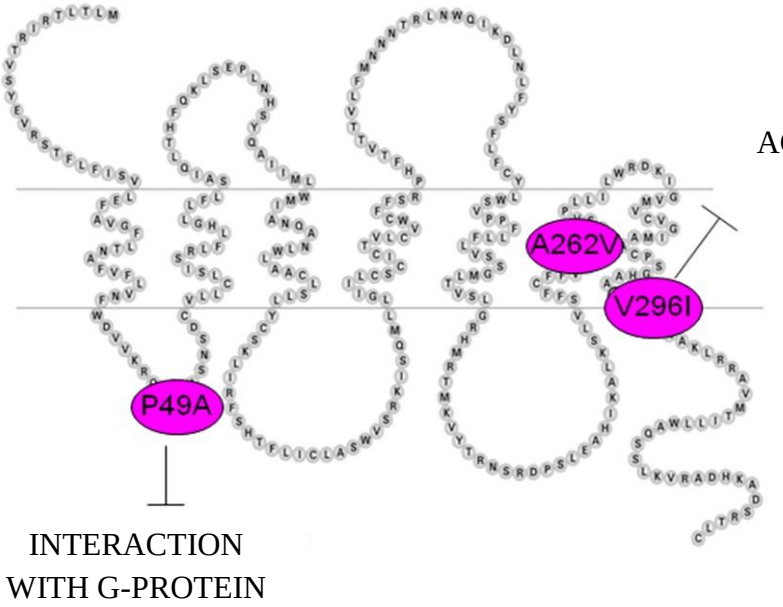


Figure 8 - Schematic representation of TAS2R38 receptor and the three polymorphisms associated with different level of sensibility.

Indeed, evidence supporting the presence of modifying genes comes from studies using a variety of approaches (family segregation, family-based linkage and genome-wide association studies [122-124].Nevertheless, studies have consistently reported that supertasters have a higher density of fungiform papillae on the anterior tongue surface when compared to the other groups [125-128] (Figure 9).

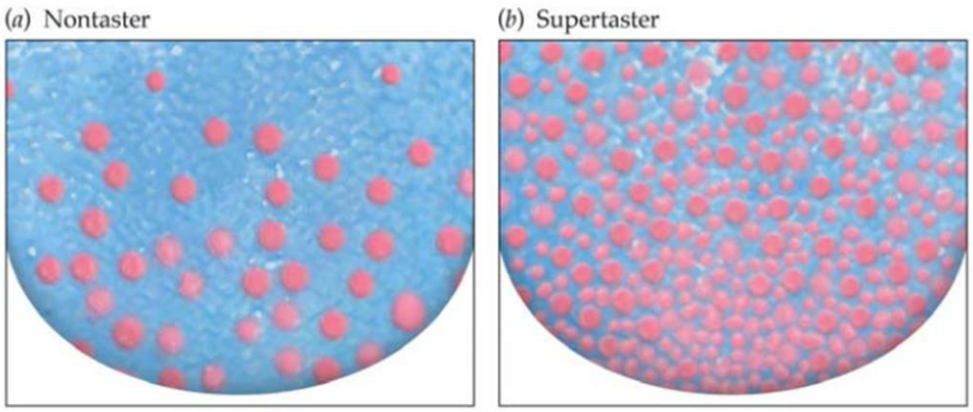


Figure 9 - Schematic representation of anatomical differences in the anterior surface of the tongue in the PROP non-tasters and PROP super-tasters.

These anatomical differences could partially explain the greater oral responsiveness of supertasters to a range of oral sensations that are not mediated via bitter taste receptors. Recently, has been studying the role of the gustin (CA6) gene, a trophic factor for taste bud development, in PROP tasting [129].

We showed that polymorphism rs2274333 (A/G) of the gustin gene led to a modification gustin's primary structure which is crucial for zinc binding and full functionality of the protein [129]. The AA genotype (associated with a fully-functional protein) was more frequent in supertasters, whereas the GG genotype (associated with a disruption in the protein) was more frequent in nontasters. These data suggest that variation in gustin may be associated with differences in papillae densities and oral chemosensory abilities across PROP phenotypic groups.

Contemporary studies in human nutrition have also revealed that PROP bitterness might also serve as a general marker for oral sensations and food preferences. This assumption is based on data showing that those who perceive PROP/PTC as more bitter are also more responsive than nontasters to various oral stimuli, including other bitter-tasting compounds [109,113,130-134], sweet substances [135], chemical irritants [114,136], and fats [111,112]. Given the nutritional value of dietary lipids, the relationship between PROP bitterness intensity and acceptance or perception of fats is of particular interest. Several studies reported that PROP nontasters had a lower ability to distinguish fat content in foods, showed a higher acceptance of dietary fat [112,137-140] and consumed more servings of discretionary fats per day than did tasters [138]. These findings have led to the hypothesis of an inverse correlation between PROP status and body mass index (BMI) which is supported by several studies [107,141-143]. However, other reports show no associations between PROP taster status and these variables [127,144-147]. This lack of consensus suggests that other factors contribute to feeding behaviour, food perception and preference in PROP taster groups. Given that PROP phenotype may have broad implications for nutritional status, it would be of great interest to characterise other factors that may contribute to differences in the genetic predisposition to taste thiourea compounds.

Outline of the work

The studies described in the present section of thesis deal with two main fields: 1) the identification and characterization of factors that may contribute to differences in the genetic predisposition to taste PROP and 2) the identification of confounding variables which may explain the controversial data in the literature about the relationship between PROP taste sensitivity andBMI.

The first point was focus on investigate for other factors that may contribute to PROP phenotype. Since that the taste stimulus occurs in the mouth, before the PROP comes in contact with the receptor sites, it must be dissolved in saliva.We hypothesized that variations of saliva composition could be correlated with taste differences and thus investigated the possible relationship between PROP bitter taste responsiveness and the salivary proteome in subjects genotyped for *TAS2R38* and gustin gene polymorphisms (**chapter 1**). To extend this to physiological mechanisms by which the specific salivary proteins facilitate the perception of PROP bitterness, we evaluated the role of these proteins and free amino acids that selectively interact with the PROP molecule, in modulating bitter taste responsiveness (**chapter 2**). Furthermore, to understand if *rs2274333* (A/G) polymorphism of gustin (CA6) gene affect the PROP sensitivity by acting on the protein function as growth factor of taste buds, we analyzed the relationship between the *TAS2R38* and gustin gene polymorphism and density and morphology of fungiform papillae, and, in *in vitro* experiments, we examined the effect of treatment with saliva collected from individuals with genotype AA and GG of gustin gene on cell development and metabolic activity, and the effect of treatment with isolated gustin, in the two iso-forms resulting from polymorphism, on cell metabolic activity (**chapter 3**). The last chapter focus on identification on confounding variables which, by influencing food choices, may explain the controversial data in the literature about the relationship PROP taste sensitivity/BMI. In this we investigated whether the endocannabinoid system, which also modulates hunger/satiety and energy balance, plays a role in modulating eating behavior influenced by sensitivity to PROP (**chapter 4**).

This section of thesis finishes with a general conclusion on results of described studies, a brief description of studies in progress and future perspectives.

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Responsiveness to 6-n-Propylthiouracil (PROP) Is Associated with Salivary Levels of Two Specific Basic Proline-Rich Proteins in Humans

Tiziana Cabras
Melania Melis
Massimo Castagnola
Alessandra Padiglia
Beverly J Tepper
Irene Messana
Iole Tomassini Barbarossa

PLoS ONE 2012, 7(2): e30962. doi:10.1371/journal.pone.0030962

Abstract

Thiourea tasting can be predictive of individual differences in bitter taste responses, general food preferences and eating behavior, and could be correlated with saliva chemical composition. We investigated the possible relationship between PROP bitter taste responsiveness and the salivary proteome in subjects genotyped for *TAS2R38* and gustin gene polymorphisms.

Taste perception intensity evoked by PROP and NaCl solutions was measured in sixty-three volunteers (21 males, 42 females, age 25 ± 3 y) to establish their PROP taster status, and 24 PROP super-tasters and 21 nontasters were selected to participate in the study. *TAS2R38* and gustin gene molecular analysis were performed using PCR techniques. Qualitative and quantitative determination of salivary proteins was performed by HPLC-ESI-MS before and after PROP taste stimulation. PROP super-tastings was strongly associated with the 'taster' variant (PAV haplotype) of *TAS2R38* and the A allele of rs2274333 polymorphism in the gustin gene and nontasting was associated with the minor alleles at both loci. ANOVA revealed that basal levels of II-2 and Ps-1 proteins, belonging to the basic proline-rich protein (bPRPs) family, were significantly higher in PROP super-taster than in nontaster un-stimulated saliva, and that PROP stimulation elicited a rapid increase in the levels of these same proteins only in PROP super-taster saliva.

These data show for the first time that responsiveness to PROP is associated with salivary levels of II-2 peptide and Ps-1 protein, which are products of the *PRB1* gene. These findings suggest that *PRB1*, in addition to *TAS2R38* and gustin, could contribute to individual differences in thiourea sensitivity, and the expression of the PROP phenotype as a complex genetic trait.

Introduction

Plants produce a large diversity of bitter-tasting compounds as protection against predation [1]. These substances include bitter alkaloids such as quinine and brucine, isothiocyanates from cabbage and mustard seeds, as well as certain fatty acids, amino acids and peptides, to name a few [2-4]. Since many bitter-tasting substances can be toxic, the ability of humans to detect bitterness at low concentrations represents an important evolutionary adaptation for limiting or avoiding the consumption plant foods that could be harmful [5]. On the other hand, several classes of bitter polyphenols found in tea, coffee, dark-colored fruit, citrus and chocolate [6] provide positive health benefits by acting as antibacterials and antioxidants [7].

Bitter taste is mediated by the TAS2R sub-family of G protein-coupled receptors [8,9]. Humans posses ~25 TAS2R bitter receptors encoded by clusters of genes located on chromosomes 5p, 7q, 12p [10]. So far, more than 550 ligands for human bitter receptors have been identified [11]. However, this number represents only a tiny fraction of the thousands of plant-based bitter compounds that exist in nature. Since the number of compounds greatly exceeds the number of receptors, it seems likely that individual receptors respond to more than one bitter compound type [12]. In fact, some receptors are narrowly-tuned, responding to a limited range of compounds. TAS2R8 is an example of a highly-selective receptor that has only 3 known ligands which share common structural properties. On the opposite end of the spectrum are TAS2R10, -14 and -46 which are highly promiscuous, responding to 50% of the bitter compounds applied in cell-based expression studies. TAS2R38, the receptor that binds the N-C=S moiety of the bitter thiourea compounds phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP) [13], is considered modestly restrictive as this receptor also responds to compounds without the N-C=S motif [14].

Individual variation in the perception of bitter taste is a common human trait [6] that reflects the rich allelic diversity in TAS2R receptors. For example, sequence variation in *TAS2R19* has been associated with individual differences in the bitter taste of quinine [15]. Mutations in *TAS2R31* and *TAS2R43* (to a lesser extent) may be responsible for individual responses to the bitter aftertaste of saccharin and acesulfame-k [16,17]. In addition, sequence variation in *TAS2R16*, *TAS2R19* and the haplo-block composed of *TAS2R3*, *-4*, *-5* are responsible for individual differences in the perception of alcohol, grapefruit juice and coffee, respectively [18].

Genetic variation in sensitivity to PTC and PROP, is the most- studied bitter-taste phenotype in humans [5,19]. PROP responsiveness has been used as a general index of oral chemosensory perception since it associates with the perception of a wide range of

oral stimuli including many of the bitter molecules discussed previously as well as, sweet substances, oral irritants and fatty texture [5]. PROP-related differences in chemosensory perception have been shown to influence food preferences which are the primary determinants of food selection and dietary behaviour [20-26]. Through this mechanism, PROP status is thought to play an important role in defining body composition and nutritional status [5]. Individuals can be defined tasters or nontasters based on their ability to discriminate threshold concentrations of PROP from plain water. When tested with suprathreshold (i.e., above threshold) concentrations of this compound, tasters can be further divided into those who are very sensitive, i.e. PROP super-tasters, and those who are moderately sensitive, i.e., medium tasters [27,28].

The first molecular characterization of *TAS2R38* was accomplished by Kim et al. [13]. Three variant sites in this gene result in three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile) and give rise to two common haplotypes: PAV, the dominant taster variant; and AVI, the nontaster recessive one. Individuals homozygous or heterozygous for the PAV haplotype taste PROP bitterness at low concentrations, whereas individuals who are either unable to taste PROP or who taste it only at high concentrations, are homozygous for the AVI haplotype. Other haplotypes (AAV, AAI, and PVI) that convey intermediate PROP/PTC response magnitudes have been rarely observed or limited to specific populations [29]. Since then, a growing number of studies have sought to fine-tune the genetic architecture of this phenotype [13, 29-32]. For example, studies have examined the effects of individual variant sites within the haplotype of the *TAS2R38* gene to better characterize their influence on bitter perception and to identify which sites may be critical for receptor activation [30,33,34].

Although the *TAS2R38* gene accounts for a large fraction of PROP/PTC phenotypic variation, it has become clear that other genetic loci contribute to the phenotype [15,35,36]. We recently showed that the polymorphism rs2274333 (A/G) of the gustin gene which controls the salivary protein carbonic anhydrase VI (CA6) alters the functionality of this enzyme and is strongly related to taste responsiveness to PROP [37]. In particular, allele A of this locus is strongly associated to the highest PROP responsiveness, whereas allele G is associated with the lowest one. Gustin is thought to be a taste-bud trophic factor and has long been implicated in taste function [38,39]. In another study we showed how the combination of the *TAS2R38* and gustin gene genotypes modulate PROP phenotype, partially explaining supertasting [40].

Other salivary proteins have been implicated in bitter taste sensitivity. Fox [41] first suggested that the salivary composition might be responsible for individual differences in taste among people. On the basis of experiments showing that the stimulating capacity

of a substance depends on its solubility [42], Fox hypothesized that taste blindness of nontasters may depend on the presence in their saliva of products (as proteins or colloids) which precipitate the taste substance and thus cause no taste to be perceived. It is known that salivary proline-rich proteins (PRPs) and histatins can bind and precipitate plant polyphenols in the oral cavity evoking astringency [43-45]. Genetic studies have shown that a cluster of *PRPs* genes, located at 12p13, are closely linked to a *T2R* gene cluster responsible for the ability to taste the bitterness of raffinose, quinine, cycloheximide, sucrose octaacetate and undecaacetate [46-49]. In addition, modification of the salivary proteome has been demonstrated in human responses to bitter tastants such as urea, quinine or calcium nitrate [50,51]. At present, no studies have characterized the salivary proteome in individuals who vary in taste responsiveness to PROP. Given the importance of salivary proteins in taste function and the role that the PROP phenotype may play as a general marker of food selection and dietary behaviour, such studies are warranted.

The purpose of this work was to investigate the possible relationships between PROP taste responsiveness and the salivary proteome, before and after PROP bitter taste stimulation in individuals genotyped for *TAS2R38* and gustin gene polymorphisms.

Materials and methods

Ethics statement

All subjects reviewed and signed an informed consent form. The study was approved by the Ethical Committee of the University Hospital of Cagliari, and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

Subjects

Sixty-three non-smoking volunteers (21 men and 42 women) were recruited through public advertisements at the local University. All were white, aged from 20 to 29 years and with body mass indices (BMIs) ranging from 18.6 to 25.3 kg/m^2. Selected subjects had to have a stable weight (no variation of body weight larger than 5 kg over the previous 3 months). They were not following a prescribed diet or taking medications that might interfere with taste function. None of the subjects had food allergies, or scored high on eating behaviour scales (assessed by the Three-Factor Eating Questionnaire) [62]. In order to rule out any gustatory impairment, the threshold for the 4 basic tastes (sweet, sour, salty, bitter) was determined in all subjects. At the beginning of the

protocol, each subject was verbally informed about the procedure and the aim of the study.

PROP tasting

In order to classify each subject based on his/her PROP phenotype, PROP and sodium chloride (NaCl) ratings were collected using the 3-solution test [63,64]. The test consists of three suprathreshold PROP (Sigma-Aldrich, Milan, Italy) (0.032, 0.32, and 3.2 mmol/l) and NaCl (Sigma-Aldrich, Milan, Italy) (0.01, 0.1, 1.0 mol/l) solutions dissolved in spring water. NaCl was used as a standard because taste intensity to NaCl does not change by PROP taster status in this method [63]. Solutions were prepared the day before each session and stored in the refrigerator until 1 h before testing.

Molecular analysis

Subjects were genotyped for three single nucleotide polymorphisms (SNPs) at base pairs 145 (C/G), 785 (C/T), and 886 (G/A) of the *TAS2R38* that result in three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile), and for the gustin (CA6) gene polymorphism rs2274333 (A/G) that consists of the substitution Ser90Gly. Molecular analyes were performed using PCR techniques followed by the sequencing of the fragments obtained in accord by Calò et al.[40]

Salivary protein determination

Saliva treatment

Aqueous solution of trifluoroacetic acid (1 ml, 0.2%) was immediately added to 1 ml of each salivary sample in an ice bath in a 1:1 v/v ratio, in order to preserve and stabilize the sample by inhibiting salivary proteases. The solution was then centrifuged at 8000 g, and 4°C for 15 min. The acidic supernatant was separated from the precipitate and either immediately analyzed by the HPLC-ESI-MS apparatus or stored at – 80°C until the analysis. Sample size was 100 μL, corresponding to 50 μL of saliva.

HPLC-ESI-IT-MS analysis

The HPLC-ESI-MS apparatus was a Surveyor HPLC system (ThermoFisher, San Jose, CA, USA) connected by a T splitter to a photodiode array detector and the electrospray ionization/ion trap mass spectrometer LCQ Advantage (ThermoFisher, San Jose, CA, USA). The chromatographic column was a Vydac (Hesperia, CA, USA) C8 with 5 μm particle diameter (column dimensions 150x2.1 mm). The following solutions were utilized for RP-HPLC-ESI-MS analysis: (eluent A) 0.056% (v/v) aqueous TFA

and (eluent B) 0.05% (v/v) TFA in acetonitrile-water 80/20, and the flow rate was 0.30 mL/min. Salivary proteins were eluted using a linear gradient from 0 to 54% of B in 39 min, and from 54% to 100% of B in 10 min. The T splitter permitted 0.20 mL/min to flow toward the diode array detector and 0.10 mL/min to flow toward the ESI source. The first five minutes of the RP-HPLC eluate was not transferred to the MS apparatus in order to avoid instrument damage derived from the high salt content. The photodiode array detector was set at 214 and 276 nm. Mass spectra were collected every 3 ms in the positive ion mode in the range 300-2000 m/z. The MS spray voltage was 5.0 kV, the capillary temperature was 260 °C.

Identification of salivary peptides and proteins

Deconvolution of averaged ESI-MS spectra was automatically performed by using MagTran 1.0 software to obtain the experimental mass values [65]. These values were compared with the theoretical ones reported in the Swiss-Prot Data Bank (http://us.expasy.org/tools). Structural characterization of salivary proteins and peptides of interest, based on Tandem-MS analysis and automated amino acid sequencing of entire proteins, as well as of proteolytic fragments obtained after different enzymatic treatments of pure proteins, was performed as previously shown [66-70].

The six families of salivary proteins and peptides quantified in this study are listed in Table 1. We recently characterized a protein belonging to the basic proline-rich protein (bPRP) family with molecular weight of 23460 Da (unpublished results). Determination of its amino acid sequence confirmed that it corresponds to the Ps-1 protein previously described by Azen et al. [53].

Quantitative determination of salivary peptides and proteins

Salivary peptide and protein quantification was based on the area of the RP-HPLC-ESI-MS eXtracted ion current (XIC) peaks, measured when the signal/noise ratio was at least 5. The XIC analysis reveals the peak associated with the protein of interest by searching along the total ion current chromatographic profile, the specific multiply-charged ions generated at the source by the protein. The ions used to quantify the proteins/peptides were carefully selected to exclude values in common with other co-eluting proteins, and were the same as those reported in Cabras et al. [52]. The area of the ion current peak is proportional to concentration, and under constant analytical conditions can be used to quantify and compare levels of the same analyte in different samples [71,72].

Table 1 - List of the salivary proteins and peptides quantified by RP-HPLC-ESI-MS.

Name	Swiss-Prot code[a]	Experimental average mass (Da)
Proline-rich peptide P-B	(P02814)	5792.9 ± 0.5
Basic proline-rich proteins family (bPRPs):		
P-F	(P02812)	5843.0 ± 0.5
P-J		5943.9 ± 0.5
P-D	(P010163)	6949.5 ± 0.7
P-H	(P02812/P04280)	5590.2 ± 0.5
IB8-a (Tot):		
- IB8-a (Con1+)		11887.8 ± 2
- IB8-a (Con1-)		11898 ± 2
IB-1 (Tot):		
- IB-1	(P04281)	9593 ± 1
- IB-1 nonphosphorylated		9513 ± 1
- IB-1 Des-Arg_{96}		9437 ± 1
II-2 (Tot):		
- II-2	(P04280)	7609 ± 1
- II-2 nonphosphorylated		7529 ± 1
- II-2 Des-Arg_{75}		7453 ± 1
Protein with molecular weight of 10435 Da		10434 ± 1
Ps-1		23460 ± 3
Acidic proline-rich phosphoproteins family (aPRPs):		
PRP-1 type diphosphorylated	(P02810)	15515 ± 2
PRP-1 type monophosphorylated		15435 ± 2
PRP-1 type nonphosphorylated		15355 ± 2
PRP-1 type triphosphorylated		15595 ± 2
PRP-3 type diphosphorylated	(P02810)	11161 ± 1
PRP-3 type monophosphorylated		11081 ± 1
PRP-3 type nonphosphorylated		11001 ± 1
PRP-3 type diphosphorylated Des-Arg_{106}		11004 ± 1
P-C peptide	(P02810)	4370.9 ± 0.4
Cystatin family (S-Cyst):		
Cyst S type nonphosphorylated	(P01036)	14186 ± 2
Cyst S type monophosphorylated at Ser3		14266 ± 2
Cyst S type diphosphorylated at Ser1 and Ser3		14346 ± 2
SN	(P01037)	14312 ± 2
SA	(P09228)	14347 ± 2
Statherin family (Stath):		
Stath diphosphorylated	(P02808)	5380.0 ± 0.5
Stath monophosphorylated		5299.9 ± 0.5
Stath nonphosphorylated	(P02808)	5220.5 ± 0.5
Histatin family (Hist):		
Hst-1	(P015515)	4928.2 ± 0.5
Hst-1 nonphosphorylated		4848.2 ± 0.5
Hst-6	(P15516)	3192.4 ± 0.3
Hst-5	(P15516)	3036.5 ± 0.3

Experimental procedure

The subjects were requested to abstain from eating, drinking and using oral care products or chewing gums for at least 8 h prior to testing that was carried out in three different visits. They had to be in the test room 15 min before the beginning of the session (at 9.30 AM) in order to adapt to the environmental conditions (23-24°C; 40-50% relative humidity) which were kept constant throughout the experimental session. In order to classify subjects for their PROP taster status, each subject was tested twice in different visits separated by a 1-month period. In women, testing was done on the sixth day of the menstrual cycle to avoid taste sensitivity changes due to the estrogen phase [73]. Stimuli were presented at room temperature as 10 ml samples. The order of taste stimulus presentation was reversed in the two visits. Samples within each solution type were tasted at random. Each stimulation was followed by oral rinsing with spring water. The interstimulus interval was set at 60 s. Taste intensity rating for each PROP or NaCl solution was collected using the Labeled Magnitude Scale (LMS) [74]. After tasting each sample, subjects placed a mark on the scale corresponding to his/her perception of the stimulus. The mean of the two replicates was calculated and the results were plotted for each subject. This procedure generates suprathreshold intensity functions for PROP and NaCl [63,75]. When the PROP ratings increased more rapidly across concentrations than did the NaCl ratings, the subject was classified, as a "PROP super-taster". Conversely, when the NaCl ratings increased more rapidly than did the PROP ratings, the subject was classified as a nontaster. When the PROP ratings overlapped with the NaCl ratings, subjects were classified as medium tasters. Medium tasters were excluded from participating in the proteome analysis in order to contrast the two extreme groups (PROP super-tasters and nontasters).

In the third visit, a sample (1 ml) of whole un-stimulated saliva was collected from each subject with a soft plastic aspirator as it flowed into the anterior floor of the mouth for less than 1 min, and then transferred to a plastic tube. One minute was sufficient to collect 1 ml of un-stimulated or stimulated saliva. Subjects then tasted 10 ml of PROP (3.2 mM). For complete impregnation of the oral cavity, subjects were instructed to keep the solution in the mouth for 5 s and then spit it out. After PROP taste stimulation, three samples of stimulated saliva were collected from each subject, immediately after stimulation, and at 5 and 10 min after stimulation.

Statistical analyses

Three-way analysis of variance (ANOVA) was used to compare PROP intensity ratings with NaCl intensity ratings across PROP taster groups. The Newman-Keuls test was used for post-hoc comparisons.

Fisher's method (Genepop software version 4.0; http://kimura.univ-montp2fr/~rousset/Genepop.htm) [76] was used to test *TAS2R38* and gustin gene polymorphisms allele frequencies according to PROP status.

Two-way analysis of variance (ANOVA) was used to evaluate PROP super-tester nontaster differences in basal levels (un-stimulated saliva) of the six salivary protein families (P-B, bPRP, aPRP, S-Cyst, Stath, Hist), as well as of the following nine bPRPs: P-F, P-J, P-D, P-H, IB-8a (Tot), II-2 (Tot), IB-1 (Tot), 10434 and Ps-1. Two-way analysis of variance (ANOVA) was also used to evaluate gender differences in basal levels of the same six salivary protein families, as well as the nine bPRPs. The effects of PROP taste stimulation (immediately after stimulation, at 5 and 10 min after stimulation) on the levels of the same salivary proteins in PROP super-testers and nontasters were analyzed by three-way ANOVA. Post-hoc comparisons were conducted with the Newman-Keuls test. Statistical analyses were conducted using STATISTICA for WINDOWS (version 6.0; StatSoft Inc, Tulsa, OK, USA). p values <0.05 were considered significant.

Results

Figure 1 shows the PROP and NaCl intensity ratings of subjects classified as PROP super-tasters (n=24) and nontasters (n=21). ANOVA revealed a significant three-way interaction of Taster group × Solution type × Concentration on the intensity ratings ($F_{[2,258]}$ = 37.89; p<0.001). Post-hoc comparisons confirmed that nontasters gave lower intensity ratings to the two highest PROP concentrations as compared to the two highest NaCl concentrations (p<0.001; Newman-Keuls test). Likewise, PROP super-tasters gave higher ratings to 0.32 and 3.2 mmol/l PROP as compared to the two highest NaCl concentrations (p<0.001; Newman-Keuls test).

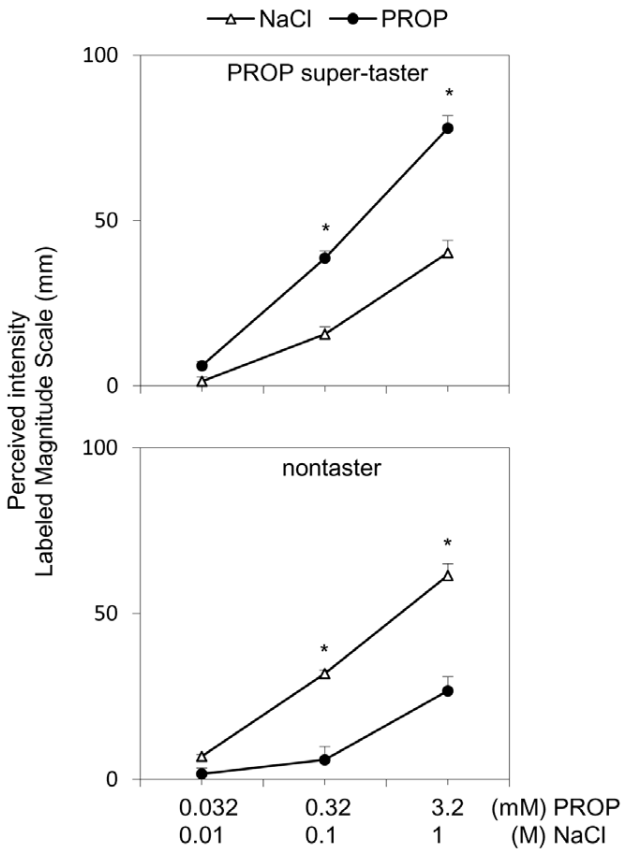

Figure 1 - Classification of subjects by PROP taster status. All values are means (± SEM). Three-way ANOVA was used to compare PROP intensity ratings with NaCl intensity ratings in PROP super-tasters (n = 24) and nontasters (n = 21) ($p<0.001$).
* indicates significant difference between PROP and the corresponding NaCl concentration ($p<0.001$; Newman-Keuls test). Medium tasters were not studied.

Molecular analysis of the *TAS2R38* SNPs and the rs2274333 (A/G) gustin gene polymorphism showed that the two PROP taster groups differed statistically based on their allelic frequencies ($\chi^2 = 32.684$; $p = 7.999e\text{-}008$; Fisher's test). In particular, PROP super-tasters had a very high frequency of haplotype PAV of *TAS2R38* (69 %) and allele A of the gustin gene (93 %), whereas nontasters had a higher frequency of haplotype AVI of *TAS2R38* (95 %) and allele G of the gustin gene (60 %).

HPLC-ESI-IT-MS analysis allowed us to demonstrate different relative concentrations of some proteins in the un-stimulated saliva of PROP super-taster subjects with respect to that of nontasters. An example of these differences is shown in Figure 2, where an HPLC profile (total ion current) of the acidic-soluble fraction of whole saliva of a representative PROP super-taster (white profile) and nontaster (grey profile) are shown in panel A. The extracted ion current (XIC) peaks of Ps-1 and II-2

proteins revealed in the two profiles are superimposed in Figure 2, panel B. The area of the Ps-1 protein peak corresponded to $3.2x10^9$ and $3.4x10^8$ arbitrary units, and the area of the II-2 protein peak corresponded to $1.8x10^9$ and $4.2x10^8$ arbitrary units in the PROP super-taster and nontaster saliva, respectively.

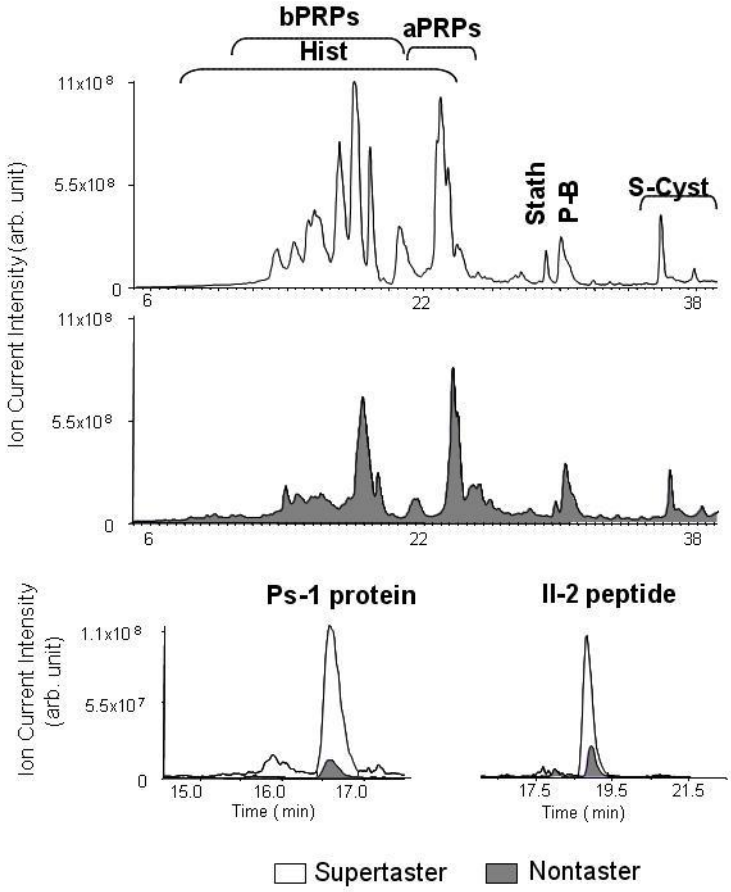

Figure 2 - Examples of HPLC-MS profiles from un-stimulated saliva and extracted ion current peaks**.** (A) HPLC-MS Total Ion Current (TIC) profiles of the acidic-soluble fraction of saliva of a representative PROP super-taster (white profile) and nontaster (grey profile). (B) The ion current (XIC) peaks of Ps-1 protein and II-2 peptide extracted from the HPLC-MS profiles of the same subjects. The XIC peaks of the PROP super-taster (white filled) are superimposed on the same XIC peaks of the nontaster (grey filled).

Basal mean values ± SEM of the XIC peak areas of the six protein families (P-B, bPRP, aPRP, S-Cyst, Stath, Hist), as well as of the nine peptides of the bPRP family (P-F, P-J, P-D, P-H, IB-8a Tot, II-2 Tot, IB-1 Tot, 10434 and Ps-1) in un-stimulated PROP super-taster and nontaster saliva are shown in Figure 3. ANOVA revealed a significant two-way interaction of Taster group × Protein type on XIC peak areas of un-stimulated

saliva proteins (the six protein families $F_{[5,258]} = 5.80$; $p<0.001$ and nine bPRPs $F_{[9,430]} = 3.086$; $p<0.002$). Post-hoc comparisons showed that, among the six protein families quantitatively determined, only the XIC peak area of bPRPs was significantly higher in PROP super-taster saliva than in nontaster saliva ($p<0.001$; Newman-Keuls test). Also, among the nine peptides of the bPRP family, only XIC peak areas of II-2 Tot and Ps-1were significantly higher in un-stimulated saliva of PROP super-tasters with respect to nontasters ($p<0.001$ and $p<0.001$, respectively; Newman-Keuls test). Importantly, the Ps-1 protein was entirely absent in 38 % of nontasters. In addition, ANOVA revealed that the levels of all salivary proteins in un-stimulated saliva were not related to gender (the six protein families $F_{[5,258]} = 0.98$; $p=0.99$ and nine bPRPs $F_{[9,430]} = 0.30$; $p=0.97$).

Stimulated mean values ± SEM of the XIC peak areas of the six protein families (P-B, bPRP, aPRP, S-Cyst, Stath, Hist), as well as of the nine peptides of the bPRP family (P-F, P-J, P-D, P-H, IB-8a Tot, II-2 Tot, IB-1 Tot, 10434 and Ps-1) in PROP super-taster and nontaster saliva are shown in Figure 4. Post-hoc comparisons subsequent to three-way ANOVA showed that, among the six protein families quantified, taste stimulation with PROP (3.2 mM) induced, in PROP super-taster saliva, a significant increase in the XIC peak area of the bPRP family with respect to basal levels (after 5 min from stimulation, $p<0.001$, and after 10 min from stimulation, $p<0.001$ respectively; Newman-Keuls test). Among the nine peptides of the bPRP family, PROP stimulation induced a significant increase in the XIC peak area of II-2 (Tot) and Ps-1 proteins with respect to basal levels in PROP super-taster saliva ($p\leq0.025$ and $p\leq0.0054$ respectively; Newman-Keuls test). No significant changes were found in stimulated saliva of nontaster subjects ($p>0.05$).

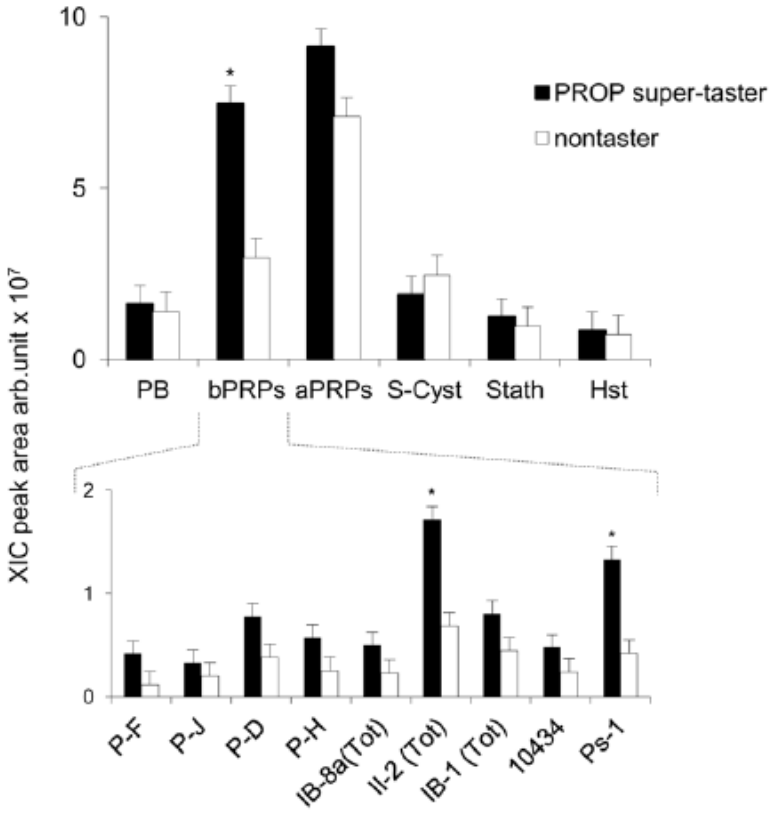

Figure 3 - Relationships between PROP taste responsiveness and the basal level of salivary proteome. Mean values ± SEM of the XIC peak areas of the six protein families (P-B, bPRP, aPRP, S-Cyst, Stath, Hist) (upper graph), and of the following individual bPRPs (P-F, PJ, P-D, P-H, IB-8a Tot, II-2 Tot, IB-1 Tot, 10434 and Ps-1) (lower graph) in PROP super-taster (n = 24) and nontaster (n = 21) un-stimulated saliva.

* = significant difference between PROP super-tasters and nontasters ($p<0.001$; Newman-Keuls test subsequent to two-way ANOVA).

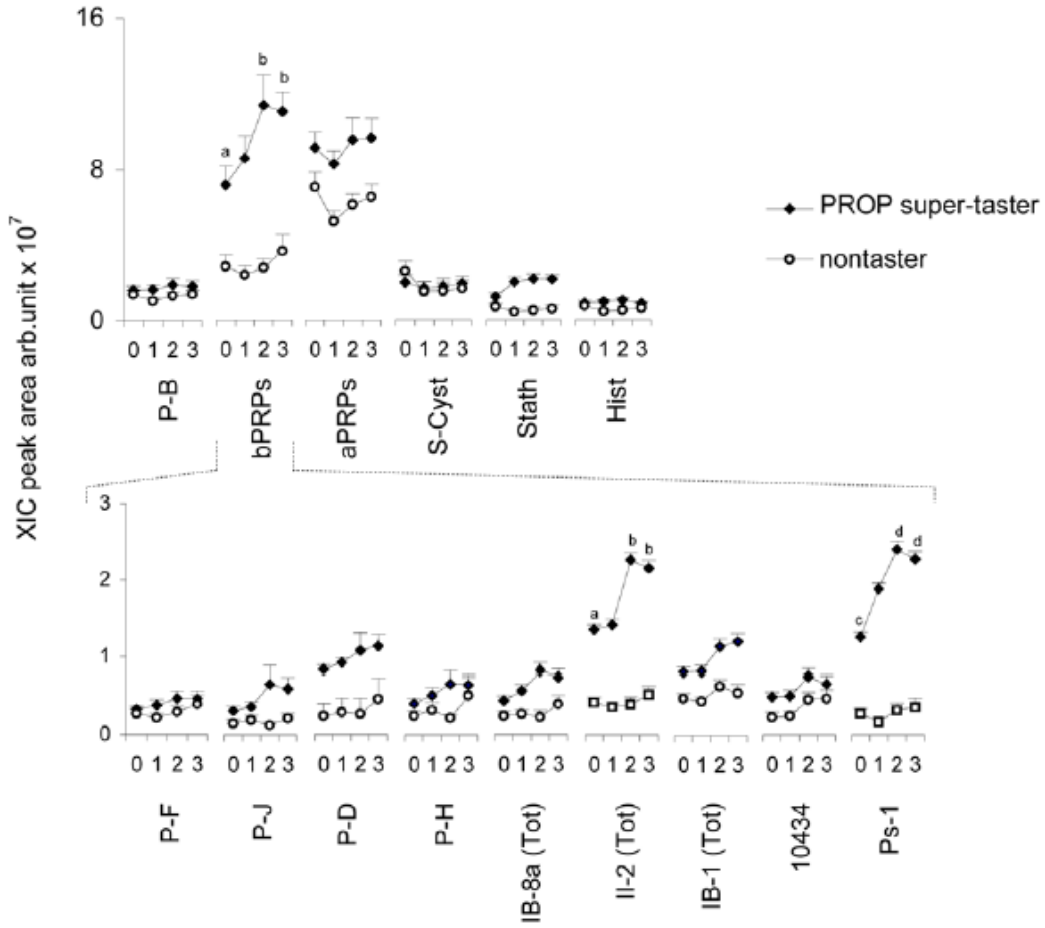

Figure 4 - Relationships between PROP taste responsiveness and the salivary proteome after PROP bitter taste stimulation. Mean values ± SEM of the XIC peak areas of the six protein families (P-B, bPRP, aPRP, S-Cyst, Stath, Hist) (upper graph), and of the following individual bPRPs (P-F, P-J, P-D, P-H, IB-8a Tot, II-2 Tot, 10434 and Ps-1) in PROP super-taster (n = 24) and nontaster (n = 21) saliva before (0 in the X-axis) and after PROP (3.2 mM) stimulation. (The numbers 1, 2, 3 on the X-axis correspond to immediately after stimulation, after 5 and 10 min from stimulation, respectively).
Different letters indicate significant difference ($p \leq 0.025$; Newman-Keuls test subsequent to three-way ANOVA).

Discussion

A primary aim of the present study was to determine if the genetic predisposition to taste the bitterness of PROP is reflected in the salivary proteome. We demonstrated for the first time that PROP status was strongly associated with basal levels of specific salivary peptides belonging to the basic proline-rich protein family. In fact, a comparative analysis of salivary protein levels in un-stimulated saliva showed that PROP super-tasting, which is strongly associated with the PAV haplotype of *TAS2R38* and the

A allele rs2274333 of the gustin gene, was also related to higher concentrations of the II-2 peptide and the Ps-1 protein, compared with PROP non-tasting which is associated with the minor alleles at both loci. None of the other proteins we analysed were related to PROP responsiveness, and no changes in the salivary proteome were related to gender. In addition, no changes in salivary protein secretion have been observed in the age range studied here [52]. Thus, neither gender nor age differences explain our findings.

The two bPRPs, that we found related to PROP status, are both encoded by the *PRB1* gene [53]. The family of *PRB* genes of chromosome 12p13.2 codes for basic and glycosylated PRPs [53,54]. Mutations in *PRB* genes (including *PRB1*) are very common and could lead to lack of expression and null phenotypes. The *PRB1* gene shows different-length and null polymorphisms. In particular, this locus exhibits four alleles named *S, Small; M, Medium; L, Large;* and *VL, Very Large*. The alleles *S*, *M* and *L* have been characterized and their expression products are pro-proteins which generate mature bPRPs by post-translational proteolytic cleavages. It is known that II-2 peptide derives from the cleavage of each pro-protein expressed by *PRB1 S*, *M*, and *L* alleles. Conversely, Ps-1 protein only derives from the *PRB1 M* allele [53].Our data on basal levels of Ps-1 protein in nontaster saliva indicate that this protein is poorly expressed (or not expressed) in these individuals, and suggest that the ability to taste PROP may be related to *PRB1*gene polymorphisms. In addition, these findings support the hypothesis that PROP super-tasting, which is related to high Ps-1 levels, might also be associated with the *M* allele of this gene. The latter assumption could also explain the specific increase in PROP super-taster saliva of the II-2 and the Ps-1 levels after PROP stimulation. By possessing a functional gene encoding the precursor for these proteins, PROP super-taster individuals may be able to secrete these proteins after stimulation, while nontasters lacking a functional gene are not able to do so.

In addition, since bPRPs are exclusively expressed by parotid glands [55,56], our data suggest that the bitter taste of PROP may specifically stimulate the rapid salivary secretion of these glands. This is in agreement with previous data showing a taste-specific secretion of parotid glands following stimulation with sour-lemon [57].

Recently, we showed that PROP responsiveness is strongly associated with gustin (CA6) salivary protein functionality [37], and that the combination of *TAS2R38* and gustin gene genotypes partially explains supertasting [40]. The results of the present study confirm that PROP responsiveness is associated to *TAS2R38* and gustin gene polymorphisms [37,40], and further extend this knowledge by examining salivary proteins which are products of the *PRB1* gene and are known to vary among individuals.

These findings suggest that the *PRB1* gene may also play a role in modulating the expression of the PROP phenotype. Future studies will examine this possibility.

The salivary proteins, primarily PRPs, have been mainly studied in relation to ingestion of tannins [43,58-61]. These salivary proteins neutralize the negative biological effects of tannins by favoring their precipitation [43]. Individuals who respond best to tannins are able to neutralize more of these compounds, as an adaptive mechanism. Having the ability to secrete high levels of these proteins would be a prerequisite to being a high-responder to tannins [59,60]. Although the focus of this study is limited to a bitter molecule, such as PROP, our results show that PROP tasting could have implications in a broader nutritional context. Future studies should examine classically-defined bitter molecules as well as tannins. These studies will help to determine if these salivary proteins serve both a permissive function, that allows the individual to taste bitterness, as well as a protective function against the negative effects of tannins.

In conclusion, these novel findings extend the understanding of the PROP phenotype by identifying new candidates in the salivary proteome to explain individual differences in the genetic predisposition to taste thiourea compounds. Our finding may have important implications for understanding taste function impairment, eating behaviour and nutritional status. Whether the results described here are uniquely related to PROP tasting is unknown. Given the complex nature of human bitter taste experience, it seems likely that variation in the salivary proteome represents an additional layer of genetic diversity contributing to individual differences in bitterness perception. Future experiments will address this question by investigating other tastants and phenotypes.

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2

Marked Increase in PROP Taste Responsiveness Following Oral Supplementation with Selected Salivary Proteins or Their Related Free Amino Acids

Melania Melis
Maria Carla Aragoni
Massimiliano Arca
Tiziana Cabras
Claudia Caltagirone
Massimo Castagnola
Roberto Crnjar
Irene Messana
Beverly J Tepper
Iole Tomassini Barbarossa

PLoS ONE 2013, 8(3): e59810. doi:10.1371/journal.pone

Abstract

The genetic predisposition to taste 6-n-propylthiouracil (PROP) varies among individuals and is associated with salivary levels of Ps-1 and II-2 peptides, belonging to the basic proline-rich protein family (bPRP). We evaluated the role of these proteins and free amino acids that selectively interact with the PROP molecule, in modulating bitter taste responsiveness. Subjects were classified by their PROP taster status based on ratings of perceived taste intensity for PROP and NaCl solutions. Quantitative and qualitative determinations of Ps-1 and II-2 proteins in unstimulated saliva were performed by HPLC-ESI-MS analysis. Subjects rated PROP bitterness after supplementation with Ps-1 and II-2, and two amino acids (L-Arg and L-Lys) whose interaction with PROP was demonstrated by 1H-NMR spectroscopy. ANOVA showed that salivary levels of II-2 and Ps-1 proteins were higher in unstimulated saliva of PROP super-tasters and medium tasters than in non-tasters. Supplementation of Ps-1 protein in individuals lacking it in saliva enhanced their PROP bitter taste responsiveness, and this effect was specific to the non-taster group.1H-NMR results showed that the interaction between PROP and L-Arg is stronger than that involving L-Lys, and taste experiments confirmed that oral supplementation with these two amino acids increased PROP bitterness intensity, more for L-Arg than for L-Lys. These data suggest that Ps-1 protein facilitates PROP bitter taste perception and identifies a role for free L-Arg and L-Lys in PROP tasting.

Introduction

The ability to detect bitterness may have evolved to protect human beings from ingesting bitter-tasting toxins from plants and the environment. Humans possess an array of ~25 bitter receptors that are capable sensing thousands of natural and synthetic compounds that impart bitter taste [1–4]. Some of these receptors are generalists, activated by many, chemically-diverse compounds (broadly tuned), whereas others are specialists, responding to only a single or a few compounds with closely-related structures [5].

Individuals vary in their perception of bitterness, and this variation is in part, genetically-determined. Genetic variability in aste sensitivity to thiourea derivatives, such as phenylthiocarbamide (PTC) and 6-n-propylthiouracil (PROP), is one of the moststudied human traits [6]. Both PROP and PTC contain a thiourea functional group (SC(NHR)2), which is responsible for their bitter taste [7–9]. The thiourea moiety is also a constituent of naturally occurring glucosinolates that are present in bitter-tasting plants of the Brassica family. Studies have shown that taste responsiveness to PTC/PROP is associated with greater perception of bitterness from glucosinolate-containing [10] and other bitter vegetables and fruits [11] as well as decreased liking and intake of these foods [12–17]. Since PROP status is also associated with individual differences in fat perception and liking, energy intake and body weight, it has often been used as an oral marker for general food preferences and dietary behavior with subsequent links to body composition [11,18–21]. Other taste receptor variants have been identified in humans that are important for bitter taste perception and liking [22–24]. However, these variants do not function as broad-based genetic markers of chemosensory responsiveness as has been attributed to PROP phenotype.

Individuals can be classified into three PROP taster categories: non-tasters, medium tasters, and PROP super-tasters based on suprathreshold measures at higher concentrations [18,19,21 25–31]. Non-tasters and PROP super-tasters illustrate the extremes of the phenotype with non-tasters showing little or no taste responsiveness to PROP, and PROP super-tasters experiencing intense bitter sensation from the compound. Medium tasters experience moderate bitterness sensation. Other reports suggest that PROP tasting may be a more continuous phenotype [18,19,27,28 32].

A growing literature in this field has focused on understanding and identifying the factors contributing to these large phenotypic differences in PROP bitter taste perception [32–35]. The ability to taste PROP is associated with haplotypes of the TAS2R38 gene. Three amino acid substitutions (Pro49Ala, Ala262Val, and Val296Ile) in the sequence of this gene express variants of the receptor that bind the C= S moiety of the thiourea group

[27,32,36]. Individuals homozygous for the AVI haplotype experience total taste blindness to PROP or a mild bitterness, whereas those homozygous or heterozygous for the PAV haplotype can taste PROP bitterness even at low concentrations. Other haplotypes (AAV, AAI, and PVI) have been observed rarely or are limited to specific populations [6]. Allelic diversity in TAS2R38 accounts for the majority but not all of the phenotypic variation in PROP bitterness, thus implying the involvement of other factors [11,28,36,37]. Indeed, family segregation, family-based linkage and genome-wide association studies suggest that other modifying genes may play a role in individual differences in PROP sensitivity [23,38,39]. Recent studies demonstrate that polymorphism rs2274333 (A/G) in the gene that codes for the salivary protein gustin (CA6) is also associated with PROP taster status in an ethnically homogeneous cohort [35,40]. Specifically, the majority of PROP super-tasters also expressed the AA (active) form of gustin, whereas the majority of non-tasters expressed the GG (inactive) form of gustin. Gustin is thought to be a trophic factor for taste bud development and maintenance [41]. PROP phenotype is modulated by the apparent cooperation between TAS2R38 and gustin polymorphisms, and the latter may explain why PROP super-tasters have a greater density of fungiform taste papillae which may contribute to their heightened oral chemosensory responsiveness.

As early as 1932, Fox [7] speculated that the inability to taste PTC/PROP was due to the presence of a product (perhaps a protein) in the saliva of non-tasters that precipitated the PROP molecule and interfered with its perception. This hypothesis received partial and indirect support from experiments indicating that the stimulating capability of a taste stimulus depends on its solubility [42]. However, other evidence suggests that PTC nontaster condition is unlikely to depend on the lack a salivary component that permits PTC to be tested [43–45]. Our laboratory has been studying the involvement of salivary proteins in PROP tasting [46]. We recently showed that PROP status is associated with basal levels of two salivary peptides belonging to the basic proline-rich protein family (bPRP), namely Ps-1 and II-2, which are both encoded by the PRB1 gene [47]. In particular, we demonstrated that greater PROP bitterness was related to higher concentrations of the Ps-1 protein and II-2 peptide compared with lower PROP bitterness. The functional significance of these two proteins in the saliva of PROP super-tasters is currently unknown. The best-known function of PRPs is their ability to precipitate and neutralize the negative biological effects of tannins during the development of oral astringency [48–53]. Establishing another role for PRPs in PROP bitterness perception would extend our understanding of their biological functions and demonstrate their importance within a broader nutritional context.

The purpose of this work was to gain insight into the physiological mechanisms by which Ps-1 and II-2 facilitate the perception of PROP bitterness in subjects classified by PROP phenotype. We administered Ps-1 and II-2 to individuals who lacked these proteins to determine if oral supplementation would lead to greater bitterness perception from PROP. ^{1}H-NMR spectroscopy was used to chemically probe the interaction between PROP and the free amino acids present in the Ps-1 and II-2 sequences. This experiment identified two amino acids (L-arginine and L-lysine) involved in the local binding of these peptides to the PROP molecule. We then administered L-arginine and L-lysine to subjects to determine if oral supplementation with these amino acids enhanced the bitterness of PROP. The overall design of the study is depicted in Figure 1.

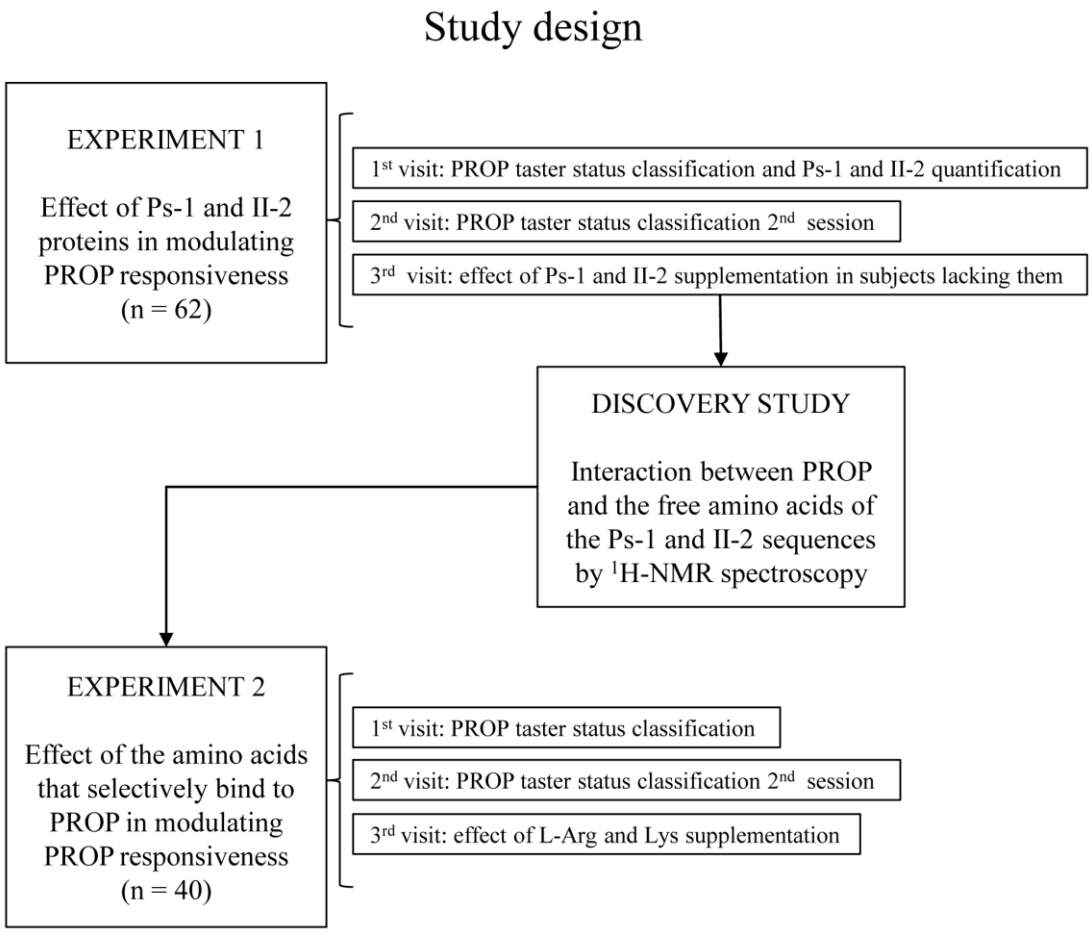

Figure 1 - Graphic diagram representing the study design.

Materials and Methods

Ethics Statement

Ethics approval was obtained from the Ethical Committee of the University Hospital of Cagliari, and the study has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. All subjects reviewed and signed an informed consent form.

Subjects

One hundred and two non-smoking subjects were recruited through public advertisements at the University of Cagliari. All were healthy white men (n= 35) and women (n =67), their average age being 27.6 y ± 61.2 y and with a body mass index (BMI) ranging from 18.6 to 25.3 kg/m2. They had no variation in body weight larger than 5 kg recorded over the previous 3 months, and were not following a prescribed diet or taking medications that might interfere with taste function. Subjects neither had food allergies, nor scored high on eating behaviour scales (assessed by the Three- Factor Eating Questionnaire) [54]. In order to rule out any gustatory impairment, thresholds for sweet, sour, salty, and bitter tastes were determined for all participants. None of the participants was ageusic. At the beginning of the protocol, before signing an informed consent form, each subject was verbally instructed about the procedure and the aim of the study.

Experimental Procedures

All subjects were requested to abstain from eating, drinking and using oral care products or chewing gums for at least 8 h prior to taste tests that were completed in three visits in both experiments (1 and 2). They had to be in the test room 15 min before the beginning of the trials (at 9.30 AM) in order to adapt to the environmental conditions (23–24°C; 40–50% relative humidity) which were kept constant throughout the experimental sessions. In the first visit, before starting taste assessments, 1 mL sample of whole unstimulated saliva was collected for the Ps-1 and II-2 quantitative determination by HPLC-ESI-IT-MS analysis as described below. In women, the taste assessments and saliva collection were done on the sixth day of the menstrual cycle to minimize taste sensitivity changes and value fluctuations due to the estrogen phase [55,56].

For all taste assessments, the solutions were prepared the day before each session and stored in the refrigerator until 1 h before testing. Stimuli were presented at room temperature. The taste intensity rating for each solution was recorded by using the Labeled Magnitude Scale (LMS) [57] in which each subject placed a mark on the scale corresponding to his/her perception of the stimulus. The LMS scale gives subjects the

freedom to rate the intensity of a stimulus relative to the ‘‘strongest imaginable’’ oral stimulus they have ever experienced in their life.

PROP Screening and Taster Status Classification

Subjects were assessed for PROP taster status using the 3- solution test [30,58]. Taste intensity ratings were collected for three suprathreshold PROP (Sigma-Aldrich, Milan, Italy) (0.032, 0.32, and 3.2 mM) and sodium chloride (NaCl, Sigma-Aldrich, Milan, Italy) (0.01, 0.1, 1.0 M) solutions dissolved in spring water. NaCl was used as standard as previously done in other studies [12,25,30]. Subjects were classified for PROP taster status in two visits that were separated by a 1-month period. The presentation order of the two taste stimuli (10 mL) (PROP or NaCl) was reversed in the two visits, and concentrations within each solution type were tasted in a random order. An oral rinsing with spring water followed each stimulation. The interstimulus interval was set at 60 s.

The mean of ratings in the two replicates was calculated and perceived taste intensity functions for PROP and NaCl for each subject were generated from the results [12,30]. When intensities of PROP ratings increased more steeply across concentrations than those of NaCl ratings, the subject was classified as a ‘‘PROP super-taster’’ (n= 36). On the contrary, when the NaCl ratings increased more steeply than did the PROP ratings, the subject was classified as a non-taster (n= 35). When the PROP ratings overlapped with the NaCl ratings, the subject was classified as a medium taster (n= 31). ANOVA was used to document the presence of the three taster groups (see Table S1). According to the study design (see Figure 1), subjects were divided into two pools. The first subject pool (n= 62) was composed of 24 PROP super-tasters; 17 medium tasters and 21 non-tasters who rated the bitterness of PROP after oral supplementation with PS-1 or II-2. The second pool (n= 40) was composed of 12 PROP super-tasters; 14 medium tasters and 14 non-tasters who rated the bitterness of PROP after oral supplementation with L-Arg and L-Lys.

Table S1 - Ratings of perceived taste intensity in response to three concentrations of PROP and NaCl in the taster groups

	super-tasters (*n*=36)	medium tasters (*n*=31)	non-tasters (*n*=35)
PROP			
0.032 mM	6.83 ± 0.93	3.91 ± 1.37	1.77 ± 0.50
0.32 mM	38.51 ± 1.76*	22.80 ± 1.60	5.80 ± 0.76*
3.2 mM	80.88 ± 2.83*	49.81 ± 3.28	27.72 ± 2.79*
NaCl			
0.01 M	1.63 ± 0.43	2.88 ± 1.10	7.15 ± 1.37
0.1 M	16.17 ± 1.09*	21.21 ± 2.07	34.87 ± 2.86*
1 M	40.05 ± 1.99*	54.72 ± 3.37	65.01 ± 3.13*

Values are means ± SEM. n = 102.Three-way ANOVA was used to compare PROP intensity ratings with NaCl intensity ratings across groups ($F_{[4,594]}$ = 37.166; p<0.00001).

* = significant difference between PROP and the corresponding NaCl concentration (p<0.00001; Newman-Keuls test).

Ps-1 and II-2 Salivary Protein Analyses

Saliva collection and treatment

A sample (1 mL) of whole unstimulated saliva was collected from sixty-two subjects with a soft plastic aspirator as it flowed into the anterior floor of the mouth for less than 1 min, and then transferred to a plastic tube.

Each sample was immediately mixed with an equal volume of aqueous trifluoroacetic acid (0.2%) in an ice bath, in order to preserve and stabilize the sample by inhibiting salivary proteases. The solution was then centrifuged at 8000 g, and kept at 4uC for 15 min. The acidic supernatant was separated from the precipitate and then immediately stored at -80°C until the HPLC-ESI-ITMS analysis.

HPLC-ESI-IT-MS analysis

Ps-1 and II-2 proteins were identified and quantified in each of the sixty-two samples, by HPLC-ESI-IT-MS according to Cabras et al. [46]. 100 mL of the acidic soluble fraction corresponding to 50 mL of whole unstimulated saliva was used. Identification was based on the chromatographic behavior and comparison of the experimental mass values with the theoretical ones reported in the Swiss-Prot Data Bank (http://us.expasy.org/tools). The quantification of Ps-1 and II-2 proteins was based on the area of the RP-HPLC-ESI-MS extracted ion current (XIC) peaks. The XIC analysis reveals the peak associated with the protein of interest by searching along the total ion

current chromatographic profile of the specific multicharged ions generated at the source by the protein. The area of the ion current peak is proportional to concentration, and under constant analytical conditions it may be used to perform relative quantification of the same analyte in different samples [59,60].

Ps-1 and II-2 proteins purification

To purify Ps-1 and II-2 proteins, a volume of 35 mL of whole saliva was collected from a single healthy female volunteer in our laboratory after she signed an informed consent. The whole saliva was treated as previously described and the volume of the acidic soluble fraction reduced by lyophilization to ca 2 mL was stored at -80°C until purification. The concentrated acidic soluble fraction of 35 mL of whole saliva was submitted to gel-filtration on a Sephadex-G 75 column (44 x 3 cm) equilibrated with 20 mM sodium acetate buffer, pH 4.8, at a flow rate of 0.35 μL/min. Fractions of 1 mL were collected and checked at 214 and 276 nm. Six pools were collected on the basis of the elution profile. Each pool was concentrated by lyophilization and then dialyzed against ultra-pure deionized water. HPLC-ESI-MS revealed that pool 2 contained almost pure Ps-1 and pool 5 almost pure II-2. The XIC peak area/mL was measured for both proteins.

PROP bitterness assessments after supplementation with Ps-1 and II-2 proteins

The concentration of each bPRP added to PROP solution (3.2 mM) corresponded to the average amount of the protein determined in 1 mL of the PROP super-taster unstimulated saliva, as established on the basis of the XIC peak area (Ps-1:1.33 x 10^9 and II-2:1.55 x 10^9 a.u.).

In a third visit, the effect of the Ps-1 or II-2 supplementation on PROP bitterness was assessed in subjects of the first pool who were lacking in Ps-1 (n= 20) or II-2 (n= 7), respectively. Briefly, all rinsed their mouth with spring water before starting. Each subject was presented, in a random order, with 2 cups (4 mL samples) one containing only PROP and the other PROP supplemented with Ps-1 or II-2. They were instructed to swish the entire contents of one cup in their mouth for 10 s and then to spit it out. Each stimulation was followed by oral rinsing with spring water. The interstimulus interval was set at 5 min. After 1 h each subject was presented with two other cups (controls) one containing only the Ps-1 protein and the other only the II-2 peptide at the same concentrations previously used. The intensity rating for each solution was collected by having the subject place a mark on the LMS scale corresponding to his/her perception of the stimulus.

^{1}H-NMR Spectroscopy-PROP/Amino acid binding

The interaction between PROP and the free form of amino acids present in the Ps-1 and II-2 sequences was investigated by ^{1}H-NMR spectroscopy. This technique is a powerful analytical tool capable of identifying and quantifying a large number of compounds having hydrogen atoms, and it has been already employed in evaluating the interaction of proteins and/or specific amino-acid sequences with tannins and polyphenols [61 Charlton et al 1996]. A proton involved in the interaction with an external molecule experiences a modification in its chemical surrounding that implies a field-shift and a change of the corresponding ^{1}H-NMR signal. Thus, when such an interaction occurs, a variation of the chemical shift of the protons belonging to the amino acids of the protein directly involved in the local binding is expected. We individually recorded the ^{1}H-NMR spectra of all the amino acids present in the Ps-1 and II-2 sequences before and after the addition of an equimolar amount of PROP.

All experiments were recorded at 300 K using a Varian Inova 500 MHz FT-NMR system.

Spectra were processed and displayed using the MestReNova program. The experiments were performed by preparing 0.5 mL of a 5 mM solution of each amino acid in D_2O and then recording the corresponding spectrum. Afterwards, an equimolar amount of a PROP solution in D_2O was added to each amino acid solution and the ^{1}H-NMR spectrum recorded. Chemical shifts for ^{1}H NMR are reported in parts per million (ppm), calibrated to the residual solvent peak set, with coupling constants reported in Hertz (Hz).The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, m= multiplet, dd = doublet of doublets. The ^{1}H-NMR chemical shift change for the PROP ring proton in the absence and in the presence of each amino acid was determined in terms of $\Delta = (|(\delta' - \delta_0)|/ \delta_0) \cdot 100$, which represents the absolute value of the difference between the ^{1}H-NMR signal (ppm) of the PROP ring proton in the absence (δ_0) and in the presence (δ') of the amino acid, normalized for δ_0 and expressed as a percentage.

PROP bitterness assessments after supplementation with L-Arginine and L-Lysine

On the basis of the results obtained in the ^{1}H-NMR spectroscopy binding study, the effect of L-Arg and L-Lys supplementation on PROP (3.2 mM) bitterness was assessed in a third visit of second pool subjects. Each subject was presented, in a random order, with 3 cups (4 mL samples): one containing only PROP, one with PROP supplemented with L-Arg, and one with PROP supplemented with L-Lys. After 1 h, each subject was presented with two more cups, one containing only L Arg and the other containing only

L-Lys. The procedure for collecting the taste intensity ratings was the same as the one described for the supplementation of Ps-1 and II-2 proteins. Concentrations of L-Arg (prepared from the hydrochloride salt, Sigma-Aldrich, Milan, Italy) and L-Lys (Sigma-Aldrich, Milan, Italy) were 3.2 mM.

Statistical Analyses

The Kruskal-Wallis test was used to compare the concentrations of the Ps-1 protein and II-2 peptide in unstimulated saliva of PROP super-tasters, medium tasters and non-tasters, and to evaluate gender differences. The Fisher exact test was used to compare the percentage of subjects lacking Ps-1 or II-2 across PROP taster groups. Repeated measures ANOVA was used to analyse the effects of supplementation with the two proteins (Ps-1 and II-2) or the two amino acids (L-Arg and L-Lys) on PROP bitterness intensity. Post-hoc comparisons were conducted with the Newman-Keuls test. Statistical analyses were conducted using STATISTICA for WINDOWS (version 7; StatSoft Inc, Tulsa, OK, USA). P values < 0.05 were considered significant.

Nomenclature

When genes and the encoded proteins share the same acronym, the name of the gene is identified in italics, while its corresponding encoded protein by plain text.

Results

Ps-1 or II-2 Oral Supplementation

Figure 2 shows the distributions of the relative concentrations of the Ps-1 protein and II-2 peptide determined by HPLC-ESI-ITMS analysis in unstimulated saliva of PROP super-tasters, medium tasters and non-tasters. The Kruskal-Wallis test showed that mean values of the extract ion current (XIC) peak areas of Ps-1 and II-2 depend on PROP taster status (Ps-1: $H[2,62]= 7.573$, $p =0.02$ and II-2: $H[2,62] = 14.958$, $p= 0.0006$). Pairwise comparisons showed that Ps-1 concentration was significantly lower in saliva of nontasters than in PROP super-tasters (Ps-1: $p= 0.0216$), and that of II-2 was significantly lower in saliva of non-tasters than in PROP super-tasters and medium tasters ($p\leq0.004$). The figure also shows that several individuals were lacking these proteins. The Ps-1 protein was undetected in a total of 20 subjects, while the II-2 peptide was undetected in only 7 subjects. Additionally, the percentage of non-tasters lacking Ps-1 (43%) was higher from that of PROP super-tasters (17%) although at the limit of statistical significance ($p = 0.053$), while the percentage of medium tasters (41%) was not different from that of the other taster groups ($p\geq0.08$). The same pattern was

observed for II-2. The percentage of non-tasters lacking II-2 (24%) was statistically different from PROP super-tasters (all had II-2) (p= 0.017), while medium tasters (12%) were not different from the other taster groups (p≥0.08). No changes in the salivary proteome were related to gender (Ps-1: H[1,62] = 0.148, p= 0.700 and II-2: H[2,62] = 0.144, p= 0.704).

Repeated measures ANOVA revealed that PROP bitterness intensity of individuals lacking Ps-1 protein significantly increased after supplementation of this protein (F[1,17] = 7.2273; p= 0.0155) (Figure 3). Post-hoc comparisons showed that Ps-1 supplementation significantly increased the PROP bitterness intensity in non-tasters (p= 0.0367; Newman-Keuls test), but not in the other two taster groups (p.0.05) (Figure 2, lower graph). The solution containing only protein did not evoke any taste perception (data not shown). The supplementation of II-2 peptide did not produce the same effect (p.0.05) (data not shown).

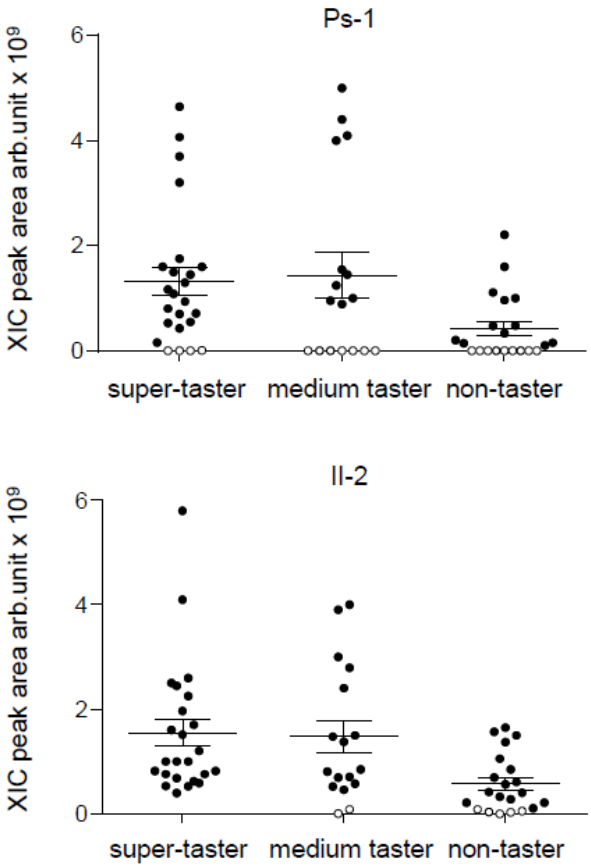

Figure 2 - Relative concentrations of Ps-1 and II-2 in the PROP taster groups in unstimulated (resting) saliva. Distribution of the XIC peak areas of Ps-1 and II-2 and mean values 6 SEM for each taster group are reported. Ps-1 mean values were lower in non-tasters than in PROP super-tasters and those of II-2 were lower in non-tasters relative to the other groups (Ps-1: $p = 0.0216$; II-2: $p \leq 0.004$; Kruskal-Wallis test). Out of 62 subjects, n = 21 non-tasters, n = 17 medium tasters and n = 24 PROP super-tasters. Subjects lacking Ps-1 (n = 20) or II-2 (n = 7) in their saliva are identified by white circles.

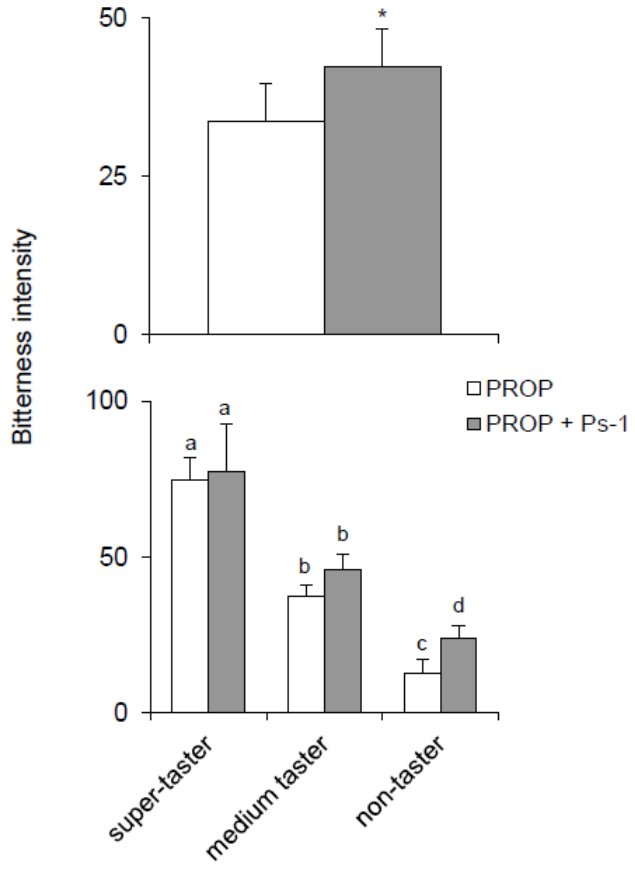

Figure 3 - Effect of the Ps-1 protein on PROP bitterness intensity. Mean (6 SEM) bitterness intensity evoked by PROP andPROP+Ps-1 solutions (upper graph) in 20 subjects lacking Ps-1. The same data are shown in the lower graph for each taster group (n = 9 non-tasters; n = 7 medium tasters; n = 4 PROP super-tasters). The solution containing only Ps-1 (control) is not shown as it did not evoke any taste perception. * = significant difference ($F[1,17] = 7.2273$, $p = 0.0155$; repeated measures ANOVA). Different letters indicate significant differences ($p \leq 0.0012$; Newman-Keuls test subsequent to repeated measures ANOVA).

^{1}H-NMR Spectroscopy – PROP Binding

^{1}H-NMR spectroscopy allowed us to determine the proton assignments for all analyzed amino acids before and after the addition of an equimolar amount of PROP (Table 1). It is interesting to note that after PROP addition, a chemical shift variation occurs only in the protons belonging to L-Arg and L-Lys, while the 1H-NMR signals for the other amino acids remained unchanged. Accordingly, the ring proton in the PROP molecule undergoes a chemical shift in the 1H-NMR signal in the presence of L-Arg and L-Lys only (Figure 4).

Table 1 - ^{1}H-NMR assignments (ppm) for the amino acids of Ps-1 and II-2 sequences, and PROP[a].

←	←^{1}H-Cα	←^{1}H-Cβ	←^{1}H-Cγ	←^{1}H-Cδ	←^{1}H-Cε	←^{1}H-Cβ'
←Ala	←3.83 (q)	←1.53 (d)	←	←	←	←
←Arg	←3.26t	←1.66-1.73 (m)	←1.66-1.73 (m)	←3.38t	←	←
←	←(3.28t)[b]	←(1.67-1.75m)[b]	←(1.67-1.75m)[b]	←(3.6br)[b]	←	←
←Asn	←4.06 (q)	←2.95 (dd)	←	←	←	←
←Asp	←4.10 (t)	←3.03 (dd)	←	←	←	←
←Gly	←3.61 (s)	←	←	←	←	←
←Glu	←3.86 (t)	←2.15-2.26 (m)	←2.59-2.63 (m)	←	←	←
←Gln	←3.82 (t)	←2.19 (q)	←2.48-2.55 (m)	←	←	←
←Ile	←3.72 (d)	←2.00-2.05 (m)	←1.32-1.82 (m)	←1.00 (t)	←	←1.06 (d)
←Leu	←3.78 (t)	←1.71-1.82 (m)	←1.71-1.82 (m)	←1.01 (t)	←	←
←Lys	←3.61t	←1.76-1.85 (m)	←1.40.1.60 (m)	←1.74t	←3.06 (t)	←
←	←(3.8br)[b]	←(1.92-2.00 m, br)[b]	←(1.40-1.60 m, br)[b]	←(1.77t)[b]	←(3.07t)[b]	←
←Pro	←4.18 (t)	←2.38-2.53 (m)	←2.04-2.15 (m)	←3.37-3.50 (m)	←	←
←Ser	←3.94-4.06 (m)	←3.89 (t)	←	←	←	←
←Val	←3.66 (d)	←2.29-2.36 (m)	←1.07 (dd)	←	←	←

[a] PROP assignments (ppm): 1.00t [$\mathbf{CH_3}(CH_2CH_2)$]; 1.70q [$(CH_3)\mathbf{CH_2}(CH_2)$; 2.25t [$(CH_3CH_2)\mathbf{CH_2}$]; 5.985s H(**CH**).

[b] Chemical shifts upon PROP addition are reported in parentheses only when changed.

NMR signal descriptions: s (singlet); d (doublet); t (triplet); q (quadruplet); m (multiplet); br (broad signal); dd (doublet of doublets).

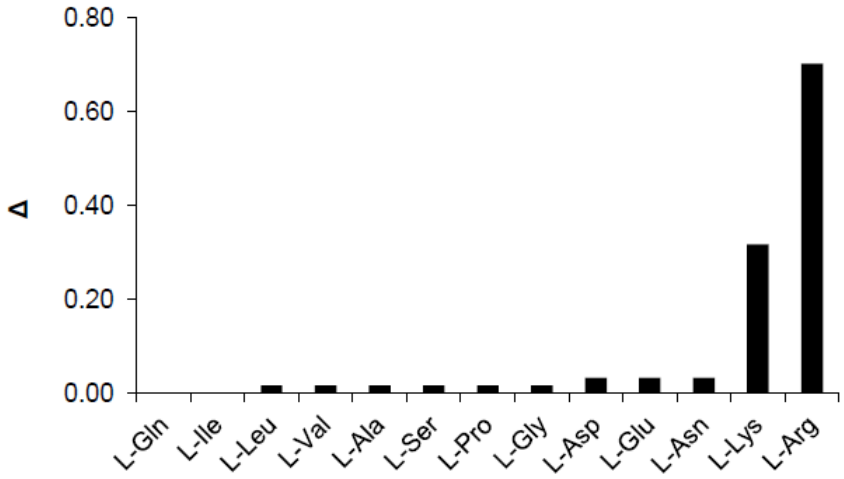

Figure 4 - PROP ring proton 1H-NMR chemical shift variation upon amino acids addition reported as Δ. $\Delta = (|(\delta' - \delta_0)|/\delta_0) \cdot 100$ represents the absolute value of the difference between the ^{1}H-NMR signal (ppm) of the PROP ring proton in the absence (δ_0) and in the presence (δ') of the amino acid of the Ps-1 and II-2 sequences, normalized for d0 and expressed as a percentage. For each amino acid, two spectra were recorded in 0.5 mL of 5 mM D_2O solution before and after the addition of an equimolar amount of PROP.

L-Arg or L-Lys Oral Supplementation

The effect of L-Arg or L-Lys supplementation on PROP bitterness intensity in 40 subjects of experiment 2 is shown in Figure 5. Repeated measures ANOVA revealed that PROP bitterness intensity significantly increased after supplementation with either of the two amino acids (L-Arg: $F[1,37] = 27.124$, $p = 0.00001$ and L-Lys: $F[1,37] = 5.949$, $p = 0.0196$) (upper graph). Post-hoc comparisons showed that L-Arg supplementation significantly increased the PROP bitterness intensity in non-tasters and medium tasters ($p \leq 0.0012$; Newman-Keuls test), but not in PROP super-tasters ($p > 0.05$). Instead, post hoc comparison showed no significant differences in the case of L-Lys supplementation ($p > 0.05$). The solutions containing only L-Arg or L-Lys did not evoke any taste perception (data not shown).

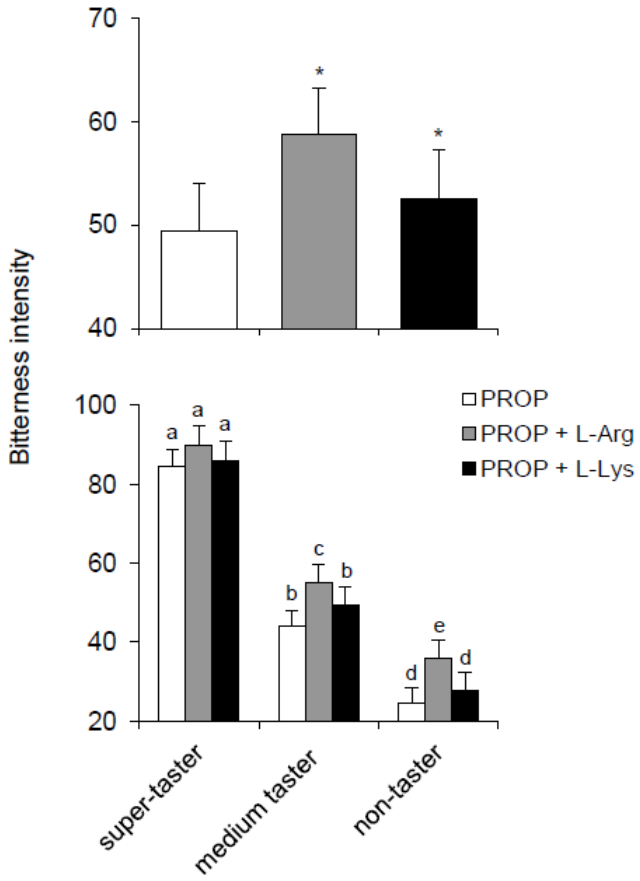

Figure 5 - Effect of L-Arg or L-Lys supplementation on PROP bitterness intensity. Mean values 6 SEM of bitterness intensity evoked by PROP, PROP+L-Arg and PROP+L-Lys solutions in a group of 40 subjects (upper graph). The same data are shown in the lower graph for each taster group (14 non-tasters; 14 medium tasters; 12 PROP super-tasters). Control solutions containing only L-Arg or L-Lys are not shown as they did not evoke any taste perception. * = significantly different from PROP (PROP +L-Arg: $F[1,37] = 27.124$, $p = 0.00001$ and PROP+L-Lys: $F[1,37] = 5.949$, $p = 0.0196$; repeated measures ANOVA). Different letters indicate significant differences ($p \leq 0.0012$; Newman- Keuls test subsequent to repeated measures ANOVA).

Discussion

The best-known function of salivary PRPs is their ability to bind and precipitate tannins in the oral cavity during astringency perception [48–53]. The present data provide new insights into the roles of Ps-1, II-2 and their constituent amino acids in PROP taste perception. First, in agreement with our previous findings [46], we showed that non-tasters had the lowest concentration of Ps-1 and II- 2 proteins in their saliva compared to PROP supertasters who had the highest concentrations. In addition, we

found that many non-tasters and medium tasters lack the two proteins in their saliva, while all or almost all PROP super-tasters have them. The lack of these two proteins in a large number of medium tasters (50% for Ps-1) is consistent with the moderate PROP responsiveness of individuals in this group.

Importantly, oral supplementation with Ps-1 in individuals lacking this protein in saliva enhanced their PROP bitter taste responsiveness, and this effect was most potent in non-tasters (Fig. 3). Since relatively few subjects (~11%) lacked salivary II-2, we could not test the effects of supplementation with this peptide on PROP bitterness.

To better understand the mechanism by which the Ps-1 protein increases PROP bitterness, we investigated the interaction between PROP and the free form of the constituent amino acids of the Ps-1 and II-2 sequences by ^{1}H-NMR spectroscopy. Our results indicate that only L-Arg and L-Lys, among all the amino acids in the sequences of the two proteins, interact with the PROP molecule, and the interaction between PROP and L-Arg is stronger than that involving L-Lys. Since L-Lys and L-Arg are the only amino acids displaying terminal amino-groups among those we studied, the ^{1}H-NMR measurements suggest that the interaction could involve these terminal groups and the carbonyl/thiocarbonyl groups of the PROP heterocycle.

Our psychophysical data strongly support the ^{1}H-NMR results, showing that L-Arg enhances the bitterness intensity of PROP more than L-Lys. Moreover, similar to our observations for Ps-1 supplementation, the effect of L-Arg on PROP bitterness intensity was restricted to non-tasters and medium tasters, and not PROP super-tasters. No changes in bitterness perception were related to PROP status in the case of L-Lys supplementation. It is worth noting that the bitter taste ratings of these amino acids are very low at high concentrations [62], and we found that L-Arg and L-Lys were tasteless at the concentrations used in this study.

The present findings may have implications for understanding the structural features and binding properties of the TAS2R38 receptor. According to recent studies, the predicted binding sites and binding affinity for PROP and PTC to the TAS2R38 receptor vary across TAS2R38 haplotypes [32,33,63]. Specifically, the hydrogen bond interaction between the transmembrane domain (TM) 3 and amino acid 262 in TM6 is involved in the interhelical network that permits the activation of the G protein-coupled receptor (GPCR) in the PAV haplotype but not in the AVI haplotype. Furthermore, the H bond between the PROP molecule and residue 262 in the PAV haplotype is involved in bitter tasting.

Based on these considerations and our own findings, we can speculate that the Ps-1 protein could be involved in orienting the PROP molecule within the binding pocket to optimize its binding when the receptor has the PAV form. The fact that supplementation

with Ps-1 in non-tasters increased PROP bitterness suggests that, even without an optimal binding pocket, the Ps-1 protein can help the PROP molecule twist and turn in order to facilitate its binding with the receptor in the AVI form. Considering that we observed a similar effect with L-Arg supplementation, and that this amino acid is highly represented in the protein sequence (7 occurrences), we suppose that the permissive function of the Ps-1 protein on PROP tasting might be carried out via L-Arg. In order to confirm this hypothesis, future studies will analyze the three dimensional structure of the protein in order to verify whether the spatial positions of the arginine residues are suitable for binding the stimulus.

In conclusion, this work further elucidates the role of the salivary proteome in PROP taste perception and highlights the importance of the Ps-1 protein and its constituent amino acids (L-Arg and L-Lys) in receptor binding and activation. Future studies will have to determine if the effects of Ps-1 on bitter taste enhancement are unique to PROP tasting or whether Ps-1 has broader effects on bitter taste function.

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The Gustin (CA6) Gene Polymorphism, rs2274333 (A/G), as a Mechanistic Link between PROP Tasting and Fungiform Taste Papilla Density and Maintenance

Melania Melis
Elena Atzori
Stefano Cabras
Andrea Zonza
Carla Calò
Patrizia Muroni
Mariella Nieddu
Alessandra Padiglia
Valeria Sogos
Beverly J Tepper
Iole Tomassini Barbarossa

PLoS ONE 2013, 8(9): e74151. doi:10.1371/journal.pone

Abstract

Taste sensitivity to PROP varies greatly among individuals and is associated with polymorphisms in the bitter receptor gene *TAS2R38*, and with differences in fungiform papilla density on the anterior tongue surface. Recently we showed that the PROP non-taster phenotype is strongly associated with the G variant of polymorphism rs2274333 (A/G) of the gene that controls the salivary trophic factor, gustin. The aims of this study were 1) to investigate the role of gustin gene polymorphism rs2274333 (A/G), in PROP sensitivity and fungiform papilla density and morphology, and 2) to investigate the effect of this gustin gene polymorphism on cell proliferation and metabolic activity. Sixty-four subjects were genotyped for both genes by PCR techniques, their PROP sensitivity was assessed by scaling and threshold methods, and their fungiform papilla density, diameter and morphology were determined. *In vitro* experiments examined cell proliferation and metabolic activity, following treatment with saliva of individuals with and without the gustin gene mutation, and with isolated protein, in the two iso-forms. Gustin and *TAS2R38* genotypes were associated with PROP threshold (p=0.0001 and p=0.0042), but bitterness intensity was mostly determined by *TAS2R38* genotypes (p<0.000001). Fungiform papillae densities were associated with both genotypes (p<0.014) (with a stronger effect for gustin; p=0.0006), but papilla morphology was a function of gustin alone (p<0.0012). Treatment of isolated cells with saliva from individuals with the AA form of gustin or direct application of the active iso-form of gustin protein increased cell proliferation and metabolic activity (p<0.0135). These novel findings suggest that the *rs2274333* polymorphism of the gustin gene affects PROP sensitivity by acting on fungiform papilla development and maintenance, and could provide the first mechanistic explanation for why PROP super-tasters are more responsive to a broad range of oral stimuli.

Introduction

Individual variability in sensitivity to the bitter taste of phenythiocarbamide was first recognized by Fox more than eight decades ago [1]. Since that time, steady progress has been made in elucidating the psychophysical features [2-5], population genetics [6,7] and molecular basis of this trait [8,9]. PTC/PROP tasting has also gained considerable attention as an oral marker for food preferences and eating habits that ultimately impacts nutritional status and health [10]. This role is based on data showing that the PROP phenotype associates with variation in perception and preference for fat [11-13], energy intake and body weight [14,15], selection of fruits and vegetables [16-18], plasma antioxidant status [19] and the risk of colon cancer [20-22]. This involvement remains controversial since some studies have failed to show the expected associations between PTC/PROP status and health outcomes [23-25]. These controversies could also be explained by confounding factors (such as cognitive control of eating behavior or the endocannabinoid system) that may play a prominent role in determining these associations [26,27].

The bitterness of PTC /PROP is due to the presence of the N-C=S group within these molecules. The human gene that expresses receptors that bind this chemical group is known as *TAS2R38*. Individuals can be divided into three taster groups (non-taster, medium taster and super-taster) based on behavioral testing assessing their PTC/PROP sensitivity. The percentage of non-taster individuals greatly varies among populations: from less than 7% to more than 40% [28]. There are two classes of screening methods: threshold determinations and suprathreshold measures that address stimulus detection and responsiveness at higher concentrations, respectively [2,10,13,14,29-36].

Allelic diversity in the *TAS2R38* bitter receptor gene is primarily responsible for PROP tasting [8,9]. Three polymorphic sites in the *TAS2R38* sequence, result in amino acid substitutions at positions Pro49Ala, Ala262Val, and Val296Ile, giving rise to two common haplotypes: PAV, the dominant (taster) variant and AVI, the recessive (non-taster) one. PROP-taster individuals possess the PAV/PAV or PAV/AVI diplotype, whereas non-tasters are homozygous for the recessive haplotype (AVI/AVI). Rare haplotypes (AAV, AAI, PVI, and PAI) have also been observed [6]. *In vitro* experiments [9] and receptor modelling [37,38] suggest that the PAV variant defines the active binding site of the receptor.

TAS2R38 is reported to account for majority (50-85%) of the variation in the phenotype [8,9], but a variety of observations suggest that other genes [39,40] may also be involved. On the other hand, a recent genome-wide association study revealed that only loci within the *TAS2R38* gene were associated with the perception of PROP [5].

This latter finding is consistent with the idea that the TAS2R38 receptor is specific for thiourea substances, and is not activated by bitter compounds lacking the thiourea group [41,42]. Nevertheless, recent data suggest that salivary proteins may complement the direct effects of DNA sequence variation in *TAS2R38* on PROP tasting, further refining bitterness perception. Specifically, Cabras et al. [43] showed that PROP super-tasting was associated with higher salivary levels of Ps-1 and II-2 peptides belonging to the basic proline-rich protein (bPRP) family of peptides, and that oral supplementation with Ps-1 peptide enhanced the bitterness of PROP [44]. These data are consistent with the role of bPRPs as modifiers of taste and astringent molecules [45–47].

Our laboratory has also been studying the role of the zinc dependent salivary protein, gustin (also known as carbonic anhydrase VI (CA6)), in PROP tasting [48,49]. Gustin/CA6 is a 42 kDa protein secreted by the parotid, submandibular and von Ebner glands [50–52]. Gustin is considered a trophic factor that promotes growth and development of taste buds since disruptions in this protein are known to decrease taste function [53]. Padiglia et al. [48] showed that the rs2274333 (A/G) polymorphism of the gustin gene results in an amino acid substitution at position Ser90Gly in the peptide, leading to a structural modification of the gustin active site, reduced zinc binding, and the accumulation of zinc ions in saliva. This gustin polymorphism is also strongly associated with PROP tasting [48] such that PROP super-tasters more frequently carried the AA genotype of gustin and expressed the native form of the protein, whereas PROP non-tasters more frequently carried the GG genotype and expressed the less functional form [49]. PROP super-tasters have a greater density of fungiform taste papillae on the anterior surface of the tongue [2,34,54–56]. Considering gustin's role in taste bud development and the close association between the rs2274333 polymorphism of gustin and PROP tasting, it is plausible that the relationship between papillae density and PROP status is mediated by gustin. To date, no studies have examined the effects of gustin on taste papilla morphology and physiology, particularly with respect to PROP taster status.

The objectives of this study were to investigate the effect of gustin gene polymorphism rs2274333 (A/G) and *TAS2R38* polymorphisms on PROP sensitivity and fungiform papillae density and morphology in a genetically homogeneous cohort. In addition, *in vitro* experiments, examined 1) the effect of treatment with saliva collected from individuals with genotype AA and GG of polymorphism rs2274333 on cell development and metabolic activity, and 2) the effect of treatment with isolated gustin, in the two iso-forms resulting from this polymorphism, on cell metabolic activity.

Materials and Methods

Ethical statement

All subjects was verbally informed about the procedure and the aim of the study. They reviewed and signed an informed consent form. The study was conformed to the standards set by the latest revision of Declaration of Helsinki and the procedures have been approved by the Ethical Committee of the University Hospital of Cagliari, Italy.

Subjects

Sixty-three non-smoking Caucasian healthy, young subjects (22 males, 42 females, age 25 ± 3 y) from Sardinia, Italy were recruited at the local University. They had a normal body mass index (BMI) ranging from 18.6 to 25.3 kg/m2 and showed no variation of body weight larger than 5 kg over the previous 3 months. None were following a prescribed diet or taking medications that might interfere with taste perception. Subjects neither had food allergies, nor scored high on eating behavior scales (assessed by the Three-Factor Eating Questionnaire [57]). Thresholds for the 4 basic tastes (sweet, sour, salty, bitter) were evaluated in all subjects in order to rule out any gustatory impairment.

PROP taste sensitivity assessments

The PROP phenotype of each subject was assessed by both threshold and suprathreshold measures. PROP (Sigma-Aldrich, Milan, Italy) thresholds were determined using a variation of the ascending-concentration, 3-alternative forcedchoice (3-AFC) procedure [58]. PROP solutions in spring water ranged from 0.00001 to 32 mM in quarter-log steps. Taste intensity ratings for a single suprathreshold PROP (3.2 mM) solution [49] were collected using the Labeled Magnitude Scale (LMS) [59] in which subjects placed a mark on the scale corresponding to his/her perception of the stimulus. The LMS scale gave subjects the freedom to rate the PROP bitterness relatively to the "strongest imaginable" oral stimulus they had ever experienced in their life.

For both methods, the solutions were prepared the day before each session and stored in the refrigerator until 1 h before testing. The stimuli were presented at room temperature as 10ml samples.

Molecular analysis

Subjects were genotyped for polymorphism rs2274333 (A/G) of the gustin (CA6) gene that consists of a substitution of amino acid Ser90Gly. They were also genotyped for three single nucleotide polymorphisms (SNPs) at base pairs 145 (C/G), 785 (C/T), and 886 (G/A) of the *TAS2R38* locus. The *TAS2R38* SNPs give rise to 3 non-synonymous coding exchanges: proline to alanine at residue 49; alanine to valine at residue 262; and valine to isoleucine at residue 296. These substitutions result in two major haplotypes (PAV and AVI) and three rare (AAI, PVI and AAV). The DNA was extracted from saliva samples using the Invitrogen Charge Switch Forensic DNA Purification kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified DNA concentration was estimated by measurements of OD260. PCR techniques were employed to amplify the gustin gene region including rs2274333 polymorphism, and the two short region of the *TAS2R38* gene including the three polymorphisms of interest.

To genotype gustin gene polymorphism rs2274333, a fragment of 253 bp was amplified with forward 5'TGACCCCTCTGTGTTCACCT3' and reverse 5'GTGACTATGGGGTTCAAAGG3' primers. The reaction mixtures (25 μL) contained 250 ng DNA, 10 pmol of each primer, 1.5 mM MgCl2, 100 mM Tris-HCl at pH 8.3, 50 mM KCl, 200 μM of dNTP mix, and 1.5 units of Hot Master Taq Eppendorf. Thermal cycles of amplification were carried out in a Personal Eppendorf Master cycler (Eppendorf, Germany). The amplification protocol included an initial denaturation at 95° C for 5 min, followed by 35 cycles of denaturation at 95° C for 30 s, annealing at 54° C for 30 s, and then extension at 72° C for 30 s. A final extension was carried out at 72° C for 5 min. Amplified samples were digested with HaeIII enzyme at 37° C for 4 hours. The digested fragments were electrophoresed on 2% agarose gel and stained with ethidium bromide. To determine *TAS2R38* haplotypes, PCR amplification followed by restriction analysis using *Hae*III for SNP detection at the 145 nucleotide position, and direct sequencing (using forward and reverse primers) for SNPs identification at the 785 and 886 nucleotide position. The following primer set was used to amplify a fragment of 221 bp including the first of three SNPs: F5-CTTCGTTTTCTTGGTGAATTTTTGGGATGTAGTGAAGAGG C**G**G-3' R 5'-AGGTTGGCTTGGTTTGCAATCATC-3'. The forward primer binds within

the *TAS2R38* gene, from nucleotides 101‑144. There is a single mismatch at position 143, where the primer has a G (underlined in bold) and the gene has an A. This mismatch is crucial to the PCR experiment, because the A nucleotide in the *TAS2R38* gene sequence, is replaced by a G in each of the amplified products. This creates the first G of the *Hae*III recognition sequence GGCC, allowing the amplified taster allele to be cut. The amplified non taster allele reads GGGC and is not cut. The

PCR reaction mixtures (25 μL) contained 250 ng DNA, 10 pmol of each primer, 1.5 mM MgCl2, 100 mM Tris-HCl at pH 8.3, 50 mM KCl, 200 μM of dNTP mix, and 1.5 units of Hot Master Taq Eppendorf. Thermal cycles of amplification were carried out in a Personal Eppendorf Master cycler (Eppendorf, Germany). The amplification protocol consisted of initial denaturation at 95° C for 5 min, followed by 35 cycles of denaturation at 95° C for 30 s, annealing at 64° C for 45 s, and then extension at 72° C for 45 s. For the analysis of the polymorphism G/C at position 143, a 3 μl aliquot of PCR products was mixed with a 17 μl solution containing 2 μl 10 × NE Buffer (50mM NaCl, 10mM Tris‑HCl, 10mM MgCl2, 1mM dithiothreitol, pH 7.9), 0.2 μ HaeIII (10 000 U ml-1; Sigma-Aldrich, St Louis, MO), and 14.8 μl sterile deionized H2O. The solution was incubated at 37° C for 4 h. The digest was mixed with 5 ml of loading buffer and electrophoresed on a 10% vertical polyacrylamide gel. The DNA bands were evidenced by ethidium bromide staining. The PCR 100 bp Low Ladder DNA was used as Mr markers (Sigma-Aldrich). Polymorphisms at the 785 and 886 nucleotide position were identified by a single PCR reaction using the sense primer 5’-TCGTGACCCCAGCCTGGAGG-3’ and the antisense primer 5’-GCACAGTGTCCGGGAATCTGCC-3’ delimiting a 298 bp fragment. The PCR reaction mixtures (25 μL) contained 250 ng DNA, 10 pmol of each primer, 1.5 mM MgCl2, 100 mM Tris-HCl at pH 8.3, 50 mM KCl, 200 μM of dNTP mix, and 1.5 units of Hot Master Taq Eppendorf. Thermal cycles of amplification were carried out in a Personal Eppendorf Master cycler (Eppendorf, Germany). The amplification protocol consisted of initial denaturation at 95° C for 5 min, followed by 30 cycles of denaturation at 95° C for 30 s, annealing at 60° C for

30 s, and then extension at 72° C for 30s. PCR products were sequenced with an ABI Prism automated sequencer. Nucleotide and deduced amino acid sequence analyses were performed with the OMIGA version 2.0 software (Oxford Molecular, Madison, WI).

Fungiform papillae identification and measurements

The method to identify fungiform papillae was similar to that developed by Shahbake et al. [56] and is briefly described as follows. The tip of the anterior tongue surface was dried with a filter paper and stained by placing (for 3 s) a piece of filter paper (circle 6 mm in diameter) that contained a blue food dye (E133, Modecor Italiana, Italy) at the left side of the midline. Photographic images of the stained area were taken using a Canon EOS D400 (10 megapixels) camera with lens EFS 55-250 mm. Three to ten photographs were taken of each subject, and the best image was analyzed. The digital images were downloaded to a computer and were analyzed using a "zoom" option in the Adobe Photoshop 7.0 program. The fungiform papillae were identified from the digital images by their mushroom-shape, they were readily distinguished from filiform papillae by their very light staining with the food dye compared to the latter papillae which stained dark [60].

The number of papillae in the stained area was counted for each subject, and the density in (1 cm^2) was calculated. The diameter of each papilla was measured in 4 dimensions (at 0, 45, 90 and 135°) and the standard deviation (SD) was calculated. This procedure was repeated for all papillae in a counting area. A fungiform papilla was considered distorted when the SD was ≥ 0.088. This value corresponded to 2 SDs. The grand mean of diameters, the mean of SDs, and the percentage of distorted fungiform papillae were determined for each subject. Papillae were separately evaluated by three trained observers who were blind to the PROP status of the subjects. The final measurements were based on the consensus assessment of all observers.

Experimental procedure

Subject testing was carried out in three visits on different days separated by a 1-month period. Subjects were requested to abstain from eating, drinking and using oral care products or chewing gums for at least 8 h prior to testing. They had to be in the test room 15 min before the beginning of the session (9.00 AM) in order to adapt to the constant environmental conditions (23-24°C; 40-50% relative humidity). In the first visit, a 3 ml sample of whole saliva was collected from each subject, into an acid-washed polypropylene test tube by means a soft plastic aspirator. Samples were stored at -80°C until molecular analyses were completed as described above. After 15 min, subjects rinsed their mouth with distilled water, then the tongue was dried and stained as described above, and photographs of the tip of the tongue were recorded (Figure S1).

Taste assessments were carried out in the 2nd and 3rd visits. In women, visits were scheduled around the sixth day of the menstrual cycle to avoid taste sensitivity changes due to the estrogen phase [61]. In the second visit, after rinsing the mouth with spring water, subjects were instructed to swish the entire contents of one cup (10 mL of PROP 3.2 mM) in their mouth for 10 s and then to spit it out. After tasting, the subjects evaluated bitterness intensity of the solution using the LMS. PROP thresholds were determined for each subject at the third visit. All rinsed their mouth with spring water before the experimental session. They were presented with 3 cups positioned in a random order, one with a given PROP concentration and two containing spring water. They were instructed to swish the entire contents of one cup in their mouth for 5 s and then to spit it out. Before moving onto the next cup, they rinsed their mouth with spring water. After tasting all 3 samples, they were asked to choose which one was different from the other two samples. The detection threshold was designated as the lowest concentration at which the subject correctly identified the target stimulus on three consecutive trials. The inter-stimulus interval as well as inter-trial interval was set at 60 s.

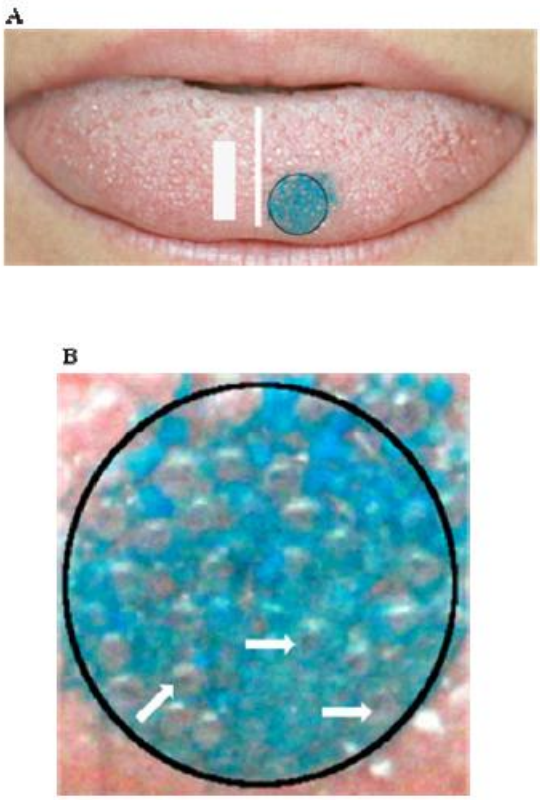

Figure S1 - Fungiform papillae in a human tongue. (A) Tongue with midline highlighted with a white line and showing the 6 mm diameter stained area where papilla counts were conducted and the 10 mm scale. (B) Images of stained area obtained with digital camera. In extension are well clear the fungiform papillae with a typical mushroom-shape which were readily distinguished from filiform papillae by their very light staining with the blue food dye compared to the latter papillae which stained dark. The arrows indicate typical fungiform papillae.

In vitro experiments

Two cell-based experiments were conducted. The first experiment tested the effects of treatment with saliva collected from individuals with genotype AA and GG of polymorphism *rs2274333* on cell proliferation and metabolic activity. The second one tested the effects of treatment with the two gustin iso-forms isolated from saliva of donors homozygous for AA and GG, on cell metabolic activity.

Cell cultures

A fetal goat tongue-derived epithelial cell line (ZZ-R 127) supplied by the Collection of Cell Lines in Veterinary Medicine of the Friedrich Loeffler Institute was used [62 Brehm et al 2009]. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA) plus 10% (v/v) fetal calf serum (FCS, Gibco) at 37° C in a humidified atmosphere of 5% CO2. Cells were plated in 24-well plates at a density of 8x104 cells/ well. After 24 h, cells in DMEM plus 10% FCS were treated for 72 h with 10% saliva from donors (or gustin iso-forms) depending on the experimental conditions.

Effects of saliva on growth and metabolic activity

For the first experiment, saliva was collected from a total of 24 subjects; 12 subjects with genotype AA at the gustin locus (*TAS2R38* genotypes were as follows: 8 heterozygous and 4 PAV homozygous) and 12 subjects with genotype GG at the gustin locus (*TAS2R38* genotypes were: 6 AVI homozygous, 4 heterozygous and 2 PAV homozygous). Saliva was collected on the same day as the *in vitro* experiments, and centrifuged at 12,000 RPM for 10 minutes. The supernatant was filtered with a sterile 0.22-μm pore filter, and then added to the cell cultures, as described below. Gustin protein was still present in filtered supernatants, as demonstrated by immunoblot experiments (data not shown). Three experimental treatments were used: (1) saliva from subjects with genotype AA; (2) saliva from subjects with genotype GG; and (3) control (DMEM plus 10% FCS alone). Saliva from each subject was assayed separately. After 72 h treatment, cells were trypsinized and counted with a hemocytometer under inverted microscope.

Cell metabolic activity was determined by the resazurin system (Tox-8 assay kit, Sigma, USA) in which metabolically active cells convert resazurin into a fluorescent dye, resorufin, by the intracellular reduction enzymes. This assay represents a simple, accurate and reproducible tool for measuring the metabolic activity of living cells [63]. After 72h treatment with saliva, resazurin dye solution was added to cells in an amount equal to 10% of the culture medium volume (100 μl/well) and cells were cultured for a further 4 h. Fluorescence of converted dye was measured using a fluorescent microplate reader (VICTOR X

Multilabel Plate Readers, PerkinElmer) at a wavelength of 590 nm using an excitation wavelength of 560 nm.

Mean values of cell number and fluorescence emission after treatments with saliva of subjects with genotype AA (n=12) and genotype GG (n=12) were calculated and are presented graphically.

Effects of gustin iso-forms on metabolic activity

In the second experiment, cells were treated with isolated gustin in the two iso-forms resulting from the polymorphism *rs2274333* (A/G). Saliva was collected from one super-taster donor homozygous for the AA form of gustin (*rs2274333*) and from one non-taster donor homozygous for the GG form (both heterozygous for *TAS2R38*), and used to purify the two isoforms of carbonic anhydrase VI. The preparation of saliva samples and all purification steps were conducted using the method of Murakami and Sly [64]. The same experimental procedure was used for the purification of each iso-form. Volunteers expectorated in a frozen bottle containing 2 ml of 0.2 M benzamidine (Sigma-Aldrich, St. Louis, MO) in 0.1 M Tris-SO4, and 0.2 M sodium sulfate, at pH 8.7. Saliva samples were collected after lunch, because food intake enhances the secretion of saliva from the parotid glands which are the primary site for gustin protein production [65]. Samples of whole saliva were collected from each subject, after stimulation with citric acid. This produced large amounts (~40 mL) per collection. The collection procedure was repeated in different days until a pooled sample of 250 ml of saliva for each genotype was obtained. Samples were stored at -80c then thawed and centrifuged (16,000 x g 15 min) to remove foreign material. The supernatant was diluted to 1 liter with 0.1 M Tris- SO4, and sodium sulfate 0.2 M at pH 8.7.

The purification of carbonic anhydrase VI was carried out through the use of affinity chromatography, preparing the column matrix as reported by Khalifah et al. [66]. Specifically, carboxy methyl Bio-Gel A (Bio-Rad Laboratories, Richmond, CA) was linked to the sulfonamide inhibitor paminomethylbenzenesulfonamide (Gallade Chemical; Newark, CA). EDAC [1-(3-dimethylamionpropyl)-3-ethyl carbodiimide hydrochloride] obtained from Sigma-Aldrich (St. Louis, MO), was used to activate the column matrix carboxyl groups. The purified fractions containing the carbonic anhydrase VI were

collected based on spectrophotometric absorbance values at 280 nm. Then, as reported by Murakami and Sly [64], fractions containing the protein were applied to a diethylaminoethyl - sephacel (Sigma-Aldrich, St. Louis, MO) ion-exchange column. The concentration of purified protein was quantified by the method of Lowry et al. [67] using bovine serum albumin as a standard, and its purity was determined by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). SDSPAGE (12% acrylamide) was performed according to Laemmli [68]. Sigma, Marker product code C 4236 (Sigma-Aldrich, St. Louis, MO) with range 8-210 kDA was employed as a standard in electrophoresis. The gel was stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich, St. Louis, MO), using the typical Coomassie staining procedure [69]. The yield of the purification was approximately 1 mg of protein starting from 250 ml of whole saliva.

The mean concentration of gustin in human saliva is about 5 ± 0.2 μg/ml [70]. Since gustin binds an ion of Zn with a stoichiometry of 1:1 [50], we used a protein concentration of 8 μ g/ml corresponding to 0.2 nmoles, and 0.2 nmoles of added Zn. Four experimental treatments were used: (1) gustin Ser90 + Zn; (2) gustinGly90 + Zn; (3) control (DMEM plus 10% FCS alone); and (4) control + Zn. The Tox8 assay (previously described) was used to obtain fluorescence emissions using the same procedures as the saliva experiment. Since we were able to obtain a large amount of isolated protein, each treatment was repeated 33 times (to maximize the reliability of the assay) and the mean values of the replicates are presented graphically.

Statistical analyses

Hardy Weinberg equilibrium for the *TAS2R38* gene and polymorphism 2274333 (A/G) of the gustin gene was verified through the Markov Chain test (Genepop software version 4.0; http://kimura.univ-montp2fr/~rousset/Genepop.htm). Linkage disequilibrium (LD) between the two loci was verified by the Markov Chain algorithm (Genepop software version 4.0.5.3; http://kimura.univ-montp2fr/~rousset/Genepop.htm). We stratified our sample based on *TAS2R38* and gustin genotypes, and tested both the additive and

dominant models for the PAV and A variants, respectively, with the Chi square test to show the two genes are independent.

Main effects ANOVA was used to examine the effects of the *TAS2R38* gene and polymorphisms *2274333* (A/G) of the gustin gene on PROP threshold, bitterness intensity rating (PROP 3.2 mM), and fungiform papilla density and diameter. Main effects ANOVA was used to assess the first-order (noninteractive) effects of multiple categorical independent variables.

One-way ANOVA was used to compare the SD of diameter of fungiform papillae and the percentage of distorted fungiform papillae across gustin gene genotypes, and the effect of treatments on cell metabolic activity. Post-hoc comparisons were conducted with the Newman-Keuls test.

Stepwise, multiple linear regression was used to predict PROP phenotype (threshold and bitterness intensity rating), fungiform papilla density and morphology using gustin and *TAS2R38* genotypes, gender and age as predictor variables. The relative contribution of each significant variable and semipartial correlations (*sr*) for each variable are reported in the tables. Cell growth (expressed as percentage of control values) was compared between cells treated with saliva from individuals with genotype AA and GG of the gustin gene using the Student's *t*-test. Statistical analyses were conducted using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). *p*-values <0.05 were considered significant.

Results

The Markov Chain test showed that the population meets the Hardy Weinberg equilibrium both for *TAS2R38* and gustin gene (p=0.6154 and p=0.1174, respectively). The distribution of the *TAS2R38* and gustin gene genotype associations is shown in Table 1. Markov Chain algorithm showed that the two loci were not in linkage disequilibrium (p=0.1782). Chi square test showed that carriers of the taster form of *TAS2R38* were not more likely to have the functional variant of the gustin gene in either the additive (χ^2=6.5; p=0.17) or the dominant model (χ^2=2.54; p=0.11).

Table 1 - Number of occurrences of each combination of the *TAS2R38* and gustin gene genotypes in a genetically homogeneous cohort.

Genotype	Subjects (n)
AVI/AVI - GG	5
AVI/AVI - AG	6
AVI/AVI - AA	9
PAV/AVI - GG	1
PAV/AVI - AG	12
PAV/AVI - AA	20
PAV/PAV - GG	2
PAV/PAV - AG	2
PAV/PAV – AA	6

PROP Thresholds and Bitterness Intensity

Molecular analysis for polymorphism *rs2274333* (A/G) of the gustin (CA6) gene allowed us to identify the genotype of sixtythree subjects: 35 were homozygous AA, 20 were heterozygous and 8 were homozygous GG. The analysis at the three SNPs of the *TAS2R38* locus identified 10 subjects who were PAV homozygous, 33 were heterozygous and 20 were AVI homozygous.

PROP threshold values and bitterness intensity ratings (PROP 3.2 mM) of individuals with genotypes AA, AG and GG of the gustin gene and with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38* are shown in Figure 1A and B. Main effects ANOVA revealed a strong association between PROP threshold and the gustin gene polymorphism (F[2,58] = 10.502; p=0.00013). Post-hoc comparisons showed that thresholds were statistically higher in individuals with genotype GG of the gustin gene than in the other genotypes ($p \leq 0.000119$; Newman-Keuls test), but not different between AA and AG individuals ($p > 0.05$). Although thresholds were variable in those with the GG genotype, thresholds were more than 10-fold higher in these individuals than in the other groups. Main effects ANOVA also showed an association between PROP threshold and *TAS2R38* genotypes (F[2,58] = 6.0189; p=0.0042). Thresholds of individuals with the AVI/AVI genotype were higher than those of individuals with genotypes PAV/PAV and PAV/AVI ($p \leq 0.00158$; Newman-Keuls test), that did not differ from each other ($p > 0.05$).

PROP bitterness intensity ratings (3.2 mM) were strongly associated with *TAS2R38* genotypes (F[2,58] = 32.468; $p < 0.000001$) and less so with the gustin gene polymorphism (F[2,58] = 3.4330; p=0.038). *TAS2R38* bitterness ratings of PAV/PAV individuals were statistically higher than those of heterozygous individuals ($p \leq 0.0173$;

Newman-Keuls test) who in turn gave higher intensity ratings to PROP than individuals with the AVI/AVI genotype (p=0.00011; Newman-Keuls test). In the case of gustin, post hoc comparisons showed that PROP bitterness was statistically higher in individuals with genotype AA than in those with the other genotypes ($p \leq 0.0471$; Newman-Keuls test), but not different between GG and AG individuals ($p > 0.05$).

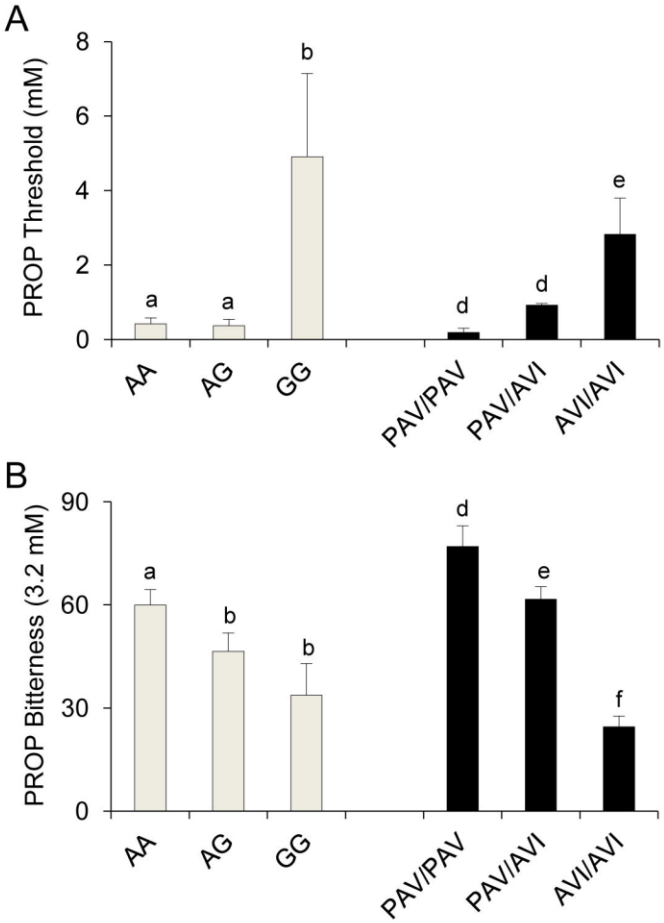

Figure 1 - Relationship between PROP phenotype and gustin gene and *TAS2R38* polymorphisms. PROP threshold (A) and bitterness intensity ratings (3.2 mM) (B) of individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism *rs2274333*(A/G), and of individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38*. All values are mean (± SEM). n=63. Different letters indicate significant difference ($p \leq 0.0471$; Newman-Keuls test subsequent to main effects ANOVA).

Papillae Density and Morphology

Figure 2 shows the mean densities (± SEM) of fungiform papillae on the anterior part of the tongue of individuals with genotypes AA, AG and GG of the gustin gene (upper graph) and of individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38* (lower graph). Also shown are representative images of the tongue tip stained area where measures were taken. ANOVA calculations showed that fungiform papillae density on the anterior part of the tongue was strongly associated with the gustin gene (F[2,58] = 8.5270; p=0.00057) and less so with *TAS2R38* polymorphisms (F[2,58] =4.6147; p=0.0138). In the case of gustin, fungiform papillae density values were lower in individuals with the GG genotype than in those with genotypes AG and AA ($p \leq 0.0379$; Newman-Keuls test). Papillae density was not different between AA and AG individuals ($p > 0.05$). In the case of *TAS2R38* genotypes, post hoc comparison showed that individuals with the PAV/PAV genotype had a higher fungiform papillae density than those with PAV/AVI and AVI/AVI genotypes ($p \leq 0.0094$; Newman-Keuls test); the density values of the latter two groups were not different from each other ($p > 0.05$).

ANOVA revealed that mean fungiform papilla diameter was associated with the gustin gene polymorphism (F[2,58] = 7.5920; p=0.00118), but not with *TAS2R38* genotypes (F[2,58] = 0.7191; p=0.491). Post-hoc comparisons showed that mean papilla diameter determined in those with genotypes AA and AG were lower than those of homozygous GG individuals ($p \leq 0.00053$; Newman-Keuls test) (Figure 3).

ANOVA was also used to examine relationships between fungiform papilla morphology and gustin and *TAS2R38* genotypes. However, only associations between these features and gustin were statistically significant. In fact, both the SD of papilla diameter (Figure 4A) and the percentage of distorted papillae (Figure 4B) depended on gustin genotype (F[2,60] =11.765; p=0.00005 and F[2,60] = 9.787; p=0.00021, respectively). Post-hoc comparisons showed that individuals with the GG genotype had papillae with greater variation in shape (higher SDs in papilla diameter) as well as a higher percentage of distorted papillae than the other genotypes ($p \leq 0.00019$ and $p \leq 0.00017$; Newman-Keuls test). No differences were found between AA and AG individuals ($p > 0.05$).

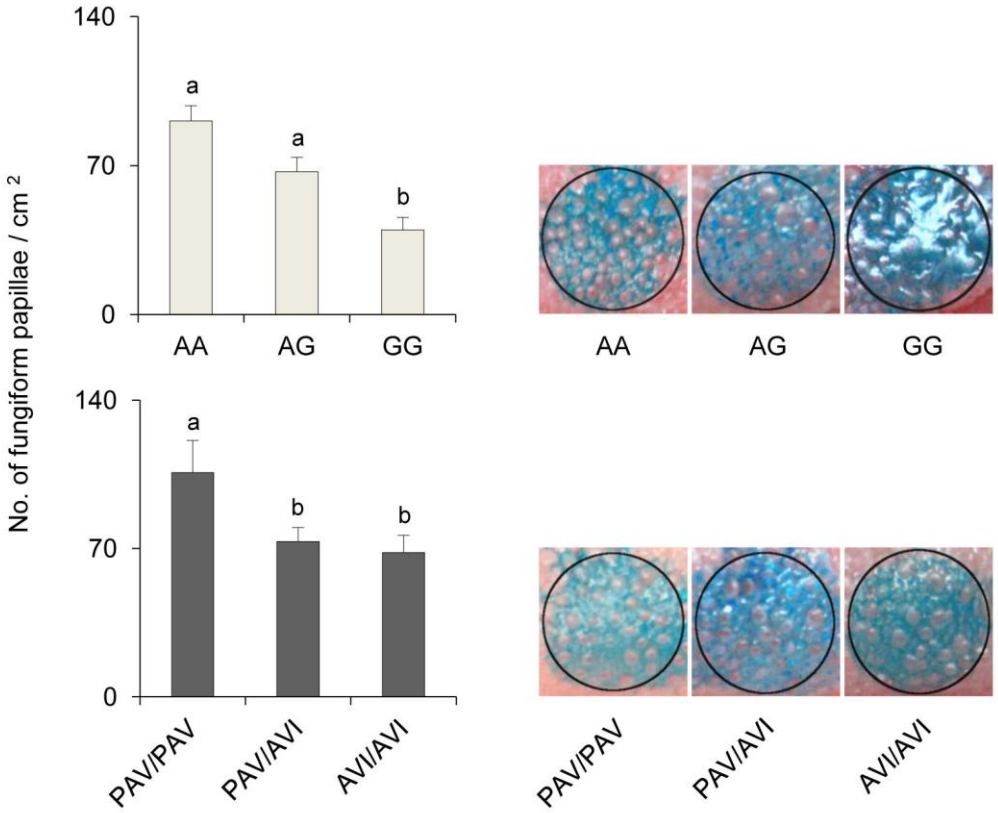

Figure 2 - Relationship between density of fungiform papillae and gustin gene and *TAS2R38* polymorphisms. Mean values ± SEM of density of fungiform papillae (No. /cm2) on the anterior part of the tongue of individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism rs2274333 (A/G) (upper graph) and of individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38* (lower graph). n=63. Different letters indicate significant difference ($p \leq 0.0379$; Newman-Keuls test subsequent to main effects ANOVA). Examples of the 6-mm-diameter stained area of the tongue tip where measures were taken are shown to the right of the graphs.

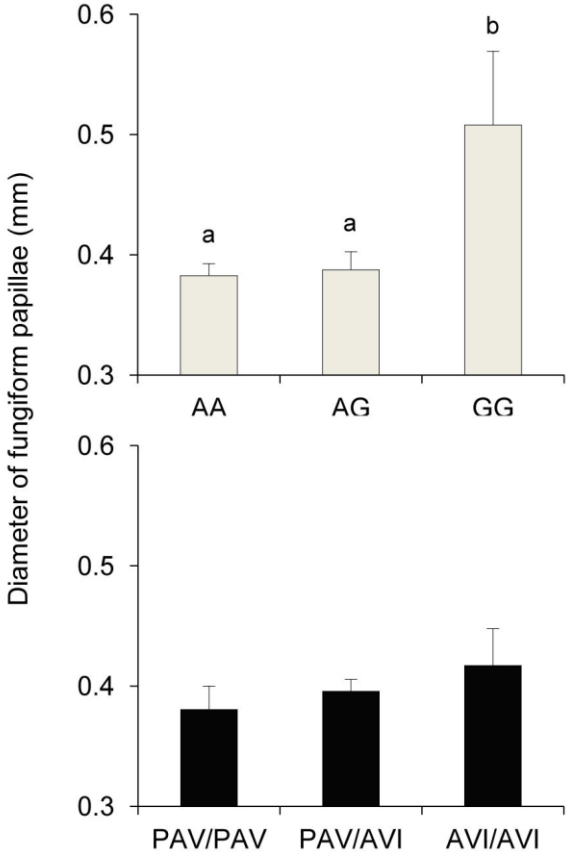

Figure 3 - Relationship between fungiform papillae diameter and gustin gene and *TAS2R38* polymorphisms. Mean values ±SEM of the diameter of fungiform papillae of individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism rs2274333 (A/G) (upper graph) and of individuals with genotypes PAV/PAV, PAV/AVI and AVI/AVI of *TAS2R38* (lower graph). n=63. Different letters indicate significant difference ($p \leq 0.00053$; Newman-Keuls test subsequent to main effects ANOVA).

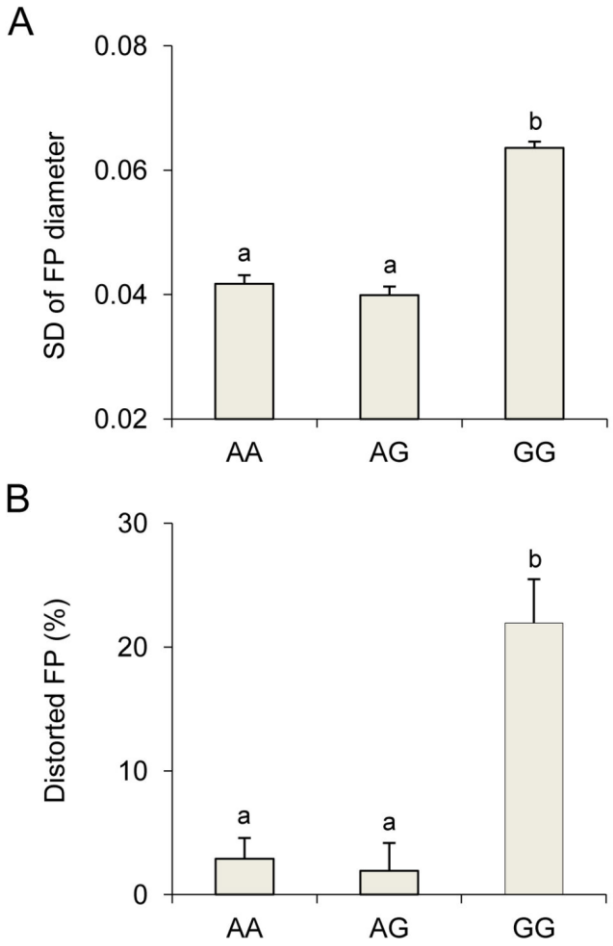

Figure 4 - Relationship between fungiform papillae distortion and gustin gene polymorphism. Standard deviation (SD) of diameter of fungiform papillae (A) and percentage of distorted fungiform papillae (B) in individuals with genotypes AA, AG and GG of gustin (CA6) polymorphism rs2274333 (A/G). All values are means (± SEM). n=63. Different letters indicate significant difference ($p \leq 0.00019$; Newman-Keuls test subsequent to one-way ANOVA).

Multiple Regression Modeling

Multiple linear regression was used to assess the relative contributions of gustin and *TAS2R38* polymorphisms to PROP tasting and papillae density and morphology (Tables 2 and 3). Accordingly, gustin genotypes, *TAS2R38* genotypes and age were significant predictors of PROP threshold, with each factor contributing 17.72%, 11.18% and 5.45%, respectively, to the model. The overall model predicted 31.01% of the variance in threshold sensitivity. In the case of PROP bitterness intensity, *TAS2R38* and gustin genotypes were the only significant contributors in the model, predicting 55.16% of the variance in PROP bitterness intensity. However, *TAS2R38* genotype was a much

stronger predictor in this model (49.75% variance) than was gustin genotype (6.18% variance).

Gustin genotypes and age were the only significant contributors to fungiform papillae density with the overall model explaining 30.90% of the variance. Finally, gustin genotype was the only significant contributor to fungiform papillae diameter, SD of papilla diameter and percentage distortion. However, the predictive power of these models were relatively low, explaining 13.2-16.11% of the variance in these measures.

Table 1 - Stepwise forward multiple regression models for PROP phenotype (threshold and Bitterness).

PROP phenotype	**Variable**	**Overall model**		**Parameter estimate**		**Each step**
		(adj R^2)	**(*P*)**	**(*sr*)**	**(*P*)**	**(R^2)**
Threshold	Gustin			-0.38	<0.001	0.1772
	TAS2R38	0.3101	<0.001	-0.31	0.005	0.2890
	Age			0.23	0.031	0.3435
Bitterness	*TAS2R38*	0.5516	<0.001	0.65	<0.001	0.4975
	Gustin			0.26	0.004	0.5593

Independent variables for both models included: Gustin genotypes, *TAS2R38* genotypes, age and gender. Only the significant variables are indicated. Adj, adjusted; sr, semipartial correlation.

Table 2 - Stepwise forward multiple regression models for fungiform papilla density and morphology (diameter of papillae, SD of diameter and percentage of distorted papillae).

	Variable	**Overall model**		**Parameter estimate**		**Each step**
		(adj R^2)	**(*P*)**	**(*sr*)**	**(*P*)**	**(R^2)**
Density of papillae	Gustin	0.3090	<0.001	0.48	<0.001	0.1952
	Age			-0.36	0.004	0.3060
Diameter of papillae	Gustin	0.1218	0.007	-0.36	0.004	0.1320
SD of diamenter	Gustin	0.1342	0.005	-0.34	0.005	0.1358
% of distorted papillae	Gustin	0.1538	0.002	-0.38	0.002	0.1611

Independent variables for all models included: Gustin genotypes, *TAS2R38* genotypes, age and gender. Only the significant variables are indicated. Adj, adjusted; sr, semipartial correlation.

In vitro experiments

The effect of gustin gene polymorphism rs2274333 (A/G) from the *in vitro* experiments is shown in Figure 5. The number of cells, expressed as a percentage of control, treated with the saliva of subjects with genotype AA (n=12) was higher than the number of cells treated with saliva of subjects with genotype GG (n=12) (p=0.0135; Student's *t* test) (Figure 5A). ANOVA showed that the fluorescence emission at a wavelength of 590 nm, as a function of cell metabolic activity, depended on treatments performed with the saliva of subjects with different genotypes for the polymorphism in the gustin gene (F[2,33] = 16.628; p=0.00001) (Figure 5B). Post hoc comparisons showed a higher emission of fluorescence from cells treated with saliva of subjects with genotype AA than that obtained from cells treated with saliva of genotypes GG (p=0.000137; Newman- Keuls test) or control (p=0.000229; Newman-Keuls test). No differences were found between treatment with saliva of genotypes GG and control (p>0.05).

ANOVA also showed that the fluorescence emission depended on treatments performed with the two iso-forms of gustin (gustin Ser90 or gustin Gly90) (F[3,128] = 10.463; P < 0.00001) (Figure 5C). Pairwise comparisons showed that cells treated with gustin 90Ser + Zn emitted a higher fluorescence than those treated with gustin 90Gly + Zn or with control + Zn or control ($P \leq 0.0067$; Newman-Keuls test). No differences were found between these last three treatments $P > 0.05$.

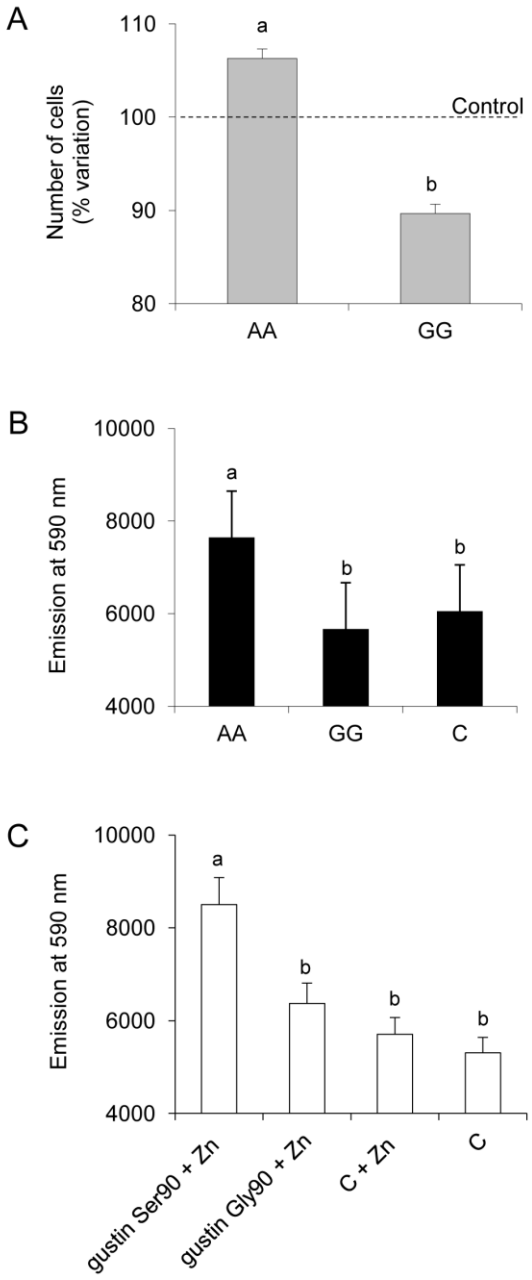

Figure 5 - Effect of gustin gene polymorphism *rs2274333* (A/G) *in vitro* experiments. (A) Number of cells, expressed as percentage of control, after treatments with saliva of subjects with genotype AA (n=12) or with saliva of subjects with genotype GG (n=12); different letters indicate significant difference (p=0.0135; Student's t test). (B) Fluorescence emission at a wavelength of 590 nm obtained from cells treated for 72 h with saliva of subjects with genotype AA, genotype GG and control; n=12; different letters indicate significant differences ($p \leq 0.00023$; Newman-Keuls test subsequent to one-way ANOVA). (C) Fluorescence emission at a wavelength of 590 nm obtained from cells treated for 72 h with the two iso-forms of isolated gustin (gustin Ser90 or gustin Gly90) + Zn, control + Zn, or control; n=33; different letters indicate significant differences ($p \leq 0.00067$; Newman-Keuls test subsequent to one-way ANOVA).

Discussion

One aim of the present study was to determine the effects of *TAS2R38* genotypes and the rs2274333(A/G) polymorphism in the gustin gene on PROP tasting, fungiform papillae density and morphology. Results showed that PROP thresholds and bitterness intensity ratings were associated with *TAS2R38* and gustin gene genotypes, as reported previously [49]. Importantly, those who were homozygous GG for the gustin SNP had thresholds that were more than 10-fold higher than those who carried either the AA or AG forms suggesting that gustin has a fundamental role in the ability to taste PROP at low concentration. Both gustin and *TAS2R38* genotypes were associated with fungiform papillae density with a stronger effect for gustin than for *TAS2R38*. However, only gustin was associated with morphological changes in fungiform papillae such as larger size, greater variation in shape and more distortions.

Regression modelling permitted us to assess the relative contributions of gustin and *TAS2R38* genotypes to these same outcomes. Both genes contributed to threshold acuity, however, *TAS2R38* polymorphisms made a much greater contribution to PROP bitterness intensity than did gustin. These data confirm the findings of Calò et al. [49] showing a much stronger effect of *TAS2R38* genotypes on suprathreshold intensity than threshold sensitivity. The reasons for these differential effects are unclear, but we can speculate that at low stimulus concentrations, that are further diluted in the oral cavity, both papillae features (as determined by gustin) and the presence of the functional, PAV form of the TAS2R38 receptor are critical for tasting PROP. At higher concentrations, when there is a higher probability that the stimulus molecules arrive at the receptor site, the number of functional (PAV) receptors may be more important for enhancing peripheral nerve signaling than the number of taste cells that are present. This explanation may be overly simplistic as it fails to account for a number of factors that affect taste function such as smoking, damage to taste nerves [71,72] and variability in TAS2R38 receptor expression. These factors need to be considered in future studies to obtain a more complete picture of the physiological mechanisms contributing to PROP tasting.

Our data showed that *TAS2R38* genotypes were associated with papillae number, and PAV homozygous individuals had a higher papillae number with respect to other genotypes. However, in the regression analysis, that looks at multiple variables at the same time, the *TAS2R38* genotypes were not significant predictors of papillae number or their other morphological features. It is important to note however, that gustin genotypes predicted only a small percentage of the variance in papillae size, and shape, suggesting that other factors define these morphological characteristics. We did not investigate brain-derived neurotrophic factor (BDNF) which has also been implicated in papillae

development and maintenance [73-75], and this also needs to be pursued in future investigations.

Numerous studies have report greater papillae densities in PROP super-tasters compared to those who perceive PROP as less intense [2,34,54-56,76]. In agreement with these studies we found that homozygous individuals for the sensitive allele (PAV) of *TAS2R38*, who perceived the highest PROP bitterness, had higher papillae densities compared to those who perceived PROP as less intense. Our results complement these earlier observations by also showing that a single A allele in the gustin gene was sufficient to increase papillae density. In addition, we studied for the first time, the relationship between papillae distortion, which seems to be a measure of functionality [53], and genotypes for the two loci. We found that a single A allele in the gustin gene produced small papillae with a regular morphology; these effects were not found for *TAS2R38* genotypes.

Hayes et al. [35] reported no association between *TAS2R38* genotypes and papillae densities. In our previous work [49] we found that *TAS2R38* and the gustin gene had independent effects in modulating PROP phenotype in an ethnically homogeneous population where the majority of PAV homozygotes also carried the AA (functional) form of the gustin *rs24743333* polymorphism. In contrast, a majority (55%) of AVI homozygotes carried the GG (less functional) form. In the present study, fewer AVI homozygotes (25%) carried the GG form. Nevertheless, the presence of the AA form of gustin was more common in those with at least one PAV allele for *TAS2R38*. Thus, it is plausible that the higher papillae densities we observed in PAV homozygotes (although the sample size for this group was low) may better reflect the actions of gustin rather than *TAS2R38* genotypes. Future studies will have to confirm this finding. Our results should not lead to the conclusion that *TAS2R38* genotypes predict gustin genotypes. The two loci are independent (not in linkage disequilibrium) and, in fact, reside on different chromosomes. Why these two discrete loci appear to have functional overlap in defining PROP tasting and papillae density and morphology is presently unknown. The answer to this question cannot be resolved here and will come from more comprehensive genetic studies.

Up to now, only few populations have been tested for variants in the gustin gene, but the allele frequencies in these populations are not known. Variations in the frequency of gustin A and G alleles across populations could produce discrepant findings across studies, and could explain why a genome wide phenotype-genotype association study of PROP threshold failed to detect a relationship with variants in the gustin gene [5]. Both confounding and heterogeneity of populations are common contributors to the problem

of non replication in genetic studies of complex traits [77]. On the other hand, the study of ethnically homogeneous populations can be expected to reduce noise in genetic association studies by diminishing ancestral diversity [77‑79]. The genetic homogeneity of the population we studied might have allowed us to observe the effect of the gustin gene as growth factor of taste buds. We also found that in regression analysis, *TAS2R38* accounted for less variance in the threshold response to PROP than in previous studies [8,35,80]. This finding could also reflect underlying differences in population characteristics.

For more than 40 years, gustin has been described as a trophic factor responsible for the growth and maintenance of taste buds [50]. This role was based on observations of patients with taste loss who exhibited pathological changes in taste buds accompanied by low salivary gustin and zinc levels. Administration of zinc to a subset of these patients improved taste function, increased salivary gustin and normalized taste bud morphology [53]. However, direct evidence that gustin increases cell growth has been lacking. Our *in vivo* studies showed that treatment of cells with saliva from individuals with the AA genotype of gustin resulted in increased cell proliferation and metabolic activity, whereas similar treatment with saliva from individuals with the GG genotype did not. Furthermore, direct treatment of cells with the active iso-form of the protein (gustin 90Ser) increased cellular metabolic activity, while treatment with the inactive iso-form (gustin 90 Gly) failed to do so. These novel findings confirm, for the first time, a role for gustin in cell proliferation and maintenance.

In conclusion, our findings in an genetically homogeneous cohort suggest that the gustin (CA6) gene polymorphism, *rs2274333* (A/G), affects PROP tasting by acting on the density and maintenance of fungiform papillae, and that between the two protein iso-forms that result from this polymorphism, gustin 90Ser exhibits full functional activity, compared to the gustin 90Gly iso-form. In addition, the results of this work, if confirmed in different populations, will provide a mechanistic explanation of why PROP super-taster individuals have a higher density of fungiform papillae than PROP non-tasters, and why they show greater oral responsiveness to a wide range of stimuli that are not mediated via the TAS2R38 bitter taste receptor.

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4

Taste sensitivity to 6-n-propylthiouracil is associated with endocannabinoid plasma levels in normal-weight individuals.

Iole Tomassini Barbarossa
Gianfranca Carta
Elisabetta Murru
Melania Melis
Andrea Zonza
Claudia Vacca
Patrizia Muroni
Vincenzo Di Marzo
Sebastiano Banni

Nutrition 29 (2013) 531–536

Abstract

Objective: A decreased sensitivity to 6-n-propylthiouracil (PROP) has been shown to be associated with increased energy intake and therefore an increased body mass index, although other studies have not confirmed this association, suggesting the involvement of other factors. We investigated whether the endocannabinoid system, which also modulates hunger/satiety and energy balance, plays a role in modulating eating behavior influenced by a sensitivity to PROP.

Methods: The plasma profile of the endocannabinoids 2-arachidonoylglycerol (2-AG), anandamide (AEA), and congeners of AEA, palmitoylethanolamide and oleylethanolamide (OEA), was determined in normal-weight PROP supertasters (STs) and PROP non-tasters (NTs). A cognitive eating behavior disorder was assessed by the Three-Factor Eating Questionnaire, which estimates dietary restraint, disinhibition, and perceived hunger.

Results: The disinhibition score of NTs was higher than those of STs ($P = 0.02$). Moreover, in NTs, OEA was inversely correlated to the perceived hunger score ($r = -0.7$, $P = 0.002$), and AEA was positively correlated to the restraint score ($r = 0.5$, $P = 0.04$) and negatively to the perceived hunger score, although the latter correlation was at the limit of statistical significance ($r = -0.47$, $P = 0.05$). In addition, we found lower concentrations of AEA and 2-AG in the plasma of NT compared with ST subjects (AEA, $P = 0.034$; 2-AG, $P = 0.003$).

Conclusions: Our data suggest that a higher disinhibition behavior in NTs may be compensated in part, in normal-weight subjects, by the decrease of peripheral endocannabinoids to downregulate the hunger–energy intake circuitry.

Introduction

It is common knowledge that weight gain and obesity have increased during recent decades in most of the Western world [1], thus becoming a serious health problem. This is the result of the current eating environment that offers easy access to a wide variety of energy- and/or fat-dense, highly palatable foods, which encourages overeating [2]. Strategies designed to identify mechanisms and risk factors that contribute to determine overeating behaviors can be of great interest to avoid weight gain and promote healthier lifestyles. Taste perception and food preferences have been extensively reported as factors influencing eating behavior and body mass [3,4]. In recent decades, a large body of work has investigated the role of the genetic taste variation in 6-n-propylthiouracil (PROP) sensitivity in influencing the ability to taste a wide range of oral sensory qualities and, hence, guiding general food preferences, dietary behavior, and nutritional status [5,6]. PROP taster individuals differ from PROP non-taster individuals (NTs) because of their capacity to detect low concentrations of this chemical. In addition, based on PROP responsiveness, tasters can be further divided in medium tasters who perceive PROP as moderately bitter and supertasters (STs) who perceive PROP as extremely bitter [7,8]. Although most PROP phenotypic variations are dependent on the allelic diversity of the bitter receptor TAS2R38 [9,10], this cannot explain PROP taster status-related differences in the perception to a wide range of oral stimuli. Rather, these differences could be explained by the demonstrated association of the high density of fungiform taste papillae and buds on the anterior tongue with the PROP ST phenotype [8,11]. This interpretation is supported by the association between the NT phenotype and a mutation in the gene encoding the taste bud trophic factor, gustin, as previously shown [12].

Several studies have reported that the PROP NT phenotype is associated with the susceptibility to prefer and consume highfat/ high-energy foods [2,13–16] and that the lower oral sensitivity of NTs is associated with a stronger preference for high-fat and strong-tasting foods compared with tasters [5,17,18]. These findings support some studies that have shown an inverse correlation between the capability of perceiving PROP and the body mass index (BMI) [5,19–21]. However, others have not confirmed this association [22–26]. Indeed, it is known that cognitive control of eating behaviors can play a prominent role in determining this relation [12,21,27]. In addition, one more confounding factor may result from gender differences in eating behavior, BMI, and above all, PROP sensitivity [8]. In turn, changes in BMI might be directly related to alterations in the hunger/satiety cycles. It is well known that hunger and satiety are also strongly influenced by the endocannabinoid system [28], the activation of which, like a decreased sensitivity to PROP, is associated to an increased appetite for fat [29,30]. In

addition, it has been recently shown that hedonic eating, defined as the consumption of food just for pleasure and not to maintain energy homeostasis, increases the plasma levels of the endocannabinoid, 2-arachidonoylglycerol (2-AG) [31]. Increased circulating levels of endocannabinoids have also been associated with abdominal obesity [32].

Based on this background, we hypothesized that different sensitivities to PROP might be associated with alterations in the endocannabinoid system, which may play a role in determining the eating behavior of NTs and their body weight. To test this hypothesis, we evaluated the plasma levels of the endocannabinoids and some of their congeners in male and female normalweight PROP STs and NTs in relation to the cognitive control of eating behaviors.

Materials and methods

Subjects

Sixty volunteers were recruited through public advertisements at the local university. Participants were healthy man and women (mean age 27.58 ± 1.16 y). They showed no variation in body weight greater than 5 kg in the previous 3 mo. No participant was following a prescribed diet or taking medications. No participant had food allergies. To rule out any taste impairments, thresholds for the four basic tastes (sweet, sour, salty, and bitter) were evaluated in each subject. All subjects were verbally informed about the procedure and the aim of the study. They reviewed and signed an informed consent form. The study conformed to the standards set by the most recent revision of the Declaration of Helsinki and the procedures were approved by the ethical committee of the University Hospital of Cagliari, Italy.

PROP responsiveness screening

The subjects were screened and classified by PROP taster status using the three-solution test [33,34]. This method assesses subjective PROP responsiveness by taste-intensity ratings of three suprathreshold PROP solutions (0.032, 0.32, and 3.2 mmol/L; Sigma-Aldrich, Milan, Italy) and sodium chloride (NaCl; 0.01, 0.1, and 1.0 mol/L; Sigma-Aldrich) in spring water. NaCl was used as a standard because taste sensitivity to NaCl is not influenced by the PROP taster status in this method [34]. The taste-intensity rating for each PROP or NaCl solution at room temperature (10-mL samples) was recorded using the Labeled Magnitude Scale [35]. After tasting each solution, subjects placed a mark on the scale corresponding to his/her perception of the stimulus. The mean of the two replicates was calculated and the results were plotted for each subject. This procedure generates perceived taste-intensity functions for PROP and NaCl [21,34]. When PROP ratings increased more rapidly across concentrations than did the NaCl

ratings, subjects were classified as super-tasters. Conversely, when NaCl ratings increased more rapidly than did the PROP ratings, subjects were classified as NTs. When PROP ratings overlapped with NaCl ratings, subjects were classified as medium tasters. To compare the two extreme groups, only super-tasters (n = 17) and NTs (n = 19) were invited to further participation, whereas medium tasters (n = 24) were excluded.

Procedure

Subject testing was carried out in three visits on different days. In women, testing and blood collection were done on the sixth day of the menstrual cycle to avoid blood value fluctuations and taste sensitivity changes owing to the estrogen phase [36]. All volunteers were requested to abstain from eating, drinking, and using oral care products or chewing gums for at least 8 h before the trials. They had to be in the test room 15 min before the beginning of the session to adapt to the environmental conditions, which were kept constant throughout the experimental session (23–24°C, 40–50% relative humidity). At the first visit, weight (kilograms) and height (meters) were recorded for each subject to calculate the BMI (kilograms per meter squared). Subjects were assessed for cognitive control of eating behaviors using the Three-Factor Eating Questionnaire (TFEQ) [37].The questionnaire estimates three aspects of the cognitive control of eating behavior: dietary restraint, disinhibition, and perceived hunger.

For PROP taste assessments, each subject was tested twice in 2 days separated by a 1-month period. In the two visits, the order of the presentation of the two stimuli was reversed, and samples of each stimulus were tasted in random order. Each stimulation was followed by oral rinsing with spring water. The interstimulus interval was set at 60 s.

At the third visit (08:00 h), a 4-mL sample of blood was collected from each subject. Three female subjects (one super-taster and two non-tasters) dropped out before the blood sampling, and these subjects were excluded from all analyses. Samples were immediately stored at -80°C until analyses were completed, as described below.

Lipid analyses

Total lipids were extracted from plasma using chloroform/methanol (2:1 [v/v]) [38]. Aliquots were mildly saponified as previously described [39] to obtain free fatty acids for high-performance liquid chromatographic analysis. The separation of fatty acids was carried out with an Agilent 1100 high-performance liquid chromatographic system (Agilent, Palo Alto, CA, USA) equipped with a diode array detector, as previously reported [40]. Determinations of 2-AG, anandamide (AEA), and of AEA congeners,

palmitoylethanolamide (PEA) and oleylethanolamide (OEA), were performed as previously described [41].

Statistical analyses

The mean values ± standard error of PROP and NaCl intensity ratings were calculated for non-tasters and super-tasters. Three-way analysis of variance (ANOVA) was used to compare PROP intensity ratings with NaCl intensity ratings across taster groups. The Tukey test was used for post hoc comparisons. The PROP sensitivity differences related to gender were evaluated by one-way ANOVA. The BMI, TFEQ, endocannabinoids, and fatty acid plasma levels differences between the super-tasters and non-tasters were evaluated according to gender by two-way ANOVA. A main effects ANOVA was used to analyze the first-order (non-interactive) effects of multiple categorical independent variables (factors). Pearson linear correlation analyses were carried out between the three factors of the TFEQ and endocannabinoids plasma levels. Statistical analyses were conducted using Statistica 6.0 for Windows (StatSoft, Inc., Tulsa, OK, USA). $P < 0.05$ was considered statistically significant.

Results

The perceived taste intensities for each stimulus concentration by 17 subjects classified as non-tasters and 16 as super-tasters are shown in Figure 1. ANOVA showed a significant three-way interaction of taster group by solution type by concentration on the intensity ratings ($F_{2,185}$ =34.866, $P < 0.000001$). Post hoc comparisons showed that NTs gave lower intensity ratings to the two highest PROP concentrations compared with the two highest NaCl concentrations ($P = 0.00002$, Tukey test). Super-tasters gave higher ratings to PROP 0.32 and 3.2 mmol/L compared with the two highest NaCl concentrations ($P = 0.0002$, Tukey test).

One-way ANOVA in the present cohort showed that the PROP sensitivity was not related to gender ($F_{1,31}$ =0.8322, $P = 0.3686$).

Two-way ANOVA showed that the BMI did not change according to gender ($F_{1,29}$ = 1.2393, $P = 0.2747$), whereas it was slightly higher, although not significantly, in non-tasters compared with super-tasters ($F_{1,29}$ = 2.3734, $P = 0.1342$; Table 1).

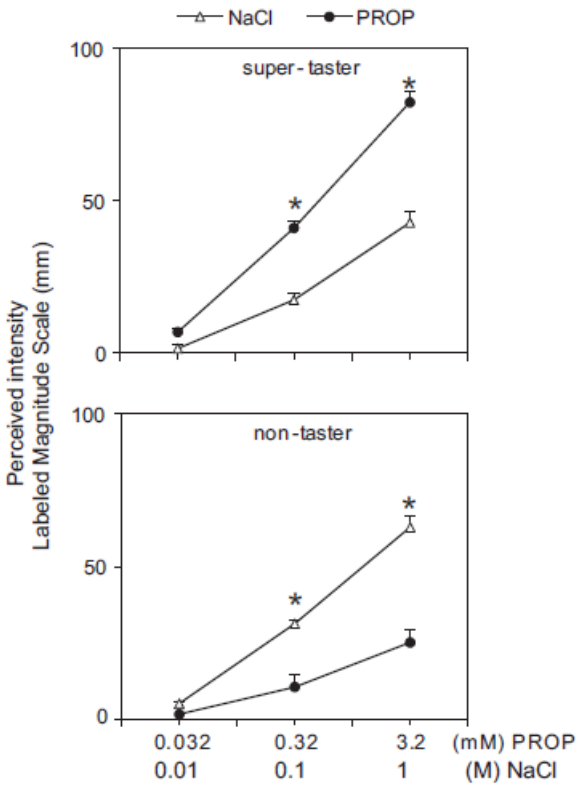

Fig. 1 - Relation betweenperceived taste intensityand stimulus concentration in non-tasters (n = 17) and super-tasters (n = 16). All values are presented as mean ± SE.* Significant difference between PROP and the corresponding NaCl concentration (P = 0.0002, Tukey test subsequent to three-way analysis of variance). NaCl, sodium chloride; PROP, 6-n-propylthiouracil.

Table 1 - Subject characteristics

	STs (n=16)	NTs (n=17)	All subjects (n=33)
Caucasian ethnicity	16	17	33
BMI (Kg/m^2)	21.27 ±0.64	23.42 ±0.86	22.38 ±0.56
Age (y)	25.68 ±0.98	29.44 ±1.786	27.58 ±1.16

BMI, body mass index; NT, non-tasters; ST, super-tasters

Values are presented as mean ± SE.

Figure 2 shows the scores relative to dietary restraint, disinhibition, and hunger determined by the TFEQ in super-taster and non-taster individuals. Two-way ANOVA showed that the score relative to disinhibition was dependent on PROP taster status, being higher in non-taster than in super-taster ($F_{1,29}$ = 7.3574, P = 0.0111), and gender, being higher in women than in men ($F_{1,29}$ = 6.3891, P = 0.01719). No interaction was

found between the two factors ($F_{1,29}$ = 0.00487, P = 0.9448). In addition, main effects ANOVA showed that disinhibition was affected more by PROP taster status (P < 0.008) than by gender (P = 0.015). No differences related to PROP taster status or gender for dietary restraint and hunger scores were found (P > 0.05).

As presented in Table 2, no differences related to PROP taster status in the profile of plasma fatty acids were found. In addition, the plasma ω-3 high polyunsaturated fatty acid (HPUFA) score, which is the percentage of ω-3 highly unsaturated fatty acids among total highly unsaturated fatty acids as a reliable biomarker of the ω-3 HPUFA status in tissues [42], did not change significantly. In contrast, two-way ANOVA showed that plasma levels of AEA and 2-AG were lower in non-tasters compared with super-tasters (AEA, $F_{1,29}$ = 8.2028, P = 0.0077; 2-AG, $F_{1,29}$ = 11.854, P = 0.00177; Fig. 3), but did not vary with gender (AEA, $F_{1,29}$ = 2.1544, P =0.1529; 2-AG, $F_{1,29}$ = 2.1576, P = 0.1526). The profile of OEA and PEA levels did not differ in relation to PROP taster status or gender (data not shown).

Linear correlation analyses between endocannabinoid and congener plasma levels and scores relative to the cognitive control of eating behavior factors assessed by the TFEQ showed that, in non-tasters, OEA was inversely correlated to the score relative to perceived hunger (r = -0.7, P = 0.002) and AEA was positively correlated to restraint (r = 0.5, P = 0.04) and negatively to perceived hunger, although the latter correlationwas at the limit of statistical significance (r = -0.47, P = 0.05). No such correlation was found in the super-taster group (Table 3). No correlation was found for 2-AG and PEA levels, irrespective of PROP taster status and/or cognitive control of eating behavior factors (data not shown).

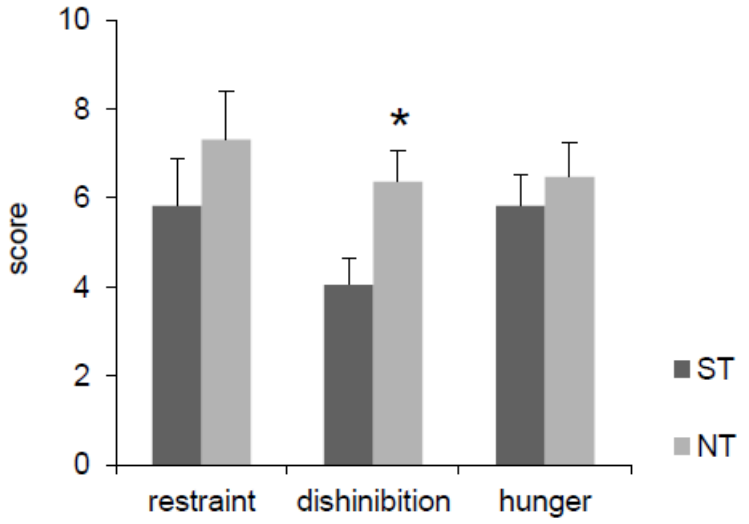

Figure 2 - Scores relative to dietary restraint, disinhibition, and perceived hunger of super-tasters (n = 16) and non-tasters (n = 17) were determined by the Three-Factor Eating Questionnaire [37]. All values are presented as mean ± SE.

* Significant difference (P = 0.02, two-way analysis of variance). NT, non-taster; ST, super-taster.

Table 2 - Concentration (mol %) of plasma fatty acids in super-tasters (STs) and non-tasters (NTs).

	STs		NTs	
	Mean	**SE**	**Mean**	**SE**
ω-3 fatty acids				
18:3	0.30	0.03	0.29	0.02
20:5	0.44	0.06	0.52	0.13
22:6	1.70	0.11	1.68	0.15
ω-6 fatty acids				
18:2	22.10	0.92	21.85	0.87
18:3	0.35	0.04	0.34	0.03
20:3	1.73	0.16	1.63	0.13
20:4	6.55	0.29	6.59	0.34
ω-9 fatty acids				
16:1	1.66	0.29	1.32	0.13
18:1	18.54	0.75	18.99	0.55
20:3	0.09	0.01	0.09	0.01
SFAs				
14:0	1.37	0.14	1.41	0.15
15:0	0.38	0.04	0.41	0.03
16:0	33.78	1.14	33.87	0.95
18:0	8.64	0.41	8.78	0.36
Total ω-3	2.44	0.18	2.49	0.28
Total ω-6	30.72	0.94	30.41	0.88
ω-3 HPUFA score *	19.72	1.15	20.27	1.98
Total SFAs	45.67	1.43	45.86	1.13
Total MUFAs	20.25	0.89	20.36	0.60
Total PUFAs	33.91	0.97	33.58	0.93

HPUFA, high polyunsaturated fatty acid; MUFAs, monounsaturated fatty acids; NT, non-testers; PUFAs, polyunsaturated fatty acids; SFAs, saturated fatty acids; ST, super-tasters

* The u-3 HPUFA score was calculated as described previously [42].

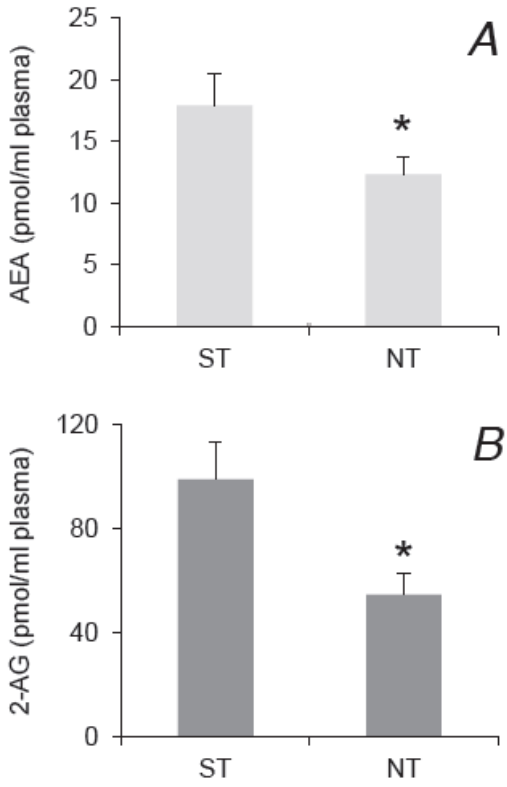

Figure 3 - Mean values ± SE of AEA (A) and 2-AG (B) plasma levels determined in STs (n = 16) and NTs (n =17). * Significant difference (AEA, P = 0.034; 2-AG, P = 0.003, two-way analysis of variance). AEA, anandamide; 2-AG, 2-arachidonoylglycerol; NT, non-taster; ST, super-taster.

Table 3 - Linear correlation analyses between AEA or OEA and scores* relative to dietary restraint, disinhibition, and perceived hunger in STs and NTs.

	NTs		**STs**	
	r	***P***	**r**	***P***
AEA				
Restraint	0.502	0.040	-0.312	0.239
Disinhibition	-0.121	0.640	-0.349	0.185
Hunger	-0.474	0.050	0.134	0.621
OEA				
Restraint	0.320	0.211	-0.313	0.239
Disinhibition	-0.244	0.344	0.284	0.286
Hunger	-0.699	0.002	0.216	0.421

AEA, anandamide; NTs, non-tasters; OEA, N-oleoylethanolamide; STs, super-tasters * Scores were determined by the Three-Factor Eating Questionnaire (n = 33) [37].

Discussion

The observation that NTs gain more weight than STs was first described in 1966 by ischer et al. [43]. However, only several decades later, a strong relation between body weight and the ability to taste PROP, especially in women, was discovered [5,27]. Other studies could not always confirm the association between the PROP taster status [22–26] or the genotypes of the taste receptor that binds PROP [24,44] and body weight. Therefore, it is likely that other genetic and non-genetic factors may contribute to the increased predisposition of higher energy intake in NT subjects. In our cohort, the analysis of the cognitive control of eating behaviors showed that NTs reported an almost two-fold higher disinhibition compared with STs. Disinhibition is usually associated not only with a higher BMI and obesity but also with higher intake of palatable food, which contributes to overweight/obesity [45]. In addition, the exposure to and consumption of highly palatable foods has been associated with increased plasma 2-AG levels [31]. The present study shows that the disinhibition behavior of NT normal-weight individuals, which may lead to increased food intake and, eventually, a higher BMI (NTs exhibited a trend toward a higher BMI than STs in the present study), is accompanied by a downregulated peripheral endocannabinoid system. This observation seems to be at odds with previous data showing that peripheral endocannabinoid (namely 2-AG) levels are increased in obese subjects [46–48]. However, in obesity, plasma endocannabinoids correlate with waist circumference and visceral fat rather than with BMI [46,47]. Furthermore, no positive association between BMI and plasma endocannabinoid levels has been observed in lean individuals [46] such as those participating in the present study. We propose that the lower AEA and 2-AG levels observed in NTs represent an adaptive mechanism attempting to normalize feeding behavior components, such as increased hunger and decreased restraint, in these subjects, thus explaining in part why they still exhibit a lean phenotype despite their preference for fat. In fact, restraint and hunger did not differ significantly between NTs and STs, but only in NTs and counterintuitively, these were positively and negatively correlated to AEA, respectively. Therefore, what could appear as a contradictory result may indicate a physiologic mechanism to maintain a normal body composition within certain limits. Interestingly, and, in this case, not surprisingly, OEA, a well-known anorexic mediator [49], was inversely correlated to hunger only in NT subjects. This suggests that other players may control eating behavior in this population of individuals who are less sensitive to PROP. It is tempting to hypothesize that lower AEA and 2-AG levels may represent an adaptive response that counterbalances a potentially decreased appetite inhibition by OEA in certain NT subjects. In this cohort we did not find any differences related to gender in the parameters studied, validating our results as specifically associated to PROP taster status.

However, we cannot rule out that gender differences may emerge in larger cohorts. Several studies have found that endocannabinoid biosynthesis is influenced by dietary fat [50], which causes a modification of fatty acid incorporation into phospholipids and, hence, may alter the availability of biosynthetic precursors for endocannabinoids and related mediators [41]. However, it has been suggested that NTs may have an increased dietary fat intake [18], and this may have influenced the plasma levels of these endogenous compounds. In a recent study, we found that decrease of AEA was associated to a higher u-3 HPUFA score in human plasma after the intake of an enriched cheese [51]. In contrast, in the present study, the lack of differences in this parameter and plasma fatty acids, which were evaluated here as a marker of changes of dietary fat [52], suggested that decrease of endocannabinoids were not linked to different dietary regimens. From the original findings of this study, it is tempting to speculate that the peculiar eating behavior characteristics of NTs, which may lead to increased energy intake and body weight, are modulated in normal-weight subjects by endocannabinoids and AEA-related mediators in a way to maintain a physiologic body composition. Besides endocannabinoids, several other factors control feeding behavior and body composition homeostasis. Among them, ghrelin has been recently shown to play a crucial role [53] in these mechanisms and it has been found to correlate to 2-AG during human hedonic eating [31]. Therefore, it would be very interesting to evaluate in future studies whether ghrelin levels are modified during hedonic eating in NTs compared with STs and the possible correlation of ghrelin with endocannabinoid levels.

Conclusions

If our present results are confirmed in a larger cohort, a personalized dietary approach, possibly targeting endocannabinoid and OEA biosynthesis [50,54–57], could be envisaged to maintain an optimal body composition in NTs.

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General conclusions

The primary aim of the studies described in this section of the thesis was to identify and characterize factors that may contribute to differences in the genetic predisposition to taste thiourea compounds. The findings described in first three chapters indicate that, in addition to the variants of TAS2R38 with its varying affinity for the stimulus, other factors are involved in the PROP phenotype determination. In particular, results of **chapter 1** and **2** suggest that two specific salivary peptides, belonging to the basic proline-rich protein family (Ps-1and II-2), could be involved in the PROP molecule twist and turn, in order to facilitate its binding with the receptor (also when is in the form with low affinity for the stimulus), and that this permissive function might be carried out via L-Arginine amino acid. Furthermore, results of **chapter 3** suggest that, in a genetically homogenous cohort, the *rs2274333* (A/G) polymorphism of gustin (CA6) gene affects the PROP sensitivity by acting on the protein function as growth factor of taste bud. This observation, if confirmed in different populations, will provide a mechanistic explanation of the reason PROP super-taster individuals have a higher density of fungiform papillae than PROP non-tasters, and why they are more responsive to a wide range of stimuli that are not mediated via the specific bitter receptor.

Regarding the identification of confounding variables which may explain the controversial data in the literature about the relationship between PROP sensitivity and BMI, results of **chapter 4** suggest that the endocannabinoid system plays an important role, and in normo-weight subjects, it seems to act as a mechanism of homeostatic balance between signals of hunger and satiety. This study opens new perspectives by evaluating a factor involved in the physiology of eating behavior control, which, by counteracting impaired eating behaviors triggered by PROP sensitivity, may restore the physiological balance of nutritional status and health of the individual.

Studies in progress

Although the aim of the studies in chapter 1 and 2 was to show salivary proteome involved in bitter taste sensitivity, the analysis was limited to only one bitter molecule, such as PROP. Studies in progress are analyzing the relationships between salivary proteins and sensitivity to classically-defined bitter molecules as well as tannins. These studies will help to determine if these salivary proteins serve both a permissive function, that allows the individual to taste bitterness, as well as a protective function against the negative biological effects of tannins.

The results of chapter 2 suggested that the permissive function of PROP tasting of the two salivary peptides (Ps-1and II-2) might be carried out via L-Arginine amino acid

present in their sequence. Since it is likely that the free-form of L-Arginine is accumulated in whole saliva, is a result of the splitting of proteins by salivary proteases and peptidases. To better understand the role of L-Arginine in PROP tasting, we are determining the relationship between levels of salivary L-Arginine and PROP sensitivity and evaluating the effects of oral supplementation of increasing concentrations of L-Arginine on PROP bitterness intensity and latency.

Furthermore, in order to confirm, in an ethnically-mixed population, the role of the gustin gene polymorphism in PROP sensitivity and fungiform papilla density already showed in a genetically homogenous population (chapter 3), we genotyped for gustin gene and *TAS2R38* gene 91 subjects of Rutgers University of New Jersey, and their fungiform papilla density were determined. A preliminary analysis showed that as expected, PROP bitterness ratings were lower in homozygous individuals for the insensitive allele of *TAS2R38* compared with those homozygous or heterozygous individuals for the sensitive allele. However, no differences in PROP bitterness were found among genotypes of the gustin gene. Fungiform papillae densities were higher in homozygous individuals for the sensitive allele of the gustin gene than in those homozygous recessive for this allele, but no differences in the density of fungiform papillae related to *TAS2R38* genotypes were found. The distribution of *TAS2R38* genotypes within each gustin genotype group was markedly different from that of the genetically homogeneous population studied by Calò et al (2011), and the occurrence of recessive alleles at both loci was rare in the present sample. These findings confirm that density of fungiform papillae is related to gustin genotypes in an ancestrally heterogeneous population, and suggest that variations in the frequency of allele combinations for these two genes could explain discrepant findings for gustin gene effects across populations.

The results shown in chapter 4 suggested that, in normal- weight individual, the endocannabinoid system may play an important role in maintaining the physiological balance between food intake and energy metabolism. Now we are determining the relationship between endocannabinoid system and taste sensitivity in obese patients.

Finally, we analyze the brain activation patterns during PROP bitter taste perception using functional magnetic resonance imaging (fMRI). Up to now, we examined one super-taster subject and one non-taster subject and we found a significant difference of cortical activation in the dorsolateral-prefrontal region during the administration of PROP. This preliminary data suggest that the dorsolateral prefrontal cortex is involved in the conscious perception of PROP, which gives rise to a pattern of activity consistent with individual differences in the ability to taste this compound.

CURRICULUM VITAE

I was born on December 21, 1985 in Cagliari.

EDUCATION

1999- 2004 Social-Psycho-Pedagogical High School "E. D'Arborea", Cagliari, Italy. High School diploma.

2004-2008 Dissertation work for the First level degree in Experimental Biology Laboratory of General Physiology, Dept. of Experimental Biology, University of Cagliari, Italy (Supervisor: Prof Iole Tomassini Barbarossa).

2008-2010 Dissertation work for the Second level degree in Experimental and Applied Biology Laboratory of General Physiology, Dept. of Experimental Biology, University of Cagliari, Italy. Graduated Magna Cum Laude (Supervisor: Prof Iole Tomassini Barbarossa).

2011-present. PhD student in Morphological and Functional Sciences, University of Cagliari, Italy (Supervisor: Dr Paolo Solari).

SCIENTIFIC ACTIVITIES

March 2009 – June 2009: research internship in the General Physiology laboratories of the Department of Experimental Biology, University of Cagliari, Italy.

February 2009 – July 2010: research internship in the General Physiology laboratories of the Department of Experimental Biology, University of Cagliari, Italy. Collaboration with Department of Science Applied to Biosystems, Biochemistry and Molecular Biology Division, University of Cagliari, Italy.

November 2010 – February 2011: Research collaborator of the Laboratory of General Physiology, Dept. of Experimental Biology, University of Cagliari, Italy.

March 2011 - present: PhD student in Morphological and Functional Sciences, Section of General Physiology, University of Cagliari, Italy. Collaboration with Department of Science Applied to Biosystems, Biochemistry and Molecular Biology Division, University of Cagliari, Italy.

January 2013 – June 2013: research internship as a visiting student researcher in the Sensory Evaluation/Nutrition laboratory, Department of Food Science, Rutgers University of New Jersey (Supervisor: Beverly J Tepper).

PUBLICATIONS

1. Cabras T, Melis M, Castagnola M, Padiglia A, Tepper BJ, Messana I, Tomassini Barbarossa I. (2012) **Responsiveness to 6-n-Propylthiouracil (PROP) is associated with salivary levels of two specific basic Proline-rich proteins in Humans.** PLOS One 7(2): e30962.

2. Tomassini Barbarossa I, Carta G, Murru E, Melis M, Zonza A, Vacca C, Muroni P, Di Marzo V, Banni S. **Taste sensitivity to 6-n-propylthiouracil is associated with endocannabinoid plasma levels in normal-weight individuals.** Nutrition. 2013 Mar; 29(3):531-536.

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ABSTRACT AT CONGRESS

1. Cabras T, Melis M, Padiglia A, Castagnola M, Messana I, Tomassini Barbarossa I. **Human salivary proteome and bitter taste: the reciprocal influence**. 6th ItPA Annual National Conference- Turin 21-24, June 2011

2. Melis M, Sollai G, Masala C, Sabatini A, Palmas F, Crnjar R, Solari P. **Chemoreception and feeding behaviour in the red swamp crayfish Procambarus clarkii.** XXI ECRO Conference- Manchester, 7-10 September 2011, Chem. Senses 31: A1–A45, 2011

3. Melis M, Cabras T, Messana I, Crnjar R, Tomassini Barbarossa I. **Human bitter perception and salivary proteins.** 62nd National Congress of the Italian Physiological Society (SIF), Sorrento, Italy 25-27 September 2011. Acta Physiologica. 2011, 203 (688): 122.

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6. Melis M, Sollai G, Masala C, Sabatini A, Palmas F, Crnjar R, Solari P. **Chemoreception and feeding behaviour in the red swamp crayfish Procambarus clarkii**. FENS-IBRO Schools "Chemical Senses: Neurobiology and Behavior; Bertinoro - Italy, 3-8 June 2012.

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11. Melis M, Sollai G, Masala C, Sabatini A, Palmas F, Crnjar R, Solari P**. Leg chemoreceptor sensitivity in the red swamp crayfish Procambarus clarkii.** ECRO Conference 2013, 26-29 August 2013, Leuven-Belgium.

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