Project Summary

The development of food preferences as a result of dietary experience is a common phenomenon amongst diverse animal species that may have provided selective advantages during evolution. The dietary experience of juveniles may impact adult food preferences providing a theoretical link to factors that affect obesity, a significant risk factor for many human diseases. However, the mechanisms of gustatory plasticity are not well understood. The role of the central nervous system in experience-dependent food preference, particularly flavor conditioning, is well established. However, plasticity of the peripheral gustatory system has not been investigated. A labeled-line model of vertebrate and insect taste that is currently emerging supports the plasticity of gustatory receptor gene expression as one mechanism of behavioral plasticity. Food acceptance is in part a balance between sweet and bitter stimuli. Mice and insects can be stimulated to feed simply by activating specific “sweet-labeled” cells in the peripheral sensory system. Aversive behavior is similarly triggered by activating “bitter-labeled” cells. For example, transgenic mice miss-expressing a bitter receptor in the sweet cells become behaviorally attracted to the bitter ligand. A small number of sugar receptor genes have been conserved within vertebrate and insect genomes, while the number and diversity of bitter receptors has expanded. Significantly, many or all of the bitter receptor genes are expressed together in the same bitter sensitive taste cells. Therefore, the activity of bitter sensitive taste cells and the aversive behaviors they mediate result from the ensemble activity of many different bitter receptors. Altering the composition and/or level of gustatory receptor expression necessarily alters taste coding and provides a mechanism for plasticity in response to dietary experience. This hypothesis will be tested using an insect model species. Specifically, gustatory receptor gene expression in sugar and bitter sensitive neurons will be compared between control animals and those induced to prefer alternate foods through experience. This objective directly supports NIDCD's goals to understanding the fundamental mechanisms of neural plasticity including associations between chemosensory disorders and altered food intake.

Project Narrative

This research seeks to understand the basic mechanisms that underlie experience related changes in food preference, something that may ultimately influence dietary choice and thus obesity. It will contribute to the general understanding of how taste functions at the molecular level that therefore contribute to the body of knowledge that is used to develop taste related medical therapies.

Plasticity of the Peripheral Gustatory System in Response to Feeding Experience

This proposal fits into the priority category for newer investigators. The principal investigator, Dr. Kevin Wanner, has not previously received any funding from NIH.

(1) Specific Aims

This study will evaluate plasticity of the peripheral gustatory system as one mechanism of induced feeding preference. Vertebrate and insect animals can display gustatory preferences based upon previous feeding experience, particularly during juvenile development. Despite theoretical connections between dietary learning and obesity, and the significance of obesity to human health, the underlying mechanisms of induced feeding preferences have not been thoroughly investigated. Conditioning experiments clearly establish the role of the central nervous system (CNS) in some forms of dietary learning. However, plasticity of peripheral sensory system has not been evaluated. Emerging labeled-line models of vertebrate and insect taste coding clearly point to chemoreceptor gene expression as one conserved mechanism of behavioral plasticity. Such plasticity has been demonstrated in the nematode *Caenorhabditis elegans*. Similarly, the electrophysiological response of some insect gustatory neurons can change in response to sensory experience. Therefore, experience dependent changes in gustatory receptor gene expression may modify feeding preference and represent a conserved mechanism of gustatory plasticity. The silkworm *Bombyx mori* is a new model organism that is uniquely suited to investigating this question. Gustatory receptor gene expression in silkworm larval taste cells will be compared between control (non-induced) and treated (preference-induced) individuals. Changes to gustatory receptor expression will be correlated to larval feeding behavior.

(2) Background and Significance

Feeding is a complex context dependent behavior that involves several different senses including taste, smell, touch and vision. However, taste is the final arbitrator of food acceptance and sustained ingestion of a food depends in part on the balance between stimulating sweet and deterring bitter tastes. Rapid advances in our understanding of the molecular and neurological mechanisms of the chemical senses have revealed a conserved logic that underlies the sensory systems of diverse animal phylogenies. However, the molecular mechanisms of sensory plasticity are not fully understood although dietary experience during juvenile development may influence adult dietary choices that contribute to obesity and obesity-related diseases (Brunstrom 2005).

2.1 Feeding experience influences vertebrate and insect feeding preferences.

Gustatory preference based upon previous feeding experience has been observed both in vertebrate and insect species. New born vertebrates are believed to discriminate only between the basic innate taste modalities; acceptance of sweet and rejection of bitter and sour tastes (Myeres and Sclafani 2006). The subsequent taste preferences and aversions of vertebrate adults have been attributed to learning, reviewed by Myeres and Sclafani (2006) and Brunstrom (2005). Therefore, the phrases “dietary learning” and “learned flavor preferences” are commonly used to describe this phenomenon in vertebrate species. While taste preferences in adulthood remain plastic and are able to be modified by gustatory experience, critical periods of plasticity during juvenile development may strongly influence adult feeding behavior (Brunstrom 2005). The role of the CNS and pavlovian conditioning responses in conditioned vertebrate feeding responses has been clearly demonstrated, particularly in adults. For example, many studies have demonstrated the ability to condition an animal’s feeding behavior (acceptance/avoidance) by associating flavor cues with positive or negative postingestive effects (Myeres and Sclafani 2006). However, changes in the peripheral sensory system during development or in response to feeding experience have not been investigated.

For many years it has been observed that phytophagous insects feeding on a particular food for a period of time afterwards demonstrate increased preference for that food (Reviewed in Jermy chapter 9). Since the mechanism was unknown and it did not fit into the usual categories of learning the phenomenon was termed “induced feeding preference”. Four to 24 hours of feeding experience is sufficient to induce a preference that can then last for several instar stages.

In some cases the induction can be extreme and appears to be nonadaptive. Insect larvae can be induced to prefer a less acceptable plant to the degree that they will reject the previously acceptable food sources and starve to death! This rigid behavioral response is unlikely to be governed solely by the CNS (Jermy chapter 9). The range of acceptable food sources is believed to be largely genetically fixed for particular insect species. However, within their normal food range naïve neonate caterpillars are less restricted then later stages that have formed food preferences. Therefore, the feeding preferences of both vertebrate and insect species appear to be more amenable during juvenile stages.

2.2 Evidence for plasticity of the peripheral sensory system.

Schoonhoven (1969) concluded that induced feeding preference within insects involves both central and/or peripheral modifications as a result of experience. The fact that food aversion learning has been demonstrated using insects points to the CNS as one mechanism. However, sensory input from taste receptors was also determined to be a contributing factor. Larval gustatory neurons became less sensitive to a deterrent compound after it was experienced during feeding as determined by electrophysiology (Schoonhoven 1969). *Manduca sexta* caterpillars exhibit strong preferences for solanaceous plants after experiencing them. The induced feeding preference was attributed to the increased sensitivity of gustatory neurons to a specific host recognition cue, indioside D (Campo et al., 2001; Campo and Miles 2003). After experiencing indioside D more neurons in the caterpillar taste hairs responded to this stimulus and their sensitivity increased. Similarly, caterpillars exposed to a behaviorally important compound (an alkaloid sequestered for defensive purposes) exhibited increased electrophysiological responses in a specific gustatory neuron tuned to that compound, after experience (Chapman et al., 2003). Other neurons in the same sensillum were not affected.

The expression levels of three nematode (*Caenorhabditis elegans*) chemoreceptor genes changed in response to specific sensory stimulation (Peckol et al., 2001). This led the Bargmann lab to conclude that “experience dependent changes in chemosensory receptor gene expression may modify [nematode] olfactory behavior”. Clearly evidence exists to support experience dependent plasticity of the peripheral sensory system. But is this a mechanism for behavioral plasticity that is generally conserved among diverse animal species such as vertebrates and mammals? I believe that the answer is yes, based on the labeled-line model of insect and vertebrate taste that is emerging from current research.

2.3 Labeled-lines code vertebrate and insect taste perception.

2.3.1 A small number of conserved sweet receptors, many more divergent bitter receptors.

At the periphery, the dendrites of specialized bipolar insect gustatory neurons extend into hollow hair-like sensilla that have a single terminal pore through which taste stimuli enter (termed contact chemosensilla). In contrast, taste buds on the mammalian tongue are composed of 50-100 cells with taste sensitive microvillae that extend into a taste pore cavity. Upon stimulation the taste cells release neurotransmitters that activate the neurons that innervate taste buds. Therefore, unlike the insect system, mammalian gustatory neurons are not in direct contact with the external chemical environment (a review by Scott 2005 contrasts insect and mammalian taste). G-protein coupled receptors (GPCRs) expressed in the taste cells/neurons bind taste chemicals and trigger signal transduction pathways.

The term chemoreceptor (Cr) refers collectively to odorant and gustatory receptors (Ors and Grs) (Robertson et al., 2003). The landmark discovery of the first mammalian Ors by Buck and Axel (1991) and the advent of whole genome sequencing facilitated the discovery of chemoreceptor genes from insects and vertebrates. The Zuker and Margolskee labs among others have identified and characterized two mammalian Gr families; the T1Rs that detect sugars and amino acids and the T2Rs that detect bitter stimuli, reviewed by Scott (2005) and Sugita (2006). The mouse and human genomes encode three members of the T1R family, T1R1, T1R2 and T1R3. Experiments using transgenic animals and heterologous expression systems have demonstrated that T1R1, T1R2 and T1R3 detect sugars by forming heterodimers (Nelson et al.,

2001; 2002; Zhao et al., 2003 among others, reviewed in Scott 2005 and Sugita 2006). T1R2 & T1R3 is broadly tuned to various sugars and sweet tasting molecules while T1R1 & T1R3 detects most of the L-amino acids. The T2R family of taste receptors detects bitter tastants such as cyclohexamide (Chandrashekar et al., 2000). The mouse and human genomes encode many more T2R bitter receptors, 35 and 25 respectively (Scott 2005).

The completion of the dog, cow, opossum, chicken, frog and three fish genome sequences has enabled an evolutionary analysis of the vertebrate taste receptor family. Interestingly, the number of sweet and umami T1R receptors, and their amino acid sequences, has remained relatively well conserved reflecting the consistent nature of the nutrients they detect in the environment (Figure 1) (Shi and Zhang, 2005). Conversely, the number T2R bitter receptors, and their amino acid sequences, is much more variable, presumably reflecting a greater variety of bitter substances encountered in the environment by these species (Shi and Zhang, 2005; Go, 2006). Frogs have 49 T2R genes and mammals have 12-37. Fish have only 4-6 and chickens only 3 bitter T2R receptors (Figure 1). Whether some animal phylogenies have evolved alternate gene families to detect bitter tastes remains to be determined.

Insect and vertebrate Crs represent separate gene families. The fruit fly genome encodes 62 Grs and 68 Ors (Robertson et al., 2003). The *Drosophila* sequences have been used in bioinformatics approaches to annotate the Or and Gr families from the recently sequenced genomes of the mosquito *Anopheles gambiae* (Hill et al., 2002) and the honey bee *Apis melifera* (Robertson and Wanner, 2006). Dr. Robertson is an acknowledged expert in the annotation of insect chemoreceptors. Working in Dr. Robertson's lab I have annotated more then 40 new Ors from the silkmoth *Bombyx mori* (Wanner et al*. in press*) as well as 80 Ors from the *Nasonia vitripennis* genome (unpublished results). The silkworm Grs will be annotated by the spring of 2007 as a component of my current research. Due to the high rates of sequence divergence and the presence of numerous introns, automated gene prediction software has not successfully annotated the silkworm Grs (or any other Or and Gr family from insect genomes sequenced to date). Therefore, manual approaches described in Robertson et al. (2003), Robertson and Wanner (2006) and Wanner et al. (In Press) are used. Briefly, the published fly, mosquito and honey bee Gr sequences (as well as unpublished beetle Gr sequences from our lab) are used to perform tblastn searches of the silkworm genome sequence entered on GenBank. The gene models are then constructed in a text editor based upon homology with know insect Ors, online software that predicts intron splice sites and familiarity with the subtle characteristics of insect Or and Gr families that comes from experience.

Few insect Grs have been functionally characterized. One notable exception is the identification of a taste receptor tuned to the sugar trehelose, DmGr5a (Dahanukar et al., 2001; Chyb et al., 2003). DmGr5a groups together with similar fly (8), mosquitoe (8), bee (2) and moth (2) Grs to form a conserved sugar receptor (putative) subfamily (Figure 1)(Robertson and Wanner 2006, Kent and Robertson unpublished results). Unlike mammals, electrophysiology indicates that insects can discriminate between different sugars. Of the approximately 50 remaining *Drosophila* Grs most are believed to be bitter receptors. In contrast, the honey bee does not require enhanced bitter perception due to its symbiotic relationship with plants (provides pollination in return for nectar) and its genome encodes only 10 Gr sequences. We expect moth genomes to encode at least 60 or more Gr genes since they are herbivores that must decode complex plant deterrent chemistry. Only three moth Grs have been reported to date (Figure 1) from the private 1X genome sequence of *Heliothis virescens*. Diverse animal species (insects, frogs and mammals) appear to have evolved small conserved taste receptor families to detect constant nutrients in the environment, and larger more diverse taste receptor families to detect more variable bitter deterrent stimuli. The expansion of bitter Gr families is more pronounced in herbivores as compared to carnivores or species that have symbiotic relationships with plants.

A) Insect Grs **B) Vertebrate Grs**

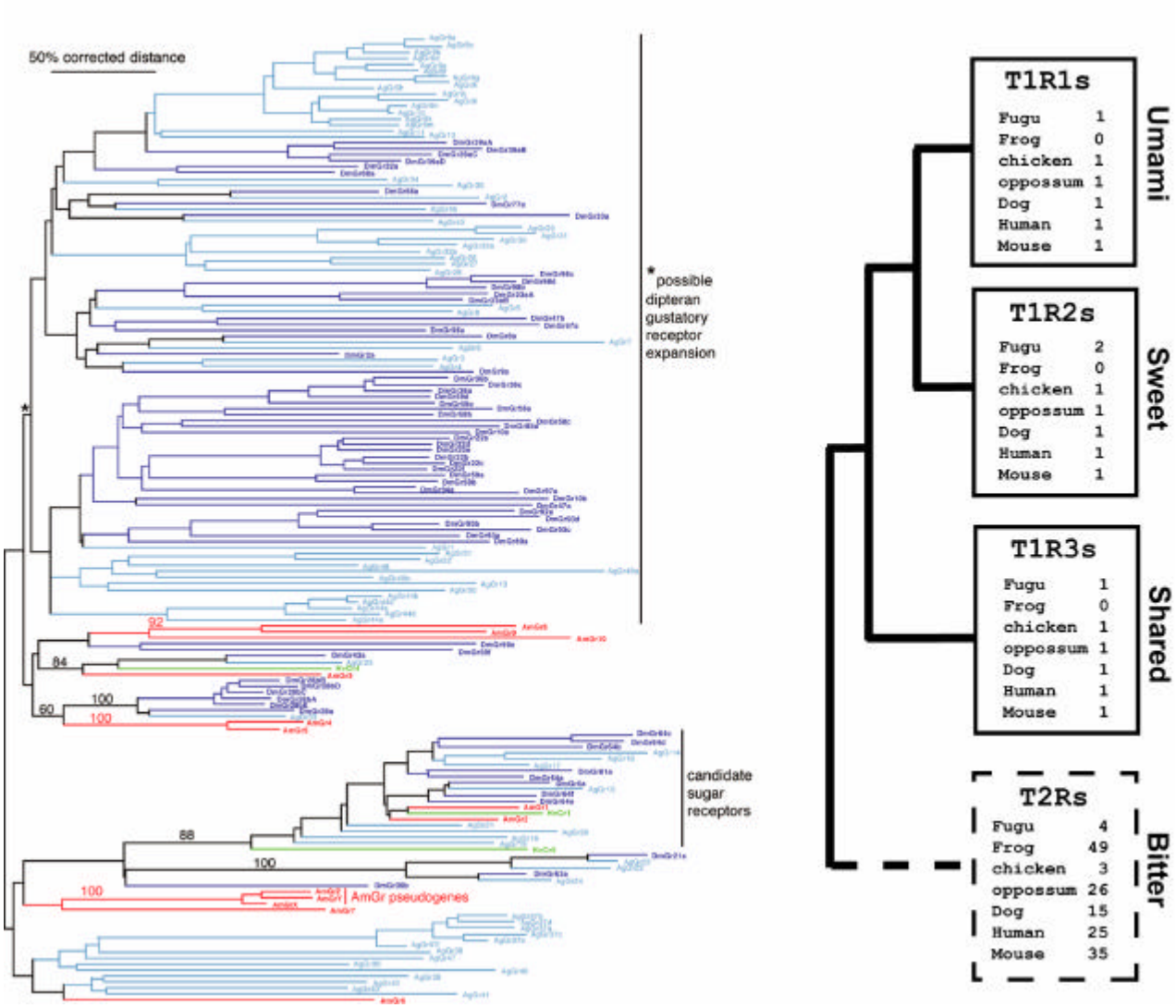

Figure 1. **A)** Neighbor-joining phylogenetic tree of Insect Grs from Robertson and Wanner (2006). Blue lines represent flies (*D. melanogaster*) and mosquitoes (*A. gambiae*), red for honey bees (*A. mellifera*) and green for moths (*H. virescens*). Bootstrap support is indicated at significant branch points. **B)** Schematic representation of vertebrate Gr phylogeny adapted from Shi and Zhang (2006). The number of fugu (fish), frog, chicken, opossum, dog, human and mouse receptors within each family are indicated.

2.3.2 Labeled-line cells expressing sweet and bitter receptors encoded taste modalities. Receptors at the periphery perceive chemical stimuli in the environment and neuronal circuits relay the information to the CNS. Several different models of taste coding have been proposed, one of which is the labeled-line model (reviewed in Scott and Giza 2000). The labeled- line model suggests that specific taste qualities are governed by specific sensory cell pathways. Segregated cells that detect a particular taste relay the information to the CNS without integration or processing along the way. Therefore, different taste modalities are encoded by the selective activation of non overlapping cells. Alternate models suggest that the activity of many different cell types, or the temporal pattern of action potentials, encodes taste quality. Emerging evidence

supports a primary role for labeled-line coding of vertebrate and insect sweet and bitter tastes that are tightly linked to feeding. As predicted by the labeled-line model, T1R receptors that detect sweet tasting ligands are expressed in discrete taste cells that do not overlap with taste cells expressing bitter T2R receptors (Adler et al., 2000; Mueller et al., 2005). In a convincing set of experiments using transgenic mice Mueller et al. (2005) demonstrate that T2R receptors are necessary and sufficient for the perception of bitter compounds. For example, transgenic mice expressing a human T2R receptor specific to glucopyranosides become behaviorally averse to this compound whereas wild type mice are indifferent. Furthermore, mice engineered to express a ***bitter*** receptor in the ***sweet*** cells become behaviorally attracted to the bitter ligand. This experiment demonstrates the tight labeled-line link between activation of the sweet cells and phagostimulation. Significantly, most of the 35 mouse T2R genes appear to be expressed together in the same bitter sensitive cells. Therefore, bitter sensitive cells in the periphery are broadly tuned and wired directly to aversive feeding behavior (Mueller et al., 2005).

Electrophysiological studies using caterpillars (the larval stage of the insect order Lepidoptera) in the 1960's and 70's provided evidence for labeled-line coding of insect taste. Caterpillars were used as models due to the simplicity of their gustatory system. Eight gustatory neurons in two contact taste sensilla located on each side of the caterpillar mouth primarily mediate feeding behavior. The two taste sensilla (medial and lateral styloconica) are prominent and discrete (Figure 2) allowing relatively easy access for single sensillum electrophysiology. Furthermore, the activity of each of the four gustatory neurons within each sensilla can be distinguished by spike frequency. Extensive studies using more then 20 different caterpillar species have identified four primary types of gustatory neuron, those tuned to sugars, bitter substances, inositol or salts (comprehensively reviewed by Schoonhoven and van Loon, 199_; 2002). The sugar sensitive gustatory neurons are narrowly tuned to one or a few sugars, typically glucose, sucrose and fructose. The bitter responsive neurons however are broadly tuned to many different compounds. Furthermore, feeding behavior is tightly correlated to the balance of activity between phagostimulating sugar and inositol receptive neurons and the deterring bitter receptive neurons, leading to the conclusion that caterpillar taste is coded by labeled-lines (REF). As with mammals, labeled-line models predict non-overlapping expression of sugar and bitter receptors in sugar and bitter sensitive insect neurons; recent fruit fly experiments confirm this prediction.

Gustatory neurons that express the *Drosophila* trehelose sugar receptor Gr5a were either selectively inactivated by tetanus toxin, or selectively activated by capsaicin sensitive TRP channels. Sensory neurons expressing DmGr5a mediate behavioral attraction to sweet stimuli but not behavioral aversion to bitter stimuli (Thorne et al., 2004; 2005; Marella et al., 2006). Similarly, gustatory neurons expressing the caffeine bitter receptor DmGr66a (Moon et al. 2006) mediate aversion to bitter stimuli, and not attraction to sugars (Thorne et al., 2004; 2005; Marella et al., 2006). One study suggests the existence of subpopulations of labeled-lines within each modality (ie specific combinations of Grs expressed in specific neuron subpopulations), such that different sugars or different bitter chemicals can be discriminated within each modality (Amrein and Thorne, 2005). This agrees with electrophysiological data from caterpillars but contrasts the studies using transgenic mice.

2.4 Peripheral mechanisms of sensory plasticity.

Sensory plasticity could result from changes in perception (peripheral), changes in circuitry (synapses) or changes to the processing (CNS). Food acceptance is in part a balance between the attractive quality of sweet and the deterring effect of bitter taste. Bitter sensitive cells are broadly tuned because they co-express many different bitter receptors in the same cell. Modulating the type and/or levels of bitter receptor genes in response to feeding experience would therefore provide a mechanism for plasticity and induced feeding preference. Nematode olfactory neurons also express more then one type of receptor, and the Bargman group suggested that changes in nematode Cr gene expression could account for plasticity of olfactory

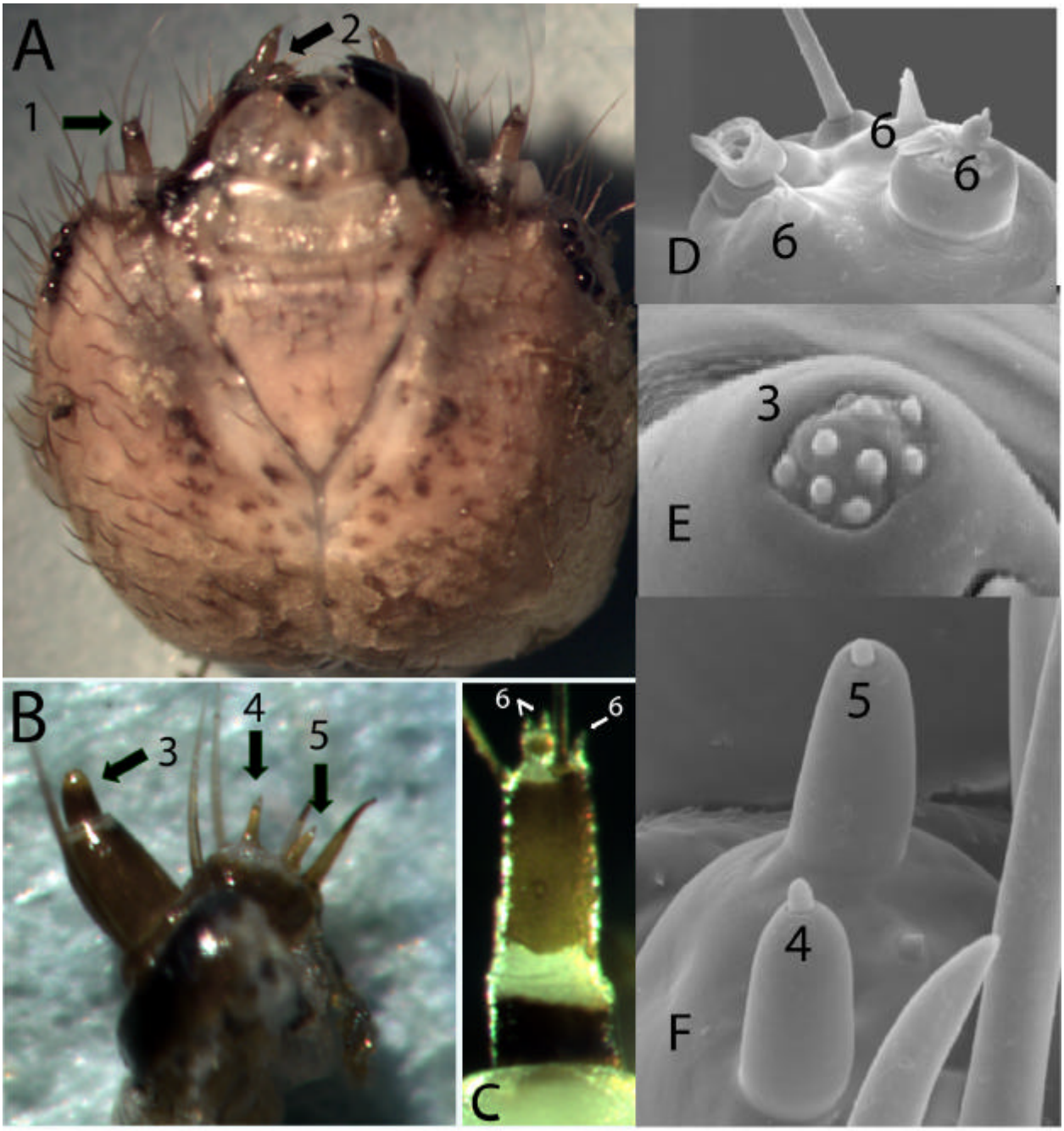

Figure 2. A) Dorsal view of the head of a 5th instar silkworm larva. An Antenna (1) on each side of the head mediates short range olfactory behavior. Sensory organs that mediate feeding are located on the maxilla (2) on each side of the mouth. **B)** The maxilla is divided into two parts, the maxillary palp (3) that probes food surfaces and mediates test biting, and the galea on which the the lateral (4) and medial (5) styloconic taste sensilla that control feeding behavior are situated. **C&D)** The tip of the antenna has three multiporous basoconic sensilla (6) that house approximately 16 olfactory neurons. **E)** The tip of the maxillary palp (3) has three multiporus olfactory sensilla that house about 12 olfactory neurons and five uniporous taste sensilla that house 15-30 gustatory neurons. **F)** The lateral (4) medial (5) styloconic taste sensilla that each house four gustatory that regulate feeding behavior are situated atop of dome shaped appendages on the galea. Images in **D-F** are scanning electron micrograms provided complements of Dr. Frank Hanson.

behaviors (Peckol et al. 2001). Alternatively, plasticity can result from changes to the circuitry (such as synaptic strength). However, due to labeled-line coding of taste, circuitry changes are less likely since they would alter the response to many taste stimuli indiscriminately.

2.5 Caterpillars are excellent gustatory models; feeding behavior is mediated by only eight gustatory neurons located on each side of the mouth.

A relatively simple set of gustatory neurons regulating relatively complex feeding behaviors made caterpillars a model insect system for studying taste and led to a wealth of information regarding their taste coding, comprehensively reviewed in Schoonhoven and van Loon (2002). Approximately 16 olfactory neurons on each antenna mediate short range attraction to host volatiles (Figure 2)(Schoonhoven 1987). Approximately 12 olfactory and 15-30 gustatory neurons on the maxillary lobe perceive host chemicals that mediate test biting (Figure 2)(Schoonhoven 1968). However, eight gustatory neurons in two uniporous styloconic taste sensilla on each side of the mouth regulate the acceptance or rejection of food. Data compiled from more then 20 different caterpillar species indicates that individual neurons are tuned to one of four main taste classes; sugars, bitters, inositol and salts (Schoonhoven and van Loon 2002). Furthermore, this simple gustatory system has evolved to decipher complex mixtures of bitter compounds produce by plants to deter herbivore feeding. By comparison, fruit fly larvae are not as amenable to electrophysiology and surprisingly little is known about bitter taste perception in either larvae or adults (Amrein and Thorne 2005). Amrein and Thorne (2005) question whether bitter perception is important to insects that feed primarily on yeast and fruit. Therefore, silkworm caterpillars, an emerging model species, are particularly suited to evaluating plasticity of Gr gene expression in response to feeding experience.

2.6 The silkworm Bombyx mori is an emerging molecular genetic model species

The silkworm *Bombyx mori* (Order Lepidoptera; Family Bombycidae) was domesticated in China approximately 4500 years ago from its wild ancestor *Bombyx mandarina* (Hamamura 2001). Due to the economic value of the global silk industry considerable research has been conducted on the physiology of *B. mori*. Its genome was sequenced in 2004 (Mita et al., 2004; Xia et al., 2004) and it is rapidly becoming a model species for basic research (reviewed by Goldsmith et al., 2005). More then 400 mutant strains have been described and maintained in stock centers in China and Japan, *B. mori* can form fertile hybrids with its wild ancestor *B. mandarina* allowing the introgression of visible mutations, genetic and molecular linkage maps are available and successful germline transformation using transposon-based vectors are a few examples of the molecular genetic tools that are available (Goldsmith et al., 2005). Interestingly, silkworm insects have become readily available for purchase in North America due to their use as feeder insects in the pet food industry (Mulberry farms for example, www.mulberryfarms.com/).

The feeding behavior of silkworm caterpillars was studied extensively in the Japan during the period from 1935-1970 in order to develop less expensive artificial diets. Much of this research was conducted by Yasuji Hamamura and is nicely reviewed in a book entitled “Silkworm Rearing on Artificial Diet (Translated from Japanese)” edited by Yasuji Hamamura. Hamamura and others identified many chemicals that attracted the larvae (primarily citral, linalool, linalyl acetate and terpinyl acetate) and that stimulated feeding (primarily B-sitosterol, isoquercitrin and morin). Subsequently, the electrophysiological response of the gustatory neurons to these stimulants was determined as well as to several bitter compounds.

(3) Research Design and Methods

Hypothesis: Feeding experience alters the composition and/or levels of gustatory receptor gene expression in the peripheral taste sensilla resulting in altered food preference or acceptance.
The following three objectives will determine: i) Whether silkworm Gr gene expression supports labeled-line coding of sweet and bitter taste in caterpillars and ii) Whether experience-dependent feeding preferences can result from alterations to Gr gene expression in peripheral gustatory neurons*Objective 1) Characterize Gr gene expression in gustatory neurons located in the medial and lateral taste (styloconic) sensilla of the silkworm Bombyx mori*.

Bitter sensitive and sugar sensitive gustatory neurons are found together in both the medial and lateral taste sensilla of most caperpillar species. Significantly, the silkworm is an exception. The medial taste sensilla of silkworms house four gustatory neurons, two tuned to salts, one to water and one to a broad range of bitter stimuli. The lateral taste sensilla also house four gustatory neurons, one sensitive to glucose, one to insositol, one to salts and one to several different sugars (Hamamura 2001; Schoonhoven and van Loon 2002). Therefore, the ***broadly*** tuned bitter sensitive neuron in the medial taste sensilla will be identified by the expression of a ***large*** number of different Gr receptor genes. Conversely, the **narrowly** tuned sugar sensitive neurons in the lateral taste sensilla will be identified by the expression of a **limited** number of Gr genes that should belong to the putative sugar receptor subfamily (Figure 1). Three approaches will be used:
A) Quantitative real-time PCR (qPCR)
The relative method of qPCR as described in Wanner and Robertson (2006) and Wanner et al. (*In Press*) will be used to quantify Gr gene expression in the larval antennae, maxillary palp and galea (Figure 2). The sensory organs will be dissected from 50 newly molted 5th instar larvae and the total RNA isolated using Trizol (Invitrogen) reagent with the addition of linear polyacrylamide to assist precipitation. Total RNA will be quantified by absorption at a wavelength of 260 nm and its quality assessed on a 1% agarose gel. Genomic DNA is digested using DNAseI (DNA-Free kit, Ambion) prior to 1st strand cDNA using a SuperScript™ III First-Strand Synthesis System for RT-PCR kit (Invitrogen) and an oligo dT_{18} primer. ABI Primer Express 2.0 software (Applied Biosystems) is used to design primers optimized for qPCR that are close to the 3' end of the gene, and that span introns where possible. qPCR is performed using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) and SYBR Green dye (SYBR Green PCR Master Mix, Applied Biosystems). The efficiency of each primer set is first validated by constructing a standard curve. No-template and no-reverse transcriptase controls will be used to exclude artifacts, and amplicon purity will be assessed using dissociation curves and by analyzing the products on a 2% agarose gel. All reactions are performed in triplicate and Gr gene expression levels are calculated relative to control genes such as *B. mori* ribosomal protein S3 using the $2^{-?CT}$ formula (Livak and Schmittgen, 2001). At least three biological replications are included and statistical differences between tissues are tested using nested Analysis of Variance (ANOVA) and the SPSS for Windows Release 11.0.01 statistical package (SPSS inc., Chicago, IL). Technical replicates (n = 3 replicated wells on each qPCR plate) are nested within each biological replicate.
B) Laser capture microdissection of gustatory neurons coupled with quantitative real-time PCR.
Precise cells can now be routinely captured using laser capture microdissection (LCM), their RNA extracted, and gene expression profiled by quantitative real-time PCR (Pinzani et al. 2006). LCM can be performed on fresh, frozen and fixed tissue, but snap-frozen material typically yields better quality RNA. Total RNA can be extracted from the dissected tissue using Trizol reagent (Invitrogen) with a carrier to improve the efficiency of precipitation. Alternatively, column based products such as the RNA micro kit (Qiagen) are specifically designed for micro dissection and they incorporate features such as on-column digestion of genomic DNA, carrier to facilitate RNA recovery and low volume elution. Following RNA isolation, standard qPCR procedures such as those outlined in the previous section are employed.

The new Institute for Genomic Biology (http://www.igb.uiuc.edu/) on the University of Illinois Urbana-Champaign campus has purchased a new Veritas LCM system (Molecular Devices Corporation) (Personal Communication, Glenn Fried, Director of Core Facilities, Institute for Genomic Biology). Beginning in December of 2006 I will receive training in the use of the Veritas LCM system. In addition, a collaborator (Dr. Jim Frazier, Entomology Department, Pennsylvania State University) has participated in demonstrations that used LCM techniques to capture gustatory neurons from caterpillar mouthparts and is interested in collaborating to further establish these methods. Individual gustatory neurons will be dissected from the medial and lateral taste sensilla of 5th instar silkworm caterpillars and Gr gene expression characterized.

C) In situ hybridization.
Visualizing cell specific RNA expression by hybridizing labeled nucleic acid probes to specific genes in sectioned tissues is a standard molecular technique. However, detecting insect chemoreceptor genes using in situ hybridization has proved difficult due to the low levels of receptors transcript expression and the hard insect exoskeleton that makes high quality tissue sections difficult. Fortunately, methodology has been developed in our department by Rodrigo Velarde. The in situ hybridization methodology described in Velarde et al. (2005) is routine, but it is the specific experience with insect tissue and chemoreceptor genes that is indispensable. This has included in situ localization of a putative pheromone Or receptor in moth antennae (Patch, Verlarde and Robertson, unpublished data). Briefly, digoxigenin-labeled RNA probes for in situ hybridization are prepared for the gene of interest by transcription with T3 or T7 RNA polymerase from a PCR product or plasmid vector using Roche RNA Labeling Mix. Dissected tissues are transferred to Bright Cryo-M-Bed embedding compound, frozen onto cryostat chucks using powdered dry ice, sectioned at 10 µm, and thaw-mounted onto FisherPlus slides. After overnight air-drying, sections are fixed in 4% paraformaldehyde, deproteinized with proteinase K, and treated with acetic anhydride prior to hybridization with the probe (1000 ng/ml) at 50 °C overnight in 50% formamide. Following posthybridization rinses, sections are incubated with a sheep anti-digoxigenin-alkaline phosphatase antibody (Roche), treated with levamisole to block endogenous alkaline phosphatase activity, and developed in NBT/BCIP (Vector Laboratories). Developed slides are coverslipped with CrystalMount (Biomeda) or glycerol. Sense strand probes are used as controls. Alternatively, RNA probes can be fluorescently labeled allowing for two-color in situ hybridization. Dr. Larry Zwiebel who collaborates with our lab is currently using two-color in situ hybridization to characterize mosquito Or and Gr gene expression (Larry Zwiebel, personal communication). I will visit Dr. Zwiebel's lab in order to receive further training.

Objective 2) Assay for changes in Gr gene expression between control larvae (non-induced) and treated larvae (induced to prefer an alternate food or accept a previously unfavorable food).
Silkworms develop optimally on white mulberry (*Morus alba*) leaves, their preferred food. Dietary research has focused on improving the growth, yield and quality of silkworm cocoons for silk production (Hamamura 2001). The fact that silkworms are oligophagous and will feed on several species in the plant family Moraceae has received less attention and in some cases is not recognized. Importantly, induced feeding preferences have been demonstrated for oligophagous caterpillars (Schoonhoven __). First, I will determine the innate preference of naïve neonate silkworm larvae for the leaves of host plants found commonly in North America. Since the maternal food source could affect neonate feeding choices through chemical legacy, I will use silkworms from two separate colonies, one reared on white mulberry leaves and one reared on artificial diet ingredients that do not contain mulberry (Hamamura 2001). White mulberry (*M. alba*) has been introduced to North America and its distribution is now widespread (USDA Plants Database, www.plants.usda.gov/index.html). Leaves are available locally from late April through to the end of October. Currently I continuously rear the Nistari strain of silkworm that does not undergo diapause.

Standard leaf disk feeding choice bioassays (Wanner et al., ___ and _____) will be used to rank the feeding preference of neonate silkworm larvae. Briefly, fresh circular leaf disks are cut with a cork borer instrument and placed into a Petri dish with naïve neonate silkworm larvae. After 24 and 48 hours, the amount of feeding leaf material consumed and fecal pellets produced is quantified. Experiments can be carried out in choice and no-choice conditions. Texas mulberry (*M. microphylla*) and red mulberry (*M. rubra*) are native to North America while black mulberry (*M. nigra*) can be found as an introduced species (USDA Plants Database). Silkworms also feed on at least three other plant genera from the family Moraceae that are commonly found in North America. A native species, Osage-Orange (*Maclura pomifera*) is a common landscape hedge, and silkworm thorn (*Cudrania tricuspidata*) is an introduced species that is also used in

landscaping. Paper mulberry (*Broussonetia papyrifera*) also introduced to North America is closely related to the *Morus* genus (USDA Plants Database). Using a forced induction regime, _____ was able to rear silkworm through the fourth instar stage on elm leaves, an entirely unconventional silkworm food source, therefore, I will also attempt to use *Ficus* species are commonly available as house plants and that also belong to the Moraceae family.

Neonate and newly molted 4th instar larvae will be induced to feed on non-preferred food without choice. The leaves of alternative less preferred host species characterized in the section above will be used for silkworms reared on white mulberry. In a second approach, larvae reared on artificial diet will be induced to accept artificial diet amended with specific deterrents (Table __) at thresholds concentrations. The threshold concentrations of various bitter deterrent stimuli can be determined in feeding choice assays as described in the section above using diet plugs in place of leaf disks. After a period of induction (24 hours and/or one complete instar stage) feeding choice assays will be used to test for induced preferences to the new food source as compared to.

iii) Assaying for changes in Gr gene expression after feeding induction.

Larvae that can be induced to prefer alternate foods over their normally preferred diet will be assessed for changes in Gr gene expression. My hypothesis is that positive post ingestive effects will provide feedback that alters the composition and/or abundance of Gr gene expression in the taste sensilla, particularly in the deterrent neuron. Induced larvae will be compared to control larvae that were maintained continuously on the control diet. First, quantitative real-time PCR using whole sensory organs and individual gustatory neurons from laser capture microdissection will be used to screen for overall changes in gene expression levels. Then candidate genes whose expression has changed will further be characterized using in situ hybridization.

Objective 3) Correlate changes in Gr gene expression to changes in taste electrophysiology and feeding behavior.

RNA interference (RNA_i) is commonly used to knock down the expression of specific genes. I will use RNAi to knock down a putative sugar receptor and a putative bitter receptor to demonstrate label-line coding of sweet and bitter taste in caterpillars. I am currently using BmOr2 (Dm83b ortholog that has broad spectrum effects on olfaction) as a control gene to develop RNAi protocol for silkworm Ors. I have cloned a 500 bp fragment of BmOr2 into the vector Litmus 28i (T7 promoter on each side of the MCS) and synthesized dsRNA by transcription with T7 RNA polymerase. The dsRNA is hybridized (heated to 85°C and cooled slowly) and can be directly used for injection or first digested into short inhibiting siRNA using the Dicer RNAi kit (Invitrogen). One key to success is the delivery and translocation of double stranded RNA to the target tissue. In the case of the four beetle *Tribolium castaneum*, female pupae can be injected to achieve a so called “maternal effect” where the dsRNA enters the developing eggs and phenotypic effects manifest during embryo development. In an attempt to knock down Gr gene expression in neonate silkworm larvae I will inject female pupae and recently fertilized eggs. I will also directly inject the taste organs of developing 4th instar larvae and assay for phenotypes after they molt to the final 5th instar. Knockdown efficiency will be quantified by laser capture microdissection and qPCR. The response of sugar and bitter sensitive neurons from control and treated larvae will be assayed using single sensillum electrophysiology. This will be accomplished through collaboration with an insect taste physiologist, Dr. Jim Frazier at Pennsylvania State University. Finally, feeding choice assays will be used to determine the behavioral sensitivity of control and treated animals to sugars and bitter stimuli.