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Identification of taste receptors and proteomic characterization of the antenna and legs of *Tribolium brevicornis*, a stored food product pest

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Abstract

Chemoreception plays an important role in mediating a diverse range of behaviours, including predation and food selection. In the present study, we combined anatomical observations, electrophysiology and proteomics to investigate sensilla that mediate chemoreception on the antenna and the legs of *Tribolium*. Scanning electron microscopy was used to differentiate the coxal and trochanteral segments of the pro-, meso- and metathoracic legs by the presence of sensilla trichoidea and chaetica, while the antennae were covered with five types of sensilla (chaetica, basiconica, trichoidea, squamiformia and coeloconica). Antenna morphology and ultrastructure were similar in both sexes. Electrophysiological recordings allowed us to characterize a row of small sensilla basiconica on the terminal segment of the antenna as taste receptors, responding to sucrose and NaCl. Proteomics investigations of antennae and legs yielded several proteins with specific interest for those involved in chemoreception. Odorant-binding proteins were antenna-specific, while chemosensory proteins were detected in both tissues.

Keywords: *Tribolium*, chemosensory, electrophysiology, proteomic, sensilla.

Introduction

Tribolium beetles belong to the Tenebrionidae family, and include 36 species, eight of which represent the most prominent stored food product pests worldwide (Nakakita, 1982; Angelini & Jockusch, 2008; Angelini *et al*., 2009). Classified as secondary colonizers, these beetles have spread widely as a result of international shipments of infested grain and flour. Although Tenebrionidae can also infest sound, whole grains and cereal seeds, their growth is optimum in flour and other processed cereal products (Aitken, 1975). The largest species, *Tribolium brevicornis* ranges in size from 10 to 12 mm with elongated shapes, and parallel, evenly coloured brown to blackish stripes on the elytra. The antennae end in gradual clubs. The beetle is a recurrent pest of stored products in California, USA, and causes significant economic damage in Idaho, USA as a predator of the immature stages of leaf-cutting bees reared to pollinate alfalfa (Polk, 1977).

One of the most intriguing *Tribolium* beetle behaviours is cannibalism, and a number of studies have investigated this attribute (Alabi *et al*., 2008; 2009, 2011; Alabi, 2010). Cannibalism and predation are widespread among *Tribolium* species, and have been well described (Park *et al*., 1964, 1965; Stevens, 1989; Alabi *et al*., 2008). There is mounting evidence that different *Tribolium* species display varying propensities for cannibalism and predation, suggesting different selective/evolutionary forces operate for these behaviours among species and strains (Sokoloff, 1972; Alabi *et al*., 2008). Some beetles, which are of primary economic importance because of the adverse impact of the species on food storage, exhibit such levels of cannibalism that laboratory stocks would become extinct without removing adults to permit eggs to

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hatch into larvae and the larvae to become pupae. Pupae are subsequently isolated to obtain parents to initiate new laboratory cultures (Sokoloff, 1972). Other species, including *T. brevicornis*, which has recently been recognized as a potential stored products pest, avoid consuming conspecific pupae, and instead devour heterospecifics (Alabi *et al*., 2008, 2011). The evolution of prey discrimination plays an important role in species viability.

In general, predator–prey relationships (e.g. whereby insects locate and select prey, and escape predation) in association with specific environmental factors have been one of several processes leading to the origin of insect species. The environment may also reinforce aversive cannibalistic behaviours. Mertz & Robertson (1970) hypothesized that high rates of egg cannibalism in the laboratory suggest *Tribolium* use a stimulus to locate eggs in flour. Craig (1986) confirmed that chemosensory information might affect egg cannibalism.

Contact chemoreception, whereby sources of the chemical stimulant are in contact with receptors, sends information regarding foods and toxins (Ozaki & Tominaga, 1999). Insects perceive environmental stimuli through their olfactory and chemosensory organs, including antennae, legs and mouthparts. Stimuli detection provides insects with information about food, predator location and mating opportunities. Selection has favoured specific morphological structures in *Tribolium* species because of its distribution in confined spaces with no particular need for rapid locomotion, i.e. the beetle possesses very short legs. The legs are potentially used to make contact with their prey. Antennae are sensory structures that house hearing, olfactory and tactile perception organs. In *Tribolium*, the antennae are crucial for the species to interact with its environment (Snodgrass, 1935; Chapman, 1998). The scape, pedicel and flagellum comprise the three segments of *Tribolium* antennae.

Tribolium species locate and optimize their search for prey disseminated in flour using a combination of sensory cues. In the present work, we concentrated on chemical cues which are detected by olfactory and taste receptors, mostly located on the beetle's antennae mouthparts and legs (Merivee *et al*., 2004). The aims of the present study were: (1) to determine the distribution of antennal and leg sensilla using scanning electron microscopy (SEM); (2) to confirm the gustatory role of a conspicuous group of sensilla on the antenna using electrophysiological methods; and (3) to perform a proteomics analysis of the antennae and legs using two-dimensional (2D) electrophoresis followed by matrix-assisted laser desorption/ionization (MALDI)/mass spectrometry (MS) and liquid chromatography tandem MS (LC-MS/MS) analysis.

The *Tribolium castaneum* genome has been entirely sequenced and *Tribolium* beetles represent important model organisms in insect development, evolution, comparative genomics and pest science. Furthermore, because of its notable economic importance, *T. brevicornis* is an appropriate model to examine the capacity of flour beetles to interact with a diverse chemical environment via chemoreceptive proteins, and several other biochemical functions (metabolic pathways, defence and contractile apparatus, among others).

Results

Anatomy

The legs of *T. brevicornis* are composed of the coxa, the trochanter, the femur, and five tarsi in the fore and mid legs and four tarsi in the hind legs, which are equipped with sensilla unequally distributed. We found sensilla trichodea and chaetica on the coxal and trochanteral segments of each leg (Fig. 1).

The antenna of *T. brevicornis* are composed of a scape, a pedicel and a club-shaped elongated funiculus comprising 11 segments (Fig. 2). The most abundant type of sensilla was sensilla chaetica, which were found both in males and females, and distributed on all antennal

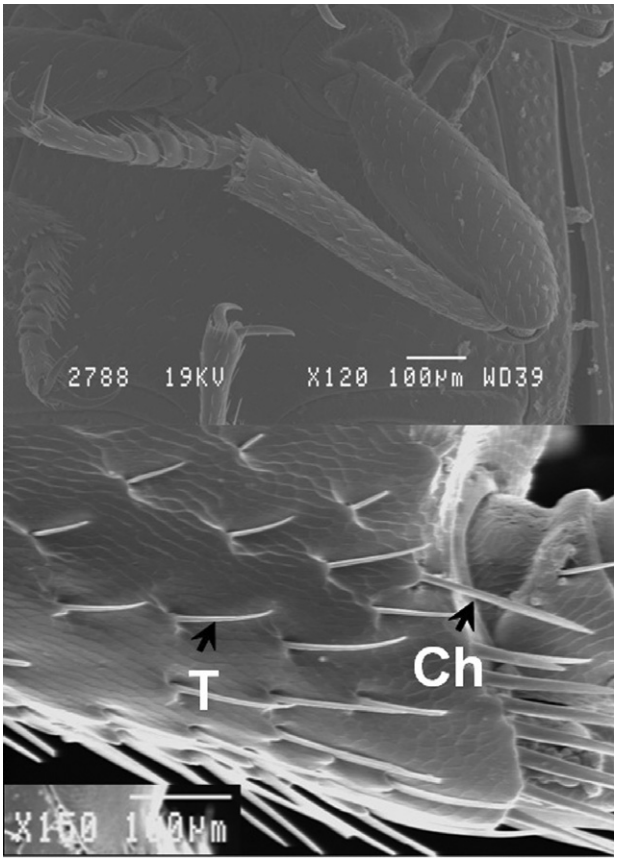

Figure 1. Scanning electron microscopy of ventral and dorsal leg views representative of adult *Tribolium brevicornis* showing distinctive features of sensilla trichoidea (T) and chaetica (Ch).

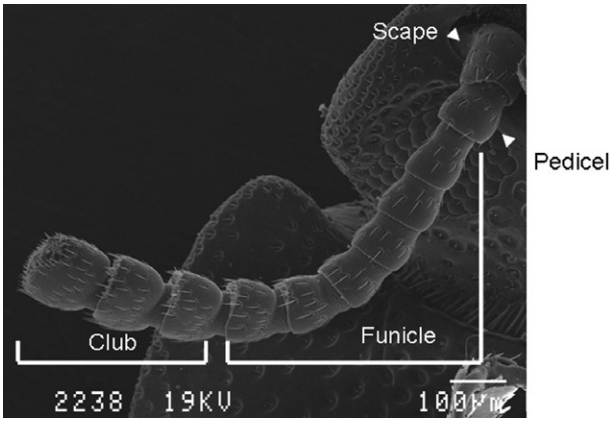

Figure 2. Scanning electron microscopy showing longitudinal view of an adult *Tribolium brevicornis* antenna.

segments, and especially on the club. These sensilla were straight with longitudinal grooves and their cuticle appeared thick and nonporous, suggesting a nonchemosensory function. Sensilla chaetica varied in length from ~18 to 24 μm. The sensilla base was inserted into a socket, which was slightly depressed into the cuticle.

On the club of the antenna of both sexes, we found five additional sensillum types: chaetica (L = 18–24 μm), basiconica (L = 2.30–3.80 μm), trichodea (L = 28–57 μm), squamiformia (L = 14.5 μm), and coeloconica (L = 3.15 μm) (Fig. 3). A circular row of sensilla basiconica was found at the periphery of the club. Given their morphology and their location, we hypothesized that these sensilla had a taste function, notably because these sensilla exhibit a pore at their tip (Fig. 3F). We presume that the other type of sensilla have an olfactory function.

Electrophysiological responses to NaCl and sucrose

Using fine-tipped electrodes containing 0.1 M NaCl, we first probed the antenna and legs under a stereomicroscope, to find hairs with which we could establish an electrical contact. This initial survey allowed us to establish that we could obtain a reliable contact with most sensilla of a crown of sensilla chaetica disposed at the tip of the club of the antenna; however, given the small size of these sensilla, it was technically difficult to stimulate each individual sensilla as the liquid contained in the stimulus electrode would often flow over the antenna by capillarity. A similar situation has been noted on antennal taste hairs of the honeybee (de Brito Sanchez *et al*., 2005).

We monitored the responsiveness of 1–5 sensilla from this crown, chosen because of their proper orientation, from about 10 different insects. These sensilla were stimulated with NaCl and sucrose at four different concentrations, as salts and sugars are tastants generally detected by phytophagous and other insects (Chapman, 2003).

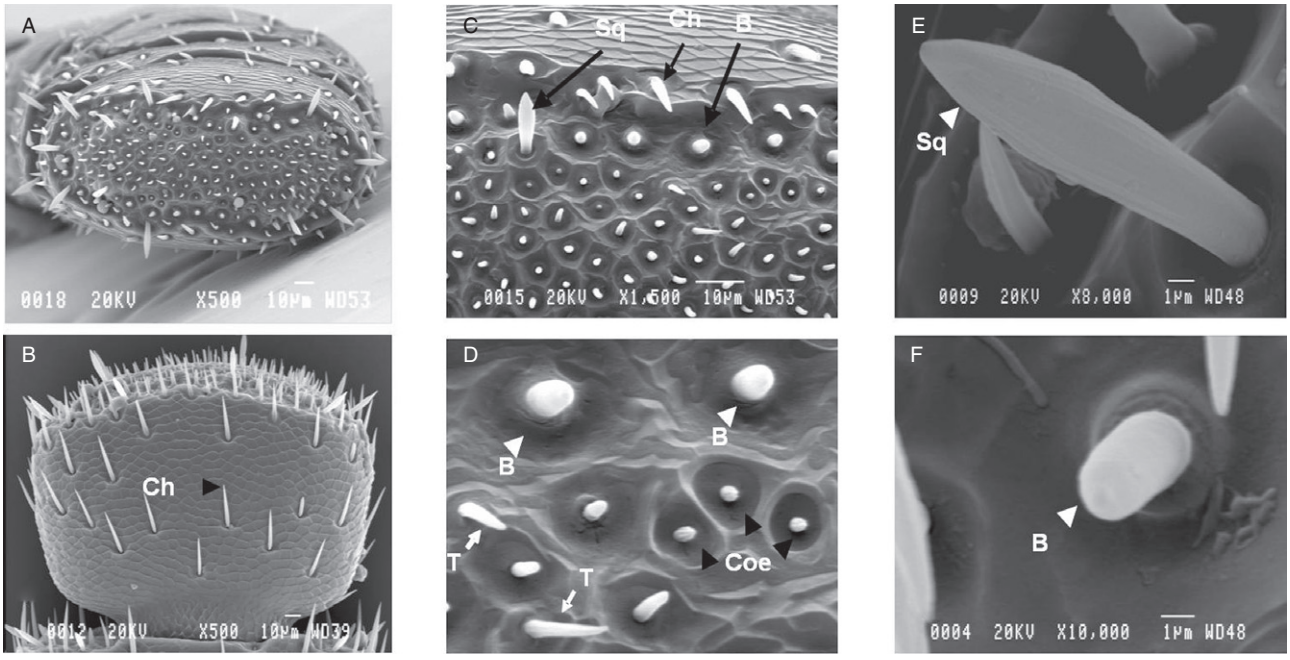

Figure 3. (A) Scanning electron microscopy (SEM) depicting crown of the first club segment; scale bar = 10 μm. (B) SEM providing a ventral view of the first club segment; scale bar = 10 μm. (C) Micrographs of sensilla squamiformium (Sq), trichoideum, basiconicum (B) and chaetica (Ch), scale bar = 10 μm. (D) SEM close-up view of the flagellum crown with sensilla basiconica (B, white arrowheads), sensilla trichoideum (T, white arrows), and sensilla coeloconica (Coe, black arrowheads). (E) Micrograph central view of sensilla squamiformia (Sq), scale bar = 1 μm. (F) SEM representative sensilla basiconicum (B) from which successful tip recordings were obtained from *Tribolium brevicornis*.

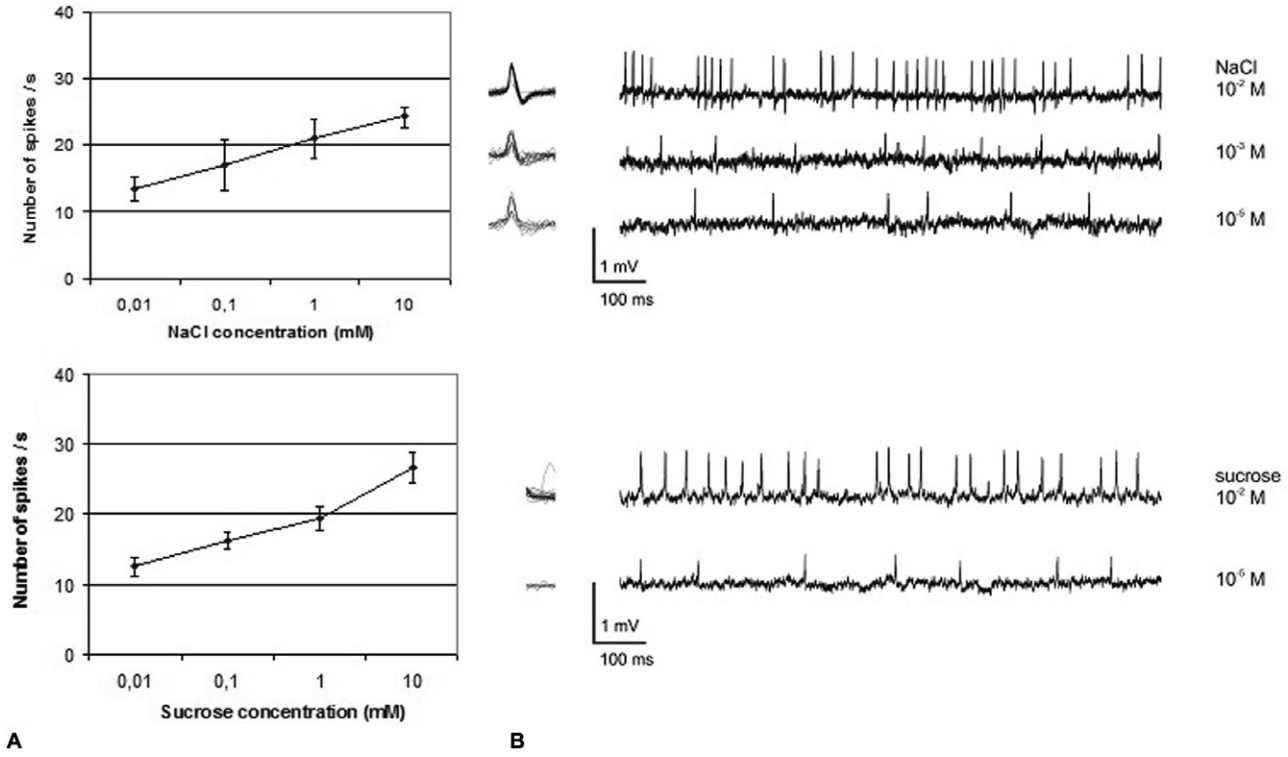

Figure 4. (A) Mean concentration-response curve from salt and sugar-sensitive neurons in the basiconicum sensilla to sodium chloride and sucrose concentrations ranging from 10^{-5} M to 10^{-2} M. The abscissa displays the stimulus solution concentration, and the ordinate the number of spikes occurring in consecutive 50-ms bins. Bars represent the mean SE. (B) s. basiconicum response to 10^{-5} M, 10^{-3} M, and 10^{-2} M NaCl, and 10^{-5} M and 10^{-2} M sucrose solution samples. Trace duration = 2 s; vertical bar = 1 mV; horizontal bar = 100 ms.

NaCl and sucrose activated at least one nerve cell which increased its firing in response to increased concentrations of these tastants (Fig. 4). These observations confirm the hypothesis that these sensilla have a taste function and that they contain several chemosensitive neurons.

General proteomic profiles of Tribolium brevicornis *legs and antennae*

The antennal protein profiles of *T. brevicornis* were well represented in 2D gels (Fig. 5). Proteomics approaches to characterizing the *T. brevicornis* proteome were optimized

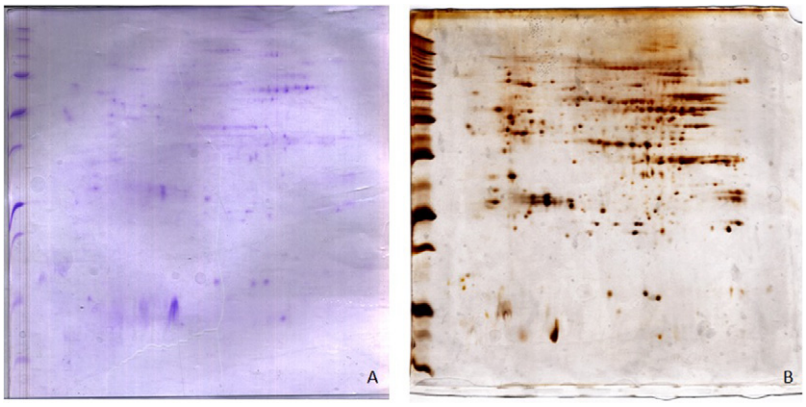

Figure 5. *Tribolium brevicornis* antenna 2DE gels stained with Coomassie (A) and silver (B). Proteins were subjected to isoelectric focusing on pH 3–11 non-linear immobiline dry strips (IPG; GE Healthcare), and subsequently separated with 12% (w/v) polyacrylamide SDS-PAGE. The images are consistent with results from those obtained in three gels from three independent experiments.

by comparing the capacity of two different stains to detect proteins separated by 2D electrophoresis. After 2D electrophoresis, antenna proteins in gels run under identical conditions were detected with either silver stain (Fig. 5) or colloidal Coomassie blue (Fig. 5). More than 100 protein spots were detected in the 2D maps for samples isolated from *T. brevicornis* antenna preparations. Three replicate gels confirmed the reproducibility of the protein patterns in leg and antenna tissues. A total of 125 proteins spots were considered from legs and antennae, ranging in molecular weight from 10 to 250 kD, from 3 to 11 pI, using PROGENESIS image analysis software. These detected protein spots were excised and trypsin-digested, and the derived peptides were analysed as peptide mass fingerprints using MALDI-time-of-flight (TOF)/MS. In addition, LC-MS/MS analysis resulted in sequencing proteins detected with MALDI. Of the total detected proteins, 54 were confidently identified based on multiple peptide hits with the combination of MALDI-TOF/MS and LC-MS/MS (Table 1). Mascot scores >50 suggested protein identity, or extensive homology, at $P < 0.05$. In both tissues, the most acidic protein was troponin C (pI 4.21 and Mw 18795), whereas the most basic was the ribosomal S4 protein (pI 10.57 and Mw 24360). The proportion of alkaline proteins was equal to that of acid proteins. The application of the metazoan subset of National Center for Biotechnology Information (NCBI) databases, including the red flour beetle genome sequence, showed 100% of the proteins identified were from insects, and 91% aligned with *T. castaneum* sequences. Many spots on the 2D gels produced the same database matches, suggesting protein spot redundancies. Proteins involved in chemoreception, energy and nucleic acid metabolism, transport, defence, detoxification, muscle contraction, cytoskeleton organisation, exoskeleton, protein metabolism, signal transduction, and transcription regulation were identified from *T. brevicornis* legs and antennae.

Discussion

Previous studies explored cannibalism and predation in seven *Tribolium* species, and examined the potential function of cuticular hydrocarbons as a chemical deterrent to cannibalism and predation behaviour (Alabi *et al*., 2008, 2009, 2011). Results from our laboratory indicated *Tribolium spp*. cultures significantly differed in their intrinsic propensities for cannibalism and predation. The highest egg and pupae cannibalism and predation rates observed in *Tribolium* suggested that stimuli were used for prey location in flour. Consequently, the first step in studying chemoreception in *Tribolium*, following the recognition that *Tribolium* responds to a chemical attractant in a flour medium, consists of chemosensory organ localization, and characterization of the potential protein receptors involved in chemoreception; therefore, we examined the morphology and sensory functions of adult *T. brevicornis* antennae and legs using a multi-technique approach incorporating SEM, electrophysiology and proteomics. *T. brevicornis* legs were predominantly characterized by sensilla trichoidea and chaetica, while antennae comprised five sensilla types (chaetica, basiconica, trichoidea, squamiformia and coeloconica). We could confirm the taste function of a crown of short sensilla basiconica located at the tip of the antenna. These sensilla house neurons responding in a dose-dependent way to NaCl and to sucrose, but most certainly other stimuli are also excitatory. Similarly to other studies assessing the electrophysiological recordings of the general functional morphology of insects (Ochieng *et al*., 2000; Maher & Thiery, 2003; Pitts & Zwiebel, 2006), our results were consistent in demonstrating that sensilla basiconica on the antennae are involved in hydrophilic compound chemoreception. In addition, the antennae house a number of sensilla which are most likely olfactory. Previous electrophysiological studies provided evidence of antennal responses to olfactory stimuli in *Tribolium* species, including the common aggregation pheromone 4,8-dimethyldecanal (DMD) and 1,6-pentadecadiene (Verheggen *et al*., 2007).

In addition to the electrophysiological observations that we performed on the antenna, we conduced proteomics investigations on the antenna and the legs. Using MS, we identified and clustered proteins into functional groups. Proteins involved in chemoreception, energy and nucleic acid metabolism, transport, defence, detoxification, muscle contraction, cytoskeleton organization, exoskeleton, protein metabolism, signal transduction and transcription regulation were identified from *T. brevicornis* legs and antennae (Fig. 6).

Chemoreception proteins were particularly abundant in antennae, indicating that odorant reception occurs primarily in this structure. These proteins comprised 7% of the total proteins identified and were represented by olfaction proteins, such as odorant-binding proteins (OBPs) established in the antennae, and proteins involved in contact chemosensation (also known as chemosensory or sensory appendage proteins), such as the OS-D-like protein expressed in *T. brevicornis* antennae and legs. These observations indicate that legs are equipped with chemoreceptor sensilla that were missed in our preliminary electrophysiological survey. It must be stressed that the small size of these insects represents a clear limitation using current electrophysiological techniques.

Previous studies described the expression of these proteins in various insect tissues (Angeli *et al*., 1999; Wanner *et al*., 2004; Pelosi *et al*., 2005). The presence of these proteins in chemosensory organs such as antennae and legs can only be elucidated by focusing attention on the

Table 1. List of identified proteins from the legs and antennae of *Tribolium brevicornis* by a combination of MALDI-TOF and liquid chromatography tandem mass spectrometry LC-MS/MS

Protein name	Organism	Accession	Peptides	Mass (D)	pI	Max score	Sequence similarity	E-value	Function
Cuticular protein 66D CG32029-PA	*Tribolium castaneum*	XP_001816349.1	K.ALSNCV	19 642	9.48	78.2	25	0.050	Exoskeleton
Arginine kinase	*T. castaneum*	XP_971800.2	R.AKLEEIAGK.F R.GTRGEHTEAEGGIYDISNK.R	40 325	5.66	607	100	5e-172	Amino acid metabolism, phosphotransferase
ATP synthase subunit β, mitochondrial	*T. castaneum*	NP_001164361.1	R.SHAAKAAK.A R.AIAELGIYPAVDPLDSTSR.I	53 544	5.19	811	100	0.0	ATP formation
Annexin IX CG5730-PC	*T. castaneum*	XP_967931.1	R.TIAQFYENMYGK.S K.WGTEESQFNQILITR.S	36 032	5.21	636	100	1e-180	Calcium binding/ signal transduction
Glyceraldehyde 3-phosphate dehydrogenase	*T. castaneum*	XP_974181.1	K.VIHDNFEIVEGLMTTVHATTATQK.T R.LGKPATYDDIK.A	35 705	8.43	592	100	1e-167	Carbohydrate metabolism
Enolase CG17654-PB	*T. castaneum*	XP_975274.1	R.SGETEDTFIADLVVGLSTGQIK.T K.NIILPVPAFNVINGGSHAGNK.L	47 124	6.48	705	99	0.0	Carbohydrate metabolism
Malate dehydrogenase	*T. castaneum*	XP_969151.1	R.YGQNVLIQFEDFGNHNAFR.Y K.GLAFTLEER.Q	69 372	6.72	1251	100	0.0	Carbohydrate metabolism, pyruvate catabolism
Glycoprotein 93 CG5520-PA	*T. castaneum*	XP_971540.1	K.SEGLDMNDMIGQFGVGFYSAFLVADR.V K.ISALTDLMDVIER.L	57 651	5.72	400	49	3e-109	Chaperone
OS-D like protein, OS-D2b	*Megoura viciae*	CAG25436.1	K.KPCTPEGAELR.K K.DYDAEWKQLLDK.W	12 831	8.86	86.7	100	7e-16	Chemoreception
Cuticular protein Ld-CP3	*T. castaneum*	XP_973909	R.GAYRQPQPGQPIAILR.Q R.QPQPGQPIAILR.Q	7 878	4.94	146	100	6e-34	Chitin sclerotization
*Putative esterase	*T. castaneum*	CAH60165.1	K.DMVMALKWVQTNIK.Y K.VWADIYNSNPLFTQ.-	58 107	7.14	994	100	0.0	Cholinesterase activity
Cytochrome b	*T. castaneum*	NP_203166.1	R.NIIIDLTN	21 337	10.39	63.5	33	2e-09	Respiratory chain complex component
Actin-87E isoform 2	*T. castaneum*	XP_975870.1	R.TTGIVLDSGDGVTHTVPIYEGYALPHAILR.L K.AGFAGDDAPR.A	38 199	5.36	690	100	0.0	Cytoskeleton organization
Actin	*T. castaneum*	XP_001814869	R.IMKEK.L NNASGASNR.S	19 681	5.25	315	100	1e-84	Cytoskeleton organization
†RNA guanine-9-methyltransferase domain containing 2	T. castaneum	XP_972690.1	MSACDDTTRTPPPNPDVIPAK.K K.AAFEMILPK.R	34 701	9.24	567	100	7e-160	Energy metabolism
Sim. G protein-coupled receptor kinase 2	*Apis mellifera*	XP_394109.2	QFHIYNMGGEPGLDIAR.A LGAINSFTSTTWVENRDWTLR.G	28 977	9.91	249	63	3e-64	Epithelial cell migration/ phosphorylation
Fructose-bisphosphate aldolase isoform A	*Nasonis vitripennis*	XP_001601054	K.ADDGTPFPELLK.Q K.KDGCHFAK.W	40 120	7.60	644	100	0.0	Fructose and mannose metabolism
Pyrroline-5-carboxylate dehydrogenase	*T. castaneum*	XP_969408.1	K.TVIQAEIDSAAELIDFFR.L K.YLAGINFTGSVPTFTR.L	63 484	8.67	1117	100	0.0	Glutamate and proline metabolism
Glutamate dehydrogenase	*T. castaneum*	XP_968936.1	K.DIVHSGLDYTMER.S R.IINDESVQESLER.R	62 794	8.26	872	100	0.0	Glutamate catabolism
Succinyl-CoA synthetase β chain	*Aedes aegypti*	XP_001661866.1	R.ICNAVMVAER.K K.VHAILVNIFGGIMR.C	48 733	6.97	639	99	0.0	Ligase activity
cellular FABP-like protein isoform 2	*T. castaneum*	NP_001164131.1	MVDAHLGKKY K.LASSENFDE	15 029	7.79	156	96	2e-37	lipid and carbohydrate metabolisms
Sim. RhoGAP71E CG32149-PA	*T. castaneum*	XP_974494.2	K.LNKEAPYR.K	28 359	9.47	389	84	1e-106	Magnesium ion binding/ metabolism
NADH dehydrogenase (ubiquinone) 1α subcomplex, 13	*Bombyx mori*	NP_001040176.1	R.NRDEEAK.L	18 841	9.30	188	87	2e-46	Mitochondrial electron transport
Heat shock cognate 70, isoform 1	*T. castaneum*	EFA12382.1	K.NQVAMNPNNTIFDAK.R R.IINEPTAAAIAYGLDK.K	71 322	5.33	1128	94	0.0	Molecular chaperone (stress response)
Heat shock 70 kD protein cognate	*T. castaneum*	XP_970569.1	R.VEIIANDQGNR.I R.ITPSYVAFTADGER.L	63 256	5.45	1042	97	0.0	Molecular chaperone (stress response)
Heat shock protein 20.6	*T. castaneum*	XP_973685.1	K.LGDFSVIDTEFSSIR.E K.DGVLTVEAPLPAITAGETLIPIQH.	21 829	5.36	348	100	2e-94	Molecular chaperone (stress response)

Tropomyosin 1 isoform 1	*T. castaneum*	XP_967128.1	K.TLTNAEAEMASLNR.K K.LAFVEDELEVAEDR.V	32 360	4.77	486	100	1e-135	Muscle contraction
Muscle protein 20-like protein	*T. castaneum*	XP_975000.1	K.FPPGELYEDVIR.D R.AGESIIGLQAGQNK.G	20 314	8.91	371	100	2e-101	Muscle contraction
Troponin C type IIIa	*T. castaneum*	XP_970615.1	-.INNNFLKK.K	19 546	4.45	271	85	2e-71	Muscle contraction
Troponin C	*T. castaneum*	XP_973979.1	K.SGSIPCDMVSDILRLMGQPFDK.K R.LEFEEFVTLAAKFIVEEDDEAMQK.E	18 795	4.21	302	92	6e-81	Muscle contraction
*Odorant receptor 153	*T. castaneum*	EEZ99175.1	R.QALIGGAGHTVMLLKALFLTTK.K R.LKPEIDK.A	46 659	8.95	62	43	1e-07	Olfactory system
*Odorant binding protein C13	*T. castaneum*	CM000285.2	R.NHEDVHDPKLDEHGFCILK.K	16 157	6.67	87.8	68	3e-16	Olfactory system
*Odorant binding protein C13	*T. castaneum*	CM000285.2	R.NHEDVHDPKLDEHGFCLLK.T	15 407	5.88	79.7	73	9e-14	Olfactory system
ATP synthase	*B. mori*	NP_001040233.1	R.TGAIVDVPVGDELLGR.V R.EAYPGDVFYLHSR.L	59 612	9.09	938	99	0.0	Oxidative phosphorylation ATP formation
Sim. mCG129107 (ubiquitin)	*T. castaneum*	XP_974406.1	K.GGPISTRR.L	18 730	9.65	219	63	1e-55	Protein metabolism
Splicing factor 45	*T. castaneum*	XP_975149.1	INICLVKM	29 201	6.93	335	68	4e-90	Protein metabolism splicing
Putative nucleoside diphosphate kinase	*T. castaneum*	XP_967503.2	R.VMLGATNPADSASGTIR.G	15 164	8.54	249	86	6e-65	Purine and pyrimidine metabolism
Pyruvate kinase isoform	*T. castaneum*	XP_966698.1	R.LSGIICTIGPASR.D K.MMETGMNIAR.L	58 685	6.84	1013	100	0.0	Pyruvate catabolism
Sim. ribosomal protein S4	*T. castaneum*	XP_969262.1	SQDGARSK.K R.KLGNSSA.	24 360	10.57	48.9	10	4e-04	RNA binding activity
Sim. Mod(mdg4)-heS00531	*T. castaneum*	EFA08224.1	K.DVAHDNMKDILEFMYMGEVNVLR.E	17 919	6.75	209	64	6e-53	Spliceosome assembly pathway
Ribosomal protein S8	*T. castaneum*	XP_967339	R.NPLRK.K	25 710	10.29	316	84	9e-103	Structural constituent of ribosome
*Glutamate receptor, ionotropic kainate 1, 2,3 (glur5, glur6,glur7)	*T. castaneum*	XP_974933	R.INEALLR.L	14 947	5.91	132	85	1e-29	Synaptic transmission regulation
Coiled-coil domain containing 99	*T. castaneum*	XP_966464.1	K.SQGNSLFAEVDDRR.V K.DVPPSASGMMKYFDNMLAMK.N	29 533	6.29	491	100	4e-137	Transcription; structural identity of cells
Vasa RNA helicase	*T. castaneum*	NP_001034520	R.APGTGDER.L R.EAGVHATR.G	23 139	9.94	40.4	27	0.14	Transcription of multiple downstream mRNAs
Sim. CG3655 CG3655-PB	*T. castaneum*	EFA05385.1	R.HETLNNHGSSSLGLSR.Y K.YSGNQGK.I	69 498	8.89	713	80	0.0	Unknown
†Hyp. protein TcasGA2_TC007256	*T. castaneum*	EFA01682	HLAIINECLFIKHGLPGRPLSPPTR.H K.IMTIYCLSFSDCTIL K.NASLEISK.F	26 781	9.86	199	47	3e-49	Unknown
Hyp. protein TcasGA2_TC002082	*T. castaneum*	EFA12376.1	R.KLHLSDNTNVNVNEICENLSR.S	21 317	8.54	373	100	5e-102	Unknown
Hyp. protein TcasGA2_TC008519	*T. castaneum*	EFA02782	K.LNHIAIWSK.N	25 482	9.81	92.4	19	3e-17	Unknown
*Hyp. protein TcasGA2_TC013119	*T. castaneum*	EFA03199.1	K.DDKPVKINK.W	23 188	9.69	245	56	2e-63	Unknown
Hyp. protein TcasGA2_TC000638	*T. castaneum*	EEZ98206.1	R.IGGRMVTSK.S R.SYPMVKATNPLFPLPK.A	16 939	9.13	112	32	1e-23	Unknown
*Hyp. protein TcasGA2_TC007808	*T. castaneum*	EFA02155	R.TGYESSMNHR.I K.MTFVIIGACMGALGLMILTVGCLATGATRH	18 736	10.20	70.5	58	5e-11	Unknown
Hyp. protein TcasGA2_TC030674	*T. castaneum*	EFA02969.1	R.QSLTLQNTTSE	21 545	10.08	51.2	11	6e-05	Unknown
Sim. CG5065-PA	*T. castaneum*	XP_973431.1	K.TVFITGGTGFMGKVLLEK.L	21 985	9.30	165	40	3e-39	Unknown
Sim. CG11876 CG11876-PB	*T. castaneum*	XP_966664.1	K.TNVWVQLMK.Q	21 145	10.36	78.6	20	3e-13	Unknown

*Proteins found in antenna only.
†Proteins found in legs only.
Hyp., hypothetical; Sim., similar to.

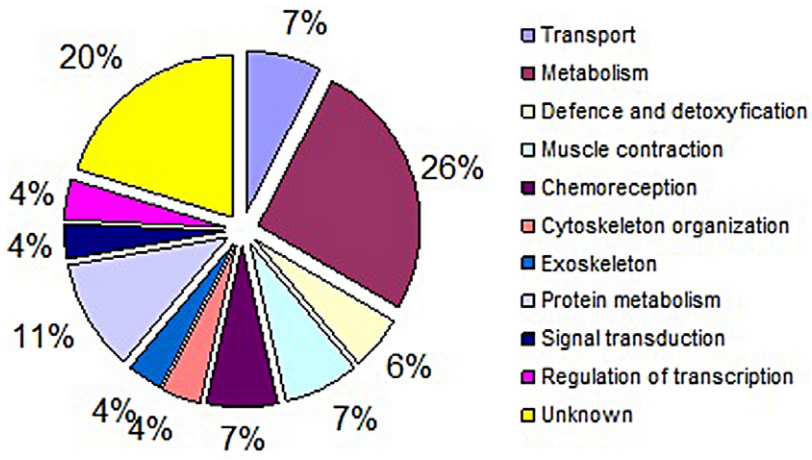

Figure 6. Distribution of proteins identified in *T. brevicornis* legs and antennae based on biochemical functions.

specific organ function. Indeed, OBPs and chemosensory proteins are crucial for odours to deliver a signal to the olfactory receptors, where the signal transduction is initiated, and a specific behaviour is induced in the insect (Kaissling, 2001; Picimbon, 2003). OBPs have also been found to be necessary for contact chemoreception (Galindo & Smith, 2001; Xu *et al*., 2005; del Campo *et al*., 2011).

Chemoreception is important to all insects, particularly in *T. brevicornis;* it may initiate behavioural responses, including the search for prey, feeding, escape, mating and oviposition (Gillott, 1980; Chapman, 2003; Jin *et al*., 2006).

The highest proportion of leg and antennal proteins were related to metabolism, representing 26% of the proteome, including proteins involved in energy production (carbohydrate, lipid, and amino acid metabolism) and sugar metabolism (glycolysis and tricarboxylic acid) pathways. The metabolic proteins identified consisted of included enolase, pyruvate kinase, malate dehydrogenase, succinyl-CoA synthetase, beta chain and fructose 1,6-bisphosphate aldolase, fatty acid binding protein, and glyceraldehyde 3-phosphate dehydrogenase. Results suggested important lipid metabolism roles in *Tribolium* by the presence of fatty acid binding proteins (FABPs) in *T. brevicornis*. FABPs are members of a highly conserved protein family responsible for protecting the delicate lipid balance of cells and are thought to facilitate the transfer of fatty acids between extra- and intracellular membranes.

Based on current knowledge of *Tribolium* beetles, we hypothesize that these three metabolic pathways (carbohydrates, lipids and amino acids) are involved in energy production in the legs and antennae of *T. brevicornis*; however, in-depth kinetic investigations of metabolic enzymes to identify which substrate is preferentially utilized, in addition to studies applying deprivation of these different energy sources, are necessary to test this hypothesis.

The third group of proteins identified were involved in muscle and cytoskeleton activity. Legs represent the primary source of locomotion in *Tribolium* species. The contraction of leg muscles mediates locomotion. In general, insect muscle is very similar to vertebrate striated muscle in function, and is characterized by several proteins that contribute to muscle contraction (Southgate *et al*., 1989). These proteins include muscle protein 20 (mp20), actin, tropomyosin and troponine. Our proteomics study provided evidence of these proteins in *Tribolium*.

In addition to the functional groups already described, the *T. brevicornis* leg and antennae proteome contained protective proteins, including 70 kDa heat shock protein cognate 4 (Hsc 70-4), and small heat shock protein (Hsp) 20.6. In laboratory culture conditions described in previous reports (Arnaud *et al*., 2005; Alabi *et al*., 2008), high *Tribolium* beetle population densities can act as an environmental stressor. Beetles synthesize Hsps in response to stress (Mahroof *et al*., 2005).

The proteomes of both tissues examined in the present study also included proteins involved in transport, particularly the electron and proton transport in various types of metabolism. Proteins associated with adenosine triphosphate (ATP) synthesis-coupled proton transport represented 8% of the total identified proteins (Clark & Baumann, 1997; Kidd *et al*., 2005).

Proteins associated with protein metabolism represented the sixth most important group of proteins we established. This group (11% of total proteins identified) comprised ribosomal protein S4, ribosomal protein S8, mCG129107, splicing factor 45, Mod(mdg4)-heS00531, and Vasa RNA helicase.

In addition to the six functionally dominant groups described, the *T. brevicornis* antennae and legs contained a smaller proportion of proteins representative of other biological processes (Table 1).

In conclusion, the present study examined whether specific proteins involved in chemoreception were expressed

in the antennae and legs of the beetle *T. brevicornis*, a stored food product pest by combining morphological, electrophysiological and proteomics techniques. Results provided details characterizing the ultrastructure of different sensillum types located in antennae and legs, and electrophysiological evidence that sensilla basiconica organized in a crown at the tip of the antenna respond to sucrose and NaCl solutions. In addition, a database of antennal and leg proteins was generated by this study. The results of our investigation make a valuable contribution to the understanding of insect chemoreception, and consequently show great promise in reducing the economic losses caused by *Tribolium*. Also, the multidisciplinary – morphological, electrophysiological and proteomics – approach provided robust data to enhance our knowledge of chemoreception in the *T. brevicornis* beetle.

Leg and antenna examination in *T. brevicornis* served to elucidate important morphological structural elements of the cuticular sensilla of both tissues. In addition to providing an extensive database of antennal and leg proteins in this beetle, our electrophysiological recordings contribute to the knowledge of antennal chemoreception characteristics in *T. brevicornis*, which are relevant to potential involvement in feeding and other behaviours.

Experimental procedures

Animals

Flour beetles, *T. brevicornis*, were reared in Petri dishes (140 mm) maintained in an incubator under complete darkness at a constant temperature (25 °C) and relative humidity (65 ± 5%). The insects were fed a mixture of wheat flour and powdered Brewer's yeast according to Alabi *et al.* (2008).

Protein extraction and two-dimensional gel electrophoresis

Crude protein extracts from 300 legs (100 mg) and 2500 antennae (37 mg) of *T. brevicornis* were separately homogenized using a polytron apparatus (PT 1200) in 20 mM phosphate buffer, pH 6.8, containing protease inhibitors (Roche, Indianapolis, IN, USA), followed by centrifugation at 15 000 × *g* for 10 min at 4 °C. The supernatant was collected, and proteins were subsequently isolated using the 2D Clean-Up Kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's guidelines. Protein concentrations were determined using the 2D Quant Kit from GE Healthcare. Immobiline dry strips (IPG strips, GE Healthcare; pH 3–11, 18 cm) were rehydrated before isoelectric focusing with 180 and 150 g of leg and antenna protein samples, respectively at 25 °C overnight (9 h) at a constant voltage (50 V). Four focusing steps were performed as follows: the initial step at 200 V for 2 h, step 2 at 1000 V for 4 h, step 3 at 10 000 V for 1 h, and the final step at 10 000 V for 3 h 30 min. The maximum current setting was limited to 50 μA per IPG strip. Focused IPG strips were equilibrated in reducing buffer for 15 min (375 mM Tris [pH 8.8], 6 M urea, 20% v/v glycerol, 2% w/v sodium dodecyl sulphate (SDS) and 130 mM DTT), and alkylating buffer for an additional 15 min (135 mM iodoacetamide, 375 mM Tris [pH 8.8], 6 M urea, 20% v/v glycerol, 2% w/v SDS). The equilibrated IPG strips were fixed with 0.5% (w/v) agarose in SDS running buffer overlaid on a 15% acrylamide gel sandwiched between glass plates for the second electrophoresis dimension. Electrophoresis using denaturing conditions was performed using a Bio-Rad PROTEAN II xi 2-D Cell apparatus (Hercules, CA, USA) at 1 W/gel overnight at room temperature (Rabilloud, 1999; Francis *et al.*, 2006).

Protein staining and image analysis

Electrophoresced proteins were detected using Coomassie colloidal blue and silver staining. For Coomassie staining, the 2D gels were fixed in 50% ethanol and 3% phosphoric acid for at least 3 h at room temperature. The gels were then washed three times in distilled water for 20 min before pre-incubation for 1 h in 34% (v/v) methanol, 3% (v/v) phosphoric acid, and 17% (w/v) ammonium sulphate. Coomassie G250 powder (0.36 g/l) was added in an equilibrated solution, and gels were incubated with the stain for 4–5 days at room temperature on a rotary shaker. Finally, the gels were washed in water to remove background stain (Westermeier, 2006).

For silver staining, gels were immediately fixed with 40% methanol (v/v) and 10% acetic acid. Thick, high-percentage polyacrylamide gels were used, therefore gels were fixed overnight at room temperature (Walker, 2005). Following fixation, Milli-Q water (Millipore, Billerica, MA, USA) was used to rehydrate gels and remove methanol through washing. Gels were then incubated in a sensitization solution (0.02% v/v sodium thiosulphate, 6.8% v/v sodium acetate, and 20% v/v methanol). Gels were washed twice (1 min per wash) in Milli-Q water to remove excess sensitization solution. Gels were then incubated in a staining solution (0.1% w/v silver nitrate and 0.04% v/v formaldehyde) for 20 min. Gels were washed twice (1 min per wash), incubated in development solution (0.04% v/v formaldehyde and 2% v/v sodium carbonate) until an acceptable signal-to-noise ratio was achieved, then transferred to stopping solution (5% acetic acid).

After gel scanning and gel image editing, raw images were processed and analysed using PROGENESIS V.3.0 software (GE Healthcare).

In-solution digestion and protein identification

Protein extracts collected from *T. brevicornis* antenna and legs using the 2D Clean Up kit were resuspended in 20 μl of 50 mM NH_4HCO_3, pH 8.0. Cysteines were reduced with 1 μl of a 200 mM DTT solution in 100 mM NH_4HCO_3 for 10 min at 50 °C followed by alkylation for 45 min with 0.8 μl of a 50 mM iodoacetamide solution in 100 mM NH_4HCO_3 at room temperature in the dark. Alkylation was terminated by neutralizing the remaining iodoacetamide via the addition of 4 μl of 200 mM DTT in 100 mM NH_4HCO_3 at room temperature for 45 min. Digestion was performed overnight with 0.1 μg of trypsin in water. The resulting peptides were dried in a vacuum centrifuge twice (Walker, 2005; Harmel *et al.*, 2008).

Peptide separation by reverse-phase liquid chromatography was performed on an Ultimate LC system (LC Packings, Amsterdam, The Netherlands) complete with a Famos autosampler and a Switchos II micro-column switching device for sample clean-up and pre-concentration. Each sample (30 μl) was loaded in dupli-

cate at a flow rate of 200 nL/min on a micro-precolumn cartridge (300 μm, inner diameter [i.d.] x 5 mm, packed with C18 PepMap resin, 5 μm, 10 nm). After 5 min, the precolumn was connected to the separating nano-column (75 μm i.d. x 15 cm, packed with C18 PepMap100, 3 μm, 10 nm), and the gradient flow was initiated. The elution gradient varied from 0 to 30% in buffer B (0.1% formic acid in acetonitrile/water, 20:80 v/v) for 30 min. Buffer A consisted of 0.1% formic acid in acetonitrile/water (2:98 v/v). The LC system outlet was directly connected to the nano-electrospray source of an Esquire HCT ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass data acquisition was performed in the mass range of 50 to 1700 m/z using the standard-enhanced mode (8100 m/z per s). For each scan, a data-dependent scheme selected the four most abundant doubly or triply charged ions to be isolated and fragmented in the trap, and the resulting fragments were mass analysed using the Ultra Scan mode (50–3000 m/z at 26 000 m/z per s).

Data processing and database searching

Data processing and database searching was facilitated by a database containing the whole genome of the related species *T. castaneum*, a widespread stored product pest, which was sequenced by the *Tribolium* genome sequencing consortium. Gene sequences are available from the BCM-HGSC website, https://www.hgsc.bcm.edu/content/genome-data.

Peptide sequences obtained from tryptic-digested excised 2D gel fragments, and identified by the MALDI bio-tool (Bruker-Daltonics) were matched to peptides from the metazoan subset of the NCBI database (a nonredundant database composed of full-length protein sequences, including the translation of nucleotide sequence databases), and the *T. castaneum* genome using Mascot Software (Matrix Science, London, UK; Perkins *et al.*, 1999; Mascot server http://139.165.204.202/mascot/x-cgi/ms-review.exe). Expected fragment ions were calculated for each peptide in the above database, where the mass was similar to that observed. Finally, a score calculated from the observed fragment ions matching the expected number, and the accuracy of the calculation, was generated.

Scanning electron microscopy

Adult *T. brevicornis* isolated from nutritional wheat flour were cleaned with a fine paintbrush, and starved for 24 h before SEM processing. After starvation, insects were dehydrated in subsequent 70% and 90% alcohol baths, for 60 min each. Insects were then fixed on a stub, and sputter-coated with gold three times during 60 s with stub rotation. SEM was performed with a Joel JSM840 electron microscope at 15 kV.

Electrophysiological recordings

Adult *T. brevicornis* beetles were immobilized on a carved micropipette tip fragment using strips of adhesive tape. Beetles were oriented with the ventral side exposed upwards. For testing antenna, they were oriented so that the crown sensilla of the first club article was exposed and directed upward. The preparation was then mounted on a magnetic ball-joint, and oriented under a stereo-microscope (Leica Wild M10, France). Recordings were performed from sensilla located on the antenna, using the tip-recording method (Hodgson *et al.*, 1955). Each recording was obtained by capping a single sensilla for ~2 s under visual control, with a capillary electrode containing the stimulus (mixed with 1 mM KCl to carry the electrical contact). A second electrode (0.8 mm silver wire), connected to the ground, was inserted into the abdomen and closed the circuit. The tip-recording electrode was connected to an amplifier (TasteProbe DTP02, Syntech, Kirchzarten, DE; Marion-Poll & van der Pers, 1996). Borosilicate glass electrodes (O.D. 1 mm) were pulled to a tip diameter ~10 μm (P77 horizontal electrode puller, Sutter Instruments Co., Novato, CA, USA), and filled with a stimulating solution immediately prior to recording. The electrical signals were further amplified (x 500–1000) and filtered (0.1–30 to 2800 Hz band-pass filter) using a programmable amplifier (CyberAmp320; Axon Instruments, Foster City, CA, USA). Data were recorded and stored on a computer with a 16-bit A/D conversion card (DT9803; Data Translation, Malboro, MA, USA) controlled by custom software (dbWave: http://taste.versailles.inra.fr\deterrents\tk\dbwave\; Marion-Poll, 1996). Each recording lasted 2 s, and was activated by a pulse delivered by the amplifier on initial electrode contact with the sensillum.

Each set of recordings was performed by stimulating the gustatory sensilla of adults of both sexes with increasing concentrations of sucrose and NaCl stimuli (from 10^{-5} to 10^{-2} M). NaCl and sucrose purchased from Sigma-Aldrich Corp. (Saint-Quentin Fallavier, France) were dissolved in distilled water (for sucrose with 1 mM KCl), and stored at −20 °C. Stimulation solutions were maintained at 4 °C for less than 1 week. Each stimulus was applied twice, with a time interval of 2 min to avoid fatigue between stimuli. The responses were quantified by the number of spikes elicited during the first second of each recording.

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