1 **Taste receptor plasticity in relation to feeding history in two congeneric species of**
2 **Papilionidae (Lepidoptera)**
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11

12 **Abstract**

13 The spike activity of the maxillary taste chemosensilla in the larvae of two related species of
14 Lepidoptera (*Papilio machaon* L. and *Papilio hospiton* Géné) raised on different host plants, was
15 recorded with electrophysiological techniques after stimulation with simple stimuli (sugars, bitters
16 and inorganic salt) and host plant saps, with the aim of cross-comparing their response patterns and
17 evaluating any effects of different feeding histories. For this purpose the larvae were raised each on
18 their preferential host plant and, in addition, *P. machaon* larvae was also raised on *Ferula*
19 *communis*, the host plant preferred by *P. hospiton*. The GRN spike activity of the lateral and medial
20 sensilla of each test group was measured in response to simple and complex stimuli. The taste
21 discrimination capabilities and modalities of the two species were measured and cross-compared
22 with the aim of studying convergence and/or divergence linked to the insect feeding history. The
23 results show that: a) the GRN responsiveness of both sensilla in *P. machaon* raised on *F. communis*
24 differs significantly from that of *P. machaon* on *F. vulgare*, but is not different from *P. hospiton* on
25 *F. communis*; b) *P. machaon* larvae raised on ferula exhibit response spectra somewhat intermediate
26 between those of *P. machaon* on fennel and of *P. hospiton* on ferula, the latter two exhibiting a
27 wider difference from each other; c) for both species, the coding modality involved in the detection
28 of plant saps, is mostly an "ensemble code" of the across-neuron pattern type. The data support the
29 hypothesis that diet-related factors may influence peripheral chemosensitivity in lepidopterous
30 larvae.

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32 **Key Words:** taste receptor plasticity, sensory coding, discrimination, insect, Papilio

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36 **1. Introduction**

37 In insects, host selection behaviour both in terms of search of food sources and of oviposition sites,
38 is strongly influenced by sensory input arising from their chemical senses (Biolchini et al., 2017;
39 Chapman, 2003; Dethier, 1976; del Campo and Miles, 2003; Masala et al., 2008; Ozaki et al., 2011;
40 Solari et al., 2007; Sollai et al., 2007; Sollai et al., 2010). In particular, the taste sensory system
41 plays a key role in identifying and evaluating the presence, in potential foods, of both nutrients and
42 deterrents that promote and inhibit feeding, respectively (Bernays et al., 2000; Cocco and
43 Glendinning, 2012; Dethier, 1973; Masala et al., 2009). Insects offer several advantages to study the
44 sense of taste: signal transduction is effected by gustatory receptor neurons (GRNs), typically 4 per
45 sensillum, whose axons project directly to the sub-esophageal ganglion; sensilla have an apical pore
46 that allows to record the neural activity arising from each GRN; the spikes recorded by each
47 chemoreceptor differ in shape and amplitude and are species- and sensillum-type specific regardless
48 of recording conditions (Asaoka, 2002; Dethier, 1976; Hodgson et al., 1955; Solari et al., 2010;
49 Tang et al, 2014). In the larvae of lepidopterans most of the electrophysiological studies have been
50 focused on the lateral and medial styloconic sensilla, since they are considered the sensory organs
51 primarily involved in feeding (Dethier and Crnjar, 1982; Martin and Shields, 2012; Schoonhoven,
52 1987; Sollai et al., 2017a). In general, each of them has at least one sugar and one deterrent cell; the
53 specific stimuli for the other cells are species dependent and include inositol, amino acids, water,
54 salt, etc. (for a review see Schoonhoven and van Loon, 2002).

55 Variability in GRN responsiveness depends on larval instar (Panzuto and Albert, 1997, 1998),
56 developmental stage (Simmonds et al., 1991), physiological state (Blaney et al., 1986), time of day
57 (Schoonhoven et al., 1991), experience (Wieczorek, 1976) and feeding history (Schoonhoven,
58 1969). This indicates that taste cells of larvae are not rigid systems, and may even possess a
59 "peripheral memory" (Schoonhoven and van Loon, 2002). Variability in taste sensitivity related to
60 feeding history has been extensively studied in several lepidopterous species, as well as in other
61 insects (Abisgold and Simpson, 1988; Bernays et al., 2004; Blaney et al., 1986; del Campo et al.,

62 2001; Glendinning et al., 1999; Milanovic et al., 2016; Renwick, 2001; Simmonds et al., 1991;
63 Simmonds et al., 1992a, 1992b; Simpson et al., 1991; Zhou et al., 2009). However, comparative
64 studies on sensitivity profiles in phylogenetically related oligophagous species with different ranges
65 of food source acceptance, are not yet available.

66 Aim of this work was to study the peripheral plasticity of the taste sensory system of lepidopterous
67 larvae in relation to the different feeding history of the insect. To this end we used two closely
68 related species of Papilionidae: *Papilio hospiton* Géné, endemic of the islands of Sardinia and
69 Corsica and the Sardinian population of the Holarctic species *Papilio machaon* L. The two species
70 are oligophagous, using various plants in the Apiaceae and Rutaceae families as hosts, and larvae
71 do not feed on plants outside of these two families. In Sardinia, larvae of *P. machaon* are found on
72 several Apiaceae and a few Rutaceae: its preferential plant is *Foeniculum vulgare,* but larvae are
73 often on *Ferula communis* and rarely on *Daucus carota*. Instead, for *P. hospiton, Ferula communis*
74 is an almost exclusive host plant: only if *F. communis* is unavailable two other plants are used, one
75 narrow endemic (*Ferula arrigonii)* and the other rare (*Ruta lamarmorae*) (Bacchetta et al., 2006).
76 This suggests that *P. hospiton* is more specialized in selecting its host plants than *P. machaon* and
77 that different degrees of acceptance of food plants between the two species could reflect differences
78 in the sensitivity profiles of their gustatory receptor neurons (Sollai et al., 2014). The results from
79 that study showed that *P. hospiton* larvae exhibit a greater sensitivity for all classes of tested
80 chemicals (phagostimulants, deterrents and inorganic salt). Therefore, these two lepidopterous
81 species represent a good model for testing the extent of convergence or divergence in the taste
82 response profiles to pure and complex stimuli, in relation to different feeding histories.

83 As a first step, we evaluated whether the responses of the lateral and medial sensilla of *P. machaon*
84 larvae to several chemicals, such as bitter compounds, sugars and salts, as well as the GRN patterns
85 of activity in response to complex stimuli such as plant saps, changed in relation to the rearing diet.
86 Then, both response profiles to single compounds and to complex stimuli were compared with those
87 of *P. hospiton* in order to evaluate the possibility of a convergence linked to the type of host plant

88 fed on by *P. machaon* larvae. We also examined the response profiles to the different host plants
89 with the aim of identifying the neural code underlying food selection behaviour (Blaney, 1975;
90 Blaney et al., 1986; Dethier, 1973; Dethier and Crnjar, 1982; Glendinning et al., 2006; Sollai et al.,
91 2015). We previously found that *P. hospiton* larvae are able to discriminate between different host
92 plants by means of both ensemble and spatio-temporal code (Sollai et al., 2017a). Thence, we
93 extended this analysis, on the neural discrimination code, to *P. machaon* larvae fed on the two host
94 plants and cross-compared the results with those on *P. hospiton*.
95

96 **2. Materials and Methods**

97 **2.1 Insects and rearing**

98 *Papilio hospiton* Géné larvae were obtained from eggs laid on potted giant fennel (*Ferula*
99 *communis* L.) in the butterfly oviposition annex (a 3 x 3 x 3m cage) at the Physiology Laboratories
100 (University of Cagliari) from lab stock adult females. *Papilio machaon* L. larvae were reared from
101 eggs obtained from adult females collected in the spring of 2015-16 in Cagliari, Sardinia.
102 Caterpillars were reared at the insectary annex of our laboratories (University of Cagliari) in 1500-
103 ml plastic cups (4-5 per cup) kept in an environmental growth chamber (24-25 °C, 70% R.H.,
104 16L/8D photoperiodic regime) and checked daily until fit for the experiments. All *P. hospiton* larvae
105 were reared on leaves of *F. communis*, while *P. machaon* ones were divided into two groups: one
106 reared on *F. communis* and the other on *Foeniculum vulgare* Mill. These are the three test groups
107 used in this study and will be hereafter referred to as "hFER", "mFER" and "mFEN", respectively;
108 *P. hospiton* was raised only on *F. communis,* as preliminary attempts to raise it on *F. vulgare* failed.
109 In fact, after hatching, most larvae refused to feed or if they did, they seldom reached the 3rd instar.
110 Fresh foliage of both *F. communis* and *F. vulgare* came from plants grown in a yard adjacent to the
111 butterfly cage and was available ad libitum each day.
112

113 **2.2 Electrophysiological experiments**

114 Spike activity from GRNs was recorded, by means of the "tip-recording" technique (Hodgson et al.,
115 1955), from the tip of the medial (M) and lateral (L) maxillary styloconic sensilla on fifth instar
116 larvae two days after moulting (Simmonds et al., 1991). The reference electrode, a thin Ag/AgCl,
117 was inserted into the head through the "foramen magnum" and pushed into the maxillary-labial
118 complex to fix the maxillae in a prognathous position. The recording electrode, a glass micropipette
119 (tip diameter 20 μm), filled with the stimulating solution, was positioned over the sensillum tip. All
120 signals were recorded with a high input impedance (10^{15} Ω) electrometer (WPI, Duo 773), band-
121 pass filtered (0.1 - 3 KHz), digitized by means of an Axon Digidata 1440A A/D acquisition system
122 (sampling rate 10 KHz) and stored on PC for later analysis (Sollai et al., 2008; Sollai et al., 2012).
123 In the case of pure chemicals, stimuli and concentrations, except inositol, were chosen on the basis
124 of previous results, obtained in our laboratory (Sollai et al, 2014), as the ones for which the two
125 species showed significant response differences.10 mM inositol was instead selected as it is the
126 only stimulus that activates the phagostimulant M1 GRN in the lateral sensillum of *P. machaon*
127 (Sollai et al., 2014). Medial sensilla were tested with aqueous solutions of fructose 250 mM, 10 mM
128 of *myo*-inositol and nicotine, and 500 mM NaCl, while lateral sensilla were tested with nicotine 10
129 mM and glucose 250 mM. All compounds, except NaCl, were dissolved in a 50 mM KCl
130 conducting solution which was also tested as a control.We decided not to show the spike activity of
131 the lateral M2 and S GRNs because no significant differences were previously found between the
132 two species (Sollai et al., 2014). In addition, both sensilla were tested with three complex stimuli
133 represented by leaf freshly-pressed extracts of the following Apiaceous plants: *Ferula communis* L.
134 (giant fennel; hereafter ferula) primary host plant of *P. hospiton* and secondary host plant of *P.*
135 *machaon* in Sardinia, *Foeniculum vulgare* Mill. (fennel) primary host of *P. machaon* in Sardinia and
136 *Daucus carota* L. (wild carrot; hereafter carrot) a rarely used host plant of *P. machaon* in Sardinia.
137 The plant extracts were obtained according to Dethier & Crnjar (1982) and Sollai et al. (2017a), and
138 were tested within 30 s after being pressed. Plant saps were replenished before each stimulation.

139 Stimuli were applied to the sensilla for 2-3 s, in a randomized sequence, and a 3 min interval was
140 allowed between consecutive stimulations to minimize adaptation phenomena. KCl was instead
141 tested at the beginning and the end of each sequence, to assess any shift in chemosensillar
142 responsiveness: whenever significant variations were found, the experiment was discarded. In order
143 to avoid any drift in solution concentration due to evaporation, a clean, dry piece of filter paper was
144 used to draw a small amount of solution from the electrode tip just before each stimulation. After
145 each test, the mouthparts of the insect were rinsed with distilled water and blotted dry. Finally, we
146 recorded only from sensilla of one maxilla for each larva (N=11-15 for chemicals; N=30 for plant
147 saps) and no preparation was used in more than one experiment.
148
149 **2.3 Data analysis**
150 Recordings typically lasted 2-3 s, but spike analysis was performed within the interval 10-1010 ms,
151 the first 10 ms being skipped as containing the contact artifact. The first second of the discharges
152 was chosen as representative of the phasic/phasic-tonic portions of the response (Dethier and
153 Crnjar, 1982; Inoue et al., 2009). Spike sorting and counting were performed by means of the
154 Clampfit 10.0 software, based on earlier studies (Biolchini et al., 2017; Dolzer et al., 2003; Dulcis
155 and Levine, 2005; Pézier et al., 2007; Sollai et al., 2014; Sollai et al., 2017b).
156
157 **2.4 Statistical analysis**
158 One-way ANOVA was used to analyze the relationship between the spike activity of each GRN and
159 the stimulus, in the case of pure chemicals, while two-way ANOVA was used to compare
160 differences, between the three experimental groups, of the spike frequency in the first second of
161 discharges of each GRN ("L", "M1", "M2" and "S") in the lateral and medial sensilla in response to
162 plant saps.
163 Subsequently, we checked if the larvae (individually for each test group) were able to discriminate
164 between the different plant saps. To this end we evaluated the presence of a rate, ensemble,

165 temporal or spatiotemporal code. Main effects ANOVA was used to verify if any two taste stimuli
166 generated a different rate code, i.e. a different number of spikes per time unit (frequency code), for
167 each test group separately. Thus, we counted the total number of spikes generated by each bitter-
168 sensitive GRN in the first second of response and we inferred a difference in rate code, e.g. between
169 ferula and fennel, whenever the main effect on the spike frequency was the taste stimulus. Two-way
170 ANOVA was used to verify if any two taste stimuli produced: a) a different ensemble code, that is a
171 different response pattern across all active GRNs. We inferred a difference in ensemble code (e.g.,
172 between ferula and fennel) if there was a significant interaction of Stimulus × GRN on the spikes
173 frequency generated by each GRN in the first second of response; b) a different temporal code, that
174 is a different distribution of neural activity over time. Time-intensity (T-I) curves (i.e. the number of
175 action potentials in successive 100 ms bins during the first second of activity) were obtained
176 separately for each taste stimulus and GRN. A difference in temporal code was inferred (e.g.,
177 between ferula and fennel), if a significant interaction of Time × Stimulus was found; c) a different
178 spatio-temporal code, according to which stimulus identity is encoded by the time course of the
179 action potential frequency of each GRN activated by the same stimulus. Time-intensity curves (T-I)
180 of each GRN were considered separately for each stimulus, and we determined if the T-I curve
181 produced by a GRN was different from the one produced by the other GRNs. We inferred a
182 difference in spatio-temporal code (e.g., between ferula and fennel), if the curves T-I of a taste
183 stimulus produced a significant interaction of Time × GRN, while those of another stimulus
184 produced a non-significant interaction (Sollai et al., 2015). Finally, two-way ANOVA was used to
185 verify if larvae (separately for each plant) produced different neural codes. We inferred a difference
186 in ensemble and/or temporal code (e.g., between hFER and mFEN), if there was a significant
187 interaction of Test group × GRN or Test group × Time on the spike frequency, respectively.
188 Data were checked for the assumptions of homogeneity of variance and normality. Post-hoc
189 comparisons were conducted with the Tukey test, unless the assumption of homogeneity of variance

was violated, in which case Duncan's test was used. Statistical analyses were performed using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). P values < 0.05 were considered significant.

2.5 Permits

Required permits were obtained for *Papilio hospiton.* Specimens were collected in Sardinia in the spring of 2012, in compliance with the permit issued on 28 May 2012 (Ref. # 0010888) to Roberto Crnjar and his co-workers, by the "Ministero dell'Ambiente e della Protezione del Territorio e del Mare" (Italian Board of Environment and Protection of Land and Sea), in derogation from the provisions set out in the regulation DPR 357/97 concerning the application of the "Council Directive 92/43/EEC of 21 May 1992 on conservation of natural habitats and of wild fauna and flora". No specific permits were required for *Papilio machaon* and all host plants tested, as they are not endangered or protected species.

205 **3. Results**

206 **3.1 Taste sensitivity to pure compounds**

207 Samples of spike activity and the mean values ± s.e.m. of spike frequency of each GRN responding
208 to its best stimulus (L-lat and M2-med GRNs in response to 10 mM nicotine, L-med in response to
209 250 mM fructose, M1-lat and M1-med in response to 10 mM inositol and S-med in response to 500
210 mM NaCl), are shown in figures 1 and 2.

211 We investigated if the spike activity of each GRN depends on the test group, by means of one-way
212 ANOVA. This analysis was used to verify if differences exist in the taste sensitivity of *P. machaon*
213 raised on two different diets (mFER and mFEN) and if the response profile of mFER was similar to
214 that of hFER. One-way ANOVA showed a significant effect of the test group on the spike
215 frequency of each GRN in response to its preferred stimulus, except for the case of M1-lat GRN (L-
216 lat $F_{[2,42]} = 4.0995$, $P = 0.02364$; M1-lat $F_{[2,42]} = 0.01718$, $P = 0.98297$; L-med $F_{[2,31]} = 6.3779$, $P =$
217 0.00479; M1-med $F_{[2,39]} = 5.3165$, $P = 0.00908$; M2-med $F_{[2,40]} = 3.2323$, $P = 0.04998$; S-med $F_{[2,37]}$
218 $= 16.411$, $P = 0.00001$). Post-hoc comparisons showed that the GRN spike frequency of mFEN was
219 lower than mFER in both lateral and medial sensilla. In detail, we found statistically significant
220 differences in the activity of: L-lat and M2-med in response to nicotine (Tukey test $P < 0.05$ and
221 Duncan's test $P < 0.05$, respectively), L-med in response to fructose (Duncan's test $P < 0.05$), M1-
222 med in response to inositol (Tukey test $P < 0.05$) and S-med in response to NaCl (Tukey test
223 $P<0.05$). Post-hoc comparisons also revealed that the taste sensitivity of hFER in response to all
224 chemicals tested was higher than mFEN (Tukey test $P < 0.05$ for L-lat, M1-med and S-med;
225 Duncan's test $P < 0.05$ for L-med and M2-med). Finally, no difference was found in the GRN spike
226 frequency between hFER and mFER ($P > 0.05$). These results confirm that hFER has an higher
227 taste sensitivity than mFEN for all tested stimuli and indicate that mFER has an intermediate
228 sensitivity, although closer to that of *P. hospiton*.

229

230 **3.2 Effect of plant saps on the spike frequency of lateral and medial GRNs**

231 Samples of spike discharges of the GRNs, recorded from the lateral and medial styloconic sensilla
232 of hFER, mFER and mFEN, in response to complex stimuli like leaf extracts of host plants, are
233 shown in figures 3 and 4. The mean values ± s.e.m. of spike frequency of each GRN in response to
234 plant saps tested for each test group are shown in figure 5. In order to assess if the spike activity
235 pattern elicited by each plant sap tested depends on the test group, we tested for a significant
236 interaction of Stimulus × Test group on the neural activity of each GRN, analyzing the spike
237 response evoked in the first second of the discharge for each GRN (“L”, “M1”, “M2” and “S”) in
238 both lateral and medial sensilla, by using two-way ANOVA.
239 For the lateral styloconic sensillum (Fig. 5), two-way ANOVA showed a significant interaction of
240 Stimulus × Test group on the spike frequency of the L GRN ($F_{[4,261]} = 5.1483$, $P = 0.00052$) and
241 post-hoc comparisons showed that the spike frequency of mFEN in response to ferula was lower
242 than those of both hFER and mFER, but only that of hFER in response to fennel ($P < 0.01$; Duncan
243 test). Post-hoc comparisons also revealed a lower response of the M2 GRN of mFEN ($P < 0.01$;
244 Tukey test) in response to fennel than the two other test groups, and a higher response to all plant
245 saps for S GRN ($P < 0.05$; Duncan test). No difference was found in the spike frequency of M1
246 GRN among species ($P > 0.05$).
247 Also for the medial sensillum (Fig. 5), two-way ANOVA revealed a significant interaction of
248 Stimulus × Test group on the spike frequency of the L GRN ($F_{(4,\ 261)} = 11.607$, $P = 0.00000$) and
249 post-hoc comparisons showed that the spike frequency of mFEN in response to ferula and fennel is
250 lower than those of hFER and mFER ($P < 0.05$; Duncan test). Moreover, post-hoc comparisons
251 showed siginificant differences in the responsiveness between mFEN and the other two test groups:
252 a lower spike activity was found for M1 GRN in response to ferula ($P < 0.05$; Duncan test) and for
253 M2 GRN in response to carrot and fennel ($P < 0.05$; Duncan test), but a higher sensitivity in
254 response to ferula for S GRN ($P < 0.05$; Duncan test). Finally, no difference was found in the spike
255 frequency between GRNs of hFER and mFER in both lateral and medial sensilla ($P > 0.05$).

256 These findings indicate that the response profiles of both lateral and medial sensilla in PmFER
257 appear to converge to a common sensitivity pattern with hFER (different species on the same host
258 plant) and to diverge from that of mFEN (same species on different host plants).
259

260 **3.3 Sensory code mediating plant discrimination**

261 In this section we have investigated if hFER, mFER and mFEN can discriminate among different
262 plant saps and which neural code/s may be used (rate, ensemble, temporal and/or spatio-temporal
263 code). The results about a difference in rate code show that taste stimulus is not the main effect on
264 the spike frequency, for both lateral and medial styloconic sensilla, and for all test groups (Tab. 1),
265 thus indicating that the tested plant saps do not generate different frequency codes. To verify a
266 difference in ensemble code, we analyzed the total number of spikes evoked in the first second of
267 the discharge in response to each plan sap, for each GRN and test group separately, by means of
268 two-way ANOVA. A significant interaction of Stimulus × GRN on spike frequency was found in
269 the plant saps comparison for both lateral ($F_{[6,348]} > 2.2793$; $P < 0.05$) and medial sensillum ($F_{[6,348]}$
270 > 4.0857; $P < 0.001$) in all three experimental groups considered (Fig. 6). In detail, the results
271 presented in table 2A, indicate that: in both sensilla of hFER, ferula, carrot and fennel generated
272 different ensemble codes; in both sensilla of mFER, ferula generated a different ensemble code
273 from carrot and fennel, but no difference was found between the latter two; in mFEN ferula
274 generated a different ensemble code from carrot and fennel in medial sensillum, and only from
275 carrot in lateral sensillum. In order to verify a difference in temporal code, we analyzed the T-I
276 curves for each plant sap and evaluated the presence of a significant interaction of Stimulus × Time
277 by using two-way ANOVA, for each test group separately. A non-significant interaction of
278 Stimulus × Time was found in both lateral ($F_{[18,3570]} < 1.0217$; $P > 0.05$) and medial sensillum
279 ($F_{[18,3570]} < 0.8301$; $P > 0.05$) in all three experimental groups considered (Fig. 7). This means that
280 the time courses of spike frequency in response to plant saps do not differ from one another, for
281 each test group considered. Finally, to check for a difference in spatio-temporal code, we analyzed

282 the T-I curves produced by each GRN separately for each taste stimulus and test group. For the
283 lateral sensillum, a significant interaction of Time × GRN was found for all stimuli tested and for all
284 test groups: this result shows that each stimulus evoked non-parallel T-I curves in all GRNs.
285 Instead, for the medial sensillum, the interaction of Time × GRN was not significant for all stimuli
286 tested and for all test groups. These findings indicate that, in hFER, mFER and mFEN, the plant
287 saps do not generate different spatio-temporal codes (Tab. 2B).
288 Once established that larvae were able to discriminate between plant saps by means of an ensemble
289 code, we evaluated if the response pattern across all active GRNs was alike or different among the
290 test groups, separately for each plant sap, by means of two-way ANOVA. A significant interaction
291 of Test group × GRN on spike frequency was found for both lateral ($F_{[6,348]} > 2.6359$; $P < 0.05$) and
292 medial sensillum ($F_{[6,348]} > 2.8909$; $P < 0.01$) (Fig. 8). In detail, the results presented in table 3,
293 indicate that in both sensilla and for each plant sap considered, a difference in the activity pattern
294 across all active GRNs was found when comparing hFER/mFEN and mFER/mFEN, but not
295 between hFER and mFER. Finally, despite the fact that the larvae were not able to discriminate
296 among plants by using a temporal code, we still checked if each plant sap generates different time-
297 courses between test groups, by means of two-way ANOVA. A non-significant interaction of Test
298 group × Time on spike frequency was found for both lateral ($F_{[18.3570]} < 1.0840$; $P > 0.05$) and
299 medial sensillum ($F_{[18,3570]} < 0.75899$; $P < 0.01$) (Fig. 9), indicating that each plant sap does not
300 generate different time-courses of spike discharge among the test groups.
301
302
303

304 **4. Discussion**

305 Previous investigations have shown that food intake and taste sensitivity vary in relation to feeding
306 history in several lepidopterous species, as well as in other insects (Abisgold and Simpson, 1988;
307 Bernays et al., 2004; Blaney et al., 1986; del Campo et al., 2001; Glendinning et al., 1999;
308 Milanovic et al., 2016; Renwick, 2001; Simmonds et al, 1991; Simmonds et al., 1992a, 1992b;
309 Simpson et al., 1991; Zhou et al., 2009). However, little comparative information is available on the
310 neural and temporal coding mechanisms used in taste discrimination of host plants and on their
311 functional plasticity in relation to endogenous or exogenous events, such as feeding history. This
312 bears special relevance when comparing the taste profiles of phylogenetically closely related
313 species, with a different range width of host plants accepted (Schoonhoven and van Loon, 2002).
314 To this end, we compared the taste responses of *P. hospiton* and *P. machaon* fed on their preferred
315 host plants, ferula and fennel respectively with those elicited from *P. machaon* larvae fed on ferula,
316 by evaluating their responsiveness, taste sensitivity profiles and neural discrimination ability. The
317 first aim of the study was to compare the responsiveness to simple compounds of the lateral and
318 medial GRNs between *P. machaon* larvae fed on different plants in order to find shifts related to
319 feeding history. Our electrophysiological results show that responsiveness is statistically different
320 between the two test groups of *P. machaon*: both deterrence and acceptance GRNs show a higher
321 sensitivity in mFER than in mFEN, except in the case of the lateral phagostimalant GRN (M1-lat),
322 for which no difference was found. Several studies have documented that feeding history can induce
323 variations in the taste sensitivity of lepidopterous larvae to simple compounds: increased sensitivity
324 of gustatory neurons is observed when insects come in contact with compounds that act as token
325 stimuli, as in the case of indioside D in *Manduca sexta* (del Campo et al., 2001), or when they are
326 raised on diets that are lacking in token stimuli, such as the case of sucrose in *Spodoptera littoralis*
327 (Simmonds et al., 1991). Conversely, a reduced taste sensitivity has been observed towards
328 compounds to which larvae are continuously exposed (Glendinning et al., 1999; Renwick, 2001;
329 Schoonhoven 1969, 1987; Simmonds and Blaney, 1983; van Loon, 1990; Zhou et al., 2009). We

330 then looked for the presence of any convergence with larvae of a different, although
331 phylogenetically related species, but with the same feeding history. Our findings show that the GRN
332 responsiveness of mFER is not statistically different from hFER, for both sensilla. This supports the
333 idea that feeding history may alter peripheral sensitivity to the point where two different species
334 converge towards a same sensitivity. The fact that no difference between mFER and mFEN was
335 found in the responsiveness of the M1-lat GRN, is justified by the fact no difference was previously
336 found between hFER and mFEN (Sollai et al., 2014). The mechanism underlying these changes in
337 peripheral sensitivity is not known, but it has been suggested that the diet may lead to a variation in
338 the number of receptor sites on the dendritic membrane (Dethier, 1976). Zhou et al. (2009) found
339 that the medial sensillum of *Pieris rapae* larvae raised on artificial diet showed a reduced sensitivity
340 to strychnine when compared to those grown on cabbage, and suggested that this is related to down
341 regulation in the expression of the receptor sites for flavonoids in the medial sensillum.
342 Another aim of the work was to evaluate differences in the response pattern to complex stimuli,
343 such as plant saps, between genetically related species that differ for the host-plants (hFER *vs.*
344 mFEN), and if larval feeding history may modify the peripheral taste sensitivity in the same species
345 (mFER *vs.* mFEN). Our results show that plant saps activate all GRNs in both sensilla of each test
346 group (hFER, mFER and mFEN), although with different intensity. Besides, by cross-comparing
347 the spike activity evoked in each GRN it emerges that the taste sensitivity of mFER tends to be
348 more similar to hFER rather than mFEN.
349 In addition, we investigated if hFER, mFER and mFEN can discriminate among the different plants,
350 and by which neural code. In particular, we tested whether differences exist between *P. machaon*
351 larvae raised on two different host plants or similarities exist with *P. hospiton* when both are fed on
352 the same host plants. We found that all larvae are able to discriminate by means of an ensemble
353 code. However, the discrimination capability is maximal in hFER, which can generate different
354 response patterns across all active GRNs for each plant considered, both in the medial and lateral
355 sensilla; the discrimination ability is minimal in mFEN, which generates different ensemble codes

356 in the ferula/carrot comparison in both sensilla and only in the medial sensillum between ferula and
357 fennel. Finally, the discriminating ability by mFER falls into an intermediate position. These
358 findings suggest the following conclusions: 1) discrimination ability is highest in hFER, that has a
359 narrower range of accepted host plants than mFEN; 2) hFER does not provide a single standard
360 response pattern to non-host plants; in fact, different ensemble codes were generated by stimulation
361 with carrot and fennel, both plants being rejected by hFER; 3) mFEN, while accepting all the plants
362 considered in this study, does not generate the same ensemble code for each of them, as observed in
363 other Papilionid species (Dethier, 1973); 4) feeding history may affect the peripheral taste
364 sensitivity of oligophagous larvae, such as those of *P. machaon*. The possibility that diet
365 modifications could induce plasticity phenomena in the gustatory sensitivity of insects has been
366 already reported. In *Grammia geneura* (Bernays et al., 2004) and *Spodoptera litoralis* (Simmonds
367 et al., 1992b) conditioning on different artificial diet was found to change the taste receptor cell
368 sensitivity.
369 Finally, since each test group uses ensemble codes to discriminate among plants, we sought
370 differences or similarities among the test groups, by comparing the across neuron patterns generated
371 by each plant. We found that the response patterns evoked across all active GRNs in both lateral
372 and medial sensilla, differ in the hFER/mFEN and mFER/mFEN comparisons, while no difference
373 was found between hFER and mFER. This suggests that different feeding histories drive mFER to
374 assume a neural discriminatory profile that appears to converge toward that of hFER, while
375 diverging from the mFEN one. The design of our experiments let us investigate the plasticity issue
376 in a double approach: on the one hand, by exploring the possible convergence of two different
377 species fed on the same host plant and, on the other, the divergence of the same species fed on two
378 different host plants.
379 In conclusion, the analysis of our results raises important considerations about the discriminatory
380 capabilities and on the phenomenon of peripheral plasticity in lepidopterous larvae. Caterpillars do
381 not normally choose their host plants: the choice of a proper host plant is accomplished by their

382 parent adult egg-laying female. In the case of oviposition mistakes (Larsson and Ekbom, 1995), if
383 nutritional conditions are met, the plasticity of the system could help the misplaced larva to bridge
384 the gap toward the chemical profile of a potentially novel host plant with which it is confronted.
385 Feeding history can modify the taste sensitivity of lepidopterous larvae to such an extent that two
386 separate species feeding on the same host plant tend to provide converging response profiles, but
387 diverging ones when a single species feeds on different host plants, both in terms of discrimination
388 capability between stimulus pairs and neural codes used.
389
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394
395 **Conflict of interest**
396 There are no financial and personal relationships with other people or organizations that may lead to
397 a conflict of interest.
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544 **Legends of figures**

545 **Figure 1** - Samples of spike discharges and mean spike frequency values ± s.e.m. of "L" and "M1"
546 GRNs in the lateral sensilla of *P. hospiton* raised on ferula (hFER) and *P. machaon* raised on ferula
547 (mFER) or fennel (mFEN), responding to 10 mM nicotine and inositol, respectively. N = 15 for
548 both stimuli and all test groups. Different letters indicate significant differences among larvae
549 ($p<0.05$; Tukey test).

550

551 **Figure 2** - Samples of spike discharges and mean spike frequency values ± s.e.m. of "L", "M1",
552 "M2" and "S" in the medial sensilla of of *P. hospiton* (hFER), raised on ferula and *P. machaon*
553 (mFEN) raised on fennel and on ferula (mFER), responding to 250 mM fructose, 10 mM nicotine
554 and inositol, 500 mM NaCl, respectively. hFER: N = 12, 13, 14 and 14 for fructose, inositol,
555 nicotine and NaCl, respectively. mFER: N = 11, 14, 14 and 15 for 15 for fructose, inositol, nicotine
556 and NaCl, respectively. mFEN: N = 11, 15, 14 and 11 for fructose, inositol, nicotine and NaCl,
557 respectively. Different letters indicate significant differences among test groups (M1 and S GRN:
558 $p<0.05$, Tukey test; L and M2 GRNs: $p<0.05$; Duncan's test).

559

560 **Figure 3** - Sample traces showing spike frequency of a lateral sensillum of *P. hospiton* (hFER),
561 raised on ferula and *P. machaon* (mFEN) raised on fennel and on ferula (mFER), following
562 stimulation with leaf saps of *F. communis* (ferula), *D. carota* (carrot) and *F. vulgare* (fennel).

563

564 **Figure 4** - Sample traces showing spike frequency of a medial sensillum of of *P. hospiton* (hFER),
565 raised on ferula and *P. machaon* (mFEN) raised on fennel and on ferula (mFER), following
566 stimulation with leaf saps of *F. communis* (ferula), *D. carota* (carrot) and *F. vulgare* (fennel).

567

568 **Figure 5** - Mean values ± s.e.m. of number of spikes evoked in each GRN of the lateral and medial
569 sensillum of of *P. hospiton* (hFER), raised on ferula and *P. machaon* (mFEN) raised on fennel and

570 on ferula (mFER), during the first second of stimulation with leaf sap of *F. communis* (ferula), *D.*
571 *carota* (carrot) and *F. vulgare* (fennel). N=30 for each stimulus and larva. Different letters indicate
572 significant differences within the same GRN among larvae.

573

574 **Figure 6** - Significant interaction of the Stimulus × GRN on the spike frequency of each
575 experimental test group separately, elicited by *F. communis* (ferula), *D. carota* (carrot) and *F.*
576 *vulgare* (fennel). N=30 for each stimulus and larva.

577

578 **Figure 7** - Significant interaction of the Stimulus × Time on the spike frequency of each test group
579 separately, elicited by *F. communis* (ferula), *D. carota* (carrot) and *F. vulgare* (fennel). N=30 for
580 each stimulus and larva.

581

582 **Figure 8** - Significant interaction of the Larva × GRN on the spike frequency elicited by *F.*
583 *communis* (ferula), *D. carota* (carrot) and *F. vulgare* (fennel). N=30 for each stimulus and larva.

584

585 **Figure 9** - Significant interaction of the Larva × Time on the spike frequency elicited by *F.*
586 *communis* (ferula), *D. carota* (carrot) and *F. vulgare* (fennel). N=30 for each stimulus and larva.

587

588 **Table 1** - Neural code used by each larva to discriminate between two plant saps. Rate code
589 analysis: we inferred a difference in rate code, e.g. between ferula and fennel, if the main effect on
590 the total number of spikes generated by each GRN in the first second of response was the taste
591 stimulus rather than the GRN (in red). L=lateral sensillum; M=medial sensillum.

592

593 **Table 2** - Neural code used by each larva to discriminate between two plant saps. (A) Ensemble
594 code analysis: we inferred a difference in ensemble code, e.g. between ferula and fennel, if there

595 was a significant interaction of the Stimulus × GRN on the spike frequency during the first second
596 of stimulation (red typing). (B) Spatio-temporal code analysis: we inferred a difference in spatio-
597 temporal code (e.g., between ferula and fennel), if the Time-Intensity curves of a taste stimulus
598 produced a significant interaction of Time × GRN (in red), while those of a different stimulus
599 produced a non-significant interaction (in black).

600

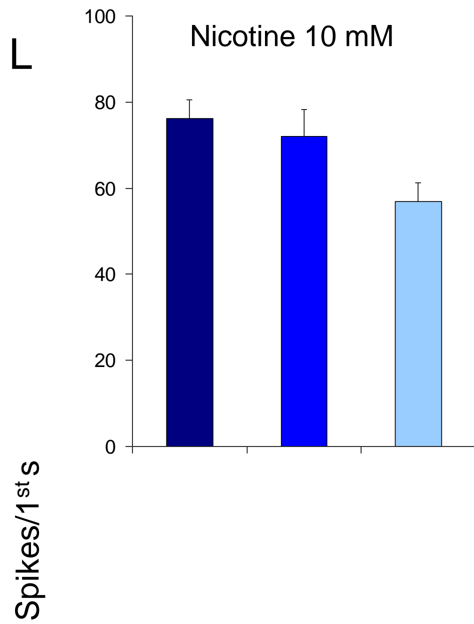
601 **Table 3** - Comparison between ensemble codes generated by each larva in response to the same
602 taste stimulus. We inferred a difference in ensemble code, e.g. between P. hospiton and P. machaon
603 *raised on fennel*, if there was a significant interaction of the Stimulus × GRN on the spike frequency
604 during the first second of stimulation with each plant sap (in red).

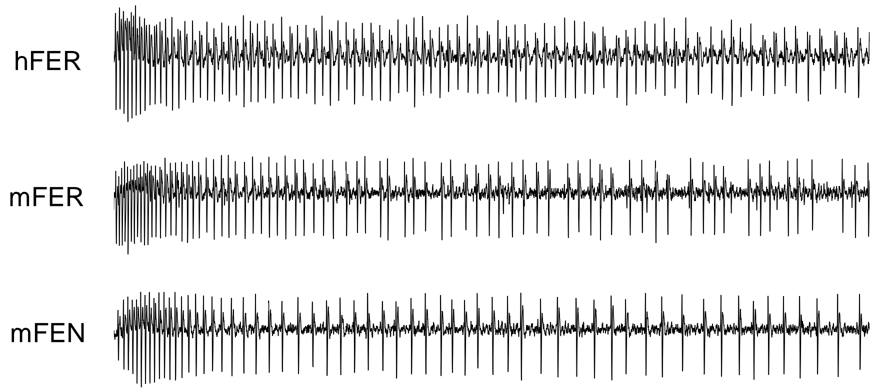
605

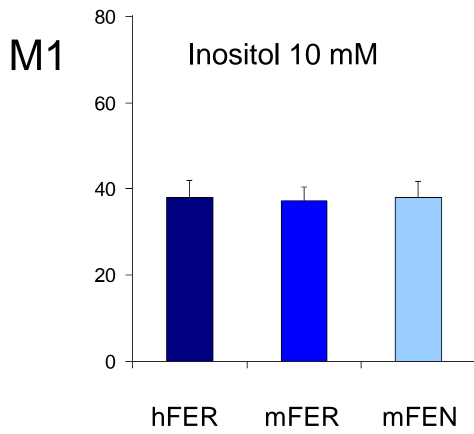
606

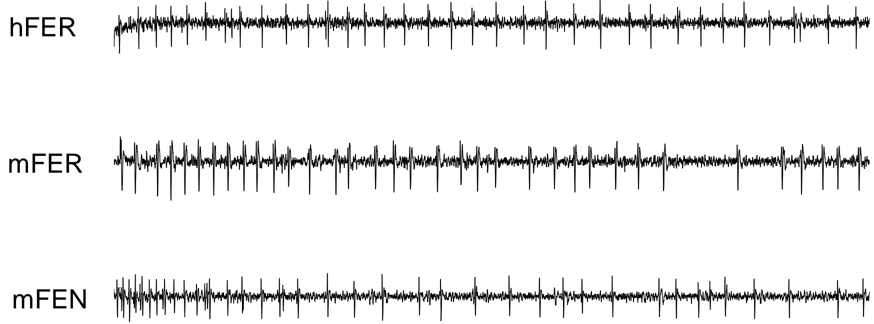
607

Lateral sensillum









5 mV

0.1 s

Figure 1

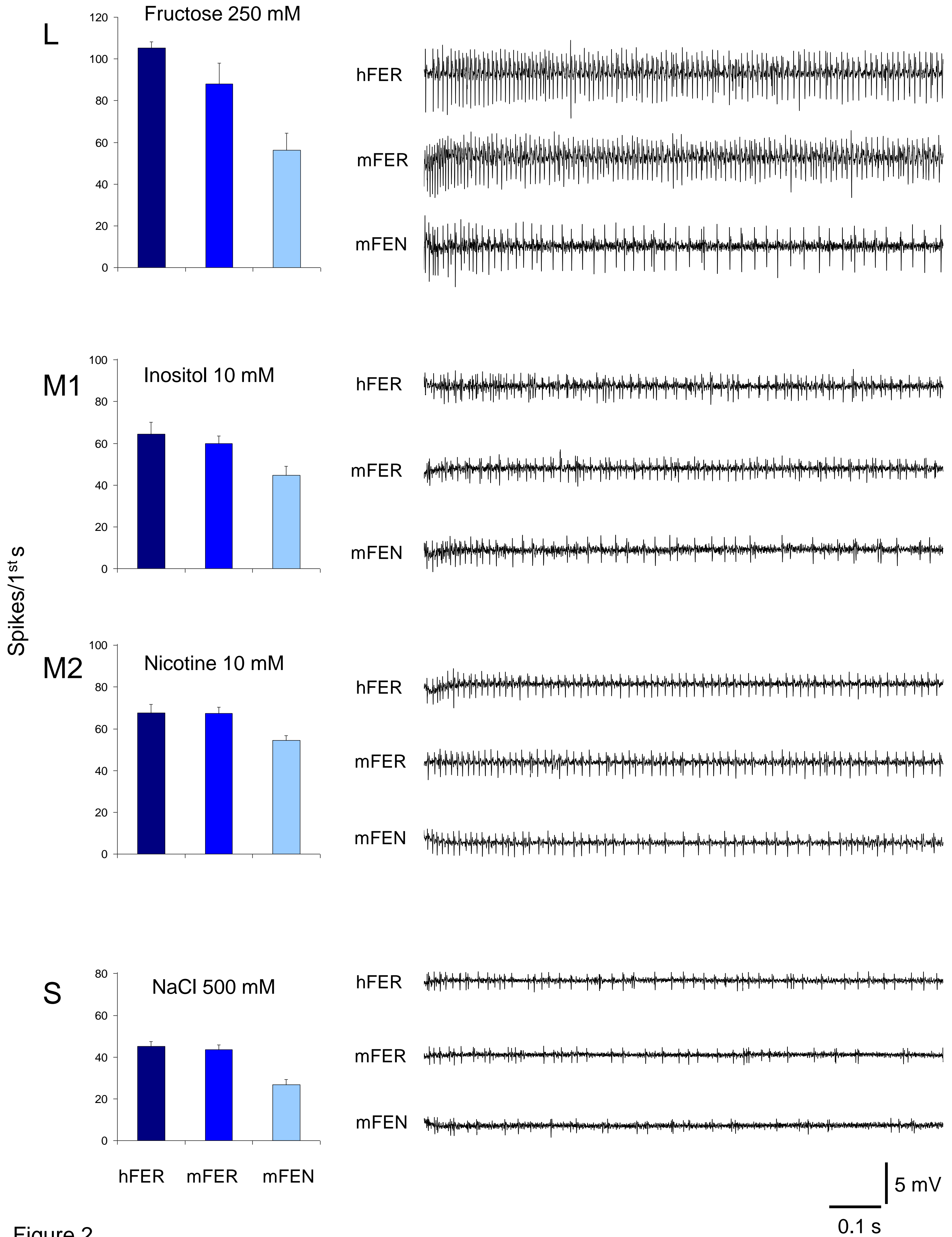
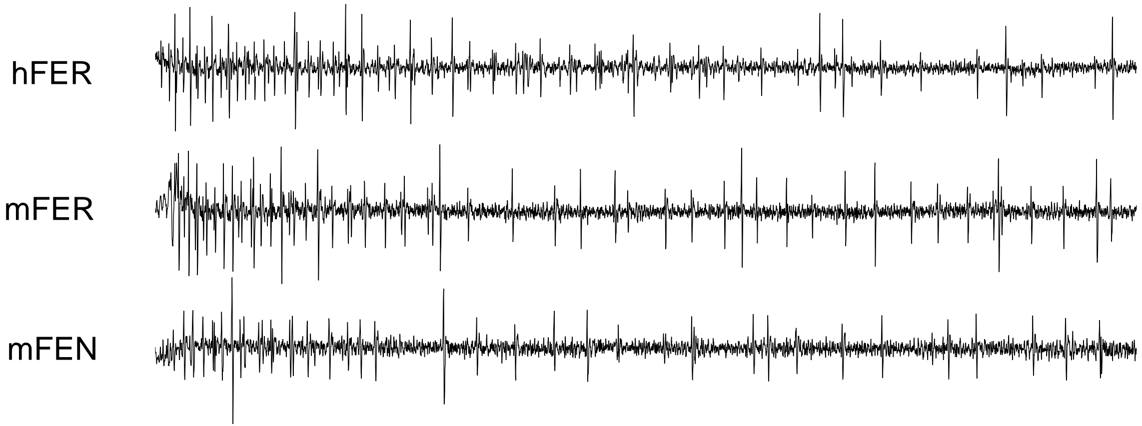
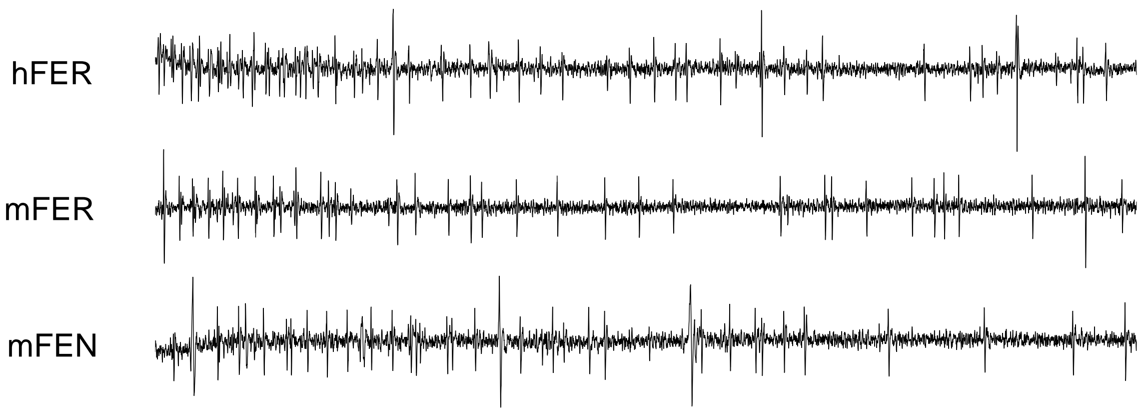


Figure 2

Lateral sensillum





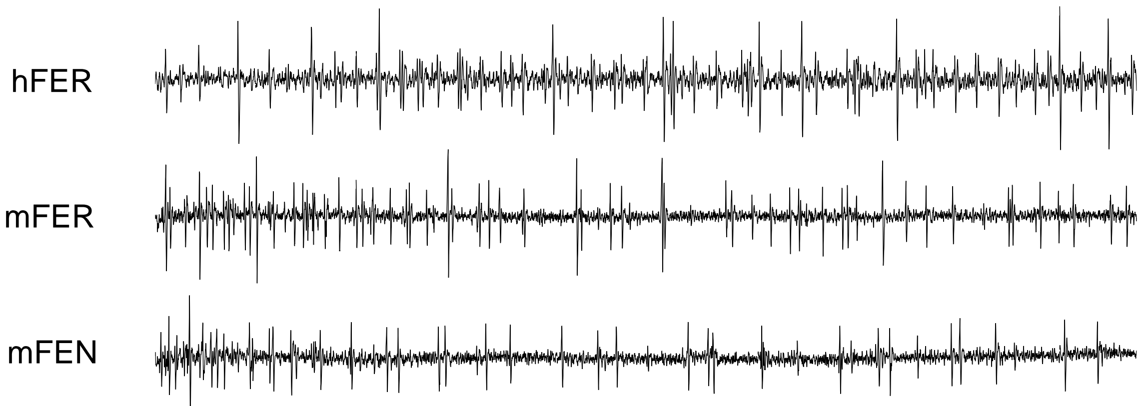
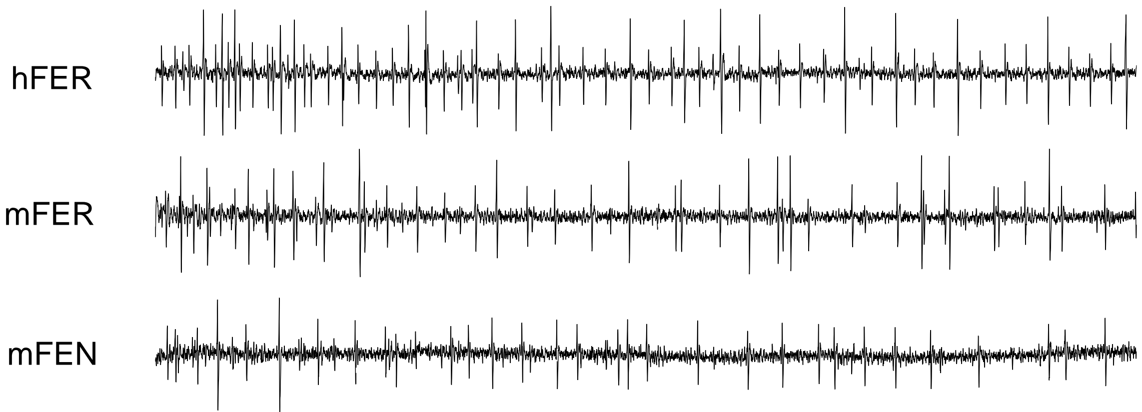
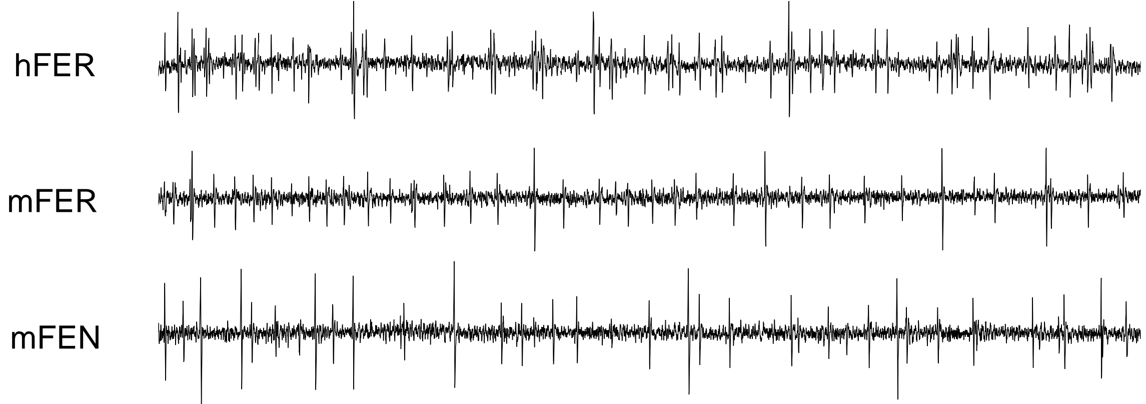


Figure 3

Medial sensillum





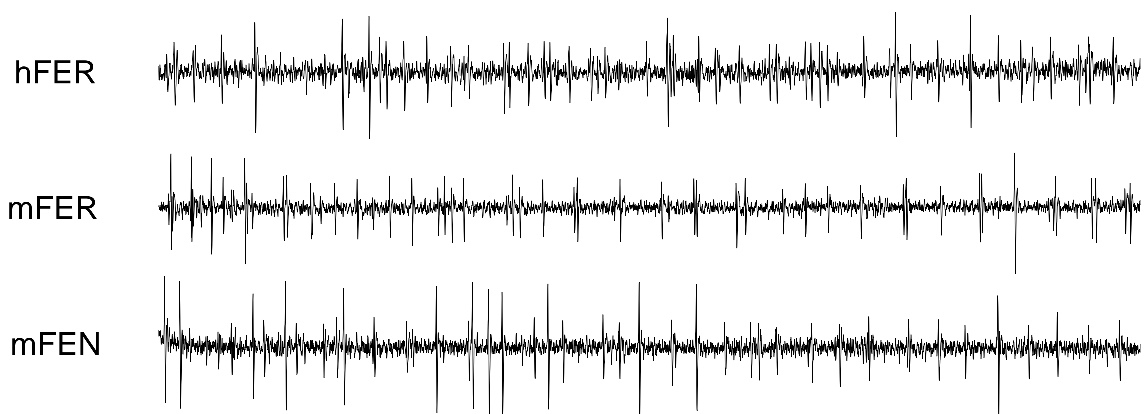


Figure 4

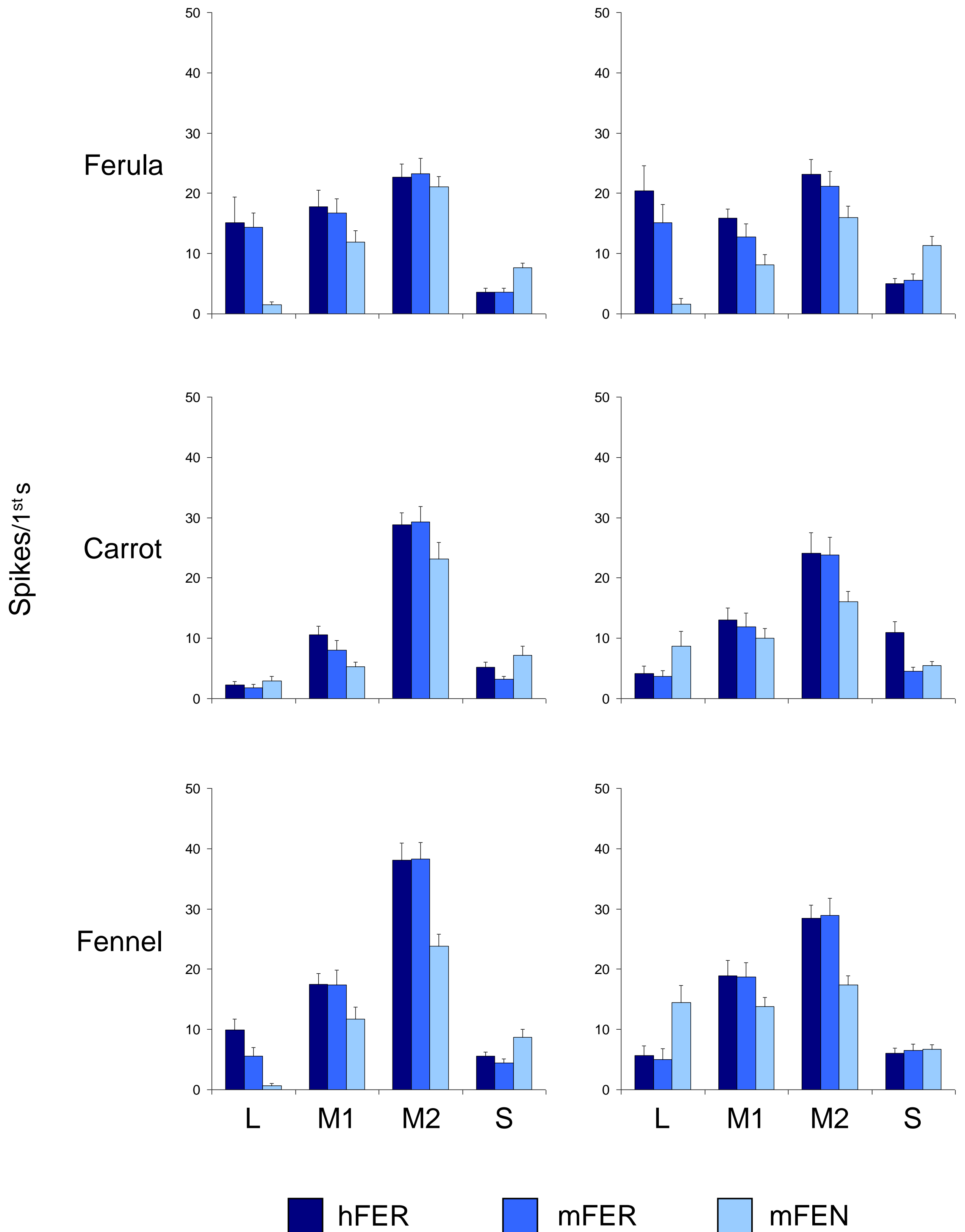


Figure 5

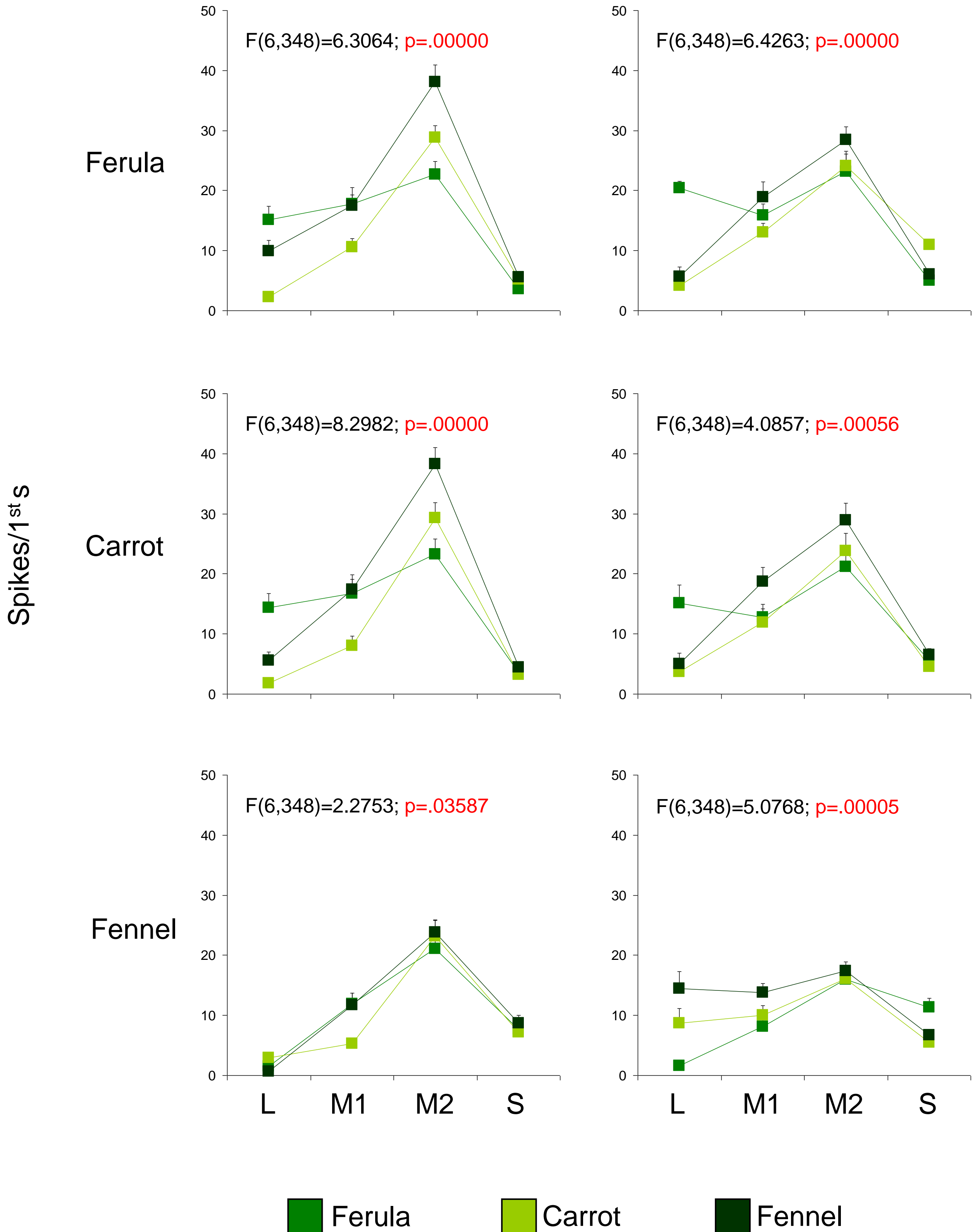
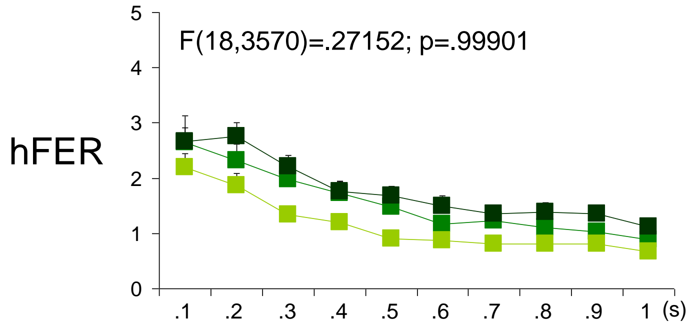


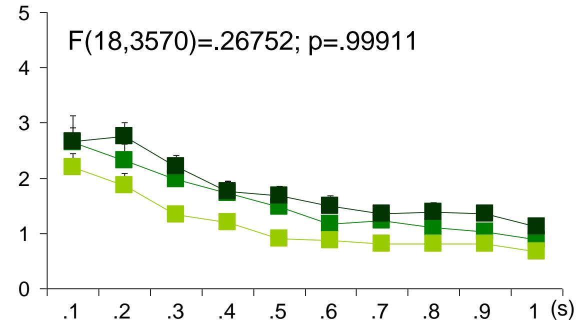
Figure 6

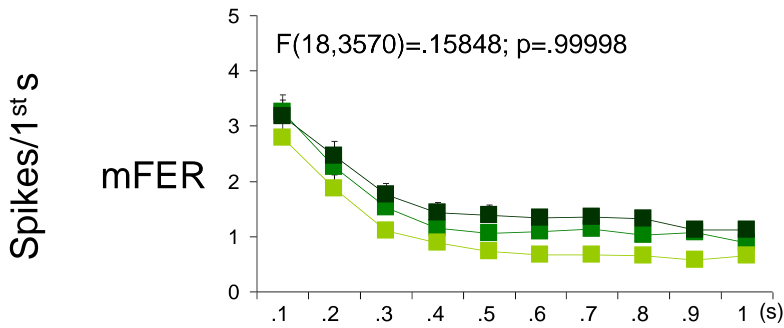
Lateral sensillum

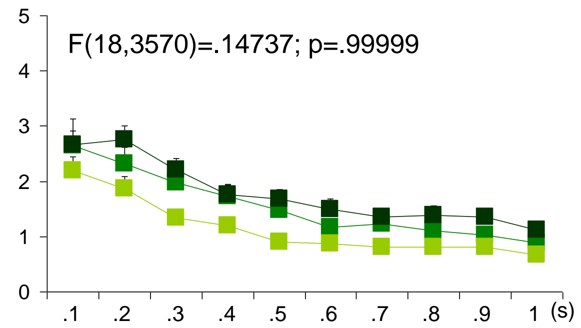
Medial sensillum

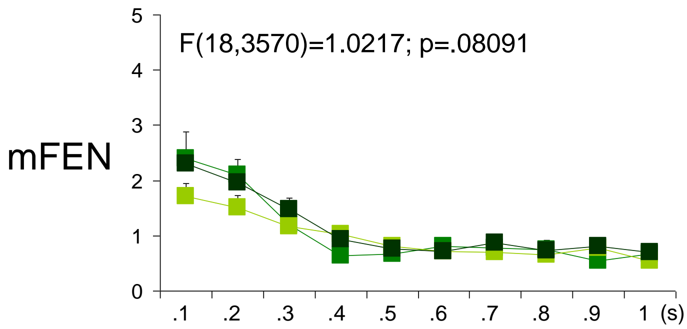
Spikes/1st s

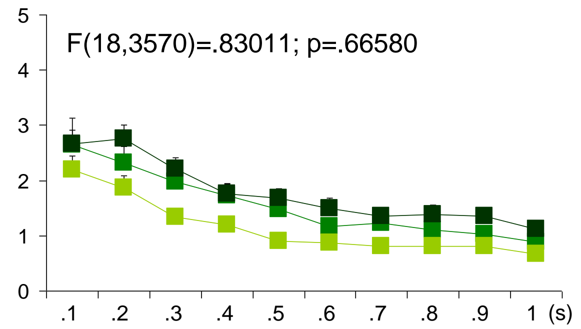












Ferula Carrot Fennel

Figure 7

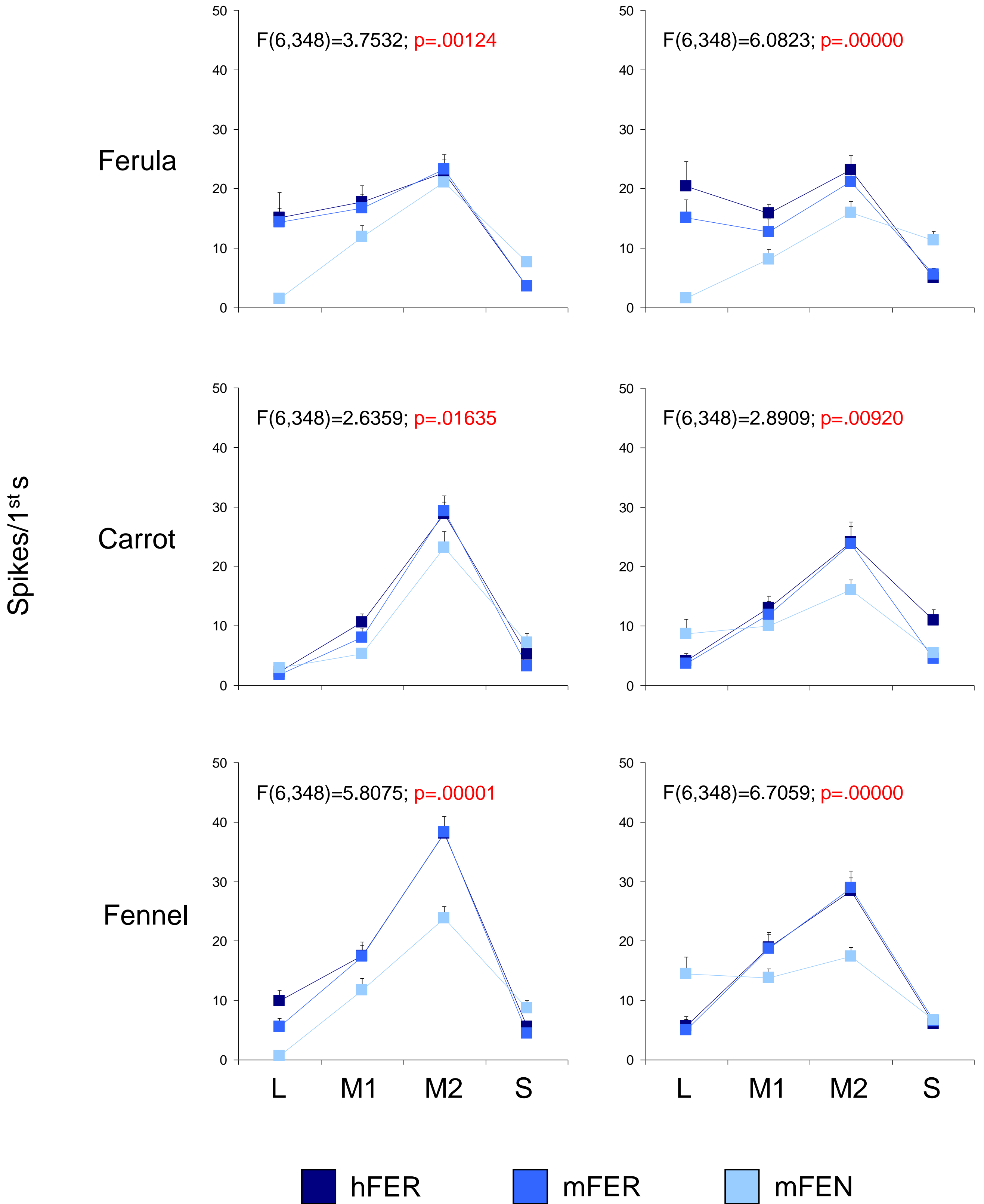
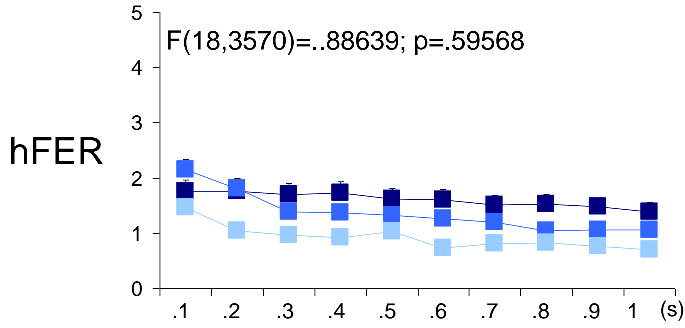
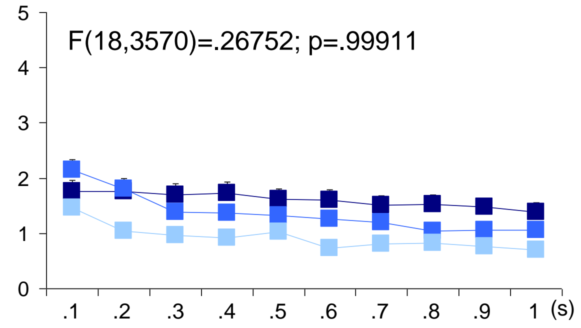


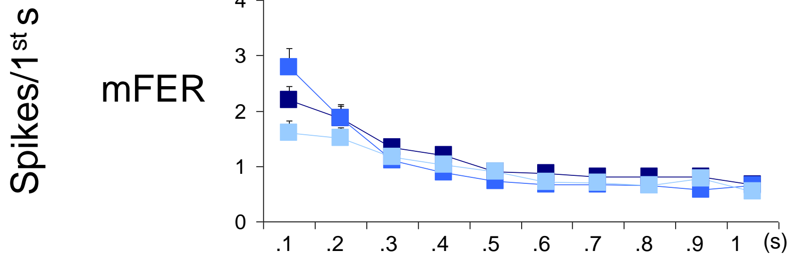
Figure 8

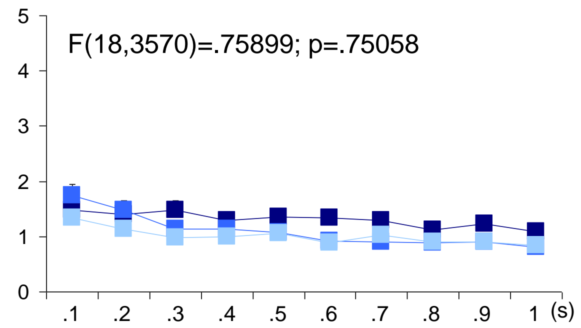
Lateral sensillum

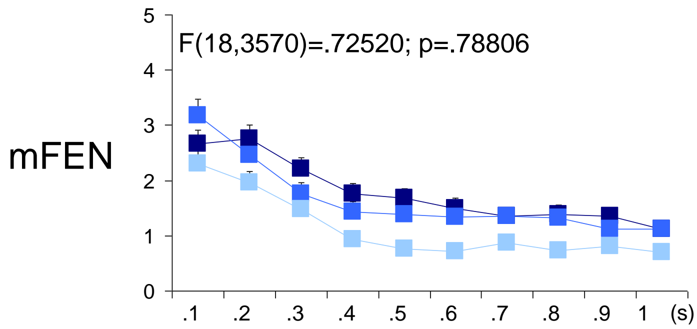
Medial sensillum

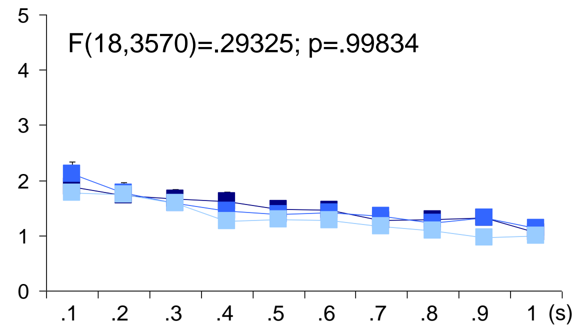












hFER mFER mFEN

Figure 9

Test group	Stimulus pair		Stimulus	Neuron
hFER	Ferula-Carrot	L	F(1,235)=3,6644; p=,05680	F(3,235)=33,061; p=,00001
		M	F(1,235)=2,9316; p=,08818	F(3,235)=13,825; p=,00001
	Ferula-Fennel	L	F(1,235)=2,9105; p=,08933	F(3,235)=38,248; p=,00001
		M	F(1,235)=,63746; p=,42544	F(3,235)=25,646; p=,00001
	Carrot-Fennel	L	F(1,235)=26,555; p=,00001	F(3,235)=123,81; p=,00001
		M	F(1,235)=1,3292; p=,25012	F(3,235)=40,772; p=,00001
mFER	Ferula-Carrot	L	F(1,235)=7,9055; p=,00534	F(3,235)=50,400; p=,00001
		M	F(1,235)=3,1266; p=,07832	F(3,235)=23,786; p=,00000
	Ferula-Fennel	L	F(1,235)=1,5446; p=,21223	F(3,235)=55,013; p=,00001
		M	F(1,235)=,48735; p=,48580	F(3,235)=26,658; p=,00000
	Carrot-Fennel	L	F(1,235)=20,915; p=,00001	F(3,235)=122,48; p=,00000
		M	F(1,235)=7,1832; p=,00788	F(3,235)=52,154; p=,00001
mFEN	Ferula-Carrot	L	F(1,235)=,72843; p=,39426	F(3,235)=61,484; p=,00001
		M	F(1,235)=,49172; p=,48385	F(3,235)=14,765; p=,00000
	Ferula-Fennel	L	F(1,235)=,48608; p=,48637	F(3,235)=78,736; p=,00001
		M	F(1,235)=9,3229; p=,00252	F(3,235)=9,5321; p=,00001
	Carrot-Fennel	L	F(1,235)=2,0114; p=,15745	F(3,235)=64,795; p=,00001
		M	F(1,235)=5,8612; p=,01624	F(3,235)=12,344; p=,00000

Table 1

A

Test group	Stimulus pair	Lateral	Medial
hFER	Ferula-Carrot	F(3,232)=7,6602; p=,00007	F(3,232)=7,7683; p=,00006
hFER	Ferula-Fennel	F(3,232)=6,8500; p=,00019	F(3,232)=8,0511; p=,00004
hFER	Carrot-Fennel	F(3,232)=2,7795; p=,04187	F(3,232)=2,6356; p=,04937
mFER	Ferula-Carrot	F(3,232)=10,076; p=,00000	F(3,232)=4,1649; p=,00674
mFER	Ferula-Fennel	F(3,232)=11,236; p=,00000	F(3,232)=6,7737; p=,00021
mFER	Carrot-Fennel	F(3,232)=2,4821; p=,06168	F(3,232)=,83288; p=,47699
mFEN	Ferula-Carrot	F(3,232)=3,4766; p=,01675	F(3,232)=5,3147; p=,00147
mFEN	Ferula-Fennel	F(3,232)=,59596; p=,61823	F(3,232)=9,5206; p=,00001
mFEN	Carrot-Fennel	F(3,232)=2,5453; p=,05682	F(3,232)=,76824; p=,51285

B

Test group	Stimulus	Lateral	Medial
hFER	F. communis	F(27,1160)=1,8101; p=,00696	F(27,1160)=1,3057; p=,13629
hFER	D. carota	F(27,1160)=4,5487; p=,00000	F(27,1160)=,48593; p=,98800
hFER	F. vulgare	F(27,1160)=2,2108; p=,00037	F(27,1160)=1,0433; p=,40426
mFER	F. communis	F(27,1160)=3,3582; p=,00000	F(27,1160)=,05069; p=,98355
mFER	D. carota	F(27,1160)=7,1908; p=,00000	F(27,1160)=,55829; p=,96746
mFER	F. vulgare	F(27,1160)=2,5200; p=,00003	F(27,1160)=,85138; p=,68464
mFEN	F. communis	F(27,1160)=7,4409; p=,00000	F(27,1160)=,80350; p=,75122
mFEN	D. carota	F(27,1160)=3,2010; p=,00000	F(27,1160)=,55147; p=,97008
mFEN	F. vulgare	F(27,1160)=7,7842; p=,00000	F(27,1160)=1,1781; p=,27274

Table 2

Stimulus	Test group pairs	Lateral	Medial
F. communis	hFER-mFER	F(3,232)=,04284; p=,98817	F(3,232)=,09130; p=,68866
	hFER-mFEN	F(3,232)=5,8501; p=,00072	F(3,232)=11,596; p=,00000
	mFER-mFEN	F(3,232)=7,8570; p=,00005	F(3,232)=8,1819; p=,00003
D. carota	hFER-mFER	F(3,232)=,45088; p=,71692	F(3,232)=,98549; p=,40032
	hFER-mFEN	F(3,232)=3,4147; p=,01818	F(3,232)=3,7378; p=,01187
	mFER-mFEN	F(3,232)=3,6969; p=,01253	F(3,232)=4,3731; p=,00512
F. vulgare	hFER-mFER	F(3,232)=,54967; p=,64885	F(3,232)=,04194; p=,98854
	hFER-mFEN	F(3,232)=8,9661; p=,00001	F(3,232)=10,410; p=,00000
	mFER-mFEN	F(3,232)=9,1828; p=,00001	F(3,232)=10,344; p=,00000

Table 3