Olfactory sensitivity to some sex pheromone components in *Ceratitis capitata* is related to mate and circadian rhythm

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Abstract

The Mediterranean fruit fly, *Ceratitis capitata* Wied., is a worldwide pest for horticulture given its high biological potential, the difficulty of its control and the broad polyphagy, mainly addressed, in Southern Europe, to pomaceous and citrus cultures. Recent studies have both characterized the chemical composition of the sex pheromone and identified 17 unique odor binding protein (OBPs) genes, 5 of which seem to be putative pheromone binding proteins (PBPs). The release of sex pheromone and the expression of these OBP genes appear to be modulated both by mating and time of the day. Based on these considerations, we measured, by electroantennogram (EAG) and electropalpogram (EPG) recordings, the olfactory sensitivity of antennae and palps of *C. capitata* in both sexes, in different physiological states (virgin and mated), and at different times of the day (morning and afternoon) following stimulation with some components of the male sex-pheromone: α-farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate. The results show that the EAG amplitude values in response to all stimuli are higher in the morning than in the afternoon for both sexes and in both virgin and mated insects. Besides, in both sexes, the olfactory sensitivity of virgin insects is higher than in mated ones. The EPG amplitude in response to all stimuli is higher in the morning in mated females than in virgin females and higher in the morning than in the afternoon in both mated sexes. The effect of sex, physiological state and time of day on the olfactory sensitivity of *C. capitata* is discussed.

Key Words: olfaction; *Ceratitis capitata*; virgin; mated; time of day.

1. Introduction

In invertebrates, several complex behaviors such as courtship, aggregation, associative learning, food searching and selection are mediated by information arising from their chemical senses (Biolchini et al., 2017; Dethier, 1976; Lebreton et al., 2017; Masala et al., 2008; Masala et al., 2009; Merivee et al., 2002; Solari et al., 2010; Solari et al., 2015; Solari et al., 2017; Sollai et al., 2017a,c; Sollai et al., 2018a; Thiel and Breithaupt, 2011; Walker et al., 2016; Yarmolinsky et al., 2009; Zhang et al., 2013). Insects are equipped with olfactory sensilla capable of detecting pheromones, kairomones and food odors, as well as repellents and insecticides: they are mainly located in the antennae, maxillary palps and ovipositor (Anton et al., 2003; Galizia and Roessler, 2010; Martin et al., 2013; Sollai et al., 2010). Olfactory sensilla house the olfactory sensory neurons (OSNs), that transduce the information content of volatile stimuli into action potentials and project their axons into the antennal lobes, the first center in the brain where olfactory information is processed (Solari et al., 2016; Strausfeld and Hildebrand, 1999). In each sensillum, odorant molecules are trapped by small luminal proteins called odorant binding proteins (OBPs) that represent the first step of the olfaction machinery and are responsible to ship the stimuli to OSNs (Fan et al., 2011; Leal et al., 2013). The OBPs have been shown to be critical in odor discrimination (Swarup et al., 2011) and receptor activation (Biessmann et al., 2010; Laughlin et al., 2008), rather than merely serving as passive odorant shuttles (Zhong et al., 2012). For this reason, OBPs have been studied in several species such as *Drosophila melanogaster*, *Aedes aegypti*, *Culex pipiens quinquefasciatus*, *Bombyx mori*, *Manduca sexta, Apis mellifera* (Foret and Maleska, 2006; Gong et al., 2009; Grosse-Wilde et al., 2011; Pelletier and Leal, 2009; Zhou et al., 2004), and more recently, *obp* genes have been identified in *Bactrocera dorsalis* (Zheng et al., 2013) and *Ceratitis capitata* (Siciliano et al., 2014).

The Mediterranean fruit fly *Ceratitis capitata* Wied., is one of the most relevant agricultural pests both for its broad polyphagy and for the difficulty of controlling its biological potential (De Meyer et al., 2008; Diamantidis et al., 2011; Gasperi et al., 2002; Malacrida et al., 2007). It has become an

adopted model for fruit fly studies on invasive processes and to improve innovative and environmentally compatible pest control programs, such as those involved in the Sterile Insect Technique (SIT) and mass-trapping (Carey and Liedo, 2002; Robinson, 2002). These techniques largely depend on the activation of the olfactory system by odorants that act as species-specific attractants. Understanding the functional properties of the peripheral olfactory system in relation to physiological state and environmental parameters could provide an insight into the mechanisms involved in odor information processing. In the past years the olfactory sensitivity of *C. capitata* towards different compounds of interest has been widely studied: electrophysiological tests have shown that the antennae of adult females are more sensitive to some *Citrus* peel oils and volatile compounds than those of males (Hernandez et al., 1996; Levinson et al., 1990; Light et al., 1988; Light et al., 1992). Instead, in behavioral tests males exhibit a stronger preference than females for the volatiles of orange flavedo (Katsoyannos et al., 1997). In females, odor preference may be affected by mating behavior: during early sexual maturity females are more attracted by the pheromone produced by males than by the ripe guava odor, and this preference is inverted after mating (Jang, 1995). Sexually mature males emit sex pheromones from their everted rectal ampoule, which is both attractive to females and able to call other males to the "lek" site (Eberhard, 2000; Whittier et al., 1992). Males form loose leks on the leaves of the host plants and perform sexual signaling through the emission of the pheromone. Leks increase the total amount of pheromone released by males, giving them an advantage in terms of female calling capability. Receptive, sexually mature, females visit the leks and choose a partner for mating during their courtship performance, which involves chemical, visual and acoustic signals (Baker et al., 1985; Eberhard, 2000; Féron, 1962; Flath et al., 1993; Landolt et al., 1992; Light et al., 1999; Prokopy and Hendrichs, 1979; Yuval and Hendrichs, 2000).

Recently, several studies have been directed both to characterizing the chemical composition of the sex pheromone and to identifying the PBPs involved in the detection of these compounds (Vanickova et al., 2012; Siciliano et al., 2014). As regards the components of the male sex

pheromone, several studies reported that the compounds can be classified as major constituents (i.e. (E,E)-α-farnesene and geranyl acetate), intermediate constituents (i.e. β-myrcene and linalool) or minor constituents (i.e. 2,3-butanediol and linalool) (Baker et al., 1985; Flath et al., 1993; Jang et al., 1989; Light et al., 1999; Vanickova et al., 2012). Instead, 5 out of the 17 unique OBP genes identified in *C. capitata* seem to be putative pheromone binding proteins: CcapObp69a, CcapObp19d-1, CcapObp83a-1, CcapObp83a-2, CcapObp28a (Siciliano et al., 2014). Besides, both the release of the male sex pheromone and the expression of the PBP genes appear to be modulated both by mating and time of day (morning and afternoon) (Flath et al., 1993; Siciliano et al., 2014). Based on these findings, the aim of this study was to examine the effects of sex (males and females), physiological state (virgin and mated) and time of the day (morning and afternoon) on the olfactory sensitivity of adult insects of *C. capitata* to some components of the male sex pheromone in both antennae and palps.

2. Materials and methods

2.1 Insects

All the experiments were performed on adult sexually mature (4-6 days old) medflies of *C. capitata* (Wied.) of both sexes, kindly supplied by the Dept. of Animal Biology of the University of Pavia (Italy) at the pupal stage, and reared under controlled conditions (22 ± 1° C, 60-70% relative humidity, 12:12 h light:dark cycle) in a climatic chamber. Immediately after eclosion, males and females were sexed and divided into two groups: for the experiments with virgin insects, males and females were kept separate to avoid reciprocal exposure, while for the experiments with mated ones, insects were kept in the same cage to allow mating. Briefly, as copulating pairs formed, these were removed from the cage and kept in small vials. Only the insects that remained in copula for at least 100 min were used for the experiments, according to Siciliano et al. (2014). Adult flies were fed a mixture of sugar and yeast (4:1) (Solari et al., 2016) and had free access to fresh water.

112 *2.2 Electrophysiology*

113 Recordings were performed separately both on antennae and maxillary palps by means of the
114 electroantennogram (EAG) technique (Crnjar et al., 1989) and the electropalpogram (EPG)
115 technique, respectively. A glass micropipette (20 μm tip diameter) filled with saline solution (NaCl
116 0.9%, KCl 0.02%, $CaCl_2$ 0.02%, $NaHCO_3$ 0.01%, final pH 6.9; Willhoeft and Franz, 1996) was
117 inserted into the isolated head through the "foramen magnum" and acted as the reference electrode
118 in EAG or EPG recordings. The antennae or palps, one per fly, were positioned in such a way as to
119 expose the largest surface to the stimulus-bearing airstream. An air-stimulus control unit (model
120 CS-55, Syntech, Hilversum, the Netherlands) was used for air and odor delivery, according to Solari
121 et al. (2007a) and Sollai et al. (2007). All signals were recorded with a high input impedance (10^{15}
122 Ω) electrometer (WPI, Duo 773), band-pass filtered (DC-1 kHz), digitized by means of an Axon
123 Digidata 1440A A/D acquisition system (sampling rate 10 kHz) and stored on PC for later analysis
124 (Sollai et al., 2008). The absolute EAG or EPG amplitudes during the 2-s stimulation period were
125 calculated by means of Axoscope 10.0 software (Sollai et al., 2012; Sollai et al., 2014a). For the
126 experiments, the variables considered were: sex, mated or unmated state, time of day (morning 8
127 AM÷12 PM and afternoon 2 PM÷6 PM) and the sensory organ tested (antenna or palp).
128
129 *2.3 Stimuli*

130 The following compounds were tested as olfactory stimuli: (E,E)-α-farnesene, (E)-β-farnesene, β-
131 myrcene, 2,3-butanediol, linalool and geranyl acetate. These compounds were selected on the basis
132 of their relative abundance in the sex pheromone mixture and their binding affinity with one of the
133 PBPs of *C. capitata* (Jang et al., 1989; Siciliano et al., 2014).

134 Each compound was first dissolved (100 μg/ml) in dichloromethane (CH_2Cl_2) and then a 50 μl
135 volume of solution was pipetted onto a pleated strip of filter paper (80x5 mm), to yield a final
136 dosage of 5 μg. The other (lower) concentrations were obtained by decadic dilution in CH_2Cl_2 and
137 50 μl of each solution was pipetted onto filter paper strips to obtain a 0.5 μg and a 0.05 μg load on

filter paper; compounds were tested in increasing sequence of a total of 3 concentrations (Sollai et al., 2010).

The CH_2Cl_2 was evaporated before the experiments started; preliminary electrophysiological experiments indicated that blank filter paper, after CH_2Cl_2 evaporation, was not stimulating. Before each stimulation in each experiment, the response to air as a control was also tested in order to measure the presence of a possible mechanoreceptor response component and, if present, its value was subtracted from those of the test stimuli. Stimuli were presented in a randomized sequence, with a blank interstimulus interval long enough to allow for complete repolarization. Insects were used only once for each electrophysiological experiment. Each test was replicated 10 times.

2.4 Statistical analysis

Repeated measures ANOVA was adopted to analyze the effect of increasing concentration of odor stimuli ((E,E)-α-farnesene, (E)-β-farnesene, β-myrcene, 2,3-butanediol, linalool and geranyl acetate) on the EAG or EPG amplitudes of the antennal or palpal sensilla respectively, separately for each stimulus, sex, physiological state (mated or virgin) and time of day (morning or afternoon). One-way ANOVA was used to test the effect of sex, physiological state (virgin or mated) and time of day (morning or afternoon) on the sensitivity of antennae and palps in response to each stimulus. Data were checked for the assumptions of normality, homogeneity of variance and sphericity (when applicable). When the sphericity assumption was violated, a Greenhouse-Geisser correction or Huynh-Feldt correction was applied in order to modify the degrees of freedom (Sollai et al., 2014b). Post-hoc comparisons were conducted with Tukey test, unless the assumption of homogeneity of variance was violated, in which case Duncan's test was used (Sollai et al., 2015; Sollai et al., 2017b). Statistical analyses were performed using STATISTICA for WINDOWS (version 7.0; StatSoft Inc, Tulsa, OK, USA). *P* values < 0.05 were considered significant.

3. Results

164 *3.1 Dose-response relationship in the EAG and EPG amplitudes*

165 Samples of EAG and EPG recordings, obtained from the antennal and palpal olfactory sensilla of
166 both males and females, virgins and mated, in the morning and afternoon, in response to 5 μg of the
167 tested stimuli: (E,E)-α-farnesene, (E)-β-farnesene, β-myrcene, 2,3-butanediol, linalool and geranyl
168 acetate, are shown in figures 1-2 and 3-4, respectively.

169 To test for a dose-response relationship, we analyzed the EAG amplitudes evoked in the antennal
170 preparations (Figs. 5, 6) and the EPG ones elicited in the palps (Figs. 7, 8) to increasing
171 concentrations of each volatile, by means of repeated-measures ANOVA.

172 A significant effect of concentration on the EAG values resulted in response to increasing
173 concentrations of all olfactory stimuli tested, both in the morning and in the afternoon, for: a) virgin
174 males (morning: $F_{[1.24,15.30]} > 82.489$; $P < 0.00001$; afternoon: $F_{[1.04,13.48]} > 40.305$; $P < 0.00001$);
175 b) mated males (morning: $F_{[2,18]} > 38.168$; $P < 0.00001$; afternoon: $F_{[1.03,11.37]} > 6.7018$; $P < 0.01$);
176 c) virgin females (morning: $F_{[1.09,16.34]} > 39.487$; $P < 0.00001$; afternoon: $F_{[1.03,11.34]} > 27.254$; $P <$
177 0.00001); d) mated females (morning: $F_{[1.01,13.19]} > 70.322$; $P < 0.00001$; afternoon: $F_{[1.42,14.02]} >$
178 13.028; $P < 0.001$).

179 Repeated-measures ANOVA showed a significant effect of concentration on the EPG values in
180 response to all olfactory stimuli tested for: a) virgin males, both in the morning ($F_{[1.15,15.01]} >$
181 73.888; $P < 0.00001$) and in the afternoon ($F_{[1.03,9.28]} > 29.656$; $P < 0.00001$); b) mated males, both
182 in the morning ($F_{[1.17,10.54]} > 21.678$; $P < 0.0001$) and in the afternoon ($F_{[1.03,11.34]} > 8.1457$; $P <$
183 0.01); c) virgin females, both in the morning ($F_{[1.21,12.26]} > 11.576$; $P < 0.01$) and in the afternoon (F
184 $_{[1.22,13.51]} > 19.840$; $P < 0.0001$); d) mated females, both in the morning ($F_{[1.28,12.64]} > 5.9818$; $P <$
185 0.05) and in the afternoon ($F_{[1.36,12.23]} > 20.323$; $P < 0.0001$).

186 These results, together with the sample EAG and EPG traces showed in figures 1-4, indicate that the
187 antennae and palps of both males and females are stimulated by components of male pheromone.

188

189 *3.2 Effects of sex, physiological state and/or time of day on olfactory sensitivity*

We also investigated whether the olfactory sensitivity of both antennae and palps at the highest dose of each chemical tested depends on sex, physiological state and/or time of day, by means of one-way ANOVA.

A significant effect of sex was found on EAG amplitude recorded from mated insects in response to α-farnesene and linalool in the afternoon, in the EAG responses of the virgin insects to 2,3-butanediol and β-myrcene in the afternoon, and to linalool at both times of day (Table 1A). As regards the sensitivity of palps, table 1B shows a significant effect of sex on EPG responses by mated insects to all chemicals tested, except for α-farnesene, and only to β-farnesene in virgin insects in the afternoon. In detail, post-hoc comparisons show that both antennal and palpal sensitivity of females was statistically higher than that of males in all cases for which a significant effect of the sex was found (Fig. 9).

For male insects, the effect of physiological state on EAG amplitude in response to all chemicals tested resulted significant at both times of the day, except for linalool in the afternoon (Table 1A). We also found an effect of physiological state in palps responding to β-myrcene in the morning, to 2,3-butanediol in the afternoon and to geranyl acetate at both times of day (Table 1B). For females, one-way ANOVA showed a significant effect of physiological state on antennal responses to β-myrcene and geranyl acetate, at both times of day considered, and in response to 2,3-butanediol and linalool in the morning (Table 1A). Physiological state was also found to affect EPG amplitudes in response to geranyl acetate at both times of day and in response to β-farnesene, linalool and β-myrcene in the morning (Table 1B). For both males and females post-hoc comparisons revealed that olfactory sensitivity of virgins was significantly higher than that of mated insects, in both antennae and palps (Fig. 10).

Finally, one-way ANOVA showed a significant effect of the time of day on both EAG and EPG amplitudes in response to all chemicals tested in both sexes and physiological states investigated (Table 1A, B), and post-hoc comparisons revealed that olfactory sensitivity was significantly higher in the morning than in the afternoon, in both sensory organs considered (Fig. 11).

4. Discussion

Chemoreception plays a key role in *C. capitata*, by regulating essential behaviors such as localisation and discrimination of host plants, detection of pheromones during recognition and location of partner for mating and discrimination between suitable and already visited hosts for oviposition (Siciliano et al., 2014; Solari et al., 2018; Sollai et al., 2018b). The goal of this work was to evaluate the olfactory sensitivity of male and female medflies towards a few chemical compounds previously indicated as major, intermediate and minor (or in trace) components of the sex-pheromone released by mature males of *C. capitata* during calling behaviour. In particular, we aimed at studying whether the olfactory sensitivity of both antennae and palps of the insects depends on the sex (males or females), the physiological state (virgins or mated) and the time of day (morning or afternoon). The dose-response relationships we found show that both males and females have an olfactory sensitivity to the tested chemicals, suggesting that the sex pheromone emitted by males during calling may be sensed by both sexes. These results are in agreement with the data reported in the literature: in fact, a large number of compounds of the male sex pheromone have been suggested to play a functional role both in attracting females and triggering consequent courtship behavior, and in inducing lekking, competition or aggregation behaviors in males themselves (Arita and Kaneshiro, 1985).

We found that most of the compounds tested evoked very similar EAG responses in both males and females, regardless of physiological state or time of day. This result is in agreement with the findings reported both for *C. capitata* and other tephritid species, in which some "major" components of the male sex pheromone (i.e., geranyl acetate and α-farnesene) and host plant volatiles also elicit behavioral responses in females (Fein et al. 1982; Jang et al., 1989; Light and Jang, 1987; Light et al., 1988; Robacker et al., 1986; Robacker and Hart, 1987; Van der Pers et al., 1984). Instead, as regards the responses of palps, the results showed a higher sensitivity in females than in males, especially in the morning and among the mated insects. This may be due to the

242 different activity or presence of specific OBPs and/or olfactory receptors, which may be involved in
243 olfactory responses to volatiles including those from host plants (Siciliano et al., 2014).
244 The results we obtained show that among males, virgin medflies evoke higher EAG responses than
245 mated ones, both in the morning and the afternoon, for all stimuli tested, while smaller differences
246 were found in the case of the EPG responses. These data are not surprising considering that
247 antennae are known to be the main olfactory organ in the medfly (Bigiani et al., 1989). Besides,
248 previous findings about the impact of sexual maturation and mating on the PBP expression, have
249 reported a reduction of the transcript of the genes for CcapOBP83a-2 and CcapOBP28a during
250 maturation, and for CcapOBP69a after mating (Siciliano et al., 2014). In fact, since courtship is an
251 energetically expensive activity, males may need a period of time to recover after mating and the
252 weak reduction in PBPRP transcription could be the result of a redistribution of resources to restore
253 the reserves used during courtship (Siciliano et al., 2014). Instead, as for females, few differences
254 were found between virgin and mated insects both in the morning and in the afternoon, suggesting
255 that the olfactory sensitivity both of the antennae and palps is little influenced by the physiological
256 state. These results are in agreement with the fact that: 1) mated females undergo a drastic
257 behavioral change, shifting their attention from the smell of the male pheromone to the odors of the
258 host plant where they oviposit; 2) being that many components of the male pheromone blend are
259 derived from host plant chemical precursors, females still detect the volatiles as components of the
260 host fruits used for oviposition (Jang, 1995; Papadopoulos et al., 2006; Siciliano et al., 2014).
261 Finally, in both sexes and in both physiological states (virgins and mated), the sensitivity of
262 antennae and palps is higher in the morning than the afternoon, in response to all the chemicals
263 tested. The medfly seems to display a bimodal pattern of sexual activity during the day, with one
264 peak at approximately 08:00-12:00 hrs and a second minor peak at approximately 14:00-16:00 hrs.
265 Similar results have been obtained in other insects: in *Drosophila* sp. chemoreception, feeding,
266 courtship, mating and oviposition are known to undergo circadian regulation (Emery and Francis,
267 2008; Kyriacou and Hall, 1980; Sakai and Ishida, 2001); evidence for a circadian rhythm has been

found in relation to EAG amplitude in forager honeybees (Nagari et al., 2017); in some moth species, the time of day in which males show the highest sensitivity to sex pheromone is aligned with the time of day when females release it, which is also under circadian regulation (Baker and Cardé, 1979; Gadenne et al., 2016; Rosen, 2002; Solari et al., 2007b). Besides, the decreased olfactory sensitivity to odorants requiring OBPs coincide with a general decreasing trend in the abundance of the transcript of the CcapOBPs in the afternoon as compared to the morning, in males and females alike (Siciliano et al., 2014). A correspondence between the OBP abundance and the olfactory sensitivity had already been found in the electrophysiological investigations from the antennae of *Anopheles gambiae*, suggesting a role for OBPs in modulating temporal changes in olfactory sensitivity (Rund et al., 2013).

In conclusion, since pheromones and odorant molecules controlling both mating and oviposition behaviors are recognized by olfactory organs, these results provide important knowledge on the variability of the olfactory sensitivity of the medflies, thus providing supporting evidence for the identification of active compounds in relation to sex, physiological state and time of day, with the aim of improving pest management strategies for *C. capitata* populations.

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Conflict of interest

There are no financial and personal relationships with other people or organizations that may lead to a conflict of interest.

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535

Figure legends

Fig. 1 – Sample EAG recordings from male antennal preparations in different physiological state (virgin or mated) and time of day (morning or afternoon) following stimulation with α-farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate at 5 μg.

Fig. 2 – Sample EAG recordings from female antennal preparations in different physiological state (virgin or mated) and time of day (morning or afternoon) following stimulation with α-farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate at 5 μg.

Fig. 3 – Sample EPG recordings from male palpal preparations in different physiological state (virgin or mated) and time of day (morning or afternoon) following stimulation with α-farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate at 5 μg.

Fig. 4 – Sample EPG recordings from female palpal preparations in different physiological state (virgin or mated) and time of day (morning or afternoon) following stimulation with α-farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate at 5 μg.

Fig. 5 – Dose-response relationship between EAG amplitudes and different olfactory stimuli of virgin or mated males in the morning and in the afternoon. All values are mean (± s.e.m.); N=10 antennae (1 antenna/medfly). Filled symbols indicate significant differences between a concentration and the next lower. In the case of virgin males significant differences are for α-farnesene, 2,3-butanediol, β-myrcene both in the morning and afternoon, β-farnesene, geranyl acetate in the morning ($P < 0.001$, Tukey test); for linalool at both times of day, for β-farnesene, geranyl acetate in the afternoon ($P < 0.01$, Tukey test). In the case of mated males: for β-myrcene at both times of day, for α-farnesene, 2,3-butanediol, linalool in the morning, for β-farnesene in the

afternoon ($P < 0.001$, Tukey test); for β-farnesene, geranyl acetate in the morning ($P < 0.01$, Tukey test); for α-farnesene, 2,3-butanediol, linalool, geranyl acetate in the afternoon ($P < 0.05$, Tukey test).

Fig. 6 – Dose-response relationship between EAG amplitudes and different olfactory stimuli of virgin or mated females in the morning and the afternoon. All values are mean (± s.e.m.); N=10 antennae (1 antenna/medfly). Filled symbols indicate significant differences between a concentration and that next lower. In the case of virgin females significant differences are for β-farnesene, 2,3-butanediol in the morning, linalool in the afternoon, β-myrcene at both times of day ($P < 0.001$, Tukey test); for α-farnesene, geranyl acetate at both times of day, β-farnesene in the afternoon ($P < 0.01$, Tukey test); for 2,3-butanediol, linalool in the morning ($P < 0.05$, Tukey test). In the case of mated females: for β-myrcene at both times of day, for α-farnesene, β-farnesene, geranyl acetate in the morning ($P < 0.001$, Tukey test); for 2,3-butanediol, linalool at both times of day, α-farnesene in the afternoon ($P < 0.01$, Tukey test); for β-farnesene, geranyl acetate in the afternoon ($P < 0.05$, Tukey test).

Fig. 7 – Dose-response relationship between EPG amplitudes and different olfactory stimuli of virgin or mated males in the morning and in the afternoon. All values are mean (± s.e.m.); N=10 palps (1 palp/medfly). Filled symbols indicate significant differences between a concentration and that next lower. In the case of virgin males significant differences are for β-myrcene at both times of day, for β-farnesene, geranyl acetate in the morning; for α-farnesene, 2,3-butanediol in the afternoon ($P < 0.001$, Tukey test); for α-farnesene, 2,3-butanediol, linalool in the morning; for β-farnesene, geranyl acetate in the afternoon ($P < 0.01$, Tukey test); for linalool in the afternoon ($P < 0.01$; Tukey test). In the case of mated males: for α-farnesene, 2,3-butanediol, β-myrcene, geranyl acetate in the morning, β-farnesene in the afternoon ($P < 0.001$, Tukey test); for linalool, β-myrcene

586 in the afternoon ($P < 0.01$, Tukey test); for β-farnesene, linalool in the morning, geranyl acetate in
587 the afternoon ($P < 0.05$, Tukey test).
588
589 **Fig. 8 –** Dose-response relationship between EPG amplitudes and different olfactory stimuli of
590 virgin or mated females in the morning and in the afternoon. All values are mean (± s.e.m.); N=10
591 palps (1 palp/medfly). Filled symbols indicate significant differences between a concentration and
592 that next lower. In the case of virgin females significant differences are for α-farnesene at both
593 times of day; for linalool, β-myrcene in the afternoon ($P < 0.001$, Tukey test); for 2,3-butanediol,
594 geranyl acetate at both times of day, β-myrcene in the morning ($P < 0.01$, Tukey test); for β-
595 farnesene at both times of day, linalool in the morning ($P < 0.05$, Tukey test). In the case of mated
596 females: for 2,3-butanediol both in the morning and afternoon, for linalool and β-myrcene in the
597 morning ($P < 0.001$, Tukey test); for β-farnesene at both times of day, β-myrcene in the afternoon
598 and geranyl acetate in the morning ($P < 0.01$, Tukey test); for α-farnesene at both times of day,
599 linalool and geranyl acetate in the afternoon ($P < 0.05$, Tukey test).
600
601 **Fig. 9 –** EAG or EPG mean amplitude values ± s.e.m. elicited by stimulation with 5 μg of α-
602 farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate in both males and
603 females. N=10 antennae or palps (1 antenna or palp/medfly). Symbols indicate significant
604 differences between the sexes. Tukey test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Duncan test: # P
605 < 0.05, ## $P < 0.01$.
606
607 **Fig. 10 –** EAG or EPG mean amplitude values ± s.e.m. elicited by stimulation with 5 μg of α-
608 farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate in both virgin and
609 mated insects. N=10 antennae or palps (1 antenna or palp/medfly). Symbols indicate significant

differences between the physiological states. Tukey test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Duncan test: # $P < 0.05$, ## $P < 0.01$.

Fig. 11 – EAG or EPG mean amplitude values ± s.e.m. elicited by stimulation with 5 μg of α-farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate at both times of day. N=10 antennae or palps (1 antenna or palp/medfly). Symbols indicate significant differences between morning and afternoon. Tukey test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Duncan test: # $P < 0.05$, ## $P < 0.01$.

Table 1 – Significant effect of sex, physiological state and time of day on EAG (A) and EPG (B) amplitude following stimulation with 5 μg of α-farnesene, β-farnesene, 2,3-butanediol, linalool, β-myrcene and geranyl acetate. Abbreviations: Vir = virgins; Mat = mated; Mor = morning; Aft = afternoon; Mal = males; Fem = females. Red typing = significantly different ($P < 0.05$).

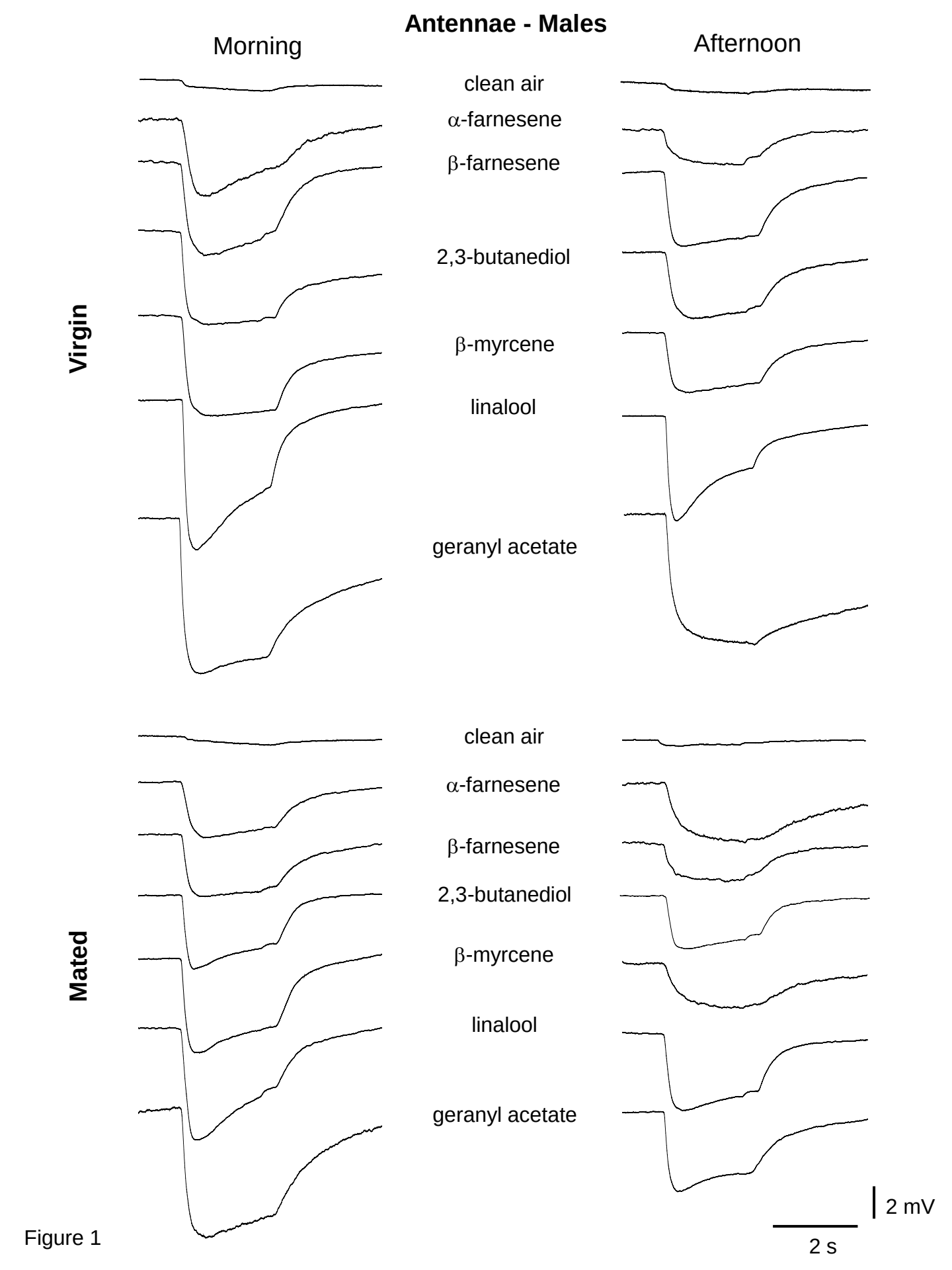
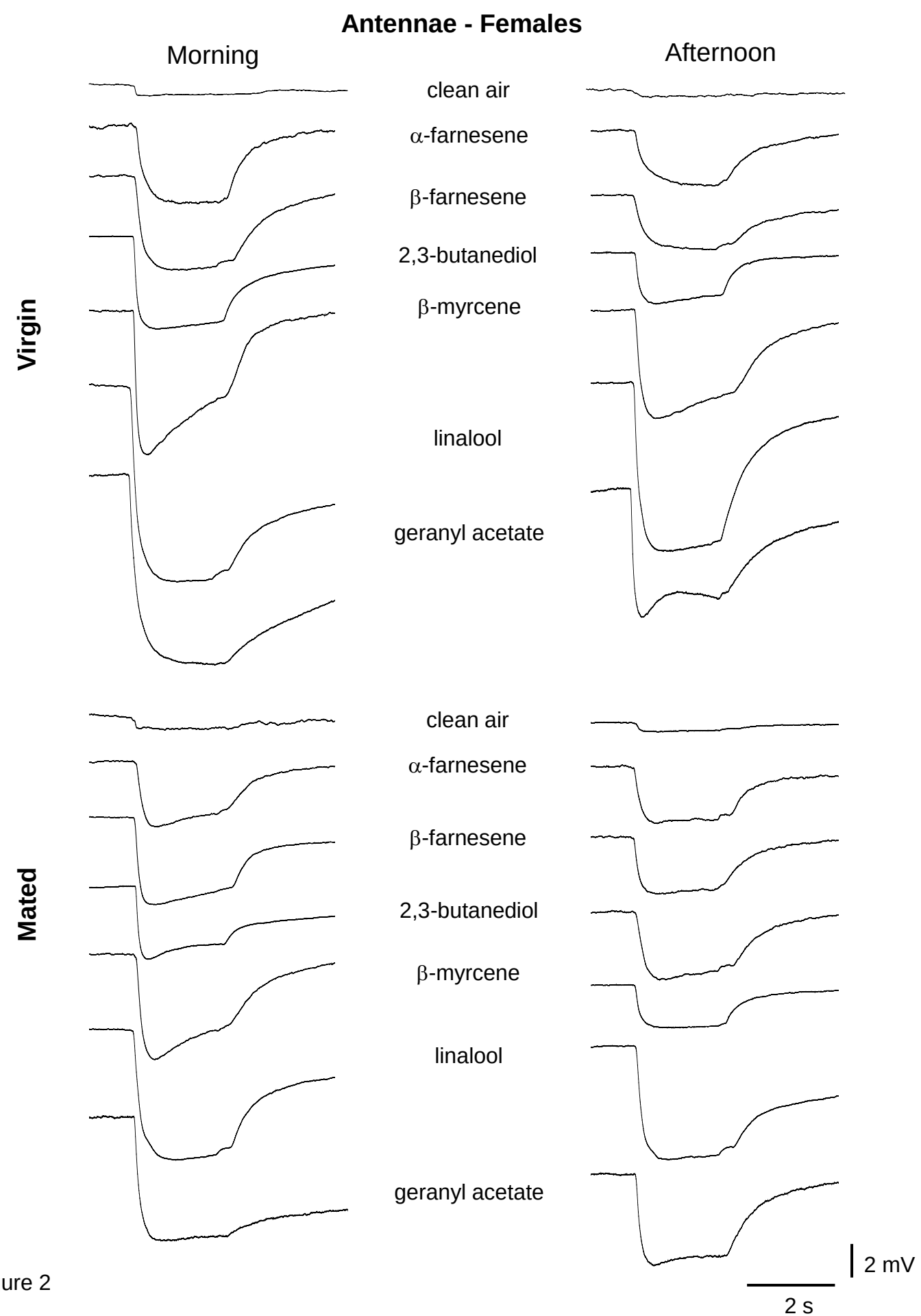

Antennae - Males
Morning
Afternoon
Virgin
Mated
clean air
α-farnesene
β-farnesene
2,3-butanediol
β-myrcene
linalool
geranyl acetate
clean air
α-farnesene
β-farnesene
2,3-butanediol
β-myrcene
linalool
geranyl acetate
2 mV
2 s

Figure 1


Antennae - Females
Morning
Afternoon
Virgin
Mated
clean air
α-farnesene
β-farnesene
2,3-butanediol
β-myrcene
linalool
geranyl acetate
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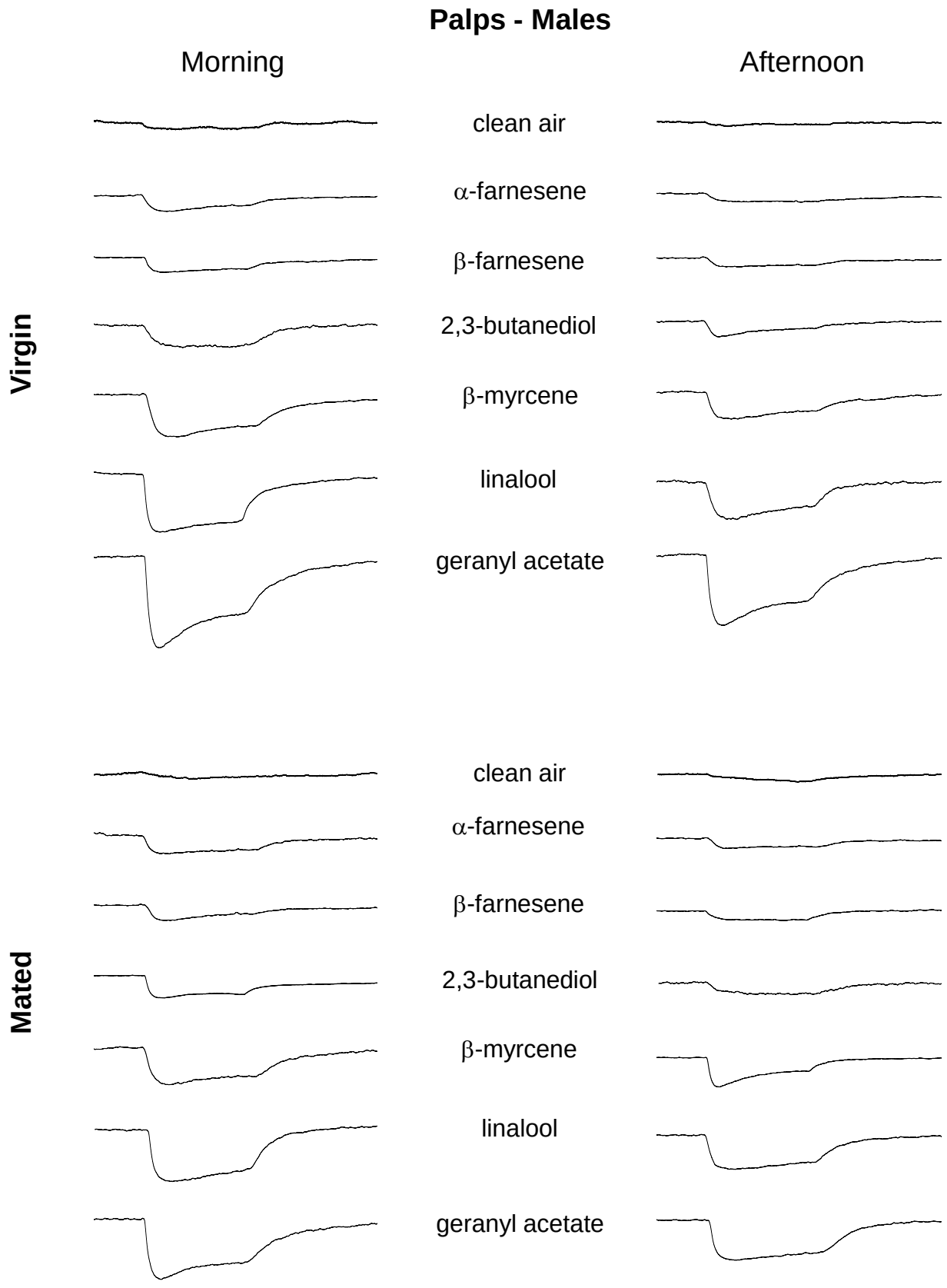
β-myrcene
linalool
geranyl acetate
2 mV
2 s

Figure 2


Palps - Males
Morning
Afternoon
Virgin
Mated
clean air
α-farnesene
β-farnesene
2,3-butanediol
β-myrcene
linalool
geranyl acetate
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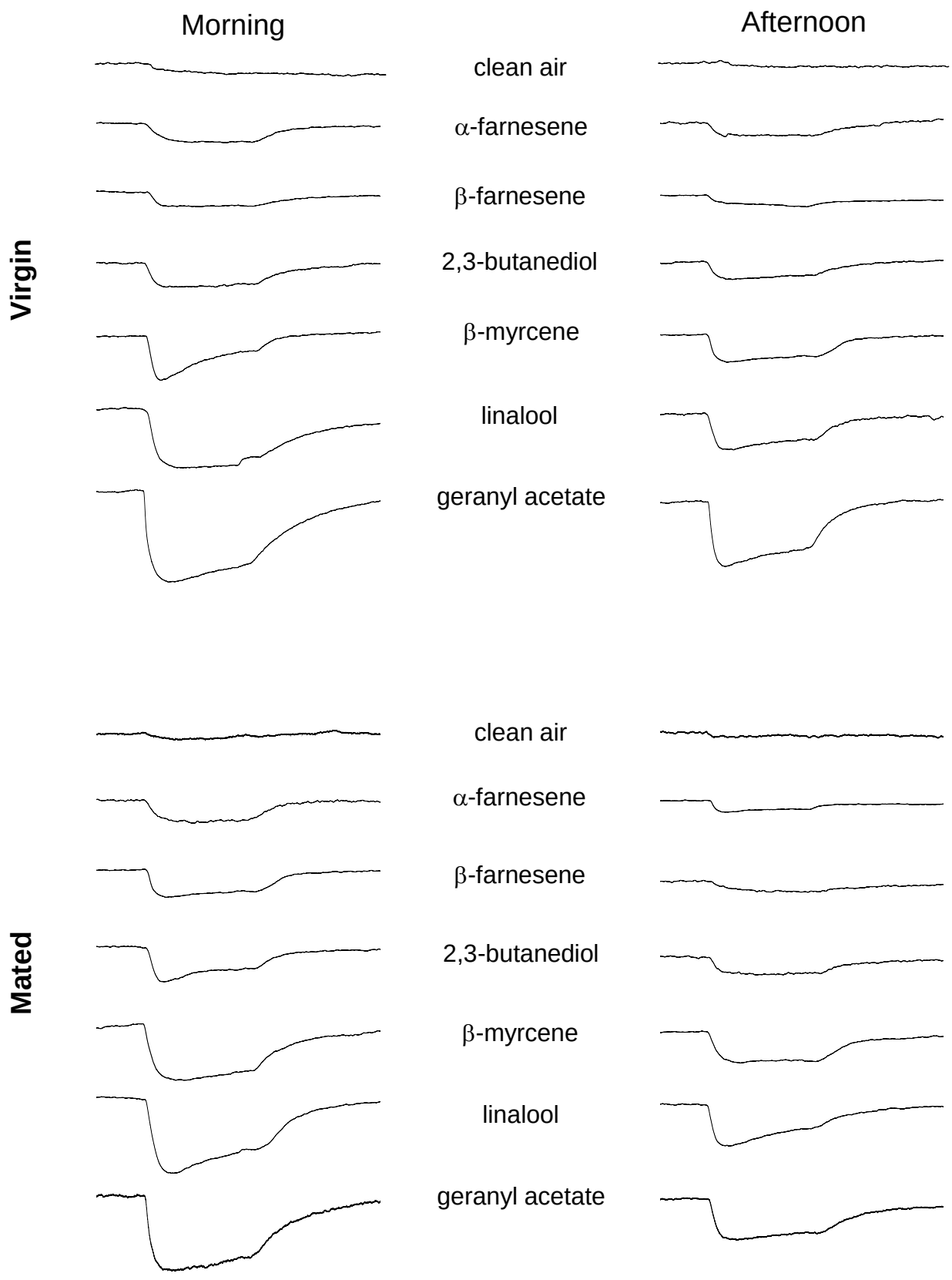
β-myrcene
linalool
geranyl acetate
2 mV
2 s

Figure 3


Palps - Females
Morning
Afternoon
Virgin
Mated
clean air
α-farnesene
β-farnesene
2,3-butanediol
β-myrcene
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geranyl acetate
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2 mV
2 s

Figure 4

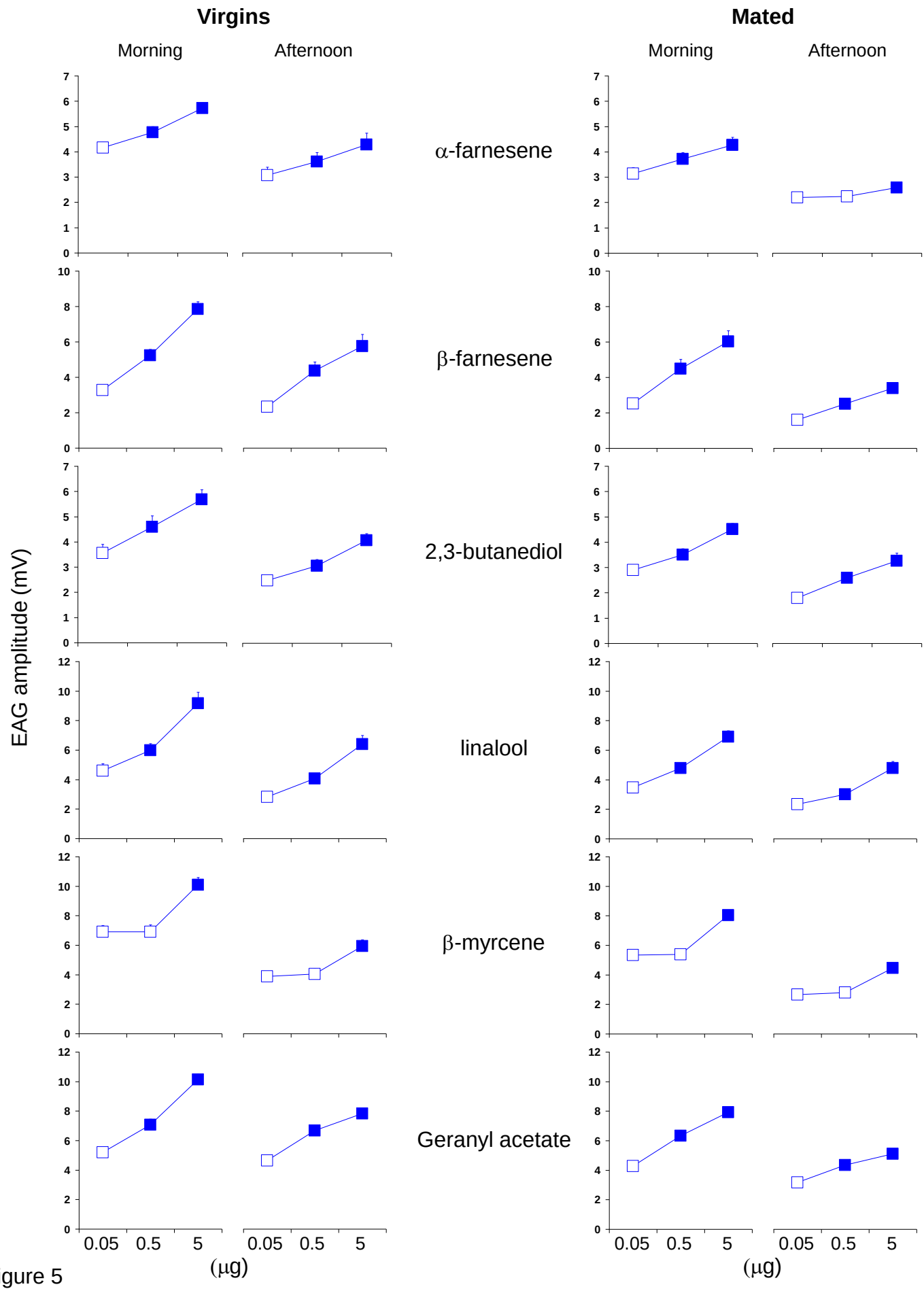
Virgins
Mated
Morning
Afternoon
Morning
Afternoon
α-farnesene
β-farnesene
2,3-butanediol
linalool
β-myrcene
Geranyl acetate
EAG amplitude (mV)
0.05
0.5
5
(µg)

Figure 5

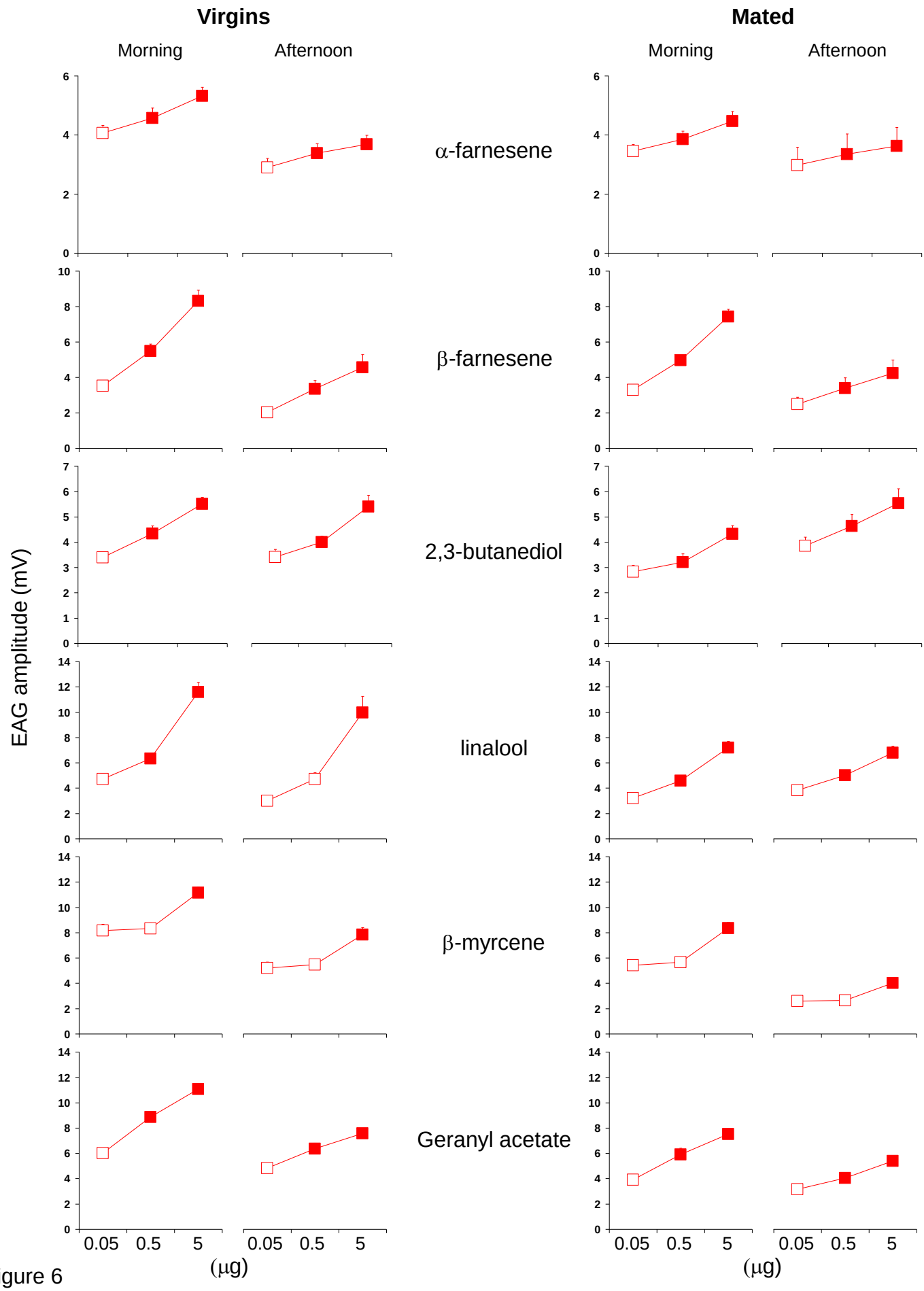
Virgins
Mated
Morning
Afternoon
Morning
Afternoon
α-farnesene
β-farnesene
2,3-butanediol
linalool
β-myrcene
Geranyl acetate
EAG amplitude (mV)
0.05
0.5
5
(μg)

Figure 6

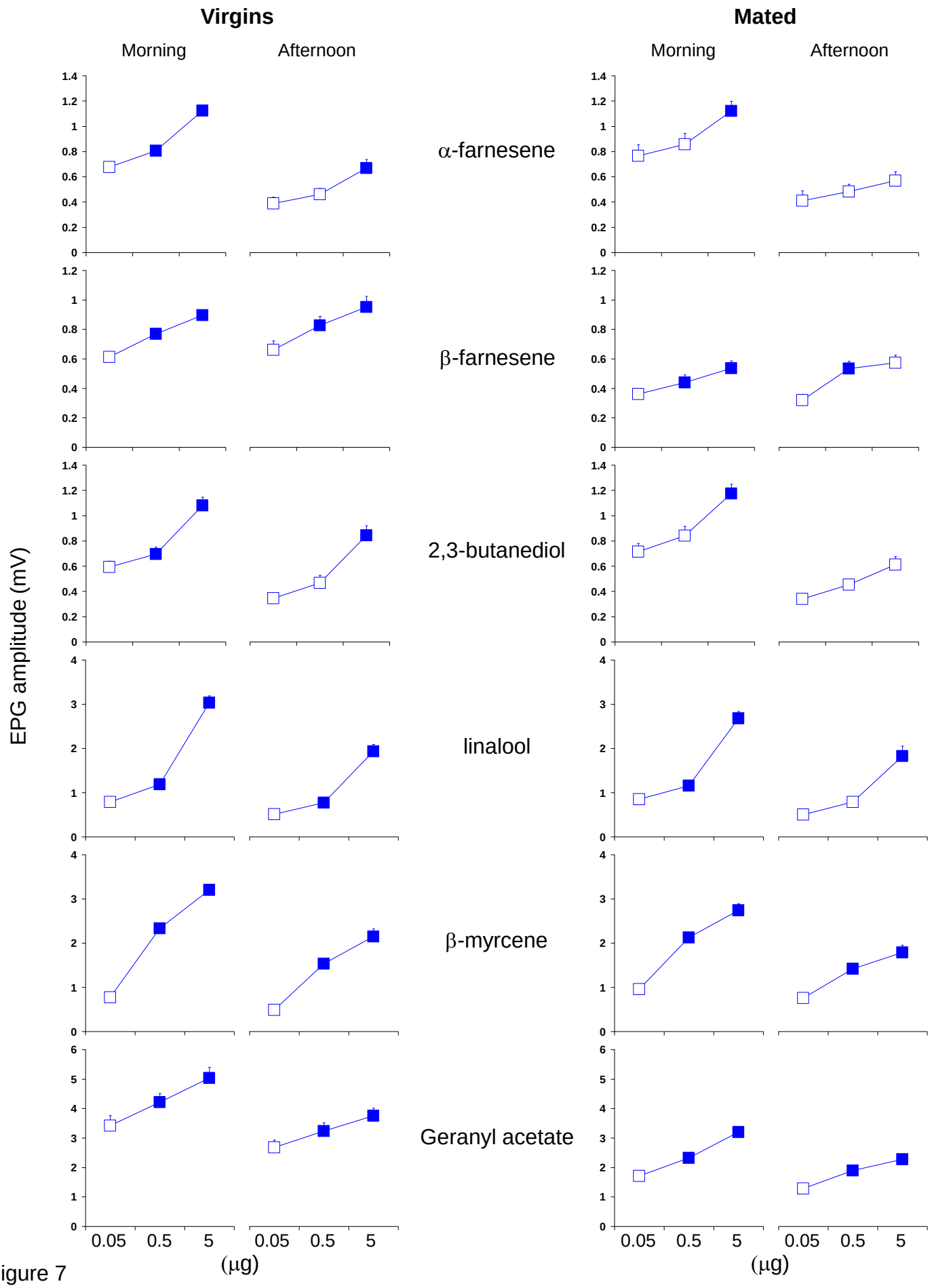
Virgins
Mated
Morning
Afternoon
Morning
Afternoon
α-farnesene
β-farnesene
2,3-butanediol
linalool
β-myrcene
Geranyl acetate
EPG amplitude (mV)
0.05
0.5
5
(µg)

Figure 7

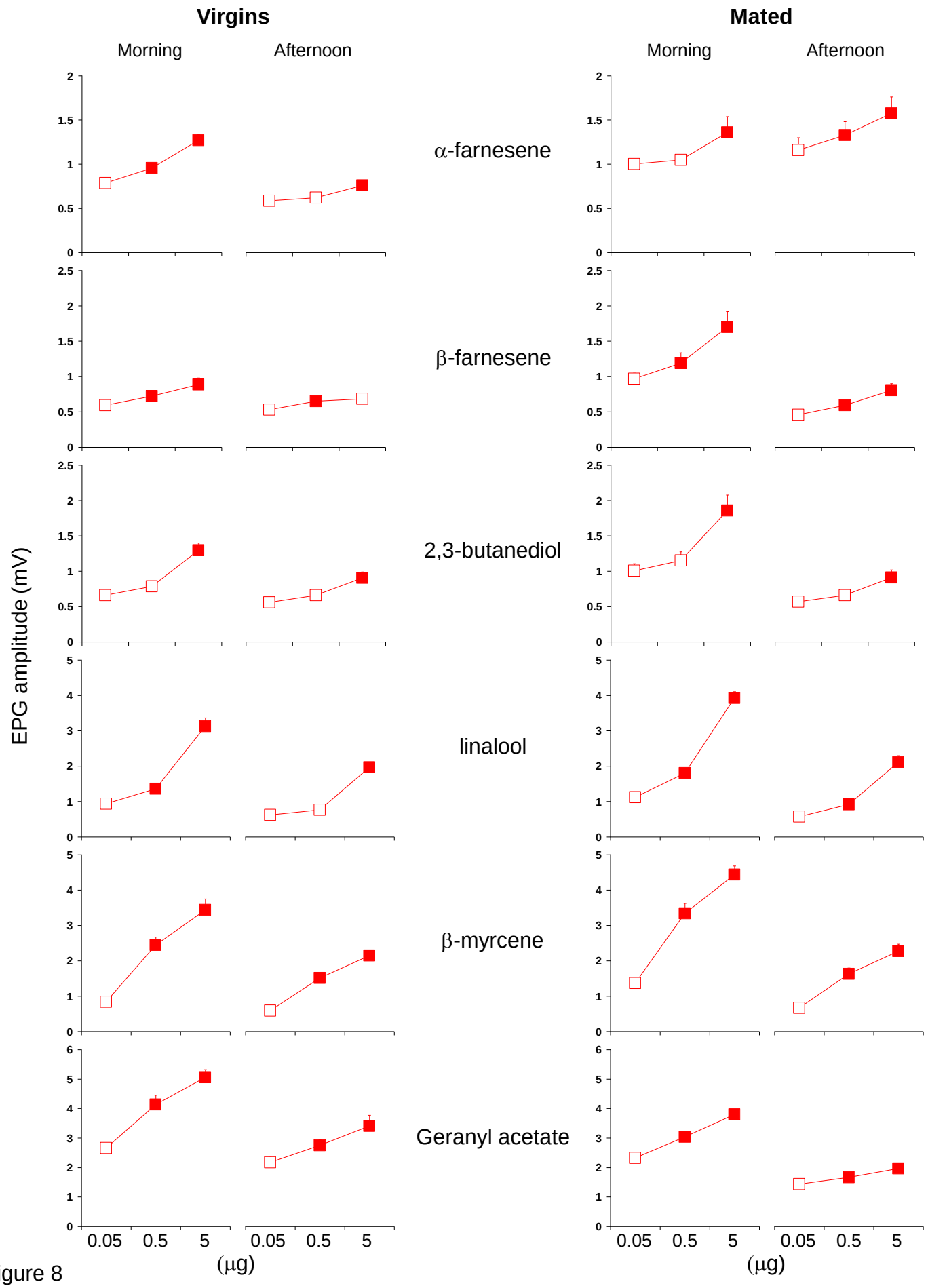
Virgins
Mated
Morning
Afternoon
Morning
Afternoon
α-farnesene
β-farnesene
2,3-butanediol
linalool
β-myrcene
Geranyl acetate
EPG amplitude (mV)
0.05
0.5
5
(µg)

Figure 8

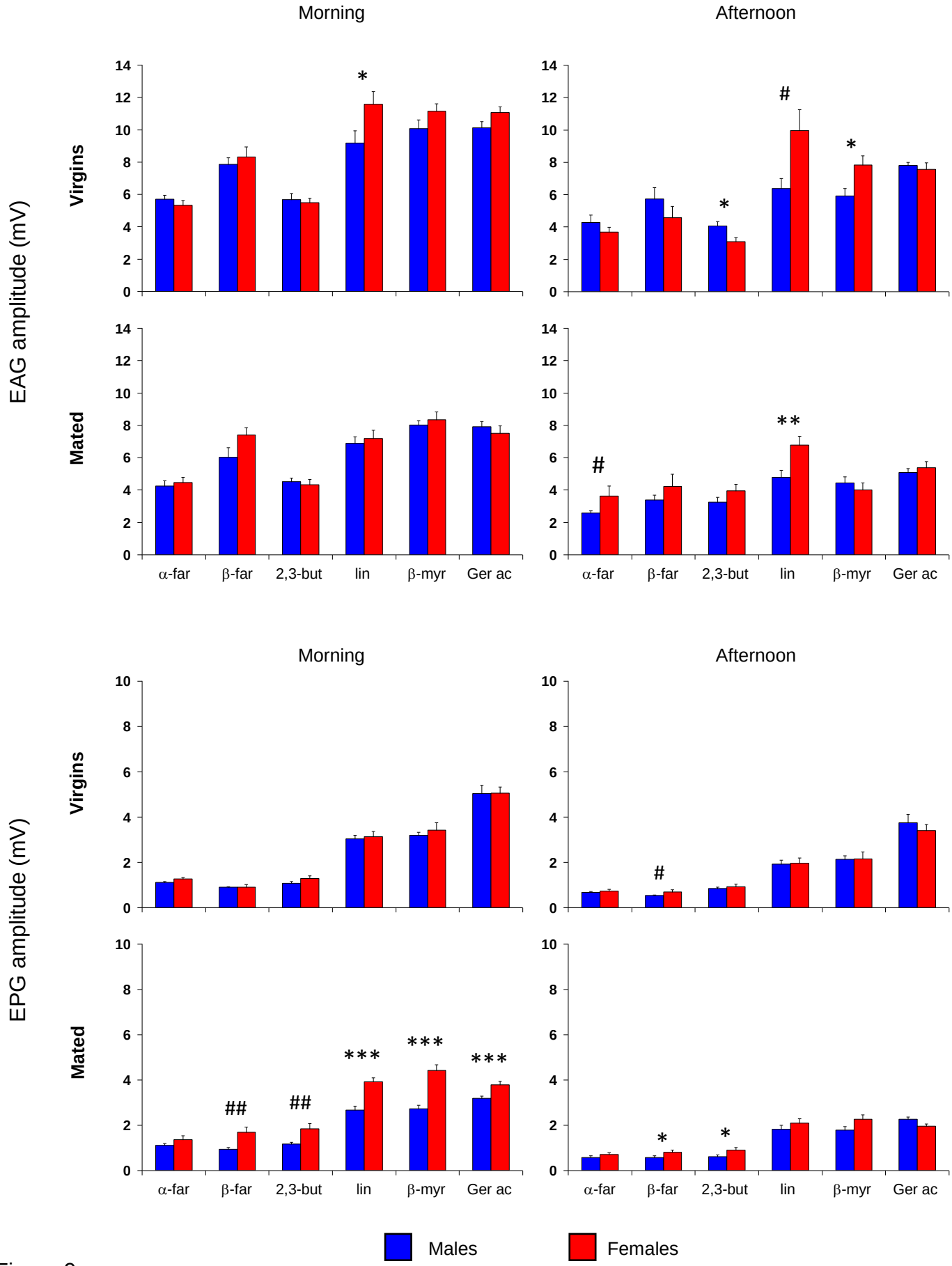
Morning
Afternoon
EAG amplitude (mV)
Virgins
Mated
EPG amplitude (mV)
α-far
β-far
2,3-but
lin
β-myr
Ger ac
Males
Females

Figure 9

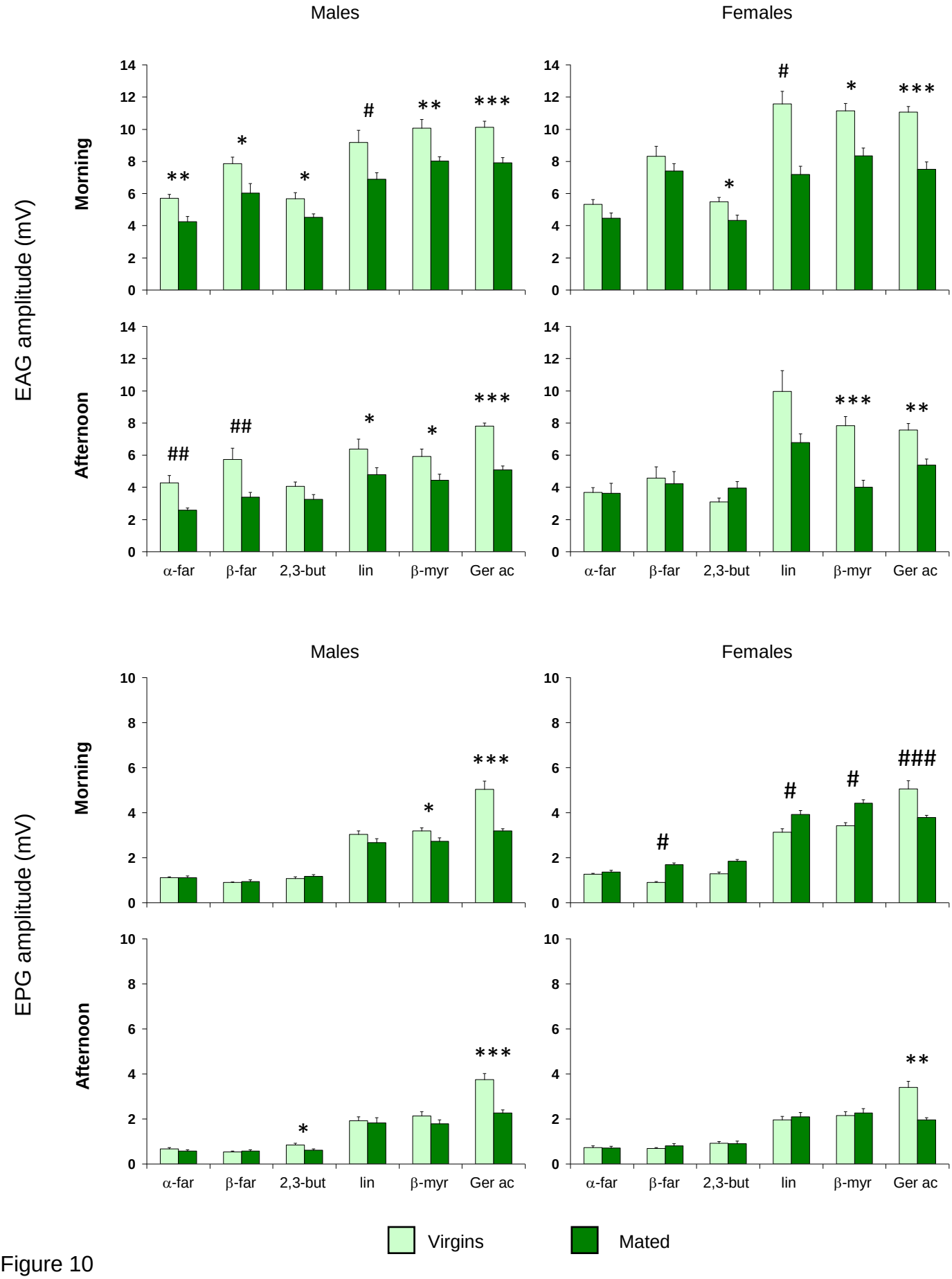
Males
Females
Morning
Afternoon
EAG amplitude (mV)
EPG amplitude (mV)
α-far
β-far
2,3-but
lin
β-myr
Ger ac
Virgins
Mated

Figure 10

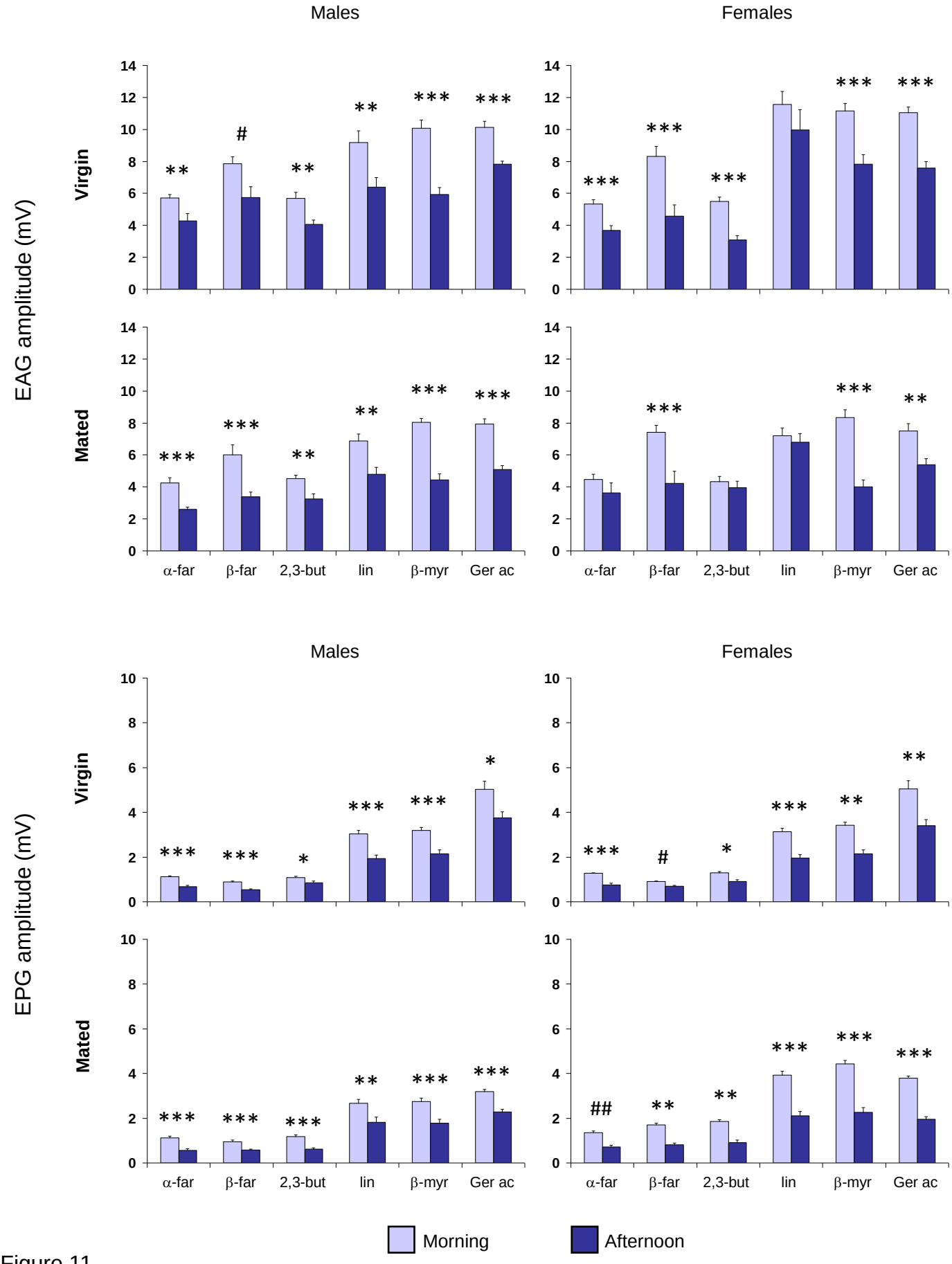
Males
Females
EAG amplitude (mV)
Virgin
Mated
EPG amplitude (mV)
α-far
β-far
2,3-but
lin
β-myr
Ger ac
Morning
Afternoon

Figure 11

A

Sensory organ: Antennae						
Stimulus	**Males vs. Females**		**Virgins vs. Mated**		**Morning vs. Afternoon**	
α-farnesene	Vir-Mor	F(1,18)=1.0982; p=0.3033	Mal-Mor	F(1,18)=14.166; p=0.0011	Mal-Vir	F(1,18)=8.2816; p=0.0077
	Vir-Aft	F(1,18)=1.1069; p=0.3032	Mal-Aft	F(1,18)=11.040; p=0.0029	Mal-Mat	F(1,18)=31.289; p=0.0000
	Mat-Mor	F(1,18)=0.1749; p=0.6802	Fem-Mor	F(1,18)=3.8408; p=0.0600	Fem-Vir	F(1,18)=14.468; p=0.0008
	Mat-Aft	F(1,18)=4.7511; p=0.0446	Fem-Aft	F(1,18)=0.0078; p=0.9307	Fem-Mat	F(1,18)=1.7360; p=0.2042
β-farnesene	Vir-Mor	F(1,18)=0.3664; p=0.5497	Mal-Mor	F(1,18)=6.2263; p=0.0210	Mal-Vir	F(1,18)=7.0136; p=0.0134
	Vir-Aft	F(1,18)=1.4003; p=0.2483	Mal-Aft	F(1,18)=8.7690; p=0.0068	Mal-Mat	F(1,18)=18.428; p=0.0004
	Mat-Mor	F(1,18)=3.5753; p=0.0732	Fem-Mor	F(1,18)=1.3227; p=0.2591	Fem-Vir	F(1,18)=15.738; p=0.0005
	Mat-Aft	F(1,18)=1.2710; p=0.2736	Fem-Aft	F(1,18)=0.1026; p=0.7523	Fem-Mat	F(1,18)=15.328; p=0.0008
2,3-butanediol	Vir-Mor	F(1,18)=0.1497; p=0.7022	Mal-Mor	F(1,18)=5.9450; p=0.0268	Mal-Vir	F(1,18)=11.947; p=0.0025
	Vir-Aft	F(1,18)=6.0959; p=0.0199	Mal-Aft	F(1,18)=3.7201; p=0.0668	Mal-Mat	F(1,18)=8.7138; p=0.0085
	Mat-Mor	F(1,18)=0.1503; p=0.7023	Fem-Mor	F(1,18)=7.7317; p=0.0096	Fem-Vir	F(1,18)=41.403; p=0.0000
	Mat-Aft	F(1,18)=1.9225; p=0.1816	Fem-Aft	F(1,18)=3.3982; p=0.0772	Fem-Mat	F(1,18)=0.4887; p=0.4922
linalool	Vir-Mor	F(1,18)=4.7255; p=0.0380	Mal-Mor	F(1,18)=4.3888; p=0.0485	Mal-Vir	F(1,18)=8.0622; p=0.0085
	Vir-Aft	F(1,18)=6.8385; p=0.0152	Mal-Aft	F(1,18)=4.3129; p=0.0487	Mal-Mat	F(1,18)=10.829; p=0.0041
	Mat-Mor	F(1,18)=0.1699; p=0.6846	Fem-Mor	F(1,18)=19.987; p=0.0001	Fem-Vir	F(1,18)=1.2313; p=0.2773
	Mat-Aft	F(1,18)=8.3856; p=0.0093	Fem-Aft	F(1,18)=4.0373; p=0.0589	Fem-Mat	F(1,18)=0.2777; p=0.6037
β-myrcene	Vir-Mor	F(1,18)=2.4498; p=0.1284	Mal-Mor	F(1,18)=11.456; p=0.0023	Mal-Vir	F(1,18)=35.164; p=0.0000
	Vir-Aft	F(1,18)=6.8587; p=0.0157	Mal-Aft	F(1,18)=6.4099; p=0.0190	Mal-Mat	F(1,18)=61.480; p=0.0000
	Mat-Mor	F(1,18)=0.2769; p=0.6034	Fem-Mor	F(1,18)=17.322; p=0.0003	Fem-Vir	F(1,18)=20.899; p=0.0001
	Mat-Aft	F(1,18)=0.5802; p=0.4547	Fem-Aft	F(1,18)=27.760; p=0.0000	Fem-Mat	F(1,18)=40.516; p=0.0000
geranyl acetate	Vir-Mor	F(1,18)=3.1294; p=0.0938	Mal-Mor	F(1,18)=18.643; p=0.0004	Mal-Vir	F(1,18)=28.193; p=0.0001
	Vir-Aft	F(1,18)=0.3114; p=0.5837	Mal-Aft	F(1,18)=81.724; p=0.0000	Mal-Mat	F(1,18)=49.360; p=0.0000
	Mat-Mor	F(1,18)=0.5635; p=0.4626	Fem-Mor	F(1,18)=36.856; p=0.0000	Fem-Vir	F(1,18)=42.204; p=0.0000
	Mat-Aft	F(1,18)=0.4377; p=0.5166	Fem-Aft	F(1,18)=15.924; p=0.0009	Fem-Mat	F(1,18)=12.494; p=0.0024

B

Sensory organ: Palps						
Stimulus	**Males vs. Females**		**Virgins vs. Mated**		**Morning vs. Afternoon**	
α-farnesene	Vir-Mor	F(1,18)=3.7751; p=0.0629	Mal-Mor	F(1,18)=0.0023; p=0.9623	Mal-Vir	F(1,18)=40.384; p=0.0000
	Vir-Aft	F(1.18)=2.1508; p=0.1523	Mal-Aft	F(1,18)=0.8921; p=0.3609	Mal-Mat	F(1,18)=22.567; p=0.0003
	Mat-Mor	F(1,18)=1.6126; p=0.2212	Fem-Mor	F(1,18)=0.2978; p=0.5910	Fem-Vir	F(1,18)=27.246; p=0.0000
	Mat-Aft	F(1,18)=1.6971; p=0.2137	Fem-Aft	F(1,18)=0.1752; p=0.6811	Fem-Mat	F(1,18)=11.856; p=0.0031
β-farnesene	Vir-Mor	F(1,18)=0.0272; p=0.8706	Mal-Mor	F(1,18)=0.5888; p=0.4510	Mal-Vir	F(1,18)=47.569; p=0.0000
	Vir-Aft	F(1.18)=7.0217; p=0.0169	Mal-Aft	F(1,18)=0.2303; p=0.6365	Mal-Mat	F(1,18)=17.346; p=0.0005
	Mat-Mor	F(1,18)=10.983; p=0.0041	Fem-Mor	F(1,18)=8.4103; p=0.0116	Fem-Vir	F(1,18)=5.1540; p=0.0395
	Mat-Aft	F(1,18)=5.0659; p=0.0358	Fem-Aft	F(1,18)=1.3368; p=0.2636	Fem-Mat	F(1,18)=14.742; p=0.0013
2,3-butanediol	Vir-Mor	F(1,18)=3.0558; p=0.0966	Mal-Mor	F(1,18)=0.8278; p=0.3728	Mal-Vir	F(1,18)=5.4285; p=0.0294
	Vir-Aft	F(1.18)=0.4825; p=0.4995	Mal-Aft	F(1,18)=5.3787; p=0.0311	Mal-Mat	F(1,18)=32.636; p=0.0000
	Mat-Mor	F(1,18)=9.2027; p=0.0075	Fem-Mor	F(1,18)=4.3342; p=0.0562	Fem-Vir	F(1,18)=5.9397; p=0.0375
	Mat-Aft	F(1,18)=6.0005; p=0.0236	Fem-Aft	F(1,18)=0.0007; p=0.9785	Fem-Mat	F(1,18)=15.466; p=0.0011
linalool	Vir-Mor	F(1,18)=0.1128; p=0.7406	Mal-Mor	F(1,18)=2.3143; p=0.1424	Mal-Vir	F(1,18)=22.724; p=0.0001
	Vir-Aft	F(1.18)=0.0138; p=0.9078	Mal-Aft	F(1,18)=0.1353; p=0.7169	Mal-Mat	F(1,18)=8.4163; p=0.0088
	Mat-Mor	F(1,18)=26.186; p=0.0001	Fem-Mor	F(1,18)=7.5069; p=0.0159	Fem-Vir	F(1,18)=19.200; p=0.0007
	Mat-Aft	F(1,18)=0.8351; p=0.3717	Fem-Aft	F(1,18)=0.3515; p=0.5616	Fem-Mat	F(1,18)=47.445; p=0.0000
β-myrcene	Vir-Mor	F(1,18)=0.6331; p=0.4360	Mal-Mor	F(1,18)=4.8851; p=0.0378	Mal-Vir	F(1,18)=22.245; p=0.0001
	Vir-Aft	F(1.18)=0.0001; p=0.9916	Mal-Aft	F(1,18)=2.0575; p=0.1669	Mal-Mat	F(1,18)=16.801; p=0.0006
	Mat-Mor	F(1,18)=34.012; p=0.0000	Fem-Mor	F(1,18)=6.1868; p=0.0261	Fem-Vir	F(1,18)=14.210; p=0.0023
	Mat-Aft	F(1,18)=3.3232; p=0.0833	Fem-Aft	F(1,18)=0.1939; p=0.6656	Fem-Mat	F(1,18)=44.954; p=0.0000
geranyl acetate	Vir-Mor	F(1,18)=0.0017; p=0.9678	Mal-Mor	F(1,18)=23.520; p=0.0001	Mal-Vir	F(1,18)=8.0052; p=0.0111
	Vir-Aft	F(1.18)=0.5594; p=0.4642	Mal-Aft	F(1,18)=24.699; p=0.0001	Mal-Mat	F(1,18)=36.343; p=0.0000
	Mat-Mor	F(1,18)=11.077; p=0.0037	Fem-Mor	F(1,18)=16.260; p=0.0008	Fem-Vir	F(1,18)=13.194; p=0.0019
	Mat-Aft	F(1,18)=3.7143; p=0.0699	Fem-Aft	F(1,18)=14.603; p=0.0013	Fem-Mat	F(1,18)=93.904; p=0.0000

Table 1