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# Contact chemoreceptive mate recognition in *Cerambyx welensii* Küster (Coleoptera: Cerambycidae)

# Mediación de la quimiorrecepción por contacto en el apareamiento en *Cerambyx welensii* Küster (Coleoptera: Cerambycidae)

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## Abstract

1. *Cerambyx welensii* Küster (Coleoptera: Cerambycidae) is a wood-borer responsible for the decline of Mediterranean oaks in open woodlands.
2. To establish that contact pheromones are involved in mate recognition of *C. welensii*, we extracted the cuticular hydrocarbons by solid phase microextraction at pre-reproductive, beginning and ending of the reproductive period, and by solvent extraction of prothorax and elytra. The extracts were analysed by GC–MS under electron impact and chemical ionization conditions. Cuticular hydrocarbon profiles varied according to the reproductive period, although differences between sexes were not significant. Two compounds, 11/13-methylheptacosane and 11-methylnonacosane were more abundant in females at the beginning and ending of the reproductive periods. Compound 11/13-methylheptacosane was also more representative in female prothorax than in males, and 2-methyloctacosane was richer in male elytra than in females.
3. We also studied the role of cuticular hydrocarbons in mate recognition in arena bioassays. Treatment of solvent-washed dead females and glass dummies with one female equivalent (FE) of cuticular extract elicited mating responses in males, especially at the beginning of the reproductive period, with copulation attempts reaching 61.9% on solvent-washed dead females and 23.8% on dummies. The successive treatment with synthetic compounds approaching a male cuticular profile inhibited male response.
4. Our results confirm that contact pheromones mediate mate recognition in *C. welensii*. Knowledge of the precise role played by the major compounds 11/13-methylheptacosane and 11-methylnonacosane and other minor compounds representative in female prothorax may contribute to the development of novel management strategies against *C. welensii*.

KEYWORDS
11/13-methylheptacosane, 11-methylnonacosane, Cerambycidae, *Cerambyx welensii*, cuticular hydrocarbons, mating behaviour, nonacosane

# INTRODUCTION

Mediterranean oak open woodlands (*dehesas* in Spanish, *montados* in Portuguese) are cited in the literature as a model example of a *High Natural Value* farming system, protected under the Habitats Directive 92/43/EEC (Council of the European Communities, 1992). In the last two decades, *Quercus* species are experiencing a severe decline, mainly in Mediterranean oak open woodlands, due to environmental stress, regeneration problems and/or incidence of biotic agents, such as soil born and leaf pathogens, or defoliating pests (Correia et al., 2014; Domínguez et al., 2022; Duque-Lazo & Navarro-Cerrillo, 2017). Physiological deterioration of oak trees has favoured conditions for the development of wood borer pests, such as *Cerambyx welensii* Küster (Duque-Lazo & Navarro-Cerrillo, 2017).

*Cerambyx welensii* is a large (up to 60 mm long) cerambycid, with a broad range of tree hosts, mainly from the genus *Quercus*. Adults have crepuscular and early nocturnal habits, with a flight period that spans from mid-May to late-July after following a semi-voltine cycle that requires 3 years to complete a generation. The larvae turn into pupae after summer (August–October) and adults cross a phase of ripeness remaining inside the tree during the winter and spring (from August–October to May).

Although feeding habits of adults usually do not cause an adverse impact on tree health, however, larvae bore into the wood causing breaks in trunks and tree branches, increasing the risk of infection by wood-decaying fungi and plant pathogens (Martín et al., 2005). The inflicted damage can also facilitate colonization by other large cerambycids, such as *Cerambyx cerdo* L. and *Prinobius myardi* Mulsant (Sallé et al., 2014). When *C. welensii* population levels increase, trees can be colonized regardless of their age and physiological status, and larval damage can become severe (Domínguez et al., 2022; Torres-Vila et al., 2022). These reasons have led to consider *C. welensii* one of the most problematic pests for Mediterranean oak open woodlands (Domínguez et al., 2022; Sallé et al., 2014; Torres-Vila et al., 2022).

Knowledge of the effect of semiochemical compounds in insect behaviour has become one of the pillars of IPM (Allison et al., 2004; Reddy et al., 2020; Smart et al., 2013). Among other effects, semiochemicals can influence host selection for oviposition and location of congeners, the use of pheromones being an integral part of current IPM strategies (Rizvi et al., 2021). Pheromones are considered an essential factor in the mating behaviour of cerambycid beetles, and an effective distance of pheromones varies among species, with some pheromones acting only after direct contact between both sexes (Fukaya et al., 1999). Insects' cuticle functions as a protection agent of internal organs, anchorage for muscles providing safety against biotic and abiotic factors (pathogens, desiccation, excessive humidity), and prevention of entry of chemicals (Ginzel & Blomquist, 2016). In this context, many insect cuticular hydrocarbons (CHCs) act as contact pheromones, and provide information on the insect age, sex and reproductive status (Ginzel, 2010). In Cerambycidae, the presence of contact pheromones has been confirmed in more than 20 species (Allison et al., 2004; Millar & Hanks, 2017; Xu & Teale, 2021). Determination of the factors associated with cuticular hydrocarbon variation in insects have been proposed as an important step towards their potential utility in pest monitoring and management strategies (Ngumbi et al., 2020). As example, a new control method for *Anoplophora* beetles has been proposed by using a contact sex pheromone in baited traps containing an entomopathogenic fungus (Fujiwara-Tsujii et al., 2019).

Preference of *C. welensii* to colonize weak, old trees has been widely observed; and, thus, aggregation phenomena have been reported on cork oak trees showing damage and/or cortical exudates (López-Pantoja et al., 2008). Both sexes meet while they wander on the host trees, and mate when males recognize females by antennal contact, followed by a typical sequence consisting of dashing towards the female, licking her body surface and bending the abdomen for mating (Sánchez-Osorio et al., 2012). Neither visual nor morphological features have been yet reported as important factors in mate recognition of *C. welensii*, but preliminary tests suggested the possible mediation of contact chemoreception in the mate recognition of this species (López-Manzano et al., 2020). To test this hypothesis, in this paper we have studied the hydrocarbon profiles of the cuticle of elytra plus prothorax in both sexes by solid phase microextraction (SPME) along three phenological periods: pre-reproductive (PR), beginning (BR), and ending of the reproductive period (ER). Solid phase micro extraction is a non-destructive sampling method that has been proposed to yield a more representative profile of cuticular hydrocarbons which can be detected by male antennae (Lacey et al., 2008). However, since SPME does not provide quantification of the CHCs, hexane extracts of adults at the ER period were also considered. To assess the degree to which hydrocarbon profiles could vary across body regions with eventual relevance in *C. welensii* mating sequence, the hexane extracts were prepared from isolated prothorax and elytra at the ER period. In addition, we have conducted behavioural bioassays to determine the role of CHCs in mate recognition at BR and ER periods using solvent-washed dead females and glass dummies coated with one female equivalent (FE) of cuticular extract. Validation of a mate recognition mechanism in *C. welensii* based on contact chemoreception would contribute to understand its behavioural activity, particularly the processes involved in the reproductive success, that could help design new IPM strategies for this species (Sánchez-Osorio et al., 2016; Sánchez-Osorio et al., 2019; Torres-Vila et al., 2012).

# MATERIALS AND METHODS

## Insects

*Cerambyx welensii* adults were collected in the field from *Quercus suber* L. logs in 'Las Espechillas' dehesa (37°20′34.55″N, 6°19′16.58″O, 67 m.a.s.l. Huévar, Sevilla, Spain), in October 2011 to obtain CHCs at the PR period and May 2012 to get them at BR and ER periods. Overwintering specimens usually stay in their pupal chamber between September and May before starting the flight period. Insects were placed individually in covered PVC tubes fitted with perforated PVC plugs, which were kept in methacrylate cages (50 × 40 × 20 cm; 10 insects per cage), and maintained in semi-darkness at 20–28°C. They were allowed to emerge from the PVC tubes, and to interact with others freely in a similar way as they would expect in the wild. Insects were supplied with 10% sucrose solution and pieces of cork.

For the behavioural assays, insects were collected between May–June 2018 in 'Las Espechillas' dehesa and 'Los Centenales' dehesa (37°22′10.96″N, 6°53′37.69″O, 34 m.a.s.l. Gibraleón, Huelva, Spain). Specimens were separated by sex and placed in cages (three specimens per cage) similar to those described above and under the same laboratory conditions (55%–70% relative humidity and semi-darkness). Beetles were provided with 10% sucrose solution, and maintained in the cages for at least 48 h before the bioassays. The mating history of field-captured beetles was unknown.

## Chemical analyses

*Cuticular hydrocarbon extraction*. Sampling of CHCs was carried out by two methods: solid phase microextraction (SPME) and solvent extraction. The cuticle of elytra plus prothorax of both sexes was sampled by SPME along the following three phenological periods: the pre-reproductive stage (PR) (10–14 January), beginning of the reproductive period (BR) (1–6 June), and ending (ER) of the reproductive period (27–29 June). For each period nine males ($N_m$) and nine females ($N_f$) were sampled.

Solvent extraction was used to study possible differences in CHC contents between isolated prothorax and elytra of adults at the ER period (Nm = 5; Nf = 5). For SPME, a polydimethylsiloxane-coated fibre (1 cm long × 100 μm wide, Sigma-Aldrich, Madrid) was gently wiped over the cuticle of elytra (three times along their entire length) and prothorax (two times through the low ridged basal pronotum area), rotating the fibre between wipes. This procedure ensured no saturation of the SPME fibre. The fibre was then ready for analysis by coupled gas chromatography–mass spectrometry (GC–MS). Before each SPME operation, the fibre was conditioned for at least one hour into the GC injector at 250°C. For solvent extraction, the isolated cuticles from the prothorax and elytra of each insect were repeatedly rinsed (three times during 3 min each) with 4 mL of hexane (purity >99.5%, Sigma-Aldrich, Madrid) in a glass tube. The extracts of each prothorax and elytra were combined separately, concentrated under a slight nitrogen flow to 80 μL, and 2 μL aliquots injected for GC–MS analysis.

*Identification of cuticular hydrocarbons*. SPME fibres and solvent extracts were subjected to analysis by GC–MS at the Institute for Advanced Chemistry of Catalonia (CSIC, Barcelona, Spain) under electron impact (EI) and chemical ionization (CI) conditions. The EI analyses were done on a Thermo Finnigan Trace 2000 GC system coupled to a Trace MS quadrupole mass spectrometry (ThermoFischer Scientific, Madrid, Spain) equipped with a Thermo TR-5MS column (30 m × 0.25 mm ID × 0.25 μm) and working under EI mode (70 eV) in splitless mode and with helium as carrier gas (1 mL $min^{-1}$). Scanning range was 40–500 m/z at 2 scans $s^{-1}$. The injection temperature was 250°C and the oven was programmed from 40°C for 1 min, and then increased at 5°C $min^{-1}$ to 190°C, followed by 10°C $min^{-1}$ to 300°C and hold for 5 min at this temperature. For CI analyses, samples were injected into an Agilent 5973 Network MSD coupled to an Agilent GC 6890 Series using an HP-5MS column (30 m × 0.25 mm ID × 0.25 μm) (Agilent Technologies, Madrid, Spain) under identical chromatographic conditions to those used for the EI analyses. Samples were subjected to the CI mode (15 eV) with methane as the reagent gas. The linear n-alkanes were identified by comparison of their mass spectra and retention times with those of standards ($n$-$C_{10}$-$C_{40}$ alkane solution). The retention times of the identified *n*-alkanes were used to calculate the Kovats Index (KI) of the remaining peaks. Branched chain and other unknown compounds were identified by comparison of their mass spectra and their retention indices with those from literature (Lockey, 1985; Nelson, 1993), and/or commercial library (NIST Registry of Mass Spectral Data, 2005; Wiley, 2000).

For quantification purposes, a hexanic solution of dodecyl acetate (50 ng $\mu l^{-1}$) was used as external standard. One aliquot of 1 μL of the external standard was injected in GC at the beginning and end of the series corresponding to each sex. The amounts of CHCs per individual were quantified relative to the average peak area of the respective two injections of the standard. This procedure was applied both in SPME experiments and in hexane extracts.

## Behavioural assays

To investigate the role of CHCs in *C. welensii* mate recognition, two bioassays were carried out. The first bioassay was adapted from Ginzel et al. (2003) and aimed to study the mating behaviour of males in the presence of solvent-washed (2 × 10 mL hexane) dead females (freeze-killed at −20°C for 4 h). In the second bioassay glass dummies were used (hexagonal-head glass stoppers, pointed bottom, 60 mm total length, DD Biolab S.L., Barcelona), following a procedure adapted from Ibeas et al. (2008). Both bioassays were carried out in two phenological periods: BR ($N_m = 21$) and ER ($N_m = 18$). In each bioassay and phenological period the mating responses of the same male to the following treatments were recorded: (1) untreated solvent-washed dead females and dummies separately (hereafter called 'control' in both cases); (2) the same solvent-washed dead females and dummies coated with one female cuticular extract equivalent (1 FE) (López-Manzano et al., 2020); (3) the same treatment as in (2) plus a 0.1 mL aliquot of a synthetic hydrocarbon blend (see below) that approached the male cuticular profile; and (4) the same as in (3) plus another 0.3 mL aliquot of the synthetic hydrocarbon blend (a total 0.4 mL of the hydrocarbon solution was applied). We considered a time delay between treatments of 10–20 min, sufficient to ensure the complete evaporation of the solvent (hexane).

Female cuticular extracts were obtained by immersion of each freeze-killed insect in 10 mL of hexane for 3 min. This was repeated twice, the hexane extracts (20 mL) were combined and concentrated under nitrogen up to 1.2 mL to provide a 'one female equivalent' (1 FE) solution.

Since a well-established strategy for recognizing cues of contact chemoreception in other Cerambycidae species has been to test the bioactivity of the compounds that are predominant in one of the sexes although detected in both (Lacey et al., 2008; Silk et al., 2011), we prepared a mixture of 100 μg ml$^{-1}$ in hexane of commercially available synthetic hydrocarbons that were dominant in males' cuticle. The compounds considered were heptacosane (C27), as major component in male prothorax (21.2% of relative presence), and nonacosane (C29), hentriacontane (C31) and hexacosane (C26) with 3.1, 1.4 and 1.1%, respectively, of presence in males' cuticle at the ER period, where CHCs profiles showed higher differences between sexes. These hydrocarbons were mixed in similar ratio as found in *C. welensii* males, that is, C27:C29:C31:C26 79:11.5:5.3:4.2.

Due to the passivity that isolated males usually show in presence of live females in arena bioassays (López-Manzano et al., 2020), the behaviour of the three males of each cage was recorded simultaneously. Once a male had established contact with a female or dummy, the male was taken away. A new solvent-washed dead female or glass dummy was presented to each cage for a total of six cages for the ER period, and seven cages for the BR period. All solvent-washed dead females had similar size (4.3 ± 0.37 cm long, 1.24 ± 0.15 cm base-elytra width), and proceeded from individuals of the same period (PR or ER) than the males used in the bioassays.

Observations were made at twilight, when the insects started to be active, and at night, when they were readily active (generally between 9:00 p.m. and 2:00 a.m.), under an adjustable-intensity red light, and were videotaped. The observation time for each group of males was 20 min. A positive male response was considered when he performed at least the first of the following actions: (i) dashing after antennal contact followed by alignment of male's body with that of the female or dummy, (ii) beginning of licking, and (iii) abdominal bending to attempt mating (Lacey et al., 2008; Silk et al., 2011).

## Statistical analyses

*Identification of cuticular hydrocarbons*. Statistical differences of the mean relative abundance of each hydrocarbon between sexes along the three reproductive periods as well as from isolated prothorax and elytra extracts, were analysed using the Yuen–Welch test based on 20% trimmed means ($\text{mean}_{0.2} =$ mean obtained after removing the 20% of the top and bottom values). Change in CHCs profile between phenological periods and between prothorax and elytra was further investigated with a discriminant analysis. To achieve this and to reduce the number of variables, peaks representing less than 1% of the total area of all detected peaks were excluded, remaining 21 peaks in the SPME and 14 peaks in the hexane extract datasets. Prior to the discriminant analysis and since relative proportions are dependent variables, peak areas were standardized according to the formula of Reyment (1989), modified to avoid undefined values for peaks of zero areas: $Z_{i,j} = \text{Log}_{10}[A_{i,j}/g(A_j) + 1]$, where $Z_{i,j}$ is the corrected area of the peak i for insect j, $A_{i,j}$ is the area of the peak i for insect j, and $g(A_j)$ is the geometric mean of the areas of all peaks for insect j.

*Behavioural assays*. To evaluate the proportion of males responding to both bioassays in each of the two phenological periods, data were analysed by Cochran's Q test for dichotomous data from repeated measures, separately for each type of behaviour (dashing, licking or abdominal bending, see above) exhibited by males, and followed by Marascuilo and McSweeney procedure for pairwise comparisons. The Cochran–Mantel–Haenszel test (CMH) together with the Woolf test to analyse the homogeneity of the Odds Ratios were used to determine whether the phenological period affects the sexual responses of males. The exact binomial test with Yate's correction was used when one-proportion test was necessary. Statistical analyses were performed using the R framework (version 3.6.0) with $\alpha = 0.05$ for significance.

# RESULTS

## Identification of cuticular hydrocarbons

A total of 45 compounds were detected across all SPME and hexanic extract samples (Figures 1 and 2, Table 1). Predominant compounds were branched alkanes, particularly those in the range C27-C31; only 10 compounds were straight-chain alkanes and 3 were alkenes, one of them branched. The CHCs profiles of prothorax and elytra obtained by SPME varied with the phenological period considered (Figure 1, Table 2), particularly in the PR period (23 compounds) relative to the BR or ER periods (38 and 42 compounds, respectively). Compared with the latter periods, the CHC profile in PR period had fewer amounts of the shortest (C20-C24) and the longest (>C32) chain hydrocarbons identified. Interestingly, 11/13-methylheptacosane (**24**), which was the most relevant compound in BR and ER samples and in extracts of prothorax and elytra (relative abundance >32% in females and > 22% in males), was absent in PR samples. In contrast, the most abundant peak in PR was absent in both BR and ER samples (compound **33**, possibly a branched C29 hydrocarbon of $M^+$ 422). Extracts from prothorax and elytra showed a similar amount of CHCs (34 and 33, respectively) (Figure 2 and Table 2). The total amount of CHCs varied among individuals and sampling methods (Table S1). In hexane extracts, the average amounts of all CHCs per insect at ER period were 18.14 μg/male and 5.45 μg/female in elytra, and 1.33 μg/male and 1.29 μg/female in prothorax. The amounts of all CHCs collected in SPME samples dropped from PR to ER periods, averaging 563.66 ng/male and 227.33 ng/female at PR, 217.38 ng/male and 216.72 ng/female at BR, and 44.02 ng/male and 176.82 ng/female at ER period.

Some differences were found among the identified compounds in solvent extraction versus SPME. Only one minor compound (**11**) of very low relative abundance (<0.5%) and unknown structure was detected in hexane extracts but not in SPME samples. However, up to seven compounds, most of them with very low relative abundance,

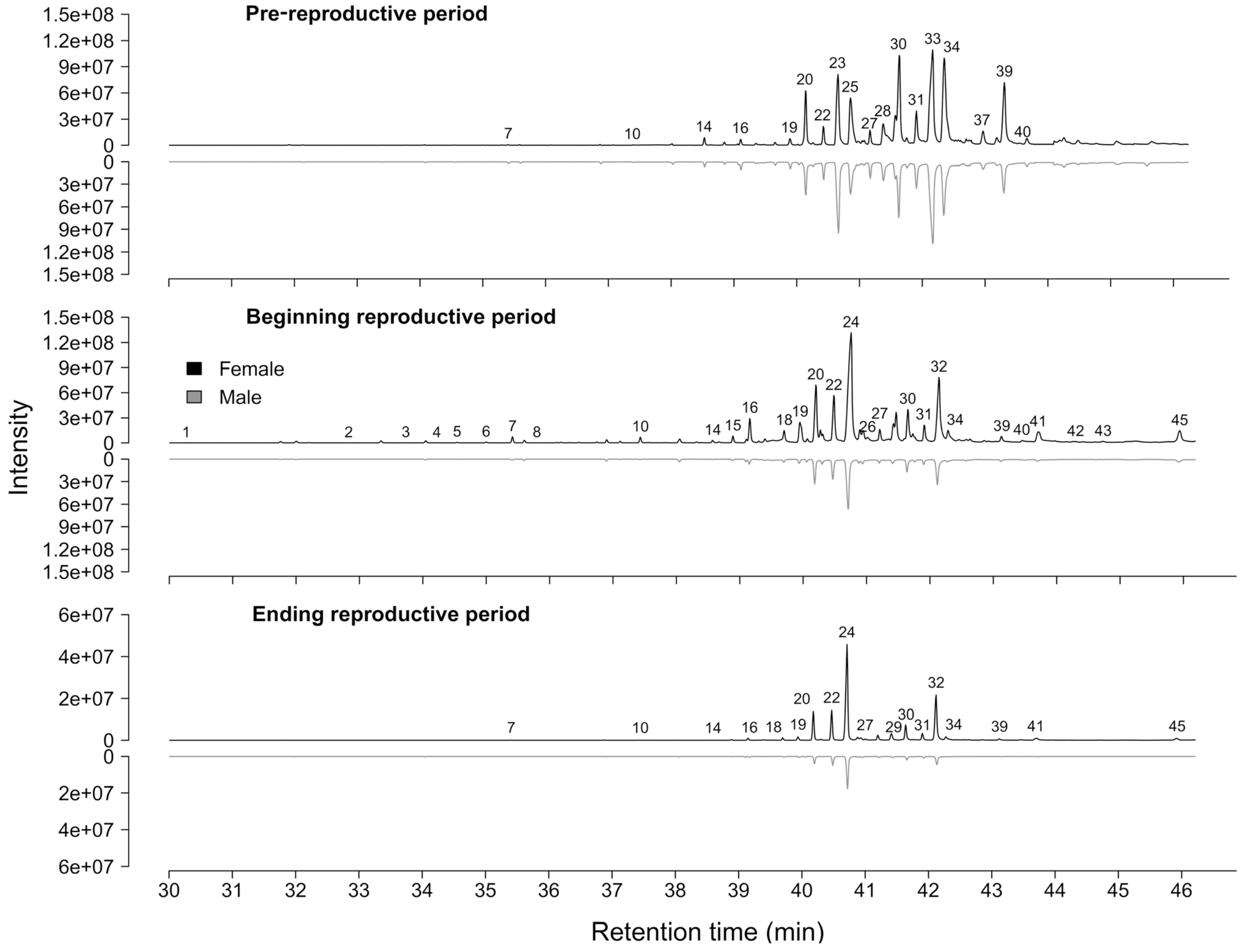

**FIGURE 1** Representative total ion chromatograms of cuticular hydrocarbon profiles in SPME extracts of prothorax and elytra of *Cerambyx welensii* males and females obtained along three phenological periods: pre-reproductive (upper plot), beginning of reproductive period (center plot), and ending of the reproductive period (lower plot). Identification of peaks is described in Table 1. For improving readability, labels corresponding to some minor peaks are omitted.

were detected in SPME but not in hexane extracts. In addition, heptacosane (**22**) and 2-methyloctacosane (**30**) were preferentially found in hexane extracts (>10% overall) than in SPME (<8% overall); in contrast, 11-methylnonacosane (**32**) reached higher proportions in SPME (>13% overall) than in hexane extracts (≤8% overall). Interestingly, all compounds from one sex detected by SPME were also present in the other; however, in prothorax extracts five minor compounds were only detected in females: heptacosene-9 (**21**), 12/14/16-methyltriacontane (**37**), 11/12/13-methyldotriacontane (**43**), and unknowns 5 (**29**) and 8 (**42**).

In SPME samples and over BR and ER periods, only two compounds, 11/13-methylheptacosane (**24**) and 11-methylnonacosane (**32**), exceeded 10% relative abundance, comprising together >49% in females and > 39% in males. In solvent extracts of prothorax and elytra, 11/13-methylheptacosane (**24**) was the most prominent compound, along with heptacosane (**22**), 2-methyloctacosane (**30**) and 11-methylnonacosane (**32**). The first three compounds surpassed 10% relative abundance, and together comprised a presence >57% of the total CHCs in females and > 54% in males.

Discriminant analysis applied to the three periods showed that 85.3% of the between-class variance can be explained by the first linear discriminant function (LD1) (Figure 3, upper biplot). The scaling coefficients showed that LD1 was mostly defined by unknown 6 (**33**), 11/13-methylheptacosane (**24**) and 11-methylnonacosane (**32**), with lesser contribution of 13/14/15/16-methylhentriacontene (**41**), 11/13-methylpentacosane (**16**) and 2-methylhexacosane (**20**). Function LD2, which explained lower between-class variance than LD1 (9.75%), was mostly defined by 11/13-methylheptacosane (**24**), unknown 4 (**23**), and hentriacontane (**40**). The distribution of insects based on their CHC profiles showed a clear differentiation by phenological periods, with PR phase being segregated by LD1 and BR and ER periods by LD2 particularly. The CHCs profiles showed slight differences between males and females at the PR period (Figure 3, upper biplot), probably due to the significant differences between sexes found in the relative abundance of two prominent compounds: unknown 4 (**23**, relative abundance 16.59% and 10.02% in males and females, respectively) (Yuen test: $t = 2.658$, df $= 9.60$, $p = 0.025$),

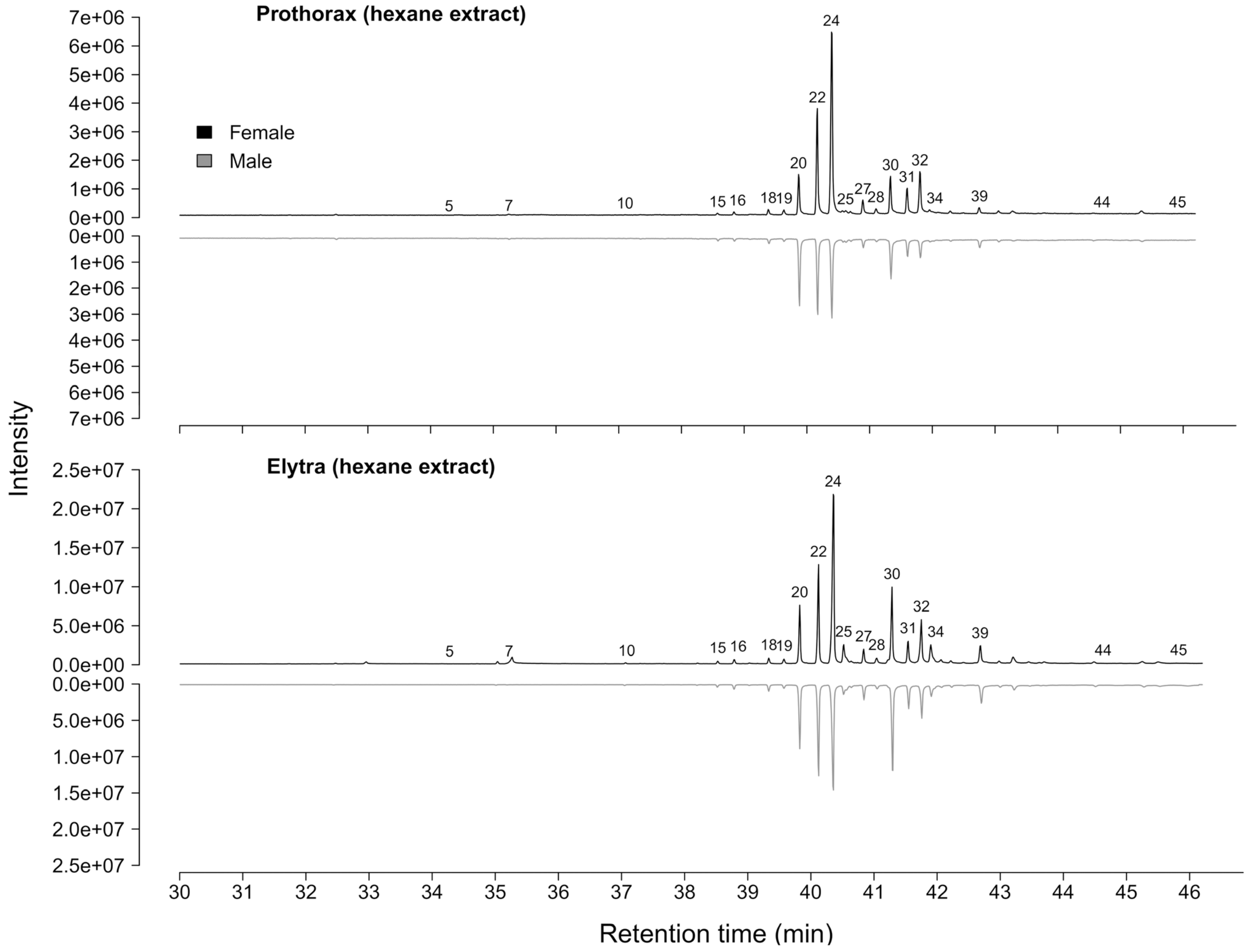

**FIGURE 2** Representative total ion chromatograms of cuticular hydrocarbon profiles in hexane extracts of separated prothorax and elytra of *Cerambyx welensii* males and females obtained at the end of the reproductive period (ER). Identification of peaks is described in Table 1. For improving readability, labels corresponding to some minor peaks are omitted.

and heptacosane (**22**, relative abundance 3.71% and 1.74% in males and females, respectively) (Yuen test: $t = 2.825$, df $= 7.80$, $p = 0.023$).

The discriminant analysis showed no difference between sexes in CHCs profiles at the BR period (Figure 3, upper biplot). In fact, only unknown 8 (**42,** relative abundance <0.2%) showed a significant higher presence in males (Yuen test: $t = 3.225$, df $= 8.58$, $p = 0.011$). In the ER period, however, although discriminant analysis did not clearly separate CHCs of both sexes, compounds 11-methylnonacosane (**32**) and unknown 3 (**12**) presented significantly higher relative abundance in females, with former compound being richer (relative abundance 17.46% and 13.88% in females and males, respectively) (Yuen test: $t = 3.346$, df $= 10.36$, $p = 0.007$). In addition, nine compounds (pentacosane, **15**; 2-methylpentacosane, **17**; hexacosane, **18**; 11/12/13-methylhexacosane, **19**; octacosane, **27**; nonacosane, **31**; triacontane, **36**; 2-methyltriacontane, **39**; and hentriacontane, **40**) showed higher abundance in males (Table 2), with nonacosane (**31**) being the most relevant (relative abundance 3.09% and 2.49% in males and females, respectively) (Yuen test: $t = 3.704$, df $= 8.44$, $p = 0.005$).

Discriminant analysis applied to the CHCs present in prothorax and elytra separately showed that 98.66% of the between-class variance was explained by the first linear discriminant function (LD1) (Figure 3, lower biplot). The scaling coefficients showed that LD1 was mostly defined again by 11/13-methylheptacosane (**24**), with lower contribution of 11,15-dimethylnonacosane (**34**), 2-methylhexacosane (**20**), 11/12/13-methylhexacosane (**19**) and 11,15-dimethylheptacosane (**25**). The LD2 function was mostly defined by 11/13-methylheptacosane (**24**), 2-methylhexacosane (**20**), 2-methyloctacosane (**30**), octacosane (**27**) and 11/12/13-methylhexacosane (**19**). The LD1 function clearly discriminated between sexes in prothorax and elytra, with 11/13-methylheptacosane (**24**) having a high contribution to characterize a female CHC profile (the peak 24 vector points to the female group, and its length indicates a larger relative contribution than the other compounds). Differences in CHC profiles between prothorax and elytra were less clear based in the LD2 function. Once again, 11/13-methylheptacosane (**24**) apparently play a relevant role as prothoracic female hydrocarbon(s). In elytra extracts, 2-methyloctacosane (**30**) showed a significantly higher abundance in males (16.56%) versus females (10.22%) (Yuen test: $t = 3.297$,

**TABLE 1** Identification, retention time (RT), retention index (Kovats Indices, KI), and diagnostic m/z ions detected in SPME and hexane extracts of prothorax and elytra of *Cerambyx welensii* males and females by GC–MS analysis.

| Peak | Identification | RT (min) | KI exp.[a] | KI lib.[b] | Diagnostic m/z ions |
|---|---|---|---|---|---|
| 1 | unknown 1 | 30.24 | nd | | M +? |
| 2 | 9-Me-C19 | 32.81 | 1939 | 1936 | 140/141; 168/169; 282 (M+) |
| 3 | C20 | 33.72 | 2000 | 2000 | 282 (M+) |
| 4 | 10-Me-C20 | 34.21 | 2038 | 2041.6 | 154/155; 168/169; 296 (M+) |
| 5 | 2-Me-C20 | 34.52 | 2063 | 2063 | 253/254; 296 (M+) |
| 6 | C21 | 35.0 | 2100 | 2100 | 296 (M+) |
| 7 | 11-Me-C21 | 35.4 | 2138 | 2139 | 168/169; 310 (M+) |
| 8 | C22-1-ene | 35.8 | 2176 | 2175 | 308 (M+) |
| 9 | C23 | 37.1 | 2300 | 2300 | 324 (M+) |
| 10 | 11-Me-C23 | 37.44 | 2338 | 2338 | 168/169; 196/197; 338 (M+) |
| 11 | unknown 2 | 37.64 | 2360 | | M +? |
| 12 | unknown 3 | 38.02 | 2402 | | M +? |
| 13 | 12-Me-C24 | 38.31 | 2434 | 2434 | 182/183; 352 (M+) |
| 14 | 2-Me-C24 | 38.59 | 2466 | 2465 | 352 (M+) |
| 15 | C25 | 38.9 | 2500 | 2500 | 352 (M+) |
| 16 | 11/13-Me-C25 | 39.17 | 2533 | 2533 | 168/169; 224/225; 366 (M+) |
| 17 | 2-Me-C25 | 39.38 | 2559 | 2560 | 323/324; 366 (M+) |
| 18 | C26 | 39.71 | 2600 | 2600 | 366 (M+) |
| 19 | 11/12/13-Me-C26 | 39.94 | 2630 | 2629 | 168/169; 182/183; 196/197; 224/225; 238/239; 380 (M+) |
| 20 | 2-Me-C26 | 40.19 | 2662 | 2662 | 337/338;380 (M+) |
| 21 | C27-9-ene | 40.31 | 2678 | 2678 | 97/98; 111/112; 378 (M+) |
| 22 | C27 | 40.48 | 2700 | 2700 | 380 (M+) |
| 23 | unknown 4 | 40.66 | 2725 | | 97/98; 168/169; 182/183; 196/197; 210/211; 224/225; 238/239; 252/253; 392 (M+) |
| 24 | 11/13-Me-C27 | 40.71 | 2732 | 2731 | 168/169; 196/197; 224/225; 252/253; 394 (M+) |
| 25 | 11,15-DiMe-C27 | 40.88 | 2755 | 2756 | 168/169;196/197;239/240;267/268;408 (M+) |
| 26 | 3-Me-C27 | 41.05 | 2778 | 2775 | 364/365; 394 (M+) |
| 27 | C28 | 41.21 | 2800 | 2800 | 394 (M+) |
| 28 | 11/12/13/14-Me-C28 | 41.41 | 2829 | 2829 | 168/169;182/183;196/197;210/211; 224/225; 238/239;252/253;266/267;408 (M+) |
| 29 | unknown 5 | 41.59 | 2855 | | 408 (M+) |
| 30 | 2-Me-C28 | 41.64 | 2862 | 2864 | 365/366; 408 (M+) |
| 31 | C29 | 41.9 | 2900 | 2900 | 408 (M+) |
| 32 | 11-Me-C29 | 42.12 | 2930 | 2930 | 168/169; 280/281; 422 (M+) |
| 33 | unknown 6 | 42.19 | 2940 | | 422 (M+) |
| 34 | 11,15-DiMe-C29 | 42.28 | 2952 | 2950 | 168/169; 224/225; 239/240; 295/296; 436 (M+) |
| 35 | unknown 7 | 42.43 | 2973 | | 422 (M+) |
| 36 | C30 | 42.63 | 3000 | 3000 | 422 (M+) |
| 37 | 12/14/16-Me-C30 | 42.84 | 3026 | 3030 | 182/183; 210/211; 224/225; 238/239; 252/253; 280/281; 436 (M+) |
| 38 | 12,16-DiMe-C30 | 43.05 | 3051 | 3055 | 182/183; 224/225; 253; 295 |
| 39 | 2-Me-C30 | 43.12 | 3060 | 3060 | 393/394; 421/422; 436 (M+) |
| 40 | C31 | 43.45 | 3100 | 3100 | 436 (M+) |
| 41 | 13/14/15/16-Me-C31-ene[c] | 43.69 | 3125 | 3126 | 97/98; 111/112; 224/225; 252/253; 448 (M+) |
| 42 | unknown 8 | 44.26 | 3184 | | 450 (M+)? |

**TABLE 1** (Continued)

| Peak | Identification | RT (min) | KI exp.[a] | KI lib.[b] | Diagnostic m/z ions |
|---|---|---|---|---|---|
| 43 | 11/12/13-Me-C32 | 44.72 | 3224 | 3225 | 168/169; 182/183; 196/197; 464 (M+) |
| 44 | unknown 9 | 45.01 | 3247 | | 464 (M+) |
| 45 | unknown 10 | 45.93 | 3319 | | 478 (M+) |

[a] KI experimental.

[b] KI from library (PubChem, Pherobase).

[c] Position of the unsaturation is unknown.

df = 3.72, $p = 0.033$), whereas the relative abundance of 11-methylheneicosane (**7**) resulted higher in females (0.27%) than in males (0.12%) (Yuen test: $t = 3.108$, df = 3.39, $p = 0.045$). In contrast, 11 compounds were found either only in female prothorax or in higher abundance in females (Table 2), the most remarkable being 11/13-methylheptacosane (**24**) (relative abundance of 35.41% and 26.58% in females and males, respectively) (Yuen test: $t = 2.941$, df = 3.61, $p = 0.048$), and 11-methylnonacosane (**32**) (8.02% and 4.74% in females and males, respectively) (Yuen test: $t = 3.884$, df = 2.99, $p = 0.030$).

## Role of contact chemoreception

Male responses to untreated solvent-washed dead females were very low (dashing response: 3.70% and 6.88% at BR and ER periods, respectively) in all behaviours considered (dashing, licking and abdominal bending), and no response at all was noticed to untreated glass dummies (Figure 4). However, when a solvent-washed dead female was treated with 1 FE extract, 71% of males at the BR period dashed to her after touching with the antennae. In addition, 62% of males licked the prothorax and elytra cuticle before bending the abdomen to attempt copula (Figure 5). However, at the ER period the number of males performing dashing behaviour dropped to 11%, and neither licking nor abdominal bending behaviours were displayed (Cochran–Mantel–Haenszel: $M^2 = 23.972$, 22.882 and 20.083, for dashing, licking and abdominal bending, respectively, df = 3, $p < 0.001$). When the same FE-coated dead female was successively treated with 0.1 mL (1.9 MEs) of the synthetic hydrocarbon blend potentially approaching the male cuticular profile, males responded equally well (71%) to the dashing and licking behaviours at the BR period, but in bending behaviour the response of males dropped to 43%. The difference was not significant. In contrast, a clear reduction in male responses was noticed when the FE-coated dead females were successively treated with 0.4 mL (7.7 MEs) of the hydrocarbon blend wherein the percentage of male responses dropped to 43% (dashing) and 33% (licking and bending) (Figure 4). When the same sequence of treatments was applied to a glass dummy at BR period, application of 1 FE extract also resulted in a high male response to the dashing (67%) (Figure 5), although moderate to the licking (33%) and bending (24%). Here again, the successive addition of 0.1 and 0.4 mL of the hydrocarbon blend potentially approaching the male cuticular profile resulted in a sharp loss of male response (Figure 4). When males were assayed at the ER period, they showed a similar pattern of behaviour to solvent-washed dead females than at the BR period, with a clearly lower percentage of males displaying all behaviours (Figure 4). When glass dummies were used, very low responses by males were observed after application of 1 FE extract.

# DISCUSSION

Cuticular hydrocarbons have been reported to act as highly multifunctional pheromones, with diverse roles in intraspecific social interactions (Ferveur & Cobb, 2010; Ingleby, 2015; Steiger et al., 2007). In Cerambycidae, the CHCs implied in mate recognition through contact chemoreception in many cases comprise *n*-alkanes and mono-methyl branched alkanes from 25 to 28 carbon atoms (Ginzel, 2010). In a number of species, single CHCs have been reported to display contact pheromonal activity, such as *n*-pentacosane in *Xylotrechus colonus* F. (Ginzel et al., 2003), 7-methylheptacosane in *Neoclytus acuminatus* F. (Lacey et al., 2008), or 11-methylheptacosane in *Tetropium fuscum* F. (Silk et al., 2011), but more complex blends can display that activity as well, f.i., the blend (Z)-9-tricosene, (Z)-9-pentacosene, (Z)-7-pentacosene, (Z)-9-heptacosene and (Z)-7-heptacosene produced by *Anoplophora glabripennis* Motschulsky (Zhang et al., 2003), or *Anoplophora malasiaca* (Thomson) females (Fukaya et al., 1999). The cues for host selection and mate location in *C. welensii* are not well known. This insect has been found to be attracted by fermentation-related odours emitted by weak trees or damaged plant tissues (Sánchez-Osorio et al., 2016; Torres-Vila et al., 2012), and a foliar emission profile from host trees could also mediate in adult encounters (Sánchez-Osorio et al., 2019).

Male antennal contacts have been found to promote intraspecific encounters with females for mating (Ginzel & Hanks, 2003; Hanks et al., 1996). In our bioassays, *C. welensii* males exhibited a mating behaviour similar to that described for other Cerambycidae species, including antennal contact, dashing towards the female, licking of pronotum and basal area of elytra, and copulation attempt (Ibeas et al., 2008; Kiriyama et al., 2018). The antennal contact is favoured in *C. welensii* by the male antennal length which doubles the body length (Torres-Vila et al., 2018), as found in other longhorn beetles (Hanks et al., 1996). In this context, the maxillary and/or labial palps of males could also play a role in the mating process in *C. welensii*, since when their antennae were coated with parafilm the dashing behaviour was

**TABLE 2** Relative abundance (%, mean$_{0.2}$ ± error$_{0.2}$) of cuticular hydrocarbons of *Cerambyx welensii* females and males identified in SPME samples (elytra and prothorax. $N_m = 9$, $N_f = 9$) obtained in the pre-reproductive (PR), beginning (BR), and ending of the reproductive period (ER), and hexane extracts (prothorax and elytra separately at the ER period, $N_m = 5$, $N_f = 5$), by GC–MS analysis.

| | Relative abundance (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SPME (PR) | | SPME (BR) | | SPME (ER) | | Hexane extracts (prothorax) (ER) | | Hexane extracts (elytra) (ER) | |
| Peak | Female | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| 1 | nd | nd | 0.01 ± 0.001 | 0 ± 0.001 | 0.02 ± 0.007 | 0.03 ± 0.008 | nd | nd | nd | nd |
| 2 | nd | nd | 0.01 ± 0.002 | 0.01 ± 0.002 | 0.02 ± 0.006 | 0.01 ± 0.002 | 0.74 ± 0.491 | 0.38 ± 0.056 | nd | nd |
| 3 | nd | nd | 0.01 ± 0.002 | 0.01 ± 0 | 0.02 ± 0.006 | 0.02 ± 0.007 | nd | nd | nd | nd |
| 4 | nd | nd | 0.01 ± 0.002 | 0.01 ± 0.002 | 0.01 ± 0.007 | 0.02 ± 0.006 | nd | nd | nd | nd |
| 5 | nd | nd | 0.03 ± 0.015 | 0.03 ± 0.007 | 0.02 ± 0.008 | 0.03 ± 0.01 | nd | nd | nd | nd |
| 6 | nd | nd | 0.05 ± 0.019 | 0.04 ± 0.007 | 0.05 ± 0.017 | 0.12 ± 0.033 | nd | nd | nd | nd |
| 7 | 0.13 ± 0.040 | 0.37 ± 0.189 | 0.49 ± 0.216 | 0.61 ± 0.175 | 0.11 ± 0.015 | 0.25 ± 0.155 | **0.32 ± 0.053** | 0.08 ± 0.017 | **0.27 ± 0.02** | 0.12 ± 0.036 |
| 8 | nd | nd | 0.01 ± 0.003 | 0.01 ± 0.002 | 0.02 ± 0.009 | 0.03 ± 0.004 | nd | nd | nd | nd |
| 9 | nd | nd | nd | nd | 0.06 ± 0.016 | 0.09 ± 0.018 | nd | nd | nd | nd |
| 10 | 0.04 ± 0.011 | 0.18 ± 0.087 | 0.36 ± 0.142 | 0.36 ± 0.085 | 0.10 ± 0.016 | 0.12 ± 0.018 | 0.16 ± 0.057 | 0.06 ± 0.025 | 0.20 ± 0.073 | 0.07 ± 0.011 |
| 11 | nd | nd | nd | nd | nd | nd | 0.31 ± 0.274 | 0.13 ± 0.024 | 0.08 ± 0.067 | 0.03 ± 0.004 |
| 12 | nd | nd | nd | nd | **0.80 ± 0.126** | 0.18 ± 0.043 | 0.04 ± 0.005 | 0.06 ± 0.023 | 0.05 ± 0.007 | 0.04 ± 0.008 |
| 13 | nd | nd | nd | nd | 0.05 ± 0.008 | 0.07 ± 0.021 | nd | nd | nd | nd |
| 14 | 0.25 ± 0.126 | 0.33 ± 0.078 | 0.14 ± 0.057 | 0.20 ± 0.034 | 0.07 ± 0.016 | 0.13 ± 0.035 | 0.09 ± 0.02 | 0.10 ± 0.041 | 0.10 ± 0.005 | 0.08 ± 0.018 |
| 15 | 0.16 ± 0.032 | 0.30 ± 0.099 | 0.5 ± 0.09 | 0.50 ± 0.085 | 0.31 ± 0.027 | **0.47 ± 0.066** | 0.41 ± 0.017 | **0.62 ± 0.028** | 0.44 ± 0.072 | 0.42 ± 0.039 |
| 16 | 0.4 ± 0.143 | **1.39 ± 0.426** | 1.53 ± 0.709 | 2.37 ± 0.277 | 0.82 ± 0.078 | 1.05 ± 0.126 | 0.82 ± 0.095 | 0.71 ± 0.052 | 0.80 ± 0.29 | 0.70 ± 0.043 |
| 17 | nd | nd | nd | nd | 0.15 ± 0.064 | **0.37 ± 0.036** | 0.08 ± 0.038 | 0.11 ± 0.014 | 0.10 ± 0.022 | 0.14 ± 0.027 |
| 18 | 0.27 ± 0.072 | **0.64 ± 0.183** | 0.97 ± 0.153 | 0.98 ± 0.120 | 0.86 ± 0.027 | **1.10 ± 0.032** | 1.03 ± 0.039 | 1.41 ± 0.102 | 0.89 ± 0.094 | 1.21 ± 0.040 |
| 19 | 0.52 ± 0.055 | **1.61 ± 0.398** | 1.84 ± 0.422 | 2.18 ± 0.230 | 1.31 ± 0.116 | **1.52 ± 0.067** | **1.0 ± 0.014** | 0.71 ± 0.032 | 0.77 ± 0.057 | 0.72 ± 0.037 |
| 20 | 4.53 ± 0.520 | 5.92 ± 0.608 | 8.36 ± 0.536 | 8.92 ± 0.663 | 8.53 ± 0.300 | 9.44 ± 0.535 | 8.33 ± 0.518 | 13.38 ± 1.512 | 7.94 ± 0.09 | 10.19 ± 0.932 |
| 21 | 0.20 ± 0.036 | 0.64 ± 0.113 | 0.3 ± 0.079 | 0.56 ± 0.117 | 0.31 ± 0.027 | 0.63 ± 0.107 | **0.11 ± 0.006** | nd | 0.11 ± 0.030 | 0.08 ± 0.019 |
| 22 | 1.74 ± 0.299 | **3.71 ± 0.823** | 7.28 ± 1.051 | 6.20 ± 0.728 | 3.76 ± 1.902 | 5.81 ± 2.348 | 15.73 ± 1.206 | **21.22 ± 0.350** | 14.54 ± 0.227 | 15.2 ± 1.270 |
| 23 | 10.02 ± 1.558 | **16.59 ± 2.635** | 0.05 ± 0.031 | 0.04 ± 0.020 | 5.72 ± 1.157 | 6.91 ± 0.684 | nd | nd | nd | nd |
| 24 | nd | nd | 32.39 ± 3.096 | 27.73 ± 0.902 | 32.01 ± 3.122 | 25.80 ± 1.375 | **35.41 ± 1.587** | 26.58 ± 2.232 | 32.91 ± 2.640 | 22.50 ± 0.739 |
| 25 | 6.48 ± 1.307 | 5.85 ± 0.919 | 1.68 ± 0.1 | 1.98 ± 0.179 | 1.29 ± 0.151 | 1.56 ± 0.04 | 0.73 ± 0.150 | 0.50 ± 0.101 | 1.67 ± 1.010 | 2.01 ± 0.458 |
| 26 | 0.40 ± 0.154 | 0.58 ± 0.115 | 1.91 ± 0.313 | 1.99 ± 0.270 | 0.79 ± 0.036 | 0.82 ± 0.081 | 0.25 ± 0.061 | 0.43 ± 0012 | 0.24 ± 0.058 | 0.27 ± 0.015 |
| 27 | 1.60 ± 0.215 | 1.95 ± 0.128 | 2.64 ± 0.135 | 2.69 ± 0.216 | 1.95 ± 0.142 | **2.25 ± 0.091** | 2.58 ± 0.429 | 3.22 ± 0.298 | 2.16 ± 0.311 | 2.99 ± 0.332 |
| 28 | 2.75 ± 0.289 | 3.33 ± 0.239 | 0.36 ± 0.064 | 0.72 ± 0.219 | 3.00 ± 0.146 | 2.11 ± 0.275 | **1.09 ± 0.055** | 0.68 ± 0.019 | 0.95 ± 0.088 | 0.86 ± 0.019 |
| 29 | nd | nd | nd | nd | 0.22 ± 0.077 | 0.29 ± 0.086 | **0.37 ± 0.101** | nd | 0.34 ± 0.102 | 0.28 ± 0.011 |

**TABLE 2** (Continued)

| | Relative abundance (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | SPME (PR) | | SPME (BR) | | SPME (ER) | | Hexane extracts (prothorax) (ER) | | Hexane extracts (elytra) (ER) | |
| Peak | Female | Male | Female | Male | Female | Male | Female | Male | Female | Male |
| **30** | 6.55 ± 0.871 | 6.17 ± 0.801 | 6.01 ± 1.09 | 7.05 ± 0.79 | 6.38 ± 0.454 | 6.15 ± 0.217 | 10.05 ± 1.928 | 14.73 ± 1.947 | 10.22 ± 1.400 | **16.56 ± 1.060** |
| **31** | 2.33 ± 0.325 | 3.12 ± 0.495 | 2.77 ± 0.508 | 2.98 ± 0.53 | 2.49 ± 0.078 | **3.09 ± 0.119** | 4.77 ± 0.369 | 4.79 ± 0.172 | 4.14 ± 0.496 | 4.64 ± 0.232 |
| **32** | nd | nd | 16.89 ± 0.781 | 14.14 ± 1.575 | **17.46 ± 0.689** | 13.88 ± 1.001 | **8.02 ± 0.685** | 4.74 ± 0.354 | 7.82 ± 0.379 | 7.35 ± 0.656 |
| **33** | 19.65 ± 2.425 | 17.97 ± 1.012 | nd | nd | nd | nd | nd | nd | nd | nd |
| **34** | 14.3 ± 2.587 | 8.18 ± 1.691 | 2.97 ± 0.163 | 4.05 ± 0.904 | 1.70 ± 0.137 | 1.57 ± 0.109 | 0.54 ± 0.085 | 0.27 ± 0.031 | 2.08 ± 0.135 | 3.02 ± 0.387 |
| **35** | nd | nd | 0.48 ± 0.125 | 0.62 ± 0.108 | 0.66 ± 0.153 | 0.83 ± 0.171 | 0.12 ± 0.019 | 0.04 ± 0.010 | 0.37 ± 0.082 | 0.46 ± 0.090 |
| **36** | nd | nd | 0.45 ± 0.07 | 0.38 ± 0.037 | 0.43 ± 0.093 | **0.97 ± 0.063** | 0.49 ± 0.076 | 0.37 ± 0.074 | 0.43 ± 0.087 | 0.44 ± 0.037 |
| **37** | 1.29 ± 0.209 | 1.48 ± 0.383 | 0.31 ± 0.044 | 0.30 ± 0.055 | 0.47 ± 0.14 | 0.41 ± 0.099 | **0.12 ± 0.003** | nd | 0.15 ± 0.023 | 0.18 ± 0.015 |
| **38** | 0.54 ± 0.167 | 0.37 ± 0.092 | 0.09 ± 0.034 | 0.14 ± 0.038 | nd | nd | nd | nd | nd | nd |
| **39** | 4.46 ± 1.243 | 3.99 ± 0.974 | 1.19 ± 0.506 | 1.91 ± 0.519 | 1.01 ± 0.078 | **1.76 ± 0.101** | 2.34 ± 0.682 | 2.69 ± 0.148 | 3.09 ± 0.421 | 4.05 ± 0.257 |
| **40** | 0.34 ± 0.098 | **0.68 ± 0.158** | 0.24 ± 0.073 | 0.23 ± 0.029 | 0.68 ± 0.047 | **1.43 ± 0.233** | 0.54 ± 0.139 | 0.67 ± 0.009 | 0.57 ± 0.060 | 0.45 ± 0.029 |
| **41** | nd | nd | 3.10 ± 0.747 | 2.00 ± 0.488 | 1.59 ± 0.451 | 2.23 ± 0.22 | **0.75 ± 0.077** | 0.21 ± 0.041 | 1.68 ± 0.078 | 1.58 ± 0.334 |
| **42** | nd | nd | 0.08 ± 0.017 | **0.17 ± 0.022** | 0.26 ± 0.039 | 0.21 ± 0.04 | **0.09 ± 0.016** | nd | 0.05 ± 0.016 | 0.07 ± 0.005 |
| **43** | nd | nd | 0.17 ± 0.042 | 0.14 ± 0.022 | 0.38 ± 0.121 | 1.45 ± 1.018 | **0.06 ± 0.004** | nd | 0.09 ± 0.031 | 0.09 ± 0.008 |
| **44** | nd | nd | 0.03 ± 0.014 | 0.06 ± 0.014 | 0.33 ± 0.082 | 0.31 ± 0.051 | 0.17 ± 0.018 | 0.27 ± 0.105 | 0.34 ± 0.023 | 0.33 ± 0.064 |
| **45** | nd | nd | 0.10 ± 0.036 | 0.17 ± 0.038 | 1.82 ± 0.235 | 1.44 ± 0.357 | 0.60 ± 0.191 | 0.35 ± 0.121 | 0.80 ± 0.145 | 0.65 ± 0.031 |

*Note*: Values in bold indicate either significant differences between sexes or a compound detected in only one sex (Yuen–Welch test, based on 20% trimmed means).

Abbreviations: nd, not detected.

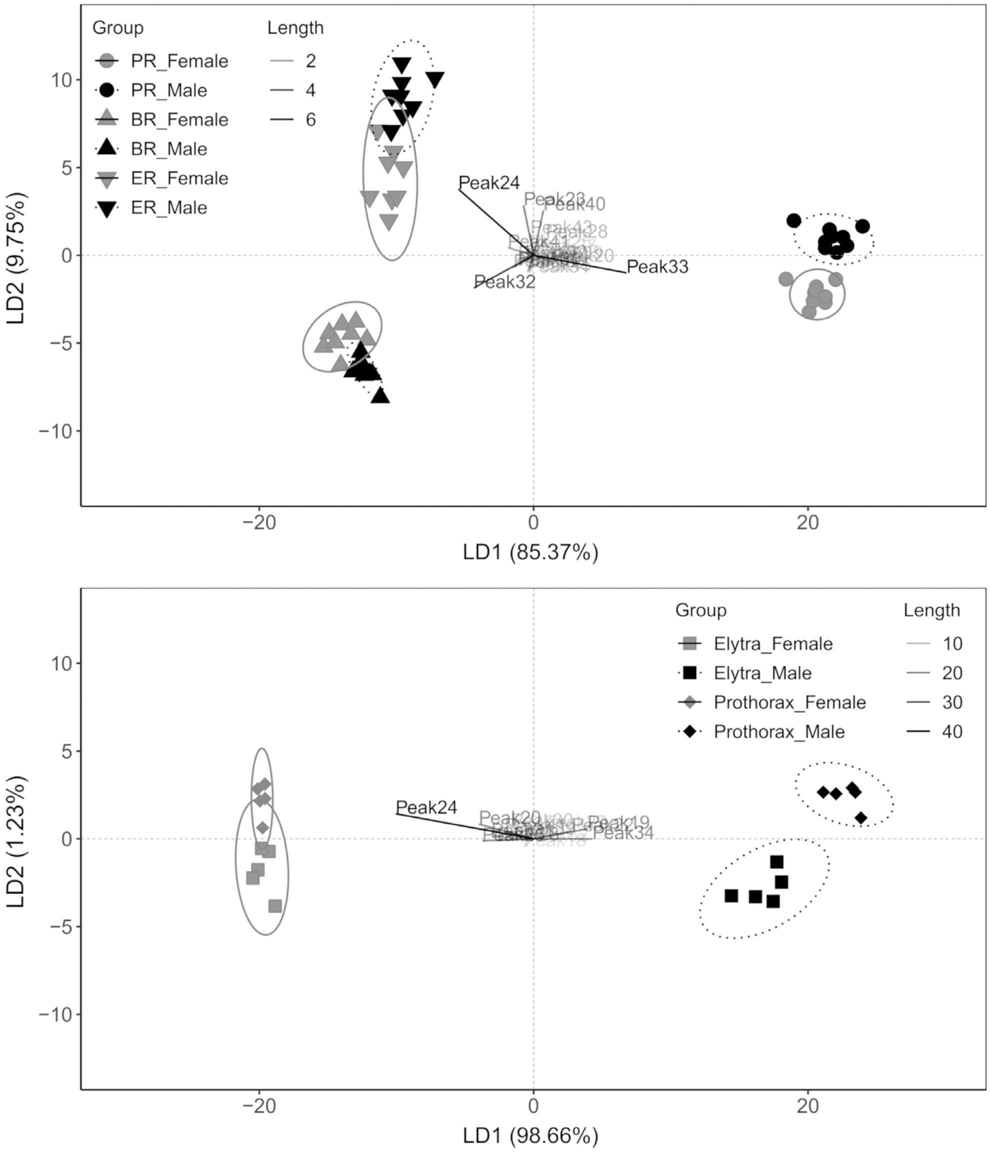

**FIGURE 3** Discriminant analysis (biplot) of cuticular hydrocarbon profiles of *Cerambyx welensii* males and females. Biplots represent the projection of variables (peaks) onto the plane defined by the first two discriminant functions LD1 and LD2, and the clustering of both sexes based on the same functions. Upper biplot shows results for the three phenological periods studied: pre-reproductive (PR), beginning of reproductive period (BR), and ending of reproductive period (ER); lower biplot shows results corresponding to the hexane extracts obtained from prothorax and elytra separately. Number of peaks is shown in Table 1. Length represents the relative contributions of the compounds (in grey colour).

impaired, but up to 80% of the copulation attempts were recovered when males were allowed to contact females with their palps (Sánchez-Osorio et al., 2012).

A visual recognition as part of the mating system has been found previously within Cerambycidae, f.i., in *A. malasiaca*, wherein the visual stimulus acted synergistically to short range pheromones (Fukaya et al., 2004). In contrast, a low role of visual stimulation has been found in cerambycids without colour dimorphism, as occurs in *C. welensii*, but with short-range or contact sex pheromones (Wang, 2002). Further research is needed to determine if visual recognition could play a role in mating in *C. welensii*.

In preliminary experiments, we suggested the implication of contact chemoreception in mate recognition by *C. welensii*, when males attempted to mate with conspecific dead females but did not respond to the same females after being washed with hexane, suggesting that females might produce a cuticular contact pheromone which had been removed by the solvent (López-Manzano et al., 2020). In the current paper, we report that the very low or null responses of males to solvent-washed dead females or untreated glass dummies were mostly recovered after treating them with 1 FE extract. In fact, ca. 70% of males at the beginning of the reproductive period dashed to solvent-washed dead females treated with female cuticular extracts after touching them with their antennae, and 62% of them licked the prothorax and elytra cuticle and bent the abdomen for the copula. This effect was neither increased nor further maintained by the follow-up treatment of the female with a synthetic hydrocarbon blend

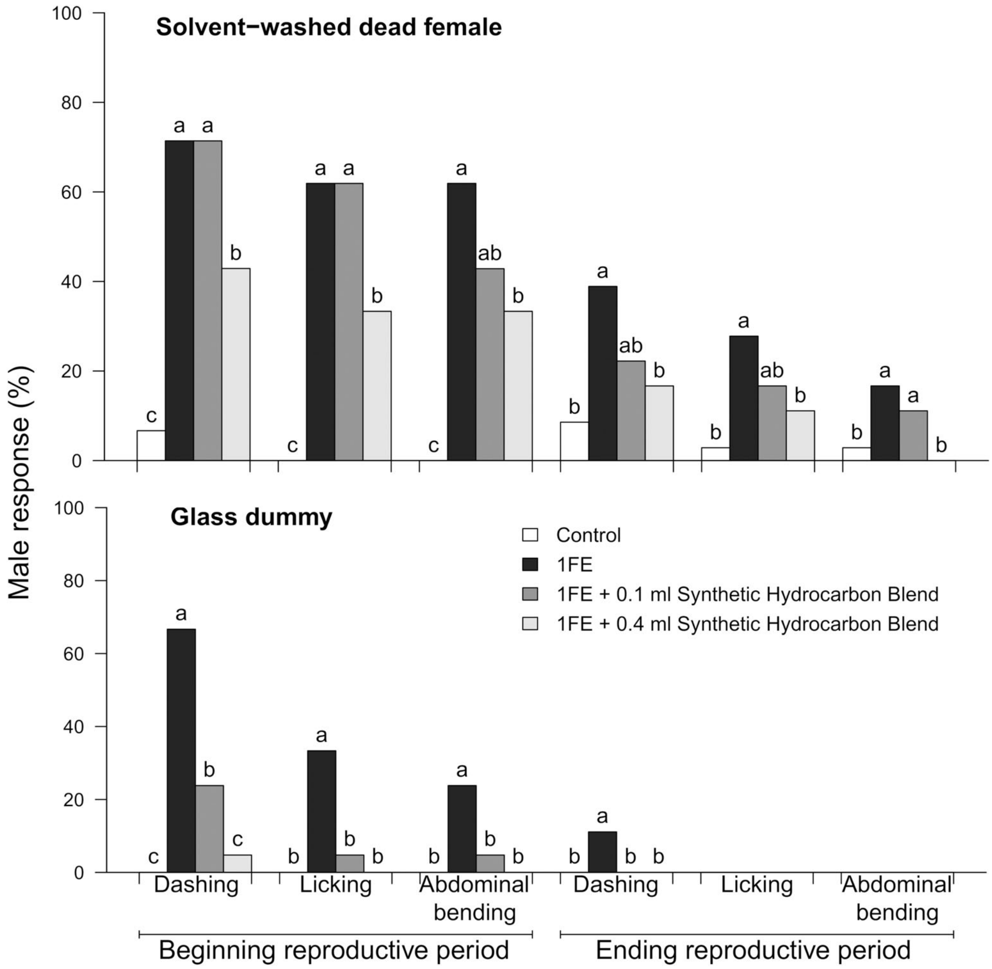

**FIGURE 4** Responses of *Cerambyx welensii* males to solvent-washed dead females and glass dummies at the beginning and ending reproductive periods, to the following treatments: no action (control), and successively coating with one female equivalent (1 FE) extract, and 0.1/0.4 mL (1.9/7.7 male equivalents) of a synthetic hydrocarbon blend that potentially approaches the male cuticular profile (100 μg ml$^{-1}$ of hydrocarbons blend in hexane). The hydrocarbons blend included heptacosane (C27), nonacosane (C29), hentriacontane (C31), and hexacosane (C26) in 79:11.5:5.3:4.2 ratio. For each male action (dashing after antennal contact, licking and abdominal bending) different letters indicate significant differences in male response to the different treatments (Cochran's Q test followed by Marascuilo and McSweeney procedure for pairwise comparisons, $N_m = 21$ and 18 for BR and ER periods, respectively).

that potentially approached the male cuticular profile. Similar type of behavioural effects was observed when glass dummies were coated with female cuticular extracts, which induced males to attempt copulation with them as with a live female. Our results confirm that long chain non-polar hydrocarbons from the female cuticle play an essential role for mate recognition in this species. In addition, male mating responses varied along its reproductive period, with higher rate of positive responses being found at the beginning of the reproductive period.

In order to deep into the nature of compounds responsible for the activity in mate recognition, the CHCs of elytra and prothorax of both sexes were obtained by SPME and solvent extraction, and chemically identified at three stages of the reproductive period. Differences in the relative amounts of compounds obtained by both methods can vary among cerambycid species. For example, whereas comparable results were found in *Neoclytus acuminatus* (F.) and *Xylotrechus colonus* F (Silliman, 2014), marked differences were noticed in *Megacyllene robiniae* (Förster) (Ginzel et al., 2003). In our study, the CHC profiles obtained from both methods showed differences in minor compounds of less than 22 carbon atoms. In addition, n-heptacosane and 2-methyloctacosane showed higher proportions in hexane extracts than in SPME, while 11-methylnonacosane appeared to be higher in SPME than in hexane extracts. The CHCs obtained by SPME have been proposed to yield a representative profile of the components of the cuticular wax layer that are actually detected by a male antenna (Ginzel et al., 2003; Lacey et al., 2008).

Cuticular hydrocarbons as contact pheromones can be affected by physiological factors, such as age and mating status. Also, temperature and other environmental factors, such as relative humidity can affect hydrocarbon profiles over relatively short periods of time (Ingleby, 2015; Noorman & Otter, 2002). Our results show that the CHC profiles of both sexes differ from the pre-reproductive phase

**FIGURE 5** A *Cerambyx welensii* male showing attempts to mate with a dummy (glass stopper) coated with one female equivalent (1 FE) extract in hexane.

and across the entire reproductive period, both qualitative and quantitatively. The relative abundance data at the end of the reproductive period followed by discriminant analyses suggest that 11/13-methylheptacosane and 11-methylnonacosane, the most relevant hydrocarbons in female cuticle along the entire reproductive period, could play a major role in the maturity of insects, being absent in the pre-reproductive phase and highly abundant at the beginning and ending reproductive periods. Differences in CHC profiles between sexes were particularly noticed at the end of their respective reproductive periods, with 11-methylnonacosane and the unknown three compound having higher relative abundance in females, and the former particularly abundant in female prothorax. In contrast, nine minor compounds showed a higher relative abundance in males than in females, with nonacosane being the most relevant. The causes of this variation have not been identified. In other Cerambycidae, variation in the relative abundance of CHCs during the seasonal flight period was explained in terms of differences in host quality in *X. colonus* (Silliman, 2014), or variation in the feeding behaviour in *Monochamus scutellatus* Say females (Brodie et al., 2012).

Understanding the regulation mechanism involved in CHC profiles can be relevant to identify potential vulnerabilities related to pest ecology and to develop novel pest management strategies (Li et al., 2021). As example, the fungus *Beauveria bassiana* (Bals.) Vuillemin has been shown to have a potential use for biological control of *C. welensii* (Morales-Rodríguez et al., 2015). In this context, the cuticular hydrocarbons could play a role in the adhesion processes of *B. bassiana* conidia to the insect cuticle, due to the hydrophobic changes induced on it by these compounds (Brückner et al., 2017; Silva, 2014).

In summary, we have confirmed that in *C. welensii* mate recognition is mediated by contact chemoreception with males orienting to solvent-washed dead females or glass dummies treated with 1 FE extract after antennal contact, and subsequent copulatory behaviours. In addition, we have characterized a number of cuticular long straight-chain and methyl-branched saturated hydrocarbons present in the prothorax and elytra of both sexes of *C. welensii*, and studied the CHC profiles across the pre-reproductive, beginning and ending of the insect reproductive period. Some of the hydrocarbons present in the female cuticle may function as contact pheromones of the insect, but further work is necessary to elucidate which are the key compounds responsible for the activity. Particularly relevant would be to determine the precise role played by the major compounds 11/13-methylheptacosane and 11-methylnonacosane either alone or in blends with other minor compounds, particularly prothoracic CHCs present exclusively in females or at significant higher rates than in males.

## AUTHOR CONTRIBUTIONS

**Israel Sánchez Osorio:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; writing – original draft; writing – review and editing. **Gloria López-Pantoja:** Conceptualization; funding acquisition; investigation; methodology; resources; writing – review and editing. **Luis Domínguez Nevado:** Conceptualization; funding acquisition; investigation; methodology; resources; writing – original draft; writing – review and editing. **María R. López-Manzano:** Data curation; investigation; methodology. **Gloria Rosell:** Formal analysis; investigation; methodology; resources; supervision. **Ángel Guerrero:** Formal analysis; investigation; methodology; resources; supervision; writing – original draft; writing – review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data presented in this study will be openly available in Open Science Framework repository.

## ORCID

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Table S1.** Average amounts (ng, mean ± standard error) of cuticular hydrocarbons of *Cerambyx welensii* females and males identified in SPME samples (elytra and prothorax. $N_m = 9$, $N_f = 9$) obtained in the pre-reproductive (PR), beginning (BR), and ending of the reproductive period (ER), and hexane extracts (prothorax and elytra separately at the ER period, $N_m = 5$, $N_f = 5$), by GC–MS analysis. nd = not detected.

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