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MULTIPLE ROLES OF A MALE-SPECIFIC COMPOUND IN THE SEXUAL BEHAVIOUR OF THE DRIED BEAN BEETLE, ACANTHOSCELIDES OBTECTUS (COLEOPTERA: CHRYSOMELIDAE, BRUCHINAE)

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RESPONSE TO THE EDITOR`S AND THE REVIEWERS` COMMENTS

The authors are very grateful for the supportive comments. Below are our responses to these.

Reviewer #1:

Line 89: `ten` replaced by `10`
Line 300: reference edited

Reviewer #2:

A supplementary table and gas chromatogram with tentative cuticular compound identifications are provided.

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Abstract-Males of *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae, Bruchinae) emit methyl (*E,R*)-2,4,5-tetradecatrienoate that attracts females for mating. This study identifies further roles for this compound in the sexual behaviour of *A. obtectus*. Earlier observations revealed that males were touching females with their antennae while tandem-running with them and initiating mounting and copulation, whereas they showed no such behaviour towards other males. A series of subsequent laboratory choice tests were set up to establish if certain cuticular compounds aid contact sex recognition in *A. obtectus*. Males chose virgin females above other males. The activity towards females could be eliminated by rinsing with hexane, but was re-gained by application of female extract onto previously rinsed females. Gas chromatographic (GC) comparison of hexane extracts revealed the presence of two male-specific compounds, methyl (*E,R*)-2,4,5-tetradecatrienoate and octadecanal, which were absent from the behaviourally active female samples. Of the two compounds, methyl (*E,R*)-

2,4,5-tetradecatrienoate was found to be responsible for the inhibition of male sexual behaviour, similar to that observed only with crude male extracts when applied onto virgin females. Furthermore, when comparing male sexual behaviour towards mated and virgin females, males preferred the latter. GC analyses revealed the presence of methyl (*E,R*)-2,4,5-tetradecatrienoate in extracts of mated females in amounts sufficient to curtail mating attempts. It appears that methyl (*E,R*)-2,4,5-tetradecatrienoate, besides being a male-produced sex pheromone, acts as a male-recognition signal in *A. obtectus*. Males also transfer it onto females upon mating, which will then be avoided by courting males.

Key Words-Contact chemoreception; Abstinon; Semiochemical parsimony; *Acanthoscelides obtectus*; Bruchinae; Sexual conflict

Introduction

The dried bean beetle, *Acanthoscelides obtectus* Say (Coleoptera: Chrysomelidae, Bruchinae), is a widely distributed oligophagous seed predator specialising on *Phaseolus* spp. (Fabaceae) (Imura 1990). Mainly due to its status as a pest of stored products (Southgate 1979; Abate and Ampofo 1996), *A. obtectus* received the early attention of chemical ecologists in search of novel semiochemical-based management methods. An unusual allenic ester, methyl (*E,R*)-2,4,5-tetradecatrienoate, was identified as a male-produced sex pheromone more than 40 years ago (Hope 1967; Horler 1970; Halstead 1973). More recently, octadecanal was described from males, and found to synergize the activity of the ester as a sex attractant for females (Annoscia et al. 2010). Other studies on *A. obtectus* suggested that males use contact chemoreception to recognise sexes. They actively tap conspecifics with their antennae, which then results in the sequence of chasing, mounting and copulation with females (Halstead 1973; Á. Szentesi unpublished). Savković et al. (2012) and Stojković et al. (2014) found differences in the cuticular hydrocarbon profiles of *A. obtectus* females and males, and proposed the role of such hydrocarbon compounds in mate recognition. In the closely related *Callosobruchus* spp., for example, female beetles produce contact sex pheromones, comprising a blend of C_{25}–C_{35} straight chain and methyl-branched hydrocarbons and dicarboxylic acids, that induce male sexual behaviour (Tanaka et al. 1981; Nojima et al. 2007).

As the process of mate recognition is poorly understood in *A. obtectus*, we aimed to shed light on this communication system, assuming the role of contact chemical signals on the body surface of beetles. We used a series of bioassays to test this assumption, as well as gas chromatography and gas chromatography-coupled mass spectrometry to analyse cuticular profiles of *A. obtectus* surface extracts. Resulting findings may become important in developing management strategies that intercept the sexual behaviour of *A. obtectus*, thereby leading to increased control efficiency.

Methods and Materials

Insects Acanthoscelides obtectus beetles for the tests were obtained from a laboratory population in Hungary, and reared on dry *Phaseolus vulgaris* `Cannellini` beans. The original laboratory population was from a natural infestation on *Ph. vulgaris* in Hungary. Maintenance conditions were the same at the Rothamsted and the Hungarian labs (artificial lighting with a 16:8 h L:D photoperiod, T=20 °C, 60% RH). In order to obtain virgin insects, seeds were kept individually in wells of an Eppendorf rack and covered with a piece of transparent acetate sheet until beetle emergence, when sexes were separated immediately for the experiments.

Preparation of Extracts We extracted surface chemicals (for Tests 3, 4 and 6, see list below) from 20 female and male *A. obtectus* specimens. Samples were prepared by first freezing the beetles on dry ice, then soaking them in 200 µl freshly distilled hexane (Fisher Scientific, UK) for 10 min. The extracts were filtered through a glass wool plug, transferred into a 1.1 ml conical vial (Kinesis, UK) and evaporated under gentle nitrogen stream to the appropriate level to obtain ca. 1 beetle equivalent per 2 µl concentrations, which were determined by gas chromatographic (GC) quantification of extracts. Samples were kept at -20 °C until use.

To determine the amount of methyl (*E,R*)-2,4,5-tetradecatrienoate from males, 10 male beetles were individually extracted in 50 µl hexane for 10 min, and extracts were analysed by GC. Ten mated females were similarly extracted in hexane immediately after the 24 h copulation period. Another two groups of 10 females were separated from males after 24 h, and extracted either 72 or 144 h later. An extract from 10 virgin females served as control.

To estimate the amount of methyl (*E,R*)-2,4,5-tetradecatrienoate on different parts of the male *A. obtectus* cuticle, the heads (with antennae and palpi), thoraxes (with legs only), elythrae and abdominal tergites, pygidia and sternites of 10 males were soaked in 50 µl hexane in a 1.1 ml conical vial for 10 min. Extracts were filtered through a glass wool plug,

transferred into a 1.1 ml conical vial (Kinesis, UK) and evaporated under nitrogen to 20 µl, and were kept at -20 °C until used for GC analyses.

Gas Chromatography (GC) and GC-Mass Spectrometry (GC-MS) Analyses of Extracts For the analysis of extracts, an Agilent 6890A GC (Agilent Technologies, USA) equipped with a cool on-column injector, FID, and a 50 m × 0.32 mm i.d. HP-1 column, was used. The oven temperature was maintained at 30°C for 0.1 min, then programmed to increase at 10°C/minute to 250°C, and was then held at this level for 38 min.

For compound identification, a HP-1 capillary GC column (50 m × 0.32 mm i.d. × 0.52 µm film thickness), equipped with a cool on-column injector, was directly coupled to a mass spectrometer (Micromass Autospec Ultima, Waters/Micromass, USA). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 minutes and then programmed to increase at 5°C/minute to 250°C. Tentative identification by GC-MS was confirmed by comparing retention indices of peaks with those of synthetic standards and by peak enhancement on GC by co-injection with authentic compounds (Pickett 1990), using an Agilent 6890A GC with 50 m × 0.32 mm i.d. HP-1 column, as well as a 30 m × 0.32 mm i.d. DB-WAX column. The carrier gas was helium. Quantification of compounds was achieved using known amounts of external standards (a series of C7-C22 alkanes).

Chemicals For behavioural assays of the two male-specific compounds, enantiomerically pure methyl (*E,R*)-2,4,5-tetradecatrienoate was synthesized following the protocol by Mori (2012). Octadecanal was synthesized from octadecanol by TPAP-mediated oxidation (Griffith et al. 1987).

Behavioural Assays All experiments were carried out under the same conditions used for the maintenance of bruchid cultures. Petri dishes (55 mm diameter, 13 mm height) with a 55 mm diameter filter paper on the bottom served as test arenas. Two pencil marks 35 mm apart on the filter paper indicated the places for the test (t) and control (c) *A. obtectus* individuals (3-8

days old). These were freeze-killed on dry ice before their use in the experiments, and were laid on their side. If soaked in hexane, the solvent was allowed to evaporate prior to tests. One male *A. obtectus* (3-11 days old) was put in each Petri dish arena (representing 1 replication), and the number of copulation attempts (mounting and penis extruded) towards the test and the control freeze-killed individuals was recorded. Beetles were observed for 20 min. Ten replications per each test were done.

Test 1 was carried out to confirm earlier observations on male mating preference for female conspecifics. Freeze-killed virgin females (t) and males (c) were compared.

Test 2 investigated if the mounting and copulation-initiating cues are certain chemicals found on the surface of females. Virgin freeze-killed females (t) were tested against females soaked in hexane for 10 min (c) (termed as `hexane-washed` hereafter).

Test 3 assessed the effect of treating hexane-washed females with female extract (t). Hexane-washed females served as controls (c). Test females were coated with 2 µl (ca. 1 female equivalent) hexane extract of 20 females, using glass micropipettes (Brand GmbH, Germany), by evenly spreading the extract on the entire dorsal surface of the insect.

Test 4 compared the activity towards virgin freeze-killed females treated with male extract (t) versus hexane-washed females (c). Test females were coated with 2 µl (ca. 1 male equivalent) hexane extract of 20 males.

To eliminate the effect of solvent that might move compounds around the cuticle of otherwise stimulatory females (Coates and Langley 1982), thereby mimicking the inhibitory effect of the male extract, in Test 5, virgin freeze-killed females were treated with 2 µl hexane (t). Hexane-washed females served as control (c).

Also, to ensure that the inhibitory effect of the male extract was not a result of unnaturally high doses of cuticular compounds jointly present in both sexes' extracts, in Test 6, unwashed females were treated with 2 µl (ca.1 beetle equivalent) of female extract (t), thereby doubling the concentration of compounds normally present on the cuticle of one female. Hexane-washed females were used as controls (c). The female extract was prepared from 20 individuals.

We then focussed on the two male-specific cuticular compounds to assess their behavioural roles. Test 7 compared the behavioural activity towards virgin freeze-killed females treated with ca. 1 male equivalent of methyl (*E,R*)-2,4,5-tetradecatrienoate (1000 ng) plus octadecanal (200 ng) dissolved in 2 µl hexane (t) with that towards hexane-washed females (c).

Further tests assessed, on male sexual activity, the effect of treating virgin freeze-killed females with either 200 ng octadecanal or 1000 ng methyl (*E,R*)-2,4,5-tetradecatrienoate in 2 µl hexane (t) (Test 8 and 9, respectively), compared to hexane-washed females (c).

There are reports in insects of the reduction of female attractiveness to males after mating due to (*i*) the delivery of certain compounds via contact from the male cuticle onto the female cuticle during courtship or after mating, or (*ii*) transfer of compounds via the spermatophore or mating plug into the female (Thomas 2011). As a mechanism similar to (*i*) could also occur in *A. obtectus* in the light of the mating-inhibitory compound methyl (*E,R*)-2,4,5-tetradecatrienoate, male sexual responses to freeze-killed virgin (t) and mated (c) females were directly compared in Test 10. Mated females were obtained by pairing a virgin female and male *A. obtectus* in a glass vial (10 mm diameter and 50 mm length), closed with a cotton wool plug, for 24 h to ensure copulation, which usually occurred within 30 min from the start of pairing.

Subsequent tests compared male sexual activity towards virgin freeze-killed females treated with ca. 1 mated female equivalent of either methyl (*E,R*)-2,4,5-tetradecatrienoate (100 ng) or octadecanal (20 ng) in 2 µl hexane (t) with that towards hexane-washed female (c) (Test 11 and 12, respectively).

Statistics To analyse the effect of treatment in each behavioural experiment separately given paired data, the difference in count was taken for each of the ten replicate arenas. These values were modelled assuming a Poisson distribution with a log link function, fitting a generalized linear model (McCullagh and Nelder 1989) of the form

$$\log(count_i) = \mu$$

where μ is a constant and i=1,…10. We test whether the mean difference in counts is significantly different from 0 on 9 degrees of freedom (being the number of arenas used less 1) at the α=0.05 level of significance. The predicted mean difference was then output with appropriate standard error.

Results

After an initial period of 1-2 min, *A. obtectus* males started to explore the arena and investigated both freeze-killed conspecifics by intensive antennation.

In Test 1, males did not attempt to copulate with freeze-killed males, but freeze-killed virgin females were readily mounted. Clearly, motion was not required to elicit mating (Fig. 1).

Males did not copulate with virgin females that had their cuticular chemicals removed by solvent (Test 2, Fig. 1). However, re-instating the female extract in amounts similar to those found on one female stimulated male copulation attempts (Test 3, Fig. 1).

Comparative GC and GC-MS analyses of hexane extracts of both sexes revealed a high level of similarity between the extracts (Supplementary material), apart from two compounds that were specific to males (Fig. 2). These were identified by GC-MS and co-injection with authentic standards as methyl (*E*,*R*)-2,4,5-tetradecatrienoate and octadecanal in 999.5±263.8 and 205.5±51.72 (mean±SE) ng respective amounts per male.

When the male extract was applied in physiologically relevant amounts onto unwashed females in Test 4, it resulted in the abolishment of male courtship (Fig. 1). Hexane treatment of unwashed females resulted in no reduction of male sexual behaviour (Test 5, Fig. 1). Also, males showed the same type of response towards females with increased dose of female cuticular chemicals that was observed towards females with the naturally occurring concentrations (Test 6, Fig. 1).

Total inhibition of mating behaviour was observed when methyl (*E,R*)-2,4,5-tetradecatrienoate and octadecanal were added to unwashed females, in Test 7, in amounts extractable from one male *A. obtectus* (Fig. 1). However, when testing these chemicals individually, octadecanal did not prevent male mating attempts (Test 8, Fig. 1), whereas methyl (*E,R*)-2,4,5-tetradecatrienoate completely inhibited such behaviour (Test 9, Fig. 1). The distribution of methyl (*E,R*)-2,4,5-tetradecatrienoate on the male *A. obtectus* body was found to be as follows: head 3.4%, thorax (with legs only) 37.5, elythrae 39.1%, abdominal tergites 0%, pygidium 6.4%, abdominal sternites 13.6% of a total amount per insect of ca. 1000 ng.

Males attempted to mate with virgin females significantly more than with mated ones (Test 10, Fig. 1). Comparison of GC profiles of hexane extracts of females 24 h after mating revealed the presence of both methyl (*E,R*)-2,4,5-tetradecatrienoate and octadecanal in 102.3±15.67 and 20±4.1 (mean±SE) ng respective amounts per female, while they were absent in virgin females (Fig. 1). In Test 11, application of methyl (*E,R*)-2,4,5-tetradecatrienoate in amounts extractable from 1 mated female on virgin females eliminated male mating attempts completely (Fig. 1), whereas octadecanal had no such effect (Test 12, Fig. 1). GC analyses of hexane extracts of mated females 72 or 144 h after the initial 24 h copulation period revealed the absence of methyl (*E,R*)-2,4,5-tetradecatrienoate and octadecanal.

Discussion

We demonstrated here that *A. obtectus* males achieve recognition of other males, and thus reduce the frequency of same-sex mating attempts, by utilizing a male-specific chemical signal, methyl (*E,R*)-2,4,5-tetradecatrienoate, on the cuticle, which is perceived by contact chemoreception. The lack of this compound indicates the presence of a virgin female and initiates mating.

Methyl (*E*,*R*)-2,4,5-tetradecatrienoate is known as a male sex pheromone of *A. obtectus* that attracts virgin females (Hope 1967; Horler 1970; Halstead 1973). Its role as a contact signal that contributes to sex recognition and aids mate choice is somewhat unexpected, as earlier, contact pheromones with more than 20 carbons were identified (Buckner 2010). It was previously demonstrated that methyl (*E*,*R*)-2,4,5-tetradecatrienoate can be obtained from the cuticle after wiping the surface of male *A. obtectus* with silica gel, then extracting the silica gel with diethyl ether (Vuts et al. unpublished). GC profiles of silica gel extracts showed qualitative and quantitative similarities to those of direct solvent extracts, indicating that methyl (*E*,*R*)-2,4,5-tetradecatrienoate is indeed present on the cuticular surface. Interestingly, spiders utilize the saturated structural analogue, methyl tetradecanoate, as a contact sex pheromone (e.g. Prouvost et al. 1999), although further conclusions about functional analogy are unlikely to be drawn between such distant taxa. Johansson and Jones (2007) suggest that "signals used in species recognition could evolve from signals with mate recognition or mate assessment functions". Long-distance signalling by highly specific female-emitted sex pheromones aids reproductive isolation in many insects even among closely related species (Löfstedt 1993; Smadja and Butlin 2009). However, pheromone compounds can also be used to fine-tune the reproductive behaviour of individuals of a given species. Some of these, unlike sex pheromones, are predicted not to be under the selective pressure to evolve species-specificity (Brent and Byers 2011), and can comprise ubiquitous chemicals active in a number of biological systems. In the light of this, male-produced methyl (*E*,*R*)-2,4,5-tetradecatrienoate could originally be utilized only for mate recognition, but a new role, assigned to species recognition, has emerged.

Contact sex pheromones, mostly comprising hydrocarbons, have been described from females of a number of insects (Howard and Blomquist 2005), including two bruchid beetles, where females produce mating elicitors that guide male sexual behaviour (Tanaka et al. 1981; Nojima et al. 2007). To our knowledge, only a few reports of male-produced contact compounds that play a role in sexual communication exist. These chemicals, termed as abstinons, block mating attempts by conspecific males, and have been identified from tsetse

flies (*Glossina* spp.) and the house fly, *Musca domestica* L. (Diptera) (Schlein et al. 1980; Nelson et al. 1981; Carlson and Schlein 1991). Kim et al. (1993) describe a blend of saturated and unsaturated hydrocarbons that serves as an abstinon in males of the longhorn beetle *Semanotus japonicus* Lacordaire (Coleoptera: Cerambycidae).

In addition to its new role as a male-recognition signal, methyl (*E,R*)-2,4,5-tetradecatrienoate appears to be used in *A. obtectus* for rendering mated females unattractive to males. It was isolated from mated, but not from virgin, females, indicating that it is transferred onto the female cuticle during mating most probably by physical contact, similar to tsetse flies (Carlson and Schlein 1991) and crickets (Weddle et al. 2013). In the latter, it is the females that mark males during mating with their cuticular hydrocarbons to identify previous mates in order to avoid them in future mating, but nonetheless, the mechanism itself might be similar in *A. obtectus*. Males mate with more than one female during their lifetime, whereas females show a pronounced resistance to re-mating and kick off mounting males, similar to another bruchid species, the cowpea seed beetle (*Callosobruchus maculatus* Fabricius) (Huignard 1974; Crudgington and Siva-Jothy 2000). This conflict between the sexes could partially be due to a toxic seminal compound in the spermatophore that is transferred during mating and which shortens female longevity (Das et al. 1980), or be due to direct physical injuries to females during copulation (Crudgington and Siva-Jothy 2000; Lange et al. 2013). The amount of methyl (*E,R*)-2,4,5-tetradecatrienoate isolated from one female 24 h post-mating was enough to cause total inhibition of mounting and copulation attempts by males. However, observations of female *A. obtectus* re-mating 3-4 days after the initial mating (Maklakov et al. 2007) could indicate the erosion of female unattractiveness. In this process, disappearance of the transferred male anti-aphrodisiac, methyl (*E,R*)-2,4,5-tetradecatrienoate, ca. 48 h after mating by yet unknown mechanisms was observed in the present study, a phenomenon that was also described in *Pieris* butterflies (Andersson et al. 2000). So although females still display resistant behaviour to re-mating, they are no longer unattractive to males, which then can perform traumatic mating.

It seems that the costs and benefits of polygamy may differ between the sexes of *A. obtectus*, which then create a potential for sexual conflict due to the divergence in the interests of females and males (Maklakov et al. 2007). The use of male-deposited scent signals on the female cuticle may relax such conflict by conferring selective advantage on both sexes. Multiple mating by females creates an opportunity for sperm competition (Parker and Pizzari 2010), but males would be able to reduce its probability by marking mated females with methyl (*E,R*)-2,4,5-tetradecatrienoate, thereby imposing monandry, as well as invoking preferential selection of unmated females. Studies by Maklakov et al. (2007) also suggest that "males that succeed in mating with a virgin female will father all eggs produced by this female for, typically, at least a few days." Thus, males would be expected to evolve an ability to discriminate female mating status, for which, chemical signals provide a feasible solution (Thomas 2011). Females would benefit from male-produced anti-aphrodisiacs through reduced male harassment, similar to the green-veined white butterfly, *Pieris napi* L. (Lepidoptera: Pieridae), that uses a male-produced volatile anti-aphrodisiac, methyl salicylate (Andersson et al. 2000), or to the Western tarnished plant bug, *Lygus hesperus* Knight (Heteroptera: Myridae), which utilizes myristyl acetate (Brent and Byers 2011). Theoretically, *A. obtectus* females could benefit from re-mating because males also transfer a nutritious substance in their spermatophore during mating (Das et al. 1980) that prolongs female lifespan (Tucić et al. 1996). Also, according to Slatyer et al. (2012), re-mating could hypothetically increase female reproductive success via the avoidance of inbreeding depression, a factor described in isolated populations of *C. maculatus* (Tran and Credland 1995). However, studies by Maklakov et al. (2006, 2007) indicate that costs of re-mating outweigh any benefits for females of *A. obtectus*.

The results presented here provide a better insight into the sexual behaviour of *A. obtectus*. On one hand, they confirm the role of cuticular compounds in mate recognition proposed by Savković et al. (2012) and Stojković et al. (2014); on the other hand, they demonstrate the parsimonious use of a compound in intra-species chemical communication.

Further studies are required to elucidate fully the pheromone biology of *A. obtectus*, thereby creating a platform for investigations of more applied nature.

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Conflict of interest

The authors declare no conflict of interest.

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Legends to figures

Figure 1. Results of Petri dish arena bioassays. The behaviour of one male *Acanthoscelides obtectus*/arena was observed for 20 min and the number of copulation attempts (mounting and penis extruded) towards each freeze-killed individual was counted. (no. replications/test = 10). Ester = methyl (*E,R*)-2,4,5-tetradecatrienoate, Oct = octadecanal, P.m.d. = Predicted mean difference, n.s. = not significant, P>0.05 (α=0.05)

Figure 2. Gas chromatographic (GC) profiles of *Acanthoscelides obtectus* hexane extracts. Beetles were extracted in 50 µl hexane for 10 min, then samples compared by GC (50 m × 0.32 mm i.d. HP-1 column; temperature programme: 30^{o}C for 0.1 min, then increased 10^{o}C/minute to 250^{o}C and held at this level for 38 min). Traces are on the same scale for comparison. Amounts (mean±SE) of methyl (*E,R*)-2,4,5-tetradecatrienoate and octadecanal on males: 999.5±263.8 and 205.5±51.72 ng, on mated females: 102.3±15.67 and 20±4.1 ng

Figure

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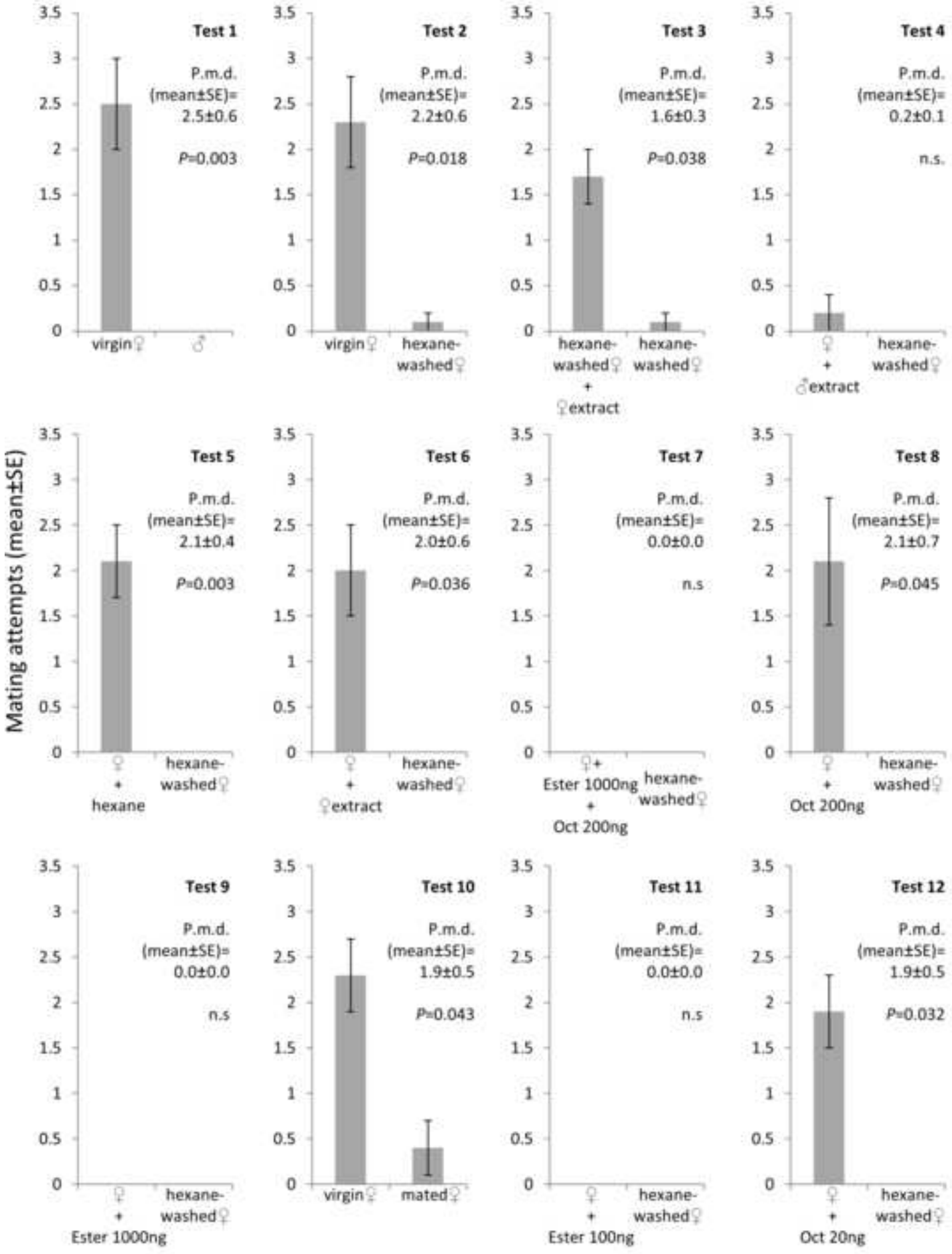

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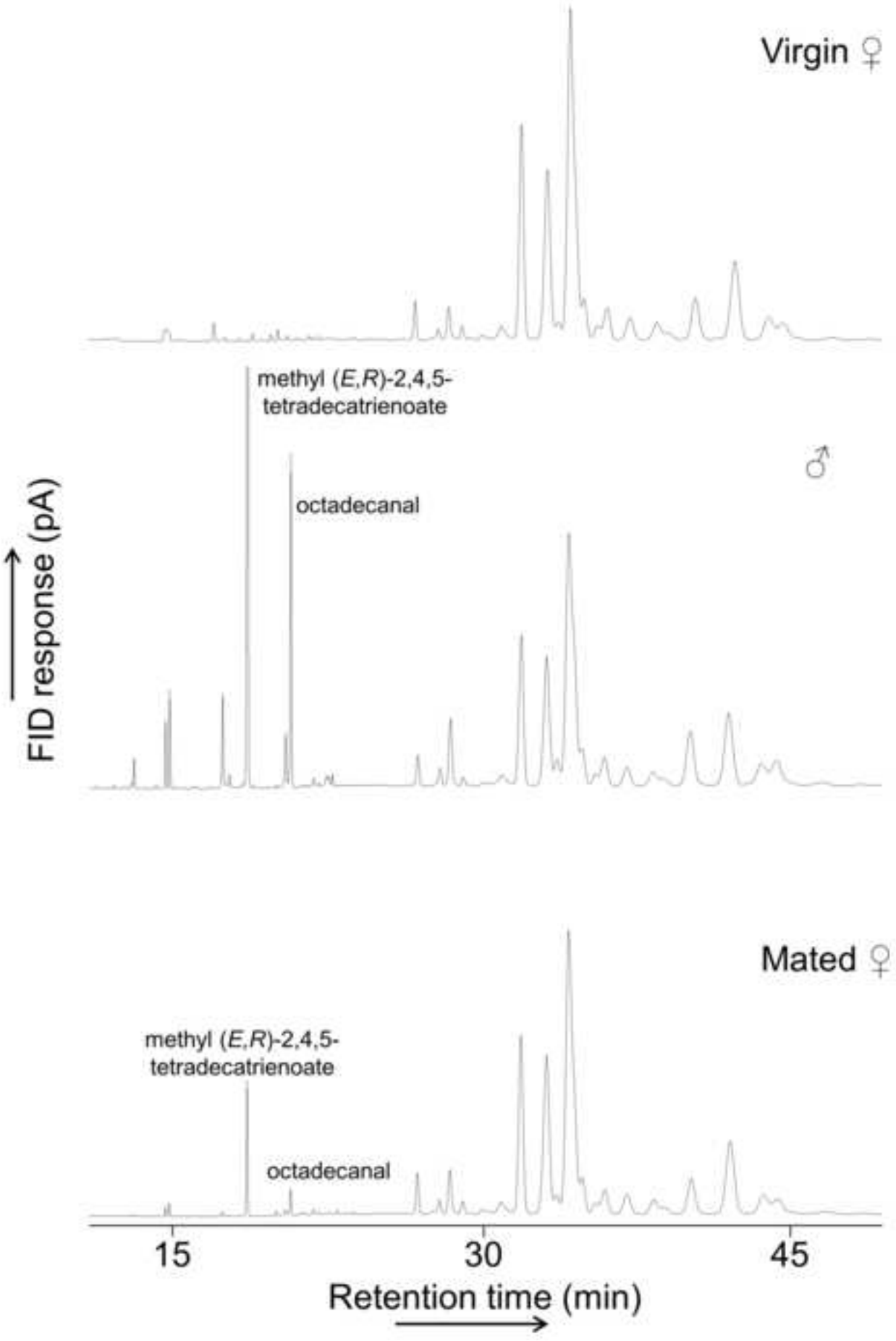

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