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Predominant accumulation of a 3-hydroxy- γ -decalactone in the male rectal gland complex of the Japanese orange fly, *Bactrocera tsuneonis*

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**Predominant accumulation of a 3-hydroxy- γ -decalactone in the male
rectal gland complex of the Japanese orange fly, *Bactrocera tsuneonis***

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Running Head: A γ -decalactone from the Japanese orange fly

24 ABSTRACT

25 The Japanese orange fly, *Bactrocera tsuneonis*, infests various citrus crops. While male
26 pheromone components accumulated in the rectal glands are well-characterized for
27 *Bactrocera*, but information regarding the chemical factors involved in the life cycles of
28 *B. tsuneonis* remains scarce. Herein, several volatile chemicals including a γ -decalactone,
29 (3*R*,4*R*)-3-hydroxy-4-decanolide [(3*R*,4*R*)-HD], were identified as major components,
30 along with acetamide and spiroketals as minor components in the rectal gland complexes
31 of male *B. tsuneonis* flies. The lactone (3*R*,4*R*)-HD was also identified in female rectal
32 gland complexes. The amount of this compound in mature males was significantly higher
33 than those observed in females and immature males. The lactone (3*R*,4*R*)-HD was
34 detected in flies fed with sucrose only, indicating that this lactone is not derived from
35 dietary sources during adulthood, but biosynthesized *in vivo*. The predominant
36 accumulation of (3*R*,4*R*)-HD in mature males also suggests a possible role in reproductive
37 behavior.

38

39 KEYWORDS

40 (3*R*,4*R*)-3-hydroxy-4-decanolide; *Bactrocera tsuneonis*; Tephritidae; rectal gland
41 complex; pest fruit fly

42

Many dacine fruit fly species comprising a major subfamily of Tephritidae are destructive pests of fruits and vegetables [1]. The Japanese orange fly, *Bactrocera tsuneonis* (Miyake), is an important pest of citrus fruits, in particular mandarin orange [2,3]. *B. tsuneonis* is a univoltine and oligophagous species widely distributed in southwestern China, Taiwan, and Japan. In Japan, the distribution of *B. tsuneonis* was restricted in the southern island Kyushu, but it has recently spread into the adjacent areas of western Honshu and the Shikoku islands. Hence, an effective lure to monitor *B. tsuneonis* population is urgently needed. However, little is known about the semiochemical factors of the life cycles of *B. tsuneonis*.

In most dacine tephritid fly species, males furnish a glandular complex in the rectum, known as the rectal gland, that is considered to be a secretory organ of male sex pheromones [4]. Various compounds, including aliphatic and aromatic volatiles, have been identified from the rectal glands [5]. In many dacine species, males acquire pheromonal components from plant secondary metabolites during adulthood [6]. For example, males of the Oriental fruit fly, *Bactrocera dorsalis*, are strongly attracted to a specific phenylpropanoid, methyl eugenol (ME), which is an essential oil component of various plants, and subsequently feed voraciously on the compound. [7,8]. The ingested ME is then biotransformed into two sex pheromone components *in vivo*, and these metabolites are subsequently sequestered in the male rectal gland to attract conspecific females [9,10]. Because of its robust attractiveness to male flies, ME has been used as a lure for pest management programs [11,12]. In the olive fly, multiple female sex pheromones are secreted by the female rectal gland [13]. Thus, the identification of chemical substances in the rectal glands of tephritid fruit flies is very important to understand the biological significance of these compounds in their life cycles and to

develop effective lures for pest managements [6].

To characterize the semiochemicals involved in *B. tsuneonis* life cycles, we analyzed the volatile chemical composition of their rectal tissues. We identified a predominant and unique hydroxy γ -lactone, along with an acetamide and a series of spiroketals in both males and females. The stereochemistry of the γ -lactone was determined and the rectal constituents were quantified in the context of maturation and sex differences. We also determined whether the γ -lactone was synthesized from dietary sources during adulthood.

Results and discussion

Identification of rectal gland components

A rectal gland complex was dissected from a mature male fly. An ethanolic extract of the tissue was analyzed by gas chromatography-mass spectrometry (GC-MS) (Figure 1). A major component (**1**) and several characteristic minor components, **2-5**, as well as general insect wax components including higher hydrocarbons were identified. The EI-MS and CI-MS of compound **1** afford major ions of $[M-H_2O]^+$ at m/z 168 and $[M+H]^+$ at m/z 187, respectively, for a possible molecular formula of $C_{10}H_{18}O_3$. We dissected rectal gland complexes from 117 laboratory-eclosed males and extracted their contents to isolate **1** for further analyses. Approximately 0.7 mg of **1** was obtained by a silica gel column chromatography. The ^{13}C -NMR, DEPT, and HMQC spectra revealed 10 carbon signals. These signals were assigned to one methyl carbon (δ 14.2), six methylene carbons (δ 39.6, 31.8, 29.3, 28.4, 25.7 and 22.7), two methine carbons (δ 84.7 and 69.3), and one carbonyl carbon (δ 175.3) from an ester/lactone group. The 1H -NMR spectrum revealed characteristic signals derived from two protons adjacent to oxygens (δ 4.49 and 4.37) and

one pair of geminal protons (δ 2.80 and 2.56). The ^1H - ^1H COSY spectrum indicated a connectivity of H2-H3-H4-H5-H6 and HMBC spectrum provided the following correlations: from H2 to C1, C3 and C4; from H3 to C1 and C4; from H4 to C3; and from H10 to C8 and C9. Therefore, we assigned the structure of **1** to be a γ -decalactone, 3-hydroxy-4-decanolide (HD). Because **1** has four possible stereoisomers, including two pairs of enantiomers due to the two chiral centers at C-3 and C-4, we synthesized a racemic mixture of *erythro*-lactones, and two optical isomers of *threo*-HD to determine the unambiguous structure of **1**. We synthesized racemic *erythro*-lactones—(3*R*,4*S*)-HD and (3*S*,4*R*)-HD—from (*E*)-3-decenoic acid via epoxidation and subsequent lactonization using amberlyst-15 [15] (Figure S1A). In addition, (3*R*,4*R*)- and (3*S*,4*S*)-HD were synthesized via Sharpless asymmetric dihydroxylation of methyl (*E*)-3-decenoate using AD-mix- β and AD-mix- α , respectively [16,17] (Figure S1B). Comparison of the ^1H -NMR and ^{13}C -NMR spectra of **1** with those of the racemic *erythro*-lactones and each enantiomeric *threo*-lactone indicated that the configuration of **1** was a *threo*-HD, either (3*R*,4*R*)-HD or (3*S*,4*S*)-HD (Figure S5 and S10). Because the optical rotation of **1** ($[\alpha]_{\text{D}}^{23}$ +61.5) corresponded to that of (3*R*,4*R*)-HD ($[\alpha]_{\text{D}}^{22}$ +40.1), rather than that of (3*S*,4*S*)-HD ($[\alpha]_{\text{D}}^{21}$ -35.1), the absolute structure was confirmed as (3*R*,4*R*)-HD. To the best of our knowledge, this is the first identification of **1** from insect species, as this compound has been reported only from culture media as a fermented yeast product (*Yarrowia lipolytica*) derived from an artificially added lipid [18].

We identified the minor components of the rectal volatiles to be *N*-(3-methylbutyl)acetamide (**2**), (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**), and (*E,Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**4**) by comparing their retention times and mass spectra with those of the corresponding synthetic and authentic samples. In

addition, **5** was putatively identified as 2-methyl-8-ethyl-1,7-dioxaspiro[5.5]undecane by comparing its mass spectrum with a mass spectrum library (Wiley7Nist05.L). The mass spectral data are provided in Table S1 and Figure S11. These compounds were previously identified from the male rectal glands of several tephritid species [5].

Quantification of rectal volatile components

In the Dacini genera, male pheromones are accumulated in the rectal gland and play a vital role in mating behavior [6]. Therefore, the major components in the rectal tissues, **1** and **2**, were compared between sexes to determine whether these compounds were distributed in a sexually dimorphic pattern. Female rectal gland complexes were dissected as previously described [14] and subsequently extracted by ethanol. The amounts of **1** and **2** were compared between wild males and females captured in a mandarin orange orchard. While **1** and **2** were detected in both wild males and females, the amount of **1** in males was significantly higher than that in females (Figure 2). The mean **1** content in the male samples was approximately 16 µg/gland, but the quantities varied among individuals (ranging from 0.87 to 33.2 µg/gland). The quantities of **2** were smaller than those of **1**, with mean of 496 and 135 ng/gland in males and females, respectively. While the amount of **2** in males was higher than that in females, no significant difference was detected between the sexes. The variation in **1** content among wild males can be ascribed to age. Indeed, morphological changes in the rectal gland are observed with age, and the storage and secretion in the reservoir of the gland increase with male sexual maturation [4,19]. Therefore, we quantified **1** and **2** in flies eclosed in a laboratory at different ages: within three days after eclosion (0–3 d AE) for sexually immature flies; as well as 7–10 d AE and 21–24 d AE for mature flies. A small amount of **1** was detected in males at 0–3 d AE

and significant increases were observed in both mature males at 7–10 and 21–24 d AE (Figure 3). The mean **1** contents were 0.30, 6.73 and 4.00 $\mu\text{g/gland}$ in males at 0–3, 7–10, and 21–24 d AE, respectively, indicating that **1** increased by > 20 times with maturation. Similarly, significant enhancement of **2** was observed in females at 7–10 d AE. It should be noted that the quantities of this compound varied widely in the 7–10 d AE females, i.e., more than 1.9 $\mu\text{g/gland}$ of **1** was detected in 7 females, while $< 0.4 \mu\text{g/gland}$ was detected in 6 females ($n = 13$). These results indicate that **1** accumulates in males as a function of maturation. Small amounts of **2** were detected in both immature males and females. While the various quantities of **2** (ranging from 0 to 1.57 $\mu\text{g/gland}$) were observed in all male groups, a significant enhancement in its content was observed in males at 7–10 d AE. Similarly, an enhancement of **2** was observed in females at 7–10 d AE, but two distinct patterns were observed, i.e., 7 females contained $> 670 \text{ ng/gland}$, but 6 females contained $< 130 \text{ ng/gland}$ ($n = 13$). This dimorphic pattern was observed for both **1** and **2**, so we examined relationship of the contents of these compounds among individual females. The correlation between the contents of **1** and **2** showed that some females contain significant amounts of these compounds, while others contain only small quantities (Figure S2).

Because the male sex pheromones of dacine species are accumulated or are synthesized from dietary pheromone or pheromone precursors, sequestered pheromones can only be detected from male rectal glands after feeding the pheromone or its precursor directly [20]. We further examined whether *B. tsuneonis* biosynthesized **1** and **2** from dietary sources or *de novo*. The flies were fed exclusively with sucrose solution to prevent ingestion of complex food as a dietary source for components in the rectal tissues. Even the flies were fed with only sucrose after eclosion, **1** and **2** were detected in both males and females at 7–10 d AE (Figure 4). The amounts of **1** in flies fed with only sucrose were

similar to those in flies fed with normal food in both males and females. The amounts of **2** in flies fed with only sucrose were smaller than those in flies fed with normal food, but no significant difference was observed between feeding groups. The contents of **1** and **2** in males and females regardless of their dietary ingredients indicate that these compounds are not directly derived from ingested food but are synthesized *in vivo*.

In preliminary indoor behavioral experiments, we examined the attractiveness of sexually mature male and female flies to an extract of the rectal gland complex, **1**, **2**, and a synthetic mixture of **1** and **2**, in a small screen cage. However, neither mature male nor female flies were attracted to the tested samples. Further, we conducted a field trap experiment with synthetic mixtures of **1**, **2**, **3**, and **4** in various combinations/doses in orange orchards in Yamaguchi Prefecture during the outbreak season of the adult flies. However, no *B. tsuneonis* adults were captured by those traps (unpublished data). Although a physiological function of these volatile components in the rectal gland complex could not be determined, **1** was accumulated in mature males, similar to the male sex pheromones of other *Bactrocera* fruit flies. The distinct pattern in the contents in mature females suggest that this compound may play a role in reproduction of *B. tsuneonis*, such as an indicator of sexual maturation and chemical signal during mating events. Elucidation of the roles of these components in the rectal tissues could provide a clue to control this hardly controllable pest fruit fly.

Experimental

Insects

Last instar larvae of *B. tsuneonis* immediately before pupariation were obtained from mandarin fruits in the local citrus orchards of Suo-Oshima Island, Yamaguchi, and kept

in a laboratory in the Yamaguchi Prefectural Agriculture and Forestry General Technology Center, Yamaguchi, Japan. The emerged pupae were kept indoors under ambient temperatures from November to June of the next year. The adults eclosed at the first half of June were provided with water and a diet of four parts sucrose and one part dry yeast AY-65 (Asahi Food & Healthcare, Ltd., Tokyo, Japan) at 25 °C and subjected to a 16 h light/8 h dark cycle. For analysis of the rectal glands from the sugar-only feed group, male and female flies were fed with 2 % sucrose solution after eclosion. For analysis of the rectal gland volatiles of wild fruit flies, males and females were captured in August 2018 in the local citrus orchards of Suo-Oshima Island, Yamaguchi.

Instruments

GC-Mass spectra were measured using an Agilent 5975 inert XL MSD mass spectrometer coupled with an Agilent 6890 gas chromatograph equipped with a capillary column (HP-5MS, 29 m × 0.25 mm, 0.25 µm film thickness, helium as a carrier gas) programmed from 60 °C (2 min holding) to 290 °C at a rate of 10 °C/min. GC quantification was performed using an HP-6850 gas chromatograph equipped with an Agilent HP-5MS 5% phenyl methyl siloxane-coated capillary column (15 m × 0.25 mm, 0.25 µm film thickness) with a flame ionization detector using the same program for GC-MS analyses for the GC oven. The ¹H-NMR and ¹³C-NMR spectra were measured using a Bruker Avance III 500 spectrometer with TMS as an internal standard. The optical rotations were measured using a JASCO P-1010 spectropolarimeter.

Rectal sample preparation

The rectal gland complexes were dissected from adult flies. Contents of the tissue were

extracted with 250 μ L of ethanol per gland for GC quantification or GC-MS analysis.

Extraction and purification of compound 1 from male rectal glands

Rectal gland complexes were dissected from 117 males eclosed indoor and extracted with ethanol. The combined extract (17 mg) was subjected to chromatography on a silica gel (500 mg) and eluted with 20% methyl acetate in hexane to isolate the major rectal volatile, compound **1** (yield: approximately 700 μ g). $[\alpha]_D^{23} +61.5$ ($c = 0.065$, CH_3OH). EI-MS: m/z (%) 168 (3, $[\text{M}-\text{H}_2\text{O}]^+$), 158 (1), 139 (12), 126 (7), 115 (40), 97 (85), 83 (19), 69 (23), 55 (100), 43 (64). CI-MS (CH_4): m/z (%) 187 (24, $[\text{M}+\text{H}]^+$), 169 (74), 151 (63), 127 (100), 109 (55). $^1\text{H-NMR}$ (CDCl_3): δ 4.49 (1H, m, H-3), 4.37 (1H, m, H-4), 2.80 (1H, dd, $J = 17.6, 5.6$ Hz, H-2), 2.56 (1H, dd, $J = 17.6, 1.0$ Hz, H-2), 1.88 (1H, m, H-5), 1.71 (1H, m, H-5), 1.55–1.25 (8H, m, H-6, H-7, H-8 and H-9), 0.90 (3H, t, $J = 7.0$ Hz, H-10). $^{13}\text{C-NMR}$ (CDCl_3): δ 175.3 (C-1), 84.7 (C-4), 69.3 (C-3), 39.6 (C-2), 31.8 (C-8 or C-9), 29.3 (C-7), 28.4 (C-5), 25.7 (C-6), 22.7 (C-8 or C-9), 14.2 (C-10).

Synthesis of a racemic mixture of erythro-lactones

First, mCPBA (6.65 g) was added to a solution of (*E*)-methyl dec-3-enoate (2.76 g) in CH_2Cl_2 (10 mL), and the mixture was stirred at room temperature for 20 h. The reaction was quenched by addition of a 5% aqueous Na_2SO_3 solution. After filtration and evaporation, the reaction mixture was extracted using diethyl ether, washed with water and brine, and subsequently dried. Epoxy ester (2.04 g, yield: 67%) was obtained as a racemic mixture. Amberlyst-15 (1.0 g) was added to a solution of epoxy ester (2.0 g) in benzene (50 mL), and the mixture was stirred at room temperature for 18 h. After filtration and evaporation, the reaction mixture (1.35 g) was subjected to silica gel chromatography

eluted with 50% methyl acetate in hexane to afford a racemic mixture of *erythro*-lactones—(3*R*,4*S*)-HD and (3*S*,4*R*)-HD—(720 mg, yield: 39%) with a minor *threo*-lactone (Figure S1). The racemic *erythro*-lactone was further purified via HPLC on a silica gel column (YMC-Pack SIL, 5 μ m, 10 $\times$ 300 mm, YMC Co., Ltd., Japan, flow rate of 2.5 mL/min with 60% ethyl acetate in hexane; yield: 21 mg). The spectral data are provided in Table S2.

Synthesis of each enantiomeric threo-lactone

First, (*E*)-3-decenoic acid was esterified with methanol containing 1% H₂SO₄ to yield methyl (*E*)-3-decenoate. AD-mix- α or - β (0.7 g) was added to 50% aqueous *tert*-BuOH (5 mL) and stirred at room temperature until two clear phases appeared. CH₃SO₂NH₂ (49 mg) was added to this solution and subsequently cooled to 0 °C. Methyl (*E*)-3-decenoate (90 mg) was added to the solution and the reaction mixture was stirred at 0 °C for 24 h. The reaction was quenched by addition of NaHSO₃ and extracted with EtOAc. The extract was washed with 2 N NaOH and brine, and subsequently dried. (3*R*,4*R*)-3-HD (85 mg, yield: 82%) and (3*S*,4*S*)-3-HD (82 mg, yield: 80%) were obtained from the reaction with AD-mix- β and AD-mix- α , respectively. (Figure S1B). The spectral data are provided in Table S2.

Synthesis of the minor components of the rectal gland

First, *N*-(3-methylbutyl)acetamide (**2**) was obtained by acetylation of 3-methylbutylamine using acetic anhydride. Subsequently, (*E,E*)- and (*E,Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecanes (**3** and **4**) were synthesized as previously described [21] and the corresponding mass spectral data are shown in Table S3.

259

260 *Quantification of rectal volatiles*

261 A 1 or 5 μ L portion of each rectal tissue extract (1 gland/250 μ L) was subjected to GC
262 quantifications with 1-pentadecanol (Wako Pure Chemical Industries, Japan) as an
263 internal standard. The contents of the relevant compounds in the rectal tissues were
264 determined by comparing FID intensities with those of standard samples with known
265 concentrations.

266

267

268 **Author contributions**

269 HO and RN conceived and designed research. HO, MN, SO, JO, TK, IT, YH, IN and RN
270 conducted experiments and analyzed data. HO and RN wrote the manuscript. All authors
271 read and approved the manuscript.

272

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278

279 **Disclosure statement**

280 No potential conflict of interest was reported by the authors.

281

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- 339

Figure captions

Figure 1. Representative total ion chromatogram of an ethanol extract from a male rectal gland of *Bactrocera tsuneonis*. The structures of the main components are also shown. **1**: (3*R*,4*S*)-3-Hydroxy-4-decanolide, **2**: *N*-(3-Methylbutyl)acetamide, **3**: (*E,E*)- and (*E,Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane, **4**: (*E,Z*)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane, **5**: 2-Methyl-8-ethyl-1,7-dioxaspiro[5.5]undecane.

Figure 2. The amounts of compounds **1** and **2** in the wild male and female rectal gland complexes of *Bactrocera tsuneonis* where each value is plotted as a dot ($n = 5-7$). The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers). Welch's *t*-test: $p < 0.01$.

Figure 3. The amounts of compounds **1** and **2** in immature (0–3-d AE) and mature (7–10 or 21–24-d AE) flies of *Bactrocera tsuneonis* where each value is plotted as a dot ($n = 6-20$). Boxes with different letters are significantly different at $p < 0.05$ as determined by Steel-Dwass test.

Figure 4. The amounts of compounds **1** and **2** in mature flies of *Bactrocera tsuneonis* 7–10-d AE fed with normal food or sucrose solution only where each value is plotted as a dot ($n = 6-15$). The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers). Boxes with different letters are significantly different at $p < 0.05$ as determined by Steel-Dwass test.

1

2

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4

5

Fig. 1

1

2

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4

5

1: γ -Lactone

2: Acetamide

B

Fig. S1

Fig. S3

CI-MS

A

B

C

C-1

C-4

C-3

C-8 or C-9

C-2

C-7

C-5

C-6

C-8 or C-9

C-10

200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 ppm

A (3*R*,4*R*)-3-Hydroxy-4-decanolide

B (3*R*,4*S*)- and (3*S*,4*R*)-3-Hydroxy-4-decanolide

A

C

B

D

SUPPLEMENTARY MATERIAL

Predominant accumulation of a 3-hydroxy- γ -decalactone in the male rectal gland complex of the Japanese orange fly, *Bactrocera tsuneonis*

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Contents:

Table S1. EI-MS spectral data of the extract of the male rectal gland complexes.

Table S2. Spectral data of the synthesized 3-hydroxy- γ -decalactones.

Table S3. EI-MS spectral data of the synthesized minor components in the rectal gland.

Figure S1. Synthetic routes for 3-hydroxy- γ -decalactone isomers. (A) racemic ($\pm$)-*erythro*- γ -lactone. (B) (+)- and (–)-*threo*- γ -lactones: (3*R*,4*R*)-3-hydroxy-4-decanolide and (3*S*,4*S*)-3-hydroxy-4-decanolide.

Figure S2. Correlation diagram between amounts of **1** ((3*R*,4*S*)-3-hydroxy-4-decanolide) and **2** (*N*-(3-methylbutyl)acetamide) in individual females from 7- to 10-days after eclosion. Data are derived from Figure 2B.

Figure S3. CI-Mass spectra of (3*R*,4*R*)-3-hydroxy-4-decanolide (**1**) isolated from *Bactrocera tsuneonis* (A), and synthesized **1** (B).

Figure S4. EI-Mass spectra. (A) (3*R*,4*R*)-3-hydroxy-4-decanolide (**1**) isolated from *Bactrocera tsuneonis*. (B) synthesized **1**. (C) racemic (3*R*,4*S*)- and (3*S*,4*R*)-3-hydroxy-4-decanolide.

Figure S5. ¹H NMR spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S6. ¹³C NMR spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S7. ¹H-¹H COSY spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S8. HMQC spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S9. HMBC spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S10. ^1H NMR spectra of synthesized 3-hydroxy-4-decanolide. (A) (3*R*,4*R*)-3-hydroxy-4-decanolide. (B) racemic (3*R*,4*S*)- and (3*S*,4*R*)-3-hydroxy-4-decanolide.

Figure S11. EI-Mass spectra of the minor components (**2-5**) contained in the rectal gland complexes of *Bactrocera tsuneonis*. (A) *N*-(3-methylbutyl)acetamide (**2**). (B) (*E,E*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**3**). (C) (*E,Z*)-2,8-dimethyl-1,7-dioxaspiro[5.5]undecane (**4**). (D) 2-methyl-8-ethyl-1,7-dioxaspiro[5.5]undecane (**5**).

Table S1. EI-MS spectral data of the extract of the male rectal gland complexes.

Compound	<i>Rt</i> (min)	Mass fragments [<i>m/z</i> (%)]
1	16.7	168 (2, [M–H ₂ O] ⁺), 158 (1), 139 (8), 126 (5), 115 (39), 97 (84), 83 (15), 69 (23), 55 (100), 43 (60).
2	9.31	129 (8, M ⁺), 114 (17), 86 (33), 73 (98), 72 (87), 60 (38), 55 (22), 43 (100).
3	9.49	184 (11, M ⁺), 169 (3), 140 (17), 125 (11), 115 (94), 112 (100), 97 (51), 83 (14), 69 (27), 55(26), 43 (23).
4	10.6	184 (6, M ⁺), 169 (3), 140 (6), 125 (8), 115 (100), 112 (47), 97 (57), 83 (9), 69 (34), 55(23), 43 (16).
5	10.8	198 (15, M ⁺), 183 (2), 169 (24), 154 (12), 140 (20), 129 (56), 126 (45), 115 (100), 112 (89), 97 (48), 83 (55), 69 (39), 55 (40), 44 (48).

Table S2. Spectral data of the synthesized 3-hydroxy- γ -decalactones.

A mixture of (3*R*,4*S*)- and (3*S*,4*R*)-3-hydroxy-4-decanolide

EI-MS: m/z (%) 168 (0.2, [M–H₂O]⁺), 158 (2), 139 (2), 126 (3), 115 (53), 97 (100), 83 (10), 69 (18), 55 (81), 43 (40).

¹H-NMR (CDCl₃): δ 4.37 (1H, m, H-4), 4.27 (1H, m, H-3), 2.82 (1H, dd, J = 18.0, 6.7 Hz, H-2), 2.51 (1H, dd, J = 18.0, 3.6 Hz, H-2), 1.68-1.54 (2H, m, H-5), 1.50-1.24 (8H, m, H-6, H-7, H-8 and H-9), 0.89 (3H, t, J = 6.9 Hz, H-10).

¹³C-NMR (CDCl₃): δ 175.9 (C-1), 88.3 (C-3), 71.6 (C-4), 37.7 (C-2), 33.1, 31.6, 29.0, 25.2, 22.5, 14.0 (C-10).

(3*R*,4*R*)-3-Hydroxy-4-decanolide

$[\alpha]_D^{25}$ +40.1 (c = 1.1, CH₃OH, 22°C)

EI-MS: m/z (%) 168 (0.3, [M–H₂O]⁺), 158 (2), 139 (3), 126 (3), 115 (53), 97 (100), 83 (11), 69 (22), 55 (99), 43 (51).

CI-MS: m/z (%) 187 (31, [M+H]⁺), 169 (82), 151 (63), 127 (100), 109 (51).

¹H-NMR (CDCl₃): δ 4.48 (1H, m, H-3), 4.37 (1H, m, H-4), 2.80 (1H, dd, J = 17.7, 5.5 Hz, H-2), 2.56 (1H, dd, J = 17.7, 0.5 Hz, H-2), 1.87 (1H, m, H-5), 1.72 (1H, m, H-5), 1.55-1.25 (8H, m, H-6, H-7, H-8 and H-9), 0.89 (3H, t, J = 7.0 Hz, H-10).

¹³C-NMR (CDCl₃): δ 175.5 (C-1), 84.8 (C-4), 69.1 (C-3), 39.5 (C-2), 31.6 (C-8 or C-9), 29.1 (C-7), 28.3 (C-5), 25.5 (C-6), 22.6 (C-8 or C-9), 14.0 (C-10).

(3*S*,4*S*)-3-Hydroxy-4-decanolide

$[\alpha]_D^{25}$ –35.1 (c = 1.1, CH₃OH, 21°C)

Table S3. EI-MS spectral data of the synthesized minor components in the rectal gland.

Compound	Mass fragments [m/z (%)]
<i>N</i> -(3-Methylbutyl)acetamide (2)	129 (12, M ⁺), 114 (22), 86 (35), 73 (100), 72 (85), 60 (37), 55 (20), 43 (84).
(<i>E,E</i>)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (3)	184 (13, M ⁺), 169 (3), 140 (22), 125 (12), 115 (96), 112 (100), 97 (55), 83 (17), 69 (31), 55 (29), 43 (24).
(<i>E,Z</i>)-2,8-Dimethyl-1,7-dioxaspiro[5.5]undecane (4)	184 (7, M ⁺), 169 (3), 140 (6), 125 (7), 115 (100), 112 (45), 97 (54), 83 (9), 69 (35), 55(23), 43 (17).

Figure S1. Synthetic routes for 3-hydroxy- γ -decalactone isomers. (A) racemic ($\pm$)-*erythro*- γ -lactone. (B) (+)- and (–)-*threo*- γ -lactones: (3*R*,4*R*)-3-hydroxy-4-decanolide and (3*S*,4*S*)-3-hydroxy-4-decanolide.

Figure S2. Correlation diagram between amounts of **1** ((3*R*,4*S*)-3-hydroxy-4-decanolide) and **2** (*N*-(3-methylbutyl)acetamide) in individual females from 7- to 10-days after eclosion. Data are derived from Figure 2B.

CI-MS

Figure S3. CI-Mass spectra of (3*R*,4*R*)-3-hydroxy-4-decanolide (**1**) isolated from *Bactrocera tsuneonis* (A), and synthesized **1** (B).

EI-MS

Figure S4. EI-Mass spectra. (A) (3*R*,4*R*)-3-hydroxy-4-decanolide (**1**) isolated from *Bactrocera tsuneonis*. (B) synthesized **1**. (C) racemic (3*R*,4*S*)- and (3*S*,4*R*)-3-hydroxy-4-decanolide.

Figure S5. ^1H NMR spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S6. ^{13}C NMR spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S7. ^1H - ^1H COSY spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S8. HMQC spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S9. HMBC spectrum of **1** isolated from *Bactrocera tsuneonis*.

Figure S10. ^1H NMR spectra of synthesized 3-hydroxy-4-decanolide. (A) (3*R*,4*R*)-3-hydroxy-4-decanolide. (B) racemic (3*R*,4*S*)- and (3*S*,4*R*)-3-hydroxy-4-decanolide.

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