Supplementary Information for 'Mandibular glands secrete 24-methylenecholesterol into honey bee (*Apis mellifera*) food jelly'

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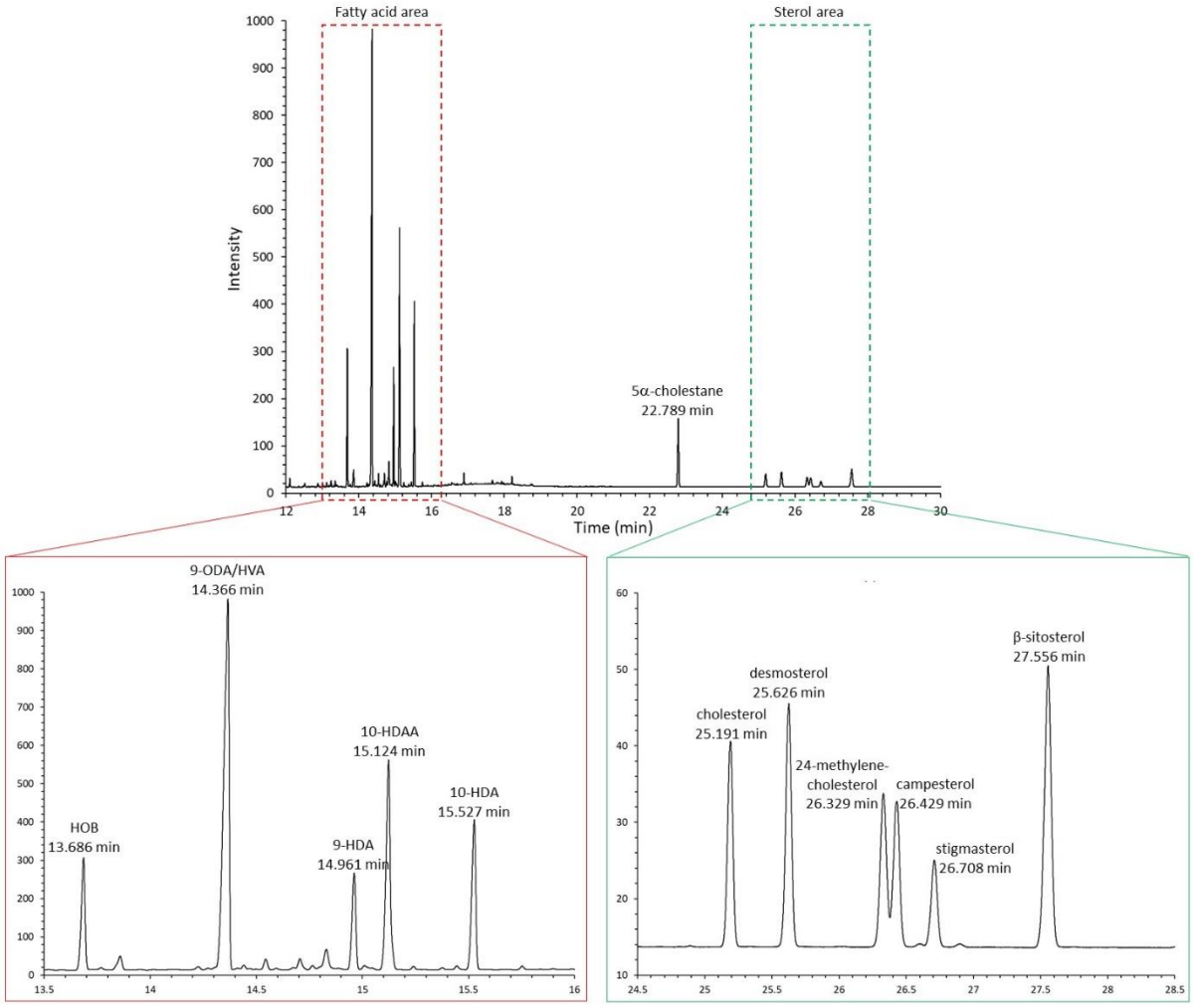


Figure S1: Chromatogram of the external standards. Besides the detection of the sterols (green boxes) and the fatty acids (red boxes), the method also allows for detection of the aromatic mandibular gland compound HOB. HVA does in the current method co-elute with 9-ODA. However, whereas workers of the African subspecies *A. m. scutellata* are indeed known to produce minor amounts of the queen pheromone 9-ODA, HVA has never been reported in workers of any *A. mellifera* subspecies as it is exclusive to queens (for review see (Mumoki et al., 2021; Mumoki & Crewe, 2021)). Thus, the peak eluting after 14.366 min in the samples was considered to be 9-ODA only.

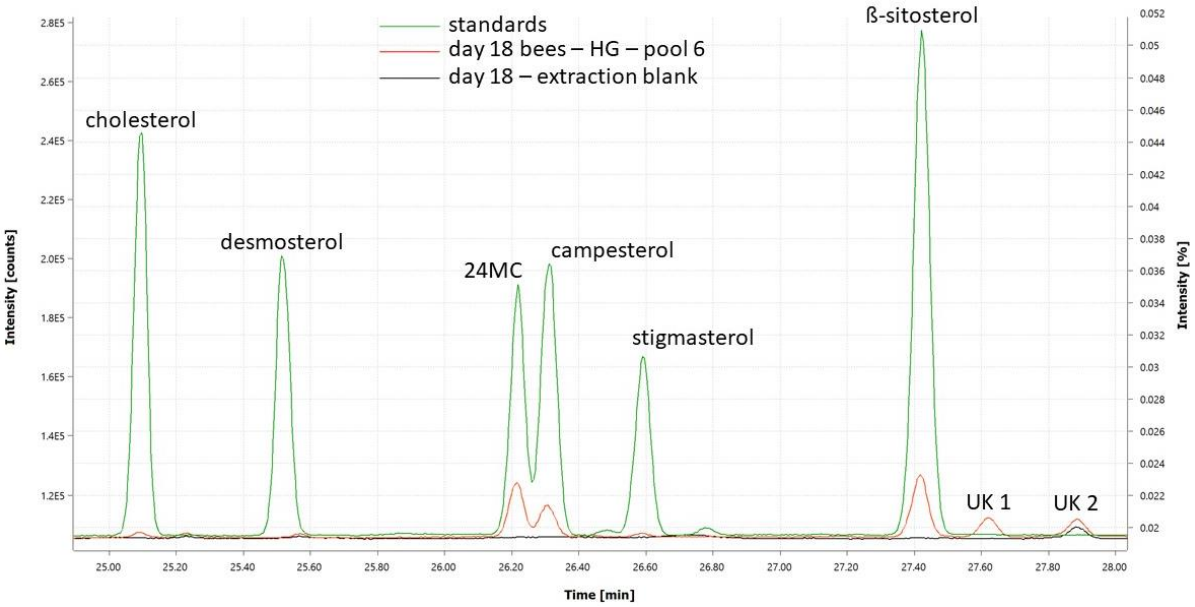
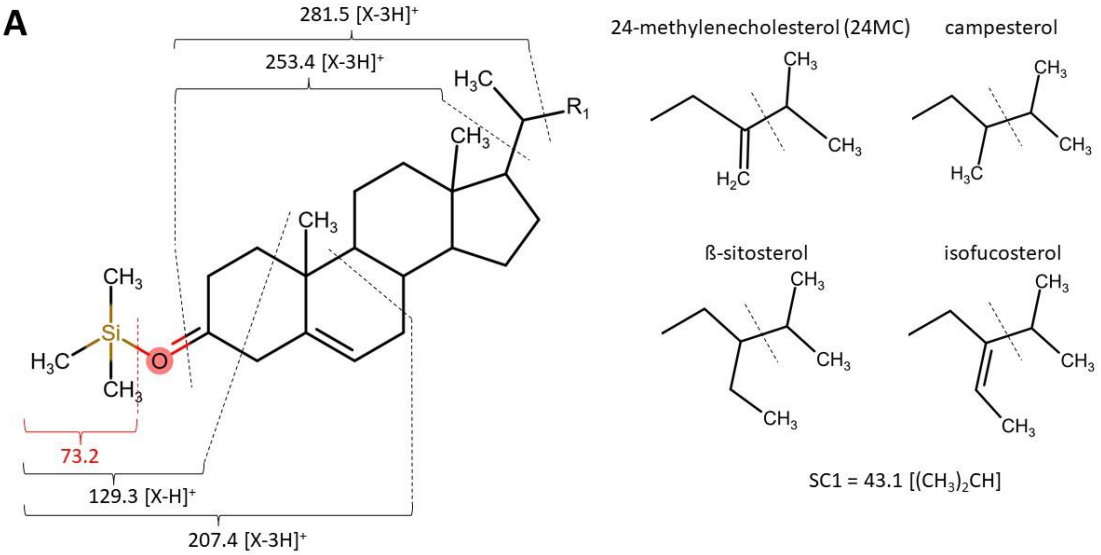


Figure S2: Chromatogram of the sterol area of the hypopharyngeal glands of 18 days old honey bees (red line), the concomitant blank extraction (black line) and the external standard run. The first unknown peak (UK1 – 27.618 min) did only elute in the samples, whereas the second unknown peak (UK2 – 27.884 min) was also found in the extraction blanks.

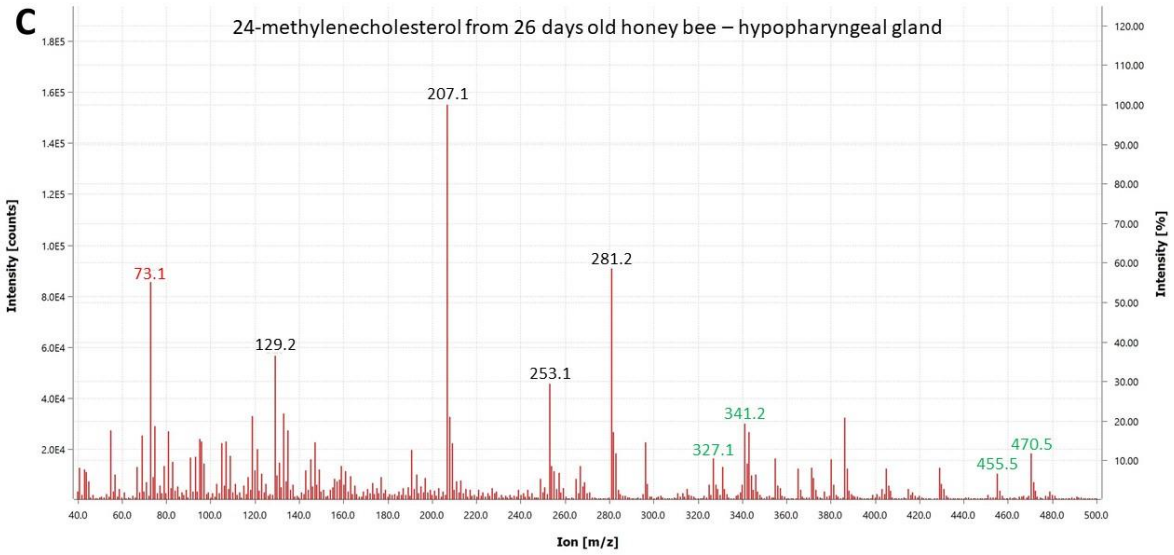
A

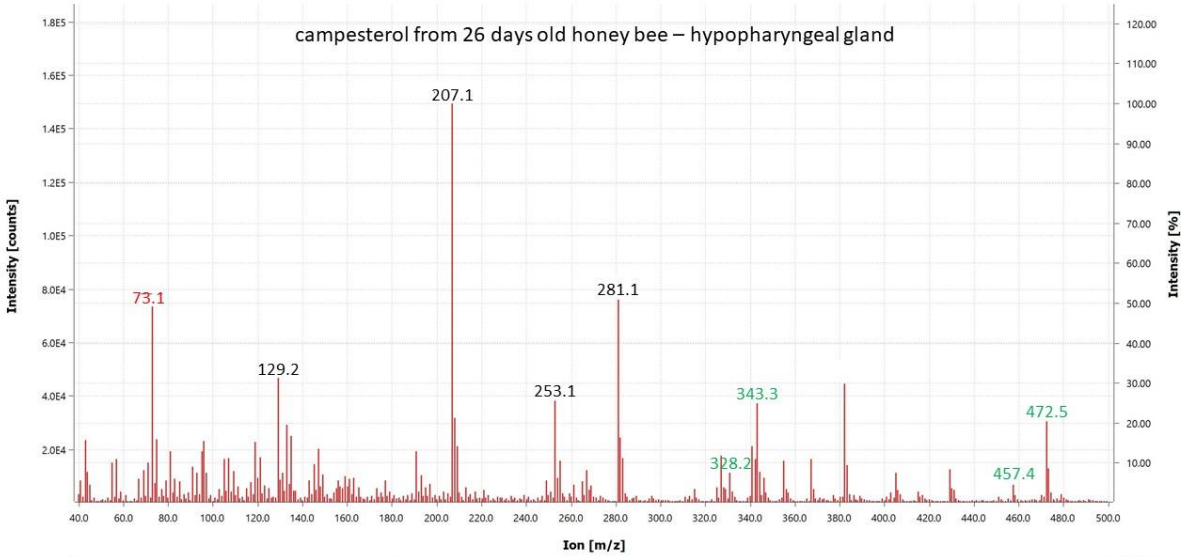


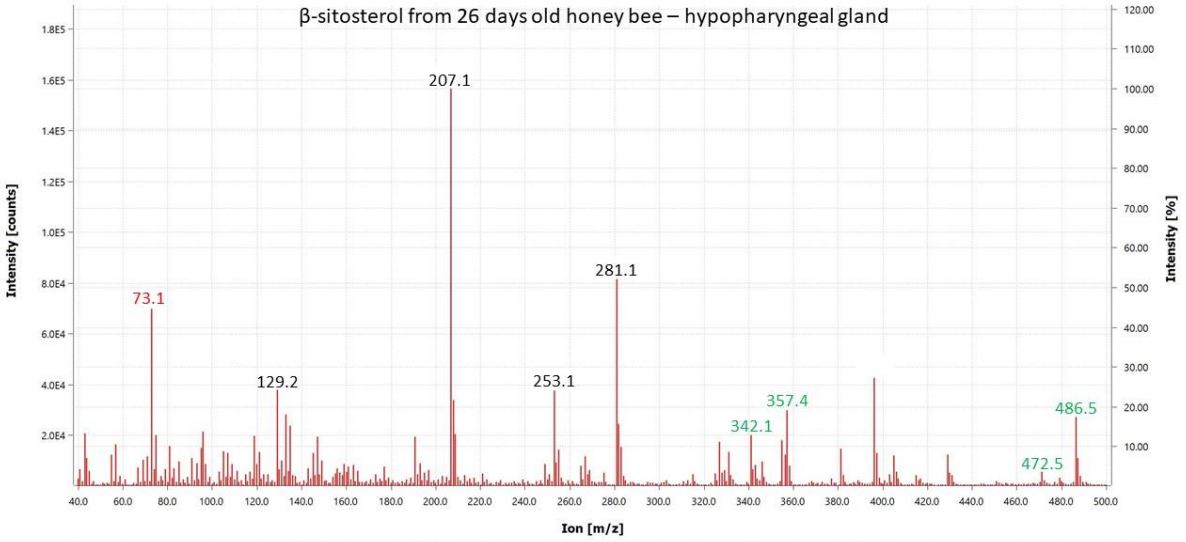
B

Sterol	24MC	campesterol	β-sitosterol	isofucosterol
M^+ (molecular ion)	470.9	472.9	486.9	484.9
Side chain (SC)	97.2	99.2	113.2	111.2
$[M-129.3-CH_3]^+$	326.6	328.6	342.6	340.6
$[M-129.3]^+$	341.6	343.6	357.6	355.6
$[M-SC1]^+$	427.8	429.8	443.8	441.8
$[M-CH_3]^+$	455.9	457.9	472.9	469.9

C




campesterol from 26 days old honey bee – hypopharyngeal gland
207.1
73.1
281.1
129.2
253.1
343.3
472.5
328.2
457.4
Intensity [counts]
Intensity [%]
Ion [m/z]


β-sitosterol from 26 days old honey bee – hypopharyngeal gland
207.1
73.1
281.1
129.2
253.1
357.4
342.1
486.5
472.5
Intensity [counts]
Intensity [%]
Ion [m/z]

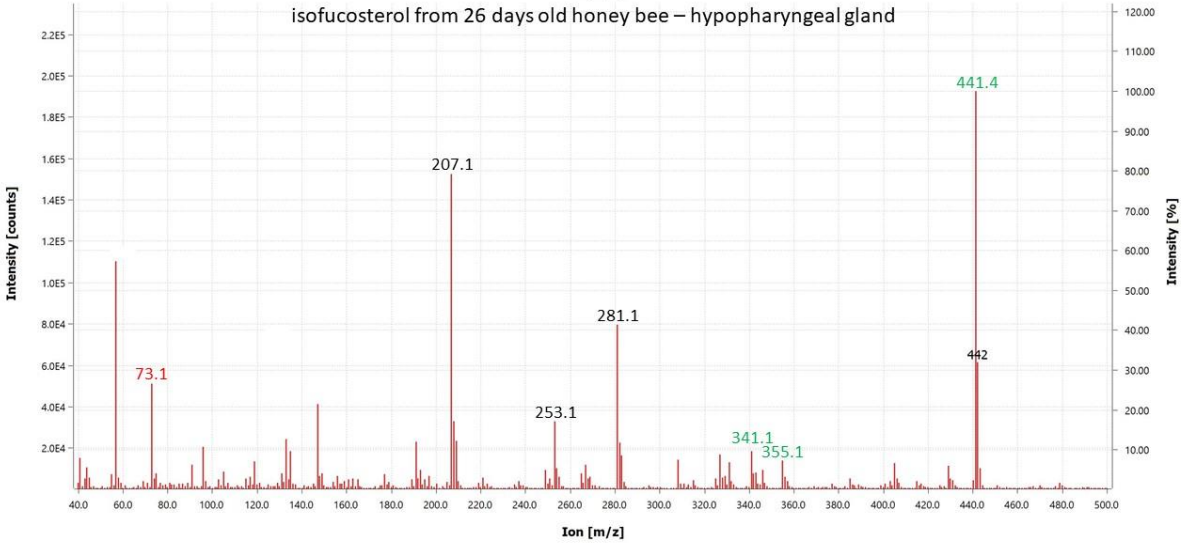

isofucosterol from 26 days old honey bee – hypopharyngeal gland
441.4
207.1
281.1
73.1
442
253.1
341.1
355.1
Intensity [counts]
Intensity [%]
Ion [m/z]

Figure S3: Identification of the sterols via GC-MS. A) Structure of the silylated sterols with molecular weights of fragment ions. B) Molecular weights of phytosterol-specific molecular and fragment ions. Verified ions are highlighted in green. C) Mass spectra of 24MC, campesterol, β-sitosterol and isofucosterol sample peaks. Fragment ions specific for the silylation shown in red, specific for the sterol backbone in black and specific for the respective phytosterol in green. For 24MC, campesterol and β-sitosterol the molecular ion was found. For isofucosterol, the molecular ion could not be identified but the fragment ion missing the $(CH_3)_2CH$ of the side chain was the base peak. (Experimental details: GCMS-QP2010 SE (Shimadzu, Kyōto, Japan); column: Rtxi-5MS (5% diphenyl / 95% dimethyl polysiloxane, 30 m × 0.25 mm × 0.25 µm) (Zebron-phenomenex, Torrance, CA, USA); program as described in section 2.3.)

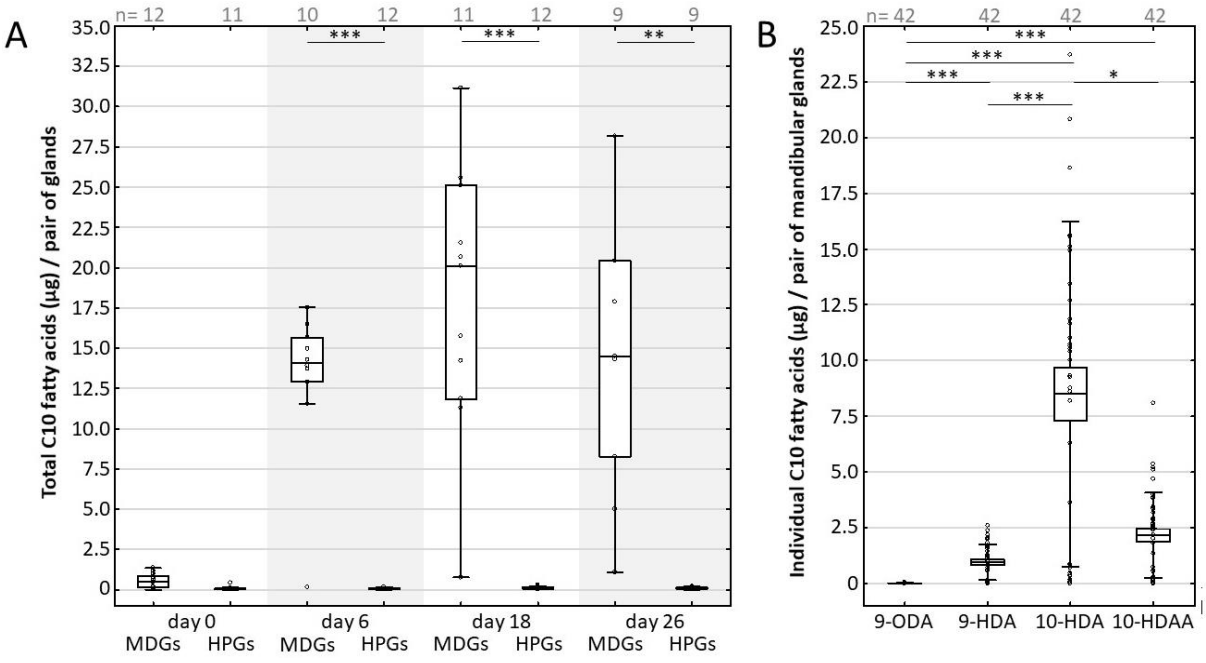


Figure S4: C10 fatty acids in the glands. A) Total C10 fatty acids (µg) per pair of glands. Boxes show medians ± IQR and whiskers show the non-outlier range. Statistics have been performed using Kruskal Wallis ANOVA. B) Individual C10 fatty acids (µg) within the mandibular glands across age groups. Boxes show means ± SE and whiskers show SD. Statistics have been performed using one-way ANOVA with post hoc Bonferroni tests (Box-Cox-transformed data). Individual data points are shown as open circles. Significant differences are indicated by asterisks (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). HPGs, hypopharyngeal glands; MDGs, mandibular glands; 9-ODA, 9-oxo-2-decenoic acid; 9-HDA, 9-hydroxy-2-decenoic acid; 10-HDA, 10-hydroxy-2-decenoic acid; 10-HDAA, 10-hydroxy-decanoic acid; n, sample size of analysed pools (each pool contained glands of three bees).

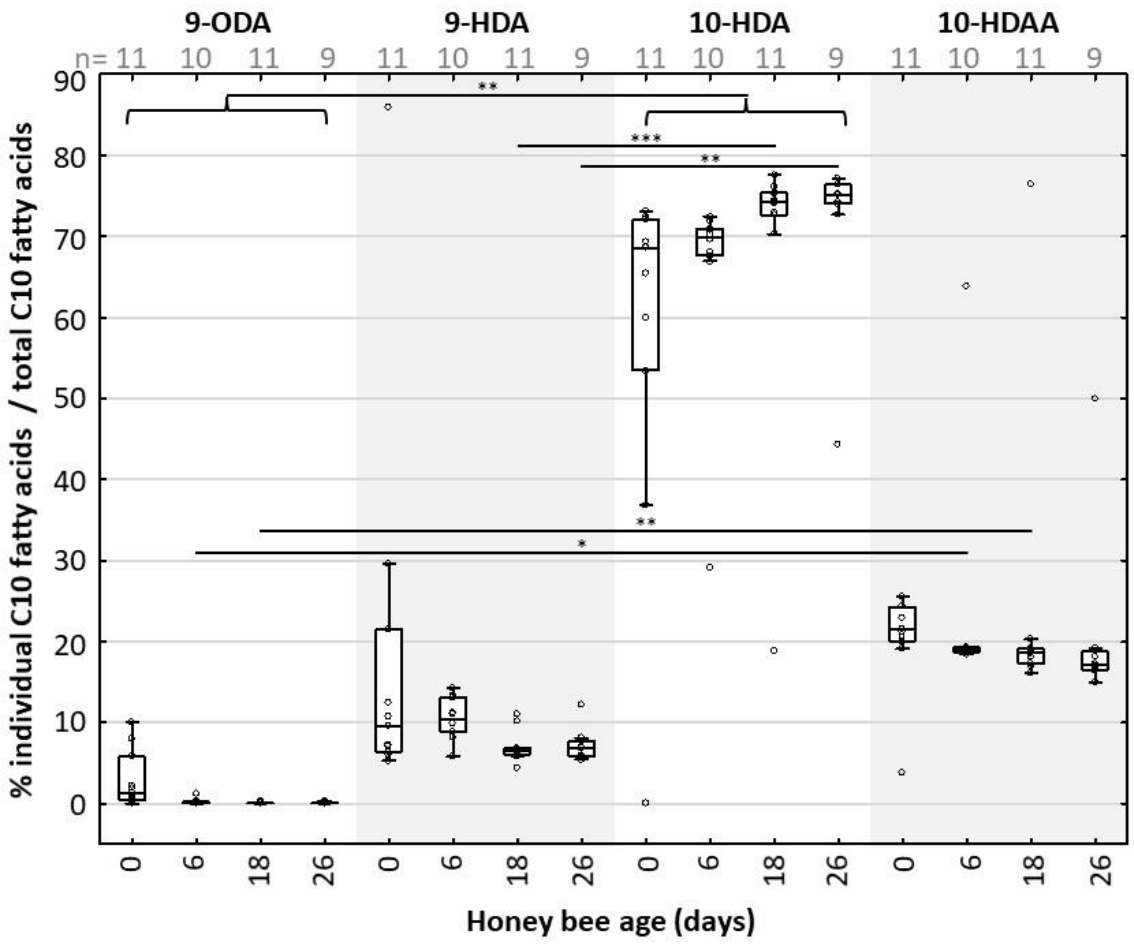


Figure S5: Percentage of individual fatty acids per total sterols in the mandibular glands. Boxes show median ± IQR and whiskers show the non-outlier range. Individual data points are shown as open circles. Statistics have been performed using Kruskal Wallis ANOVA. Significant differences are indicated by asterisks (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). 9-ODA, 9-oxo-2-decenoic acid; 9-HDA, 9-hydroxy-2-decenoic acid; 10-HDA, 10-hydroxy-2-decenoic acid; 10-HDAA, 10-hydroxy-decanoic acid; n, sample size of analysed pools (each pool contained glands of three bees). The sample size differs in freshly hatched bees from Figure 2 (11 compared to 12), as in one of the pools none of the C10 fatty acids was detected and this pool was omitted from the percentage analysis.

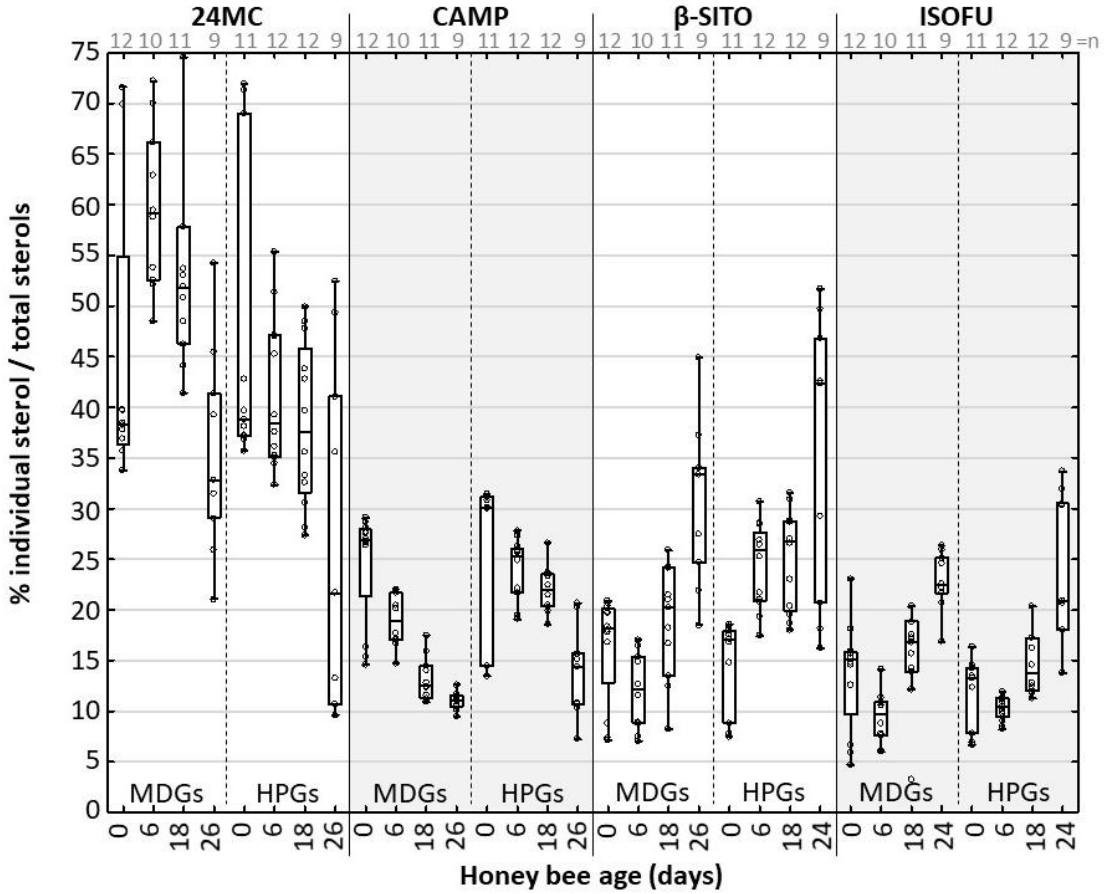


Figure S6: Percentage of individual sterols per total sterols. Boxes show median ± IQR and whiskers show the non-outlier range. Individual data points are shown as open circles. HPGs, hypopharyngeal glands; MDGs, mandibular glands; 24MC, 24-methylenecholesterol; CAMP, campesterol; β-SITO, β-sitosterol; ISOFU, isofucosterol; n, sample size of analysed pools (each pool contained glands of three bees).

Table S1: Amounts (ng) of indicated substances in the samples detected by gas chromatography. FH, freshly hatched bees; N6, 6 days old nurse bees; F18, 18 days old nurse bees; F26, 26 days old nurse bees; PF, pollen forager; MA, mandibular glands; HG, hypopharyngeal glands; MAS, mandibular gland secretion; WJ, worker jelly.

Sample	HOB	9-ODA/HVA	9-HDA	10-HDAA	10-HDA	Cholesterol	Desmosterol	24MC	Campesterol	Stigmasterol	b-Sitosterol	Isofucosterol
FH_MA1	10.6	25.0	66.9	72.3	92.4	trace	0.0	204.8	138.9	0.0	94.6	76.0
FH_MA2	10.6	12.9	30.4	65.5	121.1	trace	0.0	208.7	156.8	0.0	97.3	83.4
FH_MA3	9.0	9.9	11.8	22.9	16.5	trace	0.0	150.5	116.4	0.0	88.0	67.1
FH_HG1	6.9	11.2	24.6	29.3	18.1	trace	0.0	338.8	238.1	0.0	116.7	97.2
FH_HG2	33.9	61.7	169.6	97.3	134.6	trace	0.0	269.7	212.6	0.0	131.4	93.9
FH_HG3	8.5	14.7	35.8	34.2	25.1	trace	0.0	255.1	213.9	0.0	120.5	97.6
Blank 1	5.2	11.6	34.4	33.6	35.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FH_MA4	39.4	51.1	162.6	158.8	400.9	trace	0.0	182.7	129.6	trace	90.5	57.9
FH_MA5	26.6	15.2	62.1	135.4	397.5	trace	0.0	132.8	100.5	0.0	62.1	50.2
FH_MA6	22.8	18.8	57.4	177.0	481.5	trace	0.0	166.6	118.2	0.0	87.4	69.0
FH_HG4	21.9	16.5	19.3	35.7	22.9	trace	0.0	242.4	183.0	0.0	104.2	80.4
FH_HG5	21.4	11.3	9.0	40.5	21.5	trace	0.0	169.6	146.6	0.0	81.3	77.7
FH_HG6	15.5	20.0	37.5	42.5	36.9	trace	0.0	240.4	186.5	0.0	111.2	82.2
Blank 2	6.3	19.8	20.6	44.0	14.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FH_MA7	4.2	8.5	39.5	136.2	344.5	0.0	0.0	92.2	71.9	0.0	45.9	62.9
FH_MA8	6.6	6.0	17.4	54.5	128.1	trace	0.0	109.8	90.1	0.0	66.3	59.0
FH_MA9	4.9	6.2	9.0	15.7	13.7	trace	0.0	107.6	77.7	0.0	60.4	45.7
FH_HG7	Run	failed										
FH_HG8	12.5	30.7	61.0	37.1	60.1	trace	0.0	146.0	123.9	0.0	72.9	53.4
FH_HG9	4.3	7.7	12.1	15.3	11.1	trace	0.0	135.8	114.8	0.0	61.5	53.0
Blank 3	1.4	5.8	6.1	13.1	5.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
FH_MA10	28.3	14.2	67.6	229.0	722.9	trace	0.0	380.8	81.4	0.0	37.8	31.7
FH_MA11	14.2	15.9	95.3	257.5	863.1	trace	0.0	321.9	73.3	0.0	33.2	21.1
FH_MA12	37.9	38.4	151.1	305.0	877.1	trace	0.0	318.4	66.4	0.0	40.1	30.6
FH_HG10	13.9	14.3	23.9	35.0	54.7	trace	0.0	401.1	83.7	0.0	51.1	45.5
FH_HG11	13.6	9.4	6.5	29.3	22.8	trace	0.0	389.2	78.9	0.0	40.9	36.4
FH_HG12	4.8	11.0	13.3	30.5	37.5	trace	0.0	387.5	72.5	0.0	41.7	37.2
Blank 4	4.3	12.3	15.6	25.5	26.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N6_MA1	14.5	34.7	2057.5	2947.2	10664.8	0.0	0.0	567.2	217.7	0.0	177.2	117.5

N6_MA2	11.7	18.8	1579.9	2698.0	10040.6	trace	0.0	436.2	183.9	0.0	153.5	126.8
N6_MA3	17.7	61.1	1993.2	2584.0	9330.8	trace	0.0	451.1	183.8	0.0	129.0	74.1
N6_HG1	5.9	16.2	31.2	44.5	140.8	trace	0.0	771.4	574.7	0.0	626.0	219.7
N6_HG2	4.0	11.3	18.0	29.2	50.1	trace	0.0	636.0	507.9	0.0	603.8	219.8
N6_HG3	2.7	14.0	15.8	39.7	74.3	trace	0.0	842.9	557.5	0.0	637.1	203.2
Blank 5	0.0	8.1	5.9	23.4	22.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N6_MA4	Run	failed										
N6_MA5	14.7	17.6	1451.8	3396.8	12705.5	0.0	0.0	278.8	116.0	0.0	79.4	60.6
N6_MA6	11.6	29.6	1844.5	2603.0	9277.4	trace	0.0	284.5	106.5	0.0	56.1	36.5
N6_MA7	10.4	12.6	1649.6	3200.9	11680.7	0.0	0.0	374.9	107.2	0.0	79.8	68.2
N6_HG4	1.9	10.2	16.0	38.7	79.4	trace	0.0	479.0	380.0	trace	345.6	162.3
N6_HG5	4.3	14.8	11.5	34.2	36.3	trace	0.0	432.0	343.0	0.0	336.3	141.1
N6_HG6	3.5	14.3	17.2	30.3	26.4	trace	0.0	454.6	323.2	0.0	338.5	142.1
N6_HG7	5.3	21.2	29.2	47.7	114.9	trace	0.0	600.4	396.1	0.0	404.0	129.4
Blank 6	2.6	19.1	9.5	29.3	35.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N6_MA8	24.1	19.6	1150.6	2480.6	9285.1	trace	0.0	600.7	168.9	0.0	84.3	100.5
N6_MA9	13.2	36.0	1155.1	2205.6	8213.6	trace	0.0	614.0	158.8	trace	82.8	72.5
N6_MA10	Run	failed										
N6_MA11	18.3	20.9	1682.8	2874.2	10443.8	0.0	0.0	731.2	173.7	0.0	77.7	62.3
N6_HG8	4.5	8.9	11.1	30.4	50.7	trace	0.0	898.7	341.5	0.0	339.3	170.4
N6_HG9	8.4	15.7	24.6	36.0	70.9	trace	0.0	1462.9	504.4	trace	460.3	216.5
N6_HG10	4.9	10.3	14.3	35.4	51.1	56.1	0.0	1161.2	534.3	trace	517.0	252.5
N6_HG11	3.5	7.5	8.4	21.7	15.3	trace	0.0	1206.3	556.5	0.0	533.9	269.5
Blank 7	0.0	7.7	5.5	19.7	23.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F18_MA1	15.6	25.0	1234.8	3875.1	15590.8	0.0	0.0	134.7	38.9	0.0	74.0	57.5
F18_MA2	24.6	22.2	698.5	1848.6	8797.0	0.0	0.0	149.1	44.8	0.0	93.1	73.2
F18_MA3	32.8	27.8	2032.6	5391.9	23729.9	0.0	0.0	241.0	51.2	0.0	83.2	79.7
F18_HG1	7.1	7.1	13.5	30.1	78.4	trace	0.0	262.0	220.8	0.0	287.9	159.8
F18_HG2	6.1	6.9	10.0	39.5	82.2	0.0	0.0	319.3	213.4	0.0	299.6	212.1
F18_HG3	27.8	30.3	66.6	63.3	113.4	trace	0.0	367.1	241.5	trace	274.0	150.0
Blank 8	1.3	7.6	3.6	22.2	20.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F18_MA4	24.0	47.1	997.1	2885.9	11881.7	trace	0.0	180.1	58.6	trace	55.9	40.8
F18_MA5	17.6	24.4	2401.7	4006.3	15137.8	trace	0.0	153.7	34.7	0.0	64.0	64.5
F18_MA6	0.0	12.7	43.4	608.0	165.4	0.0	0.0	227.3	42.7	0.0	25.0	trace

F18_HG4	8.0	12.0	34.3	67.8	232.5	trace	0.0	290.0	157.3	trace	197.1	86.9
F18_HG5	1.9	10.1	14.0	33.1	62.5	trace	0.0	315.0	168.0	0.0	145.9	89.6
F18_HG6	3.5	10.2	19.2	48.8	127.7	trace	0.0	391.0	195.7	trace	210.4	117.3
Blank 9	0.0	10.5	32.9	45.7	203.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F18_MA7	12.1	19.0	1302.8	3858.8	14967.6	trace	0.0	202.5	50.7	trace	47.3	49.7
F18_MA8	21.7	19.5	1721.8	5214.0	18671.3	trace	0.0	321.8	88.4	0.0	69.1	77.5
F18_MA9	Run	failed										
F18_HG7	3.7	5.9	9.4	29.8	91.8	trace	0.0	342.1	140.3	trace	138.0	85.5
F18_HG8	15.4	26.4	56.4	29.7	78.6	32.9	trace	570.0	230.7	trace	212.6	128.2
F18_HG9	7.2	12.2	15.0	25.1	29.8	34.3	0.0	306.3	144.3	0.0	115.6	75.4
Blank 10	0.0	6.7	5.4	13.5	6.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F18_MA10	0.0	20.5	978.7	2737.7	10566.6	trace	0.0	130.6	35.0	0.0	68.2	48.7
F18_MA11	4.3	19.4	1224.6	2041.9	8629.6	0.0	0.0	168.5	36.1	0.0	71.3	55.8
F18_MA12	11.4	34.3	1725.8	4726.5	18665.6	trace	0.0	173.4	38.5	0.0	70.2	52.3
F18_HG10	5.2	10.4	14.7	49.2	132.3	trace	0.0	122.2	119.0	0.0	128.7	77.3
F18_HG11	1.3	7.1	9.6	29.6	52.4	0.0	0.0	139.8	79.8	0.0	135.5	74.1
F18_HG12	12.7	33.7	49.4	37.1	69.1	trace	trace	202.3	143.8	trace	164.2	98.7
Blank 11	0.0	3.9	0.0	12.7	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F26_MA1	21.3	34.0	1452.1	3365.3	15632.7	0.0	0.0	89.8	28.9	0.0	95.4	71.8
F26_MA2	13.7	19.8	618.3	767.5	3645.4	0.0	0.0	52.9	23.7	0.0	113.0	61.8
F26_MA3	13.1	24.3	578.9	1386.7	6321.2	trace	0.0	80.7	32.5	0.0	92.7	71.8
F26_HG1	2.5	14.9	11.8	38.6	48.4	0.0	0.0	42.4	42.8	0.0	186.4	127.3
F26_HG2	18.9	53.8	70.5	50.8	91.0	trace	trace	105.7	73.6	trace	208.2	100.9
F26_HG3	16.0	16.5	14.9	38.1	29.7	trace	0.0	54.5	58.5	0.0	281.9	172.4
Blank 12	2.2	6.9	6.7	21.9	40.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F26_MA4	13.2	17.5	986.0	3448.7	13471.1	trace	0.0	154.7	29.8	trace	52.7	48.1
F26_MA5	37.5	32.4	2619.5	8131.9	32384.0	trace	0.0	108.8	26.3	0.0	52.3	51.8
F26_MA6	22.9	24.6	2190.3	5146.2	20860.1	trace	0.0	118.0	32.8	0.0	70.4	64.2
F26_MA7	0.0	10.9	70.0	556.8	493.8	trace	0.0	60.8	17.2	0.0	42.6	34.3
F26_HG4	7.9	16.5	26.2	33.5	66.4	trace	0.0	267.0	80.1	trace	92.1	70.0
F26_HG5	4.8	9.5	14.3	42.0	138.8	12.6	0.0	196.0	81.9	trace	64.3	54.6
F26_HG6	4.3	12.2	14.7	41.6	69.5	trace	0.0	170.1	83.8	0.0	85.7	74.6
F26_HG7	2.7	10.4	14.5	42.4	85.3	trace	0.0	109.0	43.9	0.0	89.8	64.0
N6_MAS1	14.3	13.2	1006.2	1901.9	6474.6	trace	0.0	69.5	18.7	0.0	25.1	13.1

N6_MAS2	14.7	39.4	690.6	1027.4	3088.0	trace	trace	122.7	46.6	trace	52.1	27.1
Blank 13	0.0	6.2	2.1	14.8	18.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
F26_MA8	32.8	61.2	1181.8	2515.7	10771.5	trace	trace	93.5	36.0	trace	97.0	58.9
F26_MA9	5.2	23.9	852.7	2444.7	11051.7	trace	0.0	47.0	19.1	0.0	67.5	47.7
F26_HG8	0.0	9.1	0.0	27.3	4.5	trace	0.0	22.2	18.0	0.0	71.0	56.4
F26_HG9	0.0	15.0	22.1	56.6	184.1	trace	0.0	30.7	20.8	0.0	149.0	88.0
N6_MA12	0.0	11.9	20.2	139.0	73.9	trace	0.0	355.7	72.3	0.0	34.7	29.9
N6_HG12	8.1	23.7	27.1	46.4	102.3	40.4	0.0	768.9	375.8	trace	367.7	185.8
Blank 14	0.0	14.6	73.0	77.5	565.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
PF_MAS1	0.0	12.9	177.8	963.7	1534.5	0.0	0.0	trace	trace	0.0	22.0	trace
PF_MAS2	24.7	52.3	447.7	1052.3	4579.9	trace	trace	17.2	14.0	trace	23.1	trace
PF_MAS3	33.2	26.0	1488.5	2959.4	12120.4	trace	0.0	11.9	8.1	0.0	16.0	7.4
PF_MAS4	0.0	3.9	25.3	164.2	54.0	0.0	0.0	trace	trace	0.0	trace	0.0
N6_MAS3	7.5	16.1	1080.7	2020.7	6630.5	trace	0.0	72.8	28.1	0.0	27.9	20.5
N6_MAS4	9.0	14.8	668.4	1263.4	4248.1	0.0	0.0	118.2	46.3	0.0	46.0	30.2
Blank 1	0.0	6.8	6.0	22.7	36.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Blank 2	0.0	9.4	3.2	23.5	11.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
WJ_1	124.8	241.8	1281.7	3108.8	9064.0	trace	0.0	2011.6	471.1	0.0	287.5	182.0
WJ_2	0.0	186.8	2305.3	8093.0	27876.7	trace	0.0	1561.2	451.9	0.0	388.5	241.9
WJ_3	147.4	272.5	1713.0	4173.8	13052.3	trace	0.0	706.9	182.2	0.0	174.5	106.1