COMPARISON OF AGE-DEPENDENT QUANTITATIVE CHANGES IN THE MALE LABIAL GLAND SECRETION OF *Bombus terrestris* AND *Bombus lucorum*

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Abstract - Quantity of components of the labial gland secretion of bumblebee males changes significantly during ageing of males. In *B. terrestris*, compounds showing the EAG-activity to virgin queens are ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadeca-9,12,15-trienol, and geranylcitronellol. The EAG-active compounds found in *B. lucorum* are ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadeca-9,12-dienol, octadeca-9,12,15-trienol and octadecanol. Quantification of these compounds based on a calibration showed that the main components were present in milligram amounts in both species. Different concentration pattern of active compounds was observed in the species studied. Compounds reached maximal amounts about seventh day after eclosion. Then, a rapid concentration decrease occurs in *B. terrestris* whereas the amounts of active compounds stay approximately constant or decreases only slowly in *B. lucorum*. These results were also confirmed by means of optical microscopy. *B. terrestris* secretory cells undergo apoptosis since the fifth to the tenth day of life, whilst they live and show secretory activity through the whole life of *B. lucorum* males.

Key Words - Bumblebee, *Bombus terrestris*, *Bombus lucorum*, labial gland, male marking pheromone, sex pheromone, GC-EAD.

INTRODUCTION

Bumblebee species *Bombus terrestris* and *B. lucorum* are related and belong to the *Bombus* s. str. subgenus. They are among the most common species of European bees. Likewise most other bumblebee species during their premating behavior, males scent-mark various substrates on their flight routes with a sexual pheromone (patrolling behaviour; Calam, 1969; Schremmer, 1972; Svensson, 1980; Morse, 1982; Lloyd, 1981; Villalobos and Shelly, 1987; Kindl et al., 1999). Components of the sexual pheromone are produced in the acini of cephalic part of the labial gland (Kullenberg, 1973; Bergman and Bergström, 1997).

The species specific labial gland secretion attracts conspecific unmated queens (Kullenberg et al., 1970; Bergström et al., 1981; Bergman, 1997). The pheromone is a complex mixture comprising many compounds in various proportions, usually with one or two major components (Valterová and Urbanová, 1997; Terzo et al., 2003). The composition of male sex pheromones of closely related species partially overlap, but have often one specific component and different blend proportions. Currently, the composition of male cephalic gland secretion is used as the most efficient tool for taxonomic identification, species and subspecies discrimination (Paterson, 1985; Terzo et al., 2005; Rasmont et al., 2005; Coppée et al., 2008). However, a great individual variability in the production of pheromonal components was observed within a single species (Svensson and Bergström, 1977; Ågren et al., 1979; Šobotník et al., 2008), which makes taxonomic application sometimes a difficult task, especially in related species. Recently, changes of the cephalic labial gland ultrastructure related to age of males were reported in *B. terrestris* (Šobotník et al., 2008). It was found that the secretory activity of the gland cells is high in newly emerged males and it drops with the ageing. After the fifth day of bumblebee life, the synthetic activity stops, secretory cells disintegrate and are removed by apoptosis (Šobotník et al., 2008). Morphological changes in the labial gland are associated with changes in pheromone secretion and biological activity in terms of responses of queen antennae to equal amounts of extracts obtained from labial gland of males of different age. The maximum of both secretion and antennal activity was observed in 2-7 old males. In older males, the pheromone production and antennal activity gradually decrease (Šobotník et al., 2008). GC-EAD experiments conducted to detect EAG-active compounds in labial gland secretion revealed that at least six compounds are antennally active

e.g. ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadecatrienol, and geranylcitronellol. These compounds are considered to play an important role in male sex pheromone signalling (Šobotník et al., 2008). Reported study demonstrated changes in volume of pheromone production and its antennal activity, however no quantitative data were provided.

In *B. lucorum*, analysis of the labial gland extract has been reported (Bergström et al., 1973; Urbanová et al., 2001), however, no attention has been paid to the age of males. Scarce reports on seasonal variations in the secretion composition in some other species appeared in the literature (Kullenberg et al., 1970; Svensson and Bergström, 1977; Ågren et al., 1979) but this phenomenon has not been studied systematically so far.

In the present study, we report on age-dependent quantitative changes of major EAG-active components of labial gland secretion in two related bumblebee species, *B. lucorum* and *B. terrestris*. The selected species belong to the most common species of European bees and represent good model species for such detail studies. In addition, their close relatedness allowed us to study, whether the time limited pheromone secretion activity observed in *B. terrestris* is of an universal nature within subgenus *Bombus* sensus stricto.

METHODS AND MATERIALS

Insects Colonies of *Bombus lucorum* and *Bombus terrestris terrestris* (L.) were established by the well known two-queens cascade method (Ptáček *et al*., 2000) to stimulate egglaying in the laboratory. All mother queens were taken from their natural habitat during the nest-searching period in order to minimize the possible negative influence of artificial conditions on their progeny. Bumblebee colonies were kept in plastic boxes of 0.6 to 1 litre volume and fed with honeybee pollen pellets and concentrated sugar solution (sucrose:fructose 1:1). When colonies started to produce males, male cocoons were removed from the parental hives and left to ripen separately under the care of several workers supplied with food (Ptáček, 1999). Freshly emerged males were removed and kept assorted according to their age. Animals of the following age were studied. *Bombus terrestris*: shortly after eclosion, 1, 2, 3, 4, 5, 7, 10, 12, 17, 20, 24, and 33 days old (5 specimens of each age); *Bombus lucorum*: shortly after eclosion, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 and 30 days old (5-6 specimens of each age). Males were killed by freezing and kept deep frozen prior to dissection. Labial glands were dissected and extracted with hexane (100 µl per gland) containing 1-bromodecane as internal

standard (1.79 mg/ml for *B. terrestris*, 2.13 mg/ml for *B. lucorum*). The glands were shaken for 30 min, then the extracts were transferred to clean vials and kept at -18 ° C prior analyses.

Identification of compounds The extracts were analyzed using a gas chromatograph with a splitless injector (200 °C), mass detector (200 °C, Fisons MD 800) and autosampler AI3000 (Thermo). A DB-5ms column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies) and helium gas (constant flow 1 ml/min) were used for separations. The temperature programs differed for samples of different species. *B. terrestris*: the program started at 70 °C (2 min delay) after which the temperature of the oven was increased to 320 °C at the rate of 10 °C/min. *B. lucorum*: the temperature program started at 70 °C (2 minutes delay) after which the temperature of the oven was increased to 140 °C at the rate of 40 °C/min, then to 240 °C at the rate of 2 °C/min, and finally to 320 °C (15 minutes delay) at the rate of 4 °C/min. The identification of compounds was based mostly on their mass spectra compared to those in the National Institute of Standards and Technology Library (NIST, U.S.A.) and on the co-chromatography with synthetic or commercially available standards.

Gas chromatography-electroantennography (GC-EAD) GC-EAD experiments were performed on a 5890 A Hewlett-Packard gas chromatograph equipped with a DB-5 column (30 m x 0.25 mm, film thickness 0.25 µm, J & W Scientific). The column was split by a Graphpack 3D/2 four-arm splitter. The splitter led the eluate to FID and EAD detectors. N_2 make-up gas at 20 ml/min flow rate was introduced via one arm of the splitter to compensate the flow reduction due to splitting. Labial gland extracts (1-5 µl) were injected splitless. The GC was operated at an initial temperature of 50 ^{o}C (2 min), 30 ^{o}C min^{-1} to 270 ^{o}C (10 min). The temperature of injector and FID were set to 230 and 260 ^{o}C, respectively. The EAD detector consisted of queen antenna connected via two glass Ag/AgCl electrodes to universal AC/DC Probe (Syntech). EAD signal was 10 times amplified. Both EAD and FID signals were fed to a PC via the serial IDAC interface box (Syntech) and analyzed using GC-EAD software (Syntech). The antennae were exposed to compounds eluting from GC via the Effluent Conditioner Tube (Syntech) heated to 180 ^{o}C. Virgin queens (N=4) used for GC-EAD recording were kept at low temperature (5 ^{o}C) and high humidity until use. Isolated antennae with the very antennal tips cut off were used for GC-EAD recordings. Each antenna was used only once.

Chemicals The following standards were used for quantifications of EAG-active components of the labial gland secretions: (*E*)-farnesol (Firmenich), geranylgeraniol (ICN), ethyl tetradec-9-enoate (Nu-Check-Prep). (*Z,Z,Z*)-Octadeca-9,12,15-trien-1-ol, ethyl dodecanoate, hexadecan-1-ol, octadecan-1-ol, (*Z,Z*)-octadeca-9,12-dien-1-ol, and ethyl hexadec-9-enoate were purchased from Sigma. (*Z*)-Hexadec-9-enal was prepared earlier in our laboratory.

Quantitative analyses Only EAD-active compounds were quantified. *Bombus terrestris:* 2,3-dihydrofarnesol, 2,3-dihydrofarnesal, geranylgeraniol, geranylcitronellol, (*Z,Z,Z*)-octadeca-9,12,15-trien-1-ol, and ethyl dodecanoate; *Bombus lucorum:* ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadecan-9,12-dienol, octadecan-9,12,15-trienol, octadecanol. Purity of all standards used for the calibration was checked by GC-MS. External calibration was used. Quantification was carried out in Total Ion Current mode (TIC) of mass detector and based on peak areas. Compounds were present in the sample in different concentrations, thus, we had to avoid overloading the mass detector with the most abundant component. Separation of ethyl tetradec-7-enoate and ethyl tetradec-9-enoate under conditions used was very poor. Therefore, these two isomers were quantified together according to the external calibration on ethyl tetradec-9-enoate. Previous studies (Urbanová et al., 2001) showed that the concentration ratio of these isomers in the labial glands extracts was: $\text{area}_{\text{ethyl tetradec-9-enoate}}/\text{area}_{\text{ethyl tetradec-7-enoate}}=187.5$. We also assumed that the difference in responses of these isomers in mass detector was less than 5 % and the concentration ratio remained constant over the bumblebees' life.

Calibration curve for (*Z*)-hexadec-7-enal was carried out by means of (*Z*)-hexadec-9-enal as the correct isomer was not available. 2,3-Dihydrofarnesol and 2,3-dihydrofarnesal were quantified as farnesol and geranylcitronellol as geranylgeraniol due to unavailability of standards. We assume that the quantification error should be inconsiderable in comparison to other error sources (such as individual differences in groups of animals of the same age) with regard to close similarities in structures between calibrants and calibrated compounds. Octadecan-9,12-dienol and octadecan-9,12,15-trienol were also difficult to reach baseline separation, thus, we used the same rule for determination of their peak areas in the samples and calibration.

Internal standard of a comparable concentration was used in both sets of samples and calibrations (1-bromodecane, ~2 mg/ml). From the area ratios (standard/IS), the calibration curves were calculated (Tables 1 and 2). A 2^{nd} degree polynomial equation was used mostly

for fitting the obtained data. From the equations of the calibration curves, the unknown concentrations in the samples were calculated and expressed in μg per gland. Median was used as a resemble average of measurements in the particular days and standard error was used for constructing error segments in the histograms.

Microscopy Dissection took place in a droplet of fixative (2% glutaraldehyde and 2.5% formaldehyde in 0.1 M phosphate buffer), in which the tissues stayed for 1 day at lab temperature. After postfixation in 1.5 % OsO_4 in 0.1 M phosphate buffer the samples were dehydrated through the series of ethanol-water mixtures (50%, 75%, 100%). Tissues were then embedded into standard Spurr resin. Semithin sections (1μm) were stained with Azure II and observed in Amplival (Zeiss) optical microscope (equipped with Canon EOS 300D camera). Ultrathin sections were studied using a Jeol 1011 transmission electron microscope.

RESULTS

Gland physiology Physiology of the cephalic labial gland differs in the compared species. Contrary to *B. terrestris*, labial glands in *B. lucorum* remain active duringh the whole life span of males. In *B. lucorum*, the secretion production by smooth endoplasmic reticulum (SER) starts earlier (already at the time of emergence) compared to the second day of life in *B. terrestris*. The secretion is continuously excluded at the cell apices, and its volume increases causing swelling of lumens and decrease in cell layer thickness (Fig. 1a-c). The active transport of precursors from hemolymph was observed in young males of both species. The transport stops during the third day of male life of *B. terrestris*, but continues in older males of *B. lucorum* (observed rarely up to 13th day of life). An observed difference is apparently associated with different fate of secretory cells in both species. The cells die after several days of secretory activity (between 5th and 10th day, Fig. 1d) in *B. terrestris* while they live and produce the secretion through the whole life of *B. lucorum* males. In older *B. lucorum* males, flattening of cells, lower volume of SER, fewer droplets of secretion in the cell cytoplasm was observed (Fig. 2a,b) indicating a decreasing rate of pheromone production.

GC-MS, GC-EAD, and quantitative analyses Although both species are considered to be closely related, composition of their sexual pheromone is significantly different. In *B. terrestris*, composition of the labial gland secretion consists of terpenic and aliphatic

compounds (most abundant is 2,3-dihydrofarnesol with smaller amounts of geranylcitronellol; Kullenberg et al., 1970; Šobotník et al., 2008) while *B. lucorum* uses a blend consisting only of aliphatic compounds with the most abundant component ethyl tetradec-9-enoate (Urbanová et al., 2001).

Hexane extracts of bumblebee labial glands contain tens of compounds (Urbanová et al., 2001; Terzo at al., 2003) which might have different physiological functions. Some of them might serve as long-range attractants, others like close-range arrestants, still others might serve as release regulators or stabilizers. To determine which compounds might serve long-range attraction of conspecific females, electroantennography coupled to gas chromatography (GC-EAD) was performed in both species. In the labial gland of *B. terrestris*, the EAD-active compounds were identified as ethyl dodecanoate, 2,3-dihydrofarnesal, 2,3-dihydrofarnesol, hexadecanol, octadeca-9,12,15-trienol, and geranylcitronellol (Šobotník et al., 2008; Fig. 3). In *B. lucorum*, ethyl dodecanoate, ethyl tetradec-7-enoate, ethyl tetradec-9-enoate, ethyl hexadec-9-enoate, hexadecanol, hexadec-7-enal, octadeca-9,12-dienol, octadeca-9,12,15-trienol, and octadecanol elicited antennal responses in conspecific queens (Fig. 4). EAG active compounds are likely to participate in the long range attraction. Since composition of bumblebee sex pheromone is supposed to be species specific, the component blend ratio is of a great importance. Synchronous changes of the EAD active compounds may provide some information about the major pheromone components.

All EAD-active compounds of both species were quantified and their quantitative changes during life of the bumblebee's males were determined. The highest pheromone volume in both species reached up to the level of several milligrams per male gland, but the pheromone gland physiology and quantitative age-dependent pattern of EAD active compounds differ profoundly in the compared species. In *B. terrestris*, the most abundant EAD active component in the extracts was 2,3-dihydrofarnesol (Fig. 5a). Males start to produce this compound since the first day of their life (20 µg/gland). The production of 2,3-dihydrofarnesol increases up to 2 mg/gland during the next two days, then 2,3-dihydrofarnesol production remains approximately on the same level for three more days. Throughout the sixth and seventh day, the concentration increases almost three times and reaches the highest point observed (6 mg/gland). During the next days, the amount of this compound decreases dramatically and after the tenth day of life it is roughly as low as on the second day (0.5 mg/gland). After the day 20, 2,3-dihydrofarnesol almost disappears from the secretion (2 µg/gland). A similar concentration pattern was observed for geranylcitronellol (1 µg/gland up to 1 mg/gland, Fig. 5b) and hexadecanol (0.5 µg/gland up to 200 µg/gland, Fig.

5c) but on a lower concentration level. Different age-dependent pattern was observed for ethyl dodecanoate that appears in the secretion later than terpenes (2 days, 0.5 µg/gland) and drops more quickly from its maximum (800 µg/gland) to 0.7 µg/gland in 13 days (Fig. 5b). The remaining EAD active compounds, octadecatrienol and 2,3-dihydrofarnesal slightly differ in pattern from the above mentioned components. They reach a maximum abundance around the fifth day (100 µg/gland and 30 µg/gland, respectively, Fig. 5d) which is earlier than the other compounds.

In *B. lucorum*, the concentration profiles of the most abundant EAD active compounds present in pheromone secretion, e.g. ethyl tetradec-7-enoate and ethyl tetradec-9-enoate (Fig. 6a) are quite different in comparison with *B. terrestris.* No significant maximum was observed for any analyzed compound, the quantities remained relatively high during the whole life of males. Ethyl tetradec-7-enoate and ethyl tetradec-9-enoate appear during the 1st posteclosion day (0.9 mg/gland). On the 3rd day, their production increases up to 1.2 mg/gland. Maximum production (1.5 mg/gland) was observed between 8th -10th days. The quantity of ethyl tetradec-7-enoate and ethyl tetradec-9-enoate gradually decrease after the fifteenth day of the male's life. The concentration remains high until the day 30 (0.8 mg/gland). Other EAD active compounds, ethyl dodecanoate and hexadecanol (Fig. 6b) are almost ten times less abundant than the ethyl tetradec-7-enoate and ethyl tetradec-9-enoate (113 and 91 µg/gland, respectively) but their concentration profile has similar pattern – no sharp maximum and no dramatic changes between fourth and tenth days. After tenth day, the amount of hexadecanol increases in comparison to ethyl dodecanoate and reaches a second maximum in 25 days old males (185 µg/gland). This is similar to ethyl hexadec-9-enoate and hexadec-7-enal (Fig. 6c). Alcohols octadeca-9,12-dienol, octadeca-9,12,15-trienol (Fig. 6d), and octadecanol (Fig. 6c) stay roughly at the same concentration level (30-70 µg/gland) and the observed changes are probably mainly due to individual variations. Described patterns in quantitative profiles of studied compounds well correspond with physiological and morphological changes in labial glands of studied species. While in *Bombus lucorum*, the secretory activity of the acinar cells clearly continues for the whole life, in *B. terrestris* the production stops in males older than 10 days.

DISCUSSION

Unexpectedly, we have found profound differences in gland physiology, pheromone composition and pheromone production of two closely related species *B. terrestris* and *B.*

lucorum. While in *B. terrestris* the pheromone production is time-restricted due to limited life span of secretory cells in the labial gland, males of *B. lucorum* produce the pheromone during the whole life and concentration of some compounds in labial glands even slightly increases in older males. *B. terrestris* males thus i) have a limited time to attract females and ii) older males might be recognized by females based on quantity and quality of pheromone deposited during marking. *B. lucorum* males on the other hand have enough pheromone during their whole life. However, young males can be recognized by conspecific females according to slightly higher proportions of ethyl tetradecenoate in pheromone blends compared to older males.

It is supposed that virgin queens searching for a mate are more attracted to intensively marked places, i.e. to places where higher amounts of secretion were deposited by patrolling males (Ågren, 1979). Thus, younger males of *B. terrestris* have an advantage over the older ones because their scent marks are stronger than those of older males. On the other hand, the age of *B. lucorum* males cannot be discriminated simply based on pheromone quantity deposited during marking. The age-dependent changes in pheromone blend ratio might be a mechanism of selection of mates.

The reasons for such profound differences in pheromone gland physiology and pheromone biosynthesis between species related as closely as *B. terrestris* and *B. lucorum* are not known. They are supposed to lie in specific life-history trait, which unfortunately remains unclear in spite of a good knowledge of biology of these species. So far available data on age-dependent changes in gland physiology based on observation of gland morphology in *B. hypnorum* and *B. lapidarius* (Ågren et al., 1979) resemble our results on *B. lucorum*. From this point of view, *B. terrestris* seems to be exceptional with regard to the development of the cephalic labial gland.

Earlier reports on variation in the pheromone composition are scarce. Kullenberg et al. (1970) mentioned considerable seasonal variations in the diterpene content in glands of *B. hortorum* and *B. hypnorum*. Svensson and Bergström (1977) found that diterpenic components in the labial gland secretion of *B. pratorum* males appear later in the season. Ågren et al. (1979) studied changes in the labial gland secretion of *B. hypnorum* males since 5 days before eclosion until day 7 after emergence. In pupae, no pheromone was produced. The compounds appeared since day 1 after emergence and their amounts were increasing rapidly to 4-7 days. In *B. lapidarius*, their observation started at day 7 and ended at day 37. After 2 weeks of the male's life, the amounts of the secretion dropped and reached zero between 19 and 29 days-old males. A similar trend was observed for *B. hortorum* males (Ågren et al.,

1979). Although this study was not systematical for one bumblebee species and the analytical technique (TLC) enabled observations of qualitative changes only, the results seem to be consistent with our observations for *B. terrestris*.

Maximum of pheromone production (activity of secretory cells) found in *B. terrestris* between 3-5 days correlates with time when males usually leave their natal nests (the fifth day after eclosion) and start to mark and patrol. Maximum of pheromone content in the gland matches with maximum of sperm content (6^{th} post-emergence day; Tassei et al., 1998). The age at which males can mate in the laboratory extended from day 6 to day 27, but the probability of successful mating drops dramatically after day 11 (Tassei et al., 1998). The optimal age for mating under laboratory conditions was found to be 12.1±1.3 days (*B. terrestris* males). Information about optimum reproduction physiology in *B. lucorum* is not available, but it would certainly be interesting to know whether differences in the pheromone production are matched with reproduction physiology.

Our results clearly show that an age-dependent variability in pheromone production and composition exists and might be remarkably high even among closely related bumblebees. From chemical point of view, *B. terrestris* shows a similar trend in the pheromone changes as *B. hypnorum*, *B. hortorum*, and *B. lapidarius* studied by Ågren et al. (1979). From the physiological point of view on the other hand, *B. lucorum* is more similar to the above mentioned species. The observed differences between bumblebees studied rise interesting questions about the biosynthetic pathways of pheromonal components and dynamics of this biosynthesis in different species. Obtained results contribute to the knowledge on the pheromonal gland development and may help in the future planning of biosynthetic experiments.

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Figure legends

Fig. 1. Development of acini in the cephalic part of the labial gland in *B. lucorum* (A-C) and *B. terrestris* (D); A, development of acini in an 1-day-old male of *B. lucorum*; B, development of acini in a 3-day-old male of *B. lucorum*. Arrowhead marks an excretory duct of labial gland; C, development of acini in a 13-day-old male of *B. lucorum*; D, development of acini in a 13-day-old male of *B. terrestris*, all cells are already dead, but the aciny are still full of secretion. Bar represents 100 µm in all figures. Abbreviations: al, acinar lumen; hc, haemocoel; sc, secretory cells.

Fig. 2. TEM micrographs of acinar secretoy cells in *B. lucorum*; A, whole secretory cell in pharate male imago. Note the active transport at the cell base occurring as pinocytotic vesicles. Bar represents 2 µm; B, detail of cytoplasm in male less than a day old. Arrowheads mark smooth endoplasmic reticulum producing a secretion. Bar represents 500 nm; C, walls of two neighbouring acini in a 13-days old male. Bar represents 2 µm. Abbreviation: al, acinar lumen; hc, hemocoel; m, mitochondria; n, nucleus; s, secretion within the cell.s

Fig. 3. GC-EAD recording of the labial gland extract of a 7 days old *B. terrestris* male. Active compounds: **1** ethyl dodecanoate; **2** 2,3-dihydrofarnesal; **3** 2,3-dihydrofarnesol; **4** hexadecanol; **5** octadeca-9,12,15-trienol; **6** geranylcitronellol.

Fig. 4. GC-EAD recording of the labial gland extract of a 7 days old *B. lucorum* male. Active compounds: **1** ethyl dodecanoate, **2** ethyl tetradec-7-enoate, **3** ethyl tetradec-9-enoate, **4** hexadec-7-enal; **5** ethyl hexadec-9-enoate; **6** hexadecanol; **7** octadeca-9,12-dienol; **8** octadeca-9,12,15-trienol; **9** octadecanol.

Fig. 5. Concentration changes of the EAG-active compounds in the cephalic labial gland of *B. terrestris* males. Each figure shows compounds present at a comparable concentration level.

Fig. 6. Concentration changes of the EAG-active compounds in the cephalic labial gland of *B. lucorum* males. Each figure shows compounds present at a comparable concentration level.