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Faculty of Landscape Architecture, Horticulture and Crop Production Science

Behavioural studies and collection of volatiles to identify the pheromone(s) of the great Capricorn (*Cerambyx cerdo*)

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Summary

This work was aimed at identifying the pheromone component(s) of the great Capricorn longhorn beetle (*Cerambyx cerdo*). Volatile samples from beetles were obtained by means of collection of headspace volatiles from the animals, and by direct solvent extraction of body parts, and analysed by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-Electroanntennography (GC-EAG). The chemical analyses were accompanied by behavioural studies. No potential pheromone candidate(s) was identified, neither in the GC-MS analyses nor the GC-EAG analyses. However, the behavioural studies in a two-way choice olfactometer using live beetles as odour sources suggest that the males emit pheromones attracting the females.

Introduction

Insects (Insecta: Arthropoda) constitute the largest class of animals and can be found virtually at any land habitat on Earth. They are important in, for example, decomposition of dead organic matter and for pollination, but can also be severe pests and vectors of both plant and animal diseases (Campbell & Reece, 2008; Hickman *et al.* 2007).

Many wood boring insects depend on the availability of suitable host material, like large old trees, living or dead, and wind-felled trees. However, in modern-day forest management old dead trees and wind-felled trees are commonly removed as well as trees being logged before reaching the proper age (Rubene *et al.* 2014). This has a negative impact on many wood boring insects.

One group of insects that is generally negatively affected by modern-day forestry is the beetle family Cerambycidae, or longhorn beetles, consisting of about 20 000 species across the world (Bily & Mehl, 1989). About a hundred species can be found in Sweden (Ehnström, 2007). Characteristic for the Cerambycidae are their very long, sometimes extremely long, antennae. Often, the antennae are as long as the body or longer. For example, the antennae of the male timberman beetle (*Acanthocinus aedilis)* may be 4 times the body length (Ehnström, 2007). The Cerambycidae lay their eggs in trees, dead or living (Gardiner, 1957 in Allison *et al.* 2004), and their larvae contribute to the decomposition of woody matters (Furniss and Carolin, 1977 in Allison *et al.* 2004). As the Cerambycidae bore into the trees, some species are also important vectors of tree diseases. The pine wilt nematode, causing the pine wilt disease with severe tree death as result, is spread by the longhorn beetle genus *Monochamus* (Sousa *et al.* 2001).

One of the threatened animal species in Sweden is the Great Capricorn longhorn beetle *Cerambyx cerdo*. For *C. cerdo*, which in Sweden is now only found at Halltorps Hage on Öland, the lack of oak trees of appropriate age is a crucial problem for the survival of the remaining Swedish population. Jeppsson & Forslund (2014) studied extinction risk factors among Cerambycidae beetles in Sweden. They found that long generation time and narrow host specialization of the larvae is related to higher risk for extinction, which is the case for *C. cerdo*. In order to preserve the Swedish population of *C. cerdo*, a breeding programme has been initiated by the county administration board (Länsstyrelsen) in Kalmar, with animals from Halltorp being bred in captivity at Nordens Ark conservation park. The aim of the breeding programme is to re-introduce the species at several former localities where it was previously found.

In order to monitor the present and future newly established populations of *C. cerdo*, it would be beneficial to utilize live trapping systems based on highly attractive species specific pheromones. Commonly, sex pheromones are used for this (sexual attractant odours), but aggregation pheromones (attract both sexes) can also be used. Several Cerambycidae aggregation pheromones are known, for example 2,3-hexanediol (Lacey *et al.* 2009; Pajares *et al*. 2010). Sex pheromones have been used extensively for several decades for monitoring and management of insect pests in agriculture and forestry (Witzgall *et al.* 2010). Recently, their practical applications have been expanded to include monitoring of rare and threatened insects whose distributions and population trends would otherwise be very difficult to follow (Larsson and Svensson 2009; Andersson *et al.* 2014). The present study constitutes a first attempt to determine if *C. cerdo* has a pheromone-based communication system, and to identify its chemical components.

Aim of this work

This work aims at investigating whether *C. cerdo* communicates by means of male- or female-produced sex pheromones, and at identifying potential pheromone component(s) of this species. A better understanding of the chemical ecology of *C. cerdo* could help its conservation and reintroduction. By identifying pheromones, individuals can be selectively captured for breeding programs and recapture studies to estimate population density, as well as monitored by pheromone-based surveys for the species.

Background

Chemical communication in insects including Cerambycidae

Insects utilize chemical, auditory, visual and tactile stimuli for communication (Hickman *et al.* 2007). Semiochemicals is a term employed for chemical compounds used for communication within or between species and further contains pheromones and allelochemicals where the later one refers to communications between species (Rathore & Nollet, 2012). A *pheromone* is defined as a compound or a set of compounds emitted from one individual that affects the behaviour of another individual, or individuals, of the same species (Chapman, 2009).

The chemical communication within the beetle family Cerambycidae is somewhat special as there tends to be a high degree of pheromonal parsimony. Related species often make use of the same or very similar pheromone components. For example, 2,3-hexanediol, 2,3-hydroxyhexanone, 2,3-octanediol, and 2,3-hydroxyoctanone are shared pheromones for many species within the subfamily Cerambycinae (Hanks & Millar 2013). It has also been shown that fuscumol works as a pheromone for many species within the cerambycid subfamily Lamiinae (Mitchell *et al.* 2011). This is an advantage when it comes to field surveys, as pheromone traps can be baited with a blend of common cerambycid pheromones to attract numerous species simultaneously. Thus, instead of using six separate pheromone lures when catching six different Cerambycidae species, one lure baited with all six pheromone can catch all six species (Hanks *et al.* 2012).

Insect chemoreception/olfaction

Insects have chemoreceptors on both their antennae and on other places on their bodies, like their palps (Chapman, 2009). Insects generally detect pheromones via chemosensory receptor organs consisting of specialized structures, called *sensillum*, on their antennae. The sensilla consist of a cuticule layer surrounding one or two to several receptor neurons. The pheromones enter the sensillum via small pores in the cuticule (Nation, 2008). As most molecules of interest to the insects will be of a non-polar character, the sensilla are covered by a lipophilic liquid that will trap the molecules and transport them to the pores. In the pores, the pheromone molecules will bind to so called “pheromone-binding proteins”, as the lipophilic compounds will not dissolve efficiently in the insect’s aquatic haemolymph (body fluid), and are transported to the olfactory receptor neuron. In the receptor neuron, an electrical signal will be initialized. Thereafter the pheromone is enzymatically degraded (Chapman, 2009).

The great Capricorn (*Cerambyx cerdo*, Coleoptera: Cerambycidae) is one of the largest longhorn beetles in Sweden with a body length of up to 5 cm. It is a night active insect (Ehnström, 2002). The species is extinct in the Swedish mainland and can only be found in Halltorps Hage at Öland (Ehnström, 2007; Buse *et al.* 2008). In Sweden, *C. cerdo* appears to be dependent on large, old oaks for breeding. During its first time as a larva, it lives under the bark but will later go into the wood. The whole development to an adult beetle takes between 4 and 5 years (Ehnström, 2002). *C. cerdo* is of particular interest as it appears to be a so called "ecosystem engineer", as suggested by recent studies (Buse *et al.* 2008). Jones *et al.* (1994) define ecosystem engineers as "organisms that directly or indirectly modulate the availability of resources to other species, by causing physical state changes in biotic or abiotic material". When the *C. cerdo* larvae develop in the trees, they make tunnels that can be used by other insect species. Work done by Buse *et al.* (2008) have shown that trees harbouring *C. cerdo* host significantly more, particularly endangered, insect species than trees without *C. cerdo* do. Thus, preservation of the lone *C. cerdo* population in Halltorps Hage, and reintroduction to new suitable areas, could help preserving other endangered insect species.

Gas chromatography- mass spectrometry

Gas chromatography (GC) is an analytical method where a mixture of organic compounds is separated based on polarity and boiling point (Simonsen, 2005). A GC-system consists of three main parts: an injector, an oven with a column, and a detector. The sample is carried through the system via an inert gas, called *carrier gas*; commonly helium or nitrogen is used. The flow through the system depends on the pressure of the gas. Because of a higher pressure inside the GC-system the sample must be injected through a rubber septum. The sample then enters the *column* which is mounted in an oven. It is in the column where the separation occurs. Based on their molecular properties (primarily polarity) the compounds will travel through the column at different rates, and thus get separated. The inside of the column might be of a polar or non-polar character. A non-polar column will better retain non-polar compounds and polar molecules will pass through faster and vice versa. Also the temperature in the oven will affect separation. A GC analysis can be performed using a temperature program or using a constant (isothermal) program (Simonsen, 2005).

After the column the sample will (optimally) be separated by compound and the individual compounds (or effluents) flowing from the column enter the detector. Many different types of GC detectors exist but in this work a mass spectrometer was mostly used and is thus described. In the MS detector, the separated analytes will be ionized, in this case, by an electron beam (electron

impact ionization), causing the molecules to fall apart into charged fragments. These fragments will be sorted by a mass filter, here a quadrupole mass filter. The quadrupole, as its name implies, consists of two pairs of rods between which a voltage is laid between each pair. By altering the voltages, only fragments of a particular mass and charge (m/z) will pass at a particular time, thus a separation of the molecular fragments are achieved. The fragments subsequently reach the detector, in this case an electron multiplier. Here, each ion fragment results in a cascade of electrons that amplifies the signal and the signal is finally registered.

For the GC-EAD analyses, a flame ionization detector (FID) was used in parallel to antennal recording. The detection principle is the detection of ions created when organic molecules are combusted. The ions will carry a current between two electrodes in the detector and the more ions, the stronger the signal (Skoog *et al.* 2006).

Gas chromatography-electroantennography detection

A convenient way to identify semiochemicals from a mixture of compounds, or to confirm physiological activity from a pure compound, is the gas chromatography-electroanntenography detection (GC-EAD). The compounds will first be separated in a GC column and the effluent is subsequently split for the FID (or sometimes MS) detector and the electroantennography (EAG) detector (Figure 1). In the EAG, an insect antenna has been connected to two electrodes, one in each end of the antenna, which is then located in the outflow from the GC. As the insect antennae contain olfactory receptor neurons, the antennae can function as a sensitive physiological detector for species-specific pheromone compounds. Responses in the FID/MS and EAG detectors will be registered concurrently and displayed in the chromatogram. All (organic) compounds will be registered by the FID/MS detector and if a compound elicits any response in the EAG detector, responses will be shown at the same time in the chromatogram (Larsson & Svensson, 2005).

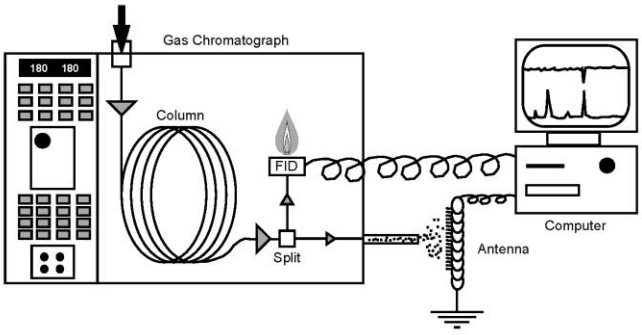

Figure 1. Instrumentation of gas chromatography-electroantennography. The sample is injected into the GC, separated in the column and the effluent is split ~50/50 to the FID/MS detector and the EAG detector (Illustration from Larsson & Svensson, 2005).

Solid phase microextraction

Solid phase microextraction (SPME) is a convenient sampling method that is based on the absorption of analytes to a thin polymer coated fibre. This fibre can have different polarities depending on the polarity of the polymer(s) (including a combination of polar and non-polar coating). If the fibre is inserted into a closed vessel, analytes in the air will be absorbed by the fibre until an equilibrium between the polymer coating and the surrounding air is reached. Normal extraction time varies from a few seconds for concentrated samples to hours for dilute samples. The SPME fibre can then be inserted, for example, into a GC-system. The warm injector (commonly 250^{o}C) will cause desorption of the analytes from the fibre and they will follow the carrier gas flow (Pawliszyn, 2000).

As only minute amounts of analytes are required for an analysis and as no further sampling preparation steps are required, loss of analytes is prevented and SPME is therefore suitable for pheromone identification.

Materials and Methods

Model organism – Cerambyx cerdo

For this work, 20 male and 20 female *C. cerdo* (Figure 2), , of Polish origin (but bred in Sweden), were provided by Nordens Ark (zoological conservation park) and kept at Linnaeus University (LNU, Faculty of Health and Life Sciences, Kalmar). Collections of volatiles were performed in a course laboratory one floor above were they were kept. The animals were kept in plastic boxes, most of them individually. For the collection of volatiles one group of males (3 individuals) and one group (usually 4-5 individuals) of females were kept together with oak branches to stimulate pheromone emission. For the other half of the behavioural studies, animals were kept with oak branches for about a day before the behavioural experiments. The animals were fed every other or third day with organic pear slices and a nectar solution provided by Nordens Ark.

Figure 2. *C. cerdo* male feeding on a piece of a pear.

Behavioural assay experiments

In order to assess which sex might emit pheromones, a two-choice bioassay was prepared. Small, non-transparent plastic boxes made from ice cream containers were connected to the short sides of a larger, rectangular plastic box (25x36x13 cm, IKEA) via non-transparent plastic pipes (Figure 3). The tubes had a diameter of 5 cm and were 12 cm long. The tube ends connected with the smaller boxes were covered by plastic net to avoid that the males and females to come in contact with each other. Two identical bioassays were manufactured. Different treatments were then applied, letting the tested sex choose between other animals or one or two controls (Table 1). A control consisted of an empty box. As the animals are drawn to light, the bioassays

were placed at the floor with curtains drawn and light turned off. The long side of the bioassays were placed parallel to the window (east) and the controls/animal were placed (approximately even number of times) with either south or north direction. By doing this, any influence of light would result in an attraction to one long side and not result in a false positive attraction to either a control or another animal. The animal's position in the bioassay were followed by using a red flashlight.

Table 1. The following combinations were tested. Each tested sex had to choose between another animal or control (empty box).

Tested sex (chooser)	Chose between
Female	Male: Control
Female	Female: Control
Female	Control 1: Control 2
Male	Female: Control
Male	Male: Control
Male	Control 1: Control 2

Figure 3. The bioassay used for the behavioural experiments (one of two). The chooser was placed in the middle of the large box and another animal was placed in one of the smaller boxes and the other box (control) was left empty.

C. cerdo is night active, so the behavioural experiments started when all individuals to be used had become active, usually between 21:20 to 22:00. The behavioural studies were conducted for about an hour. The boxes were placed at the floor to minimize light interference from windows (curtains were drawn) and all lights were turned off. The test animal was placed in the centre of the large box and allowed to freely explore the central chamber and either stay there or exit through either of the two pipes leading to the stimulus boxes. A choice was recorded when a beetle entered into one of the tubes. The position of the choosing animal was checked using a flashlight with red

light. They were usually checked every three minutes. Because of the low number of animals available, some animals were tested more than once (but only once for each combination of test stimuli).

Statistics

Three treatments for each sex were tested. Statistical tests were performed both on the first choice only either animal or control, or any particular control when the animals were subjected to only controls) And, as many individuals went in and out several times, the total number of entries into either tube during the one-hour trial. In some instances, there were too few choices to perform any statistical test. The frequency of their choices were tested with the chi-squared test using R (R Core Team, version 3.2.0). It is used to test whether an observed frequency differs from the expected frequency, which in this case would be equal numbers of choices in either tube if the null hypothesis of no effect was true. The χ^2 is calculated by Equation 1 and compared to a table value. If the χ^2_{calc} is greater than the $\chi^2_{tab,}$ the null hypothesis is rejected (in other words, there is a significant difference) (Miller and Miller, 2005).

$$\chi^2 = \Sigma_i \frac{(O_i - E_i)^2}{E_i} \qquad \text{Equation 1}$$

Where *O* is the observed number and E_i is the expected number.

Collection of volatiles

Volatiles from the *beetles* were collected using filters consisting of teflon tubing packed with ≈ 50 mg Porapak Q (mesh 50/80) or by solid phase microextraction (SPME), field samplers with fibres coated with a combination of polydimethylsiloxane (PDMS) and divinylbenzene (DVB).

The females could be kept together (up to 5 individuals) in even a small container (250 mL round-bottom flask) without problems. The males were very aggressive towards other males and tried to kill each other in smaller spaces. However, up to three males could be kept together in a larger box (the same type as the bioassays were made from) without start fighting. To collect volatiles from several males it was necessary to keep them in individual containers within the larger extraction chamber. Glass beakers, 50 mL, covered with aluminium net was used. Prior to the volatile collection, all animals had been kept in a larger box (the same as the bioassays were built from) together with branches of oak to stimulate pheromone emittance.

A system for the collection of volatiles were built (see Figure 4). First, the air passed through a filter consisting of active coal to purify the air. The airflow was between 30-40 mL/min. Thereafter, the air was split into two tubes and lead to two collection chambers. Thereafter the air passed through the filters. One container was used for the males and the other for the females (though the actual containers were altered to avoid systematic errors). By using the same air and splitting it into males and females, any reproducible differences in detected compounds could be expected to be sex specific. Collection chambers initially consisted of round-bottom glass flasks, which were later replaced with 2 larger cylindrical glass flasks to fit the beakers with the males.

All materials used for the volatile collection were washed with ethanol/acetone, water and detergent and dried in a 120^{o}C oven prior to use. The filters were washed with 1 mL each of methanol, dichloromethane and pentane directly prior to usage.

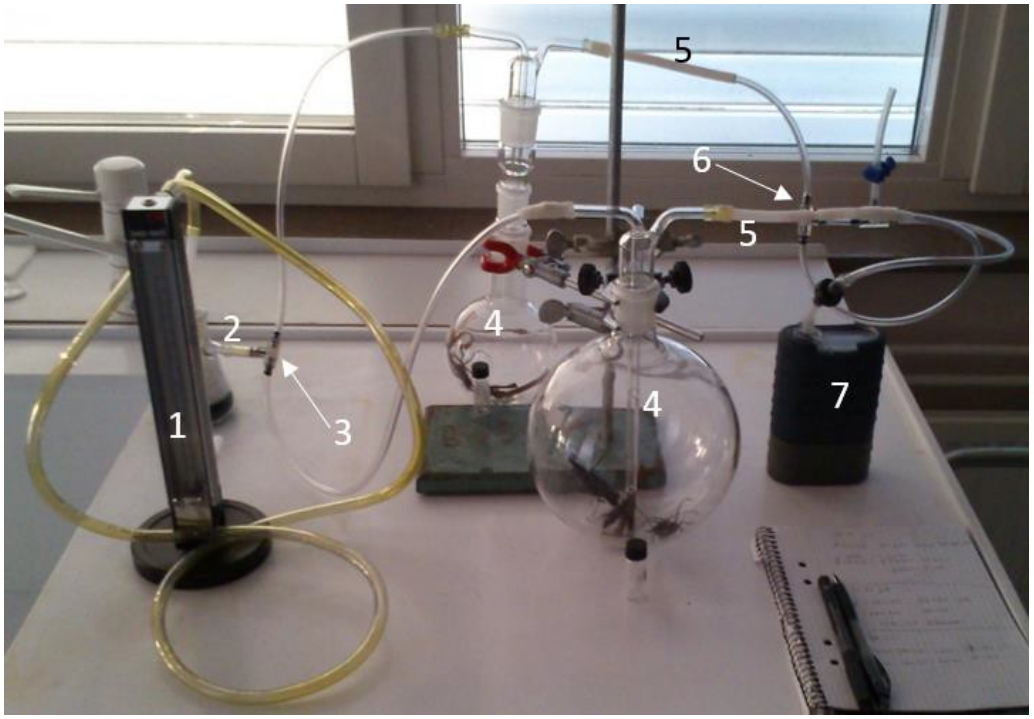

Figure 4. Setup of the volatile collection system. (1) Flowmeter and air inlet, (2) active carbon filter, (3) inlet air split (4) containers for the animals, (5) Poropak filters, (6) air merge and (7) pump. Later, the round-bottom flasks were replaced with larger cylindrical glass flasks.

Whole body extracts of insects

Whole body extracts of 3 males and 3 females were done. The animals were cut into several pieces for better extraction and then extracted, first in ca 10 mL hexane and thereafter in 10 mL ether, 30 min in both. The extracts were concentrated using nitrogen. More animals were extracted in hexane after removal of their antennas for the GC-EAG analyses. Also, one ovipositor from a female was removed and extracted in hexane.

Instrumentation

Gas chromatography-mass spectrometry

For the GC-MS analyses at LNU, an Agilent 6890 Series GC system with an Agilent 5973 Network Mass Selective Detector was used. The column was a Cyclosil-B enantioselective column (30% heptakis (2,3-di-O-methyl-6-0-t-butyl-dimethylsilyl)-β-cyclodextrin) (J&W Scientific, 30 m x 0.25 mm with 0.25 µm coating). Helium was used as carrier gas. Samples were injected in splitless mode with injector temperature of 250^{o}C. Oven temperature was held at 30^{o}C C for 3 minutes and raised 8^{o}C/minute and held at a final temperature of 250^{o}C for 10 minutes. The software Agilent MSD Chemstation was used to evaluate the chromatograms and MS-spectra.

Gas chromatography electroantennography

For GC-EAD, chromatography was performed on an Agilent 6890 series GC system with a Flame Ionization Detector (FID). The column was a non-polar HP-5 column, 30 m x 0.25 mm with 0.25 µm coating. Samples were injected in splitless mode with injector temperature 225^{o}C. Oven temperature was held at 30^{o}C for 2 minutes and raised 20^{o}C/minute and held at a final temperature of 250 ^{o}C for 10 minutes.

Electroantennographic recordings were performed by excising the distal part of the antenna (the antennas were cut off from the animals) and placing it between two glass capillaries containing Beadle-Ephrussi ringer and silver electrodes. The base of the antennal preparation was connected to ground, whereas the tip was connected to a Syntech AC/DC probe and further to a Syntech IDAC-2 digital converter which also simultaneously collected the FID signal from the GC.

The software Synthech GC-EAD 2011 v1.2.3 was used to evaluate the GC-EAG data.

Chemicals

To elute the filters used for collection of volatiles, a 1:1 mixture of pentane: diethyl ether was used (SDS, 95 % and Carlo Erba, 99.8 %, respectively). After and prior to use, the filters were washed with 1 mL of methanol (Carlo Erba, 99.9 %), 1 mL of dichloromethane (SDS, 99.9 %) and 1 mL of pentane. Whole-body extracts were made in diethyl ether and hexane.

Analyses of collected volatiles

The collected volatile extracts were analysed by GC-MS, both at LNU and SLU Alnarp and some of the extracts were also analysed by GC-EAD at SLU Alnarp. Collections of volatiles were performed simultaneously for males and females and the GC-MS chromatograms were then overlayed. Compounds consistently found in only one sex would constitute strong candidates for sex pheromones. Commonly, Porapak filters were used for the collection. In some instances, volatile collection for individuals of one sex was made by Poropak filter and collection from the other sex was made by SPME (DVB/PDMS-fibre), or both sexes were extracted by SPME. As a SPME-extraction can only be analysed once, all of those analyses were performed at LNU.

Results

Behavioural studies

The animals were tested in a two-choice bioassay were they could choose between; same sex: control, opposite sex: control and control: control. Generally, few choices were made and particularly the males were inactive. Both sexes were sensitive to light and were drawn to light sources. The first behavioural study was performed on a table close to a window and the animal was clearly drawn to the light. Both sexes were attracted to light sources. Hence, the behavioural studies had to be conducted with as little interfering light as possible. Some animals were very active and even tried to fly in the bioassay while others barely moved at all. However, almost all of the females in the male: control treatment showed interest for the males, as can be seen in Figure 5. The statistics are presented below in Table 2a and 2b. In two of the treatments for the males, too few choices were made to allow any statistic calculations.

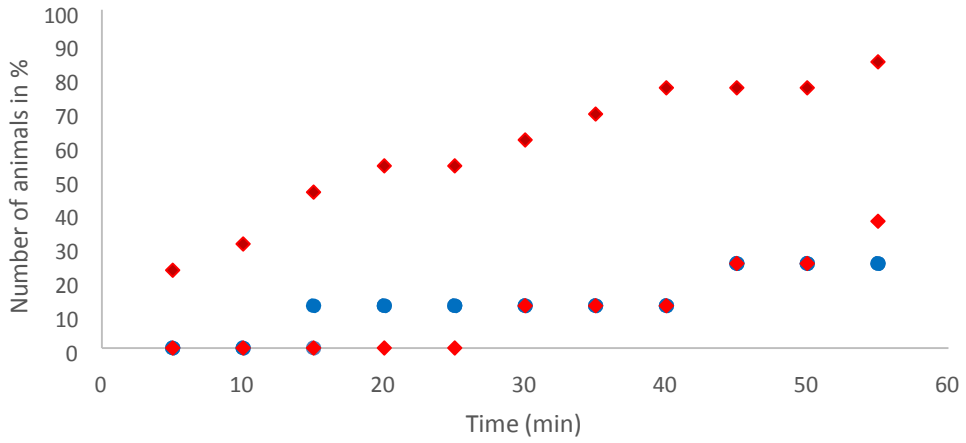

Figure 5. The Y-axis shows how many percent of the animals that have been attracted to another animal, of the same or opposite sex, as their first choice, for a treatment at a certain time (X-axis). Blue circle = males choosing between females and control (n = 5), blue square = males choosing between males and control (2), red circle = females choosing between males and control (n = 13) and red square = females choosing between females and control (n = 4). Note: only animals that did an active choice were used for the statistics. n = the number of animals in the treatment that did a choice.

Table 2a. Statistics for choosing other sex first. The values for χ^2, p, number of animals (n) and degrees of freedom (df.) are presented.

Sex	Treatment	Statistics				
		χ2	**p**	**n/N**	**df.**	**Significant**
Female	Male: Control	3.76	0.052	13/14	1	No
Female	Female: Control	1.0	0.317	4/8	1	No
Female	Control: Control (B1[a])	0	1	3/4	1	No
Female	Control: Control (B2[b])	2	0.15	5/6	1	No
Male	Female: Control	3.4	0.18	5/8	1	No
Male	Male: Control	2	0.15	2/8	1	No
Male	Control: Control (B1)	0	1	2/6	1	No
Male	Control: Control (B2)[c]	-	-	-	-	-

n = number of animals used for the statistics and N = total number of animals in the treatment. Animals that did not make a choice were excluded from the statistics.
[a] = bioassay 1, [b] = bioassay 2. [c] = Only one male entered a control.

Table 2b. Statistics for the number of entrances. The values for χ^2, p, number of animals (n) and degrees of freedom (df.) are presented.

Sex	Treatment	Statistics					
		χ2	**p**	**n/N**	**df.**	**Significant**	**Choice**
Female	Male: Control	8.16	0.004	13/14	1	Yes	Male
Female	Female: Control	0.5	0.47	4/8	1	No	Control
Female	Control: Control (B1[a])	0	1	3/4	1	No	
Female	Control: Control (B2[b])	2.0	0.15	5/6	1	No	
Male	Female: Control	0.50	0.47	5/8	1	No	Control
Male	Male: Control	1.8	0.17	2/8	1	No	Male
Male	Control: Control (B1)	1	0.31	2/6	1	No	
Male	Control: Control (B2)	2	0.15	1/4	1	No	

n = number of animals used for the statistics and N = total number of animals in the treatment. Animals that did not make a choice were excluded from the statistics.
[a] = bioassay 1, [b] = bioassay 2.

Chemical analyses

Evaluation of GC-MS chromatograms

The chromatograms from the separately sampled male(s) + females were overlayed and sex specific peaks were searched for. Male chromatograms were also compared with other male chromatograms and female chromatograms were compared with other female chromatograms. There were some differences in comparisons between individual male and female extracts sampled in parallel, but no potentially sex specific peaks were found in any other extracts from the same sex (Figure 6).

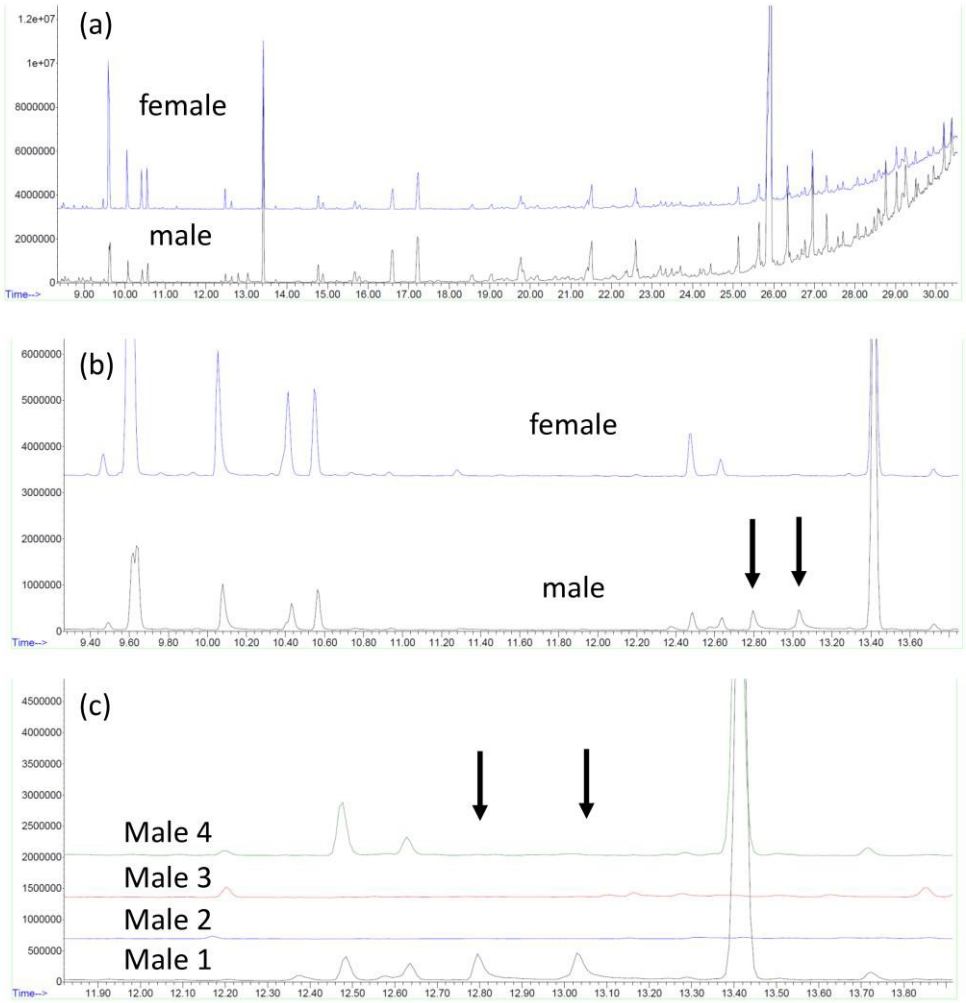

Figure 6. Comparisons between chromatograms from different *C. cerdo* volatile collections, demonstrating the general patterns found in qualitative comparisons between the sexes. Drawn to scale, with the baselines of some chromatograms offset in relation to the Y axis. (a) Overlay of one female and one male volatile chromatogram demonstrates their overall similarity. (b). A section from chromatogram (a) is zoomed in and there appears to be two male specific compounds, as indicated by arrows at ~12 min and ~13 min. (c) Comparing the male chromatogram with three other male chromatograms reveals that these compounds were not found in extracts from other males.

Evaluation of GC-EAG chromatograms

Two different male extracts gave weak apparent responses for both a male and a female antenna at about the same retention time (12.52 min) (Figure 7aand b). However, they did not induce as strong responses on the EAD as would be expected from a pheromone based on the size of the FID-signals, and neither of these responses were repeatable with the same extracts. Most analyses produced no good EAD responses, as seen in Figure 7c.

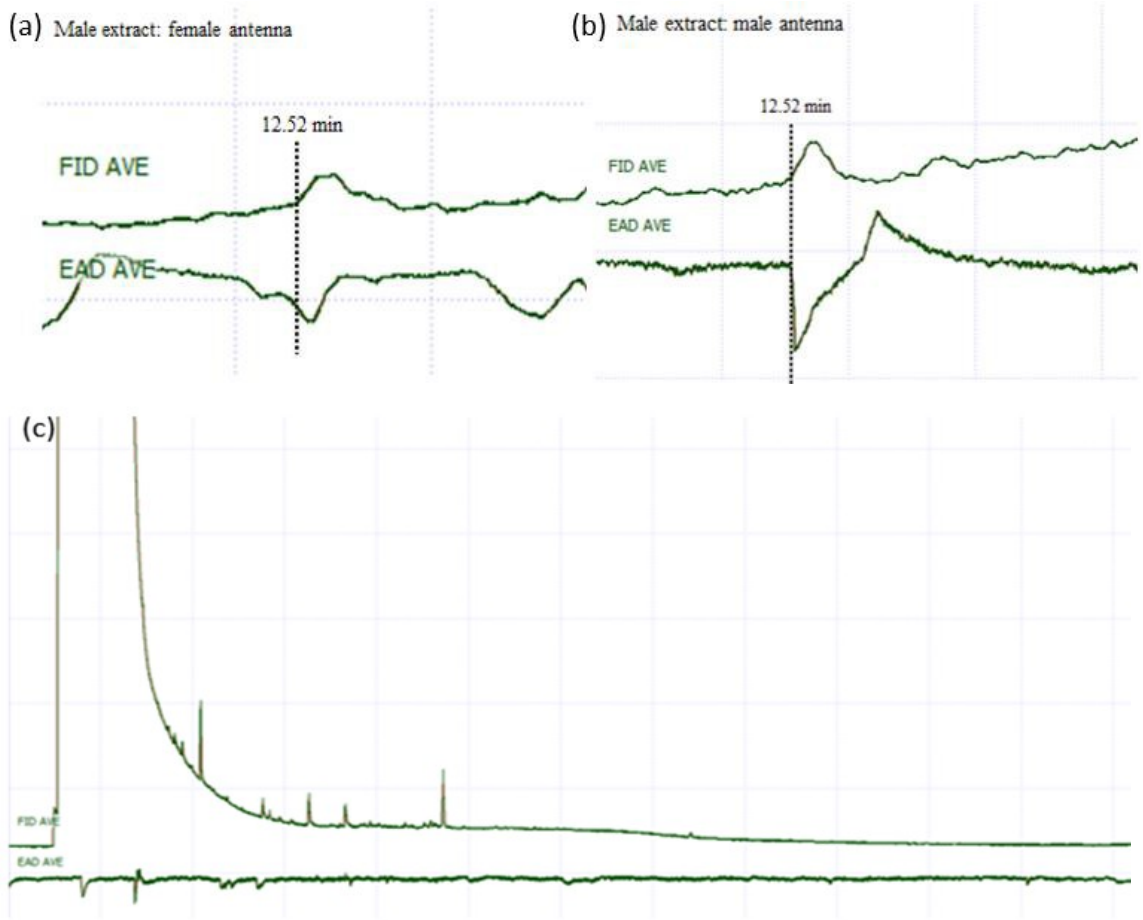

Figure 7. Repeated GC-EAD responses at retention time 12.52 min from two different male extracts and one female (a) and one male antenna (b). Otherwise, EAD responses (c) (lower trace) towards the extracts were mostly absent as seen in the recording. None of the apparent responses from the antenna were repeatable in spite of many recordings.

References for 1-hexanol and 2-hexanone were injected together as these compounds recurrently had been suggested by the MSD Chemstation library from the extracts. These compounds did not elicit any response in the EAD. As there are no known active substances for *C. cerdo*, no positive control could be performed.

Discussion

Behavioural observations in this study strongly suggested that *C. cerdo* does use pheromonal communication, with males being the pheromone emitters and females being the primary responders. During the behavioural studies, only the females appeared to show any attraction for the exit tube with the conspecific beetles as stimuli, and they consistently did so only when males constituted the stimulus. This would be entirely consistent with many other species in the subfamily Cerambycinae, in which males are typically the pheromone emitters (Mitchell *et al.* 2015; Ray *et al.* 2006)

However, despite of best efforts, no likely pheromone candidate was found. This is in some sense inconsistent with behavioural results suggesting that males release pheromones, as in other longhorn beetle species where males are the emitters the pheromones are generally released in very high quantities. In most species, the amounts of pheromones released would make them trivial to detect during laboratory collections (Lacey *et al.* 2004) (MC Larsson *personal communication*). The GC-MS and GC-EAD chromatograms were extensively screened for sex specific compounds without results. The hypothesis was that only one of the sexes would emit pheromone(s) and that this compound(s) would be exclusively found in only the chromatograms of that sex. In a few occasions, compounds with the same retention time were found exclusively for one sex. However, when analysing their MS-spectra, they were revealed to be different compounds. A difficulty with the GC-EAG analyses was that each antenna only lasted for two or three runs before it was consumed and that many animals had died prior to the GC-EAG analyses. This meant that the GC-EAD work was somewhat limited and occasionally, GC-EAD runs failed due to disturbances.

There could be several reasons for the absence of sex specific compounds. First, the animals were kept in an environment that hardly resemble their natural habitat; small plastic containers (even though they were kept in a larger box with oak branches prior to collection). The limited space might have prevented them from emitting pheromones. They were also kept at a near constant temperature (~20^{o}C) at all times. In Nature, the evening/night, when they are active, the temperature would normally be lower. As there are nothing known about their sexual behaviour, it is difficult to resemble an environment that stimulate pheromone emittance.

Secondly, they potentially utilize some natural compound for the synthesis of their pheromone, like the bark beetles (Xiao-ling *et al.* 2006), that was not available (though this is in conflict with the results from the behavioural studies suggesting that some attractant was released from males).

A third possibility, there is one, or more, sex specific compound but they are hidden under another compound. However, any hidden compound(s) would have been detected in the GC-EAD analyses. Despite the active carbon filter, most chromatograms contained a lot of noise. The addition of oak branches also increased the number of compounds in the chromatograms. To find potentially hidden compounds, different columns and different temperature programs can be tested.

Further on, pheromones could have been emitted only sporadically which make them hard to catch in the extractions. Though, almost all of the females were attracted to the males in the behavioural studies that were carried out in about the same time as the volatile collections. In that case, differences in pheromone emittance could be explained by different environments (volatile

extractions in a closed system versus a more open bioassay). Also, some insect species are extremely sensitive towards their pheromones. In certain cases, $10^{-18} – 10^{-15}$ grams of pheromone is enough for attraction (Shani, 1982). Of course, such small amounts would be hard to detect, especially in a complex mixture of compounds.

Both sexes have the capacity of doing sounds, quite loud too. The sounds might be involved in their communications (attraction of the other sex) as an addition to the suspected chemical communication. It is also possibly that the sound is solely used for defence purposes, to frighten attackers, as they only made sounds when moved.

Although the pheromone(s) of *C. cerdo* was not found in this work, it provides a starting point for the next attempt. The behavioural studies suggest the males to be pheromone emitters, which agrees with the literature that suggest that the Cerambycinae males are the primary emitters of sex and aggregation pheromone emitters (Allison *et al.* 2004; Lacey *et al.* 2009). As only the *C. cerdo* females showed an attraction to the males, it appears that the males emit sex pheromones rather than aggregation pheromones.

As the number of GC-EAD runs were limited, priority was given to the extracts. A proposed strategy for next attempts is to start the work by analyzing known Cerambycidae pheromones, particularly those of closely related species, by GC-EAD. As many reference pheromones can be injected in the same time, most known compounds can be tested in a relatively short period of time. If any compound(s) would yield a response, their behavioral response could be examined using a bioassay. These compound(s) could then also be screened for in the volatile extracts. Furthermore, to improve the detection, some extractions could use simultaneous pump extractions and SPME. A more natural environment for the *C. cerdo's* might also prove helpful.

Conclusion

In this work, an attempt to identify the pheromone(s) of the great Capricorn, *C. cerdo* was undertaken. Although no likely compound was identified the behavioural studies suggests the males to emit female-attracting sex pheromones. Based on this work, new tactics to find the pheromone(s) in the future have also been proposed.

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