Training dogs to indicate synthetic pheromone from *Plagionotus detritus*, Coleoptera, Cerambycidae to detect living beetles

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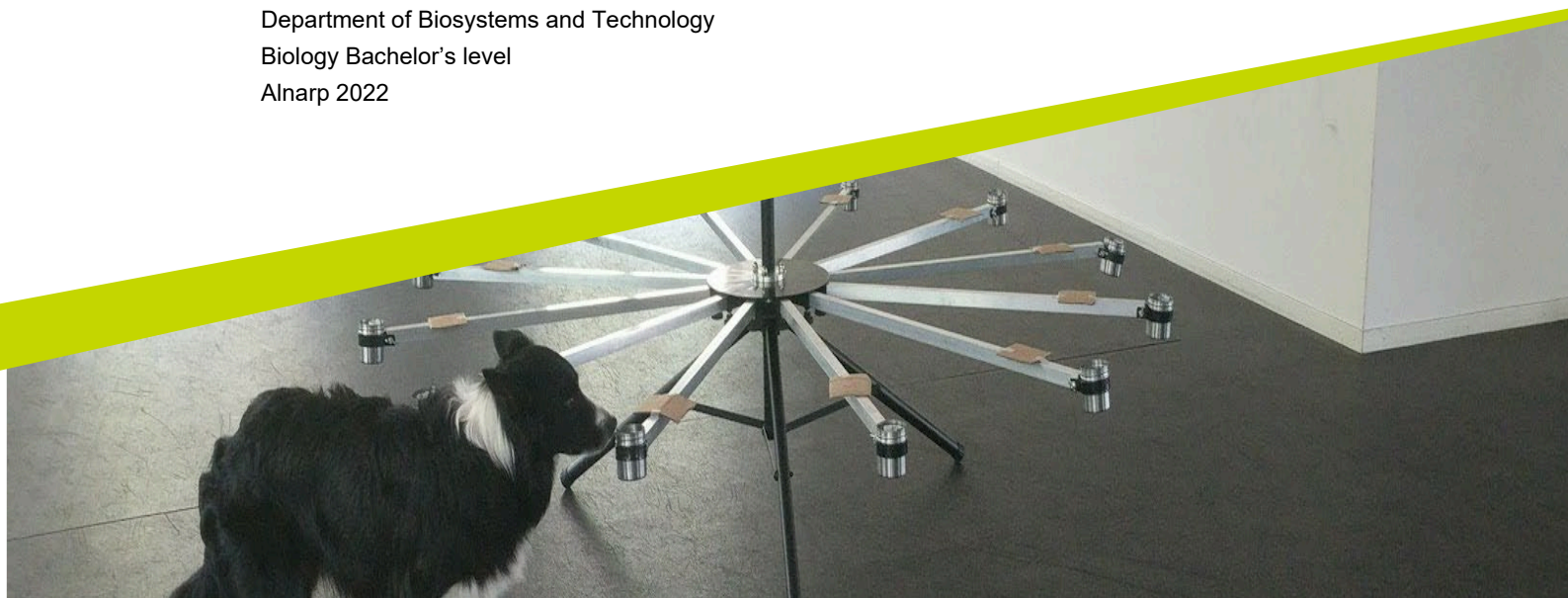

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Abstract

Using dogs to detect insects' emitted volatiles is a non-invasive procedure, and a trained detection dog is potentially a fast and accurate method to search for saproxylic insects. The longhorn beetle *Plagionotus detritus* is categorized as Endangered in the Swedish Red List of Threatened Species. The beetle is difficult to detect and monitor with conventional survey methods. Studies indicate that the beetle can be monitored using pheromone traps. Pheromone works on large scale and using a detection dog might be a good complement. Performing the training of the dog on a synthetically reproduced scent from the beetle will not require access to live specimens of the endangered target species. In this study the potential for trained dogs to indicate synthetic pheromones from *P. detritus* to detect a living male beetle was examined. The dogs were first trained to indicate synthetic pheromone from *P. detritus*. The synthetic compounds used in the study were the two pheromone components of the *P. detritus* pheromone: *(R)*-3-hydroxy-hexan-2-one as the major component and *(S)*-2-hydroxy-octan-3-one as the minor component. Secondly, the dogs were taught to discriminate the target odour from similar decoys. Both compounds in the synthetic pheromone were used separately as decoys, in addition to three other compounds found in closely related Swedish longhorn beetle species, living in the same habitat. For the final test the synthetic pheromone was replaced with a living male beetle of *P. detritus* to determine if the dog trained to detect synthetic pheromone from *P. detritus* can recognize the scent from a living male beetle. The study was conducted on five dogs. The evaluation of a dog, the imprinting of the target scent, and the tests of the pheromone were carried out indoors for one day. All dogs managed to indicate the synthetic pheromone from *P. detritus* and learned to discriminate the decoys. Three of the dogs indicated the living male beetle in at least one out of three trials against synthetic decoys, and one dog indicated the beetle in three out of three trials. This method of indoor training on synthetics could be a valuable method for training dogs to detect endangered saproxylic insects in their habitat. This is promising for advancing the use of detection dogs for monitoring and protect endangered insects.

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1. Introduction

A dog´s sense of smell is a complex airway network estimated to be up to 10 000 times greater than humans (Matthew and Relton 2021). Dogs are widely used for scent detection work, assisting in search for narcotics, mines, missing people, pest insects, and human diseases like cancer. They are also more increasingly used as detection dogs in nature conservation settings over the last years (Beebe et al. 2016). Dogs have been used to detect endangered species since the 1890´s when dogs were used to find the kiwi bird *Apteryx* sp. and the kakapo *Strigops habroptila* (Beebe et al. 2016). Over hundred years later the use of dogs for detection of rare or endangered species remains relatively unexplored (Matthew and Relton 2021) and mostly used to detect mammals or pest insects. Suma et al. (2014) showed that dogs trained to indicate scent material from red palm weevil *Rhynchophorus ferrugineus* in potted palm trees indicates 78% correct positive indications. Dogs trained to detect Asian longhorn beetle *Anoplophora glabripennis* in hollow building blocks correctly indicated 85.6% for the frass, 8% of the larva and 92.6% of the infested food + larvae (Hoyer-Tomiczek et al. 2016). Dogs trained to find Eastern subterranean termite *Reticulitermes flavipes* workers were 95.93% accurate (Brooks et al. 2003). The accurate detection of Bed bugs Cimex lectularius is even higher, 97.5% (Pfiester et al. 2008).

There are only a few studies using dogs to detect rare and endangered insects. A dog trained to detect bumblebee nests found 100% of the nests (Water 2010). Monitoring larvae of Hermit beetle *Osmoderma eremita* with a detection dog was much less time-consuming and more accurate than human wood mould sampling (Mosconi et al. 2017). Most insect survey techniques might disturb or even harm the insects since they involve catching the insect or using invasive methods for searching through the habitat. A survey using a dog detecting the insect's emitted volatiles is a non-invasive procedure, and a trained detection dog is potentially a fast and accurate method to search for saproxylic insects (insects dependent on dead wood) (Mosconi et al. 2017).

The number of red listed species in Sweden are increasing and many of the listed species are becoming more endangered (Artdatabanken 2020). More effective and non-invasive ways to detect endangered species might give more information about

the species without killing individuals, which would facilitate the conservation work. The longhorn beetle *Plagionotus detritus* is a saproxylic, rather large beetle assigned threat category Endangered in the Swedish Red List of Threatened Species (ArtDatabanken 2020). The larvae live in sun-exposed, newly dead and relatively thick oak branches or dead steams (Ehnström and Axelsson 2002). Large dead or dying oak branches are a rare commodity and are often pruned for safety reasons and removed from the area, especially when growing in public areas or in pastureland. The beetle has exhibited a dramatic reduction in distribution. It was previously found in Skåne, Blekinge, Halland, Öland, Gotland, in eastern Småland, Östergötland and Västergötland (Ehnström 2005) but today only has a single population known with certainty on Djurgården in Stockholm (Artdatabanken 2016). The beetle is difficult to detect and monitor with conventional survey methods (Molander et al. 2019a). Unpublished field studies indicate that *P. detritus* might have a wider distribution than previously noticed. An unpublished study using pheromone traps to catch and recapture *P. detritus* indicates that it is a useful method to indicate presence in an area. The attraction range is not known which make the method uncertain when finer scales like certain tree. Monitoring the insect using pheromone traps also requires the insects to be captured. Since *P. detritus* is difficult to detect and monitor with conventional survey methods (Molander et al. 2019a) an alternative method to monitor the beetle is desirable.

A problem with training a detection dog to detect endangered and elusive insects is that the training requires collection of many beetles to teach the dog the correct scent picture. Collecting endangered species should be avoided. This problem could be circumvented if the dogs could be trained on synthetic odour mimics. A study using dogs to detect the European spruce bark beetle *Ips typographus* shows that the dogs rapidly learned responding to synthetic bark beetle pheromone (Johansson et al. 2019). Furthermore, responses to synthetic pheromones could be transferred to detection of live beetles under field conditions (Johansson et al. 2019). Performing the training of the dog on a synthetically reproduced scent will not require access to live specimens of the endangered target species.

Longhorn beetles (Cerambycidae) use long-distance aggregation pheromones extensively for locating mates (Allison et al. 2004). The aggregation pheromone from *P. detritus* has been identified and tested in the field by Molander et al. (2019a) and Imrei et al. (2021). The males of *P. detritus* produce mainly *(R)*-3-hydroxy-hexan-2-one (R3C6) and *(S)*-2-hydroxy-octan-3-one (S2C8) (Molander et al. 2019a; Imrei et al. 2021), both of which can be synthetically produced. As a part of the Swedish national action plan for *P. detritus*, the species is bred at Nordens Ark, a zoo specialized in breeding endangered species. This makes it possible to perform test on living adult individuals without having to collect the endangered species from the wild.

Waters et al. (2011) argue that it is possible that a detection dog will respond to insect species that are closely related. Species with similar pheromones living in the same habitat could be used as distraction scents to investigate if the dog could distinguish the pheromone from the target species from other related species in the habitat.

Dogs perform differently, some individual dogs succeed in tasks where others perform poorly (Kerley and Alkina 2007). They have different olfactory ability and different biological, psychological, and social traits (Beebe et al. 2016), which supports the importance of performing the test on several individuals. Another important aspect when testing the performance of a detection dog is to use blind test, where neither the dog nor the handler knows where the target scent is placed (Lit et al. 2011). In a blind test, the handler depends on a clear indication from the dog.

The purpose of this study is to investigate the potential for a dog trained to indicate synthetic pheromones from *P. detritus* to detect a living male beetle. First test was to investigate if it is possible for a dog to detect and mark the synthetic pheromone from *P. detritus*. Second step was to study if the dog can learn to distinguish the scent from potential decoys. The third test was to determine if a dog trained to detect and mark synthetic pheromone from *P. detritus* can recognize the scent from a living male *P. detritus*.

2. Material and method

2.1 Study species and sample specimens

Adult wild individuals of *P. detritus* have been collected from Djurgården, Stockholm by the county administrative board in Stockholm and cultivated at the zoo Nordens Ark in Sweden since 2006. The purpose is to find methods for self-sufficient cultivation of *P. detritus* to reintroduce adults in areas where the species previously has gone extinct. The beetles used in this study originate from bred individuals who were reared in a medium based on oak wood saw dust and protein supplement at Nordens Ark and were delivered as pupae to Swedish University of Agricultural Science, SLU, at Alnarp. The pupae were kept in a climate chamber at 25°C and adult beetles emerged soon after arrival to SLU Alnarp.

Adult females and males were kept separately in two different 22 L plastic boxes (IKEA model Samla). The containers were kept in a room with natural daylight at 20-25°C. A branch of oak, a nectar solution, a piece of banana, and damp paper were placed in the container (Figure 1).

Figure 1. Plagionotus detritus were kept in a 22 L plastic boxes (IKEA model Samla) together with a branch of oak, a nectar solution, a piece of banana and damp paper. (Photo by Matilda Apelqvist)

2.2 Pheromone chemistry

2.2.1 Preparation of synthetic stimuli

The synthetic compounds used in the study were the two pheromone components of the *P. detritus* pheromone: *(R)*-3-hydroxy-hexan-2-one (R3C6) as the major component and *(S)*-2-hydroxy-octan-3-one (S2C8) as the minor component (Molander et al. 2019a; Imrei et al. 2021) (Figure 2, Table 1). In addition, three other compounds that have been found in closely related Swedish longhorn beetle species, living in the same habitat were also included as potential decoys: racemic 2-methyl-butan-1-ol (RS2MB), racemic 3-hydroxy-octan-2-one (RS3C8) and racemic 3-hydroxy-decan-2-one (3C10) (Figure 2, Table 1). 2-methyl-butan-1-ol was obtained from Sigma-Aldrich (St Louis, USA), whereas all other compounds were obtained from Jocelyn Millar at UC Riverside, California, USA. These compounds were synthetized according to methods described in the papers describing their identification as pheromone components and references therein (Molander et al. 2019a; Molander et al. 2019b, Molander et al. 2019c, Imrei et al. 2019).

Odour stimuli were made from 1,5 ml screw neck glass vials with closed butyl/PTFE seal screw caps (Skandinaviska Genetec AB, Stockholm, Sweden) with inserted cut pieces of filter paper strips (Munktell's 1002, Munktell, Falun, Sweden) approximately 15x40 mm, 40 mg and folded twice. 10 µL of each compound was pipetted on the filter paper, which completely absorbed the liquid and enabled handling the open vials without any leakage. For headspace sampling, vials were also prepared containing 10 µL each of *(R)*-3-hydroxy-hexan-2-one and *(S)*-2-hydroxy-octan-3-one. The vials were sealed with screw caps when not in use and kept in a freezer at -18°C. Vials were allowed to acclimatize to room temperature before being used for dog detection tests or headspace collections.

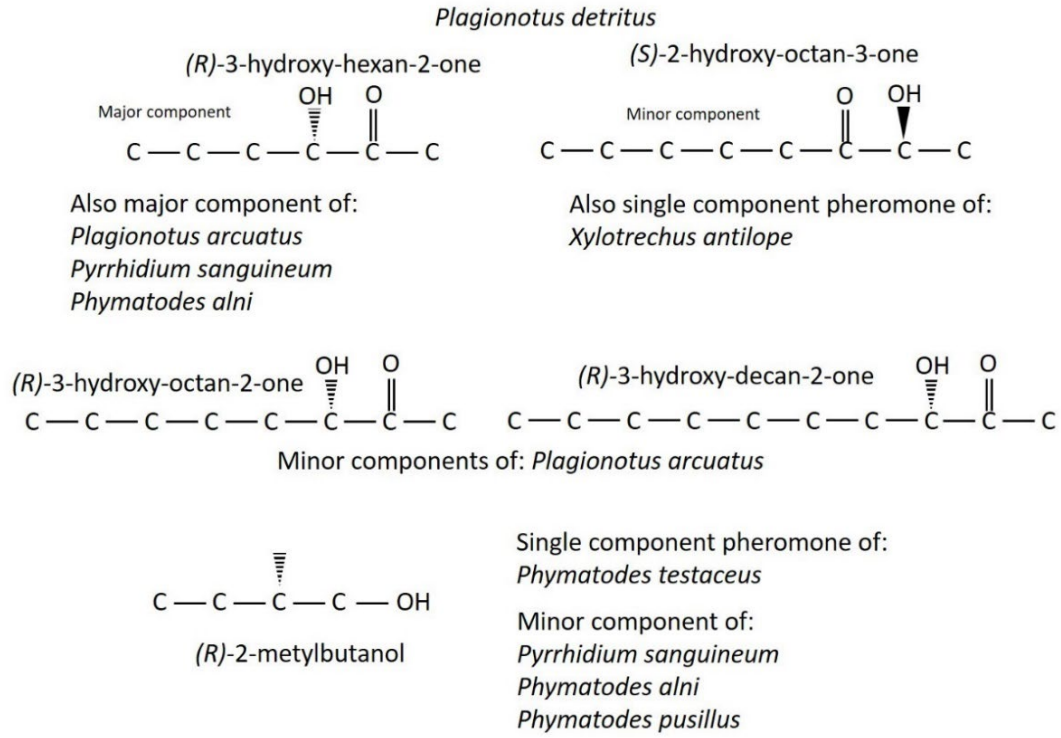

Figure 2. Illustrations of the chemical structures of some different pheromone components from Swedish longhorn beetles (Molander and Larsson 2018; Molander et al. 2019 a, b. c; Imrei et al. 2019). Note that for all compounds except the two actual pheromone components from P. detritus, racemic compounds were used.

Table 1. Compounds used in the test.

Compound	**Abbreviations**	**Decoys on the scent wheel**
(R)-3-hydroxy-hexan-2-one	R3C6	Major component in synthetic pheromone from *P. detritus*
(S)-2-hydroxy-octan-3-one	S2C8	Minor component in synthetic pheromone from *P. detritus*
Racemic 2-methyl-butan-1-ol	RS2MB	Racemate of component used by other insects living in the same habitat
Racemic 3-hydroxy-octan-2-one	RS3C8	Racemate of component used by other insects living in the same habitat
Racemic 3-hydroxy-decan-2-one	RS3C10	Racemate of component used by other insects living in the same habitat

2.2.2 Headspace collections

A headspace sample technique was used to match the relative composition of synthetic volatiles released from stimulus vials with the chemical composition of the male-produced sex aggregation pheromone from *P. detritus* (Molander et al. 2019a). Among the synthetic stimuli, headspace collections were only performed

using *(R)*-3-hydroxy-hexan-2-one and *(S)*-2-hydroxy-octan-3-one. The release rates of the other compounds used as decoys were not determined.

Headspace sampling was performed in an illuminated climate chamber at 25°C and 70% RH for 4 hours at daytime between 11.00 and 15.00. Three male and three female adults of *P. detritus* were put in separate transparent 2L plastic frying bags (ICA brand). A small piece of a living oak branch and a small piece of dead oak branch were put in the bag together with the *P. detritus* to encourage the beetle to produce pheromone (Figure 3). Two separate frying bags contained one stimulus vial with 10 µL of (R)-3-hydroxy-hexan-2-one and a separate stimulus vial with 10 µL (S)-2-hydroxy-octan-3-one. In addition, one frying bag contained a single stimulus vial with 10 µL each of the two pheromone components. Each bag had two small openings. One allowed unrestricted inflow of ambient air into the bag the other was fitted with an adsorption column consisting of Teflon® (TFE) tubing (inner diameter 3 mm length 50 mm). The TFE tubing contained 25 mg of the adsorbent polymer Porapak™ Q (mesh size 50–80; Supelco/ Sigma-Aldrich, Munich, Germany). Polypropylene wool, secured by short pieces of smaller TFE tubing (inner diameter 1.5 mm length 2 mm), was inserted into the main column on both sides of the adsorbent material to hold it in place. Polyvinyl chloride (PVC) tubing (diameter 4 mm) connected the adsorption columns to air pumps (model PM 10879 NMP 03; KNF Neuberger, Freiburg, Germany). The air pumps pulled ambient air through the frying bags and onto the adsorption columns. A flow meter (Kytölä Instruments, Muurame, Finland) and plastic valves on the PVC tubing were used to achieve a constant flow rate of about 150 mL/min through each column.

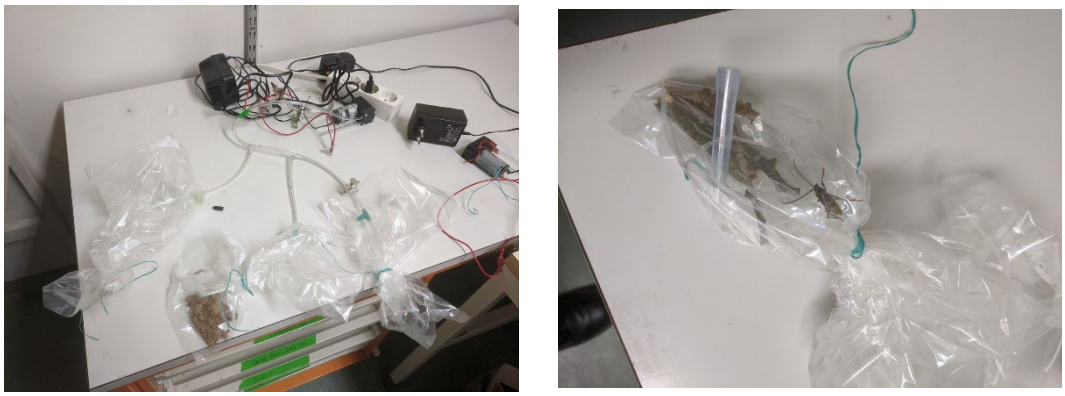

Figure 3. Headspace sampling was performed in an illuminated climate chamber at 25°C and 70% RH: Three male and three female adults of P. detritus were put in separate transparent 2L plastic frying bags (ICA brand). A small piece of a living oak branch and a small piece of dead oak branch were put in the bag together with the beetle to encourage the P. detritus to produce the pheromone. (Photo by Matilda Apelqvist)

After termination of the headspace collection procedure, each adsorbent column was extracted with 2x150 µL of hexane. The hexane was pushed through the adsorbent column into 1,5 mL glass vial with a slight nitrogen pressure. Glass vials were of the same type as the odour stimulus vials earlier. The glass vials were capped and stored in a freezer at -18°C until analysis.

2.2.3 Chemical analysis of headspace samples

Analysis of headspace samples were performed by GC-MS using a GC model 7890B interfaced to a 5977 mass selective detector (Agilent Technologies, Palo Alto, CA, USA). The GC was fitted with a DB-WAX capillary column (polyethylene glycol, 60 m × 0.25 mm inner diam., 0.25 µm film thickness: J&W Scientific, Folsom, CA, USA). Injections of 2 µL of each aeration sample were made manually in splitless mode (split vent opened after 0.5 min, injector temperature 225 °C). The carrier gas was helium with a constant flow rate of 1.9 ml min^{-1}. The oven program started at 40 °C, with a 3 min hold. Thereafter the temperature was rising by 8 °C min−1 to 230 °C, with a 10 min hold. The transfer line temperature was set at 150 °C. MS spectra were taken in electron impact ionization (EI) mode at 70 eV, with a scanning range of 29–400 m/z.

Sex-specific peaks from male *P. detritus*, consisting of (R)-3-hydroxy-hexan-2-one as the major component and (S)-2-hydroxy-octan-3-one as the minor component, were identified by matching the mass spectra and retention times to reference spectra from the synthetic compounds.

2.3 Dog's training and testing

2.3.1 The choice of dogs

Dogs have different olfactory ability and different biological, psychological, and social traits (Beebe et al. 2016). To test the ability of a dog to identify the scent and to investigate if there is any difference between different dogs, the test was conducted on five dogs. The dogs were of different ages and four different breeds. Three of the dogs were trained to detect scents and working as conservation detection dogs, one Golden Retriever, one Border Collie and one Belgian Malinois, their handlers have long experience in training and working with detection dogs. Two of the dogs had not been trained to indicate scents before, one Labrador Retriever and one Border Collie. Their handlers were new to training detection dogs. The dogs were trained as part of the study.

1. Golden Retriever – trained detection dog
2. Border Collie – trained detection dog
3. Belgian Malinois – trained detection dog
4. Labrador Retriever– new detection dog
5. Border Collie– new detection dog

2.3.2 Scent wheel

The tests were performed using a scent wheel or carousel (Figure 4). The scent wheel had a fixed centre and arms reaching out from the middle, like spokes on a wheel. At the end of each arm there was a container in which the target scent can be placed. The scent wheel is adjusted in height to make it easy for the dog to smell the container. Two different scent wheels were used. The wheel used in the test of dog number 1-3 had 11 arms and the scent wheel used in the test of dog number 4 and 5 hand 12 arms. The construction is fixed and the dog walked around the wheel and smelled the different containers.

For the detection tests, a 50 ml Falcon tube cut in half, with the open, cut-off end pointing upwards towards the dog, was placed in each of the containers of the arms of the scent wheel (Figure 4 and 5). One or two odour stimulus vials were placed in each tube, which made it possible to present individual odours or a combination of two odours in each container. Falcon tubes with different contents could also be handily exchanged without touching the vials. Each arm contained Falcon tubes with either one or two odour stimuli, or vials without any odour as controls. When live beetles were used in the containers of the arms, they were placed in a transparent vented container, normally used for transporting bee queens. All the materials were handled with gloves or a tweezer.

Figure 4. The scent wheel had a fixed centre and twelve arms reaching out from the middle. At the end of each arm there was a container in which the scent was placed. A 50 ml Falcon tube cut in half, with the open, cut-off end pointing upwards towards the dog, was placed in each of the containers of the arms of the scent wheel. (Photo by Matilda Apelqvist)

Figure 5. A 50 ml Falcon tube cut in half, with the open, cut-off end pointing upwards towards the dog. (Photo by Matilda Apelqvist)

2.3.3 Indication training

The two dogs who were new to detection training, dog number 4 and 5, were trained to indicate Kong® which is a dog toy made of natural rubber, commonly used as a target scent when training detection dogs. The dogs indicated by sitting and staring at the toy. The training was performed in different steps with smaller and smaller pieces until the dogs could identify and mark 5 times 5 mm pieces of Kong®. The pieces of Kong® were placed on a 2 times 1 meter wall made of bricks or firewood. The indication training lasted for approximately 5 weeks. For half a day the two untrained dogs were trained to search and indicate on a scent wheel. The dogs were first trained to search on three arms. The training was advancing until the dogs could search systematically on all eleven arms of the scent wheel. The dogs were trained using positive reinforcement using treat or toy as reward.

2.3.4 Basic set up for the test

The evaluation of the dog, the imprinting of the target scent and the tests of the pheromone were carried out indoors for one day. The dogs were tested individually at one task at a time, until all dogs mastered each step, and could rest between the tests. Dog number 1,2 and 3 was tested at the same day and dog number 4 and 5 were tested separately. The dogs were handled by their respective owners. The handler and the dog are here referred to as the dog and handler team.

The dog's ability to indicate a scent was evaluated before the test started. The dog and handler team needed to be able to indicate 5 times 5 mm piece of Kong® on the scent wheel three times in a row. The evaluation of the dogs and the detections-tests of the pheromone were conducted according to five guidelines:

1. An assistant was handling the test substances using gloves or a tweezer to avoid contamination of the vial or the container on the scent wheel.

2. The scent was randomly placed in one of the eleven or twelve containers of the scent wheel using a randomizer application on Google Play® called Pretty Random – Random Number® (Steven Burnett).

3. All tests where blind test, and the scent was placed by an assistant without the dog nor the handler looking. The handler depended on a clear indication from the dog.

4. The dog and handler team were considered to pass the test if the handler could tell the correct number of the container in which the target scent was placed.

5. The dog and handler team were able to indicate the target scent when they passed three blind tests in a row.

The dogs were trained to recognize the new scent, so called imprinting. After imprinting the dogs were trained to ignore different decoys in several steps with progressively more difficult combinations (Table 2.). To pass each step the dog and handler team indicated the correct number of the container in which the target scent was placed three times in a row.

Table 2. Overview of the stimuli used during the different stages of training and testing. Each individual odour was placed in a separate glass vial. When two odours were combined, two glass vials were placed in the same Falcon tube. P. detritus pher. = Synthetic pheromone components (R)-3-hydroxy-hexan-2-one (R3C6as the major component and (S)-2-hydroxy-octan-3-one (S2C8) as the minor component. RS2MB = racemic 2-methyl-butan-1-ol, RS3C8 = racemic 3-hydroxy-octan2-one, RS3C10 = racemic 3-hydroxy-decan-2-one.

	Target Scent	**Decoys on the scent wheel**							
Imprinting	R3C6+S2C8 *P. detritus pher.*								
Test 1	R3C6+S2C8 *P. detritus pher.*	RS2MB	RS3C8	RS3C10					
Test 2	R3C6+S2C8 *P. detritus pher.*	RS2MB	RS3C8	RS3C10	S2C8 Minor comp. *P. detritus*				
Test 3	R3C6+S2C8 *P. detritus pher.*	RS2MB	RS3C8	RS3C10	S2C8 Minor comp. *P. detritus*	R3C6 Major comp. *P. detritus*			
Test 4	R3C6+S2C8 *P. detritus pher.*	RS2MB	RS3C8	RS3C10	S2C8 Minor comp. *P. detritus*	R3C6 Major comp. *P. detritus*	R3C6 + RS2MB	3R3C6+ RS3C8	R3C6 + RS3C10
Final test	Male *P. detritus*	RS2MB	RS3C8	RS3C10	S2C8 Minor comp. *P. detritus*	R3C6 Major comp. *P. detritus*	R3C6 + RS2MB	3R3C6+ RS3C8	R3C6 + RS3C10

All dogs marked the target by sit and stare. If the handler was uncertain if the marking by the dog was correct, the handler commanded the dog to search again. When the handler was convinced in which container the dog thought the target scent was placed, he or she told the assistant.

2.3.5 Imprinting

A Falcon Tube was prepared with one vial with the major component, (R)-3-hydroxy-hexan-2-one, and one vial with the minor component, (S)-2-hydroxy-octan-3-one. The Falcon Tube was placed in one of the containers of the scent wheel. Falcon Tubes containing an empty vial with only a filter paper, were placed in the other containers of the scent wheel, as controls. The dog was commanded to search the wheel. The dog was rewarded when sniffing at the container containing

the target scent. The tube with the synthetic pheromone from *P. detritus* was shifted to a new, randomly selected position before every search. When the handler thought the dog had learned to identify the synthetic pheromone, the handler waited to reward the dog until he or she marked the target substance. The dog and handler team were able to indicate the scent when they managed to point out the correct container in three successive blind tests.

2.3.6 Distinguishing the full blend from decoys

After imprinting the synthetic pheromone, the decoys were added to the scent wheel. Controls were kept on the wheel during all the tests.

The dogs were trained to ignore the decoy and mark the full blend. The handler knew in which container the full blend was placed and in which there were decoys. When the dog indicated the correct container, it was rewarded by the handler. If the dog marked the decoy the behaviour was ignored. The handler decided when the dog was able to distinguish the decoy and ready to perform the test. The test was a blind test. The position of the full blend was shifted at random after every search as well as at least one decoy. The dog was able to distinguish the scent when the dog and handler team could point out the correct container in three successive blind tests. If the dog and handler team failed before it managed three times, training was re-initiated again, whereafter the dog and handler team were given a new opportunity until it succeeded.

Each step started with an evaluation whether the dog spontaneously distinguished between the imprinted target scent and the decoys. If there were mistaken identifications, the training continued until the dog successfully distinguished the target odour again.

The general aim was to determine whether the dogs ultimately learned to identify the target odour correctly, and therefore detailed notes of every hit and miss was not performed along the way during the training sessions. If any of the decoys appeared to be significant distractors with more than occasional false hits notes were taken.

2.3.7 Test with living male *Plagionotus detritus*

For the final test the synthetic pheromone, was removed but all the decoys were kept on the scent wheel. The synthetic pheromone was replaced with a living male *P. detritus*. The beetle was placed in a transparent vented container, normally used for transporting bee queens. One of the was arms of the scent wheel was chosen randomly with the randomizer application. Three blind searches were performed

without any practice before the test and the dog and handler team were not allowed to redo the test unlike when testing the synthetic full blend against the decoys. A correct identification was recorded as a success, or 1. If the dog and handler team did not manage to point out the correct container it was recorded as failure, or 0.

Dog number 1, 2 and 3 where all tested at the same time using the same beetle individual, towards the end of the day between 17.00 and 18.00. Dogs number 4 and 5 were tested two weeks later between 14:00 and 15:00 with another beetle individual.

3. Results

3.1 Headspace collections

The collections of the emitted volatile compounds from male *P. detritus* revealed the main pheromone component (R)-3-hydroxy-hexan-2-one and the minor pheromone component (S)-2-hydroxy-octan-3-one, with enantiomeric configuration assumed from previous publications, as the present GCMS analysis could not differentiate between enantiomers. The ratio between the major component and the minor component in the three collections were 1.8, 1.9 and 4.7, respectively, based on the relative areas of their respective peaks in the chromatograms (Figure 6). Corresponding ratios from two stimulus vials in one bag were 3.4 and 3.7, and the ratio from two compounds in the same vial was 5.2, respectively (see Figure 6 for examples of chromatograms).

Ratios of the two pheromone components produced by the beetles were thus variable but overlapped with the ratios from synthetic stimulus vials. No absolute amounts of compounds collected from the headspace collections were determined. However, the collections should be roughly comparable between different samples, and direct comparisons of peak areas between beetle samples and synthetic samples suggested that the amounts released from synthetic vials were approximately an order of magnitude higher than from the beetles (see Figure 6 - data not shown).

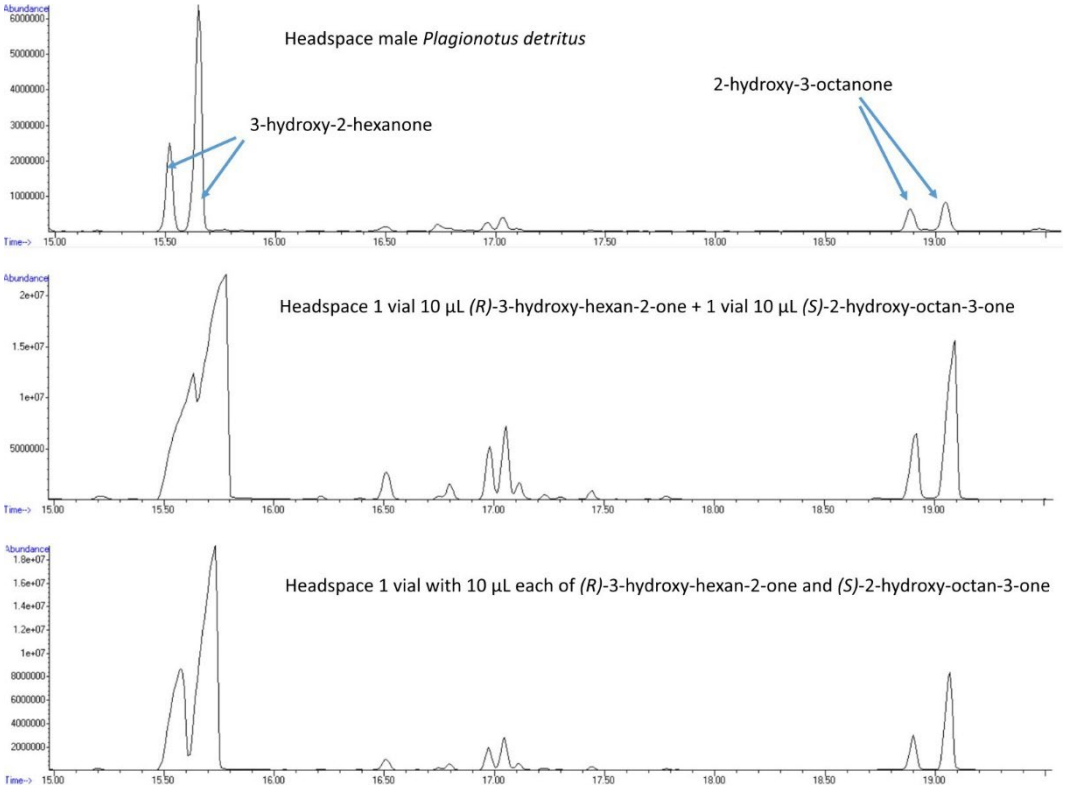

Figure 6. Representative chromatograms showing headspace collections from Plagionotus detritus male and from synthetic stimulus vials. Each of the two pheromone components are configurated during the chromatographic process and are thus represented by two peaks each.

3.2 Training evaluation and imprinting of the dogs

The training of the two dogs that were not previously trained to detect scents proceeded according to the plan. Both dogs learned how to indicate a target scent and to search on a scent wheel. All five dog and handler teams managed the evaluation to indicate 5 times 5-mm piece of Kong®, three times in a row and could be used in the test.

All five dog and handler teams managed to detect the full blend three blind tests in a row.

3.3 Distinguishing the full blend from the decoys

All five dog and handler teams learn to distinguish the full blend from all the decoys. For most dogs this required repeated rounds of training with some of the decoys before they finally managed to identify the target scent three times in a row.

During Test 1 (Table 2), the dogs generally had little problems spontaneously distinguishing the target odour from the decoys; none of which was part of the target blend. However, several dogs showed interest for racemic 3-hydroxy-2-octanone (RS3C8). It is a heterospecific pheromone component that is a close structural analogue of the minor pheromone component S-2-hydroxy-3-octanone, differing only in the relative positions of the hydroxy and keto groups. Neither 2-methyl-butanol (RS2MB) or 3-hydroxy-2-decanone (RS3C10) elicited any interest.

During Test 2 (Table 2), when the minor pheromone component (S)-2-hydroxy-octan-3-one (RSS2C8) was added as a single compound to the test array, some dogs had problems separating this single compound from the full blend. Initially, some dogs spontaneously selected the single minor component instead of the target stimulus, and only managed to indicate the full blend after training.

During Test 3 (Table 2), when the major pheromone component (R)-3-hydroxy-hexan-2-one (R3C6) was added as a single compound to the test array, this did not affect the results. The major component alone did not appear to be a significant distractor to the full blend, whereas the minor component continued to attract false markings from the dogs.

During test 4 (Table 2), here again, several dogs needed many rounds of training before they could correctly identify the target blend three times in a row. The main distraction again came from the minor component (S)-2-hydroxy-octan-3-one (S2C8) presented alone, rather than from the structural analogue 3-hydroxy-2-octanone (3C8) or the major component (R)-3-hydroxy-hexan-2-one (R3C6), or any of the blends where these compounds were included.

3.4 Test with living male *Plagionotus detritus*

The result of the final test, when the full blend was replaced by a living male *P. detritus*, differed between the different dog and handler teams (Table 3). Three out of five dog and handler teams managed to detect the beetle at least once (Table 3). Two dog and handler teams could not tell in which container the beetle was placed. One dog and handler team detected the beetle all three times. The average rate of successful indication was 40 % (Table 3).

Table 3 The result of the final test. 1 is a correct indication and 0 is failure.

Dog and handler team number	Previous training	Male *P. detritus* Test 1	Male *P. detritus* Test 2	Male *P. detritus* Test 3	Successful detection
1	Detection dog	1	0	0	33%
2	Detection dog	0	0	0	0%
3	Detection dog	0	1	1	67%
4	Untrained	1	1	1	100%
5	Untrained	0	0	0	0%

4. Discussion

The study demonstrates the potential for a dog trained to indicate synthetic pheromones from *P. detritus* to detect a living male beetle. To teach a dog to detect and mark the synthetic pheromone from *P. detritus* is a relatively fast and easy procedure. Teaching the dog to distinguish the target scent from potential decoys sems to be more difficult.

The results of the headspace from male *P. detritus* confirmed previous results (Molander et al. 2019a; Imrei et al. 2021). The major component was the enantiomer (R)-3-hydroxy-hexan-2-one, and the minor molecule was (S)-2-hydroxy-octan-3-one. These are the same components as Molander et al. (2019a) and Imrei et al. (2021) identified as the sex-aggregation pheromone. Headspace comparisons also confirmed that the synthetic vial stimuli used for training released compounds with similar ratios of the pheromone components as the range of ratios detected from male *P. detritus*, although considerably stronger (Figure 6).

Beyond the initial ability of dogs to learn the target scent, one of the most important aspects determining the usefulness of the method is the risk of false positives, if the dogs would frequently respond to other odour stimuli that they perceive as similar to the target odour. The selection of test odours likely represents a major part of the potential distractors that could be expected from Swedish longhorn beetles that are related to *P. detritus* or found in similar habitats (Figure 2). Longhorn beetle pheromones that release pheromones with these structural motifs are primarily found in the longhorn beetle subfamily Cerambycinae (Hanks and Millar 2016), which also includes *P. detritus* and the other related species referred to in this study. It is likely that our selection of compounds constitutes the most significant semiochemicals with these structural motifs from Swedish cerambycine longhorn beetles. This conclusion is partly based on direct headspace collections from many published (Molander and Larsson 2018; Molander et al. 2019a, b, c; Imrei et al. 2019) and unpublished species (M.C. Larsson et al. unpublished data). In addition, the compounds used here have been used in numerous field trials with these and other compounds in relevant habitats across southern Sweden, with significant attraction of cerambycid beetles found only for the species mentioned here (see

previous references and Molander 2019a, b, c; Celander 2020; Rönnqvist 2020; M.C. Larsson et al. unpublished data).

We only knew if the dog could distinguish between the substances that we tested. However, as was argued above, the selection of test compounds represents the most likely structurally close analogues that are likely to be found in a Swedish context.

It appeared as the dogs could readily distinguish the synthetic pheromones from *P. detritus* from the three other compounds that have been found in closely related Swedish longhorn beetle species, living in the same habitat: racemic 2-methyl-butan-1-ol (RS2MB), racemic 3-hydroxy-octan-2-one (RS3C8), and racemic 3-hydroxy-decan-2-one (RS3C10). This despite the analogue structure of some of the substances being relatively alike to pheromone components. The structural difference between minor component of the synthetic pheromone from *P. detritus*, (S)-2-hydroxy-octan-3-one (S2C8), and racemic 3-hydroxy-octan-2-one (3C8) is only the location of the closely situated keto and hydroxyl groups (Figure 2).

Another potential source of false positives could be from species sharing the same or a very similar pheromone blend, or a partially overlapping pheromone containing one of the two components from *P. detritus*. Among the two pheromone components, the major component *a priori* was assumed to be the most potentially problematic. (R)-3-hydroxy-hexan-2-one is a major pheromone component for several other species and should be the most dominant component of the odour bouquet of the combined *P. detritus* blend. In reality, it was instead obvious that the dogs perceived the minor component (S)-2-hydroxy-octan-3-one as the most important part of the recognition of the synthetic blend. The results demonstrate that initial imprinting of the synthetic odours was fast and efficient. Studies on bark-beetles *Ips typographus* shows that training dogs on synthetic pheromone components is a quick method to train a dog to detect an insect (Johansson et al. 2019). In comparison, the imprinting phase of *Osmoderma eremita* larvae was 7 months (Mosconi et al 2017).

The result shows that four of the dogs had difficulties identifying the beetle. Previous studies on dogs indicates that dogs have different olfactory ability (Beebe et al. 2016). Since three of the dogs having trouble to identify the male beetle are used as detection dogs, they are all likely to have good olfactory abilities. More dogs probably would have strengthened the results.

A reason why some dog hand handler teams could not identify the beetle might be that the dog perceived the stimuli differently based on concentration. The beetles likely released much lower amounts of pheromone than the synthetic stimuli. The headspace samples showed that the synthetic pheromone used in the study was approximately ten times higher than the amount emitted from the beetle (Figure 6).

Johansson et al. (2019) showed that correct negative responses decline with increased synthetic dose. Gradually lowering the dose of the synthetic pheromone to the same dose as emitted from the beetle during the headspace sample would clarify this.

It would be interesting to lower the dose of the synthetic pheromone to study how low dose a dog can detect. To be able to detect a living beetle in its natural habitat the dog probable needs to be able to detect very low doses of pheromone.

The release rates from the beetles at the time of the odour detection tests is not known. The circumstances, with the beetles being put in containers indoors, may not have been optimal for stimulating pheromone release. Especially during the first day of testing with dogs 1-3, the beetle was only used late in the afternoon, which may be beyond the main time of signalling. A headspace sample from the synthetic pheromones and from the living *P. detritus* performed from the scent wheel would determine the differences between the emitted compounds. It would also determine if the beetle is emitting the pheromone during the test. This would make the results more reliable since the tests was performed on different days and different beetle individuals.

Even though all dogs learned to distinguish the synthetic pheromone from *P. detritus* from the decoys of synthetic pheromones from other beetles living in the same habitat, we do not know if the dog can distinguish different living species. Replacing the synthetic pheromones with living pheromone producing individuals would clarify the dog's ability to distinguish the different species from each other. To obtain more reared beetles or collecting living individuals of the insects used as decoys was not possible in the time frame for this study.

Interesting to further investigations is to study if a dog searching for saproxylic insects is more effective or accurate than using a pheromone trap or more conventional monitoring methods. Experiments on monitoring *Osmoderma eremita* with a detection dog was much less time-consuming and more accurate than human wood mould sampling (Mosconi et al 2017). Searching in the field requires a lot more of the dog than searching on a scent wheel. The fact that training and housing a dog is costly and requires certain skills should also be taken into consideration (Beebe et al. 2016).

The use of synthetic training stimuli for indoor training could be a valuable method for training dogs to detect endangered saproxylic insects and using detection dogs for monitoring and protect endangered insects.

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