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Chemical ecology and insect conservation: optimising pheromone-based monitoring of the threatened saproxylic click beetle *Elater ferrugineus*

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Abstract *Elater ferrugineus* is a saproxylic click beetle inhabiting old deciduous trees in Europe. It is threatened throughout its area of distribution due to habitat loss. No efficient monitoring method has been available for this species, but observed attraction of females to (R)-$(+)$-γ-decalactone, which is a male-produced sex pheromone of its prey, the scarab beetle *Osmoderma eremita*, has led to the development of an odour lure for monitoring. In addition, four esters have recently been identified from the pheromone-producing gland in female *E. ferrugineus*, and a blend of these esters is highly attractive to conspecific males in the field, revealing an alternative odour-based method for monitoring this species. However, no rigorous analysis has been performed to check whether all four esters show biological activity in male *E. ferrugineus*, and whether its own sex pheromone is a more potent lure than the prey kairomone for monitoring of *E. ferrugineus*. In this study, we reinvestigated the *E. ferrugineus* sex pheromone, using electrophysiological and behavioural analyses, and found that only one of the esters, 7-methyloctyl (Z)-4-decenoate, is active. In addition, trapping experiments revealed that 7-methyloctyl (Z)-4-decenoate is a much more efficient attractant for male *E. ferrugineus* than the prey pheromone is for conspecific females, or any sex of *O. eremita*. With a very efficient odour lure at hand, novel information about current distribution, local population sizes, and dispersal ranges in *E. ferrugineus* can now be obtained, which can aid in conservation efforts to protect this threatened insect and its habitat.

Keywords Elateridae · Scarabaeidae · Sex pheromone · Kairomone · Field trapping · Conservation

Introduction

Effective conservation depends on the ability to reliably measure and monitor changes in species abundance in relation to habitat change. Pheromone-based monitoring has great potential to become an important part of many programs for assessing the conservation status of threatened insects, because sex pheromones are highly species-specific and often efficient in detecting target species at very low densities (Larsson et al. 2009). For many insects, pheromone trapping thus provides a robust and standardised way of gathering valuable information about population sizes and dispersal ranges. However, the applications of chemical ecology tools have so far been almost exclusively directed towards monitoring and control of pest insects (reviewed by Witzgall et al. 2010), with very little emphasis on the use of semiochemicals in insect conservation and biodiversity studies (but see Gandhi et al. 2009). Consequently, pheromone identifications for threatened insect species carried

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out expressly for conservation purposes are still very scarce (Larsson et al. 2003; Millar et al. 2010; Tolasch et al. 2007).

Monitoring saproxylic insects living in tree hollows is a difficult task. Both traditional methods, like pitfall trapping or wood mould sampling (Ranius 2002), as well as more innovative approaches (Bußler and Müller 2009; Svensson et al. 2003) are labour intensive and time consuming. Thus, developing efficient odour-based trapping systems that are easy to use would greatly facilitate monitoring of these species, which is needed to get more reliable data on their current conservation status. We recently initiated a research program with the aim to develop efficient odour attractants for monitoring of threatened saproxylic beetles in Europe. The first species for which the pheromone was identified was the hermit beetle, *Osmoderma eremita* Scopoli, which is considered an indicator for the species rich saproxylic insect fauna in Europe (Ranius 2002) and has been given special protection according to the European Union's Habitat Directive (Anonymous 1992). We identified (R)-$(+)$-γ-decalactone as the single constituent of the male-produced sex pheromone of *O. eremita* (Larsson et al. 2003; Svensson and Larsson 2008), and showed that females of the rare red click beetle *Elater ferrugineus* L., which is a larval predator on several other saproxylic beetles including *O. eremita*, use this compound as a kairomone (prey or habitat signal) (Svensson et al. 2004; Svensson and Larsson 2008). We have used traps baited with (R)-$(+)$-γ-decalactone to catch both beetle species, and mark-release-recapture data, using pheromone traps outside trees and pitfall traps placed inside tree hollows (Larsson and Svensson 2009), have generated more accurate estimates of population sizes and dispersal rates for *O. eremita* compared to estimates based on data from pitfall traps alone (Ranius 2001; Ranius and Hedin 2001; Hedin et al. 2008; Svensson et al. 2011). Catch data from pheromone traps over several years have also been used to analyse spatiotemporal variation in abundance of these species (Larsson and Svensson 2011).

In addition to the identified kairomone for *E. ferrugineus*, a female-produced sex pheromone of this beetle was recently reported as a blend of four esters: 7-methyloctyl 5-methylhexanoate, 7-methyloctyl octanoate, 7-methyloctyl 7-methyloctanoate, and 7-methyloctyl (Z)-4-decenoate (Tolasch et al. 2007). The four-component blend was applied in field tests and found to be very attractive to male beetles. However, no subtractive experiments were performed to check whether all compounds elicit a behavioural response in this species. The esters are unique as pheromone components in insects, and are not commercially available. It is thus important to know if all four compounds are required for efficient attraction of male beetles, or if fewer compounds constitute the sex pheromone of *E. ferrugineus*, to limit costs for the synthesis of the pheromone and develop

a cost-efficient attractant. In addition, checking whether the sex pheromone or the kairomone is most efficient in attracting the species is important to develop the best possible monitoring system.

The aims of this study were twofold: to reinvestigate the sex pheromone of *E. ferrugineus*, and to compare the efficiency of the sex pheromone and the kairomone (R)-$(+)$-γ-decalactone as lures for monitoring of this species. We show that only one of the four esters identified by Tolasch et al. (2007) constitutes the sex pheromone of *E. ferrugineus*, and that the sex pheromone is a much more powerful monitoring lure compared to the kairomone.

Materials and methods

The species

Elater ferrugineus is a large (15–25 mm) saproxylic beetle living exclusively in the hollows of old deciduous trees. Due to its cryptic lifestyle, the species is rarely observed outside the natal tree habitat, and data are still lacking regarding many fundamental aspects of its ecology. The species develops in the wood mould (a mixture of loose, rotten wood, fragments of dead insects, fungi and old bird nests) inside tree hollows. The flight period of Swedish populations is from late June to mid August. The species is a facultative larval predator on several saproxylic scarab beetles, including *O. eremita*, *Gnorimus variabilis* L., *Protaetia aerigunosa* (Drury), and *P. lugubris* (Herbst) (Hansen 1966; Tolasch et al. 2007). Although no detailed data are available on its current distribution, habitat destruction and fragmentation has most probably rendered *E. ferrugineus* threatened over its entire range in Europe. It is considered Vulnerable in the Swedish Red List of threatened species (Gärdenfors 2010).

Insect collection and electrophysiology

To obtain male beetles for electrophysiological screening, we performed small-scale trapping experiments in southern Sweden using non-destructive traps and PCR tubes loaded with the four-component blend as described below. Gas chromatography with electroantennographic detection (GC-EAD) (Larsson and Svensson 2005) was then applied to check the antennal response to the four female-produced esters reported by Tolasch et al. (2007). An antenna was cut and mounted to a PRG-2 EAG ($10\times$ gain) probe (Syntech, Kirchzarten, Germany) using conductive gel (Blågel, Cefar, Malmö, Sweden). Charcoal-filtered and humidified air passed over the antenna from a glass tube outlet positioned at 5 mm distance from the preparation. The GC effluent to the antenna passed through a heated transfer line set at 230°C. One microliter of a mixture of

7-methyloctyl 5-methylhexanoate, 7-methyloctano-ate, 7-methyloctyl 7-methyloctanoate, and 7-methyloctyl (Z)-4-decenoate was injected into an Agilent 7890A gas chromatograph (Agilent Technologies), equipped with a polar HP-INNOWax column (30 m × 0.25 mm i.d., and 0.25 μm film thickness; J&W Scientific, Folsom CA, USA). A split at the end of the column allowed a 1:1 partition of the GC effluent to the flame ionization detector (FID) and to the antenna. Hydrogen was used as carrier gas at a flow rate of 43 cm/s and injector temperature was 225°C. The column temperature was maintained at 60°C for 2 min after injection and then increased by 10°C/min to 220°C. Two concentrations of samples were used, corresponding to 2.5 or 25 ng/μl of each compound. Each antennal preparation was used only for a single GC-EAD trial. Data were analyzed with the GC-EAD Pro Version 4.1 software (Syntech, Kirchzarten, Germany).

Chemicals and dispensers

For trapping of male *E. ferrugineus*, we used esters synthesised according to a method modified from Tolasch et al. (2007). The four esters were obtained with high chemical purities (>98%) and a detailed description of the synthesis of these compounds is provided as supplementary information. Based on the results from the GC-EAD trials (see below), we used three different treatments for trapping male *E. ferrugineus*: 7-methyloctyl (Z)-4-decenoate alone, a blend of 7-methyloctyl 5-methylhexanoate, 7-methyloctyl octanoate, 7-methyloctyl 7-methyloctanoate, and 7-methyloctyl (Z)-4-decenoate, in a ratio of approximately 1:1:3:3 (four-component), and a blend of 7-methyloctyl 5-methylhex-anoate, 7-methyloctyl octanoate, and 7-methyloctyl 7-methyloctanoate, in a ratio of approximately 1:1:3 (three-component), using the same relative ratios of compounds as those reported by Tolasch et al. (2007).

Two dispenser types were used: grey rubber septa (PheroNet, Lund, Sweden) or 0.2 ml polypropylene PCR tubes (Multiply®—Pro #72.737, Sarstedt, Landskrona, Sweden). Rubber septa were used to compare attraction to lures where each individual component was presumably released at comparable absolute levels, regardless of the blend context. Solutions for rubber septa were prepared in 100 μl of distilled hexane per septum, with relative component ratios measured by weight. A septum was loaded with an ester mixture at an amount corresponding to 1 mg of 7-methyloctyl (Z)-4-decenoate, and then put in a fume hood for 1 h to allow the solvent to evaporate. PCR tubes were used for comparison with the original strategy employed by Tolasch et al. (2007). Mixing neat compounds in a container in different combinations would likely change the absolute release rate of 7-methyloctyl (Z)-4-decenoate as presented in a blend (where it is actually diluted by the

other components) versus as a pure compound. Nevertheless, we wanted to study whether the different treatments were functionally comparable in terms of trap catch. Each PCR tube was loaded with relative component ratios measured by volume: 2 μl of neat 7-methyloctyl (Z)-4-decenoate, or with one of the multi-component mixtures at a total amount of 5 μl (three-component) or 8 μl (four-component) of neat compounds [note that using different total volumes in the treatments, including 2 μl instead of 3 μl of neat 7-methyloctyl (Z)-4-decenoate in the first treatment, is not likely to significantly change the saturation level inside the PCR tubes, and thus should not affect the release rate of the compound(s)]. Prior to the field tests, the PCR tube was pierced with an insect pin size 3 to allow release of compounds (see Tolasch et al. 2007).

For trapping of female *E. ferrugineus*, as well as both sexes of *O. eremita*, we used the male-produced pheromone of *O. eremita*: (R)-(+)-γ-decalactone. The compound was purchased from Sigma-Aldrich, Sweden (97% chemical purity). Similar to earlier studies (e.g. Larsson et al. 2003; Svensson et al. 2004), 2 ml glass vials were used as dispensers, and loaded with 600 μl of neat compound to ensure a high stable release rate of the compound during the whole flight season. Cut strings of cotton dental rolls (Celluron, Paul Hartmann, S.A., France) were inserted as wicks into the glass vials.

Trapping experiments

Field trials were conducted south of Linköping, Östergöt-land province, in Bjärka-Säby (58°16′N, 15°46′E) and Brokind (58°12′N, 15°40′E), southeast Sweden, in 2009. These areas house some of largest populations of *E. ferru-gineus* and *O. eremita* reported in Sweden (Nilsson and Baranowski 1994; Larsson and Svensson 2009). Non-destructive custom-built traps (Svensson and Larsson 2008) were suspended from oak branches at 2–4 m height and at least 10 m apart. A dispenser was attached to a trap with a thin metal wire and used throughout the whole trapping experiment.

For *E. ferrugineus* males, trapping experiments with different dispenser types were performed in both study areas with two replicates each, separated by at least 100 m: tests with rubber septa from 23rd to 30th of July, and tests with PCR tubes from 31st of July to 11th of August. For *E. ferrugineus* females and *O. eremita* (both sexes), trapping experiments with (R)-(+)-γ-decalactone were performed over the whole flight season (from 29th of June to 17th of August; twelve traps in Bjärka-Säby and 16 traps in Brokind). Traps were checked approximately every second day, and after each check, the relative positions of traps within replicates were changed to avoid position effects. All captured beetles were sexed and given an individual

mark on the elytra with ink marks, which allowed for their identification in case of subsequent recapture. Captured beetles were released on the trunk of the trap tree. In the subtractive experiments with *E. ferrugineus*, trap catches per check were pooled for each treatment, and differences in catches between treatments were analysed using one-way Anova on log $(x + 1)$ transformed data, followed by multiple comparisons according to the Bonferroni post hoc test with adjusted levels for significance. Statistical analyses were performed using SPSS version 16.0.

Results

Electrophysiology

In total, twelve antennae from six male *E. ferrugineus* were used for electrophysiological screening of esters. Response to 7-methyloctyl (*Z*)-4-decenoate was observed in eight antennae. In contrast, 7-methyloctyl octanoate and 7-methyloctyl 7-methyloctanoate only elicited response in one antenna, and the response amplitudes to these esters were much smaller than that for 7-methyloctyl (*Z*)-4-decenoate (Fig. 1). No response to 7-methyloctyl 5-methylhexanoate was observed in any run.

Field trapping

In the field trials using rubber septa as dispensers, there were large overall differences in trap catch among treatments ($F = 33.6$; $df = 2$; $P < 0.001$; Fig. 2a). Traps baited with 7-methyloctyl (*Z*)-4-decenoate and the four-component blend attracted equal number of males, whereas traps baited with the three-component blend attracted significantly fewer males compared to the other two treatments. The same difference was observed when PCR tubes were used as

dispensers ($F = 26.0$; $df = 2$; $P < 0.001$; Fig. 2b). No apparent difference in mean trap catch was observed between experiments with different dispenser types. The recapture rate was 54 and 63% for the experiments with rubber septa and PCR tubes, respectively. Few beetles returned to the same trap from which they were released (traps with rubber septa: 13%; traps with PCR tubes: 18%). In addition, a large fraction of the recaptures in a new trap occurred in the other trap replicate within an oak stand (traps with rubber septa: 46%; traps with PCR tubes: 28%).

When comparing the capture and recapture patterns to different lures, clear species and sex specific differences emerged (Fig. 3). The number of captures per individual was higher for male *E. ferrugineus* to the conspecific sex pheromone than for any other combination of lure and species, in spite of the comparatively lower trap effort. Captures to the *O. eremita* pheromone were dominated by female *O. eremita*, with a much lower capture efficiency for either male *O. eremita* and female *E. ferrugineus* (Fig. 3). Similar to male *E. ferrugineus*, most of the recaptures of female *O. eremita* occurred in a new trap, with only two out of 16 individuals recaptured in the same trap from which they were released.

Discussion

Electrophysiological data (Fig. 1) and behavioural data (Fig. 2) strongly suggest that the female-produced sex pheromone of *E. ferrugineus* consists of the single compound 7-methyloctyl (*Z*)-4-decenoate. During GC-EAD screening, this compound elicited strong responses in antennae of males, whereas the other esters identified by Tolasch et al. (2007) elicited negligible responses. In addition, attraction of males to traps baited with 7-methyloctyl (*Z*)-4-decenoate alone was as high as to traps baited with

Fig. 1 Simultaneous response of flame ionisation detector (FID) and antenna (EAD) of a male *Elater ferrugineus* to a blend of 7-methyloctyl esters identified from gland extracts of conspecific females by Tolasch et al. (2007). Two different doses of compounds (2.5 and 25 ng) were used for screening. Whereas 7-methyloctyl (*Z*)-4-decenoate elicited clear antennal response in the majority of trials, negligible antennal responses were observed to the remaining three esters
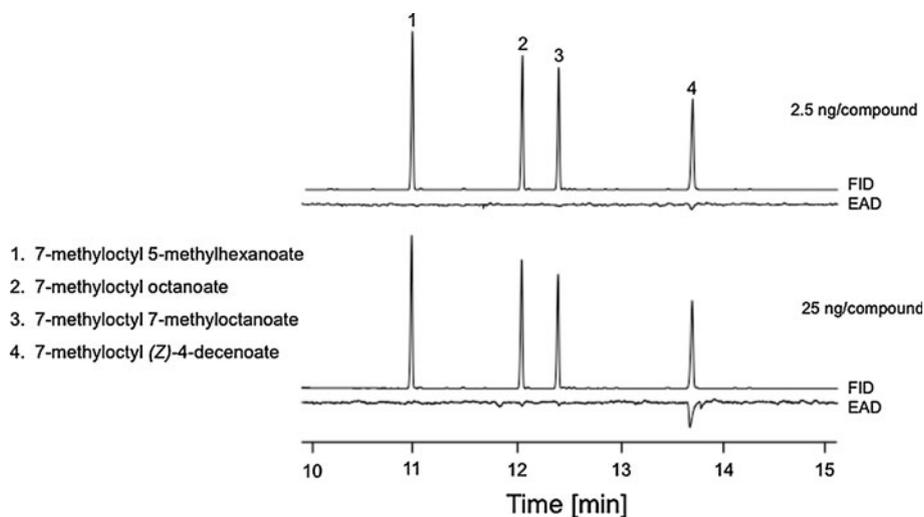
1. 7-methyloctyl 5-methylhexanoate
2. 7-methyloctyl octanoate
3. 7-methyloctyl 7-methyloctanoate
4. 7-methyloctyl (*Z*)-4-decenoate

Fig. 2 Attraction of male *Elater ferrugineus* to traps baited with different blends of 7-methyloctyl esters identified from gland extracts of conspecific females by Tolasch et al. (2007). Rubber septa or 0.2 ml PCR tubes were used as dispensers. *Bars* with *different letters* indicate significantly different catches (one-way Anova on pooled log $(x + 1)$ transformed data, followed by multiple comparisons according to the Bonferroni post hoc test)

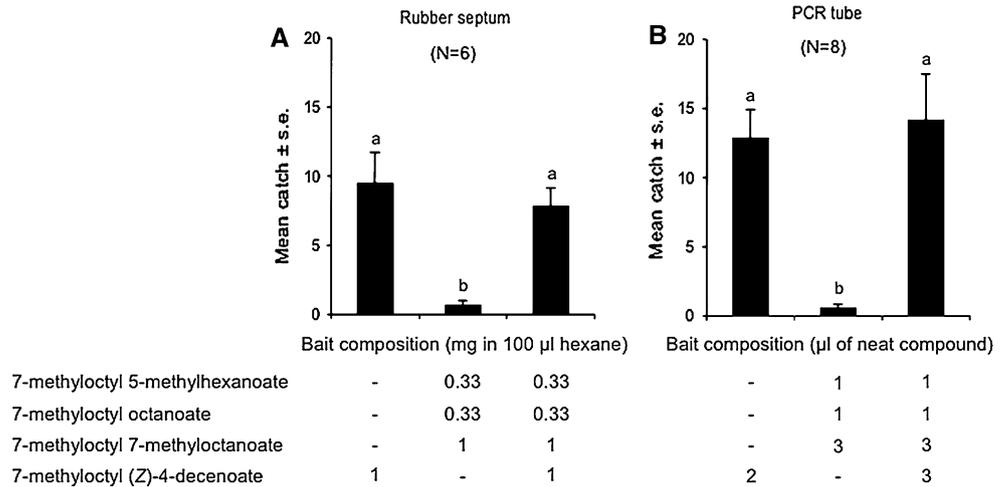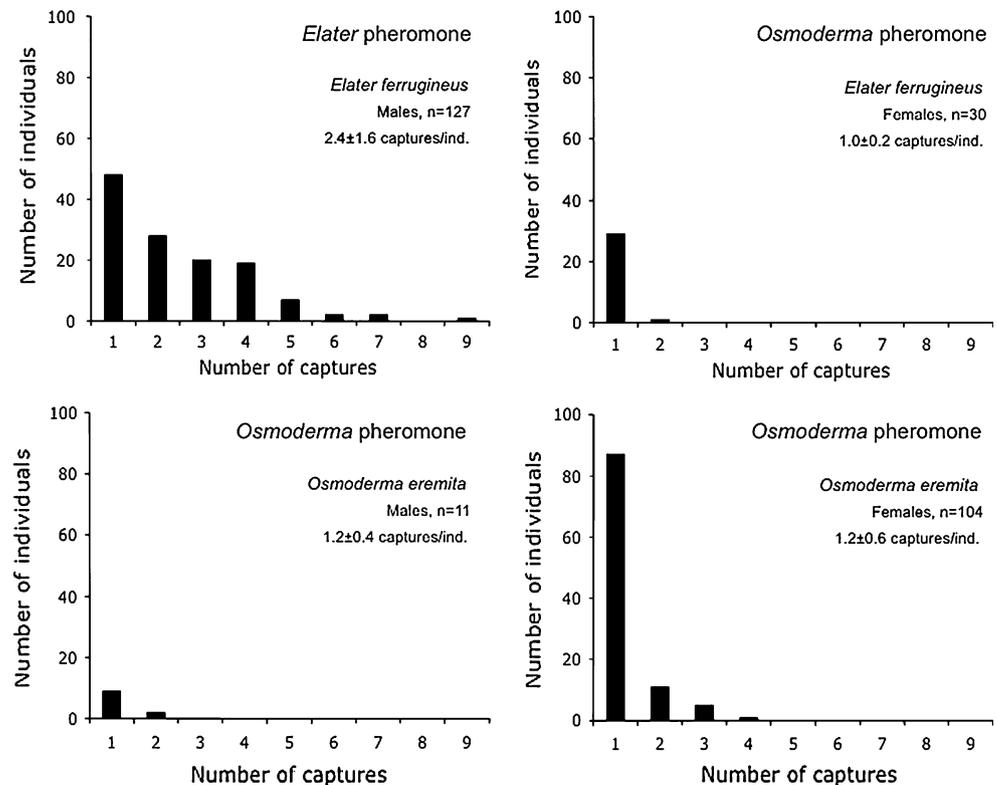|	Rubber septum (N=6)			PCR tube (N=8)		
Bait composition	(mg in 100 μl hexane)			(μl of neat compound)		
7-methyloctyl 5-methylhexanoate	-	0.33	0.33	-	1	1
7-methyloctyl octanoate	-	0.33	0.33	-	1	1
7-methyloctyl 7-methyloctanoate	-	1	1	-	3	3
7-methyloctyl (Z)-4-decenoate	1	-	1	2	-	3

Fig. 3 Number of captures of *Elater ferrugineus* and *Osmoderma eremita*, using two different odour-based trapping systems

the blend of all four esters, and significantly higher than those baited with a blend of the remaining three esters. Although a direct comparison of trapping efficiency between experiments with different dispenser types cannot be made, as they were performed at different parts of the flight season of *E. ferrugineus*, no apparent difference in trap catch of beetles was observed between these two dispensers (Fig. 2). Regardless of dispenser type used, 7-methyloctyl (Z)-4-decenoate appears to be an extremely efficient attractant for male *E. ferrugineus*. Considering the fact that until 1993 only 147 documented specimens had been recorded in Sweden (Nilsson and Baranowski 1994),

our trapping data show that the species is obviously more locally abundant in Sweden than previously recognised.

When comparing lures for monitoring of *E. ferrugineus*, the sex pheromone seems to attract male beetles far better than the kairomone attracts female beetles (Fig. 3). Individual males were on average captured more than twice as many times compared to individual females, and the difference in trap catch between sexes of *E. ferrugineus* would have been even more pronounced if the pheromone-based system for attracting males had been applied with the same number of traps and over the same time period as the kairomone-based system for attracting females. The

observed asymmetry between sexes in both the number of individuals trapped and the recapture rate should reflect a true difference in attraction to the two odour lures, assuming an even sex ratio for the target species. More than 60% of the captured males were later recaptured (Fig. 3), which is a much higher recapture rate than normally observed in mark-release-recapture studies using pheromone traps (Robbins et al. 2008; Kishita et al. 2003; Östrand et al. 2000; but see Zhang and Schlyter 1996).

Comparisons of capture-recapture patterns could provide an indication of the relative capture efficiency of different pheromone systems, also where the population densities of the target species are not known. The overall higher capture efficiency of the *E. ferrugineus* pheromone system than the *O. eremita* pheromone system is relevant from an applied perspective, as it allows for higher probability of detection with less effort. This difference could be due to innate differences in responsiveness of the two species to their respective pheromone (male-produced vs. female-produced pheromones). It could also be due to higher mobility of *E. ferrugineus* males, leading to more encounters with traps, as dispersal ranges of *O. eremita* appear to be very limited in both space and time (Hedin et al. 2008; Svensson et al. 2011). The fact that few male *E. ferrugineus* and female *O. eremita* were recaptured in the same trap from which they were released suggests that the design of the trapping experiments did not bias the overall recapture rate.

Although a highly attractive odour lure is appreciated in most monitoring programs for pest species, it may in fact cause problems when studying rare and threatened insects. In this study, individual males were trapped up to nine times during an experimental period of 3 weeks, i.e., they were prevented from mating during a significant part of their adult life. If a large fraction of males is effectively excluded from the mating pool it could have negative effects on the local population dynamics, especially in small populations, which has to be taken into consideration when designing monitoring programs for rare insects, i.e. by checking traps more frequently, using fewer traps per location, or running trapping experiments during only a small fraction of the total flight season.

Although our data show that the own pheromone is superior to the kairomone for monitoring of *E. ferrugineus*, the *O. eremita* pheromone should not be disregarded as monitoring lure for this species. In contrast to 7-methyloctyl (Z)-4-decenoate, which is not commercially available (R)-(+)-γ-decalactone can be purchased for a relatively low cost, and using traps baited with this compound is still a much better alternative for monitoring *E. ferrugineus* compared to traditional survey methods. Because such traps will also attract both sexes of *O. eremita*, programs can be developed for standardised monitoring of both beetle species, which will be less labour intensive and time consuming

compared to other methods (Ranius 2002; Svensson et al. 2003; Bußler and Müller 2009). The cost can be reduced further by using a racemic mixture of enantiomers (also commercially available) instead of pure (R)-enantiomer of γ-decalactone, because these beetles do not respond antagonistically to the (S)-enantiomer (Svensson and Larsson 2008), a phenomenon commonly observed in other beetles using chiral compounds in their chemical communication (Leal 1996; Tolasch et al. 2003; Lacey et al. 2004), and the racemic mixture is several times cheaper to purchase compared to pure (R)-enantiomer, thus lowering the total cost per lure even if twice as much substance is needed per trap. Trapping using γ-decalactone as lure seems to give a rather high capture probability for *O. eremita* when trap density is high enough (Larsson and Svensson 2009), and such traps probably constitute a rather good mode of detection even for *E. ferrugineus* females.

The initial pheromone work on *E. ferrugineus* by Tolasch et al. (2007) was performed on beetles reared from field-collected larvae in Germany. No gland extracts from local Swedish females were analysed in the present study, so the number of esters produced by Swedish beetles has not been determined. Although our data strongly suggest that only 7-methyloctyl (Z)-4-decenoate is used as sex pheromone by Swedish populations, we cannot at this point rule out the possibility that geographic variation exists in the mate signal for *E. ferrugineus*, with populations from different regions in Europe using different number of esters as sex pheromone. However, such alternative hypothesis is not supported by available data: recent trapping experiments have shown that 7-methyloctyl (Z)-4-decenoate is a very efficient attractant for *E. ferrugineus* in several countries in Europe (Bulgaria, England, Poland, Turkey; GP Svensson, MC Larsson, N Jansson, B Gueorguiev, D Harvey, A Oleksa, unpublished data), indicating that this click beetle uses a single compound as sex pheromone throughout its range of distribution. We have also noted that all three colour morphs (Hansen 1966) of the species have been attracted to traps baited with 7-methyloctyl (Z)-4-decenoate alone, showing that colour polymorphism is not correlated with differences in ester composition within this species (GP Svensson, MC Larsson, N Jansson, unpublished data).

Applied insect chemical ecology research has so far been directed towards semiochemical-based monitoring and population control of pest species, with specific focus on agriculture and forestry species (Cook et al. 2007; Witzgall et al. 2010). In contrast, there is an almost complete lack of identified attractants that could be used to monitor the distribution and abundance of rare and threatened insects. Our work on the chemical ecology of *E. ferrugineus* and *O. eremita*, together with the recent identification of the sex pheromone of the protected Spanish moon moth, *Graellsia isabellae* (Millar et al. 2010), represent the first attempts to

integrate classic insect pheromone research into the area of conservation biology. Using odour traps seems to be the only realistic method to monitor *E. ferrugineus* (Larsson and Svensson 2009), and with a very efficient pheromone lure available, the true geographic range of this species can now be delineated. In addition, mark-recapture experiments using pheromone traps will give valuable information about local population sizes and dispersal ranges of *E. ferrugineus*, which are important parameters for assessing the conservation status of this rare click beetle.

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