Neryl decanoate: female sex pheromone of the click beetle 
Dalopius marginatus

Till Tolasch | Johannes L.M. Steidle

Institut für Biologie, Universität Hohenheim, 
Chemische Ökologie 190t, Garbenstraße 30, 
Stuttgart, 70593, Germany

Correspondence 
E-mail: tolasch@uni-hohenheim.de

Abstract

Dalopius marginatus (L.) (Coleoptera: Elateridae) is a very common click beetle species developing in forest soils in an area from southwest Europe to eastern Siberia. Here, we studied the composition of the female-released sex pheromone of D. marginatus using coupled gas chromatography-mass spectrometry (GC-MS). Neryl octanoate, neryl decanoate, and neryl dodecanoate, in a ratio of approximately 1:100:3, were the only volatile compounds present in the extracts of the pheromone glands. The main compound neryl decanoate proved to be highly attractive to swarming D. marginatus males in the field, whereas adding the two trace compounds in the natural ratio did not influence its attractiveness. Although closely related to the genus Agriotes, D. marginatus is distinct in the use of neryl compounds with comparatively long acid moieties, as opposed to the use of geranyl- and/or (E,E)-farnesyl esters of shorter-chain fatty acids as sex pheromones in Agriotes species.

KEYWORDS

Dalopius marginatus, Coleoptera, Elateridae, Elaterinae, Agriotini, click beetle, sex pheromone, monitoring, neryl decanoate, GC-MS, volatile compounds, male attraction

INTRODUCTION

Dalopius marginatus (L.) (Coleoptera: Elateridae) is one of the most common forest click beetle species in Central Europe. Its huge distribution ranges from Portugal to East Siberia, including the whole of Europe from the Mediterranean area to the boreal forests of Scandinavia (Horion, 1953). Throughout Europe, D. marginatus represents the only member of its genus; other Dalopius species are known from the eastern Palearctic (Japan) and North America (Laibner, 2000; Cate, 2007). With its light brown colour, the dark longitudinal stripe on the elytrae, and a body length of 6–7.5 mm, D. marginatus is easily recognizable. Agriotes acuminatus (Stephens), which resembles D. marginatus, can be distinguished in the field with some practice by the shape of the lateral edge of the more finely punctuated and shinier pronotum (cf. Lohse, 1979).

Larvae of D. marginatus develop in the dry soil of both deciduous and coniferous forests, where they are often among the most abundant members of the soil macrofauna. Unlike most other insect species, they even inhabit the soil of intensively managed spruce plantations, where they can reach densities of several hundred larvae m⁻² (Schaerffenberg, 1942; Horion, 1953). Maybe due to this high abundance and their typical wireworm appearance, they have often been said to cause damage on roots of forest plants (e.g., Korschefsky, 1941); however, already 80 years ago Schaerffenberg (1941) pointed out that the opposite is likely – larvae of D. marginatus are beneficial by preying on sawfly cocoons and pupae of geometrid moths such as Bupalus piniarius (L.). More recently, Kozlov et al. (2020) showed experimentally that larvae of D. marginatus actually do not feed on live roots of forest plants at all, not even in the absence of other food sources. Therefore, they should not be regarded as pest insects.
Sex pheromones of click beetles are generally female-produced and attract conspecific males, often over long distances and in large numbers. Pheromones of <40 species have been investigated so far (<0.5% of the known click beetle species), most of them members of the agriculturally important genus *Agriotes* (for an overview, see Tóth, 2013). The structural features of the known click beetle pheromones largely fit the higher taxonomy (tribes, subfamilies; e.g., Tolasch et al., 2007). *Agriotes* species, the closest relatives of *Dalopius* (Elaterinae, Agriotini), mainly use geranyl- and/or (E,E)-farnesyl esters of equal chain acids (2–8 carbon atoms) as sex pheromones (Tóth, 2013). Here, the identification and synthesis of the sex pheromone of *D. marginatus* is reported. The aim of this study was to enhance the knowledge of the pheromones of central European click beetles and to check whether there is a general structural difference between sex pheromones of the genera *Dalopius* vs. *Agriotes*.

**MATERIALS AND METHODS**

**Insects and extracts**

Adults of *D. marginatus* were collected in May 2005 from beech, oak, and spruce twigs in a mixed forest near Göppingen (Baden-Württemberg, southwest Germany; 48.751°N, 9.586°E, WGS 84) using a beating tray. Beetles were sexed in the laboratory; 27 females were deep frozen (−22 °C) for 30 min and their abdomens were broken off by bending them over to the ventral side. Tergites were removed and the paired pheromone glands were excised; these glands were found to be similar to those in other members of the subfamily Elaterinae (Merivee & Erm, 1993; Tolasch et al., 2007, 2010, 2013; König et al., 2016). Two extracts, each of 10 females, were made by transferring their glands on the tip of an insect pin to 300 µl CH₂Cl₂ in a 2 ml clear glass vial (Supelco/Merck, Darmstadt, Germany), the remaining females were treated separately by extracting their glands in 100 µl CH₂Cl₂ each.

**Chemical analyses**

Extracts were analyzed by coupled gas chromatography–mass spectrometry (GC-MS) employing a 6890 GC gas chromatograph linked to a 5973N MSD (both Agilent Technologies, Santa Clara, CA, USA) in electron impact mode at 70 eV. Using helium as carrier gas (1.2 ml per min), separations were done by using an HP-5ms column (30 m x 0.25 mm inner diameter, 0.25 mm film thickness; Agilent Technologies) that was operated at 60 °C for 3 min, increased to 300 °C at a rate of 3 °C per min, and finally held at this temperature for 10 min. Retention indices (RI) for compounds were determined by comparing retention times with those of a homologous series of *n*-alkanes (C₈–C₃₀) analyzed under the same conditions. Compounds were identified by comparison of mass spectra and RI with those of authentic samples. Nuclear magnetic resonance (NMR) spectra of synthetic compounds were recorded with an INOVA 500 instrument (Varian, Palo Alto, CA, USA).

**Synthesis**

Nerol (97%), octanoyl chloride (99%), decanoyl chloride (98%), and dodecanoyl chloride (98%) were purchased from Sigma-Aldrich (Schnelldorf, Germany), solvents (≥99.5%) from Carl Roth (Karlsruhe, Germany) and Merck (Darmstadt, Germany). Conversion of nerol to the corresponding esters was achieved by employing the appropriate acyl chlorides according to known methods (Tóth et al., 2003). Purification of synthetic products was carried out by flash chromatography on silica gel (silica 32–63, 60 Å; ICN-Biomedicals, Eschwege, Germany) at 1.3 bar using mixtures of ethyl acetate and hexane, resulting in purities of ≥99%.

**Bait dispensers and traps**

Two types of lure were used: (A) 10 mg neryl decanoate (2), and (B) 0.1 mg neryl octanoate (1), 10 mg neryl decanoate (2), and 0.3 mg neryl dodecanoate (3), resembling the natural ratio of ca. 1:100:3 found in the glands. Dispensers were prepared from 0.2 ml PCR tubes (ThermoTube; Peqlab, Erlangen, Germany) as described earlier (Tolasch et al., 2007). Synthetic test substances were applied as *n*-hexane solutions into the tubes, which were pierced twice directly before use, with an insect pin (0.5 mm diameter) at the front side, 2 mm below the lid. Under laboratory conditions, such odor dispensers baited with similar compounds had a stable mean release rate of approximately 8–10 µg per day, regardless of the amount in the tubes (determined by weighing; Tolasch, 2008).

Funnel traps were the same custom-made items as described and illustrated for earlier experiments (Tolasch et al., 2007). Two transparent Plexiglas sheets (17 cm high, 20 cm wide) were arranged crosswise over a 185-mm powder funnel with its outlet (4 cm diameter) connected to a 1-l polyethylene collection bottle. Dispensers were mounted on a thread in a gap at the top between the plastic sheets. Each collecting bottle was filled with brine (250 ml) to preserve the beetles and minimize the possible attraction of further individuals to those already captured.

**Field experiments**

Two separate field tests were performed in 2007 and 2008 in the same mixed forest near Göppingen, where the samples had been taken. Predominant tree species were spruce [*Picea abies* (L.) Karst] and beech [*Fagus sylvatica* L.], with single oaks [*Quercus robur* L.] and birches [*Betula pendula* Roth] in between. In the first test, carried out at
the beginning of the flight period from 21 May to 11 June 2007, neryl decanoate (lure A) was tested against neryl decanoate + trace compounds (lure B) and empty controls to check the general attractiveness and possible synergistic effects. Thirty traps were grouped into 10 sets, each containing a trap baited with lure A, lure B, and an unbaited control. Traps were suspended ca. 1.5–2.0 m above the ground from branches at clearings and forest roads. The distance between traps within one set was about 5 m, the distance between sets was at least 50 m. Traps were checked weekly, captured beetles were removed, and the brine was replaced. The 2007 test was abandoned after 4 weeks due to unsuitable weather conditions.

A second field assay was carried out during the main flight period of *D. marginatus* in 2008 from 6 June to 31 July, under stable weather conditions. Twenty traps were grouped into 10 sets, each containing a trap baited with lure A and an empty control. Traps were placed in the same locations as in the previous year and emptied every 2 weeks.

**Statistical analysis**

The numbers of male beetles caught were analyzed using R v.3.3.2 software (R Core Team, 2016). Because data were not normally distributed and due to lack of homogeneity of variances, we calculated generalized linear mixed models, family ‘negative binomial’, with trap set number and week as random variables (Bates et al., 2015). Means were separated by post-hoc Tukey’s tests (Hothorn et al., 2008).

**RESULTS**

**Chemical analyses**

The composition of all gland extracts was the same and almost no quantitative variation was found between samples of the various females (Figure 1). The main compound, eluting at *R* = 50.4 min (RI 2124), was identified as neryl decanoate (2) by comparison with a synthetic sample. The trace compounds turned out to be the homologous esters neryl octanoate (1; *R* = 44.2 min, RI 1926, ca. 1.0-1.6% of the main compound) and neryl dodecanoate (3; *R* = 56.1 min, RI 2332, ca. 2.9-3.7%).

No other compounds were present in the extracts, not even the corresponding geranyl esters, known from numerous other click beetle species (Tóth, 2013). Neryl decanoate and neryl octanoate have been identified previously from other click beetle species (Tolash et al., 2013; Tóth, 2013; König et al., 2016).

**Field trapping experiments**

In the first set of field experiments in 2007, in total 491 *D. marginatus* males were caught in the traps with only 12 males in the empty control traps. Almost equal numbers were caught with lures A (244 males) and B (247). Beetle numbers differed significantly between controls vs. lure A (estimate ± SE = 3.09 ± 0.44; *z* = 7.05) or lure B (2.98 ± 0.44; *z* = 6.80, both *P<0.001*), but not between lures A vs. B (−0.11 ± 0.33; *z* = −0.33, *P* = 0.94). Thus, there is no additive or synergistic effect of the trace compounds neryl octanoate (1) and dodecanoate (3) towards the main compound neryl decanoate (2).

In 2008, in total 10 358 males were caught in the traps baited with lure A and 47 in the empty controls, a highly significant difference (5.38 ± 0.19; *z* = 27.86; *P<0.001*; Figure 2).

**DISCUSSION**

Our chemical analysis revealed the presence of neryl octanoate (1), neryl decanoate (2), and neryl dodecanoate (3) in a ratio of approximately 1:100:3 as the sole compounds in the pheromone glands of female *D. marginatus*. The main compound neryl decanoate (2) was highly attractive to conspecific males in the field, but catches could not be enhanced by adding the trace compounds, in contrast to other species such as *A. acuminatus* (Tolash et al., 2010). Still, even if minor compounds are not required for attraction, they may play an important role in the reproductive isolation between species as repellents, as has been shown for the click beetles *Agriotes spumator* (L.) and *Agriotes brevis* Candèze (Tóth et al., 2002).

Neryl decanoate is not known as sex pheromone of any other insect species so far, but it has previously been found to be produced by two other click beetle species. In *Agriotes bogatschevi* Dolin, native to southwest Russia, it was identified as one of six gland compounds, but its possible role as part of the pheromone was not further investigated (Yatsynin et al., 1996). In the rare central European click beetle *Betarmón bisbimaculatus* (Fabricius), neryl decanoate has been found as a component of the pheromone, which itself is not attractive, but synergistically enhances the attractive effect of neryl hexanoate (König et al., 2016).
Interestingly, when field-testing pheromone dispensers for *B. bisbimaculatus* containing a mixture of neryl hexanoate, octanoate, and decanoate in a ratio of 3:1:6, *D. marginatus* was frequently caught in the traps, but exclusively in (mainly northern) areas where *B. bisbimaculatus* does not occur (C König & T Tolasch, unpubl.). The sex pheromones of the closely related and very similar *Agriotes* species are mainly made up of one or two geranyl- and/or (E,E)-farnesyl esters (2E-configuration) of equal-chained fatty acids with 2–8 carbon atoms (Tóth, 2013). The neryl unit (2Z-configuration) of the compounds found in *D. marginatus*, as well as their comparatively long acid moieties, support its placement in a different genus.

**ACKNOWLEDGMENTS**

We thank Dr. Michael Stöffler for the generous permission to use his hunting ground for field experiments. We thank Dr. Maximilian von Fragstein for support in field work.

**AUTHOR CONTRIBUTIONS**

Till Tolasch: Conceptualization (lead); Investigation (lead); Methodology (lead); Visualization (lead); Writing – original draft (lead); Writing – review & editing (lead). Johannes L.M. Steidle: Conceptualization (supporting); Formal analysis (lead); Visualization (supporting); Writing – review & editing (supporting).

**ORCID**

Till Tolasch @ https://orcid.org/0000-0002-8236-8110

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How to cite this article: Tolasch T & Steidle JLM (2022) Neryl decanoate: female sex pheromone of the click beetle Dalopius marginatus Entomologia Experimentalis et Applicata 170: 339-343.