Home is where the heart rot is: violet click beetle, *Limoniscus violaceus* (Müller, 1821), habitat attributes and volatiles

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**Abstract.** 1. The decreasing number of veteran trees in Europe threatens old-growth habitats and the fauna they support. This includes rare taxa, such as the violet click beetle, *Limoniscus violaceus* (Müller, 1821).

2. Samples of wood mould were taken from all beech trees in Windsor Forest previously confirmed to have contained *L. violaceus* larvae, and from trees where *L. violaceus* had not previously been detected, the latter categorised as having high, medium or low likelihood of containing the beetle during recent surveys. Habitat characteristics were measured, and volatile profiles determined using gas-chromatography mass-spectrometry.

3. Water content significantly differed between tree hollows of different violet click beetle status, high-potential habitats having higher and relatively stable water content compared with habitats with medium or low potential of beetle occupancy. Several volatile organic compounds (VOCs) were significantly associated with *L. violaceus* habitats. No differences in other characteristics were detected.

4. The distinction in water regime between habitats highlights that recording this quantitatively could improve habitat surveys. Several potential *L. violaceus* attractant VOCs were identified. These could potentially be integrated into existing monitoring strategies, such as through volatile-baited emergence traps or volatile-based surveying of habitats, for more efficient population monitoring of the beetle.

**Key words.** Coleoptera, Elateridae, hollow, microhabitat, rot hole, saproxylic, volatile organic compounds.

**Introduction**

Deforestation of British woodland in the 20th Century, particularly during the war periods (1914–1918 and 1939–1945), has resulted in a generation gap between young and veteran trees (Rotherham, 2013). Veteran trees are old trees exhibiting features such as large trunk diameters, fissured bark and large hollows (Lindenmayer & Laurance, 2017). Increasing threats from habitat fragmentation and loss jeopardise the future of these trees (Harper et al., 2005; Lindenmayer et al., 2014; Ruete et al., 2016) and the organisms that depend on the microhabitats they provide, such as hollows (Gough et al., 2014).

Heart rot (fungal decay of the central heartwood) results in long-lasting hollows, or rot holes, on which many saproxylic invertebrate species are dependent (Hennon, 1995; Stokland et al., 2012; Taylor & Ranius, 2014; Siitonen & Ranius, 2015). Tree hollows can remain for many years, maintaining habitat continuity, even after the tree’s death (Rose et al., 2001; Stahlheber et al., 2015). Many invertebrate taxa require specific biotic and abiotic characteristics for their development, thus heterogeneity of these conditions is a major driver of invertebrate diversity in dead wood habitats (Quinto et al., 2014; Seibold et al., 2016). Hollow organisms such as fungi also produce habitat cues (specific stimuli which attract invertebrates), including volatile organic compounds (VOCs; Leather et al., 2014; Webster & Cardé, 2017). Given the declining numbers of veteran trees, and the long time period taken by trees to achieve this status and for hollowing to occur, the fauna associated with heart rot habitats is often endangered (Cálix et al., 2018). Understanding
the requirements of the fauna dependent on these habitats, and what makes these environments attractive, is essential for developing effective conservation strategies.

The violet click beetle, *Limoniscus violaceus* (Müller 1821), is an Annex II species within the EU Habitats Directive and has Endangered status at a European level (Cálix et al., 2018). *Limoniscus violaceus* inhabits composting wood mould in the base of hollow tree trunks, mostly in beech (*Fagus sylvatica*) and ash (*Fraxinus excelsior*) in the United Kingdom, at only three sites (Gouix et al., 2012; Gouix et al., 2015). At the time of this study, specific trees were confirmed to contain the beetle larvae in only Windsor Forest. Larvae develop over 2 years before pupation; nocturnal adult forms are thought by some to emerge, if they do, in spring, rarely dispersing from their natal habitat, with oviposition happening in late spring (Whitehead, 2003). The adult beetle is suspected to be active between April and June.

The aim of this study was to determine whether hollows known to contain *L. violaceus*, and those which did not, differed in tree diameter, hole opening size, wood mould density, percentage water content and water potential, and VOCs emitted. Two hypotheses were tested: tree hollows in which *L. violaceus* has been recorded within the previous 2 years (*H1*) exhibit specific physical characteristics, and (*H2*) have distinct volatile profiles.

**Materials and methods**

**Study sites and sample collection**

All samples and data were collected from Windsor Forest (51°26′02.5″N, 0°38′37.2″W). Windsor Forest contains many veteran trees with large hollows that afford habitat for many saproxylic invertebrates (JNCC, 2019; Fowles, 2020). *Limoniscus violaceus* was first recorded in Windsor Forest in 1937 and this location was thought to be the home of the largest UK population of the beetle at the time of this study. Windsor Forest is designated a Special Area of Conservation (SAC) under Annex II of the EC Habitats Directive.

The tree hollow microhabitats at Windsor Forest have all been assessed and each rated according to *L. violaceus* occupancy potential (i.e. habitat status): confirmed, and low, medium or high potential of beetle presence. Habitat statuses were determined in 2014–2015 by entomological experts from Natural England and Buglife contracted to survey the site. Veteran beech trees were categorised as high potential if they had basal hollows, blown tops and evidence of heart rot comprising black wood mould within the hollow. Medium potential trees lacked obvious basal hollows but had their top blown off, trunk damage or early-stage basal hollows. Low potential trees were intact beech with no obvious or accessible hollows, or substrate too dry and exposed to be *L. violaceus* habitat. Breeding populations of *L. violaceus* were confirmed in five trees by presence of larvae, the surveys taking place 1–2 years before this study, thus very likely that beetles remain given their 1–2 year larval development.

The number of trees sampled in the present study was based around the number of confirmed *L. violaceus* habitats, all of which were sampled alongside a similar number from each other survey category. Non-confirmed trees were selected based upon proximity to confirmed trees, the volume and accessibility of wood mould, and to satisfy approximate equivalence to the number of confirmed trees. Wood mould was collected from each sampled Windsor Forest tree in April, May and June 2016, the period during which adult *L. violaceus* is active (pers. comm. Sarah Henshall, Buglife). Samples were taken from 16 trees at Windsor Forest, comprising five trees with confirmed activity of *L. violaceus* (all of the confirmed *L. violaceus* habitat trees present at the time of collection), and three, four and four trees considered to have, respectively, high, medium and low likelihood of violet click beetle presence. All sampling was carried out with a Natural England habitat disturbance licence. Samples were taken from standing trees and a single fallen tree. Substrate samples of approximately 100 cm³ in volume were removed by gloved hand from basal hollows. One substrate sample (~15 cm³) was taken from each tree hollow in each month by taking wood mould from a few centimetres below the surface in a gloved hand and sealing it in a universal tube for analysis of VOCs. The tree diameter at 1.3 m from the ground, and hollow entrance dimensions halfway up and along each hole were measured. The substrate samples were transported to the laboratory at Cardiff University on the same day and stored at 4°C.

**Wood mould characterisation**

Density (oven dry mass/fresh volume; g cm⁻³), water content (% oven dry mass) and water potential (MPa) were determined for each sample. Each of these is intimately associated with the decay process: density determines the physical resistance and affects moisture relations; water content is relevant both in terms of whether there is enough for physiological processes to occur or too much, which restricts aeration, but suffers from the drawback that its value varies not only depending on the amount of water present but also on the density of the material; and water potential indicates the availability of water, the latter known to be particularly important in facilitating fungal activity. Density and water content were determined by measuring fresh volume and mass, oven-drying for 5 days at 60°C, and reweighing. Water potential of fresh wood mould samples was determined in a Decagon Devices WP4C Dew Point PotentiaMeter (METER Group Inc., Pullman, WA, USA) at 25°C.

**VOC analysis**

The VOCs present in each wood mould sample were determined using thermal desorption gas-chromatography time-of-flight mass-spectrometry (TD-GC-TOF-MS) by tipping the wood mould into plastic food-grade roasting bags and sealing them for 30 min at room temperature (~25°C), allowing the VOCs to diffuse into the bag’s headspace. Using an Easy-VOC manual hand pump (Markes International Ltd., Llantrisant, UK), 500 ml of the air from the headspace was extracted over a SafeLok™ thermal desorption tube (TenaxTA/Sulficarb, Markes International Ltd.). A control sample was prepared by

pumping 500 ml of air from the laboratory atmosphere over a thermal desorption tube for each round of sampling. A retention standard was prepared by directly loading 1 μl of C8-C20 alkane standard solution (Sigma-Aldrich, St. Louis, MO, USA) into a thermal desorption tube.

The thermal desorption tubes were placed in a Markes International TD-100 Thermal Desorber (Markes International Ltd.) which desorbed the tubes at 100°C for 5 min, followed by 280°C for 5 min with a 40 ml min⁻¹ trap flow. Trap desorption and transfer were carried out with a temperature increase of 20°C sec⁻¹, a maximum temperature of 300°C for 3 min, with a split flow of 5 ml min⁻¹. The VOCs were then separated in an Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA, USA) with helium used as a carrier gas at 2 ml min⁻¹ under constant flow conditions for 2 min at 40°C. This process was followed by a temperature increase of 5°C min⁻¹ up to a maximum temperature of 240°C, at which point the temperature remained constant for 5 min. The mass spectra of the separated VOCs were then recorded from m/z 30–350 in a time-of-flight ALMSCO BenchTOF-dx (Markes International Ltd.).

Data from GC–MS were processed using MSD ChemStation (Agilent Technologies Inc. 2005) and AMDIS, with a custom retention-indexed mass spectral library of compounds, a compiled list of compounds identified across all of these samples and others taken from wood mould that have been checked and verified against the NIST 2011 library. All VOCs scoring more than 80% in forward and backward fit in the NIST 2011 library were included in the custom library of mass spectra. Data were normalised as proportions of each volatile profile and were square root transformed to prevent large values from biasing the results.

Statistical analyses

Statistical analyses were carried out in the R statistical package v3.2.2 (R Core Team, 2020). Habitat characteristics were compared using a Kruskal–Wallis test as the data did not conform to analysis of variance (ANOVA) assumptions. Where significant differences were identified, Dunn post hoc tests were carried out with P-values adjusted by Bonferroni correction. The GC–MS data were initially analysed using permutational multivariate analysis of variance (PerMANOVA) using the ‘adonis’ function of the ‘vegan’ package (Oksanen et al., 2016) to compare the VOCs against L. violaceus habitat status, month, tree diameter, tree hollow opening size, wood mould density, water content and water potential, and relevant inter-variable interactions. Data were then analysed using canonical analysis of principal coordinates using the ‘CAPdiscrim’ function of the ‘BiodiversityR’ package (Kindt & Coe, 2005). The VOCs were separated into groups based on hierarchical clustering. Weighted correlation network analysis (WCNA) was carried out to identify whether any groups of VOCs, and the VOCs within these groups, correlated with L. violaceus habitat status and other independent variables. For WCNA, L. violaceus habitat status and tree species were represented as discrete variables (WCNA cannot analyse categorical variables), with low, medium and high potential, and confirmed habitats represented by 0, 1, 2 and 3, respectively. The soft threshold power was set to 7 and a minimum group size of 10 used. All compounds significantly associated with L. violaceus habitat status were separated and analysed in a final VOC PerMANOVA.

Results

Habitat characteristics

Overall, trees of different L. violaceus status did not have significantly different diameters, hollow hole sizes, wood mould water potentials nor wood mould densities, but did have significantly different wood mould water content (Kruskal–Wallis: \( \chi^2 = 10.553, P = 0.014 \); Fig. 1; Table 1). High potential habitats had significantly higher water content than medium (Dunn: \( Z = 2.75, \text{adjusted}-P = 0.036 \)) and low (Dunn: \( Z = 2.75, \text{adjusted}-P = 0.036 \)) potential habitats. Invertebrate communities were also coarsely compared between hollows, but no significant differences found (Supporting Information S1; Tables S1 and S2).

Habitat VOC analysis

A library of 185 compounds was compiled from all samples, with an average of 68 compounds per wood mould sample (Supporting Information Figs. S1 and S2). The volatile profiles

Figure 1. Water content (expressed gravimetrically as percentage of oven dry mass) of habitats of different L. violaceus survey status. Water content was significantly different between trees of different L. violaceus statuses, with high potential habitats having significantly higher water content than medium and low potential habitats. The water content of high potential and confirmed L. violaceus habitat wood mould is less variable than medium and low potential habitats.
Table 1. Habitat characteristics of trees with confirmed, and of high, medium and low potential of L. violaceus presence. Mean values ± standard deviations.

<table>
<thead>
<tr>
<th>Category</th>
<th>Tree diameter, cm</th>
<th>Hollow opening size, cm²</th>
<th>Wood mould density, g cm⁻³</th>
<th>Wood mould water content, % dry mass*</th>
<th>Wood mould water potential (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trees confirmed for L. violaceus</td>
<td>106 ± 23</td>
<td>4416 ± 2938</td>
<td>0.21 ± 0.06</td>
<td>74 ± 10</td>
<td>−0.25 ± 0.28</td>
</tr>
<tr>
<td>High potential trees</td>
<td>102 ± 20</td>
<td>6130 ± 7664</td>
<td>0.15 ± 0.02</td>
<td>84 ± 1</td>
<td>−0.41 ± 0.36</td>
</tr>
<tr>
<td>Medium potential trees</td>
<td>88 ± 15</td>
<td>681 ± 807</td>
<td>0.32 ± 0.27</td>
<td>56 ± 14</td>
<td>−2.97 ± 3.50</td>
</tr>
<tr>
<td>Low potential trees</td>
<td>96 ± 11</td>
<td>4701 ± 8405</td>
<td>0.22 ± 0.07</td>
<td>52 ± 22</td>
<td>−1.37 ± 2.39</td>
</tr>
</tbody>
</table>

Only water content was significantly different between habitat categories (denoted by *). The other invertebrates present in these hollows were coarse identified and compared, but did not differ significantly (Supporting Information S1; Tables S1 and S2).

were significantly different between months (PerMANOVA: \(R^2 = 0.365, df = 2, \text{pseudo-}F = 14.351, P < 0.001\)), but not between habitats of different L. violaceus statuses (Supporting Information Figure S3). Canonical analysis of principal coordinates on month and L. violaceus habitat status, combined into a single variable, resulted in correct classification of 42.9% of volatile profiles to their month and L. violaceus habitat status based on their constituent VOCs (CAP: \(P < 0.001\); Fig. 2a). April volatile profiles were distinct from other months, with large separation based on L. violaceus habitat status. Whilst more tightly clustered, May volatile profiles showed little overlap between L. violaceus habitat statuses. June volatile profiles substantially overlapped between L. violaceus statuses, but the confirmed habitat volatile overlapped little with other statuses.

Canonical analysis of principal coordinates on L. violaceus habitat status and month, separately resulted in correct classification of 46.9% (CAP: \(P = 0.008\); Fig. 2b) and 93.9% (CAP: \(P < 0.001\); Fig. 2c) of volatile profiles to their L. violaceus habitat status and month, respectively, based on their constituent VOCs. Volatile profiles of different L. violaceus habitat status substantially overlapped. Less overlap was observed between months.

Through WCNA, 17 VOCs were identified as significantly associated with L. violaceus habitat status (Table 2; Fig. S3). Of these, 3-methyl nonane was minimally detected in all but the confirmed L. violaceus habitat samples, and dimethyl-dodecane isomer 2 and 5-methyl-undecane were minimally detected in all but the confirmed and high potential L. violaceus habitat samples. A PerMANOVA with this subset of 17 associated VOCs identified significantly different profiles between habitats of different L. violaceus habitat status (PerMANOVA: \(R^2 = 0.194, df = 3, \text{pseudo-}F = 4.470, P < 0.001\)) and between habitats with different water potential (PerMANOVA: \(R^2 = 0.115, df = 1, \text{pseudo-}F = 7.939, P = 0.001\)).

A combination of these might be worth trialling in bioassays for use in attracting the beetle or for detecting additional L. violaceus habitats. Whilst no overall significant difference was found between the whole volatile profiles between habitats of different L. violaceus status, coarse distinctions were evident in the canonical analysis of principal components visualisation (Fig. 2). The substantial separation of volatile profiles based on L. violaceus habitat status in April aligns with the beetle’s suspected emergence. The distinction between volatile profiles from habitats of different L. violaceus status throughout May and June could relate to specific activity of the beetle, such as oviposition, in the confirmed habitat hollows during these months. The difference in volatile profiles between months could, however, relate to differences in the activity of other organisms. Regardless, the use of these VOCs for any downstream applications relating to L. violaceus must consider the temporal nature of these VOCs since compounds relevant in April may not be relevant in June, and vice versa, possibly relating to the temporal dynamics of the beetle’s emergence and oviposition. Of the compounds associated with L. violaceus habitat, only benzene was also associated with a specific month (April), indicating that the other compounds should be distinctly associated with L. violaceus habitat across all three months studied.

Discussion

This study has shown, for the first time, that specific VOCs are associated with the habitat of the violet click beetle. These VOCs were mostly branched alkanes, containing several methyl-dodecane isomers (Table 2; Supporting Information Fig. S3).

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The volatiles identified as associated with *L. violaceus* habitats resemble many VOCs produced by fungi, including branched alkanes and benzene derivatives (Morath et al. 2012). Of these, dimethyl-dodecane isomers (Geethalakshmi and Sareda 2013), 2,7-dimethyl-octane (Schaeffer et al. 1979) and 2,9-dimethyl-decane (O’Leary et al. 2019) are all produced by fungi, including during wood decomposition. Many such fungal VOCs are important insect attractants (Morath et al. 2012; Leather et al. 2014). The precise sources of the individual VOCs identified in this study are, however, unknown and would be extremely difficult, if not impossible, to ascertain, even with exhaustive study of volatile-organism associations since several organisms, individually or interacting, may be responsible for production of the same compounds (Hynes et al., 2007; El Aribi et al., 2016; Hiscox et al., 2018). Investigation of the fungi present in these wood mould substrates, and their volatile profiles, could, however, give a tentative indication of the sources of VOCs important for *L. violaceus*, given the importance of fungal VOCs as insect habitat cues (Leather et al., 2014; Webster & Cardé, 2017).

*Limoniscus violaceus* has been a priority of many conservation management plans and Special Areas of Conservation. The loss of hollow veteran trees, and consequently viable habitat for *L. violaceus*, is particularly catastrophic given the lack of

recruitment of such additional trees (Alexander, 2009; Gouix et al., 2009). The sensitive nature of these habitats, the paucity of beetles remaining, and their unidentifiable frass, render most population monitoring methods too invasive or ineffective (Gouix & Brustel, 2012). Wood mould sampling is effective, but destructive, whilst pitfall traps within hollows are effective but mostly lethal, which is unacceptable for such rare species (Ranius & Jansson, 2002). Emergence trapping is a relative improvement given the more selective approach, but attractants could enhance this efficiency. Live trapping (i.e. without the use of preservatives) remains the most acceptable approach, but great taxonomic experience must first be accrued by those surveying to give confident field identification. Care must also be taken to reduce any within-trap predation of these rare beetles by simultaneously trapped generalist predators such as large spiders and carabid beetles. Such events can be limited by regular monitoring the traps, with visits twice daily advisable where practical. Habitat cue VOCs known to attract L. violaceus could enhance emergence traps or be used to detect habitats occupied by the beetle (Gouix & Brustel, 2012; Hoyer-Tomiczek et al., 2016).

The significantly higher water content in high potential L. violaceus habitats could indicate a requirement for higher water content by L. violaceus or an organism on which it depends, although this distinction was not true for the confirmed L. violaceus habitats. Despite no significant difference being detected between L. violaceus habitats and other hollows, reference to these quantitative values could refine habitat surveys given the relatively stable water content between hollows for confirmed trees. Current qualitative survey approaches for L. violaceus habitats consider the moistness of the wood mould, so inclusion of quantitative guides could refine these efforts.

Habitat status was based on qualitative assessment of substrate characteristics by entomological experts familiar with the beetle’s habitat, termed microhabitat-based searching (Stokland et al., 2012). Whilst such surveys are highly effective, the suitability of habitats may decline over time, and their volatile profiles and microclimate conditions may change accordingly. There were no other confirmed habitats of L. violaceus at the time of this study, making this the most comprehensive study of British L. violaceus habitat volatiles possible at that time. Further habitat trees have been identified in the ash trees of Bredon Hill, which could confirm the results of this study if also investigated. Failure to discover additional L. violaceus habitats in this survey of Windsor Forest highlights the rarity of the beetle. Identification of a suitable attractant VOC for L. violaceus could facilitate refined population monitoring and an improved understanding of the habitat requirements of L. violaceus (Hoyer-Tomiczek et al. 2016); however, confirmation of any attractant properties of the VOCs identified in this study would need to be carried out via bioassay prior to their application. These VOCs may, however, provide some utility in identifying active habitats of L. violaceus via sensitive surveys prior to confirmation with traditional techniques, such as live trapping. Regardless, these VOCs may facilitate the development of less invasive surveys, ultimately guiding development of improved conservation plans to protect the remaining British L. violaceus populations.

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Data availability statement

All necessary data are deposited on and publicly available through Dryad: https://doi.org/10.5061/dryad.f4qrf6tq

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1: Supplementary Information

Figure S1 Example chromatograms of volatile profiles from rot of a confirmed *L. violaceus* habitat and of a habitat with low potential of *L. violaceus* occupancy. For fair comparison the most visually similar profiles from each category were selected from the same month, in this case June.

Figure S2 The example chromatograms presented in Figure S1 of volatile profiles from rot of a confirmed *L. violaceus* habitat and of a habitat with low potential of *L. violaceus* occupancy, but with expanded y axes to emphasise the differences between the profiles given the compression of most peaks due to the presence of much larger peaks. Substantial differences are highlighted with grey boxes of equivalent height along each y axis.

Figure S3 The relative intensity of the VOCs significantly associated with *L. violaceus* habitat status are given as percent-ages of the total of these intensities for each of the four habitat statuses.

References


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