**Title:** Rapid molecular methods for in-field and laboratory identification of the yellow-legged Asian hornet (Vespa velutina nigrithorax)

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**Abstract**

The yellow-legged Asian hornet (*Vespa velutina nigrithorax*) is an invasive species that presents a threat to apiculture in Europe; first introduced into France in 2004 it has subsequently spread into neighbouring European countries. There is a risk of invasion and establishment in the UK and in 2016, nests were found and destroyed in Alderney in the Channel Islands, and in Tetbury, Gloucestershire, illustrating a need for screening of suspect specimens so that invading hornets can be rapidly identified, and their nests destroyed. In this study loop-mediated isothermal amplification (LAMP) and real-time PCR assays were developed to enable both in-field and laboratory testing. Species specific identification assays and generic invertebrate control assays were developed. All the assays were validated according to the European Plant Protection Organisation standard PM 7/98. The assays were tested successfully against *V. velutina* *nigrithorax* obtained from France, Asia and the UK. Eight non-target species, that were closely related or morphologically similar to the Asian hornet, gave negative results with the species-specific assays, and positive results with the control assays. The assays could be used to detect target DNA at concentrations as low as 5 pg per reaction. LAMP was rapid, and cable of generating positive results within 10 minutes. Using simplified sample homogenisation protocols that could be performed in the field, the LAMP assay was successful when tested against all developmental stages and nest samples, assisting with identification of samples that cannot be determined morphologically and allowing detection away from the laboratory. These assays provide a valuable tool for fast and reliable detection of this invasive species, offering the ability to identify damaged/incomplete specimens and immature life-stages.

**Keywords**: Loop-mediated isothermal amplification (LAMP), real-time PCR, diagnostics, invasive species, *Apis mellifera*

**Introduction**

Human beings are responsible for the introduction of thousands of exotic species; for example, within the United Kingdom, among insects alone there are an estimated 340 alien species and approximately 170 have become established (Manchester and Bullock, 2000). The impact of an introduction of non-native species can be devastating for many native species (Manchester and Bullock, 2000). The yellow-legged Asian hornet (*Vespa velutina nigrithorax*) is an invasive species accidentally introduced into Europe, from China, in 2004. It feeds on a wide range of invertebrates but is a particular threat to apiculture due to a preference for feeding on honey bees (Monceau, Bonnard & Thiery, 2014). The hornets will hover outside the entrance to a honey bee colony and catch returning bees, remove the legs, head and abdomen and return to the nest carrying the protein rich thorax to feed developing hornet larvae (Beggs et al., 2011). European honey bees (*Apis mellife*ra) have no effective means of defence against hornets and reports from France suggest that sustained predation can devastate entire colonies (Arca et al., 2014). Surveys describe honey bee colony losses in France as a direct cause of *V. velutina nigrithorax* activity of between 5 and 7.5% (Monceau et al., 2014).

*Vespa velutina nigrithorax* has the potential for rapid long-distance dispersal and is extremely successful at colonising new areas. The French population of *V. velutina nigrithorax* was determined through mitochondrial haplotyping to have originated from a single introduction event (Arca et al., 2014). Despite starting with a small population, spread through France from 2004 until 2008 was rapid, estimated at between 75 and 82 km/year (Robinet, Suppo & Darrouzet, 2016). Subsequently it has gone on to colonise Northern Spain in 2010, Portugal and Belgium in 2011, Italy in 2012 (Lopez, Gonzalez & Goldarazena, 2011, Darrouzet, Gevar, Guigmard & Aron, 2015) and Germany in 2014 (Villemant et al., 2015). After its introduction into Italy, *V. velutina nigrithorax* occupied an area of 250 km2 in 2013, this tripled in two years to 930 km2 (Bertolino, Lioy, Laurino, Manino & Porporato, 2016). An incursion of the hornet into Korea spread at 12.4 km/year (Jung, 2012). In 2016, the first report of the yellow-legged Asian hornet was confirmed in the UK (Defra, 2016, Budge et al., 2017).

Tools for rapid and reliable identification of invasive species are crucial to mounting an effective and rapid response. There is no effective means for area-wide eradication of *V. velutina nigrithorax* so nests must be manually identified and destroyed with pesticides on a nest-by-nest basis.They are a particularly problematic species to detect as the majority of nests are constructed in very high trees, shielded from detection by the leaves. A single mated queen need only raise her nest undetected until autumn to create hundreds of sexual stages for the next generation (Monceau et al., 2014). As a result, speed in detecting and identifying the nests is important to mount an effective eradication campaign.

The life cycle of the nest for *V. velutina nigrithorax* involves a reproductive phase in the autumn, whereby the queen begins laying eggs which become drones and females that develop into virgin queens (Rome et al., 2015). Emerging reproductive individuals will overwinter as mated queens until the spring when they establish a primary nest. Rapid identification of suspect *V. velutina nigrithorax* samples may enable more swift intervention to reduce hornet spread. In the UK, suspected *V. velutina nigrithorax* samples received by the National Bee Unit are often cases of mistaken identity with morphologically similar species such as *Vespa crabro*, *Vespula germanica* and *Urocerus gigas* or primary nests of these species. Rapid identification is necessary as a confirmed discovery of *V. velutina nigrithorax* instigates an immediate contingency response to contain this species. Current methods for identification of *V. velutina nigrithorax* rely on morphological identification (Archer 1989 and Archer 1994), which is limited to particular life-stages and which requires access to highly skilled entomologists and an intact adult specimen. Partial adult specimens, immature life-stages or early nest material would be difficult to confirm rapidly using existing methods.

Molecular methods such as real-time PCR or loop-mediated isothermal amplification (LAMP) represent ideal techniques for fast identification of morphologically indeterminate samples. LAMP requires very little expertise and equipment to operate, and can be performed in the field (Hodgetts et al., 2015) on portable hand held platforms such as the Genie® (OptiGene). Real-time PCR is a laboratory gold standard in many diagnostic fields and is ideal for confirmatory testing. In the laboratory both methods can enable high-throughput screening of material using large volume instrumentation. The aim of this study was to develop specific, sensitive LAMP and real-time PCR assays for the rapid identification of *V. velutina nigrithorax* on any suspect morphologically indeterminate specimens, including nest material. The assays were then validated according to the European Plant Protection Organisation (EPPO) standard PM7/98 (EPPO, 2014) to provide evidence of their suitability for use.

**Methods**

**DNA extraction**

*Vespa velutina nigrithorax* specimens were kindly provided by Mr. Quentin Rome from the Muséum National d’Histoire Naturelle (Paris, France) and DNA samples from Dr. Florence Mougel at the Centre national de la recherche scientifique (Gif sur Yvette, France). Other invertebrate samples were sourced from collections held at the UK National Bee Unit (Sand Hutton, York) and Fera. DNA for assay development and validation was extracted using the Wizard® Magnetic DNA purification system (Promega) or DNeasy® blood and tissue kit (QIAGEN) from a single leg or abdomen. DNA was quantified using a Qubit fluorometer and dsDNA high sensitivity assay kit (Life Technologies, Carlsbad, CA, USA) or a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific) following manufacturers’ protocols.

**In-field crude sample homogenisation**

Samples of different *V. velutina nigrithorax* life-stages and hornet nest material were obtained from the nest site in Tetbury and tested using a crude homogenisation protocol. The tissue was placed in a 5 ml tube with an 11.5 mm diameter stainless-steel ball bearing and PEG buffer (60% PEG 200, 20 mM KOH, pH 13.3-13.5). The sample was homogenised by shaking vigorously by hand for 1 minute and diluted in molecular grade water prior to testing by LAMP (Chomczynski & Rymaszewski 2006). During method optimisation, the crude extract was tested at a range of dilutions; 1:5, 1:10, 1:100, 1:250 and 1:500. The buffer:sample ratio varied depending on the sample size: an egg in 0.2 ml buffer; larvae and pupae sections (approximately 2 cm2), an adult leg or a meconium (faecal) pellet in 1 ml of buffer; a cell silk cap in 0.5 ml of buffer; and interior (cell) or exterior nest paper casing in 1 ml of buffer. To assess the effect of delays/varying storage methods, a single hind leg was taken from *V. velutina nigrithorax* individuals and half of the legs were stored at -80°C, while the other half were kept at room temperature for 1 week prior to testing as described above.

**LAMP assay design and testing**

DNA sequencing of a range of species was undertaken for several loci i) a region of the *mitochondrial cytochrome oxidase subunit I* (mtCOI) gene using the universal invertebrate primers LCO 1490 and HCO 2198 (Folmer, Black M, Hoeh, Lutz & Vrijenhoek, 1994); and ii) the D10 region of the *28S ribosomal RNA* (28S rRNA) gene (Machida and Knowlton, 2012). Sequences from *V. velutina nigrithorax*, *Vespa crabro*, *Vespula germanica*, *Urocerus gigas* and *Apis mellifera* were aligned using MegAlign Ver.4.0.43 (DNASTAR). An invertebrate control assay to amplify multiple species was designed to regions of homology identified manually in the 28S rRNA gene (table S1). A *V. velutina nigrithorax* species-specific assay was designed to polymorphisms of the mtCOI gene which were identified manually (table S1). These are referred to as ‘Invertebrate control’ and ‘Vvn’ respectively. Primer characteristics were assessed using Oligo Calc (Kibbe, 2007). Primers were synthesised by Eurofins-MWG-Operon. FIP and BIP primers were HPLC purified and all other primers were HPSF purified.

LAMP reactions were comprised of 15 µl of isothermal master-mix ISO-001 (OptiGene, Horsham, UK), 2 µM each FIP and BIP primer, 1 µM each FLoop and BLoop primer and 0.2 µM each F3 and B3 primer in a 25 µl reaction. The Vvn assay was further optimised with the addition of 0.125 µl of 20x EvaGreen (Biotium), a DNA-intercalating dye which emits fluorescence upon binding with dsDNA and increases the fluorescent output. For purified DNA, 1µl of DNA was added to each reaction; for crude extracts 5µl of the diluted extract was added. A 65°C amplification step was run for 40 minutes followed by a slow anneal from 98°C to 70°C at 0.5°C per second using the OptiGene Genie®II platform. Each run included no template (water)(negative) and *V. velutina nigrithorax* purified DNA (positive) controls. All samples were tested in duplicate, and all assays were replicated, with at least two independent assay runs by 2 different operators. Results were interpreted in terms of time to positive (Tp) value and annealing temperature (Ta).

**Real-time PCR assay design and testing**

Alignments from the LAMP assay design were used to design real-time PCR primers and probes for the *mtCOI* gene and the *28S rRNA gene* (table S1). Probes were synthesised to contain the 5’ reporter FAM and the 3’ quencher TAMRA (Eurofins-MWG-Operon). Real-time PCR reactions were comprised of 1 µl DNA in 10 µl reactions containing 5 µl iTaq™ Universal probes reaction mix (BioRad), 375 nM of each primer and 125 nM probe. Reactions were carried out on a ViiA™ 7 or 7900HT real-time PCR system (ThermoFisher Scientific) using the following cycling conditions: 95°C for 2 min followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. Each run included no template (water) (negative) and *V. velutina nigrithorax* purified DNA (positive) controls. All samples were tested in duplicate and replicated with two different operators. Results were interpreted in terms of the CT (cycle threshold) value determined using the Sequence Detection Software.

**Assay validation**

Assays were validated according to guidelines within the European Plant Protection Organisation (EPPO) standard PM7/98 (EPPO, 2014). Analytical specificity was tested with DNA from invertebrate or nest samples most likely to be mistaken directly for *V. velutina nigrithorax (Vespa crabro, Vespula germanica, Vespula vulgaris, Uroceres gigas, Dolichovespula media and Dolichovespula saxonica)*. Analytical sensitivity was tested through a ten-fold serial dilution of DNA in water, starting from neat samples (ranging from 3 – 100 ng DNA per µl) to a dilution of 1:106, with each dilution tested in triplicate. The repeatability of the assay was evaluated by analysing at least 3 replicates of DNA extracts at two concentrations; neat DNA and DNA diluted to the assays limit of detection. For reproducibility testing, three replicates of neat DNA and DNA at the limit of detection were tested by two different operators on different days. For real-time PCR, reproducibility was assessed on two different instruments; 7900HT Real-Time PCR System and ViiA™ 7 Real-Time PCR System (ThermoFisher Scientific).

**Results**

**Specificity of detection of *Vespa velutina nigrithorax***

*LAMP assay*

*V. velutina nigrithorax* DNAsampleswere obtained from the UK (n=4), France (n=16) and Asia (n=10). Of the samples from Asia, two samples were from Vietnam, four samples from Indonesia (Java, Sulawesi, Flores, Sumbawa) and four samples from China (Yunnan and Zhejiang/Jiangsu). Amplification was observed in all samples using the Vvn LAMP assay with amplification achieved in 8 to 18 minutes (table 1) whereas no amplification was observed for non-target species (table 1). The invertebrate control assay, which acts to confirm the presence of amplifiable DNA, successfully amplified DNA from all invertebrate species tested. Tp values ranged from 15 to 37 minutes for *V. velutina nigrithorax* and 18 to 24 minutes for the non-target species. The assay specific annealing temperatures were 80.7 ºC (range 80.4 to 81.4 ºC) for the Vvn assay and 88.2 ºC for the invertebrate control assay, although this assay had a broader range (83.8 to 89.3 ºC) due to the diverse species tested. A Ta value outside the expected range and without a Tp value was observed in negative reactions, which was indicative of primer dimers and not considered a positive result. Both a Tp and Ta within the stipulated range are required for a sample to be called positive. There were no differences between data collected based on operator. We present the data from a single operator in the results table 1, and the second operator in table S2.

*Real-time PCR assay*

Results from the LAMP assays were compared to the real-time PCR assays for species-specific detection of *V. velutina nigrithorax* and the control assay for general invertebrate species. The *V. velutina nigrithorax* real-time PCR assay demonstrated species specificity, amplifying all samples of *V. velutina nigrithorax*, with an average CT value of 22.5 (range 15.8 to 34.0), with no amplification observed in all the non-target invertebrate samples (table 1). The invertebrate control assay successfully amplified all *V. velutina nigrithorax* samples and all non-target invertebrates with an average CT value of 19.9 (range 13.4 to 31.7). Data from the second operator is available in supplementary table 2.

**Assay sensitivity**

Purified *V. velutina nigrithorax* DNA was quantified using a Qubit fluorometer and determined to be approximately 5ng µl-1. This sample was diluted in a ten-fold serial dilution to assess the limits of detection for the assays. The Vvn LAMP assay was able to successfully amplify DNA from samples diluted to 5 pg across three technical replicates (table 2). This assay was repeated twice by two independent operators. When the DNA was diluted further, the assay only gave amplification in 2 out of 3 technical replicates at 500 fg and there was no amplification at higher dilutions. The limit of detection for the control assay was assessed using *V. velutina nigrithorax* and three invertebrate samples/species; *Vespa crabro*, *Apis mellifera* and *Dolichovespula media*. The limit of detection of the control assay was approximately 5 pg with *V. velutina nigrithorax*, 34 pg in *Apis mellifera*, 100 fg in *Vespa crabro* and 14 ng in *Dolichovespula media*.

The limit of detection was tested with the Vvn real-time PCR assay and reliable detection of *V. velutina nigrithorax* DNA was possible at 500 fg. The invertebrate control real-time PCR assay also provided reliable detection of DNA at 500 fg (table 2). When the DNA was diluted further, the Vvn assay gave positive results from 1 out of 3 technical replicates at 50 fg, whereas for the invertebrate assay, 2 out of 3 technical replicates gave positive results. No positive results occurred below 50 fg for either assay. Data from the second operator is available in supplementary table 3.

**Detection of *Vespa velutina nigrithorax* DNA from nests and life stages**

A rapid, crude, field-deployable sample homogenisation method was evaluated. Eggs, larvae, pupae and adult legs produced positive results with the VvnLAMP assay at all dilutions tested, amplifying within 15 minutes at the optimum dilution (1:50) (table 3). Crude extracts from legs stored at -80 °C successfully amplified up to a dilution of 1:250; from legs kept at room temperature, amplification was still detected up to a dilution of 1:500. Meconium and silk capping both amplified reliably at all dilutions tested (table 3). We assessed paper nest casing taken from the exterior of the nest and paper casing that formed the interior of a larval cell. We found amplification at all dilutions from the interior nest sample; however, the samples from the outer nest casing material did not amplify successfully in any reaction despite testing varying sample sizes and dilutions.

**Repeatability and reproducibility**

Repeatability and reproducibility testing of the Vvn and invertebrate control LAMP and real-time PCR assays was evaluated by analysing replicates of DNA at neat concentration and at the assays limit of detection. The assays were all found to be 100% repeatable and 100% reproducible when tested by 2 operators on different days and on different real-time PCR platforms (table 2 and S3).

**Discussion**

Identifying and eliminating any suspect nests as soon as possible is crucial to prevent hornets from spreading and becoming established. Until now, samples suspected as *V. velutina nigrithorax* could only be identified morphologically and this may not always be possible if only partial specimens or immature life-stages are recovered. Our newly described assays provide quick and unequivocal identification on partial tissue samples, as demonstrated through the amplification in samples from a single hind leg. We have demonstrated that our LAMP assay also provides fast and accurate identification on early developmental stages which may be more time consuming to identify morphologically.

In spring, queen hornets and wasps seek sites to develop their primary nests and may be in a shed or low lying area (Monceau et al., 2014), which may be encountered by members of the public. Primary nests are small and will be abandoned later in spring, as workers and the queen abscond to form the main nest; the primary nest once abandoned may be difficult to identify as adult hornets are no longer present. *Dolichovespula saxonica*, *Vespula germanica* and *Vespula vulgaris* are very common social wasp species found in the UK. *Dolichovespula saxonica* commonly builds its nests in sheds and outbuildings, as do *V. germanica* and *V. vulgaris* although to a lesser extent (Zahradník, 1991). The Vvn LAMP assay can be used to amplify DNA from the silk capping and meconium (faecal) pellet derived from the larvae from a *V. velutina nigrithorax* nest, and has been verified to not amplify the DNA from these common wasp species. Conversely the invertebrate control assay has been verified to detect all of these species (providing confirmation of the success of amplifying DNA from crude extract of possibly degraded samples); therefore, deployment of these assays in combination could be used to identify if *Vespa velutina nigrithorax* is the maker of suspect abandoned nests.

LAMP assays are robust in their ability to amplify in the presence of inhibitors that would otherwise preclude amplification in a standard PCR reaction (Hodgetts et al., 2015). Invertebrate samples submitted to the National Bee Unit for identification are sometimes degraded or damaged, and may have spent a number of days at variable temperatures in the postal system. Similarly, abandoned nests may have spent some time decomposing before their discovery. LAMP provides the possibility of species discrimination in a sample that may be too far degraded for any conventional PCR assays. As the awareness of the potential *V. velutina nigrithorax* incursion into the UK intensifies, more samples will no doubt be submitted by bee inspectors and members of the public, and both the LAMP and the real-time PCR assays give the potential to screen hundreds of samples in the space of only a few days in a laboratory setting. This fast and reliable detection is necessary for directing control and eradication efforts.

LAMP assays can be performed using crude extracts tested on small, portable instruments such as the Genie® (OptiGene) that can be used in facilities with minimal laboratory equipment such as sites of import or field stations. It can be operated and interpreted by users with basic training. In the case of *V. velutina nigrithorax*, LAMP may prove potentially useful for testing at any sites of outbreak and may expedite the eradication of nests which will contribute to slowing the spread of this damaging invasive pest.

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**Author contributions**

Authors 1 and 5 conceived the research and wrote the manuscript.

Authors 1, 2 and 5 conducted the experiments.

All authors read and approved the manuscript.

**Conflict of Interest**

The authors declare that they have no conflict of interest.

**References**

Arca M., Papachristoforou A., Mougel F., Rortais A., Monceau K., Bonnard O., Tardy P., Thiery D., Silvain J.F. & Arnold G. (2014) Defensive behaviour of *Apis mellifera* against *Vespa velutina* in France: testing whether European honey bees can develop an effective collective defence against a new predator. *Behavioural Processes, 106*, 122-129.

Archer M. (1994) Taxonomy, distribution and nesting biology of the *Vespa bicolor* group (Hym., Vespinae). *Entomologist’s Monthly Magazine,130*,149-58.

Archer M.E. (1989) *Key to World Species of the Vespinae: Part 1 - Keys, checklist and distribution*. University College of Ripon and York St John.

Beggs J.R., Brockerhoff E.G., Corley J.C., Kenis M., Masciocchi M., Muller F., Rome Q. and Villemant C. (2011) Ecological effects and management of invasive alien Vespidae. *Journal of the International Organisation of Biological Control, 56*, 505-526.

Bertolino S., Lioy S., Laurino D., Manino A. and Porporato M. (2016) Spread of the invasive yellow-legged hornet *Vespa velutina* (Hymenoptera: Vespidae) in Italy. *Applied Entomology and Zoology, 51* (4), 589-597.

Budge G.E., Hodgetts J., Jones E.P., Ostojá-Starzewski J.C., Hall J., Tomkies V., Semmence N., Brown M., Wakefield M. and Stainton K. (2017) The invasion, provenance and diversity of *Vespa velutina* Lepeletier (Hymenoptera: Vespidae) in Great Britain. *PLOS ONE, 12* (9), e0185172, DOI: 10.1371/journal.pone.0185172

Chomczynski P. and Rymaszewski M. (2006) Alkaline polyethylene glycol-based method for direct PCR from bacteria, eukaryotic tissue samples, and whole blood. *Biotechniques, 40*, 454-458.

Darrouzet E., Gevar J., Guigmard Q. and Aron S. (2015) Production of early diploid males by European colonies of the invasive hornet *Vespa velutina nigrithorax*. PLOS ONE, DOI:10.1371/journal.pone.0136680.

Defra (2016) Press release: Asian hornet outbreak contained in Gloucestershire and Somerset [text article] Retrieved from: https://www.gov.uk/government/news/asian-hornet-outbreak-contained-in-gloucestershire-and-somerset.

EPPO (European and Mediterranean Plant Protection Organization) (2014) PM 7/98 (2) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. *EPPO Bulletin, 44,* 117-147.

Folmer O., Black M., Hoeh W., Lutz R. and Vrijenhoek R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechology, 3*(5), 294-299.

Hodgetts J., Karamura G., Grant M., Studholme D.J., Boonham N., Karamura E. and Smith J.J. (2015) Rapid, specific, simple, in-field detection of *Xanthomonas campestris* pathovar *musacearum* by loop-mediated isothermal amplification. *Journal of Applied Microbiology, 119,* 1651-1658.

Jung C.E. (2012) Spatial expansion of an invasive hornet, *Vespa velutina nigrithorax* Buysson (Hymenoptera: Vespidae) in Korea. *Korean Journal of Apiculture, 27,* 87-93.

Kibbe W.A. (2007) OligoCalc: an online oligonucleotide properties calculator. Nucleic Acids Res. 35, DOI: 10.1093/nar/gkm234.

Lopez S., Gonzalez M. and Goldarazena A. (2011) *Vespa velutina* Lepeletier, 1836 (Hymenoptera: Vespidae): first records in Iberian Peninsula. *EPPO Bulletin, 41*, 439-441.

Machida R.J. and Knowlton N. (2012) PCR Primers for Metazoan Nuclear 18S and 28S Ribosomal DNA sequences. *PLOS ONE*, doi: 10.1371/journal.pone.0134314.

Manchester S.J. and Bullock J.M. (2000) The impacts of non-native species on UK biodiversity and the effectiveness of control. *Journal of Applied Ecology, 37* (5), 845-864.

Monceau K., Bonnard O. and Thiery D. (2014) *Vespa velutina*: a new invasive predator of honey bees in *European Journal of Pest Science, 87*, 1-6.

Robinet C., Suppo C. and Darrouzet E. (2016) Rapid spread of the invasive yellow-legged hornet in France: the role of human-mediated dispersal and the effects of control measures. *Journal of Applied Ecology, 54* (1), 205-215.

Rome Q., Muller F.J., Touret-Alby A., Darrouzet E., Perrard A. and Villemant C. (2015) Caste differentiation and seasonal changes in *Vespa velutina* (Hym.:Vespidae) colonies in its introduced range. *Journal of Applied Entomology, 139*, 771-782.

Villemant C., Zuccon D., Rome Q., Muller F., Poinar G.O. and Justine J.L. (2015) Can parasites halt the invader? Mermithid nematodes parasitizing the yellow-legged Asian hornet in France. *PeerJ*, DOI: 10.7717/peerj.947.

Zahradník, J. (1991) *A field guide in colour to bees and wasps*. Aventinum Publishing House, Prague.

**Table legends**

Table 1: Samples used in the study, their origin and results with the *Vespa velutina nigrithorax* (Vvn) and invertebrate control (Invt ctr) LAMP and real-time PCR assays. All values are the mean from duplicate reactions, ‘-‘ indicates a negative result. Tp: time to positive value, Ta: annealing temperature and C**T**: cycle threshold value.

Table 2: Sensitivity of the *Vespa velutina nigrithorax* (Vvn) and invertebrate control (Invt ctr) LAMP and real-time PCR assays. Each dilution was tested in triplicate, and all values are the mean from all positive reactions, ‘-‘ indicates a negative result. Tp: time to positive value, Ta: annealing temperature and C**T**: cycle threshold value.

Table 3: Results of crude sample homogenisation on *Vespa velutina nigrithorax* nest samples and different life stages, tested with the *Vespa velutina* LAMP assay. All values are the mean from duplicate reactions, ‘-‘ indicates a negative result. Tp: time to positive value, Ta: annealing temperature and C**T**: cycle threshold value.

Supplementary table 1. Sequences of primers and probes used in the study.

Supplementary table 2: Results of repeatability testing by a second operator for assay specificity. All values are the mean from duplicate reactions, ‘-‘ indicates a negative result. Tp: time to positive value, Ta: annealing temperature and C**T**: cycle threshold value.

Supplementary material table 3: User 2 data for sensitivity of the *Vespa velutina nigrithorax* (Vvn) and invertebrate control (Invt ctr) LAMP and real-time PCR assays. Each dilution was tested in triplicate, and all values are the mean from all positive reactions, ‘-‘ indicates a negative result. Tp: time to positive value, Ta: annealing temperature and C**T**: cycle threshold value.

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|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Sample/Species | **Origin** |  | **LAMP** |  | **Real-time PCR** |
| **Vvn****Tp (mm:ss)** | **Vvn****Ta (°C)** | **Invt ctr****Tp (mm:ss)** | **Invt ctr****Ta (°C)** | **Vvn** **CT** | **Invt ctr****CT** |
| *Vespa velutina* *nigrithorax* V051 | France | 11:22 | 80.67 | 16:13 | 86.67 | 25.05 | 25.89 |
| *Vespa velutina* *nigrithorax* V066 | France | 11:07 | 80.67 | 32:13 | 89.18 | 21.90 | 18.65 |
| *Vespa velutina nigrithorax* V071 | France | 13:15 | 80.66 | 15:05 | 86.94 | 25.79 | 24.60 |
| *Vespa velutina* *nigrithorax* V076 | France | 09:30 | 80.58 | 14:45 | 86.52 | 19.34 | 17.89 |
| *Vespa velutina nigrithorax* V082 | France | 10:15 | 80.67 | 37:30 | 86.30 | 22.23 | 30.03 |
| *Vespa velutina nigrithorax* V083 | France | 10:25 | 80.64 | 15:30 | 86.59 | 33.18 | 29.62 |
| *Vespa velutina nigrithorax* V116 | France | 08:22 | 80.70 | 16:20 | 87.13 | 15.83 | 18.43 |
| *Vespa velutina nigrithorax* V121 | France | 09:22 | 80.52 | 15:30 | 86.79 | 18.55 | 17.90 |
| *Vespa velutina nigrithorax* V142 | France | 09:00 | 80.60 | 18:20 | 87.15 | 20.12 | 18.65 |
| *Vespa velutina nigrithorax* V143 | France | 09:52 | 80.69 | 25:38 | 87.45 | 20.19 | 21.20 |
| *Vespa velutina nigrithorax* V164 | France | 09:52 | 80.68 | 35:05 | 87.58 | 21.18 | 23.80 |
| *Vespa velutina nigrithorax* V167 | France | 09:15 | 81.02 | 35:58 | 88.77 | 18.62 | 21.49 |
| *Vespa velutina nigrithorax* V208 | France | 08:30 | 80.74 | 27:38 | 89.17 | 17.91 | 17.00 |
| *Vespa velutina nigrithorax* V209 | France | 08:45 | 80.65 | 27:23 | 89.27 | 19.05 | 18.14 |
| *Vespa velutina nigrithorax* V217 | France | 10:00 | 80.86 | 28:15 | 89.07 | 33.99 | 31.72 |
| *Vespa velutina nigrithorax* V219 | France | 14:13 | 80.62 | 30:50 | 88.83 | 23.71 | 21.38 |
| *Vespa velutina nigrithorax* V039 | Java, Indonesia | 17:30 | 80.58 | 26:20 | 88.90 | 25.33 | 16.51 |
| *Vespa velutina nigrithorax* V133 | Vietnam | 08:52 | 80.48 | 24:15 | 88.95 | 21.70 | 18.10 |
| *Vespa velutina* *nigrithorax* V140 | Vietnam | 08:45 | 80.48 | 24:23 | 88.97 | 21.02 | 18.01 |
| *Vespa velutina* *nigrithorax* V185 | Sulawesi, Indonesia | 15:37 | 81.04 | 22:28 | 88.97 | 26.53 | 17.35 |
| *Vespa velutina nigrithorax* V188 | Yunnan, China | 13:22 | 80.56 | 26:20 | 89.03 | 22.46 | 18.68 |
| *Vespa velutina nigrithorax* V206 | Yunnan, China | 12:52 | 80.60 | 27:58 | 89.06 | 21.67 | 20.59 |
| *Vespa velutina nigrithorax* V221 | Flores, Indonesia | 16:37 | 81.02 | 25:35 | 88.29 | 26.51 | 18.08 |
| *Vespa velutina nigrithorax* V229 | Sumbawa, Indonesia | 14:37 | 81.01 | 25:35 | 88.54 | 25.45 | 16.25 |
| *Vespa velutina nigrithorax* V235 | Zhejiang/Jiangsu, China | 08:15 | 80.74 | 26:20 | 89.23 | 18.15 | 18.53 |
| *Vespa velutina nigrithorax* V260 | Zhejiang/Jiangsu, China | 08:52 | 80.75 | 26:05 | 89.16 | 23.72 | 17.31 |
| *Vespa velutina nigrithorax* EB2455 | Alderney, Channel Islands | 09:30 | 80.59 | 24:08 | 88.48 | 23.40 | 19.35 |
| *Vespa velutina* *nigrithorax* EB2483 | Tetbury, UK | 08:15 | 80.79 | 25:58 | 89.13 | 20.78 | 21.24 |
| *Vespa velutina nigrithorax* EB2485 | Tetbury, UK | 09:00 | 80.80 | 28:05 | 89.20 | 21.27 | 20.57 |
| *Vespa velutina nigrithorax* EB2511 | Somerset, UK | 09:00 | 80.79 | 27:20 | 89.08 | 19.27 | 17.36 |
| Non-target species |  |  |  |  |  |  |  |
| *Vespa crabro* | York, UK | - | 71.34 | 18:45 | 88.24 | - | 16.71 |
| *Vespa analis* | Indonesia | - | 71.36 | 23:00 | 88.34 | - | 20.86 |
| *Vespula germanica* | York, UK | - | 71.34 | 16:45 | 88.76 | - | 14.22 |
| *Vespula vulgaris* | York, UK | - | 71.34 | 19:00 | 88.20 | - | 19.73 |
| *Urocerus gigas* | York, UK | - | 71.30 | 18:15 | 88.25 | - | 13.44 |
| *Dolichovespula media* | Tetbury, UK | - | 71.30 | 23:30 | 88.11 | - | 22.75 |
| *Dolichovespula saxonica* | Dyfed, Wales, UK | - | 71.30 | 19:45 | 88.72 | - | 15.48 |
| *Apis mellifera* | York, UK | - | 71.30 | 21:15 | 83.80 | - | 18.83 |

Table 2: Sensitivity of the *Vespa velutina nigrithorax* (Vvn) and invertebrate control (Invt ctr) LAMP and real-time PCR assays. Each dilution was tested in triplicate, and all values are the mean from all positive reactions, ‘-‘ indicates a negative result. Tp: time to positive value, Ta: annealing temperature and C**T**: cycle threshold value.

|  |  |  |  |
| --- | --- | --- | --- |
| DNA concentration (per reaction) |  | **LAMP** | **Real-time PCR** |
| **Positive replicates** | **Vvn****Tp (mm:ss)** | **Vvn****Ta (°C)** | **Positive replicates** | **Invt ctr****Tp (mm:ss)** | **Invt ctr****Ta (°C)** | **Positive replicates** | **Vvn****CT** | **Positive replicates** | **Invt ctr****CT** |
| *Vespa velutina* 5.07 ng | 3 | 07:03 | 80.32 | 3 | 20:05 | 89.51 | 3 | 16.28 | 3 | 15.66 |
| *Vespa velutina* 507 pg | 3 | 08:15 | 80.38 | 3 | 26:07 | 89.44 | 3 | 20.19 | 3 | 19.51 |
| *Vespa velutina* 50.7 pg | 3 | 10:15 | 80.40 | 3 | 30:13 | 89.36 | 3 | 27.04 | 3 | 25.27 |
| *Vespa velutina* 5 pg | 3 | 12:03 | 80.43 | 3 | 33:43 | 88.14 | 3 | 30.36 | 3 | 29.15 |
| *Vespa velutina* 507 fg | 2 | 15:28 | 80.40 | 2 | 38:15 | 86.10 | 3 | 35.13 | 3 | 33.60 |
| *Vespa velutina* 50.7 fg | 0 | - | 71.57 | 2 | 38:08 | 85.72 | 1 | 37.80 | 2 | 37.10 |
| *Vespa velutina* 5 fg | 0 | - | 71.51 | 1 | 38:00 | 85.29 | 0 | - | 0 | - |

Table 3: Results of crude sample homogenisation on *Vespa velutina nigrithorax* nest samples and different life stages, tested with the *Vespa velutina* LAMP assay. All values are the mean from duplicate reactions, ‘-‘ indicates a negative result. Tp: time to positive value, Ta: annealing temperature and C**T**: cycle threshold value.

|  |  |  |
| --- | --- | --- |
| Sample/tissue type | **Vvn****Tp (mm:ss)** | **Vvn****Ta (°C)** |
| Nest: Meconium | 09:32 | 80.10 |
| Nest: Silk capping | 9:00 | 80.32 |
| Nest: paper casing (exterior) | - | - |
| Nest: paper casing (interior) | 09:07 | 79.63 |
| Egg | 07:37 | 81.31 |
| Larva | 13:00 | 80.96 |
| Pupa | 13:00 | 80.67 |
| Single leg: stored at -80 °C | 12:08 | 79.92 |
| Single leg: stored at RT | 12:17 | 80.75 |