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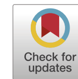

The son-killer microbe *Arsenophonus nasoniae* is a widespread associate of the parasitic wasp *Nasonia vitripennis* in Europe

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A R T I C L E I N F O

Original content: Sex ratio produced by Arsenophonus infected vs uninfected Nasonia vitripennis from the UK and Switzerland (Original data)
Presence of Nasonia wasps and the bacterium Arsenophonus nasoniae in calliphorid fly pupae collected from great tit nests in Coimbra, Portugal. (Original data)
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Keywords:
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A B S T R A C T

Heritable microbes that exhibit reproductive parasitism are common in insects. One class of these are the male-killing bacteria, which are found in a broad range of insect hosts. Commonly, our knowledge of the incidence of these microbes is based on one or a few sampling sites, and the degree and causes of spatial variation are unclear. In this paper, we examine the incidence of the son-killer microbe *Arsenophonus nasoniae* across European populations of its wasp host, *Nasonia vitripennis*. In preliminary work, we noticed two female *N. vitripennis* producing highly female biased sex ratios in a field study from the Netherlands and Germany. When tested, the brood from Germany was revealed to be infected with *A. nasoniae*. We then completed a broad survey in 2012, in which fly pupal hosts of *N. vitripennis* were collected from vacated birds' nests from four European populations, *N. vitripennis* wasps allowed to emerge and then tested for *A. nasoniae* presence through PCR assay. We then developed a new screening methodology based on direct PCR assays of fly pupae and applied this to ethanol-preserved material collected from great tit (*Parus major*) nests in Portugal. These data show *A. nasoniae* is found widely in European *N. vitripennis*, being present in Germany, the UK, Finland, Switzerland and Portugal. Samples varied in the frequency with which they carry *A. nasoniae*, from being rare to being present in 50% of the pupae parasitised by *N. vitripennis*. Direct screening of ethanol-preserved fly pupae was an effective method for revealing both wasp and *A. nasoniae* infection, and will facilitate sample transport across national boundaries. Future research should examine the causes of variation in frequency, in particular testing the hypothesis that *N. vitripennis* superparasitism rates drive the variation in *A. nasoniae* frequency through providing opportunities for infectious transmission.

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1. Introduction

Animals live in a microbial world (McFall-Ngai et al., 2013). In many cases, the microbe grows externally to the organism, within the gut or on the cuticle/skin. In other cases, the microbes live within the host as an endosymbiont, commonly exhibiting specialised systems to live in host cells or tissues (Douglas, 2010). Endosymbionts vary in their impact on the host, from being beneficial partners (e.g., supplying vitamins or amino acids or providing natural enemy protection) through to being pathogens and parasites (Hurst, 2017). These individual impacts affect the ecology and evolution of their host, altering nutritional ecology, dynamics of natural enemy-host interactions, and patterns of sexual selection.

The means of establishment of endosymbiosis vary (Hurst, 2017). In some cases, the interaction arises *de novo* each generation, with either the host acquiring the microbe from the environment or the microbe infecting the host from an environmental source. In other cases, transmission is vertical from parent to offspring. Where transmission is vertical, it selects strongly for the microbe to contribute positively to host biology because host health then correlates with microbe transmission opportunities. Nevertheless, the maternal inheritance of many of these microbes makes male hosts an evolutionary ‘dead end’. This has selected for microbes to exhibit sex ratio distorting ‘reproductive parasitisms’ that bias the host sex ratio towards the production and survival of female offspring of the host (Hurst and Frost, 2015).

Male-killing represents one class of reproductive parasitism in which the microbe specifically kills the sons of infected females. Male-killing symbionts are found in a broad range of arthropods, including pseudoscorpions, spiders, hemipteran bugs, parasitic wasps, lacewings, flies, beetles and butterflies/moths (Hurst and Frost, 2015). The microbes that have evolved to cause male-killing are likewise diverse, and include *Spiroplasma*, *Wolbachia*, *Rickettsia*, flavobacteria and members of the gammaproteobacteria (Gherna et al., 1991, Werren et al., 1994, Hurst et al., 1997, Hurst et al., 1999b, Hurst et al., 1999a), alongside some heritable viruses (Kageyama et al., 2017) and microsporidia (Andreadis, 1985). The male-killing action of the microbe either liberates resources and reduces competition for female sibling hosts that carry the infection (Hurst and Majerus, 1993), or alternately enables infectious transmission of the microbe out of the male host where vertical transmission is not possible. The former is often associated with the male host dying during embryogenesis (early male-killing) whereas infectious transmission is most commonly observed where the male host dies late in development (Hurst, 1991). Male-killers are interesting symbionts in that they can achieve high frequency. When they do they have diverse population and evolutionary impacts, altering the transmission dynamics of sexually transmitted infections that circulate in the host (Ryder et al., 2014), the pattern of sexual selection experienced by the host (Jiggins et al., 2000, Charlat et al., 2007), and selecting for host genes that restore the viability of male hosts (Hornett et al., 2022).

Arsenophonus nasoniae strain *a*Nv is a male-killing bacterium associated with the jewel wasp, *Nasonia vitripennis*. It is a member of the gammaproteobacteria and is unusual amongst heritable symbionts in that it is culturable in cell-free media and can be genetically manipulated (Werren et al., 1986, Nadal-Jimenez et al., 2019). This culturability reflects transitions in this genus from environmentally acquired infections (*Arsenophonus apicola* in honey bees, *Arsenophonus* strain *a*Pb in the blue butterfly *Polyommatus bellargus*) as well as vertically transmitted infections (*A. nasoniae* strain *a*Pv in the parasitic wasp *Pachycrepoideus vindemmiae*) (Drew et al., 2021, Nadal-Jimenez et al., 2022, Nadal-Jimenez et al., 2023). *Arsenophonus nasoniae* strain *a*Nv is itself interesting in that it has mixed modes of transmission. When the wasp stings and oviposits into a fly pupa, the microbe is injected onto the surface of the metamorphosing fly. When the wasp eggs hatch into larvae, they are exposed to the microbe through feeding. The microbe then passes through the gut to establish infection, with a pronounced tropism for the ovipositor that enables onward microbial passage when the wasp parasites a fly pupa (Nadal-Jimenez et al., 2019). This transmission mode means that when an infected and an uninfected female parasitise the same fly pupa (superparasitism), the progeny of the uninfected female can also acquire *A. nasoniae* infection (Skinner, 1985). This infectious transmission makes the dynamics of *A. nasoniae* very sensitive to rates of wasp superparasitism (Parratt et al., 2016). *Arsenophonus nasoniae* can also pass into other pteromalid wasp species when they co-parasitise fly pupae with another species (Duron et al., 2010), leading to it being found in a range of host wasp species (Duron et al., 2010, Taylor et al., 2011). It is thus possible for the wasp community context beyond *N. vitripennis* to impact the frequency of the microbe.

Studies of *A. nasoniae* in the natural environment are rather rare. A survey by Balas et al (1996) collected fly pupae from bird’s nests, allowed *N. vitripennis* to emerge and then tested lines for *A. nasoniae* through culture, hybridisation or sex ratio phenotype. This analysis indicated that infection was found in *N. vitripennis* from across USA, in 7–11% of female individuals. Later PCR-based analyses on Canadian wasp populations revealed high presence in this population (Taylor et al., 2011). This survey further revealed a number of other pteromalid wasps carry *A. nasoniae* infection. In contrast, there have been no surveys of European *N. vitripennis* to date. The difference in wasp communities between the two continents makes such a survey timely. Whilst *N. vitripennis* is the sole member of the genus in Europe, this species lives sympatrically with *N. giraulti*, *N. longicornis* and *N. oneida* in North America (Darling and Werren, 1990, Raychoudhury et al., 2010), creating the context for within community transmission.

In this study we first examined material from a previous study of *N. vitripennis* from field collected fly pupae in the Netherlands and Germany for evidence of *A. nasoniae* infection as a preliminary indicator of *A. nasoniae* presence in European *N. vitripennis*. We then completed our own survey of four European *N. vitripennis* populations for *A. nasoniae* using similar methodologies to previous studies, that is to say to collect vacated birds’ nests, extract and isolate fly pupae, and allow any wasps to emerge before testing through PCR assay. Finally, we developed a novel methodology for screening that involves simply collecting and testing fly pupae, using a highly specific PCR assay for wasp and microbe, and then applied this to test for the presence of *A. nasoniae* in a Portuguese *N. vitripennis* population.

2. Materials and methods

2.1. An initial targeted screen for A. nasoniae in N. vitripennis in Germany and Netherlands

We examined material derived from a previous study into the genetic structure of wild *N. vitripennis* populations from Grillenberger et al. (2008). In this work, host fly pupae were collected from 18 different nest boxes in two field sites in Europe (Hoge Veluwe National Park, the Netherlands and Schlüchtern, near Hessen, Germany). Wasps were allowed to emerge from the pupae (a single pupa may contain up to 100 wasps), and then collected for onward relatedness analysis with microsatellite markers. 3,550 wasps that emerged were identified as the offspring of 49 different foundresses. From the data deposited, it was possible to ascertain the sex ratio of offspring produced by each foundress, and we reasoned that two foundresses which produced very female biased broods were strong candidates to be infected with *A. nasoniae*: foundress 46 from Germany (2 males to 114 females across 12 host pupae) and foundress 21 from the Netherlands (6 males to 151 females across 5 host pupae).

DNA templates from three offspring of each of these two foundresses were obtained from the authors of the original study and screened by PCR for the presence of *A. nasoniae* using primers Ars16Sf (5′GGGT TGTAAAGTACTTTCAGTCGT) and Ars16Sr (5′CGCAGGCTCGCCTCT CTC) which were designed to be specific for the genus based on the 16S rRNA gene of *A. nasoniae* (GenBank M90801.1) and amplify an 846 bp fragment of 16S rRNA. PCR assays were performed under the following

conditions: initial denaturation (95 °C for 2 min), 30 cycles of denaturation (94 °C, 30 s), annealing (54 °C, 1 min) and extension (72 °C, 1 min) before a final extension at 72 °C for 5 min. DNA from pure cultures of *A. nasoniae*, ATCC number 49151, was used as a positive control (extraction by boiling for 15mins in ddH_2O) and DNA from an uninfected lab strain of *N. vitripennis* as a negative control. Where PCR amplicons were obtained, these were sequenced to confirm specific amplification and relatedness to *A. nasoniae*.

2.2. Assessing the frequency of A. nasoniae in N. vitripennis in Europe

Nasonia vitripennis commonly oviposits in calliphorid 'filth fly' pupae found in birds' nests. Nests of blue tit (*Cyanistes caeruleus*), great tit (*Parus major*) and other passerine birds were collected shortly following fledging from the nest and placed in bags. Collections were made in June/July 2012 from the area surrounding Harjavalta, Finland; from Lausanne, Switzerland; from Kraslava, Latvia; and from Marbury, Cheshire in the UK. Each nest was given an ID number, was manually inspected for calliphorid/protocalliphorid pupae, and these pupae removed individually to a tube for any parasitising wasps to emerge. Where more than one pupa was collected from the same nest this was noted, as pupae from the same nest may be parasitised by the same individual wasp. The pupae, which were monitored daily, were placed at 25 °C for 14 days to allow for *N. vitripennis* wasps to emerge.

For PCR assays, *N. vitripennis* DNA was prepared using the Chelex method. Template quality was assessed based on COI amplification using primers HCO/LCO (Folmer et al, (1994), with 30 PCR cycles of 93 °C for 15 s, 47 °C for 1 m, 72 °C for 1 m and an expected amplicon size of. c. 710 bp. Templates passing this quality control were then tested for *A. nasoniae* using primers amplifying the metalloprotease gene (M1f GGGTCACATACCTATTTT, M1r GTAGTCGCCTGGGTGGG), with 30 cycles of 93 °C for 15 s, 55 °C for 45 s, 72 °C for 30 s and an expected amplicon size of 594 bp. A number of amplicons for *A. nasoniae* for each positive location were selected for Sanger sequencing to confirm identity.

For the UK and Swiss populations, we additionally established the sex ratio produced by *A. nasoniae* infected females compared to uninfected females. To this end, the F1 progeny of female *N. vitripennis* that emerged from field collected pupae and allowed to oviposit were scored for sex. In this analysis, individuals producing no female offspring were discounted, as this is commonly the result of failure of the female wasp to mate and fertilize eggs.

2.3. Isolation of A. nasoniae to plate culture

Where *A. nasoniae* was detected in the above screens, the wasps maintained onward were used to attempt isolation of *A. nasoniae* to plate culture to curate a representative isolate from each population. In brief, *N. vitripennis* females were allowed to oviposit on fly pupae and those pupae maintained at 25 °C for ten days. Pupae were then 'cracked' to access the *N. vitripennis* pupae. These pupae were surface sterilized with 70% ethanol, and the interior smeared on GC agar plates with isovitalex. Because *A. nasoniae* is slow growing, bacteria were allowed to grow for 5–10 days at 30 °C, and plates were then examined. Bacterial growth in the site of smearing was then sampled with a loop and streaked to a new plate to obtain cloned *A. nasoniae*. Microbial identity was first confirmed through colony morphology in brain–heart infusion agar (cauliflower like) and then through colony PCR using the PCR primers M1f an M1r above.

2.4. PCR detection of N. vitripennis and A. nasoniae in fly pupae

Biosecurity considerations make transportation of live material from field collections across international boundaries complex. We therefore developed a method for jointly assaying for *N. vitripennis* wasps and *A. nasoniae* in ethanol-preserved fly pupal material. We first emulated this scenario with fly pupae known to be parasitised with *A. nasoniae* infected *N. vitripennis* in the laboratory. To this end, "white maggots", generally intended for fishing in freshwater, were obtained from local fish bait shops. Each 500 ml of maggots was placed in a tray measuring 38 (L) × 28 (W) × 7.5 (H) cm with a sheet of towel paper at the bottom and another sheet covering them and left to pupate at 25 °C for 4 days. Subsequently, the pupae were placed in plastic boxes with a layer of towel paper, holes on the lid to allow respiration and decrease ammonia concentration and stored at 4 °C. Two days after, the pupae were washed under tap water using a cooking sieve. The excess of water was removed, and the pupae were placed overnight to dry at 25 °C. This step decreases bacterial contamination and reduces the potential rotting of the pupae (which are metabolically very active 48–72 h after pupation). Upon this second process, the pupae are placed back in clean plastic boxes and stored at 4 °C for up to 20 days.

A time series analysis was completed to establish the capacity to detect recent wasp and *A. nasoniae* infection. To this end, one mated *N. vitripennis* strain AsymC female wasp (previously infected with *A. nasoniae a*Nv_Fin strain) was added to a plastic vial containing two fresh fly pupae; the tube was sealed with a cotton plug and placed at 25 °C in an incubator with a cycle of 12 h light/12 h darkness. This experiment was performed in triplicate for each time point (2, 4, 24, 48, and 72 h) post wasps being allowed to oviposit. Three tubes where flies were not exposed to wasps were used as a negative control (0 h). At each of the time intervals, the fly pupae were removed and kept in 1.5 ml tubes at −20 °C till the moment of DNA extraction.

For DNA extraction, the surface of the fly pupae was washed by rubbing the pupae against a clean towel paper previously sprayed with 70% ethanol. The two pupae of each tube were then homogenized with a sterile pestle. Genomic DNA (gDNA) from the homogenized samples was extracted using a Zymo Research Quick-DNA Miniprep plus kit (D4069, Zymo Research, USA) and eluted in 30 µl of molecular biology grade water (Thermo Fisher Scientific, UK). Sample concentrations were measured using a nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, UK). *N. vitripennis* was detected by PCR assay using primers NvS6KQTF1 (5′ GGCATTATCTACAGAGATTTGAAACCAG) and NvS6KQTR3 (5′ CAAAGCTATATGACCTTCTGTATCAAG), with cycling conditions 1 × 95 °C – 5 min; 30 × (94 °C – 15 s, 55 °C – 45 s, 72 °C – 1 min; 1 × 72 °C – 5 min (Bordenstein and Bordenstein, 2011). For the detection of *A. nasoniae* we designed and used novel primers based on one of the conserved *luxI* (AHL) synthase genes of this bacterium (*Ars-FIN_37250* gene in *A. nasoniae a*Nv_Fin genome). This *luxI* gene is present and fully conserved in all five sequenced *A. nasoniae* strains but has low homology to that of other bacteria including other *Arsenophonus* spp. which will minimize the rate of false positive amplicons (Frost et al., 2020). To this end, PCR primers luxI2_AN_F (5′ ATGTTAAACTTTTT-TAATGCAAAAT) and luxI2_AN_R (5′ TTACCATCTTGATA-GAAAATTTAAA) were utilized with PCR conditions were 1 × 95 °C – 5 min; 30 × (94 °C – 15 s, 55 °C – 45 s, 72 °C – 1 min; 1 × 72 °C – 5 min).

2.5. Application of the method in blowfly puparia collected in great tit nests in Portugal

During the breeding season of 2021, we sampled pupae from great tit nests at Mata Nacional do Choupal (40°13′N, 8°27′W), a mixed woodland on the periphery of the city of Coimbra, Portugal (more details of the study area in (Norte et al., 2009). Old nest material from all nest boxes had been previously removed at the end of the previous breeding season to avoid the accumulation of overwintering *Protocalliphora* spp. adults (Gold and Dahlsten, 1983). Once all fledglings had left their nests in May/June, the nests were collected in plastic bags and transported to the laboratory. Blowfly puparia were sorted manually from the nest material and were stored in 1.5 ml tubes with absolute ethanol for further genetic analysis.

To determine the presence of *N. vitripennis* and its endosymbiont *A. nasoniae*, up to five pupae collected from each nest were surface

sterilised with 70% ethanol, added to a 2 ml Eppendorf tube, crushed and homogenised using a sterile pestle. Fly pupal shells were removed with the aid of a sterile forceps and DNA was extracted from the homogenised sample using a Zymoresearch Quick DNA ™ Miniprep plus kit (Zymoresearch, USA). These were then subject to diagnostic QC PCR of the COI gene with HCO/LCO primers described above (this amplifies fly DNA in the absence of other parties, and establishes template quality). Then, PCR assays for wasp and microbe were performed as outlined above (primer pair NvS6KQTF1 & NvS6KQTR3 primer pair luxI2_AN_F/ luxI2_AN_R respectively). Amplicons for the *A. nasoniae* PCR assay were sequenced using the Sanger method to confirm identity (Accession Numbers OX459129- OX459132).

3. Results

3.1. An initial screen for A. nasoniae in N. vitripennis in Germany and Netherlands

Arsenophonus nasoniae infection was confirmed in one of the two European *N. vitripennis* lines tested. All three daughters of foundress 46 from Schlüchtern (Germany) tested positive for *A. nasoniae* infection. The sequence of the PCR amplicon detected was identical in *16S rRNA* gene sequence to the *A. nasoniae* infection of American *N. vitripennis* (GenBank M90801.1). The progeny of foundress 21 from the Netherlands did not produce an amplicon in the *A. nasoniae* PCR assay and were scored as uninfected.

3.2. Assessing the frequency of A. nasoniae in N. vitripennis in Europe

Nasonia vitripennis presence in birds' nests varied greatly between sites, explained partly by variation in the number of nests in which fly pupae were found (from 26% to 47.5% of nests), and the rate of parasitism by *N. vitripennis* where pupae were found in a nest (from 3% to 58% of nests)(Table 1). *Arsenophonus nasoniae* was detected broadly in European *N. vitripennis*, from Finland in the NE Europe to Switzerland in central Europe and the UK in Western Europe. One population, Latvia, showed no evidence of *A. nasoniae* infection in *N. vitripennis*, but this was a small sample size where *N. vitripennis* only emerged from 4 nests with 8 pupae total, so may reflect low prevalence of *A. nasoniae* rather than absence.

The frequency with which nests with *N. vitripennis* carried *A. nasoniae* and the frequency of pupae with *N. vitripennis* carrying *A. nasoniae* was heterogeneous between samples (for nests with pupae parasitised by *N. vitripennis*, null hypothesis of no heterogeneity of *A. nasoniae* presence between places: 4 × 2 Fisher exact test, $p < 0.025$; for pupae parasitised by *N. vitripennis* null hypothesis of no heterogeneity of *A. nasoniae* presence between places: 4 × 2 Fisher exact test $p < 0.001$). *Arsenophonus nasoniae* frequency in *N. vitripennis* was highest in the Harjavalta population (Finland) with over half of the sampled pupae and over half of the sampled nests being *A. nasoniae* positive. In all cases where amplicons were sequenced, the strain was identical in marker sequence to *A. nasoniae*.

We then examined the sex ratio produced by the female *N. vitripennis* that emerged from the field-collected pupae. For the UK sample, female wasps infected with *A. nasoniae* produced more female biased sex ratios than uninfected females (average proportion of female offspring from infected females = 90.1%, from uninfected females = 64.4%, N = 42 and N = 15 crosses respectively). The null hypothesis of equal sex ratios according to *N. vitripennis* infection status was rejected (Mann Whitney test $N1 = 42$, $N2 = 15$ $U = 88$, $p < 0.001$). For the Swiss sample, female wasps infected with *A. nasoniae* again produced more female biased sex ratios than uninfected females (average proportion female offspring from infected females = 100%, from uninfected females 83.3%, N = 5 and N = 22 crosses respectively). The null hypothesis of equal sex ratios according to *N. vitripennis* infection status was rejected (Mann Whitney test $N1 = 5$, $N2 = 22$ $U = 2.5$, $p < 0.001$).

We isolated *A. nasoniae* to *in vitro* culture from individuals from Finland, Switzerland and the UK, and verified the strains as being *A. nasoniae* from 16S rRNA sequence. These were designated *A. nasoniae* strain *a*Nv_Fin, *A. nasoniae* strain *a*Nv_UK and *A. nasoniae* strain *a*Nv_CH The genome sequence of *A. nasoniae* strain *a*Nv_Fin was completed and reported elsewhere (Frost et al., 2020).

3.3. Direct detection of N. vitripennis and A. nasoniae in fly pupae by PCR assay

We applied PCR methods for the detection of both *N. vitripennis* and its bacterial symbiont, *A. nasoniae*, in fly pupae collected from wild bird's nests. Our first challenge was to determine how long after introduction of a *N. vitripennis* female we could detect both wasp (oviposition) and microbe by PCR assays on the fly pupa. Under laboratory conditions, we could detect *A. nasoniae* 4 h following exposure of a fly pupa to a wasp female, and the wasp itself after 24 h (Fig. 1). This time frame corresponds to the period between egg laying and hatching. This earlier detection of *A.nasoniae* compared to the host suggests that either more DNA is available in this interval or that the PCR assay for the *luxI* gene is more sensitive than the assay for *N. vitripennis*.

3.4. Application of the method in blowfly puparia collected in great tit nests in Portugal

In the field study, one to five fly pupae were recovered from 14 great tit nests and tested for the presence of *N. vitripennis* and *A. nasoniae* by PCR assay. In 10 cases, neither *N. vitripennis* nor *A. nasoniae* were detected. In one case, *N. vitripennis* was scored as present but there was no evidence of *A. nasoniae*. In three cases, both *N. vitripennis* and *A. nasoniae* were scored as present. Where *luxI* amplicons were detected, Sanger sequence confirmed identity as *A. nasoniae* with 100% similarity to the reference sequence. Thus, pupae in four nests can be considered as being parasitised by *N. vitripennis* and in three of these at least one *Nasonia* individual harbours *A. nasoniae*.

4. Discussion

Arsenophonus nasoniae, the son-killer symbiont of *N. vitripennis*, is an unusual reproductive parasite in having mixed modes of transmission, contrasting to the purely vertical transmission of other microbes that kill male embryos. The combination of transmission modes makes the rate at

Table 1
Presence of *A. nasoniae* in *N. vitripennis* collected from fly pupae from European bird's nests.

Nest Sampling site	Nests examined	# nests with fly pupae	# nests with *N. vitripennis*	# Pupae parasitised by *N. vitripennis*	# Nests with *N. vitripennis* infected with *A. nasoniae*	# pupae with *N. vitripennis* infected with *A. nasoniae*
UK (Marbury)	NA	5	1	8	1	4
Finland (Harjavalta)	33	12	7	18	5	10
Latvia (Kraslava)	40	19	4	13	0	0
Switzerland (Lausanne)	116	48	26	126	9	14
Total	189	84	38	165	15	28

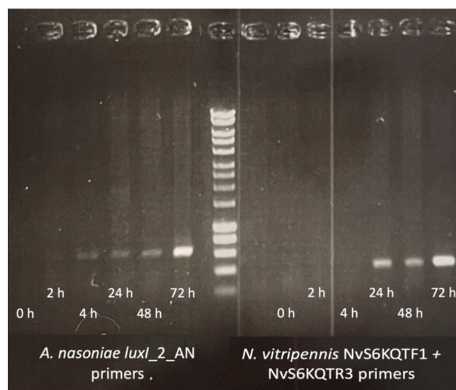

Fig. 1. Direct detection of *A. nasoniae* (left) and *N. vitripennis* (right) directly in fly pupae through PCR assay. 0 h = negative control (unexposed); 2–72 h period = time elapsed since *A. nasoniae* infected *N. vitripennis* female was put in contact with a pupa.

which wasps superparasitise – that is to say, how commonly two wasps utilize the same fly host – a critical parameter in *A. nasoniae* dynamics (Parratt et al., 2016). This difference in population biology from other male-killing microbes drove our survey of European *N. vitripennis* populations for *A. nasoniae*. Preliminary work, finding *A. nasoniae* in a German *N. vitripennis* sibship with very female biased sex ratios, indicated European *N. vitripennis* carried *A. nasoniae*. Wider sampling indicated *A. nasoniae* was present in *N. vitripennis* across Europe, with the Swiss, Finnish and UK samples being positive, In addition, we detected *A. nasoniae* infection in *N. vitripennis* in Portugal through our novel combined method for the detection of *N. vitripennis* and *A. nasoniae* in fly pupae collected from nests. Altogether, these populations include Northern and Southern locations, Eastern and Western, and we infer *A. nasoniae* is likely to be generally present in *N. vitripennis* across Europe.

The rates at which *N. vitripennis* were infected with *A. nasoniae* were heterogeneous between our samples, being high in Finland (>50% of *N. vitripennis* tested carried the infection) and relatively low in Switzerland (~10% of *N. vitripennis* carrying *A. nasoniae*). Studies in North America have recovered an overall low frequency infection in *N. vitripennis* (5–8%) (Skinner, 1985, Balas et al., 1996, Duron et al., 2010), but with some local samples having very high frequency (e.g. 47% in Lethbridge, Canada) (Taylor et al., 2011). In the latter case, the number of fly pupae collected was unclear making it hard to estimate frequency of infection in the field; our data indicate robustly that *A. nasoniae* can reach high local prevalence.

The causes of variation in frequency with which *N. vitripennis* are infected with *A. nasoniae* are currently unclear. A strong hypothesis is that *A. nasoniae* frequency in a population relates to the recent historical rates of superparasitism by the wasp host, as this was the key determinant of spread in laboratory experiments. Superparasitism rates are not widely known in *N. vitripennis*. In the most complete study, Grillenberger et al (2008) estimated that 40% of fly pupae parasitized by *N. vitripennis* in the Netherlands/Germany are subject to attack by more than one wasp female. Variation in the rate of superparasitism – which is likely to depend on local *N. vitripennis* density – would drive variation in the frequency of *A. nasoniae* infection. Further work should assess the hypothesis that *A. nasoniae* frequency is highest in areas with high *N. vitripennis* density and high rates of superparasitism by the wasp.

One drawback in surveying for *Arsenophonus* in *Nasonia* is the intensity of the work needed for proper sample collection and storage. Birds' nests are collected, and then commonly couriered to the interested scientist's laboratory. The nests are then teased apart to find pupae, these pupae isolated for wasp emergence before testing for *A. nasoniae*. Whilst this method is the 'Gold standard' – allowing live material to be obtained from which cultures can be established and onward experiments – it is commonly not feasible and may be restricted by biosecurity regulations which prevent live material from crossing International boundaries without license. We therefore established a method for joint detection of *N. vitripennis* and its endosymbiont *A. nasoniae* in preserved fly pupae, based on PCR assays. This method has the benefit that preserved dead pupae are permitted for transport and a faster assay process, with few tubes and no time spent waiting for wasps to emerge for testing. The PCR based method was able to establish the presence of wasp and *A. nasoniae* 24 h post parasitisation in the laboratory. This level is satisfactory for field collected material and should create only a very low level of false negatives for very recently parasitized fly pupae. Subsequently, we used this method to analyse fly pupae collected from nests of great tits at Mata Nacional do Choupal, near Coimbra, Portugal for the presence of both *N. vitripennis* parasitoid wasps and its bacterial endosymbiont. The method was successful at detecting *N. vitripennis* and symbiont in the material and indicates that the material can be preserved in 70% ethanol *in situ* for screens aiming to identify both wasp and symbiont in natural populations.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Gregory Hurst reports financial support was provided by Biotechnology and Biological Sciences Research Council. Gregory Hurst reports financial support was provided by Natural Environment Research Council. Eeva Tapio reports financial support was provided by Academy of Finland. Indirikis Krams reports financial support was provided by Latvian Council of Sciences. Ana Norte reports financial support was provided by Fundação para a Ciência e a Tecnologia. Gregorio Moreno-Rueda reports financial support was provided by Spanish Ministry of Education.

Data availability

Sex ratio produced by Arsenophonus infected vs uninfected Nasonia vitripennis from the UK and Switzerland (Original data) (Figshare)

Frequency of A. nasoniae in calliphorid fly pupae collected from European Bird's nests (Original data) (Figshare)

Presence of Nasonia wasps and the bacterium Arsenophonus nasoniae in calliphorid fly pupae collected from great tit nests in Coimbra, Portugal. (Original data) (Figshare)

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