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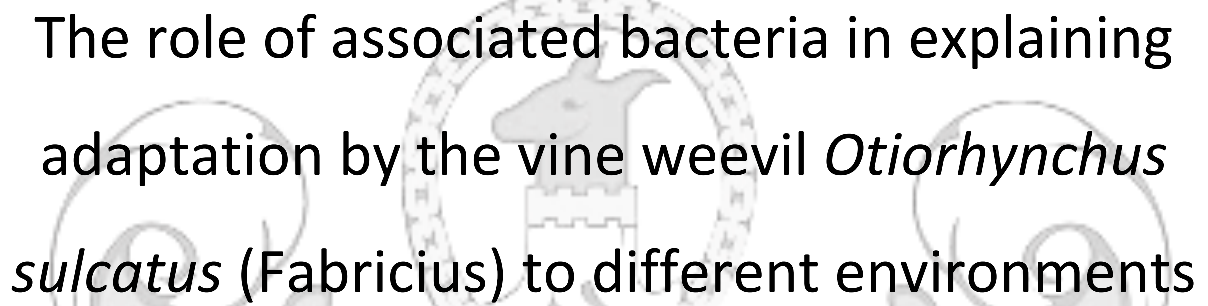

The role of associated bacteria in explaining adaptation by the vine weevil *Otiorhynchus sulcatus* (Fabricius) to different environments

by

Pilar Morera Margarit

A thesis submitted in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy

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Candidate's declaration

I, Pilar Morera Margarit, do hereby certify that this thesis has been written solely by me, and that it is the record of work carried out by me, or principally by myself in collaboration with others as acknowledged, and that it has not been submitted, in whole or part, in any previous application for a degree.

Pilar Morera Margarit

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Abstract

The vine weevil *Otiorhynchus sulcatus* (Fabricius) is a curculionid pest endemic to central Europe which has successfully invaded regions on nearly all continents. This weevil species is characterised by an ability to feed on a broad range of plant species, several of which are grown as horticultural crops. Research investigating the distinctiveness of insects inhabiting separate geographic areas or infesting different crop species is still scant. Genetically, vine weevils from different locations have been shown to display limited genomic differences. The research presented here further investigated if features other than insect genetic variation are distinct amongst vine weevil populations found at different locations. The bacterial community of adult vine weevils from different populations found at geographically distinct locations within the UK but from the same host plant species was characterised. Additionally, some of these populations were tested for variation in susceptibility to a fungal pathogen commonly included in control strategies targeting this pest, *Metarhizium brunneum* (Petch). Oviposition choice was also examined on these insects between two host plant species susceptible to vine weevil infestation, strawberry and raspberry. Additionally, the fluctuation in titre of the bacterium *Candidatus* Nardonella during vine weevil development was assessed. This prokaryote was shown to dominate the bacterial community of the vine weevil and so we aimed at identifying important functions for its host. Insect population source *per se* did not shape the bacterial microbiota or influence the insect fitness parameters measured. *Ca.* Nardonella abundance followed a pattern which may indicate its involvement in tyrosine metabolism and cuticle formation. Future research should investigate vine weevils from a wider selection of geographic locations including non-crop environments to complete this line of research. Additionally, the influence of the host plant species on the distinctiveness of vine weevil populations should also be tested. *Ca.* Nardonella has been shown to play important roles for other weevils. Hence, the function of this bacterium for the vine weevil deserves further attention.

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1 # Chapter 1

2 ## Introduction[1]

3 ### 1.1. Brief overview of the thesis

4 *Otiorhynchus sulcatus* (Fabricius), commonly known as the black vine weevil or simply the
5 vine weevil, is a beetle belonging to the weevil superfamily Curculionoidea (Alonso-Zarazaga
6 and Lyal, 2002). This curculionid species is native to central Europe although its range has
7 expanded to give an almost worldwide distribution, due mainly to increasing plant trade in the
8 last century (Kingsley, 1898; Masaki et al., 1984; Moorhouse et al., 1992; Prado, 1988). The
9 vine weevil is considered a highly polyphagous pest with more than 150 host plant species
10 susceptible to feeding by this curculionid, among them economically important soft fruit crops
11 such as strawberry and raspberry as well as ornamental crops such as roses or cyclamens
12 (Moorhouse et al., 1992; Smith, 1932; Warner and Negley, 1976). On a global scale, it is
13 difficult to estimate annual damage caused by this pest, yet, at a local scale, it was estimated
14 that vine weevil damage cost UK strawberry growers £14M in 2016 (Wynn, 2010).

15 The mechanism by which the vine weevil has been able to invade such a wide range of
16 environments remains yet to be discovered. It has been hypothesised that environmental
17 conditions met at the invaded regions pose a challenge for the survival and reproduction of
18 this insect. Nonetheless, to date this has not been empirically tested. For instance, the vine
19 weevil is able to feed and reproduce in a great variety of plants from outside its native area
20 (Masaki et al., 1984; van Tol et al., 2004). If abiotic conditions are considered, differences can
21 also be found between areas inhabited by the vine weevil. For instance, Scotland is generally
22 colder and has higher rainfall than is experienced England. The temperature difference oscillates
23 from 1.77°C and 2.8°C when minimum and maximum temperatures are considered,
24 respectively. Additionally, on average there is a difference of 59 mm of rain between these
25 two areas (data taken from www.metoffice.gov.uk database, years considered 2010 to 2019).
26 All vine weevils studied so far are triploid females, which reproduce by thelytokous or mitotic

[1] Part of the following chapter has been published in: Morera-Margarit, P, Pope, TW, Mitchell, C, Karley, AJ. Could bacterial associations determine the success of weevil species? Annals of Appiedl Biology (2020); 1– 11. https://doi.org/10.1111/aab.12625

27 parthenogenesis (Lundmark, 2010). On the one hand, clonal reproduction is advantageous as
28 it allows weevils to establish a new population from a single female and avoid energy costs
29 invested in courtship and mating (Lundmark, 2010). On the other hand, this reproduction
30 strategy provides low genetic variability and so it could be detrimental for adaptation to new
31 niches. This is compensated in other polyploid parthenogenetic species of weevil by
32 hybridisation between closely related species before experiencing polyploidisation (Kotásková
33 et al., 2018; Lundmark and Saura, 2006; Saura et al., 1993; Stenberg and Lundmark, 2004).
34 Nonetheless, unlike other polyploid weevil species, the vine weevil is a clonal polyploid lineage
35 of non-hybrid origin (Lundmark, 2010). Then, what is the secret of the vine weevil?

36 In the current PhD project I hypothesised that the vine weevil could survive and reproduce in
37 different niches via its associated bacteria, benefiting from these associations to expand the
38 distribution range. It has not previously been tested if bacteria can directly influence the
39 expansion range in other insects. Nonetheless, it has been proposed that the benefits derived
40 from the insect-bacteria associations could indirectly contribute to increase the expansion
41 range of the insect host (reviewed by Shapira, 2016 and also later in this section). The study
42 presented here was designed to investigate if the vine weevil microbiota is key in vine weevil
43 adaptation by studying insects collected from different areas within the UK. Understanding the
44 role of bacteria for the insect fitness could contribute to improve Integrated Pest Management
45 (IPM) strategies. For instance we could design strategies targeting specifically relevant
46 symbionts (explained later in this section). Additionally, in light of the results described in the
47 first chapter, the role of a specific bacteria for this weevil species was also examined. The
48 current thesis is divided into six chapters:

49 1) The first chapter contains a broad review on weevil-bacteria associations within an
50 agricultural context, followed by a more specific review on the vine weevil.

51 2) The second chapter is an experimental chapter that describes the characterisation of
52 the bacterial community associated with vine weevil adults sampled from populations at
53 different locations within the UK.

54 3) The third chapter is a method chapter that describes a) the vine weevil DNA extraction
55 optimisation process and b) the optimisation of an artificial diet for mass rearing vine weevil
56 insects.

57 4) The fourth chapter is a short experimental chapter which describes differences
58 between vine weevil populations in susceptibility to a fungal pathogen, *Metarhizium*
59 *brunneum* (Petch), commonly used in control strategies targeting this pest.

60 5) The fifth chapter is a short experimental chapter that describes differences in
61 oviposition preference of vine weevil adults from two geographically distinct populations to
62 two host plant species, strawberry and raspberry.

63 6) The sixth experimental chapter describes the variation in abundance through different
64 life stages of *Candidatus* Nardonella, a dominant bacterium in the vine weevil microbiota.

65 7) The seventh chapter comprises the general discussion and future perspectives.

66

1.2. The role of bacterial associations in determining the success of agriculturally important species of weevil

The Green revolution movement in agriculture was born in the 1940s in Mexico from where it rapidly spread to many parts of the world aiming to provide sufficient food for a growing human population (Jacques and Jacques, 2012; Smith et al., 1976; Wouw et al., 2010). The Green revolution led to what we now refer to as conventional agriculture and is characterised by substituting a wide range of genetically diverse crop landraces with fewer high-yielding modern crop varieties, which displayed a high dependency on synthetic chemical fertilisers and pesticides. These practices consequently decreased the genetic diversity of crop species and contributed to the appearance of pesticide-resistant pests and pathogens (Jacques and Jacques, 2012; Smith et al., 1976; Wouw et al., 2010). Integrated Pest Management (IPM) arose in response to the negative side-effects of a reliance on synthetic pesticides in modern conventional agriculture and advocates development of more sustainable practices with an agroecosystem multidisciplinary approach (Oerke, 2006; Oerke and Dehne, 2004; Savary et al., 2019; Smith et al., 1976). Increasingly, there is a societal shift towards the adoptions of more environmental friendly pest management options as can, for example, be seen in the encouragement from the European Commission to implement IPM practices (Remáč, 2009). Despite the many advances in the agriculture over recent years, it is estimated that at a global scale annual yield losses due to pests and pathogens on five of the major crops, i.e. wheat, rice, maize, potato and soybean, is 20–30% (Oerke, 2006; Savary et al., 2019). It is, therefore, necessary to improve our knowledge of the biology and ecology of each pest to design more effective control practices. In this context, understanding the role of insect associated bacteria could provide valuable insights to improve current IPM control methodologies.

The nature of the association between insects and bacteria has been a controversial point for decades. In the early 1900s, a few scientists started to hypothesise that the presence of bacteria in insects was not a random event as bacteria seemed to be involved in important insect processes (reviewed by Steinhaus, 1940). These studies were limited by the available techniques at that time, mainly microscopy and culturing, to study microorganism morphology and physiology. Yet, soon scientists noticed the limitations of culturing bacteria. Some microorganisms were reluctant to grow in media as they required specific nutrients, temperatures or pressures. Other microbes could not be cultured due to their intracellular nature. Consequently, the diversity of existing microbes was overlooked by the simple fact that they could not be observed growing in artificial media (reviewed by Handelsman, 2004). In the late 1990s, bacterial studies experienced a paradigm shift when Carl Woese determined the 16S rRNA gene sequence from the small prokaryotic ribosome subunit as a molecular

102 chronometer that could be used as a taxonomy identifier (Woese, 1987). This gene, shared
103 by all prokaryotes, harbours non-coding regions that accumulate sequence alterations at a
104 constant rate that can be used to classify bacteria and archaea. This new approach enabled
105 the identification of prokaryotes taxonomically from complex samples in a culture independent
106 manner. Contemporarily, the techniques employed to sequence nucleotide molecules
107 advanced rapidly. In 1977, Sanger´s chain termination or dideoxy sequencing technique was
108 developed and its automation permitted deciphering nucleotide sequences of almost one
109 kilobase in length (Sanger et al., 1977). Successively, this methodology was improved by
110 using pyrophosphate as a signal molecule to detect nucleotide incorporation (Hyman, 1988).
111 In 2005, a sequencing machine was designed to automate the pyrophosphate technique,
112 allowing the parallelisation of sequencing reactions (Margulies et al., 2005). This improvement
113 created a platform for mass sequencing at an unprecedented time/cost efficiency, which
114 represented the beginning of Next Generation Sequencing. Additional methodologies
115 appeared *a posteriori* such as Illumina (Turcatti et al., 2008), SoliD (Shendure et al., 2005) or
116 Ion torrent (Rothberg et al., 2011) for mass parallel sequencing using alternative
117 methodologies to the initial pyrophosphate (reviewed by Heather and Chain, 2016).

118 The association between multicellular organisms and bacteria has received a great deal of
119 interest in recent decades. Stable associations between two or more organisms, frequently
120 termed symbiosis, is a widespread phenomenon in nature with outcomes ranging from
121 negative to neutral to beneficial, often classified as parasitism, commensalism or mutualism,
122 respectively. Microorganisms that locate within the host cell will be classified as
123 endosymbionts while if they locate outside the cell will be classified as ectosymbionts. These
124 associations are also categorised based on the grade of dependency as primary symbionts,
125 which show strong interdependence and have typically long co-evolutionary history with the
126 host, and facultative symbionts, which show more recent association and are not strongly
127 interdependent (reviewed by Moya et al., 2008).

128 Technological innovations, together with the use of the 16S rRNA gene to identify prokaryotes,
129 have enabled remarkable progress in studies focused on insect-bacteria associations. To
130 date, many studies have demonstrated the potential importance of these partnerships for host
131 fitness. Some insects with restricted diets rely on bacteria to compensate nutritional
132 deficiencies. For instance, the pea aphid *Acyrthosiphon pisum* (Harris) is provided with
133 essential amino acids and the vitamin riboflavin by its obligate endosymbiotic bacterium
134 *Buchnera aphidicola* (Nakabachi and Ishikawa, 1999) and the tsetse fly *Glossina morsitans*
135 (Westwood) is provided with essential vitamins by the endosymbiotic bacterium
136 *Wigglesworthia glossinidia* (Nogge, 1981). Furthermore, bacteria can improve insect host
137 fitness by degrading toxic secondary metabolites produced by plants as a chemical defence.

138 This is the case for the coffee berry borer *Hypothenemus hampei* (Ferrari) which harbours
139 *Pseudomonas* bacteria that detoxify caffeine by expressing caffeine demethylase genes
140 (Ceja-Navarro et al., 2015). Importantly, certain bacteria have been shown to render their
141 insect hosts less susceptible to predators and pathogens. This has been illustrated for the pea
142 aphid, which is protected from parasitism by the parasitoid wasp *Aphidus ervi* (Haliday) when
143 aphids are infected with the bacterium *Hamiltonella defensa* (Oliver et al., 2005, 2003) and
144 from infection by the entomopathogenic fungus *Pandora neoaphidis* (Remaud & Hennebert)
145 when aphids harbour the bacterium *Regiella insecticola* (Scarborough et al., 2005). Bacteria
146 can also influence host reproduction as in the case of members from the genus *Wolbachia*,
147 which increase fecundity on the fruit fly *Drosophila simulans* (Sturtevant) (Weeks et al., 2007).

148 The weevil superfamily Curculionoidea is one of the largest insect groups with more than
149 60,000 described species (Alonso-Zarazaga and Lyal, 2002), among them agricultural pest
150 species that are distributed around the world. For instance, the red palm weevil
151 *Rhynchophorus ferrugineus* (Olivier) is an important pest of palm trees that causes
152 considerable economic losses to countries in the Gulf, Middle East and Europe (FAO, no
153 date). The sibling weevils *Sitophilus oryzae* (Linnaeus) and *S. zeamais* (Motschulsky)*,* rice
154 and maize weevil, respectively, are devastating pests of stored grain, rice, maize, barley and
155 wheat worldwide (Grahame, 2017a, 2017b). Also, the vine weevil damages a wide range of
156 horticultural crops around the world (Buxton and Pope, 2011; Wynn, 2010). Weevil species
157 are, therefore, numerous and problematic for farmers globally, however, studies focused on
158 understanding weevil bacteria associations are still scarce when compared to other insect
159 families. For instance, a Web of Science search for bacteria associated with the search terms
160 'aphid*' AND 'bacteria*' returns 1,512. However, a similar Web of Science search using the
161 terms 'weevil*' AND 'bacteria*' returns only 335 results. Therefore, the numbers of studies
162 reported does not reflect the fact that there are only 5,000 described species of aphids
163 (Blackman and Eastop, 2000) compared with over 60,000 described weevil species (Alonso-
164 Zarazaga and Lyal, 2002). In the following sections, research on weevil bacteria associations
165 is discussed and the implications of these associations for the economic importance of weevils
166 considered. The intention here is to highlight the relevance of these associations for the pest
167 status and control of weevils, underlining the existing knowledge gaps to encourage future
168 investigations in this field. Advancements in this research area will ultimately contribute to the
169 development of improved weevil pest control strategies.

1.2.1. Tougher than tough: *Candidatus* Nardonella, an important player in cuticle formation

Studies of weevil-associated bacteria have typically focused on the symbiotic association between the bacterium *Candidatus* Nardonella and different weevil species. Research started at the beginning of the 1990s with the observation of intracellular microorganisms confined in specialised cells, called bacteriocytes, in the rice weevil *Calandra oryzae* (Linnaeus), although it remained undetermined whether the observed bacteria constituted a “symbiotic organ” or were simply “accessory cells” (Mansour, 1930, 1927; Pierantoni, 1927). Further investigation combining molecular techniques and fitness measures showed that these bacteria were present in different weevil species and were involved in adult development (Campbell et al., 1992; Nardon and Grenier, 1988). Nonetheless, it was not until the beginning of the 21st century that Lefèvre et al. (2004), using a phylogenetic analysis of the 16S rRNA gene, identified this microorganism as a γ-proteobacterium and designated the new lineage *Candidatus* Nardonella.

Candidatus Nardonella has since been shown to be widespread throughout the weevil superfamily and is estimated to have become associated with weevils 125 million years ago (Conord et al., 2008; Lefèvre et al., 2004a). Nevertheless, some studies have found that *Ca.* Nardonella has been replaced in species of the genus *Curculio* and *Sitophilus*, highlighting the dynamic nature of insect-bacteria associations (Conord et al., 2008; Lefèvre et al., 2004a; Toju et al., 2010, 2013). Subsequent studies focused on identifying *Ca.* Nardonella in other weevil species and on studying other features of its biology, such as population dynamics during different insect life stages or the location of the *Ca.* Nardonella bacteriocytes in insect tissues (Conord et al., 2008; Hosokawa et al., 2015; Hosokawa and Fukatsu, 2010; Huang et al., 2016; Mansour, 1930; Nardon et al., 2002; Toju and Fukatsu, 2011). These studies have confirmed that *Ca.* Nardonella is strictly intracellular, which is an indicator of the process of co-evolution with its weevil host (reviewed by Moya et al., 2008). The bacteriocytes harbouring this bacterium are organised in an organ called bacteriome that makes petal-like lobes around the larval gut. In female adults the larval bacteriome disappears and bacteria move to the tip of the ovarioles (Anbutsu et al., 2017). Importantly, Anbutsu et al. (2017) working on the black hard weevil, *Pachyrhynchus infernalis* (Fairmaire), showed that *Ca.* Nardonella is involved in insect cuticle formation by contributing to tyrosine synthesis and its suppression, by administering antibiotics in the diet at a larval stage, produced adults with low tyrosine titres and reddish, crumpled and/or deformed elytra. Similarly, Kuriwada et al. (2010) eliminated *Ca.* Nardonella from the West Indian sweet potato weevil, *Euscepes postfasciatus* (Fairmaire), larvae by antibiotic treatment obtaining smaller adults with reddish cuticle.

205 The insect cuticle represents the first level of interaction between the insect and its
206 environment and has been shown to play an important role in intra- and interspecific
207 communication, water loss reduction and protection against predators and pathogens (Gibbs
208 et al., 1991; Hamilton et al., 2011; Hamilton and Bulmer, 2012a; Howard and Blomquist, 2005;
209 Lockey, 1988; Ortiz-Urquiza and Keyhani, 2013; Tseng et al., 2014; Turillazzi et al., 2000;
210 Weissling and Giblin-Davis, 1993). Despite the importance of the cuticle, the presence of *Ca.*
211 Nardonella in the black hard and sweet potato weevils seems not to be essential for survival
212 in laboratory conditions (Anbutsu et al., 2017; Kuriwada et al., 2010). Nonetheless, in a more
213 natural environment the developmental defects derived from the absence of this symbiont
214 likely impair the fitness of the insect. The importance of the weevil cuticle is also revealed in
215 *Sitophilus* weevils. In this weevil genus, *Ca.* Nardonella was substituted by another bacterial
216 symbiont named *Sitophilus pierantonius* or SPE. This symbiont, amongst other functions,
217 provides the weevil host with tyrosine, in a similar way to *Ca.* Nardonella. These amino acids
218 are then invested in the formation of the adult cuticle. In this way, symbionts ensure that adult
219 insects form a thick and protective cuticle in shorter time (Vigneron et al., 2014; Wicker and
220 Nardon, 1982). This similar functionality in two separate symbiont species illustrates the
221 importance of the weevil cuticle. Although tyrosine is not an essential amino acid, it is possible
222 that the extra tyrosine produced by weevils carrying symbionts provided an evolutionary
223 advantage by ensuring more rapid polymerisation of the exoskeleton even on nutritiously poor
224 diets, which may have broadened their range of host plant species.

225 Cuticle colouration was shown to be important in deterring predatory lizards in the weevils
226 *Pachyrhynchus tobafolius* (Kano) and *Kashotonus multipunctatus* (Kôno) (Tseng et al., 2014).
227 The cuticle was also shown to reduce water loss in the weevil species *Rhynchophorus*
228 *cruentatus* (Fabricius) (Weissling and Giblin-Davis, 1993). Although not a weevil, saw-toothed
229 grain beetle *Oryzaephilus surinamensis* (Linnaeus) associated symbionts were also shown to
230 be involved in cuticle melanisation and resistance to desiccation (Engl et al., 2018). The
231 subterranean termites *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) secrete β-1,3-
232 glucanase onto the cuticle that prevents infection by the pathogenic fungus *Metarhizium*
233 *brunneum* (Petch) (Hamilton et al., 2011). This fungus is commonly used in pest control
234 strategies targeting a wide variety of arthropods, including weevils. Although the role of weevil
235 cuticle in protection against pathogens has not been scrutinised yet, it has been suggested
236 that a thick cuticle could improve the mechanical defence against predators (reviewed by
237 Lemoine et al., 2020). It would therefore be interesting to test if the physical properties of the
238 cuticle as well as cuticular secretions protect weevils from such pathogens.

239 ### 1.2.2. To fly or not to fly: *Sitophilus oryzae* and *S. zeamais* bacteria involved
240 in flight activity

241 The possibility of bacteria compensating for amino acid and vitamin deficiencies in the diet of
242 the insect was investigated for the rice weevil and the maize weevil by comparison of untreated
243 insects with insects that were bacteria-free as a result of a heat treatment. In this way, Wicker
244 and Nardon (1982) and Wicker (1983) showed that bacteria in these weevils are involved in
245 the acquisition of phenylalanine and tyrosine (Wicker and Nardon, 1982) and in the synthesis
246 of the vitamins pantothenic acid, biotin and riboflavin (Wicker, 1983). A subsequent study by
247 Gasnier-Fauchet and Nardon (1987) also suggested that bacteria were involved in the
248 metabolism of the amino acid methionine and its derivates sarcosine and methionine
249 sulfoxide.

250 Several studies have shown that the influence of bacteria for host fitness goes beyond
251 compensating for poor diets. Heddi et al. (1993) observed in rice weevils that mitochondrial
252 activity was higher in weevils with bacteria compared to weevils without bacteria, although
253 mitochondrial enzymatic activities were absent in bacteria isolated from these weevils. Hence,
254 based on earlier discoveries of bacteria involved in amino acid and vitamin metabolism, it was
255 suggested that bacteria could be providing mitochondria with intermediary metabolites to
256 maintain normal activity. Grenier et al. (1994) reported drastically reduced or null flight activity
257 in both the rice weevil and the maize weevil when individuals were deprived of bacteria by
258 heat treatment, and that this effect could in some cases be partially restored by enriching diets
259 with vitamins. Flight requires a large quantity of ATP, which is provided from cellular respiration
260 via the mitochondrial Krebs cycle. Thus, in light of these and previous discoveries, it was
261 proposed that bacteria in these two species of weevil contribute to the Krebs cycle by providing
262 mitochondria with necessary coenzymes, such as pantothenic acid or riboflavin, to allow for
263 production of sufficient energy to fly.

264 Intensive pesticide usage has promoted the evolution of insecticide resistance in populations
265 of *S. zeamais* across Brazil and Mexico (Braga et al., 2011; Guedes et al., 2006, 1994; Oliveira
266 et al., 2007; Perez-Mendoza, 1999; Ribeiro et al., 2003). Resistance to synthetic chemical
267 insecticides in this weevil species may be due to detoxifying enzymatic activities (Fragoso et
268 al., 2003) but also from flying to actively avoid chemically treated areas. Guedes et al. (2009)
269 and Braga et al. (2011) examined flight take off frequency in resistant and susceptible
270 populations of *S. zeamais* in response to pyrethroid and organophosphate pesticide exposure.
271 These studies showed that, independently of physiological resistance to these chemicals,
272 weevils had the ability of escaping the sprayed zone by flying away. These findings highlight
273 the importance of considering behavioural avoidance, i.e. flying to escape the chemically

274 treated zone, when evaluating resistance to chemical control. Thus, given the role of bacteria
275 for flight activity in *S. oryzae* and *S. zeamais,* the possibility of manipulating bacteria to reduce
276 pesticide resistance could be investigated to improve integrated pest management strategies
277 targeting these pests.

278 1.2.3. The complicated case of weevil parthenogenesis: can *Wolbachia* 279 manipulate weevil reproduction?

280 Bacteria of the genus *Wolbachia* are intracellular α-proteobacteria initially observed by Hertig
281 and Wolbach (1924) in the ovaries of the mosquito *Culex pipiens* (Linnaeus). Currently,
282 *Wolbachia* is considered to be widely spread among arthropods and it has been estimated to
283 infect 66% of species within this phylum (Hilgenboecker et al., 2008). This bacterium may
284 inhabit host ovaries and testes, but it can also inhabit somatic tissues such as the brain,
285 muscles, the midgut or the salivary glands (Dobson et al., 1999). This prokaryote is transmitted
286 to the progeny vertically through the germ line, however, host-bacterium phylogenetic
287 incongruences have revealed that *Wolbachia* can also be transmitted horizontally (O'Neill et
288 al., 1992; Werren et al., 1995). The combination of these two transmission routes has enabled
289 bacteria from this genus to spread intra- and inter-specifically between arthropods coinhabiting
290 the same environment. For example, *Wolbachia* is transmitted to a parasitic wasp *Leptopilina*
291 *boulardi* (Barbotin, Carton and Keiner-Pillault) horizontally from its infected host *D. simulans*
292 and successively transmitted to the wasp offspring for at least three generations (Heath et al.,
293 1999). The success of *Wolbachia* also lies in its ability to induce host phenotypes that have
294 led to consider this bacterium as a reproductive parasite. The most common reproductive
295 manipulation is cytoplasmic incompatibility, which occurs when two individuals with different
296 *Wolbachia* cytoplasmic load, i.e. with or without *Wolbachia* or with different *Wolbachia* strains,
297 mate and as a result paternally inherited chromosomes are not transferred to the offspring.
298 *Wolbachia* can also induce asexual reproduction or parthenogenesis by aborting the first
299 mitosis that originates the haploid female gamete, consequently obtaining a diploid egg cell
300 that develops into a female (Stouthamer et al., 1999).

301 The weevil superfamily is known for harbouring many polyploid species that reproduce by
302 thelytokous apomictic parthenogenesis in which egg cells are generated by mitosis and
303 develop into female offspring (Saura et al., 1993; Suomalainen, 1962; Suomalainen et al.,
304 1987). It was initially proposed that parthenogenesis in weevils emerges as a result of a two-
305 step stochastic process. First, insects would hybridise with a closely related species
306 generating a new lineage with higher genetic diversity. This event would then be followed by
307 the fusion of unreduced gametes generated by meiotic errors consequently increasing the
308 chromosomic load of the lineage. The meiosis required for sexual reproduction would as a

309 result be more complex for polyploids with even numbers of chromosomes. By contrast,
310 polyploids with odd numbers of chromosomes would always generate non-viable gametes
311 with abnormal chromosome loads. This would consequently promote the appearance of
312 parthenogenesis as the most effective or only, for polyploids with uneven number of
313 chromosomes, possible reproduction strategy (Saura et al., 1993). This has been seen for
314 instance in *Otiorhynchus scaber* (Linnaeus) in which polyploid parthenogenetic forms arose
315 from hybridisation with *O. nodosus* (Robinson) and subsequent fusion of gametes that had
316 not undergone the meiotic chromosome reduction (Stenberg et al., 2000; Stenberg and
317 Lundmark, 2004). Nonetheless, hybridisation is not the only mechanism by which asexual
318 reproduction arose in species of weevil as for instance the parthenogenetic triploid vine weevil
319 is of non-hybrid origin (Lundmark, 2010). Which other factors could therefore promote
320 parthenogenesis in weevils? Based on the role of *Wolbachia* in inducing asexual reproduction
321 in a variety of insect species, such as thelytokous parthenogenetic spider mite species from
322 the genus *Bryobia* (Weeks and Breeuwer, 2001), the involvement of this bacterium in the
323 origin of weevil parthenogenesis has been investigated.

324 *Wolbachia* studies in weevils began when cytoplasmic incompatibility caused by this
325 proteobacterium was discovered while studying different populations of invasive alfalfa
326 weevils *Hypera postica* (Gyllenhal) in the US (C. Hsiao and Hsiao, 1985; T. H. Hsiao and
327 Hsiao, 1985; Leu et al., 1989). Subsequent studies investigated the influence of bacteria for
328 the reproduction of the rice water weevil *Lissorhoptrus oryzophilus* (Kuschel) by comparing
329 invasive populations in China, which reproduce asexually, with a native population from the
330 US, which reproduces sexually. *Wolbachia* was present in weevils from all locations studied
331 regardless of the reproduction strategy, implying it is not involved in promoting
332 parthenogenesis. Later investigations by Rodriguero et al. (2010) and Elias-Costa et al. (2019)
333 nonetheless found a strong correlation between the presence of *Wolbachia* and
334 parthenogenesis in weevils of the tribe Naupactini. By contrast, Stenberg and Lundmark
335 (2004) recorded *Wolbachia* almost exclusively in sexual forms of *O. scaber* rather than in
336 asexual forms meaning that this bacterium was not behind the origin of parthenogenesis, at
337 least for this species of weevil. Similarly, Lachowska et al. (2008) suggested that weevil
338 parthenogenesis originated from meiotic chromosome reduction failure in a sexual ancestor
339 in weevils from the subfamily Entiminae. In this study, vestiges of meiosis were observed in
340 developing eggs, indicating that apomictic parthenogenesis evolved from automictic
341 parthenogenesis, as this second type of parthenogenesis includes meiosis. This was
342 previously proposed by Saura et al. (1993). Mazur et al. (2016) studied the genetic variability
343 of the nuclear, mitochondrial and *Wolbachia* DNA in various populations of the
344 parthenogenetic weevil *Eusomus ovulum* (Germar). Nonetheless, the role of *Wolbachia* in

345 parthenogenesis in this weevil species remains controversial. On one hand, as the same
346 *Wolbachia* strain was shared by all weevil populations studied, it was suggested that this
347 prokaryote may have caused parthenogenesis. On the other hand, this *Wolbachia* strain was
348 present in other weevil species coinhabiting the same area. This, together with the low
349 bacterial genomic variation observed between the weevil populations, suggested that this
350 reproductive parasite might have been acquired recently irrespective of the origin of
351 parthenogenesis.

352 Research into the influence of *Wolbachia* on weevil reproduction has not yet reached a final
353 conclusion. Alternatively, other hypotheses to explain the prevalence of *Wolbachia* in
354 parthenogenetic weevils have been proposed. Lachowska et al. (2010) investigated the
355 presence of *Wolbachia* in 40 European weevil species, from four subfamilies within the family
356 Curculionidae, and related the infection status to adaptation and reproduction. In this study,
357 *Wolbachia* was recorded almost twice as frequently in parthenogenetic weevils compared to
358 sexual weevils. However, it was proposed that rather than inducing parthenogenesis,
359 *Wolbachia* might be benefiting from infecting these weevils by increasing its chances of
360 transmission. Kotásková et al. (2018) recorded the presence of *Wolbachia* in two sibling
361 species of weevil, the parthenogenetic weevil *Strophosoma melanogrammum* (Forster), and
362 the sexual *S. capitatum* (De Geer). Based on the higher heterozygosity of the asexual species
363 compared to the sexual species, it was proposed that parthenogenesis originated from
364 hybridization, similarly to *O. scaber* (Stenberg et al., 2000). Interestingly, the parthenogenetic
365 species harboured only one *Wolbachia* strain whereas among the sexual species three strains
366 were found, different to the strain found in the asexual counterpart, which were possibly
367 acquired through horizontal transmission. Thus, it was suggested that instead of inducing
368 parthenogenesis, *Wolbachia* may have induced speciation in an ancestor of these sibling
369 weevils by imposing a reproductive barrier. *Wolbachia* is a widespread bacterium among
370 weevils and further research should aim to investigate if it influences other fitness parameters
371 in weevils. For instance reducing *Wolbachia* titre by antibiotic treatment in eggs of the rice
372 water weevil and the vine weevil diminished the number of hatched eggs (Chen et al., 2012;
373 Son et al., 2008). Also, *Wolbachia* has been shown to protect *Drosophila melanogaster*
374 (Meigen) from pathogenic RNA viruses (Teixeira et al., 2008). Thus, future studies should also
375 focus on understanding the role of *Wolbachia* for other aspects of weevil biology.

376 *Wolbachia* has been considered in the context of agriculture with the insect incompatibility
377 technique, usually abbreviated as IIT. This is a pest control strategy that exploits the
378 cytoplasmic incompatibility induced by bacteria from the genus *Wolbachia*. Males of the target
379 pest are artificially inoculated with a *Wolbachia* strain that will create a reproductive barrier
380 with the female of the target pest. Upon mass release, males mate with wild females and as

381 a consequence of unsuccessful mating the pest population is diminished (Brelsfoard and
382 Dobson, 2009). Despite *Wolbachia* being a commonly found bacteria amongst weevils, the
383 application of IIT for the control of this insect group has been limited. Importantly, *Wolbachia*
384 can be horizontally transferred within and between species as was seen between the rice
385 weevil and the maize weevil, and between the maize weevil and its parasitoid wasp *Theocolax*
386 *elegans* (Westwood) (Carvalho et al., 2014). The horizontal transmission of the artificially
387 introduced *Wolbachia* could bridge the reproductive incompatibility and could ultimately
388 spread to target as well as to non-target organisms within the same habitat. Although it
389 remains unclear what role this bacterium plays in parthenogenesis in weevils, the application
390 of IIT could potentially inadvertently give rise to an asexual strain that may be better able to
391 spread, as has been seen for *O. scaber* (Stenberg and Lundmark, 2004). Further research is,
392 therefore, needed to clarify if *Wolbachia* can be used as an IPM strategy, such as IIT.

393 #### 1.2.4. Back from the future: bacterial community studies in weevils, a fast-
394 moving field

395 The 21st century has seen a large number of studies investigating insect bacterial
396 communities. For instance, Web of Science database search for 'Insect*' AND 'Bacteria*
397 returns 14,044 results (Web of Science, 18th of May, England, UK).This has been due largely
398 to recent improvements in the available sequencing technology. These studies have
399 taxonomically characterised bacteria inhabiting a great variety of insect species. Gut
400 microbiota has received special attention due to its importance in shaping insect-plant
401 interactions (Frago et al., 2012). Colman et al. (2012) for instance compared the gut bacterial
402 communities of 62 insect species and showed that taxonomy as well as diet are important in
403 determining the composition of gut bacterial microbiota. By contrast, Jones et al. (2013)
404 showed that diet has little influence in bacterial community composition. This study developed
405 a cross-taxon bacterial community analysis of 28 insect families and revealed that the bacterial
406 community is more similar amongst closely related taxa.

407 Weevil bacterial communities have hitherto received relatively little attention, and so studies
408 are limited to a few species in which the influence of the life stage, the diet or the geographic
409 location for the bacterial microbiota composition has been studied. The life cycle in weevils is
410 holometabolous and so they experience a complete metamorphosis. Variation has been
411 observed between the life stages mainly due to the pupal stage harbouring a less diverse gut
412 microbiota in comparison to larvae and adults for the bark beetle *Dendroctonus rhizophagus*
413 (Thomas and Bright) (Morales-Jiménez et al., 2012), the pine engraver *Ips pini* (Say)
414 (Delalibera et al., 2007) and the Chinese white pine beetle *Dendroctonus armandi* (Tsai and
415 Li) (Hu et al., 2013). Diet has been shown to be a major factor in shaping the bacterial

416 community for different weevil species in agreement with Colman et al. (2012) while in
417 contradiction with Jones et al. (2013). Merville et al. (2013) showed that four *Curculio* species
418 coinhabiting oak trees had a very similar bacterial community composition despite being
419 separate species, indicating that the food source may exert an influence on the microbiota.
420 Likewise, Berasategui et al. (2016) observed that the bacterial community of the pine weevil
421 *Hylobius abietis* (Linnaeus) was closer to other bark beetles from different locations with a
422 similar diet than to other weevils feeding on non-conifer food sources. Morera-Margarit et al.
423 (2019) also suggested that host plant may play a role in shaping the bacterial community of
424 the vine weevil. Changes in the diet in an experimental set-up were also found to alter the
425 bacterial community in the red palm weevil (Montagna et al., 2015) and the cotton boll weevil
426 *Anthonomus grandis* (Boheman) (Ben Guerrero et al., 2016). Weevil dietary enzymatic
427 requirements seemed in some cases to be provided by the bacterial community composition.
428 For instance, cellulolytic activity was found in the bacterial microbiota of the Chinese white
429 pine beetle (Hu et al., 2014) and the red palm weevil larvae (Muhammad et al., 2017), which
430 is probably required to exploit their natural food source. Interestingly, the gut microbiota of the
431 red turpentine beetle *Dendroctonus valens* (Le Conte) could be involved in the production of
432 a pheromone that depending on the concentration can act as an attractant or as a repellent
433 for conspecific beetles. Variation in bacterial community composition between weevil
434 populations collected at different locations has been studied for the bark beetles *Dendroctonus*
435 *valens* and *D. mexicanus* (Hopkins) in Mexico (Hernández-García et al., 2018) and for the
436 vine weevil in the UK (Morera-Margarit et al., 2019). Both studies found that there is a core
437 microbiota shared by all the populations regardless of the geographic origin.

438 These studies clearly show the importance of bacterial communities for weevil biology and
439 adaptation. Although still developing, this field of research is very promising and future
440 research will benefit from combining existing knowledge on weevil ecology, genetics and
441 evolutionary biology as well as technological advances, to ultimately advance understanding
442 in this area (Christian et al., 2015; Douglas and Werren, 2016). In terms of pest control, there
443 is scant research focused on applying knowledge gleaned through microbiota studies to
444 design pest control strategies. For instance, the characterization of the bacterial community
445 of various native and invasive weevil species in New Zealand identified candidate bacteria
446 involved in resistance to the parasitic wasp *Microctonus aethiopoides* (Loan), used as a
447 biological control against these species of weevil (White et al., 2015). As this is a fast-moving
448 area of research, it is likely that findings in the near future will begin to find their way into IPM
449 programmes targeting weevils that are agricultural pests around the world.

450 ### 1.2.5. The Rosetta stone: translating bacterial community knowledge into
451 ecological knowledge

452 The weevil superfamily is the largest group within the class Insecta and so the largest group
453 in the animal kingdom. This is a fascinating taxon which harbours species adapted to a great
454 variety of environments as well as species with different ploidy levels and reproduction
455 strategies. Furthermore, many species of weevil are economically damaging pests of staple
456 food such as wheat, maize or rice and so have implications for food security. Nonetheless,
457 bacterial studies on weevils are not as abundant in comparison to other insect taxa and the
458 influence of bacteria for weevil biology and pest status requires further attention. For instance,
459 in the cases in which the symbiont *Ca.* Nardonella has been substituted (Conord et al., 2008;
460 Lefèvre et al., 2004a; Toju et al., 2010, 2013), are the replacing symbionts also involved in
461 tyrosine acquisition and cuticle formation? Or, what is the mechanism used by *Wolbachia* to
462 manipulate egg development in the rice and vine weevils (Chen et al., 2012; Son et al., 2008)?
463 The technological innovations developed through studies of other groups of organisms should
464 be applied to study bacteria in weevils with a metagenomic approach to understand variation
465 in bacteria composition and abundance across life stages or between different environments.
466 This will allow the identification of candidate bacteria influencing the development and/or
467 adaptation of the weevil host. Weevil phenotypes derived from the manipulation of these
468 candidate bacteria, for example after antibiotic treatment could reveal meaningful
469 associations. Ultimately, this will allow the translation of knowledge acquired by characterising
470 weevil microbiota to an ecological understanding of these insects and will provide valuable
471 information to design more efficient and sustainable pest control strategies.

472 ## 1.3. A cosmopolitan and polyphagous pest: the vine weevil,
473 *Otiorhynchus sulcatus* (Fabricius)

474 The vine weevil or black vine weevil, *Otiorhynchus sulcatus* (Fabricius), is a member of the
475 weevil superfamily Curculionoidea and the family Curculionidae (Alonso-Zarazaga and Lyal,
476 2002). At the beginning of the 19th century the distribution of this species was limited to central
477 Europe where it is endemic, including the UK (Buxton and Pope, 2011; Moorhouse et al.,
478 1992). Nevertheless, in the last two centuries the distribution of the vine weevil has expanded
479 rapidly, mainly due to plant trade, and this species is now found in most parts of Europe and
480 in parts of North America, South America, New Zealand, and Japan (Kingsley, 1898; Masaki
481 et al., 1984; Moorhouse et al., 1992; Prado, 1988). Vine weevil larvae live below-ground where
482 they feed on plant roots stems and bulbs voraciously. This particular life stage is considered
483 the most dangerous not only because of their voraciousness, but also because the infestation

might develop unseen until the affected plant suddenly wilts and dies (Bennison et al., 2018a; Smith, 1932). Vine weevil adults are wingless beetles that live above-ground where they feed on leaves and flowers making small rounded notches (Smith, 1932). Adult insects are nocturnal and hide in dark places during the day such as plant litter or under plant pots (Buxton and Pope, 2011; Moorhouse et al., 1992). Damage caused by this life stage does not usually result in plant death although may cause problems for marketed plants and ornamentals.

The vine weevil is considered to be a very polyphagous species as together, larvae and adults, can successfully feed on more than 150 host plant species (Moorhouse et al., 1992; Smith, 1932; Warner and Negley, 1976). This host plant range was estimated based only on feeding experiments on the US and Japan (Moorhouse et al., 1992; Smith, 1932; Warner and Negley, 1976). Following experiments to test potential vine weevil plant hosts included fecundity as a fitness parameter, resulting in a narrower host range to previous studies (Tol and Visser, 1998; van Tol et al., 2004). A more recent grower survey has revealed that some of the previously considered highly susceptible plants are rarely attacked by vine weevils, although they can support vine weevil growth when artificially infested (Buxton and Pope, 2011). Vine weevils can adapt to sub-optimal hosts (Clark et al., 2012b; Johnson et al., 2010), hence this contradictory results may be explained by the fact that previous research determined the host range by inoculating plants with eggs or young larvae rather than allowing adult insects to freely oviposit (Buxton and Pope, 2011). These results therefore reflect the importance of complementing controlled experiments with field experiments which reproduce a more real situation.

1.3.1. Catch me if you can: vine weevil control with the entomopathogenic fungus *Metarhizium brunneum* (Petch)

Metarhizium brunneum (Petch), formerly classified as *M. anisopliae*, is a ubiquitous fungus with entomopathogenic activity from the Ascomycota division. This insect pathogen together with *Beauvaria bassiana* ((Bals.-Criv.) Vuill.), another entomopathogenic fungus, constitute 75% of all biocontrol products based on entomopathogenic fungi as both species possess a broad insect host spectrum (de Faria and Wraight, 2007; Jackson et al., 2010).

1.3.1.1. *Metarhizium brunneum* pathogenesis

Fungal pathogenesis in insects occurs as a step-by-step process. The insect cuticle represents the first level of interaction between the insect and its environment. Hence, the first step in fungal pathogenesis constitutes the adherence of fungal spores to the insect outer layer of the cuticle or epicuticle (Hajek and St. Leger, 1994; Moonjely et al., 2016). The insect epicuticle is hydrophobic and so fungal spore adherence is mediated by hydrophobic fungal

518 proteins named hydrophobins. The spore surface of *M. brunneum* is covered by these proteins
519 organised in rodlet layers (Ortiz-Urquiza and Keyhani, 2013; Wang and St Leger, 2007). The
520 role of hydrophobins, however, extends beyond hydrophobic cuticle attachment as these
521 proteins were also shown to be involved in sporulation, growth, spore pigmentation and
522 virulence in *M. brunneum* (Sevim et al., 2012).

523 After attachment, fungal spores start to absorb water and swell before germinating. Then, the
524 rodlet layer of hydrophobins is degraded and is subsequently substituted by a different set of
525 proteins, named adhesins, which will confer the spore a more specific attachment to the insect
526 epicuticle (Wang and St Leger, 2007). In *M. brunneum* the adhesin genes Mad1 and Mad2
527 play an important role in this second adherence step (Wang and St Leger, 2007). MAD1 and
528 MAD2 proteins have been shown to adhere to insect cuticle and plant cells, respectively.
529 MAD1 protein function, however, was also involved in cytoskeleton organisation and Mad1
530 gene disruption triggered defective germination and morphology (Wang and St Leger, 2007).

531 Once the fungal spore has attached to the insect cuticle and starts to germinate, it needs to
532 breach the physical barrier that constitutes the exoskeleton. The penetration process starts
533 with the secretion of hydrolytic enzymes that degrade the cuticular barrier (Ortiz-Urquiza and
534 Keyhani, 2013; Vilcinskas, 2010). In *M. brunneum* at least three proteinases have been
535 identified that degrade the insect cuticle, enabling hyphal penetration: the subtilisin-like serine
536 proteinase Pr1, the trypsin-like serine proteinase Pr2 and a metalloproteinase (Small and
537 Bidochka, 2005; Vilcinskas, 2010). After chemical degradation of the epicuticle surface the
538 fungus forms specialised structures that, by exerting mechanical pressure, penetrate the
539 different layers of the cuticle until the underlying epidermis to finally reach the insect haemocel
540 (Hajek and St. Leger, 1994; Kurtti and Keyhani, 2008; Ortiz-Urquiza and Keyhani, 2013).

541 The insect immune response is directed via humoral and cellular defences. Humoral defences
542 consist of soluble molecules such as antimicrobial peptides, complement- like proteins and
543 phenoloxidases. Cellular defences are mediated by haemocytes which can carry out
544 phagocytosis, encapsulation or clot formation (Lavine and Strand, 2002). Once the pathogenic
545 fungus reaches the insect haemolymph it needs to disperse throughout the insect body and
546 outcompete the insect immune system. Fungal dispersion in the haemolymph is done via
547 hyphal bodies which are yeast-like structures called blastospores and short hyphal fragments
548 (Hajek and St. Leger, 1994; Kurtti and Keyhani, 2008; Moonjely et al., 2016). To avoid
549 recognition by the insect immune system, *M. brunneum* hyphal bodies are coated with a
550 collagenous protein named MCL1. This protein forms a hydrophilic layer that masks the fungal
551 wall β-glucans from the insect haemocytes. These wall components are recognised pathogen
552 associated molecular patterns, or PAMPs, which trigger the immune response by the host.
553 Hence, the MCL1 coating hides the fungal dispersion bodies from the insect immune

554 defences. Additionally, the hydrophilic nature of this coating prevents attachment to host
555 surfaces and is thought to facilitate dispersal throughout the insect hemocoel (Wang and
556 Leger, 2006).

557 *Metarhizium brunneum* can also secrete toxic secondary metabolites, named destruxins, into
558 the host hemocoel, impairing the ability of the insect haemocytes to aggregate (Huxham et
559 al., 1989; Kershaw et al., 1999; Samuels et al., 1988). This was observed *in vivo* when
560 cockroaches *Periplaneta Americana* (Linnaeus) were injected with destruxins followed by an
561 injection with β-glucan. *In vitro,* insect haemocytes incubated with destruxins reduced the
562 humoral as well as the cellular immune response by reducing the production of phenoloxidase
563 and the ability of the cells to aggregate (Huxham et al., 1989).

564 The fungal pathogen spreads through the insect body and eventually kills its host. Insect death
565 results from a combination of factors. Mechanical cellular damage leads to water loss and
566 dehydration of the insect. Blastospores absorb insect nutrients, which in combination with gut
567 cellular damage due to destruxins leads to starvation of the insect (Branine et al., 2019;
568 Gillespie and Claydon, 1989; Kershaw et al., 1999; Samson et al., 2013; Samuels et al., 1988;
569 Shah et al., 2005; Shang et al., 2015). Destruxins can additionally induce muscle tetanic
570 paralysis followed by flaccid paralysis which, in a dose dependent manner, may also result in
571 insect death (Kershaw et al., 1999; Samuels et al., 1988). The fungus then grows
572 saprophytically on the dead insect (Samson et al., 2013) and emerges on the surface of the
573 insect corpse to sporulate (Hajek and St. Leger, 1994; Moonjely et al., 2016). *Metarhizium*
574 *brunneum* Pr1protease, which plays a role in cuticle penetration in the early stages of fungal
575 infection, is also involved in fungal emergence onto the surface of the insect before
576 sporulating. The expression of this enzyme is induced by the poor nutrient conditions
577 experienced in the insect hemocoel due to fungal growth. In this way, *M. brunneum* fine tunes
578 the process of infection to eventually emerge onto the insect surface and produce its
579 characteristic olive green powdery conidia that will infect other hosts (Moonjely et al., 2016).

580 #### 1.3.1.2. *Metarhizium brunneum* as a biopesticide to control vine weevil larvae

581 In the late 1990s, the company Bayer developed the first commercial product based on an
582 entomopathogenic fungus aimed at controlling the vine weevil. This product, named BIO1020,
583 consisted of *M. brunneum* strain F52. BIO1020 was withdrawn from the market likely due to
584 the low prices of chemical pesticides available at that time. More recently, however, the
585 pesticide market has changed with many synthetic chemical pesticides being withdrawn and
586 farmers seeking alternative tools with which to control pests. In this context, the product initially
587 developed as BIO1020 was purchased and remarketed by the company Novozymes with the

588 name Met52, and is sold around the world to control a range of pests (Bennison et al., 2014;
589 Ravensberg, 2015).

590 In commercial scale production of *M. brunneum* the fungus is allowed to colonise rice grains,
591 eventually producing spores on the surface of the grain, similar to the process occurring in
592 infected insects. The rice grains colonised by fungal hyphae and covered in spores may then
593 be packaged for sale as the rice grains allow easy incorporation of the spores into growing
594 media. This means that the Met52 product is typically used to control soil-dwelling pests.
595 Met52 can be purchased ready to be incorporated into growing media or already incorporated
596 into the compost. The fungus in the compost will continue to grow breaking down the rice
597 grains and spreading to infect soil inhabiting pests. Vine weevil larvae live below-ground
598 feeding on plant roots and so this developmental stage is the most common use of Met52
599 applications targeting this pest (Bennison et al., 2014).

600 **1.3.1.3. *Metarhizium brunneum* growth is temperature dependent**

601 The efficacy of *Metarhizium brunneum* is determined by environmental factors such as
602 temperature. *M. brunneum* is considered to be effective between 15°C and 30°C, with no
603 growth below 10°C and with the fastest growth rate at 20°C (Bennison et al., 2014, 2018a).
604 Slower fungal growth at 15°C may, however, be compensated for by applying higher doses of
605 fungal conidia (Bennison et al., 2014).

606 Soil temperatures in northerly locations where the vine weevil is problematic, such as
607 Scotland, Norway or Iceland, are usually below 20°C (Busby, 2015; Klingen et al., 2015;
608 Oddsdottir et al., 2010). There is then a need to find an alternative *M. brunneum* strain to the
609 F52 with better growth rate at colder temperatures. In France, Soares et al. (1983) studied the
610 virulence of various *M. brunneum* and *M. flavoridae* strains against vine weevil larvae. At 15°C,
611 the *M. brunneum* strain 85 treatment resulted in higher larvae mortality levels in comparison
612 with the other fungal strains and species considered. A study carried out in Iceland also
613 showed that a *M. brunneum* strain isolated from the southeast of the island displayed good
614 rates of control of vine weevil larvae, although the temperature at which the experiment was
615 carried out was not specified (Oddsdottir et al., 2010). *M. brunneum* strains isolated from
616 Norwegian soils have been shown to be effective at controlling vine weevil larvae at
617 temperatures as low as 12°C (Klingen et al., 2015). These results are promising and may
618 indicate that other *M. brunneum* strains exist that are effective at lower temperatures. Further
619 efforts are still needed to deepen our understanding and commercialise these strains. Should
620 a wider range of M. brunneum strains or other species that show entomopathogenic activity
621 become commercially available then it may also be possible to use a mixture of fungal strains,

622 or species, to cover a wider range of temperatures at which the fungi are able to infect and
623 grow.

624 1.3.1.4. *Metarhizium brunneum* in the rhizosphere

625 Ideally, biocontrol products based on entomopathogenic fungi should remain in the soil
626 conferring protection against soil-dwelling pests for long periods of time. The ability of *M.*
627 *brunneum* to persist in the soil post-application was studied by Hu and St Leger (2002) on
628 cabbage plants *Brassica oleracea* Linnaeus. Four months after fungal application, conidia
629 displayed an aggregated distribution pattern with higher densities in the areas surrounding the
630 roots or rhizosphere. Similarly, Bruck (2005) showed that *M. brunneum* conidia aggregated
631 on the rhizosphere of *Picea abies* (Linnaeus Karsten) with persistence of up to a year.
632 Interestingly, Klingen et al. (2015) showed that in a semi-field experiment carried out in
633 Norway, a *M. brunneum* strain indigenous to that country persisted for a longer period in the
634 rhizosphere of strawberry plants in comparison with a strain native to Austria.

635 The behaviour of *M. brunneum* after application deserves further attention. If rhizosphere
636 competency is a common phenomenon in the field, this could change the way the fungus is
637 applied. Bruck (2005) for instance applied conidia directly to the roots of *P. abies* and this pre-
638 treatment increased the number of vine weevil larvae infected due to root feeding. Thus, a
639 prophylactic root inoculation before planting could be a promising new approach to vine weevil
640 biocontrol.

641 1.3.1.5. *Metarhizium brunneum* as an endophyte

642 In recent years there has bee an increased interest in entomopathogenic fungi not only as
643 biopesticides but also as plant endophytes. Fungal endophytes colonise plant tissues without
644 causing apparent symptoms or harm to their host plant. This has been observed in insect
645 pathogenic fungi such as *B. bassiana* and *M. brunneum.* Plant-fungi endophytic associations
646 are relevant in an agricultural context because they have shown to provide plants with
647 increased protection against pests and pathogens as well as to improve plant growth (see
648 Jaber and Ownley, 2018; and Vega et al., 2009). In this section I will focus only on the role of
649 *M. brunneum* as endophyte conferring host plants with higher resistance to herbivorous
650 insects.

651 Ongoing research into endophytic associations is, in part, focused on investigating the use of
652 different fungal application procedures, such as foliar application, root inoculation and seed
653 inoculation. Foliar applications of *M. brunneum* conidia on alfalfa, *Medicago sativa* (Linnaeus),
654 melon, *Cucumis melo* (Linnaeus), and tomato, *Solanum lycopersicum* (Linnaeus), enabled

655 fungal colonisation of the whole plant (Resquín-Romero et al., 2016). Root inoculation on
656 tomato plants allowed the spread of the fungus through the roots but also to the aerial parts
657 (García et al., 2011; Krell et al., 2018). In potato, *Solanum tuberosum* (Linnaeus), however,
658 spraying the aerial parts of the plant with *M. brunneum* conidia resulted in better colonisation
659 of stems and leaves than of the tubers and roots (Ríos-Moreno et al., 2016). Seed inoculation
660 of haricot beans, *Phaseolus vulgaris* (Linnaeus), resulted in better *M. brunneum* colonisation
661 of the roots than of above-ground plant parts (Behie et al., 2015). Faba bean *Vicia faba*
662 (Linnaeus) seed inoculation also successfully allowed root colonisation (Akello and Sikora,
663 2012). It remains a matter of debate which application procedure is the most effective to
664 establish endophytic association and similarly undetermined as to whether further
665 investigations will lead to the development of standardised application methods or if, rather,
666 this will need to be adapted for each crop specifically.

667 *Metarhizium brunneum* endophytes improved the control of the sweet potato whitefly *Bemisia*
668 *tabaci* (Gennadius) nymphs on melon (Garrido-Jurado et al., 2017) and the beet armyworm
669 *Spodoptera littoralis* (Boisduval) larvae on tomato, melon and alfalfa plants (Resquín-Romero
670 et al., 2016). However, faba bean plants colonised by *M. brunneum* were as susceptible to
671 pea aphids *Acyrthosiphon pisum* (Harris) as uncolonized plants (Akello and Sikora, 2012). It
672 has been proposed that secondary metabolites produced by the fungus when infecting
673 insects, such as destruxins, could be produced in plant tissues and that this affects insects
674 feeding on these plants. Destruxin was found in 43% of the sweet potato whitefly nymphs
675 analysed after feeding on fungus colonised leaves (Garrido-Jurado et al., 2017), while only a
676 small percentage of beet armyworm larvae feeding on colonised plants had detectable
677 concentrations of destruxin (Resquín-Romero et al., 2016). Potato plants colonised by *M.*
678 *brunneum* also showed very low levels of destruxin in the tubers and roots and destruxin was
679 not in the leaves and stems (Ríos-Moreno et al., 2016). It is not yet clear what the mechanism
680 is by which endophytic fungi interact with herbivorous pests but the results obtained to date
681 seem to indicate that the activity of destruxins alone does not explain the observed effects on
682 herbivorous insects.

683 The use of *M. brunneum* as an endophyte against the vine weevil has not been tested yet. In
684 haricot beans, this fungal pathogen established and grew better on the roots than on the stems
685 and leaves of treated plants (Behie et al., 2015). The establishment of *M. brunneum* on roots
686 could protect plants from feeding by vine weevil larvae as well as from other soil-dwelling
687 pests. This approach for crops such as strawberry has yet to be investigated.

688 ### 1.3.2. A complete metamorphosis: the vine weevil life cycle

689 The vine weevil life cycle is holometabolous and involves six to seven larval instars, a pupation
690 and a final adult transformation (LaLone and Clarke, 1981; Smith, 1932). The development of
691 weevils during their life cycle is temperature (Smith, 1932; Son and Lewis, 2005) and
692 photoperiod (Evenhuis, 1978; Garth and Shanks, 1978; Moorhouse et al., 1992; Nielsen and
693 Dunlap, 1981) dependent, although the influence of photoperiod has been less well studied.
694 As a consequence, the vine weevil life cycle is influenced by microclimatic conditions in the
695 area where they are developing (Bennison et al., 2018a; Smith, 1932).

696 In outdoor conditions, adult emergence starts in early June and can last until December
697 (Moorhouse et al., 1992; Smith, 1932), although certain practices that increase soil or
698 substrate temperatures like mulches can accelerate pupation and adult emergence (Bennison
699 et al., 2018a; Evenhuis, 1978). Following emergence, adults start to feed and undergo a
700 reproductive maturation process after which they start to lay eggs (Smith, 1932; Son and
701 Lewis, 2005). In September and October, adults stop feeding and ovipositing (Cram, 1965;
702 Smith, 1932) Although the majority of adults die during the winter, some can successfully
703 overwinter in sheltered areas and start to oviposit the following spring (Bennison et al., 2018a;
704 Moorhouse et al., 1992; Smith, 1932). Larvae hatch from eggs laid at or just below the surface
705 of the soil or substrate and immediately move deeper before starting to feed on the host plant
706 roots. When soil temperature drops, larvae enter a dormant stage to overwinter (Smith, 1932).
707 As soil temperature increases in early spring, dormant larvae become active and start to feed
708 on roots once more (Smith, 1932). The pupation process begins in May after which a new
709 generation of adults start to emerge in June (Garth and Shanks, 1978; Moorhouse et al.,
710 1992).

711 #### 1.3.2.1. The vine weevil eggs

712 Vine weevil eggs are sub-spherical glistening pearly white with the long and the short axis
713 measuring 0.65-0.80 mm and 0.65-0.70 mm, respectively (Smith, 1932). Between one to three
714 days after the egg is laid the chorion darkens to a chestnut brown colour and becomes more
715 rigid through a process of melanisation (Smith, 1932). As a consequence of this melanisation,
716 eggs become more resistant to high temperature peaks, low humidity and fungal attacks
717 (Shanks and Finnigan, 1973b).

718 Eggs develop within a range of temperatures from 9°C to 27°C (Stenseth, 1979), with the
719 optimal temperature being 21°C (Son and Lewis, 2005). Time to hatching can range from 56
720 days at 9°C to 8.4 days at 27°C (Shanks and Finnigan, 1973b). The minimum relative humidity

721 (RH) at which melanised eggs can develop is 55% RH, however, the highest hatching rate
722 occurs at 75-97% RH (Shanks and Finnigan, 1973b).

723 **1.3.2.2. The vine weevil larvae**

724 Once the pre-neonate larva is ready to hatch it starts to move its mandibles rhythmically to cut
725 the egg shell to emerge (Smith, 1932). The body of the neonate larva is bright white covered
726 with small hairs, the head is chestnut brown and the mandibles are darker brown (Smith,
727 1932). The neonate larvae are negatively phototropic so they bury themselves in the soil or
728 substrate immediately after hatching (Moorhouse et al., 1992; Smith, 1932). This early
729 behavioural reaction is important as larvae die in a few hours if they fail to move deeper into
730 the soil (Smith, 1932).

731 Once larvae are ready to moult they stop feeding and form an earthen cell at a depth of 10 to
732 13 cm in the soil using the contents of the alimentary tract to bind the soil particles together
733 (Smith, 1932). Between one to three days from the start of the quiescent period in the earthen
734 cell, the head capsule and thoracic segments split along the median dorsal line (Smith, 1932).
735 Subsequently, the head of the larva is pushed through the opening, and the larva leaves the
736 old skin using waves of expansion and contraction (Smith, 1932). The head of the newly
737 moulted larva is milky white with the tips of the mandibles dark brown (Smith, 1932). The larva
738 remains in the cell for one to three days longer while the head hardens and turns a chestnut
739 brown colour (Smith, 1932). The larva leaves the earthen cell once the head has hardened
740 and returns to feed on the roots of the host plant (Smith, 1932). During consecutive instars,
741 larvae enlarge and their body acquires a characteristic C shape (Moorhouse et al., 1992) due
742 to the thickening of the thoracic segments (Smith, 1932). The moulting periods, especially the
743 first three, are critical stages for the larvae as many can die during this period (Smith, 1932).

744 Developmental time for larvae and pre-pupae at 12, 15 and 24°C is 198, 130 and 110 days,
745 respectively (Stenseth, 1979). Larval survival increases with increasing humidity with an
746 optimal relative humidity level ranging from 85 to 95%. Furthermore, the ability of larvae to
747 enter the soil is also humidity dependent (Shanks and Finnigan, 1973b).

748 Larvae during the first three instars feed on root hairs, but after the 4th instar they start to feed
749 on bigger roots (Smith, 1932). Larvae can feed on bulbs and the cortex of larger roots but they
750 can also tunnel into stems above the soil (Masaki and Ohto, 1995; Moorhouse et al., 1992;
751 Smith, 1932) and feed on strawberry fruits in contact with the soil (pers. obs.). Feeding by the
752 last two larval instars is rapid and the infested plant may exhibit no injury until sudden wilting
753 is observed (Smith, 1932). This is why larvae are considered the most damaging stage for
754 plants (Moorhouse et al., 1992).

755 #### 1.3.2.3. The vine weevil pupae

756 At the conclusion of the larval feeding period, larvae build pre-pupal cells by mixing their gut
757 contents with soil particles at a depth of 1.25 to 5cm (Smith, 1932). The pre-pupal weevil
758 changes to a muddy-yellow colour, and the head becomes a weathered brown colour in the
759 cell (Smith, 1932). Then the thoracic segments enlarge slightly on the dorsal side, and the
760 prothorax and head of the pupa forms there (Smith, 1932). The larval skin splits along the
761 median line of the thorax and the head capsule and is worked off posteriorly (Smith, 1932).

762 Young pupae are milky white with spines on the head, femora and abdomen, and the
763 mandibles have curved hooks (Smith, 1932). Pupae can move if disturbed by bending the
764 abdomen (Smith, 1932) describing circular movements (pers. obs.). In this way, the pupa can
765 turn its body inside the cell when exposed to light or when the cell is broken (Smith, 1932).
766 Approximately seven days before transforming into an adult, the mandibles and the snout
767 become black, the eyes, antennae, tarsi and femoral tips become a rusty brown, the prothorax
768 turns faintly rusty in colour, the legs darken at the joints, and the occiput, elytral pads and
769 abdomen turn creamy white (Smith, 1932). Then, the pupal skin splits along the median dorsal
770 line on the prothorax and occiput and is worked off posteriorly (Smith, 1932).

771 Developmental time ranges from 50 days at 15°C to 10 days at 24°C (Stenseth, 1979). So,
772 the developmental time from a newly laid egg to emergence of an adult weevil ranges from
773 200 days at 15°C to 130 days at 24°C (Stenseth, 1979).

774 #### 1.3.2.4. The vine weevil adults

775 Recently transformed adults are milky white with a black snout and eyes, rusty brown
776 antennae, coxae, femoral tips, tibiae and tarsi, and faintly rusty prothorax (Smith, 1932).
777 Around an hour after transformation the elytra expand to cover the abdomen and the edges
778 meet along the median line (Smith, 1932). In the following days the body becomes chestnut
779 brown, then black and when the adult is ready to emerge, patches of yellowish hair can be
780 seen on the elytra (Smith, 1932). Adults typically measure between 10.5 to 11.5 mm in length,
781 although Smith (1932) observed that when larvae pupated in the 5th instar, adults were only
782 8.5mm in length.

783 Adults experience a pre-oviposition period during which reproductive organs become mature
784 before they start laying eggs. This period is temperature dependent with a lower and higher
785 threshold at 6.8°C and 30°C, respectively, and an optimum at 22.7°C (Son and Lewis, 2005;
786 Stenseth, 1979). Oviposition occurs at temperatures between 6°C to 28°C, with an optimal
787 temperature at 21.8°C (Bennison et al., 2018b; Evenhuis, 1978; Son and Lewis, 2005; Zepp
788 et al., 1979). Different responses have been reported regarding the influence of humidity on

789 oviposition. Smith (1932) stated that adults would rather avoid hiding and laying eggs in humid
790 places, while Cram (1965) and Shanks (1980) stated that dry conditions would negatively
791 affect oviposition and egg viability. Additionally, Montgomery and Nielsen (1979) found that
792 females would rather choose places with high humidity to oviposit.

793 Peak oviposition activity occurs two or more hours after sunset (Stimmann et al., 1985), as
794 adults are nocturnal, and eggs are normally found on the soil or on plants (Smith, 1932). It
795 was suggested by Smith (1932), Neiswander (1953) and Breakey (1959) that adults randomly
796 drop eggs while feeding. In contrast, Garth and Shanks (1978) reported that eggs were laid
797 either on the soil or at approximate depths of 5cm and that larval survival was greater when
798 the eggs were placed into the soil. In addition, Montgomery and Nielsen (1979) reported that
799 adults could retain their eggs until finding an appropriate location to oviposit.

800 At the end of the egg laying period adults enter a quiescent stage (Cram, 1965; Smith, 1932).
801 This quiescence is not a true diapause as when environmental conditions become appropriate
802 oviposition can be re-initiated (Cram, 1965; Garth and Shanks, 1978). In outdoor conditions
803 few adults survive overwinter compared to indoor conditions (Evenhuis, 1978; Garth and
804 Shanks, 1978; Nielsen and Dunlap, 1981; Smith, 1932; Stenseth, 1979). Noticeably, under
805 laboratory conditions the oviposition period can be extended and adults have a potential life
806 span of two years (Moorhouse et al., 1992) or more (pers. obs.)

807

808 ## 1.4. Thesis aims and objectives

809 **Aims:**

810 The aim of this thesis is to investigate potential factors involved in vine weevil adaptation to
811 different environments and provide information about the bacterial microbiota associated with
812 this economically damaging pest.

813 **Objectives:**

814 1) Characterise the bacterial community of vine weevil adults collected from different host
815 plant species and locations across the UK using high throughput Illumina MiSeq
816 sequencing technology.

817 2) Optimise DNA extraction procedure and artificial diet for mass rearing of the vine weevil.

818 3) Detect differences in susceptibility to the fungal pathogen *Metarhizium brunneum*
819 (Petch) between vine weevil populations in controlled experiments.

820 4) Test oviposition preference between strawberry and raspberry plants of vine weevil
821 populations in glasshouse experiments.

822 5) Quantify abundance variation of the dominant bacterium *Candidatus* Nardonella
823 between larvae, pupae and adults using quantitative PCR analysis.

824

825

Chapter 2

826 827 828

The bacterial community associated with adult vine weevil *Otiorhynchus sulcatus* (Fabricius) in UK populations is dominated by *Candidatus* Nardonella[2]

829 **Abstract**

830 *Otiorhynchus sulcatus* (Fabricius) (Coleoptera: Curculionidae),
831 commonly known as black vine weevil or simply vine weevil, is an
832 important pest of soft fruit and ornamental crops. This species is endemic
833 to temperate areas of Europe but has spread to many other areas over
834 the last century, including North America and Australasia. The ability of
835 vine weevils to adapt to such different environments is difficult to
836 reconcile with the parthenogenetic reproduction strategy, which is likely
837 to underpin a low genetic diversity. It is therefore tempting to hypothesise
838 that weevil adaptation to different environments is mediated, at least
839 partly, by the microbial communities inhabiting these insects. As a first
840 step towards testing this hypothesis we characterised the composition of
841 the bacterial microbiota in weevils from populations feeding on different
842 host plants at separate locations across the UK. A second
843 characterisation was carried out to study the bacterial community of vine
844 weevil insects collected from strawberry plants across four
845 geographically separate locations in the UK. We performed 16S rRNA
846 gene Illumina amplicon sequencing. Ecological indices, namely Chao1
847 and Shannon, revealed that all the populations used for this study
848 harboured a low diversity and an uneven bacterial microbiota
849 composition. Furthermore, β-diversity analysis failed to identify a clear
850 association between microbiota composition and the factors host plant

[2] Part of the following chapter has been published in: Morera-Margarit, P., Bulgarelli, D., Pope, T. W., Graham, R. I., Mitchell, C., & Karley, A. J. (2019). The bacterial community associated with adult vine weevil (*Otiorhynchus sulcatus*) in UK populations growing on strawberry is dominated by *Candidatus* Nardonella. Entomologia Experimentalis et Applicata, 167(3), 186–196. https://doi.org/10.1111/eea.12757

851 species and location. Notably, a single operational taxonomic unit
852 phylogenetically related to *Candidatus* Nardonella was consistently the
853 most abundant bacterial sequence in all tested insects. Our results
854 indicate that vine weevil bacterial microbiota resembles that of other
855 insects as it has low diversity and it is dominated by few taxa. A prediction
856 of this observation is that host plant and location *per se* may not influence
857 the microbiota composition of the vine weevil. Our results will serve as a
858 reference framework to investigate other or additional hypotheses aimed
859 at elucidating vine weevil adaptation to its environment.

860

861 ## 2.1. Introduction

862 Vine weevils, *Otiorhynchus sulcatus* (Fabricius), are parthenogenetic triploid females endemic
863 to central Europe (Moorhouse et al., 1992). In the last two centuries, vine weevil distribution
864 has expanded rapidly, primarily through plant trade routes, and this species is now found in
865 most parts of Europe, and in parts of North America, South America, New Zealand and Japan
866 (Kingsley, 1898; Masaki et al., 1984; Moorhouse et al., 1992; Prado, 1988). Vine weevils have
867 been recorded developing successfully on 150 different host plant species (Moorhouse et al.,
868 1992; Smith, 1932; Warner and Negley, 1976) with particular preference for strawberry
869 (Hanula, 1988; Tol and Visser, 1998; van Tol et al., 2004, p. 200). Based on the ability of vine
870 weevil to invade and establish in different environments despite its parthenogenetic
871 reproduction mode, we hypothesised that the bacterial community associated with vine
872 weevils could play an important role in insect adaptation.

873 In the last decade, advances in sequencing and computational approaches have enabled the
874 characterisation of the microbial communities associated with both plant and animal
875 eukaryotic hosts, i.e. their microbiotas, at an unprecedented depth (Hacquard et al., 2015).
876 Perhaps not surprisingly, such advances have been exploited to gain novel insights into the
877 ecology of weevil microbiota. For instance, Hirsch et al. (2012) reports that parthenogenetic
878 species tend to harbour a less diverse bacterial community in comparison with sexual species
879 in the weevil genus *Otiorhynchus*. Why this might be the case remains is, as yet, unexplained.
880 White et al. (2015) studied the bacterial community associated with exotic and endemic
881 weevils in New Zealand and speculated that the presence of *Wolbachia* and *Rickettsia* could
882 be involved in weevil resistance to parasitoids used in biocontrol.

883 The influence of insect diet on shaping the bacterial microbiota composition was reported in
884 the red palm weevil *Rhynchophorus ferrugineus* (Olivier) and the cotton boll weevil
885 *Anthonomus grandis* (Boheman) when fed on artificial diets (Ben Guerrero et al., 2016;
886 Montagna et al., 2015). Interestingly, research developed by Berasategui et al. (2016) on the
887 bacterial community of the pine weevil *Hylobius abietis* (Linnaeus) revealed that these weevils
888 harboured a microbiota more similar to other conifer feeding beetles than to weevil species
889 feeding on other food sources, indicating the influence of diet for the microbiota structure and
890 composition (Berasategui et al., 2016). Many studies have also shown that location can affect
891 the bacterial microbiome of insects. For example, bacterial community richness and
892 composition varied significantly between *D. melanogaster* populations collected from
893 geographically separated areas of the US (Corby-Harris et al., 2007). Furthermore, collection
894 area was shown to clearly influence bacterial community assemblage of melon aphid *Aphis*
895 *gossypii* (Glover) populations sampled across four Hawaiian Islands (Jones et al., 2011).

The influence of host plant and location for the vine weevil bacterial community composition was investigated. As a first step to address this question, a pilot experiment was carried out using high-throughput sequencing to characterise the bacterial microbiota of vine weevil adult insects collected from various host plant species at separate locations across the UK. Results from this initial sequencing analysis did not reveal a clear association between the two factors considered and the microbiota composition. To simplify the interpretation of results we designed a second sequencing analysis to investigate if location alone could exert a major role in the vine weevil microbiota composition. This was done by considering vine weevil adult insects collected from strawberry crops located at geographically separated UK areas. Our results in combination indicate that location or host plant *per se* may not be influencing the vine weevil bacterial community composition and that, importantly, the vine weevil bacterial microbiota was always dominated by a single bacterial phylotype, classified as *Candidatus* Nardonella.

2.2. Pilot experiment: Characterisation of the bacterial community of vine weevil adult insects from different host plant species at separate UK locations

2.2.1. Materials and methods

2.2.1.1. Vine weevil adult populations

Vine weevil adults were collected during summer 2015 from different crop and non-crop environments at sites across the UK (Table 2.1). Collected insects were sent to the James Hutton Institute in Dundee, UK, where they were frozen with liquid N_2 and stored at -80°C until further use. Insects collected from different sites were considered as different populations for later analysis.

Table 2.1 Vine weevil populations

POPULATION	LOCATION	HOST PLANT
TP01	Berkshire	Primula and Yew (mixed host plant)
TP03	Herefordshire	Raspberry
TP04	Dorset	*Syringa vulgaris* and *Forsythia* spp. (mixed host plant)
TP06	Surrey	*Hydrangea* spp.
TP07	Shropshire	Strawberry
TP08	Staffordshire	Strawberry
CM03	Fife	Raspberry

920 **2.2.1.2. DNA extraction**

921 DNA extraction was performed on four insects from each population (one insect = one
922 replicate). Insects were taken from the freezer and surface sterilised in a 1% bleach (May and
923 Baker LTD, Dagenham, England) solution for 1 minute (Lawrence et al., 2015; Malacrinò et
924 al., 2018). To remove the remaining bleach, insects were submerged in autoclaved water 3
925 times, each time the insects were submerged for 1 minute. Surface sterilised insects were
926 ground individually using a pestle and mortar previously exposed to UV light for 10 minutes.
927 Once the whole sample was ground to a powder total DNA was extracted using the
928 NucleoSpin Kit (Macherey-Nagel, Düren, Germany) following the alternative step suggested
929 in the Kit protocol. DNA extracted was stored at -20°C in autoclaved Eppendorf tubes until
930 further use.

931 **2.2.1.3. PCR amplification of the 16S rRNA gene**

932 A fragment of the V4 hypervariable region of the 16S rRNA gene was used for the current
933 bacterial community study as it has been shown to yield optimal community analysis in
934 previous studies (Caporaso et al., 2011) and it was chosen as a reference marker for the Earth
935 Microbiome Project (EMP) (Gilbert et al., 2010). The primers used, 515F (5'-
936 GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), carry an
937 Illumina adapter, pad and linker at the 5' terminus. Additionally, the reverse primer (806R)
938 carries a unique barcode which is a 12-base error correcting Golay code to allow multiplexing,
939 i.e. sequencing different samples simultaneously. The error correcting Golay code allows the
940 correction of 3-bits errors and the detection of 7-bit errors.

941 The Kapa HiFi HotStart PCR kit (Kapa Biosystems, Wilmington, USA) was used to amplify the
942 targeted DNA fragment in a G-Storm GS1 Thermal Cycler (Gene Technologies, Somerton,
943 UK). The PCR mixture (20 µL) consisted of 4 µL of 5X Kapa HiFi Buffer, 1 µL of a 10 ng/µL
944 Bovine Serum Albumin solution (Roche, Mannheim, Germany), 0.6 µL of a 10 mM Kapa
945 dNTPs solution, 0.6 µL of a 10 µM solution of each primer, 0.25 µL of Kapa HiFi polymerase
946 (0.02 U/µL), 8 µL of sterile water and 1 µL of a 10 ng/µL solution of the template DNA. Samples
947 in the thermocycler were subjected to three minutes of DNA initial denaturation at 94°C, then
948 35 cycles of 30 seconds of DNA denaturation at 98°C, 30 seconds of primer annealing at
949 50°C, and one minute of DNA elongation at 72°C, followed by a final elongation step of 10
950 minutes at 72°C.

951 Based on the protocol described by Costello et al. (2009) and adopted by the EMP, each
952 insect replicate was PCR amplified using a specific combination of forward and reverse
953 primers with a unique, replicate-specific, barcode. For each primer pair combination, the

954 corresponding PCR reaction was performed in simultaneous triplicates to diminish
955 amplification biases, with an additional no template control. PCR reactions were combined in
956 a barcode-wise manner, i.e. amplification replicates of the same primer pair were mixed and
957 were tested on a 1.5% agarose gel with the corresponding no template control. The
958 simultaneous triplicate amplification procedure was repeated three times for each primer pair
959 combination. So, for each primer pair combination we performed nine amplifications in total.
960 Finally, all PCR products were mixed in a barcode-wise manner (nine amplifications mixed)
961 and kept at -20°C until further use.

962 **2.2.1.4. Illumina MiSeq library preparation and sequencing**

963 PCR products were purified with Agencourt AMPure XP kit (Beckman Coulter, Brea, USA)
964 using 0.7 µL AMPure XP beads per 1 µL of sample. The DNA concentration of 3 µL of each
965 PCR reaction, mixed according to their barcode, was quantified using Picogreen
966 (ThermoFisher, UK) following the manufacturer's recommendations. Next, the amplicon
967 library was generated by mixing individual barcoded replicates in an equimolar ratio. The
968 library was sequenced by the Genome technology group at the James Hutton Institute,
969 Dundee UK, using Illumina MiSeq platform with paired-end reads of 150 bp per read.

970 **2.2.1.5. Illumina MiSeq data processing with QIIME**

971 The Illumina MiSeq platform generated three FASTQ files with the forward, reverse and
972 barcode sequences. The FASTQ files and the metadata information, organised in a mapping
973 file, were processed with the open source software Quantitative Insights Into Microbial
974 Ecology (QIIME) version 1.9.0 (Caporaso et al., 2010) using the default parameters unless
975 otherwise specified.

976 Forward and reverse FASTQ files were decompressed and merged specifying a minimum
977 sequence overlap of 5 bp between pairs of reads using the command 'join_paired_ends.py'
978 The reads were quality filtered and demultiplexed with the command 'split_libraries_fastq.py'
979 specifying a minimum Phred quality score of 20. The remaining high-quality reads were
980 clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity using
981 SortMeRNA and sumaclust algorithms. As a great amount of sequencing reads were
982 discarded, we decided to take a subsampled open-reference OTU picking approach with the
983 command 'pick_open_reference_otus.py' against the chimera checked Greengenes database
984 version 13_5 (DeSantis et al., 2006). This new approach randomly subsamples the sequences
985 that were initially discarded because they failed to hit the database reference collection. These
986 sequences are then clustered amongst them at 97% similarity and these OTUs are called New
987 Reference OTUs. The output was an OTU table with the identified OTUs as rows and the

988 samples as columns, containing the abundance of each OTU per sample. The OTUs that did
989 not match by 97% similarity any bacterial sequence on the database were classified as
990 Unassigned. These OTUs were manually searched on the online SILVA database using 0.95
991 “minimum identity with query sequence” for the OTU_0 and 0.8 “minimum identity with query
992 sequence” for the OTU_1264 and OTU_1276.

993 **2.2.1.6. Data analysis with R**

994 To analyse the data with R software, the packages phyloseq version 1.19.1 (McMurdie and
995 Holmes, 2013), DESeq2 version 1.26.0 (Love et al., 2014), PMCMR version 4.3 (Pohlert,
996 2014) were installed from Bioconductor using the code ‘source
997 (“http://bioconductor.org/biocLite.R”)’ and the function ‘biocLite()’. The function ancom was
998 installed using the code ‘source(“ancom_functions.R”)’ and ‘source(“plot_ancom.R”)’. The
999 packages vegan version 2.4-5 (Dixon, 2003), ape version 5.0 (Paradis et al., 2004) and
1000 ggplot2 version 3.0.0 (Wickham, 2009) and plyr version 1.8.4 (Wickham, 2011) were installed
1001 with the function ‘install.packages’.

1002 The initial Illumina library contained a mixture of amplicons originating from samples of the
1003 present study and from samples coming from a different unrelated study. Sequencing data
1004 from the two sample sets were analysed in combination up to the creation of the phyloseq
1005 object. A new OTU table was generated from the sequencing library using the function ‘prune’
1006 to remove OTUs classified as mitochondria and chloroplast, likely representing plant and
1007 insect contamination. Next, any instances where OTUs matched sequences identified as
1008 environmental contaminants of the laboratory where we generated our sequencing library
1009 were removed from the remaining OTUs list (Pietrangelo et al., 2018). The phyloseq package
1010 was used to create the phyloseq object combining the OTU table, the taxonomy matrix, the
1011 phylogenetic tree, and the mapping file using the command ‘merge_phyloseq’. As the tree
1012 was unrooted, the most abundant OTU in the phylum Chrenarchaeota was used as an
1013 outgroup to root the phylogenetic tree generated by QIIME.

1014 Both of the datasets were then separated and singleton OTUs, which are OTUs with fewer
1015 than 2 observations in the entire dataset, present in the segregated dataset were eliminated.
1016 Then an abundance filtering step was carried out on the dataset to remove OTUs with fewer
1017 than 5 reads in at least 25% of the studied insects with the function ‘filter_taxa’. This filtering
1018 step removed poorly represented OTUs with zero reads in many samples and facilitated data
1019 analysis and the following computational steps.

1020 To study the α-diversity, i.e. within sample diversity, samples were rarefied to a similar
1021 sequencing depth. The Chao1 and Shannon indices were then calculated with the function

1022 'estimate_richness'. To study the β-diversity, i.e. between community diversity, the dataset
1023 was transformed into relative abundances and distance matrices were calculated with Bray-
1024 Curtis metrics, which considers OTU relative abundance. Distance values were represented
1025 with Principal Coordinate Analysis (PCoA) plots, in which proximity between samples indicates
1026 similarity.

1027 The New Reference OTU_0 was by far the most abundant OTU in all the studied insects.
1028 Hence, to discard the possibility of the OTU_0 masking bacterial community patterns, a new
1029 dataset without this OTU was created with the function 'prune'. The sample TP01_I had less
1030 than 2,000 reads as a consequence, so it was eliminated from this dataset with the function
1031 'prune' as this number of reads was considered too low for further analysis. The β-diversity
1032 analysis was repeated on the new dataset without considering the OTU_0 and the sample
1033 TP01_I.

1034 2.2.1.7. Statistical analysis

1035 Statistical differences in microbial composition among populations were tested using a non-
1036 parametric MANOVA with the function 'adonis'. For this test the distance matrices previously
1037 calculated using Bray-Curtis metrics were used, with and without the dominant OTU_0. OTUs
1038 showing significant differences in abundance between populations were studied by applying
1039 an analysis of composition of microbiomes with the function 'ANCOM' from the package
1040 ANCOM using the multiple correction option '1' (Weiss et al., 2017).

1041

1042 2.2.2. RESULTS

1043 2.2.2.1. Illumina MiSeq library preparation

1044 Based on Picogreen DNA concentration values, aliquots of each amplicon solution were mixed
1045 to obtain a final Illumina library with 50 ng of each amplicon. Exceptionally, a 10 μL aliquot of
1046 the TP01_I amplicon solution was used, as this sample had a DNA concentration lower than
1047 5 mg/μL.

1048 2.2.2.2. Vine weevil bacterial microbiota is composed of 63 bacterial taxa

1049 The initial sequencing library, with the combined samples from both studies, contained 10,246
1050 OTUs and 7,029,278 reads. From these OTUs, 79, 36 and 43 OTUs were classified as
1051 chloroplast, mitochondria and contaminants, respectively. The subsequent file devoid of
1052 chloroplast, mitochondria and contaminant OTUs had 10,088 OTUs and 6,697,763 reads (per

1053 sample number of reads mean= 62,016.32, max= 108,327 and min= 12,637). Thus, 95.3%
1054 and 98.5% of the initial reads and OTUs, respectively, were retained.

1055 The phyloseq object generated carried 9,583 OTUs. This number of OTUs was fewer than the
1056 initial 10,088 in the file devoid of chloroplast, mitochondria and contaminant OTUs. To track
1057 where these missing OTUs were lost, the files that formed the phyloseq object were merged
1058 individually. It was then revealed that the missing OTUs in the phyloseq object were lost after
1059 adding the phylogenetic tree file, which indicates these were chimeric OTUs.

1060 Once the phyloseq object was created, the datasets from the two different studies were
1061 separated and, after eliminating singleton OTUs, the segregated vine weevil dataset contained
1062 1,385 OTUs and 1,989,440 reads. The abundance filtering step further reduced the number
1063 of OTUs to 63 and the number of reads to 1,949,306. Although this step dramatically reduced
1064 the number of OTUs to 4.5% of its initial number, we retained 97.98% of the total number of
1065 high-quality reads. This indicates that the bacterial microbiota of the populations tested in this
1066 study comprised a relatively low number of highly abundant bacterial taxa.

1067 **2.2.2.3. Vine weevil bacterial microbiota is composed of *γ*- and *α*-proteobacteria**
1068 **and is dominated by the OTU_0**

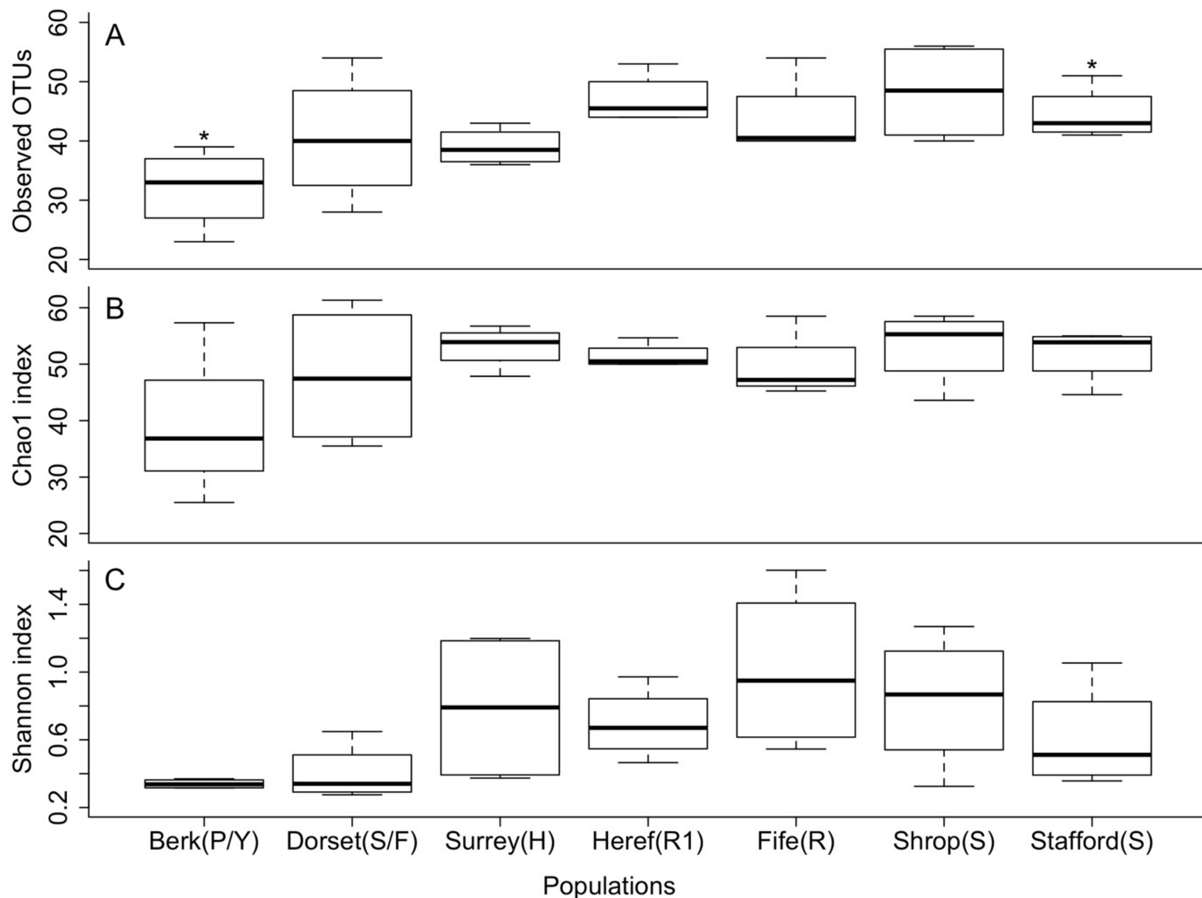
1069 Bacterial taxa identified using the chimera checked Greengenes database 13_5 are displayed
1070 in Table 2.2. To reduce the amount of data handled, only taxa with more than 100 reads and
1071 with presence in all replicates at least in one of the populations were considered. From the
1072 OTUs classified in the family Enterobacteriaceae, the OTU_52842 and the New reference
1073 OTU_32 were classified in the genus *Citrobacter*, the OTU_759061 and the New reference
1074 OTU_16 could not be identified to genus level, and the New reference OTU_7 was classified
1075 as *Plesiomonas shigelloides*. From the OTUs classified in the family Rickettsiaceae, the
1076 OTU_332714 and the New reference OTU_39 were classified in the genus *Rickettsia* and the
1077 New reference OTU_1 could not be identified to genus level. From the Unassigned OTUs, the
1078 New reference OTU_1264 matched by 89.72% the symbiont *Candidatus* Puchtella
1079 pedicinophila, a member of the Enterobacteriaceae family found in the macaque louse,
1080 *Pedicinus obtusus* (Fukatsu et al., 2009); the New reference OTU_1276 matched by 86.96%
1081 a member of the γ-proteobacteria order, classified as a secondary symbiont of psyllids (Thao
1082 et al., 2000); and the New reference OTU_0 matched by 89.72% the bacterium *Candidatus*
1083 Nardonella, an endosymbiont found in weevils that belongs to the Enterobacteriaceae family
1084 (Lefèvre et al., 2004b).

1085 **Table 2.2 Major OTUs: abundance and taxonomy.**
The average number of reads of the four replicates from each population is shown along with the taxonomy assigned to family level when possible. * symbol indicates New Reference OTUs.

OTUs	TP01	TP03	TP04	TP06	TP07	TP08	CM03	TAXONOMY
52842	1	0	0	1	5	1	4494	γ-proteobacteria
*32	6	12	6	9	23	32	827	Enterobacteriaceae
759061	1	5	2	49	16	5	1267	
*16	6	33	8	1286	17	11	396	
*7	329	551	764	1651	505	588	1614	
332714	415	2017	2030	758	5356	870	2243	α-proteobacteria
*39	20	401	283	54	373	131	231	Rickettsiaceae
*1	1062	4591	1566	1372	6864	3689	2773	
*1264	59	69	127	93	89	93	78	Unassigned
*1276	126	176	275	176	178	200	206	
*OTU_0	39,269	52,532	78,950	52,811	55,229	53,710	60,933	

1086 **2.2.2.4. Vine weevil populations harbour a low diversity bacterial microbiota**

1087 Within population diversity, or α-diversity, computed at OTU level, revealed low diversity in the
1088 bacterial communities across vine weevil populations. On average, populations harboured a
1089 bacterial community comprising 42 OTUs, a richness value (Chao1 index) of 49.45, and an
1090 evenness value (Shannon index) of 0.67 (Figure 2.1). Statistical analysis of the observed
1091 OTUs revealed that Berkshire (Primula/Yew) population tended to harbour a smaller number
1092 of OTUs while Shropshire (Strawberry) population tended to have a larger number of OTUs.
1093 There were only significant differences between these two populations which in turn were not
1094 significantly different to the rest (ANOVA, F= 2.594, df= 6, pvalue= 0.0485; Tukey HSD
1095 Berkshire-Shropshire pvalue= 0.041 Figure 2.1A). The Chao1 index was not significantly
1096 different between the populations (Kruskal-Wallis Chao1 H= 3.7, df= 6, p-value= 0.71; Figure
1097 2.1B). The Shannon index appeared as significantly different *a priori,* however, the post-hoc
1098 test did not reveal significant differences between the populations (Kruskal-Wallis test
1099 Shannon H= 12.99, df = 6, p-value = 0.04; Figure 2.1C).



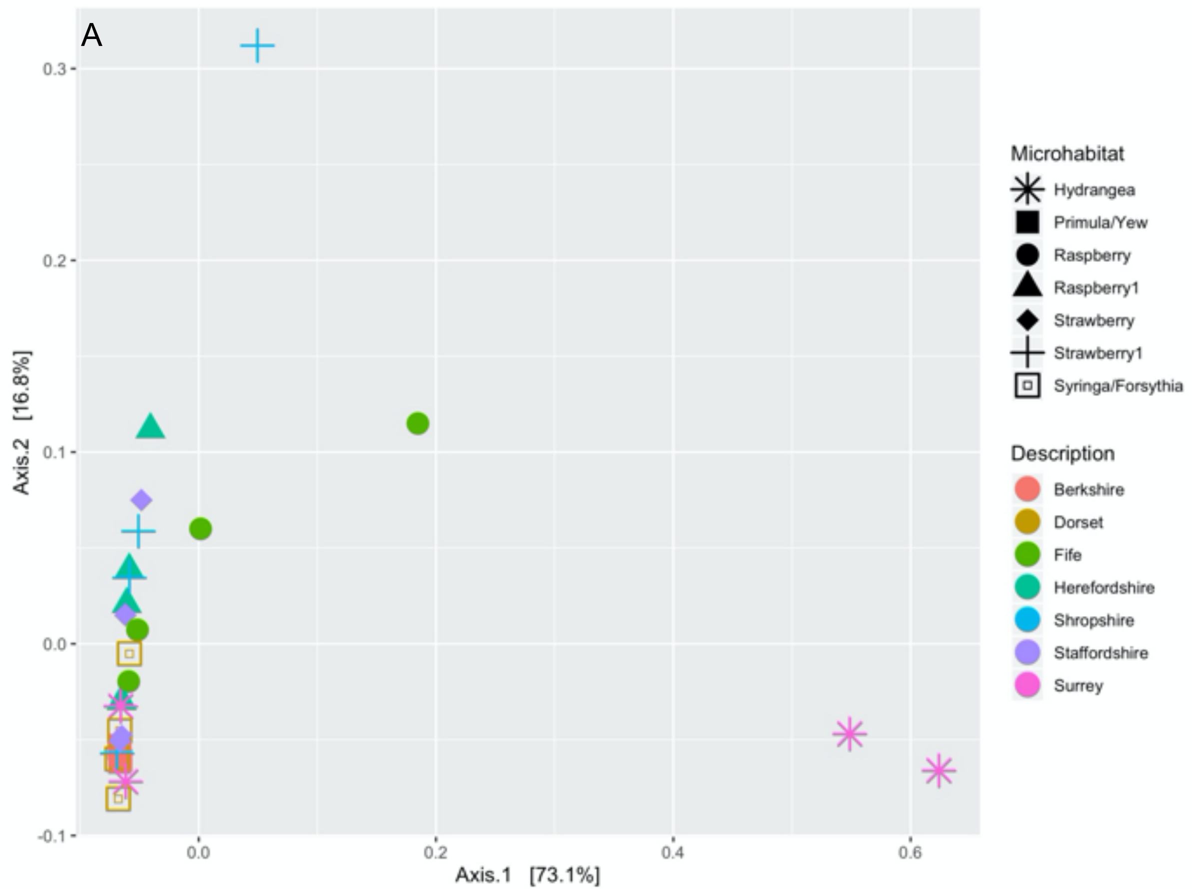
1100

1101 **Figure 2.1 Average (A) observed operational taxonomic units (OTUs), (B) richness**
1102 **(Chao1 index), and (C) evenness (Shannon index) of bacterial communities, per vine**
1103 **weevil population.**
1104 Box plots indicate the median as a thick line, the interquartile range (IQR) as a box, 1.5 IQR
1105 as whiskers and the outliers as points outside the whisker range. Population values sharing
1106 the "*" symbol were significantly different to each other but not to the rest (Tukey test: P<0.05).
1107 Berk= Berkshire, Heref= Herefordshire, Shrop= Shropshire, Stafford= Staffordshire, P/Y=
1108 Primula and Yew (mixed host plant), S/F= *Syringa vulgaris* and *Forsythia* spp. (mixed host
1109 plant species), H= *Hydrangea* spp., R= Raspberry and S= Strawberry.

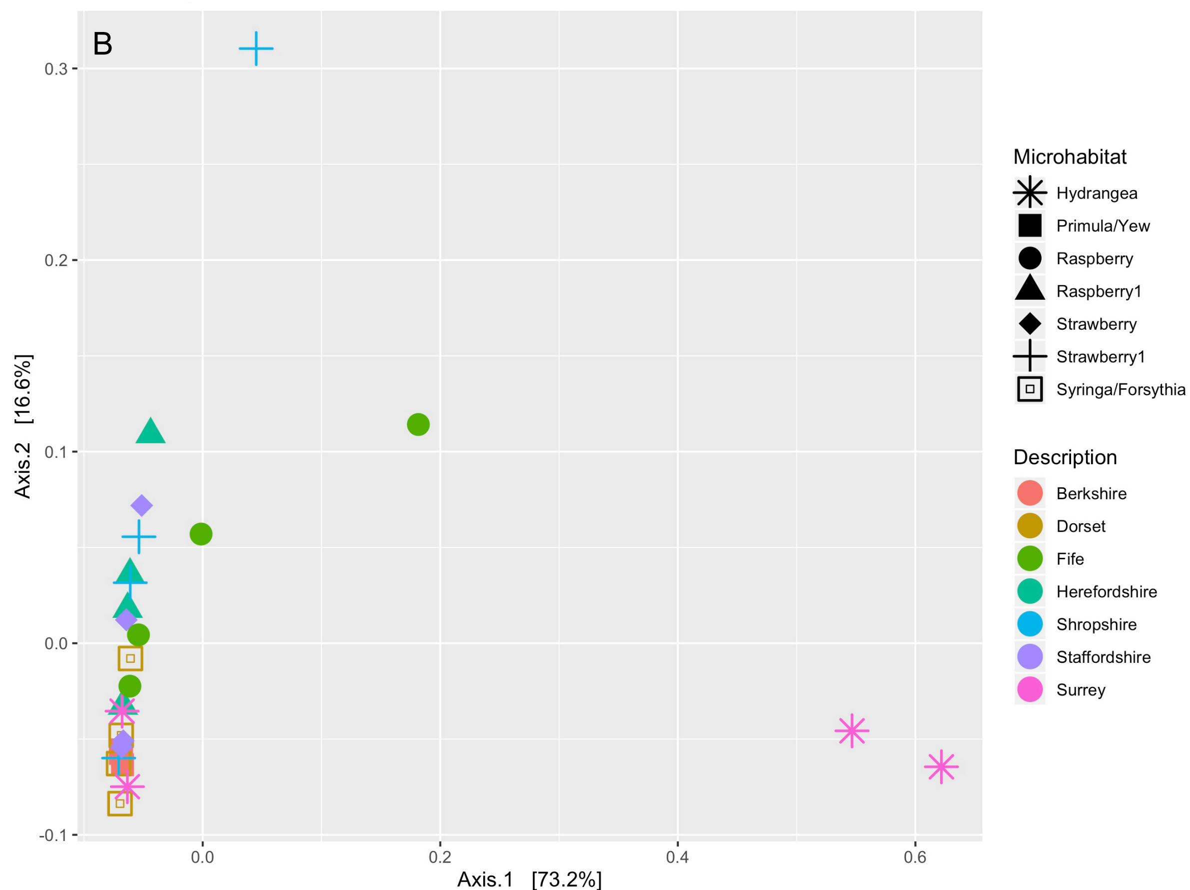
1110 **2.2.2.5. The vine weevil bacterial community assemblage does not follow a**
1111 **location nor a host plant associated pattern**

1112 The statistical analysis of between sample diversity revealed significant differences among
1113 populations for Bray-Curtis distance matrix calculation when the OTU_0 was included (Adonis
1114 test df=6, pvalue= 0.01, R2 Location= 0.41; Figure 2.2A) and when the OTU_0 was excluded
1115 (Adonis test df=6, pvalue= 0.02, R2 Location= 0.40; Figure 2.2B). Nonetheless, the ANCOM
1116 analysis did not reveal the presence of significantly different OTUs. This was likely caused by
1117 the fact that while adonis does a permutational analysis considering all the samples, ANCOM
1118 tests differences in abundance by considering mean values for each population. For instance,
1119 the Surrey (Hydrangea) population had two insects which were placed distant from the

1120 majority of data points in the PCoA. This could have caused the differences found by the
1121 Adonis test. However, as this population has two insects different from, and two insects similar
1122 to, the rest, the average used by ANCOM did not reveal significant differences. We can be
1123 certain that the OTU_0 abundance is similar between the insects studied as the elimination of
1124 this bacterial type did not change the results. This indicates that the OTU_0 does not mask
1125 location or host plant associated patterns in bacterial community composition.



1126



1127

1128 **Figure 2.2 Bray-Curtis distance for vine weevil populations with and without the OTU_0.**
1129 PCoA was constructed with the distance matrix calculated using Bray Curtis approach. A) with
1130 the abundant OTU_0 and B) without the OTU_0. Each point in the graph represents a single
1131 insect from which the shape represents the host plant and the colour the location from where
1132 the vine weevil adults were collected. The key contains the different shapes representing the
1133 host plant species (Microhabitat) and the different colours indicating the location (Description).
1134 The axis values indicate the % of variation explained by the factors location and host plant.

1135 2.3. Follow-up experiment: Characterisation of the bacterial 1136 community of vine weevil adult insects from strawberry crops at 1137 separate UK locations

1138 2.3.1.1. Vine weevil adult populations

1139 Vine weevil adults were collected during summer 2015, 2016 and 2017 from an area of
1140 approximately 50 m^2 within strawberry crops at five different sites across the UK. Insects
1141 collected at different locations were considered as different populations. Exceptionally, we
1142 considered insects collected at the Invergowrie site as two separated populations, despite
1143 coming from the same area, as they were collected in two consecutive years and could
1144 harbour different bacterial community influenced by the different environmental conditions
1145 experienced. Details of the collection sites are presented in Table 2.3 and Figure 2.3. The

1146 collection sites in Stafford were only separated by 766 m whereas the Shifnal and Woore
1147 collection sites were separated from these two sites an average distance of 30 km. The
1148 collection site in Invergowrie was 494 km distant in average from the rest of the sites. Following
1149 collection, insects were directly frozen with liquid N_2 and stored at -80°C until further use.

1150

1151 **Figure 2.3 Location of vine weevil sampling areas across the UK.**
1152 Each shape represents a population collection site (see Table 2.1).
1153
1154 **Table 2.3 Vine weevil population and years of collection.**

POPULATION	LOCATION	YEAR
Stafford_1	Stafford, Staffordshire	2017
Stafford_2	Stafford, Staffordshire	2017
Shifnal	Shifnal, Shropshire	2015
Woore	Woore, Staffordshire	2015
Invergowrie_1	Invergowrie, Dundee	2017
Invergowrie_2	Invergowrie, Dundee	2016

1155

1156 **2.3.1.2. DNA extraction**

1157 DNA extraction was performed on eight insects from each population except for the Stafford_2
1158 population in which four insects were used due to the small sample size at this site (one insect
1159 = one replicate). Insects were surface sterilised in a 1% bleach (May and Baker LTD,
1160 Dagenham, England) solution for one minute (Lawrence et al., 2015; Malacrinò et al., 2018).
1161 To remove the remaining bleach insects were submerged in autoclaved water three times,

1162 each time the insects were submerged for one minute. Surface sterilised insects were ground
1163 individually using pestle and mortar sterilised by exposing to UV light for 10 minutes. Once the
1164 whole sample was ground to a powder, total DNA was extracted using the NucleoSpin Kit
1165 (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and the
1166 alternative step suggested in the Kit protocol. An additional incubation at 70°C for 10 minutes
1167 was included, after the 10 minutes lysis step at 65°C specified in the protocol, to lyse gram
1168 negative bacterial cell walls. Extracted DNA was stored at -20°C in autoclaved Eppendorf
1169 tubes until further use.

1170 **2.3.1.3. PCR amplification of the 16S rRNA gene**

1171 A fragment of the V4 hypervariable region of the 16S rRNA gene was used for the current
1172 bacterial community study as it has been shown to yield optimal community analysis in
1173 previous studies (Caporaso et al., 2011) and it was chosen as a reference marker for the Earth
1174 Microbiome Project (EMP) (Gilbert et al., 2010). The primers used, 515F (5'-
1175 GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'), carry an
1176 Illumina adapter, pad and linker at the 5' terminus. Additionally, the reverse primer (806R)
1177 carries a unique barcode which is a 12-base error correcting Golay code to allow multiplexing,
1178 i.e. sequencing different samples simultaneously.

1179 The Kapa HiFi HotStart PCR kit (Kapa Biosystems, Wilmington, USA) was used to amplify the
1180 targeted DNA fragment in a G-Storm GS1 Thermal Cycler (Gene Technologies, Somerton,
1181 UK). The PCR mixture (20 µL) consisted of 4 µL of 5X Kapa HiFi Buffer, 1 µL of a 10 ng/µL
1182 Bovine Serum Albumin solution (Roche, Mannheim, Germany), 0.6 µL of a 10 mM Kapa
1183 dNTPs solution, 0.6 µL of a 10 µM solution of each primer, 0.25 µL of Kapa HiFi polymerase
1184 (0.02 U/µL), 8 µL of sterile water and 1 µL of a 10 ng/µL solution of the template DNA. Samples
1185 in the thermocycler were subjected to three minutes of DNA initial denaturation at 94°C, then
1186 35 cycles of 30 seconds of DNA denaturation at 98°C, 30 seconds of primer annealing at
1187 50°C, and one minute of DNA elongation at 72°C, followed by a final elongation step of 10
1188 minutes at 72°C.

1189 Based on the protocol described by Costello et al. (2009) and adopted by the EMP, each
1190 insect replicate was PCR amplified using a specific combination of forward and reverse
1191 primers with a unique, replicate-specific, barcode. For each primer pair combination, the
1192 corresponding PCR reaction was performed in simultaneous triplicates to diminish
1193 amplification biases, with an additional no template control. PCR reactions were combined in
1194 a barcode-wise manner, i.e. amplification replicates of the same primer pair were mixed and
1195 were tested on a 1.5% agarose gel with the corresponding no template control. The

1196 simultaneous triplicate amplification procedure was repeated three times for each primer pair
1197 combination. So, for each primer pair combination we performed nine amplifications in total.
1198 Finally, all PCR products were mixed in a barcode-wise manner (nine amplifications mixed)
1199 and kept at -20°C until further use.

1200 **2.3.1.4. Illumina MiSeq library preparation and sequencing**

1201 PCR products were purified with Agencourt AMPure XP kit (Beckman Coulter, Brea, USA)
1202 using 0.7 µL AMPure XP beads per 1 µL of sample. The DNA concentration of 3 µL of each
1203 PCR reaction, mixed according to their barcode, was quantified using Picogreen
1204 (ThermoFisher, UK) following the manufacturer's recommendations. Next, the amplicon
1205 library was generated by mixing individual barcoded replicates in an equimolar ratio. The
1206 library was sequenced by the Genome technology group at the James Hutton Institute,
1207 Dundee UK, using Illumina MiSeq platform with paired-end reads of 150 bp per read.

1208 **2.3.1.5. Illumina MiSeq data processing with QIIME**

1209 The Illumina MiSeq platform generated three FASTQ files with the forward, reverse and
1210 barcode sequences. The FASTQ files and the metadata information, organised in a mapping
1211 file, were processed with the open source software Quantitative Insights Into Microbial
1212 Ecology (QIIME) version 1.9.0 (Caporaso et al., 2010) using the default parameters unless
1213 otherwise specified.

1214 Forward and reverse FASTQ files were decompressed and merged specifying a minimum
1215 sequence overlap of 5 bp between pairs of reads using the command 'join_paired_ends.py'
1216 The reads were quality filtered and demultiplexed with the command 'split_libraries_fastq.py'
1217 specifying a minimum Phred quality score of 20. The remaining high-quality reads were
1218 clustered into Operational Taxonomic Units (OTUs) at 97% sequence similarity using
1219 SortMeRNA and sumaclust algorithms. OTUs were defined using a subsampled open-
1220 reference OTU picking approach with the command 'pick_open_reference_otus.py' against
1221 the chimera checked Greengenes database version 13_5 (DeSantis et al., 2006). The output
1222 was an OTU table with the identified OTUs as rows and the samples as columns, containing
1223 the abundance of each OTU per sample. The OTUs that did not match by 97% similarity any
1224 bacterial sequence on the database were classified as Unassigned.

1225 **2.3.1.6. Identification of the Unassigned OTU_0**

1226 The proportion of different Unassigned OTUs revealed that the dominant OTU was the
1227 OTU_0, which accounted for 99% (2,347,616 reads) of the total reads for Unassigned OTUs

1228 (2,364,356 reads). This OTU matched bacterial sequences found in different members of the
1229 Curculionidae family on the NCBI database. The highest matching percentage revealed
1230 similarity with bacterial sequences found in *Otiorhynchus sulcatus* (Fabricius) (vine weevil) by
1231 100% (GenBank: Accession No. JN563788.1 and JN563787.1) and in *O. salicicola* Heyden
1232 (GenBank: Accession No. JN394467.1), *O. armadillo* Rossi (GenBank: Accession No.
1233 JN394466.1) and *O. rugostriatus* Goeze (GenBank: Accession No. JN394465.1) by 98%
1234 (Hirsch et al., 2012). Furthermore, it matched bacterial sequences found in *Listronotus*
1235 *bonariensis* Kuschel by 96% (GenBank: Accession No. KJ522448.1) (White et al., 2015), in
1236 *Steriphus variabilis* Broun by 93% (GenBank: Accession No. KJ522449.1) (White et al., 2015)
1237 and a bacterial sequence classified as *Candidatus* Nardonella (γ-proteobacteria) found in
1238 *Pachyrhynchus infernalis* by 92% (GenBank: Accession No. AP018160.1) (Anbutsu et al.,
1239 2017). Hence, we have provisionally classified the OTU_0 as *Ca.* Nardonella .

1240 **2.3.1.7. Data analysis with R**

1241 To analyse the data with R software version 3.3.3 the packages phyloseq version 1.19.1
1242 (McMurdie and Holmes, 2013) and PMCMR version 4.3 were installed from Bioconductor
1243 using the code 'source ("http://bioconductor.org/biocLite.R")' and the function 'biocLite()'. The
1244 packages dendextend version 1.8.0 (Galili, 2015), vegan version 2.4-5 (Dixon, 2003), ape
1245 version 5.0 (Paradis et al., 2004) and ggplot2 version 3.0.0 (Wickham, 2009) were installed
1246 with the function 'install.packages'. The function ancom was installed using the code
1247 'source("ancom_functions.R")' and 'source("plot_ancom.R")'.

1248 First, a new OTU table was generated after filtering the initial OTU table obtained with QIIME
1249 for OTUs classified as mitochondria or chloroplast, likely representing a contamination from
1250 host tissues and/or the food source. Next, we removed from the remaining OTUs list, instances
1251 matching OTUs identified as environmental contaminants of the laboratory where we
1252 generated our sequencing library (Pietrangelo et al., 2018). After this initial filtering *in silico*,
1253 we identified the most abundant OTU in the phylum Bacteroidetes was used as an outgroup
1254 to root the phylogenetic tree generated by QIIME. Third, the phyloseq package was used to
1255 create the phyloseq object combining the new OTU table, the taxonomy matrix, the
1256 phylogenetic tree and the mapping file using the command 'merge_phyloseq'. Fourth, the
1257 dataset was filtered to discard OTUs with less than five reads in at least 10% of the studied
1258 insects with the function 'filter_taxa'.

1259 To study the α-diversity, replicates were rarefied to a similar sequencing depth of 11,207 reads
1260 with the function 'rarefy_even_depth' from the package phyloseq. The Chao1 and Shannon
1261 indices were then calculated with the function 'estimate_richness' from the package phyloseq.
1262 Normality was tested by applying a Shapiro-Wilk test with the function 'shapiro.test' which

1263 revealed that only Shannon index values were not normally distributed. Therefore, data for
1264 Observed OTUs and Chao1 index were analysed with the parametric ANOVA test paired with
1265 Tukey test for multiple comparisons with the functions 'aov' and 'TukeyHSD' from the R stats
1266 package 3.3.3. Shannon index values were analysed with the non-parametric Kruskal-Wallis
1267 test using the functions 'Kruskal.test' and 'posthoc.kruskal.dunn.test' from the package
1268 PMCMR.

1269 To study the β-diversity, the dataset was transformed into relative abundances, i.e. sample
1270 reads/total amount of reads. A distance matrix was calculated using Bray-Curtis metrics, which
1271 considers OTU relative abundance, with the function 'ordinate' from the package phyloseq. A
1272 hierarchical cluster analysis was performed with the function 'hclust' and the generated Cluster
1273 dendrogram was modified with the function 'set' within the package dendextend before
1274 plotting. Statistical differences in microbial composition among populations were tested using
1275 a permutational multivariate analysis of variance with the function 'adonis' from the package
1276 vegan (Dixon, 2003). OTUs showing significant differences in abundance between
1277 populations were revealed by applying an analysis of composition of microbiomes with the
1278 function 'ANCOM' from the package ANCOM using the multiple correction option '1' (Weiss
1279 et al., 2017).

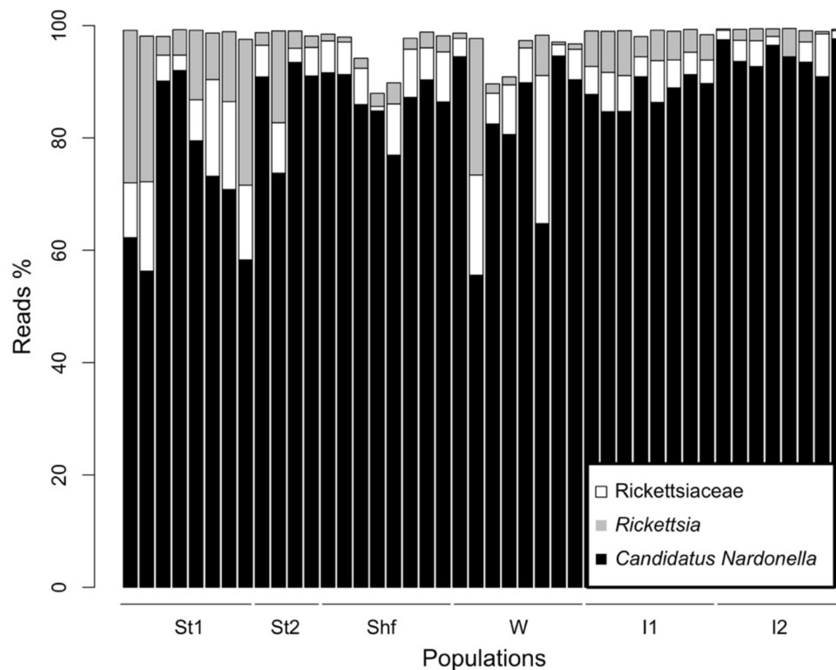
1280 2.3.2. Results

1281 2.3.2.1. Vine weevil bacterial microbiota is composed of 85 different bacterial 1282 taxa

1283 We characterised the bacterial community of six vine weevil populations collected from
1284 strawberry crops grown at different locations in the UK (Table 2.3 and Figure 2.3) using an
1285 Illumina MiSeq 16S rRNA gene sequencing approach. The sequencing library yielded
1286 3,153,991 high-quality reads which clustered in 994 Operational Taxonomic Units (OTUs) at
1287 97% similarity. OTUs classified as chloroplast and mitochondria, as well as predicted
1288 contaminant OTUs, were removed from the original file, which reduced the number of high-
1289 quality reads to 2,882,853 (per sample mean 65,519; max 199,121; and min 11,224) and the
1290 number of OTUs to 931. As a result, 91% and 93% of the original reads and OTUs,
1291 respectively, were kept for further analysis. To discard low abundance OTUs, which have low
1292 reproducibility, OTUs that had less than five reads in at least 10% of the studied insects were
1293 removed for subsequent analysis. This further reduced the number of reads to 2,871,373 and
1294 the number of OTUs to 85. Although this step reduced the number of OTUs by over 90%, we
1295 retained more than 99% of the total number of high-quality reads. This suggested that the
1296 bacterial microbiota of the populations tested in this study comprised a relatively low number
1297 of highly abundant bacterial taxa.

1298 **2.3.2.2. Vine weevil bacterial microbiota is dominated by γ-proteobacteria and α-**
1299 **proteobacteria**

1300 To investigate the taxonomic distribution at genus level, we manually annotated the OTU_0
1301 as *Candidatus* Nardonella and imposed a threshold of 1% abundance on the whole dataset
1302 for plotting purposes. As a result, only two bacterial genera and one family, that could not be
1303 classified at genus level, were considered: *Ca.* Nardonella (γ-proteobacteria) and *Rickettsia*
1304 and Rickettsiaceae (α-proteobacteria) with average relative abundance of 85%, 5.8% and
1305 6.9%, respectively (Figure 2.4). This further supports the idea that vine weevil bacterial
1306 microbiota in the sampled insects was dominated by a small number of taxa.



1307

1308 **Figure 2.4 Taxonomic classification of bacterial community members at genus level.**
1309 Y-axis represents average relative abundance (% reads). Bars represent individual insects
1310 from the population specified on the x-axis (see Table 2.1).

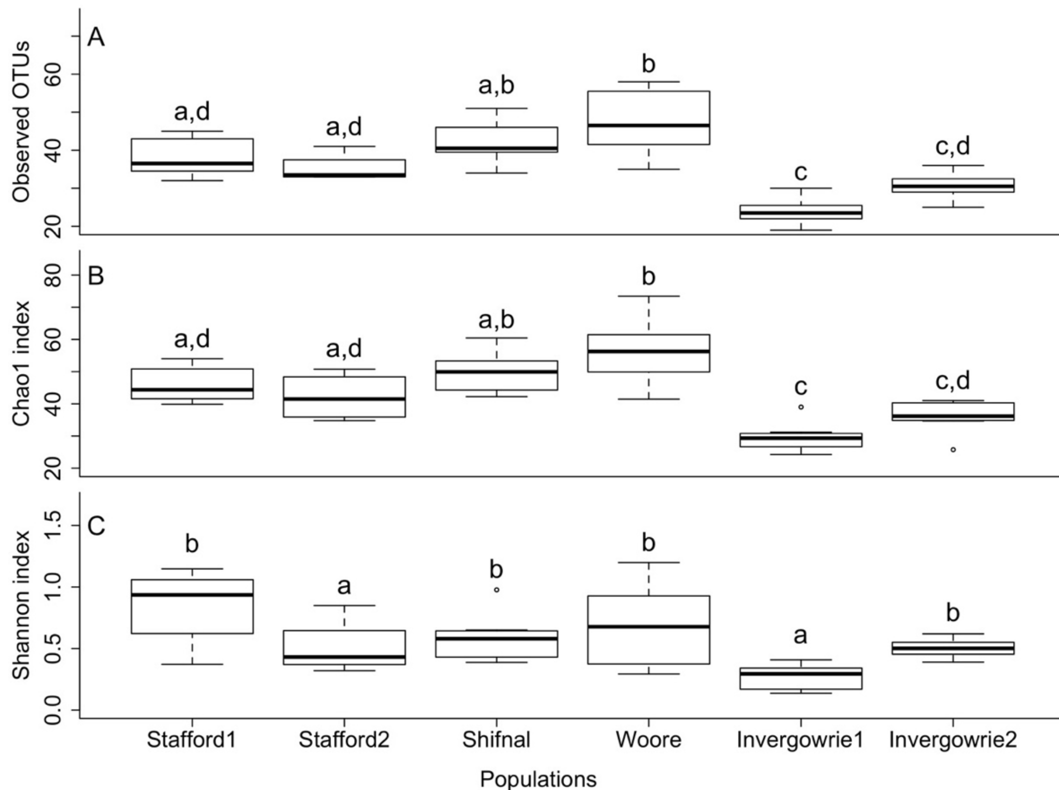
1311 **2.3.2.3. Vine weevil populations harbour a low diversity bacterial microbiota**

1312 Within population diversity, or α-diversity, computed at OTU level, revealed low diversity in the
1313 bacterial communities across vine weevil populations. On average, populations harboured a
1314 bacterial community comprising 36 OTUs, a richness value (Chao1 index) of 43 and an
1315 evenness value (Shannon index) of 0.5 (Figure 2.5). Statistical analysis of the observed OTUs
1316 revealed that Invergowrie populations tended to harbour a lower number of OTUs (Figure
1317 2.5A, ANOVA, $F= 20.16$, $df= 5$, $P< 0.05$) and lower richness index values (Figure 2.5B,
1318 ANOVA, $F= 16.89$, $df= 5$, $P< 0.05$) compared to the rest of the populations, although
1319 Stafford_2 and Invergowrie_2 populations were not significantly different. Statistical analysis
1320 of Shannon index values revealed that evenness was significantly lower only for Stafford_2

1321 and Invergowrie_1 populations, compared to the rest of the populations (Figure 2.5C, Kruskal-
1322 Wallis test, H= 19.88, df=5, P< 0.05).

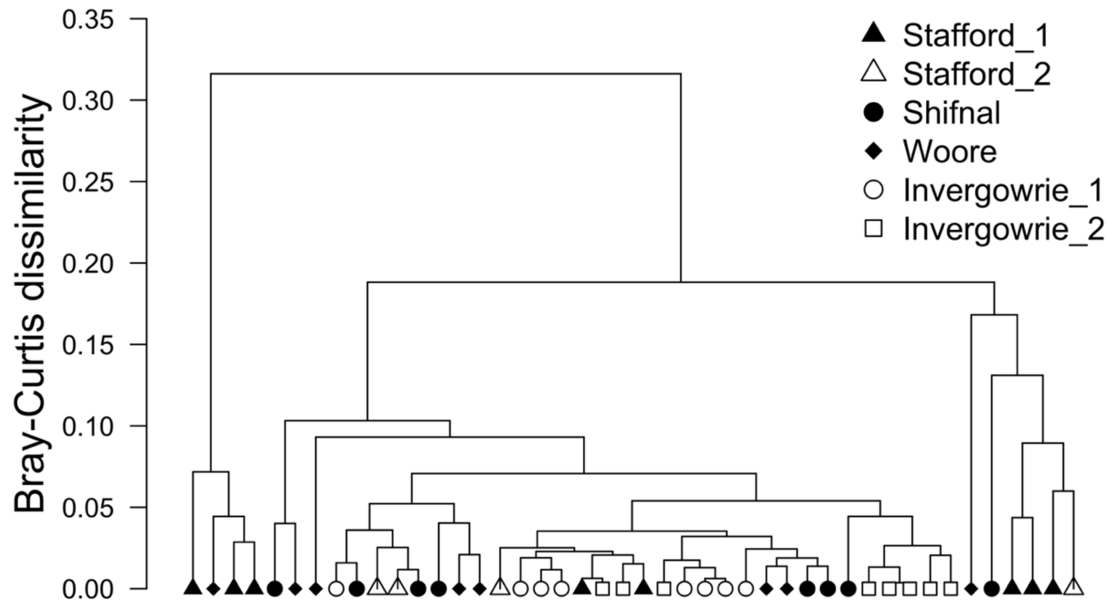
1323 **2.3.2.4. Vine weevil bacterial microbiota composition is dominated by**
1324 ***Candidatus* Nardonella**

1325 Vine weevil bacterial community diversity between populations, or β-diversity, was calculated
1326 using a Bray Curtis approach, which considers OTU relative abundance. This analysis failed
1327 to reveal a clear pattern associated with location (Figure 2.6). Closer inspection of the
1328 individual OTUs identified in our library revealed that samples were dominated by the OTU_0,
1329 classified as *Ca.* Nardonella , which represented 81% of the total sequencing reads and 84%,
1330 in average, of the sequencing reads assigned to each individual insect. Thus, the high
1331 incidence of a single bacterial phylotype classified as *Ca.* Nardonella governed the bacterial
1332 community assembly of the populations studied here.



1333

1334 **Figure 2.5 Average (A) observed operational taxonomic units (OTUs), (B) richness**
1335 **(Chao1 index), and (C) evenness (Shannon index) of bacterial communities, per vine**
1336 **weevil population.**
1337 Box plots indicate the median as a thick line, the interquartile range (IQR) as a box, 1.5 IQR
1338 as whiskers and the outliers as points outside the whisker range. Population values sharing
1339 the same letter were not significantly different (Tukey test: P<0.05 for observed OTUs and
1340 Chao1 index; Dunn's test: P<0.05 for Shannon index).



1341

1342 **Figure 2.6 Bray-Curtis cluster dendrogram of the bacterial community associated with**
1343 **vine weevils.**
1344 Y-axis represents Bray-Curtis dissimilarity values. Each dendrogram leaf represents a single
1345 insect and different shapes represent different populations.

1346 **2.3.2.5. Location specific OTUs are dominated by members of the Proteobacteria**
1347 **phylum**

1348 Statistical analysis revealed that despite the lack of location-associated pattern in the
1349 microbiota composition, we identified significant differences between populations (Adonis test,
1350 df=5, P<0.05, R2 Location= 0.37). A total number of 16 OTUs was shown to vary significantly
1351 in abundance between vine weevil populations with 11, 2 and 1 of the OTUs belonging to
1352 Proteobacteria, Bacteroidetes and Actinobacteria phyla, respectively, and 2 Unassigned
1353 OTUs (ANCOM test, P<0.01, multiple test correction). OTUs assigned to Proteobacteria
1354 phylum belonged to Sphingomonadales and Rickettsiales orders within α-proteobacteria and
1355 to Enterobacteriales, Pseudomonadales and Xanthomonadales orders within γ-
1356 proteobacteria. OTUs assigned to Bacteroidetes phylum belonged to Sphingobacteriales and
1357 Flavobacteriales orders, and OTUs assigned to Actinobacteria phylum belonged to
1358 Actinomycetales order. The average abundance for these OTUs per population was: 0.05%
1359 for Stafford_1, 0.02% for Stafford_2, 0.08% for Shifnal, 0.12% for Woore, 0.02% for
1360 Invergowrie_1 and 0.02% for Invergowrie_2. Thus, OTUs that varied in abundance between
1361 locations represented a small fraction of the total number of reads and, despite belonging to
1362 different phyla, they were biased towards members of the Proteobacteria phylum. This
1363 observation suggests that the 37% of the variance attributed to location in the analysis, is
1364 associated, at least partially, to the fluctuation of *Ca.* Nardonella across populations.

1365 ## 2.4. Conclusion based on results from both sequencing analysis

1366 The current studies characterised for the first time the bacterial community of vine weevil
1367 adults from different UK geographic areas and from different host plants. Our results showed
1368 that the bacterial microbiota composition did not follow a pattern governed by host plant nor
1369 location, as only a small fraction of the Operational Taxonomic Units (OTUs) varied in
1370 abundance between populations. Furthermore, the bacterial community was dominated by
1371 members of the Proteobacteria phylum, with remarkably high abundance of a single bacterium
1372 belonging to the γ-proteobacteria and classified as *Candidatus* Nardonella. These findings are
1373 consistent with those reported previously in insect bacterial community studies, which
1374 revealed a similarly low diversity of bacterial microbiota dominated by members of the
1375 Proteobacteria phylum, compared with analogous studies on vertebrates or soil (Bansal et al.,
1376 2014; Bili et al., 2016; Broderick et al., 2004; Chandler et al., 2011; Colman et al., 2012; Corby-
1377 Harris et al., 2007; Douglas, 2011; Fierer and Jackson, 2006; Gauthier et al., 2015; Ishak et
1378 al., 2011; Jones et al., 2011; Robertson-Albertyn et al., 2017; Vasanthakumar et al., 2006;
1379 Wong et al., 2011; Yun et al., 2014). This bacterial microbiota pattern seems to be common
1380 across insect clades even when targeting different 16S rRNA gene hypervariable regions
1381 (Baker et al., 2003; Guo et al., 2013; Suzuki and Giovannoni, 1996; Yang et al., 2016) or
1382 applying different DNA extraction procedures (Martin-Laurent et al., 2001). The reasons
1383 underlying such an intriguing pattern remain undetermined, although a number of hypotheses
1384 have been proposed to explain low microbial diversity in insects. One hypothesis suggests
1385 that the insect immune system fine tunes the bacterial microbiota composition in order to
1386 tolerate only beneficial bacteria as has been seen in *D. melanogaster* and the red palm weevil
1387 (Chandler et al., 2011; Dawadi et al., 2018; Lhocine et al., 2008; Login et al., 2011; Ryu et al.,
1388 2008). Another hypothesis, although not exclusive, suggests that low microbial diversity
1389 results from negative interactions between co-inhabiting bacteria as has been seen between
1390 *Buchnera* and *Rickettsia* in the pea aphid (Sakurai et al., 2005), between *Spiroplasma* and
1391 *Wolbachia* in *D. melanogaster* (Goto et al., 2006) and between *Bartonella* and *Rickettsia* in
1392 fleas from the genus *Oropsylla* (Jones et al., 2012). Nonetheless, the biological factors
1393 shaping insect bacterial microbiota in this characteristic manner remain speculative and open
1394 to future investigation.

1395 The findings presented here show that vine weevil bacterial community is mainly composed
1396 of members of the α and γ-proteobacteria classes with noteworthy high abundance of the OTU
1397 classified as *Ca.* Nardonella. Conversely, a previous sequencing attempt to characterise vine
1398 weevil bacterial microbiota showed that it was composed entirely of members of the α-
1399 proteobacteria order and, surprisingly, *Ca*. Nardonella abundance was very low as it could
1400 only be detected by diagnostic PCR with specific primers (Hirsch et al., 2012). Differences

1401 between the previous and the current vine weevil bacterial microbiota characterisations could
1402 be attributed to insect ontogeny as Hirsch et al. (2012) examined 24-72h old vine weevil
1403 larvae, whereas we used vine weevil adults close to maturity. Insect life stage has been shown
1404 to influence microbial community composition in several insects, for example the Hessian fly
1405 *Mayetiola destructor* (Say) (Bansal et al., 2014), species of the parasitoid wasp genus *Nasonia*
1406 (Brucker and Bordenstein, 2012), the rice water weevil *Lissorhoptrus oryzophilus* (Kuschel)
1407 (Huang et al., 2016), the southern pine beetle *Dendroctonus frontalis* (Zimmermann)
1408 (Vasanthakumar et al., 2006), the house fly *Musca domestica* (Linnaeus) (Wei et al., 2013),
1409 *D. melanogaster* (Wong et al., 2011) and the neotropical butterfly *Heliconius erato* (Linnaeus)
1410 (Hammer et al., 2014). Furthermore, *Ca.* Nardonella in rice water weevil was present at low
1411 titre in larvae and pupae whereas its abundance increased substantially upon adult
1412 emergence (Huang et al., 2016). The mechanisms triggering such developmental changes in
1413 microbial composition are unclear, although it has been proposed that adaptation to utilise
1414 different resources at different life stages could influence bacterial community composition
1415 (Hammer et al., 2014). An additional factor to consider is that Hirsch et al. (2012) used larvae
1416 hatched from surface sterilised eggs for bacterial community characterisation. Although
1417 bacterial transmission to progeny through the egg surface has not been studied in vine weevil,
1418 egg surface sterilisation could potentially eliminate an important source of bacteria for the
1419 developing insect as has been described in other members of the Coleoptera order, such as
1420 the reed beetle genus *Macroplea* (Kleinschmidt and Kölsch, 2011; Kölsch et al., 2009)
1421 (Kleinschmidt & Kölsch, 2011; Kölsch et al., 2009) and the rove beetle *Paederus sabaeus*
1422 (Erichson) (Kellner, 2002, 2001). Therefore, to clarify the differences between these studies,
1423 further research should aim to characterise vine weevil larvae bacterial microbiota in
1424 comparison with egg and adult life stages.

1425 Perhaps unexpectedly, no significantly different OTUs were found when insects from different
1426 host plants and locations were considered. When only considering insects collected at
1427 separate locations, detected location specific bacteria constituted a small fraction of the total
1428 number of reads suggesting that location has a limited role in sculpting the composition of vine
1429 weevil bacterial microbiota. However, caution should be exerted when interpreting these data.
1430 For instance, our study could be limited by considering a relatively narrow sampling area.
1431 Furthermore, Shifnal and Woore populations lacked sampling replication as I only analysed
1432 one population at each of these locations. Hence, the greater proportion of location specific
1433 OTUs from the Woore population, compared with the other populations, may be derived from
1434 the sampling design rather than the intrinsic biology of the populations. Thus, future studies
1435 should aim to collect insects from a wider geographic area, including different populations from
1436 the same area, to determine if location has an influence on bacterial community composition

1437 in vine weevil. I have carried out a study to taxonomically characterise the bacterial community
1438 harboured by vine weevil populations. Future research should aim at using a metagenomic
1439 approach to understand the metabolic capabilities of this significantly different OTUs. Only in
1440 this I could hypothesise the putative role of these bacteria for the weevil host (Whiteside et al.,
1441 2015).

1442 The high incidence of the OTU classified as *Ca.* Nardonella in all tested insects could indicate
1443 the importance of its contribution to adult development and cuticle integrity as has been
1444 demonstrated in studies of other weevil species (Anbutsu et al., 2017; Kuriwada et al., 2010).
1445 *Ca.* Nardonella is a bacterial symbiont widespread throughout the weevil superfamily located
1446 in bacteriocytes forming a specialised organ, the bacteriome, which localises at the
1447 foregut/midgut junction of larvae and at the apex of the ovarioles in adults (Conord et al., 2008;
1448 Hosokawa et al., 2015; Hosokawa and Fukatsu, 2010; Huang et al., 2016; Mansour, 1930;
1449 Nardon et al., 2002). In a recent study, the Nardonella genome was sequenced from the black
1450 hard weevil *Pachyrhynchus infernalis* revealing that it possesses an extremely small genome
1451 (0.20 to 0.23 Mb) with reduced metabolic capacity (Anbutsu et al., 2017), a characteristic
1452 feature for primary obligate symbionts (reviewed by Moya et al., 2009). Results from the same
1453 study revealed that this bacterium could influence adult development through its involvement
1454 in tyrosine production. Therefore, based on the contribution of Nardonella to adult
1455 development in other weevil species, it would be of great interest to investigate the dynamics
1456 of this bacterium at all vine weevil life stages.

1457 The findings of the present study contribute to the field of research on insect bacterial
1458 microbiota as we have comprehensively characterised vine weevil bacterial community of
1459 several insect populations by amplifying a region of the V4 hypervariable region of the
1460 prokaryotic 16S rRNA gene, paired with Illumina MiSeq sequencing technology. Moreover,
1461 our results showed that vine weevil bacterial community of the populations sampled from
1462 strawberry plants did not follow a location specific pattern and was dominated by a single
1463 bacterium identified as *Ca.* Nardonella . This study forms the basis for future research to
1464 understand the role of location-specific biotic and abiotic factors in shaping vine weevil
1465 bacterial community. An additional interesting line of research would be to study the
1466 importance of *Ca.* Nardonella for vine weevil development and or reproduction. Likewise, as
1467 innovations in sequencing technology are becoming available for experimentation, it will be
1468 interesting to accurately identify and quantify the dominance of *Ca.* Nardonella in the vine
1469 weevil microbiota with additional methodologies. This will provide valuable insights for the field
1470 of agroecology to devise new strategies for management and biocontrol of this damaging and
1471 polyphagous insect pest.

1472

Chapter 3

Methods: Optimisation of DNA extraction and artificial rearing for the vine weevil *Otiorhynchus sulcatus* (Fabricius)

3.1. Optimisation of DNA extraction for the vine weevil

DNA extraction procedures for the vine weevil have to date only been applied for the larval stage. Aiming to be able to perform DNA-based molecular studies on all the vine weevil developmental stages, i.e. eggs, larvae, pupae and adults, we evaluated the adequacy of three different DNA extraction procedures: DNeasy Blood & Tissue kit, phenol: chloroform: isoamyl alcohol protocol and the NucleoSpin kit.

3.2. Materials and methods: Optimisation of DNA extraction for the vine weevil

3.2.1. DNA extraction with DNeasy Blood & Tissue kit

DNA extraction was performed on fresh insect material using either an entire individual (larva or adult) or 20 eggs using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) (Jousselin et al., 2016). The sample was placed in a sterile Eppendorf tube and frozen by submerging the tube briefly in liquid N_2. Then, the frozen sample was ground using a pestle and mortar, previously sterilised with UV light for 10 minutes in the laminar flow cabinet. Once the whole sample was ground to a powder, 1 or 2 mL of buffer A (35 mM Tris-HCl, 25 mM KCl, 10 mM MgCl2, 25 mM sucrose; pH 7.5), depending on the amount needed to collect the whole sample, was used to dilute the sample and transfer it to another sterile Eppendorf tube. These tubes were centrifuged for 10 minutes at 5000x *g*. DNA was extracted following the manufacturer's instructions specified in the kit protocol for "pre-treatment for gram positive bacteria". The supernatant was discarded and the pellet was re-suspended in 180 µl of enzymatic lysis buffer (20 mM, Tris-HCl, 2 mM sodium EDTA, 1.2% Triton® X-100; pH 8.0). In order to lyse gram positive bacteria, 18 µL of lysozyme (10 mg/mL) (Sigma-Aldrich Ltd, St Louis, USA) was added to each sample. Samples were incubated in a water bath for 30 minutes at 37°C and were inverted every 10 minutes to mix. Subsequently, 25 µl of proteinase K and 200µl of buffer AL (without ethanol) were added to each sample and mixed by vortexing, then incubated at 56°C for 30 minutes with inversion every 10 minutes. Adult samples were

1503 centrifuged for 1 minute at 735x *g* to sediment exoskeleton fragments and the supernatant
1504 was transferred to a clean Eppendorf tube. This extra step was included to avoid blocking the
1505 column with exoskeleton parts in later steps. To each sample, 200 µl of ethanol (96–100%)
1506 was added and samples were mixed thoroughly by vortexing. Then, samples were transferred
1507 to the spin columns provided with the kit, and centrifuged at 600x *g* for 1 minute. The flow
1508 through was discarded, 500 µL buffer AW1 were added to the columns and they were newly
1509 centrifuged at 600x *g* for 1 minute. The flow through was discarded, 500 µL buffer AW2 was
1510 added to the columns and they were centrifuged at 20000x *g* for 3 minutes. The flow through
1511 was discarded and columns were transferred to clean Eppendorf tubes. Then, 200 µL of buffer
1512 AE were added to each column and they were incubated at room temperature for 1 minute.
1513 To elute the DNA these columns were centrifuged at 600x *g* for 1 minute. Eluted DNA was
1514 stored at -20°C in clean Eppendorf tubes until further use.

1515 3.2.2. DNA extraction with phenol: chloroform: isoamyl alcohol protocol

1516 DNA extraction was performed on fresh insect material using the phenol: chloroform: isoamyl
1517 alcohol protocol (Chia et al., 1985). An entire vine weevil adult was used but neither larva nor
1518 eggs were used. The sample was placed in a sterile Eppendorf tube and was frozen by
1519 submerging the tube briefly in liquid N_2. Then, the frozen sample was ground using pestle and
1520 mortar previously sterilised with UV light for 10 minutes in the laminar flow cabinet. Once the
1521 whole sample was ground to a powder, it was collected using 1.2 mL of grinding buffer (100
1522 mM Tris-HCl, 10 mM EDTA, 350 mM NaCl, 2%SDS, 7 M urea ultrapure; pH 7.5) and placed
1523 in another sterile Eppendorf tube. Then, samples were incubated in a warm water bath at 70°C
1524 for 10 minutes in order to break gram positive bacteria cell walls. Later, in the fume hood, 400
1525 µL of phenol: chloroform: isoamyl alcohol (25:24:1) (Sigma-Aldrich Ltd, St Louis, USA) were
1526 added to each tube and these tubes were mixed by inversion 100 times. Subsequently, tubes
1527 were centrifuged at 16,000x *g* for 5 minutes and the tube content separated in two layers.
1528 Then, the top layer was carefully transferred to a clean Eppendorf tube using wide end tips.
1529 This top layer was newly treated with phenol: chloroform: isoamyl alcohol repeating previous
1530 steps. The 2nd top layer was treated with 400 µL of chloroform (Fisher scientific,
1531 Loughborough, UK): isoamyl alcohol (Sigma-Aldrich Ltd, St Louis, USA) (24:1) and tubes were
1532 inverted 50 times. These tubes were centrifuged at 16,000x *g* for 2 minutes and the top layer
1533 was newly transferred to a clean Eppendorf tube. Then, 2.5X volume of ice cold 100% ethanol
1534 (Sigma-Aldrich Ltd, Dorset, UK) was added to each tube. These tubes were inverted slowly
1535 and left overnight at -20°C. On the following day, tubes were centrifuged at 16,000x *g* for 10
1536 minutes. Next, the ethanol was poured off carefully to avoid disturbing the pellet. The pellet
1537 was washed with 200 µL of 70% ethanol (ice cold) and centrifuged at 16,000x *g* for 5 minutes.
1538 Then, the ethanol was poured off carefully and tubes were left open at room temperature until

the remaining ethanol evaporated. The dry pellet was re-suspended with 100 µL of sterile water. DNA extracted was stored at -20°C in clean Eppendorf tubes.

3.2.3. DNA extraction with NucleoSpin kit

DNA extraction was performed on fresh material using the Nucleo Spin kit (Macherey-Nagel, Düren, Germany) (Kranzfelder et al., 2016). An entire vine weevil adult was used but neither larva nor eggs were used. The sample was placed in a sterile Eppendorf tube and was frozen by submerging the tube briefly in liquid N_2. Then, the frozen sample was ground using pestle and mortar previously sterilised with UV light for 10 minutes in the laminar flow cabinet. Once the whole sample was ground to a powder, the instructions specified in the NucleoSpin kit protocol were followed. So, 400 µL of buffer PL1 and 10 µL of RNase A were added to the samples and they were incubated at 65°C for 10 minutes. The kit protocol offers an alternative to this step which was tested on half of the samples aiming to find the most efficient way to extract DNA from adult vine weevils. So, 300 µL of PL2 buffer and 10 µL of RNase A were added to these samples and they were incubated at 65°C for 10 minutes. Later, 75 µL of buffer PL3 were added and the samples were kept on ice for 5 minutes. Then, all the samples were treated similarly following the kit protocol instructions. DNA extracted was stored at -20°C in clean Eppendorf tubes.

3.2.4. PCR with primers for the 16S rRNA bacterial gene

The ribosomal 16S rRNA gene is highly conserved among prokaryotes; however, it possesses some variable regions that enable taxonomic distinction. Thus, this gene is used routinely to identify bacteria present in complex samples. In our analysis, a fragment of the 16S rRNA bacterial gene was amplified by PCR using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1494R (5'-GCTCTAGAGCGGYTACCTTGTTACGACTT-3') (Lane, 1991). The PCR reaction was conducted in a total reaction mixture volume of 25 µL and comprised: 10 µL of 5xGoTaq buffer (Promega, Southampton, UK), 0.5 µL of dNTPs 12.5 mM (Promega, Southampton, UK), 1 µL of each primer 10 µM (Sigma-Aldrich Ltd, Gillingham, UK), 11 µL of sterile ultrapure water, 0.125µL of GoTaq DNA polymerase 5 u/µL (Promega, Southampton, UK) and 1 µL of DNA or water. To minimise potential contamination, Eppendorf tubes, PCR tubes, pipette tips, pipettes and water were sterilised with UV light in the laminar flow cabinet for 10 minutes, and the PCR reaction mixture was assembled in the laminar flow cabinet. Water used in the PCR reaction was first autoclaved, then sterilised by passing through a 0.22 µm filter (Millipore Ireland Ltd, Carrigtwohill, Ireland) and stored in sterile Eppendorf tubes. Each PCR screen included a negative control (1 µL of the same water used for the PCR reaction), and a positive control (1 µL of *Escherichia coli* strain O157 extracted DNA). Samples in the thermocycler were subjected to 2 minutes of DNA denaturation at 95°C, 40 cycles of 30

1574 seconds of DNA denaturation at 95°C, 30 seconds of primer annealing at 55°C and 3 minutes
1575 of DNA elongation at 72°C, and a final elongation of 7 minutes at 72°C. PCR products were
1576 used immediately or stored at 4°C for one day.

1577 3.2.5. Visualisation of DNA extracted and PCR products on agarose gel

1578 The state of the extracted DNA and the PCR products were visualised on agarose gel.
1579 Ultrapure agarose (Invitrogen, Carlbad, USA) was dissolved at 1% (w/v) in TBE buffer (89 mM
1580 Tris, 89 mM boric acid, 2 mM EDTA; pH 7.6) with heating, then 2.5% (v/v) GelRed (Invitrogen,
1581 USA) stain was added. The agarose solution was allowed to solidify in the gel mould tray at
1582 room temperature for 20 minutes covered with aluminium foil to avoid photo-degradation of
1583 the GelRed. Aliquots of 4 μL of each PCR amplification product, or extracted DNA, mixed with
1584 4 μL of loading dye, were loaded into the gel slots. An aliquot of 4 μL of a 1 Kb DNA ladder
1585 (Promega, Madison, USA) mixed with loading dye, at a proportion of 2 loading dye: 3 ladder,
1586 was loaded for size comparison with the PCR amplified products. The gel was run for about
1587 40 minutes at 60 V while covered with aluminium foil. PCR bands were visualized with a UV
1588 trans-illuminator (Alpha Innotech).

1589 3.3. Results: Optimisation of DNA extraction for the vine weevil

1590 3.3.1. DNA extraction with DNAeasy Blood & Tissue kit

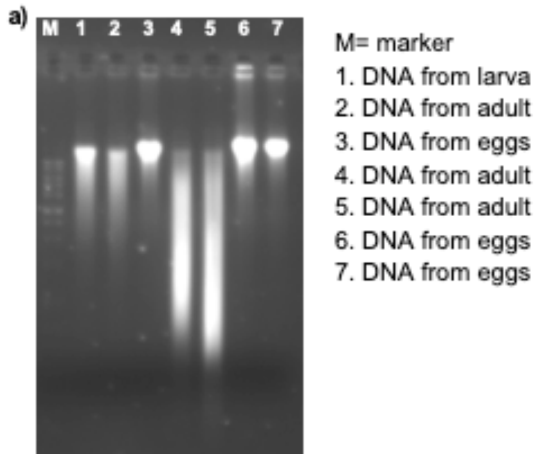
1591 DNA extracted from vine weevil larvae and eggs was considered of a good quality as a clear
1592 band was visualised in the agarose gel (Figure 3.1, a). However, when DNA extracted from
1593 adults was tested in the agarose gel a smear was visualised, meaning it was highly degraded
1594 and therefore of a poor quality (Figure 3.1, a). PCR performed using this extracted DNA as a
1595 template amplified the 16S rRNA gene when larval or egg DNA were used, as a band could
1596 be visualised on the agarose gel (Figure 3.1, b), but not when adult DNA was used, as no
1597 band could be visualised on the agarose gel (Figure 3.1, b).

1598 3.3.2. DNA extraction with phenol: chloroform: isoamyl alcohol protocol

1599 DNA extracted from vine weevil adults was highly degraded as a smear was visualised on the
1600 agarose gel (Figure 3.1, c). Additionally, when PCR was performed using this DNA as a
1601 template the 16S rRNA gene could not be amplified as no band was visualised on the agarose
1602 gel (Figure 3.1, d).

1603 3.3.3. DNA extraction with NucleoSpin kit

1604 DNA was successfully extracted from vine weevil adults as bands could be visualised on the
1605 agarose gel (Figure 3.1, e). Moreover, PCR performed on this DNA amplified the 16S rRNA
1606 gene as bands could be visualised on the agarose gel (Figure 3.1,f).



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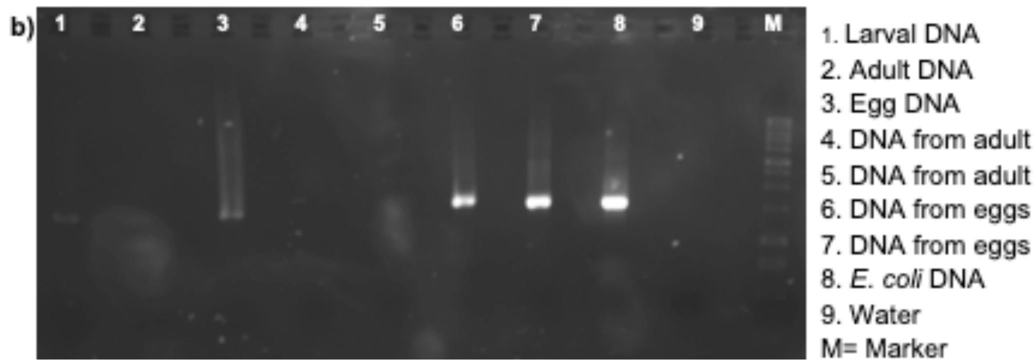

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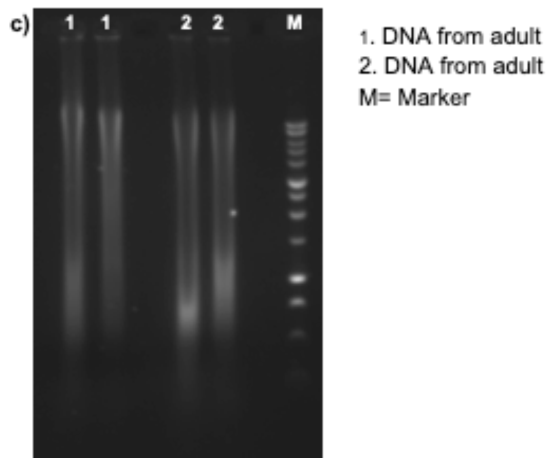

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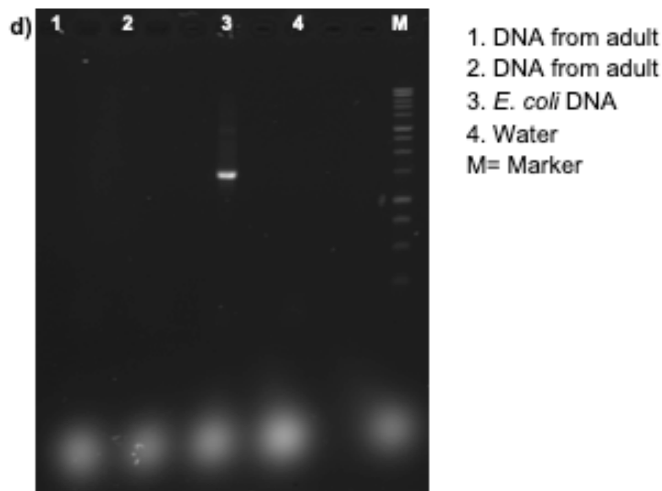

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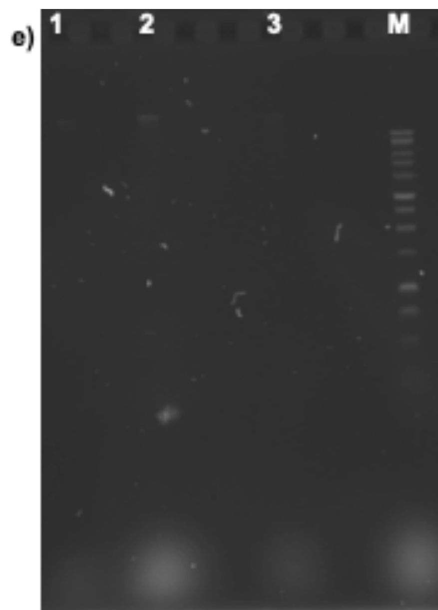

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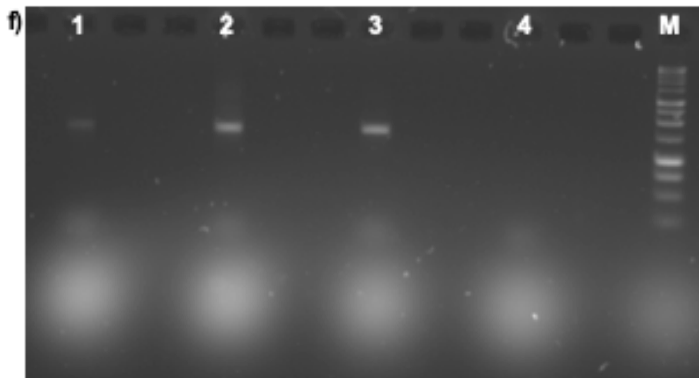


1613 **Figure 3.1 1% Agarose gels to visualise DNA quality and PCR results.**
1614 a) DNA extracted with DNeasy Blood & Tissue kit, b) PCR with primers 27F/1494R on DNA
1615 extracted with DNeasy Blood & Tissue kit, c) DNA extracted with phenol: chloroform: isoamyl
1616 alcohol protocol, d) PCR with primers 27F/1494R on DNA extracted with phenol: chloroform:
1617 isoamyl alcohol protocol, e) DNA extracted with NucleoSpin kit, f) PCR with primers
1618 27F/1494R on DNA extracted with NucleoSpin kit. *Escherichia coli* DNA and water were used
1619 as positive and negative controls respectively for the PCR amplification. M= molecular marker
1620 with bands of size: 250, 253, 500, 750, 1,000, 1,500, 2,000, 2,500, 3,000, 4,000, 5,000, 6,000,
1621 8,000 and 10,000bp.

1622 3.4. Discussion: Optimisation of DNA extraction for the vine weevil

1623 Good quality DNA was successfully extracted from larvae and eggs using the DNeasy Blood
1624 & Tissue kit. Nevertheless, adult DNA extraction was more difficult and from the three different
1625 procedures tested the NucleoSpin kit was the only one that gave non-degraded DNA. It is
1626 known that some nucleases, enzymes that break down DNA, are guided by specific RNA
1627 molecules (Bi et al., 2014; Citorik et al., 2014; Hammond et al., 2000). Therefore, it could be
1628 that the presence of RNase A, enzyme that degrades RNA, in the extraction with NucleoSpin
1629 kit, protected the weevil DNA from these enzymes. The alternative step suggested in the

1630 NucleoSpin kit protocol gave better results; therefore, in later DNA extractions the NucleoSpin
1631 kit following the alternative step was used.

1632 3.5. Optimisation of vine weevil artificial diet

1633 The vine weevil has a holometabolous life cycle whose length is temperature dependent
1634 (Smith, 1932; Son and Lewis, 2005). In outdoor conditions, the cycle is completed in a year
1635 (Moorhouse et al., 1992; Smith, 1932), although certain practices that increase soil or
1636 substrate temperatures like mulches or indoor glasshouse conditions can accelerate pupation
1637 and adult emergence consequently shortening the life cycle (Bennison et al., 2018a; Evenhuis,
1638 1978). A long developmental cycle makes the vine weevil a difficult organism to culture and
1639 consequently research applied to this insect species remains scarce. Here we aimed at
1640 optimising a previously developed artificial diet for mass rearing the vine weevil (Fisher and
1641 Bruck, 2004) by: a) eliminating the antibiotic to diminish the alteration of the microbiota, b)
1642 reducing the UV exposure time to facilitate the production of media.

1643 3.6. Materials and methods: Optimisation of vine weevil artificial diet

1644 3.6.1. Vine weevil adult lines and leaf culture

1645 Vine weevil adults were sampled during summer 2015 from different outdoor environments
1646 across the UK. Vine weevil adults collected from different locations were kept separately as
1647 lines. For each line, some of the individuals were frozen with liquid N_2 and stored at -80°C until
1648 further use, and some of the individuals were maintained as live cultures on leaves (Table
1649 3.1).

1650 Vine weevil adults in leaf culture were kept in Petri dishes lined with moist paper (Kleenex,
1651 Kimberly-Clark professional, Kent, UK) and provided with fresh strawberry leaves (*Fragaria* x
1652 *ananasa* (Duchesne), mixed varieties) as a source of food, in controlled environment rooms
1653 (18°C, 16:8 h L:D) (Figure 3.2). For each line, vine weevils were transferred to clean paper-
1654 lined Petri dishes with fresh strawberry leaves weekly. To collect eggs, the Petri dishes in
1655 which adults had fed and oviposited for a week were stored at 4°C, after transferring the adults
1656 to another dish, to slow down egg developmental changes (Fisher and Bruck, 2004), for
1657 maximum of two weeks.

1658 **Table 3.1 Vine weevil lines from different locations and host plants.**

Line	Location	Host plant
TP01	Berkshire	Primula/Yew
TP02	Norfolk	Ornamentals
TP03	Herefordshire	Raspberry

TP04	Dorset	Ornamentals *(Syringa vulgaris)*
TP06	Surrey	Ornamentals (*Forsythia* sp)
TP07	Shropshire	Strawberry
TP08	Staffordshire	Strawberry
TP10	Lincolnshire	Ornamentals
TP11	Gloucestershire	Blackcurrant
TP12	North Yorkshire	Raspberry
TP13	Lincolnshire	Ornamentals (*Photinia* sp)
CM01	Longforgan	Sorrel
CM03	Fife	Raspberry
HR01	Staffordshire	Raspberry
TP14	Swansea	*Primula* sp and *Euonymus* sp

1659

1660

1661 **Figure 3.2 Vine weevil adults in leaf culture.**

1662 3.6.2. Egg surface sterilisation

1663 Eggs were collected from the Petri dishes in which adults had fed and oviposited for a week.
1664 Eggs used in experiments were preferably one week old, although, two week old eggs were
1665 collected in lines in which the amount of eggs was not sufficient for subsequent experiments.
1666 Eggs were placed in a beaker and covered with a 1.25% (v/v) sodium hypochlorite (May and
1667 Baker Ltd, Dagenham, UK) solution for 1 minute. Then the eggs were separated from the
1668 solution by vacuum suction through a 90 mm diameter filter paper Whatman No.1 (GE
1669 Healthcare UK Ltd, Little Chalfont, UK) using a Büchner funnel. To remove all traces of sodium
1670 hypochlorite solution, eggs were washed with sterile water using the Büchner funnel-vacuum
1671 pump set up. After that, the filter paper and eggs were placed in sterile Petri dishes, which
1672 were sealed with parafilm (Azwell Inc., Osaka, Japan) and stored at 4°C for two weeks
1673 maximum.

3.6.3. Media to rear vine weevil larvae

Larvae were reared artificially with media prepared based on the recipe of Fisher and Bruck (2004) with minor modifications. Media with and without antibiotic was used to compare the effect on the vine weevil bacterial microbiota. UV exposure time required to effectively surface sterilise the media was also tested at 10, 30 and 60 minutes.

For the preparation of the media, dried butter beans, *Phaseolus lunatus,* (ASDA, Leeds, UK) were immersed in tap water and kept at room temperature overnight. After 18 hours, 96 g of soaked beans were blended in 400 mL of sterile water using a blender (Christison particle technologies, Gateshead, UK). Subsequently, 32 g of yeast, *Kluyvercomyces fragilis,* (VWR BDH Prolabo, Leuven, Belgium), 3.2 g of L-ascorbic acid (Sigma-Aldrich Inc., St Louis, USA) and 1 g of potassium sorbate (Alfa aesar, Heysham, UK), were added to the blended beans. Then, 24 g of agar (VWR BDH Prolabo, Leuven, Belgium) was dissolved in 400 mL of sterile water and added to the bean mixture. Once all media components were incorporated, the mixture was autoclaved. For artificial diet with antibiotic, 0.8 g of streptomycin sulphate (Panreac Applichem, Darmstadt, Germany) was added to the autoclaved media (800 mL) under sterile laminar flow conditions.

Plastic containers for food use with 29.5 mL capacity (Solo cup company, Illinois, USA) were opened, with lids placed upside down, and sterilised with UV light for 10 minutes, then filled with approximately 15-20 mL of sterile media inside the laminar flow cabinet. Media was allowed to solidify in the laminar flow cabinet for one hour, then subjected to surface-sterilisation using UV light for 10, 30 or 60 minutes, depending on the treatment. After this, the lids were replaced and the containers of solid sterile media were stored at 4°C for a maximum of two weeks.

3.6.4. Rearing procedure

Based on Fisher and Bruck (2004) rearing procedure, five shallow holes were made on the media surface (distributed like a five dots die side) using a dissection needle, previously sterilised with a 1.25% (v/v) sodium hypochlorite solution (Figure 3.3a). Eggs were placed in pairs inside the holes with a paint brush, previously sterilised with a 1.25% (v/v) sodium hypochlorite solution. Afterwards, containers were transferred to an incubator and maintained in complete darkness at 20°C until further manipulation. Containers with media and larvae were always manipulated inside the laminar flow cabinet.

Larvae were reared in three different media treatments 1) media with antibiotic and 60 minutes of UV light exposure, 2) media with antibiotic and 30 minutes of UV light exposure and 3) media without antibiotic and 10 minutes of UV light exposure. Each of the 15 established vine weevil lines were replicated three times on each media treatment and this experiment was

repeated twice. Growing larvae (Figure 3.3b) were transferred to fresh media every seven weeks until adult emergence (Figure 3.3c). Exceptionally, media with pupae was not manipulated to avoid disturbing pupae (Figure 3.3d). Containers in which adults emerged were kept in complete darkness at 20°C as additional adults could emerge. Adults that emerged in the media were transferred to Petri dishes under the same conditions as adults kept in leaf culture.

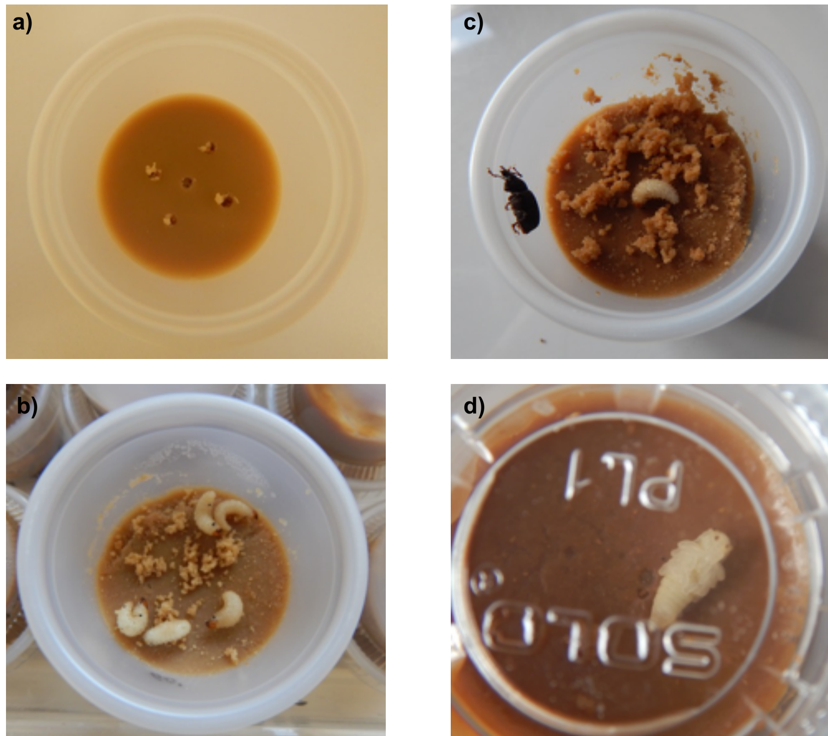


Figure 3.3 Vine weevils reared on artificial media
Picture a) eggs placed inside the holes on the media, b) larvae growing on the media, c) adult emerged on the media and d) pupa on the media.

3.6.5. Statistical analysis with Genstat

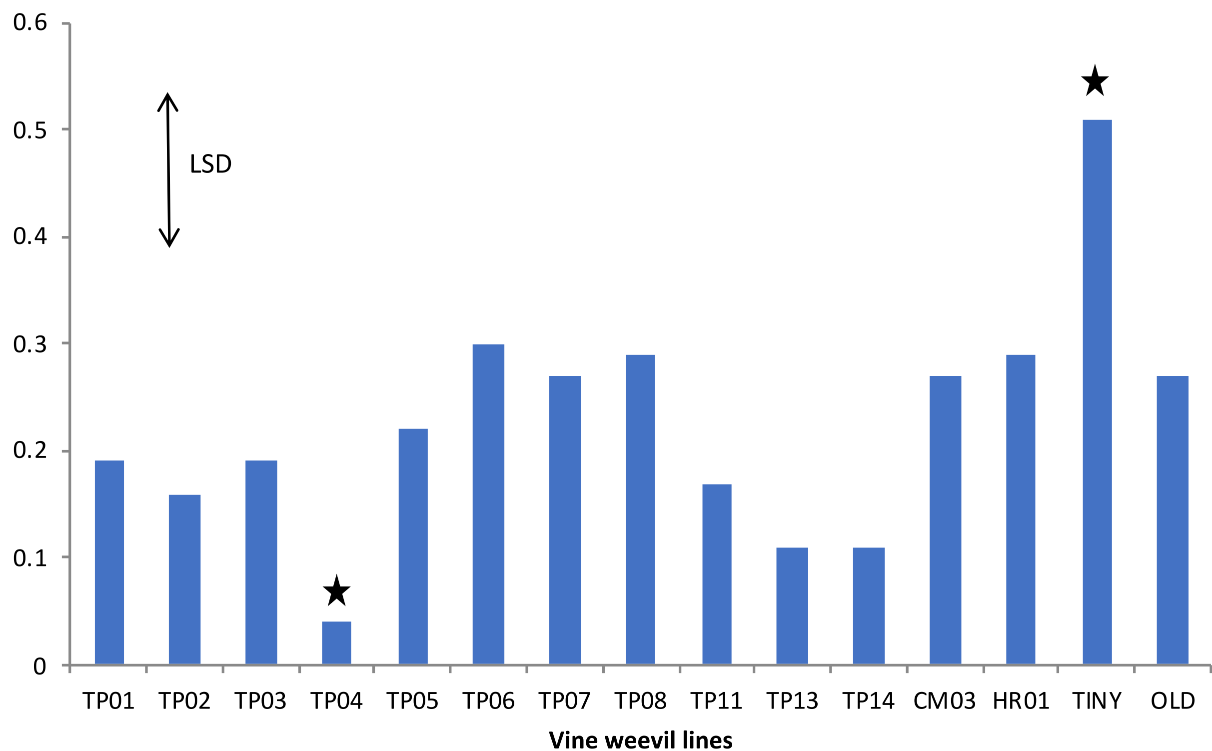
The proportion of adults emerged on each plastic containers respect to the numbers of eggs initially placed in the container was statistically analysed. Data was transformed with the function arcsine square root to obtain data normally distributed and with homogeneous variance. Transformed data was analysed with ANOVA and significant differences between lines and treatments were further investigated with the Least Significant Difference (LSD) test.

3.7. Results: Optimisation of vine weevil artificial diet

3.7.1. Adult emergence

Adult emergence started five months after eggs were placed on the artificial media and lasted for a period of two months. Statistical analysis of adult emergence showed significant

1729 differences between lines and treatments but the interaction between these factors was not
1730 significant (ANOVA line df= 14, pvalue<0.001; treatment df= 2, pvalue<0.001; line-treatment
1731 df= 27, pvalue= 0.161). We investigated which lines and treatments were significantly different
1732 between each other and found that: a) lines TP04 and TINY were significantly different to the
1733 rest and between each other, with the lowest and the highest adult emergence, respectively;
1734 and that b) the treatment with antibiotic and 60 minutes of UV had a significantly higher adult
1735 emergence with respect to the other treatments (LSD for line= 0.17; treatment= 0.076; Figure
1736 3.4 and 3.5). Importantly, some vine weevil adults emerged on the artificial media were smaller
1737 and had less hair on the elytra compared with the parental adult vine weevils, and a great
1738 number of adults displayed developmental defects with the posterior exoskeleton edge not
1739 completely closed and with the ovipositor protruding (Figure 3.6).



1740

1741 **Figure 3.4 Proportion of adults emerged for all different vine weevil lines.**
1742 There were significant differences between the vine weevil lines used. Stars above the bars
1743 indicate significant differences. TP04 and TINY were significantly different to the rest and
1744 between each other (ANOVA pvalue<0.001; LSD= 0.17)

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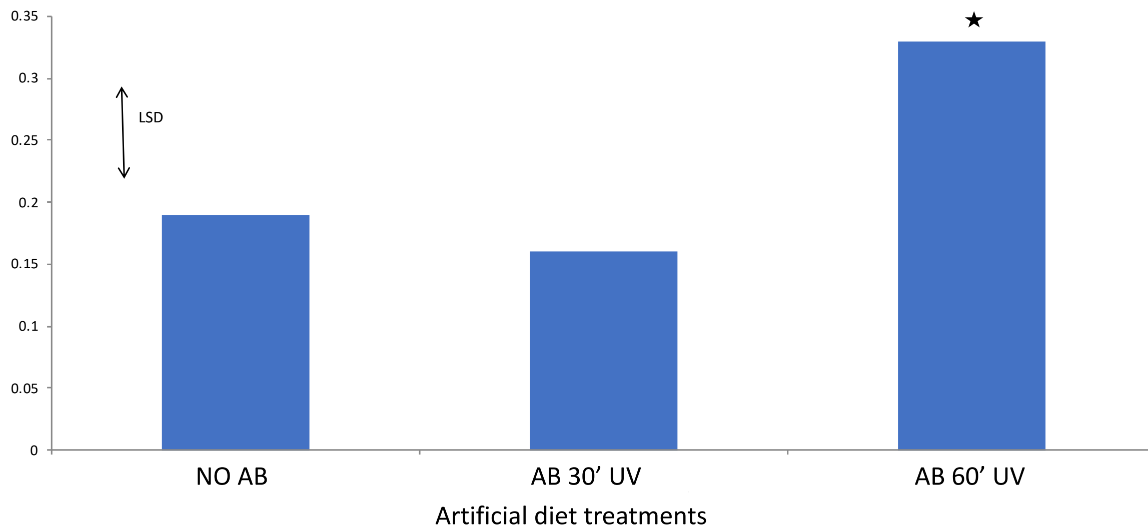
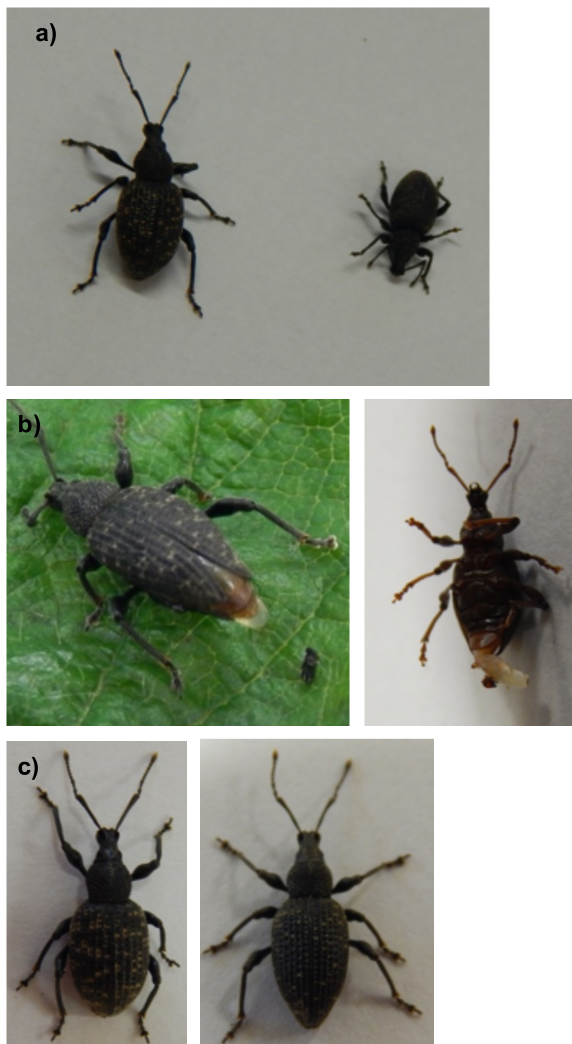


1746

1747 **Figure 3.5 Proportion of adults emerged on the three artificial media treatments.**
1748 The treatments used were: 1) NO AB: media without antibiotic and 10 minutes of UV light
1749 exposure, 2) AB 30'UV: media with antibiotic and 30 minutes of UV light exposure and 3) AB
1750 60'UV: media with antibiotic and 60 minutes of UV light exposure. The star above the bar
1751 indicates significant differences (ANOVA pvalue<0.001; LSD= 0.076).



1752

1753 **Figure 3.6 Adults emerged with developmental defects.**

1754 a) Smaller size: parental individual (left) and artificial diet emerged adult (right), both adults
1755 belong to the same line. b) Exoskeleton not completely closed and ovipositor protruding
1756 (insects were alive). c) Less hair on the elytra: parental individual (left) and artificial diet
1757 emerged adult (right), both adults belong to the same line.

1758 3.8. Discussion: Optimisation of vine weevil artificial diet

1759 3.8.1. Adult emergence

1760 Our results showed higher rates of adult emergence, which is directly related to larvae survival,
1761 on media containing antibiotic and sterilised with 60 minutes of UV light exposure. Higher
1762 larvae survival on artificial media containing antibiotic was previously reported by Shanks and
1763 Finnigan (1973). This observation was attributed to the fact that including antibiotic in the
1764 artificial media may eliminate endemic bacterial infection causing disease to the larvae.
1765 Therefore, it could be that the artificial media treatment used to rear larvae in this project with
1766 antibiotic and longer UV light exposure eliminated bacteria more efficiently, increasing the
1767 number of adults that emerged as a result. Moreover, significant differences in adult
1768 emergence between vine weevil lines may reflect differences in susceptibility to artificial
1769 growing media related to the environment from which parental adults were collected.

1770 3.8.2. Adults emerged with developmental defects

1771 It was reported by Smith (1932) that smaller adults emerged from larvae that had completed
1772 fewer moults. Therefore, differences in size observed between adults collected outdoors and
1773 adults emerged on the artificial diet could result from larvae developing more quickly and
1774 passing through fewer moults when reared artificially due to experiencing higher
1775 temperatures. Developmental abnormalities detected in adults emerging from larvae reared
1776 on the artificial diet might also be a consequence of defective moults or nutritional deficiencies
1777 on the diet as has been seen by previous groups working on vine weevil reared on artificial
1778 diets (Shanks and Finnigan, 1973a, 1971).

1779 Developmental defects found in adult vine weevils emerged from artificial diets in this study
1780 were not previously reported by Fisher and Bruck (2004), who used a similar artificial diet to
1781 rear vine weevil larvae. Fisher and Bruck (2004) collected larvae from outdoor grown crops
1782 and reared them artificially on diet until adulthood. Then, eggs laid by these adults were used
1783 for the experiments testing the quality of the diet. It is possible that there was a pre-selection
1784 of individuals that could adapt better to artificial growing conditions in the first generation,
1785 hence developmental defects were not observed in the following generation. Nonetheless, we
1786 decided to avoid using artificial diet for rearing vine weevils due to the lack of understanding
1787 of the developmental abnormalities.

1788

Chapter 4

Geographic origin does not influence vine weevil *Otiorhynchus sulcatus* (Fabricius) susceptibility to the entomopathogenic fungus *Metarhizium brunneum* (Petch)[3]

Abstract

Otiorhynchus sulcatus (Fabricius), known as the vine weevil, is a polyphagous pest that currently threatens important horticultural crops around the world. The entomopathogenic fungus *Metarhizium brunneum* (Petch) is widely used to control this pest. However, little research has investigated variation in susceptibility to this pathogen between vine weevil populations found in different locations. This study addresses this knowledge gap by comparing survival rates of larvae from adults collected in two UK geographic areas when treated with *M. brunneum*. Our experiments suggest that the efficacy of *M. brunneum* against vine weevil larvae is similar regardless of the geographic source of weevils.

[3] Part of this chapter has been published as: Pilar Morera-Margarit, Alison J. Karley, Carolyn Mitchell, Robert I. Graham & Tom W. Pope (2020) Geographic origin may not influence vine weevil *Otiorhynchus sulcatus* (Fabricius) susceptibility to the entomopathogenic fungus *Metarhizium brunneum* (Petch), Biocontrol Science and Technology, DOI: 10.1080/09583157.2020.1788507

4.1. Introduction

The vine weevil, *Otiorhynchus sulcatus* (Fabricius), is a curculionid endemic to central Europe, yet its distribution has expanded to most parts of Europe, parts of North America, South America, New Zealand and Japan. Adult weevils feed on leaves and flowers causing cosmetic damage, whereas larvae feed on plant roots, stems and bulbs, which reduces plant vigour and may cause plant death. The vine weevil is highly polyphagous and so threatens a wide range of horticultural crops around the world, among them economically important soft-fruit crops, such as strawberry where it is estimated that more than 2,000 ha were affected in the UK alone in 2016 and that losses were worth an estimated £14M (Wynn, 2010). In addition, ornamental crops, such as *Rhododendron, Photinia, Euonymus* and *Cyclamen* are also subject to damage by this pest.

All vine weevils analysed so far are triploid females which reproduce by thelytokous parthenogenesis. This reproductive strategy is expected to be detrimental for adaptation to new habitats, yet it has not hampered vine weevil range expansion. Little research has focused on the biological distinctiveness of populations of this species, which could be the key to understanding vine weevil adaptation ability. Lundmark (2010) compared weevils collected from different locations at a genetic level and found little variation. To address this question from a different angle, Morera-Margarit et al. (2019) characterised the bacterial community of vine weevils from various locations in the UK, and similarly detected little variation in bacteriome taxonomic composition. To determine the existence of variation in other phenotypic traits, we studied vine weevil populations from different locations by comparing the susceptibility of larvae to a fungal pathogen *Metarhizium brunn*eum (Petch) used in biocontrol strategies targeting this insect species. Our results suggest that for the two vine weevil populations tested here under controlled laboratory conditions, *M. brunneu*m virulence is not affected by the geographic origin of the insects.

4.2. Materials and methods

4.2.1. Vine weevil adult populations and plants

Vine weevil adults were collected from strawberry crops at two UK sites separated by 524 km: Stafford, Staffordshire, and Invergowrie, Perthshire (collection site = population). Vine weevils were kept in Petri dishes lined with moist paper (Kleenex, Kimberly-Clark professional, Kent, UK) and provided with strawberry leaves (*Fragaria* x *ananasa* (Duchesne), mixed varieties) in controlled environment rooms (18°C, 16:8 h L:D).

1838 Strawberry (*Fragaria x ananasa*, var. Elsanta) plants used for the experiment were grown in
1839 1 L pots with a 3:1 mixture of compost (peat-sand-perlite 6N: 3P: 1K; Everris Ltd, Ipswich,
1840 UK): grit sand (Arthur Bower's Ltd, Lincoln, UK).

1841 **4.2.2. Vine weevil egg collection and plant infestation**

1842 Vine weevil eggs were collected from Petri dishes in which the collected adults had fed for a
1843 week. To infest the plants, 20 eggs were gently washed into a small indentation in the surface
1844 of the compost 2 cm deep and 1 cm wide at a distance of 2 cm from the main plant stem.
1845 Compost temperature was measured with thermocrons (DS1921G-F5 thermocrons,
1846 Homechip Ltd, Milton Keynes, UK), placed 5 cm deep in the compost, and the software
1847 OneWireViewer.exe v. 0.3.19.47. Average substrate temperature was 15°C ± 6°C. Plants
1848 were arranged in a randomised block design, each block comprising two strawberry plants
1849 representing a replicate of each population. Three blocks were infested each week for 14
1850 weeks. Experiments were completed in a glasshouse (14-20°C, 16:8 h L:D).

1851 **4.2.3. Vine weevil larvae collection**

1852 Larvae were collected four to six months after the plants had been infested with eggs by
1853 removing the plants from the pots and hand searching the compost and roots for larvae. Only
1854 larvae that were between 0.045 g and 0.09 g in weight were used in this experiment. Plants
1855 from blocks infested during three consecutive weeks were grouped to ensure enough larvae
1856 were collected for a single experiment.

1857 **4.2.4. *Metarhizium brunneum* conidia suspension**

1858 *Metarhizium brunneum* isolate 275.86, strain commercialised as Met52® (Novozymes,
1859 Denmark), was provided by Warwick Crop Centre at Warwick University, UK. Cultures of this
1860 isolate were grown for 14 days in Sabouraud dextrose agar media (20 g glucose, BDH,
1861 Lutterworth, UK; 5 g mycological peptone, Oxoid, Basingstoke, UK; 10 g technical agar no.3,
1862 Oxoid, Basingstoke, UK; 500 ml deionised water) in the dark at 23°C ± 1°C. Conidia were
1863 harvested by gentle agitation in sterile 0.01% Triton solution X-100 (BDH, Lutterworth, UK).
1864 Conidia concentration was enumerated using a Neubauer improved haemacytometer and
1865 diluted with sterile water to a final concentration of 10^7 conidia/mL. This solution was diluted
1866 with 0.05% Triton solution X-100 to achieve a working concentration of 10^6 conidia/mL.

1867 **4.2.5. Vine weevil larvae treated with *Metarhizium brunneum* conidia**

1868 Vine weevil larvae treatment consisted of pipetting 25 µL of conidia suspension onto the
1869 thoracic segments close to the head of the larva (Klingen et al., 2015). Negative controls were
1870 treated with the same volume of 0.05% Triton solution X-100. *Galleria melonella* (Linnaeus)

1871 larvae (Big larvae, UK Waxworms Ltd, Sheffield, UK) were used as positive controls and were
1872 treated with 25 µL of conidia suspension in a similar way. Larvae were placed in Petri dishes
1873 (92 mm diameter) with moist compost (insecticide-free peat-sand-perlite 6N: 3P: 1K; Everris
1874 Ltd, Ipswich, UK) and thin carrot slices as a food source at 18°C, in the dark.

1875 Each experimental replicate (4 experimental replicates in total) was arranged as a randomised
1876 block design comprising four blocks, each containing at least one dish per vine weevil
1877 population, two negative controls for the corresponding population and a positive control, with
1878 randomised position within the block. Petri dishes with the same treatment, i.e. conidia-
1879 treatment or control, within the same experiment repetition always contained the same number
1880 of larvae. Final numbers of larvae per population and treatment were: Stafford-control = 28,
1881 Stafford-conidia treatment = 61, Invergowrie-control = 43 and Invergowrie-conidia treatment
1882 = 154. Petri dishes were assessed once each week in a four-week period by removing and
1883 counting dead larvae, and replacing the carrot slices.

1884 4.2.6. Statistical analysis and graphical representation

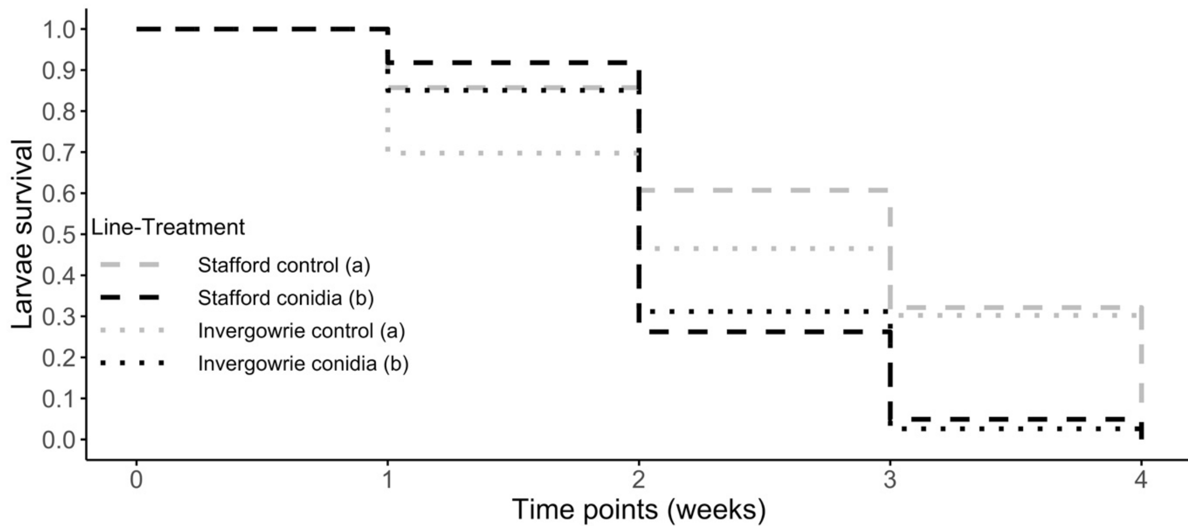
1885 Statistical analysis and graphical representation were performed using R software v. 3.3.3 and
1886 the packages ggplot2 (Wickham, 2009), survival (Therneau and Grambsch, 2000), coxme
1887 (Therneau, 2018), survminer (Kassambara and Kosinski, 2018), car (Fox and Weisberg, 2018)
1888 and plyr (Wickham, 2011). To test for significant differences a survival object was created and
1889 was tested using a mixed effect Cox model for interactions. The model included the
1890 interactions between the fixed factors vine weevil population and conidia treatment, and the
1891 random factors block and experiment replicate. A post-hoc log-rank test for multiple
1892 comparisons with Benjamini-Hochberg p-value adjustment method was performed.

1893 4.3. Results

1894 4.3.1. *Metarhizium brunneum* treatment is not influenced by location

1895 The Mixed effects Cox regression to test for the effect of *M. brunneum* on larval survival
1896 revealed a significant effect of the treatment (χ^2 = 10.15, d.f. = 1, p-value = 0.001). The Cox
1897 proportional hazard, or hazard ratio (HR), calculated for treatment indicated that treating
1898 larvae with conidia increased the mortality rate by a factor of 2.2 (HR=2.2). The hazard ratio
1899 for population also indicated that the origin of the vine weevil population did not affect larvae
1900 survival (HR=1). The post-hoc analysis revealed a significant decrease in survival of conidia-
1901 treated larvae over the experimental period within population (Stafford control-conidia
1902 treatment p-value = 0.003, Invergowrie control-conidia treatment p-value = 0.003; Figure 4.1;
1903 Table 4.1) but not between the populations. Closer inspection at the descriptive statistics
1904 revealed that: a) in the absence of conidia, there was a greater variation in the survival rate;

1905 and b) negative control larvae from Stafford population tended to have higher survival than
1906 negative control larvae from Invergowrie population (Stafford-control median = 2 weeks
1907 95%CI [2, 4]; Stafford-conidia treatment median = 2 weeks 95%CI [2,2]; Invergowrie-control
1908 median = 2 weeks 95%CI [2, 3]; Invergowrie-conidia treatment median = 2 weeks 95%CI [2,
1909 2]; Figure 4.1; Table 4.1). This could indicate that although there might be background
1910 variation in survival rate, this variation does not affect the efficiency of the entomopathogenic
1911 fungus under the conditions used for our experiments.



1912

1913 **Figure 4.1 Cox regression for survival of vine weevil larvae from different populations**
1914 **treated with *Metarhizium brunneum*.**
1915 Y-axis represents larvae survival while x-axis represents the time points considered for the
1916 study (weeks). Line-treatment combinations on the legend sharing the same letters were not
1917 significantly different (log-rank test: p-value< 0.05).

1918

1919 **Table 4.1 Probability of survival for larvae from both populations, Stafford and**
1920 **Invergowrie.**
1921 The table shows the probability of survival as a percentage of the total number of larvae
1922 throughout the experimental period given by the Cox regression model. The time points at
1923 which mortality was recorded are represented as 1st to 4th week.

Probability of survival	Stafford control	Stafford conidia	Invergowrie control	Invergowrie conidia
1st week	86%	92%	70%	85%
2nd week	61%	26%	47%	31%
3rd week	32%	5%	30%	3%
4th week	6%	0%	24%	0%

1924 ## 4.4. Discussion

1925 The present study is the first to investigate differences in susceptibility of vine weevil larvae
1926 collected at different geographic locations to the entomopathogenic fungus *M. brunneum*. Our

1927 experiments confirm that *M. brunneum* can effectively infect vine weevil larvae despite using
1928 a temperature close to the lower fungal growth threshold. We did not detect differences in
1929 susceptibility to *M. brunneum* associated with vine weevil population. The lack of variation in
1930 susceptibility is congruous with previous studies which found little variation at genetic and
1931 bacterial community levels of insects collected at separate locations (Lundmark, 2010;
1932 Morera-Margarit et al., 2019).

1933 Larvae not exposed to *M. brunneum* conidia experienced greater within population variation
1934 in mortality and this effect most pronounced for larvae from the Stafford population. These
1935 results may indicate the presence of natural variation in vine weevil larval mortality, which was
1936 higher for the Stafford population, although this may also be due to the smaller number of
1937 larvae used from this population. The overall mortality obtained in our experiments when
1938 larvae were treated with *M. brunneum* conidia was similar to values reported by Klingen et al.
1939 (2015), despite the fact that Klingen et al. (2015) applied a 10-fold higher conidia
1940 concentration. Results presented here indicate that the application of *M. brunneum* conidia
1941 had a strong effect on weevil mortality that masked the background variation observed in
1942 untreated larvae. It would be useful to test lower concentrations of conidia in future research
1943 and to test vine weevil populations collected from a wider range of locations within cropped
1944 and uncropped habitats. Additional experiments will confirm whether susceptibility to *M.*
1945 *brunneum* remains consistent regardless of the geographic origin or crop environment. The
1946 application of *M. brunneum* is currently standardised, hence the same procedure is applied
1947 irrespective of the geographic area or the crop affected. Results presented here suggest that
1948 location-specific strategies for vine weevil control using *M. brunneum* are not necessary.

1949

1950

Chapter 5

1951
1952

Limited effects of vine weevil *Otiorhynchus sulcatus* (Fabricius) source on adult oviposition preference

1953 Abstract

1954 The vine weevil *Otiorhynchus sulcatus* (Fabricius) is a curculionid
1955 endemic to central Europe but which has expanded its range to many
1956 parts of the world in the last century, mainly through plant trade routes.
1957 It has been recorded as feeding successfully on more than 150 host plant
1958 species, among them important horticultural crops. Nonetheless, despite
1959 the wide distribution area and the broad range of susceptible hosts, little
1960 is known about the variation between populations of this weevil inhabiting
1961 different locations and different crop species. The current research
1962 addresses this gap in the literature by examining differences in
1963 oviposition behaviour between insects collected at two separate
1964 locations within the same crop species. Host oviposition preference was
1965 tested by using adult vine weevils collected from two strawberry fields in
1966 a choice experiment with two host-plant species: strawberry and
1967 raspberry. The results presented here show that insects from different
1968 locations did not display a significant difference in egg laying choice and
1969 that raspberry was a preferred host to strawberry. These findings
1970 suggest that location has little effect on vine weevil variation and that
1971 other, or additional, factors should be considered in future experiments.
1972 Further research in this area is required to better understand the ability
1973 of vine weevils to adapt to different environments, such information
1974 would be valuable in the design of improved Integrated Pest
1975 Management strategies targeting this versatile pest.

1976

1977 ## 5.1. Introduction

1978 *Otiorhynchus sulcatus* (Fabricius), commonly known as the vine weevil or black vine weevil,
1979 is a wingless beetle member of the Curculionidae family. This species is endemic to central
1980 Europe but its distribution has expanded in the last century to most parts of Europe, parts of
1981 North America, South America, New Zealand and Japan (Kingsley, 1898; Masaki et al., 1984;
1982 Moorhouse et al., 1992; Prado, 1988). Adult vine weevils live above ground and feed on leaves
1983 and flowers of a wide range of horticultural crops, making small rounded notches that render
1984 plants unmarketable; while larvae live in the soil or substrate and feed on plant roots, stems
1985 and bulbs, reducing plant vigour, which can eventually result in plant death (Smith, 1932).
1986 Damage caused by this pest to agricultural crops is significant, for instance, vine weevil
1987 affected more than 2,000 ha of strawberry crops in the UK in 2016, causing damage worth an
1988 estimated £14M (Wynn, 2010).

1989 Vine weevils have typically been considered as a highly polyphagous species capable of
1990 feeding successfully on more than 150 host plant species. Smith (1932) reported adults and
1991 larvae feeding successfully on 77 and 42 different host plant species, respectively, in the USA.
1992 Additional work at the USDA (United States Department of Agriculture) added 70 more plant
1993 species to the vine weevil hosts plant list (Warner and Negley, 1976). Masaki et al. (1984)
1994 completed feeding tests on economically important plant species in Japan and reported 90
1995 and 46 host plants on which adults and larvae, respectively, could feed successfully. These
1996 studies had, however, only focused on identifying plants on which vine weevils could feed and
1997 survive.

1998 Research investigating vine weevil suitable host plant species has combined data from
1999 feeding choice tests and fecundity, by counting the number of eggs laid (Tol and Visser, 1998;
2000 van Tol et al., 2004). Similarly to previous studies by Nielsen and Dunlap (1981) and Doss
2001 (1984), Tol et al. (2004) found that *Rhododendro*n was the least preferred host for feeding by
2002 adult vine weevils. Vine weevils adults also laid comparatively fewer eggs on *Rhododendron*
2003 in comparison with the other hosts considered, including *Taxus, Euonymus*, strawberry and
2004 *Humulus. Taxus* was the second least preferred host in feeding experiments. However,
2005 oviposition was similar on *Taxus, Euonymus* and strawberry while lower on *Humulus*. This
2006 aligns with results by Hanula (1988) that showing that *Taxus* is the preferred oviposition host,
2007 followed by strawberry, in comparison with *Rhododendron* and azalea. They attributed this
2008 mismatch among preference and performance to the fact that some of the plants used in the
2009 experiment are not present in the European native vine weevil distribution (van Tol et al.,
2010 2004). Thus, van Tol et al. (2004) arrived at the conclusion that due to the lack of fecundity
2011 data in the previous studies, host plant species range had been overestimated.

Clark et al. (2012) compared vine weevil feeding and oviposition preference on red raspberry *Rubus idaeus* (Linnaeus) cultivars Glen Ample, Glen Clova, Glen Magna, Glen Moy, Glen Rosa, Malling jewel, Octavia, Tulameen and Wild accessions. Choice experiments and no-choice experiments were designed with these raspberry cultivars. The cultivar Tulameen was the preferred host followed by Glen Ample in adult feeding choice experiments. However, in no-choice experiments, when only a single cultivar is presented to the insects, Glen Moy and Glen Rosa were preferred in terms of leaf consumption. This might be related to the vine weevil ability to adapt to sub-optimal hosts by showing compensatory feeding, i.e. by consuming larger amounts of foliage (Clark et al., 2012b; Johnson et al., 2010). Oviposition preference was significantly different between cultivars and was similar in choice and no-choice experiments. Insects laid more eggs on Glen Ample and Tulameen cultivars. Additional experiments by Clark et al. (2012) tested how raspberry cultivar influences larval performance considering Glen Rosa and Glen Ample. Interestingly, when fed on Glen Rosa vine weevil larvae were 26% larger but this cultivar harboured 56% fewer larvae in comparison with Glen Ample. These results highlight the complexity of understanding host plant suitability for vine weevil in terms of host preference and consumption, as well as fecundity and progeny survival.

An additional factor that could be influencing vine weevil host plant preference is the location from where the insects are collected. To date, little research has addressed this subject. Results from a study developed by van Tol and Visser (2002), however, pointed out differences in sensitivity to some volatiles among vine weevils collected at different locations and from different plant species. The current research was designed to investigate if insect collection site affects oviposition preference. For this, adult insects were collected from strawberry fields at two different locations and a choice experiment was designed with two suitable hosts: strawberry and raspberry. My results suggest that the populations tested do not differ in their oviposition preference as insects from both sampling sites preferred to lay eggs on raspberry.

5.2. Materials and methods

5.2.1. Vine weevil adult populations and plants

Vine weevil adults were collected during summer 2017 from an area of approximately 50 m2 within strawberry crops at two sites in Stafford, UK. Collection sites were separated by an approximate distance of 766 m from each other and were named Stafford1 and Stafford2. Vine weevil adult insects were kept in Petri dishes (92 mm diameter) lined with moist paper (Kleenex, Kimberly-Clark professional, Kent, UK) and provided with strawberry leaves (Fragaria x ananasa (Duchesne), mixed varieties) as a source of food, in controlled

2046 environment rooms (18°C, 16:8 h L:D). Insects were transferred to clean paper-lined Petri
2047 dishes moistened with water and supplied with fresh strawberry leaves weekly.

2048 Strawberry (*Fragaria x ananasa*, var. Elsanta) and raspberry (*Rubus idaeus*, var. Glen Ample)
2049 plants were used for experiments. Plants were grown in 1 L pots with a 3:1 mixture of compost
2050 (peat-sand-perlite 6N: 3P: 1K; Everris Ltd, Ipswich, UK): grit sand (Arthur Bower's Ltd, Lincoln,
2051 UK). Experiments were completed in a glasshouse (14-20°C, 16:8 h L:D).

2052 5.2.2. Choice experiment

2053 Vine weevil oviposition preference was tested in a choice experiment between strawberry
2054 (*Fragaria x ananasa*, var. Elsanta) and raspberry (*R. idaeus*, var. Glen Ample). The
2055 experiment was completed in mesh cages with dimensions of 47.5 x 47.5 x 47.5 cm
2056 (BugDorm-4E4545 Insect Rearing Cage, Mega View Science Co, Ltd, Taichung, Taiwan)
2057 containing one strawberry and one raspberry allocated to a randomised position inside the
2058 cage. To allow egg recovery, a layer of fine mesh (160 µm aperture) was placed around the
2059 plant stem and was covered with Horticultural pea gravel (Deco-Pak Ltd, West Yorkshire, UK)
2060 (Clark et al., 2012b; Johnson et al., 2010). For the experiment, vine weevil adults from both
2061 populations were tested simultaneously (10 mesh cages/population). Only insects that had
2062 laid eggs for a minimum period of two weeks while in culture were used. Two vine weevil
2063 adults were introduced into each mesh cage equidistant from each plant, and insects were
2064 allowed to lay eggs during a three-week period. At the end of the experiment, the mesh and
2065 the gravel cover were submerged in a saturated KCl solution (Sigma-Aldrich, St Louis, USA)
2066 to allow eggs to float (Clark et al., 2012b; Johnson et al., 2010).

2067 5.2.3. Statistical analysis and graphical representation

2068 Statistical analysis and graphical representation were performed using R software v. 3.3.3 and
2069 the packages ggplot2 v. 3.0.0 (Wickham, 2009), car v. 3.0-2 (Fox and Weisberg, 2018),
2070 emmeans v. 1.3.1 (Russell L, 2019), lme4 v. 1.1-15 (Bates et al., 2014) and multcomp v. 1.4-
2071 8 (Hothorn et al., 2008).

2072 Plant oviposition preference was tested with generalised linear mixed effects model
2073 considering population and plant species as fixed factors and mesh cage as a random factor
2074 with the function 'glmer' using a Poisson error distribution and log-link function. Post-hoc
2075 analysis was performed with the function 'glht' with 'emm' function for pairwise comparisons
2076 of marginal means with "single step" p-value adjustment method. Boxplot graphical output was
2077 performed with the function 'ggplot'.

5.3. Results

5.3.1. Vine weevil adults prefer to lay eggs on raspberry plants

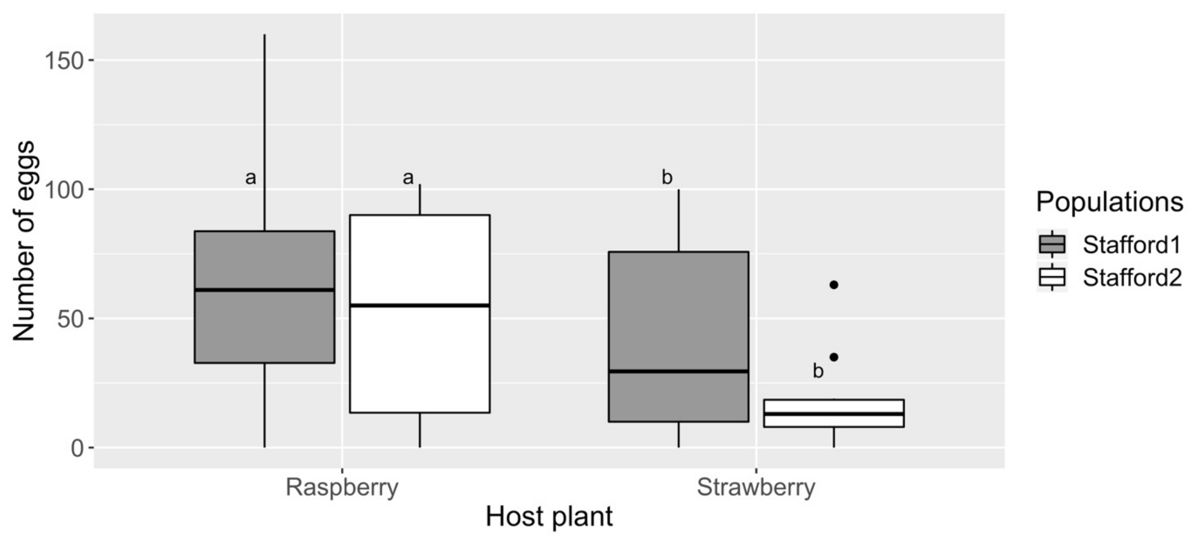
A choice experiment was used to test vine weevil host plant oviposition preference. Statistical analysis revealed that the plant host had a significant effect on the number of eggs laid ($\chi2$= 65.2; d.f.= 1; p-value< 2.2e-16; mean number of eggs laid per insect on raspberry= 60 and strawberry= 29) and that there was a significant interaction between the vine weevil population used and the host plant ($\chi2$= 25.7; d.f.= 1; p-value= 4.05e-07). The post-hoc test however, revealed only significant differences between hosts within the same population but not between populations (Stafford1 raspberry-strawberry p-value <0.001; Stafford2 raspberry-strawberry p-value <0.001; Figure 5.1). Closer inspection at the graphical representation revealed that the significance of the interaction population-host plant might have been forced by the lower number of eggs laid by the population Stafford2 on strawberry (mean number of eggs laid per insect by Stafford1 on raspberry= 67 and strawberry= 40, and Stafford2 on raspberry= 52 and strawberry=18; Table 5.1). These results suggest that despite differences between vine weevil populations in terms of quantity of eggs laid, there is no significant difference in oviposition preference as raspberry is the preferred host to strawberry in both populations tested.

Table 5.1 Vine weevil oviposition choice test using strawberry and raspberry plants
Eggs laid for both of the vine populations used, S1: Stafford1 and S2: Stafford2, with each of the replicate (one replicate= one mesh cage) for each host plant (S: strawberry, R: raspberry).

POPULATION	HOST	REPETITION	EGGS
S1	S	1	19
S1	R	1	48
S1	S	2	7
S1	R	2	160
S1	S	3	19
S1	R	3	74
S1	S	4	86
S1	R	4	142
S1	S	5	0
S1	R	5	0
S1	S	6	85
S1	R	6	77
S1	S	7	0
S1	R	7	13
S1	S	8	48
S1	R	8	86
S1	S	9	40
S1	R	9	41

S1	S	10	100
S1	R	10	30
S2	S	11	12
S2	R	11	76
S2	S	12	17
S2	R	12	84
S2	S	13	63
S2	R	13	12
S2	S	14	0
S2	R	14	5
S2	S	15	8
S2	R	15	100
S2	S	16	35
S2	R	16	102
S2	S	17	14
S2	R	17	34
S2	S	18	8
S2	R	18	0
S2	S	19	8
S2	R	19	18
S2	S	20	19
S2	R	20	92

2099



2100

2101 **Figure 5.1 Eggs laid by vine weevil adult insects from Stafford1 and Stafford2**
2102 **populations on raspberry and strawberry plants.**
2103 Boxplots display the median as a thicker line, the interquartile range (IQR) as a box, the
2104 1.5IQR as whiskers above and under the box and the values outside this range as outliers
2105 depicted as points. Boxplots sharing the same letters were not significantly different (marginal
2106 means pairwise comparison: p-value< 0.05).

2107 ## 5.4. Discussion

2108 In this study, I examined oviposition preference of adult vine weevils from two strawberry fields
2109 when offered two suitable hosts: strawberry and raspberry. The vine weevil populations were
2110 collected from a similar geographic area with collection sites separated by approximately 766
2111 m. Vine weevil adults show little dispersal behaviour within growing strawberry crops and
2112 move only 0.38 m/day (Pope et al., 2015), hence the populations used here were considered
2113 distinct from one another. My experiments showed no differences in the oviposition behaviour
2114 between the two populations tested. These results reflect the limited variation in UK
2115 populations of this insect pest as has previously been observed. Morera-Margarit et al. (2019)
2116 characterised the bacterial community of vine weevil adults collected from strawberry fields at
2117 different UK locations, including the collection sites sampled for the current research. This
2118 study revealed minimal variation in the bacterial microbiota composition amongst the
2119 collection sites. Likewise, Lundmark (2010) revealed little genetic variation between vine
2120 weevil insects collected at separate locations. Further research is required to extend the work
2121 presented here to confirm the observed lack of variation in oviposition preference by
2122 considering insects collected from a wider geographic area.

2123 Strawberry is considered a plant host preferred by the vine weevil based on performance
2124 experiments and its recurrent presence on strawberry crops (Cowles, 2004; Hanula, 1988, p.
2125 198; Moorhouse et al., 1993; Pope et al., 2015; Wynn, 2010). Raspberry has typically received
2126 less attention and to my knowledge there is only one study, by Coyle et al. (2011), comparing
2127 oviposition preference between these two crop species. This study investigated if the pre-
2128 oviposition host, which is the plant species where insects were fed before initiating the
2129 experiment, affected vine weevil oviposition choice. Coyle et al. (2011) hypothesised that vine
2130 weevils prefer to lay eggs on the same host as the pre-oviposition host. By contrast, my
2131 findings showed that previous host experience does not influence vine weevil oviposition
2132 choice, given that adult insects preferred to lay eggs on raspberry despite coming from
2133 strawberry crops. Similarly, Hanula (1988), found that previous host experience does not
2134 influence vine weevil oviposition choice. Coyle et al. (2011) tested oviposition preference over
2135 a period of 7 days and revealed little difference in the number of eggs laid between strawberry
2136 and raspberry, with graphs showing overlapping error bars. By contrast, my experiments
2137 revealed a clear difference between strawberry and raspberry, possibly due to prolonging the
2138 experimental assessment period to 21 days. To clarify the contradiction between Coyle et al.
2139 (2011) and the present study, insects from the same location but reared on different host
2140 plants should be tested.

2141 Additional research is still needed to determine how location and feeding history can influence
2142 the vine weevil oviposition host. Future findings will provide a better understanding of the vine
2143 weevil adaptation to different locations and the mechanisms by which this insect species
2144 senses host suitability. The information provided by the present study could also contribute to
2145 developing more effective control strategies of this damaging pest. For instance, if the vine
2146 weevil oviposition preference for raspberry remains consistent, the possibility of using a trap
2147 crop strategy could be evaluated. The trap crop strategy combines a crop of interest,
2148 strawberry in this case, with a crop that will serve to protect the main crop by means of
2149 attracting, diverting, intercepting and/or retaining the targeted pests, the raspberry crop in this
2150 case (Shelton and Badenes-Perez, 2006). Nonetheless, further efforts are required to be able
2151 to apply the findings of the current study in this manner.

Chapter 6

Candidatus Nardonella population dynamics across developmental stages of the vine weevil *Otiorhynchus sulcatus* (Fabricius)

Abstract

Candidatus Nardonella is an intracellular symbiotic bacterium which is involved in tyrosine metabolism and cuticle formation in some weevil species. The vine weevil *Otiorhynchus sulcatus* (Fabricius) also carries this associated bacterium although its function remains poorly understood. The study presented here investigated the titre of this symbiont in insect tissues throughout the vine weevil life cycle to identify possible roles for weevil host biology. The density of *Ca.* Nardonella was quantified for larvae, pupae and adults, including one teneral adult with immature cuticle, by quantitative PCR (qPCR) analysis. Concentration values tended to decrease as the life cycle progressed with adults harbouring a significantly lower abundance of the bacterium. However, a subgroup of pupae exhibited the highest abundance values. Interestingly, among these pupae some individuals presented signs of cuticle formation at the time of collection. So, although additional tests are required, these results could indicate that *Ca.* Nardonella is involved in cuticle formation in the vine weevil. The melt curve profile was examined to detect the amplification of fragments with different nucleotide sequence. We observed the presence of a peak common to all life stages, likely to represent the target bacterium. Two extra peaks, representing a different amplicon, appeared on the melt curve profile in some insects. These peaks could be an artefact of low primer specificity and result from amplification of DNA from other bacterial types by the same primer pair. A more comprehensive understanding of *Ca.* Nardonella at a genetic level is needed to design more accurate diagnostic tools to clarify if the occurrence of more than one peak is a result of targeting different bacteria or if, instead, it is the result of targeting various *Ca.* Nardonella strains coexisting within a single insect.

2184 ## 6.1. Introduction

2185 The vine weevil, *Otiorhynchus sulcatus* (Fabricius), is a coleopteran member of the weevil
2186 family Curculionidae (Alonso-Zarazaga and Lyal, 2002). This insect species is native to central
2187 Europe, however, it has spread to many parts of the world in the last century where it threatens
2188 a wide range of crop species (Moorhouse et al., 1992; Smith, 1932; Warner and Negley, 1976).
2189 The vine weevil has a holometabolous life cycle with six to seven larval instars followed by
2190 pupation and a final adult transformation (LaLone and Clarke, 1981; Smith, 1932). Larvae live
2191 underground and voraciously feed on the plant root system, stems and bulbs, a behaviour
2192 which may eventually result in plant death (Smith, 1932). At the end of this life stage, larvae
2193 build a cell compartment underground by mixing gut contents with the surrounding soil
2194 particles to initiate pupation (Smith, 1932). Pupae remain inactive inside this chamber while
2195 the metamorphic changes occur during the final adult transformation (Smith, 1932). A few
2196 days after adult transformation the cuticle turns from being chestnut brown to black in colour
2197 with patches of orange hair when the adult is ready to emerge to the soil surface (Smith, 1932).
2198 The reproductive organs of the adult weevil mature following emergence after which
2199 oviposition commences (Son and Lewis, 2005). Unlike the larvae and pupae, the adults live
2200 above ground where they feed on plant leaves making characteristic small round notches on
2201 the edge of the leaf (Smith, 1932). Plant damage caused by this life stage is mostly cosmetic
2202 and rarely causes plant death, nonetheless, it might render plants unmarketable (Smith,
2203 1932).

2204 *Candidatus* Nardonella is the most common symbiont found in members of the weevil
2205 superfamily, an association which is estimated to date back 125 My (Conord et al., 2008;
2206 Lefèvre et al., 2004a). *Ca.* Nardonella localises inside specialised cells called bacteriocytes
2207 organised in organ-like structures, or bacteriomes, which are normally found associated with
2208 the gut in larvae and in oocytes and the apex of the ovarioles in adults (Conord et al., 2008;
2209 Hosokawa et al., 2015; Hosokawa and Fukatsu, 2010; Lefèvre et al., 2004a; Login et al., 2011;
2210 Toju et al., 2010). The genome of this bacterium was examined in four weevil species from
2211 the family Curculionidae: the red palm weevil *Rhynchophorus ferrugineus* (Olivier), the giant
2212 weevil *Sipalinus gigas* (Fabricius), the West Indian sweet potato weevil *Euscepes*
2213 *postfasciatus* (Fairmaire), and the black hard weevil *Pachyrhynchus infernalis* (Fairmaire)
2214 (Anbutsu et al., 2017). *Ca.* Nardonella genome size and gene content in these weevil species
2215 reflected a striking reduction in metabolic capabilities with the only complete pathways
2216 dedicated to the synthesis of the aminoacid tyrosine and the cell wall peptidoglycan (Anbutsu
2217 et al., 2017). This is a distinguishing feature of primary obligate symbionts that reflects a long
2218 history of coevolution between the bacterium and the insect host (reviewed by Moya et al.,
2219 2009). More detailed experiments were designed to determine the function of *Ca.* Nardonella

2220 for the black hard weevil biology (Anbutsu et al., 2017). *In vitro* experiments confirmed that
2221 these bacteria play a major role in tyrosine production and that the bacteriome housing these
2222 bacteria resembles a tyrosine producing organ (Anbutsu et al., 2017). Further, the involvement
2223 of this bacterium in cuticle formation was shown by adults that developed defective cuticles
2224 when larvae of the black hard weevil and the West Indian sweet potato weevil were reared on
2225 diets containing an antibiotic (Anbutsu et al., 2017; Kuriwada et al., 2010).

2226 In the vine weevil, *Ca.* Nardonella was determined to be the most abundant bacterium
2227 dominating the microbiota of adult insects (Morera-Margarit et al., 2019), which may indicate
2228 that it plays an important role for its host biology. Nonetheless, research focusing on the
2229 function of this bacterium for the vine weevil has, to date, received little attention. As an initial
2230 step to study this bacterium in the context of vine weevil development, the present research
2231 was designed to investigate *Ca.* Nardonella abundance across insect life stages. The
2232 experiments presented here reveal a general decrease in the concentration of this prokaryote.
2233 These results suggest that *Ca.* Nardonella in the vine weevil could be involved in providing
2234 the host with tyrosine for larval growth and development but also for cuticle formation, hence
2235 explaining the significantly lower titre observed for adult insects with a complete cuticle.
2236 Additionally, the results from the qPCR melt curve may indicate that the primer pair used for
2237 the qPCR targeted different bacterial types whose abundance fluctuates according to the life
2238 stage. Additional experiments are necessary to define if *Ca.* Nardonella concentration
2239 oscillates in parallel with tyrosine requirements during development of the weevil and to
2240 identify if the different amplicons obtained in the qPCR belong to different species of bacteria.

2241 6.2. Materials and methods

2242 6.2.1. Vine weevil insect collection and DNA extraction

2243 Vine weevil larvae, pupae and adults were collected during 2018 from potted strawberry plants
2244 kept outdoors as well as from a crop of field grown strawberries at the James Hutton Institute
2245 in Dundee, UK. All the insects were considered as a single population as they were sampled
2246 from neighbouring areas. Following collection, insects were directly frozen with liquid N_2,
2247 weighed and stored at -80°C until further use.

2248 DNA extraction was performed on eight insects from each life stage. Exceptionally, only one
2249 teneral adult with chestnut brown cuticle was examined. Insects were first surface sterilised in
2250 a 1% bleach v/v (May and Baker LTD, Dagenham, England) solution for 1 minute (Lawrence
2251 et al., 2015; Malacrinò et al., 2018). To eliminate residual bleach, insects were submerged in
2252 autoclaved water three times, 1 minute each time. Insects were then ground individually using
2253 a pestle and mortar previously sterilised by exposing to UV light for 10 minutes. After grinding,

total DNA was extracted using the NucleoSpin Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions and the alternative step proposed in the manufacturer's procedure. An additional incubation at 70°C for 10 minutes was included, after the 10 minutes lysis step at 65°C specified in the protocol, to lyse gram negative bacterial cell walls. Extracted DNA was stored at -20°C in autoclaved Eppendorf tubes until further use.

6.2.2. Primer design for *Candidatus* Nardonella

Due to the paucity of published 16S rRNA gene sequences for *Ca.* Nardonella associated with the vine weevil, a representative sequence from a previous Next Generation Sequencing study (Morera-Margarit et al., 2019) was aligned with sequences downloaded from the NCBI database using POA (Partial Order Alignment). Using this alignment, the primers used by (Huang et al., 2016) were modified to amplify a *Ca.* Nardonella sequence in the rice water weevil, *Lissorhoptrus oryzophilus* (Kuschel), to reduce the number of mismatches with the target sequence. These newly designed primers NardvwF (ACACGGTCCAGACTTCTA) and NardvwR (TCACCCTTTACGCCCAAT) were used for all subsequent amplifications.

6.2.3. *Candidatus* Nardonella cloning

DNA extracted from four larvae as specified above was used as a template to amplify a *Ca.* Nardonella fragment with the primer pair NardvwF/NardvwR with GoTaq (Promega, Madison, USA) in a G-Storm GS1 Thermal Cycler (Gene Technologies, Somerton, UK).The PCR mixture (21.9 μL) contained 4.4 μL of 5X colourless GoTaq reaction buffer, 0.44 μL of PCR nucleotide mix (10mM each nucleotide), 1.1 μL of each of the primers, 0.11 μL of GoTaq DNA polymerase (5U/μL), 13.75 μL of sterile water and 1 μL of template DNA. Amplification conditions were 3 minutes of initial denaturation at 95°C, then 35 cycles of 20 seconds of denaturation at 95°C, 10 seconds of annealing at 56°C and 20 seconds of elongation at 72°C, followed by a final elongation of 10 minutes at 72°C.

A single bright band of approximately 200 bp was obtained for the PCR amplification which was subsequently gel-extracted with MiniElute extraction kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations. The fragment was ligated into a pGEM-T vector (Promega, Southampton UK) and transformed into *Escherichia coli* JM109 competent cells following manufacturer's instructions. After blue-white screening, five colonies were selected and their plasmids were purified using Monarch plasmid miniprep kit (New England Biolabs, Hitchin, UK). The inserts were sequenced by Sanger sequencing at the James Hutton Institute Sequencing Service (Dundee, UK) using primers M13 forward and reverse. The resulting insert sequences generated from all five plasmids were aligned to each other to confirm a single sequence had been cloned. One of these clones was used for BLAST

2288 searches (Altschul et al., 1990). The BLAST results are as follows: the highest similarity was
2289 found to be with bacteria found associated with the black hard weevil (GenBank: Accession
2290 No. AP018160.1 and LC014982.1; Max. score= 416) and the cotula weevil *Steriphus variabilis*
2291 (Broun) (GenBank: Accession No. KJ522449.1; Max. score= 416) at 96% sequence identity;
2292 followed by the Yonaguni hard weevil *Metapocyrtus yonagunianus* (Chujo) (GenBank:
2293 Accession No. LC014980.1; Max. score= 411) and the Argentine stem weevil *Listronotus*
2294 *bonariensis* (Kuschel) (GenBank: Accession No. KJ522448.1; Max. score= 401) at 95%
2295 sequence identity; the root-boring weevil *Hylobius transversovittatus* (Goeze) (GenBank:
2296 Accession No. EF434872.1; Max. score= 399) and the pine weevil *H. abietis* (Linnaeus)
2297 (GenBank: Accession No. EF434871.1; Max. score= 388) at 94% sequence identity; the West
2298 Indian sweet potato weevil (GenBank: Accession No. AP018159.1 and AB506808.1; Max.
2299 score= 377), the olive weevil *Pimelocerus perforatus* (Roelofs) (GenBank: Accession No.
2300 LC014981.1; Max. score= 377), the Asiatic palm weevil *Rhabdoscelus similis* (Chevrolat)
2301 (GenBank: Accession No. KX067892.1 and LC014986.1; Max. score= 374) and the West
2302 Indian cane weevil *Metamasius hemipterus* (Linnaeus) (GenBank: Accession No.
2303 AY126635.1; Max. score= 374) at 93% sequence identity. The first BLAST result not related
2304 to any weevil species was attributed to a bacterium associated with the Australian fig psylloid
2305 *Mycopsylla fici* (Tryon) (GenBank: Accession No. KT273275.1; Max. score= 363) at 92%.
2306 Based on these results, this clone was selected to generate the standards for the quantitative
2307 PCR (qPCR).

2308 **6.2.4. Quantitative PCR standards**

2309 The concentration of the extracted plasmid was determined with a Nanodrop ND 1000
2310 spectrophotometer and the total plasmid copy number was calculated using the Avogadro
2311 number (Lee et al., 2006). The plasmid was then linearised by incubation with the restriction
2312 enzyme NotI (New England Biolabs, Hitchin UK) at 37°C for 90 minutes followed by a step at
2313 65°C for 15 minutes to inactivate the enzyme. The solution with the linearised plasmid was
2314 diluted in 10 mM Tris-HCl pH 8.5 in a 10-fold series from 10^8 to 10^1 copy number. Serial
2315 dilutions of this plasmid were used with the qPCR to extrapolate absolute *Ca.* Nardonella
2316 fragment copy number for each of the samples.

2317 **6.2.5. Quantitative PCR**

2318 qPCR was performed in a LightCycler 480 Instrument II (Roche, Mannheim, Germany) with
2319 LightCycler 480 SYBR Green I Master (Roche, Mannheim, Germany). The qPCR mixture
2320 (20.5 μL) was done by adding 10 μL of 2X Ready mixed, 1 μL of each primer
2321 (NardvwF2/NardvwR), 6.5 μL of UltraPure DNase/RNase-Free Distilled Water (Fisher
2322 Scientific, Loughborough, UK) and 2 μL of DNA template or 2 μL of the linearised plasmid for

2323 the standards. The amplification was carried with an initial denaturation at 95°C for 15 minutes,
2324 then 40 cycles of denaturation at 95°C for 10 seconds, annealing at 54°C for 5 seconds,
2325 elongation at 72°C for 20 seconds and fluorescence acquisition at 81°C. The melt curve was
2326 performed from 65°C to 95°C with continuous acquisitions.

2327 For the qPCR incorporating all sampled weevils, the amplifications were organised on a 96
2328 well plate (LightCycler 480 plates 96 well, Roche, Mannheim, Germany). For each life stage
2329 eight biological replicates were considered, except for the teneral adult stage where there was
2330 only a single biological replicate. Each insect was amplified in triplicate to correct for
2331 concentration biases or contaminations. Also, a template-free control and the standards
2332 ranging from 10^8 to 10^4 copy number were included in triplicate. Cycle threshold values for
2333 each sample were converted to copy number values using the 2nd derivative max function
2334 within the Roche LightCycler.

2335 ### 6.2.6. Statistical analysis and graphical representation

2336 The absolute abundance of *Candidatus* Nardonella relative to body weight after being frozen
2337 with liquid Nitrogen was compared among the life stages. The statistical analysis and graphical
2338 representation were developed with R software v 3.3.3 and the packages DescTools v 0.99.23
2339 (Signorell, 2016), ggplot2 v. 3.1.1 (Wickham, 2009) and ggrepel v. 0.7.0 (Slowikowski, 2017).

2340 The abundance data were $\log_{10}$ -transformed to obtain normally distributed data. Normality
2341 was tested visually as well as with the Shapiro Wilk test. To test for differences between the
2342 life stages a one-way analysis of variance (ANOVA) was performed without considering the
2343 teneral adult as there were no replicates of this developmental stage. A post-hoc test with
2344 Bonferroni adjustment method was applied to reveal which life stages differed. Graphical
2345 representation of the concentration values was depicted with ggplot2 and ggrepel package
2346 functions whereas the melt curves were depicted with basic R functions.

2347 ## 6.3. Results

2348 ### 6.3.1. *Candidatus* Nardonella is less abundant in vine weevil adults

2349 The abundance of *Candidatus* Nardonella through the vine weevil life stages was measured
2350 by qPCR and was subsequently compared between the life stages with one-way ANOVA. The
2351 teneral adult used for the qPCR amplification was not included in the statistical analysis as it
2352 represented only a single sample of this insect developmental stage. There were significant
2353 differences between the life stages in the abundance of *Candidatus* Nardonella (ANOVA: df=
2354 2; F-value= 6.407; p-value= 0.00673). Differences between specific life stages were
2355 investigated with a post-hoc test which revealed that adults had a significantly lower

concentration of the bacterial sequence compared with the other life stages (larva-adult p-value= 0.0234; pupa-adult p-value= 0.0118). Closer inspection of the graphical representation of the *Ca.* Nardonella concentration values, including the teneral adult, revealed a trend of reduced abundance from larva to adult (Figure 6.1). However, mean concentration values did not reflect this trend, possibly due to an increase in the variation within life stage from larva to adult (mean ± variation for larva= 7.36 ± 0.06; pupa= 7.41 ± 0.14; teneral adult= 7.47; adult= 6.88 ± 0.12; Figure 6.1). Pupae could be divided into a low- and a high-abundance subgroup, with two of the three insects in the higher subgroup exhibiting signs of advanced development at the time of collection. It is possible that sampling different phases in the development within this life stage may have introduced the large degree of observed variation. Similarly, different reproductive maturation stages in adults could have caused the variation detected in adults. The maturation stage at the time of collection was not determined as insects were directly frozen to avoid possible changes in bacterial titre due to handling and removal from the native environment. Altogether, these results suggest that there is a general decrease in the abundance of *Ca.* Nardonella from larvae to adults but that also changes experienced within each life stage should be considered as this might alter the concentration of this bacterium.

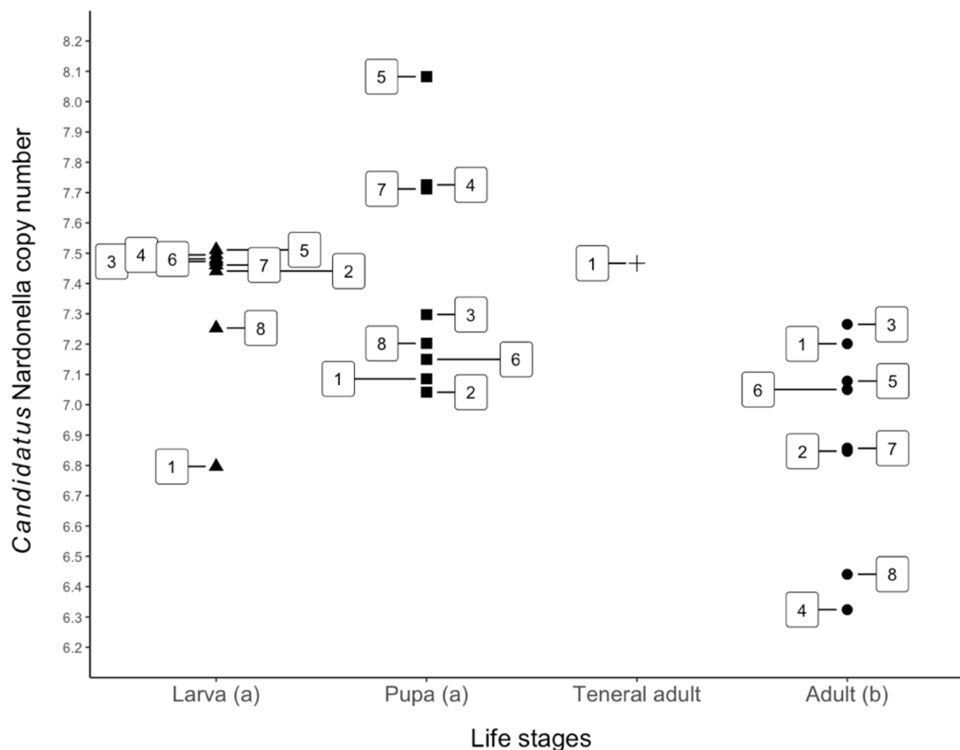
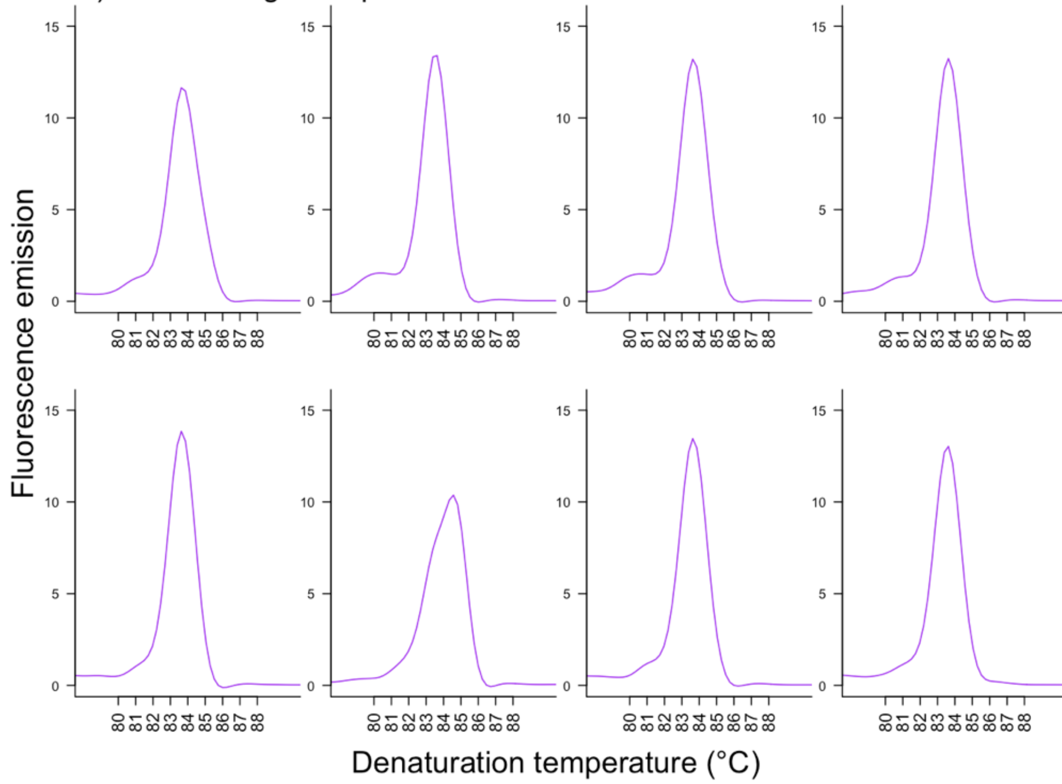


Figure 6.1 *Candidatus* Nardonella copy number per mg of body mass in the different vine weevil life stages.
Different shapes represent a distinct life stage specified in the x-axis and the associated numbers represent each biological replicate. The y-axis represents log10 of the absolute copy number of the *Ca.* Nardonella sequence fragment obtained by qPCR. Letters shared by the x-axis labels indicate no statistically significant difference (ANOVA p-value< 0.05). Teneral

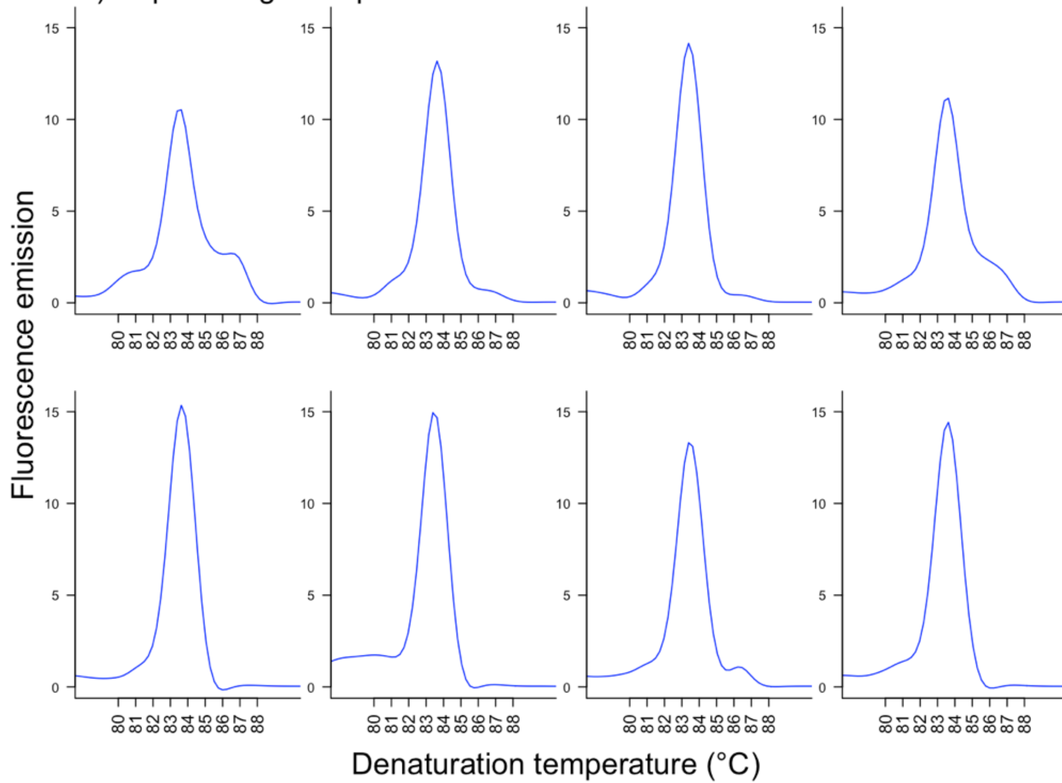
2379 adults were not included in the statistical analysis as only a single insect of this developmental
2380 stage was collected.

2381 6.3.2. QPCR may be amplifying more than one bacterial type in pupae and 2382 adults

2383 The melting temperature profile was used to detect the existence of heterogenous
2384 amplification, i.e. the presence of amplicons with different nucleotide sequence. Graphically,
2385 this is represented as peaks positioned on the x-axis at the temperature at which the amplified
2386 dsDNA fragment separates, with y-axis values reflecting the fluorescence intensity and so the
2387 amount of the specific nucleotide sequence (Figure 6.2). Through all life stages there was a
2388 peak at 83.5°C which suggests it is a product amplified from a target common to all vine weevil
2389 life stages (Figure 6.2). A second peak was observed between 85.5 to 86.5°C in some of the
2390 pupae (replicates 1-4 and 7 from Figure 6.2B) and all adults in varying proportions (Figure
2391 6.2C). For adults, replicate 5 had only a low proportion of this second peak but replicates 2, 7
2392 and 8 had a larger signal associated with this second peak than was associated with the
2393 common peak at 83.5°C (Figure 6.2C). A third peak appeared between 80 and 81°C with low
2394 fluorescence in some larvae (replicates 2-4 from Figure 5.2A), pupae (replicate 1 from Figure
2395 6.2B) and adults (replicates 1,2 from Figure 6.2C). Amplicons from the teneral adult only
2396 produced a single peak at 83.5°C and not the second peak (Figure 6.2D). However, this study
2397 is restricted by the fact that only a single insect of this developmental stage was evaluated.
2398 These findings may indicate that although there is a bacterium shared by all vine weevil life
2399 stages, likely to be *Ca.* Nardonella, additional bacterial species are detected with the same
2400 primer pair generating the additional peaks.



2401



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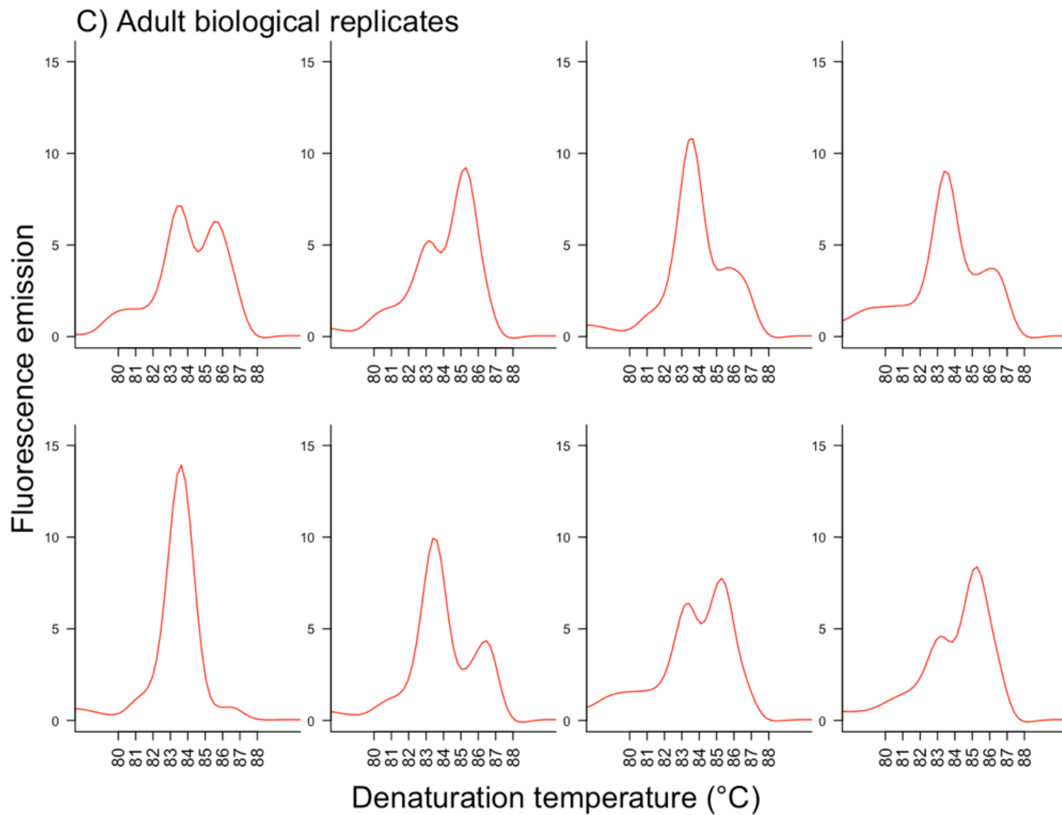
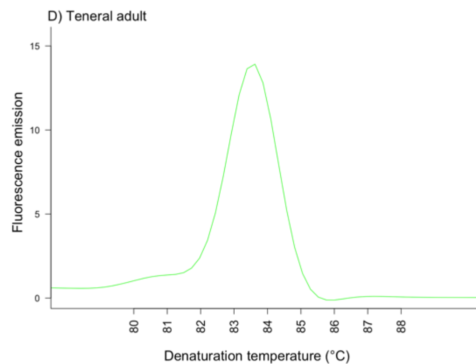


2404



2405 **Figure 6.2 Melt curve peaks for all life stages and biological replicates.**
2406 This graph depicts the melt curves for all the eight A) larvae, B) pupae and C) adult biological
2407 replicates and D) the teneral adult included in the analysis. From left to right and top to bottom
2408 replicates are ordered from 1st to 8th (see Figure 6.1). The x-axis represents the temperature
2409 at which the dsDNA amplicon separates while the y-axis represents fluorescence emission
2410 intensity, which is proportional to the quantity of fragment denatured at that specific
2411 temperature.

2412 6.4. Discussion

2413 The present study describes for the first time the population dynamics of the bacterium
2414 *Candidatus* Nardonella across the vine weevil life stages and discusses its potential function
2415 for the weevil host biology. *Ca.* Nardonella is an intracellular bacterium widely spread
2416 throughout the weevil phylogeny, although it has been replaced in weevils from the genus

2417 *Sitophilus* and *Curculio* (Conord et al., 2008; Lefèvre et al., 2004a; Toju et al., 2010). In a
2418 previous study, Morera-Margarit et al. (2019) characterised the bacterial community of adult
2419 vine weevils collected at different locations in the UK revealing *Ca.* Nardonella as the dominant
2420 bacterium in the microbiota. The investigations presented here therefore follow up previous
2421 microbiota studies on the vine weevil and provide valuable information for future investigation
2422 into the role of *Ca.* Nardonella for its vine weevil host.

2423 The population density of *Ca.* Nardonella throughout the life cycle was previously investigated
2424 for the rice water weevil (Huang et al., 2016). This study used eggs, 1st instar larvae, 3rd instar
2425 larvae, 4th instar larvae, pupae and newly emerged adults. Adults displayed a higher *Ca.*
2426 Nardonella density in comparison with larvae and pupae, although the density in eggs was not
2427 significantly different to adults. Huang et al. (2016) used a low number of samples, 13 in total,
2428 divided into these six different developmental categories. It is possible that larvae and pupae
2429 sampled were more similar to the larvae and pupae with *Ca.* Nardonella density values located
2430 at the edge of the distribution of the present study. This would explain the low *Ca.* Nardonella
2431 density of Huang et al. (2016) in comparison to the present research. Additionally, only newly
2432 emerged or teneral adults were used by Huang et al. (2016). My study consists of only of one
2433 teneral adult and so the results from the two studies cannot be compared in terms of *Ca.*
2434 Nardonella density on teneral adults.

2435 Our findings are in agreement with the results obtained by a previous study of the black hard
2436 weevil, which determined that *Ca.* Nardonella titre oscillates through the life stages depending
2437 on the tyrosine demand of the host (Anbutsu et al., 2017). The high abundance of *Ca.*
2438 Nardonella in larvae reported here may indicate that this bacterium provides vine weevil larvae
2439 with tyrosine for growth and development. Tyrosine was provided to larvae by *Ca.* Nardonella
2440 of the black hard weevil but also by the associated bacteria of the rice weevil, *Stiophilus oryzae*
2441 (Linnaeus) (Anbutsu et al., 2017; Wicker and Nardon, 1982). The importance of this amino
2442 acid for weevils is indicated by the presence of tyrosine storage in form of tyrosine rich proteins
2443 in the fat body of larvae and pupae of the rice weevil and the South American palm weevil
2444 *Rhynchophorus palmarum* (Linnaeus) (Rahbé et al., 1990). Possibly, *Ca.* Nardonella in the
2445 vine weevil is also involved in accumulating tyrosine aimed at forming the vine weevil adult
2446 cuticle as larvae from other weevil species reared on antibiotic diets developed an abnormal
2447 cuticle as adults (Anbutsu et al., 2017; Kuriwada et al., 2010). The *Ca.* Nardonella abundance
2448 pattern observed in pupae in this study may reflect different tyrosine requirements through the
2449 pupal development. Pupae within the higher abundance subgroup displayed the highest level
2450 of all life stages as was previously shown for the black hard weevil (Anbutsu et al., 2017).
2451 Interestingly, two of the insects within this subgroup presented melanised joints at the time of
2452 collection which might indicate a higher tyrosine provision compared with the other pupae. To

2453 complement these experiments, pupae at different developmental stages should be examined
2454 to determine if levels of *Ca.* Nardonella are congruent with tyrosine requirements along with
2455 measurements of free and protein tyrosine in insect tissues. The reduction in bacterial load
2456 from young adults to fully formed adults could reflect a reduced need for tyrosine due to the
2457 completion of the cuticle formation process. A decrease in tyrosine levels was also reported
2458 by Anbutsu *et al.* (2017) during cuticle pigmentation experienced by newly emerged adults
2459 until the conclusion of cuticle formation. Furthermore, in weevils from the genus *Sitophilus* a
2460 drastic reduction in the symbiont population was detected during cuticle coloration and this
2461 process was accelerated by enriching the diets with the tyrosine derivative DOPA (Vigneron
2462 et al., 2014). Nonetheless, this experiment is constrained by the fact that it was possible only
2463 to inspect only one teneral adult. So, additional tests are needed to inspect a larger sample of
2464 teneral adults to validate this decline in bacterial titre.

2465 The variation in bacterial abundance identified amongst adult weevils could be a consequence
2466 of sampling insects at different sexual maturation stages. *Ca.* Nardonella location inside the
2467 vine weevil host has not been yet identified, however, it could be hypothesised that it locates
2468 in the apex of the ovarioles and oocytes similarly to other weevil species (Conord et al., 2008;
2469 Hosokawa et al., 2015; Hosokawa and Fukatsu, 2010; Lefèvre et al., 2004a; Login et al., 2011;
2470 Toju et al., 2010; Vigneron et al., 2014). In this case, during the ovariole maturation period,
2471 which lasts approximately 50 days in a temperature dependent manner for the vine weevil
2472 (Son and Lewis, 2005), bacterial load could oscillate. Future experiments should therefore
2473 attempt to characterise *Ca.* Nardonella abundance in vine weevil adults collected at different
2474 sexual maturation phases. Also, it would be interesting to unravel the location of the *Ca.*
2475 Nardonella bacteriome over different life stages. In the light of these results it could be
2476 conjectured that the vine weevil symbiotic bacteria *Ca.* Nardonella is involved in provisioning
2477 the host with tyrosine necessary for optimal development and formation of the insect cuticle.
2478 However, additional experiments are needed to confirm these findings by examining *Ca.*
2479 Nardonella population dynamics during the different developmental changes experienced by
2480 insects at each life stage.

2481 The melt curve is usually used as a diagnostic tool to detect amplification of more than one
2482 nucleotide sequence while doing qPCR analysis, as it allows sequences with one or two
2483 nucleotide differences to be distinguished (Skow et al., 2005). This methodology has been
2484 used to distinguish closely related species of bacteria, for instance within the genera
2485 *Staphylococcus* and *Candida* (Hays et al., 2011; Skow et al., 2005). On the melt curve profile
2486 presented here, a high sharp peak was detected at 83.5°C from all insects regardless of the
2487 life stage, which is expected to represent a *Ca.* Nardonella sequence. Additionally, two extra
2488 peaks, likely representing additional bacteria, were detected ranging from 85.5°C to 86.5°C

2489 and from 80 to 81°C in some of the insects. The extra peak at 80-81°C does not follow an
2490 apparent developmental stage pattern. Interestingly, the extra peak at 85.5°C-86.5°C is small
2491 when appearing in pupae while larger and sharper when appearing in adults. This extra peak
2492 could therefore indicate the presence of an additional bacterial target which increases in
2493 density from pupae to adults. Taking this scenario, it could be proposed that in earlier
2494 developmental stages this additional bacteria is present at a low concentration and is below
2495 detectable limits with this qPCR assay.

2496 The way the vine weevil could regulate the titre of *Ca.* Nardonella through the different life
2497 stages remains as yet unresolved. Antimicrobial peptides from the coleoptericin family are
2498 important in regulating weevil symbiosis and perhaps these or other peptides regulate the
2499 fluctuation of symbiotic bacteria density in the vine weevil. In the maize weevil, a member of
2500 the coleoptericin family named Col-A controls and restricts the population location of its
2501 primary symbiont, referred as SZPE hereafter, to remaining inside the bacteriocytes (Login et
2502 al., 2011). Inhibiting the expression of *coleoptericins-A* gene with the RNAi technique resulted
2503 in endosymbiotic bacteria exiting the bacteriocytes. This coleoptericin seems to be conserved
2504 as it interacts not only with SZPE but also with *Ca.* Nardonella and with *E. coli* (Login et al.,
2505 2011). Coleoptericins are able to halt cell cytokinesis while allowing chromosome replication
2506 in *E. coli* inducing cell gigantism. Giant bacteria of SZPE and *Ca.* Nardonella were detected
2507 in the maize and red palm weevils, respectively, with a remarkable giant *Ca.* Nardonella of
2508 200 mm with 120 chromosomes (Login et al., 2011). It is possible that coleoptericins induce
2509 cell gigantism in these weevil species symbionts. Based on the ability of these peptides to
2510 interact with the cell cycle machinery, it could be proposed that the fluctuation of *Ca.*
2511 Nardonella between life stages is regulated, at least partially, by coleoptericins. Future
2512 research should aim at characterising the role of Col-A and other possible coleoptericins for
2513 the vine weevil. This could be done for instance by RNAi silencing similar to the experiments
2514 developed by Login et al. (2011).

2515 At a genomic level, there is little knowledge on the variation of *Ca.* Nardonella bacteria carried
2516 by different weevil species or populations. Hence, it cannot be discarded the possibility that
2517 rather than the various peaks being a product of targeting different species of bacteria, theses
2518 peaks could be a consequence of amplifying various *Ca.* Nardonella strains inhabiting the
2519 same insect, as was previously reported for the rice water weevil (Huang et al., 2016). In the
2520 future, additional genetic information will help to design more specific primers allowing to
2521 unravel the lineage diversity of this prokaryote for the vine weevil but also across the weevil
2522 phylogeny.

2523 In conclusion, the findings presented here may suggest that *Ca.* Nardonella is involved in the
2524 important process of cuticle formation by providing the vine weevil host with sufficient tyrosine

2525 for its polymerisation. However, this prokaryote may also be involved in additional functions in
2526 larvae and fully formed adults. Further experiments are needed to determine if tyrosine levels
2527 vary similarly to *Ca.* Nardonella abundance, reflecting the metabolic requirements for this
2528 amino acid at each developmental stage. Furthermore, rearing larvae on artificial diet
2529 containing antibiotics could reveal if the elimination of this bacterium results in abnormal
2530 cuticle development as has been reported for other weevils (Anbutsu et al., 2017; Kuriwada
2531 et al., 2010). Moreover, it would be useful to identify if the peaks obtained on the melt curve
2532 diagnostic represent various bacterial types. The identification of additional bacteria species
2533 whose concentration increases during development could pinpoint prokaryotes involved in
2534 functions specific to the more advanced developmental stages. Alternatively, the additional
2535 amplicon could represent the coexistence of different *Ca.* Nardonella lineages within the same
2536 host. Determining and identifying *Ca.* Nardonella lineage diversity for the vine weevil could
2537 provide valuable information for tracing invasion events if weevils from different locations
2538 harbour distinct lineages.

2539

Chapter 7

A puzzle with missing pieces: between population diversity for the vine weevil *Otiorhynchus sulcatus* (Fabricius)

The vine weevil, *Otiorhynchus sulcatus* (Fabricius), is a beetle member of the weevil family Curculionidae (Alonso-Zarazaga and Lyal, 2002) native to central Europe. The distribution of this curculionid species has expanded to many parts of the world in the last century as a result of human plant exchange between endemic and non-endemic regions (Kingsley, 1898; Masaki et al., 1984; Moorhouse et al., 1992; Prado, 1988). This invasive species of weevil is capable of infesting more than 150 host plant species with many of these hosts originally from outside its native distribution range.

Invasive biology is a relatively young discipline which aims at investigating the factors that influence the ability of an organism to invade a region beyond its native range, establish and spread through the ecosystem (Jeschke et al., 2012). Several theories have been proposed to explain why an invasive species can establish and sometimes outcompete native species (see reviews by Jeschke et al., 2012; Moles et al., 2012). Amongst these theories are the "enemy release theory" that posits that the exotic area is deprived of natural enemies that existed in the native area of the invasive organism. This theory, however, remains controversial as it has also been proposed that exotic species could also be more susceptible to the natural enemies present in the invaded area (Colautti et al., 2004). The "novel weapons hypothesis" explains the success of invasive species based on the fact that these species harbour new traits or "weapons" that can be used to outcompete the native populations (Jeschke et al., 2012). Another theory proposes that more diverse ecosystems are more resilient to invasion by foreign species. This, however has not been proved as results considering distinct ecosystems are contradictory (Moles et al., 2012). An added problem is that the different theories vary according to the organisms tested (Jeschke et al., 2012). These theories are therefore context-specific and the validity will depend on the traits of the invading organism and the invaded ecosystem. The lack of resolution on these theories it is likely a consequence of a too simplistic approach, i.e. by focusing on a small number of factors. It is therefore necessary to aim at understanding invasive species by designing multifactorial approaches that include biotic and abiotic factors of invader and native species as well as from the original and new ecosystems (Moles et al., 2012). Regardless of the difficulties to

2572 establish general theories in invasive biology, it is still relevant to put together the pieces of
2573 information of what we know about the vine weevil. Only in this way we can progress our
2574 understanding of its invasive nature.

2575 Firstly, it is necessary to define what is considered as a vine weevil population. Vine weevil
2576 adults are wingless beetles which move by crawling, whereas larvae and pupae live below-
2577 ground and remain close to the roots of the plant. Thus, adults are the only developmental
2578 stage capable of dispersing more than a few centimetres. A study by Pope et al. (2015)
2579 monitored vine weevil adult movement finding that in optimal crop conditions insects move
2580 around 40 cm a day. Nonetheless, when crops are disturbed vine weevils can move up to 50
2581 m in search of suitable food sources (Bennison et al., 2018a). Taking a cautious approach
2582 and assuming that vine weevils are introduced only once into infested areas, insects inhabiting
2583 locations separated a minimum distance of 100 m or from different host species could be
2584 considered as separate populations.

2585 At a genetic level, vine weevil populations from separate locations appear to be similar
2586 between each other. Vine weevils are parthenogenetic triploid females and so lack the genetic
2587 exchange attributed to sexual reproduction (Lundmark, 2010). Lundmark et al. (2010)
2588 investigated between population diversity at a genetic level by examining two small genomic
2589 regions of insects sampled from European countries within the vine weevil native distribution
2590 and from the US as a non-native region. These insects were not found to differ genetically
2591 regardless of the location. Given the low number of nucleotide substitutions it was proposed
2592 that the vine weevil parthenogenetic triploid lineage originated recently. A limitation of this
2593 study is the restricted section of the genome considered due to paucity of genetic information
2594 on the vine weevil. It would be interesting to conduct similar experiments considering a larger
2595 section of the vine weevil genome.

2596 Given the limited genetic differences between vine weevil populations, Lundmark et al. (2010)
2597 hypothesised that the "general purpose genotype" could explain the invasive nature of this
2598 insect pest. The general purpose genotype theory claims that asexual organisms might be
2599 more resistant to environmental stresses due to displaying generalist features displaying a
2600 high level of plasticity rather than conserved specialist features. In the context of a changing
2601 environment, those individuals with more generalist features will have an adaptive advantage.
2602 The selected individuals that reproduce asexually will benefit from maintaining a generalist
2603 genotype unchanged due to the lack of genetic mixing or meiotic recombination.

2604 Vine weevil populations at separate locations seem to harbour very similar bacterial
2605 communities. In the context in which the novelty required to adapt to new niches may not be
2606 acquired via genetic diversity, the possibility of associated bacteria providing the weevil host

2607 with the ability to adapt to new environments was investigated for the vine weevil (Morera-
2608 Margarit et al., 2019) (Chapter 2). Hence, the bacterial microbiota of adult vine weevils
2609 collected at various UK locations from within strawberry fields was characterised. Results from
2610 this study revealed that bacterial community of the populations tested was not significantly
2611 different. To ratify the consistency of these results across the vine weevil distribution range, it
2612 is necessary to characterise the bacterial community of insects sampled from a wider
2613 geographic area. Importantly, the lack of variation in the bacterial community composition
2614 could add power to the Lundmark et al. (2010) "general purpose genotype" hypothesis.

2615 Reanalysing the bacterial community sequencing data obtained with the newest approach,
2616 QIIME2, could contribute to confirming that vine weevil populations do not harbour location
2617 specific bacteria that were previously overlooked. QIIME2 is the newest version of QIIME. This
2618 is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA
2619 sequencing (Caporaso et al., 2010). QIIME uses the Operational Taxonomic Unit (OTU)
2620 approach, which clusters nucleotide sequences at a given percentage similarity together, 97%
2621 in most cases, generating what is referred to as an OTU. From the clustered sequences in an
2622 OTU, the most abundant is chosen as the representative to identify the taxonomy of the given
2623 OTU. QIIME2 includes statistical tools to correct for nucleotide sequence errors generating
2624 unique sequences, instead of clusters of sequences, known as amplicon sequence variants
2625 (Bolyen et al., 2018). This allows for a more comprehensive understanding of bacterial
2626 communities as the biological diversity dismissed in the OTU approach is taken into account
2627 (reviewed by Fricker et al., 2019).

2628 At a fitness level, vine weevil populations do not seem to differ in their resistance to the
2629 entomopathogenic fungus *Metarhizium brunneum* (Petch) and in their oviposition preference.
2630 Between population variation in susceptibility to the fungus *M. brunneum*, commonly used as
2631 part of Integrated Pest Management strategies to control the vine weevil, was investigated
2632 (Chapter 4). The influence of the host plant species for oviposition preference in vine weevil
2633 populations from different locations was investigated, too (Chapter 5). Larval mortality after *M.*
2634 *brunneum* treatment was not different amongst the populations tested in these experiments.
2635 Likewise, vine weevil adults from separate populations did not vary in their preference for
2636 oviposition plant host. These studies are limited by the small number of populations and the
2637 narrow geographic area considered. Further studies are required to evaluate these fitness
2638 parameters for insects collected at additional locations from a wider sampling area.
2639 Meanwhile, this information may be indicating that the vine weevil displays more generalist
2640 than specialist features.

2641 Could chemical compounds be involved in the vine weevil resistance to *M. brunneum*? The
2642 fungus *M. brunneum* is mostly used against vine weevil larvae. This pathogenic fungus when

2643 used alone is not sufficient to control the vine weevil and it is recommended that this fungal
2644 pathogen is used as part of an Integrated Pest Management programme (Bennison et al.,
2645 2018a). Temperature is an important factor affecting the growth of this fungus and its efficiency
2646 at controlling the vine weevil (Bennison et al., 2018a, 2014). Other factors, such as vine weevil
2647 chemical defences against the fungus have been overlooked to date. For example, the termite
2648 species *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) secrete β-1,3-glucanase
2649 onto the cuticle that prevents infection by *M. brunneum* (Hamilton and Bulmer, 2012b). Could
2650 vine weevil larvae secrete similar compounds to reduce pathogenicity by this fungus? The
2651 earthen cells built by larvae to moult or pupate (Smith, 1932) could also constitute a physical
2652 as well as a chemical barrier against fungal pathogens. Chemical defence in the moulting cells
2653 has been reported for the pecan weevil *Curculio caryae* (Horn). Pupal cells of this weevil
2654 species harbour antimicrobial compounds that reduce growth of the entomopathogenic
2655 *Beauvaria bassiana* ((Bals.-Criv.) Vuill.) in artificial media (Shapiro-Ilan and Mizell, 2015). The
2656 vine weevil cell compartments are built by mixing gut contents with surrounding soil or
2657 substrate particles. Gut contents of the termite *R. flavipes* inhibited growth of *M. brunneum*
2658 (Chouvenc et al., 2009). Could vine weevil gut contents have also antimicrobial properties?
2659 And, could gut bacteria produce these compounds?

2660 The results from the bacterial community study on the vine weevil revealed the presence of a
2661 dominant bacteria, identified as *Candidatus* Nardonella, which displayed a remarkable high
2662 abundance in all the studied insects regardless of the location. The changes in abundance of
2663 this specific bacterium was investigated in a single vine weevil population. These experiments
2664 revealed that adults harboured a significantly lower concentration of this prokaryote and
2665 importantly may have revealed its putative function in the acquisition of the amino acid tyrosine
2666 and the formation of the adult cuticle as has been reported for other weevil species (Anbutsu
2667 et al., 2017; Kuriwada et al., 2010) (Chapter 6). Further experiments are still needed to verify
2668 the influence of *Ca.* Nardonella in providing the host with the amino acid tyrosine and aiding
2669 in cuticle formation. The genome of the bacteria carried by the vine weevil could be
2670 investigated to see if similar to what was found in other weevils, there is a remarkable gene
2671 content reduction while genes for the tyrosine synthesis pathway are retained (Anbutsu et al.,
2672 2017). Vine weevil artificial diet containing antibiotics could be used to detect abnormal cuticle
2673 development after this symbiont is eliminated (Fisher and Bruck, 2004) (Chapter 3).
2674 Additionally, tyrosine levels in the insect haemolyph could also be measured to detect if the
2675 elimination of *Ca.* Nardonella results in a defective acquisition of this amino acid from the diet
2676 or its mobilisation from stored forms.

2677 In summary, what are the pieces required to complete the vine weevil puzzle? We have
2678 focused on location to define what a vine weevil population may be. Additional studies are

2679 necessary to complement the research presented in this thesis by examining insects from a
2680 broader geographic area. However, it remains to be determined if insects feeding on different
2681 host plants could be perceived as different populations too. Thus, in addition to testing the
2682 effect of location, future research should focus on understanding the importance of the host
2683 plant species in determining the distinctiveness of vine weevil populations. The feeding plant
2684 species has been shown to influence vine weevil host oviposition choice (Coyle et al., 2011).
2685 Moreover, the influence of the host plant in shaping the microbiota has been observed for
2686 weevil species within the genus *Curculio* (Merville et al., 2013), for the pine weevil *Hylobius*
2687 *abietis* (Linnaeus) (Berasategui et al., 2016), for the red palm weevil *Rhynchophorus*
2688 *ferrugineus* (Olivier) (Montagna et al., 2015) and for the cotton boll weevil *Anthonomus grandis*
2689 (Boheman) (Ben Guerrero et al., 2016).

2690 A deeper genomic analysis of the bacterium *Ca.* Nardonella, together with functional analysis,
2691 could explain the high abundance and ubiquity of this bacterium through the vine weevil
2692 populations. If the function of this symbiont in providing extra tyrosine invested in forming the
2693 cuticle is confirmed, two additional lines of research would open. First, it could be investigated
2694 if by feeding on a more nutritious host the titre of this bacterium could be downregulated. The
2695 tamarind weevil *Sitophilus linearis* (Herbst) lacks the typical symbiotic association found in
2696 closely related *Sitophilus* weevils (Delobel and Grenier, 1993). This weevil species, unlike the
2697 related weevil species *S. oryzae, S. zeamais and S. granarius* which feed on monocot grains,
2698 feeds on the more nutritious seeds of the leguminous tree tamarind *Tamarindus indica*
2699 (Linnaeus). Hence, it has been proposed that the richer diet of the tamarind weevil may have
2700 overcome the need for a nutritional symbiotic association. Second, the insect cuticle is
2701 involved in reducing water loss due to high temperature in some insects (Ahearn, 1970; Gibbs
2702 et al., 1991). Hence, it would be interesting to measure if vine weevil populations inhabiting
2703 warmer areas require higher abundance of *Ca.* Nardonella in comparison to populations from
2704 colder areas subjected to lower heat and drought stress.

2705 Genetically, vine weevils inhabiting different areas were shown to be rather similar. There is
2706 no information on how vine weevil from separate populations could differ at an epigenetic
2707 level. Epigenetic information directs the formation of distinct phenotypes from a common
2708 genome through DNA methylation, histone modifications or noncoding RNAs. The epigenetic
2709 information dictates for instance the development of an organism via regulated changes in the
2710 genome structure of cells that despite containing the same genetic information, will display
2711 different phenotypes. Epigenetic modifications of the genome can also be transmitted to the
2712 offspring which is of paramount importance from the point of view of evolution. For instance,
2713 intergeneration epigenetic changes seem to regulate worker defence behaviour on the
2714 honeybee *Apis mellifera* (Linnaeus) (reviewed by Glastad et al., 2019; and Yan et al., 2014;

2715 Guzman-Novoa et al., 2005). However, given the lack of variation in insect fitness between
2716 the populations tested in my experiments, there is limited evidence that there is variation at
2717 epigenetic level between vine weevil populations

2718 Further research is still needed to find the missing pieces in the vine weevil puzzle. This will
2719 reveal s, if this insect pest is adapted to each of the inhabited niches or if it is rather a generalist
2720 pest that can thrive in a wide variety of environments.

References

Ahearn, G.A., 1970. The control of water loss in desert Tenebrionid beetles. J. Exp. Biol. 53, 573–595.

Akello, J., Sikora, R., 2012. Systemic acropedal influence of endophyte seed treatment on *Acyrthosiphon pisum* and *Aphis fabae* offspring development and reproductive fitness. Biol. Control 61, 215–221. https://doi.org/10.1016/j.biocontrol.2012.02.007

Alonso-Zarazaga, M.A., Lyal, C.H.C., 2002. Addenda and corrigenda to 'A world catalogue of families and genera of Curculionoidea (Insecta: Coleoptera).' Zootaxa 63, 1. https://doi.org/10.11646/zoootaxa.63.1.1

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Anbutsu, H., Moriyama, M., Nikoh, N., Hosokawa, T., Futahashi, R., Tanahashi, M., Meng, X.-Y., Kuriwada, T., Mori, N., Oshima, K., Hattori, M., Fujie, M., Satoh, N., Maeda, T., Shigenobu, S., Koga, R., Fukatsu, T., 2017. Small genome symbiont underlies cuticle hardness in beetles. Proc. Natl. Acad. Sci. 114, E8382–E8391. https://doi.org/10.1073/pnas.1712857114

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. J. Microbiol. Methods 55, 541–555. https://doi.org/10.1016/j.mimet.2003.08.009

Bansal, R., Hulbert, S.H., Reese, J.C., Whitworth, R.J., Stuart, J.J., Chen, M.-S., 2014. Pyrosequencing reveals the predominance of Pseudomonadaceae in gut microbiome of a gall midge. Pathogens 3, 459–472. https://doi.org/10.3390/pathogens3020459

Bates, D., Mächler, M., Bolker, B., Walker, S., 2014. Fitting linear mixed-effects models using lme4. ArXiv14065823 Stat.

Behie, S.W., Jones, S.J., Bidochka, M.J., 2015. Plant tissue localization of the endophytic insect pathogenic fungi Metarhizium and Beauveria. Fungal Ecol. 13, 112–119. https://doi.org/10.1016/j.funeco.2014.08.001

Ben Guerrero, E., Soria, M., Salvador, R., Ceja-Navarro, J.A., Campos, E., Brodie, E.L., Talia, P., 2016. Effect of different lignocellulosic diets on bacterial microbiota and hydrolytic enzyme activities in the gut of the cotton boll weevil (*Anthonomus grandis)*. Front. Microbiol. 7, 2093. https://doi.org/10.3389/fmicb.2016.02093

Bennison, J., Allen, J., Atwood, J., Pope, T., 2018a. Vine weevil control in soft fruit crops (No. AHDB factsheet 09/18).

Bennison, J., Brown, S., Rose, D., Dyer, C., Pope, T., Rowley, C., Chandler, D., Prince, G., Hall, D., 2018b. Improving Vine weevil control in Hardy Nursery Stock (No. HNS 195 Second annual report).

Bennison, J., Chandler, D., Prince, G., Pope, T., Atwood, J., Talbot, D., Roberts, H., Creed, C., 2014. A review of vine weevil knowledge in order to design best-practice IPM protocols suitable for implementation in UK horticulture | AHDB (No. CP 111). ADAS, Harper Adams University and Warwick Crop Centre.

Berasategui, A., Axelsson, K., Nordlander, G., Schmidt, A., Borg-Karlson, A.-K., Gershenzon, J., Terenius, O., Kaltenpoth, M., 2016. The gut microbiota of the pine weevil is similar across Europe and resembles that of other conifer-feeding beetles. Mol. Ecol. 25, 4014–4031. https://doi.org/10.1111/mec.13702

Bi, Y., Sun, L., Gao, D., Ding, C., Li, Z., Li, Y., Cun, W., Li, Q., 2014. High-efficiency targeted editing of large viral genomes by RNA-guided nucleases. PLoS Pathog. 10. https://doi.org/10.1371/journal.ppat.1004090

Bili, M., Cortesero, A.M., Mougel, C., Gauthier, J.P., Ermel, G., Simon, J.C., Outreman, Y., Terrat, S., Mahéo, F., Poinsot, D., 2016. Bacterial community diversity harboured by interacting species. PLOS ONE 11, e0155392. https://doi.org/10.1371/journal.pone.0155392

Blackman, R.L., Eastop, V.F., 2000. Aphids on the world's crops: an identification and information guide., Ed. 2. ed. John Wiley & Sons Ltd.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E., Silva, R.D., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Michael S Robeson, I.I., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., Hooft, J.J. van der, Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., Hippel, M. von, Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2018. QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science (No. e27295v2). PeerJ Inc. https://doi.org/10.7287/peerj.preprints.27295v2

Braga, L.S., Corrêa, A.S., Pereira, E.J.G., Guedes, R.N.C., 2011. Face or flee? Fenitrothion resistance and behavioral response in populations of the maize weevil, *Sitophilus zeamais*. J. Stored Prod. Res. 47, 161–167. https://doi.org/10.1016/j.jspr.2010.11.001

Branine, M., Bazzicalupo, A., Branco, S., 2019. Biology and applications of endophytic insect-pathogenic fungi. PLoS Pathog. 15. https://doi.org/10.1371/journal.ppat.1007831

Breakey, E.P., 1959. Control of root weevils in strawberry plantings. J. Econ. Entomol. 52, 1117–1119. https://doi.org/10.1093/jee/52.6.1117

Brelsfoard, C.L., Dobson, S.L., 2009. *Wolbachia*-based strategies to control insect pests and disease vectors. Asia Pac J Mol Biol Biotechnol 17, 55–63.

Broderick, N.A., Raffa, K.F., Goodman, R.M., Handelsman, J., 2004. Census of the bacterial community of the gypsy moth larval midgut by using culturing and culture-independent methods. Appl. Environ. Microbiol. 70, 293–300. https://doi.org/10.1128/AEM.70.1.293-300.2004

Bruck, D.J., 2005. Ecology of *Metarhizium anisopliae* in soilless potting media and the rhizosphere: implications for pest management. Biol. Control 32, 155–163. https://doi.org/10.1016/J.BIOCONTROL.2004.09.003

Brucker, R.M., Bordenstein, S.R., 2012. The roles of host evolutionary relationships (genus: Nasonia) and development in structuring microbial communities. Evolution 66, 349–362. https://doi.org/10.1111/j.1558-5646.2011.01454.x

Busby, J., 2015. UK shallow ground temperatures for ground coupled heat exchangers. Q. J. Eng. Geol. Hydrogeol. 48, 248–260. https://doi.org/10.1144/qjegh2015-077

Buxton, J., Pope, T., 2011. Host plant range of vine weevil (No. HDC Factsheet 18/10).

Campbell, B.C., Bragg, T.S., Turner, C.E., 1992. Phylogeny of symbiotic bacteria of four weevil species (coleoptera: curculionidae) based on analysis of 16S ribosomal DNA. Insect Biochem. Mol. Biol. 22, 415–421. https://doi.org/10.1016/0965-1748(92)90136-3

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336. https://doi.org/10.1038/nmeth.f.303

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522. https://doi.org/10.1073/pnas.1000080107

Carvalho, G.A., Corrêa, A.S., de Oliveira, L.O., Guedes, R.N.C., 2014. Evidence of horizontal transmission of primary and secondary endosymbionts between maize and rice weevils (*Sitophilus zeamais* and *Sitophilus oryzae*) and the parasitoid *Theocolax elegans*. J. Stored Prod. Res. 59, 61–65. https://doi.org/10.1016/j.jspr.2014.05.004

Ceja-Navarro, J.A., Vega, F.E., Karaoz, U., Hao, Z., Jenkins, S., Lim, H.C., Kosina, P., Infante, F., Northen, T.R., Brodie, E.L., 2015. Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee. Nat. Commun. 6. https://doi.org/10.1038/ncomms8618

Chandler, J.A., Lang, J.M., Bhatnagar, S., Eisen, J.A., Kopp, A., 2011. Bacterial communities of diverse Drosophila species: Ecological context of a host–microbe model system. PLOS Genet. 7, e1002272. https://doi.org/10.1371/journal.pgen.1002272

Chen, S.-J., Lu, F., Cheng, J.-A., Jiang, M.-X., Way, M.O., 2012. Identification and biological role of the endosymbionts *Wolbachia* in rice water weevil (Coleoptera: Curculionidae). Environ. Entomol. 41, 469–477. https://doi.org/10.1603/EN11195

Chia, W., Savakis, C., Karp, R., Pelham, H., Ashburner, M., 1985. Mutation of the Adh gene of *Drosophila melanogaster* containing an internal tandem duplication. J. Mol. Biol. 186, 679–688. https://doi.org/10.1016/0022-2836(85)90388-2

Chouvenc, T., Su, N.-Y., Robert, A., 2009. Inhibition of *Metarhizium anisopliae* in the alimentary tract of the eastern subterranean termite *Reticulitermes flavipes*. J. Invertebr. Pathol. 101, 130–136. https://doi.org/10.1016/j.jip.2009.04.005

Christian, N., Whitaker, B.K., Clay, K., 2015. Microbiomes: Unifying animal and plant systems through the lens of community ecology theory. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00869

Citorik, R.J., Mimee, M., Lu, T.K., 2014. Sequence-specific antimicrobials using efficiently delivered RNA-guided nucleases. Nat. Biotechnol. 32, 1141–1145. https://doi.org/10.1038/nbt.3011

Clark, K.E., Hartley, S.E., Brennan, R.M., Jennings, S.N., McMenemy, L.S., McNicol, J.W., Mitchell, C., Johnson, S.N., 2012a. Effects of cultivar and egg density on a colonizing vine weevil (Otiorhynchus sulcatus) population and its impacts on red raspberry growth and yield. Crop Prot. 32, 76–82. https://doi.org/10.1016/J.CROPRO.2011.10.008

Clark, K.E., Hartley, S.E., Brennan, R.M., MacKenzie, K., Johnson, S.N., 2012b. Oviposition and feeding behaviour by the vine weevil *Otiorhynchus sulcatus* on red raspberry: effects of cultivars and plant nutritional status. Agric. For. Entomol. 14, 157–163. https://doi.org/10.1111/j.1461-9563.2011.00554.x

Colautti, R.I., Ricciardi, A., Grigorovich, I.A., MacIsaac, H.J., 2004. Is invasion success explained by the enemy release hypothesis? Ecol. Lett. 7, 721–733. https://doi.org/10.1111/j.1461-0248.2004.00616.x

Colman, D.R., Toolson, E.C., Takacs-Vesbach, C.D., 2012. Do diet and taxonomy influence insect gut bacterial communities? Mol. Ecol. 21, 5124–5137. https://doi.org/10.1111/j.1365-294X.2012.05752.x

Conord, C., Despres, L., Vallier, A., Balmand, S., Miquel, C., Zundel, S., Lemperiere, G., Heddi, A., 2008. Long-term evolutionary stability of bacterial endosymbiosis in Curculionoidea: Additional evidence of symbiont replacement in the Dryophthoridae family. Mol. Biol. Evol. 25, 859–868. https://doi.org/10.1093/molbev/msn027

Corby-Harris, V., Pontaroli, A.C., Shimkets, L.J., Bennetzen, J.L., Habel, K.E., Promislow, D.E.L., 2007. Geographical distribution and diversity of bacteria associated with natural populations of *Drosophila melanogaster*. Appl. Environ. Microbiol. 73, 3470–3479. https://doi.org/10.1128/AEM.02120-06

Costello, E.K., Lauber, C.L., Hamady, M., Fierer, N., Gordon, J.I., Knight, R., 2009. Bacterial community variation in human body habitats across space and time. Science 326, 1694–1697. https://doi.org/10.1126/science.1177486

Cowles, R.S., 2004. Susceptibility of strawberry cultivars to the vine weevil *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Agric. For. Entomol. 6, 279–284. https://doi.org/10.1111/j.1461-9555.2004.00233.x

Coyle, D.R., Clark, K.E., Raffa, K.F., Johnson, S.N., 2011. Prior host feeding experience influences ovipositional but not feeding preference in a polyphagous insect herbivore. Entomol. Exp. Appl. 138, 137–145. https://doi.org/10.1111/j.1570-7458.2010.01083.x

Cram, W.T., 1965. Fecundity of the root eeevils *Brachyrhinus sulcatus* and *Sciopithes obscurus* on strawberry in the laboratory and outdoors. Can. J. Plant Sci. 45, 169–176. https://doi.org/10.4141/cjps65-027

Dawadi, B., Wang, X., Xiao, R., Muhammad, A., Hou, Y., Shi, Z., 2018. PGRP-LB homolog acts as a negative modulator of immunity in maintaining the gut-microbe symbiosis of red palm weevil, *Rhynchophorus ferrugineus* Olivier. Dev. Comp. Immunol. 86, 65–77. https://doi.org/10.1016/j.dci.2018.04.021

de Faria, M.R., Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control 43, 237–256. https://doi.org/10.1016/j.biocontrol.2007.08.001

Delalibera, I., Vasanthakumar, A., Klepzig, K.D., Raffa, K.F., 2007. Composition of the bacterial community in the gut of the pine engraver, *Ips pini* (Say)(Coleoptera) colonizing red pine. Symbiosis 43, 97–104.

Delobel, B., Grenier, A.M., 1993. Effect of non-cereal food on cereal weevils and tamarind pod weevil (Coleoptera: Curculionidae). J. Stored Prod. Res. 29, 7–14. https://doi.org/10.1016/0022-474X(93)90014-U

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72, 5069–5072. https://doi.org/10.1128/AEM.03006-05

Dixon, P., 2003. VEGAN, a package of R functions for community ecology. J. Veg. Sci. 14, 927–930. https://doi.org/10.1111/j.1654-1103.2003.tb02228.x

Dobson, S.L., Bourtzis, K., Braig, H.R., Jones, B.F., Zhou, W., Rousset, F., O'Neill, S.L., 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem. Mol. Biol. 29, 153–160. https://doi.org/10.1016/S0965-1748(98)00119-2

Doss, R.P., 1984. Role of glandular scales of lepidote rhododendrons in insect resistance. J. Chem. Ecol. 10, 1787–1798. https://doi.org/10.1007/BF00987361

Douglas, A.E., 2011. Lessons from studying insect symbioses. Cell Host Microbe 10, 359–367. https://doi.org/10.1016/j.chom.2011.09.001

Douglas, A.E., Werren, J.H., 2016. Holes in the hologenome: Why host-microbe symbioses are not holobionts. mBio 7, e02099-15. https://doi.org/10.1128/mBio.02099-15

Elias-Costa, A.J., Confalonieri, V.A., Lanteri, A.A., Rodriguero, M.S., 2019. Game of clones: Is *Wolbachia* inducing speciation in a weevil with a mixed reproductive mode? Mol. Phylogenet. Evol. 133, 42–53. https://doi.org/10.1016/j.ympev.2018.12.027

Engl, T., Eberl, N., Gorse, C., Krüger, T., Schmidt, T.H.P., Plarre, R., Adler, C., Kaltenpoth, M., 2018. Ancient symbiosis confers desiccation resistance to stored grain pest beetles. Mol. Ecol. 27, 2095–2108. https://doi.org/10.1111/mec.14418

Evenhuis, H.H., 1978. Bionomics and control of the black vine weevil *Otiorrhynchus sulcatus*. Meded. Van Fac. Landbouwwet. Rijksuniv. Gent 43, 607–611.

FAO, no date. Red Palm Weevil | Food Chain Crisis | Food and Agriculture Organization of the United Nations [WWW Document]. URL http://www.fao.org/food-chain-crisis/how-we-work/plant-protection/red-palm-weevil/en/

Fierer, N., Jackson, R.B., 2006. The diversity and biogeography of soil bacterial communities. Proc. Natl. Acad. Sci. 103, 626–631.

Fisher, J.R., Bruck, D.J., 2004. A technique for continuous mass rearing of the black vine weevil, *Otiorhynchus sulcatus*. Entomol. Exp. Appl. 113, 71–75.

Fox, J., Weisberg, S., 2018. An R companion to applied regression. SAGE Publications.

Frago, E., Dicke, M., Godfray, H.C.J., 2012. Insect symbionts as hidden players in insect–plant interactions. Trends Ecol. Evol. 27, 705–711. https://doi.org/10.1016/j.tree.2012.08.013

Fragoso, D.B., Guedes, R.N.C., Rezende, S.T., 2003. Glutathione S-transferase detoxification as a potential pyrethroid resistance mechanism in the maize weevil, *Sitophilus zeamais*. Entomol. Exp. Appl. 109, 21–29. https://doi.org/10.1046/j.1570-7458.2003.00085.x

Fricker, A.M., Podlesny, D., Fricke, W.F., 2019. What is new and relevant for sequencing-based microbiome research? A mini-review. J. Adv. Res., Special Issue on Plant Microbiome 19, 105–112. https://doi.org/10.1016/j.jare.2019.03.006

Fukatsu, T., Hosokawa, T., Koga, R., Nikoh, N., Kato, T., Hayama, S., Takefushi, H., Tanaka, I., 2009. Intestinal endocellular symbiotic bacterium of the macaque louse *Pedicinus obtusus*: Distinct endosymbiont origins in anthropoid primate lice and the old world monkey louse. Appl. Environ. Microbiol. 75, 3796–3799. https://doi.org/10.1128/AEM.00226-09

Galili, T., 2015. dendextend: an R package for visualizing, adjusting and comparing trees of hierarchical clustering. Bioinformatics 31, 3718–3720. https://doi.org/10.1093/bioinformatics/btv428

García, J.E., Posadas, J.B., Alejandro, P., Roberto, L., 2011. *Metarhizium anisopliae* (Metschnikoff) Sorokin promotes growth and has endophytic activity in tomato plants. Adv. Biol. Res. 5, 22–27.

Garrido-Jurado, I., Resquín-Romero, G., Amarilla, S.P., Ríos-Moreno, A., Carrasco, L., Quesada-Moraga, E., 2017. Transient endophytic colonization of melon plants by entomopathogenic fungi after foliar application for the control of *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). J. Pest Sci. 90, 319–330. https://doi.org/10.1007/s10340-016-0767-2

Garth, G.S., Shanks, C.H., 1978. Some actors affecting infestation of strawberry fields by the black vine weevil in western Washington. J. Econ. Entomol. 71, 443–448. https://doi.org/10.1093/jee/71.3.443

Gasnier-Fauchet, F., Nardon, P., 1987. Comparison of sarcosine and methionine sulfoxide levels in symbiotic and aposymbiotic larvae of two sibling species, *Sitophilus oryzae* L. and *S. zeamais* Mots. (Coleoptera: Curculionidae). Insect Biochem. 17, 17–20. https://doi.org/10.1016/0020-1790(87)90138-7

Gauthier, J.-P., Outreman, Y., Mieuzet, L., Simon, J.-C., 2015. Bacterial communities associated with host-adapted populations of pea aphids revealed by deep sequencing of 16S ribosomal DNA. PLOS ONE 10, e0120664. https://doi.org/10.1371/journal.pone.0120664

Gibbs, A., Mousseau, T.A., Crowe, J.H., 1991. Genetic and acclimatory variation in biophysical properties of insect cuticle lipids. Proc. Natl. Acad. Sci. 88, 7257–7260. https://doi.org/10.1073/pnas.88.16.7257

Gilbert, J.A., Meyer, F., Antonopoulos, D., Balaji, P., Brown, C. Titus, Brown, Christopher T., Desai, N., Eisen, J.A., Evers, D., Field, D., Feng, W., Huson, D., Jansson, J., Knight, R., Knight, J., Kolker, E., Konstantindis, K., Kostka, J., Kyrpides, N., Mackelprang, R., McHardy, A., Quince, C., Raes, J., Sczyrba, A., Shade, A., Stevens, R., 2010. Meeting report: The terabase metagenomics workshop and the vision of an Earth microbiome project. Stand. Genomic Sci. 3, 243–248. https://doi.org/10.4056/sigs.1433550

Gillespie, A.T., Claydon, N., 1989. The use of entomogenous fungi for pest control and the role of toxins in pathogenesis. Pestic. Sci. 27, 203–215. https://doi.org/10.1002/ps.2780270210

Glastad, K.M., Hunt, B.G., Goodisman, M.A.D., 2019. Epigenetics in Insects: Genome Regulation and the Generation of Phenotypic Diversity. Annu. Rev. Entomol. 64, 185–203. https://doi.org/10.1146/annurev-ento-011118-111914

Goto, S., Anbutsu, H., Fukatsu, T., 2006. Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. Appl Env. Microbiol 72, 4805–4810. https://doi.org/10.1128/AEM.00416-06

Grahame, J., 2017a. Maize (greater grain) weevil (339). Pacific pests and pathogens (No. Factsheets. Pacific pests and pathogens).

Grahame, J., 2017b. Rice (lesser grain) weevil (338). Pacific pests and pathogens (No. Factsheets.).

Grenier, A.M., Nardon, C., Nardon, P., 1994. The role of symbiotes in flight activity of *Sitophilus* weevils. Entomol. Exp. Appl. 70, 201–208. https://doi.org/10.1111/j.1570-7458.1994.tb00748.x

Guedes, N.M.P., Guedes, R.N.C., Ferreira, G.H., Silva, L.B., 2009. Flight take-off and walking behavior of insecticide-susceptible and – resistant strains of *Sitophilus zeamais* exposed to deltamethrin. Bull. Entomol. Res. 99, 393–400. https://doi.org/10.1017/S0007485309006610

Guedes, R.N.C., Lima, J.O.G., Santos, J.P., Cruz, C.D., 1994. Inheritance of Deltamethrin resistance in a Brazilian strain of maize weevil (*Sitophilus zeamais* Mots.). Int. J. Pest Manag. 40, 103–106. https://doi.org/10.1080/09670879409371863

Guedes, R.N.C., Oliveira, E.E., Guedes, N.M.P., Ribeiro, B., Serrão, J.E., 2006. Cost and mitigation of insecticide resistance in the maize weevil, *Sitophilus zeamais*. Physiol. Entomol. 31, 30–38. https://doi.org/10.1111/j.1365-3032.2005.00479.x

Guo, F., Ju, F., Cai, L., Zhang, T., 2013. Taxonomic precision of different hypervariable regions of 16S rRNA gene and annotation methods for functional bacterial groups in biological wastewater treatment. PLOS ONE 8, e76185. https://doi.org/10.1371/journal.pone.0076185

Guzman-Novoa, E., Hunt, G.J., Page, R.E., Uribe-Rubio, J.L., Prieto-Merlos, D., Becerra-Guzman, F., 2005. Paternal Effects on the Defensive Behavior of Honeybees. J. Hered. 96, 376–380. https://doi.org/10.1093/jhered/esi038

Hacquard, S., Garrido-Oter, R., González, A., Spaepen, S., Ackermann, G., Lebeis, S., McHardy, A.C., Dangl, J.L., Knight, R., Ley, R., Schulze-Lefert, P., 2015. Microbiota and host nutrition across plant and animal kingdoms. Cell Host Microbe 17, 603–616. https://doi.org/10.1016/j.chom.2015.04.009

Hajek, A.E., St. Leger, R.J., 1994. Interactions between fungal pathogens and insect hosts. Annu. Rev. Entomol. 39, 293–322. https://doi.org/10.1146/annurev.en.39.010194.001453

Hamilton, C., Bulmer, M.S., 2012a. Molecular antifungal defenses in subterranean termites: RNA interference reveals *in vivo* roles of termicins and GNBPs against a naturally encountered pathogen. Dev. Comp. Immunol. 36, 372–377. https://doi.org/10.1016/j.dci.2011.07.008

Hamilton, C., Bulmer, M.S., 2012b. Molecular antifungal defenses in subterranean termites: RNA interference reveals in vivo roles of termicins and GNBPs against a naturally encountered pathogen. https://doi.org/10.1016/j.dci.2011.07.008

Hamilton, C., Lay, F., Bulmer, M.S., 2011. Subterranean termite prophylactic secretions and external antifungal defenses. J. Insect Physiol. 57, 1259–1266. https://doi.org/10.1016/j.jinsphys.2011.05.016

Hammer, T.J., McMillan, W.O., Fierer, N., 2014. Metamorphosis of a butterfly-associated bacterial community. PLOS ONE 9, e86995. https://doi.org/10.1371/journal.pone.0086995

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404, 293–296. https://doi.org/10.1038/35005107

Handelsman, J., 2004. Metagenomics: Application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev 68, 669–685. https://doi.org/10.1128/MMBR.68.4.669-685.2004

Hanula, J.L., 1988. Oviposition preference and host recognition by the black vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Environ. Entomol. 17, 694–698. https://doi.org/10.1093/ee/17.4.694

Hays, C., Duhamel, C., Cattoir, V., Bonhomme, J., 2011. Rapid and accurate identification of species belonging to the *Candida parapsilosis* complex by real-time PCR and melting curve analysis. J. Med. Microbiol. 60, 477–480. https://doi.org/10.1099/jmm.0.026633-0

Heath, B.D., Butcher, R.D.J., Whitfield, W.G.F., Hubbard, S.F., 1999. Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. Curr. Biol. 9, 313–316. https://doi.org/10.1016/S0960-9822(99)80139-0

Heather, J.M., Chain, B., 2016. The sequence of sequencers: The history of sequencing DNA. Genomics 107, 1–8. https://doi.org/10.1016/j.ygeno.2015.11.003

Heddi, A., Lefebvre, F., Nardon, P., 1993. Effect of endocytobiotic bacteria on mitochondrial enzymatic activities in the weevil *Sitophilus oryzae* (Coleoptera : Curculionidae). Insect Biochem. Mol. Biol. 23, 403–411. https://doi.org/10.1016/0965-1748(93)90024-M

Hernández-García, J.A., Gonzalez-Escobedo, R., Briones-Roblero, C.I., Cano-Ramírez, C., Rivera-Orduña, F.N., Zúñiga, G., 2018. Gut bacterial communities of *Dendroctonus valens* and *D. mexicanus* (Curculionidae: Scolytinae): A metagenomic analysis across different geographical locations in México. Int. J. Mol. Sci. 19, 2578. https://doi.org/10.3390/ijms19092578

Hertig, M., Wolbach, S.B., 1924. Studies on rickettsia-like micro-organisms in insects. J. Med. Res. 44, 329.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J.H., 2008. How many species are infected with *Wolbachia*? – a statistical analysis of current data. FEMS Microbiol. Lett. 281, 215–220. https://doi.org/10.1111/j.1574-6968.2008.01110.x

Hirsch, J., Strohmeier, S., Pfannkuchen, M., Reineke, A., 2012. Assessment of bacterial endosymbiont diversity in Otiorhynchus spp. (Coleoptera: Curculionidae) larvae using a multitag 454 pyrosequencing approach. BMC Microbiol. 12 Suppl 1, S6. https://doi.org/10.1186/1471-2180-12-S1-S6

Hosokawa, T., Fukatsu, T., 2010. *Nardonella* endosymbiont in the west Indian sweet potato weevil *Euscepes postfasciatus* (Coleoptera: Curculionidae). Appl. Entomol. Zool. 45, 115–120. https://doi.org/10.1303/aez.2010.115

Hosokawa, T., Koga, R., Tanaka, K., Moriyama, M., Anbutsu, H., Fukatsu, T., 2015. *Nardonella* endosymbionts of Japanese pest and non-pest weevils (Coleoptera: Curculionidae). Appl. Entomol. Zool. 50, 223–229. https://doi.org/10.1007/s13355-015-0326-y

Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363. https://doi.org/10.1002/bimj.200810425

Howard, R.W., Blomquist, G.J., 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. Annu. Rev. Entomol. 50, 371–393. https://doi.org/10.1146/annurev.ento.50.071803.130359

Hsiao, C., Hsiao, T.H., 1985. *Rickettsia* as the cause of cytoplasmic incompatibility in the alfalfa weevil, *Hypera postica*. J. Invertebr. Pathol. 45, 244–246. https://doi.org/10.1016/0022-2011(85)90016-3

Hsiao, T.H., Hsiao, C., 1985. Hybridization and cytoplasmic incompatibility among alfalfa weevil strains. Entomol. Exp. Appl. 37, 155–159. https://doi.org/10.1111/j.1570-7458.1985.tb03467.x

Hu, G., St Leger, R.J., 2002. Field studies using a recombinant mycoinsecticide (*Metarhizium anisopliae*) reveal that it is rhizosphere competent. Appl. Environ. Microbiol. 68, 6383–7. https://doi.org/10.1128/AEM.68.12.6383-6387.2002

Hu, X., Wang, C., Chen, H., Ma, J., 2013. Differences in the structure of the gut bacteria communities in development stages of the Chinese white pine beetle (*Dendroctonus armandi*). Int. J. Mol. Sci. 14, 21006–21020. https://doi.org/10.3390/ijms141021006

Hu, X., Yu, J., Wang, C., Chen, H., 2014. Cellulolytic bacteria associated with the gut of *Dendroctonus armandi* larvae (Coleoptera: Curculionidae: Scolytinae). Forests 5, 455–465. https://doi.org/10.3390/f5030455

Huang, X., Huang, Y., Zhang, J., Lu, F., Wei, J., Jiang, M., 2016. The symbiotic bacteria *Nardonella* in rice water weevil (Coleoptera: Curculionidae): Diversity, density, and associations with host reproduction. Ann. Entomol. Soc. Am. 109, 415–423. https://doi.org/10.1093/aesa/saw015

Huxham, I.M., Lackie, A.M., McCorkindale, N.J., 1989. Inhibitory effects of cyclodepsipeptides, destruxins, from the fungus *Metarhizium anisopliae*, on cellular immunity in insects. J. Insect Physiol. 35, 97–105. https://doi.org/10.1016/0022-1910(89)90042-5

Hyman, E.D., 1988. A new method of sequencing DNA. Anal. Biochem. 174, 423–436. https://doi.org/10.1016/0003-2697(88)90041-3

Ishak, H.D., Plowes, R., Sen, R., Kellner, K., Meyer, E., Estrada, D.A., Dowd, S.E., Mueller, U.G., 2011. Bacterial diversity in *Solenopsis invicta* and *Solenopsis geminata* ant colonies characterized by 16S amplicon 454 pyrosequencing. Microb. Ecol. 61, 821–831. https://doi.org/10.1007/s00248-010-9793-4

Jaber, L.R., Ownley, B.H., 2018. Can we use entomopathogenic fungi as endophytes for dual biological control of insect pests and plant pathogens? Biol. Control, When insect endosymbionts and plant endophytes mediate biological control outcomes 116, 36–45. https://doi.org/10.1016/j.biocontrol.2017.01.018

Jackson, M.A., Dunlap, C.A., Jaronski, S.T., 2010. Ecological considerations in producing and formulating fungal entomopathogens for use in insect biocontrol. BioControl 55, 129–145. https://doi.org/10.1007/s10526-009-9240-y

Jacques, P.J., Jacques, J.R., 2012. Monocropping cultures into ruin: The loss of food varieties and cultural diversity. Sustainability 4, 2970–2997. https://doi.org/10.3390/su4112970

Jeschke, J.M., Gómez Aparicio, L., Haider, S., Heger, T., Lortie, C.J., Pyšek, P., Strayer, D.L., 2012. Support for major hypotheses in invasion biology is uneven and declining.

Johnson, S.N., Petitjean, S., Clark, K.E., Mitchell, C., 2010. Protected raspberry production accelerates onset of oviposition by vine weevils (*Otiorhynchus sulcatus*). Agric. For. Entomol. 12, 277–283. https://doi.org/10.1111/j.1461-9563.2010.00473.x

Jones, R.T., Bernhardt, S.A., Martin, A.P., Gage, K.L., 2012. Interactions among symbionts of *Oropsylla* spp. (Siphonoptera: Ceratophyllidae). J. Med. Entomol. 49, 492–496. https://doi.org/10.1603/ME11244

Jones, R.T., Bressan, A., Greenwell, A.M., Fierer, N., 2011. Bacterial communities of two parthenogenetic aphid species cocolonizing two host plants across the Hawaiian islands. Appl. Environ. Microbiol. 77, 8345–8349. https://doi.org/10.1128/AEM.05974-11

Jones, R.T., Sanchez, L.G., Fierer, N., 2013. A cross-taxon analysis of insect-associated bacterial diversity. PLOS ONE 8, e61218. https://doi.org/10.1371/journal.pone.0061218

Jousselin, E., Clamens, A.-L., Galan, M., Bernard, M., Maman, S., Gschloessl, B., Duport, G., Meseguer, A.S., Calevro, F., D'acier, A.C., 2016. Assessment of a 16S rRNA amplicon Illumina sequencing procedure for studying the microbiome of a symbiont-rich aphid genus. Mol. Ecol. Resour. 16, 628–640. https://doi.org/10.1111/1755-0998.12478

Kassambara, A., Kosinski, M., 2018. survminer: Drawing survival curves using "ggplot2." R Package Version 043.

Kellner, R.L.L., 2002. Molecular identification of an endosymbiotic bacterium associated with pederin biosynthesis in *Paederus sabaeus* (Coleoptera: Staphylinidae). Insect Biochem. Mol. Biol. 32, 389–395. https://doi.org/10.1016/S0965-1748(01)00115-1

Kellner, R.L.L., 2001. Suppression of pederin biosynthesis through antibiotic elimination of endosymbionts in *Paederus sabaeus*. J. Insect Physiol. 47, 475–483. https://doi.org/10.1016/S0022-1910(00)00140-2

Kershaw, M.J., Moorhouse, E.R., Bateman, R., Reynolds, S.E., Charnley, A.K., 1999. The role of destruxins in the pathogenicity of *Metarhizium anisopliae* for three species of insect. J. Invertebr. Pathol. 74, 213–223. https://doi.org/10.1006/jipa.1999.4884

Kingsley, R.I., 1898. On the occurrence of the black vine weevil (*Otiorhynchus sulcatus*) in Nelson. Trans. Proc. New Zealnd Inst. 22, 338–340.

Kleinschmidt, B., Kölsch, G., 2011. Adopting bacteria in order to adapt to water—how reed beetles colonized the wetlands (Coleoptera, Chrysomelidae, Donaciinae). Insects 2, 540–554. https://doi.org/10.3390/insects2040540

Klingen, I., Westrum, K., Meyling, N.V., 2015. Effect of Norwegian entomopathogenic fungal isolates against *Otiorhynchus sulcatus* larvae at low temperatures and persistence in strawberry rhizospheres. Biol. Control 81, 1–7. https://doi.org/10.1016/J.BIOCONTROL.2014.10.006

Kölsch, G., Matz-Grund, C., Pedersen, B.V., 2009. Ultrastructural and molecular characterization of endosymbionts of the reed beetle genus *Macroplea* (Chrysomelidae, Donaciinae), and proposal of "*Candidatus* Macropleicola appendiculatae" and "*Candidatus* Macropleicola muticae." Can. J. Microbiol. 55, 1250–1260. https://doi.org/10.1139/W09-085

Kotásková, N., Kolasa, M., Kajtoch, Ł., 2018. Contrasting patterns of molecular diversity and *Wolbachia* infection in bisexual and parthenogenetic *Strophosoma* weevils (Coleoptera: Curculionidae). Entomol. Sci. 21, 385–395. https://doi.org/10.1111/ens.12317

Kranzfelder, P., Ekrem, T., Stur, E., 2016. Trace DNA from insect skins: a comparison of five extraction protocols and direct PCR on chironomid pupal exuviae. Mol. Ecol. Resour. 16, 353–363. https://doi.org/10.1111/1755-0998.12446

Krell, V., Jakobs-Schoenwandt, D., Vidal, S., Patel, A.V., 2018. Encapsulation of *Metarhizium brunneum* enhances endophytism in tomato plants. Biol. Control, When insect endosymbionts and plant endophytes mediate biological control outcomes 116, 62–73. https://doi.org/10.1016/j.biocontrol.2017.05.004

Kuriwada, T., Hosokawa, T., Kumano, N., Shiromoto, K., Haraguchi, D., Fukatsu, T., 2010. Biological role of *Nardonella* endosymbiont in its weevil host. PLOS ONE 5, e13101. https://doi.org/10.1371/journal.pone.0013101

Kurtti, T.J., Keyhani, N.O., 2008. Intracellular infection of tick cell lines by the entomopathogenic fungus Metarhizium anisopliae. Microbiology, 154, 1700–1709. https://doi.org/10.1099/mic.0.2008/016667-0

Lachowska, D., Kajtoch, Ł., Knutelski, S., 2010. Occurrence of *Wolbachia* in central European weevils: Correlations with host systematics, ecology, and biology. Entomol. Exp. Appl. 135, 105–118. https://doi.org/10.1111/j.1570-7458.2010.00974.x

Lachowska, D., Rożek, M., Holecová, M., 2008. New data on the cytology of parthenogenetic weevils (Coleoptera, Curculionidae). Genetica 134, 235–242. https://doi.org/10.1007/s10709-007-9230-x

LaLone, R.S., Clarke, R.G., 1981. Larval development of *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) and effects of larval density on larval mortality and injury to Rhododendron. Environ. Entomol. 10, 190–191. https://doi.org/10.1093/ee/10.2.190

Lane, D.J., 1991. 16S/23S rRNA sequencing. Nucleic Acid Tech. Bact. Syst. 115–175.

Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity. Insect Biochem. Mol. Biol., Recent Progress in Insect Molecular Biology 32, 1295–1309. https://doi.org/10.1016/S0965-1748(02)00092-9

Lawrence, A.L., Hii, S.-F., Chong, R., Webb, C.E., Traub, R., Brown, G., Šlapeta, J., 2015. Evaluation of the bacterial microbiome of two flea species using different DNA-isolation techniques provides insights into flea host ecology. FEMS Microbiol. Ecol. 91. https://doi.org/10.1093/femsec/fiv134

Lee, C., Kim, J., Shin, S.G., Hwang, S., 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J. Biotechnol. 123, 273–280. https://doi.org/10.1016/j.jbiotec.2005.11.014

Lefèvre, C., Charles, H., Vallier, A., Delobel, B., Farrell, B., Heddi, A., 2004a. Endosymbiont Phylogenesis in the Dryophthoridae Weevils: Evidence for Bacterial Replacement. Mol. Biol. Evol. 21, 965–973. https://doi.org/10.1093/molbev/msh063

Lefèvre, C., Charles, H., Vallier, A., Delobel, B., Farrell, B., Heddi, A., 2004b. Endosymbiont phylogenesis in the Dryophthoridae weevils: Evidence for bacterial replacement. Mol. Biol. Evol. 21, 965–973. https://doi.org/10.1093/molbev/msh063

Lemoine, M.M., Engl, T., Kaltenpoth, M., 2020. Microbial symbionts expanding or constraining abiotic niche space in insects. Curr. Opin. Insect Sci., Behavioural ecology * Pests and resistance 39, 14–20. https://doi.org/10.1016/j.cois.2020.01.003

Leu, S.-J.C., Li, J.K.-K., Hsiao, T.H., 1989. Characterization of *Wolbachia postica*, the cause of reproductive incompatibility among alfalfa weevil strains. J. Invertebr. Pathol. 54, 248–259. https://doi.org/10.1016/0022-2011(89)90035-9

Lhocine, N., Ribeiro, P.S., Buchon, N., Wepf, A., Wilson, R., Tenev, T., Lemaitre, B., Gstaiger, M., Meier, P., Leulier, F., 2008. PIMS modulates immune tolerance by negatively regulating *Drosophila* innate immune signaling. Cell Host Microbe 4, 147–158. https://doi.org/10.1016/j.chom.2008.07.004

Lockey, K.H., 1988. Lipids of the insect cuticle: origin, composition and function. Comp. Biochem. Physiol. – Part B Biochem. 89, 595–645. https://doi.org/10.1016/0305-0491(88)90305-7

Login, F.H., Balmand, S., Vallier, A., Vincent-Monegat, C., Vigneron, A., Weiss-Gayet, M., Rochat, D., Heddi, A., 2011. Antimicrobial peptides keep insect endosymbionts under control. Science 334, 362–365. https://doi.org/10.1126/science.1209728

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8

Lundmark, M., 2010. *Otiorhynchus sulcatus*, an autopolyploid general-purpose genotype species? Hereditas 147, 278–282. https://doi.org/10.1111/j.1601-5223.2010.02198.x

Lundmark, M., Saura, A., 2006. Asexuality alone does not explain the success of clonal forms in insects with geographical parthenogenesis. Hereditas 143, 23–32. https://doi.org/10.1111/j.2006.0018-0661.01935.x

Malacrinò, A., Campolo, O., Medina, R.F., Palmeri, V., 2018. Instar-and host-associated differentiation of bacterial communities in the Mediterranean fruit fly *Ceratitis capitata*. PloS One 13, e0194131.

Mansour, K., 1930. Memoirs: Preliminary studies on the bacterial cell-mass (accessory cell-mass) of *Calandra oryzae* (Linn.): The rice weevil. J. Cell Sci. s2-73, 421–435.

Mansour, K., 1927. Memoirs: The development of the larval and adult mid-gut of *Calandra oryzae* (Linn.): The rice weevil. J. Cell Sci. s2-71, 313–352.

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.-J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.-B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F., Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. Nature 437, 376–380. https://doi.org/10.1038/nature03959

Martin-Laurent, F., Philippot, L., Hallet, S., Chaussod, R., Germon, J.C., Soulas, G., Catroux, G., 2001. DNA extraction from soils: Old bias for new microbial diversity analysis methods. Appl. Environ. Microbiol. 67, 2354–2359. https://doi.org/10.1128/AEM.67.5.2354-2359.2001

Masaki, M., Ohmura, K., Ichinohe, F., 1984. Host range studies of the black vine weevil, *Othiorhynchus sulcatus* (Fabricius) (Coleoptera : Curculionidae). Appl. Entomol. Zool. 19, 95–106. https://doi.org/10.1303/aez.19.95

Masaki, M., Ohto, K., 1995. Effects of temperature on development of the black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae). Res. Bull. Plant Prot. Serv. Jpn.

Mazur, M.A., Holecová, M., Lachowska-Cierlik, D., Lis, A., Kubisz, D., Kajtoch, Ł., 2016. Selective sweep of *Wolbachia* and parthenogenetic host genomes – the example of the weevil *Eusomus ovulum*. Insect Mol. Biol. 25, 701–711. https://doi.org/10.1111/imb.12255

McMurdie, P.J., Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLOS ONE 8, e61217. https://doi.org/10.1371/journal.pone.0061217

Merville, A., Venner, S., Henri, H., Vallier, A., Menu, F., Vavre, F., Heddi, A., Bel-Venner, M.-C., 2013. Endosymbiont diversity among sibling weevil species competing for the same resource. BMC Evol. Biol. 13, 28. https://doi.org/10.1186/1471-2148-13-28

Moles, A.T., Flores-Moreno, H., Bonser, S.P., Warton, D.I., Helm, A., Warman, L., Eldridge, D.J., Jurado, E., Hemmings, F.A., Reich, P.B., Cavender-Bares, J., Seabloom, E.W., Mayfield, M.M., Sheil, D., Djietror, J.C., Peri, P.L., Enrico, L., Cabido, M.R., Setterfield, S.A., Lehmann, C.E.R., Thomson, F.J., 2012. Invasions: The trail behind, the path ahead, and a test of a disturbing idea. J. Ecol. 100, 116–127. https://doi.org/10.1111/j.1365-2745.2011.01915.x

Montagna, M., Chouaia, B., Mazza, G., Prosdocimi, E.M., Crotti, E., Mereghetti, V., Vacchini, V., Giorgi, A., Biase, A.D., Longo, S., Cervo, R., Lozzia, G.C., Alma, A., Bandi, C., Daffonchio, D., 2015. Effects of the diet on the microbiota of the red palm weevil (Coleoptera: Dryophthoridae). PLOS ONE 10, e0117439. https://doi.org/10.1371/journal.pone.0117439

Montgomery, M.E., Nielsen, D.G., 1979. Embryonic development of *Otiorhynchus sulcatus*: Effect of temperature and humidity. Entomol. Exp. Appl. 26, 24–32. https://doi.org/10.1111/j.1570-7458.1979.tb02893.x

Moonjely, S., Barelli, L., Bidochka, M.J., 2016. Chapter four - Insect pathogenic fungi as endophytes, in: Lovett, B., St. Leger, R.J. (Eds.), Advances in Genetics, Genetics and Molecular Biology of Entomopathogenic Fungi. Academic Press, pp. 107–135. https://doi.org/10.1016/bs.adgen.2015.12.004

Moorhouse, E.R., Charnley, A.K., Gillespie, A.T., 1992. A review of the biology and control of the vine weevil, *Otiorhynchus sulcatus* (Coleoptera: Curculionidae). Ann. Appl. Biol. 121, 431–454.

Moorhouse, E.R., Gillespie, A.T., Charnley, A.K., 1993. The development of Otiorhynchus sulcatus (Fabricius) (Coleoptera: Curculionidae) larvae on a range of ornamental pot-plant species and the potential for control using Metarhizium anisopliae. J. Hortic. Sci. 68, 627–635. https://doi.org/10.1080/00221589.1993.11516394

Morales-Jiménez, J., Zúñiga, G., Ramírez-Saad, H.C., Hernández-Rodríguez, C., 2012. Gut-associated bacteria throughout the life cycle of the bark beetle *Dendroctonus rhizophagus* Thomas and Bright (Curculionidae: Scolytinae) and their cellulolytic activities. Microb. Ecol. 64, 268–278. https://doi.org/10.1007/s00248-011-9999-0

Morera-Margarit, P., Bulgarelli, D., Pope, T.W., Graham, R.I., Mitchell, C., Karley, A.J., 2019. The bacterial community associated with adult vine weevil (*Otiorhynchus sulcatus*) in UK populations growing on strawberry is dominated by *Candidatus* Nardonella. Entomol. Exp. Appl. 167, 186–196. https://doi.org/10.1111/eea.12757

Moya, A., Gil, R., Latorre, A., 2009. The evolutionary history of symbiotic associations among bacteria and their animal hosts: a model. Clin. Microbiol. Infect. 15, 11–13. https://doi.org/10.1111/j.1469-0691.2008.02689.x

Moya, A., Peretó, J., Gil, R., Latorre, A., 2008. Learning how to live together: genomic insights into prokaryote–animal symbioses. Nat. Rev. Genet. 9, 218–229. https://doi.org/10.1038/nrg2319

Muhammad, A., Fang, Y., Hou, Y., Shi, Z., 2017. The gut entomotype of red palm weevil *Rhynchophorus ferrugineus* Olivier (Coleoptera: Dryophthoridae) and their effect on host nutrition metabolism. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.02291

Nakabachi, A., Ishikawa, H., 1999. Provision of riboflavin to the host aphid, *Acyrthosiphon pisum*, by endosymbiotic bacteria, *Buchnera*. J. Insect Physiol. 45, 1–6. https://doi.org/10.1016/S0022-1910(98)00104-8

Nardon, P., Grenier, A.M., 1988. Genetical and biochemical interactions between the host and its endocytobiotes in the weevils *Sitophilus* (Coleoptere, Curculionidae) and other related species, in: Scannerini, S., Smith, D., Bonfante-Fasolo, P., Gianinazzi-Pearson, V. (Eds.), Cell to Cell Signals in Plant, Animal and Microbial Symbiosis, NATO ASI Series. Springer Berlin Heidelberg, pp. 255–270.

Nardon, P., Lefevre, C., Delobel, B., Charles, H., Heddi, A., 2002. Occurrence of endosymbiosis in Dryophthoridae weevils: cytological insights into bacterial symbiotic structures. Symbiosis 33, 227–41.

Neiswander, R.B., 1953. Control of the black vine weevil. J. Econ. Entomol. 46, 234–237. https://doi.org/10.1093/jee/46.2.234

Nielsen, D.G., Dunlap, M.J., 1981. Black vine weevil: Reproductive potential on selected plants. Ann. Entomol. Soc. Am. 74, 60–65. https://doi.org/10.1093/aesa/74.1.60

Nogge, G., 1981. Significance of symbionts for the maintenance of an optimal nutritional state for successful reproduction in hematophagous arthropods, in: Parasitology. CAMBRIDGE UNIV PRESS 40 WEST 20TH STREET, NEW YORK, NY 10011-4211, pp. 101–104.

Oddsdottir, E.S., Eilenberg, J., Sen, R., Halldorsson, G., 2010. The effects of insect pathogenic soil fungi and ectomycorrhizal inoculation of birch seedlings on the survival of *Otiorhynchus* larvae. Agric. For. Entomol. 12, 319–324. https://doi.org/10.1111/j.1461-9563.2010.00482.x

Oerke, E.-C., 2006. Crop losses to pests. J. Agric. Sci. 144, 31–43. https://doi.org/10.1017/S0021859605005708

Oerke, E.-C., Dehne, H.-W., 2004. Safeguarding production—losses in major crops and the role of crop protection. Crop Prot. 23, 275–285. https://doi.org/10.1016/j.cropro.2003.10.001

Oliveira, E.E., Guedes, R.N.C., Tótola, M.R., De Marco, P., 2007. Competition between insecticide-susceptible and -resistant populations of the maize weevil, *Sitophilus zeamais*. Chemosphere 69, 17–24. https://doi.org/10.1016/j.chemosphere.2007.04.077

Oliver, K.M., Moran, N.A., Hunter, M.S., 2005. Variation in resistance to parasitism in aphids is due to symbionts not host genotype. Proc. Natl. Acad. Sci. 102, 12795–12800. https://doi.org/10.1073/pnas.0506131102

Oliver, K.M., Russell, J.A., Moran, N.A., Hunter, M.S., 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc. Natl. Acad. Sci. 100, 1803–1807. https://doi.org/10.1073/pnas.0335320100

O'Neill, S.L., Giordano, R., Colbert, A.M., Karr, T.L., Robertson, H.M., 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. 89, 2699–2702. https://doi.org/10.1073/pnas.89.7.2699

Ortiz-Urquiza, A., Keyhani, N., 2013. Action on the surface: entomopathogenic fungi versus the insect cuticle. Insects 4, 357–374. https://doi.org/10.3390/insects4030357

Paradis, E., Claude, J., Strimmer, K., 2004. APE: Analyses of phylogenetics and evolution in R language. Bioinformatics 20, 289–290. https://doi.org/10.1093/bioinformatics/btg412

Perez-Mendoza, J., 1999. Survey of insecticide resistance in Mexican populations of maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae). J. Stored Prod. Res. 35, 107–115. https://doi.org/10.1016/S0022-474X(98)00017-4

Pierantoni, U., 1927. L'organo simbiotico nello sviluppo di *Calandra oryzae*. Rend R. Acad Sci Fis Mat Napoli 35, 244–250.

Pietrangelo, L., Bucci, A., Maiuro, L., Bulgarelli, D., Naclerio, G., 2018. Unraveling the Composition of the Root-Associated Bacterial Microbiota of Phragmites australis and Typha latifolia. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.01650

Pohlert, T., 2014. The pairwise multiple comparison of mean ranks package (PMCMR)[Internet]. R Packag. 2015 Feb 05 Available Httpcran R-Proj. OrgwebpackagesPMCMRvignettesPMCMR Pdf.

Pope, T., Gundalai, E., Elliott, L., Blackshaw, R., Hough, G., Wood, A., Bennison, J., Prince, G., Chandler, D., 2015. Recording the movement of adult vine weevil within strawberry crops using radio frequency identification tags. J. Berry Res. 5, 197–206. https://doi.org/10.3233/JBR-150102

Prado, E., 1988. Notas sobre insectos de importancia agrícola en Chile. Agric. Téc. Chile 48, 51–54.

Rahbé, Y., Delobel, B., Guillaud, J., Nardon, C., 1990. Storage proteins in coleoptera: A new class of tyrosine-rich proteins from the pupae of two weevils, *Sitophilus oryzae* and

Rhynchophorus palmarum (Coleoptera: Curculionidae). Insect Biochem. 20, 331–341. https://doi.org/10.1016/0020-1790(90)90052-V

Ravensberg, W.J., 2015. Commercialisation of microbes: Present situation and future prospects, in: Lugtenberg, B. (Ed.), Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture. Springer International Publishing, Cham, pp. 309–317. https://doi.org/10.1007/978-3-319-08575-3_32

Remáč, M., 2009. European Comission. Directive on the sustainable use of pesticides (No. 128/EC).

Resquín-Romero, G., Garrido-Jurado, I., Delso, C., Ríos-Moreno, A., Quesada-Moraga, E., 2016. Transient endophytic colonizations of plants improve the outcome of foliar applications of mycoinsecticides against chewing insects. J. Invertebr. Pathol. 136, 23–31. https://doi.org/10.1016/j.jip.2016.03.003

Ribeiro, B.M., Guedes, R.N.C., Oliveira, E.E., Santos, J.P., 2003. Insecticide resistance and synergism in Brazilian populations of *Sitophilus zeamais* (Coleoptera: Curculionidae). J. Stored Prod. Res. 39, 21–31. https://doi.org/10.1016/S0022-474X(02)00014-0

Ríos-Moreno, A., Garrido-Jurado, I., Resquín-Romero, G., Arroyo-Manzanares, N., Arce, L., Quesada-Moraga, E., 2016. Destruxin A production by *Metarhizium brunneum* strains during transient endophytic colonisation of *Solanum tuberosum*. Biocontrol Sci. Technol. 26, 1574–1585. https://doi.org/10.1080/09583157.2016.1223274

Robertson-Albertyn, S., Alegria Terrazas, R., Balbirnie, K., Blank, M., Janiak, A., Szarejko, I., Chmielewska, B., Karcz, J., Morris, J., Hedley, P.E., George, T.S., Bulgarelli, D., 2017. Root hair mutations displace the barley rhizosphere microbiota. Front. Plant Sci. 8. https://doi.org/10.3389/fpls.2017.01094

Rodriguero, M.S., Confalonieri, V.A., Guedes, J.V.C., Lanteri, A.A., 2010. *Wolbachia* infection in the tribe Naupactini (Coleoptera, Curculionidae): association between thelytokous parthenogenesis and infection status. Insect Mol. Biol. 19, 631–640. https://doi.org/10.1111/j.1365-2583.2010.01018.x

Rothberg, J.M., Hinz, W., Rearick, T.M., Schultz, J., Mileski, W., Davey, M., Leamon, J.H., Johnson, K., Milgrew, M.J., Edwards, M., 2011. An integrated semiconductor device enabling non-optical genome sequencing. Nature 475, 348.

Russell L, 2019. emmeans: Estimated marginal means, aka least-squares means. R package version 1.3.3. https://doi.org/10.1080/00031305.1980.10483031

Ryu, J.-H., Kim, S.-H., Lee, H.-Y., Bai, J.Y., Nam, Y.-D., Bae, J.-W., Lee, D.G., Shin, S.C., Ha, E.-M., Lee, W.-J., 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in *Drosophila*. Science 319, 777–782. https://doi.org/10.1126/science.1149357

Sakurai, M., Koga, R., Tsuchida, T., Meng, X.-Y., Fukatsu, T., 2005. *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: Novel cellular tropism, effect on host fitness, and interaction with the essential symbiont *Buchnera*. Appl. Environ. Microbiol. 71, 4069–4075. https://doi.org/10.1128/AEM.71.7.4069-4075.2005

Samson, R.A., Evans, H.C., Latge, J.-P., 2013. Atlas of Entomopathogenic Fungi. Springer Science & Business Media.

Samuels, R.I., Charnley, A.K., Reynolds, S.E., 1988. The role of destruxins in the pathogenicity of 3 strains of *Metarhizium anisopliae* for the tobacco hornworm *Manduca sexta*. Mycopathologia 104, 51–58. https://doi.org/10.1007/BF00437924

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467.

Saura, A., Lokki, J., Suomalainen, E., 1993. Origin of polyploidy in parthenogenetic weevils. J. Theor. Biol. 163, 449–456. https://doi.org/10.1006/jtbi.1993.1130

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N., Nelson, A., 2019. The global burden of pathogens and pests on major food crops. Nat. Ecol. Evol. 3, 430–439. https://doi.org/10.1038/s41559-018-0793-y

Scarborough, C.L., Ferrari, J., Godfray, H.C.J., 2005. Aphid protected from pathogen by endosymbiont. Science 310, 1781–1781. https://doi.org/10.1126/science.1120180

Sevim, A., Donzelli, B.G.G., Wu, D., Demirbag, Z., Gibson, D.M., Turgeon, B.G., 2012. Hydrophobin genes of the entomopathogenic fungus, *Metarhizium brunneum*, are differentially expressed and corresponding mutants are decreased in virulence. Curr. Genet. 58, 79–92. https://doi.org/10.1007/s00294-012-0366-6

Shah, F.A., Wang, C.S., Butt, T.M., 2005. Nutrition influences growth and virulence of the insect-pathogenic fungus Metarhizium anisopliae. FEMS Microbiol. Lett. 251, 259–266. https://doi.org/10.1016/j.femsle.2005.08.010

Shang, Y., Feng, P., Wang, C., 2015. Fungi That Infect Insects: Altering Host Behavior and Beyond. PLOS Pathog. 11, e1005037. https://doi.org/10.1371/journal.ppat.1005037

Shanks, C.H., 1980. Strawberry and yew as hosts of adult black vine weevil and wffects on oviposition and development of progeny. Environ. Entomol. 9, 530–532. https://doi.org/10.1093/ee/9.5.530

Shanks, C.H., Finnigan, B., 1973a. An Artificial Diet for *Otiorhynchus sulcatus* larvae. Ann. Entomol. Soc. Am. 66, 1164–1166. https://doi.org/10.1093/aesa/66.5.1164

Shanks, C.H., Finnigan, B.F., 1973b. Temperature and relative humidity effects on eggs and first-stage larvae of the black vine weevil, *Otiorhynchus sulcatus*. Environ. Entomol. 2, 855–858. https://doi.org/10.1093/ee/2.5.855

Shanks, C.H., Finnigan, B.F., 1971. Development of Thoracic Legs on Black Vine Weevil Larvae Fed on a Powdered Milk Diet. Ann. Entomol. Soc. Am. 64, 1340–1341. https://doi.org/10.1093/aesa/64.6.1340

Shapira, M., 2016. Gut Microbiotas and Host Evolution: Scaling Up Symbiosis. Trends Ecol. Evol. 31, 539–549. https://doi.org/10.1016/j.tree.2016.03.006

Shapiro-Ilan, D.I., Mizell, R.F., 2015. An insect pupal cell with antimicrobial properties that suppress an entomopathogenic fungus. J. Invertebr. Pathol. 124, 114–116. https://doi.org/10.1016/j.jip.2014.12.003

Shelton, A.M., Badenes-Perez, F.R., 2006. Concepts and applications of trap cropping in pest management. Annu Rev Entomol 51, 285–308.

Shendure, J., Porreca, G.J., Reppas, N.B., Lin, X., McCutcheon, J.P., Rosenbaum, A.M., Wang, M.D., Zhang, K., Mitra, R.D., Church, G.M., 2005. Accurate multiplex polony sequencing of an evolved bacterial genome. Science 309, 1728–1732.

Signorell, A., 2016. DescTools: Tools for descriptive statistics. R Package Version 099 18.

Skow, A., Mangold, K.A., Tajuddin, M., Huntington, A., Fritz, B., Thomson, R.B., Kaul, K.L., 2005. Species-level identification of staphylococcal isolates by real-time PCR and melt curve analysis. J. Clin. Microbiol. 43, 2876–2880. https://doi.org/10.1128/JCM.43.6.2876-2880.2005

Slowikowski, K., 2017. ggrepel: Repulsive text and label geoms for ‘ggplot2.’ R Pakcage Version 07 0.

Small, C.-L.N., Bidochka, M.J., 2005. Up-regulation of Pr1, a subtilisin-like protease, during conidiation in the insect pathogen Metarhizium anisopliae. Mycol. Res. 109, 307–313. https://doi.org/10.1017/S0953756204001856

Smith, F.F., 1932. Biology and control of the black vine weevil. US Department of Agriculture.

Smith, R.F., Apple, J.L., Bottrell, D.G., 1976. The origins of Integrated Pest Management concepts for agricultural crops, in: Apple, J.L., Smith, R.F. (Eds.), Integrated Pest Management. Springer US, Boston, MA, pp. 1–16. https://doi.org/10.1007/978-1-4615-7269-5_1

Soares, G.G., Marchal, M., Ferron, P., 1983. Susceptibility of *Otiorhynchus sulcatus* (Coleoptera: Curculionidae) larvae to *Metarhizium anisopliae* and *Metarhizium flavoviride* (Deuteromycotina: Hyphomycetes) at two different temperatures. Environ. Entomol. 12, 1887–1891. https://doi.org/10.1093/ee/12.6.1887

Son, Y., Lewis, E.E., 2005. Effects of temperature on the reproductive life history of the black vine weevil, *Otiorhynchus sulcatus*. Entomol. Exp. Appl. 114, 15–24. https://doi.org/10.1111/j.0013-8703.2005.00233.x

Son, Y., Luckhart, S., Zhang, X., Lieber, M.J., Lewis, E.E., 2008. Effects and implications of antibiotic treatment on *Wolbachia*-infected vine weevil (Coleoptera: Curculionidae). Agric. For. Entomol. 10, 147–155. https://doi.org/10.1111/j.1461-9563.2008.00369.x

Steinhaus, E.A., 1940. The microbiology of insects. Bacteriol. Rev. 4, 17–57.

Stenberg, P., Lundmark, M., 2004. Distribution, mechanisms and evolutionary significance of clonality and polyploidy in weevils. Agric. For. Entomol. 6, 259–266. https://doi.org/10.1111/j.1461-9555.2004.00231.x

Stenberg, P., Terhivuo, J., Lokki, J., Saura, A., 2000. Clone diversity in the polyploid weevil *Otiorhynchus scaber*. Hereditas 132, 137–142. https://doi.org/10.1111/j.1601-5223.2000.00137.x

Stenseth, C., 1979. Effects of temperature on development of <i>Otiorhynchus sulcatus<(i> (Coleoptera: Curculionidae). Ann. Appl. Biol. 91, 179–185. https://doi.org/10.1111/j.1744-7348.1979.tb06488.x

Stimmann, M., Kaya, H., Burlando, T., Studdert, J., 1985. Black vine weevil management in nursery plants. Calif. Agric. 39, 25–26.

Stouthamer, R., Breeuwer, J.A., Hurst, G.D., 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53, 71–102.

Suomalainen, E., 1962. Significance of parthenogenesis in the evolution of insects. Annu. Rev. Entomol. 7, 349–366. https://doi.org/10.1146/annurev.en.07.010162.002025

Suomalainen, E., Saura, A., Lokki, J., 1987. Cytology and evolution in parthenogenesis. CRC Press.

Suzuki, M.T., Giovannoni, S.J., 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62, 625–630.

Teixeira, L., Ferreira, Á., Ashburner, M., 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLOS Biol. 6, e1000002. https://doi.org/10.1371/journal.pbio.1000002

Thao, M.L., Clark, M.A., Baumann, L., Brennan, E.B., Moran, N.A., Baumann, P., 2000. Secondary endosymbionts of psyllids have been acquired multiple times. Curr. Microbiol. 41, 300–304. https://doi.org/10.1007/s002840010138

Therneau, T.C., 2018. Mixed effects cox models. R package version 2.2-10; 2018.

Therneau, T.M., Grambsch, P.M., 2000. Modeling Survival Data: Extending the Cox Model, Statistics for Biology and Health. Springer New York, New York, NY. https://doi.org/10.1007/978-1-4757-3294-8

Toju, H., Fukatsu, T., 2011. Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. Mol. Ecol. 20, 853–868. https://doi.org/10.1111/j.1365-294X.2010.04980.x

Toju, H., Hosokawa, T., Koga, R., Nikoh, N., Meng, X.Y., Kimura, N., Fukatsu, T., 2010. "*Candidatus Curculioniphilus buchneri*," a novel clade of bacterial endocellular symbionts from weevils of the genus *Curculio*. Appl Env. Microbiol 76, 275–282. https://doi.org/10.1128/AEM.02154-09

Toju, H., Tanabe, A.S., Notsu, Y., Sota, T., Fukatsu, T., 2013. Diversification of endosymbiosis: replacements, co-speciation and promiscuity of bacteriocyte symbionts in weevils. ISME J. 7, 1378–1390. https://doi.org/10.1038/ismej.2013.27

Tol, R.W.H.M. van, Visser, J.H., 1998. Host-plant preference and antennal responses of the black vine weevil (*Otiorhynchus sulcatus*) to plant volatiles. Entomol. Exp. Appl. 9, 35–40.

Tseng, H.-Y., Lin, C.-P., Hsu, J.-Y., Pike, D.A., Huang, W.-S., 2014. The functional significance of aposematic signals: Geographic variation in the responses of widespread lizard predators to colourful invertebrate prey. PLOS ONE 9, e91777. https://doi.org/10.1371/journal.pone.0091777

Turcatti, G., Romieu, A., Fedurco, M., Tairi, A.-P., 2008. A new class of cleavable fluorescent nucleotides: synthesis and optimization as reversible terminators for DNA sequencing by synthesis. Nucleic Acids Res. 36, e25–e25. https://doi.org/10.1093/nar/gkn021

Turillazzi, S., Sledge, M.F., Dani, F.R., Cervo, R., Massolo, A., Fondelli, L., 2000. Social hackers: Integration in the host chemical recognition system by a paper wasp social parasite. Naturwissenschaften 87, 172–176. https://doi.org/10.1007/s001140050697

van Tol, R.W.H.M., Dijk, N.V., Sabelis, M.W., 2004. Host plant preference and performance of the vine weevil *Otiorhynchus sulcatus*. Agric. For. Entomol. 6, 267–278. https://doi.org/10.1111/j.1461-9555.2004.00232.x

van Tol, R.W.H.M., Visser, J.H., 2002. Olfactory antennal responses of the vine weevil *Otiorhynchus sulcatus* to plant volatiles. Entomol. Exp. Appl. 102, 49–64. https://doi.org/10.1046/j.1570-7458.2002.00924.x

Vasanthakumar, A., Delalibera, I., Handelsman, J., Klepzig, K.D., Schloss, P.D., Raffa, K.F., 2006. Characterization of gut-associated bacteria in larvae and adults of the southern pine beetle, *Dendroctonus frontalis* Zimmermann. Environ. Entomol. 35, 1710–1717. https://doi.org/10.1093/ee/35.6.1710

Vega, F.E., Goettel, M.S., Blackwell, M., Chandler, D., Jackson, M.A., Keller, S., Koike, M., Maniania, N.K., Monzón, A., Ownley, B.H., Pell, J.K., Rangel, D.E.N., Roy, H.E., 2009. Fungal entomopathogens: new insights on their ecology. Fungal Ecol. 2, 149–159. https://doi.org/10.1016/j.funeco.2009.05.001

Vigneron, A., Masson, F., Vallier, A., Balmand, S., Rey, M., Vincent-Monégat, C., Aksoy, E., Aubailly-Giraud, E., Zaidman-Rémy, A., Heddi, A., 2014. Insects recycle endosymbionts when the benefit is over. Curr. Biol. 24, 2267–2273. https://doi.org/10.1016/j.cub.2014.07.065

Vilcinskas, A., 2010. Coevolution between pathogen-derived proteinases and proteinase inhibitors of host insects. Virulence 1, 206–214. https://doi.org/10.4161/viru.1.3.12072

Wang, C., Leger, R.J.S., 2006. A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses. Proc. Natl. Acad. Sci. 103, 6647–6652. https://doi.org/10.1073/pnas.0601951103

Wang, C., St Leger, R.J., 2007. The MAD1 adhesin of *Metarhizium anisopliae* links adhesion with blastospore production and virulence to insects, and the MAD2 adhesin enables attachment to plants. Eukaryot. Cell 6, 808–16. https://doi.org/10.1128/EC.00409-06

Warner, R.E., Negley, F.B., 1976. The genus *Otiorhynchus* in America north of Mexico (Coleoptera: Curculionidae). Proc. Entomol. Soc. Wash. 78, 240–262.

Weeks, A.R., Breeuwer, J. a. J., 2001. *Wolbachia*–induced parthenogenesis in a genus of phytophagous mites. Proc. R. Soc. Lond. B Biol. Sci. 268, 2245–2251. https://doi.org/10.1098/rspb.2001.1797

Weeks, A.R., Turelli, M., Harcombe, W.R., Reynolds, K.T., Hoffmann, A.A., 2007. From parasite to mutualist: Rapid evolution of *Wolbachia* in natural populations of Drosophila. PLOS Biol. 5, e114. https://doi.org/10.1371/journal.pbio.0050114

Wei, T., Hu, J., Miyanaga, K., Tanji, Y., 2013. Comparative analysis of bacterial community and antibiotic-resistant strains in different developmental stages of the housefly (Musca domestica). Appl. Microbiol. Biotechnol. 97, 1775–1783. https://doi.org/10.1007/s00253-012-4024-1

Weiss, S., Xu, Z.Z., Peddada, S., Amir, A., Bittinger, K., Gonzalez, A., Lozupone, C., Zaneveld, J.R., Vázquez-Baeza, Y., Birmingham, A., Hyde, E.R., Knight, R., 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5, 27. https://doi.org/10.1186/s40168-017-0237-y

Weissling, T.J., Giblin-Davis, R.M., 1993. Water loss dynamics and humidity preference of *Rhynchophorus cruentatus* (Coleoptera: Curculionidae) adults. Environ. Entomol. 22, 93–98. https://doi.org/10.1093/ee/22.1.93

Werren, J.H., Zhang, W., Guo, L.R., 1995. Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc. R. Soc. Lond. B Biol. Sci. 261, 55–63. https://doi.org/10.1098/rspb.1995.0117

White, J.A., Richards, N.K., Laugraud, A., Saeed, A., Curry, M.M., McNeill, M.R., 2015. Endosymbiotic candidates for parasitoid defense in exotic and native New Zealand weevils. Microb. Ecol. 70, 274–286. https://doi.org/10.1007/s00248-014-0561-8

Whiteside, S.A., Razvi, H., Dave, S., Reid, G., Burton, J.P., 2015. The microbiome of the urinary tract—a role beyond infection. Nat. Rev. Urol. 12, 81–90. https://doi.org/10.1038/nrurol.2014.361

Wicker, C., 1983. Differential vitamin and choline requirements of symbiotic and aposymbiotic *S. oryzae* (Coleoptera: Curculionidae). Comp. Biochem. Physiol. A Physiol. 76, 177–182. https://doi.org/10.1016/0300-9629(83)90311-0

Wicker, C., Nardon, P., 1982. Development responses of symbiotic and aposymbiotic weevils *Sitophilus oryzae* L. (Coleoptera, Curculionidae) to a diet supplemented with aromatic amino acids. J. Insect Physiol. 28, 1021–1024. https://doi.org/10.1016/0022-1910(82)90008-7

Wickham, H., 2011. The split-apply-combine strategy for data analysis. J. Stat. Softw. 40, 1–29.

Wickham, H., 2009. Ggplot2 : Elegant graphics for data analysis. Springer-Verlag New York.

Woese, C.R., 1987. Bacterial evolution. Microbiol. Rev. 51, 221–271.

Wong, C.N.A., Ng, P., Douglas, A.E., 2011. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. Environ. Microbiol. 13, 1889–1900. https://doi.org/10.1111/j.1462-2920.2011.02511.x

Wouw, M. van de, Kik, C., Hintum, T. van, Treuren, R. van, Visser, B., 2010. Genetic erosion in crops: Concept, research results and challenges. Plant Genet. Resour. 8, 1–15. https://doi.org/10.1017/S1479262109990062

Wynn, S., 2010. Impact of changing pesticide availability on horticulture (No. Defra Project IF01100).

Yan, H., Simola, D.F., Bonasio, R., Liebig, J., Berger, S.L., Reinberg, D., 2014. Eusocial insects as emerging models for behavioural epigenetics. Nat. Rev. Genet. 15, 677–688. https://doi.org/10.1038/nrg3787

Yang, B., Wang, Y., Qian, P.-Y., 2016. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinformatics 17, 135. https://doi.org/10.1186/s12859-016-0992-y

Yun, J.-H., Roh, S.W., Whon, T.W., Jung, M.-J., Kim, M.-S., Park, D.-S., Yoon, C., Nam, Y.-D., Kim, Y.-J., Choi, J.-H., Kim, J.-Y., Shin, N.-R., Kim, S.-H., Lee, W.-J., Bae, J.-W., 2014. Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. Appl. Environ. Microbiol. 80, 5254–5264. https://doi.org/10.1128/AEM.01226-14

Zepp, D.B., Dierks, A.Z., Sanders, D.J., 1979. Effects of Diflubenzuron on black vine weevil oviposition, egg viability, and adult longevity (Coleoptera: Curculionidae). J. Kans. Entomol. Soc. 52, 662–666.