Gut microbiota of the pine weevil degrades conifer diterpenes and increases insect fitness

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Insect gut symbionts degrade plant defenses

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30 **Abstract**

31 The pine weevil (*Hylobius abietis*), a major pest of conifer forests throughout Europe,
 32 feeds on the bark and cambium, tissues rich in terpenoid resins that are toxic to
 33 many insect herbivores. Here we report the ability of the pine weevil gut microbiota to
 34 degrade the diterpene acids of Norway spruce. The diterpene acid levels present in
 35 ingested bark were substantially reduced on passage through the pine weevil gut.
 36 This reduction was significantly less upon antibiotic treatment, and supplementing
 37 the diet with gut suspensions from untreated insects restored the ability to degrade
 38 diterpenes. In addition, cultured bacteria isolated from pine weevil guts were shown
 39 to degrade a Norway spruce diterpene acid. In a metagenomic survey of the insect's
 40 bacterial community, we were able to annotate several genes of a previously
 41 described diterpene degradation (*dit*) gene cluster. Antibiotic treatment disrupted the
 42 core bacterial community of *H. abietis* guts and eliminated nearly all *dit*-genes
 43 concordant with its reduction of diterpene degradation. Pine weevils reared on an
 44 artificial diet spiked with diterpenes, but without antibiotics, were found to lay more
 45 eggs with a higher hatching rate than weevils raised on diets with antibiotics or
 46 without diterpenes. These results suggest that gut symbionts contribute towards host
 47 fitness, but not by detoxification of diterpenes, since these compounds do not show
 48 toxic effects with or without antibiotics. Rather the ability to thrive in a terpene rich
 49 environment appears to allow gut microbes to benefit the weevil in other ways, such
 50 as increasing the nutritional properties of their diet.

51

52

53 **Introduction**

54 The interactions between plants and insects are often mediated by plant secondary
 55 metabolites. In addition to acting as feeding attractants, these molecules can also be
 56 involved in plant defense acting as feeding deterrents or toxins that disrupt gut
 57 membranes, impede digestion, hinder normal metabolism, or block ion and nutrient
 58 transport, among other effects (Mithoefer and Boland 2012). Herbivores have, in
 59 turn, evolved different mechanisms to overcome the noxious effects of plant
 60 defenses (Hammer and Bowers 2015). These involve modification of feeding
 61 behavior to avoid ingesting high amounts, manipulation of plant defenses to lower
 62 their concentrations, increased excretion rates, sequestration away from sensitive
 63 processes, target site insensitivity and metabolic degradation (Després et al. 2007).

64 In addition, insects do not face the threat of plant defenses alone, but together with
 65 their gut microbes (Hammer and Bowers 2015). Symbiotic microorganisms have
 66 repeatedly been demonstrated to influence interactions between plants and
 67 herbivores by supplementing essential nutrients or degrading complex dietary
 68 polymers (Douglas 2009). Recently, these microbial functions have been expanded
 69 to encompass the manipulation (Chung et al. 2013) and degradation of plant
 70 secondary metabolites (Hammerbacher et al. 2013, De Fine Licht et al. 2012 Kohl et
 71 al. 2014, Ceja-Navarro et al. 2015, Welte et al. 2015). However, gut microbes are
 72 also susceptible to some plant toxins (Bakkali et al. 2008) and might even be their
 73 primary target (Mithoefer and Boland 2012).

74 Many herbivores exploit conifers as a food source. Feeding on conifer tissues is not
 75 only problematic nutritionally due to their low concentrations of nitrogen, phosphorus,
 76 vitamins and sterols, but conifers typically also contain high concentrations of
 77 defensive phenolic compounds and terpenoid resins composed mainly of
 78 monoterpene olefins and diterpene resin acids (Keeling and Bohlmann 2006). These

79 defenses are constitutively expressed and can also be induced upon herbivory. With
 80 more than 50,000 compounds, terpenes are the most diverse family of plant
 81 defenses described to date (Conolly and Hill 1991). Their protective effects include
 82 antimicrobial properties (Rastogi et al. 1998, Lunde et al. 2000) as well as feeding
 83 deterrence and toxicity against insects, and thus might be involved in conifer
 84 resistance against herbivores. Although the exact mode of terpene action is unknown
 85 in most cases (Gershenzon and Dudareva 2007), they seem to derive some of their
 86 toxic properties from the disruption of gut membranes due to their lipophilic nature or
 87 by causing neural damage through compromised ion channels (Keeling and
 88 Bohlmann 2006).

89
 90 Although many insects are able to perform well in the presence of low terpene
 91 concentrations, higher amounts can act as deterrents or growth inhibitors (Zhao et al.
 92 2011). A number of terpenes have been associated with toxicity to conifer-feeding
 93 insects (Cook and Hain 1988, Raffa and Smalley 1995, Werner 1995). For example,
 94 monoterpenes and diterpenes are correlated with white and Sitka spruce resistance
 95 against the white pine weevil (*Pissodes strobi*) (Harris et al. 1983, Tomlin et al. 1996,
 96 2000) and diterpenes are correlated with Jack pine resistance against sawflies (Ikeda
 97 et al. 1977). Likewise, the Douglas fir pitch moth (*Synanthedon novaroensis*) is more
 98 successful attacking lodgepole pines containing low amounts of the monoterpene
 99 delta-3-carene (Rocchini et al. 2000). Similarly, the application of methyl jasmonate
 100 on seedlings, which increases chemical defenses including terpenes in many conifer
 101 species (Martin et al. 2002, Heijari et al. 2005, Schmidt et al. 2005, Moreira et al.
 102 2009) correlates with higher resistance against the pine weevil (Heijari et al. 2005,
 103 Erbilgin et al. 2006, Sampedro et al. 2011).

104
 105 Some environmental bacteria are known to degrade terpenes. For instance,
 106 *Pseudomonas abietaniphila* BKME-9 (Martin et al. 2000) and *Burkholderia*

107 *xenovorans* (Smith et al. 2007) have been reported to degrade diterpene resin acids.
 108 However, the ability to degrade and utilize terpenes is not limited to environmental
 109 microorganisms, but also occurs in symbiotic microbes associated with vertebrates
 110 and invertebrates. Goat rumen-associated bacteria can degrade several
 111 monoterpenes (Malecky et al. 2012), and some members of the gut community of
 112 bark beetles are capable of *in vitro* degradation of monoterpenes and diterpenes
 113 (Boone et al. 2013, Xu et al. 2015). However, whether bacterial degradation of
 114 terpenes occurs within the insects as well has not been explored.

115

116 The pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae: Molytinae), feeds on
 117 bark and phloem of several conifer species. It is considered the most damaging
 118 conifer pest in managed forests in Europe (Leather et al. 1999, Nordlander et al.
 119 2011) given its devastating impact on newly planted seedlings (Petersson and
 120 Orlander 2003). While adults feed both above and below ground, larvae complete
 121 their development underground tunneling in the bark of stump roots (Nordlander et
 122 al. 2005, Wallertz et al. 2006). Therefore, pine weevils encounter high concentrations
 123 of resin terpenes throughout their life cycle. Despite many reports on the noxious
 124 effects of terpenoids on herbivores, the pine weevil seems able to cope well with
 125 these compounds. Weevils feeding on Sitka spruce show a positive correlation
 126 between adult feeding, larval development and high concentrations of both
 127 carbohydrates and resin (Langström and Day 2004), suggesting that the pine weevil
 128 is adapted to a wide range of terpene concentration. However, high resin content
 129 often deters them from feeding in Scots pine (Ericsson et al. 1988). At present, it is
 130 not known what mechanisms pine weevils employ to circumvent these compounds,
 131 and whether they do so on their own or through association with symbiotic
 132 microorganisms.

133 Here we investigate whether the microbial associates of the pine weevil can play a
 134 role in overcoming conifer defenses. The gut microbiota of the pine weevil is
 135 geographically stable across Europe, especially within the most abundant bacterial
 136 family, the Enterobacteriaceae (Berasategui et al. 2016). The core microbiota of this
 137 insect consists of members of the genera *Erwinia*, *Rahnella*, and *Serratia*. This
 138 community assembly seems to be shaped, at least in part, by the nutritional resource
 139 these insects exploit (i.e. conifer bark and cambium). It extends beyond the pine
 140 weevil, being similar in other conifer feeding beetles, including several species of the
 141 genus *Dendroctonus* and *Ips pini* (Cardoza et al. 2006, Adams et al. 2013, Hu et al.
 142 2014, Berasategui et al. 2016, Dohet et al. 2016) as well as wood-feeding wasps
 143 (Adams et al. 2011), but it is absent in weevils feeding on non-conifer food sources
 144 such as crops or ornamental plants (Berasategui et al. 2016). Among the different
 145 members of this conserved community, species of the genera *Pseudomonas*,
 146 *Rahnella* and *Serratia* have been described based on metagenomics data (Adams et
 147 al. 2013) or phylogenetic inference (Berasategui et al. 2016) to contain many genes
 148 involved in diterpene degradation.

149
 150 In this communication, we follow up on our broad survey of the gut community in the
 151 pine weevil and ask whether this consortium of microbes is involved in conferring
 152 resistance against plant terpenoid defenses encountered by their insect host. After
 153 first finding that diterpene resin acids are reduced during passage through the pine
 154 weevil, we manipulated the gut microbiota using antibiotics to determine if microbes
 155 are involved in degradation. We also used cultured pine weevil microbes to establish
 156 their diterpene degradative capacity outside the insect. Additionally, we sequenced
 157 the bacterial metagenome of individuals feeding on different diets in order to explore
 158 the taxonomic changes occurring under antibiotic treatment and whether the
 159 bacterial community contains the aforementioned diterpene-degrading *dit*-gene

cluster. Finally, we performed bioassays to assess the effect of both diterpenes and gut microbes on the fitness of the insect host.

Results

Diterpene levels after passage through weevils

We first determined whether diterpene resin acids were degraded during transit through the pine weevil gut. Insects were fed on Norway spruce branches overnight and their feces were collected after 24 hours. The diterpenes in both food and feces were identified and quantified by gas chromatography-mass spectrometry (GC-MS) of the methyl ester derivatives. Our results show that total diterpene content was reduced by 80% in feces relative to ingested material (Fig. 1A. All individual diterpenes detected were reduced by more than 50%, except dehydroabietic acid (DHAA), which was reduced by only 7% (Fig. 1B). These results demonstrate substantial diterpene degradation during gut passage.

Figure 1. Amounts of (A) total and (B) individual diterpenes ingested by *H. abietis* feeding on Norway spruce bark (62 insects in 16 hours) compared to the amounts in their feces after digestion. The diterpene content of bark and feces samples was analyzed by GC-MS. The amount of bark ingested was determined by weighing twigs offered to the insect before and after the feeding period. Experiment was repeated twice with similar results.

181

182 **Diterpene degradation by bacteria in weevils**

183 To test whether microorganisms mediate weevil degradation of the diterpene resin
 184 acids observed after passage through the pine weevil gut, we manipulated the gut
 185 microbes through the application of the broad-spectrum antibiotic rifampicin to a
 186 semi-artificial diet, and subsequently assessed the diterpene content in the feces of
 187 the experimental animals by GC-MS. We observed an increase in the amounts of all
 188 the major diterpenes detected in the feces of antibiotic-treated individuals relative to
 189 the untreated control group (Fig. 2) ($P=0.001$ in all cases). Reinfection of the gut with
 190 the native community by supplementing the diet with a gut suspension of untreated
 191 individuals into the diet of antibiotic-treated insects rescued the insect's
 192 biodegradative capacity, suggesting that gut microbes are responsible for the
 193 breakdown of such compounds within the host. This re-acquired degradation
 194 capacity resulted in the complete elimination of all diterpenes.

195

196

197

198 **Figure 2.** Amount (µg) of major diterpenes in feces of pine weevils feeding on different diets.
199 Color of boxes signifies the experimental treatment: Control, semi-artificial diet with ground
200 Norway spruce for 14 days; Antibiotic, diet amended with rifampicin for 14 days, Reinfected,
201 after being fed with an antibiotic diet for 7 days, these insects were fed diet amended with a
202 weevil gut suspension for 7 days; Lines represent medians, boxes comprise the 25–75th
203 percentiles, and whiskers denote the range.

204

205 Diterpene degradation by cultured weevil microbes

206 To test whether the gut community of *H. abietis* can degrade terpenes, we isolated
207 bacteria from the guts of the weevil and cultivated them in LB medium overnight.
208 Bacteria were inoculated into medium amended with DHAA (sodium salt) at a
209 concentration of 500 µg/ml, which is in the range of that found in spruce tissue. This
210 compound was chosen because of its commercial availability in high purity and
211 comparatively greater stability in solution, as compared to other diterpene acids from
212 Norway spruce. Sterile, uninoculated medium amended with DHAA served as the
213 control. Although no significant differences in concentration were detected after 1 day
214 of treatment (ANOVA, $P=0.98$), we observed a significant reduction in the amount of

215 DHAA in the presence of bacteria compared to controls at five days (ANOVA,
216 $P=0.03$) (Fig. 3).

217

218 **Figure 3.** Degradation of the sodium salt of dehydroabiatic acid (DHAA) by cultured bacteria
219 isolated from *H. abietis* after 1 and 5 days of growth in LB medium. The initial concentration of
220 DHAA was 14.8µg/mL. Lines represent medians, boxes comprise the 25–75 percentiles, and
221 whiskers denote the range. (ANOVA, $P=0.03$).
222

223 Metagenomic insights into diterpene degradation

224 To characterize the genetic basis of bacterial-mediated diterpene degradation in *H.*
225 *abietis*, we sequenced the bacterial metagenome of weevils feeding on their natural
226 food source (Norway spruce) compared to weevils feeding on an artificial diet, as
227 well as an artificial diet supplemented with antibiotics. Each library contained on
228 average 15.3 million base pairs (Table S1), and assemblies resulted in an average of
229 49.983 contigs per library.

230

231 Of the contigs from the metagenome assembly of weevils fed on spruce, 72.1% were
232 assigned to a bacterial origin, and 27.5% to an eukaryotic origin (Table S2).
233 Consistent with our previous 16S rRNA-based survey of the insect's microbiome
234 (Berasategui et al. 2016), community profiling using protein-coding genes revealed
235 the gut microbiota of weevils feeding on a coniferous diet to be dominated by

236 gammaproteobacterial associates that could be assigned to various
 237 Enterobacteriaceae genera (91.3%; Fig. 4A) including *Erwinia*, *Rahnella* and *Serratia*
 238 (Fig. 4B). All genera that appear in more than 1% abundance belonged to the
 239 Enterobacteriaceae family, and nine out of fourteen were previously reported to be
 240 present in the pine weevil's community (Berasategui et al. 2016).

241

242 The gut bacterial community of weevils reared on the artificial diet was qualitatively
 243 very similar to that fed on spruce (Fig. 4A). The Enterobacteriaceae was the most
 244 abundant family (87.9%) followed by the Streptococcaceae and Clostridiaceae
 245 families (1.7% in total) (Fig. 4A). Additionally, all Enterobacteriaceae genera present
 246 in more than 1% abundance in spruce-fed weevils were present in artificial diet-fed
 247 insects, with only the addition of *Streptococcus*. However, supplementing antibiotics
 248 into the artificial diet altered the microbiota, rendering it much more diverse (Fig. 4A),
 249 with five families having abundances higher than 1%. In this group, the
 250 Xanthomonadaceae family is present in the highest level (45.5%), followed by the
 251 Enterobacteriaceae (31.5%), Streptococcaceae (3.29%), Clostridiaceae (0.54%),
 252 Anaplasmaeae (specifically *Wolbachia*) (2.5%) and others (16.65%). The
 253 Enterobacteriaceae family is the most susceptible to antibiotic treatment, suffering a
 254 drastic reduction in overall abundance compared to insects reared on their natural
 255 diet or an artificial diet devoid of antibiotics (Fig. 4B).

Figure 4. A) Family classification of the bacterial metagenome of pine weevils fed on Norway spruce, artificial diet, and artificial diet with antibiotics, respectively. B) Abundance of different genera in the gut microbiota of weevils feeding on the three diets. Grey dots depict taxa previously found in the gut microbiota of the pine weevil (Berasategui et al. 2016). The dashed box indicates members of the Enterobacteriaceae family.

In order to explore the genetic basis of symbiont-mediated diterpene degradation in the pine weevil, the three bacterial metagenomes sequenced in this study were screened for the presence of a gene cluster (*dit*) predicted to be involved in the degradation of diterpenes in *Pseudomonas abietaniphila* BKME-9 (Adams et al, 2013, Martin and Mohn 2000, Smith et al. 2007, 2008). Genes encoding enzymes that have been previously implicated in diterpene degradation were identified using BLASTn. The metagenome of beetles feeding on Norway spruce contained 10 out of 19 *dit* genes (Fig. 5), and the same was true for beetles reared on the artificial diet, consistent with the minimal changes observed in the composition of the microbial

community. However, supplementing antibiotics into the artificial diet led to a near complete loss of *dit*-genes in addition to disrupting the composition of the bacterial associates of *H. abietis* (Fig. 5).

275

276

277 **Figure 5.** Diterpene gene cluster (Martin and Mohn, 2000) present in the metagenome of pine
278 weevils feeding on different diets: Norway spruce, artificial diet, and artificial diet amended
279 with antibiotics. Coloring: turquoise, present; grey, absent.
280

281 **Effect of diterpenes and gut bacteria on weevil fitness**

282 In order to assess the effect that diterpenes and microbially-mediated diterpene
283 degradation might have on pine weevil fitness, we measured survival and fecundity
284 of weevils feeding on different types of artificial diet. Insects were fed on diet with a
285 natural mixture of diterpene acids, with the antibiotic rifampicin, with both diterpenes
286 and antibiotic, and without any supplementation.

287 We observed no difference in survival rates depending on treatment (Mantel-Cox
288 $P=0.18$; Breslow $P=0.27$; Tarone-Ware $P=0.19$) or sex (Mantel-Cox $P=0.56$; Breslow
289 $P=0.67$; Tarone-Ware $P=0.66$) after 30 days (Fig. 6A). However, we observed
290 differences between treatments in relation to the number of eggs laid (Fig. 6B).
291 Individuals that fed on diet with diterpenes only (no antibiotic added) laid more eggs
292 than individuals in any of the other three groups ($P=0.05$). Likewise, hatching rates of

eggs laid by diterpene-fed mothers with their native microbiota were significantly higher than in any of the three other groups ($P=0.01$, Fig. 6C).

Figure 6. Fitness parameters of pine weevils fed on artificial diet containing no additives (black), 0.3% (w/v) of the antibiotic rifampicin (red), 3% (w/w) of a natural diterpene mixture (grey), and the antibiotic plus diterpenes (dashed). In the box plots, lines represent medians, boxes comprise the 25–75 percentiles, and whiskers denote the range. (a) Survival: treatment (Mantel-Cox $P=0.18$; Breslow $P=0.27$; Tarone-Ware $P=0.19$); sex (Mantel-Cox $P=0.56$; Breslow $P=0.67$; Tarone-Ware $P=0.66$). (b) Number of eggs laid (ANOVA, $P=0.05$) and (c) egg hatching rate (ANOVA, $P=0.01$).

306 **Discussion**

307 Microorganisms associated with herbivorous insects have been shown to mediate
 308 the outcome of insect-plant interactions across a number of insect groups in different
 309 ways (Tsuchida et al. 2004, Hosokawa et al. 2007). For example, microorganisms
 310 can aid in the exploitation of plant resources by supplementation of essential
 311 nutrients, degradation of complex structural metabolites (Douglas 2009) and
 312 manipulating (Chung et al. 2013) or overcoming (Hammer and Bowers 2015) plant
 313 defenses. Microbial symbionts of herbivorous animals have long been suspected to
 314 aid in the detoxification of plant secondary metabolites and thereby contribute to host
 315 fitness, but direct experimental evidence remains scarce (but see Kohl et al. 2014,
 316 Ceja-Navarro et al. 2015). Here we show that diterpene resin acids, a group of plant
 317 defense compounds abundant in the bark of conifer trees, are degraded in the pine
 318 weevil after ingestion. Gut microbes of the pine weevil contain genes encoding
 319 enzymes that catalyze the degradation of diterpene acids. These microbes were
 320 found to degrade diterpenes in the insect and when cultured separately on medium
 321 containing natural concentrations of one diterpene acid. Diterpene supplementation
 322 of the pine weevil diet in the presence of the native gut microbiota enhances the
 323 weevil's fecundity and egg hatching rate.

324

325 **Diterpene-degrading genes in the pine weevil gut bacterial metagenome nearly** 326 **eliminated by antibiotic treatment**

327 In agreement with our previous report using 16S rRNA profiling (Berasategui et al.
 328 2016), taxonomic classification of the gut bacterial metagenome of the pine weevil
 329 revealed a community dominated by the Enterobacteriaceae family. Specifically,
 330 members of *Erwinia*, *Pantoea*, *Serratia*, and *Yersinia*, encompassed 90% of the
 331 whole microbiota. Although we detected *Wolbachia* species in all three
 332 metagenomes sequenced, their abundance was markedly lower than that previously

333 reported using 16S rRNA data (Berasategui et al. 2016). While differences in
 334 *Wolbachia* titers within a single insect population have been previously described
 335 (Müller et al. 2013), it is possible that variation in this case arises from bias in the
 336 PCR reaction required for 16S pyrosequencing, which is not required for
 337 metagenome sequencing. The very low abundance of Firmicutes in the present study
 338 is consistent with that of weevils previously collected in the same location (Spain)
 339 which were devoid of this bacterial family (Berasategui et al. 2016). However,
 340 members of the Enterobacteriaceae were as dominant as in the previous study
 341 supporting their role as stable and conserved constituents of the pine weevil gut
 342 microbiota in contrast to the variability affecting other members of the community.
 343 The conserved fraction of the pine weevil microbiota overlaps with that of other
 344 conifer-feeding beetles as well as a conifer-feeding wasp (Adams et al. 2011)
 345 suggesting that this is shaped by a shared environment and may be adaptively
 346 significant (Berasategui et al. 2016).

347 The pine weevil microbial community remained unaltered after two weeks of
 348 beetles feeding on an artificial diet (Fig. 4A), indicating that it is also resilient to
 349 changes in diet. However, the addition of antibiotics did alter community composition,
 350 reducing the relative abundance of most Enterobacteriales (with the exception of
 351 *Enterobacter* sp., *Escherichia* sp., and the bacteriome-localized endosymbionts), and
 352 increasing that of *Stenotrophomonas* sp., *Xanthomonas* sp., and *Wolbachia* (Fig.
 353 4B).

354 Our previous inference of metagenomic functions via PICRUSt (Berasategui
 355 et al. 2016) suggested the enrichment of a gene cluster known as *dit* among *H.*
 356 *abietis*' bacterial associates that is potentially involved in diterpene degradation
 357 based on studies of free-living bacteria (Adams et al. 2013, Martin et al. 2000, Smith
 358 et al. 2007, 2008). Functional annotation of the bacterial metagenome in the present
 359 study revealed the presence of ten out of the 19 known *dit* genes in weevils feeding
 360 on their natural food source as well as on an artificial diet (Fig. 5). However, the

supplementation of antibiotics in the artificial diet resulted in the loss of all but one *dit* gene (Fig. 5).

Even with only 10 of 19 reported *dit* genes, the members of the *H. abietis* microbial community may still be able to degrade diterpene resin acids. Not all 19 genes are required for bacterial catabolism of diterpenoids by *Pseudomonas abietaniphila* BKME-9 (Martin and Mohn 2000, Smith et al. 2004, 2007). For example, knocking out *ditR* does not impair the growth of this *Pseudomonas* strain on diterpene-rich media (Martin and Mohn 2000). Conversely, knocking out *ditQ*, restricts growth of *P. abietaniphila* on dehydroabietic acid but not on abietic acid (Smith et al. 2004). Moreover, *ditI* and *ditH*, both reported as essential genes for diterpene degradation, were found to be present in our metagenomic survey of the *H. abietis*' gut bacterial community (Fig. 5). Parallel to previous findings (Adams et al. 2013), phylogenetic binning of *dit*-gene sequences annotated within our metagenomes revealed that most of the sequences belonged to taxa from the Enterobacteriaceae, strongly supporting the involvement of this bacterial family in the degradation of diterpenes within *H. abietis*' gut. Despite also belonging to the Enterobacteriaceae, the primary endosymbiont of pine weevils, *Nardonella* sp. (Conord et al. 2008), is not likely to play a major role in this degradation given that it is not affected by antibiotic treatment (Fig. 4B), and thus is not responsible for either the differential occurrence of *dit* genes or the reduction in terpene concentration observed in this study.

Pine weevil gut microbes degrade diterpenes in the insect and in isolated cultures

The possibility of microbial degradation of diterpenes in the pine weevil was first suggested by our measurements showing an 83% decrease in total diterpene content between the spruce bark ingested by the weevil and the frass. To determine if the microbiota degrades diterpenes inside the insect, we manipulated the gut

community through the addition of antibiotics to the insect diet. Our results indicated that the degradation of diterpenes decreased upon addition of antibiotics (Fig. 2), and these capabilities were rescued in antibiotic-treated insects upon supplementing their native bacterial community through the diet. We then tested the ability of isolated weevil microbes to degrade dehydroabietic acid (DHAA) in solution at concentrations found in spruce bark, and found a significant decline (Fig. 3). Thus experiments with both weevils and isolated bacterial illustrate the contribution of gut bacteria towards diterpene breakdown. The correlation of reduced breakdown rates with a reduction in the expression of diterpene catabolizing genes under antibiotic treatment is another line of evidence linking the pine weevil gut bacterial community to diterpene degradation.

Symbiotic bacteria have been previously demonstrated to breakdown conifer resin terpenes. For example, close relatives of *Serratia*, *Rahnella* (both Enterobacteriales), *Pseudomonas*, and *Brevundimonas* isolated from the gut of bark beetles (*Dendroctonus ponderosae* and *D. valens*) were found to degrade monoterpenes and diterpenes (Boone et al. 2013, Xu et al. 2015). Free-living microbes isolated from conifer pulp mill wastewater and forest soil, such as *Pseudomonas abietaniphila* BKME-9 (from which the *dit* gene cluster was reported) and *Burkholderia xenovorans* LB400, are also described to degrade diterpenes (Smith et al. 2008). These bacteria are also able to utilize diterpenes as their sole carbon source (Martin and Mohn 2000, Morgan and Wyndhan 2002, Smith et al. 2007).

The degradation of plant secondary metabolites by symbiotic bacteria is not limited to terpenes, nor to insects. For instance, members of the gut community of the cabbage root fly (*Delia radicum*) harbor a plasmid (*saxA*) involved in metabolizing the isothiocyanates of the host plant (Welte et al. 2016). The coffee bean borer (*Hypotenemus hampei*) relies on at least *Pseudomonas fulva*, a member of its gut bacterial community, to degrade the alkaloid caffeine, thereby increasing the insect's

fitness (Ceja-Navarro et al. 2015). Likewise, desert woodrats (*Neotoma lepida*) harbor a gut microbial assembly that allow their hosts to exploit the toxic creosote bush (*Larrea tridentata*) through the degradation of phenolic compounds (Kohl et al. 2014, 2016). In addition to bacteria, symbiotic fungi can also degrade plant secondary metabolites for their hosts. *Lasioderma serricorne*, the cigarette beetle harbors a symbiotic yeast (*Symbiotaphrina kochii*) in its digestive system that is able to degrade several plant toxins and use them as sole carbon sources (Dowd and Shen 1990, Shen and Dowd 1991). Similarly, the phenolics that leaf cutter ants (*Acromyrmex echinator*) encounter in leaves, are detoxified by a laccase produced by the ant fungal cultivar, *Leucocoprinus gongylophorus* (De Fine Licht et al. 2012).

Gut bacteria increase pine weevil fitness, but not by direct detoxification of diterpenes

To assess the impact of gut bacteria on the pine weevil performance, we measured several fitness parameters on weevils feeding on diets with and without the diterpenes and with and without the broad-spectrum antibiotic rifampicin. We observed a fitness benefit for the weevil as evidenced by higher fecundity and hatching rate only when insects fed on diterpene-containing diet with a full spectrum of bacteria (without antibiotics) (Fig. 6B and 6C). Different hypotheses might explain how gut bacteria can enhance host fitness through the metabolism of terpenes. For example, removal of diterpenes could reduce the levels of compounds toxic to the weevils, but our bioassays show that insects that lack their full microbial complement do not suffer higher adult mortality when feeding on diterpene-containing diet than insects with all of their native microbes, suggesting that insects have an intrinsic mechanism of circumventing diterpenoid toxins. Microbes could conceivably assist insects in degrading terpene toxins (Raffa 2014), but such a contribution does not seem important in the case of *H. abietis*.

Another explanation for the fitness benefits of microbes is that they could improve the nutritional properties of the pine weevil diet allowing females to allocate more resources to egg production (Wainhouse et al. 2001). Degraded terpenes might be directly employed as substrates for insect respiration. Or, if terpenes are readily used as respiratory substrates by microbes (DiGuistini et al. 2011, Wang et al. 2014), they could enhance microbial growth and so increase the breakdown of refractory carbohydrate polymers or increase supplies of nitrogenous compounds, vitamins or sterols (Douglas 2009, McCutcheon and Moran 2007, Salem et al. 2014), all scarce resources in conifer bark and phloem. For example, Morales-Jiménez and colleagues (2009, 2012) demonstrated that members of the gut community of the conifer-feeding bark beetles *D. ponderosae*, (*Rahnella* sp. *Pantoea* sp and *Stenotrophomonas* sp.) and *D. rhizophagous* (*Pseudomonas* sp, *Rahnella* sp. and *Klebsiella* sp.) can fix nitrogen (Morales-Jimenez et al. 2012) while free-living fungal associates of bark beetles can supply *Dendroctonus* beetles with sterols (Bentz and Six, 2006).

Hence for *H. abietis* the fitness benefits of its gut microbes may not be derived directly from terpene degradation. Rather, the ability to degrade terpenes may allow microbes to thrive in a terpene-rich environment and enhance pine weevil growth by providing critical nutrients and vitamins. Given that the taxonomical composition and functional capabilities of the weevil microbial assembly strongly resemble those of other bark beetles, wood-feeding wasps, and sawflies exploiting very similar ecological niches (Berasategui et al. 2016, Adams et al. 2011, 2013, Boone et al. 2013, Whittome et al. 2007), these other insect groups may also accrue such fitness benefits from their gut microbiota.

470 **Material and methods**

471 **Insect collection and maintenance**

472 Adult pine weevils (*Hylobius abietis*) were collected near Neustadt, Lower Saxony
 473 (Germany) and in Galicia (Spain). In Germany weevils were collected by leaving a
 474 recently cut log near a clear cut for some days and manually collecting the insects
 475 that were attracted. In Spain beetles were collected with clean pit-fall traps baited
 476 with α -pinene and ethanol (Nordlander 1987). Vials without lids were filled with
 477 ethanol, and the entrance blocked with bait-impregnated paper towels or cotton and
 478 placed leaning towards a pine branch. Once in the lab, the beetles were stored in
 479 darkness at 10°C in boxes of 50 individuals with moist paper, a container with soil
 480 and Norway spruce (*Picea abies*) twigs. Insects were brought to the lab bench one
 481 week before each experiment for acclimatization.

482 **Semi-artificial and artificial diet preparation**

483 **Semi-artificial diet.** Bark and cambium of Norway spruce branches were manually
 484 removed from the wood and frozen in liquid nitrogen. Needles were removed with a
 485 scalpel and the remaining tissues were homogenized manually with a mortar and
 486 pestle under liquid nitrogen. Agar (1.60 g, Roth, Karlsruhe, Germany) was diluted in
 487 50 mL of water and let cool to ~ 60°C. This mixture was added to 18.75 g of the
 488 homogenized spruce tissue and stirred until homogeneous. The antibiotic containing
 489 semi-artificial diet was prepared by adding the broad spectrum antibiotic rifampicin to
 490 the diet to a final concentration of 0.3% (w/v).

491 **Complete artificial diet.** The diet used for seed-feeding pyrrhocorid bugs described
 492 in Salem et al. (2014) was modified with the addition 6 mL of sunflower seed oil
 493 instead of 20 mL.

494 **Degradation of diterpenes on passage through weevils**

495 Pine weevils (62 individuals) were kept without food in plastic boxes for 4 days and
 496 sprayed with water daily to maintain humidity. After this starvation period, insects
 497 were allowed to feed on Norway spruce twigs *ad libitum*. After 16 hours, insects were
 498 transferred to glass petri dishes and allowed to defecate for 24 hours. Feces were
 499 collected and suspended in 5 ml water. We added the suspended feces to *tert*-butyl
 500 methyl ether (1:5, v/v) in a total of 25 ml and let the mixture shake for 20 hours, after
 501 which 5 ml of 0.1 mM (NH₄)₂CO₃, pH 8.0, were added to the mixture and vortexed
 502 (Schmidt et al. 2011). The ether layer was removed and concentrated to 0.5 ml and
 503 analyzed for diterpenes by GC-MS (see below) alongside the ether fraction of the
 504 bark of Norway spruce twigs prepared in a similar manner. The quantity of bark
 505 ingested by the weevils in the feeding period was determined by weighing the twigs
 506 before and after with a correction for water loss.

507 **Manipulation of the gut microbiota and *in vivo* degradation of diterpenes**

508 To assess the potential role of the gut bacteria in the degradation of terpenoids within
 509 the beetle gut, six weevils (three males and three females) in individual petri dishes
 510 were allocated to each of three treatments: (i) control, (ii) antibiotic, and (iii)
 511 reinfected. Control individuals were fed on control semi-artificial diet for 14 days,
 512 whereas antibiotic-treated individuals were fed on semi-artificial diet amended with
 513 the antibiotic rifampicin for 14 days. Reinfected individuals were fed on the antibiotic
 514 diet for half of the experiment and then switched to a semi-artificial diet amended
 515 with a gut suspension of untreated weevils for the remaining time. We generated this
 516 suspension by crushing the guts of four untreated weevils in 1 ml PBS and vortexing.
 517 Insects were provided with 100 mg of diet every day that was supplemented with 10
 518 µL of gut suspension in the case of the reinfected treatment or PBS in the control.
 519 Feces were collected daily and frozen at -20°C. Feces from the last experimental day

were extracted with 200 μ L *tert*-butyl methyl ether, shaken overnight and prepared for diterpene analysis via GC-MS as described below.

Degradation of diterpenes by cultured gut microbes

Six beetles were dissected under sterile conditions. Individual guts were suspended in a vial of 1 mL of PBS (phosphate-buffered saline). To separate bacteria from the gut walls, tissues were sonicated (50/60 Hz, 117 V, 1.0 Amp) for 30 s, macerated with a pestle and vortexed at medium speed for 10 s. Vials were centrifuged at 3000g to pellet host tissues and the supernatant was filtered using 10 μ m syringe filters.

A 10 μ L quantity of each bacterial suspension was inoculated in 10 mL LB medium and grown overnight shaking at 220 rpm at room temperature. Overnight cultures were diluted to an optical density of 0.1 at 600 nm (OD_{600}) with additional LB medium. To test whether the gut community of the pine weevil is able to degrade diterpenes, we inoculated 10 μ L of the diluted bacterial culture in a vial of 990 μ L LB medium amended with dehydroabietic acid (DHAA) as the sodium salt. In the same way, 10 μ L LB medium was inoculated in control vials of medium amended with DHAA. The experiment was carried out in a 24-well flat bottom plate (Sarstedt, Nümbrecht Germany), replicated six times, and allowed to grow for 5 days. Samples were taken for analysis on the 1st and 5th days. Upon sampling, the content of each well was transferred to Eppendorf tubes and centrifuged at 3000g for 5 min to pellet bacterial cells. The supernatant was then transferred to glass vials for diterpene analysis by HPLC rather than GC-MS to detect the sodium salts directly.

The sodium salt of DHAA was produced by dissolving dehydroabietic acid (Sigma, 1.33 g) in 12 mL MeOH. This mixture was amended with an equimolar amount of $NaHCO_3$ (372 mg) dissolved in 3 mL of water. The mixture was left standing in a closed vial for 14 days at room temperature to allow the reaction to proceed. Subsequently, the solution was filtered with a syringe filter and evaporated

547 to dryness under nitrogen. In order to prepare the experimental medium, 750 mg of
548 DHAA in this form was added to 1.5 L of LB medium. After autoclaving (121°C, 20
549 min), the solution was vacuum-filtered to remove un-dissolved particles.

550 **DNA extraction and Illumina-based metagenome sequencing**

551 For one week, a group of six weevils were reared on one of three different diets: (1)
552 Norway spruce (*Picea abies*) twigs, (2) artificial diet (AD), or (3) artificial diet
553 amended with the antibiotic rifampicin at 0.3% (w/v). Through dissection, the midgut
554 region was harvested from every individual using sterile forceps and iris scissors.
555 Once dissected, DNA was extracted from the midgut using the Microbiome Kit
556 (Qiagen, Hilden, Germany) following the manufacturer's instructions. Equimolar
557 concentrations of samples from the same treatment were pooled, resulting in a single
558 sample per treatment. Sequencing of a 150-bp library was conducted for each
559 treatment using an Illumina Genome Analyzer IIx at the Genome Center of the Max
560 Planck Institute in Cologne (Germany). The assembly was generated with Meta-
561 Velvet v1.0.19 (Namiki et al. 2012) based on ~40 million quality-filtered read pairs
562 and subjected to a gap-closing analysis. For annotation of gene content as well as
563 taxonomic assignment, we used the Meta Genome Rapid Annotation using
564 Subsystem Technology (MG-RAST) (Meyer et al. 2008).

565 We determined the community profile in the metagenomic dataset using the
566 "Best Hit Classification" tool of MG-RAST to classify CDSs on the basis of best
567 BLASTP hits with a minimum identity cutoff of 60%, and a maximum e-value cutoff of
568 10^{-5} . Sequences were classified at the genus level (95% identity), and taxa with <1%
569 abundance were removed. To examine the enrichment patterns of genes encoding
570 diterpene degradation genes across the different treatments, we performed BLASTP
571 against a custom data set of proteins belonging to the *dit* diterpene acid degradation
572 pathway of *Pseudomonas abietaniphila* BKME-9, as previously described by Adams
573 et al. (2013).

574 **Fitness assays**

575 Weevils (80 male and 80 female) were randomly distributed as pairs in plastic boxes.
 576 Each box contained one piece of moisture paper and an Eppendorf tube lid as
 577 container for artificial diet prepared as described above. Each box was randomly
 578 assigned to one of four treatments: (i) diet without additions, (ii) diet with the
 579 antibiotic rifampicin at 0.3% (w/v), (iii) diet with a natural mixture of diterpene acids
 580 and (iv) diet with both antibiotic and diterpene acids. The diterpene acids were from a
 581 natural conifer mixture, sold as “gum rosin” (Sigma), which was dissolved in
 582 methanol, added to the diet as 3% (w/w), and the methanol allowed to fully evaporate
 583 before using. Weevils were subjected to the treatments for one month. As fitness
 584 parameters, survival, number of eggs laid and number of eggs hatched were
 585 recorded every 24 hours.

586 **Statistics**

587 ***In vitro* assays.** Differences in the concentration of terpenes in liquid cultures were
 588 assessed in SPSS with an ANOVA.

589 ***In vivo* assays.** Differences in the amount of terpenes in the feces of weevils were
 590 analyzed using a Kruskal–Wallis test.

591 **Fitness assays.** The number of eggs laid and the hatching rate were analyzed in R
 592 with a generalized linear model. Survival was analyzed in SPSS using three different
 593 statistical tests: Mantel-Cox, Breslow, and Tarone-Ware.

594

595 **Chemical analyses**

596 **GC-MS analysis of diterpene resin acids**

597 Diterpene resin acids of Norway spruce bark and pine weevil feces were analyzed as
 598 described in Schmidt et al. (2011). In brief, *tert*-butyl methyl ether extracts that had
 599 been washed with (NH₄)₂CO₃ to remove small organic acids were methylated with N-

600 trimethylsulfonium hydroxide in methanol and concentrated under nitrogen.
601 Separation was accomplished on a polyethylene glycol stationary phase by split
602 injection. Compounds were identified by comparison with methyl esters produced
603 from authentic diterpene acid standards and quantified with an internal standard of
604 methylated dichlorodehydroabietic acid.

605 **HPLC analysis of diterpene resin acids (sodium salts)**

606 Samples were analyzed on an HPLC (1100 series equipment, Agilent Technologies,
607 Santa Clara, CA, USA), coupled to a photodiode array detector (Agilent
608 Technologies). Separation was accomplished on a Nucleodur Sphinx RP column
609 (250 x 4.6 mm, 5 µm particle size, Macherey-Nagel, Düren, Germany). The mobile
610 phase consisted of 0.2% formic acid in water (A) and acetonitrile (B) with a flow of 1
611 ml per minute with the following gradient: start 20% B, 0-20 min 20-100% B, 20-23
612 min 100% B, 23.1-28 min 20% B. For diterpene quantification, peaks were integrated
613 at 200 nm and an external standard curve with an authentic standard of DHAA
614 (Sigma, Taufkirchen, Germany) was created.

615

616 **Conflict of interests**

617 The authors declare no conflict of interests.

618

619 **Author Contributions**

620 AB carried out the microbiological and chemical lab work, performed the data and
621 statistical analyses, and wrote the manuscript; HS carried out the molecular work and
622 metagenome analysis; CP synthesized the DHAA salt; VMS aided in chemical lab
623 work; AS provided reagents; AB, AS, MK, JG and conceived of the study. All authors
624 gave critical comments on the manuscript and gave final approval for publication.

625

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887 **Data Accessibility**

888 Accession numbers will be provided upon acceptance.