

1 **Ancient symbiosis confers desiccation resistance to stored grain pest beetles**

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14 **Abstract**

15 Microbial symbionts of insects provide a range of ecological traits to their hosts that are beneficial in the  
16 context of biotic interactions. However, little is known about insect symbiont-mediated adaptation to the  
17 abiotic environment, e.g. temperature and humidity. Here we report on an ancient (~400 Mya) clade of  
18 intracellular, bacteriome-located Bacteroidetes symbionts that are associated with grain and wood pest  
19 beetles of the phylogenetically distant families Silvanidae and Bostrichidae. In the saw-toothed grain beetle  
20 *Oryzaephilus surinamensis*, we demonstrate that the symbionts affect cuticle thickness, melanization and  
21 hydrocarbon profile, enhancing desiccation resistance and thereby strongly improving fitness under dry  
22 conditions. Together with earlier observations on symbiont contributions to cuticle biosynthesis in weevils,  
23 our findings indicate that convergent acquisitions of bacterial mutualists represented key adaptations  
24 enabling diverse pest beetle groups to survive and proliferate under the low ambient humidities that  
25 characterize dry grain storage facilities.

26

27 **Introduction**

28 Microbial mutualists are a major driving force of evolution (Klepzig et al., 2009), as they confer a variety of  
29 ecological benefits to their host (Feldhaar, 2011, Oliver and Martinez, 2014). In insects, numerous studies  
30 yielded evidence for symbiont-provided benefits in the context of biotic interactions, particularly through the  
31 supplementation, degradation, or detoxification of the diet (Moran, 2006, Douglas, 2009, van den Bosch and  
32 Welte, 2017) or by defending the host against natural enemies (Florez et al., 2015). However, comparatively  
33 little is known about symbiont-mediated adaptations to the abiotic environment. Notably, several studies  
34 reported on symbionts that enhance resistance of insects to high temperatures (Russell and Moran, 2006,  
35 Montllor et al., 2002, Brumin et al., 2011). In most of these cases, however, facultative secondary symbionts  
36 ameliorate heat susceptibility of primary obligate symbionts rather than directly altering the host physiology  
37 and thereby extending the viable temperature range (Wernegreen, 2012, Corbin et al., 2017). Nevertheless,  
38 under selective conditions for heat resistance, during hot summers in desert sites, the abundance of  
39 protective, secondary symbionts was indeed found to increase in pea aphids, presumably reflecting  
40 adaptation to higher temperatures (Harmon et al., 2009).

41 Stored grain pest insects profit from an excess of food but face the challenge of low environmental humidity  
42 that is maintained in storage facilities to prevent the growth of mould fungi (Hagstrum et al., 1996). Several  
43 groups of beetles independently managed to invade the same ecological niche of stored grain and dried plant  
44 products despite the considerably lower humidity compared to the ancestral habitat associated with a

45 fungivorous or saprophagous state of living under bark (Crowson, 1981, Hunt et al., 2007). Most of these  
46 groups were described to harbor facultative intracellular bacterial symbionts. Weevils of the genus *Sitophilus*  
47 engage in a symbiosis with the  $\gamma$ -proteobacterium *Sodalis pierantonius*, and the silvanid saw-toothed grain  
48 beetle *Oryzaephilus surinamensis* and several bostrichid beetles are associated with as yet unidentified  
49 symbionts (Koch, 1931, Mansour, 1934, Koch, 1936a, Buchner, 1965, Nardon and Grenier, 1988, Heddi et al.,  
50 1999, Kleespies et al., 2001). In all cases, the symbionts can be experimentally depleted or removed without  
51 disrupting the hosts' life cycle in laboratory settings, and some populations of *O. surinamensis* were even  
52 found to contain aposymbiotic individuals in the field (Koch, 1936b, Huger, 1956, Vigneron et al., 2014).  
53 However, *S. pierantonius* was shown to provide precursors for cuticle biosynthesis that are especially  
54 important during beetle development, and symbiont cells are actively degraded in adults (Vigneron et al.,  
55 2014). Symbiont-free (aposymbiotic) weevil populations suffer from a lower growth rate due to higher  
56 mortality and reduced fecundity, but are viable and able to reproduce (Nardon and Grenier, 1988). The  
57 symbionts of both *O. surinamensis* and *Rhizopertha dominica* are likewise not obligate, and past studies were  
58 unable to establish any evidence for a physiological or ecological benefit of their presence (Koch, 1956, Huger,  
59 1956).

60 Here, we investigate the hypothesis that engaging in mutualistic associations with bacteria represents a  
61 (pre)adaptation in several beetle families to exploit stored grain products as a food source. To that end, we  
62 characterized the intracellular symbionts of five bostrichid and three silvanid species of grain and wood pest  
63 beetles, revealing a shared and ancient symbiosis related to the intracellular Bacteroidetes symbionts of  
64 cicadas (*Sulcia*), cockroaches and termites (*Blattabacterium*). For the saw-toothed grain beetle  
65 *O. surinamensis*, experimental symbiont elimination resulted in reduced cuticle melanization and thickness as  
66 well as increased cuticular hydrocarbon biosynthesis upon drought stress. Concordantly, aposymbiotic  
67 beetles suffered from lower population growth rates and were more susceptible to desiccation and drought-  
68 inflicted mortality, indicating that the symbiosis enhances desiccation resistance and thereby likely played,  
69 besides in other important functions, like the defense against natural enemies, a key role for the adaptation  
70 of phylogenetically diverse beetles to conditions of low ambient humidity in mature grain and especially  
71 stored grain facilities.

72

## 73 **Results**

### 74 **Bostrichid and silvanid pest beetles harbor ancient *Sulcia*-like intracellular symbionts**

75 By PCR amplification, cloning and sequencing of the bacterial 16S rRNA gene, we characterized the symbionts  
76 associated with five bostrichid beetles (*Lyctus brunneus*, *Rhizopertha dominica*, *Prostephanus truncatus*,  
77 *Dinoderus bifoveolatus* and *Dinoderus porcellus*) and three silvanid beetles (*Ahasverus advena*, *Oryzaephilus*  
78 *mercator* and *Oryzaephilus surinamensis*). While *L. brunneus* feeds on seasoned hard wood, all others are  
79 serious pests of diverse stored grain products. Surprisingly, despite the phylogenetic distance of Bostrichidae  
80 and Silvanidae (about 240 mya, see Hunt et al., 2007), the symbionts of all eight species were assigned to the  
81 same clade of Bacteroidetes bacteria that also contained *Sulcia muelleri*, the symbionts of Auchenorrhyncha,  
82 and *Blattabacterium*, the symbionts of cockroaches and some termites (Fig. 1, Supplemental Fig. 1). While  
83 the three silvanid species and two of the bostrichids (*R. dominica* and *P. truncatus*) contained a single  
84 symbiont, *L. brunneus* and the two *Dinoderus* species additionally displayed a second, more basally branching  
85 clade of symbionts (Fig. 1). Based on diagnostic PCRs, the derived and in all species maintained symbiont  
86 could be detected in 68%-99% (Supplemental Table 2) of tested adult beetles except *A. advena*, whereas the  
87 ancestral symbiont only associated with *Lyctus* and *Dinoderus* was detected in 68% -90%. In total, 95%-100%  
88 of the tested individuals were positive for at least one of both symbionts. In *O. surinamensis* and *R. dominica*,  
89 the degradation of symbionts in old individuals (particularly in males) has been reported (Huger, 1956),  
90 probably accounting for the low apparent infection frequencies across host species. Infection rates estimated  
91 by diagnostic PCR and Fluorescence in situ hybridization (FISH) in *A. advena* were with 30% considerably lower  
92 (N=10). Due to rare and low levels of infections (Fig. 2d) symbionts in *A. advena* were probably formerly not  
93 detected (Buchner, 1965). Consistently, despite being usually also considered as a stored grain pest, *A. advena*  
94 actually feeds on fungal infestations of grain, requires the addition of yeast extract in artificial grain diets and  
95 also relatively high environmental humidities of 70% (Thomas and Leschen, 2009).

96 A phylogenetic dating analysis based on the partial symbiont 16S rRNA gene of ~1250bp and two calibration  
97 points, the origin of the cicada-*Sulcia* symbiosis (260-280Mya; Moran et al., 2005) and the cockroach-  
98 *Blattabacterium* symbiosis (150-300Mya; Patino-Navarrete et al., 2013), revealed an ancient origin of the  
99 clade of Bacteroidetes endosymbionts 494 Mya ago (Fig1, Supplemental Fig 1 and Supplemental Table 3). The  
100 mutualistic Bacteroidetes group comprising *Blattabacteria*, the beetle symbionts and *Sulcia* dates back  
101 around 394 Mya, with the split of the ancestral beetle symbiont clade and the split between *Sulcia* and the  
102 other beetle symbionts around 355Mya ago and the split of the *Oryzaephilus* and bostrichid symbionts around  
103 331 Mya ago (Fig1, Supplemental Fig 1 and Supplemental Table 3). Different nucleotide substitution models  
104 had little impact on node ages with a mean node age for all endosymbionts varying between 489-503 Mya,  
105 and the mutualistic symbionts between 404-414 Mya, whereas strict clock models resulted in younger node  
106 ages (423-432 Mya and 384-392 Mya, respectively, see Supplemental Table 3). Omitting one calibration point

107 shifted the divergence times to a considerably earlier time of 593 Mya, if only the origin of *Blattabacterium*  
108 was included, and to 474 Mya with only the origin of *Sulcia* included (Supplemental Table 3).

109 The bacterial symbionts were all located intracellularly, in bacteriomes located between gut, fat body and  
110 reproductive organs, but without direct connection to any of these tissues. While the Silvanid beetles  
111 contained two pairs of bacteriomes, both with the same single symbiont strain (Fig. 2a-d; see also Koch,  
112 1936a), *R. dominica* and *P. truncatus* contained only one bacteriome pair with a single strain (Fig. 2e+f). In  
113 contrast, both *Dinoderus* species contained one pair of bacteriomes for each symbiont strain, which were  
114 anatomically separated from each other (Fig. 2g+h). *L. brunneus* harbored a pair of bacteriomes, each  
115 composed of a central bacteriome containing the ancestral symbiont strain surrounded by three bacteriomes  
116 harboring the derived symbiont strain (Fig. 2i+j; see also Koch, 1936a).

117

#### 118 **Symbiont association is not obligate for *O. surinamensis***

119 As described previously, the *O. surinamensis* symbiont titers could be reduced by either heat or tetracycline  
120 treatment (Koch, 1936b, Huger, 1956). Treatment of adult beetles over three months with tetracycline  
121 resulted in a complete and stable elimination of the symbiont, while heat treatment only reduced symbiont  
122 titers (Supplemental Fig. 2). In the tetracycline treatment group, the native symbiont could neither be  
123 detected by quantitative PCR (Supplemental Fig. 2a) nor by FISH (16 eggs and 8 adults tested per time point,  
124 100% lacked a symbiont in the treatment group, whereas 100% in the control group showed infection).  
125 Although the qPCRs occasionally resulted in off-target amplification in the absence of the native symbiont,  
126 none of the amplification products in the tetracycline-treated group matched the melting profile of the native  
127 symbiont amplicon (Supplemental Fig. 2a). In contrast, the offspring (eggs) of beetles that were exposed to  
128 36°C as either adults or larvae showed slightly, but not significantly lower symbiont titers (Mann-Whitney-U  
129 tests; heat treated larvae vs. control U=30, N=10 each, p=0.243, heat treated adults vs. controls U=101, N=30  
130 and 10, p=0.269; Supplemental Fig. 2b). In the next generation, symbionts were significantly reduced and  
131 often completely absent (Mann-Whitney-U test, F2 of heat treated adults vs control, U=10.5, N=10 each,  
132 p=0.003; Supplemental Fig. 2b), but less consistently so than in the tetracyclin treatment group.

133 Due to the successful elimination of symbionts by tetracycline treatment, the offspring of tetracycline treated  
134 beetles were maintained in a continuous laboratory culture over two years to perform all following  
135 experiments. Despite overall good performance of aposymbiotic cultures under optimal growth conditions of  
136 60% RH at 30°C, we observed a significant reduction of cuticle melanization (exact 2-sided Mann-Whitney-U

137 test,  $U=154$ ,  $p<0.001$ , Supplemental Fig. 3a) and a significantly lower population growth of aposymbiotic  
138 beetles (exact 2-sided Mann-Whitney-U test,  $U=30$ ,  $p=0.004$ , Supplemental Fig. 3b).

139

#### 140 **Symbionts contribute to cuticle thickness and melanization in *O. surinamensis***

141 Melanization of the cuticle increases its physical strength and contributes to desiccation resistance (Gibbs and  
142 Rajpurohit, 2010). Given the observed impact of symbiont elimination on cuticle melanization, we set out to  
143 assess the contribution of the *O. surinamensis* symbiont to cuticle formation and melanization in more detail,  
144 by exposing replicate symbiotic and aposymbiotic populations to dry (30-40%RH) and humid (60% RH)  
145 conditions. In adult beetles, both symbiont absence and reduced environmental humidity significantly  
146 reduced cuticle melanization (Table1, Fig. 3a) and cuticle thickness (Table1, Fig. 3b) with both a thinner endo-  
147 and exocuticle (Table1, Supplemental Fig. 4a+b). Aposymbiotic adults exhibited overall a less melanized and  
148 on average 26% thinner cuticle than their symbiotic counterparts. In 4<sup>th</sup> instar larvae, symbiont absence, but  
149 not the humidity regime, resulted in a significant reduction in cuticle thickness by about 20% (Table1,  
150 Supplemental Fig. 4c).

151

#### 152 **Effect of symbionts on the epicuticular hydrocarbon profile**

153 By adapting their cuticular hydrocarbon (CHC) profile, insects including *O. surinamensis* can rapidly change  
154 the water permeability of their epicuticle (Howard et al., 1995, Gibbs and Rajpurohit, 2010). We exposed  
155 adult beetles from all four treatments (full factorial design of dry and moist, symbiotic and aposymbiotic) to  
156 severe desiccation stress (one day at <2% RH) or not (control) and measured their respective CHC profiles.  
157 Symbiont presence and environmental humidity during rearing had a significant influence on the total amount  
158 of CHCs, with symbiont elimination and dry conditions resulting in an increased amount of CHCs (Table1).  
159 While acute drought stress alone did not affect CHC amounts (Table1), the interaction with symbiont status  
160 did, with aposymbiotic beetles applying more CHCs to their cuticle under both chronic and acute desiccation  
161 stress (Table1, Fig 4a). In addition, symbiont absence and long term exposure to low humidity significantly  
162 increased the proportion of unsaturated hydrocarbons (Table1,  $p<0.001$ ), and acute desiccation stress  
163 increased the proportion of unsaturated hydrocarbons in beetles adapted to low humidity, but decreased it  
164 for beetles adapted to high humidity (Table1, Fig. 4b). Furthermore, symbiont absence itself and its  
165 interaction with environmental humidity also affected the average chain length of CHCs on the cuticle with  
166 aposymbiotic beetles carrying shorter chain CHCs, which is even enhanced under low humidity (Table1, Fig.

167 4c), whereas neither long-term low humidity *per se* nor acute desiccation stress affected CHC chain length  
168 (Table1, Fig 4c). These results demonstrate that aposymbiotic beetles perceive desiccation stress significantly  
169 more strongly than symbiotic beetles, especially if they were already kept under low humidity, and mount a  
170 physiological response to improve their epicuticular properties, as higher amounts of hydrocarbons provide  
171 a better evaporation protection. Shorter and less saturated hydrocarbons are generally considered to offer  
172 less protection against desiccation. However these conclusions are derived by studying the behavior of CHC  
173 mixtures at different temperatures, not at a fixed temperature with varying humidities (Gibbs and Rajpurohit,  
174 2010). Accordingly, beetles without symbionts and under chronic, low humidity seem not to be able to keep  
175 the potentially more protective composition of their CHCs, but rather rely on the protective effect of a thicker  
176 hydrocarbon layer, whereas symbiotic beetles reared in a more humid environment are able to shift their  
177 CHC profile to a more favourable composition under acute desiccation stress.

178

#### 179 **Symbionts confer desiccation resistance to *O. surinamensis***

180 In order to test whether the observed symbiont-mediated changes in cuticular thickness, melanization, and  
181 CHC composition affect desiccation resistance in *O. surinamensis*, we measured water loss and mortality of  
182 symbiotic and aposymbiotic beetles under desiccation stress. Indeed, aposymbiotic beetles reared at high or  
183 low humidity desiccated faster than their symbiotic counterparts (Table1, Fig. 5a; measured as proportional  
184 decrease in weight as beetle dry mass differed between treatments; see Supplemental Fig. 5). Concordantly,  
185 symbiont-free beetles also exhibited higher mortality upon acute drought stress, independent of the humidity  
186 they experienced during development (Cox Mixed-Effect Model, N=400 (8 replicates with 50 individuals per  
187 treatment), Table1, Fig. 5b). Similarly, survival from oviposition until emergence of adults was significantly  
188 lower in the absence of symbionts under low humidity ( $\chi^2$  homogeneity test at 30% RH: 10.5% for  
189 aposymbiotic beetles, N=114, vs 27.5% for symbiotic beetles, N=40,  $\chi^2=6.71$ ,  $p=0.013$ ), but not at high  
190 humidity ( $\chi^2$  homogeneity test at 60% RH: 31.4% for aposymbiotic beetles, N=296 vs 41.7% for symbiotic  
191 beetles, N=103,  $\chi^2=3.63$ ,  $p=0.057$ ). The differential susceptibility to desiccation was also reflected in the  
192 beetles' population growth over three months, with a significant influence of symbiont absence, ambient  
193 humidity as well as their interaction (Table1, Fig. 6).

194

#### 195 **Discussion**

196 We showed that grain pest beetles in the beetle families Bostrichidae and Silvanidae engage in a symbiotic  
197 association with a group of Bacteroidetes bacteria that is closely related to *Sulcia* and *Blattabacterium*, the  
198 obligate nutritional mutualists of cicadas and cockroaches, respectively. However, in contrast to these (Lo et  
199 al., 2003, Takiya et al., 2006), the beetle symbiont 16S rRNA sequences revealed multiple acquisition,  
200 exchange and/or loss events, and the bacterial partners exhibited higher sequence divergences, indicating  
201 older associations and/or faster evolutionary rates than the *Sulcia* and *Blattabacterium* associations (Silva and  
202 Santos-Garcia, 2015). The common ancestor of *Blattabacterium*, *Sulcia* and the symbionts of the bostrichid  
203 and silvanid beetles was estimated to have lived around 394 Mya, predating the evolution of the beetles  
204 (Coleoptera) (Hunt et al., 2007). Considering that the common ancestor of Auchenorrhyncha, Blattodea,  
205 Silvanidae and Bostrichidae existed around 380 to 390 Mya and gave rise to all holometabolous and most  
206 hemimetabolous insects (Misof et al., 2014), an ancient infection with the Bacteroidetes symbiont and  
207 subsequent losses in all but these four taxa seems to be an unlikely scenario. The more parsimonious  
208 explanation, especially considering the estimated age of the Bostrichidae (~150Mya) and Silvanidae  
209 (~180Mya; Hunt et al. 2007) involves at least six independent acquisition events (one in Auchenorrhyncha,  
210 one in Blattodea, two in Silvanidae, and two in Bostrichidae) (Fig. 1 and Supplemental Fig. 1) and is reminiscent  
211 of the repeated acquisition of symbionts from a few clades of intracellular gamma-proteobacteria across  
212 diverse insect taxa (Husnik et al., 2011). Possibly, particular clades of bacteria were adapted to an insect-  
213 associated (possibly parasitic) lifestyle and – akin to extant *Wolbachia* infections – successfully spread through  
214 mixed vertical and horizontal transmission, but only those associations evolving towards mutual benefits  
215 proved to be stable over evolutionary timescales, resulting in the patchy distribution we observe today.  
216 Supporting this hypothesis, a basal clade of the bacteroidetes endosymbionts actually consists of three  
217 species that are described as male-killing endosymbionts in different ladybug beetles (Fig.1; Hurst et al., 1997,  
218 Hurst et al., 1999).

219 Within the bostrichid-Bacteroidetes association, we discovered three cases of multipartite symbioses with  
220 two different strains of the same bacterial clade, located in different bacteriome organs or compartments.  
221 While co-obligate symbionts have been described repeatedly across sap-feeding Hemiptera, they are usually  
222 co-localized in adjacent bacteriocytes to facilitate exchange of metabolic products or intermediates for jointly  
223 synthesized products (McCutcheon and von Dohlen, 2011, Wu et al., 2006). Alternatively, they are intermixed  
224 in the same bacteriocytes, as is the case in the fragmented *Hodgkinia* genomes in several cicada species (Van  
225 Leuven et al., 2014, Campbell et al., 2015). Interestingly, some of the Bostrichid species seem to have lost  
226 their ancestral symbiont, a theory already formulated by Huger (1956) who also occasionally observed the  
227 formation of additional, uninfected bacteriomes in *R. dominica*. This raises not only the question of the



228 individual contribution of the single strains, but also the physiological and especially ecological consequence  
229 of symbiont acquisition, replacement or loss events for the respective beetle groups (Joy, 2013, Sudakaran et  
230 al., 2015, Sudakaran et al., 2017). Ancestrally, both groups of beetles inhabit rather humid environments.  
231 While Bostrichid beetles are described to inhabit and feed on sapwood, dying or dead trees, Silvanid beetles  
232 feed presumably on fungal detritivores of decomposing wood (Hunt et al., 2007, Thomas and Leschen, 2009).  
233 Finally, while the ecological niches of the investigated beetles are all characterized by low humidity, they  
234 differ widely in the available nutrient composition of their diet. Dry wood inhabited by the genus *Lyctus*  
235 probably represents the resource that is poorest in nitrogen (Hoadley, 1998); dried fruit and roots as  
236 preferred by *Dinoderus spp.* may be similarly unbalanced (Nations, 1990), whereas the germ tissue of  
237 different grains may contain sufficient nitrogen sources (Souci et al., 2009), which could have contributed to  
238 the loss of the basal symbiont lineage in those beetles. The insect cuticle is in general composed of chitin  
239 fibrils – a polymer of N-acetyl-glucosamine, which is synthesized from glucose, glutamine and acetyl-  
240 coenzyme A (Muthukrishnan et al., 2012) – in a complex with proteins (Hackman, 1974). The outer layer of  
241 the cuticle, the exocuticle, can be melanized and sclerotized and thereby becomes harder, darker and  
242 supposedly more water proof (Hackman, 1974, Gibbs and Rajpurohit, 2010). The fact, that, unlike in *S. oryzae*  
243 (Vigneron et al., 2014), the thickness of both endo- and exocuticle of *O. surinamensis* is reduced in the absence  
244 of the symbiont, suggests a more general contribution of nutrients than specifically Dopa or a similar  
245 precursor for cuticle melanization and sclerotization (Klein et al., 2016). Thus, nutritional benefits provided  
246 by Bacteroidetes symbionts might likely reach beyond individual amino acids or their precursors for cuticle  
247 melanization as in *S. oryzae* (Vigneron et al., 2014) to nitrogen recycling and essential amino acid and vitamin  
248 provisioning, as in cockroaches and the Auchenorrhyncha (Sabree et al., 2009, McCutcheon and Moran, 2010)  
249 or in carpenter ants (Gil et al., 2003, Degnan et al., 2005) and *Nardonella* harboring weevils (Kuriwada et al.,  
250 2010, Hosokawa et al., 2015). The individual contributions and interactions between the symbiont strains are  
251 interesting topics for future studies, especially given the facultative nature of the intracellular symbiosis and  
252 possibility for experimental manipulation of symbiont infection status.

253 In addition to identifying an ancient group of symbiotic *Sulcia*-related bacteria in diverse grain and wood pest  
254 beetles, we demonstrated an ecological benefit in the saw-toothed grain beetle *O. surinamensis* conferred by  
255 their non-obligate, yet prevalent, intracellular symbiont. By supporting cuticle synthesis, the bacteria confer  
256 desiccation resistance to their host, which constitutes a significant fitness benefit for the beetles, particularly  
257 under the dry conditions that characterize their anthropogenic habitat of granaries and other storage facilities  
258 (Hagstrum et al., 1996). Interestingly, grain weevils that occupy an almost identical ecological niche evolved  
259 a symbiotic association with  $\gamma$ -proteobacteria (*Sodalis pierantonius*) that supports cuticle biosynthesis in a

260 similar manner (Heddi et al., 1999, Vigneron et al., 2014) and may hence contribute to drought tolerance.  
261 Likewise, carpenter ants of the genus *Camponotus* and the invasive ant *Cardiocondyla obscurior* evolved  
262 symbioses with the  $\gamma$ -proteobacteria *Blochmannia* and *Candidatus Westeberhardia cardiocondylae*,  
263 respectively, that support cuticle melanization through the synthesis of essential amino acids or tyrosine  
264 precursors, respectively (*Blochmannia*, de Souza et al., 2011, *Candidatus Westeberhardia*, Klein et al., 2016).  
265 However, in which way these ants benefit from enhanced cuticle melanization remains unclear. Ants as well  
266 as beetles my generally benefit from a thicker and harder cuticle as a first line of defense against many natural  
267 enemies, like predators, parasitoids and pathogens. For the grain beetles, we hypothesize that the symbioses  
268 with Bacteroidetes and  $\gamma$ -proteobacteria, respectively, constituted (pre)adaptations that enabled the three  
269 families to independently invade their niches of originally individual items of dried grain, fruit and wood, but  
270 proved to be especially advantageous to invade recent, anthropogenic stores of dry grain and fruit, as well as  
271 seasoned wood.

272

## 273 **Materials & Methods**

### 274 **Insect cultures**

275 *O. surinamensis*, *A. advena*, *R. dominica*, *P. truncatus*, *D. bifoveolatus*, and *D. porcellus* insect cultures were  
276 obtained from the Julius-Kühn-Institute / Federal Research Centre for Cultivated Plants (Berlin, Germany), *O.*  
277 *mercator* from Fera Science Ltd (Darlington, UK) and *L. brunneus* from the Federal Institute for Material  
278 Research and Testing (Berlin, Germany). Continuous symbiotic and aposymbiotic (see below) *O. surinamensis*  
279 cultures were maintained in 1.8L plastic containers, filled with 50g oat flakes, at 30°C and ambient humidity  
280 between 40% and 60% in the dark.

281

### 282 **DNA extraction, 16s rDNA cloning and sequencing**

283 Total DNA was isolated individually from 25-30 adults per species using the Epicentre MasterPure™ Complete  
284 DNA and RNA Purification Kit (Illumina Inc., Madison, Winsconsin, USA).

285 Total 16s rDNA was amplified from 5-12 DNA extracts per species using universal eubacterial primers fD1 and  
286 rP2 (Welsburg et al. 1991). Reaction mixtures for PCR amplification consisted of 6.4 $\mu$ l distilled water, 1.25 $\mu$ l  
287 PCR Buffer, 0.25 $\mu$ l MgCl<sub>2</sub>, 1.5 $\mu$ l dNTPs, 1 $\mu$ l of each primer (each 10 pmol/ $\mu$ l), 0.1 $\mu$ l Taq polymerase (5U/ $\mu$ l)  
288 and 1 $\mu$ l template in a final volume of 12.5 $\mu$ L. The PCR temperature profile was 95°C for 3 minutes, followed

289 by 30 cycles of 95°C, 60°C, and 72°C for 40s each, and a subsequent elongation step at 72°C for 10min. PCR  
290 products were purified with the Analytik Jena innuPREP PCRpure Kit (Jena, Germany).

291 Bacterial 16S rRNA amplicons from five individuals per beetle species were cloned with a pSC-A-amp/kan  
292 vector (Strata Clone PCR Cloning Kit, Agilent Technologies, Santa Clara, California, USA) into *Escherichia coli*  
293 K12. Vector insertion sequences of successfully transformed colonies were amplified by another PCR using  
294 the flanking primer pair M13\_fwd and M13\_rev. The PCR parameters and purification were identical as  
295 described above, except that an annealing temperature of 65°C was used, and entire cells from clone colonies  
296 were added to the PCR reaction mix as template. Bidirectional Sanger sequencing was performed in house to  
297 obtain the full sequence of the amplified 16S fragments using the M13\_fwd/rev primers.

### 298 **Infection frequencies**

299 Diagnostic PCRs were conducted to assess symbiont infection frequencies in all eight insect species. Specific  
300 oligonucleotides (Supplemental Table 1) were designed based on an alignment of *Sulcia muelleri*,  
301 *Blattabacterium sp.* and free-living Bacteroidetes 16S rDNA sequences. Specificity of the primer sequences  
302 for Bacteroidetes was assessed *in silico* using the Ribosomal Database Project 16S rDNA collection (Cole et al.,  
303 2014). Specificity was further tested by trying to amplify fragments from DNA extracts of the beetle species  
304 that should not carry the focal symbiont as well as European firebug *Pyrrhocoris apterus* and European  
305 beewolf *Philanthus triangulum* guts DNA extracts that similarly are known to lack Bacteroidetes bacteria.

### 306 **Phylogenetic inference**

307 Beetle symbiont 16S rDNA sequences were aligned to representative *Sulcia muelleri*, *Blattabacterium* and  
308 free living Bacteroidetes 16S rDNA sequences obtained from the NCBI database, using the SILVA algorithm  
309 (Quast et al., 2013, Yilmaz et al., 2014) . A maximum-likelihood phylogenetic tree was reconstructed with  
310 PHYML (Guindon and Gascuel, 2003) as implemented in Geneious Pro 9.1.5 (Drummond AJ, 2011), using the  
311 GTR model with uniform substitution rates per site. The initial tree for the heuristic search was obtained  
312 automatically by applying Neighbour-Joining and BioNJ algorithms to a matrix of pairwise distances estimated  
313 using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log  
314 likelihood value. Bootstrap values were obtained from 10,000 replicates. A second tree was reconstructed by  
315 Bayesian inference applying a GTR +G +I model using MrBayes 3.2 (Huelsenbeck et al., 2001, Huelsenbeck and  
316 Ronquist, 2001, Ronquist and Huelsenbeck, 2003). The analysis ran for 2,000,000 generations, with trees  
317 sampled every 1,000 generations. After confirming that split frequencies converged to less than 0.01, we used  
318 a “Burnin” of 20%. Both trees were visualized with FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>).

319

320 **Phylogenetic dating**

321

322 Divergence time estimations were inferred using BEAST v1.8.4. MCMC analyses (Drummond A. J. and  
323 Rambaut, 2007) with HKY, GTR and TN93 nucleotide substitution models (empirical or estimated base  
324 frequencies, various site heterogeneity models [none, G, I+G]). Analyses were conducted under a strict clock  
325 (using a single rate of sequence evolution across the phylogeny) and an uncorrelated lognormal relaxed clock  
326 model (allowing variable substitution rates; Drummond A. J. et al., 2006). In each analysis, 100 million steps  
327 were performed, and trees were sampled every 100,000 steps. The phylogenetic tree from the Bayesian  
328 inference analysis (see previous section) was used a fixed input tree in all analyses. Nucleotide substitution  
329 priors were determined with jmodeltest v2.1.9 (Guindon and Gascuel, 2003, Darriba et al., 2012).

330 The age of the cockroach-*Blattabacterium* (normal distribution, mean±SD=220±25; Patino-Navarrete et al.,  
331 2013) and cicada-*Sulcia muelleri* (normal distribution, mean±=270±3.5; Moran et al., 2005) symbioses were  
332 used as calibration points for the dating analyses.

333 Selection of best fitting model was performed by path and stepping stone sampling (Baele and Lemey, 2013)  
334 with 100 steps logged every 1 million generations. The analysis with the best fitting model (GTR+I+G) was  
335 repeated twice with only one of both calibration points. Models were evaluated using Tracer v1.6  
336 (<http://beast.bio.ed.ac.uk/Tracer>), consensus trees were generated with TreeAnnotator v1.8.4 (Drummond  
337 A. J. and Rambaut, 2007) using a burnin of 10% and a posterior probability limit of 0.3 and visualized with  
338 FigTree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree/>).

339

340

341 **Fluorescence *in situ* hybridization (FISH)**

342 Whole mount FISH was performed on *O.surinamensis* larvae, standard FISH on squashed, fixed eggs and on  
343 sections of adult beetles. Fresh or frozen beetle eggs were fixed by slightly squashing them on a glass slide  
344 and incubation for three minutes in 70% ethanol and another 3 minutes in 96% ethanol. Whole larvae, pupae  
345 and adults were briefly washed in diethylether and fixed for at least three days in 4% paraformaldehyde in  
346 PBS. Adults were then dehydrated and embedded in Technovit 8100 (Heraeus-Kulzer, Germany), and 10µm  
347 sections were cut on a microtome Microtome (Microm HM355S, Leica, Germany) and mounted on diagnostic  
348 microscope slides.

349 Probes were designed based on specific primers (see Supplemental Table 1) and were labelled with the  
350 cyanine dyes Cy3 or Cy5. Sections of adults and whole eggs were covered with hybridization buffer containing  
351 0.9M NaCl, 0.02M Tris/HCl, 0.01% SDS, 0.5µM of each labelled oligonucleotide probe and 5µg/ml of the

352 general DNA stain DAPI. Hybridization was performed at 50°C for 60min. The samples were subsequently  
353 washed twice with washing buffer consisting of 0.1M NaCl, 0.02M Tris/HCl, 5mM EDTA and 0.01% SDS and  
354 incubated at 50°C for 20min in washing buffer, followed by a washing step with distilled water. After drying,  
355 the sections or eggs were covered with Vectashield (Vector Laboratories, Burlingame, CA, USA) and a cover  
356 slip.

357 Whole mount fish was performed by staining entire larvae at 50°C overnight in the same hybridization buffer.  
358 Afterwards, samples were washed twice for two hours with pre-warmed washing buffer at 50°C and twice for  
359 20min with distilled water at room temperature, before mounting on 2-well slides and covering with  
360 Vectarshield for fluorescence microscopy. ). Images were acquired with an AxioImager Z2 equipped with an  
361 Apotome.2 (Zeiss, Germany) and a SOLA light engine LED light source (Lumencor, OR, USA) under 200-400x  
362 magnification with the Z-stack option.

363

#### 364 **Elimination of *O. surinamensis* symbionts**

365 In order to obtain symbiont-free *O. surinamensis*, 150 adults were kept for three months on oat flakes that  
366 were soaked in a tetracycline solution (30mg tetracycline hydrochloride / g oat flakes; Sigma-Aldrich,  
367 Germany) and dried at 60°C. 200 adult offspring individuals of these beetles were then transferred back to a  
368 standard oat flake diet. A control group experienced the same conditions except that the tetracycline was  
369 omitted from the oat flake soaking step. Efficiency of symbiont elimination was verified by qPCR of both eggs  
370 and adults immediately after the tetracycline treatment as well as three and twelve months later,  
371 respectively. For all time points, eggs were also collected and squashed onto slides, fixed for 10min with 95%  
372 and 70% ethanol subjected to FISH as described above to verify symbiont absence. Furthermore, eight female  
373 adults of the F2-F3 generation (three months after the tetracycline treatment) were sectioned and subjected  
374 to FISH as described above.

375 Quantitative PCRs were carried out in 25µL reactions using the Qiagen QuantiTect-SYBR-Green-PCR mix  
376 (Qiagen, Venlo, The Netherlands), including 0.5µM of each primer and 1µL template DNA. To compensate for  
377 host developmental stage and size as well as DNA extraction efficiency, all qPCR samples were additionally  
378 subjected to a PCR with primers targeting the host 28S rRNA gene, and the resulting delta Ct values were used  
379 for relative quantification of bacterial 16S rRNA copies per host 28S rRNA copy (Pfaffl, 2001).

380

#### 381 **Physiological response and fitness of symbiotic and aposymbiotic *O. surinamensis* lines**

382 Eight symbiotic and aposymbiotic *O. surinamensis* populations were founded from one year old aposymbiotic  
383 and symbiotic control cultures and reared at 30-40% RH and 60% RH in a full-factorial design to measure  
384 cuticle thickness of 4<sup>th</sup> instar larvae, melanization and thickness of the adult cuticle, cuticular hydrocarbon  
385 profiles of adults, desiccation resistance measured as water loss, as well as survival and population growth.  
386 For each replicate, 50 beetles were transferred to a box with oat flakes (20g) that were pre-conditioned for  
387 one week to the experimental conditions. Replicate populations were kept at 28°C and the two different  
388 humidity conditions in the dark for three months. In parallel to the replicate treatments, individual females  
389 from the basic cultures of all four treatments were separated into 12 well plates, eggs collected and the  
390 offspring individually reared in 48-well plates provided with one oat flake and incubated under above  
391 mentioned conditions. Survival until emergence of adults was monitored daily to assess mortality during  
392 development.

393 To evaluate the impact of humidity and symbiont elimination on cuticle melanisation, we determined the  
394 inverse red values (Vigneron et al., 2014) of 24 beetles from each treatment group. Photographs were taken  
395 with a Sony NEX 5 camera coupled to a Motic dissection stereoscope (Wetzlar, Germany) under identical  
396 conditions. Average red values were measured within an elliptic area covering the ventral thorax with the  
397 histogram tool in ImageJ 1.50a (Rasband, 1997-2016) and transformed into the inverse red values. To  
398 measure cuticle thickness, 9-14 adult beetles and ten 4<sup>th</sup> instar larvae per treatment group were fixated in 4%  
399 paraformaldehyde in PBS. These beetles, as well as larvae from collected after three months from the eight  
400 replicate populations were embedded in epoxy resin (Epon\_812 substitute, Sigma-Aldrich, Germany) and  
401 1µm cross sections of the thorax next to the second pair of legs were cut on a Microtome (Microm HM355S,  
402 Leica, Germany) with a diamond blade and mounted on silanized glass slides with Histokitt (Roth, Germany).  
403 Images to measure cuticle diameter were taken with an AxioImager Z2 (Zeiss, Germany) under 200x  
404 magnification and differential interference contrast. Mean cuticle diameter was measured for one randomly  
405 chosen dorsal, ventral and lateral point, respectively, with the ZEN software distance tool (Zeiss, Germany).

406 Living adult beetles of each population were counted manually after three months to measure population  
407 growth. Immediately after counting, two batches of 50 beetles of each population were transferred to  
408 separate containers that were either empty or provided with three dried oat flakes, to measure water loss  
409 and survival, respectively. At the same time, two samples of 30 beetles each were transferred to 1.4mL glass  
410 vials to measure cuticular hydrocarbon profiles before and after desiccation stress, respectively.

411 Desiccation resistance was measured as water loss and survival rates by incubating containers of 50 beetles  
412 in a chamber that was covered with a layer of silica gel. The chamber was aerated with a constant air stream  
413 of 1 mL/min that was guided through a column of silica gel to reduce it's humidity. The humidity inside the

414 chamber was thereby reduced to below 2% RH within 30min after closing the box. One group of containers  
415 with 50 beetles was weighed daily to measure water loss keeping dead beetles in the container. From the  
416 other group, dead beetles were counted and removed daily to monitor survival.

417 To assess the impact of low humidity, symbiont elimination, and acute desiccation stress on CHC profiles,  
418 glass vials containing 30 symbiotic or aposymbiotic beetles that had been reared under high (60% RH) or low  
419 humidity (30-40% RH) were incubated for 24h in a desiccation chamber as described above and subsequently  
420 given another 24h to recover under their respective rearing conditions. Control groups were kept for 48h  
421 under the respective rearing conditions. Afterwards, all beetles from each vial were freeze-killed and  
422 extracted for 10 min with 100 $\mu$ L hexane HPLC-grade (Roth, Germany) containing 2 $\mu$ g octadecane (Sigma-  
423 Aldrich, Germany) as internal standard. After removal of beetles, extracts were concentrated to  $\sim$ 30 $\mu$ L, and  
424 5 $\mu$ L were analysed on a Varian 450GC gas chromatograph coupled to a Varian 240MS ion-trap mass  
425 spectrometer (Agilent Technologies, Böblingen, Germany) using a split/splitless injector at 250°C with the  
426 purge valve opened after 60s. The GC was equipped with a DB5-ms column (30 m $\times$ 0.25 mm ID; 0.25  $\mu$ m df;  
427 Agilent, Santa Clara, CA, USA) and programmed from 150 to 320°C at 5°C/min with a 5 min. final isothermal  
428 hold. Helium was used as carrier gas, with a constant flow rate of 1ml/min. Mass spectra were recorded using  
429 electron ionization (EI-MS) with an ion trap temperature of 90°C. Data acquisition and quantifications were  
430 achieved with MS Workstation Version 6.9.3 Software (Agilent Technologies). Hydrocarbons were identified  
431 by retention index and fragmentation pattern in accordance with Howard et al. (1995). CHCs were  
432 automatically quantified using the Varian MS Workstation 6.9.3 software with manual correction. For  
433 analysis, we calculated total amount of CHCs per beetle (based on the amount of internal standard), the  
434 proportion of saturated CHCs, and a carbon chain length index (sum of the proportions of compounds  
435 multiplied by their respective number of carbon atoms).

#### 436 **Statistical procedures**

437 Symbiont abundance ( $\Delta C_T$  (symbiont 16s rDNA/host 28S rDNA)), initial test of cuticle melanization and  
438 population growth were tested between treatment and control by exact, 2-sided Mann-Whitney-U tests in  
439 SPSS 23 (IBM, Armonk, NY).

440 Influence of symbiont infection, rearing humidity, and in case of CHC profile also desiccation stress, and their  
441 interaction effects was tested with generalized linear models (GLMs) in SPSS 23. For beetle melanization,  
442 cuticle diameter of adults and larvae, all CHC measurements and water loss, we used linear scale response  
443 models with a normal distribution. For the population size counts, we used a Poisson distribution and a log  
444 link function. Model parameters were estimated by the quasi-likelihood method and accepted if the full

445 factorial model showed a significantly better fit than the intercept-only model (in all cases  $p < 0.001$ ). Wald  $\chi^2$   
446 statistics were calculated for the models and single factors. Boxplots were also visualized with SPSS 23. Water  
447 loss over time and across treatments was tested with generalized linear models with repeated measures, also  
448 in SPSS23 and visualized using the 'ggplot2' (Wickham and Chang, 2016) package in R studio version 3.1.1.

449 Mortality of *O. surinamensis* adults was analysed using a Cox mixed effects model with symbiont infection and  
450 rearing humidity and a random intercept per replicate population. The analysis was carried out using the  
451 package 'coxme' (Therneau, 2012) in R studio version 3.1.1. Survival probability of treatments was plotted  
452 based on Kaplan-Meier models using the 'rms' package (Harrell and Frank, 2013).

453 Survival during juvenile development (measured as percentage of individuals successfully developing from  
454 eggs to adults) was compared by manually calculating  $\chi^2$  homogeneity tests.

#### 455 **Data availability**

456 Partial symbiont 16S rDNA sequences are deposited in Genbank under accession numbers MF183956-  
457 MF183966.

#### 458 **Contributions**

459 T.E. and M.K. designed the project and wrote the manuscript, C.A. and R.P. provided beetle cultures and  
460 specimen, N.E., C.G. and T.K. characterized the symbionts of *O. surinamensis*, T.S. performed *O. surinamensis*  
461 larval survival assays, T.E. performed all other experiments and analyzed the data.

#### 462 **Acknowledgements**

463 M.K. and T.E. acknowledge funding from the Max-Planck-Society. We thank Benjamin Weiss, Dagmar Klebsch  
464 and Christiane Stürzbecher for technical assistance.

#### 465 **Competing interests**

466 The authors declare no competing financial interests.

467

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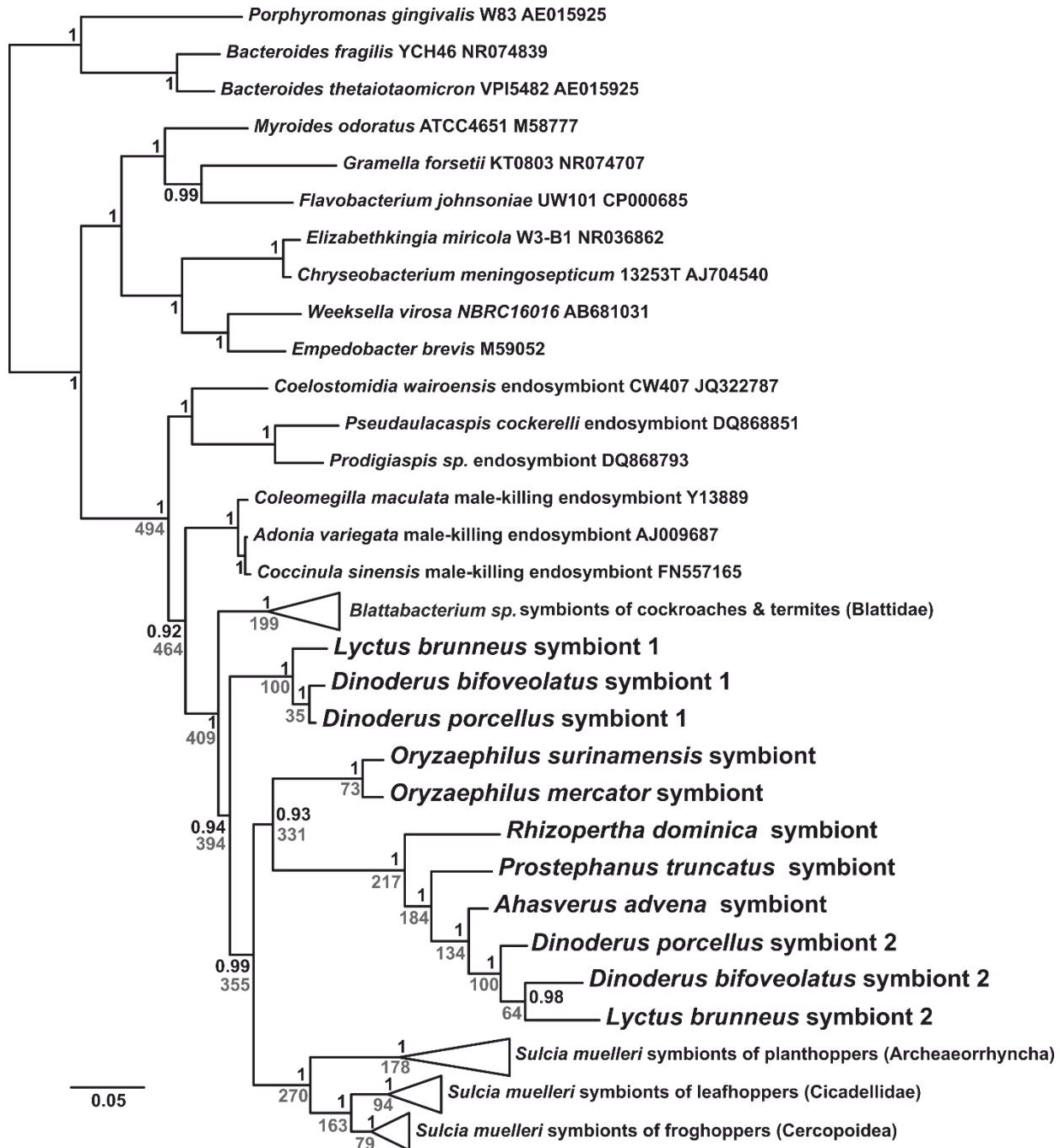
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670 **Table 1** Statistical test results of generalized linear and cox-mixed effects models describing the influence of  
 671 symbiont presence/absence, environmental humidity and acute desiccation stress on variable beetle  
 672 parameters. Tests are in order as they appear in the manuscript.  $\chi^2 = \chi^2$  Wald factors significantly influencing  
 673 a parameter are highlighted in bold.

	test	symbiont presence/absence	Environmental humidity	acute desiccation stress	symbiont* humidity	symbiont* stress	humidity* stress	all three factors
Melanization	GLM	<b><math>\chi^2=92.3</math>, d.f.=1, p&lt;0.001</b>	<b><math>\chi^2=94.1</math>, d.f.=1, p&lt;0.001</b>		$\chi^2=0.5$ , d.f.=1, p=0.488			
Cuticle thickness	GLM	<b><math>\chi^2=52.5</math>, d.f.=1, p&lt;0.001</b>	<b><math>\chi^2=4.5</math>, d.f.=1, p=0.034</b>		$\chi^2=0.837$ , d.f.=1, p=0.360			
Endocuticle thickness	GLM	<b><math>\chi^2=32.6</math>, d.f.=1, p&lt;0.001</b>	<b><math>\chi^2=6.8</math>, d.f.=1, p=0.009</b>		$\chi^2=1.7$ , d.f.=1, p=0.197			
Exocuticle thickness	GLM	<b><math>\chi^2=49.5</math>, d.f.=1, p&lt;0.001</b>	$\chi^2=0.07$ , d.f.=1, p=0.790		$\chi^2=0.04$ , d.f.=1, p=0.843			
Larval cuticle thickness	GLM	<b><math>\chi^2=17.8</math>, d.f.=1, p&lt;0.001</b>	$\chi^2=0.2$ , d.f.=1, p=0.621		$\chi^2=0.5$ , d.f.=1, p=0.473			
Total CHC amounts	GLM	<b><math>\chi^2=14.6</math>, d.f.=1, p&lt;0.001</b>	<b><math>\chi^2=83.5</math>, d.f.=1, p&lt;0.001</b>	$\chi^2=3.5$ , d.f.=1, p=0.061	<b><math>\chi^2=6.2</math>, d.f.=1, p=0.013</b>	<b><math>\chi^2=9.2</math>, d.f.=1, p=0.002</b>	$\chi^2=0.7$ , d.f.=1, p=0.391	<b><math>\chi^2=5.4</math>, d.f.=1, p=0.02</b>
Proportion unsaturated CHCs	GLM	<b><math>\chi^2=14.6</math>, d.f.=1, p&lt;0.001</b>	<b><math>\chi^2=83.5</math>, d.f.=1, p&lt;0.001</b>	<b><math>\chi^2=4.8</math>, d.f.=1, p=0.029</b>	$\chi^2=1.9$ , d.f.=1, p=0.169	$\chi^2=0.7$ , d.f.=1, p=0.418	$\chi^2=5.9$ , d.f.=1, p=0.015	$\chi^2=0.8$ , d.f.=1, p=0.366
Average CHC chain length	GLM	<b><math>\chi^2=13.3</math>, d.f.=1, p&lt;0.001</b>	$\chi^2=0.02$ , d.f.=1, p=0.903	$\chi^2=0.9$ , d.f.=1, p=0.356	<b><math>\chi^2=7.1</math>, d.f.=1, p=0.008</b>	$\chi^2=1.3$ , d.f.=1, p=0.259	$\chi^2=0.5$ , d.f.=1, p=0.490	$\chi^2=0.3$ , d.f.=1, p=0.567
Water loss	repeated measures GLM	<b><math>\chi^2=35.8</math>, d.f.=1, p&lt;0.001</b>	<b><math>\chi^2=217.2</math>, d.f.=1, p&lt;0.001</b>		$\chi^2=0.6$ , d.f.=1, p=0.449			
Mortality	Cox mixed-effects model	<b>z=-2.11,</b> p=0.034	z=-1.22, p=0.220		z=0.73, p=0.470			
Population growth	GLM	<b><math>\chi^2=431.3</math>, d.f.=1, p&lt;0.001</b>	<b><math>\chi^2=926.5</math>, d.f.=1, p&lt;0.001</b>		<b><math>\chi^2=74.1.1</math>, d.f.=1, p&lt;0.001</b>			

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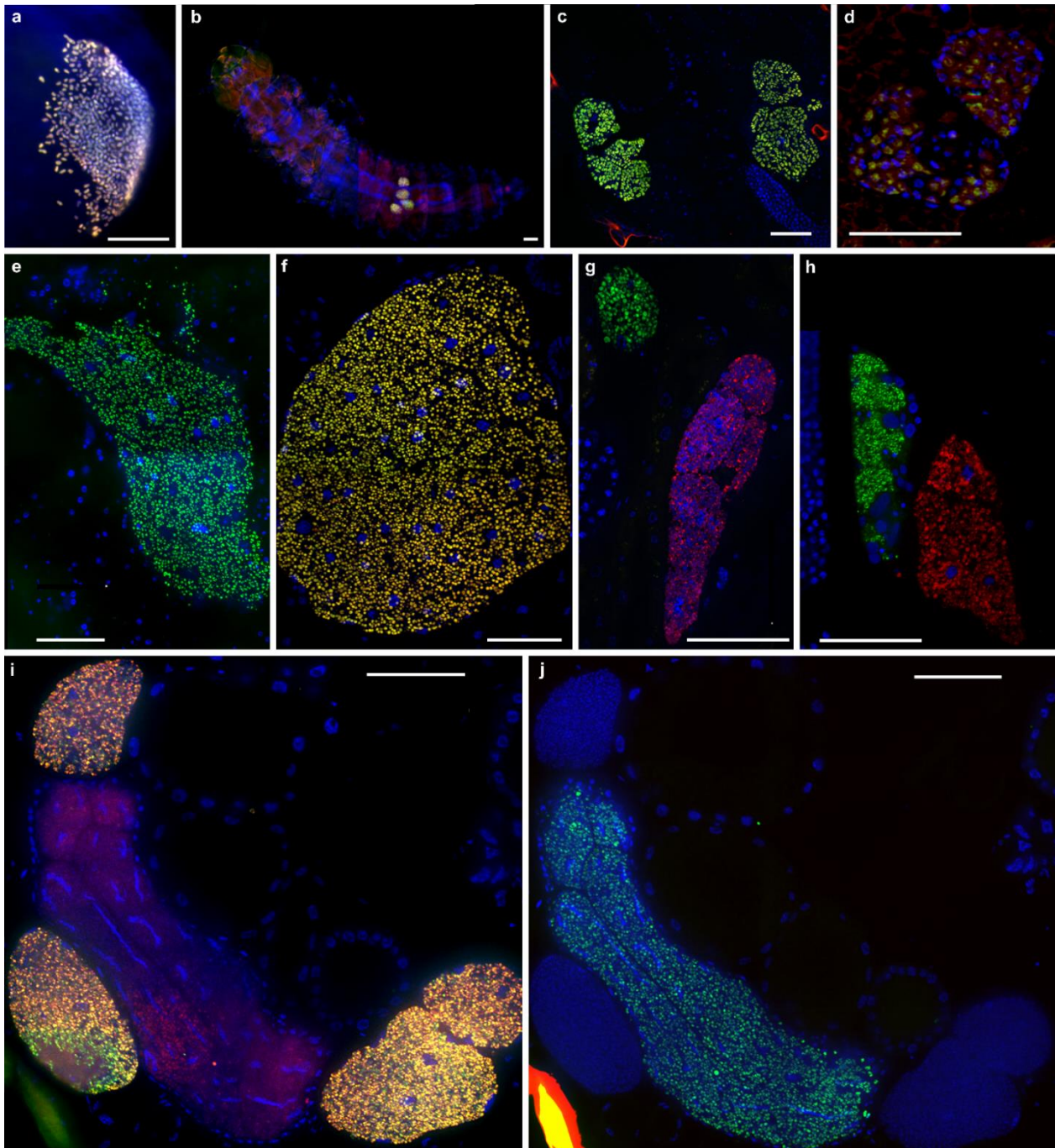




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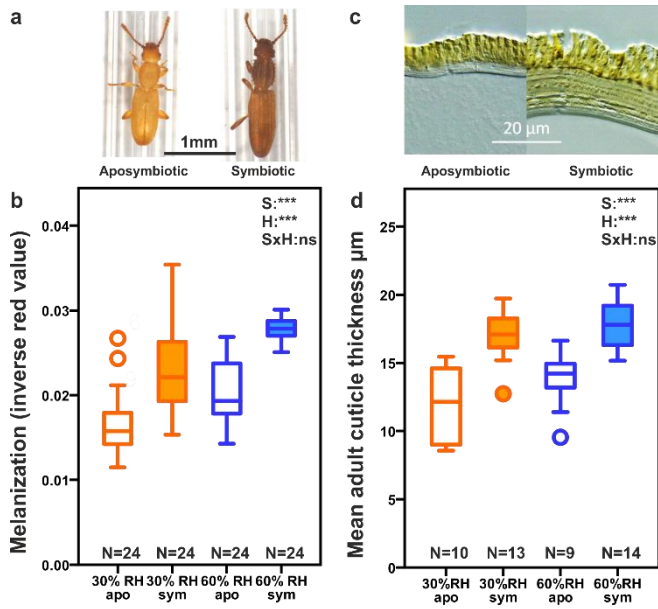
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677 **Figure 1** Phylogenetic placement of intracellular symbionts in silvanid and bostrichid grain pest beetles  
 678 within the Bacteroidetes, and their close association to endosymbionts of cockroaches and cicadas. The  
 679 phylogeny was reconstructed using Bayesian inference, and black node values represent Bayesian  
 680 posteriors. Grey values represent mean node ages in Mya, based on a phylogenetic dating analysis with the  
 681 age of *Blattabacterium* and *Sulcia* as calibration points.



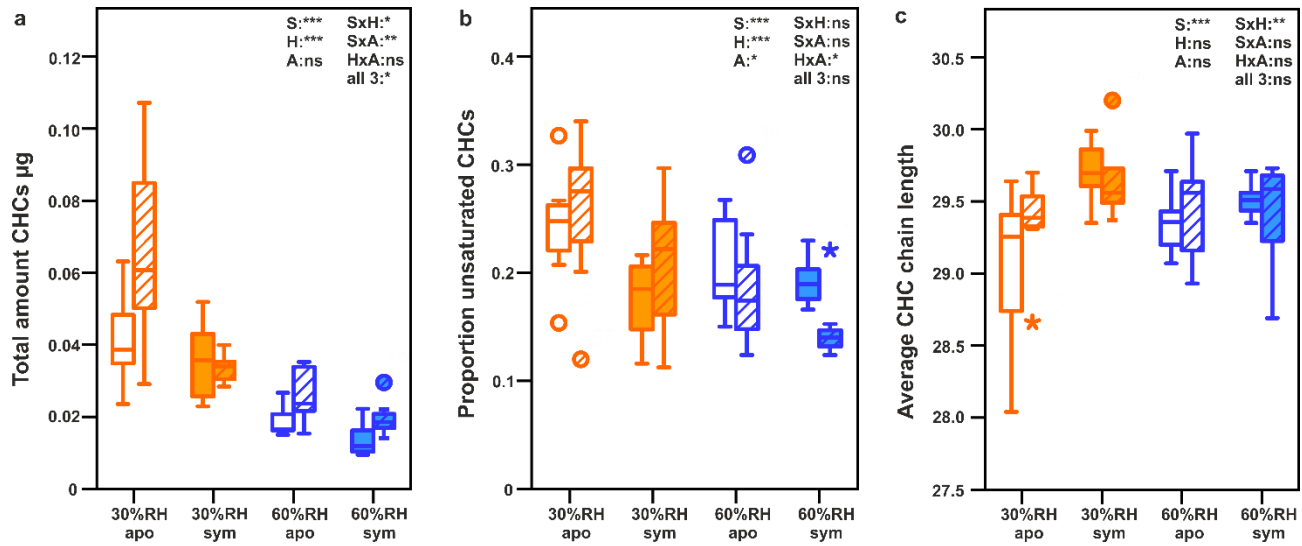
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683 **Figure 2** Bacteroidetes symbiont localization in silvanid and bostrichid beetle bacteriomes. Whole mount  
684 Fluorescence *in situ* hybridization (FISH) of *O. surinamensis* (a) egg, (b) larva, and (c) a longitudinal section of  
685 an adult female stained with EUB338-Cy3 (red) and OsurSym16S-Cy5 (green). (d) Cross section of an *A.*  
686 *advena* adult stained with Eub338-Cy3 and CFB563-Cy5. Longitudinal sections of (e) *R. dominica* and (f) *P.*  
687 *truncatus* adults, stained with EUB338-Cy3 (red; did not work in e) and Bostrichidae\_Sym2-Cy5 (green), of  
688 adult (f) *D. bifoveolatus* and (g) *D. porcellus*, stained with Bostrichidae\_Sym1-Cy3 (red) and  
689 Bostrichidae\_Sym2\_Cy5 (green). Cross sections of a *L. brunneus* female stained with (i) Eub338-Cy3 (red)  
690 and Bostrichidae\_Sym1-Cy5 (green) and (j) Bostrichidae\_Sym2-Cy5 (green). DAPI was used as a general DNA  
691 stain for all experiments (blue). Scale bars represent 50µm.



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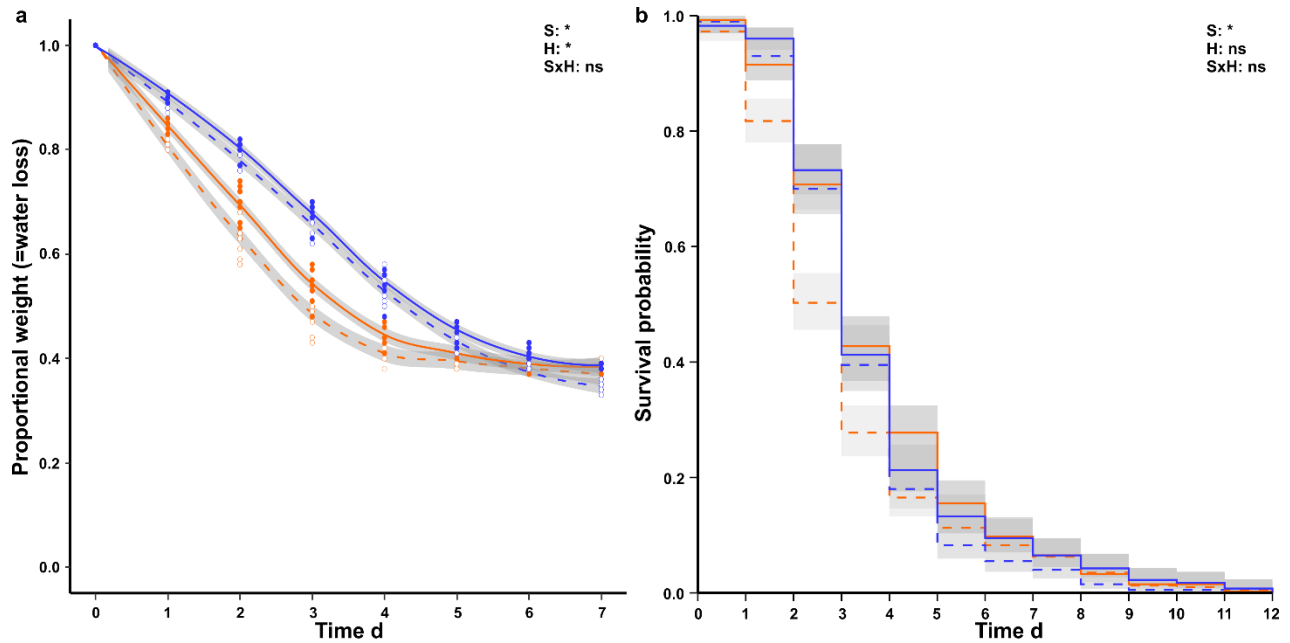
693 **Figure 3** Melanization and cuticle thickness of symbiotic and aposymbiotic *O. surinamensis* adults. (a)  
 694 Photographs of 2 day old aposymbiotic and symbiotic *O. surinamensis* adults and (b) melanization measured  
 695 as thorax coloration of aposymbiotic and symbiotic adults reared at different humidities. (c) Ventral,  
 696 thoracal cuticle sections of aposymbiotic and symbiotic *O. surinamensis* adults and (d) cuticle thickness of  
 697 aposymbiotic and symbiotic adults reared at different humidities. Both symbiont presence (S) and  
 698 environmental humidity (H; GLM,  $p < 0.001$ ), but not their interaction (S\*H; GLM,  $p > 0.05$ ) had a highly  
 699 significant influence on cuticle melanization and thickness. Boxplots show medians, quartiles and  
 700 minima/maxima. Sample size is given under each box. Filled boxes represent symbiotic and empty ones  
 701 aposymbiotic beetles, orange boxes indicate rearing at 30% RH, blue ones at 60% RH



702

703 **Figure 4** Rapid desiccation hardening (changes in the cuticular hydrocarbon profile) of *O. surinamensis* as a  
 704 response to symbiont loss, environmental humidity and acute desiccation stress. (a) Total amounts of  
 705 cuticular hydrocarbons per beetle (average calculated from 30 pooled beetle extracts), (b) proportion of  
 706 unsaturated hydrocarbons and (c) average hydrocarbon chain length show physiological counter-  
 707 adaptations of beetles to long-term exposure to low environmental humidity, but especially to acute  
 708 desiccation stress. Statistical results report on different factors and their interaction affecting CHC  
 709 parameters (GLM, S=symbiont presence, H=environmental humidity, A=acute desiccation stress, \*\*\*:  
 710  $p < 0.001$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ , n.s.:  $p \geq 0.05$ ). Filled boxes represent symbiotic and empty ones aposymbiotic  
 711 beetles, orange boxes indicate rearing at 30% RH, blue ones at 60% RH, and hatched boxes show the  
 712 respective changes after acute desiccation stress.

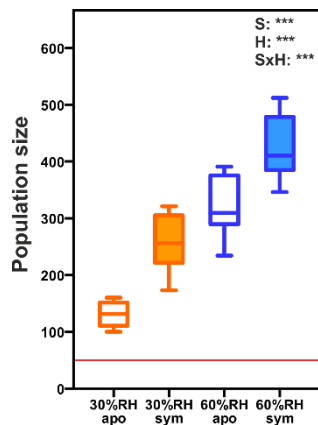
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715 **Figure 5** Water loss and survival of *O. surinamensis* adults under acute desiccation stress. (a) Water loss is  
716 significantly influenced by symbiont presence as well as rearing humidity (S,H; GLM,  $p < 0.05$ ), but not their  
717 interaction (S\*H; GLM,  $p > 0.05$ ), whereas (b) mortality is only significantly influenced by symbiont presence  
718 (S; Cox Mixed-Effect Model,  $p < 0.05$ ). Lines show mean, and shaded areas 99% confidence intervals of (a) 80  
719 pooled beetles for eight replicate populations per treatment and (b) 50 individual beetles from eight  
720 replicate populations per treatment. Continuous lines represent symbiotic and dashed lines aposymbiotic  
721 beetles, orange lines indicate rearing at 60% RH, blue lines at 30% RH

722



723

724 **Figure 6** Population growth of *O. surinamensis* over three months from starting populations of 50 beetles.  
725 Symbiont presence, environmental humidity and their interaction have a significant influence on population  
726 growth (S, H, S\*H; GLM,  $p < 0.001$ ). The red line indicates the initial population size. Boxplots show median,  
727 quartiles, and minimum/maximum of eight replicate populations per treatment. Filled boxes represent  
728 symbiotic and empty ones aposymbiotic beetles, orange boxes indicate rearing at 30% RH, blue ones at 60%  
729 RH