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► **To cite this version:**

Seonghan Jang, Peter Mergaert, Tsubasa Ohbayashi, Kota Ishigami, Shuji Shigenobu, et al.. Dual oxidase enables insect gut symbiosis by mediating respiratory network formation. *Proceedings of the National Academy of Sciences of the United States of America*, 2021, 118 (10), pp.e2020922118. 10.1073/pnas.2020922118 . hal-03374649

HAL Id: hal-03374649

<https://hal.science/hal-03374649>

Submitted on 13 Oct 2021

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Main Manuscript for

Dual oxidase enables insect gut symbiosis by mediating respiratory network formation

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Author contributions: SJ, PM, and YK designed the study. SJ, TO, and HI performed symbiont inoculation tests. SJ and SS performed RNA-seq analysis. HI performed phylogenetic analysis. SJ and KI performed DTN staining experiment and qPCR. SJ performed other experiments. SJ, PM and YK wrote the manuscript.

Competing Interest Statement: The authors declare that they have no competing interests.

Classification: Biological Sciences/Evolution

Keywords: Symbiosis, Duox, *Burkholderia*, *Riptortus pedestris*, trachea

This PDF file includes:

Main Text
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Abstract

1 Most animals harbor a gut microbiota that consists of potentially pathogenic, commensal and
2 mutualistic microorganisms. Dual oxidase (Duox) is a well-described enzyme, involved in
3 gut mucosal immunity by the production of reactive oxygen species, that antagonizes
4 pathogenic bacteria and maintains gut homeostasis in insects. However, despite its non-
5 specific harmful activity on microorganisms, little is known about the role of Duox in the
6 maintenance of mutualistic gut symbionts. Here we show that in the bean bug *Riptortus*
7 *pedestris*, Duox-dependent ROS did not directly contribute to epithelial immunity in the
8 midgut in response to its mutualistic gut symbiont, *Burkholderia insecticola*. Instead, we
9 found that the expression of Duox is tracheae-specific and its downregulation by RNAi
10 results in the loss of dityrosine crosslinks in the tracheal protein matrix and a collapse of the
11 respiratory system. We further demonstrated that the establishment of symbiosis is a strong
12 oxygen sink triggering the formation of an extensive network of tracheae enveloping the
13 midgut symbiotic organ as well as other organs, and that tracheal breakdown by Duox RNAi
14 provokes a disruption of the gut symbiosis. Downregulation of the hypoxia-responsive
15 transcription factor Sima or the regulators of tracheae formation Trachealess and Branchless
16 produces similar phenotypes. Thus, in addition to known roles in immunity and in the
17 formation of dityrosine networks in diverse extracellular matrices, Duox is also a crucial
18 enzyme for tracheal integrity, which on its turn is crucial to sustain mutualistic symbionts and
19 gut homeostasis. We expect that this is a conserved function in insects.

20

21

22

23 **Significance statement**

24 The insect respiratory system consists in tubular tracheae that transport oxygen to the organs.
25 We show that in the insect pest *Riptortus pedestris*, the establishment of an essential
26 symbiosis in the gut with the aerobic bacterial species *Burkholderia insecticola* triggers the
27 development of an extensive tracheal network enveloping the gut. Genetically blocking the
28 trachea formation prevents this gut symbiosis. We further discovered that the reactive oxygen
29 species-generating enzyme Duox is crucial for the formation and stabilization of tracheae by
30 forming protein cross-links in the tracheal matrix. Reactive oxygen species generated by
31 Duox can be scavenged with antioxidants such as N-acetylcysteine and feeding insects with
32 this compound prevents tracheal formation and symbiosis, suggesting that antioxidants can
33 be used as novel insecticides.

34

35 **Introduction**

36 Many animals possess a gut microbiota that is composed of pathogenic, commensal and
37 mutualistic microorganisms (1, 2). In insects, gut symbiotic microorganisms play a broad
38 range of physiological roles in host metabolism and immunity through digestion of food,
39 supplementation of essential nutrients that are scarce in food, degradation of phytotoxins and
40 pesticides, and prevention of pathogen invasion by stimulation of the host immune system,
41 production of antibiotics or competition for limited nutrients and colonization sites (1, 3–6).
42 Keeping symbiotic populations in the gut poses specific challenges: the hosts should
43 specifically sustain the beneficial microbes, while at the same time and same place, they need
44 to winnow out pathogens and parasites acquired through feeding. To maintain gut
45 homeostasis, some insects have evolved a symbiont sorting organ or filter in the gut (7, 8), or
46 develop a specific gut compartment called crop or crypt to harbor beneficial microbes (9).
47 Specific physiological conditions in the gut lumen, such as pH, osmotic pressure, and oxygen
48 concentration probably also contribute to create the favorable conditions for gut symbiosis.
49 Except for gut symbionts in wood-feeding insects and honeybees, most insects' gut
50 symbionts are aerobic bacteria belonging to the Proteobacteria (10, 11), suggesting that
51 oxygen supplementation is critical to maintain quality and quantity of the gut symbiont
52 population. However, it is poorly understood how the oxygen supply to the gut is established
53 and how it affects the gut symbiosis.

54

55 In addition, studies in *Drosophila* have revealed that gut mucosal immunity plays an
56 important role to eliminate unfavorable bacteria and to keep gut homeostasis (12, 13).
57 Ingested bacteria, especially pathogens that come in contact with the gut epithelia, activate
58 immune responses such as the production of antimicrobial peptides (AMPs) and reactive
59 oxygen species (ROS) (14, 15). The activated gut immunity maintains the gut homeostasis by

60 eliminating the invading environmental microbes, which may be harmful to the host.
61 Especially, bactericidal ROS, produced by the membrane-bound enzyme dual oxidase (Duox)
62 is thought to antagonize bacterial growth and to play a major role in gut mucosal immunity
63 (12, 13, 16–20). Duox generates ROS via its C-Terminal NADPH oxidase domain that
64 produces H₂O₂ in the gut lumen and its N-terminal peroxidase-homology domain that
65 converts the H₂O₂ into highly bactericidal hypochlorous acid. In *Drosophila* fed with a gut
66 pathogen, silencing of *Duox* led to an increased pathogen load in the gut and the death of the
67 insect (12). It was proposed that the Duox and ROS-dependent immunity in the gut is
68 dominant over AMP production that serves as a fail-safe system in case of infection with
69 ROS-resistant microbes (21).

70

71 In addition to the direct immune function, Duox-mediated ROS also contributes to gut
72 homeostasis in insects by stabilizing the peritrophic membrane of the midgut through the
73 formation of covalent dityrosine bonds between matrix proteins (22, 23). The peritrophic
74 matrix of insects forms a layer composed of chitin and glycoproteins that lines the midgut
75 lumen. This layer protects the midgut epithelium from abrasive food particles and microbes.
76 In the mosquito *Anopheles gambiae*, *Duox*-RNAi leads to the disruption of a dityrosine
77 network (DTN), which cross-links proteins in the peritrophic membrane, and a loss of the
78 matrix's barrier function (23). Consequently, gut microbes gain direct contact with gut
79 epithelial cells and activate chronic AMP expression, which provokes on its turn a dramatic
80 reduction of the bacterial midgut population. A similar function of Duox was reported in the
81 tick *Ixodes scapularis* (24). Thus, Duox can interact in the insect gut with ingested microbes
82 in two ways, by producing bactericidal ROS or by generating a physical barrier. In contrast to
83 these well-described functions in mucosal immunity, little is known about the impact of the
84 Duox enzyme on mutualistic bacteria.

85

86 The bean bug *Riptortus pedestris* (Fig. 1A), a notorious pest of legume crops, has a particular
87 symbiotic organ located in the posterior midgut (25–27) (Fig. 1B). This region, called M4, is
88 composed of several hundred crypts, organized in two parallel rows. The lumen of these
89 crypts is entirely filled with a specific gut symbiont of the genus *Burkholderia*. This is a
90 nutritional symbiosis based on recycling by the gut symbiont of insect metabolic waste into
91 high-value metabolites used by the host (28). This interaction is essential for optimal
92 development and reproduction of the bean bug host (26). Every new generation of *R.*
93 *pedestris* acquires the symbiont from the environmental soil by oral intake during feeding and
94 drinking. A narrow channel located in the midgut, at the entrance of the M4, as well as
95 microbe-microbe competition in the gut crypts sorts out the specific partner from the ingested
96 soil microbiota, resulting in the specific colonization of the symbiotic organ (7, 29). The few
97 hundreds of bacterial cells that are taken up by the symbiotic organ during infection multiply
98 in a few days to a population of several ten millions that completely occupy the luminal space
99 of all the crypts in the M4 (30). The crypts control this very large symbiont population using
100 immune-related AMPs as well as M4-specific symbiotic peptides (28, 31–33).

101

102 Given the cardinal role of ROS produced by Duox in insect gut immunity and the fact that the
103 *Burkholderia* symbionts stay in direct contact with the crypt epithelial cells (34), we
104 postulated in this study that besides the AMPs, Duox and ROS are also involved in the
105 management of the symbiosis in the midgut crypts of *R. pedestris*. However, our findings did
106 not support an antimicrobial immune function of Duox, but revealed an unanticipated pivotal
107 role for the enzyme in the formation of a tracheal network enveloping the symbiotic organ,
108 which is on its turn essential for supplying sufficient oxygen for respiration to the symbiotic
109 organ and the aerobic gut symbiont.

110

111 **Results**

112 **Gut symbionts induce the generation of rDuox-dependent ROS in the *R. pedestris*** 113 **midgut**

114 To determine if the gut symbiont *Burkholderia insecticola* induces ROS in the symbiotic
115 organ of *R. pedestris*, we measured the relative ROS level in the lysate of dissected gut
116 compartments of apo-symbiotic (Apo) and symbiotic (Sym) insects using the fluorescent dye
117 2',7'-Dichlorofluorescein Diacetate. An approximately threefold higher level of ROS was
118 detected in the symbiotic organ of Sym insects compared to that of Apo insects, confirming
119 that the colonization of the crypts by the *B. insecticola* symbiont stimulates ROS production
120 (Fig. 1C). Moreover, the ROS level in the digestive tract of the midgut, the M1, M2 and M3
121 anterior regions (Fig. 1B), equally increased after symbiont colonization, indicating that the
122 colonization by *B. insecticola* induces systemic high ROS levels throughout the whole
123 midgut regions even if the symbiont resides only in the posterior symbiotic midgut region M4
124 (Figs. 1B and C). To demonstrate whether Duox generates the symbiont-mediated ROS, the
125 homologous gene of *R. pedestris* (*rDuox*) was down-regulated by RNAi (*SI Appendix*, Fig.
126 S1A). The *rDuox* transcript was identified based on homology with the *Drosophila*
127 *melanogaster* protein in a *R. pedestris* transcriptome dataset generated from the infected
128 symbiotic organ. We found one *rDuox* copy in *R. pedestris*, displaying 74.7% homology and
129 the encoded protein had all the functional domains of typical Duox proteins from insects to
130 humans (22) (Fig. 1D). *rDuox*-RNAi reduced the ROS level in the symbiotic organ, as well
131 as in the other midgut regions of Sym insects, to a similar level of Apo insects (Fig. 1C),
132 demonstrating that induction of ROS by symbiont colonization is due to Duox activity.
133 Similarly, the feeding of Sym insects with the antioxidant compound *N*-acetylcysteine (NAC)
134 (35), added to the drinking water, also decreased the ROS levels of the midgut regions to

135 similar concentrations as in Apo insects (Fig. 1C). Taken together, these results demonstrate
136 that the infection of *B. insecticola* induces production of ROS in the entire midgut region via
137 the rDuox enzyme.

138

139 **rDuox-produced ROS is important for the gut symbiosis but not as a microbicidal**
140 **molecule**

141 The production of infection-associated ROS is compatible with a mucosal immune response
142 directed against the gut symbiont in the symbiotic organ. To test this hypothesis, we
143 estimated the population size of symbionts in the M4 of Sym, *rDuox*-RNAi (Sym^{*rDuox*-RNAi}),
144 and NAC-fed (Sym^{NAC-fed}) insects by qPCR. In *Drosophila*, silencing of *Duox* led to a
145 reduced microbicidal ROS production and a concomitant increased total number of gut
146 microbes (12). However, contrary to *Drosophila*, the titer of the *Burkholderia* symbiont in the
147 M4 of *R. pedestris* was decreased more than 100 times in *rDuox*-RNAi insects compared to
148 Sym insects (Fig. 1E). Also feeding of the antioxidant NAC to Sym insects reduced the
149 number of gut symbionts to similarly low levels (Fig. 1E). Microscopy analysis confirmed
150 that *rDuox*-RNAi and NAC-feeding caused the collapse of the symbiosis (Fig. 1F). Laser
151 scanning microscopy (LSM) and transmission electron microscopy (TEM) images showed
152 that in the symbiotic organ of *rDuox*-RNAi and NAC-fed insects, gut symbionts were present
153 close to the epithelial surface of the midgut crypts, but few symbionts were observed in the
154 interior part of the crypt lumen (Fig. 1F). These results indicate that rDuox plays a pivotal
155 role in controlling gut symbiosis but by a mechanism that is unrelated to a bactericidal action
156 of ROS against the symbiont.

157

158 We next investigated the possible relation of rDuox activity and bacterial infection and
159 multiplication in the anterior digestive regions of the midgut. Although the ROS-level was

160 significantly decreased in M3 by *rDuox*-RNAi (Fig. 1C), we found that the survival rate of *R.*
161 *pedestris* after oral infection with the entomopathogen *Serratia marcescens* was not affected
162 (Fig. 2A), suggesting that removing Duox-mediated ROS production did not render the
163 insects more susceptible to the pathogen. Accordingly, the number of *S. marcescens* cells in
164 the M3 region of the midgut was not significantly changed after *rDuox* RNAi (Fig. 2B).
165 Moreover, the number of *B. insecticola* cells that were transiently present in the M3 region
166 after symbiont ingestion was also not altered in *rDuox*-RNAi insects (Fig. 2C). Together,
167 these results demonstrate that Duox-mediated ROS generation is not important to regulate the
168 bacterial titer of pathogens or symbionts in the non-symbiotic midgut regions.

169

170 **Immune stimulation by *rDuox*-RNAi is not responsible for symbiosis collapse**

171 In addition to its bactericidal activity, Duox-mediated ROS is also known to contribute to the
172 construction of the midgut peritrophic membrane, as shown in *A. gambiae* (23). Knock-down
173 of *duox* in *A. gambiae* leads to a decreased barrier function of the peritrophic matrix and
174 enhanced immune stimulation and AMP expression by gut microbes coming in close contact
175 with epithelial cells. However, in contrast with the majority of insects, most hemipteran
176 insects, including *R. pedestris*, does not possess a peritrophic membrane in the midgut (36).
177 Nevertheless, the gene expression profile comparison between the symbiotic organs of Sym
178 and *rDuox*-RNAi insects showed that expression of immunity-related genes, especially genes
179 encoding AMPs (37), was increased in the midgut of *rDuox*-RNAi insects (Figs. 2D and E),
180 but not in the fat body (Fig. 2F). It should be noted however that this up-regulated AMP gene
181 expression in the midgut by *rDuox*-RNAi was significantly lower than in immune-stimulated
182 insects by septic injury (Figs. 2E and F). Because induced immunity in the symbiotic organ
183 could antagonize the gut symbiont and thus in principle be at the origin of the symbiosis
184 defect in *rDuox*-RNAi insects, we aimed at knocking down the expression of the AMP genes

185 in the bean bug. In the brown-winged green bug *Plautia stali*, a stinkbug related to *R.*
186 *pedestris* in the infraorder Pentatomomorpha but belonging to a different family, the NF- κ B
187 transcription factor Relish of the Imd pathway is an essential regulator of AMP gene
188 expression (38). *rRelish*-RNAi in *R. pedestris* reduced the expression of all three tested AMP
189 genes in the symbiotic organ as well as the fat body, indicating that also in *R. pedestris* Relish
190 is a critical regulator of the expression of the AMP immune effectors (Figs. 2E and F). After
191 simultaneously silencing of both *rRelish* and *rDuox*, the AMP expression was not up-
192 regulated anymore in the symbiotic organ (Fig. 2E), showing that the induced AMP
193 expression in the M4 by knockdown of *rDuox* is dependent on the canonical Imd immune
194 pathway. Importantly, the GFP signal of *Burkholderia* gut symbionts in double
195 *rDuox/rRelish*-RNAi insects were not restored to control levels but remained similar to single
196 *rDuox*-RNAi insects (Fig. 2G and *SI Appendix*, Fig. S2). Therefore, we can conclude that the
197 up-regulation of the immune response does not directly contribute to disrupting the symbiotic
198 relationship in *rDuox*-RNAi insects.

199

200 **Symbiont colonization triggers tracheal development and rDuox-mediated ROS**
201 **stabilizes the respiratory network by forming a dityrosine network**

202 Thus, contrary to our initial expectation, the role of rDuox and the ROS produced by it in the
203 *R. pedestris*–*Burkholderia* symbiosis is not related to mucosal gut immunity. To find a clue
204 leading to the specific function of *rDuox* in the symbiosis, we measured its tissue-specific
205 expression by RT-qPCR. Surprisingly, the expression of the *rDuox* gene in Apo and Sym
206 insects was incomparably high in the tracheae relative to all other tested tissues, suggesting
207 that the rDuox-mediated generation of ROS may have an important function in the
208 respiratory system for maintaining gut symbiosis (Fig. 3A). Although the expression level of
209 *rDuox* in Sym insects was slightly higher than in Apo insects, the gene was also very strongly

210 expressed in the tracheae of the latter insects, indicating that *rDuox* is constantly expressed in
211 the tracheal network (Fig. 3A). The insect tracheal system consists of a tubular epithelial
212 network containing a luminal chitin and protein matrix (23, 39–41). High *rDuox* expression
213 levels in the tracheae could be in agreement with the previously documented function of
214 Duox-mediated ROS in shaping extracellular matrices by forming dityrosine cross-links
215 between proteins and thereby creating a DTN (22). Immunostaining of a dissected midgut by
216 a dityrosine-specific antibody indeed revealed the presence of a DTN enveloping the
217 symbiotic organ of *R. pedestris* that likely corresponds to a respiratory network composed of
218 tracheae and tracheoles (Fig. 3B). In addition to protein, chitin is another major constituent of
219 the tracheal matrix (41). Chitin staining with calcofluor-white Fluorescent Brightener 28
220 revealed a tracheal network around the symbiotic organ that colocalized with the network
221 revealed with the dityrosine-specific antibody (Fig. 3C), confirming that the DTN highlights
222 tracheae and that dityrosines are abundant in them. Interestingly, both chitin and dityrosine
223 staining revealed that the tracheal network enveloping the symbiotic organ and the tracheoles
224 connecting inside the midgut crypt tissue were much stronger developed in Sym insects
225 compared to Apo insects (Fig. 3D). This morphological response suggests that colonization
226 of the crypts by the large number of the *Burkholderia* gut symbiont is an oxygen-requiring
227 process that induces the development of the respiratory network to supply enough oxygen to
228 the symbiotic organ. Moreover, the tracheal network, especially the ramified tracheoles, did
229 develop in response to the colonization of the gut symbiont *Burkholderia* not only around the
230 crypt-bearing M4 but also in the other midgut regions as well as the fat body (*SI*
231 *Appendix*, Fig. S3A and B), indicating that the activity of the symbiotic organ provokes a
232 systemic oxygen stress in the whole insect body. This is furthermore in agreement with the
233 ROS accumulation in all midgut regions (Fig. 1C). Accordingly, the higher ROS levels in the
234 midgut of Sym insects compared to Apo insects, resulted from the strongly developed

235 attached tracheal network since their careful removal from the symbiotic organ significantly
236 reduced the detected ROS level (*SI Appendix*, Fig. S1B). Strikingly, the DTN as well as the
237 chitin mesh in the symbiotic organ of Sym insects was strongly reduced by *rDuo*x-RNAi or
238 feeding of the antioxidant NAC (Fig. 3D). Thus, *rDuo*x is indispensable for the establishment
239 of the symbiosis-induced tracheal network in *R. pedestris* and the increased ROS level in the
240 midgut, produced by *rDuo*x after symbiont infection, is associated with the development of a
241 tracheal network along the different midgut regions. The RNAi inactivation of *rDuo*x also
242 abolished the formation of the enhanced tracheal network in the digestive, anterior midgut
243 regions M1, M2 and M3 (*SI Appendix*, Fig. S3A) as well as the fat body (*SI Appendix*, Fig.
244 S3B). These observations are in agreement with the suppression of *rDuo*x expression in the
245 entire insect body, as can be expected from the double stranded RNA injection procedure to
246 induce gene silencing.

247

248 **Destabilization of the respiratory network by *rDuo*x-RNAi causes hypoxia in the midgut**

249 Based on these findings, we made the hypothesis that the collapse of the respiratory system
250 by *rDuo*x-RNAi will cause a hypoxic environment in the symbiotic organ. We first measured
251 in the crypt epithelial cells the activity of the mitochondria using the MitoTracker dye. In
252 Sym insects, a high number of active mitochondria was detected in the epithelial cells of the
253 crypts of the symbiotic organ, which was drastically decreased after *rDuo*x-RNAi (Fig. 4A).
254 Correspondingly, the expression of genes encoding the mitochondrial respiratory chain was
255 down-regulated in *rDuo*x-RNAi insects (Fig. 4B), confirming that the symbiotic organ
256 entered into a hypoxic condition after *rDuo*x-RNAi. Moreover, staining the symbiotic organ
257 with the Image-IT dye, which is a real-time hypoxia indicator, revealed a high fluorescence in
258 the epithelia of the midgut crypts after *rDuo*x-RNAi compared with control Sym insects,
259 further supporting that the disorganization of the respiratory network by *rDuo*x-silencing

260 causes hypoxia of the symbiotic organ (Fig. 4A). Of note, our finding that AMP-encoding
261 genes are up-regulated in the hypoxic M4 of *rDuox*-RNAi insects in a Relish dependent
262 manner (Figs. 2D and E), mirrors previous findings in *Drosophila*, demonstrating that a
263 hypoxic environment activates the expression of AMPs via the Toll and IMD pathways (42,
264 43).

265

266 Hypoxia could reduce the metabolic activity (43) of the crypt epithelial cells and their
267 capacity to feed the bacterial symbiont, thereby provoking the collapse of the symbiosis.
268 Some insect genes related to glycolysis and most genes of the tricarboxylic acid (TCA) cycle
269 were down-regulated in the M4 after *rDuox*-RNAi, indicating that the metabolic activity of
270 the crypt epithelial cells was indeed lower in *rDuox*-RNAi insect than in Sym insects (*SI*
271 *Appendix*, Fig. S4A). Furthermore, hypoxia is expected to affect also directly the proliferation
272 of the *Burkholderia* symbiont. Species of the genus *Burkholderia* are obligate aerobic
273 bacteria, although they can tolerate microaerobic and anaerobic conditions (44–46).
274 Accordingly, the *Burkholderia* symbiont grew up normally in normoxia (21% O₂) but did not
275 grow well in hypoxia (6-12% O₂) and anoxia (<0.1% O₂) (*SI Appendix*, Fig. S4B). However,
276 the bacteria incubated first in hypoxia or anoxia grew up again when transferred to normoxia
277 and cultured for an additional 48 hours (*SI Appendix*, Fig. S4B), suggesting that *B.*
278 *insecticola* needs enough oxygen for proliferation *in vitro* as well as in the midgut crypts.
279 Furthermore, a *B. insecticola* mutant in the *resB* gene, essential for cytochrome c maturation
280 and respiration, cannot properly colonize the M4 crypts (Fig. 4C). A reduced flux of oxygen
281 and nutrients into the lumen of the crypts of *rDuox*-RNAi insects could create a rapid
282 depletion of these molecules resulting in a gradient from the surface of the crypts towards
283 their interior. Such an oxygen and nutrient gradient could explain the presence of a relatively
284 high number of symbionts close to the epithelial surface of the midgut crypts, and a lack of

285 them in the interior part of the crypt lumen, as observed by TEM (Fig. 1F). Thus, the
286 symbiosis defect in the *rDuox*-RNAi insects is likely due to a lack of sufficient oxygen in the
287 symbiotic organ that can sustain the respiratory activity of the symbiont.

288

289 Conversely, the high bacterial respiration in the M4 is the probable trigger of the extensive
290 tracheal network in Sym insects. *B. insecticola* mutants in *fliC*, encoding the flagellin protein,
291 and *uppP*, involved in cell wall synthesis, cannot colonize the M4 (7, 47), while a mutant in
292 *purL*, involved in purine biosynthesis, can only weakly colonize the crypts (48) (Fig. 4C).
293 The formation of tracheae was not or less stimulated in insects infected with these mutants
294 (Fig. 4C), confirming that the mere presence of *Burkholderia* in the midgut is not sufficient
295 and that bacterial proliferation in the M4 crypts is required to trigger tracheal development.

296

297 **Increased oxygen supply compensates the lack of tracheal development and restores**
298 **symbiosis in *rDuox*-RNAi insects**

299 Next, we confirmed the crucial role of oxygen in gut symbiosis by rearing Sym and *rDuox*-
300 RNAi insects in hypoxia (6-12% O₂), normoxia (21% O₂) and hyperoxia (39-42% O₂)
301 conditions (*SI Appendix*, Fig. S4C). The GFP signal of gut symbionts was diminished in Sym
302 insects reared in a low oxygen environment (Fig. 4D). In agreement with the microscopy
303 observation, qPCR confirmed the decreased number of *Burkholderia* in the M4 crypts (*SI*
304 *Appendix*, Fig. S4D). In contrast, when Sym insects were reared in hyperoxia, the number of
305 gut-colonizing symbionts was not significantly increased, suggesting that the oxygen supply
306 to maintain symbiosis is sufficient in normoxia (Figs. 4D and *SI Appendix*, Fig. S4D).

307

308 Contrary to Sym insects reared in normoxia, those reared in hyperoxia did not much develop
309 an enhanced tracheal network in the symbiotic organ compared to Apo insects (Fig. 4E).

310 These results indicate that the available oxygen in the hyperoxia condition is sufficient to
311 support symbiosis without the need for more tracheae and that the high oxygen consumption
312 by symbiont colonization in the midgut is the trigger of the enhanced tracheal development in
313 Sym insects in normoxia.

314

315 As described above, *rDuoX*-RNAi insects harbor only few symbiotic bacteria under normoxia,
316 and when they were reared in hypoxia, their symbiont titer was decreased even further (Figs.
317 4D and *SI Appendix*, Fig. S4D). However, the M4 crypt population of gut symbionts in
318 *rDuoX*-RNAi insects considerably recovered in a hyperoxia environment (Figs 4D and *SI*
319 *Appendix*, Fig. S4D). These results demonstrate that the availability of sufficient oxygen in
320 the symbiotic organ is critical for a successful symbiosis and that a deficient oxygen supply is
321 the major factor underpinning the symbiotic defect of *rDuoX*-RNAi insects.

322

323 **Silencing of regulators of tracheal development simulates the collapse of symbiosis**

324 To confirm the importance of oxygen distribution by the respiratory network in gut symbiosis,
325 we silenced *branchless* (*rBnl*) and *trachealess* (*rTrh*), two known genes important for the
326 tracheal development in *Drosophila* (49–51), as well as *rSima*, encoding the Hypoxia-
327 Inducible Factor (HIF)-1 α , which is a central regulator of the transcriptional response to
328 hypoxia (52) and which controls the expression of *Bnl* and *breathless* (*Btl*), another tracheal
329 regulator (53). Thus, *Sima*, *Trh*, *Bnl* and *Btl* constitute a regulatory module that controls
330 tracheal development under low oxygen. The *rTrh*-RNAi insects showed a high mortality (*SI*
331 *Appendix*, Fig. S5B), but the alive insects and those silenced for the other two genes had an
332 underdeveloped respiratory network in the M4 (Fig. 5), in agreement with the expected role
333 of *rBnl* and *rTrh* and further showing that oxygen-depletion signaling via *Sima* controls the
334 tracheal development in the symbiotic organ. Crucially, the defect in the tracheal

335 development in these RNAi insects is accompanied with a failure to establish the symbiosis
336 with a full infection of the crypts as revealed by the strongly decreased GFP-signal derived
337 from gut symbionts in the symbiotic organ (Fig. 5 and *SI Appendix*, Fig. S5A). Thus, the
338 downregulation of these three regulators of the respiratory system affected symbiosis in a
339 very similar way as rDuox-RNAi. The enhanced expression of *rSima*, *rBnl* and *rBtl* in the
340 crypts of the *rDuox*-RNAi insects (Fig. 4B) is in agreement with *Sima* being still active after
341 *rDuox* RNAi and thus with rDuox acting downstream of the *Sima/Btl/Trh/Bnl* regulatory
342 module.

343

344 **Discussion**

345 Our results demonstrate that the large population of the *Burkholderia* symbiont present in the
346 body of the bean bug stimulates the sprouting of tracheal branches towards the symbiont-
347 infected M4 crypts. We highlight a previously unknown function of the Duox enzyme in this
348 process, catalyzing the formation of a newly discovered tracheal DTN, which is essential for
349 the stabilization of the respiratory network (Fig. 6). The tracheal system consists of a tubular
350 epithelial network containing an extracellular luminal chitin and protein matrix (39, 40).
351 Chitin is an essential component of this matrix, providing rigidity to the tracheal tubes, and
352 mutants in chitin synthesis genes form misshapen tracheal tubes in *Drosophila* (41, 54). The
353 matrix contains also proteins, including the zona pellucida domain proteins Piopio (Pio) and
354 Dumpy (Dp), chitin-binding and remodeling proteins and other unidentified protein
355 components. They are thought to provide, together with the chitin scaffold, a structural
356 network in the luminal space, regulating tube length, width, branching and integrity (55–57).
357 The requirement for Duox in the formation of new tracheae indicates that dityrosine-cross-
358 linking these matrix proteins is crucial for their structural function. Similar as in *R. pedestris*,
359 we found that the expression of *Duox* is much higher in the tracheae than in the midgut in the

360 phylogenetically diverse insects *Gryllus bimaculatus* (Orthoptera), *Bombyx mori*
361 (Lepidoptera), *Tribolium castaneum* (Coleoptera), and *D. melanogaster* (Diptera) (*SI*
362 *Appendix*, Fig. S6). This conserved expression pattern suggests that the role of Duox in
363 tracheae is common in the insects.

364

365 The here proposed pivotal role of Duox-mediated ROS in the formation of the tracheal
366 cuticle adds up to a number of other structures composed of extracellular matrices requiring
367 Duox for DTN formation. These structures include the earlier mentioned peritrophic matrix
368 of the gut in the mosquito *A. gambiae* as well as in the tick *Ixodes scapularis* (23, 24), the
369 eggshell of the kissing bug (58), the cuticle structure of wings in *Drosophila* (59, 60), the
370 collagenous extracellular cuticle matrix that forms the outer covering of nematodes (61) and
371 the hardened extracellular matrix of sea urchin eggs after fertilization (62). Therefore, the
372 importance of Duox in forming DTNs as part of the biogenesis of extracellular cuticle
373 structures is conserved and not limited to the insects. This function could be the ancestral and
374 primary function of Duox enzymes.

375

376 The formation of dityrosine cross-links in cuticle requires a NADPH oxidase to generate
377 H₂O₂ and a heme peroxidase to catalyze the formation of a covalent bond between two
378 neighboring tyrosine residues via tyrosyl radical formation and the reduction of the H₂O₂.
379 Potentially both these functions are provided by Duox, via its C-terminal NADPH oxidase
380 domain and N-terminal peroxidase homology domain, respectively. In *Caenorhabditis*
381 *elegans*, Duox is such a self-contained catalyst that mediates tyrosine cross-linking
382 independently (63). However, in mosquito, fly and ticks, the peroxidase domain is only
383 partially functional in DTN formation and an additional secreted heme peroxidase protein is
384 required for the formation of the dityrosine cross-links (23, 24, 59). The weak peroxidase

385 activity of Duox in these organisms is believed to result from a mutation in a glutamic acid
386 residue in the N-terminal peroxidase domain, involved in heme binding (24). In the case of
387 the *R. pedestris* rDuox protein, the same point mutation is present, suggesting that the
388 functionality of rDuox may be similar to the tick, fly and mosquito Duox enzymes. Therefore,
389 we predict that the rDuox enzyme works in concert with an additional, unidentified heme
390 peroxidase to form the dityrosine bridges in the matrix proteins.

391

392 Often, the production of bactericidal ROS is presented as the principal function of Duox in
393 insect physiology (14, 15, 22). In *Drosophila*, knock-down of *Duox* or *Mesh*, a positive
394 regulator of *Duox* expression, leads to an increase of the gut microbiome load or to the
395 persistence in the gut after oral infection of the entomopathogenic bacterium *Erwinia*
396 *carotovora* ssp. *carotovora* strain *Ecc15* (20, 64). A similar role in direct killing of gut
397 microbes was proposed in *C. elegans* (19). In striking contrast, we did not observe after
398 *Duox*-RNAi in the bean bug an increase of the pathogen *S. marcescens* in the M3 digestive
399 region of the midgut or of the symbiont in M3 or the M4 crypts (Figs. 1E, 2B and 2C). On the
400 contrary, we noticed a strongly reduced symbiotic population in M4 (Figs. 1E and F). In the
401 mosquito *A. gambiae* and the tick *I. scapularis*, silencing *Duox* or the gene encoding the
402 Duox partner-peroxidase, decreased the microbial load in the midgut rather than increasing it
403 (23, 24). Together, these results indicate that the direct bactericidal activity of Duox as
404 proposed originally in *Drosophila* is not universal in the insects and other arthropods. In light
405 of this conclusion, we propose that the exclusive emphasis on the role of Duox in the
406 production of antimicrobial ROS in the gut of *Drosophila* (14, 15, 22) should be revisited. It
407 is likely that *Drosophila* Duox is involved also in the synthesis of the peritrophic matrix in
408 the midgut and contributes through this activity as well to gut immunity. In agreement with
409 this hypothesis, a mutation of the *Drosophila dcy* gene, encoding the Drosocrystallin chitin-

410 binding protein, results in a thinner but not completely abolished peritrophic matrix, which
411 renders the flies more sensitive to gut infections with entomopathogenic bacteria (65, 66).
412 This phenotype reminds the Duox RNAi flies although the latter were more severely affected
413 in the immune response (12). Transglutaminase is another enzyme that affects protein-protein
414 cross-linking and permeability in the peritrophic matrix of *Drosophila*, as well as the
415 tolerance of the flies to the gut microbiota (67). Thus, Duox could have a dual contribution to
416 the gut immune homeostasis in the case of *Drosophila*. On the other hand, the load of aerobic
417 bacteria in the *Drosophila* midgut is much lower than in the *R. pedestris* symbiotic organ (68).
418 Therefore, Duox-mediated development of tracheae enveloping the midgut could possibly
419 have a lesser importance for sustaining the microbiota or permitting pathogen development in
420 *Drosophila*.

421

422 Nutritional cues, via insulin-like and neuropeptide signaling, give rise to changes in the
423 tracheal architecture in the *Drosophila* midgut (69). Conversely, trachea-derived morphogens
424 control midgut development by stimulating intestinal stem cells (70). Therefore, we can
425 speculate that bacterial or host signals, for example warning for enhanced nutritional
426 requirements, are the triggers for the formation of the respiratory network enveloping the
427 crypts, while simultaneously, the growing tracheae produce morphogens that allow the
428 swelling and opening of the crypts needed for bacterial colonization. On the other hand,
429 hypoxia is the major known regulator of tracheal plasticity in *Drosophila* (53, 71). Therefore,
430 low oxygen levels created by the metabolic activity of the proliferating symbionts and the
431 crypt epithelial cells that nurture the symbiont, could be the direct cause of tracheae genesis
432 (Fig. 6). In agreement with the view of the symbiotic organ as an oxygen sink, we find no
433 symbiosis-stimulated development of tracheae in insects reared in high oxygen levels (Fig.
434 4E) or when the insects were infected with symbiont mutants that have weak or no crypt

435 colonization capacity (Fig. 4C). Moreover, Sima, Trh and Bnl are required for tracheae
436 development in a similar way as Duox. The *bnl* gene in *Drosophila* encodes a fibroblast
437 growth factor homolog whose expression is highly increased in oxygen-starved non-tracheal
438 cells. Bnl exerts chemoattraction of tracheal cells via binding to the trachea-specific receptor
439 Btl that controls the ramification of tracheae towards the Bnl-producing cells (49, 71). The
440 transcription factor Trh is a tracheal cell identity regulator, controlling trachea-specific gene
441 expression, including *btl* (50, 51). The Hypoxia-Inducible Factor (HIF)-1 α homolog Sima in
442 *Drosophila* is a central regulator of the transcriptional response to hypoxia. The Sima
443 transcription factor is actively degraded in normoxia, dependent on the oxygen sensor Fatiga,
444 but is stable and active in hypoxia (52, 72). Active Sima in hypoxia up-regulates the
445 expression of *btl* in tracheal cells and *bnl* in non-tracheal cells, thereby stimulating the
446 development of the respiratory network (53). Thus in *R. pedestris*, oxygen depletion in the
447 symbiotic organ triggered by symbiont colonization is potentially enough as a signal to
448 stabilize Sima in both the crypt epithelia and the local tracheal cells. Sima subsequently
449 activates the transcription of respectively *rBnl* and *rBtl*, the latter necessitating also the
450 tracheae-specific transcription factor Trh. This signaling cascade controls the expression of
451 the genes required for the development of the tracheal network around the crypts, which most
452 likely also include *rDuox*.

453

454 In a remarkable analogous process to the tracheal development in the colonized midgut of *R.*
455 *pedestris*, colonization of the mammalian small intestine by microbiota promotes
456 angiogenesis and the development of the blood vessel network around the gut epithelia. This
457 enhanced vascularization increases oxygenation of the villi, which have a modified
458 morphology after colonization and an increased nutrient absorption activity (73–75). Thus
459 from insects to mammals, colonization of the gut by symbionts can create a local oxygen sink,

460 triggering an analogous postembryonic developmental response.

461

462 Besides the midgut crypts of *R. pedestris* and related stinkbugs, other types of symbiotic
463 organs in other insects, such as bacteriomes (9, 76), are equally enveloped by a tracheal
464 network, presumably to provide oxygen to the respiring symbiotic bacteria (77, 78). These
465 symbioses might therefore depend on Duox activity as well. Feeding *Drosophila* and *C.*
466 *elegans* with different types of antioxidant chemicals inhibits Duox-produced ROS (19, 64).
467 Here, we show that feeding *R. pedestris* with the antioxidant NAC is sufficient to prevent the
468 accumulation of ROS produced by Duox and the sprouting of the tracheae and this blocks the
469 establishment of symbiosis. Since many insects obligatorily depend on their symbioses,
470 triggering its collapse by the specific inhibition of the respiratory network with antioxidants
471 could be a new route to fight insect pests.

472

473 **Materials and Methods**

474 **Insect rearing and infection with symbiont**

475 The *R. pedestris* strain used in this study was originally collected from a soybean field in
476 Tsukuba, Ibaraki, Japan, and maintained in the laboratory for over ten years. The bean bugs
477 were reared in Petri dishes (90 mm in diameter and 20 mm high) under a long-day regimen
478 (16 h light and 8 h dark) at 25°C and fed with soybean seeds and distilled water containing
479 0.05% ascorbic acid (DWA). To infect insects with symbionts, *B. insecticola* (RPE75), GFP-
480 expressing *B. insecticola* (RPE225), or mutant *Burkholderia* strains (*SI Appendix*, Table S1)
481 were orally administrated to second instar nymphs by supplying the bacteria at a density of
482 10^7 cells/ml to the DWA. To feed NAC, DWA with 10 mg/ml of NAC was supplied to the
483 bean bugs. *T. castaneum* was a gift of Dr. Ryo Futahashi (AIST Tsukuba Center, Japan). *G.*
484 *bimaculatus* and *B. mori* were purchased from a pet shop (Sapporo, Japan).

485

486 **Detection of ROS levels**

487 Fourth instar nymphs of bean bugs were dissected in phosphate-buffered saline (PBS), pH 7.4,
488 using fine forceps under a dissection microscope (S8APO, Leica; MZ FZ III, Leica), and
489 each part of their midguts was transferred to 1.5 ml microcentrifuge tubes and homogenized
490 by a pestle. The homogenized midgut samples were centrifuged at 15,000 rpm for 1 min to
491 remove tissue debris, and the supernatant was transferred to the 96-well black polystyrene
492 microplate and incubated with 50 μ M 2',7'-dichlorofluorescein diacetate (DCF-DA) (Thermo
493 Fisher Scientific) for 20 min in the dark. The ROS level in each sample was measured by a
494 microplate reader, Infinite F200 PRO (Tecan), using an excitation wavelength of 488 nm and
495 an emission wavelength of 535 nm.

496

497 **Synthesis of dsRNA and RNA-interference**

498 Gene sequences of *Duox*, *rRel*, *rTrh*, *rBnl* and *rSima* were identified by an RNA-sequencing
499 analysis of symbiotic insects and BLAST identification using the *Drosophila* proteins as
500 search term (Accession number MT270146-MT270150). Fragments of target genes for RNAi
501 knock down were obtained from *R. pedestris* cDNA. The fifth instar nymphs of bean bugs
502 were dissected in PBS and total RNA was extracted from the M4 using the RNAiso Plus
503 (Takara Bio Inc) and the RNeasy Mini Kit (Qiagen Inc). The cDNA was synthesized from 1
504 μ g of extracted total RNA by PrimeScriptTM RT reagent Kit with gDNA Erase (Takara Bio
505 Inc).

506

507 To synthesize dsRNA for RNAi, approximately 500 bp fragments of the target gene were
508 amplified from cDNA with primers listed in *SI Appendix*, Table S2. The PCR products were
509 cloned in the pT7Blue T-vector and transformed into *Escherichia coli* DH5a cells. Clones

510 were verified by sequencing. Next, the target gene was amplified by PCR from these
511 plasmids using specific upstream and downstream primers, each extended with the T7
512 promoter sequences (*SI Appendix*, Table S2), to create a template for RNA polymerization.
513 The dsRNA was synthesized from these PCR products by the MEGAscript™ RNAi kit
514 (Thermo Fisher Scientific).

515

516 To silence a target gene, 100 ng of the corresponding dsRNA was injected into the
517 hemolymph, between the torso and hind leg of the symbiont-infected second instar nymph of
518 bean bugs after 2 days of symbiont infection, using a glass capillary and a Femtojet
519 microinjector (Eppendorf). For triggering a septic injury, 10^7 *E. coli* DH5a cells were
520 similarly injected in the hemolymph. The phenotypic alteration in the insects was observed a
521 week after RNAi. The detailed timeline of the experiment is described in (*SI Appendix*, Fig.
522 S7).

523

524 **Estimation of infection levels of symbiotic organs by quantitative PCR**

525 To measure the number of symbionts present in the M4, DNA was extracted from the
526 dissected M4 by the QIAamp DNA Mini Kit (Qiagen Inc) and real-time quantitative PCR
527 was performed using primers BSdnaA-F and BSdnaA-R (*SI Appendix*, Table S2), which
528 target the *dnaA* gene of the *Burkholderia* symbiont, the KAPA SYBR FAST qPCR Master
529 Mix Kit (KAPA Biosystems Inc) and the Roche LightCycler® 96 System (Roche). The
530 number of gut symbionts was calculated based on a standard curve for the *dnaA* gene
531 containing 10 , 10^2 , 10^3 , 10^4 , 10^5 , 10^6 , and 10^7 copies per reaction of the target PCR product.

532

533 **Gene expression determination by reverse transcriptase-quantitative PCR**

534 To measure the mRNA expression level of the target genes, total RNA was extracted from the

535 the salivary gland, the midgut regions, the fat body, or the tracheae of the Apo and Sym bean
536 bugs and reverse transcribed to cDNA as described above. The real-time quantitative PCR for
537 target genes was performed as described above using primers listed in *SI Appendix*, Table S2.
538 The efficiency of the RNAi process on target genes and change of gene expression patterns of
539 immune genes was measured three days after RNAi (*SI Appendix*, Fig. S7). The mRNA
540 levels were normalized by the elongation factor 1-alpha (EF1 α) housekeeping gene.

541

542 **Microscopy analyses**

543 To analyze the infection level of control, RNAi or NAC treated insects, the bean bugs were
544 infected with GFP-expressing *Burkholderia* symbionts and injected with dsRNA or fed with
545 NAC (*SI Appendix*, Fig. S7). After a week, the M4 organs of fourth instar bean bugs were
546 dissected in PBS, transferred to a glass-bottom dish (Matsunami) and covered with a cover
547 glass. The GFP-signal derived from the M4-colonizing gut symbionts was observed with an
548 epifluorescence microscope (DMI4000B, Leica).

549

550 Alternatively, to observe the colonization of the M4 by the gut symbiont with confocal
551 microscopy, the bean bugs harboring GFP-expressing symbionts were dissected in PBS and
552 the midgut was transferred to 2 ml microcentrifuge tube. The gut samples were fixed with 4%
553 paraformaldehyde (pH 7.4) for 10 min at 25°C. The fixed samples were washed three times
554 by PBS and permeabilized by PBS with 0.1% Triton X-100 (PBST) for 10 min at 25°C. After
555 permeabilization, the gut samples were washed again three times by PBS and stained with
556 4',6-diamidino-2-phenylindole (DAPI) and Alexa Fluor™ 647 Phalloidin for 30 min at 25°C,
557 to stain respectively the nucleus and cytoskeleton of host cells. Then, the samples were
558 transferred to a glass-bottom dish, mounted with ProLong™ Gold Antifade Mountant
559 (Thermo Fisher Scientific), and observed under a laser scanning confocal microscope (TCS

560 SP8, Leica).

561

562 Dityrosine networks were revealed by immunostaining. The whole gut of the fourth instar of
563 control or RNAi bean bugs was dissected in PBS and fixed with 4% paraformaldehyde. The
564 fixed midgut was permeabilized and incubated with blocking buffer (1% BSA in PBST) for
565 30 min at 25°C. Then, the gut was stained with 1:400 dilution of anti-dityrosine monoclonal
566 antibody (MyBioScience) overnight at 4°C. Subsequently, the midgut was washed three
567 times by PBS, each for 10 min, and then stained with 1:500 dilution of goat anti-mouse IgG
568 H&L conjugated with Alexa Fluor[®] 555 (Abcam) for 1 hour at 25°C in the dark. The midgut
569 was washed three times by PBS, each for 10 min, and then incubated with DAPI and
570 phalloidin. The midgut was transferred to a glass-bottom dish, mounted, and observed with a
571 laser scanning confocal microscope. The 3D image of the dityrosine network was
572 reconstructed from z-stacked images using the Leica LAS X software.

573

574 To observe the tracheal network by chitin fluorescent labelling, dissected midguts were
575 stained with the fluorescent brightener 28 (Sigma-Aldrich) at a final concentration of 0.1
576 mg/ml for 5 min in the dark. Then, the midgut was washed three times by PBS, transferred to
577 a glass-bottom dish, and observed with a confocal or epifluorescence microscope.

578

579 To analyze the hypoxic condition of the midgut, dissected M4 were stained with 10 μ M
580 Image-iT[™] Green Hypoxia Reagent for 30 min at 25°C and observed with a confocal
581 microscope. To label active mitochondria, dissected midguts were incubated with 100 nM
582 MitoTracker[™] Green FM for 30 min at 25°C and observed with a confocal microscope.

583

584 For transmission electron microscopy (TEM) observations, the bean bugs were dissected with

585 fine forceps in the fixative solution (0.1 M sodium phosphate buffer containing 2.5%
586 glutaraldehyde, pH 7.4). The isolated M4 was pre-fixed in the fixative solution at 4°C
587 overnight and post-fixed in the 2% osmium tetroxide at 4°C for 1 hour. After a series of
588 dehydration steps by ethanol, the gut was embedded in Epon812 resin (TAAB Ltd). Ultrathin
589 sections were made by using an ultramicrotome (EM UC7, Leica), mounted on a copper
590 mesh, stained with uranyl acetate and lead citrate, and observed under a transmission electron
591 microscope (H-7600, Hitachi).

592

593 **Colony forming unit assay**

594 Cultured *S. marcescens* (10^7 cells/ml) were orally administrated to the third instar of Sym and
595 *rDuox*-RNAi bean bugs. The midgut M3 regions were dissected 3 days after infection and
596 homogenized with a pestle in 100 µl of PBS. After the serial dilution of these M3-lysates, 10
597 µl of the dilutions were spotted on a LB agar plate with 30 µg/ml of rifampicin and plates
598 were incubated at 30°C for 1 day to obtain bacterial growth. To measure the symbiont titer in
599 the M3 of Sym and *rDuox*-RNAi bean bugs, dsRNA of *rDuox* was injected to second instar
600 nymphs of Apo insects, and then newly molted third instar nymphs were infected with
601 cultured *Burkholderia* symbionts (10^7 cells/ml) and the M3 was dissected 24 h after infection.
602 The dissected M3 was homogenized and dilution series were spotted on a YG agar plate with
603 30 µg/ml of rifampicin, which was incubated for 2 days at 30°C.

604

605 **Bacterial growth and insect rearing in hypoxia and hyperoxia conditions**

606 To culture the *Burkholderia* symbiont in anaerobic or microaerophilic conditions, agar plates
607 were placed in a 2.5 L W-zip standing pouch jar (Mitsubishi Gas Chemical), containing
608 respectively an Anaero Pack-Anaero ($O_2 < 0.1\%$) or an Anaero Pack-MicroAero ($6\% < O_2 <$
609 12%) (Mitsubishi Gas Chemical). Plates were incubated for 48 h at 30°C and subsequently

610 for a further 48 h at 30°C in normoxia condition.

611

612 To rear insects in a hypoxia environment, Petri dishes containing second instar nymphs were
613 placed in the 2.5 L rectangular jar containing an Anaero Pack-MicroAero. To create the
614 hyperoxia condition, Petri dishes containing second instar nymphs were placed in a culture
615 jar and oxygen was injected daily via a valve in order to maintain 39-42% oxygen as shown
616 in (*SI Appendix*, Fig. S4C). The oxygen content (%) in the jar was monitored by an oxygen
617 indicator OXY-M (JICKO).

618

619 Additional methods are provided in the *SI Appendix*, Supplementary text.

620

621 **Acknowledgements**

622 We thank H. Ooi (AIST) for insect rearing; X.-Y. Meng and M. Kanno (AIST) for help with
623 electron microscopy; and Y. Matsuura (The University of the Ryukyus), K. Takeshita (Akita
624 Prefecture University), and S. Kanie (AIST) for critical comments on the manuscript. This
625 study was supported by JSPS Research Fellowships for Young Scientists to SJ (201911493)
626 and to TO (20170267 and 19J01106); by the Ministry of Education, Culture, Sports, Science
627 and Technology (MEXT) KAKENHI to YK (18KK0211 and 20H03303); and by a JSPS-
628 CNRS Bilateral Open Partnership Joint Research Project and the CNRS IRP project “Ménage
629 à Trois” to YK and PM.

630

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820 **Figure legends**

821 **Fig. 1. rDuox-mediated ROS is important for the gut symbiosis.** (A) An adult male *R.*
822 *pedestris* feeding on soybeans. (B) The dissected midgut of *R. pedestris*. (C) Relative ROS
823 level in the different midgut regions of Apo (Apo^{control}), Sym (Sym^{control}), *rDuox*-RNAi
824 (Sym^{rDuox-RNAi}), and NAC-fed (Sym^{NAC}) insects. All values were normalized by the ROS
825 level of Apo insects. (D) The predicted domains of the rDuox protein (top). Maximum
826 likelihood phylogeny based on amino acid sequences (bottom). Numbers in parentheses
827 indicate accession numbers. Bootstrap values (> 60%) are shown near each node. (E) The
828 number of symbionts colonizing the M4. Data shown in (C and E) are mean ± s.d. *n* = 9 and
829 6 insects, respectively. Error bars indicate statistically significant differences (*P* < 0.05). The
830 statistical significance of differences between samples was analyzed by a Kruskal-Wallis test
831 with Bonferroni correction. (F) Microscopy images of the M4. Epifluorescence microscopy
832 (first row). Laser scanning confocal microscopy (second row). Green, symbionts; blue, host
833 nuclei; red, host cytoskeleton; white arrowheads, midgut crypts. Transmission electron
834 microscopy (third row). lu; luminal region of the midgut crypt.

835 **Fig. 2. rDuoX function is not related to gut mucosal immunity in the bean bug. (A)** The
836 Survival rate of Sym (Sym^{control}) and *rDuoX*-RNAi (Sym^{rDuoX-RNAi}) insects after oral infection
837 with 10⁷ cells/ml of *Serratia marcescens*. *n* = 20 insects. **(B)** Number of *S. marcescens*
838 colonies from the M3 region of third instar nymph Sym^{control} and Sym^{rDuoX-RNAi} insects. **(C)**
839 The number of *Burkholderia* symbionts in the M3 of the third instar nymph Sym^{control} and
840 Sym^{rDuoX-RNAi} insects; CFU, colony-forming units. **(D)** Expression profile from RNA-seq data
841 of immunity-related genes in the M4 of Sym^{control} and Sym^{rDuoX-RNAi} insects. Each column is a
842 replicate experiment. **(E)** The expression level of AMPs in the M4 of Sym insects without
843 immune stimulation (Sym^{control}), Sym^{control} with septic injury by *E. coli* injection in the
844 hemolymph (Sym^{control-E. coli}), *rRelish*-RNAi with *E. coli* injection (Sym^{rRelish-RNAi-E.coli}),
845 *rDuoX*-RNAi without bacterial injection (Sym^{rDuoX-RNAi}), and double *rDuoX/rRelish*-RNAi
846 insects without bacterial injection (Sym^{rDuoX/rRelish-RNAi}). **(F)** The expression level of AMPs in
847 the fat body. Data shown in **(B and C)** are *n* = 5 and **(E and F)** are *n* = 4 insects. Different
848 letters indicate statistically significant differences (*P* < 0.05). The statistical significance was
849 analyzed by the Mann-Whitney *U* test **(B and C)** or Kruskal-Wallis test with Bonferroni
850 correction **(E and F)**. Error bars indicate SDs; ns, not statistically significant. **(G)** Effect of
851 *rDuoX/rRelish*-double RNAi on symbiosis. Green color indicates the GFP signal from gut-
852 colonizing *Burkholderia* symbionts.

853 **Fig. 3. Symbiont colonization triggers tracheal development and *rDuox*-mediated ROS**
854 **stabilizes it by forming a dityrosine network. (A)** The relative expression of *rDuox* in
855 different tissues of Apo and Sym insects. Data shown are mean \pm s.d. $n = 4$ insects. Different
856 letters indicate statistically significant differences ($P < 0.05$). The statistical significance of
857 differences between samples was analyzed by a Kruskal-Wallis test with Bonferroni
858 correction. **(B)** 3D image of the tracheal network in the M4 of a Sym^{control} insect. **(C)** Staining
859 of the chitin and dityrosine network (DTN) of tracheae detached from the M4. **(D)** The
860 tracheal network in the M4 of Apo (Apo^{control}), Sym (Sym^{control}), *rDuox*-RNAi (Sym^{rDuox-RNAi}),
861 and NAC-fed (Sym^{NAC}) insects. The tracheal network, stained with fluorescent brightener 28
862 (calcofluor-white) to visualize chitin (first row); DTN in the internal section of the M4
863 (second row); DTN located on the surface of the M4 (third row); 3D reconstitution of DTN
864 of the tracheal network (fourth row). The colors in the images of panels **(B-D)** are: white,
865 chitin; yellow, the DTN; red, host cytoskeleton; blue, host nuclei; green, GFP signal derived
866 from gut-colonizing symbionts.

867 **Fig. 4. Destabilization of the respiratory network by rDuox-RNAi causes hypoxia in the**
868 **midgut. (A)** Active mitochondria in Sym (Sym^{control}) and *rDuox*-RNAi (Sym^{rDuox-RNAi})
869 insects stained by Mitotracker, a membrane potential fluorescence dye, which stains active
870 mitochondria in live cells. Green, Mitotracker; blue, host nuclei (top). Hypoxia in the M4
871 revealed by staining with the Image-IT hypoxia fluorescence dye, a real-time oxygen detector
872 (green, bottom). **(B)** Heatmap of relative expression levels of genes related to the host
873 respiratory chain, *Sima*, *Branchless* (bnl), and *Breathless* (btl) in Sym^{control} and Sym^{rDuox-RNAi}
874 insects. Each column is a replicate experiment. **(C)** Colonization level of the wild type, *resB*-,
875 *purL*-, *uppP*-, and *fliC*-deficient *B. insecticola* mutants in the symbiotic organ (top panel).
876 The DTN of the tracheal network from the symbiotic organs infected with wild type and
877 *Burkholderia* mutants (lower panel). Yellow color indicates the DTN signal of the tracheal
878 network. **(D)** Observation by epifluorescence microscopy of the midgut of Sym^{control} and
879 Sym^{rDuox-RNAi} insects reared in hypoxia (6-12% O₂), normoxia (21% O₂), and hyperoxia (39-
880 42% O₂) environments. Green is the GFP signal derived from *Burkholderia* gut symbionts.
881 **(E)** Tracheal network in the M4 of Apo^{control} and Sym^{control} insects reared under the normoxia
882 and hyperoxia condition.

883 **Fig. 5. Oxygen supply to the symbiotic organ via the tracheal network is important for**
884 **gut symbiosis.** Chitin staining (first row), 3D reconstruction of the dityrosine network
885 (second row), colonization by *Burkholderia* (third row) and crypt morphology (fourth row) in
886 the M4 of Sym (Sym^{control}), *rBnl*-RNAi (Sym^{rBnl-RNAi}), *rTrh*-RNAi (Sym^{rTrh-RNAi}), and *rSima*-
887 RNAi (Sym^{rSima-RNAi}) insects. Images were obtained by epifluorescence microscopy in the
888 first and third row and by laser scanning confocal microscopy in the second and fourth rows.
889 White, chitin; yellow, DTN; green, gut symbiont; blue, host nuclei; red, host cytoskeleton.

890 **Fig. 6. rDuox mediates symbiosis and tracheal network stability.** Tracheal network in the
891 M4 before symbiont infection (top right). Development of the tracheal network is stimulated
892 to supply oxygen to the M4 when colonized by the symbiont, which consumes high levels of
893 oxygen (middle left and right); red, newly ramified tracheoles; green, gut symbiont; grey
894 epithelial cells experiencing reduced oxygen levels and attracting growing tracheoles. The
895 branched tracheoles are stabilized by *rDuox* (bottom right). *rDuox* stabilizes the DTN of the
896 tracheal cuticle layer, which is critical for structurally maintaining trachea (bottom left). The
897 developmental order is in the direction of the arrows.











