1 Original Research Article

2 **Ploidy dynamics in aphid host cells harboring bacterial symbionts**

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24

25 **Abstract**

26 Aphids have evolved bacteriocytes or symbiotic host cells that harbor the obligate
 27 mutualistic bacterium *Buchnera aphidicola*. Because of the large cell size (approximately
 28 100 μm in diameter) of bacteriocytes and their pivotal role in nutritional symbiosis,
 29 researchers have considered that these cells are highly polyploid and assumed that
 30 bacteriocyte polyploidy may be essential for the symbiotic relationship between the aphid
 31 and the bacterium. However, little is known about the ploidy levels and dynamics of
 32 aphid bacteriocytes. Here, we quantitatively analyzed the ploidy levels in the
 33 bacteriocytes of the pea-aphid *Acyrtosiphon pisum*. Image-based fluorometry revealed
 34 the hyper polyploidy of the bacteriocytes ranging from 16- to 256-ploidy throughout the
 35 lifecycle. Bacteriocytes of adult parthenogenetic viviparous females were mainly
 36 64-128C DNA levels, while those of sexual morphs (oviparous females and males) were
 37 consisted of 64C, and 32-64C cells, respectively. During post-embryonic development of
 38 viviparous females, the ploidy level of bacteriocytes increased substantially, from 16-32C
 39 at birth to 128-256C in actively reproducing adults. These results suggest that the ploidy
 40 levels are dynamically regulated among phenotypes and during development. Our
 41 comprehensive and quantitative data provides a foundation for future studies to
 42 understand the functional roles and biological significance of the polyploidy of insect
 43 bacteriocytes.

44 **Introduction**

45 Endopolyploidy or somatic polyploidy is generated through endoreduplication cycles, in
 46 which the nuclear genome is repeatedly replicated without mitotic cell division^[1–4].
 47 Polyploid cells are typically observed in tissues with high metabolic demand and are
 48 considered not only to play essential roles in normal development but also to influence
 49 ecologically important traits, such as body or organ size, growth rate, and nutrient
 50 storage^[5, 6]. Polyploidization is often attributed to increasing cellular size, metabolic rate,
 51 and gene expression levels owing to the increasing availability of DNA templates for
 52 transcription; these ideas have been supported by experiments in model organisms^[3, 7, 8].
 53 Nevertheless, relatively few empirical studies have focused on endopolyploidy in
 54 evolutionary and ecological contexts^[6].

55 Many insects, because of their unbalanced diet, live in symbiotic associations
 56 with microorganisms, which can provide host insects with nutrients that cannot be
 57 synthesized by insects or obtained from their diets^[9–11]. Such endosymbiotic
 58 microorganisms are frequently harbored in insect-specific cells or tissues
 59 (endosymbiosis; ^[9, 12–14]). So far, many researchers have pointed out that symbiotic host
 60 cells (mycetocytes or bacteriocytes) have large nuclei and thus, would be polyploid in
 61 various insect species^[11–14]. Considering the well-known effects of polyploidy such as
 62 cell enlargement and upregulation of gene expression, we can predict that
 63 polyploidization of symbiotic host cells may have critical roles in endosymbiosis
 64 between insects and microorganisms; however, very few studies have quantified the
 65 ploidy levels of symbiotic host cells in insects. Exceptionally, Nakabachi *et al.* (2010)
 66 revealed that in psyllid insects, *Pachypsylla venusta* (Osten-Sacken), symbiotic host cells
 67 were 16-ploid in both adult males and females^[15]. There are other reports on the ploidy

68 levels of symbiotic host cells, but they are incomplete^[16–18].

69 Aphids (Hemiptera: Aphididae) have evolved symbiotic relationships with the
70 gamma-proteobacterium *Buchnera aphidicola*, which supplies the host with vitamins and
71 essential amino acids that aphids cannot synthesize or derive insufficiently from their diet,
72 the plant phloem sap^[10, 19, 20]. *B. aphidicola* lives within large aphid cells, called
73 “bacteriocytes.” The cells and another type of cells (small and flattened cells, called
74 “sheath cells”) are grouped into organ-like structures, referred to as “bacteriomes”
75 (Figure 1a, b, ^[21]). Bacteriocytes were long thought to be polyploid because of their large
76 cellular and nuclear sizes (ca. 100 and 20 µm in diameter, respectively)^[12, 13, 22]. Moreover,
77 aphid bacteriocytes should be highly metabolically active, because they provide *B.*
78 *aphidicola* with a large amount of essential amino acids and other metabolites that the
79 symbiont can no longer produce, owing to the massive gene losses^[23–25]. Therefore,
80 polyploidy may be a key element in the maintenance of the symbiotic system, as has been
81 shown in between leguminous plants and rhizobium bacteria^[26–28]; however, information
82 about the ploidy levels of aphid bacteriocytes remains scarce.

83 Pea-aphid, *Acyrtosiphon pisum* (Harris), is one of the best-studied aphid species
84 in terms of life history (Figure 2, ^[29, 30]), polymorphism (Figure S1, ^[30–32]), embryonic
85 development^[33–35], nutritional symbiosis^[23–25, 36–38], and genomics^[39–41]. Detailed
86 descriptions have focused on bacteriocyte development during aphid embryogenesis^{[21,}
87 ^{22]}, and it has been revealed that the number and volume of pea-aphid bacteriocytes
88 increase during post-embryonic development (^[38, 42] but see ^[43, 44]). These observations
89 imply that aphid bacteriocytes are already polyploid at the end of embryogenesis and
90 become hyper polyploid during post-embryonic development. However, no quantitative
91 data in this aspect have been reported. It should also be noted that most of the previous

92 studies on aphid bacteriocytes characterized only viviparous insects, excluding other
93 morphs (e.g., oviparous females and males)—presumably because of the ease of rearing
94 viviparous insects in the laboratory (Figure S1).

95 In this study, we investigated the pattern of polyploidization and the cellular
96 features of pea-aphid bacteriocytes. We first observed the cytological features, such as
97 cell size and nucleolar number/size of bacteriocytes from adults of viviparous/oviparous
98 females and males, and other developmental stages of viviparous insects. Then, using
99 image-based fluorometry established in the present study, we determined the ploidy
100 levels of bacteriocytes and compared these levels among morphs (adult
101 viviparous/oviparous females and males). We also performed ploidy analysis on the cells
102 at each stage of post-embryonic development (from first-instar nymphs to senescent
103 adults in viviparous insects). Finally, based on our observation of the cytological features
104 of bacteriocytes, we discussed the potential effects of bacteriocyte polyploidy on the
105 aphid/*Buchnera* intracellular symbiosis.

106

107 **Results**

108 *General observation and methods for ploidy analysis on aphid bacteriome cells*

109 Consistent with previous observations^[9, 21, 22, 40], the bacteriome of viviparous aphids
110 consisted of two types of cells: bacteriocytes and sheath cells (Figure 3). Bacteriocytes
111 contained *Buchnera* cells and were much larger than sheath cells. Sheath cells exhibited a
112 flattened morphology and surrounded the bacteriocytes. Both cell types possessed a
113 single nucleus. Bacteriocytes had a single prominent nucleolus, which was not stained
114 using DAPI, but using “Nucleolus Bright Red” staining (Figure 3). Most sheath cells also
115 had a single nucleolus, yet a small number had two. “Nucleolus Bright Red” also stained

116 the peripheral region of *Buchnera*, probably because of the richness of RNA around
117 *Buchnera* cells.

118 To determine the most suitable methods for ploidy analysis of aphid bacteriocytes,
119 three types of methods, flow cytometry, Feulgen densitometry, and fluorometry were
120 compared. First, flow cytometry successfully detected the nuclei of bacteriome cells and
121 heads, and distinct peaks were present (Figure S2). There were several peaks, which can
122 be categorized as ploidy classes based on head peaks, assuming that the smallest peaks
123 correspond to a diploid population. We recognized peaks up to 256C (256-ploidy) cells
124 but could not distinguish cell types (i.e., bacteriocytes or sheath cells) in this method due
125 to a lack of cytological information. Note that “C” means haploid genome size, for
126 example, 2C = diploid and 8C = octoploid. Second, Feulgen densitometry also showed
127 several ploidy levels of up to 128C (Figure S3) in bacteriocytes. Sheath cells mainly
128 consisted of 16-32C cells. However, we found that many cells were lost during the
129 experimental procedures because the number of observed nuclei was too small.

130 We found the third method, image-based fluorometry for isolated nuclei, the best
131 for quantitative ploidy analysis of aphid bacteriocytes (Figure 4). Fluorometry showed
132 distinct peaks of integrated fluorescence intensity, and they could be categorized as each
133 ploidy class based on the intensity of the smallest peak in head cells (diploid population).
134 The results were consistent with other methods; ploidy levels were 32C-256C in
135 bacteriocytes and 16C-32C in sheath cells. In this analysis, the nucleolus size was used to
136 discriminate between cell types. During cytological observation, we obtained the size
137 distribution of the nucleolus, and it was revealed that the nucleolus of bacteriocytes was
138 always larger than that of sheath cells (Figure S4). Based on the results, we determined
139 the threshold of the size of the nucleolus. More specifically, in viviparous females, nuclei

140 that have nucleoli larger than $20 \mu\text{m}^2$ were categorized into bacteriocytes. Note that the
141 peaks of sheath cells were not distinct or reliable for categorizing their ploidy class;
142 therefore, we showed results focusing on bacteriocytes in the following sections.

143

144 *Cellular features of bacteriome cells in viviparous and oviparous females, and males*

145 The cellular features were generally consistent among adults of three morphs, viviparous
146 and oviparous females, and males (Figure 3). *Buchnera*-absence zones in the cytoplasm
147 of bacteriocytes, which are considered to be degeneration of *Buchnera*^[45], and
148 bacteriocytes degeneration^[46] were both observed more frequently in male bacteriocytes
149 than in females (Figure 3). The cell size of bacteriocytes was significantly different
150 among morphs (LM with type II test, $F = 286.15$, $df = 2$, $p < 0.001$, Figure S5).
151 Viviparous females had significantly larger bacteriocytes [449847.05 ± 21583.0 (mean $\pm$
152 SEM) μm^3 , $n = 22$] than other types of aphids (oviparous females, 298989.9 ± 16196.6
153 μm^3 , $n = 24$, and males, $29020.35 \pm 3001.6 \mu\text{m}^3$, $n = 37$) (Tukey's test, $p < 0.05$, Figure
154 S5). The size of nucleoli was significantly different between bacteriocytes and sheath
155 cells, regardless of aphid morphs (LMM with type II test; viviparous females, $\chi^2 = 618.4$,
156 $df = 1$, $p < 0.001$, oviparous females, $\chi^2 = 1,430.4$, $df = 1$, $p < 0.001$, males, $\chi^2 = 261.37$, df
157 $= 1$, $p < 0.001$, Figure S3). There was no overlap in the nucleolus size between cell types
158 (Figure S4). Based on these data, we determined the threshold of the size of the nucleolus
159 to discriminate between bacteriocytes and sheath cells. Specifically, in viviparous and
160 oviparous females, and males, nuclei that have nucleoli larger than $20 \mu\text{m}^2$, $20 \mu\text{m}^2$, $8 \mu\text{m}^2$,
161 were categorized into bacteriocytes, respectively.

162

163 *Ploidy analysis on the bacteriocyte of viviparous and oviparous females, and males*

164 Ploidy analysis of the adult bacteriocytes revealed that the cells were highly polyploid
165 (from 32C to 256C) in all phenotypes (Figure 5). We found variation in the level of
166 ploidy; bacteriocytes of viviparous females, oviparous females, and males mainly
167 consisted of 64-128C (45% for each), 64C (70%), and 32-64C (30% and 47%),
168 respectively. There were significant differences in the degree of polyploidy (median
169 ploidy level) in bacteriocytes among the three aphid phenotypes (Brunner–Munzel test
170 with Bonferroni adjustment; viviparous females vs. oviparous females, $p < 0.05$;
171 viviparous females vs. males, $p < 0.05$; oviparous females vs. males, $p < 0.05$; Figure 5).

172

173 *Fecundity and longevity of viviparous and oviparous females*

174 Viviparous females in this strain laid 95.25 ± 24.75 (mean $\pm$ SD, $n = 12$) nymphs during
175 their lifetime, while oviparous females oviposited 28.83 ± 6.52 ($n = 12$) eggs (Figure S6).
176 The total number of nymphs laid by viviparous females was significantly higher than that
177 of eggs oviposited by oviparous females (GLMM with type II test, $\chi^2 = 449.74$, $df = 1$, $p <$
178 0.001). Viviparous females lived longer [44.33 ± 4.38 (mean $\pm$ SEM) days] than
179 oviparous ones (26.08 ± 2.31 days), yet there was no interaction between female types
180 and their lifetime (GLMM, with type II test; female type, $\chi^2 = 118.13$, $df = 1$, $p < 0.001$,
181 lifetime, $\chi^2 = 69.32$, $df = 1$, $p < 0.001$, and the interaction $\chi^2 = 0.74$, $df = 1$, $p = 0.39$).
182 Viviparous females started reproducing from days 2-3 and the rate of larviposition peaked
183 during days 3-20 but slowed down during days 21-28. They lived at most 50-55 days,
184 although most of them stopped the larviposition after day 30. In oviparous females, first
185 oviposition and mating with males were observed on days 3-4. They actively laid eggs
186 until day 14, but their death was observed almost simultaneously (Figure. S6).

187

188 *Cellular features of bacteriome cells in each stage of post-embryonic development*

189 At 16 °C, viviparous (and apterous) aphids reached adult stage approximately 14 days

190 after birth [13.73 ± 0.32 (mean $\pm$ SEM), $n = 16$, Figure 6a]. In particular, N1, N2, N3, and

191 N4 periods lasted for 3.2 ± 0.11 , 3.0 ± 0.09 , 3.3 ± 0.12 , and 4.2 ± 0.14 days (mean $\pm$ SEM,

192 $n = 16$, Figure 6a), respectively. Adult aphids started reproducing 2 or 3 days after

193 eclosion (molt for an adult) and continued larviposition for approximately 4 weeks

194 (Figure S6). Based on these data, A7 aphids (7 days after eclosion) could be categorized

195 as actively reproducing individuals. A21 aphids (21 days after eclosion) were categorized

196 as senescent individuals, although they continuously produced offspring. During the

197 nymphal stages of viviparous aphids, the morphology of bacteriome cells was generally

198 consistent; all bacteriocytes and most sheath cells were uninuclear (Figure 6b), but very

199 few of the latter cells had several small nuclei. Notably, there were drastic morphological

200 changes in adult stages; bacteriocyte and sheath cell nuclei of A21 individuals were

201 irregularly shaped in comparison with those of young (A0) and reproducing (A7)

202 individuals. Furthermore, in A21, we frequently observed bacteriocytes in which the

203 signals of DAPI and nucleolus bright red signals on *Buchnera* were weak (Figure 6b).

204 These changes were consistent with symptoms of *Buchnera* degeneration and cell

205 senescence, which have been previously reported ^[45, 46]. Developmental stages had a

206 significant effect on bacteriocyte size (LMM with type II test, $\chi^2 = 338.73$, $df = 6$, $p <$

207 0.001). During post-embryonic development, the size of bacteriocytes consistently

208 increased (Tukey's test: $N1 = N2 = N3 < N4 < A0 = A7 < A21$, $p < 0.05$, Figure S7). The

209 size of nucleoli was significantly different between bacteriocytes and sheath cells,

210 regardless of the post-embryonic developmental stages (LMM with type II test; N1, $\chi^2 =$

211 891.82 , $df = 1$, $p < 0.001$, N2, $\chi^2 = 294.04$, $df = 1$, $p < 0.001$, N3, $\chi^2 = 842.31$, $df = 1$, $p <$

0.001, N_4 , $\chi^2 = 817.18$, $df = 1$, $p < 0.001$, old adults, $\chi^2 = 1,405.6$, $df = 1$, $p < 0.001$, Figure S8). There was no overlap in the nucleolus size between cell types (Figure S8). Based on these data and the data from young adults, we determined the threshold of the size of the nucleolus for ploidy analysis (in N_1 and N_2 , $10 \mu\text{m}^2$, and later stages, $25 \mu\text{m}^2$).

Ploidy dynamics of aphid bacteriocytes along with post-embryonic development

During post-embryonic development of viviparous females, the ploidy level of bacteriocytes gradually increased; bacteriocytes were 16-32C at the time of birth (N_1) and reached the highest ploidy level in actively reproducing adults (A7, 128-256C) (Figure 7). All stages of viviparous females except senescence stage A21 showed significant differences in ploidy levels (Brunner–Munzel test with Bonferroni adjustment, $N_1 < N_2 < N_3 < N_4 < A_0 < A_{21} < A_7$, $p < 0.05$, Figure 7). The highest dominant ploidy class was observed in A7 aphids (256C, 43%) (Figure 7). A similar pattern was observed in oviparous aphids (Figure S9), and the highest dominant ploidy was also observed in A7 individuals (but 128C, 47%).

Relationship between the size of the nucleolus and ploidy levels in aphid bacteriocytes

There were significant effects of ploidy class on the size of the nucleolus in adult bacteriocytes of each morph (LM with type II test; viviparous females, $F = 62.94$, $df = 2$, $p < 0.001$, oviparous females, $F = 23.97$, $df = 2$, $p < 0.001$; males, $F = 6.44$, $df = 3$, $p < 0.001$, Figure 8a). Note that 16C and 256C viviparous bacteriocytes were excluded from the analysis due to their small number. Similarly, 16C and 8C cells of females and males, respectively, were excluded from the analysis. In viviparous females, the size of the nucleolus consistently increased from 32C to 128C (Tukey's test, $p < 0.001$). In oviparous

236 females, 128C cells had larger nucleoli than 32C and 64C cells ($p < 0.001$ each), yet the
 237 difference between 32C and 64C cells was marginally non-significant ($p = 0.06$). In males,
 238 the size of the nucleolus of 128C cells was significantly larger than that of 16C and 32C
 239 cells ($p < 0.001$ each), but we did not find any significant difference among other
 240 comparisons (16C vs. 32C, $p = 0.71$, 16C vs. 64C, $p = 0.10$, 32C vs 64C, $p = 0.08$, 64C vs
 241 128C, $p = 0.18$) (Figure 8a). A significant effect of ploidy class on the nucleolus size was
 242 also detected in the data from each developmental stage of viviparous aphids (LMM with
 243 type II test; $\chi^2 = 788.83$, $df = 5$, $p < 0.001$, Figure 8b). Note that 4C and 512C
 244 bacteriocytes were excluded from the analysis because of the small number of
 245 observations. The size of the nucleolus consistently increased from 8C to 256C (Tukey's
 246 test, $p < 0.05$, Figure 8b).

247

248 Discussion

249 This study presented quantitative data on ploidy levels and ploidy dynamics in the
 250 bacteriocytes, which are pivotal cells in aphid/*Buchnera* endosymbiosis. The method
 251 developed for ploidy analysis of aphid bacteriocytes in this study (Figure 4) revealed the
 252 hyper polyploidy of the bacteriocytes ranging from 16- to 256-ploidy throughout the
 253 lifecycle. We also found significant differences in the ploidy levels among morphs in
 254 adult stages (viviparous females > oviparous females > males, Figure 5) and
 255 developmental stages in viviparous females (reproducing adults > senescent adults >
 256 pre-reproducing adults > nymphs, Figure 7). Considering that viviparous adults exhibited
 257 a high rate of reproduction, which was at a maximum in the first three weeks (Figure S6),
 258 more metabolically active (actively reproducing) individuals may show higher
 259 polyploidy in their bacteriocytes. We observed a similar pattern of bacteriocyte

260 polyploidy in oviparous aphids (Figure S9), yet the highest ploidy level was lower than
261 viviparous females. These results provide fundamental information to understand the
262 functional significance of polyploidy in aphid bacteriocytes.

263 Our findings in this study raise the possibility that bacteriocyte polyploidy can
264 enhance nutritional symbiosis between aphids and *B. aphidicola*, wherein both supply
265 each other with nutrients that they cannot synthesize on their own^[10, 19, 20]. Specifically,
266 we predict that genes involved in amino acid metabolism, related to transport, and for
267 symbiont regulation (e.g., lysozymes or cysteine-rich secreted proteins targeting bacteria),
268 which have been reported to be highly expressed in the aphid bacteriomes^[24, 25, 38, 40], may
269 be upregulated in a ploidy-dependent manner^[6, 7, 47]. On the other hand, it is commonly
270 known that cell volumes change depending on ploidy levels^[6, 48]. In fact, our data
271 suggested a positive correlation between bacteriocyte volume and ploidy level in the
272 aphid bacteriocytes (Figure S5 and S7). Bacteriocyte enlargement may simply increase
273 the number of *B. aphidicola* that can be harbored, which should lead to enhanced
274 nutritional symbiosis. In order to examine the effect of polyploidization on the
275 aphid/*Buchnera* symbiosis, an integrated approach including gene expression analysis on
276 a per-nucleus basis, and monitoring cell phenotypes such as ploidy-level and cell/nuclear
277 size is required.

278 In this study, we also found positive correlations between the ploidy class of
279 bacteriocytes and the size of their nucleoli, regardless of the morphs and developmental
280 stage (Figure 8). The main function of the nucleolus is ribosomal biogenesis, and the size
281 and morphology of nucleoli are linked to nucleolar activity, such as transcription and
282 ribosomal RNA production rates^[49–51]. In plants, there is evidence that more polyploid
283 nuclei not only exhibit larger nucleolar size but also exhibit increased transcription of

284 rRNA and mRNA; a positive correlation between DNA content and transcriptional
285 activity has been identified in the polyploid tomato fruit pericarp^[8]. Our data are
286 consistent with the hypothesis that aphid bacteriocytes with higher ploidy levels produce
287 more ribosomal RNA, leading to higher metabolic activity; a direct relationship between
288 ploidy levels and nucleolar activity needs to be examined in future studies.

289 Aphid bacteriocytes have long been considered to be polyploid [*Myzus persicae*
290 (Sulzer)^[18], *Pemphigus spyrothecae* Passerini^[22], and *Cinara* species^[52]]. Furthermore, it
291 has been concluded that bacteriocytes are polyploid in many other insects, such as
292 psyllids, whiteflies, scale insects, weevils, bark beetles, termites, and cockroaches^{[11, 12, 15,}
293 ^{53–56]}, although quantitative data were lacking, except for psyllids^[15]. In this study, by
294 comprehensively describing the patterns of polyploidization, we demonstrated, for the
295 first time, that high metabolic demand such as active reproduction is associated with
296 higher polyploidy levels in aphid bacteriocytes. It would be valuable to investigate this
297 relationship in intracellular symbiosis in various insect species. Accumulating
298 information on bacteriocyte polyploidy will help us gain a better understanding of the
299 maintenance and evolution of mutual relationships between host and symbionts because
300 polyploidization in the symbiotic host cells is a common rule in insect-microorganism
301 intracellular symbioses^[12, 13, 57].

302 In the present study, we compared three methods for ploidy level quantification
303 and found an image-based fluorometry the best for the analysis of aphid bacteriomes
304 (Figure 4), because it could distinguish between cell types (e.g., bacteriocytes and sheath
305 cells) by the size of nucleolus, unlike flow cytometry (Figure S2). In addition, it was
306 timesaving compared with Feulgen densitometry (Figure S3). Our results from
307 fluorometry showed that sheath cells mainly consisted of 16C and 32C in adult

308 viviparous aphids, although peaks in the histogram were not clear (Figure 4); therefore,
 309 nuclei exhibiting more than 32C can be reasonably assumed to be bacteriocytes. These
 310 approach, the combination of several methods such as the fluorometry and flow
 311 cytometry would be applicable to other symbiotic systems of insects with
 312 microorganisms, wherein the bacteriome frequently contains several types of cells (e.g.,
 313 primary or secondary bacteriocytes, and sheath cells)^[13, 14, 21].

314 In conclusion, we comprehensively described the patterns of polyploidization in
 315 aphid bacteriocytes, which has long been assumed to be polyploid, yet there have been no
 316 quantitative studies^[9, 10, 14]. Based on the patterns and cytological features observed in
 317 this study, we suggest that hyper polyploidy may enhance gene expression levels and
 318 increase cell size, contributing to the nutritional symbiosis with the bacterial symbiont *B.*
 319 *aphidicola*. This study provides a foundation for further molecular-level analysis of the
 320 functions and underlying mechanisms of polyploidy in insect symbiotic host cells.

321

322 **Material and methods**

323 *Aphids*

324 In this study, we used a long-established parthenogenetic clone of the pea-aphid, *A. pisum*,
 325 ApL strain, which was originally collected in Sapporo, Hokkaido, Japan (referred to as
 326 Sap05Ms2 in^[58]). We confirmed that this strain only harbors the primary endosymbiont *B.*
 327 *aphidicola* by diagnostic PCR, as described in a previous study^[59]. Viviparous insects
 328 were maintained on young, broad bean plants (*Vicia faba* L.) in a 16 °C incubator, with a
 329 photoperiod of 16 h light:8 h dark (long-day conditions). Sexual morphs (oviparous
 330 females and males) were induced by short-day conditions (e.g., 8 h light:16 h dark)
 331 (Figure 2, S1, modified from^[58, 60, 61]). Viviparous females were randomly selected from

the synchronized source populations and reared on young broad bean plants at 16 °C under long-day conditions. Newly larviposited aphids (G0, first-instar nymphs, Figure S1) were then transferred onto the leaves of the bean plants in a 15 °C incubator with a photoperiod of 8 h light:16 h dark. After these G0 nymphs grew into adults, they started to produce G1 offspring, which are morphologically identical to apterous/viviparous females but produce sexual individuals (Figure S1). G1 adults produce G2 offspring that contains sexual individuals (oviparous females and males), but they also produce a few viviparous females (Figure S1). In this study, all oviparous females and males were G2 offspring, and all viviparous individuals were apterous. To reveal the fecundity and longevity of both viviparous and oviparous aphids, female aphids were reared separately and observed daily (see supplementary information “SI Methods”).

Size and morphology of aphid bacteriome cells

Aphid bacteriomes consist of two types of cells: bacteriocytes containing *Buchnera* cells in their cytoplasm and sheath cells without them (Figure 1b, ^[9, 21, 40]). To gain more detailed cellular features of aphid bacteriocytes, we performed a morphological analysis of bacteriomes using a confocal laser-scanning microscope (CLSM; FV1000, Olympus, Japan) on three adult morphs (viviparous/oviparous females and males). These adults were within 5 days of eclosion. We also observed the bacteriome at each stage of viviparous females [nymphs N1 (first-instar nymph), N2 (second-instar nymph), N3 (third-instar nymph), and N4 (fourth-instar nymph), and young adults (3-5 days after eclosion), and old adults (approximately 21 days after eclosion)]. Each stage of viviparous females was used on the day of molting, but teneral insects were not used). Aphid bacteriomes were dissected in phosphate-buffered saline (PBS: 33 mM 143

356 KH_2PO_4 , 33 mM Na_2HPO_4 , pH 6.8) under a stereomicroscope (SZ61, Olympus, Japan),
 357 with fine forceps, and their bacteriome cells (bacteriocytes and sheath cells) were
 358 surgically isolated from the aphid abdomen. The cells were fixed with 4%
 359 paraformaldehyde in PBS for 30 min. Fixed bacteriome cells were washed three times in
 360 0.3% Triton X-100 in PBS (PBS-T) for 15 min for permeabilization. The cells were then
 361 stained with 4,6-diamidino-2-phenylindole (DAPI) (1 $\mu\text{g}/\text{mL}$; Dojindo, Japan) for the
 362 nuclei and Alexa FluorTM 488 phalloidin (66 nM; Thermo Fisher Scientific, USA) for the
 363 cytoskeleton (F-actin), respectively. The nucleolus, which is the site of both ribosomal
 364 RNA (rRNA) synthesis and the assembly of ribosomal subunits^[49], was visualized using
 365 Nucleolus Bright Red (1 mM; Dojindo, Japan). Nucleolus Bright Red dyes are small
 366 molecules that electrostatically bind to RNA in the nucleolus to emit fluorescence. After 1
 367 h at room temperature (from 20 to 25 °C), the cells were washed three times with PBS-T
 368 for 15 min and mounted with VECTASHIELD antifade mounting medium (Vector
 369 Laboratories, USA). The morphology of the bacteriocytes and sheath cells was visualized
 370 using fluorescent staining and differential interference contrast microscopy. The captured
 371 images were processed using the image analysis software ImageJ (NIH,
 372 <http://rsb.info.nih.gov/ij/>). The diameter of each bacteriocyte (D) was measured at its
 373 widest point. The approximate bacteriocyte volume (V) was calculated using the standard
 374 formula: $V = \left(\frac{\pi}{6}\right) D^3$. The nucleolus size (area) of both the bacteriocytes and sheath cells
 375 was also recorded.

376

377 *Establishment of the method for ploidy analysis on aphid bacteriocytes*

378 To establish a method for ploidy analysis of aphid bacteriocytes, we used three methods:
 379 flow cytometry, Feulgen densitometry, and fluorometry. Young adult viviparous aphids

(3-5 days after eclosion) or late instar (third-fourth instar) of nymphs were used. In all analyses, not only bacteriome cells but also head cells were used as diploid controls, which were confirmed as diploid by preliminary analysis of sperm cells (haploid). We first performed flow cytometry, which is an efficient and commonly used method for nuclear DNA-content analysis^[62, 63]. For this analysis, bacteriome cells dissected from five aphids were suspended and repeatedly pipetted in 250 μ L of trypsin buffer (0.11% Nonidet P40, 0.1% sodium citrate, 0.05% spermine tetrahydrochloride, 0.01% Tris base, and 0.003% trypsin in distilled water). Heads were ground with tight-fitting pestles in the trypsin buffer. After incubation at room temperature (from 20 to 25 °C) for 10 min, 5 μ L of trypsin inhibitor solution (trypsin inhibitor, from soybean, 25 mg/mL) and 1 μ L of RNase A (100 mg/mL) were added. After the mixture was incubated at room temperature (from 20 to 25 °C) for 10 min, 250 μ L of dilution buffer (0.11% Nonidet P40, 0.1% sodium citrate, 0.17% spermine tetrahydrochloride, and 0.01% Tris base in distilled water) was added, and the mixture was filtered through a 48 μ m nylon mesh. Isolated nuclei were stained with DAPI (1 μ g/mL). The filtered mixture was incubated at room temperature for at least 10 min and stored on ice until use. Stained nuclei were analyzed for DNA-DAPI fluorescence using a Cell Sorter SH800 (SONY, Japan) at an excitation wavelength of 405 nm. Each experiment was performed in triplicates.

Second, we conducted Feulgen densitometry, which have been widely used for DNA content analysis^[64]. This method was used in the ploidy analysis of psyllid bacteriome cells^[15]. This analysis was performed according to the protocol of Hardie *et al.* (2002)^[64]. Briefly, the bacteriome cells and heads were dissected from an aphid and then smeared on glass slides. The smears were fixed in MFA (methanol, formalin, acetic acid = 85:10:5 v/v) for 24 h, hydrolyzed in 5.0 N HCl for 2 h, and stained with Schiff

404 reagent for 2 h. Images of stained nuclei were captured with a BX-61 microscope
405 (Olympus, Japan) and a DS-Fi1 CCD camera (Nikon, Japan). All the steps were
406 performed at room temperature (from 20 to 25 °C). Using ImageJ, the green channel was
407 extracted, and the integrated optical density (IOD) of the Feulgen stain in the nuclei was
408 measured. Background signal intensity was measured in an area adjacent to each nucleus
409 and deduced from the nuclear IOD. Each experiment was performed in triplicates. In this
410 analysis, the nuclei of bacteriome cells were not isolated; therefore, we can categorize
411 cell types based on their cytoplasmic status [*Buchnera* presence (bacteriocyte) or absence
412 (sheath cells)].

413 Third, image-based fluorometry of the isolated nuclei was conducted.
414 Bacteriome cells were dissected from three individuals in PBS, and the PBS droplets
415 containing the cells were transferred onto glass slides by careful pipetting. Nuclear
416 isolation buffer (10 µL), identical to “dilution buffer,” was added to the cells on the glass
417 slide. The nuclei of these cells were isolated by agitation using fine needles (insect pins,
418 Shiga Konchu Fukyusha, Japan) in the droplets. This step was completed using an SZ61
419 stereomicroscope. After drying the nuclei on the glass slide, they were fixed in MFA for
420 30 min at room temperature (from 20 to 25 °C). The slides were washed thrice with
421 distilled water. Nuclei were stained with DAPI solution (1 µg/mL DAPI and 2 mg/mL
422 RNase A) for 1 h at room temperature (from 20 to 25 °C). The slides were washed three
423 times with distilled water and mounted with VECTASHIELD antifade mounting medium.
424 The same devices used for the Feulgen densitometry were used for image capturing.
425 Using ImageJ, the blue channel was extracted, and the integrated fluorescent intensity
426 (IFI), which is the integrated gray value in the region of interest, was measured for each
427 nucleus. The background signal intensity was measured in an area adjacent to each

nucleus and deduced from nuclear IFI. The experiment was performed in duplicates. In this analysis, the nucleolus size (area) was also measured and compared with the data from confocal microscopy to discriminate cell types (bacteriocytes and sheath cells).

Ploidy analysis for each morph, and each developmental stage of viviparous aphids

Bacteriome cells and heads were dissected in PBS and processed with the abovementioned “fluorometry” method. First, to assess the variation in the degree of polyploidy among morphs, young adult individuals (3-5 days after adult eclosion) of viviparous/oviparous females and males were used. Three individuals were pooled for each morph. Nucleolus sizes were recorded to discriminate cell types. Second, to elucidate the dynamics of polyploidization and post-embryonic development of aphids, bacteriocytes of viviparous aphids at different developmental stages were analyzed. Specifically, the following stages were used: nymphs N1, N2, N3, and N4, and adults at three distinct time points: A0 (0 days after eclosion) as pre-reproductive adults, A7 (7 days after eclosion) as actively reproducing adults, and A21 (21 days after eclosion) as senescent individuals. All viviparous individuals were apterous. Each stage of viviparous females was used on the day of molting, but teneral insects were not used. To discriminate cell types, the nucleolus size (area) was measured and compared with the data obtained by confocal microscopy. Nucleolus sizes have also been used as indicators of the translation activity of cells^[8, 49]. Three individuals were used in each stage. Additionally, each developmental stage of oviparous females (nymphs N1, N2, N3, and N4, and A0 and A7 adults) was also analyzed. For discrimination of cell types in oviparous females, data from viviparous females were used, because we preliminarily confirmed that the size of the nucleolus of bacteriocytes in oviparous females was not significantly different from

452 that in viviparous females. Three individuals were included in each stage.

453

454 *Statistical analysis*

455 To compare the size of bacteriocytes among aphid morphs (young adults of viviparous
456 females, oviparous females, and males), we used a linear model (LM), followed by
457 Tukey’s post hoc test. In this analysis, morphs were treated as fixed effects. For analysis
458 of cell size among the developmental stages of viviparous females, we used linear mixed
459 models (LMM), followed by Tukey’s post hoc test. In the analysis, developmental stages
460 and individuals were included as fixed and random effects, respectively. For pairwise
461 comparisons of ploidy levels of bacteriocytes among morphs and developmental stages
462 of viviparous/oviparous females, we used the Brunner–Munzel test, which is a
463 non-parametric test that adjusts for unequal variances. Significant *p*-values were adjusted
464 using Bonferroni’s correction. To compare the size of the nucleolus in bacteriocytes,
465 which was recorded during fluorometry, among ploidy classes, we used LMs and LMMs,
466 followed by Tukey’s HSD post hoc test. In these analyses, ploidy classes were treated as
467 factorial fixed effects. All analyses were performed for each morph. In the analysis of
468 developmental stages, data from each stage were pooled, and individuals were included
469 as a random effect. All analyses were conducted using the “car”, “emmeans”, “lawstat”,
470 “lme4”, and “multcomp” packages in R software v4.1.1 (<https://www.r-project.org/>).

References

1. Nagl, W. DNA endoreduplication and polyteny understood as evolutionary strategies. *Nature* **261**, 614–615 (1976). DOI: [10.1038/261614a0](https://doi.org/10.1038/261614a0), Pubmed: [934306](https://pubmed.ncbi.nlm.nih.gov/934306/).
2. Edgar, B. A. & Orr-Weaver, T. L. Endoreplication cell cycles: more for less. *Cell* **105**, 297–306 (2001). DOI: [10.1016/S0092-8674\(01\)00334-8](https://doi.org/10.1016/S0092-8674(01)00334-8), Pubmed: [11348589](https://pubmed.ncbi.nlm.nih.gov/11348589/).
3. Edgar, B. A., Zielke, N. & Gutierrez, C. Endocycles: A recurrent evolutionary innovation for post-mitotic cell growth. *Nat. Rev. Mol. Cell Biol.* **15**, 197–210 (2014). DOI: [10.1038/nrm3756](https://doi.org/10.1038/nrm3756), Pubmed: [24556841](https://pubmed.ncbi.nlm.nih.gov/24556841/).
4. Shu, Z., Row, S. & Deng, W. M. Endoreplication: the good, the bad, and the ugly. *Trends Cell Biol.* **28**, 465–474 (2018). DOI: [10.1016/j.tcb.2018.02.006](https://doi.org/10.1016/j.tcb.2018.02.006), Pubmed: [29567370](https://pubmed.ncbi.nlm.nih.gov/29567370/).
5. Lee, H. O., Davidson, J. M. & Duronio, R. J. Endoreplication: polyploidy with purpose. *Genes Dev.* **23**, 2461–2477 (2009). DOI: [10.1101/gad.1829209](https://doi.org/10.1101/gad.1829209), Pubmed: [19884253](https://pubmed.ncbi.nlm.nih.gov/19884253/).
6. Neiman, M., Beaton, M. J., Hessen, D. O., Jeyasingh, P. D. & Weider, L. J. Endopolyploidy as a potential driver of animal ecology and evolution. *Biol. Rev. Camb. Philos. Soc.* **92**, 234–247 (2017). DOI: [10.1111/brv.12226](https://doi.org/10.1111/brv.12226), Pubmed: [26467853](https://pubmed.ncbi.nlm.nih.gov/26467853/).
7. Galitski, T., Saldanha, A. J., Styles, C. A., Lander, E. S. & Fink, G. R. Ploidy regulation of gene expression. *Science* **285**, 251–254 (1999). DOI: [10.1126/science.285.5425.251](https://doi.org/10.1126/science.285.5425.251), Pubmed: [10398601](https://pubmed.ncbi.nlm.nih.gov/10398601/).
8. Bourdon, M. *et al.* Evidence for karyoplasmic homeostasis during endoreduplication and a ploidy-dependent increase in gene transcription during tomato fruit growth. *Development* **139**, 3817–3826 (2012). DOI: [10.1242/dev.084053](https://doi.org/10.1242/dev.084053), Pubmed: [22991446](https://pubmed.ncbi.nlm.nih.gov/22991446/).
9. Buchner, P. *Endosymbiosis of Animals with Plant Microorganisms* (Interscience Publishers, New York, 1965).
10. Douglas, A. E. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic

- 495 bacteria *Buchnera*. *Annu. Rev. Entomol.* **43**, 17–37 (1998). DOI: [10.1146/annurev.ento.43.1.17](https://doi.org/10.1146/annurev.ento.43.1.17),
496 Pubmed: [15012383](https://pubmed.ncbi.nlm.nih.gov/15012383/).
- 497 11. Baumann, P., Moran, N. A., Baumann, L. & Dworkin, M. Bacteriocyte-associated
498 endosymbionts of insects in *The Prokaryotes* (ed. Dworkin, M., Falkow, S., Rosenberg, E.,
499 Schleifer, K. H. & Stackebrandt, E.). DOI: [10.1007/0-387-30741-9_16](https://doi.org/10.1007/0-387-30741-9_16) (Springer, New York,
500 2006).
- 501 12. Koch, A. Intracellular symbiosis in insects. *Annu. Rev. Microbiol.* **14**, 121–140 (1960). DOI:
502 [10.1146/annurev.mi.14.100160.001005](https://doi.org/10.1146/annurev.mi.14.100160.001005), Pubmed: [13757312](https://pubmed.ncbi.nlm.nih.gov/13757312/).
- 503 13. Douglas, A. E. Mycetocyte symbiosis in insects. *Biol. Rev. Camb. Philos. Soc.* **64**, 409–434
504 (1989). DOI: [10.1111/j.1469-185X.1989.tb00682.x](https://doi.org/10.1111/j.1469-185X.1989.tb00682.x), Pubmed: [2696562](https://pubmed.ncbi.nlm.nih.gov/2696562/).
- 505 14. Moran, N. A. & Telang, A. Bacteriocyte-associated symbionts of insects. *BioScience* **48**,
506 295–304 (1998). DOI: [10.2307/1313356](https://doi.org/10.2307/1313356).
- 507 15. Nakabachi, A., Koshikawa, S., Miura, T. & Miyagishima, S. Genome size of *Pachypsylla*
508 *venusta* (Hemiptera: Psyllidae) and the ploidy of its bacteriocyte, the symbiotic host cell that
509 harbors intracellular mutualistic bacteria with the smallest cellular genome. *Bull. Entomol. Res.*
510 **100**, 27–33 (2010). DOI: [10.1017/S0007485309006737](https://doi.org/10.1017/S0007485309006737), Pubmed: [19302725](https://pubmed.ncbi.nlm.nih.gov/19302725/).
- 511 16. Brown, S. W. The Comstockiella system of chromosome behavior in the armored scale insects
512 (Coccoidea: Diaspididae). *Chromosoma* **14**, 360–406 (1963). DOI: [10.1007/BF00326785](https://doi.org/10.1007/BF00326785).
- 513 17. Dasch, G. A., Weiss, E. & Chang, K. P. Endosymbionts of insects in *Bergey's Manual of*
514 *Systematic Bacteriology*, vol. I (ed. Krieg, N. R.) (881–883) (Williams and Wilkins, Baltimore,
515 1984).
- 516 18. Blackman, R. L. Reproduction, cytogenetics and development. Aphids: their Biology, Natural
517 Enemies and control **2**, 163–195 (1987).
- 518 19. Shigenobu, S. & Wilson, A. C. Genomic revelations of a mutualism: the pea aphid and its

- 519 obligate bacterial symbiont. *Cell. Mol. Life Sci.* **68**, 1297–1309 (2011). DOI:
520 [10.1007/s00018-011-0645-2](https://doi.org/10.1007/s00018-011-0645-2), Pubmed: [21390549](https://pubmed.ncbi.nlm.nih.gov/21390549/).
- 521 20. Hansen, A. K., Pers, D. & Russell, J. A. Symbiotic solutions to nitrogen limitation and amino
522 acid imbalance in insect diets. *Adv. Insect Physiol.* **58**, 161–205 (2020). DOI:
523 [10.1016/bs.aiip.2020.03.001](https://doi.org/10.1016/bs.aiip.2020.03.001).
- 524 21. Koga, R., Meng, X. Y., Tsuchida, T. & Fukatsu, T. Cellular mechanism for selective vertical
525 transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. *Proc. Natl*
526 *Acad. Sci. U. S. A.* **109**, E1230–E1237 (2012). DOI: [10.1073/pnas.1119212109](https://doi.org/10.1073/pnas.1119212109), Pubmed:
527 [22517738](https://pubmed.ncbi.nlm.nih.gov/22517738/).
- 528 22. Braendle, C. *et al.* Developmental origin and evolution of bacteriocytes in the aphid–*Buchnera*
529 symbiosis. *PLOS Biol.* **1**, e21 (2003). DOI: [10.1371/journal.pbio.0000021](https://doi.org/10.1371/journal.pbio.0000021).
- 530 23. Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y. & Ishikawa, H. Genome sequence of the
531 endocellular bacterial symbiont of aphids *Buchnera* sp. *APS. Nature* **407**, 81–86 (2000). DOI:
532 [10.1038/35024074](https://doi.org/10.1038/35024074), Pubmed: [10993077](https://pubmed.ncbi.nlm.nih.gov/10993077/).
- 533 24. Nakabachi, A. *et al.* Transcriptome analysis of the aphid bacteriocyte, the symbiotic host cell that
534 harbors an endocellular mutualistic bacterium, *Buchnera*. *Proc. Natl Acad. Sci. U. S. A.* **102**,
535 5477–5482 (2005). DOI: [10.1073/pnas.0409034102](https://doi.org/10.1073/pnas.0409034102), Pubmed: [15800043](https://pubmed.ncbi.nlm.nih.gov/15800043/).
- 536 25. Hansen, A. K. & Moran, N. A. Aphid genome expression reveals host–symbiont cooperation in
537 the production of amino acids. *Proc. Natl Acad. Sci. U. S. A.* **108**, 2849–2854 (2011). DOI:
538 [10.1073/pnas.1013465108](https://doi.org/10.1073/pnas.1013465108), Pubmed: [21282658](https://pubmed.ncbi.nlm.nih.gov/21282658/).
- 539 26. Kondorosi, E. & Kondorosi, A. Endoreduplication and activation of the anaphase-promoting
540 complex during symbiotic cell development. *FEBS Lett.* **567**, 152–157 (2004). DOI:
541 [10.1016/j.febslet.2004.04.075](https://doi.org/10.1016/j.febslet.2004.04.075), Pubmed: [15165909](https://pubmed.ncbi.nlm.nih.gov/15165909/).
- 542 27. Kondorosi, E., Mergaert, P. & Kereszt, A. A paradigm for endosymbiotic life: cell differentiation

- 543 of *Rhizobium* bacteria provoked by host plant factors. *Annu. Rev. Microbiol.* **67**, 611–628 (2013).
- 544 DOI: [10.1146/annurev-micro-092412-155630](https://doi.org/10.1146/annurev-micro-092412-155630), Pubmed: [24024639](https://pubmed.ncbi.nlm.nih.gov/24024639/).
- 545 28. Suzuki, T. *et al.* Endoreduplication-mediated initiation of symbiotic organ development in *Lotus*
- 546 *japonicus*. *Development* **141**, 2441–2445 (2014). DOI: [10.1242/dev.107946](https://doi.org/10.1242/dev.107946), Pubmed: [24850853](https://pubmed.ncbi.nlm.nih.gov/24850853/).
- 547 29. Brisson, J. A. & Davis, G. K. Pea aphid in *Genome Mapping and Genomics in Arthropods*
- 548 (59-67) (Springer, Berlin, Heidelberg, 2008).
- 549 30. Ogawa, K. & Miura, T. Aphid polyphenisms: trans-generational developmental regulation
- 550 through viviparity. *Front. Physiol.* **5**, 1 (2014). DOI: [10.3389/fphys.2014.00001](https://doi.org/10.3389/fphys.2014.00001), Pubmed:
- 551 [24478714](https://pubmed.ncbi.nlm.nih.gov/24478714/).
- 552 31. Braendle, C., Davis, G. K., Brisson, J. A. & Stern, D. L. Wing dimorphism in aphids. *Heredity* **97**,
- 553 192–199 (2006). DOI: [10.1038/sj.hdy.6800863](https://doi.org/10.1038/sj.hdy.6800863), Pubmed: [16823401](https://pubmed.ncbi.nlm.nih.gov/16823401/).
- 554 32. Ogawa, K. & Miura, T. Two developmental switch points for the wing polymorphisms in the pea
- 555 aphid *Acyrtosiphon pisum*. *EvoDevo* **4**, 30 (2013). DOI: [10.1186/2041-9139-4-30](https://doi.org/10.1186/2041-9139-4-30), Pubmed:
- 556 [24175956](https://pubmed.ncbi.nlm.nih.gov/24175956/).
- 557 33. Miura, T. *et al.* A comparison of parthenogenetic and sexual embryogenesis of the pea aphid
- 558 *Acyrtosiphon pisum* (Hemiptera: Aphidoidea). *J. Exp. Zool. B Mol. Dev. Evol.* **295**, 59–81
- 559 (2003). DOI: [10.1002/jez.b.3](https://doi.org/10.1002/jez.b.3), Pubmed: [12548543](https://pubmed.ncbi.nlm.nih.gov/12548543/).
- 560 34. Chang, C. C., Lee, W. C., Cook, C. E., Lin, G. W. & Chang, T. Germ-plasm specification and
- 561 germline development in the parthenogenetic pea aphid *Acyrtosiphon pisum*: Vasa and Nanos as
- 562 markers. *Int. J. Dev. Biol.* **50**, 413–421 (2006). DOI: [10.1387/ijdb.052100cc](https://doi.org/10.1387/ijdb.052100cc), Pubmed:
- 563 [16525937](https://pubmed.ncbi.nlm.nih.gov/16525937/).
- 564 35. Chung, C. Y., Cook, C. E., Lin, G. W., Huang, T. Y. & Chang, C. C. Reliable protocols for
- 565 whole-mount fluorescent *in situ* hybridization (FISH) in the pea aphid *Acyrtosiphon pisum*: A
- 566 comprehensive survey and analysis. *Insect Sci.* **21**, 265–277 (2014). DOI:

- 567 [10.1111/1744-7917.12086](https://doi.org/10.1111/1744-7917.12086), Pubmed: [24850784](https://pubmed.ncbi.nlm.nih.gov/24850784/).
- 568 36. Price, D. R. *et al.* Aphid amino acid transporter regulates glutamine supply to intracellular
569 bacterial symbionts. *Proc. Natl Acad. Sci. U. S. A.* **111**, 320–325 (2014). DOI:
570 [10.1073/pnas.1306068111](https://doi.org/10.1073/pnas.1306068111), Pubmed: [24367072](https://pubmed.ncbi.nlm.nih.gov/24367072/).
- 571 37. Feng, H. *et al.* Trading amino acids at the aphid–*Buchnera* symbiotic interface. *Proc. Natl Acad.*
572 *Sci. U. S. A.* **116**, 16003–16011 (2019). DOI: [10.1073/pnas.1906223116](https://doi.org/10.1073/pnas.1906223116), Pubmed: [31337682](https://pubmed.ncbi.nlm.nih.gov/31337682/).
- 573 38. Pers, D. & Hansen, A. K. The boom and bust of the aphid’s essential amino acid metabolism
574 across nymphal development. *G3 (Bethesda)* **11** (2021). DOI: [10.1093/g3journal/jkab115](https://doi.org/10.1093/g3journal/jkab115),
575 Pubmed: [33831149](https://pubmed.ncbi.nlm.nih.gov/33831149/).
- 576 39. International Aphid Genomics Consortium. Genome sequence of the pea aphid *Acyrtosiphon*
577 *pisum*. *PLOS Biol.* **8**, e1000313 (2010). DOI: [10.1371/journal.pbio.1000313](https://doi.org/10.1371/journal.pbio.1000313), Pubmed:
578 [20186266](https://pubmed.ncbi.nlm.nih.gov/20186266/).
- 579 40. Shigenobu, S. & Stern, D. L. Aphids evolved novel secreted proteins for symbiosis with bacterial
580 endosymbiont. *Proc. Biol. Sci.* **280**, 20121952 (2013). DOI: [10.1098/rspb.2012.1952](https://doi.org/10.1098/rspb.2012.1952).
- 581 41. Li, Y., Park, H., Smith, T. E. & Moran, N. A. Gene family evolution in the pea aphid based on
582 chromosome-level genome assembly. *Mol. Biol. Evol.* **36**, 2143–2156 (2019). DOI:
583 [10.1093/molbev/msz138](https://doi.org/10.1093/molbev/msz138), Pubmed: [31173104](https://pubmed.ncbi.nlm.nih.gov/31173104/).
- 584 42. Simonet, P. *et al.* Direct flow cytometry measurements reveal a fine-tuning of symbiotic cell
585 dynamics according to the host developmental needs in aphid symbiosis. *Sci. Rep.* **6**, 19967
586 (2016). DOI: [10.1038/srep19967](https://doi.org/10.1038/srep19967), Pubmed: [26822159](https://pubmed.ncbi.nlm.nih.gov/26822159/).
- 587 43. Douglas, A. E. & Dixon, A. F. G. The mycetocyte symbiosis of aphids: variation with age and
588 morph in virginoparae of *Megoura viciae* and *Acyrtosiphon pisum*. *J. Insect Physiol.* **33**,
589 109–113 (1987). DOI: [10.1016/0022-1910\(87\)90082-5](https://doi.org/10.1016/0022-1910(87)90082-5).
- 590 44. Douglas, A. E. & Wilkinson, T. L. Host cell allometry and regulation of the symbiosis between

- 591 pea aphids, *Acyrtosiphon pisum*, and bacteria, *Buchnera*. *J. Insect Physiol.* **44**, 629–635 (1998).
- 592 DOI: [10.1016/s0022-1910\(98\)00030-4](https://doi.org/10.1016/s0022-1910(98)00030-4), Pubmed: [12769945](https://pubmed.ncbi.nlm.nih.gov/12769945/).
- 593 45. Nishikori, K., Kubo, T. & Morioka, M. Morph-dependent expression and subcellular localization
- 594 of host serine carboxypeptidase in bacteriocytes of the pea aphid associated with degradation of
- 595 the endosymbiotic bacterium *Buchnera*. *Zoolog. Sci.* **26**, 415–420 (2009). DOI:
- 596 [10.2108/zsj.26.415](https://doi.org/10.2108/zsj.26.415), Pubmed: [19583501](https://pubmed.ncbi.nlm.nih.gov/19583501/).
- 597 46. Simonet, P. *et al.* Bacteriocyte cell death in the pea aphid/*Buchnera* symbiotic system. *Proc. Natl*
- 598 *Acad. Sci. U. S. A.* **115**, E1819–E1828 (2018). DOI: [10.1073/pnas.1720237115](https://doi.org/10.1073/pnas.1720237115), Pubmed:
- 599 [29432146](https://pubmed.ncbi.nlm.nih.gov/29432146/).
- 600 47. Nagymihály, M. *et al.* Ploidy-dependent changes in the epigenome of symbiotic cells correlate
- 601 with specific patterns of gene expression. *Proc. Natl Acad. Sci. U. S. A.* **114**, 4543–4548 (2017).
- 602 DOI: [10.1073/pnas.1704211114](https://doi.org/10.1073/pnas.1704211114), Pubmed: [28404731](https://pubmed.ncbi.nlm.nih.gov/28404731/).
- 603 48. Orr-Weaver, T. L. When bigger is better: the role of polyploidy in organogenesis. *Trends Genet.*
- 604 **31**, 307–315 (2015). DOI: [10.1016/j.tig.2015.03.011](https://doi.org/10.1016/j.tig.2015.03.011), Pubmed: [25921783](https://pubmed.ncbi.nlm.nih.gov/25921783/).
- 605 49. Shaw, P. & Doonan, J. The nucleolus: playing by different rules? *Cell Cycle* **4**, 102–105 (2005).
- 606 DOI: [10.4161/cc.4.1.1467](https://doi.org/10.4161/cc.4.1.1467), Pubmed: [15655371](https://pubmed.ncbi.nlm.nih.gov/15655371/).
- 607 50. Buchwalter, A. & Hetzer, M. W. Nucleolar expansion and elevated protein translation in
- 608 premature aging. *Nat. Commun.* **8**, 328 (2017). DOI: [10.1038/s41467-017-00322-z](https://doi.org/10.1038/s41467-017-00322-z), Pubmed:
- 609 [28855503](https://pubmed.ncbi.nlm.nih.gov/28855503/).
- 610 51. Tiku, V. *et al.* Small nucleoli are a cellular hallmark of longevity. *Nat. Commun.* **8**, 16083 (2017).
- 611 DOI: [10.1038/ncomms16083](https://doi.org/10.1038/ncomms16083), Pubmed: [28853436](https://pubmed.ncbi.nlm.nih.gov/28853436/).
- 612 52. Manzano-Marín, A. *et al.* Serial horizontal transfer of vitamin-biosynthetic genes enables the
- 613 establishment of new nutritional symbionts in aphids' di-symbiotic systems. *ISME J.* **14**,
- 614 259–273 (2020). DOI: [10.1038/s41396-019-0533-6](https://doi.org/10.1038/s41396-019-0533-6), Pubmed: [31624345](https://pubmed.ncbi.nlm.nih.gov/31624345/).

- 615 53. Luan, J., Sun, X., Fei, Z. & Douglas, A. E. Maternal inheritance of a single somatic animal cell
616 displayed by the bacteriocyte in the whitefly *Bemisia tabaci*. *Curr. Biol.* **28**, 459–465.e3 (2018).
617 DOI: [10.1016/j.cub.2017.12.041](https://doi.org/10.1016/j.cub.2017.12.041), Pubmed: [29395925](https://pubmed.ncbi.nlm.nih.gov/29395925/).
- 618 54. Tremblay, E. & Caltagirone, L. E. Fate of polar bodies in insects. *Annu. Rev. Entomol.* **18**,
619 421–444 (1973). DOI: [10.1146/annurev.en.18.010173.002225](https://doi.org/10.1146/annurev.en.18.010173.002225).
- 620 55. Nardon, P., Lefevre, C., Delobel, B., Charles, H. & Heddi, A. Occurrence of endosymbiosis in
621 Dryophthoridae weevils: cytological insights into bacterial symbiotic structures. *Symbiosis* **33**,
622 227–241 (2002).
- 623 56. Bandi, C. & Sacchi, L. Intracellular symbiosis in termites in *Termites: Evolution, Sociality,*
624 *Symbioses, Ecology* (261–273) (Springer, Dordrecht, 2000).
- 625 57. Heddi, A. & Nardon, P. *Sitophilus oryzae* L.: a model for intracellular symbiosis in the
626 Dryophthoridae weevils (Coleoptera). *Symbiosis* **39**, 1–11 (2005).
- 627 58. Kanbe, T. & Akimoto, S. I. Allelic and genotypic diversity in long-term asexual populations of
628 the pea aphid, *Acyrtosiphon pisum* in comparison with sexual populations. *Mol. Ecol.* **18**,
629 801–816 (2009). DOI: [10.1111/j.1365-294X.2008.04077.x](https://doi.org/10.1111/j.1365-294X.2008.04077.x), Pubmed: [19207245](https://pubmed.ncbi.nlm.nih.gov/19207245/).
- 630 59. Tsuchida, T., Koga, R., Shibao, H., Matsumoto, T. & Fukatsu, T. Diversity and geographic
631 distribution of secondary endosymbiotic bacteria in natural populations of the pea aphid,
632 *Acyrtosiphon pisum*. *Mol. Ecol.* **11**, 2123–2135 (2002). DOI:
633 [10.1046/j.1365-294x.2002.01606.x](https://doi.org/10.1046/j.1365-294x.2002.01606.x), Pubmed: [12296954](https://pubmed.ncbi.nlm.nih.gov/12296954/).
- 634 60. Ishikawa, A. *et al.* Juvenile hormone titre and related gene expression during the change of
635 reproductive modes in the pea aphid. *Insect Mol. Biol.* **21**, 49–60 (2012). DOI:
636 [10.1111/j.1365-2583.2011.01111.x](https://doi.org/10.1111/j.1365-2583.2011.01111.x), Pubmed: [21988597](https://pubmed.ncbi.nlm.nih.gov/21988597/).
- 637 61. Matsuda, N., Kanbe, T., Akimoto, S. I. & Numata, H. Transgenerational seasonal timer for
638 suppression of sexual morph production in the pea aphid, *Acyrtosiphon pisum*. *J. Insect Physiol.*

- 639 **101**, 1–6 (2017). DOI: [10.1016/j.jinsphys.2017.06.008](https://doi.org/10.1016/j.jinsphys.2017.06.008), Pubmed: [28610748](https://pubmed.ncbi.nlm.nih.gov/28610748/).
- 640 62. Nozaki, T. & Matsuura, K. Termite queens have disproportionately more DNA in their fat body
- 641 cells: reproductive division of labor and endoreduplication. *Entomol. Sci.* **19**, 67–71 (2016). DOI:
- 642 [10.1111/ens.12156](https://doi.org/10.1111/ens.12156).
- 643 63. Nozaki, T. & Matsuura, K. Evolutionary relationship of fat body endoreduplication and queen
- 644 fecundity in termites. *Ecol. Evol.* **9**, 11684–11694 (2019). DOI: [10.1002/ece3.5664](https://doi.org/10.1002/ece3.5664), Pubmed:
- 645 [31695878](https://pubmed.ncbi.nlm.nih.gov/31695878/).
- 646 64. Hardie, D. C., Gregory, T. R. & Hebert, P. D. From pixels to picograms: a beginners' guide to
- 647 genome quantification by feulgen image analysis densitometry. *J. Histochem. Cytochem.* **50**,
- 648 735–749 (2002). DOI: [10.1177/002215540205000601](https://doi.org/10.1177/002215540205000601), Pubmed: [12019291](https://pubmed.ncbi.nlm.nih.gov/12019291/).

649

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658

659 **Author contributions**

660 T. N. and S. S. designed research; T. N. performed the experiment and analyzed data; T. N.

661 and S. S. wrote the original draft of the paper and both authors will contribute

662 substantially to revisions.

663

664 **Declarations of interest**

665 There are no conflicts of interest to declare.

666 Figures and legends

667
668 **Figure 1.** Intracellular symbiosis in the pea-aphid, *Acyrthosiphon pisum*. **a** a photograph
669 of pea-aphids (viviparous females). **b** Aphids harbor their bacterial symbionts in the
670 specialized organ, bacteriome. The organ contains two types of cells, bacteriocytes and
671 sheath cells. Bacteriocytes are symbiotic host cells that harbor *Buchnera aphidicola* in
672 the cytoplasm and are remarkably large (approximately 100 μm in diameter); sheath cells
673 are much smaller than bacteriocytes and do not present *Buchnera*. The function of sheath
674 cells has not been well studied. Illustrations were drawn according to Koga *et al.*^[21].

675
676 **Figure 2.** The life cycle and polymorphism of pea-aphids. In the field, viviparous
677 females are observed from spring to summer on their host plants and rapidly reproduce
678 through viviparous parthenogenesis. N1-4 represents the first to fourth instar nymphs.
679 Larviposited nymphs grow up to adults after molting four times. Oviparous females and
680 males emerge in autumn; they are induced by short-day photoperiod. Oviparous females
681 mate with males and produce eggs (sexual reproduction). All aphid morphs harbor *B.*
682 *aphidicola* inside of their bacteriocytes. Aphid morphs and developmental stages that we
683 have used for ploidy analysis of their bacteriocytes were bold lettered. Illustrations were
684 drawn according to Ogawa & Miura^[30].

685
686 **Figure 3.** Morphology of bacteriocytes and sheath cells from each morph of aphids
687 visualized using DAPI/Phalloidin/Nucleolus Bright Red staining. DNA and F-actin were
688 stained by DAPI (gray or blue) and Phalloidin (green), respectively. The nucleolus, which
689 is the site of ribosome biogenesis, was visualized by Nucleolus Bright Red (red). This dye
690 binds RNA electrostatically, therefore the cytoplasm of bacteriocytes and *Buchnera* cells
691 were also stained. Bacteriocytes (*white arrows*) had single prominent nucleolus, and the
692 cell sizes were much larger than sheath cells (*white arrowheads*) in all aphid morphs.

693

694 **Figure 4.** Ploidy analysis of aphid bacteriocytes using DAPI-fluorometry. A
695 representative result from the analysis of adult viviparous females is presented. Isolated
696 nuclei of bacteriome cells were stained using DAPI, image-captured with a CCD camera,
697 and their integrated fluorescence intensity was measured using ImageJ software. Nuclei
698 were categorized into “bacteriocytes” or “sheath cells,” based on the size distribution of
699 nucleolus (see Material and methods). Relative ploidy levels were calculated based on the
700 data from head cells which are mainly diploid. Bacteriocytes of adult viviparous aphids
701 consisted of 16C-256C cells, and 64-128 cells were dominant, while sheath cells
702 exhibited lower ploidy levels (mainly 16C). “C” means haploid genome size, for example,
703 2C = diploid and 8C = octoploid.

704

705 **Figure 5.** Ploidy distribution of bacteriocytes among aphid morphs, viviparous females,
706 oviparous females, and males. Sample size (numbers of bacteriocyte nuclei) is shown at
707 the right. Different letters with aphid categories indicate significant differences in the
708 median ploidy class (Brunner–Munzel test with Bonferroni adjustment, $p < 0.05$).
709 Bacteriocytes of adult viviparous females in the pea-aphid were highly polyploid and
710 were mainly 64-128C cells. The cells of oviparous females and males were mainly 64C
711 and 32-64C, respectively. The degree of polyploidy levels was highest in viviparous
712 females.

713

714 **Figure 6.** Developmental scheme and morphological changes of bacteriocytes during
715 post-embryonic development in viviparous females. **a** First-instar nymphs of viviparous
716 aphids molt four times during 13.73 ± 0.32 (mean $\pm$ SEM, $n = 16$) days at 16 °C. After
717 adult eclosion, aphids live approximately 1 month under 16 °C. N1-4 represents the first
718 to fourth instar nymphs. A0, A7, and A21 mean adult aphids at the day of eclosion, at 7
719 and 21 days after eclosion, respectively. **b** Confocal microscopic images of bacteriocytes
720 and sheath cells in each stage of viviparous females. N1 and N3 were selected and
721 presented as representatives of nymphal stages. A0 and A21 adults were presented as
722 young adults and senescent adults, respectively. DNA and F-actin were stained using
723 DAPI (blue) and Phalloidin (green), and nucleolus was visualized by Nucleolus Bright
724 Red (red). *White arrows*; nuclei of bacteriocytes, *white arrowheads*; nuclei of sheath

725 cells.

726

727 **Figure 7.** Ploidy distribution of bacteriocytes of each developmental stage of viviparous
728 aphids. Sample size (numbers of bacteriocyte nuclei) is shown below each column. N1-4
729 represents the first to fourth instar nymphs, and A0, A7 and A21 indicate 0-, 7-,
730 21-day-old adults. Different letters with aphid stages indicate significant differences in
731 the median ploidy class (Brunner–Munzel test with Bonferroni adjustment, $p < 0.05$).
732 Bacteriocytes of adult viviparous females (A0-A21) in the pea-aphid were highly
733 polyploid (64-256C), while those of N1 nymphs were mainly 16C and 32C cells.

734
735 **Figure 8.** The relationship between the size of nucleolus and ploidy levels of aphid
736 bacteriocytes. In the boxplots, central bold lines represent the medians, boxes comprise
737 the 25–75 percentiles and whiskers denote the range. **a** The size of nucleolus was
738 significantly different among ploidy class in all aphid morphs (LM with type II test; $p <$
739 0.001 each). Different letters indicate significant differences (Tukey's test, $p < 0.05$).
740 Note that 16C/256C, 16C, and 8C bacteriocytes in viviparous, oviparous females and
741 males, respectively, were excluded from these analyses due to the small sample size. **b**
742 Nucleolus size was significantly different among ploidy class in viviparous females
743 (LMM with type II test; $p < 0.001$). Different letters indicate significant differences
744 (Tukey's test, $p < 0.05$). Note that 4C and 512C were excluded from this analysis due to

745 the small number.