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Variation and asymmetry in host-symbiont dependence in a microbial symbiosis

--Manuscript Draft--

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Abstract:	<p>Background Symbiosis is a major source of evolutionary innovation and, by allowing species to exploit new ecological niches, underpins the functioning of ecosystems. The transition from free-living to obligate symbiosis requires the alignment of the partners' fitness interests and the evolution of mutual dependence. While symbiotic taxa are known to vary widely in the extent of host-symbiont dependence, rather less is known about variation within symbiotic associations.</p> <p>Results Using experiments with the microbial symbiosis between the protist <i>Paramecium bursaria</i> and the alga <i>Chlorella</i>, we show variation between strains in host-symbiont dependence, encompassing facultative associations, mutual dependence and host dependence upon the symbiont. Facultative associations displayed higher symbiotic growth rates and higher per host symbiont loads than those with greater degrees of dependence.</p> <p>Conclusions These data show that the <i>Paramecium-Chlorella</i> interaction exists at the boundary between facultative and obligate symbiosis, and furthermore suggest that the host is more likely to evolve dependence than the algal symbiont.</p>	
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1 **Variation and asymmetry in host-symbiont dependence in a microbial symbiosis**

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28 **Abstract**

29 *Background*

30 Symbiosis is a major source of evolutionary innovation and, by allowing species to exploit new
31 ecological niches, underpins the functioning of ecosystems. The transition from free-living to obligate
32 symbiosis requires the alignment of the partners' fitness interests and the evolution of mutual
33 dependence. While symbiotic taxa are known to vary widely in the extent of host-symbiont
34 dependence, rather less is known about variation within symbiotic associations.

35 *Results*

36 Using experiments with the microbial symbiosis between the protist *Paramecium bursaria* and the
37 alga *Chlorella*, we show variation between strains in host-symbiont dependence, encompassing
38 facultative associations, mutual dependence and host dependence upon the symbiont. Facultative
39 associations displayed higher symbiotic growth rates and higher per host symbiont loads than those
40 with greater degrees of dependence.

41 *Conclusions*

42 These data show that the *Paramecium-Chlorella* interaction exists at the boundary between facultative
43 and obligate symbiosis, and suggest that the host is more likely to evolve dependence than the algal
44 symbiont.

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46

47

48 **Background**

49 Symbiosis —the intimate living together of unlike organisms— is a major source of evolutionary
50 innovation, providing interacting species with new functions and thus facilitating the evolution of
51 complex life (1,2). Symbioses are common in nature, and, by allowing species to exploit otherwise
52 inaccessible ecological niches, underpin the diversity and functioning of natural ecosystems (3–5).
53 Yet understanding the origins and evolutionary stability of symbioses remains a major challenge for
54 evolutionary biologists. The evolutionary transition from free-living to obligate symbiosis requires
55 that the fitness interests of interacting species be aligned, and that the species evolve to become
56 mutually dependent (6–10). However, while famous examples of obligate symbiotic partnerships
57 exist, many symbioses are facultative wherein species retain the ability to survive in the free-living
58 state (11,12). Comparative evolutionary analysis suggests that this variation among lineages in their
59 degree of host-symbiont dependence is at least partially explained by the types of benefits exchanged
60 between symbiotic partners and the mode of symbiont inheritance, with mutual dependence being
61 more common in vertically-inherited, nutritional symbioses (13). These macroevolutionary patterns
62 cannot reveal, however, the extent of variation in host-symbiont dependence available to natural
63 selection within symbioses, nor the potential for asymmetries in dependence among partners in a
64 symbiosis.

66 Photosynthetic endosymbioses (photosymbioses), typically between eukaryotic algae and animal or
67 protist hosts, are a classic example of widespread and ecologically important symbiosis (5,12,14) and
68 therefore represent a useful model system for understanding evolutionary transitions in symbiosis.
69 Photosymbioses are typically based upon the reciprocal exchange of nutrients in the form of fixed
70 carbon from symbiont to host, and nitrogen compounds from host to symbiont (15). Photosymbioses
71 vary widely in their degree of host-symbiont dependence, from ancient and obligate organelles (*e.g.*
72 primary, secondary, and tertiary plastids in eukaryotic algae, see Keeling (16)), to facultative
73 symbioses where symbiotic partners are also able to survive in the free-living state (*e.g.*
74 *Symbiodinium* and anthozoan corals (17,18)). Across the extant eukaryotic tree of life, transitions
75 from facultative to obligate photosymbiosis have occurred independently a number of times

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76 (16,19,20), yet facultative photosymbioses are arguably both more common and more diverse (12,21).

77 Little is known, however, about variation in host-symbiont dependence within facultative
78 photosymbioses and the ecological drivers selecting for maintenance of the facultative habit.

79
80 The microbial photosymbiosis between the host *Paramecium bursaria* —a heterotrophic ciliate— and
81 the symbiont *Chlorella* sp. —a green alga— is a tractable model system (22–27) where the fitness
82 effects of symbiosis relative to free-living can be directly quantified (28). The *P. bursaria-Chlorella*
83 (Pb-C) symbiosis is widespread in shallow freshwater habitats, and is primarily based upon provision
84 of nitrogen compounds from host heterotrophy to the symbiont, and of maltose and oxygen derived
85 from symbiont photosynthesis to the host (29–32). The Pb-C symbiosis has evolved multiple times,
86 such that, whilst each Pb-C strain contains a clonal population of *Chlorella*, multiple origins of
87 symbiotic lineages occur across the *Chlorella* clade (33,34). Within the *Chlorella* clade, *C. vulgaris*
88 are found in both the free-living and symbiotic states, whereas *C. variabilis* is more typically
89 associated with symbiosis (35). We have previously shown for a single Pb-C strain that the fitness
90 effects of symbiosis are environmentally context dependent and highly asymmetric: For hosts,
91 symbiosis is costly in the dark but becomes increasingly beneficial with increasing irradiance,
92 whereas, for symbionts, symbiosis is not beneficial and becomes increasingly costly with increasing
93 irradiance (28). Hosts exert tight control over symbiont load (i.e., the number of symbionts per host
94 cell), regulating symbiont number in relation to light to maximise the benefit-to-cost ratio of
95 symbiosis (28,36). Accordingly, symbiont load peaked at low light levels but was reduced both in
96 the dark, where symbionts are not beneficial, and at high light levels, where per symbiont benefits are
97 highest (28). Given the inherent conflict between these symbiotic partners, and the strong
98 environmental context dependence of the fitness effects of symbiosis, we hypothesise that selection to
99 retain free-living growth should be stronger for *Chlorella* than *P. bursaria* due to the asymmetries in
100 the fitness benefits of symbiosis.

101
102 Here we experimentally investigate natural variation in host-symbiont dependence by comparing free-
103 living versus symbiotic growth among five strains of Pb-C. We report variation in both the fitness

104 effects of symbiosis and host-symbiont dependence between Pb-C strains. Among the five Pb-C
105 strains, we observed fully facultative associations, an association displaying mutual dependence, and
106 associations in which hosts alone displayed dependence. Notably, symbiotic growth rates were higher
107 in Pb-C strains that retained the fully facultative lifestyle and maintained higher symbiont loads. Our
108 data therefore show that Pb-C strains vary in the degree of host-symbiont dependence, and suggest
109 that *Paramecium* is more likely to evolve dependence than *Chlorella*.

110

111 **Materials & Methods**

112 *Paramecium* strains and culturing conditions

113 Experiments were performed using five *Paramecium bursaria* strains along with their naturally
114 occurring *Chlorella* symbionts. These Pb-C strains are designated 186b, HA1, HK1, CT39, and Dd1.
115 Strain 186b (CCAP 1660/18) was obtained from the Culture Collection for Algae and Protozoa
116 (Oban, Scotland) and isolated in the UK, whilst the remaining four strains were all obtained from the
117 *Paramecium* National Bio-Resource Project (Yamaguchi, Japan) and were all isolated in Japan.
118 Further details of the strains used are provided in Table S1. All experiments were performed by
119 culturing in bacterized Protozoan Pellet Media (PPM, Carolina Biological Supply, NC, USA) which
120 was made to a concentration of 0.66 g L⁻¹ with Volvic natural mineral water, and inoculated
121 approximately 20 hours prior to use with *Serratia marscesens* from frozen glycerol stocks. All stock
122 cultures were maintained at 25 °C with 50 μE m⁻² s⁻¹ of light and a 14:10 L:D cycle. Stock cultures
123 were maintained by batch culture, where cultures were diluted by half every 2-3 weeks with fresh
124 bacterized PPM. Unless otherwise stated, experiments were performed under the same culture
125 conditions.

126

127 *Symbiotic and apo-symbiotic host growth rates in response to light*

128 Growth rates of hosts were compared across a light gradient and in the presence (symbiotic) or
129 absence (apo-symbiotic) of *Chlorella* symbionts. Apo-symbiotic cell cultures were established by
130 treating symbiotic cells with a combination of paraquat (10 μg mL⁻¹) and cyclohexamide (10 μg mL⁻¹)
131 and exposing the cells to high light intensities (> 50 μE m⁻² s⁻¹) for a period of between four and seven

132 days, until host cells were visibly symbiont free. Apo-symbiotic cell cultures were verified by
133 monitoring the colour of host cells on the microscope, and observing that re-greening by *Chlorella* did
134 not occur over three weeks.

135

136 Both symbiotic and apo-symbiotic *P. bursaria* cells were washed and concentrated using sterile
137 Volvic and re-suspended in bacterized PPM. Cells were acclimated to 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ light for two days
138 before being washed once again, re-suspended in fresh bacterized PPM. Cells were then acclimated to
139 their treatment light condition (0, 1.5, 3, 6, 12, 25, & 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) for five days before being washed
140 and re-suspended in bacterized PPM at a target cell density of approximately 350 cells mL^{-1} . To
141 estimate growth rates, cell densities were determined at 0, 24, and 48 hours by fixing 350 μL of each
142 cell culture, in triplicate, in 1% v/v glutaraldehyde in 96-well flat bottomed micro-well plates. Images
143 of each well after settling were recorded using a Nikon D600 camera mounted to an inverted
144 microscope through a 4 \times objective lens. Cell counts for each well were recorded using an automated
145 image analysis macro in ImageJ v1.50i (37).

146

147 *Free living Chlorella growth*

148 Free-living symbiont cultures were established in triplicate by washing 10 mL of stock culture in
149 approximately 200 mL of sterile Volvic on an 11 μm nylon mesh. Host cells retained by the mesh
150 were re-suspended in 1.5 mL Volvic and ultra-sonicated using a Fisherbrand Q500 Sonicator (Fisher
151 Scientific, NH, USA), at a power setting of 20% for 10 seconds. Ultra-sonication resulted in lysis of
152 host cells (confirmed by visual inspection) and release of symbionts into the surrounding media.
153 Symbionts were separated from host cell lysate by centrifugation, re-suspended in 5 mL Bold's Basal
154 Medium (BBM) (38), and cultured in 30 mL glass tubes under the same conditions as for host stock
155 cultures but with the addition of shaking at 130 rpm. The dynamics of these populations was tracked
156 for five days. Cell densities were estimated each day using a CytoFLEX S flow cytometer (Beckman
157 Coulter Inc., CA, USA), and manually gating *Chlorella* events for each individual sample using
158 CytExpert2.0 (Beckman Coulter Inc., CA, USA). Specifically, *Chlorella* cells were distinguished

159 from other particles on the basis of their fluorescence and size characteristics, which were initially
1 determined by visual inspection of a subset of the flow cytometry data.

161

162 *Host symbiont load in response to light*

163 *P. bursaria* cells were washed and concentrated using sterile Volvic and re-suspended in bacterized
164 PPM. Cells were evenly split into 28 microcosms each containing 5 mL of bacterized PPM and
165 microcosms were randomly assigned to one of seven light treatment groups (n=4). Microcosms were
166 acclimated to their light treatment (0, 1.5, 3, 6, 12, 25, & 50 $\mu\text{E m}^{-2} \text{s}^{-1}$) for approximately 6 days prior
167 to flow cytometry analysis.

168

169 Host symbiont loads were estimated using a CytoFLEX S flow cytometer (Beckman Coulter Inc., CA,
170 USA) by measuring the intensity of chlorophyll fluorescence for individual *P. bursaria* cells
171 (excitation 488 nm, emission 690/50 nm). Data are presented as relative fluorescence, and are
172 calibrated against 8-peak beads, to reduce variation between samples run in separate sessions.

173

174 *Data analysis*

175 All statistical analyses were performed in R v.2.3.4 (R Core Development Team, 2016). Host growth
176 rates were analysed treating light as either a continuous variable or a factor (the results of both
177 analyses were qualitatively similar). In the first analysis, strain, symbiont presence/absence, and light
178 were treated as factors. In the second analysis, since the relationship between growth and light
179 differed markedly for symbiotic and apo-symbiotic hosts, we analysed these responses separately to
180 detect strain-specific differences in growth using linear and non-linear regression for apo-symbiotic
181 and symbiotic responses, respectively. Symbiotic host growth responses were modelled as:

182
$$r = \frac{r_{max}(L - p')}{k + (L - p')}$$

183 where r is growth rate at a given light intensity (L), r_{max} is the light dependent maximum growth rate,
184 k is the half saturation constant and p' is the threshold light concentration (i.e. light concentration
185 when growth is zero). Free living symbiont growth rates were analysed by One Way ANOVA.

186
187 Host symbiont loads were analysed by non-linear regression and non-linear mixed effects models
188 (NLME) with the function

$$\phi = \frac{a (L - l')}{b + (L - l')^c}$$

189
190 where ϕ equals the mean host symbiont load (relative units of chlorophyll host⁻¹) at a given light
191 intensity (L), a , b , c , and l are parameters.

192

193 Results

194 To examine natural variation in the effect of symbionts on host growth, we grew multiple independent
195 strains of *P. bursaria* across a light gradient, both with and without symbionts. Growth rates for hosts
196 with symbionts increased with light, whereas growth rates for hosts without symbionts were
197 unaffected by light levels (light by symbiosis interaction, $F_{1,213}=69.3$, $P<.001$), and the effect of
198 symbionts on host growth varied between strains (strain by symbiosis interaction, $F_{3,213}=3.5$,
199 $P=0.009$). To further understand these patterns, growth responses of hosts with and without symbionts
200 were analysed separately (Supplementary Information). For all strains, symbiotic host growth rates
201 were either zero or negative in the dark and increased as a function of light, in most strains this
202 response was asymptotic reaching a maximum growth rate at high light levels (Fig 1). Symbiotic host
203 strains HA1 and 186b had the highest maximum growth rates (r_{max}) and were significantly higher
204 than in symbiotic host strain HK1 (two-sample t-tests: HK1 vs HA1, $t = 3.104$, $P = 0.039$; HK1 vs
205 186b, $t = 3.097$, $P = 0.036$). Host growth rates without symbionts varied between symbiont-free host
206 strains (ANOVA, $F_{3,94}=15.0$, $P<0.001$), from low (HA1 & 186b) to negative growth rates (CT39 &
207 Dd1), and did not respond to light (ANOVA, $F_{6,88}=0.57$, $P=0.757$). For one strain, HK1, hosts without

208 symbionts did not survive. These data suggest that hosts varied both in the benefit derived from
209 symbiosis and in their dependence upon symbionts for growth and survival.

210

211 To estimate survival of algal symbionts in the free-living state, *Chlorella* were isolated from their host
212 and grown for one week in $50 \mu\text{E m}^{-2} \text{s}^{-1}$ light and population densities measured daily. Algal strains
213 varied in their free-living growth rate (Fig 2, ANOVA, $F_{5,12}=767$, $P<0.001$). Four algal strains
214 displayed positive growth rates, whereas algae isolated from Dd1 were unable to grow in the free-
215 living state (Fig 2). Taken together with the data for free-living host growth rates, these data suggest
216 that whereas some strains were facultative, wherein both the host and symbiont were capable of free-
217 living (186b and HA1), other strains displayed some degree of dependence. For example, both the
218 host and symbiont from the strain Dd1 were mutually dependent (i.e., unable to sustain free-living
219 growth), while in CT39 and HK1 the symbionts were capable of free-living but the hosts were not,
220 suggesting host dependency.

221

222 We previously showed that hosts tightly regulate symbiont load in relation light level to maximise the
223 benefit-to-cost ratio of symbiosis (28). To test whether host control varied among Pb-C strains and
224 was related to the degree of host-symbiont dependence we measured the per host symbiont load of
225 each Pb-C strain across a light gradient. Consistent with our previous finding, across all hosts,
226 symbiont loads were lowest in the dark, peaked at low light intensities ($2-8 \mu\text{E m}^{-2} \text{s}^{-1}$), and declined
227 to intermediate levels at high light intensities (Fig 3). While this pattern of symbiont load was broadly
228 consistent among hosts, we did observe minor variations in the estimated parameters of the fitted
229 curves (NLME, $\chi^2_6=118$, $P<0.001$; see Supplementary Information). Specifically, host strain 186b had
230 a higher symbiont load than HK1 independent of light level (i.e. parameter a). Peak symbiont load
231 occurred at lower light intensities in host strains 186b and Dd1 than HK1 and CT39 (i.e. parameter l),
232 potentially suggesting differences in the light environment to which the strains were adapted in nature.
233 Host strains Dd1 and HA1 reduced symbiont load at high light intensities to a greater extent than
234 strain CT39 (i.e. parameter c), suggesting variation in the intensity of host regulation of symbiont
235 load. These data suggest that host control is a broadly conserved trait across *P. bursaria*, but show no

236 clear association between host control parameters and host-symbiont dependence, except that
237 symbiont load was highest in the most facultative host (186b) and lowest in the host least able to
238 survive without its symbionts (HK1).

239

240 **Discussion**

241 The transition from facultative to obligate symbiosis, and thus the evolution of mutual dependence
242 constitutes a major evolutionary transition in individuality (8,9), and underpins the evolution of
243 cellular organelles such as chloroplasts (1). The evolutionary transition to mutual dependence
244 requires there to be variation in host-symbiont dependence available for natural selection to act upon,
245 and for mutual dependence to be associated with higher symbiotic fitness (6–10). Using experiments
246 with the microbial photosymbiosis between the ciliate host, *P. bursaria*, and the green alga, *Chlorella*
247 sp., we demonstrate variation in host-symbiont dependence ranging from strains that are fully
248 facultative to those that display either mutual dependence or dependence of hosts upon symbionts.
249 Thus the *P. bursaria-Chlorella* interaction appears to exist on the boundary between facultative and
250 obligate symbiosis. Moreover, since symbiotic growth rates of facultative Pb-C strains were higher
251 than those showing greater degrees of dependence, indeed the host HK1 which was unable to survive
252 without symbionts showed the lowest symbiotic growth rate, it seems likely that facultative symbiosis
253 may be favoured by selection. Interestingly, this is consistent with the distribution of symbiotic strains
254 across the predominantly free-living *Chlorella* clade (34), which suggests repeated transitions from
255 free-living to symbiosis and a long evolutionary history of its association with *P. bursaria* being
256 facultative. Furthermore, Pb-C strains that were more recently isolated from natural populations (186b
257 and HA1 were isolated in 2006 and 2010, respectively), were more facultative than those with longer
258 histories of laboratory culture (HK1 and Dd1 were isolated in 1990 and 1995, respectively). This
259 could suggest that host-symbiont dependence is a derived trait among lab-adapted Pb-C strains,
260 whereas in natural populations the facultative state is more common, however more extensive studies
261 of natural populations will be required to test this.

262

263 Dependence was more commonly observed in *P. bursaria* than in *Chlorella*, possibly suggesting an
264 asymmetry in selection for dependence between the host and the symbiont in this system. This would
265 be consistent with our previous work, which showed that this is an exploitative symbiotic interaction,
266 wherein hosts benefit more than symbionts from engaging in symbiosis (28). This underlying conflict
267 between host and symbiont would be expected to select for the retention of free-living ability,
268 particularly in the symbiont. The fitness benefit of symbiosis to hosts increases with increasing light
269 intensity and with decreasing availability of heterotrophic food (28), suggesting that selection for
270 dependence in hosts is likely to be environmentally context dependent. We would predict therefore
271 that *P. bursaria* should be more likely to evolve dependence on their symbionts in high light, low
272 food habitats, but less likely in low light, high food habitats, or in environments that are highly
273 variable in terms of light intensity and/or food availability. Indeed, in variable environments the
274 facultative nature of the photosymbiosis may allow for partner-switching whereby hosts could acquire
275 locally-adapted symbionts to promote their invasion of new habitats.

276
277 We observed similar responses among hosts in their regulation of symbiont load across light
278 gradients. Consistent with our previous data (28) and a mathematical model of this system (36), we
279 observed that symbiont load per host peaked at low light levels. This occurs because hosts adjust
280 symbiont number to maximise the benefit-to-cost ratio of symbiosis. In the dark, hosts reduce their
281 symbiont load as their maintenance is costly and they provide no benefit to host growth through
282 photosynthesis. At very low light intensities, hosts need many symbionts in order to gain a growth
283 benefit, which albeit costly in terms of demand for nitrogen leads to a peak in symbiont load. As light
284 increases, the per symbiont benefit to hosts increases and so hosts need fewer symbionts to provide
285 the same photosynthetic output, allowing hosts to reduce their N costs. Above a given maximal light
286 level, the per-symbiont benefit saturates leading to an asymptotic relationship between symbiont load
287 and light. The response of symbiont load to light was broadly conserved among our host strains, and
288 our empirical estimates of this trait closely matched the theoretical predictions in Dean *et al.* (36).

289 Minor variations in the parameters of the fitted curves were observed but were not associated with

290 variation in dependency, with the exception that symbiont load was highest in the fastest growing and
291 most facultative host (186b) and lowest in the host that was slowest growing and least able to survive
292 without its symbionts (HK1). This suggests that while all host strains have the ability to control
293 symbiont load, an overall higher symbiont load favoured faster symbiotic growth, whereas lower
294 symbiont loads may have evolved in more highly dependent associations, presumably to minimise the
295 costs of symbiosis.

296

297 **Conclusions**

298 Comparative evolutionary analysis suggests that host-symbiont dependence varies widely between
299 symbiotic lineages across the tree of life (13). Data from the study presented here show that the
300 degree of host-symbiont dependence also varies within symbiotic partnerships, and asymmetrically
301 for hosts and symbionts. Where symbiosis is based upon exploitation, as here, our data suggest that
302 the evolution of dependence is less likely in the exploited symbiotic partner, in this case, *Chlorella*.
303 Mutual dependence was associated with lower rates of symbiotic growth, thus while transitions to
304 obligate mutual dependence appear to occur frequently at the individual level, these may be unlikely
305 to outcompete facultative strains, favouring the evolutionary maintenance of facultative symbiosis.

306

307 **Abbreviations**

308 PPM - Protozoan Pellet Media

309 L:D – Light:Dark

310 BBM – Bold's Basal Medium

311

312 **Declarations**

313 • **Ethics approval and consent to participate**

314 Not applicable

315 • **Consent for publication**

316 Not applicable

317 • **Availability of data and material**

1
2 318 All data generated or analysed during this study are included in this published article and its
3 319 supplementary information files.

4 320 • **Competing interests**

5
6 321 The authors declare that they have no competing interests

7
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15
16 328 • **Authors' contributions**

17 329 EJAM, CDL, AJW, DDC and MAB designed the study; EJAM and MESS performed the
18 330 experiments; EJAM analysed the data; EJAM, DDC and MAB drafted the manuscript; all authors
19 331 contributed to the submitted manuscript.

20
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432 **Legends**

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4 434 Figure 1. Reaction norms for host growth rate (day^{-1}) in response to light ($\mu\text{E m}^{-2} \text{s}^{-1}$), for both
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6 435 symbiotic (green) and aposymbiotic (open) hosts, with fitted models (mean growth for aposymbiotic,
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8 436 non-linear regression for symbiotic). Each panel shows data for a different strain. Dotted line
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11 437 indicates where host growth equals zero.

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15 439 Figure 2. Growth rate of extracted *Chlorella* symbionts in 7-day cultures grown in Bold's Basal
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17 440 Medium immediately following mechanical liberation from *Paramecium bursaria* hosts. Boxes show
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19 441 median and ranges for three independent culture replicates, dotted line indicates zero growth.

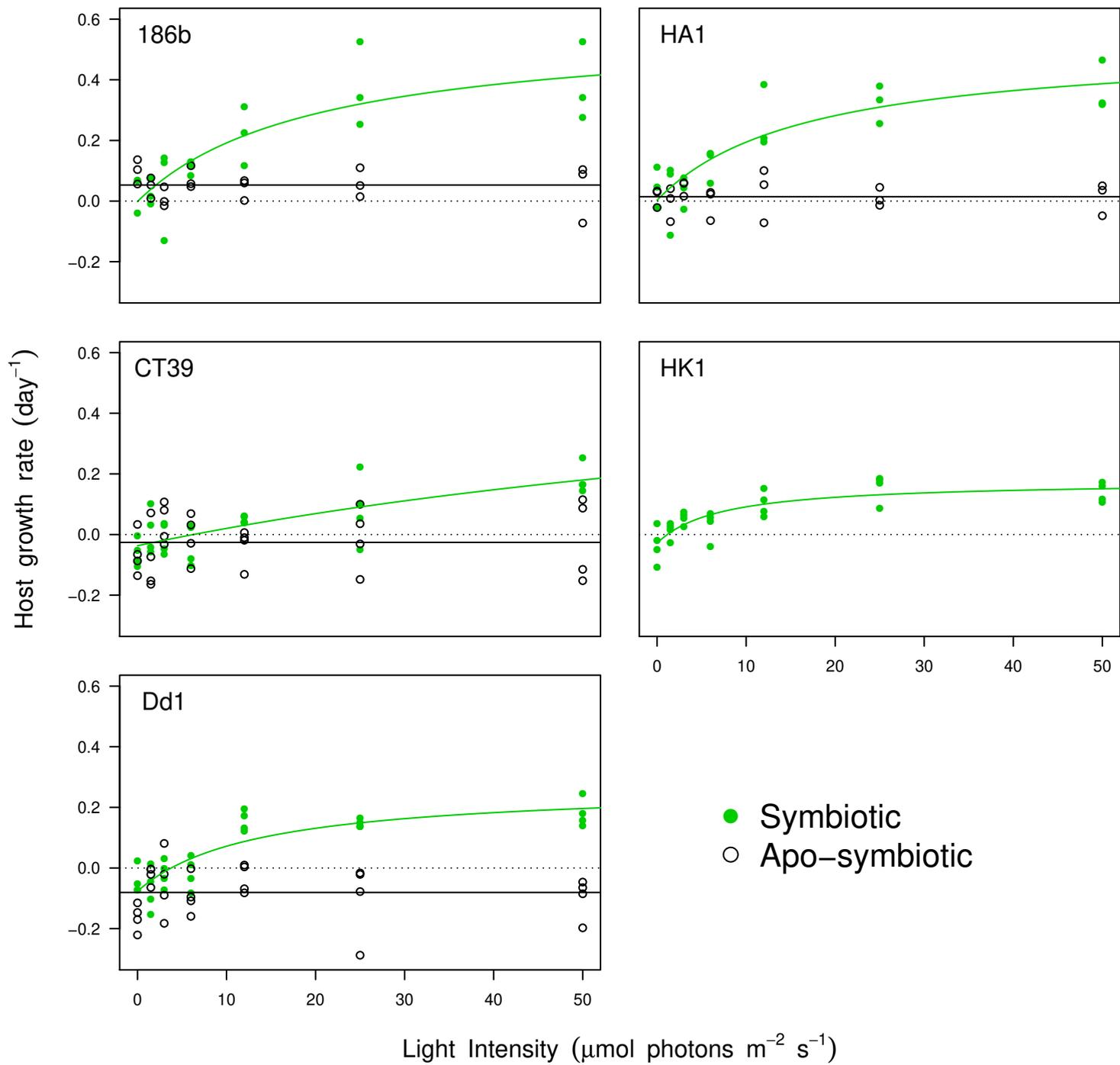
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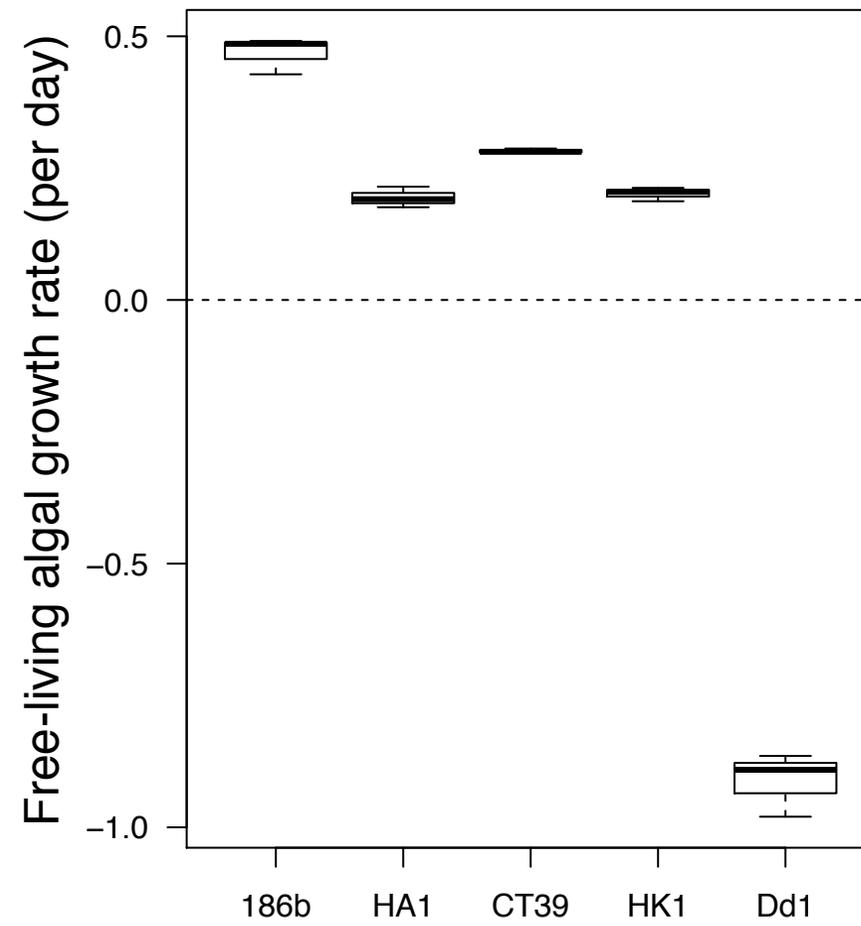
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24 443 Figure 3. Reaction norms of mean host symbiont load (estimated from individual host chlorophyll
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26 444 fluorescence, scale is relative fluorescence) in response to light ($\mu\text{E m}^{-2} \text{s}^{-1}$), for symbiotic hosts. Each
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28 445 panel shows data for a different strain.

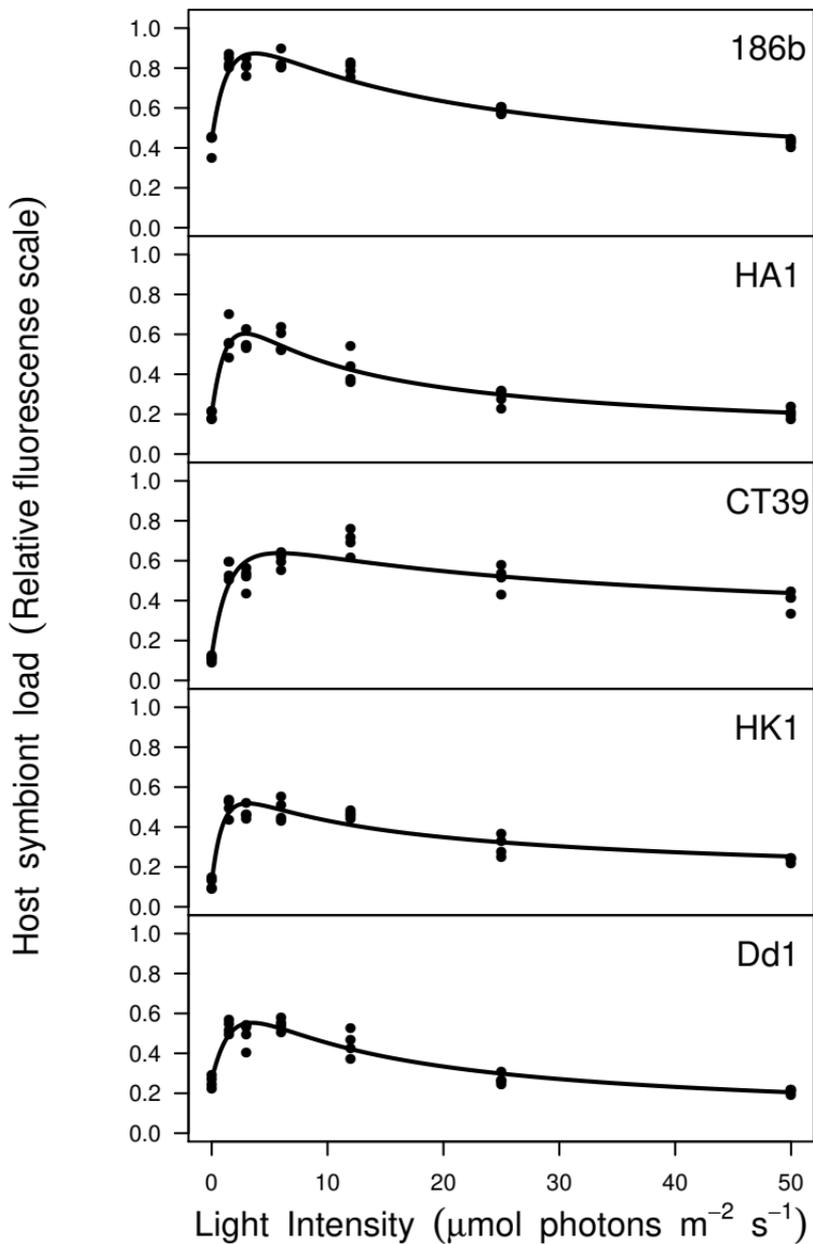
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Figure 1

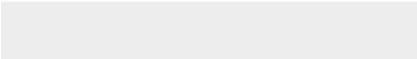
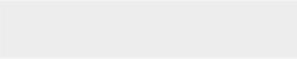
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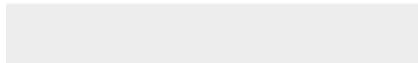


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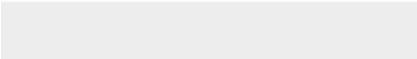
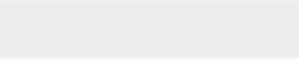


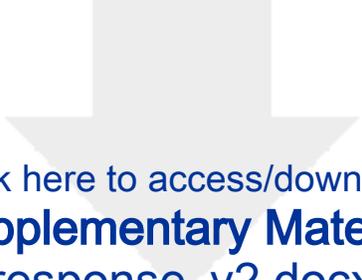
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