INTRODUCTION

Global warming causes biodiversity crises, which impact organisms not only directly but also indirectly through other organisms with which they interact (Bellard et al., 2012; Blois et al., 2013; Harley, 2011; Penuelas et al., 2013; Ullah et al., 2018). Symbiosis is important for global biodiversity, ecosystem services, and agriculture (Soka & Ritchie, 2015; Wernegreen, 2012; Werner et al., 2018). In recent years, the possibility that elevated temperatures resulting from global warming may substantially affect biodiversity through disrupting mutualistic associations such as the coral–dinoflagellate symbiosis (Hoegh-Guldberg et al., 2007; Pandolfi et al., 2011), insect–bacteria symbioses (Kikuchi et al., 2016; Wernegreen, 2012), and plant–pollinator interactions (Eckert et al., 2010; Hegland et al., 2009) has been highlighted. The coral–dinoflagellate model, which is an obligate symbiotic relationship, showed that thermal stress could lead to coral bleaching (corals’ loss of zooxanthellae that provide up to 90% of host nutritional requirements) (Baker et al., 2018; Ferrier-Pages et al., 2018). The stable, long-term mutualistic relationship between insects and their carried symbionts is also vulnerable to thermal stress (Kiers et al., 2010). However, empirical investigations of facultative mutualism under global warming have been scarce and mostly focus on insects (Burke et al., 2010; Wernegreen, 2012). Research on facultative symbiosis is needed.

Abstract

Anthropogenic global change is increasingly raising concerns about collapses of symbiotic interactions worldwide. Therefore, understanding how climate change affects symbioses remains a challenge and demands more study. Here, we look at how simulated warming affects the social ameba Dictyostelium discoideum and its relationship with its facultative bacterial symbionts, Paraburkholderia hayleyella and Paraburkholderia agricolaris. We cured and cross-infected ameba hosts with different symbionts. We found that warming significantly decreased D. discoideum’s fitness, and we found no sign of local adaptation in two wild populations. Experimental warming had complex effects on these symbioses with responses determined by both symbiont and host. Neither of these facultative symbionts increases its hosts’ thermal tolerance. The nearly obligate symbiont with a reduced genome, P. hayleyella, actually decreases D. discoideum’s thermal tolerance and even causes symbiosis breakdown. Our study shows how facultative symbioses may have complex responses to global change.

KEYWORDS

bacterial symbionts, Dictyostelium discoideum, global warming, Paraburkholderia, symbiosis
The symbiosis between social amebae and certain *Paraburkholderia* bacterial species is a promising system for gaining insight into how facultative mutualisms respond to global warming. The soil-dwelling ameba *Dictyostelium discoideum* is a good model to address eukaryote–microbe interactions because of its dynamic relationship with bacteria. In a nutrient-rich environment, *D. discoideum* lives as an independent haploid ameba that reproduce by binary fission. When food is scarce, cAMP-mediated aggregation occurs, leading to the formation of multicellular slugs that move to a favorable location to develop into fruiting bodies. In these fruiting bodies, approximately 20% of the cells die to form a long thin stalk that supports a spherical structure called the sorus, while the remaining 80% ascend into the sorus and turn into spores (Kessin, 2001). *D. discoideum* is a predator of bacteria and a popular model for studying biological phenomena, including multicellularity, chemical signaling, and social phenomena (Chen et al., 2016; DiSalvo et al., 2015; Ho et al., 2013; Kessin, 2001; Shu et al., 2018; Strassmann & Queller, 2011; Zhang et al., 2016).

In addition to eating bacteria, *D. discoideum* can also form symbiotic associations with some bacterial species (Brock et al., 2011; DiSalvo et al., 2015; Strassmann & Shu, 2017). About one-third of wild-collected clones of *D. discoideum*, which are referred to as “primitive farmers,” have stable associations with their symbiotic bacteria throughout their life cycle (Brock et al., 2011). These farmer clones can carry bacteria during spore dispersal and seed them as new food sources (Figure 1). Later studies found that farming status is induced by symbiotic bacteria belonging to the genus *Paraburkholderia* (DiSalvo et al., 2015; Haselkorn et al., 2019; Shu et al., 2018) (named *P. agricolaris*, *P. hayleyella*, and *P. bonniea* (Brock et al., 2018)). These *Paraburkholderia* are not edible themselves, but they facilitate further carriage of food bacteria that on their own would be digested. The inedible symbionts actively find their ameba hosts through chemotaxis, reside within food vacuoles, and form very stable associations (Figure 1) (Shu et al., 2015; Haselkorn et al., 2019; Shu et al., 2018; Shu et al., 2018). Therefore, we also define their association as “bacterial carriage” by social ameba.

Both *D. discoideum* and their *Paraburkholderia* symbionts can live independently, making them facultative symbioses. However, *P. hayleyella* shows three indications of being more obligate than *P. agricolaris*. First, it is a sister species comprising a very long branch in the phylogeny, suggesting that it has been associated with amebas for a long time (Brock et al., 2018; Haselkorn et al., 2019). Second, consistent with greater dependence on the host, it grows slowly on its own under laboratory conditions compared to *P. agricolaris*. *P. hayleyella* also has greatly reduced carbon usage compared to *P. agricolaris* (Brock et al., 2020). Finally, it shows the genome size reduced by over one half compared to close relatives (Brock et al., 2018). This system gives us an opportunity to investigate how increased temperatures associated with global warming could potentially affect facultative symbioses.

Facultative symbioses could be more vulnerable to global warming compared to obligate symbioses because their relationships are less stable. Alternatively, facultative symbioses may be more resilient to global warming because both partners can live on their own and therefore may be more resilient to environmental changes. We will test whether these facultative symbionts help or harm their hosts under warming, and also whether the symbiosis is less or more resilient with the more facultative species *P. agricolaris* versus the more obligate species *P. hayleyella*. We first tested the thermal tolerance of social amebas using common garden experiments. Then, we mixed and matched social ameba hosts with different *Paraburkholderia* symbionts (Figure 2a) to investigate how different combinations respond to simulated global warming.

## 2 MATERIALS AND METHODS

### 2.1 *D. discoideum* clones and culture conditions

We used wild *D. discoideum* isolates (Table 1) collected at Mountain Lake Biological Station in Virginia (N37°21′, W80°31′), Houston Arboretum and Nature Center in Texas (N29°77′, W95°45′) and Little Butt’s Gap in North Carolina (35°46′ N, 82°20′ W). These clones were uninfected (called naïve hosts in this paper) or infected with either *P. agricolaris* or *P. hayleyella* (called native hosts in this paper). We grew *D. discoideum* from previously frozen spores on SM/5 agar plates (2 g glucose, 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g MgCl₂, 1.9 g KH₂PO₄, 1 g K₂HPO₄, and 15 g agar per liter) with food bacterium *Klebsiella pneumoniae* (obtained from the Dicty Stock Center) at room temperature (21°C).

### 2.2 Symbionts

We used *D. discoideum*-associated *Paraburkholderia* symbionts isolated and described in previous studies (Brock et al., 2011; DiSalvo et al., 2015; Haselkorn et al., 2019; Shu et al., 2018; Shu et al., 2018). Therefore, we also define their association as “bacterial carriage” by social ameba.

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![FIGURE 1](image) Scheme summarizing the social ameba–*Paraburkholderia* symbiosis. Figure courtesy of Susanne DiSalvo
et al., 2015; Haselkorn et al., 2019; Shu et al., 2018). P. agricolaris strains were isolated from QS70, QS159, and NC21 D. discoideum hosts, while P. hayleyella strains were isolated from QS11, QS21, and NC28 D. discoideum hosts, respectively. Specific isolates used in this study are listed in Table 1.

2.3 | Choosing experimental temperature for simulating warming

We wanted to choose an experimental temperature that is stressful to social amebae but does not cause complete death. We tested growth conditions of D. discoideum (three clones: QS11, QS70, and QS9) under different temperatures ranging from 21 to 30°C. We found that almost no clone can survive above 28°C, while there were drastic changes between 27 and 28°C (Figure 2b). Therefore, we chose 27.5°C as the thermal stress temperature for this experiment.

We want to test how extreme warming event (from D. discoideum ameba's perspective) affects the social ameba symbiosis and whether its bacterial symbionts could help.

2.4 | Effects of thermal stress on two wild D. discoideum populations

We used two D. discoideum populations from geographic and climate divergent locations Texas (N29°46′, W95°27′; elevation, 11 m; annual temperatures: 5.7–34.7°C; average temperatures: 20.6°C) and Virginia (N37°21′, W80°31′; elevation, 1,160 m; annual temperatures: −15–25°C; average temperatures: 5.2°C) to investigate how D. discoideum responds to simulated thermal stress and whether they could locally adapt to it. We randomly chose 10 Texas clones and 10 Virginia clones of wild D. discoideum and plated those (2 × 10⁵ spores) in association with K. pneumoniae (200 μl, OD1.5) on SM/5 plates.
We incubated these clones at room temperature 21°C (control) and 27.5°C (thermal stress treatment), respectively. We harvested spores from each plate after one week. We flooded the plate with 10 ml KK2 + 0.1%NP-40 and collected spores into 15 ml falcon tubes. We counted spores on a hemocytometer using a light microscope. This design resulted in a total of 2 (populations) × 10 (clones) × 2 (temperatures) × 3 (replicates) = 120 experimental units. The mean of three replicates was used for further statistical analyses.

### 2.5 Effects of thermal stress on *D. discoideum–Paraburkholderia* symbiosis

We generated symbiont-free native host clones (QS70C, QS159C, NC21C, QS11C, QS21C, and NC28C) by curing them of their bacteria with tetracycline, or by ampicillin–streptomycin treatment as previously described (Brock et al., 2011; DiSalvo et al., 2015; Shu, et al., 2018). We confirmed the loss of infection status by plating

| Clones  | Location  | Host types  | Symbionts                  | Choosing test temperature | Amoebae under warming | Symbioses under warming |
|---------|-----------|-------------|----------------------------|---------------------------|-----------------------|-------------------------|
| QS177   | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS198   | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS323   | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS325   | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS600   | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS68    | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS71    | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS74    | Texas     | Naïve host  |                            |                           | √                     | √                       |
| QS76    | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS78    | Texas     | Naïve host  |                            |                           | √                     |                         |
| QS1010  | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS1041  | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS1068  | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS1072  | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS1080  | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS17    | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS18    | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS4     | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS6     | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS9     | Virginia  | Naïve host  |                            |                           | √                     | √                       |
| QS1     | Virginia  | Naïve host  |                            |                           | √                     |                         |
| QS70    | Virginia  | Naïve host  | *P. agricolaris* B1qs70    |                           | √                     | √                       |
| QS159   | Virginia  | Naïve host  | *P. agricolaris* B1qs159   |                           | √                     |                         |
| NC21    | North Carolina | Naïve host | *P. agricolaris* B1nc21   |                           | √                     |                         |
| QS11    | Virginia  | Naïve host  | *P. hayleyella* B2qs11    |                           | √                     | √                       |
| QS21    | Virginia  | Naïve host  | *P. hayleyella* B2qs21    |                           | √                     |                         |
| NC28    | North Carolina | Naïve host | *P. hayleyella* B2nc28   |                           | √                     |                         |
| QS70C   | Virginia  | Cured native host |                       |                           | √                     |                         |
| QS159C  | Virginia  | Cured native host |                   |                           | √                     |                         |
| NC21C   | North Carolina | Cured native host |                   |                           | √                     |                         |
| QS11C   | Virginia  | Cured native host |                   |                           | √                     |                         |
| QS21C   | Virginia  | Cured native host |                   |                           | √                     |                         |
| NC28C   | North Carolina | Cured native host |                   |                           | √                     |                         |
them out on bacteria-free plates and confirming that the social ameba could not proliferate, a test we call a spot test (Brock et al., 2011). We mixed and matched (Figure 2) *D. discoideum* (naïve hosts: QS1, QS5, and QS74; native hosts: QS70C, QS159C, NC21C, QS11C, QS21C, and NC28C) with two facultative symbionts *P. agricolaris* (B1qs70, B1qs159, and B1nc21) and *P. hayleyella* (B2qs11, B2qs21, and B2nc28) to investigate how thermal stress affects their symbiotic relationships. We tested four combinations under two temperature treatments (21 and 27.5°C): native hosts—*P. agricolaris*, naïve hosts—*P. agricolaris*, native hosts—*P. hayleyella* and naïve hosts—*P. hayleyella* with three replicates.

To set up each experiment, we plated $2 \times 10^5$ spores in association with *K. pneumoniae* (200 µl, OD1.5) on SM/5 plates. For experiments adding *Paraburkholderia*, we mixed the specified *Paraburkholderia* (OD1.5) clones at 3% (6 µl) and *K. pneumoniae* at 97% (194 µl) vol and plated *D. discoideum* spores ($2 \times 10^3$) with 200 µl of the bacterial mixture on SM/5 plates. We incubated these clones at room temperature 21°C (control) and 27.5°C (thermal stress treatment), respectively. We harvested spores from each plate after one week and flooded the plate with 10 ml KK2 and plated them out on bacteria-free plates and confirming that the social ameba could not proliferate, a test we call a spot test (Brock et al., 2011).

### 2.6 Statistical analyses

#### 2.6.1 Effects of thermal stress on two *D. discoideum* populations

We analyzed the data (N = 40) with a general linear mixed model in IBM SPSS Statistics 24. In these analyses, population (two levels: Texas and Virginia), temperature (two levels: 21 and 27.5°C), and their interactions were used as fixed factors. *D. discoideum* clone was nested within population and used as a random factor. The data passed the normality test (Kolmogorov–Smirnov test) and tested for homogeneity of variance (Levene’s test).

We analyzed spore production (outcome variable) as a measure of ameba fitness. A significant temperature main effect would indicate thermal stress affects *D. discoideum*’s fitness, a significant population type main effect would indicate that populations differ in their fitness, and a significant population × temperature interaction would indicate adaptive divergence in thermal tolerance in two populations.

#### 2.6.2 Effects of thermal stress on *D. discoideum*-*Paraburkholderia* symbiosis

We analyzed and plotted four combinations separately (native hosts—*P. agricolaris*, Figure 3a; naïve hosts—*P. agricolaris*, Figure 3b; native hosts—*P. hayleyella*, Figure 3c and naïve hosts—*P. hayleyella*, Figure 3d). Native hosts—*P. agricolaris* (N = 12), naïve hosts—*P. agricolaris* (N = 24), and native hosts—*P. hayleyella* (N = 12) data were log-transformed to improve normality. Transformed data passed the normality test (Kolmogorov–Smirnov test) and tested for homogeneity of variance (Levene’s test). We analyzed these data with general linear models. Naïve *P. hayleyella* data (N = 24) were analyzed with a generalized linear model (GLM) with Negative binomial distribution in IBM SPSS Statistics 24.

We used spore production as a measure of ameba fitness. A significant temperature main effect indicates that thermal stress can affect *D. discoideum* fitness. A significant symbiont main effect indicates that the presence of a symbiont can affect *D. discoideum* fitness. A significant temperature × symbiont interaction will indicate that the presence of symbiont can affect *D. discoideum* fitness under thermal stress.

### 3 RESULTS

#### 3.1 The pattern of local adaptation to thermal stress in *D. discoideum*

Increased temperature decreased the fitness of both the Texas clones and the Virginia clones (Figure 2c), as indicated by the significant temperature main effect (GLM, $F_{1,18} = 351.25$, $P < .001$). Virginia clones outperformed Texas clones at both temperatures (Figure 2c). However, we found no variation in thermal tolerances of Texas and Virginia populations, as shown by the nonsignificant population × temperature interaction (GLM, $F_{1,18} = 2.141$, $P = .161$). These results suggest that increased temperature significantly decreases *D. discoideum*’s fitness. We did not find adaptive divergence to thermal stress in two wild populations of *D. discoideum* from locations that differed in ambient temperature.

#### 3.2 The complex effects of simulated warming on *D. discoideum*-*Paraburkholderia* symbioses

##### 3.2.1 *P. agricolaris* had no effect on *D. discoideum’s* thermal tolerance

When *P. agricolaris* clones were mixed with their native hosts, thermal stress decreased *D. discoideum*’s fitness, as indicated by the significant temperature main effect (GLM, $F_{1,20} = 20.188$, $P < .001$, Figure 3a). However, adding *P. agricolaris* made no difference to host fitness (GLM, $F_{1,20} = 2.406$, $P = .137$, Figure 3a). The effect of thermal stress did not change with the addition of *P. agricolaris*, as indicated by the nonsignificant temperature × symbiont interaction (GLM, $F_{1,20} = 0.427$, $P = .521$, Figure 3a).

When *P. agricolaris* clones (n = 3) were mixed with naïve hosts (n = 3), the pattern is the same (Figure 3b). Thermal stress decreased *D. discoideum*’s fitness (General linear model, $F_{1,8} = 82.087$, $P < .001$, Figure 3b), while adding *P. agricolaris* made no difference to host’s fitness (GLM, $F_{1,8} = 1.803$, $P = .216$, Figure 3b). Also, there was no significant temperature × symbiont interaction.
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(General linear model, $F_{1,8} = 0.004, p = .953$, Figure 3b), indicating that the effect of thermal stress did not change with the addition of P. agricolaris.

Overall, these results suggest that the more facultative P. agricolaris neither helps nor harms D. discoideum under thermal stress. In addition, there is no difference between native and naïve hosts.

3.2.2 | P. hayleyella decreased D. discoideum’s thermal tolerance and caused a symbiosis breakdown when mixed with naïve hosts

When P. hayleyella clones ($n = 3$) were mixed with their native hosts ($n = 3$), thermal stress decreased D. discoideum’s fitness, as indicated by the significant temperature main effect (GLM, $F_{1,8} = 44.747, p < .001$, Figure 3c). We also found that adding P. hayleyella decreased host fitness (GLM, $F_{1,8} = 17.287, p = .003$, Figure 3c). There was no significant temperature*symbiont interaction (GLM, $F_{1,8} = 2.624, p = .144$, Figure 3c), indicating that adding P. hayleyella did not further decrease the native host’s fitness under thermal stress (Figure 3c).

When P. hayleyella clones ($n = 3$) were mixed with naïve hosts ($n = 3$), both adding P. hayleyella (Negative binomial GLM, $\chi^2 = 6.73, p = .009$) and thermal stress (Negative binomial GLM, $\chi^2 = 8.471, p = .004$) decreased D. discoideum’s fitness (Figure 3d). There was also a significant temperature*symbiont interaction (Negative binomial GLM, $\chi^2 = 4.958, p = .026$, Figure 3d), indicating that adding P. hayleyella further decreased naïve host’s fitness under thermal stress. In addition, 2 out of 3 tested naïve hosts showed zero growth under thermal stress when mixed with P. hayleyella, indicating symbiosis breakdown, while this did not happen in any of the native hosts.

Taken together, these results suggest that adding P. hayleyella, like thermal stress, can decrease D. discoideum’s fitness. In addition, it further decreases host fitness under thermal stress. We also found evidence of symbiosis breakdown when P. hayleyella was mixed with naïve hosts, while this does not happen in the native hosts. This indicates potential partner adaptation between P. hayleyella and their native hosts.

4 | DISCUSSION

Overall, we show that increased temperature affects symbiotic interactions. Increased temperature can significantly decrease D. discoideum’s fitness. We found no adaptive divergence to thermal stress in two wild populations. Neither symbiont increased its hosts’
thermal tolerance. Our study shows that facultative symbioses can also have complex responses to warming.

Previous studies found that facultative symbionts provide greater flexibility in response to temperature change compared to obligate symbioses (Burke et al., 2010; Renoz et al., 2019). For example, facultative bacterial symbionts benefit aphids under heat stress (Montllor et al., 2002) and may protect both host and obligate symbiont from thermal stress (Burke et al., 2010). However, in the social ameba symbiosis system, we find no evidence that facultative Paraburkholderia symbionts increase D. discoideum hosts’ thermal tolerance.

We find that different symbionts behave differently within the same host under simulated warming, and we also find evidence of host adaptation. Of the two symbionts, the more facultative P. agricolaris has no effects on the thermal tolerance of either native or naïve D. discoideum hosts. On the other hand, the more obligate P. hayleyella induces a significant difference to the host’s thermal tolerance, imposing a higher cost to D. discoideum. Our study shows that the addition of P. hayleyella to its native host decreases host fitness at both temperatures indicating that native hosts suffer a fitness cost when they carry P. hayleyella. In addition, P. hayleyella harms and even kills naïve hosts exposed to thermal stress, disrupting the symbiosis. The more severe fitness costs exerted by P. hayleyella colonization in naïve hosts compared to native hosts suggest potential host adaptation between P. hayleyella and their native host clones.

One potential drawback of this study is that we did not monitor the population dynamics of K. pneumoniae and Paraburkholderia symbionts under different temperatures. Simulated warming can directly affect the interactions between food bacteria and symbionts, which in turn affects the growth of amebae. Indeed, a recent study reported that the optimal growth temperature for both Paraburkholderia symbionts is 30°C, and P. agricolaris grows faster than P. hayleyella (Brock et al., 2020). Therefore, in this study, both food bacterium Klebsiella pneumoniae and Paraburkholderia symbionts grow faster under warming conditions. However, we argue that their interactions may have little effect on host fitness. First, K. pneumoniae grows much faster than symbionts, and their starting proportion is very high (97%) compared to symbionts (3%). Second, the faster-growing symbiont, P. agricolaris, did not change host’s fitness in both temperatures, indicating its frequency has little effect on host fitness. Moreover, P. hayleyella colonization in naïve hosts compared to native hosts suggest potential host adaptation between P. hayleyella and their native host clones.

The authors declare no conflicts of interest.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTION
Longfei Shu: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Visualization (lead); Writing-original draft (lead). Xinye Qian: Data curation (equal); Formal analysis (equal); Visualization (equal); Writing-review & editing (equal). Debra A. Brock: Data curation (equal); Formal analysis (equal); Writing-original draft (equal). Katherine S. Geist: Data curation (equal); Formal analysis (equal); Visualization (equal); Writing-review & editing (equal). David C. Queller: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Supervision (lead); Writing-review & editing (equal). Joan E. Strassmann: Conceptualization (lead); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Supervision (lead); Writing-original draft (equal); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT
All data are available from the Mendeley Data: http://dx.doi.org/10.17632/fjj9mbm6hw.1

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