Natural History Note

Developmental and Ecological Benefits of the Maternally Transmitted Microbiota in a Dung Beetle

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abstract: To complete their development, diverse animal species rely on the presence of communities of symbiotic microbiota that are vertically transmitted from mother to offspring. In the dung beetle genus *Onthophagus*, newly hatched larvae acquire maternal gut symbionts by the consumption of a maternal fecal secretion known as the pedestal. Here, we investigate the role of pedestal symbionts in mediating the normal development of *Onthophagus gazella*. Through the stepwise removal of environmental and maternal sources of microbial inoculation, we find that pedestal microbiota can enhance both overall growth and developmental rate in *O. gazella*. Further, we find that the beneficial effects of symbionts on developmental outcomes are amplified in the presence of ecologically relevant temperature and desiccation stressors. Collectively, our results suggest that the pedestal may provide an adaptive function by transmitting beneficial microbiota to developing dung beetle larvae and that the importance of microbiota for developmental and fitness outcomes may be context dependent.

Keywords: microbiome, *Onthophagus*, developmental symbiosis, nongenetic inheritance, stress.

Introduction

One long-standing goal of evolutionary ecology is to understand the ecological causes and evolutionary consequences of interactions between hosts and their symbionts. While much of this work has traditionally been undertaken in adults, it is increasingly clear that many groups of animals often associate with rich communities of microbial symbionts throughout their development. Although in some cases these symbionts can present substantial challenges to host development and survival (e.g., as pathogens), in others they constitute a well-integrated feature of diverse ontogenetic processes, ranging from preembryonic development to life-history transitions (Dethlefsen et al. 2007; Gilbert 2016). For example, microbial symbionts have been shown to generate cytoplasmic incompatibility across most major arthropod taxa (O'Neill et al. 1992; Hoffmann and Turelli 1997; Werren et al. 2008), contribute to the establishment of the anterior-posterior axis in embryonic nematodes (Landmann et al. 2014); influence tissue and organ development in a wide array of taxa including cephalopods (McFall-Ngai 2014), fish (Rawls et al. 2004), and mammals (Hooper and Gordon 2001; Stappenbeck et al. 2002); and shape key life-history traits such as growth and maturation (e.g., metamorphosis; Hadfield 2011; Shikuma et al. 2014). Such observations of developmental symbiosis demonstrate the diverse and often essential nature of host-microbe interactions throughout host ontogeny.

Both during host development and into adulthood, symbiont populations can buffer against challenging environmental conditions and may even facilitate adaptation and diversification into these environments over evolutionary time (e.g., Sudakaran et al. 2015). Microbial metabolisms, for instance, can help condition the developmental environment of their hosts by aiding in digestion and generating limited or unavailable nutrients that are essential for host growth and health. For example, *Buchnera aphidicola* provisions sap-feeding pea aphids with essential amino acids (Douglas 1998), *Wigglesworthia morsitans* synthesizes vitamin B for blood-feeding tsetse flies (Rio et al. 2012), and diverse symbionts aid in the digestion of cellulose in the termite gut (Tokuda and Watanabe 2007; Douglas 2009).

In many cases, symbiont-derived benefits are assumed to be outcomes of coevolution that are invariant features of host biology, making studies of the context dependency of these interactions relatively rare. Yet it is becoming increasingly clear that the function and strength of association between hosts and their microbial symbionts are highly contingent on environmental conditions. For example, in pea aphids, the presence of secondary microbial symbionts substantially enhances the fitness of nymphs (e.g., survival, development time) when exposed to a heat shock but has no effect on fitness under permissive temperatures (Russell and Moran 2006). In addition, the pea aphid defensive symbiont, *Hamiltonella de-*

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fensa, is favored and maintained in the presence of parasitoid wasps but lost in their absence, and the infection frequency of a mutualistic secondary symbiont of the red gum lerp psyllid is strongly positively correlated with parasitoid presence across an environmental gradient (Hansen et al. 2007; Oliver et al. 2008). Therefore, a more complete understanding of the causes and fitness consequences of developmental symbioses requires an appreciation of the range of environmental contexts in which they occur.

At the same time, stable host-microbe associations require reliable mechanisms by which hosts can acquire microbiota anew in each generation. Although microbial colonization often occurs via horizontal acquisition from social interactions with conspecifics (e.g., Marsh et al. 2014) or through sampling of the external environment (e.g., Kikuchi et al. 2007; McFall-Ngai et al. 2012), in many animal species, symbionts are transgenerationally inherited from mother to offspring (Salem et al. 2015; Gilbert 2016). Among insects, maternal transmission is commonly mediated by passaging of obligate intracellular microorganisms (e.g., *Wolbachia*) both through the germline and through egg and oviposition site inoculation (Salem et al. 2015). These mechanisms ensure both the initiation and specificity of host-microbe interactions across generations and may provide the foundation for developmental symbiosis and its evolution (Nyholm and McFall-Ngai 2014). Here we explore whether maternally transmitted microbiota influence normal development in dung beetles and the ecological conditions under which such influence is most likely to manifest.

Dung beetles (Scarabaeidae: Scarabaeinae) are a clade of >5,000 species that feed primarily on the dung of large herbivores (Monaghan et al. 2007) and may be a promising system for studying the developmental, ecological, and evolutionary consequences of the maternal transmission of microbial symbionts (Estes et al. 2013). Throughout their life span, these beetles feed on dung, which is generally low in nutrition, depleted of essential amino acids, and commonly >80% cellulose (Muller 1980). During the reproductive season, females of many species colonize dung pads and construct subterranean brood (i.e., dung) balls in which larvae develop to adulthood. In the genus *Onthophagus*, adult females generate a hollow brood chamber for larval development within one pole of each brood ball and provision each chamber with their own excrement, forming a pedestal onto which a single egg is oviposited and thereby anchored into the brood chamber (Halffter and Edmonds 1982; Estes et al. 2013). Upon hatching, larvae immediately consume the pedestal before feeding on any other portion of the brood ball. In *Onthophagus taurus*, previous work has demonstrated that the pedestal and brood chamber facilitate the transgenerational passage of maternal gut microbiota to offspring (Estes et al. 2013). As with other insects that feed on a nutritionally poor diet, dung beetles have long been thought to utilize the physiological capabilities of microorganisms in order to derive key vitamins, amino acids, and other essential nutrients from their environments (Goidanich and Malan 1964; Douglas 2009). Given that the pedestal has previously been shown to facilitate the faithful transmission of microbiota from mother to offspring (Estes et al. 2013), it has been hypothesized that this structure is an essential source for the transmission of microbiota needed for larvae to feed and develop on dung (Byrne et al. 2013). Yet the contributions of these microbiota toward larval survival and developmental outcomes, if any, remain to be identified.

Here, we investigate the role of the maternally transmitted pedestal and associated microbiota in promoting normal growth and development in a rapidly developing species of *Onthophagus*, *Onthophagus gazella*, using two sets of experiments. In the first set of experiments, we sought to identify the nature, source, and developmental consequences of microbiota found within the pedestal and dung and did so through the stepwise removal of potential sources of microbial inoculation. In light of the transgenerational inheritance of gut microbiota in *O. taurus*, we predicted that the pedestal-associated microbiota would have a substantial benefit for developmental outcomes (e.g., increasing total growth and developmental rate). In the second set of experiments, we assessed the environmental contingency of developmental symbiosis and did so by exposing developing larvae to two ecologically relevant stressors (i.e., desiccation and temperature fluctuations) throughout larval development. Given previous work on the environmental contingency of host-microbe interactions, we predicted that the presence of either stressor would exacerbate the disparity of the developmental responses between larvae provisioned with and without their pedestal-derived microbiota.

Material and Methods

Beetle Collection and Husbandry

Adult *Onthophagus gazella* were collected from cow dung pads at Kualoa Ranch in Kaneohe, Hawaii (21°31′15″N, 157°50′14″W), from June 2014 to January 2015 and shipped to Bloomington, Indiana, for rearing. All beetles were maintained as a single colony in the laboratory at 28°C and a 16L:8D cycle and fed homogenized cow dung ad lib. following an established protocol (Moczek et al. 2002). Dung was collected from Marble Hill Farm in Bloomington (39° 3′8″N, 86°36′12″W). Marble Hill Farm is an organic farm whose pastures are home to several abundant *Onthophagus* species.

In order to obtain offspring, beetles were allowed to breed in plastic containers (25 cm tall × 20 cm diameter) filled 3:4 with a moist sand:soil mixture. Each week, six female and three male beetles were added to each of two breeding con-

tainers and provisioned with ~0.5 L of homogenized cow dung. Beetles were allowed to breed for approximately 2 days, at which point they were recaptured and brood balls were collected and placed in separate containers for experimentation. Approximately 25 brood balls were produced per breeding container per week. Brood balls containing eggs were then allocated haphazardly to different experimental treatment groups.

For most treatments (but see experiment 3), eggs were rapidly transferred into individual wells within 12-well tissue culture plates (Dot Scientific), provisioned with ad lib. dung, and maintained at 28°C and 16L : 8D cycle inside stand-alone incubators, as established previously (Shafiei et al. 2001). This method allows a large number of eggs and larvae to be reared in a standardized manner and facilitates both observation and manipulation of offspring. Immature *Onthophagus* complete all developmental transitions from egg to larva, pupa, and adult inside the well, similar to brood ball–reared larvae (Shafiei et al. 2001). To minimize the effects of potential microclimatic variation within and among 12-well plates, we placed different treatment groups at either end of each plate and rotated the shelf position of each plate every 2 days within each incubator.

Manipulating the Microbial Environment of O. gazella

We executed three successive experiments to characterize the nature, source, and possible developmental benefits of the maternally derived pedestal and the symbiotic microbiota that live within each brood ball. We then assessed the environmental contingency of host-microbe interactions in two additional experiments.

Experiment 1: Rearing with Standing and Reduced Microbial Communities

To assess the significance, if any, of host-associated microbiota for normal development in *O. gazella*, we sought to rear larvae under experimental conditions that either attempted to reduce the microbial community (M(−)) or maintain standing microbial communities (M(+); see table A1 for an appendix of all experimental conditions). These conditions excluded or included, respectively, sources of microbial inoculation from the egg surface, maternally derived pedestal, and dung that filled the wells in which larvae developed. Under the M(−) condition ($n = 35$), all eggs were surface sterilized using 100 μL of a 1% sodium hypochlorite/0.1% Triton-X 100 solution and rinsed twice with 1 mL sterilized water (as established previously in Estes et al. 2009, 2013). Using a sterilized paintbrush, we transferred eggs into sterile 12-well plates containing sterilized dung, which had been previously autoclaved at 15 PSI and 121°C for 20 min. A thin layer of this dung was used to cover eggs within wells, in order to mitigate desiccation stress. It is unlikely that all microbiota were eliminated, but this 20-min autoclave cycle was sufficient to remove all culturable microbiota on Luria-Bertani (LB) and brain-heart infusion (BHI) agar grown overnight at 28°C under both aerobic and anaerobic conditions (induced by the BD GasPak anaerobic system; data not shown).

Under the M(+) condition ($n = 32$), we aimed to include all possible natural sources of microbial inoculation, including (*a*) the pedestal containing maternally transmitted microbiota and (*b*) the dung within the brood ball (Estes et al. 2013). For *a*, we supplied nonsterilized eggs (i.e., rinsed with 2.1 mL of sterilized water alone) with the dissected pedestal corresponding to its brood ball using a sterile scalpel. The egg was placed into wells containing nonsterilized dung, transferred on top of the corresponding pedestal, and covered by a layer of nonsterile dung. Larvae were then reared to adulthood within incubators at 28°C on a 16L : 8D cycle.

Experiment 2: Distinguishing between Pedestal and Dung as Putative Microbiota Sources

We next investigated whether the developmental responses observed in experiment 1 were primarily due to provisioning M(+) larvae with (*a*) their pedestal or (*b*) nonsterile dung during larval development. In order to disentangle these effects, we provisioned surface-sterilized eggs with two possible sources of microbial inoculation: in one treatment, sterilized eggs were assigned to wells containing sterilized dung but a (nonsterile) dissected pedestal (P(+)D(−); $n = 43$); in the other, eggs were assigned to wells containing nonsterilized dung but no pedestal (P(−)D(+); $n = 40$). Larvae were reared to adulthood as in the first experiment.

Experiment 3: Inoculating with Pedestal Microbiota

We next investigated whether the developmental responses observed from pedestal transmission were due to (*a*) the effect of symbiotic microbiota living within the pedestal or (*b*) the effect of nutrients sequestered within the pedestal (as suggested in Byrne et al. 2013). To test whether the presence of symbiotic microbiota alone could recover the responses observed in experiments 1 and 2, we transferred surface-sterilized eggs into wells containing sterile dung and treated those wells with one of three inoculates: (*a*) microorganisms cultured from the pedestal (PI; $n = 19$), (*b*) microorganisms cultured from a soil sample obtained from a pasture naturally inhabited by diverse dung beetle species to assess whether host responses are specific to a particular subset of cultured microbiota (SI; $n = 24$), and (*c*) sterile phosphate-buffered saline (PBS; $n = 20$) containing no microbiota as a negative control.

To culture microbiota from the pedestal, we haphazardly selected three brood balls from each breeding container.

Eggs were removed from each brood ball using a sterile paintbrush, and pedestals were dissected and vortexed into suspension in 9 mL sterile PBS. This suspension was serially diluted and plated onto LB and BHI agar. Colonies were grown overnight at 28°C on both media types and under both aerobic and anaerobic conditions, as in experiment 1. To generate a soil inoculate, we collected a soil sample from the pastures of Marble Hill Farm in Bloomington, and microbiota from this sample were cultured as above. For both treatments, colonies were scraped from plates containing 200–300 colony-forming units and resuspended in ~1.5 mL of PBS to form a homogenate.

For larvae receiving treatment with either the pedestal or soil cultures, respectively, each well of sterilized dung received 60 μL of homogenate from each plate type (i.e., LB and BHI) and each condition (i.e., aerobic and anaerobic), for a total of 240 μL of inoculate in each well. The responses of larvae to these conditions were compared to those of control larvae whose wells were treated with 240 μL of PBS alone. Egg and dung microbial communities were sterilized and larvae reared to adulthood as in experiment 1.

Experiment 4: Assessing the Environmental Contingency of Host-Microbe Interactions

Last, we sought to investigate the role of the maternally transmitted microbiota in buffering larvae against two common ecological stressors known to affect larval performance: desiccation and temperature fluctuations. In nature, both stressors emerge as a consequence of variation in the depth at which brood balls are buried by mothers (Snell-Rood et al. 2016).

To examine the contribution of microbiota in buffering against desiccation stress, we exposed larvae reared under M(+) and M(−) conditions to either low or high levels of this stressor. This experiment was carried out in parallel with experiment 1 described above. M(+) ($n = 26$) and M(−) ($n = 34$) larvae exposed to high desiccation stress were reared similarly to low-stress larvae from experiment 1 but with one modification: eggs were no longer covered with a thin layer of moist dung. Failure to cover these eggs exposes larvae to the relatively low humidity at the dung-air interface, similar to brood balls buried naturally at shallow depths or near the soil-dung pad interface, as is the case for a subset of dung beetle species (Halffter and Edmonds 1982; Hanski and Cambefort 1991).

To examine the contribution of microbiota in buffering against temperature stress, we next reared larvae under PI ($n = 96$) and PBS ($n = 103$) conditions (as in experiment 3) and exposed them to either constant ($n = 101$) or fluctuating ($n = 98$) temperature conditions. PI- and PBS-treated larvae exposed to the constant temperature treatment were reared similarly to larvae in experiment 3 and at a constant temperature of 25°C. In south-central Indiana, including Marble Hill Farm, this temperature corresponds to natural temperature conditions experienced by larvae whose brood balls are buried approximately 25 cm underground during midsummer (Snell-Rood et al. 2016). PI- and PBS-treated larvae exposed to the fluctuating temperature treatment experienced circadian fluctuations (19° [12 h] to 31°C [12 h]) around the same mean temperature (25°C), which in turn corresponds to natural temperature conditions experienced by larvae buried near the soil-dung interface. Exposure to this range of temperatures decreases larval growth and increases the length of the larval stage (Snell-Rood et al. 2016).

Data Collection and Analysis

For each experiment, we collected the following developmental data: larval body mass at 6 days after hatching (most larvae enter the third and final instar around this time), time to and mass at pupation, survival to adulthood, adult body size (measured as pronotum width, following Emlen 1994), and time to adulthood (i.e., developmental rate). Across all treatments and experiments, pupal mass was highly correlated with adult body size ($R^2 = 0.961$, $P < .001$), and we therefore present results for adult body size only. Similarly, because the number of days between pupation and adult eclosion is highly conserved (i.e., 6 days), here we present results pertaining only to total time to adulthood.

We conducted unpaired two-tailed *t*-tests and one- or two-way ANOVAs on all dependent variables except for mortality, which was analyzed using χ^2 or Fisher's exact tests. Before these tests, data were tested for normality using a Shapiro-Wilk test and for equality of variance using Levene's test. Where these assumptions were not met, we conducted nonparametric tests (i.e., Mann-Whitney *U*-test, Kruskal-Wallis test). For all ANOVAs, the type of microbial provisioning treatment (e.g., M(−) or M(+)) was treated as a fixed effect. For the temperature stress experiment, we used a general linear mixed model for numeric variables (e.g., time to adulthood) and a generalized linear mixed model for mortality. For the desiccation stress experiment, we used a generalized linear model for mortality. Temperature (constant 25°C or fluctuating 19°–31°C) and microbial inoculation (PI or PBS) and their interaction were modeled as fixed effects, and replicate batches within each experiment were treated as a random effect. General linear and generalized linear mixed models were conducted using the lme4 package in the R computing environment, version 3.1.2 (Bates et al. 2014; R Core Development Team 2014); all other statistical analyses were completed using SPSS statistical software, version 22 (IBM 2013). All data are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.9m2n7 (Schwab et al. 2016).

Results

Response to Pedestal and Pedestal Microbiota Transfer

We first sought to test the hypothesis that microbial inoculation via the brood ball environment was beneficial to normal larval development and predicted that larvae reared under standing microbial community (M(+)) conditions would outperform larvae reared under reduced microbial community (M(−)) conditions by all developmental measures (experiment 1). In support of our prediction, M(+) larvae had a greater mass than M(−) larvae on day 6 of larval development ($\bar{x}_{M(+)} = 0.192$ g, $\bar{x}_{M(-)} = 0.102$ g; *t*-test: $t = 5.578$, $P < .001$), reached adulthood faster ($\text{median}_{M(+)} = 22$ days, $\text{median}_{M(-)} = 28$ days; Mann-Whitney *U*: $U = 53.5$, $P < .001$), and exhibited larger body sizes as adults ($\text{median}_{M(+)} = 6.001$ mm, $\text{median}_{M(-)} = 5.207$ mm; Mann-Whitney *U*: $U = 45.5$, $P < .001$; fig. 1*A*). However, there was no difference in mortality between either treatment ($\text{survival}_{M(+)} = 67\%$, $\text{survival}_{M(-)} = 57\%$; χ^2 test: $\chi^2 = 0.311$, $P = .577$). Combined, these data suggest that the nonsterilized components of the pedestal, dung, or both benefit the developmental environment of larval *Onthophagus*.

We then sought to test whether this beneficial, nonsterilized component was contributed by the maternally derived pedestal or, alternatively, the dung (experiment 2). In this experiment, larvae were exposed to one of two treatments: in the P(+)D(−) treatment, larvae were reared with a pedestal and on sterilized dung; in the P(−)D(+) treatment, larvae were reared without a pedestal but on nonsterilized dung. We predicted that if the maternally derived pedestal is a beneficial component of the larval developmental environment, then larvae reared on the P(+)D(−) treatment would develop faster and to larger body sizes than P(−)D(+) larvae. In contrast to our predictions, we found no significant difference in mass at day 6 of larval development ($\text{median}_{P(+)D(-)} = 0.259$ g, $\text{median}_{P(-)D(+)} = 0.225$ g; Mann-Whitney *U*: $U = 359.5$, $P = .091$), time to adulthood ($\text{median}_{P(+)D(-)} = 20$ days, $\text{median}_{P(-)D(+)} = 20$ days; Mann-Whitney *U*: $U = 349.5$, $P = .975$), or adult body size ($\bar{x}_{P(+)D(-)} = 6.054$ g, $\bar{x}_{P(-)D(+)} = 6.010$; *t*-test: $t = 0.331$, $P = .742$). However, we did recover a trend toward higher mortality in the P(−)D(+) treatment ($\text{survival}_{P(+)D(-)} = 74\%$, $\text{survival}_{P(+)D(-)} = 55\%$; χ^2 test: $\chi^2 = 3.438$, $P = .064$). In addition, we detected a highly significant difference in variance between the treatments, with greater variance in the P(−)D(+) treatment for both mass at day 6 of larval development ($\sigma^2_{P(+)D(-)} = 0.003$, $\sigma^2_{P(-)D(+)} = 0.008$; Levene's test: Levene's statistic = 9.003, $P = .004$) and time to adulthood ($\sigma^2_{P(+)D(-)} = 1.468$, $\sigma^2_{P(-)D(+)} = 5.457$; Levene's test: Levene's statistic = 13.089, $P < .001$; fig. 1*B*). This increased variability manifested in a disproportionate number of small larvae at day 6 and larvae with increased times to adulthood, an effect consistent with developmental stress (Debat and David 2001). These results therefore suggest at least some role of the pedestal in both enhancing survival and decreasing developmental variability.

We next investigated whether these pedestal-associated benefits correspond to a specific set of microbiota sequestered within the pedestal (experiment 3). We predicted that larvae reared on sterile dung that was inoculated with pedestal microbiota (PI) would develop faster and to larger body sizes than larvae reared on either sterile dung inoculated with randomly sampled soil microbiota (SI) or PBS alone. Consistent with this prediction, we found that rearing conditions significantly influenced mass on day 6 of larval development ($\text{median}_{PI} = 0.438$ g, $\text{median}_{SI} = 0.284$ g, $\text{median}_{PBS} = 0.346$ g; Kruskal-Wallis ANOVA: $H = 24.720$, $P < .001$), such that PI larvae were larger than both SI- and PBS-reared larvae (Dunn's test: PI vs. SI $P < .001$, PI vs. PBS $P < .001$, SI vs. PBS $P = .610$; fig. 1*C*). Similarly, we found that rearing conditions significantly influenced time to adulthood ($\bar{x}_{PI} = 17.56$ days, $\bar{x}_{SI} = 20.14$ days, $\bar{x}_{PBS} = 19.84$ days; one-way ANOVA: $\bar{x}_{PBS} = 19.84$, $F = 16.922$, $P < 0.001$) and adult body size ($\text{median}_{PI} = 6.704$ mm, $\text{median}_{SI} = 6.231$ mm, $\text{median}_{PBS} = 6.543$ mm; Kruskal-Wallis ANOVA: $H = 14.722$, $P < .001$). PI larvae outperformed SI and PBS larvae for both measures, developing faster (Fisher's LSD: PI vs. SI $P = .005$, PI vs. PBS $P = .015$, SI vs. PBS $P = .733$) and to larger adult body sizes (Dunn's test: PI vs. SI $P < .001$, PI vs. PBS $P = .024$, SI vs. PBS $P = .262$). In addition, we detected significantly greater variation in the SI treatment than in PI and PBS treatments for mass at day 6 ($\sigma^2_{PI} = 0.002$, $\sigma^2_{SI} = 0.012$, $\sigma^2_{PBS} = 0.003$; Levene's test: Levene's statistic = 9.406, $P < .001$) and adult body size ($\sigma^2_{PI} = 0.036$, $\sigma^2_{SI} = 0.284$, $\sigma^2_{PBS} = 0.079$; Levene's test: Levene's statistic = 10.219, $P < .001$; fig. 1*C*). However, there was no difference in mortality between any of these treatments ($\text{survival}_{PI} = 95\%$, $\text{survival}_{SI} = 88\%$, $\text{survival}_{PBS} = 95\%$; χ^2 test: $\chi^2 = 0.258$, $P = .879$). Collectively, these results suggest that maternally transmitted pedestal microbiota significantly enhance larval growth and developmental buffering.

Response to Stressors

Last, we investigated whether brood ball–derived microorganisms influence larval development in response to two ecologically relevant stressors: desiccation stress and temperature fluctuations. For both experiments we predicted that the presence of a stressor should exacerbate the disparity of the developmental responses between M(+) and M(−) and PI- and PBS-treated larvae as seen in experiments 1 and 3, respectively. We found at least partial support for our hypothesis for both types of stressors.

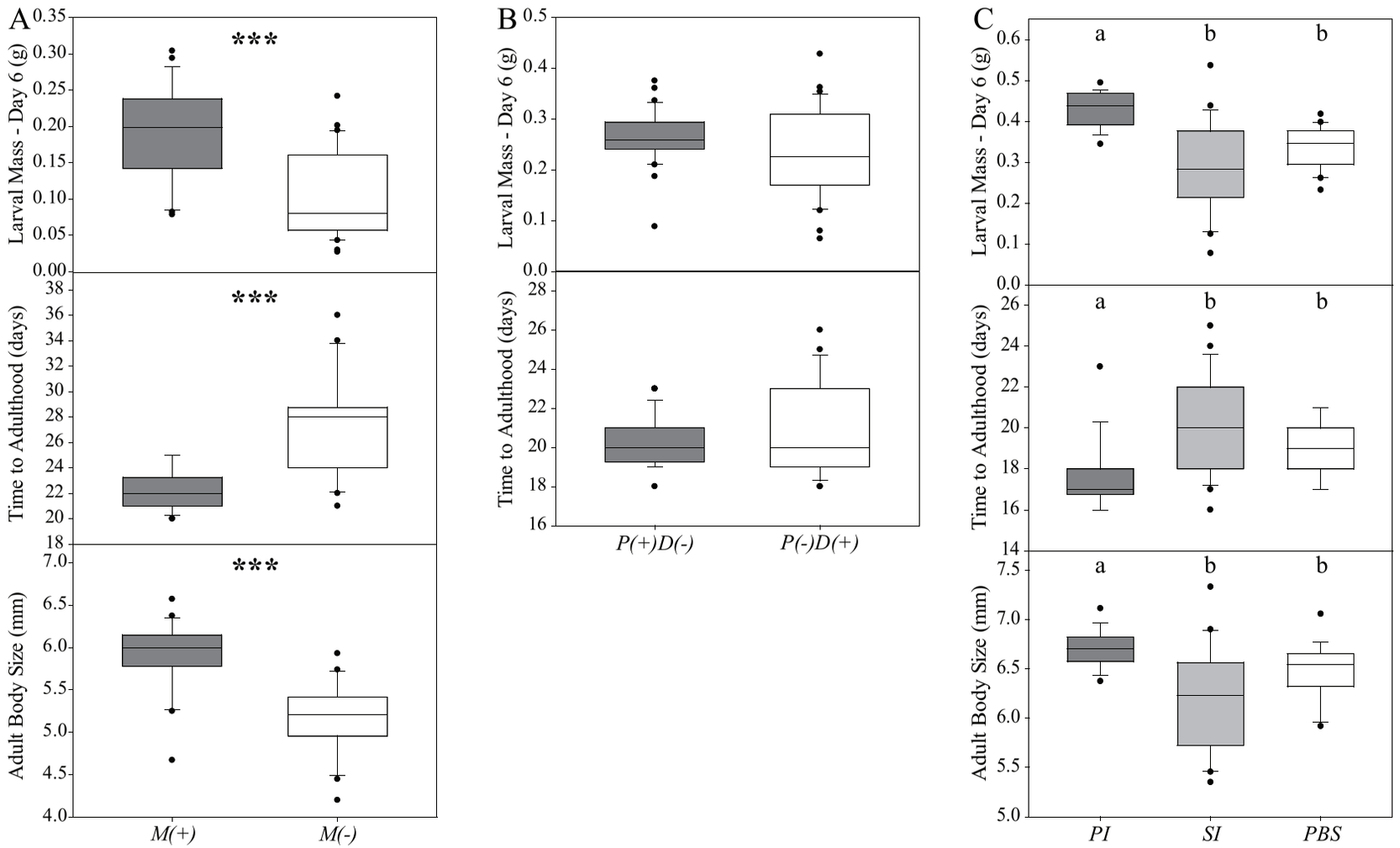

Figure 1: Developmental responses of larvae, including larval mass, time to adulthood, and size at adulthood, to experimental treatments. *A*, Larvae reared under standing (M(+)) and reduced (M(−)) microbial conditions are shown (experiment 1). Dark gray boxes indicate responses of larvae reared under M(+) conditions. White boxes indicate responses of larvae reared under M(−) conditions. Asterisks indicate significant differences among treatment groups. *B*, Larvae reared with a pedestal and on sterilized dung (P(+)D(−)) or without a pedestal but on nonsterilized dung (P(−)D(+); experiment 2). Dark gray boxes indicate responses of larvae reared under P(+)D(−) conditions. White boxes indicate responses of larvae reared under (P(−)D(+)) conditions. There was no significant difference in mean response but significantly higher variance under P(−)D(+) conditions for larval mass and time to adulthood. *C*, Larvae reared with an inoculum of pedestal microbiota (PI), soil microbiota (SI), or sterile phosphate-buffered saline (PBS; experiment 3). Responses of PI larvae are shown in dark gray boxes, SI larvae in light gray boxes, and PBS larvae in white boxes. Letters indicate significant differences among treatment groups. One outlier was removed from SI.

Desiccation Stress. In experiment 1 (i.e., in the absence of desiccation stress) M(+) larvae grew faster and to larger adult body sizes but did not exhibit enhanced survival relative to M(−) larvae (fig. 1*A*). Replicating this experiment in the presence of desiccation stress revealed significantly higher survival rates in the M(+) treatment than in the M(−) treatment. Specifically, none of 34 M(−) larvae survived, whereas 9 of 26 (34.6%) of the M(+) larvae survived to adulthood, resulting in a significant interaction between desiccation stress and microbial treatment when compared with experiment 1 (generalized linear model: $Z = 2.037$, $P = .041$; fig. 2*A*). When surviving desiccation-stressed M(+) beetles were compared to beetles from experiment 1 that were reared in the absence of desiccation stress, these surviving adults were significantly smaller ($\bar{x}_{\text{desiccation M(+)}} = 5.18$ mm, $\bar{x}_{\text{experiment 1 M(+)}} = 5.91$ mm, $\bar{x}_{\text{experiment 1 M(−)}} = 5.18$ mm; one-way ANOVA: $F = 20.070$, $P < .001$) and took longer to reach adulthood ($\bar{x}_{\text{desiccation M(+)}} = 26.89$, $\bar{x}_{\text{experiment 1 M(+)}} = 22.31$, $\bar{x}_{\text{experiment 1 M(−)}} = 27.15$ days; one-way ANOVA: $F = 17.940$, $P < .001$) than experiment 1 M(+) beetles (Fisher's LSD: $P < .001$, both comparisons) and phenocopied experiment 1 M(−) beetles for both adult body size (Fisher's LSD: $P = .988$) and time to adulthood (Fisher's LSD: $P = .818$).

Temperature Fluctuations. In experiment 3, PI larvae grew faster and to larger body sizes than SI- or PBS-inoculated individuals but showed no difference in mortality (fig. 1*C*). We

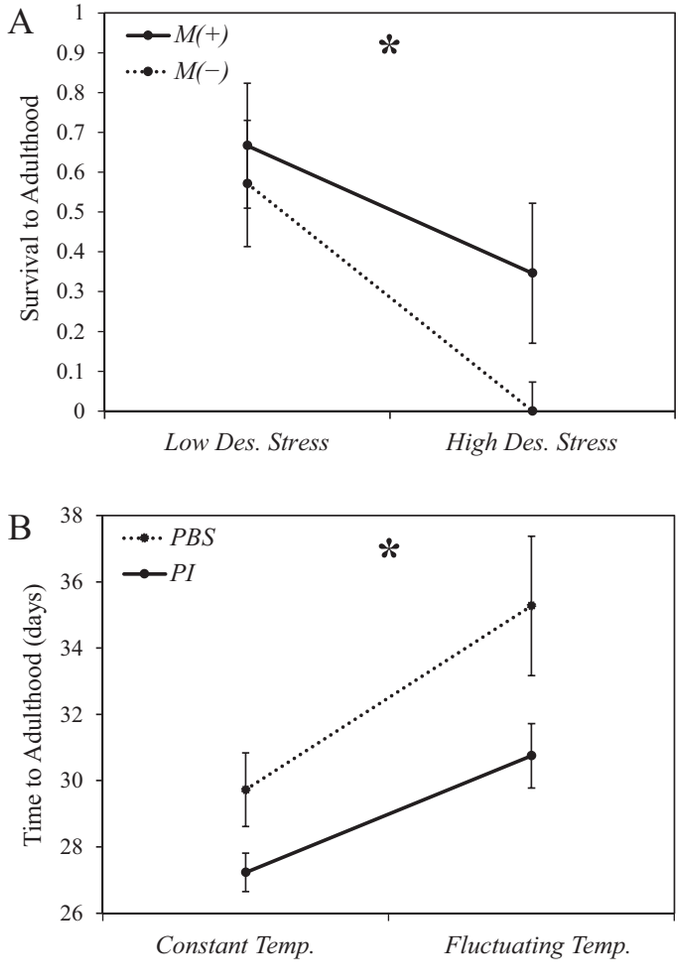

Figure 2: Interaction plots for desiccation and temperature stressors. Significant interaction effects between microbial treatment and stress level were recovered for desiccation stress on survival to adulthood (*A*) and temperature stress on time to adulthood (*B*). Disparity of developmental responses between microbial treatment groups (M(+)/M(−) or inoculum of cultured pedestal microbiota/phosphate-buffered saline) were much greater under high levels of these stressors. Error bars represent 95% confidence intervals. Asterisks indicate significant interaction effects.

predicted that the effect size and benefit of this treatment would be enhanced under stressful, fluctuating temperature conditions. After replicating the experiment in the presence or absence of temperature stress, we found significant main effects of both microbial inoculation and temperature stress on mass at day 6 of larval development and adult body size but not mortality (table 1), demonstrating that inoculation treatment and temperature stress affect larval performance independently. In addition, and in support of our main hypothesis, we recovered a significant interaction between temperature and microbial inoculation for time to adulthood but not any of the other metrics, including mortality (fig. 2*B*; table 1). Furthermore, we detected a highly significant difference in variance between the treatments for time to adulthood ($\sigma^2_{\text{PBS, constant}} = 12.92$, $\sigma^2_{\text{PBS, fluctuating}} = 46.15$, $\sigma^2_{\text{PI, constant}} = 3.85$, $\sigma^2_{\text{PI, fluctuating}} = 8.82$; Levene's test: Levene's statistic $= 13.37$, $P < .001$) and a slightly nonsignificant trend for adult body size ($\sigma^2_{\text{PBS, constant}} = 0.328$, $\sigma^2_{\text{PBS, fluctuating}} = 0.396$, $\sigma^2_{\text{PI, constant}} = 0.224$, $\sigma^2_{\text{PI, fluctuating}} = 0.193$; Levene's test: Levene's statistic $= 2.55$, $P = .058$) with greater variance in the fluctuating PBS treatment for both variables.

Discussion

Understanding the causes, nature, and consequences of host-microbe interactions has recently become a central research goal of both evolutionary and ecological developmental biology (e.g., see Gilbert et al. 2015). Symbioses are increasingly understood to be a well-integrated feature of organismal development and have the potential to shape interactions between developing organisms and their environments (Lee and Brey 2013; Nyholm and McFall-Ngai 2014; Gilbert 2016). Here, we investigated the contribution of the maternally derived pedestal in promoting the normal development of *Onthophagus gazella*, as well as its environmental contingency. Our results support the hypotheses (i) that the pedestal contains beneficial microbiota that enhance larval growth and developmental rate, (ii) that pedestal microbiota and not those found in the dung or soil have this effect on developing larvae, and (iii) that the degree to which microbiota enhance developmental outcomes is itself contingent on environmental conditions. Below, we discuss the most important implications of our results.

Developmental Symbiosis Contributes to Normal Development in Onthophagus

Consistent with our hypothesis that the maternal pedestal provisions offspring with beneficial microbial symbionts, we found through a series of experiments that pedestal microbiota can improve developmental outcomes for *O. gazella*, enhancing developmental responses in some cases while yielding greater phenotypic robustness in others (e.g., experiment 2; fig. 1*B*). Previous work in *Onthophagus taurus* has demonstrated that the pedestal and brood chamber facilitate the transgenerational passage of a subset of maternal gut microbiota to offspring (Estes et al. 2013). Among microbiota sampled from whole guts, the most common families across life stages and populations were the Pseudomonadaceae, Enterobacteriaceae, and Comamonadaceae. Members of these families (e.g., *Enterobacter* spp.) have been implicated in cellulose degradation and nitrogen conservation within the guts of host insects feeding on similar plant-based diets, and the brood balls of two dung beetle species have previously been shown to contain bacteria capable of metabolizing cellulose in vitro (Goidanich and Malan 1964; Ben-Yosef et al. 2008; Douglas 2009; Huang et al. 2012). However, while our study demonstrates the beneficial role of pedestal microbiota in the normal development of *O. gazella*, the potential metabolic functions provided by these microbiota are presently unclear. Elucidating these functions in *Onthophagus* and other dung beetle taxa will require the use of additional methodologies, such as transcriptomic and metabolic analyses (e.g., see Lee et al. 2015), which are presently under way.

In addition, it is presently unclear whether and how developmentally important intra- and extracellular endosymbionts are able to colonize and persist within *Onthophagus* beetles. In many insect orders, specialized crypts known as bacteriomes or mycetomes house bacterial and fungal symbionts, respectively, within the gut (Schwemmler and Gassner 1990; Douglas 2015). While these structures are present in several families of Coleoptera (e.g., Dowd 1989; Heddi et al. 1999; Grünwald et al. 2010), surveys of beetles in the family Scarabaeidae have largely failed to locate any such structures along the mid- and hindguts (Nardon and Grenier 1989), though the hindgut is expanded to form an anoxic fermentation chamber in some species and methanogen-containing, lobe-like structures are present in larvae of the grass grub, *Costelytra zealandica* (Huang et al. 2010). Preliminary inspections of the larval guts of multiple *Onthophagus* species have thus far failed to locate any such structures (D. B. Schwab, unpublished observations). Instead, scarab endosymbionts appear primarily to adhere to the intestinal lumen, where they have previously been shown to form biofilms (Nardon and Grenier 1989; Egert et al. 2005).

Alternatively, or in addition, beneficial microbiota may exert their effects externally to developing larvae. Throughout their development, *Onthophagus* larvae feed on, defecate throughout, and then refeed on their own feces within the brood chamber, potentially spreading maternally transmitted microbiota throughout the brood ball. Supplementation of the larval diet may therefore be mediated not solely by gut or intracellular symbionts but also by the spreading of microbiota throughout the brood ball, whose collective metabolisms may then establish an external rumen (Swift et al. 1979). For example, larvae of the xylem-feeding woodwasp,

Table 1: General linear mixed model comparing larval mass at day 6, time to adulthood, and adult body size and generalized linear mixed model for mortality

	Larval mass (day 6)					Time to adulthood					Adult body size					Mortality			
	Estimate	SE	df	*t*	*P*	Estimate	SE	df	*t*	*P*	Estimate	SE	df	*t*	*P*	Estimate	SE	*Z*	*P*
Intercept	.098	.016	9.36	6.124	<.001	30.144	1.043	11.46	28.903	<.001	5.765	.123	10.31	46.689	<.001	−1.712	.653	−2.622	<.001
Microbiota	−.045	.011	154.35	−3.935	<.001	2.364	.773	149.46	3.06	.002	−.322	.1	149.45	−3.207	.002	−.892	.588	−1.515	.13
Temperature	−.039	.012	154.33	−3.278	.001	3.563	.796	149.45	4.478	<.001	−.212	.103	149.44	−2.055	.042	.143	.517	.277	.782
Microbiota × temperature	.011	.016	154.4	.676	.5	2.292	1.124	149.55	2.04	.043	−.211	.146	149.55	−1.447	.15	.622	.789	.788	.431
Batch	…	.003	…	…	…	…	.196	…	…	…	…	.024	…	…	…	…	.099	…	…

Note: Microbial inoculation, temperature, and their interaction were treated as fixed effects. Batch was treated as a random effect. Indicated are the parameter estimate, the standard error (SE), the degrees of freedom (df), the *t* and *Z* ratio of the test statistic, and the *P* value for each factor and interaction in the model.

Sirex noctilio, develop inside tree holes provisioned with the fungus *Amylostereum areolatum*. Throughout larval development, *S. noctilio* feeds exclusively on xylem that has been previously degraded by *A. areolatum*, providing the wasp its sole source of dietary sterols (Thompson et al. 2013). However, these effects may not be limited to diet supplementation but may extend to the suppression of parasitic or pathogenic microorganisms that threaten host health. Indeed, symbiotic actinomycete bacteria suppress the growth of fungal parasites in the fungus gardens of leaf-cutting ants and ambrosia beetles, and both Formosan subterranean termites and wood cockroaches incorporate the antimicrobial compounds of symbiotic bacteria and protozoa into their dung, suppressing the germination of common soil pathogens such as *Metarhizium anisopliae* in their nests (Currie 2001; Scott et al. 2008; Chouvenc et al. 2013; Rosengaus et al. 2013). While it is additionally possible that larvae derive nutrition by directly feeding on bacteria growing along the brood ball walls, this may only partially explain the growth effects in our study, since the addition of nonspecific soil bacteria in the SI treatment failed to rescue larval growth relative to the control PBS treatment (fig. 1*C*). Regardless, the *Onthophagus* system has the potential to contribute to a growing number of studies aimed at understanding the role of larval behaviors and microbiota in shaping their developmental environment (McNally and Brown 2015).

Symbiont Benefits to Onthophagus *Development Are Environmentally Contingent*

Throughout development, larvae of many *Onthophagus* species must contend with several natural stressors, such as the desiccation of brood ball dung and daily fluctuations in temperature. The degree to which these stressors impinge on larval development is in part a consequence of the depth at which brood balls are buried by mothers; brood balls buried at shallow depths or near the soil-dung pad interface tend to experience these stressors most strongly, decreasing adult body size and increasing time to emergence (Snell-Rood et al. 2016). In numerous insect species, smaller adult body sizes and longer development times are associated with reduced fitness (i.e., lower fecundity and increased generation time; Brown et al. 1993; Kingsolver and Huey 2008).

Consistent with our predictions, we observed that laboratory analogs of these natural stressors exacerbated the disparity of developmental responses between larvae provisioned with and without pedestal microbiota, suggesting a critical role of the maternally transmitted microbiota in buffering larvae against environmental stress. Larvae reared under conditions of high desiccation stress were smaller and took longer to reach adulthood than larvae reared under low stress, and this effect was most pronounced in the M(−) treatment in which larvae suffered complete mortality (fig. 2*A*). Similarly, larvae reared under stressful temperature conditions took disproportionately longer to reach adulthood when reared in the absence of pedestal microbiota and were far more variable than any other treatment group, suggesting an increased ability for these stressors to decanalize developmental processes in the absence of microbiota (fig. 2*B*). Importantly, we cannot fully exclude the possibility that the responses of M(+) desiccation-stressed larvae could have been mediated by additional sources (e.g., nonsterile dung) of microbiota and that these same sources could potentially have benefitted larvae under temperature stress, even though the results of experiments 1–3 suggest pedestal-derived microbiota as the most likely drivers of this response. The ability of obligate and facultative microbial symbionts to shape the desiccation or thermal tolerance of their hosts has been documented in multiple animal and plant species (Gilbert et al. 2010; Rolli et al. 2015). For instance, the thermal environment of hosts can strongly shape both the composition and gene expression of their associated microbial communities (Thurber et al. 2009; Lokmer and Wegner 2015; Enders and Miller 2016), and stressful temperature conditions have been shown to favor the retention and proliferation of symbionts that confer heat tolerance to hosts in natural populations (e.g., Montllor et al. 2002; Berkelmans and van Oppen 2006). While the mechanisms underlying increased thermotolerance are poorly understood, an increasing number of studies suggest that symbionts may contribute to host thermotolerance by inducing the expression of host heat shock or cytoskeletal proteins that contribute to heat tolerance (Dunbar et al. 2007; McLellan et al. 2007; Brumin et al. 2011). Alternatively, or in addition, diet supplementation via symbiont metabolic processes may increase larval health and enable larvae to mount the physiological responses necessary to maintain normal development. Regardless of the mechanisms involved, the results presented here suggest that the outcomes of host-microbe interactions may best be studied on an environment-by-environment basis.

Evolutionary Implications of Developmental Symbiosis in Onthophagus

In this study, we have demonstrated that maternally transmitted symbionts are a beneficial component of the developmental environment of dung beetles, enhancing developmental outcomes in fitness-related life-history traits and canalizing the development of these traits in the presence of significant ecological stressors. Importantly, the frequency and degree to which developing beetles are exposed to these stressors may vary with the reproductive ecology of the species and population under study (Halffter and Edmonds 1982; Hanski and Cambefort 1991). For instance, dwellers such as *Aphodius* spp. exclusively deposit their eggs directly

into dung pads aboveground, constitutively exposing larvae to the threat of desiccation and temperature stress. Even in subterranean tunneling species such as *Onthophagus*, which generally bury brood balls deep underground, there is substantial variation among both species and populations in the mean depth at which brood balls are buried (Macagno et al. 2016). This variation in burial depth may influence or be facilitated by the evolution of symbiont community composition, as well as the importance of these symbionts for host fitness outcomes, and presents exciting opportunities for future research.

The results presented here additionally demonstrate that the maternal pedestal represents an adaptive route for the extracellular transmission of beneficial microbiota from parent to offspring and may in part be responsible for the fixation and evolution of this developmental symbiosis in *O. gazella* and other dung beetle species that construct brood balls (Estes et al. 2013; Salem et al. 2015). Although the pedestal is highly pronounced in many species of *Onthophagus*, in other genera such as *Euoniticellus*, it is composed only of a smear of maternal feces that anchors the egg to the brood chamber. In *Euoniticellus*, this reduced pedestal was suggested to serve as a predigested, highly nutritious meal for newly hatched larvae but shown not to be a source of beneficial microbiota (Byrne et al. 2013). While the precise nutritional value of the pedestal alone is unclear in *Onthophagus*, this differential reliance on symbiont populations may have important evolutionary implications for these lineages, not only shaping the source of selectable, phenotypic variation in metabolic and physiological (e.g., thermotolerance) traits of host dung beetles but also altering the nature of selection acting on these traits (e.g., see Sabree et al. 2012).

It has been hypothesized that the effects of parental care not only may benefit offspring under the normal range of environmental conditions but additionally could facilitate niche expansion into novel or stressful environments (e.g., West-Eberhard 2003; Uller 2008). For instance, the evolution of the salt beetle, *Bledius spectabilis*, into intertidal habitats may in part have been facilitated by maternal care: mothers lay their eggs within specially constructed burrows that prevent flooding and maintain normoxic conditions at high tide (Wyatt 1986). Whether the maternal transmission of microbial symbiont communities can similarly facilitate range expansion into novel or stressful environments, such as those studied here, is presently unclear but presents exciting opportunities for future research, including in *Onthophagus* where recent work has documented rapid differential niche expansion among several exotic populations (Silva et al. 2016). At the same time, it is worth noting that the species richness of *Euoniticellus* is nearly two orders of magnitude lower than that of *Onthophagus* (Cambefort 1991). This raises the intriguing possibility that the expansion of pedestal function from a food supplement and/or anchor to a reliable microbial reservoir may have facilitated the evolutionary diversification of a subset of dung beetle taxa onto novel food sources or into stressful environments such as those studied here (Janson et al. 2008).

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APPENDIX

Supplementary Table

Table A1: Index of treatment abbreviations and all experimental conditions for experiments 1–3

	Experimental components			
Experiment and abbreviation	Egg	Pedestal	Dung	Components cultured
Experiment 1:[a]				
M(+)	Nonsterilized	Transferred	Nonsterilized	NA
M(−)	Sterilized	Not transferred	Sterilized	NA
Experiment 2:				
P(+)D(−)	Sterilized	Transferred	Sterilized	NA
P(−)D(+)	Sterilized	Not transferred	Nonsterilized	NA
Experiment 3:[b]				
PI	Sterilized	Not transferred	Sterilized	Pedestal
SI	Sterilized	Not transferred	Sterilized	Soil
PBS	Sterilized	Not transferred	Sterilized	NA

Note: NA = not applicable; PBS = phosphate-buffered saline; PI = inoculum of cultured pedestal microbiota; SI = inoculum of cultured soil microbiota.

[a] Conditions in experiment 4, desiccation stressor.

[b] Conditions in experiment 4, temperature stressor.

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