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Reciprocal microbiome transplants differentially rescue fitness in two syntopic dung beetle sister species (Scarabaeidae: *Onthophagus*)

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Abstract. 1. Microbial symbionts play a crucial role in the development, health, and homeostasis of their hosts. However, the eco-evolutionary conditions shaping these relationships and the evolutionary scale at which host–microbiome interactions may diverge warrant further investigation, especially in non-model systems. This study examines the impact of reciprocal gut microbiome transplants between two ecologically very similar, sympatric, and syntopic dung beetle sister species.

2. *Onthophagus vacca* and *Onthophagus medius* were specifically used to compare the growth, development, and fitness outcomes of individuals that were either (i) reared in the presence of a microbiome provided by a mother of the same species ("self-inoculated"), (ii) forced to develop with a microbiome derived from a heterospecific mother ("cross-inoculated"), or (iii) reared without a maternally transmitted microbiome.

3. This study found that individuals reared in the absence of a maternally derived gut microbiome incur detrimental changes in survival, as well as in several metrics signalling normative development. Furthermore, such negative effects are only partly rescued through inoculation with a heterologous microbiome.

4. Collectively, this study's results suggest that inoculation with a species-specific, maternally transmitted microbiome is critical for normative development, that the significance of maternally derived microbiota for host survival differs across species, and that the phenotypic outcomes resulting from host–microbiome interactions may diverge even between closely related, ecologically similar host species.

Key words. developmental symbiosis, gut microbiota, host–symbiont evolution, Onthophagini, survival analysis.

Introduction

The realisation that microbial symbionts are often critical for their host's development, health, and homeostasis has opened diverse novel avenues of investigation into how hosts and their microbiomes interact in ways that are able to shape each other's evolutionary history (Gilbert *et al.*, 2012; McFall-Ngai *et al.*, 2013). In particular, research has demonstrated that exactly what kind of host–microbial associations are able to form, and their respective phenotypic outcomes, may depend greatly on context (e.g. the microbial environment: Vautrin & Vavre, 2009; Schubert *et al.*, 2015; the external, abiotic environment: Corbin *et al.*, 2017; Renoz *et al.*, 2019; Lemoine *et al.*, 2020; and the nutritional environment: Douglas, 2009; Feldhaar, 2011). However, the evolutionary scale at which host–microbiome interactions may diversify remains poorly understood.

Partial progress toward addressing this issue has emerged through the rapidly increasing application of high-throughput sequencing, which has facilitated an explosion in large-scale taxonomic comparisons of microbial communities. Such efforts have permitted an assessment of how host relatedness correlates with microbial community similarity (Brooks *et al.*, 2016; Kohl *et al.*, 2018; Lim & Bordenstein, 2020) or how the microbiome of introduced species may shift to resemble those of native species (Gundale *et al.*, 2016; Parker *et al.*, 2020). In contrast, analyses of the phenotypic outcomes emerging from

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host–microbiome interactions in the context of host development and health have been limited to a select few systems. Particular foci to date include manipulating the relationships between long-term obligate symbionts and their hosts to assess symbiont function (e.g. aphids and *Buchnera aphidicola*: Moran *et al.*, 1993; Moran & Yun, 2015; Chong & Moran, 2018; leafhoppers: Bennett & Moran, 2013; Koga *et al.*, 2013; Sudakaran *et al.*, 2017), exploring the developmental consequences of microbiome disruption in model systems such as *Drosophila* (Broderick *et al.*, 2014; Bing *et al.*, 2018; Morimoto *et al.*, 2019; Nguyen *et al.*, 2020), and comparing the phenotypic outcomes of host–microbe interactions between distantly related and ecologically divergent taxa (Brooks *et al.*, 2016; Parker *et al.*, 2019). As a consequence, relatively little is known about how early in host diversification, under what types of ecological conditions, and in what systems the phenotypic outcomes emerging from host–microbiome interactions may actually diversify in the wild. Here, we investigate the phenotypical significance of host-associated microbiota in two highly ecologically similar, sympatric sister species of dung beetles in the genus *Onthophagus* through a reciprocal transplant experiment.

With over 2300 extant species found in a variety of habitats and on every continent save Antarctica, *Onthophagus* represents one of the most species-rich and widespread genera in the animal kingdom (Tarasov & Solodovnikov, 2011). Most species utilise the dung of mammals for feeding and breeding. Beetles excavate tunnels underneath droppings and provision dung for offspring in the form of buried 'brood balls', each containing a single developing individual (Halffter & Edmonds, 1982). Importantly, *Onthophagus* females vertically transmit gut microbial communities to their offspring through the *pedestal* – a maternal faecal secretion on which mothers oviposit their eggs within individual brood balls, which is consumed by larvae upon hatching (Estes *et al.*, 2013). Recent work has shown that (i) these pedestal microbiota are crucial for normative development of the host as depriving juvenile *Onthophagus* beetles of their pedestals leads to a marked reduction in adult body size and prolonged development time (Schwab *et al.*, 2016) and that (ii) these negative effects are exacerbated under stressful rearing conditions but can be rescued through re-inoculation with cultured pedestal bacteria (Schwab *et al.*, 2016). In addition to this documented reliance on vertically transmitted microbiota, the genus *Onthophagus* includes many closely related sister species and species complexes (e.g. Pizzo *et al.*, 2006; Macagno *et al.*, 2011; Breeschoten *et al.*, 2016; Roy *et al.*, 2016; Joaqui *et al.*, 2019), which offer the opportunity to investigate conservation and diversification in host–microbiome interactions over a range of phylogenetic distances, including across recently evolved host species. Here, we explored one such system – the sister species *Onthophagus medius* and *Onthophagus vacca* – to probe the eco-evolutionary contexts that may shape the early stages of diversification in host–microbiome interactions.

O. vacca and *O. medius* are estimated to have last shared a common ancestor in the late Miocene (~8.7 million years ago), thereafter undergoing allopatric speciation followed by secondary contact (Roy *et al.*, 2016). To date, both species have broadly overlapping western Palearctic distributions (Roessner *et al.*, 2010) and occupy highly similar ecological niches. While reproductively isolated via post-mating/post-zygotic barriers, individuals are frequently found in the same locations, feeding in the same dung pads, with no reported local aggregation patterns and partial phenological overlap (Roy *et al.*, 2016). In this study, we tested whether such closely related and ecologically similar species also share interchangeable microbial symbiont communities. Using syntopic populations (i.e. populations coexisting in close proximity within the same habitat: Rivas, 1964), we compared the growth, development, and fitness outcomes of individuals forced to develop with the pedestal-derived microbiome of the other species ("cross-inoculated") with those reared with their own pedestal ("self-inoculated") and to individuals reared without a pedestal. Based on previous research (Schwab *et al.*, 2016, and see above), we predicted that beetles reared without a pedestal would suffer the greatest reduction in fitness-related growth metrics and survival. Furthermore, we predicted that if divergence in the phenotypic outcomes resulting from host–microbiome interactions already accompanies descent from a common ancestor, cross-inoculation should fail to fully rescue the fitness of developing hosts compared to those receiving their species-specific microbiome.

Materials and methods

Beetle collection and husbandry

Parental *O. medius* and *O. vacca* were field collected as adults from pastures within the *Pantano della Zittola* peat bog (Isernia province, Italy) in early May 2019, were sorted by species, and were shipped to Bloomington, IN. All beetles were transferred to species-specific colonies upon arrival in the lab, where they were maintained in a sand/soil mixture at 22 °C and fed antibiotic-free cow dung weekly, per Moczek (2006). After a 2-week acclimation period in the lab, 20 females per species were provided with *ad libitum* dung and allowed to oviposit for 2–3 weeks until egg depletion in individual ovipositing containers as detailed below. Brood balls containing developing F1 individuals were harvested and incubated at 22 °C. Once developed to adulthood, individuals of the F1 generation were harvested and housed in monospecific colonies and then subjected to a vernalisation protocol similar to that described in Roy *et al.* (2016). Specifically, the temperature in the incubator housing the F1 colonies was lowered weekly by 4 °C, from 22 to 10 °C, over the course of 3 weeks. The colonies were maintained at 10 °C for 1 month, and then, the temperature was again increased to 22 °C over a 3-week span. Beetles were maintained at 22 °C for three additional weeks and subsequently used for experiments as follows.

Experimental design

Seven to 10 adult *O. medius* and *O. vacca* females were removed from each colony weekly (total *n O. medius* mothers = 29; *n O. vacca* = 27) and placed individually in plastic ovipositing containers (27 cm X 8 cm X 8 cm) filled with a compacted sand/soil mixture and provided with *ad libitum* dung on top. Brood balls were collected from each ovipositing container

after a week and carefully opened with gloved hands. Eggs and pedestals were extracted using sterilised paintbrushes and scalpels, respectively. Eggs were then surface-sterilised with one rinse of 100 µl of 1% bleach and 0.1% Triton-X 100 solution followed by two rinses of 1 mL of deionised water. After sterilisation, eggs were placed at the centre of an artificially constructed brood ball within the well of a 12-well plate, either on top of an extracted pedestal or on top of the same kind of dung forming the artificial brood ball, depending on treatment. Eggs from each species, and mother, were haphazardly assigned to one of three treatment groups: a self-inoculated treatment where each sterile egg was placed back on its own pedestal, a cross-inoculated treatment where eggs were placed on a pedestal from the other species, or an absent treatment where eggs were placed into a well with no pedestal. These six resultant treatment groups were blocked within each 12-well plate so that each plate contained two replicates of each group (e.g. two replicates of cross-inoculated *O. medius*), and their order in each plate was randomised to minimise random within-plate effects.

Plates were then stored at 22 °C for all of the development duration and checked weekly on days 3, 5, and 7 following their initial set-up to assess animal growth and developmental stage. After each check, plates were rotated 180°, and their placement within the incubator was changed to further minimise any potential microclimatic variation within the incubator. Final sample sizes for *O. medius* were 82 cross-inoculated, 83 not inoculated, and 77 self-inoculated and for *O. vacca* were 91 cross-inoculated, 126 not inoculated, and 124 self-inoculated.

Data collection

To assess the impact of our pedestal manipulation protocol on the growth and survival of our experimental animals, we collected the following data: (i) mass at the third (and final) larval instar (L3) and at the pupal stage; (ii) time from hatching of the egg to the onset of the third larval instar, to the onset of the pupal stage, and to adulthood; (iii) adult size; and (iv) survival to adulthood. L3 mass was measured 7 days after an animal was first scored as a third-instar larva as a proxy of each individual's ability to maximise mass gain in the critical rapid growth stage before reaching peak larval mass (Moczek & Nijhout, 2002; pers. obs.). Pupal mass was measured 48 hours after an individual was first scored as a pupa – this measurement served as an estimate of the final body mass attained by an individual following its gut purge and successful larval to pupal moult. Pupal mass also serves as a close correlate with adult body size in *Onthophagus* (Moczek, 2006). All mass measurements were recorded to the nearest 0.0001 g with a Mettler Toledo AL54 (Mettler, Columbus, Ohio, USA) scientific scale. Adult body size was measured as the width of the pronotum to the nearest 0.01 cm using a digital calliper. All individuals were sexed at the pupal stage - when the genital protrusion is clearly visible in males.

Data analysis

All statistical analyses were performed in R v3.5.3 (R Core Team, 2013) and RStudio v1.2.1335 (RStudio Team, 2015) using the packages *car* (Fox *et al.*, 2012), *GGally* (Schloerke *et al.*, 2017), *ggplot2* (Wickham, 2016), *lme4* (Bates *et al.*, 2015), *survival* (Therneau, 2015), *survminer* (Kassambara *et al.*, 2019), and *visreg* (Breheny & Burchett, 2017).

To determine the influence of our pedestal manipulation treatment on the growth, development, and survival of our experimental animals, we constructed a series of linear (growth, development) and generalised linear (survival, binomial family) mixed models, regressing our measured variables on all possible combinations of the fixed effects of pedestal treatment, species, and sex (included in models considering response variables measured in the pupal and adult stages), as well as their interactions. In each model, plate code was included as a random effect to account for any potential random error introduced by our experimental design. Furthermore, most models also included the unique code of each experimental individual's mother as a random effect to account for any random variation introduced from maternal line alone. This random effect was not included in models regressing either size at the third larval instar or time needed to reach the third larval instar. In these cases, the variance explained by mother's ID was zero, and mother's ID was therefore dropped to avoid overfitting. The regressors in each model were validated using Wald χ^2 tests, and non-significant interaction terms were removed. No interactions were ever found to be significant, nor was the factor sex, so every final model consisted only of the main effects of species and pedestal treatment along with random effects. Standard regression diagnostics were performed on each final model.

Finally, survival curves for each of our six treatment groups were calculated using the Kaplan–Meier estimator (Kaplan & Meier, 1958), and the resultant curves were compared using the non-parametric log-rank test.

Results

Using a reciprocal transplant experiment, we sought to assess whether two dung beetle sister species with a long history of sympatry, syntopy, and broadly overlapping ecological niches utilise interchangeable gut microbiomes or, alternatively, may have diverged in the phenotypic outcomes of host–microbiome interactions. Our results support the latter hypothesis, as detailed below.

Cross-inoculation differentially rescues survival in Onthophagus vacca *and* O. medius

Our three pedestal treatments had marked effects on survival in both focal species. Specifically, when reared without a pedestal, individuals survived at the lowest rate and showed the most precipitous early decline in survival compared to self-inoculated individuals, which survived at a significantly higher rate and did not experience a comparable drop in survival early on (Table 1; Fig. 1). However, both *O. vacca* and *O. medius* were differentially affected by cross-inoculation: when cross-inoculated with *O. vacca* pedestals, *O. medius* survived at intermediate rates, significantly different from both self-inoculation (log-rank test: $P = 0.028$) and absence of a

Table 1. Coefficients of mixed models testing for the significance of pedestal treatment and species on fitness-related developmental metrics.

		Species	Pedestal
Days to L3	χ^2	17.22	32.55
	P	**<0.001**	**<0.001**
	$\beta \pm$ SE	-2.19±0.51 (*vacca*)	2.51 ± 0.56 (none), −0.33 ± 0.5 (self)
L3 mass	χ^2	5.64	7.03
	P	**0.018**	**0.03**
	$\beta \pm$ SE (g)	−0.012 ± 0.0051 (*vacca*)	−0.0029 ± 0.0051 (none), 0.0087 ± 0.0046 (self)
Days to pupa	χ^2	48.44	9.94
	P	**<0.001**	**0.007**
	$\beta \pm$ SE	−7.96±1.14 (*vacca*)	1.086 ± 1.023 (none), −1.75 ± 0.91 (self)
Pupal mass	χ^2	0.053	4.48
	P	0.82	0.11
	$\beta \pm$ SE	0.0008 ± 0.0033 (*vacca*)	0.0003 ± 0.0038 (none), 0.0061±0.0033 (self)
Total development time	χ^2	31.36	8.19
	P	**<0.001**	**0.02**
	$\beta \pm$ SE (days)	−8.55±1.53 (*vacca*)	1.35 ± 1.18 (none), 1.67 ± 1.01 (self)
Adult size	χ^2	0.983	4.34
	P	0.32	0.11
	$\beta \pm$ SE	−0.082±0.083 (*vacca*)	0.029± 0.086 (none), 0.14 ±0.074 (self)
Survival	χ^2	5.54	22.59
	P	**0.019**	**<0.001**
	$\beta \pm$ SE (prob.)	0.33±0.31 (*vacca*)	0.68 ± 0.26 (none), 0.41 ± 0.24 (self)

The 12-well plate code and maternal ID were used as random effects in each model (except for days to third instar larva (L3) and larval weight, where maternal ID was dropped – see Methods for details). All non-significant interactions were removed. Rows show Chi-square (χ^2) test statistics values, the resulting test probabilities, and estimated effect sizes plus or minus standard error for each response variable. Notations in parentheses following β and standard error (SE) estimates reflect the change in value from one category to another (e.g. −2.19±0.51 (*vacca*) means that "Days to L3" decrease by 2.19±0.51 for *O. vacca* compared to *O. medius*).

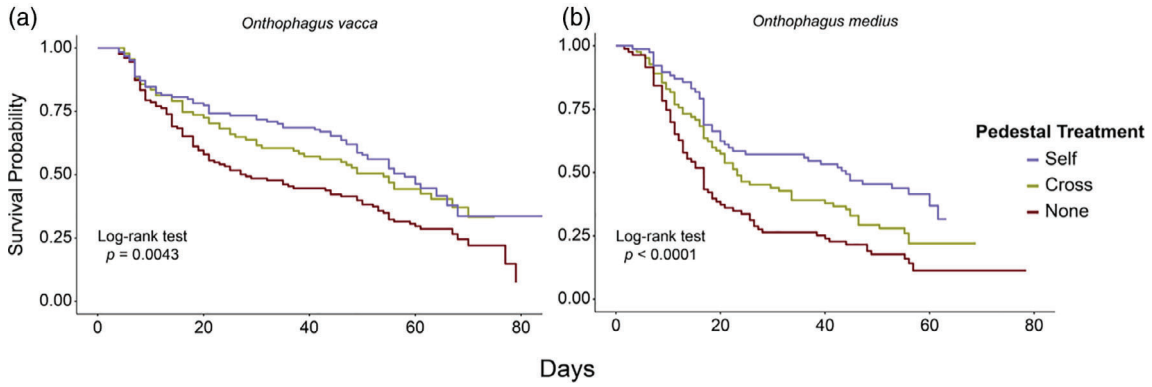

Fig. 1. Effect of pedestal manipulation on survival of *Onthophagus vacca* and *O. medius*. Survival curves of *O. vacca* (a) and *O. medius* (b) who received their own pedestal (self-inoculated), the other species' pedestal (cross-inoculated), or no pedestal (none). Curves were calculated using the Kaplan–Meier estimator, and the distributions of the curves were compared using non-parametric log-rank tests. In both species, self-inoculated animals showed the greatest survival rate throughout the course of the experiment, while animals receiving no pedestal showed the lowest. In *O. vacca*, cross-inoculation rescued fitness compared to the no pedestal treatment to the extent that final survival rate was indistinguishable from self-inoculated animals. In *O. medius*, cross-inoculation also improved survival compared to no pedestal but not to the extent seen in the self-inoculated treatment. [Colour figure can be viewed at wileyonlinelibrary.com].

pedestal (log-rank test: $P = 0.027$) (Fig. 1b). In contrast, in *O. vacca*, cross-inoculation with *O. medius* pedestals restored survival sufficiently that it became significantly different only from that of pedestal-free individuals but statistically indistinguishable from *O. vacca* inoculated with *O. vacca* pedestals (log-rank test: $P = 0.5$) (Fig. 1a). Combined, these results suggest that cross-inoculation with a heterologous pedestal is sufficient to largely restore survival in *O. vacca* but not in *O. medius*.

Pedestal-free rearing reduces growth, delays development, and is only partly reversed through inoculation with a heterologous microbiome

In both *O. vacca* and *O. medius*, our pedestal manipulations negatively impacted a range of growth and developmental metrics tied to fitness in insects (Moczek, 1998; Kingsolver & Huey, 2008). Specifically, while *O. vacca* generally developed

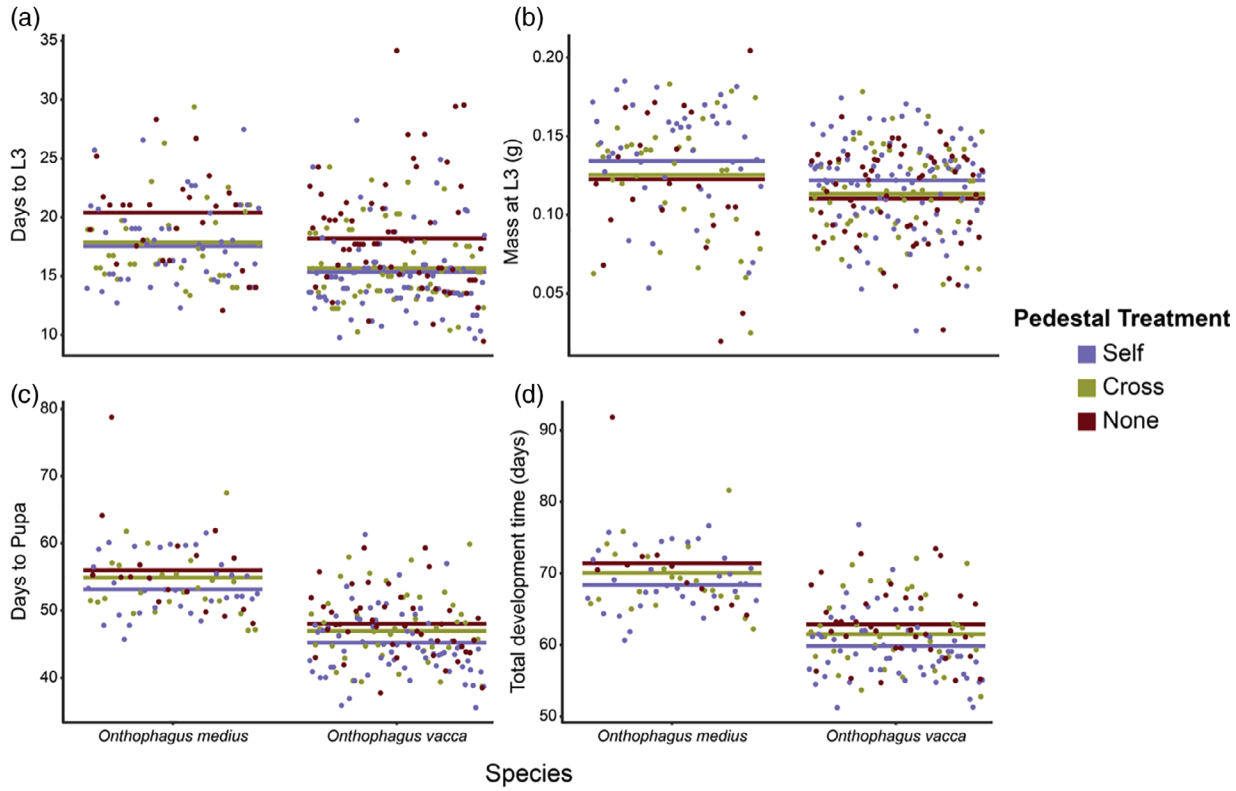

Fig. 2. Effect of pedestal manipulation on developmental metrics. Effects plots showing the estimated influence of pedestal treatment and species on (a) days until the third (and final) larval instar ($n = 333$), (b) mass in grams on day 7 of the third larval instar ($n = 304$), (c) days until the pupal stage ($n = 228$), and (d) total developmental time as days until adult eclosion ($n = 195$). All plots were derived from linear mixed models containing the factors pedestal treatment and species, as well as the random factors of plate code and identity of mother (omitted from (a), (b) because the variance of this effect was zero). In general, *Onthophagus vacca* develop faster and are smaller during the larval stage than *Onthophagus medius*. Furthermore, self-inoculated animals have the fastest development and are the heaviest as larvae, while animals receiving no pedestal develop the slowest and are the lightest. Cross-inoculated animals are generally intermediate between these groups. Points indicate partial residuals, and horizontal coloured lines indicate predicted values in each plot. [Colour figure can be viewed at wileyonlinelibrary.com].

faster and achieved higher larval mass, in both species, the absence of a maternally derived pedestal significantly prolonged development and lowered mass as measured on day 7 of the third (=last) larval instar (Table 1; Fig. 2a,b). Cross-inoculation with a heterologous pedestal partly reversed a subset of these effects to roughly comparable degrees in both species. That is, we found that cross-inoculated animals reached the third larval instar faster than pedestal-free animals and at a rate indistinguishable from self-inoculated animals in both *O. vacca* (Wald chi-square: $\chi^2 = 1.16$, $P = 0.28$) and *O. medius* (Wald chi-square: $\chi^2 = 0.014$, $P = 0.91$) (Table 1; Fig. 2a). However, this acceleration of development caused by cross-inoculation disappeared during later timepoints. Cross-inoculated *O. vacca* (Wald chi-square: $\chi^2 = 0.027$, $P = 0.87$) and *O. medius* (Wald chi-square: $\chi^2 = 2.64$, $P = 0.10$) both reached the pupal stage at the same rate as pedestal-free animals but slower than self-inoculated individuals (Table 1; Fig. 2c). Similarly, cross-inoculated *O. vacca* (Wald chi-square: $\chi^2 = 0.601$, $P = 0.44$) and *O. medius* (Wald chi-square: $\chi^2 = 0.927$, $P = 0.34$) completed development at the same reduced rate as pedestal-free animals (Table 1; Fig. 2d).

Furthermore, we found an overall significant effect of our pedestal manipulation on L3 mass (i.e., mass as measured on day 7 of the third larval instar) (Wald chi-square: $\chi^2 = 7.034$, $P = 0.03$) (Table 1). Specifically, in both species, cross-inoculation reduced L3 mass by an extent similar to pedestal deprivation ($\beta = -0.0029 \pm 0.0051$), while self-inoculation resulted in individuals reaching comparatively greater L3 mass ($\beta = 0.0087 \pm 0.0046$) (Table 1, Fig. 2b). Together, these results suggest that inoculation with a heterologous pedestal and corresponding microbiota is insufficient to fully restore growth and development time during larval ontogeny of either species. Finally, despite these differences found in developmental rate and mass during early development, we failed to find a significant effect of either species or pedestal treatment on both pupal mass and adult body size (as shown by non-significant results for these two factors in Table 1).

Discussion

We investigated whether two ecologically overlapping and geographically co-occurring dung beetle sister species utilise

interchangeable gut microbiomes. Using a reciprocal transplant experiment, we found that individuals reared in the absence of a maternally derived gut microbiome suffer reduced survival, as well as detrimental changes in several fitness-relevant developmental metrics. Furthermore, we found that such negative effects are only partly rescued through inoculation with a heterologous microbiome (i.e. a pedestal derived from a heterospecific mother), suggesting that developmentally significant divergences in the phenotypic outcomes resulting from host–microbiome interactions may already manifest during sister species formation and in spite of highly similar ecological conditions. Below, we discuss the most significant implications of our results.

Inoculation with a species-specific, maternally transmitted microbiome is critical for normative development

In line with previous research (Schwab *et al.*, 2016), we found that animals reared without access to a pedestal performed worse than animals that provided their own, species-specific pedestal in a host of fitness-relevant developmental metrics (Fig. 2; Table 1). In addition, while overall *O. medius* developed slower than *O. vacca*, both pedestal-free and cross-inoculated animals took longer to reach the pupal and adult stages than self-inoculated individuals, revealing that inoculation with a heterologous pedestal slows development in both species. Interestingly, only pedestal-free, but not cross-inoculated, animals showed a significant increase in the time needed to reach the final (=third) larval instar when compared to self-inoculated animals (Fig. 2a; Table 1). That is, cross-inoculated individuals developed at the same pace as self-inoculated individuals up until the third larval instar but slowed down significantly thereafter, ultimately reaching the pupal and adult stages at the same rate as pedestal-free animals. In addition, cross-inoculated animals had significantly lower mass 7 days into the final larval instar than self-inoculated larvae (Fig. 2b; Table 1). Our data therefore demonstrate that cross-inoculation has little impact on the developmental rate during the early larval stages but does significantly slow growth in the third larval instar – a period critical for rapid mass gain in *Onthophagus* (Moczek & Nijhout, 2002) – possibly leading to subsequent developmental delays as cross-inoculated animals must spend more time feeding as larvae in order to gain sufficient mass for the onset of pupation to occur (Shafiei *et al.*, 2001). These results thus lend further support to the idea of dung beetle microbiota as a host species-specific nutritional symbiont (Estes *et al.*, 2013; Schwab *et al.*, 2016; Shukla *et al.*, 2016; Parker *et al.*, 2019).

Host species differ in their reliance on maternally transmitted microbiome for survival

Pedestal-free rearing not only reduced growth and delayed development but also substantially affected survival rates. That is, while, overall, *O. vacca* survived at higher rates than *O. medius*, pedestal-free rearing severely reduced survival during development in both species (Fig. 1; Table 1). Previous research showed a similar reduction in survival in pedestal-free individuals of a different *Onthophagus* species but only when reared under stressful environmental conditions (high desiccation stress and temperature fluctuations; Schwab *et al.*, 2016). In comparison, in *O. vacca* and *O. medius*, the negative effects of pedestal removal were obvious even under the relatively benign rearing conditions used in this study. At the same time, in both *O. vacca* and *O. medius*, cross-inoculation improved survival compared to pedestal-free rearing. However, while *O. vacca* reared with heterologous pedestals showed survival rates statistically indistinguishable from those reared with their own pedestal, *O. medius* receiving a heterologous pedestal survived at a rate higher than pedestal-free, but still lower than self-inoculated, animals (Fig. 1b). Our pedestal exchange experiment therefore provides further support for differential, host species-specific reliance on pedestal microbiota (also see Parker *et al.*, 2019). In particular, our results suggest that, even though reliance on maternally transmitted microbiota for normative host development may be a general feature in *Onthophagus*, different host species within this genus may nevertheless diverge in the extent of this reliance.

Divergence in the phenotypic outcomes resulting from host–microbiome interactions is detectable even in closely related, ecologically similar species

To date, few studies have investigated the potential for divergence in the phenotypic consequences of host–microbiome interactions across host species (Brooks *et al.*, 2016; Sudakaran *et al.*, 2017; van Opstal & Bordenstein, 2019). Among dung beetles, such putative interspecific differentiation was detected by swapping pedestals between *Onthophagus sagittarius* and *Digitonthophagus gazella* (Parker *et al.*, 2019). While both are tunnelling dung beetle species belonging to the tribe Onthophagini, they are phylogenetically much more distant than the sister species used in the present study (37 MYA: Breeschoten *et al.*, 2016). Furthermore, *O. sagittarius* and *D. gazella* derive from different continents and have only had a very recent history of sympatry following artificial introductions into Australia in the 1970s as part of a biocontrol programme (Edwards, 2007). Reciprocal microbiome transplants across these focal species similarly affected developmental metrics and survival in a host-specific manner. Yet, diversification of distantly related hosts in their reliance into non-interchangeable microbial communities could simply be a product of their great phylogenetic distance and biogeographic separation. In contrast, our finding that pedestal cross-inoculation between *O. vacca* and *O. medius* fails to fully rescue the fitness of developing individuals suggests that divergence in the phenotypic outcomes of host–microbiome interactions may indeed already accompany descent from a common ancestor and manifest over much shorter evolutionary time periods. Moreover, these sister species appear to rely on non-interchangeable microbiomes *despite* their long history of sympatry/syntopy and broadly overlapping autecologies (Roy *et al.*, 2016), raising questions regarding exactly what evolutionary and ecological dynamics may have driven, and are now maintaining, host-specific microbiome divergences.

It is currently hypothesised that *O. vacca* and *O. medius* speciated in allopatry and only subsequently established their

present-day sympatric ranges as a result of secondary contact (Roessner *et al.*, 2010; Roy *et al.*, 2016). Stochastic (e.g. priority and founder effects) or deterministic forces (e.g. host selection and environmental pressure), both of which have the potential to significantly impact microbiome assemblies (Maignien *et al.*, 2014; Schmidt *et al.*, 2015; Vecchi *et al.*, 2018; Parker *et al.*, 2020), may therefore have shaped distinct host–microbiome interactions already during the allopatric stage of species formation. If true, the results of our study reflect a relatively deep divergence, established during speciation and then maintained throughout secondary contact. In addition, divergence in the phenotypic outcomes of host–microbiome interactions might also have arisen, or been emphasised, once the two species re-established contact. In this scenario, the establishment of diverging host–symbiont relationships – possibly combined with differential microhabitat specialisations – may have facilitated the maintenance of the two sister species in syntopy, avoiding competitive exclusion (Levin, 1970; Schoener, 1974; Scriven *et al.*, 2016). Finally, given that (i) *O. vacca* and *O. medius* can interbreed in captivity but form low-fitness hybrids (Roy *et al.*, 2016) and that (ii) research on other insects has established that such post-mating hybrid lethality can be attributed directly to the maternally transmitted microbiome (Brucker & Bordenstein, 2013), it is also possible that reliance on a non-interchangeable microbiome may contribute to sympatric speciation via reinforcement (i.e. selection against hybridisation) in these sister species. If true, *O. vacca* and *O. medius* would join a growing list of examples illustrating the potential of microbial symbionts to contribute to speciation of their hosts (Sharon *et al.*, 2010; Lizé *et al.*, 2013; Morimoto *et al.*, 2017; Leftwich *et al.*, 2018). Further studies are needed to confirm or reject this possibility.

Conclusions

Progressing beyond taxonomic descriptions of the microbiome towards a more comprehensive understanding of the emergent properties of the complex interactions between microbial symbionts and their hosts, and of the ecological and evolutionary conditions shaping these relationships, remains a crucial goal, especially in non-model systems. Previous work documented that dung beetle species may associate with non-interchangeable microbiota (Parker *et al.*, 2019), yet the phylogenetic scope and ecological conditions that facilitate such divergences remained to be characterised. Here, we have shown that sister species may rely on non-interchangeable microbiomes to support their development and enhance their survival. Importantly, our observations suggest that such disparate, non-equivalent host–microbiota associations may be maintained despite a long history of coexistence in the same geographical areas and overlapping host autecologies. Vertical transmission appears to be perhaps the most plausible strategy to maintain such associations, although host-specific differential horizontal acquisition of selected strains from the environment cannot be currently excluded as an alternate, or additional, mechanism (Moran & Sloan, 2015; Shapira, 2016). Further investigations into the phenotypic significance of maternally transmitted microbial symbionts of closely related host species in both sympatry and allopatry, coupled with an analysis of their potential for the maintenance of the hosts' reproductive barriers, may shed more light on how hosts and their microbiomes interact in ways able to shape each other's evolutionary history. In addition, molecular-based insights into the composition and vertical transmission of pedestal-inoculated microbiota would greatly help elucidate the functions provided by microbial symbionts and whether microbiome divergences may precede, parallel, or follow speciation events of *Onthophagus* hosts.

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Data availability statement

Data available from the authors upon request.

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