Transgenerational developmental effects of species-specific, maternally transmitted microbiota in *Onthophagus* dung beetles

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Abstract. 1. The significance of host–microbe interactions is increasingly appreciated across biological disciplines, yet to what extent these interactions influence developmental outcomes within and across generations remains poorly understood.

2. This study investigated the putative role of host–microbe interactions in the adaptive diversification of *Onthophagus* dung beetles, one of the most species-rich and ecologically successful genera of insects. *Onthophagus* mothers vertically transmit growth- and fitness-enhancing gut symbionts to their offspring through a faecal secretion known as the pedestal.

3. Pedestals were reciprocally exchanged between two ecologically similar congeneric *Onthophagus* species to assess the degree to which pedestal microbiota from one species can substitute for those of another.

4. It was found that the presence of a heterospecific pedestal delays development and increases mortality, and that the fitness costs of non-host-specific microbiota are maintained transgenerationally.

5. Collectively, these results support the hypothesis that *Onthophagus* beetles maintain, interact with, and are dependent upon host species-specific microbial communities to support normal growth and development. The implications of these results are discussed in the context of host microbiota coevolution.

Key words. developmental symbiosis, holobiont, host–symbiont interactions.

Introduction

Understanding the processes that enable and shape the generation of biodiversity is a major objective of evolutionary biology. Traditionally, these processes have been explained as being the result of divergent selective pressures acting on generations of individuals. However, increasing appreciation of the vital role of host-associated microbial symbionts has added complexity to this notion of individuality, as microbial associates have been shown to exert a diverse array of influences on their animal hosts, including nutritional supplementation (reviewed in Douglas, 2009), life-history decisions [such as metamorphosis induction (Hadfield, 2011; Shikuma *et al.*, 2014), reproductive timing (Leonardo & Mondor, 2006), and modification of survival–reproduction trade-offs (Emelianoff *et al.*, 2008)], or instruction of embryonic and post-embryonic tissue development [e.g. as seen in nematodes (Landmann *et al.*, 2014), cephalopods (McFall-Ngai, 2014), fish (Rawls *et al.*, 2004), and mammals (Hooper & Gordon, 2001; Stappenbeck *et al.*, 2002)].

Even though awareness and appreciation of the importance of host–microbiota interactions in structuring developmental outcomes have been increasing broadly (reviewed in Gilbert *et al.*, 2012), the evolutionary consequences of disruptions to these interactions remain poorly understood. The importance of the microbiota for normal development, especially in partnerships involving strict, vertical transmission of microbial symbionts [e.g. in aphids (Koga *et al.*, 2012), shield bugs (Salem *et al.*, 2015), and leafhoppers (Watanabe *et al.*, 2014)], has raised the possibility that alteration or disruption of these communities could result in fitness consequences within and across generations, thereby affecting host evolution. Indeed, recent work suggests that gut microbiota disruptions in *Drosophila* lead to transgenerational alterations in development and mate preferences (Sharon *et al.*, 2010; Morimoto *et al.*, 2017); however, some of these effects have been difficult to replicate (Leftwich

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et al., 2017). Overall, empirical studies addressing this hypothesis remain rare and more work is needed to determine the broader validity of this perspective. Here, we investigate the role of gut microbiota in influencing the development and potential fitness of dung beetles within and across generations. We focus on beetles in the genus *Onthophagus*, one of the most dramatic radiations of terrestrial invertebrates, and assess the extent to which different *Onthophagus* host species have evolved to utilise host-specific microbial partners.

Onthophagus are considered true dung beetles in that they rely on dung throughout their life cycle. With over 2000 extant species (Tarasov & Kabakov, 2010), *Onthophagus* ranks among the most species-rich genera in the animal kingdom. This diversity is in part attributed to resource specialisation (Davis & Sutton, 1997; Emlen *et al.*, 2005), as extant *Onthophagus* have radiated onto a remarkable diversity of dung types [e.g. ungulates (Emlen *et al.*, 2005), arboreal monkeys (Estrada *et al.*, 1999), kangaroos, and wallabies (Matthews, 1971)]. However, the same type of dung is often utilised by multiple *Onthophagus* species (Emlen *et al.*, 2005), and several species complexes exist, especially in Europe (Pizzo *et al.*, 2006; Angus, 2008; Macagno *et al.*, 2011), comprising closely related and morphologically highly similar yet non-hybridising species, suggesting that specialisation on discrete dung types cannot be the sole ecological mechanism underlying *Onthophagus* diversity. At the same time, all dung types constitute challenging food sources that are generally low in nutritional value, lack essential amino acids, and comprise mainly hard-to-digest cellulose (Muller, 1980). Recent work suggests that *Onthophagus* beetles utilise this food source through the association with symbionts that are vertically transmitted by mothers through the pedestal, a maternal faecal pellet onto which an egg is oviposited and which is consumed upon hatching of the larva (Estes *et al.*, 2013; Schwab *et al.*, 2016, 2017). Experimental removal of maternally transmitted symbionts in *Onthophagus* results in delayed development and reduced growth, as well as elevated mortality under stressful conditions, whereas re-inoculation with experimentally cultured pedestal microbiota restores normative development (Schwab *et al.*, 2016). The significance of vertically transmitted symbionts to normal development in *Onthophagus*, coupled with the remarkable diversity seen among ecologically similar *Onthophagus* species, raises the possibility that the diversification of *Onthophagus* has been at least partially shaped by the developmental symbioses formed between beetle hosts and their symbionts. Here we assess an important prediction of this hypothesis.

Specifically, we aimed to examine whether dung beetle host species have evolved to interact with, and benefit from, a host-specific microbial community. To test this, we exchanged the maternally provisioned microbiota between two ecologically similar, congeneric, generalist feeding dung beetle species, *Onthophagus gazella* (Fabricius, 1787) and *Onthophagus sagittarius* (Fabricius, 1775), derived from a location where both species occur in sympatry. We hypothesised that if the microbiota of each species provides functionally unique benefits to its host, beetles receiving a mismatched (i.e. heterospecific) microbiota should have compromised growth, as well as developmental and survival outcomes compared with individuals provided

their own, host-species specific microbiota. We further predicted that, if microbiota–host mismatches cause adverse fitness consequences, these effects should be transgenerational, as at least a subset of the mismatched microbes would be vertically transmitted to the next generation.

Materials and methods

Beetle collection and husbandry

Onthophagus gazella and *O. sagittarius* beetles were field collected in Gatton and Imbil near Brisbane, Queensland, Australia, and shipped to Bloomington, Indiana. *Onthophagus sagittarius* and *O. gazella* are native to Southeast Asia and much of Africa, respectively, and in their native ranges do not occur in sympatry. However, both species were introduced into Australia *c*. 50 years ago as part of a biocontrol effort aimed to enhance the management of dung and dung-breeding flies (Edwards, 2007). Both species are now well established and broadly overlapping throughout their exotic range.

Colonies of both species were maintained in a sand/soil mixture at 28 °C and fed antibiotic-free cow manure twice a week as described in Moczek (2006). Experimental animals were generated by breeding six females and three males in a small sand/soil-filled container with *ad libitum* food. Following protocols established in Schwab *et al.* (2016, 2017), brood balls produced by adult females were collected after 5 days and carefully opened by hand. Eggs were extracted using autoclave sterilised paintbrushes. After removal, eggs were then surface-sterilised with 100 μl of 1% bleach (final concentration of 0.0525% sodium hypochlorite) and 0.1% Triton-X 100 solution followed by two rinses with 1 ml of deionised water. Concurrently, the interior surface upon which the egg had been positioned by the mother (referred to as the pedestal) was extracted using a flame-sterilised surgical blade following Schwab *et al.* (2016). The pedestal was then placed at the centre of an artificial brood ball within the well of a 12-well plate prepared as described in Shafiei *et al.* (2001) and Schwab *et al.* (2016). A sterile paintbrush was then used to place a single sterile egg on the dissected pedestal, after which the artificial brood ball containing egg and pedestal were gently covered with dung to prevent desiccation. Eggs obtained from each species were haphazardly assigned to one of two treatments: a self-inoculated treatment wherein each surface-sterilised egg was placed on its own pedestal or a cross-inoculated treatment wherein each surface-sterilised egg was placed on a pedestal obtained from the other species, resulting in two treatment groups per species, and a total of four treatments. For logistical reasons we could only accommodate two treatment groups, and chose the control group that would permit the most extreme contrast between microbiota origins (different species versus entirely self). A maximum of three individuals per treatment were placed in rows at random locations within each of the 12-well plates used.

Twelve-well plates were then transferred to a 28 °C incubator and checked once every 48 h to assess larval progression in development as described below. The orientation and position of plates within the incubator were changed every 48 h to minimise

the effects of any potential microclimatic variation within the incubator.

Assessing the effects of microbiota swapping on growth, development and survival

To assess potential effects of microbiota swapping on growth, developmental rate, and survival we collected the following measurements for each individual: time (in days) to: (i) final (third) larval instar, (ii) pupation, and (iii) adulthood. We measured larval peak mass, operationally defined as the larval mass 48 h after a given individual was first scored as a third instar (i.e. approximately day 3 or 4 of the final instar, or roughly 12 days since egg treatment for a self-inoculated animal), as well as pupal mass. All mass measurements were obtained to the nearest 0.0001 g using a Mettler Toledo AL54 (Mettler, Columbus, Ohio) scientific scale. Animals were sexed as pupae to enable the analysis of sex bias. Lastly, we measured survival to adulthood as well as adult body size. Adult size was measured as thorax (pronotum) width using a two-dimensional morphometric setup consisting of a Leica dissecting microscope (Wetzlar, Germany), a Scion digital camera (Frederick, Maryland) and ImageJ v1.44p software as previously described (Moczek, 2006).

Assessing transgenerational effects of microbiota manipulations

To assess whether microbiota manipulations affected fitness beyond the initial generation of larvae, we used surviving F_1 adults to rear a second generation of beetles. However, because not a single *O. sagittarius* female receiving a *O. gazella* pedestal survived to reach adult sexual maturity (out of a total of 23; see below), this experiment was restricted to *O. gazella* only.

To obtain second-generation individuals, virgin adult F_1 *O. gazella* females were maintained in two female-only, treatment-specific colonies following the protocol described above for at least 3 weeks to allow for sexual maturation. Then, male *O. gazella* from the original field-collected colony were added to each female treatment-specific colony to generate an approximately 2:1 female:male ratio. Male and female *O. gazella* were maintained together for 6 days, after which females were separated and placed individually in single-female breeding containers and provided *ad libitum* dung. After 5 days, brood balls containing F_2 offspring were collected. Each brood ball was weighed and then uniquely labelled and stored in an individual small soil/sand-filled container until adult emergence. Emerging F_2 beetles were sexed and then measured for body size as described earlier.

Statistical analyses

The effects of microbiota swapping on F_1 beetle development and fitness was assessed using a Welch's *t*-test to compare means of the two treatment groups for *O. gazella*. Differences in survival to adulthood between treatments was assessed using Pearson's χ^2 tests.

To investigate the specific influence of the transgenerational effect of microbiota swapping on F_2 beetle development and fitness, we constructed linear and generalised linear (binomial family, logit link) models regressing F_2 adult body size, developmental time, and survival on all possible main effect combinations of maternal size, brood ball weight, sex, and microbiota identity. In each of these models, we tested for a possible interaction between maternal size and brood ball weight, as previous work has shown a correlation between these two measures (Hunt & Simmons, 2002; Macagno *et al.*, 2018). The regressors included in each model were validated using *F*-tests, and regression diagnostics were performed to assess assumptions related to the constancy of variance and normality of the residuals, and to identify any outlying or otherwise particularly influential single points. All analyses were executed in R using RStudio and the *car*, *realimpo*, and base packages (Fox *et al.*, 2012; Grömping, 2006; RCore Team, 2013; RStudio Team, 2015). All figures were generated in RStudio using the *ggplot2*, *gridextra*, *ggsignif*, and *visreg* packages (Auguie, 2016; Wickham, 2016; Ahlmann-Eltze, 2017; Breheny & Burchett, 2017).

Results

Microbiota swapping prolongs development time, reduces growth, and affects survival in both Onthophagus *species*

We sought to determine whether the vertically transmitted microbiotas of *O. gazella* and *O. sagittarius* provide benefits specific to each host species. To do so, we performed reciprocal swaps of the maternally provisioned pedestal, i.e. the main conduit through which mothers bequeath maternal microbiota unto their larval offspring (Schwab *et al.*, 2016). We then measured several metrics related to growth, development, and survival among treatment groups. We predicted that if *Onthophagus* microbiota provide host species-specific benefits, this should manifest in prolonged development, reduced maximal growth, or decreased survival among cross-inoculated compared with self-inoculated individuals. Our results provide partial support for these predictions.

Specifically, *O. gazella* individuals that received an *O. sagittarius* pedestal took significantly longer to reach both pupation and adulthood, yet weighed significantly less as pupae than *O. gazella* individuals which were provided their original pedestal (Welch's *t*-test: $t_{pupation} = 3.6965$, $P_{pupation} = 0.0012$; $t_{adulthood} = 2.9875$, $P_{adulthood} = 0.0076$; $t_{weight} = 2.373, P_{weight} = 0.0288$; Fig. 1). None of these treatment effects differed significantly among sexes ($t_{pupation} = 1.3532$, $P_{pupation} = 0.1834$; $t_{adulthood} = 1.0755$, $P_{adulthood} = 0.2886$; $t_{weight} = 0.1895$, $P_{weight} = 0.8506$). Conversely, we detected no significant treatment effect with respect to peak larval weight ($P = 0.2807$), time needed to reach the third larval instar ($P = 0.3185$), or adult body size ($P = 0.1371$). Further, even though survival to adulthood was lower in cross-inoculated individuals (48%) compared with self-inoculated individuals (71%) this effect was not statistically significant ($\chi^2 = 1.7145$, $P = 0.1904$; Fig. 2).

By contrast, we recovered a significant effect of pedestal origin on survival to adulthood in *O. sagittarius*: when reared on
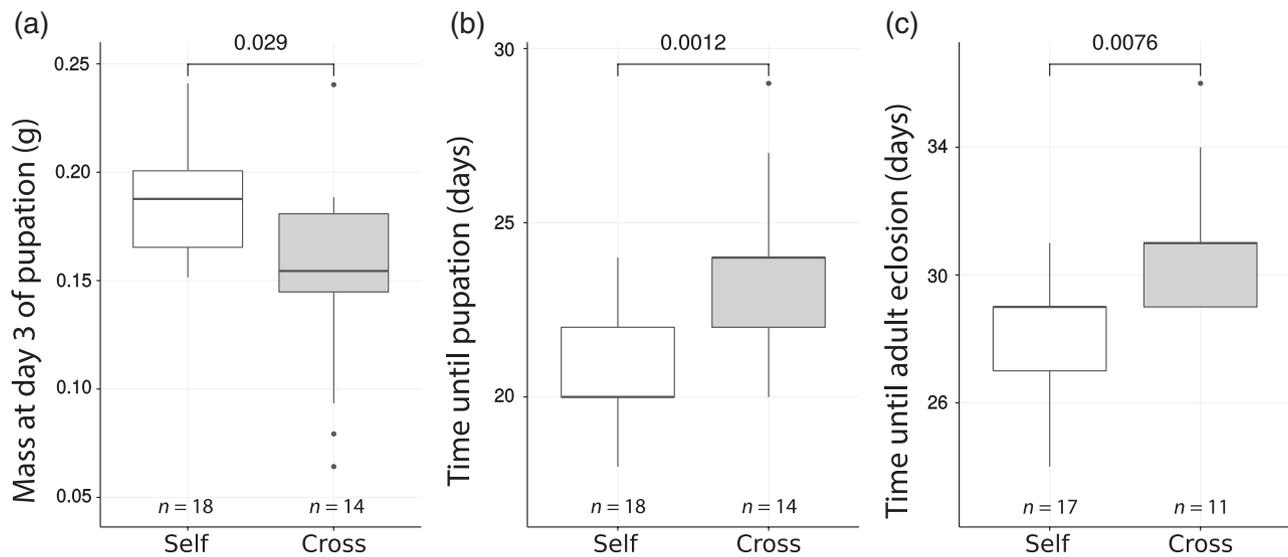
Fig. 1. Developmental consequences of inoculation with species-specific (self) or heterospecific (cross) microbiota in the dung beetle *Onthophagus gazella*. Individuals receiving their own *O. gazella*-specific microbiota are shown in white, and individuals receiving microbiota derived from the congener *Onthophagus sagittarus* are shown in grey. (a) Pupal mass on day 3 during pupation. Cross-inoculated pupae reached a lower mass. (b) Number of days from egg to pupation. Cross-inoculated *O. gazella* larvae took longer to reach pupation. (c) Number of days from egg until eclosion of adults. Cross-inoculated larvae took longer to reach adulthood. Numbers below median lines of each box plot represent sample sizes for each group. Numbers above brackets are *P*-values obtained from a Welch's t-test comparing means of the two treatment groups.

their original *O. sagittarius* pedestal, 16/32 (50%) individuals survived to pupa and 14 (44%) to adulthood. However, when reared on an *O. gazella* pedestal, only 5/23 (22%) individuals survived to the final larval instar, of which only three individuals (two males, one female; 13%) reached the pupal and subsequent adult stages ($\chi^2 = 4.5580$, $P = 0.0328$; Fig. 2). Unfortunately, this high mortality in the cross-inoculated treatment group severely reduced sample sizes for comparisons of growth and developmental time metrics, precluding meaningful statistical inference for this species.

Negative effects of microbiota swapping are transgenerational

Lastly, we sought to investigate whether any developmental or fitness effects due to heterospecific microbiota inoculation would recur in subsequent generations, as would be predicted if functionally relevant microbes were vertically inherited. Specifically, we tested the prediction that the offspring of mothers who as larvae were forced to develop utilising a heterospecific pedestal would exhibit reduced growth performance and survival compared with the offspring from mothers who as larvae had access to their species-specific pedestal. Further, if such an effect existed, we sought to compare it with other factors identified by previous work to influence adult body size, in particular maternal size and brood ball weight (Hunt & Simmons, 2002). Due to the high mortality seen in one of the two *O. sagittarius* treatments, we were only able to execute this experiment in *O. gazella*.

In contrast to earlier studies (e.g. Macagno *et al.*, 2018), we did not detect a significant effect of brood ball weight, maternal size, or their interaction when added to models already

containing offspring sex and microbiota origin. Furthermore, the two variables were not correlated (Pearson's correlation coefficient: $r = -0.03$). However, we did find a significant effect of offspring sex (one-way ANOVA: $F = 80.5198$, $P < 0.0001$), and, to a slightly lesser extent, microbiota origin ($F = 21.6109$, $P < 0.0001$), on adult F_2 offspring size. That is, after controlling for the effects of offspring sex, offspring of *O. gazella* larvae inoculated with their species-specific microbiota ($n = 39$) developed to significantly larger adult sizes compared with offspring of parents inoculated with microbiota derived from *O. sagittarius* ($n = 34$; Fig. 3). By contrast, no such effect was seen in survival rate or development time to adulthood. These results suggest that at least some of the developmental consequences introduced through microbiota swapping persist across generations.

Discussion

A large body of work demonstrates the diverse benefits that hosts derive from their microbial partners (as reviewed in McFall-Ngai *et al.*, 2013). These findings have motivated the hypothesis that host development may in part be shaped by the symbiotic partnerships that hosts form, and that evolutionary outcomes may in part be influenced by these interactions (Gilbert *et al.*, 2015). However, the role and significance of host microbiota influences on long-term evolutionary trajectories of their hosts remain poorly understood. Here we investigate the possible role of the pedestal microbiota in the diversification of dung beetles in the genus *Onthophagus*, one of the most dramatic radiations of insects, by assessing the extent to which *Onthophagus* host species have evolved to utilise specific microbial partners. We
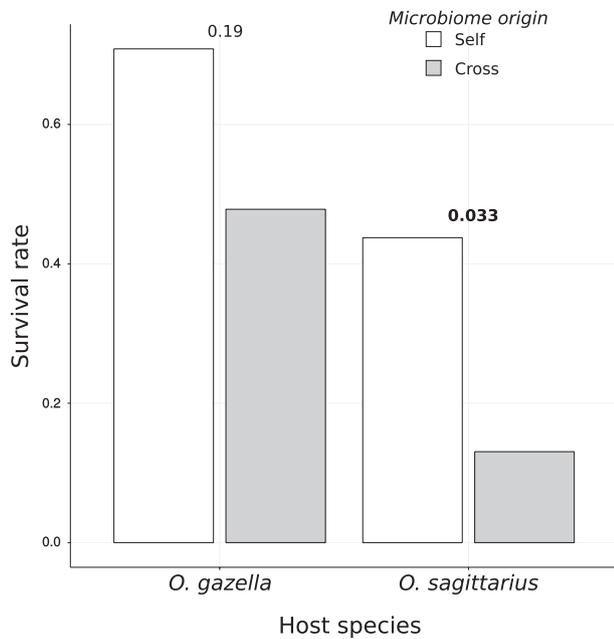
Fig. 2. Survival to adulthood of *Onthophagus gazella* and *Onthophagus sagittarius* inoculated as larvae with their species-specific microbiota (shown in white) or cross-inoculated with microbiota derived from their respective congener (shown in grey). Cross-inoculated individuals showed lower survival rates in both species; however, this effect was significant only in *O. sagittarius* ($P = 0.0328$) but not *O. gazella* ($P = 0.1904$), where *P*-values represent the results of χ^2 tests. A general relationship between treatment and species was also seen from a χ^2 test for independence ($P = 0.004\,82$).

find: (i) that individuals provisioned with the microbiota of a congeneric beetle host exhibit lower fitness compared with individuals raised on their species-specific microbiota; and (ii) that a subset of fitness reductions seen in cross-inoculated individuals is maintained transgenerationally. In the following, we discuss the most important implications of our results.

Inoculation with non-host-specific microbiota reduces fitness

We found that individuals inoculated with microbiota derived from a heterospecific host exhibit significantly reduced growth and prolonged development time (*O. gazella*) or elevated mortality (*O. sagittarius*). These results support the hypothesis that these two relatively distantly related *Onthophagus* species, which diverged roughly 37 Ma (see Emlen *et al.*, 2005, Fig. 3; Breeschoten *et al.*, 2016), are adapted to utilise host-specific microbial communities (Figs. 1 and Fig. 2). Importantly, these results were obtained from populations collected from a relatively recently established range in which both species have been occurring in sympatry and frequently in syntopy, i.e. the same dung pads, for *c.* 50 years, or roughly 100 generations. These observations suggest that even though these species now occur in extremely close proximity, both appear to have maintained disparate, and functionally non-equivalent microbiota. However, it is worth noting that our choice of a control group – eggs

returned to their own, original pedestal – does not allow us to separate host species-specific and maternal line-specific contributions to the role of the microbiota in determining offspring fitness and development. To address this issue, future work should specifically contrast the developmental outcomes of individuals provided with microbiota of strictly maternal origin and those receiving microbiota from unrelated adults.

Furthermore, we found that the magnitude of fitness reductions seen in the F_1 generation was not fully equivalent in both species. Cross-inoculated *O. sagittarius* beetles survived at an extremely low rate (only 13% reached adulthood) as compared with both the self-inoculated treatment and the cross-inoculated *O. gazella* beetles. Although this low survival rate precluded statistical comparisons of other developmental metrics between the two *O. sagittarius* treatment groups, the difference in survival rates between the two species suggests that *O. sagittarius* beetles might generally be more sensitive to microbiota disruptions. It is unclear at this point whether this reduced survival is due to the loss of beneficial microbes or the introduction of microbes that become pathogenic in a new host. To clarify this, future work is needed to characterise: (i) the composition of the undisrupted gut microbial communities of these two beetle species; (ii) how the compositions of these communities are altered through pedestal transplanting; and (iii) if transplanting allows latent pathogenic bacteria to overwhelm their new hosts. Regardless of this current ambiguity, these results support the hypothesis that ecologically similar *Onthophagus* beetles have evolved to obtain host-specific benefits from diverse and functionally non-equivalent microbial communities.

Fitness costs due to non-host-specific microbiota are transgenerational

Previous work has shown that in a third species, *Onthophagus taurus* (Schreber, 1759), mothers vertically transmit gut microbes to their offspring through the pedestal (Estes *et al.*, 2013). We reasoned, therefore, that negative fitness effects due to host−microbiota mismatching seen in the F_1 generation of *O. sagittarius* and *O. gazella* should be at least partially maintained in subsequent generations, as cross-inoculated larvae would, as adults, pass on the non-host-specific microbiota through their own pedestal. In support of our prediction, we found that the offspring of cross-inoculated *O. gazella* mothers were significantly smaller as adults than were the offspring of self-inoculated *O. gazella* mothers. The effect of microbiota origin on F_2 size remained significant in a model that also accounted for the effects of sex, brood ball size, and maternal size (Fig. 3). This transgenerational effect of pedestal swapping provides additional support for the hypothesis that vertical transmission of host-specific microbiota is a common feature of *Onthophagus* beetles. Moreover, it provides further support for the prediction that the benefits provided by these vertically inherited communities are the result of a history of coevolution.

Surprisingly, F_2 males were found to be significantly larger than F_2 females (Fig. 3), yet no such effect was seen in the F_1 generation. To our knowledge, this is the first demonstration of such a pronounced sex-biased body size dimorphism in
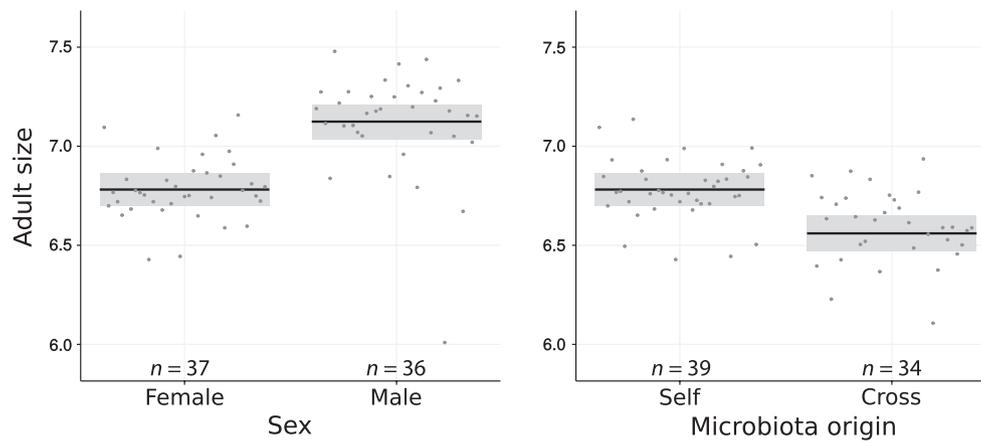
Fig. 3. Effect plots showing the predicted values (black lines) and confidence intervals (grey shading) for two explanatory variables when regressed against variation in adult size of F_2 *Onthophagus gazella*. We found that offspring sex and, to a lesser extent, microbiota origin significantly explain the variation seen in adult size; that is, after controlling for the effects of all other regressors, male larvae ($n = 36$) developed to a larger adult size than did female larvae ($n = 37$), and offspring ($n = 39$) of self-inoculated mothers developed to larger adult sizes than did offspring ($n = 34$) of cross-inoculated mothers. Sex and microbiota origin explained 46.8% of the variation seen in body size when together (model $R^2 = 0.468$), and the relative importance values of the two regressors, from the 'lmg' decomposition method, were 33.7% and 13.1%, respectively (Grömping, 2006).

Onthophagus generally, and *O. gazella* specifically, although the mechanism underlying this result is unclear. One potential explanation could be that pre-adult mortality in our study primarily affected small males, yet sex ratios of survivors were even (36 males vs. 39 females) and the mass distribution of the brood balls of individuals that did not reach adulthood, a measure which is generally tightly correlated with both offspring body size and male mortality rates (Hunt & Simmons, 2002; House *et al.*, 2011), was not significantly different from those of the survivors, failing to support this hypothesis.

Transgenerational effects of gut microbes in insects have also been uncovered by other studies. Recent work in *Drosophila melanogaster* (Meigen, 1830), for example, shows that daughters of parents inoculated with *Acetobacter pomorum* are significantly smaller than daughters of parents inoculated with other bacterial strains (Morimoto *et al.*, 2017). Furthermore, body size in *D. melanogaster* is positively associated with numerous fitness metrics, such as fecundity, fertilisation success, and attractiveness, all of which are related to the strength of sexual selection in populations (Bonduriansky, 2001; Byrne & Rice, 2006; Morimoto *et al.*, 2016). Similarly, our work shows that both daughters and sons of cross-inoculated parents are significantly smaller as adults compared with offspring of self-inoculated parents. As in *D. melanogaster*, body size in *Onthophagus* beetles is positively associated with fecundity, survival, and offspring quality in females (Hunt & Simmons, 2000, 2002), and fighting success in males (Moczek & Emlen, 2000). Thus, our findings lend additional support to the hypothesis that the maintenance of host-specific gut microbiota associations may be critical for many life-history traits, and conversely, that disruptions of these associations may affect population health and persistence. Future studies should aim to directly assess the impact of gut microbiota disruptions on *Onthophagus* population dynamics over time.

Diverse ecological factors may structure host-specific microbiota in Onthophagus

Dung constitutes a challenging diet for insects given the abundance of macromolecules that are hard to digest, such as cellulose and lignin, and the simultaneous absence of key nutrients such as essential amino acids (Muller, 1980). It is therefore conceivable that dung beetle hosts utilise specific microbiota in order to meet the challenges imposed by such a diet, and that over time such partnerships result in interactions and dependencies specific to a given host beetle species and microbiota. Another role for the microbiota in *Onthophagus* could be the production of antimicrobial compounds, as *Onthophagus* beetles develop in close proximity to diverse fungi, including entomophagic taxa such as *Metarhizium* (Chouvenc *et al.*, 2013; Estes *et al.*, 2013; Rosengaus *et al.*, 2013; Shukla *et al.*, 2016). Recent work showed that experimental inoculation with maternal microbiota significantly reduces mortality in *Onthophagus* dung beetles following standardised exposure to *Metarhizium* spores compared with inoculation with random soil microbes or phosphate-buffered saline alone (Schwab *et al.*, Unpublished). This raises the possibility that host species also associate with microbiota to aid in defence against fungal attacks, providing additional pressure to maintain associations between beetle hosts and their microbial partners.

Other research has suggested that most species acquire their microbiota not through strict vertical or horizontal transmission, but through some combination of the two (Shapira, 2016; Moran & Sloan, 2015). Our findings suggest that this is probably also the case for the two dung beetle species examined here. Cross-inoculated F_1 *O. gazella* beetles survived to adulthood at higher rates than cross-inoculated *O. sagittarius* beetles, but in neither case did the loss of these specific microbial communities lead to the complete mortality of a host line. This suggests both species were able to assemble functionally compensatory microbial communities in the absence of their vertically transmitted

microbiota, although *O. sagittarius* appeared less able to do so for reasons currently unclear to us. We speculate that this may be due to a greater reliance on strict vertical transmission of the microbiota in this species. The observation of compensation in the absence of normally vertically transmitted microbiota is not surprising; even extremely tight host−symbiont associations, such as between aphids and *Buchnera aphidicola*, can break down and be successfully replaced by newly acquired symbionts (Chong & Moran, 2018). Future research could leverage the *Onthophagus* system to explore the drivers of microbial community assembly by examining the extent to which different *Onthophagus* species rely on vertical or horizontal transmission, or varying combinations thereof, and under what conditions transmission routes may change.

Onthophagus beetles as a model system for the study of the evolutionary ecology of symbiosis

The hologenome theory of evolution suggests that selection acting on a host and its associated microbiota together (a holobiont) should lead to phylosymbiosis, or a microbiota community assembly concordant with host phylogenetic distance (Zilber-Rosenberg & Rosenberg, 2008; Theis *et al.*, 2016). This, in turn, predicts that increasing host phylogenetic distance will correlate with increasing levels of host−microbiota incompatibility (Brooks *et al.*, 2016). In this study we have shown that two *Onthophagus* species whose last common ancestor existed approximately 37 Ma exhibit measurable adverse fitness and developmental effects when subjected to cross-inoculation. Future work could leverage the number of experimentally accessible species across both greater and narrower phylogenetic distances (Emlen *et al.*, 2005) to test this prediction in a comparative fashion across multiple clade members.

At the same time, the history of accidental and deliberate introductions seen in numerous *Onthophagus* species, coupled with the experimental tractability of this genus, offers exciting opportunities to test if and how hosts, their microbiota, and the interactions between them evolve in novel environments (Edwards, 2007; Silva *et al.*, 2016). Studies examining the role of host−symbiont interactions in potentially structuring range expansions into novel, challenging, habitats are particularly well suited for study within this system. Current work is investigating if and how *Onthophagus* gut microbiota change when host populations invade novel environments, knowledge that will further our understanding of the potential role of gut microbiota in shaping the ecological radiation of *Onthophagus* species and populations.

Acknowledgments

Author contributions

References

Ahlmann-Eltze, C. (2017) *ggsignif: Significance Brackets for 'ggplot2'*. R package version 0.4.0.

Angus, R.B. (2008) A chromosomal analysis of the *Onthophagus similis-opacicollis-fracticornis* species group (Coleoptera: Scarabaeidae). *Tijdschrift voor Entomologie*, **151**, 235−244.

Auguie, B. (2016) *gridExtra: Miscellaneous Functions for "Grid" Graphics*. R package version 2.2.1.

Bonduriansky, R. (2001) The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biological Reviews*, **76**, 305−339.

Breeschoten, T., Doorenweerd, C., Tarasov, S. & Vogler, A.P. (2016) Phylogenetics and biogeography of the dung beetle genus *Onthophagus* inferred from mitochondrial genomes. *Molecular Phylogenetics and Evolution*, **105**, 86−95.

Breheny, P. & Burchett, W. (2017) Visualization of regression models using visreg. *The R Journal*, **9**, 56−71.

Brooks, A.W., Kohl, K.D., Brucker, R.M., van Opstal, E.J. & Bordenstein, S.R. (2016) Phylosymbiosis: relationships and functional effects of microbial communities across host evolutionary history. *PLoS Biology*, **14**, e2000225.

Byrne, P.G. & Rice, W.R. (2006) Evidence for adaptive male mate choice in the fruit fly *Drosophila melanogaster*. *Proceedings of the Royal Society of London B: Biological Sciences*, **273**, 917−922.

Chong, R.A. & Moran, N.A. (2018) Evolutionary loss and replacement of *Buchnera*, the obligate endosymbiont of aphids. *ISME Journal*, **12**, 898−908.

Chouvenc, T., Efstathion, C.A., Elliott, M.L. & Su, N.Y. (2013) Extended disease resistance emerging from the faecal nest of a subterranean termite. *Proceedings of the Royal Society of London B: Biological Sciences*, **280**, 20131885.

Davis, A.J. & Sutton, S.L. (1997) A dung beetle that feeds on fig: implications for the measurement of species rarity. *Journal of Tropical Ecology*, **13**, 759−766.

Douglas, A.E. (2009) The microbial dimension in insect nutritional ecology. *Functional Ecology*, **23**, 38−47. http://doi.org/10.1111/j .1365-2435.2008.01442.x.

Edwards, P. & Landcare Australia (2007) *Introduced dung beetles in Australia 1967−2007: current status and future directions*. Landcare Australia, Maleny, Australia.

Emelianoff, V., Chapuis, E., le Brun, N., Chiral, M., Moulia, C. & Ferdy, J.B. (2008) A survival-reproduction trade-off in entomopathogenic nematodes mediated by their bacterial symbionts. *Evolution*, **62**, 932−942.

Emlen, D.J., Marangelo, J., Ball, B. & Cunningham, C.W. (2005) Diversity in the weapons of sexual selection: horn evolution in the beetle genus *Onthophagus* (coleoptera: Scarabaeidae). *Evolution*, **59**, 1060–1084.

Estes, A.M., Hearn, D.J., Snell-Rood, E.C., Feindler, M., Feeser, K., Abebe, T. *et al.* (2013) Brood ball-mediated transmission of microbiome members in the dung beetle, *Onthophagus taurus* (Coleoptera: Scarabaeidae). *PLoS One*, **8**, 1–15.

Estrada, A., Anzures, D. & Coates-Estrada, R. (1999) Tropical rain forest fragmentation, howler monkeys (*Alouatta palliata*), and dung beetles at Los Tuxtlas, Mexico. *American Journal of Primatology*, **48**, 253–262.

Fox, J., Weisberg, S., Adler, D., Bates, D., Baud-Bovy, G., Ellison, S. *et al.* (2012) *Package 'car'*. R Foundation for Statistical Computing, Vienna, Austria.

Gilbert, S.F., Sapp, J. & Tauber, A.I. (2012) A symbiotic view of life: we have never been individuals. *The Quarterly Review of Biology*, **87**, 325–341.

Gilbert, S.F., Bosch, T.C.G. & Ledón-Rettig, C. (2015) Eco-Evo-Devo: developmental symbiosis and developmental plasticity as evolutionary agents. *Nature Reviews Genetics*, **16**, 611–622.

Grömping, U. (2006) Relative importance for linear regression in R: the package relaimpo. *Journal of Statistical Software*, **17**. [WWW document]. URL http://www.jstatsoft.org/.

Hadfield, M.G. (2011) Biofilms and marine invertebrate larvae: what bacteria produce that larvae use to choose settlement sites. *Annual Review of Marine Science*, **3**, 453–470.

Hooper, L.V. & Gordon, J.I. (2001) Commensal host-bacterial relationships in the gut. *Science*, **292**, 1115–1118.

House, C.M., Simmons, L.W., Kotiaho, J.S., Tomkins, J.L., & Hunt, J. (2011) Sex ratio bias in the dung beetle Onthophagus taurus: adaptive allocation or sex-specific offspring mortality? *Evolutionary ecology*, **25**, 363–372.

Hunt, J. & Simmons, L.W. (2000) Maternal and paternal effects on offspring phenotype in the dung beetle *Onthophagus taurus*. *Evolution*, **54**, 936–941.

Hunt, J. & Simmons, L.W. (2002) The genetics of maternal care: direct and indirect genetic effects on phenotype in the dung beetle *Onthophagus taurus*. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 6828–6832.

Koga, R., Meng, X.Y., Tsuchida, T. & Fukatsu, T. (2012) Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. *Proceedings of the National Academy of Sciences of the United States of America*, **109**, E1230–E1237.

Landmann, F., Foster, J.M., Michalski, M.L., Slatko, B.E. & Sullivan, W. (2014) Co-evolution between an endosymbiont and its nematode host: *Wolbachia* asymmetric posterior localization and AP polarity establishment. *PLoS Neglected Tropical Diseases*, **8**, e3096.

Leftwich, P.T., Clarke, N.V.E., Hutchings, M.I. & Chapman, T. (2017) Gut microbiomes and reproductive isolation in drosophila. *Proceedings of the National Academy of Sciences of the United States of America*, **114**, 12767–12772.

Leonardo, T.E. & Mondor, E.B. (2006) Symbiont modifies host life-history traits that affect gene flow. *Proceedings of the Royal Society of London B: Biological Sciences*, **273**, 1079–1084.

Macagno, A.L., Pizzo, A., Rolando, A. & Palestrini, C. (2011) Size and shape interspecific divergence patterns partly reflect phylogeny in an *Onthophagus* species-complex (Coleoptera: Scarabaeidae). *Zoological Journal of the Linnean Society*, **162**, 482–498.

Macagno, A.L., Zattara, E.E., Ezeakudo, O., Moczek, A.P. & Ledón-Rettig, C.C. (2018) Adaptive maternal behavioral plasticity and developmental programming mitigate the transgenerational effects of temperature in dung beetles. *Oikos*, **127**, 1319–1329.

Matthews, E.G. (1971) A revision of the scarabaeine dung beetles of Australia. I. Tribe Onthophagini. *Australian Journal of Zoology Supplementary Series*, **19**, 3–330.

McFall-Ngai, M.J. (2014) The importance of microbes in animal development: lessons from the squid-vibrio symbiosis. *Annual Review of Microbiology*, **68**, 177–194.

McFall-Ngai, M., Hadfield, M.G., Bosch, T.C., Carey, H.V., Domazet-Lošo, T., Douglas, A.E. *et al.* (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 3229–3236.

Moczek, A.P. (2006) Pupal remodeling and the development and evolution of sexual dimorphism in horned beetles. *The American Naturalist*, **168**, 711–729.

Moczek, A.P. & Emlen, D.J. (2000) Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: do alternative reproductive tactics favour alternative phenotypes? *Animal Behaviour*, **59**, 459–466.

Moran, N.A. & Sloan, D.B. (2015) The hologenome concept: helpful or hollow? *PLoS Biology*, **13**, 1–10.

Morimoto, J., Pizzari, T. & Wigby, S. (2016) Developmental environment effects on sexual selection in male and female *Drosophila melanogaster*. *PLoS One*, **11**, e0154468.

Morimoto, J., Simpson, S.J. & Ponton, F. (2017) Direct and trans-generational effects of male and female gut microbiota in *Drosophila melanogaster*. *Biology Letters*, **13**, 20160966.

Muller, Z. O. (1980) *Feed from Animal Wastes: State of Knowledge*. FAO Animal Production and Health Paper, 18. Rome, Food and Agriculture Organization of the United Nations, Rome, Italy.

Pizzo, A., Roggero, A., Palestrini, C., Cervella, P., Del Pero, M. & Rolando, A. (2006) Genetic and morphological differentiation patterns between sister species: the case of *Onthophagus taurus* and *Onthophagus illyricus* (Coleoptera, Scarabaeidae). *Biological Journal of the Linnean Society*, **89**, 197–211.

Rawls, J.F., Samuel, B.S. & Gordon, J.I. (2004) Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 4596–4601.

RCore Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Rosengaus, R.B., Mead, K., Du Comb, W.S., Benson, R.W. & Godoy, V.G. (2013) Nest sanitation through defecation: antifungal properties of wood cockroach feces. *Naturwissenschaften*, **100**, 1051–1059.

RStudio Team (2015) *RStudio: Integrated Development for R*. RStudio, Inc., Boston, Massachusetts [WWW document]. URL http://www.rstudio.com [accessed on 26 April 2018].

Salem, H., Florez, L., Gerardo, N. & Kaltenpoth, M. (2015) An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. *Proceedings of the Royal Society of London B: Biological Sciences*, **282**, 20142957.

Schwab, D.B., Riggs, H.E., Newton, I.L.G. & Moczek, A.P. (2016) Developmental and ecological benefits of the maternally transmitted microbiota in a dung beetle. *American Naturalist*, **188**, 679–692.

Schwab, D.B., Casasa, S. & Moczek, A.P. (2017) Evidence of developmental niche construction in dung beetles: effects on growth, scaling and reproductive success. *Ecology Letters*, **20**, 1353–1363.

Shapira, M. (2016) Gut microbiotas and host evolution: scaling up symbiosis. *Trends in Ecology and Evolution*, **31**, 539–549.

Shafiei, M., Moczek, A.P. & Nijhout, H.F. (2001) Food availability controls the onset of metamorphosis in the dung beetle Onthophagus taurus (Coleoptera: Scarabaeidae). *Physiological Entomology*, **26**, 173–180.

Sharon, G., Segal, D., Ringo, J.M., Hefetz, A., Zilber-Rosenberg, I. & Rosenberg, E. (2010) Commensal bacteria play a role in mating

preference of *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 4852.

Shikuma, N.J., Pilhofer, M., Weiss, G.L., Hadfield, M.G., Jensen, G.J. & Newman, D.K. (2014) Marine tubeworm metamorphosis induced by arrays of bacterial phage tail-like structures. *Science*, **343**, 529–533.

Shukla, S.P., Sanders, J.G., Byrne, M.J. & Pierce, N.E. (2016) Gut microbiota of dung beetles correspond to dietary specializations of adults and larvae. *Molecular ecology*, **25**, 6092–6106.

Silva, D.P., Vilela, B., Buzatto, B.A., Moczek, A.P. & Hortal, J. (2016) Contextualized niche shifts upon independent invasions by the dung beetle *Onthophagus taurus*. *Biological Invasions*, **18**, 3137–3148.

Stappenbeck, T.S., Hooper, L.V. & Gordon, J.I. (2002) Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 15451–15455.

Tarasov, S.I. & Kabakov, O.N. (2010) Two new species of *Onthophagus* (Coleoptera: Scarabaeidae) from Indochina, with a discussion of some problems with the classification of *Serrophorus* and similar subgenera. *Zootaxa*, **2344**, 17–28.

Theis, K.R., Dheilly, N.M.M., Klassen, J.L., Brucker, R.M., Baines, J.F., Bosch, T.C.G. *et al.* (2016) Getting the hologenome concept right: an eco-evolutionary framework for hosts and their microbiomes. *Msystems*, **1**, e00028–16.

Watanabe, K., Yukuhiro, F., Matsuura, Y., Fukatsu, T. & Noda, H. (2014) Intrasperm vertical symbiont transmission. *Proceedings of the National Academy of Sciences of the United States of America*, **111**, 7433–7437.

Wickham, H. (2016) *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag, New York, New York.

Zilber-Rosenberg, I. & Rosenberg, E. (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiology Reviews*, **32**, 723–735.