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Phenotypic integration in an extended phenotype: among-individual variation in nest-building traits of the alfalfa leafcutting bee (*Megachile rotundata*)

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Abstract

Structures such as nests and burrows are an essential component of many organisms' life-cycle and requires a complex sequence of behaviors. Because behaviors can vary consistently among individuals and be correlated with one another, we hypothesized that these structures would 1) show evidence of among-individual variation, 2) be organized into distinct functional modules, and 3) show evidence of trade-offs among functional modules due to limits on energy budgets. We tested these hypotheses using the alfalfa leafcutting bee, *Megachile rotundata*, a solitary bee and important crop pollinator. *M. rotundata* constructs complex nests by gathering leaf materials to form a linear series of cells in pre-existing cavities. In this study, we examined variation in the following nest construction traits: reproduction (number of cells per nest and nest length), nest protection (cap length and number of leaves per cap), cell construction (cell size and number of leaves per cell), and cell provisioning (cell mass) from 60 nests. We found a general decline in investment in cell construction and provisioning with each new cell built. In addition, we found evidence for both repeatability and plasticity in cell provisioning with little evidence for trade-offs among traits. Instead, most traits were positively, albeit weakly, correlated ($r \sim 0.15$), and traits were loosely organized into covarying modules. Our results show that individual differences in nest

construction are detectable at a level similar to that of other behavioral traits and that these traits are only weakly integrated. This suggests that nest components are capable of independent evolutionary trajectories.

Keywords:

Animal personality; behavioral syndromes; extended phenotype; nesting behavior; *Megachile rotundata;* leafcutting bee, phenotypic integration

Introduction

Many animals build structures that are a vital component of their life-cycle and reproductive fitness. These animal structures serve a variety of functions including protection from predators, thermoregulation of microenvironment, reproduction and courtship, and traps for prey. Animal structures are examples of extended phenotypes wherein an individual's genes modify and control its environment (Dawkins, 1978, Dawkins, 1982). Animal structures are often composed of functionally different units varying from fairly simple to extremely intricate. For example, many spider webs are composed of non-sticky silk threads that provide support for the structure, and sticky threads that facilitate prey capture (Foelix, 2011). Deer mice burrows are composed of an entrance, main chamber, and an escape tunnel– each serving a different function (i.e. access to the structure, shelter and thermoregulation, and protection from predator intrusion, respectively) (Sumner & Karol, 1929, Wolfe & Esher, 1977). Similarly, the nests of social insect colonies are composed of various chambers used for food storage or brood care connected by an intricate network of tunnels (Tschinkel, 2004, Moreira et al., 2004). Building these nests requires a corresponding suite of complex behaviors, and variation in the structure's shape can in turn influence behavioral expression (Pinter-Wollman, 2015, Montiglio & DiRienzo, 2016). As the individual functional components that make up animal structures all contribute to the animal's

fitness (Bult & Lynch, 1997), we expect the structures to coevolve with behaviors and to show some degree of integration among functional components.

While most of the research on phenotypic integration has been investigated within the context of morphological traits (Wagner & Altenberg, 1996, Mitteroecker & Bookstein, 2007 Wagner et al. 2007), the topic of *extended* phenotypic integration has received surprisingly little attention. For many organisms, the question of whether functionally-distinct components of animal structures are tightly integrated, or, alternatively, organized into independent modules remains unexplored. The degree of integration (or modularity) can shed light on the rate of coevolution among these individual units and their associated behaviors. When integration is strong, selection on a functional component will carry over to all related structural and behavioral traits. Thus, strong correlations among components can impose constraints on the evolutionary trajectories of animal structures, especially if multiple traits are controlled by the same genes (Dochtermann & Dingemanse, 2013). Furthermore, strong integration means that certain combinations of traits will be less common, and populations may take longer to reach a fitness optimum. Alternatively, traits may be arranged into distinct functional modules with each trait within a module being highly correlated to other traits in the module, and different modules being relatively independent. This configuration, by contrast, would allow functional components of animal structures to evolve independently (West-Eberhard, 2003, Blows & Hoffmann, 2005, Araya-Ajoy & Dingemanse, 2014). In general, integration among traits is expected because animal structures are energetically costly, and animals have limited resources and energy budgets. These factors should lead to trade-offs among different functional components. For example, under limited prey densities, black widows will invest more into the capture components of the web than into the structural or protective components (Blackledge & Zevenbergen, 2007).

An important outstanding question is how much of the variation in these animal structures is due to genetic *vs.* environmental influences. Individual differences in behavior are extremely common in nature (Sih et al., 2004a, Sih et al., 2004b, Réale et al., 2007); for example, some individuals consistently take more risks or more thoroughly explore their environment than other individuals. Because animal structures are intimately linked to the behaviors that generate them, one would expect to observe similar amounts of individual variation in the constructions themselves as in the specific behaviors. Alternatively, if animal structural components are under intense selection due to their influence on fitness, the genetic variation underpinning behavioral variation may be reduced over time. In this case, variation in animal structures would be mostly generated through environmental variation and behavioral plasticity.

To date there have been relatively few studies that have investigated patterns of among-individual variation and trait integration in animal structures. From the few studies that have investigated this topic, there is some evidence that among-individual differences in constructions, particularly nests, do exist. For example, in Western black widows (*Latrodectus hesperus*), web components showed repeatable differences among individuals ranging from 0.35 to 0.52, and accounted for ~ 20% of the variation in prey aggression and boldness (DiRienzo & Montiglio, 2016, Montiglio & DiRienzo, 2016). Similar amounts of among-individual differences have also been found in the nest-building behaviors of the male three-spined sticklebacks (Rushbrook et al., 2008) and Southern masked weavers (Walsh et al., 2011). However, these examples represent only a small fraction of animal constructions found in nature, the majority of which remain relatively unexplored.

Burrowing rodents provide perhaps the most comprehensive body of studies on the genetic architecture of an extended phenotype. The repeatability of burrowing length in deer mice (genus *Peromyscus*) has been shown to vary between 0.16 and 0.54 depending on the species considered. Interestingly, species with more complex burrowing architecture had greater among-individual differences in burrowing length compared to species with more simple burrowing behaviors (Weber

& Hoekstra, 2009). Further analyses have shown that burrowing architecture is highly modular, with entrance and escape tunnel being genetically uncoupled, and tunnel length being a polygenic trait (Weber et al., 2013). In domestic mice (*Mus musculus*), nest building has been shown to be moderately heritable ($h^2 \sim 0.2$; Lee, 1973, Lynch, 1994), and exhibits characteristics of a trait under intense selection with multiple genes each having a small effect on the resulting phenotype (Sauce et al., 2013). Taken together, these results suggest that a signal for individual differences in animal structure is indeed representative of underlying genetic variation and that modularity between different functional units of an extended phenotype are to be expected, at least in rodents. More data from different clades is needed to determine the generality of this pattern.

In the present study, we examined among-individual variation and modularity in nest architectural features of a cavity nesting bee: the alfalfa leafcutting bee (*Megachile rotundata*). This species is an excellent study organism for examining the integration among components of an extended phenotype because nests are composed of architectural features (hereafter referred to as "traits") with distinct functions. Females construct their nests in long, narrow cavities into which they build a series of cylindrical-shaped cells made from leaf clippings (Fig. 1) (Stephen, 1962, Pitts-Singer & Cane, 2011). Within each cell, the female provides pollen and nectar for a single egg (Stephen, 1962, Pitts-Singer & Cane, 2011). After building a series of cells, the cavity is capped by a plug made from a variable number of leaf clippings, which may help to regulate internal nest temperature and light and protect against predators and parasites (Richards, 1984). As such, traits associated with the nest cap (e.g. number of leaves per cap) may comprise one distinct module, traits measured at the cell level (e.g. cell mass or size) may be organized into a second module, and traits measured at the nest level (e.g. number of cells per nest) organized into a third distinct module. The specific ways in which each of these components are integrated or organized into functional modules has yet to be empirically tested in this species. Finally, as these bees build cells sequentially within the nest, we can examine how cell construction and provisioning change over time. Here, we aim to answer the following questions: (1) How much of the phenotypic variation in

nest architecture is due to among vs. within-individual variation? (2) Is there evidence for plasticity in cell provisioning strategies over an individual's nest-building period? (3) How strongly are nest traits integrated together? and (4) Is there evidence for distinct modules consisting of different nest functional components (i.e. cell-level traits, nest-level traits, and cap traits), and (5) are there trade-offs between these modules?

Methods

Bee nest collection

We collected bee nests during June and July 2016 from an alfalfa field located near Fargo, North Dakota (46°55'02.9"N 96°50'43.6"W). Alfalfa leafcutting bees, initially obtained from JWM Leafcutters, Inc. (Nampa, ID) in the early spring of 2016, were stored as overwintering prepupae in a 6º C environmental chamber to maintain quiescence throughout late winter and early spring 2016. At four different time points in May and June 2016, groups of approximately 600-2,000 prepupa were removed from cold storage and allowed to develop to adults in a fluctuating thermal regime to minimize cold storage damage (see Rinehart et al., 2013, 2016). Upon emergence, these adult bees were released at the field site on 13, 20, and 30 June and on 16 July 2016. We only released bees after no nesting activity had been observed at the nesting block for at least two consecutive days. In the field where the bees were released, we placed a 90 cm wide $\times$ 60 cm high $\times$ 7.8 cm deep polystyrene nesting block (Beaver Plastics of Acheson, Alberta) composed of 3200 nest holes. Each nest hole measured 6 mm in diameter and contained a 7.5 mm long paper straw for easy nest removal. This nest box was placed inside a 3-sided wooden shelter, with the open side covered with wire fencing to protect the nest from rain, wind, and predators. The wooden shelter was elevated 30 cm off the ground and placed facing southeast following standard practices (Stephen, 1962, Pitts-Singer & Cane, 2011).

After releasing the bees, the nest box was monitored for signs of female nesting, and completed nests were collected daily. Nests were considered to be complete when females sealed off the outside of the nest with a cap made from leaf clippings. We began nest collection on 30 June 2016, shortly after the first release of bees, and completed nest collection on July 23rd 2016, when little to no activity had been observed around the nest box for multiple consecutive days. To analyze among-individual variation in nest construction, we needed to be sure that individual nests belonged to different females. As females typically do not lay more than one egg per day under field conditions (Klostermeyer et al., 1973, Pitts-Singer & Cane, 2011), they are therefore unlikely to complete more than one nest consisting of an average of 6 cells in a one-week period. We therefore made the assumption that all nests initiated and completed in a one-week period belonged to different females.

Nest Dissection

All 60 collected nests were stored at room temperature (22°C) prior to dissection and were left inside their straw casing to prevent desiccation of leaf and cell provision materials. Nest dissections generally occurred within 3-4 days after collection and no nest was dissected more than 7 days post collection. We removed nests from their paper straw casings and collected the following data for each nest: nest length (mm) and the number of cells per nest (nest-level traits), cap length (mm) and the number of individual leaf clippings per cap (cap-level traits), and cell length (mm), cell mass (mg), and the number of individual leaf clippings per cell (cell-level traits). We examined the number of leaf clippings per cap and per cell as an indication of the relative effort put into nest protection and cell construction, respectively. We measured cell mass as an indication of the relative effort put into nest provisioning, as nectar and pollen provisions comprise the majority of the cell mass (Klostermeyer et al., 1973) and have significant effects on offspring size (Owen & McCorquodale, 1994, Klostermeyer et al., 1973). We measured nest, cap, and cell lengths to the nearest mm and weighed each cell to the nearest 0.001 mg using a microbalance (UMT2, Mettler Toledo, Columbus, OH).

Statistical Analysis

All analyses were performed using R version 3.4.1 (R Core Team). We used the package lme4 (Bates et al., 2014) for univariate mixed models and MCMCglmm (Hadfield, 2010) for Bayesian multivariate mixed models. The phenotype network was assessed using the qgraph and assortnet packages (Epskamp et al., 2012, Farine, 2014). All response variables were expressed as standard deviation units to facilitate model convergence, and some response variables (number of leaves per cell and number of leaves per cap) were square root transformed to match the assumptions of data normality. Additionally, the number of cells per nest was treated as a Poisson-distributed response variable, since square-root transformation did not achieve normality.

Testing for variation in nest traits at the population, among-individual, and within-individual levels

To partition nest variation into among and within-individual components, we used a reaction norm approach with random regressions (Dingemanse et al., 2010). This allowed us to quantify the amount of variation in cell construction within a nest as a function of cell position in the nest. This was achieved using univariate mixed models where all traits varying at the cell level (i.e. cell mass, cell length, and the number of leaves per cell) were included as response variables. We included cell position in the nest and collection date (both centered around the population average) as fixed effects. Nest identity and cell position within the nest were fitted as random intercepts and random slopes respectively. Cell position was coded such that cell number 1 corresponded to the first cell being built (the one furthest from the nest cap).This approach allowed us to partition the phenotypic variance in cell-level traits into the following components:

- The amount of variation due to population-level trends for the effects of cell position and collection date on cell-level traits ($V_{Fixed\ effects}$)
- The among-nest variance (V_{Nests}), which represents how average cell-level traits for each nest differ from the population intercept for that trait

- The slope variation among nests (V_{Slopes}) for the effect of cell position on cell-level traits, representing how much individual nests vary from the overall population slope for the effect of cell position
- The residual – or within-nest variance ($V_{Residuals}$) represents variation in cell-level traits from cell to cell within individual nests or due to measurement error
- The slope-intercept correlation ($r_{Slopes \times Nests}$), which indicates whether nests with higher than average trait values tend to have larger (positive correlation) or smaller (negative correlation) random slope values

From these parameters, we estimated trait repeatability (τ), calculated as:

$$(1)\ \tau = \frac{V_{Nests}}{\left(V_{Nests} + V_{Slopes} + V_{Residuals}\right)}$$

We also calculated the proportion of the variance explained (R^2) by each variance component and fixed effects following (Nakagawa & Schielzeth, 2013). We assessed significance based on likelihood ratio tests for fixed effects, and calculated 95 % confidence intervals using likelihood profile for each random effect. We also tested the significance of the random slope and slope-intercept correlation terms by comparing our model with models where these parameters were set to 0 using likelihood ratio tests.

Correlations among nest traits

When repeated measures are taken on multiple traits, the correlation between two phenotypic traits (hereafter phenotypic correlation) may be split into among and within-individual (or error) components. A strong between-individual correlation indicates correlation among individual mean trait values, while within-individual correlations represent correlated changes attributable to phenotypic plasticity or error correlation (Dingemanse et al., 2012, Dingemanse & Dochtermann, 2013).

We used a series of bivariate mixed models to estimate the strength of phenotypic, among and within-nest (or residual) correlations for all nest traits. For traits measured at the cell level (cell mass, number of cell leaves and cell length) for which multiple measurements per nest were obtained, we specified three bivariate models with cell position and collection date as fixed effects and nest identity as random effects. From these models, we obtained estimates for the among and within-nest correlations as well as the phenotypic correlations after controlling for fixed effects calculated according to Dingemanse and Dochtermann (2013) as:

$$(2)\ r_{P(x,y)} = r_{Nest(x,y)} \times \sqrt{\tau_x \times \tau_y} + r_{WN(x,y)} \times \sqrt{(1-\tau_x) \times (1-\tau_y)}$$

Where $r_{P(x,y)}$ is the phenotypic correlation between trait x and y, $r_{Nest(x,y)}$ and $r_{WN(x,y)}$ are the among and within-nest correlations between x and y; and τ_x and τ_y represents the repeatability of x and y respectively. For trait combinations where one trait was measured only once per nest (e.g. traits measured at the nest level including nest and cap length, number of leaves per cap, and number of cells per nest) and the other trait had multiple measurements per nest (e.g. cell-level traits), the within-nest correlation was not estimable. We therefore specified twelve bivariate models as above but fixed the within-nest correlation to 0. The phenotypic correlation was calculated using equation (2) by weighting the among-nest correlation by the repeatability of the cell-level trait. Finally, for trait combinations where only one measurement per nest was obtained, we specified six bivariate models as above but fixed the among-nest correlation at 0, such that the within-nest correlation corresponded to the phenotypic correlation. We used a Bayesian implementation for all these models and specified Markov Chain Monte Carlo (MCMC) chains with 1.3×10^6 iterations, 300,000 burn-in period, a thinning interval of 1,000, and used a prior that was flat for trait covariances. All chains had appropriate convergence based on visual inspection and low autocorrelation (< 0.1). We used 95 % credibility intervals and posterior modes for the correlation estimates to assess the precision and magnitude of these correlations. To assess the significance of these correlations, we reported the probability that a given correlation excluded 0 (hereafter:

Pmcmc), calculated as the proportion of posterior estimates excluding 0.

Testing for distinct nest-building modules

Given that we had little *a priori* expectations of how nest-building traits would be correlated, we used a phenotype network approach to test the degree of phenotypic integration among traits and whether these traits were organized into distinct modules (Wilkins et al. 2015), rather than test the fit of specific models of modularity as is common in behavioral syndrome studies (Araya-Ajoy & Dingemanse, 2014, Royauté et al., 2015). We therefore calculated the following network metrics: the average correlation strength: $|\overline{r_P}|$ – a measure of overall strength of the phenotypic integration varying between 0 and 1 – calculated as the mean of the absolute value of all pairwise correlations; the network's density *d*, which corresponds to the proportion of significant phenotypic correlations and indicates whether the phenotype network is highly redundant (high *d* value means that all traits are strongly correlated to all other traits); and the network's assortativity r_d, which indicates the degree to which traits are organized into modules. This last metric compares the strength of correlations within and among modules. Modular networks have assortativity values closer to 1, indicating that correlations are stronger within modules than among modules. In order to calculate network assortativity, we classified nest traits into three distinct categories: cell-level traits (cell mass, number of cell leaves, and cell length), cap-level traits (cap length and number of cap leaves) and nest-level traits (nest length and number of cells per nest). We then calculated the observed weighted coefficient of assortativity, which we compared to the expected coefficient based on randomly generated networks by permuting nodes across modules for 1,000 iterations (Wilkins et al., 2015). Finally, in order to determine whether certain traits had a disproportionate influence on the phenotype network, we calculated a trait's average correlation strength $|\bar{r}|$ (the average of all pairwise correlations for a given trait) its connectivity degree (the number of significant correlations associated with a given trait) and betweenness centrality (the number of shortest paths going through a given trait).

Results

Population trends

On average, bees built nests composed of six cells, measuring 50 - 60 mm in total length, and with a cap representing about 17 % of the total nest length (Table S1). We found a general declining trend in cell investment as the position of the cell in the nest sequence increased. This trend was most pronounced for cell mass (estimate ± SE; β = -0.41 ± 0.06, $\chi^2_{df:1}$= 37.51, P < 0.001) and cell length (β = -0.32 ± 0.06, $\chi^2_{df:1}$= 24.23, P < 0.001), while the number of leaves per cell also showed a negative, but not statistically significant relationship with position in the sequence (β = -0.11 ± 0.06, $\chi^2_{df:1}$= 24.23, P = 0.06) (Table 1, Fig. 2). It is possible that this pattern of cell provisioning may be confounded with offspring sex since females are often larger and laid earlier in the cell sequence (Stephen & Osgood, 1965, Klostermeyer et al., 1973). While we were not able to obtain information on offspring sex for the primary data set, a subsequent collection of 29 nests for which offspring sex was determined revealed a similar pattern of larval mass decline with cell position in the nest (Supplementary Materials – Appendix S1, Table S2), but with no evidence for a sex × cell position interaction ($\chi^2_{df:1}$= 0.44, P = 0.5), (Fig. S1). This supplemental data suggests that the decline in cell mass over time (i.e. with cell position) is unrelated to trends in offspring sex over time.

Trait repeatability and variation at the among and within-individual levels

The repeatability of cell construction traits was relatively weak (cell length and the number of leaves per cell, τ < 0.15), while the repeatability of cell provisioning (cell mass) was moderate (estimate ± CI; τ = 0.33 ± [0.18; 0.50]). We also found evidence of significant variation in nest reaction norms (i.e. random slopes variance) for both cell mass (R^2 = 0.11, $\chi^2_{df:2}$= 25.06, P < 0.00001) and cell length (R^2 = 0.09, $\chi^2_{df:2}$= 14.09, P < 0.001), but not for the number of leaves per cell (P = 0.20). In addition, we found evidence of a negative slope-intercept correlation for cell length (*r* = -0.63 ± [-1.00; -0.21], $\chi^2_{df:1}$= 5.03, P < 0.05), but not for cell mass or nest length (P > 0.05). This indicates that there is substantial variation in cell construction and cell provisioning strategies

among individuals, and that nests that are built with larger cells on average tend to experience a larger decline in size with each new cell constructed (Table 1, Fig. 2c). Perhaps surprisingly, the population trends for cell position and collection date accounted for less than 20 % of the observed phenotypic variation in construction and provisioning traits, with most of the variation instead observed at the within-nest level ($0.45 < R^2 < 0.81$) (Table 2, Fig. 3). This implies that unobserved sources of variation (e.g. temporal variation in weather or floral resources) were responsible for the majority of the variation in cell construction and provisioning.

Phenotype network structure and correlations among nest traits

We found weak to moderate patterns of trait correlations at the among and within-individual levels. At the among-individual level, cell length and the number of leaves per cell were significantly negatively correlated (r_{Nests} = -0.34, Pmcmc = 0.95), indicating that larger cells were constructed with fewer leaves. This correlation was also significant at the phenotypic level (r_P = -0.21, Pmcmc = 0.99) with little contribution from the within-nest correlation (r_{WN} = -0.15, Pmcmc = 0.97) (Table 3), suggesting that most of the correlation between cell length and the number of leaves per cell is underpinned by its among-individual correlation. At the within-individual level, cell mass and cell length were positively correlated (r_{WN} = 0.31, Pmcmc = 1.00), indicating that for a given nest, cells that were built larger than the nest average also tended to be heavier than the nest average (Table 3b).

At the phenotypic level, notable correlations included positive correlations among nest length, cap length, cell mass, and the number of leaves per cap (r_P > 0.20); between the number of cells per nest and nest length (r_P = 0.50, Pmcmc = 1.00), and between the number of cap leaves and cap length (r_P = 0.80, Pmcmc = 1.00). We found little evidence for trade-offs among nest traits as evidenced by the general lack of significant negative correlations, with the exception of the cell length × number of leaves per cell correlation mentioned above (Table 3).

The network analysis indicated that nest traits had a weak degree of integration (average correlation strength: $|\bar{r_P}| = 0.16$) and moderate redundancy (network density: d = 0.43) (Fig. 3). We also found that the degree of assortativity was significantly higher than expected compared to random networks, although the value of the assortativity coefficient was moderate (estimate ± SE; observed r_d = 0.43 ± 0.20, expected r_d = -0.22, P < 0.05). This indicates that cap, cell-level, and nest-level traits were only weakly integrated into modules. All traits were significantly correlated to at least one other trait (connectivity > 1) with nest length and cell mass showing highest connectivity and nest length having the highest betweenness. Nest length, along with cap length and cap leaves, also had the highest average correlation strength ($|\bar{r}| > 0.20$) and thus potentially had the most influence on the phenotype network (Table 4).

Discussion

We sought to investigate how phenotypic variation in nest architectural traits was structured at different levels, including the degree of within-individual and among-individual variation and how strongly nest architectural traits were integrated into functional modules. We found evidence for among-individual differences in nest architecture as well as among-individual plasticity in cell-provisioning traits (e.g., cell mass). When investigating how cell-, cap-, and nest-level traits were interrelated, we found a surprisingly weak signal for phenotypic integration, although the phenotype network was also characterized by a moderate level of redundancy and some modularity among cell-, cap-, and nest-level traits. Taken together, these results indicate that individual variation of certain components of an extended phenotype, bee nests, is on par with that observed in other behavioral traits. However, specific functional components of nest structure were only weakly integrated, suggesting the potential for independent evolutionary trajectories. We consider these results and their implications in greater depth below.

Variation in nest building

Our results give important insight into how nest construction and provisioning likely vary among female alfalfa leafcutting bees. There was a general tendency for females to build lighter cells composed of fewer leaves as nest construction progressed, regardless of offspring sex. This decline in investment in cell construction and provisioning could represent a type of "front-loading" strategy where reproductive investment is prioritized for the first few offspring. Alternatively, females could also have increasingly depleted energy budgets as nest construction progresses, leading to smaller investment in each new offspring. Females of other Megachilidae species decrease reproductive investment as they age, reducing the body-size and reproductive fitness of subsequent offspring (Sugiura & Maeta, 1989, Kim, 1997). However, in this study, we were only capable of collecting one nest per female while *M. rotundata* typically builds 2-3 nests over their lifetime. Therefore, a full investigation of reproductive trade-offs would require comparing investment in cell provisioning across multiple nests per female including early and late nests.

Although we found evidence for a decline in provisioning effort at the population level, we also found evidence for substantial amounts of individual variation and plasticity in some nest traits. Cell mass was repeatable at a level expected for behavioral traits ($\tau \sim 0.37$, Bell et al. 2009), meaning that some females consistently provision cells with more pollen and nectar, while other females consistently provision cells with less. There is also indication based on previous studies that the relative investment in pollen vs. nectar provisioning can change depending on the position of a cell within a nest (Klostermeyer et al., 1973). However, the degree of individual differences in these provisioning strategies and their consequences on offspring development remains to be investigated.

We also found evidence for plasticity in cell construction and provisioning suggesting that, although investment generally declined with each new cell built, some nests showed a faster decrease in investment than others (Fig. 2). In the case of variation in cell length, we also found evidence for a negative slope × intercept correlation, meaning that individual variation in cell length is greater for the first few cells constructed, but rapidly converges to similar values as nest construction progresses (Fig. 2c).

Most studies investigating variation in animal constructions have either focused on differences expressed at the species or population/colony level (Weber & Hoekstra, 2009, Pinter-Wollman, 2015) or have shown evidence of population-level plasticity in response to experimental manipulation (e.g. food availability influence on web building; Blackledge & Zevenbergen, 2007, Blamires, 2010). Relatively few studies have been able to partition such variation further into among- and within-individual (or among- and within-colony) components (but see Rushbrook et al., 2008, Walsh et al., 2011, DiRienzo & Montiglio, 2016, DiRienzo & Dornhaus, 2017), and therefore estimate the repeatability of traits involved in animal constructions. This is important because repeatability typically sets the upper bound for a trait's heritability, as among-individual variation in a phenotype arises in part through genetic variation (Boake, 1989, Dingemanse & Dochtermann, 2014, Dochtermann et al., 2015). That animal structures have repeatabilities similar to other behavioral traits suggests the presence of additive genetic variation (h^2 ~ 0.5 × repeatability; Dochtermann et al. 2015). The data on the repeatability *and* heritability of extended phenotypes remains sparse, with only a handful of studies having investigated these questions in detail (Sauce et al., 2013, Weber et al., 2013).

Overall, our results indicate that cell provisioning (as indicated by cell mass) varies among females and that individual females also show moderate amounts of plasticity in terms of adjusting their cell provisioning with each additional offspring. Individual variation in provisioning strategies has also been observed in nesting birds (Westneat et al., 2011). In contrast to our results however,

parents typically tended to increase provisioning effort as offspring age. The patterns observed in our data suggest potential trade-offs wherein females reduce their investment in each new offspring due to energy reserves or ageing. An important way forward will be to investigate whether these differences in nest provisioning strategies are indicators of individual trade-offs between current and future reproduction. This could be tested by comparing the slopes of early and late nests built by the same females when multiple nests per female can be collected and accurately identified.

Our results must be interpreted with caution due to a number of caveats. First, we did not attempt to track individual female behaviors in the field, as the few females that we could have accurately followed would have reduced the sample size too low to reliably estimate variance components. Instead, we released large numbers of bees and sampled individual nests as a proxy for individual females. Our estimation of among-individual variance therefore assumes that each collected nest was built by a unique female, which we believe is a fair assumption given that nests were collected immediately after they were completed. Females generally do not complete more than one nest in a one-week period (Klostermeyer et al., 1973, Pitts-Singer & Cane, 2011), and nest usurpation is estimated to be only 2% for *M. rotundata* (McCorquodale & Owen, 1994). Another important issue is that we were unable to obtain female body size prior to collecting nests. It is therefore possible that part of the variation in cell provisioning is attributable to larger females being able to provision their nests with more pollen and nectar. We hope that improvements in automatic tracking technology will allow us to reliably link a mother's phenotypic attributes (body size, foraging range) along with components of the nest and help link those components to offspring survival and performance in the field.

Patterns of integration among nest-building traits

When investigating patterns of phenotypic integration among nest traits, we found a surprising lack of trade-offs between nest traits as evidenced by the absence of negative correlations. Cell length × number of cell leaves was the only negative correlation identified at the

phenotypic level (r_P = -0.21). This correlation is not indicative of a trade-off, however, but rather simply shows that larger cells need fewer leaves to be constructed, presumably because large cells are composed of only a few large leaves. The presence of trade-offs among nest components in solitary bees has received mixed support so far. For example, Kim & Thorp (2001) found evidence for trade-offs between offspring size and offspring numbers in a closely related species, while O'Neill et al. (2010) found no evidence of such trade-offs between *M. rotundata*, similarly to our results. Note that many of these studies investigated a very narrow set of nest traits. In addition, these correlations were primarily estimated at the phenotypic level, while trade-offs are mostly expected to be identified at the within-individual level according to resource acquisition allocation theory (Van Noordwijk & de Jong, 1986). In the present case, many traits could be measured only once per nest, making within-individual correlations impossible to estimate. As mentioned above, collection of multiple nests per female would allow for a more robust examination of whether such trade-offs occur.

Apart from structurally expected correlations, such as large caps being composed of more leaves, ($r_P = 0.80$) and larger nests being composed of more cells ($r_P = 0.50$), other nest traits were only weakly to moderately correlated ($r_P \sim 0.20$). This indicates that *M. rotundata* nests are composed of relatively independent traits, as evidenced by a low average correlation strength ($|\bar{r}_P| = 0.16$). From an evolutionary perspective, our results indicate that the degree of constraints imposed by among-trait correlations will be moderate and that each of these nest traits can evolve relatively independently from other traits. Regardless of weak overall integration among nest traits, selection on certain key traits may still have the potential to reshape nest architecture as a whole. For example, we found that three traits potentially had a disproportionate influence on the rest of the phenotype network: nest length, number of cap leaves, and cell mass. This means that selection acting on any of these three traits may have important direct and indirect effects on nest architecture as a whole given the centrality of these traits within the network (high betweenness and connectivity) and their average correlation strength ($0.15 < |\bar{r}| < 0.25$). Structural correlations

are also likely to play a strong role on the degree of constraints imposed on the evolutionary trajectory of nest architecture. For example, selection for a larger cap will invariably select for more leaves per cap, and therefore more leaf collection trips. Note that our inference is based on correlations estimated at the phenotypic level which conflates genetic and environmental sources of variation. While phenotypic correlations are known to generally match genetic correlations in sign, they do not always correctly predict magnitude (Roff, 1995, Roff, 1996, Kruuk et al., 2008, Dochtermann, 2011). Further inference on the evolutionary trajectories of nest architectural traits will therefore require estimates based on genetic correlations in order to differentiate between genetic and environmental levels of integration (Klinbergen 2014).

While testing for evidence of network modularity, we found that that our classification into cell-, cap-, and nest-level modules was supported and stronger than compared to randomized networks. However, this loose classification may not be the only way to characterize nest architecture networks. For example, if we only consider correlations > 0.20 as biologically meaningful, the phenotype network could instead be characterized by two main modules: one including cell construction (cell length and cell leaves) and another composed of nest size, cell provisioning, and cap-level traits (i.e. number of cells per nest, nest length, number of cap leaves, cap length, and cell mass). This type of trait organization into quasi-independent modules has received mixed support in the literature. For example, metabolic rate and exploratory behaviors are independent in domestic crickets, contrary to expectations of integration among physiology, life-history, and behavior (Réale et al., 2010, Royauté et al., 2015). In mice, despite weak evidence for overall trait integration ($|\bar{r}| < 0.10$), traits involved in nest building quality, maternal care, offspring survival, and offspring "thrifty phenotype" (i.e. patterns of fat accumulation as a result of food deprivation) are arranged into weakly related modules while traits involved in stress reactivity form their own independent module (Sauce et al., 2017). In contrast, plant functional traits show strong evidence of overall integration, showing a phenotype network where traits involved in resource acquisition, sap transport, mechanical support and canopy architecture are interconnected in

complex ways (Messier et al., 2017). Ultimately our approach can be used to generate testable *a priori* predictions on which combination of traits is most likely to respond rapidly to changes in selective pressures or impose constraints on evolutionary trajectories.

Implications for pollinator management and pollination services

M. rotundata is utilized in commercial pollination for alfalfa seed production and other crops and is therefore reared in large, managed populations (Pitts-Singer & Cane, 2011). An important implication of this research is that the nest architecture itself can be a biomarker of individual female health and performance. Likewise, examining the variation in nest construction among females within a population can indicate variation in female health and performance, with potential consequences for the pollination services provided by these females. For example, the number of cells per nest can indicate the fecundity of individual females, with effects on population size, while cell mass can indicate female provisioning rates, which may be correlated to their foraging rates and crop pollination efficacy. Therefore, examining nest constructions may offer insights into whether a population of *M. rotundata* is relatively healthy and performing efficiently as a crop pollinator. Development of these ideas as a management tool will require a deeper understanding of how nest quality relates to female and offspring phenotypes, but could yield a potential way to diagnose a population through nest characteristics.

Conclusion

By measuring multiple traits within an extended phenotype, solitary bee nests, we demonstrate that nests show among-individual variation, particularly in terms of the amount of pollen and nectar provisions provided to each cell. Cell construction and provisioning also showed evidence of plasticity, indicating that females vary in the intensity of construction and provisioning rates over time. Focusing on individual variation in nest architecture can therefore help us to understand patterns of variation in reproductive investment. By treating the extended phenotype as

a network of inter-related traits, we found support for a modular phenotype network with cap, cell, and nest-level traits being relatively weakly integrated with one another. This study is a first step in building a larger understanding of the connection between variation in behavior and variation in the resulting extended phenotype. When paired with data on both female behavior and morphology, and offspring survival and sex ratio, this approach can resolve important evolutionary questions on how complex behavioral phenotypes arise and potentially change across environments and populations.

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Table 1. Summary of univariate mixed models testing for the effects of cell position and date on cell construction and provisioning traits. All estimates come from random intercept-random slope models with nest identity set as the random intercept (V_{Nests}) and cell position as the random slope (V_{Slopes}). All response variables were expressed as standard deviation units. Cell leaves and cell length were square-root transformed to fit assumptions of normality. Statistical inference for fixed effects, random slopes, and slope-intercept correlation for the random effects is based on likelihood ratio tests following a Chi-square distribution.

	Fixed effects					Random effects				
	Estimate	SE	t	χ^2 (df)	P		Variance	[95 % CI]	χ^2 (df)	P
Cell mass										
Intercept	-0.04	0.08	-0.51			V_{Nests}	0.30	[0.18; 0.46]		
Cell position	-0.41	0.06	-0.97	**37.51 (1)**	**$<10^{-5}$**	V_{Slopes}	0.12	[0.05; 0.20]	**25.06 (1)**	**$<10^{-5}$**
Collection date	-0.01	0.01	-1.27	1.63 (1)	0.20	$V_{Residuals}$	0.49	[0.41; 0.58]		
						$r_{Nest\times Slope}$	0.12	[-0.27; 0.47]	0.37 (2)	0.54
Cell leaves										
Intercept	0.09	0.13	0.71			V_{Nests}	0.14	[0.05; 0.30]		
Cell position	-0.11	0.06	-1.87	3.47 (1)	0.06	V_{Slopes}	0.04	[0.00; 0.11]	3.19 (1)	0.20
Collection date	0.02	0.02	0.90	0.80 (1)	0.37	$V_{Residuals}$	0.81	[0.68; 0.99]		
						$r_{Nest\times Slope}$	-0.82	[-1.00; 1.00]	3.06 (2)	0.08
Cell length										
Intercept	-0.01	0.06	-0.10			V_{Nests}	0.10	[0.02; 0.17]		

Cell position	-0.32	0.06	-5.55	**24.23 (1)**	**$<10^{-5}$**	V_{Slopes}	0.09	[0.03; 0.20]	**14.09 (1)**	**<0.001**
Collection date	0.01	0.01	1.56	1.56 (1)	0.12	$V_{Residuals}$	0.71	[0.61; 0.85]		
						$r_{Nest \times Slope}$	-0.63	[-1.00; -0.21]	**5.03 (1)**	**<0.05**

Table 2. Repeatability (τ) of cell construction and provisioning traits and the proportion of the phenotypic variation (R^2) explained by each variance component included in the random regression analyses.

Variance component	Cell mass		Cell leaves		Cell length	
	τ [95 % CI]	R^2	τ [95 % CI]	R^2	τ [95 % CI]	R^2
V_{Nest}	0.33 [0.18; 0.50]	0.28	0.14 [0.04; 0.30]	0.14	0.11 [0.02; 0.21]	0.10
V_{Slope}		0.11		0.04		0.09
$V_{Fixed\ effects}$		0.17		0.02		0.11
$V_{Residuals}$		0.44		0.80		0.70

Table 3. (a) Among-nest (below diagonal elements) and within-nest (above diagonal elements) correlations among nest construction and provisioning traits, and (b) phenotypic correlations among nest construction and provisioning traits. Bold values indicate significant correlations with >95 % of posterior estimates excluding zero (Pmcmc > 0.95), while bold and italicized values indicate correlations with >90 % of posterior estimates excluding zero (Pmcmc > 0.90).

(a) Among and within-nests correlations

	Cell mass	Cell leaves	Cell length
Cell mass		***0.17*** ***[-0.05; 0.26]***	**0.31** **[0.17; 0.37]**
Cell leaves	0.02 [-0.39; 0.27]		**-0.15** **[-0.29; 0.01]**
Cell length	-0.08 [-0.35; 0.27]	**-0.34** **[-0.62; 0.04]**	

(b) Phenotypic correlations

	Nest length	Cap length	Cap leaves	Cell numbers	Cell mass	Cell leaves	Cell length
Nest length		N = 45	N = 60	N = 60	N = 60	N = 42	N = 60
Cap length	**0.16** **[0.01; 0.23]**		N = 45	N = 45	N = 45	N = 27	N = 45
Cap leaves	**0.17** **[0.08; 0.27]**	**0.80** **[0.76; 0.83]**		N = 60	N = 60	N = 42	N = 60
Cell numbers	**0.50** **[0.38; 0.65]**	0.09 [-0.13; 0.27]	***0.15*** ***[-0.07; 0.33]***		N = 60	N = 42	N = 60
Cell mass	**0.23** **[0.06; 0.36]**	**0.21** **[0.02; 0.36]**	**0.22** **[0.03; 0.35]**	0.05 [-0.10; 0.23]		N = 42	N = 60
Cell leaves	***-0.14*** ***[-0.29; 0.04]***	0.06 [-0.18 0.19]	0.04 [-0.12; 0.22]	-0.04 [-0.18; 0.12]	0.02 [-0.09; 0.21]		N = 42
Cell length	0.04 [-0.11; 0.18]	0.00 [0.15; 0.13]	-0.08 [-0.19; 0.09]	-0.04 [-0.15; 0.11]	**0.18** **[0.08; 0.31]**	**-0.21** **[-0.34; -0.05]**	

Table 4. Network metrics for traits' importance within the phenotypic network. Correlation strength is calculated as the average value for each pairwise correlation associated with a given trait, connectivity represents the number of pairwise correlations that are statistically significant for a given trait, and betweenness represents the number of shortest paths passing through a given trait. Bold indicates the trait with the highest value for each metric.

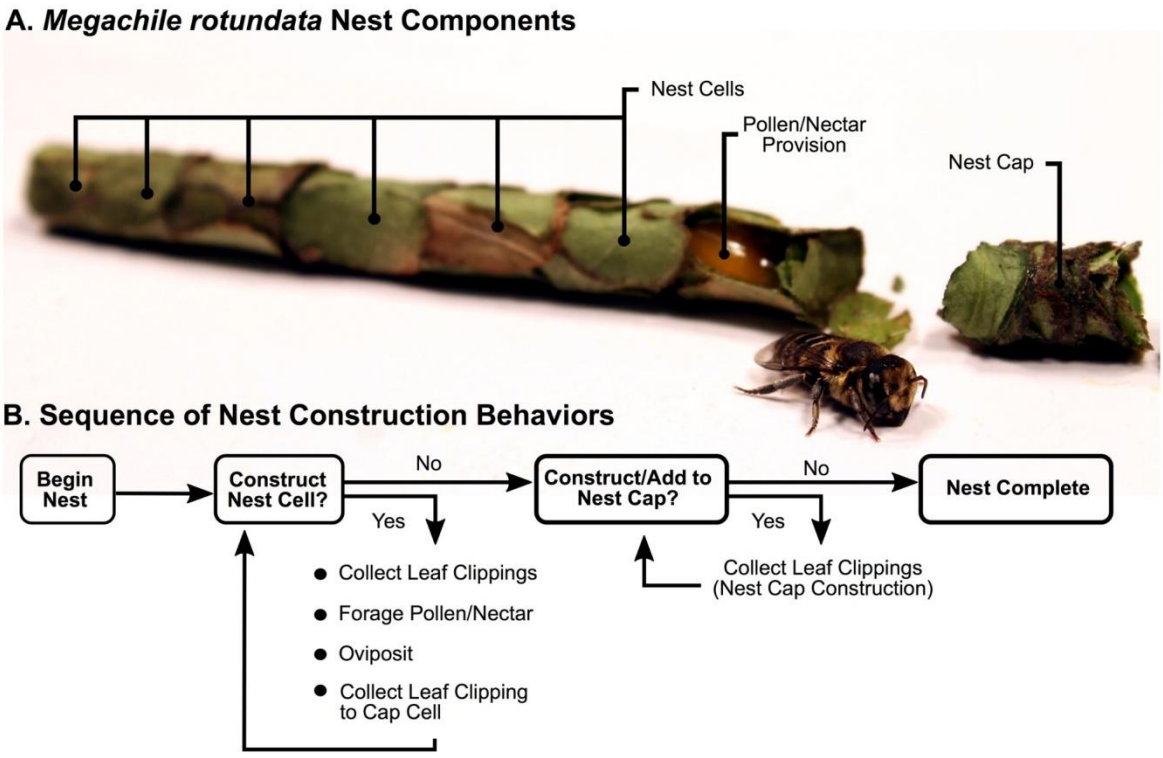
Trait	Correlation strength ($\lvert\bar{r}\rvert$)	Connectivity (degree)	Centrality (betweenness)
Nest length	0.21	**4**	**5**
Cap length	0.22	3	0
Cap leaves	**0.24**	3	1
Cell numbers	0.13	1	0
Cell mass	0.15	**4**	4
Cell leaves	0.02	1	0
Cell length	0.09	2	1

Figures Legends

Fig. 1 (a) Female *M. rotundata* with a nest including a cell that has been dissected open to reveal nectar and pollen provisions for offspring, and (b) the complex sequence of behaviors required to build a complete nest including individual nest cells and the nest cap

Fig. 2 Reaction norms showing patterns of maternal investment in cell construction and provisioning as a function of cell position in the nest. Cell position corresponds to the order in which cells were built with cell number 1 being the first to be built and furthest from the nest cap. The black line represents the population trend, and each nest is represented by a grey line. Points represent nest random intercepts.

Fig. 3 Phenotype network representation of correlations among nest building traits. Circle nodes represent nest-level traits, square nodes represent cap traits and diamond nodes represent cell-level traits. Edge size is proportional to correlation strength, positive correlations are represented in blue, and negative correlations are shown in red. Significant edges (based on non-overlap of 95 % credible intervals with 0) correspond to those associated with numeric values.



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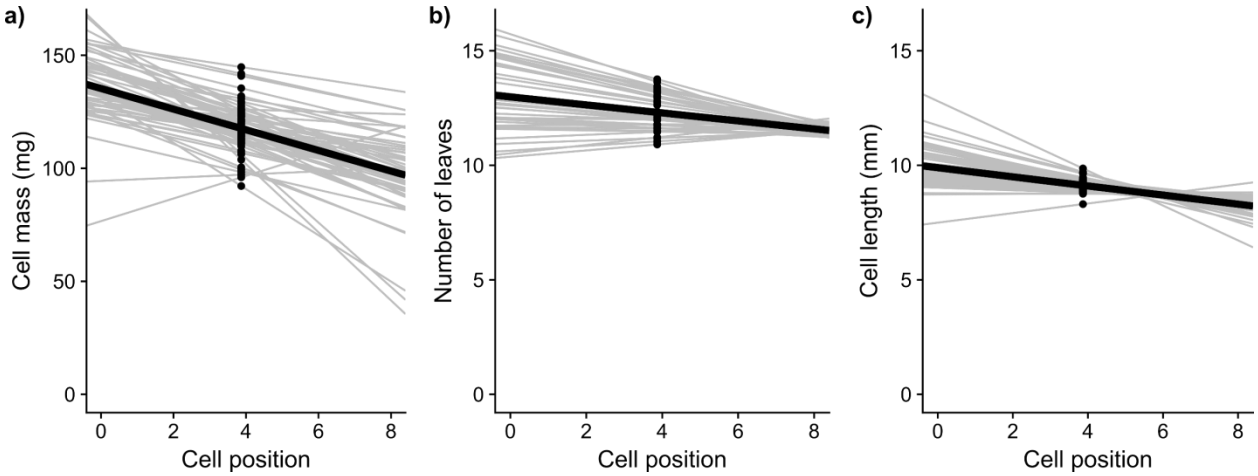

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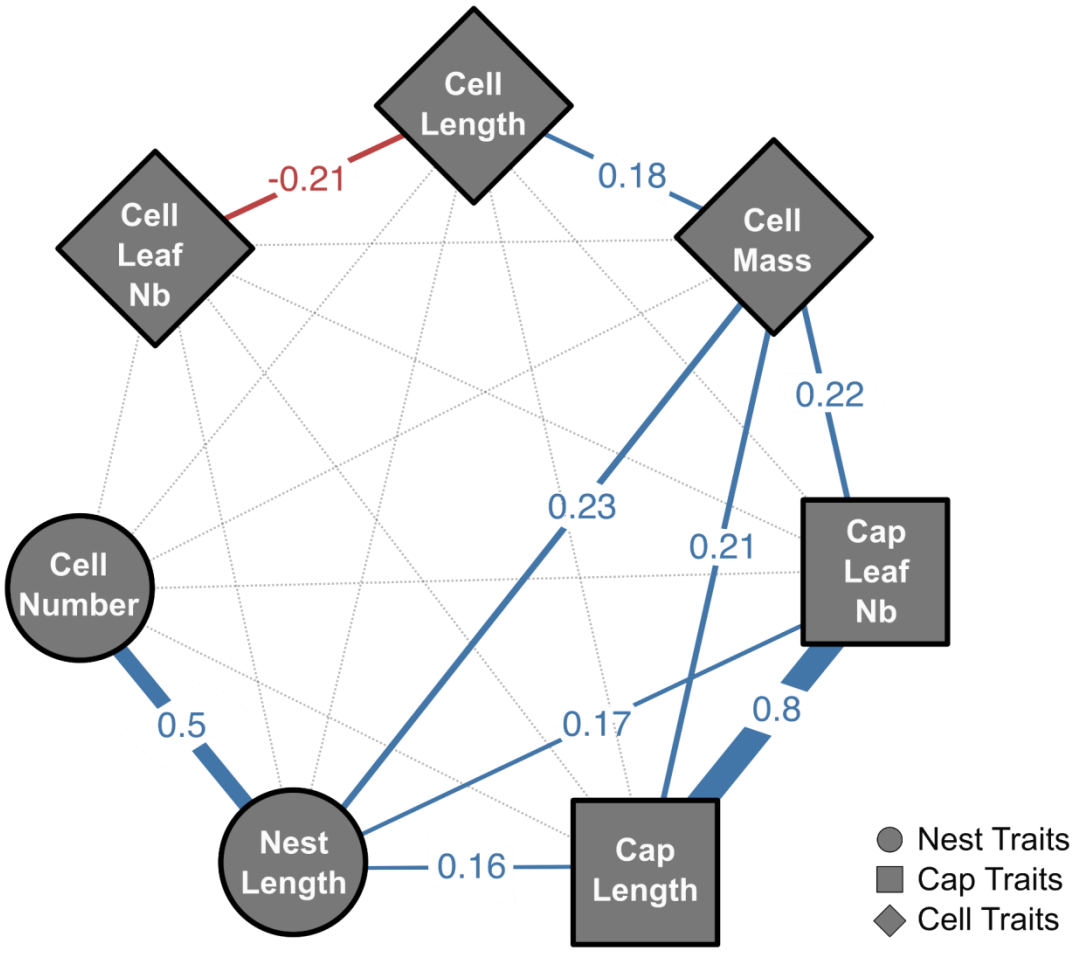

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