E-Natural History Note

Rapid Divergence of Nesting Depth and Digging Appendages among Tunneling Dung Beetle Populations and Species

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Abstract: Many dung beetle communities are characterized by species that share very similar morphological, ecological, and behavioral traits and requirements yet appear to be stably maintained. Here, we document that the morphologically nearly indistinguishable, sympatric, and syntopic tunneling sister species Onthophagus taurus and Onthophagus illyricus may be avoiding competitive exclusion by nesting at remarkably different soil depths. Intriguingly, we also find rapid divergence in preferred nesting depth across native and recently established O. taurus populations. Furthermore, geometric morphometric analyses reveal that both inter- and intraspecific divergences in nesting depth are paralleled by similar changes in the shape of the primary digging appendages, the fore tibiae. Collectively, our results identify preferred nesting depth and tibial shape as surprisingly evolutionarily labile and with the potential to ease interspecific competition and/or to facilitate adaptation to local climatic conditions.

Keywords: competitive exclusion, fossorial limb, geometric morphometrics, nesting behavior, native and introduced Onthophagus.

Introduction

The competitive exclusion principle (Hardin 1960), also known as Gause’s law, is a fundamental determinant of ecosystem dynamics. It postulates that two or more species that compete for the same resources cannot coexist in a constant environment, and it predicts that one of the species that occupy the same ecological niche (sensu Whittaker et al. 1973) will always engage in competitive interactions with the other, leading in the long term to either extinction or niche displacement of the competitor. At the same time, because niche space is determined by the abiotic features of the environment as well as by other members of the community, each species that successfully invades a community makes the niche space of that community more diverse. As interactions among species become more complex, new niches and species diversity may be generated in a positive feedback fashion (Caswell 1976; Pfennig and Pfennig 2012).

Dung beetle communities are a conspicuous example of this mechanism, where a variety of adaptations in the way dung is utilized have evolved to minimize competition for feeding and breeding resource (Hanski and Cambefort 1991a, 1991b). For example, in these communities, “dwellers” that live inside the droppings in both the adult and larval stages may compete for both space and food. Adult “rollers,” by comparison, avoid competition for space by transporting dung balls away from the source (Halfifer and Edmonds 1982; Hanski and Cambefort 1991a, b), whereas adult “tunnelers” excavate tunnels underneath droppings and provision dung for offspring in the form of brood balls at the blind end of each tunnel (Halfifer and Edmonds 1982; Moczek and Emlen 2000). Tunnels are dug roughly perpendicular to the interface between soil and dung, resulting in interference competition for nesting space underneath dung pads, especially in areas where tunnels branch out into nesting chambers (e.g., Halfifer and Edmonds 1982; Hanski and Cambefort 1991b; Moczek 2009). Among tunneling species, very large and very small species tend to bury their brood balls at deeper and shallower depth, respectively, which is thought to help reduce overall competition for nesting space (Hanski and Cambefort 1991a; Rougon and Rougon 1991; Hernández et al. 2011). However, to our knowledge, similar displacement mechanisms have never been documented over narrow phylogenetic distances (i.e., among sister species or populations).

At the same time, there appear to be several exceptions to Gause’s law of competitive exclusion in dung beetle communities, most notably among closely related species that coexist at both regional and local scales despite sharing similar body size, body shape, and most other morphological, behavioral, and ecological traits investigated (e.g., Halfifer...
and Matthews 1966; Binaghi et al. 1969; Martín Piera and Zunino 1986; Hanski and Cambefort 1991a; Baraud 1992; Giller and Doube 1994; Dellacasa and Dellacasa 2006; Hernández et al. 2011; Tocco et al. 2011). This is the case of the tunneling sister species Onthophagus taurus and Onthophagus illyricus (Balthasar 1963; Martín-Piera and López-Colón 2000), the only two scarab species of the subgenus Onthophagus s.s. occurring in Europe (Zunino 1979). These species are almost indistinguishable on the basis of external morphology (reviewed in Pizzo et al. 2006), whether inter- and intraspecific, and can be found feeding in the same dung pads. We also collected O. taurus from two exotic areas where this species was introduced ~50 years ago as part of a biocontrol program (Australia; Tyndale-Biscoe 1996) as well as an accidental introduction (eastern United States; Fincher and Woodruff 1975) and where O. illyricus is not present (Moczek and Nijhout 2003). Specifically, individuals were collected in Monroe County (Indiana, in the eastern United States: ~400 individuals, collected May 2014) and Busselton (Western Australia: ~400 individuals, collected December 2012 and maintained in the laboratory for several generations as described in Beckers et al. 2015). For details on the natural history and life-history characteristics of O. taurus in both exotic ranges, see Moczek (2003), Beckers et al. (2015), and Macagno et al. (2015a). Individuals of the four populations were placed in separate colony containers with loose, shallow soil (10 cm), given unlimited access to dung, and maintained and reared in an environmental chamber at 24°C, 40% humidity, and a 16L:8D cycle. Experimental common-garden breeding started after at least 1 month acclimation under these conditions and occurred in the same time frame of ~3 months for the four laboratory colonies. All data collected (see below) are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.nc66n (Macagno et al. 2015b).

### Brood Ball Burial Depth in O. taurus and O. illyricus

We aimed to investigate differences in brood ball burial depth across native and exotic populations of O. taurus and one native population of O. illyricus. To do so, we selected adults at random from the parental colonies and placed them in plastic 26-cm-tall, 20-cm-diameter containers, filled up to 21 cm with a moist 2:1 mixture of sand and topsoil. This soil was added to the containers in three 7-cm layers, each firmly packed with a dumbbell weight. We placed 5–7 females and 2–5 males (for a total of 8 ± 1 individuals) per container and provided them with 0.5 L of thawed homogenized cow dung. We covered these breeding containers (O. illyricus Italy [ILLY]; n = 7; O. taurus Italy [IT]; n = 14; O. taurus Indiana [IN]; n = 11; O. taurus Western Australia [WA]; n = 7) with window screen and perforated black plastic foil, and we incubated them for 8 days at the environmental conditions described above. At the end of the breeding period, and after removing the
dung, we carefully separated the three soil layers (top, center, and bottom) and sifted them separately to retrieve brood balls made within each. Adult beetles were returned to the parental colonies. To compare the number of brood balls laid in the top, center, and bottom layer of soil across populations of *O. illyricus* (ILLY) and *O. taurus* (IT, IN, WA; brood ball numbers reported in Table 1), we performed pairwise comparisons across populations within each layer with Z-tests for the comparison of proportions, using Holm-Bonferroni corrections for multiple comparisons.

Shape and Size Differentiation of Digging Appendages across *O. taurus* and *O. illyricus*

In beetles of the genus *Onthophagus*, the prothoracic tibiae are modified into robust scraping devices adapted for digging tunnels in hard soil, equipped with an apical spur and four prominent teeth on the anterior border (Fig. 1). To inspect whether brood ball burial depth divergence in *O. taurus* and *O. illyricus* might be accompanied by differentiation of tibial morphology, we analyzed inter- and intraspecific shape variation of the tibia in 12 randomly selected females per population (IN, WA, IT, ILLY), using landmark-based geometric morphometrics (Bookstein 1991; Rohlf and Slice 1990; Dryden and Mardia 1998; Zelditch et al. 2004). Landmarks were digitized with TpsDig 2.10 (Rohlf 2006) on 2-D calibrated images of the right tibia (Fig. 1). We avoided placing landmarks on the apices of the tibial teeth, because these wear out with use (Tyndale-Biscoe 1978; González-Megías and Sánchez-Piñero 2004).

We used generalized procrustes analysis (GPA) to discard all geometrical information related to translation, rotation, and scale and to compare tibiae exclusively on the basis of their shape (Rohlf and Slice 1990). After Procrustes superimposition, each structure (defined by its landmark configuration) corresponds to a point on a curved, non-Euclidean shape space (Kendall 1981, 1984). We performed an orthogonal projection onto a Euclidean space tangential to a reference point in Kendall’s shape space (Dryden and Mardia 1998; Rohlf 1999) and then looked for quantitative differences between populations with a canonical variate (CV) analysis of shape coordinates. This analysis produces a set of CVs that are uncorrelated both within and among groups and account in sequence for the maximum amount of among-group shape difference relative to within-group variance (Klingenberg and Monteiro 2005). We expressed the degree of divergence across populations by means of Mahalanobis distances (Mardia et al. 1979) and assessed their significance with permutation tests (10,000 permutation rounds). Analyses were performed in MorphoJ (Klingenberg 2011).

We estimated tibial size of each specimen as the centroid size (CS) of the landmark configuration (Bookstein 1991). This measure is approximately uncorrelated with shape for small isotropic landmark variation (Bookstein 1991; Dryden and Mardia 1998; Mitteroecker and Gunz 2009). Additionally, we used pronotum width as a proxy for body size (Emlen 1996; Moczek 2003; Macagno et al. 2011a, 2011b) and measured it using a stereoscope (Leica MZ-16, Bannockburn, IL), a digital camera (Scion, Frederick, MD), and the software ImageJ (Rasband 2014). We compared the tibia to body size ratio, log(tibia CS):log(pronotum width), across populations using a one-way ANOVA and Tukey honestly significant difference post hoc tests. The assumption of homoscedasticity was checked using the Levene test. Analyses were performed in SPSS 22.0.

Brood Ball Mass and Size of Offspring in Native *O. taurus* and *O. illyricus*

We aimed to investigate any association between brood ball burial depth and brood ball mass and adult size of offspring in native populations of the two sister species. To do so, the brood balls harvested from each layer of soil were counted and weighted to the closest 0.0001 g using a Mettler Toledo (AL 54) scale and then incubated separately in 32-oz plastic containers filled with sterile soil and covered with clear plastic wrap. A plastic 3-oz cup was placed in the soil in each brood ball container as a pitfall trap for emerging adult offspring. We checked these traps every other day for 60 days after brood ball harvesting. Newly emerged adult beetles were removed on the day that they

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<th>Table 1: Percentage of brood balls (BBs) harvested from three layers of soil (bottom, center, and top) in breeding containers</th>
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<td><strong>Species</strong></td>
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Note: Within each layer, all pairwise comparisons across populations are significant at $P < .05$ (Z-tests for the comparison of proportions, Holm-Bonferroni correction for multiple comparisons applied).
Figure 1: Top, landmark configuration chosen to analyze shape variation of the tibia; scatterplot graph showing ordination of the samples along the first two canonical variate axes derived from a canonical variate (CV) analysis of shape variables (populations are color-coded; blue = *Onthophagus illyricus*, Italy [ILLY]; green = *Onthophagus taurus*, Italy [IT]; yellow = *O. taurus*, Indiana [IN]; red = *O. taurus*, Western Australia [WA]); and table reporting shape divergence of tibia, expressed as Mahalanobis distances across populations analyzed. Significance is marked with two asterisks ($P < .001$). Bottom, shape changes along the first two CVs, shown as deformations (dark blue) with respect to the mean shape along the CV (light blue), using wireframe graphs connecting landmarks. Within each shape change, the figure on the left shows a negative deviation from the mean along the CV, and the figure on the right shows a positive deviation. Shape changes are emphasized to make visualization easier.
were found in the traps. Their pronotum width, measured as described above, was used as a proxy for body size (Emlen 1996; Moczek 2003; Macagno et al. 2011a, 2011b).

We compared brood ball mass and body size of emerging offspring between Italian O. taurus and O. illyricus across the three soil layers using GLMs including species, layer, and their interaction as factors. We then repeated these analyses within each species, comparing brood ball mass and body size of emerging offspring across layers using a one-way ANOVA and LSD post hoc tests (O. taurus) or t-tests (O. illyricus). The assumption of homoscedasticity was checked using the Levene test. To randomize maternal effects, analyses were performed on subsets of brood balls (n = 8 to 15 per species and layer) and newly emerged offspring (n = 8 to 19 per species and layer) chosen at random from several breeding containers.

Results
Brood Ball Burial Depth
Onthophagus illyricus showed a marked tendency to position brood balls in the most superficial level of soil, with ~80% of brood balls retrieved from 7 cm or less below soil surface. Approximately 20% of brood balls were found in the center layer, and none were harvested from the bottom of the breeding containers. By comparison, native O. taurus (IT) positioned brood balls almost exclusively within the bottom (~51%) and center layer (~43%) of soil in the breeding containers. Partly similar differences were detected among exotic O. taurus populations. Eastern US (IN) O. taurus buried 70% of the brood balls in the bottom layer, yet none in the top layer, whereas WA O. taurus were the most likely to build brood balls in the center layer (~58%; table 1).

Tibial Morphology
Based on the shape variation described by the first and second CVs (explaining ~59% and ~28%, respectively, of the amount of scaled between-group shape variation; fig. 1), shallow-nesting O. illyricus females had the narrowest and most elongated tibia of all populations analyzed. Tibial shape divergence was greatest between native, deep-nesting O. taurus (IT) and shallow-nesting O. illyricus (Mahalanobis distance: 3.49). Notably, a roughly similar pattern of divergence was found between deep-nesting native IT O. taurus and shallow-nesting exotic WA O. taurus both quantitatively (Mahalanobis distance: 3.47) and qualitatively along the CV1 axis. IN O. taurus exhibited a tibial shape intermediate to the shorter and stockier-appearing IT O. taurus and the comparatively more elongated WA O. taurus.

The effect of population on the tibia to body size ratio was significant in a one-way ANOVA (F3,47 = 4.64, P < .01).

However, subsequent Tukey’s HSD post hoc tests showed that only O. illyricus had greater tibia to body size ratio (mean ± SD: 0.48 ± 0.03) compared with WA O. taurus (0.43 ± 0.04), whereas all other contrasts, including IT O. taurus (0.45 ± 0.04) and IN O. taurus (0.45 ± 0.02), were not significant (P > .05).

Brood Ball Mass and Size of Offspring in Native O. taurus and O. illyricus
Brood ball mass and body size of emerging adult offspring of native O. taurus and O. illyricus depended on a combination of species and layer of soil (fig. 2; effect of species × layer on brood ball mass: F1,81 = 12.13, P < .01; effect on body size of offspring: F3,65 = 12.40, P < .01). In O. taurus, the effect of layer was significant in a one-way ANOVA for both response variables (brood ball mass: F3,74 = 3.92, P = .03; body size of offspring: F3,65 = 13.52, P < .01). LSD post hoc tests showed that brood ball mass and offspring size were significantly lower in the top layer of soil, whereas the center and bottom layers did not differ statistically. In O. illyricus, data suggested brood ball mass and size at emergence were elevated in the top layer compared with the center layer. This difference was significant for brood ball mass (t26 = 2.76, P = .01) but not for offspring size (t26 = 1.50, P = .15).

Discussion
In dung beetle communities, the coexistence of species with broadly similar morphological, ecological, and behavioral traits (e.g., Halfter and Matthews 1966; Binaghi et al. 1969; Martín Piera and Zunino 1986; Hanski and Gamberg 1991a; Baraud 1992; Giller and Doube 1994; Dellacasa and Dellacasa 2006; Hernández et al. 2011; Tocco et al. 2011) appears to pose a challenge to Gause’s law of competitive exclusion (Hardin 1960). However, aspects of their biology that have been overlooked so far may facilitate the occurrence of these species in syntopy. Here, we focused on the tunneling sister species Onthophagus taurus and Onthophagus illyricus and found that, despite their apparent equivalence in size, shape, and ecological requirements (Pizzo et al. 2006b), they appear to nest at different depths. Specifically, O. illyricus prefers the layer of soil immediately underneath the dung pad, whereas O. taurus buries brood balls substantially deeper. Higher investment in brood ball mass (and consequently in offspring size) at the favored depth for both species further supports the existence of divergence in microhabitat choice relating to nesting depth. Furthermore, we found that brood ball burial depth is also surprisingly evolutionarily labile across populations, having diverged between native and introduced O. taurus populations within ~100 generations. Last, we found that evolutionary
changes in nesting depth have occurred alongside inter- and intraspecific changes in the shape of the primary digging appendages and that the type and magnitude of this divergence across native and exotic populations of *O. taurus* is reminiscent of that between *O. taurus* and *O. illyricus* in their native range. Below we discuss the most notable implications of our results.

**Interspecific Divergence in Nesting Depth**

Under the competitive exclusion principle, we hypothesized that *O. taurus* and *O. illyricus* might have diverged in nesting depth to minimize competition for nesting space. Brood ball burial depth was indeed different between the two sister species in their native range, with *O. illyricus* colonizing primarily the top 7 cm of soil, whereas *O. taurus* buried the majority of brood balls at or below 14 cm (for scale, note that adults are roughly 1 cm in length). It is notable that these differences were detected in a common-garden experiment after acclimation in monospecific colonies, indicating that differences in nesting behavior may be genetically fixed rather than displayed in response to the presence or absence of heterospecific competitors. Moreover, for both species, the preferred layers also housed the heaviest brood balls and gave rise to the largest offspring. Combined, our results are consistent with the hypothesis that, in nature, both species specialize in different nesting depths, thereby potentially minimizing interference competition in syntopy.

**Brood Ball Burial Depth Is Evolutionarily Labile**

We detected considerable divergence in brood ball burial depth, not only between *O. illyricus* and *O. taurus* (IT) in syntopy, but also between populations of *O. taurus* that were introduced into Western Australia and the eastern United States (IN) in the 1970s, approximately 100 generations ago. Here, WA *O. taurus* built by far the most superficial brood balls, whereas IT and, to an even greater extent, IN *O. taurus* buried their brood balls considerably deeper. The exact mechanism that may have driven the divergence in nesting depth across native and exotic *O. taurus* populations is unclear at present, although three main scenarios can be hypothesized. First, several studies have highlighted that, in Western Australia, introduced dung beetles can reach densities far higher than those occurring in the eastern United States or Europe (Doube et al. 1991). Because dung is removed by competitors extremely rapidly in these conditions (Moczek 2003; Beckers et al. 2015), WA *O. taurus* may be under higher pressure to bury brood balls at a shallower depth to minimize nesting time, thereby maximizing access to dung. Alternatively, the pattern highlighted could result from local adaptations to climatic conditions. Snell-Rood et al. (2015) demonstrated that burial depth decreases daily temperature fluctuations, enabling mothers to buffer temperature fluctuations experienced by the offspring’s juvenile stages through deeper burial. If harsher, more variable climate conditions select for increased buffering behav-

![Figure 2: Boxplots of brood ball mass produced by the parental generation and body size of emerging offspring across layers of soil in native *Onthophagus illyricus* and *Onthophagus taurus* (Italy). In *O. taurus*, brood ball mass and offspring size are lower in the top layer of soil, whereas the center and bottom layers do not differ statistically. In *O. illyricus*, brood ball mass is elevated in the top layer compared with the center layer; offspring size at emergence shows a similar pattern, but this difference is not statistically significant.](image-url)
ior, nesting depth is expected to be positively correlated with the magnitude of temperature fluctuations in the field. This was the case in our study, as brood balls were buried deeper the larger the variation in annual temperature experienced by *O. taurus* populations in the sampling areas (Bloomington, Indiana: 26°C; Pont Canavese, Italy: 20°C; Busselton, Western Australia: 9.8°C; Climate-Data.org 2015). Last, we cannot exclude the alternative explanation that differences in preferred burial depth simply reflect nonadaptive founder effects during the early stages of *O. taurus* invasion. Collectively, our results identify nesting depth as surprisingly evolutionarily labile and with the potential to ease interspecific competition and/or facilitate adaptation to local climatic conditions.

*Inter- and Intraspecific Shape Differentiation of Digging Appendages Parallels Divergence in Brood Ball Burial Depth*

In tunneling dung beetles, the tibiae of the first pair of legs are modified into robust rake-like devices equipped with an apical spur and four prominent teeth on the anterior border, adapted for digging tunnels in hard soil. Because nesting requires considerable tunneling efforts, primarily by females (Moczek 1999, 2009), we examined whether inter- and intraspecific differentiation in brood ball depth has occurred alongside changes in the morphology of the female tibiae. We detected a modest size differentiation and a more marked shape differentiation between sister species and across native and introduced *O. taurus* populations. Faster divergence of shape versus size has been highlighted in earlier studies, suggesting that these two components of morphology may be developmentally and genetically decoupled enough to evolve independently of each other (Macagno et al. 2011a, 2011b).

Specifically, we found that the size of the tibia is likely not associated with brood ball burial depth performance in these beetles, because *O. illyricus* and WA *O. taurus* had the maximum divergence in tibial size despite sharing a preference for burying brood balls in shallow soil, whereas we found no difference in tibial size across *O. taurus* populations that were, in turn, substantially different in brood ball burial depth. By comparison, divergence of tibial shape was in line with that of brood ball burial depth: shallow-nesting *O. illyricus* and WA *O. taurus* had the narrowest tibiae, whereas the populations burying their brood balls deeper (IT and IN *O. taurus*) had the widest. Female digging appendages of deep nesters appeared enlarged and shovel-like, thus seemingly better suited to displace larger quantities of soil. Interestingly, these results are in keeping with a recent, preliminary finding that dung removal performance in some tunneling beetles (*Anoplotrupes stercorarius*, *Geotrupes stercorarius*, *Trypocopris pyrenaicus*, and *Onthophagus fracticornis*) is negatively correlated to the length but positively correlated with the width of the distal part of the digging appendages (B. Nervo, personal communication), consistent with rapid adaptive coevolution of brood ball burial depth and tibial shape. On the other hand, our data do not allow us to rule out the possibility that phylogenetic dependence may also have contributed to drive the divergence pattern highlighted (Pizzo et al. 2006, 2008), because the deformation described by the CV2 in our analysis of tibial shape (fig. 1) mainly separated *O. illyricus* from both the native and, to an even greater extent, the exotic populations of *O. taurus*. Additional studies are needed to disentangle the relative contribution of adaptive and nonadaptive evolutionary mechanisms to the rapid and parallel divergence of brood ball burial depth and the shape of digging appendages in these beetles. Future research may also seek to experimentally manipulate nesting depths in mixed-species colonies to measure the fitness consequences of sharing and avoiding nesting depths with heterospecific competitors.

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*Onthophagus taurus* female. Photo credit: Anna L. M. Macagno.