



# The role of ancestral phenotypic plasticity in evolutionary diversification: population density effects in horned beetles

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Plasticity's role in shaping phenotypic diversification continues to receive considerable attention. One especially debated issue concerns the significance of genetic accommodation in diversification, and the proposed role of ancestrally plastic responses in facilitating or biasing subsequent genetically canalized differentiation among taxa. Here, we investigated whether pre-existing plasticity in response to variation in population density present in the ancestral Mediterranean range of the bull-headed dung beetle *Onthophagus taurus* may have mediated previously documented rapid canalized divergences among descendent exotic populations that have been subject to dramatically different levels of competition for mates and resources in the field. We focused on two maternal behavioural traits, two life history traits and two morphological traits. We find that (1) Mediterranean *O. taurus* exhibited plasticity in response to adult densities for four of our six focal traits; (2) in two of those, plastic responses matched the direction of canalized divergences among natural populations; and (3) the presence and direction of plasticity appeared unrelated to trait type. More generally, our results provide partial support for the hypothesis that evolution by genetic accommodation could have contributed to the very early stages of population differentiation in a subset of traits in *O. taurus*.

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Phenotypic plasticity is well established as a mechanism enabling organisms to maintain high fitness in the face of fluctuating environments (Schlichting & Pigliucci, 1998). Furthermore, plasticity is increasingly being recognized as impacting the persistence of lineages by influencing populations' ability to colonize novel habitats or to resist extinction in the face of major environmental perturbations (Hendry, 2016; Yeh & Price, 2004). What is much less well understood, however, is plasticity's roles in more directly shaping phenotypic diversification and phenotypic innovation, in particular through the process of genetic accommodation (reviewed in Moczek et al., 2011; Pfennig et al., 2010; Wund, 2012). Genetic accommodation is defined most broadly as gene frequency change due to selection on the regulation of an environmentally induced response (West-Eberhard, 2003) and has received most attention because it proposes a mechanism whereby initially environmentally induced traits may become at least partly genetically canalized, or in other words, for phenotypic changes due to plastic responses to environmental conditions to precede corresponding genetic changes within populations. Such a

'plasticity-first' scenario may be possible, for instance, if plastic responses to environmental conditions make visible to selection previously cryptic genetic variation that was allowed to accumulate without resulting in selectable phenotypic variation (Ledón-Rettig, Pfennig, Chunco, & Dworkin, 2014; Paaby & Rockman, 2014).

The concept of genetic accommodation grew historically out of a broadening of the concept of 'genetic assimilation', now recognized as an extreme form of accommodation, whereby an initially environmentally determined phenotype becomes constitutively expressed. Initially focused on behavioural plasticity and learning (Baldwin, 1986, 1902), it has now grown to encompass all forms of plasticity, as well as all changes in the regulation of an environmentally induced response (Renn & Schumer, 2013; Waddington, 1953; West-Eberhard, 2003). Evidence in support of genetic accommodation initially derived primarily from environmental perturbation and artificial selection experiments in the laboratory, demonstrating that novel or stressful perturbations may elicit developmental responses that free up previously unexpressed genetic and phenotypic variation able to fuel rapid responses to artificial selection in the laboratory (*Drosophila*: Dworkin, 2005; Rutherford & Lindquist, 1998; Waddington, 1953; *Manduca sexta*: Suzuki & Nijhout, 2006; *Caenorhabditis*: Sikink, Reynolds, Ituarte, Cresko, & Phillips, 2014; *Arabidopsis*: Queitsch, Sangster, &

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Lindquist, 2002; fungi: Cowen & Lindquist, 2005; cyanobacteria: Walworth, Lee, Fu, Hutchins, & Webb, 2016). More recently, work on natural populations and species has also begun to accumulate evidence consistent with pre-existing plasticity as an initial mediator of subsequent genetic differentiation in the wild, including examples of morphological as well as behavioural plasticity: gut morphology and time of development in spadefoot toad tadpoles from diverse genera, including *Spea* and *Scaphiopus* (Gomez-Mestre & Buchholz, 2006; Ledón-Rettig, Pfennig, & Nascone-Yoder, 2008), trophic morphology, body form, size and behaviour in threespine sticklebacks, *Gasterosteus aculeatus* (Robinson, 2013; Shaw, Scotti, & Foster, 2007; Wund, Baker, Clancy, Golub, & Foster, 2008), melanization in *Daphnia melanica* (Scoville & Pfrender, 2010), reproductive physiology and behaviour in house finches, *Haemorrhous mexicanus* (Badyaev, 2009), and loss of eyes in cavefish, *Astyanax mexicanus* (Rohner, et al. 2013).

However, more studies remain necessary before the evolutionary significance of genetic accommodation can be fully assessed (Ehrenreich & Pfennig, 2016; Levis & Pfennig, 2016). For example, we need to learn more about the degree of phenotypic change that evolution by genetic accommodation may be capable of mediating, as well as potential biases it may exert toward certain trait types over others, possibly depending on their inherent sensitivity to the environment (Beldade, Mateus, & Keller, 2011; Foster, 2013; Foster, Wund, Graham et al., 2015; Ghalambor, McKay, Carroll, & Reznick, 2007). For instance, many types of behaviour exhibit extreme evolutionary lability (e.g. flight and flashing behaviour in fireflies: reviewed in Lewis & Cratsley, 2008; songs in birds and crickets: Desutter-Grandcolas & Robillard, 2003; Podos, Huber, Taft, 2004; Zuk, Rotenberry, & Simmons, 2001; nest building in social hymenoptera: Turner, 2002). Furthermore, behavioural plasticity has been documented extensively across a wide range of taxa (Foster & Endler, 1999), and shown to vary across populations of the same species (Foster, 1999; Foster & Endler, 1999). Yet, further comparative work is needed to assess whether behavioural traits are, as has been hypothesized (Allf, Durst, Pfennig, & McPeck, 2016; West-Eberhard, 1986, 2003), indeed more likely to undergo evolution by genetic accommodation compared to other traits that exhibit reduced lability and plasticity. Similarly, little is known about the speed with which initial, plasticity-mediated changes in phenotype expression may be accommodated into genetically canalized divergences, insights that may necessitate the study of the very early stages of population differentiation. Here we seek to contribute to a better understanding of both scope and speed of genetic accommodation by exploring the role of ancestral plasticity in mediating the early stages of rapid population differentiation in morphological, life history and maternal behavioural traits in recently established exotic populations of the bull-headed dung beetle *Onthophagus taurus*. We selected this species because it unites several features that make it a promising study organism to assess the role of phenotypic plasticity in the earliest stages of evolutionary diversification, most notably a diversity of ecologically relevant and experimentally accessible traits that are undergoing rapid diversification in recently established exotic populations (Beckers, Anderson, & Moczek, 2015; Moczek & Nijhout, 2003).

Adult *O. taurus* colonize dung pads of primarily cows and horses, establish tunnels underneath and provision dung in the form of brood balls at the blind end of each tunnel (Fincher & Woodruff, 1975; Halffter & Edmonds, 1982). Females oviposit a single egg in each brood ball, which then constitutes the entire food supply larvae have available to complete development to adult (Moczek & Emlen, 1999). Brood ball quantity and quality strongly affect offspring adult body size as well as sexual and male dimorphism: only male *O. taurus* develop a pair of long, curved horns on their

heads, and only if larval feeding conditions allow male larvae to metamorphose to an adult body size above a critical threshold value (Moczek & Emlen, 1999). Males smaller than this threshold size grow only rudimentary horns, whereas all females regardless of size develop a minor ridge instead. The resulting male horn polyphenism is paralleled by alternative reproductive tactics, where large horned males fight to gain access to females using horns as weapons, while small males rely on nonaggressive sneaking tactics to gain access to females (Moczek & Emlen, 2000). In the late 1960s *O. taurus* was introduced from its native range in the Mediterranean (Balthasar, 1963) to Western Australia to help control cow dung and dung-breeding flies (Tyndale-Biscoe, 1996), as well as into the eastern United States by accident (Fincher & Woodruff, 1975). Since introduction, both populations have diverged rapidly in diverse traits, attributed to substantial differences in local dung beetle densities and the resulting divergent intensities of mate and resource competition: *O. taurus* densities in the eastern United States rarely exceed a few individuals per dung pad, and competition from heterospecific species is essentially nonexistent (Moczek, 2003). As a consequence, competition among females for dung is minimal (most dung dries out above ground before being processed by adults), and male–male competition for females is moderate. In contrast, *O. taurus* densities in Western Australia reach into the hundreds to low thousands of individuals per dung pad, and densities from competing species can be similarly high (Moczek, 2003). As a consequence, competition among females within and across species for dung is severe (dung pads may be removed by beetle activity within hours) and intraspecific male–male competition for females is extreme. Earlier work posited that these extreme differences in competitive environments may have driven phenotypic divergences in a wide range of traits, including those in the following three categories. (1) Morphology: adults in Western Australia are consistently and significantly smaller than adults in the eastern United States. At the same time, the adult size threshold needed for horn induction has increased among males in Western Australia, but decreased in males in the eastern United States. Both divergences are maintained in common garden environments (Moczek & Nijhout, 2003; Moczek, Hunt, Emlen, & Simmons, 2002). (2) Maternal behavioural traits: females in Western Australia produce heavier brood balls, which are buried at a more shallow level compared to those in the eastern United States, and both divergences are again maintained in common garden environments (Beckers et al., 2015; Macagno, Moczek, & Pizzo, 2016). (3) Life history traits: females in Western Australia produce a much higher number of brood balls when given a breeding opportunity compared to their eastern United States counterparts and the resulting offspring exhibit significantly greater eclosion success. Of these life history divergences, only differences in brood ball number are retained in common garden conditions, whereas differences in eclosion success disappear in the F2 generation (Beckers et al., 2015). Interestingly, *O. taurus* from the ancestral range exhibit trait values intermediate to those described for Western Australia and the eastern United States (Macagno et al., 2016; Moczek, 2003; Moczek & Nijhout, 2003) for at least a subset of traits, suggesting that establishment of exotic populations was followed by rapid divergences in both exotic ranges, yet in opposite directions.

In this study, we sought to investigate whether pre-existing behavioural and/or morphological plasticity in response to adult densities present in the ancestral *O. taurus* population may have mediated these rapid divergences among descendent exotic populations. Specifically, we used a Mediterranean population obtained from Spain as a proxy for the ancestral population to quantify presence and direction of plastic responsiveness to high and low conspecific densities to answer the following three questions. (1)

Do Mediterranean *O. taurus* exhibit plasticity in response to adult densities for some or all the traits known to have diverged among exotic populations? (2) Are plastic responses in a direction matching those of canalized divergences among natural populations? (3) Are presence, absence and direction of plasticity dependent on trait type? By exposing *O. taurus* derived from the ancestral Mediterranean range to low or high adult densities, we demonstrate significant plasticity in four of six focal traits, including two where the direction of the response matches the direction of canalized divergences among exotic populations. More generally, our results provide partial support for the hypothesis that evolution by genetic accommodation could have contributed to the early stages of population differentiation in a subset of traits in *O. taurus*.

## METHODS

### Beetle Collection and Husbandry

Adult *O. taurus* were collected around Seville, Spain, and shipped to our laboratory in Bloomington, Indiana, U.S.A. Beetles were maintained as moderate-density laboratory colonies (~200–300 beetles) at 24 °C in a sand/soil mixture on a 16:8 h light:dark cycle and fed homogenized cow dung twice per week for 2 weeks to facilitate acclimation prior to the experiment (Moczek et al., 2002).

### Population Density Manipulation

Following acclimation, adult beetles were assigned to one of two treatments: low or high population density. Low-density treatment consisted of placing one male and one female in round plastic containers (approximately 1.9 litre volume, 15 cm diameter, 12 cm depth) filled with a sand/soil mixture to depth of ~7 cm. High-density treatment consisted of placing 10 males and 10 females into a container set up in an identical fashion. A total of 19 low-density containers and six high-density containers were generated. Each container was provided with approximately 200 ml of cow dung equilibrated at room temperature. Dung was collected earlier from a local organic farm, homogenized, frozen, and defrosted just prior to the experiment. We replaced dung every 4 days and counted the number of beetles to ensure intended densities were maintained. Low-density containers found to contain a dead female were either combined with other low-density containers where a male had died ( $N = 1$ ), or discarded ( $N = 1$ ). Four high-density containers in which two or more beetles per container had died were combined to maintain 18–20 beetles per container. This resulted in a total of three high-density containers at the end of the density manipulation phase.

Beetles were maintained in density manipulation containers for 3 weeks, after which females from each treatment were then allowed to breed individually in separate breeding containers. Breeding containers consisted of modified pasta containers (9 cm diameter, 28 cm depth), packed with a moist sand/soil mixture and provided with ~200 ml of cow dung. After 7 days, breeding containers were turned upside down and all contents were carefully emptied. For each brood ball produced, we recorded burial depth to the nearest 2 cm using a ruler, as well as weight using a Mettler Toledo analytical balance. Brood balls were then placed in individual cups within a moist sand/soil mixture and maintained at 24 °C until adults emerged.

### Allometric Measurements

Adults (parental and F1) were measured using a two-dimensional morphometric set-up consisting of a Leica dissecting

microscope, a Scion digital camera and ImageJ v1.44p software (ImageJ, National Institutes of Health, Bethesda, MD, U.S.A., <http://rsbweb.nih.gov/ij/>). Thorax width was used as a measure of body size and head horn size was measured as previously described (Moczek, 2003; Moczek & Nijhout, 2003). Measurements were taken to the nearest 0.001 mm.

### Statistical Analyses

We performed generalized linear models (GLMs) and generalized linear mixed model (GLMMs) analyses using R statistical software (R Foundation for Statistical Software, Vienna, Austria). Model selection was done by simplifying and removing nonsignificant variables as determined by chi-square tests or by *F* tests when the data were overdispersed (Bolker et al., 2009).

Because females will sometimes produce partial brood balls or fail to oviposit into brood balls, we used measures of brood ball burial depth and weight only from those brood balls that yielded adult offspring. We used separate GLMMs with gamma distributions to assess brood ball weight and brood ball burial depth, with replicate container included as a random variable. Fixed variables included maternal size, population density treatment, offspring sex and their interactions.

The total number of brood balls produced per mother was modelled as a response variable using count data, and a GLM, with maternal size and population density treatment as fixed variables. We used a negative binomial distribution for these data after a visual examination and confirmation by Akaike's information criterion (AIC). To determine whether maternal size or population density had an effect on offspring survival or offspring emergence success to adulthood, we used the number of individuals that emerged as adults as a proportion of the total number of brood balls produced per mother. We used a GLM with a binomial distribution; explanatory variables were maternal size and population density and the response variable was the proportion of offspring that survived per mother.

We analysed offspring body size using a GLMM (normal error distribution) with replicate container as a random variable and maternal body size, population density, offspring sex, brood ball weight and their interactions as fixed variables. Following several previous studies (Emlen, 2001; Nijhout & Emlen, 1998; Parzer & Moczek, 2008), we analysed possible effects on the sigmoidal horn length–body size scaling relationship of males by fitting a four-parameter nonlinear regression of the form

$$\text{horn length} = y_0 + \frac{a(\text{body size}^b)}{c^b + (\text{body size}^b)}$$

(using Sigma Plot 12.5, SigmaPlot, SPSS Inc., Chicago, IL, U.S.A.;  $y_0$  represents the minimum horn length,  $a$  describes the range of horn lengths,  $b$  the slope coefficient and  $c$  specifies the inflection point of the sigmoidal curve) to horn length–body size data obtained from offspring of mothers exposed to low and high density, respectively, and used Welch's *t* test to compare parameter means (amplitude, slope, inflection point and *y* intercept).

In addition, we calculated horn length residuals by fitting the same model as previously described to the combined data set and calculated the difference between observed and expected horn length for each individual male, which were then contrasted using a Mann–Whitney *U* test.

### Ethical Note

This research adhered to the ASAB/ABS Guidelines for the use of animals in research, and animals were kept in the best

possible conditions based on the biology of this species. We also adhered to the legal requirements of the U.S.A. and Indiana University. Experimental animals were preserved in ethanol and stored.

## RESULTS

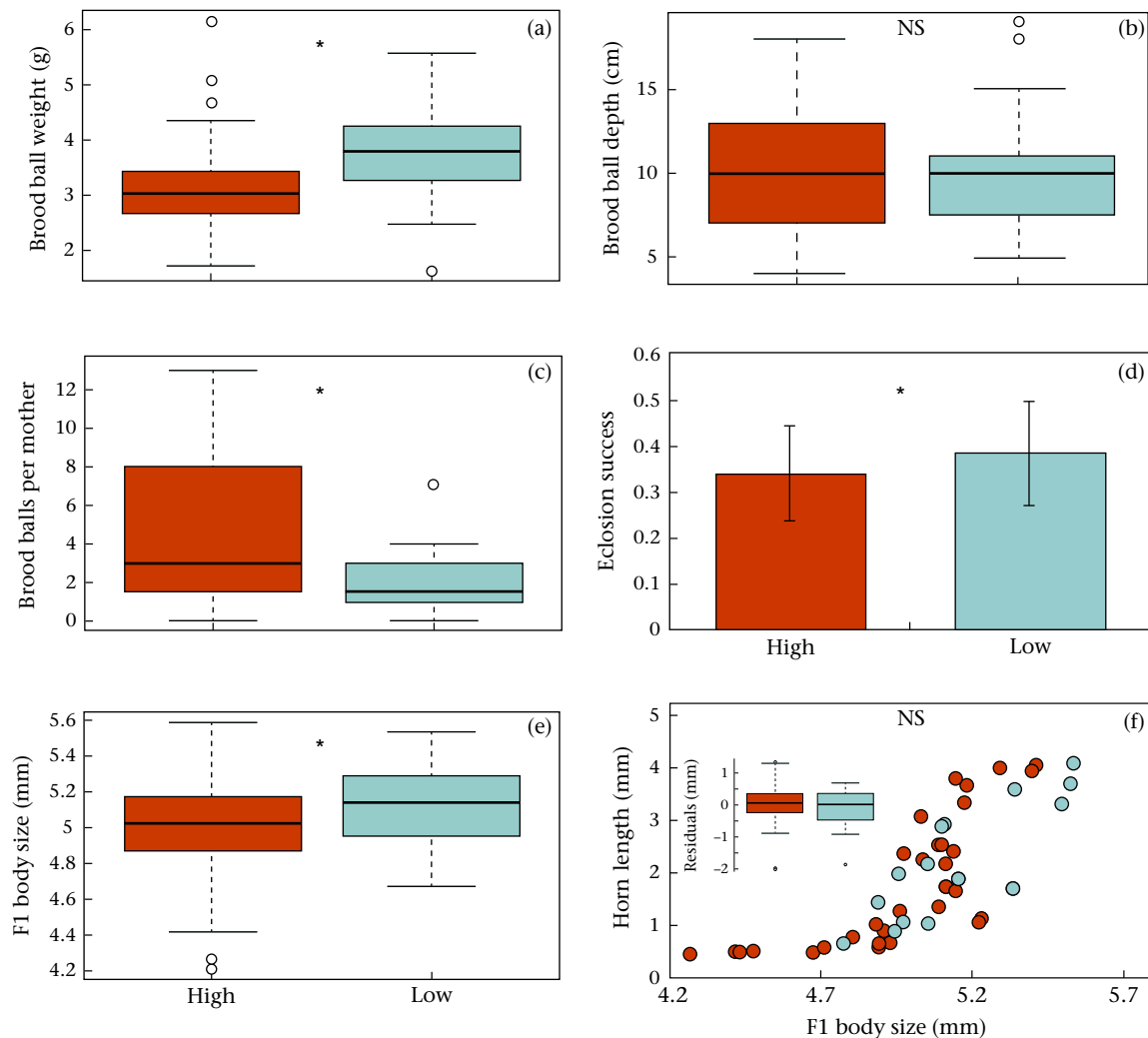
In this study, we sought to investigate whether ancestral plasticity in response to population densities may have contributed to the rapid divergence between exotic *Onthophagus* populations. To do so we exposed adult *O. taurus* collected from within the native range of the species to high and low adult densities, and measured the effects of this treatment on six traits previously documented to have diverged among derived populations that exhibit dramatic population density differences in the field. We focused on two maternal behavioural traits (brood ball weight and brood ball burial depth), two life history traits (brood ball number and emergence success) and two offspring morphological traits (body size and relative horn length). Our results provide mixed support for the hypothesis that ancestral plasticity may bias rapid responses to selection.

### Maternal Behavioural Traits

#### Brood ball weight, but not burial depth, was reduced in response to high density

First, we investigated whether brood ball weight and brood ball burial depth, two measures of maternal investment, differed between females exposed to low and high densities as adults. Recall that previous work established that Western Australian (high-density) populations bury relatively heavier brood balls at relatively shallower depths than their eastern United States (low-density) counterparts, and that these divergences are maintained in a common garden environment (Beckers et al., 2015; Macagno et al., 2016). In contrast, in our experiment, *O. taurus* females exposed to high densities produced significantly lighter brood balls than those exposed to low densities ( $\chi^2_1 = 4.023$ ,  $P = 0.045$ ; Fig. 1a), while brood ball burial depth was unaffected ( $\chi^2_1 = 0.09$ ,  $P = 0.765$ ; Fig. 1b).

We also recovered a marginally significant effect of offspring sex (Appendix, Fig. A1), as well as a significant interaction effect between offspring sex and maternal size (Appendix, Table A1); mothers tended to invest more in brood balls that resulted in male



**Figure 1.** Effect of low and high adult density on maternal behavioural traits (a: brood ball weight; b: brood ball depth; see Appendix, Tables A1, A2), life history traits (c: number of brood balls per mother; d: offspring eclosion success) and offspring morphological traits (e: adult body size; f: horn length–body size relationship; see Appendix, Tables A3, A4). Inset in (f) is a box plot of horn length residuals calculated as the difference between observed and expected horn lengths for a given body size of male offspring from parents exposed to high (■●) or low (□○) densities. Box plots show 25% and 75% quartiles (boxes), medians (lines dividing the boxes), outermost values excluding outliers (whiskers) and outliers (circles). \* $P < 0.05$ .

offspring compared to female offspring ( $\chi^2_1 = 3.773$ ,  $P = 0.052$ ), and this effect increased with maternal size ( $\chi^2_1 = 4.155$ ,  $P = 0.042$ ).

### Life History Traits

*At high adult densities, mothers produced more brood balls, but offspring showed reduced emergence success*

We then asked whether females exposed to higher densities produced more brood balls with increased emergence success, as is characteristic of Western Australian *O. taurus* populations (Beckers et al., 2015). Recall that when reared under common garden conditions, brood ball number remains elevated in Western Australian populations compared to (low-density) eastern United States populations for multiple generations, while emergence success equilibrates after one generation (Beckers et al., 2015). Matching predictions, we found a significant increase in brood ball number when mothers were exposed to high densities (likelihood ratio:  $\chi^2_1 = 4.868$ ,  $P = 0.027$ ; Fig. 1c). In contrast, and opposite to our prediction, the emergence success to adulthood was significantly lower for offspring from mothers that experienced high densities as adults ( $P = 0.023$ ; Fig. 1d). Furthermore, we recovered a significant interaction between maternal size and density on offspring survival; the negative effect of maternal density exposure on offspring emergence success increased with maternal size ( $P = 0.0243$ ).

### Offspring Morphological Traits

*Offspring body size decreased in response to maternal high density exposure, but relative horn length was unaltered*

Lastly, we investigated whether maternal exposure to high or low adult densities affected two critical morphological traits in their offspring: body size and relative male horn length. Recall that compared to low-density eastern United States populations, high-density Western Australian populations exhibit lower mean body sizes and relatively smaller horns for a given body size, manifest in an increase in the population-wide body size threshold separating alternate male morphs. Furthermore, these differences are maintained across several generations in common garden experiments (Beckers et al., 2015; Moczek et al., 2002). We therefore hypothesized that exposure to high densities would result in decreased offspring size, reduced relative horn length, and thus an increase in the body size–horn length threshold. Our hypothesis received partial support; we found a significant decrease in the body sizes of offspring of mothers exposed to high densities ( $\chi^2_1 = 4.226$ ,  $P = 0.04$ ; Fig. 1e) but failed to find a significant effect on relative horn length and the corresponding body size–horn length threshold (Fig. 1f).

We also found that maternal size ( $\chi^2_1 = 10.53$ ,  $P = 0.001$ ) and brood ball weight ( $\chi^2_1 = 5.845$ ,  $P = 0.016$ ; Appendix, Table A3) affect offspring body size, paralleling previously published findings (Hunt & Simmons, 2002a) as well as a significant positive interaction between the two ( $\chi^2_1 = 5.202$ ,  $P = 0.023$ ). Furthermore, we found significant interactions between density and maternal size ( $\chi^2_1 = 5.542$ ,  $P = 0.019$ ) and brood ball weight ( $\chi^2_1 = 8.419$ ,  $P = 0.004$ ), respectively, such that offspring derived from mothers exposed to high densities were more affected by these factors (i.e. they suffered more from small maternal sizes and low brood ball weight) than those derived from mothers exposed to low densities.

## DISCUSSION

The role of phenotypic plasticity in evolutionary diversification remains controversial (Laland et al., 2014; Moczek et al., 2011; Pfennig et al., 2010; Whitman & Agrawal, 2009). Traditionally,

plasticity has been viewed as a product of adaptive evolution, enabling organisms to maintain high fitness in the face of fluctuating selective conditions (reviewed in Snell-Rood, Van Dyken, Cruickshank, Wade, & Moczek, 2010). However, current theoretical and empirical work has indicated that plasticity might also facilitate the colonization of novel habitats, or delay extinction in the face of drastic environmental change, two effects that have the potential to feed back and shape subsequent evolutionary trajectories (reviewed in Pfennig et al., 2010). Finally, recent work has begun to explore the role of pre-existing, ancestral plasticity in biasing or facilitating subsequent genetic differentiation, perspectives often subsumed under the plasticity-first hypothesis (Levis & Pfennig, 2016; Schlichting & Wund, 2014; West-Eberhard, 2003).

Critical support for the potential of plasticity in shaping evolutionary trajectories derived initially from laboratory studies (e.g. Cowen & Lindquist, 2005; Dworkin, 2005; Queitsch et al., 2002; Rajakumar et al., 2012; Rutherford & Lindquist, 1998; Sikink et al., 2014; Suzuki & Nijhout, 2006; Waddington, 1953; Walworth et al., 2016), as well as increasingly from studies on natural populations (e.g. Aubret & Shine, 2009; Diggle & Miller, 2013; Ledón-Rettig et al., 2008; Losos et al., 2000; Schlichting & Wund, 2014; Susoy, Ragsdale, Kanzaki, & Sommer, 2015). Collectively, these studies present diverse data consistent with the hypothesis that ancestral plasticity guided phenotypic differentiation in several taxa. However, relatively few studies have examined the potential of plastic responses in shaping the very early stages of population differentiation (Badyaev, 2009; Badyaev, Potticary, & Morrison, 2017; Fischer, Ghalambor, & Hoke, 2016; Huizinga, Ghalambor, & Reznick, 2009; Levis, Serrato-Capuchina, & Pfennig, 2017; Rohner et al., 2013; Scoville & Pfrender, 2010). Furthermore, it is at present unclear whether plasticity-mediated differentiation may be more likely for certain trait classes than others (Levis & Pfennig, 2016).

Here we take advantage of the rapid recent divergence between exotic dung beetle populations of the bull headed dung beetle *O. taurus* in Western Australia and the eastern United States, respectively. Both introductions derive from a common ancestral population native to the Mediterranean region, including Spain (Moczek & Nijhout, 2003). Derived populations in Western Australia and the eastern United States exhibit strikingly different densities and commensurate levels of mate and resource competition (Moczek, 2003), and have undergone rapid heritable divergence in a diversity of morphological, behavioural, physiological and life history traits in directions consistent with adaptive differentiation (hormone physiology: Moczek & Nijhout, 2002; ovarian maturation: Macagno, Beckers, & Moczek, 2015; diverse life history traits: Beckers et al., 2015; horn allometries: Moczek & Nijhout, 2003; genital morphology: Macagno et al., 2011; tibial shape: Macagno et al., 2016). Using beetles derived from the ancestral Mediterranean range, and focusing on two maternal behavioural traits, two life history traits and two offspring morphological traits, we examined the role of ancestral plasticity in mediating rapid divergences in descendent exotic populations. Specifically, we asked whether exposure to low or high levels of adult densities in laboratory mesocosms is sufficient to induce phenotypic differences in these traits in mothers or their offspring and, if so, whether plastic responses occurred in a direction that matched evolved differences seen among derived exotic populations.

We find that differences in adult densities indeed possess the potential to induce plastic responses in a subset of traits. However, which trait was affected and, in what direction, varied and had to be determined on a trait-by-trait basis. Furthermore, we find partial support for the hypothesis that ancestral plasticity may precede and bias rapid responses to selection in at least one morphological trait (offspring body size) and one life history trait (brood ball

number), while two other traits (brood ball weight, emergence success) exhibited significant plasticity yet in a direction opposite to divergence patterns observed in derived populations. Below we discuss the most important implications of our results.

Our treatments failed to reveal a significant plastic response in two of six traits studied: maternal brood ball burial depth and the body size–horn length scaling relationship of male offspring, respectively. This lack of responsiveness to adult densities could reflect the general developmental robustness of both traits and their corresponding insensitivity to variation in adult densities. In fact, a lack of allometric responsiveness was also detected in a previous study that exposed the derived Western Australian population to varying densities (Buzatto, Tomkins, & Simmons, 2012). Alternatively, lack of plasticity in both traits may reflect limitations of our experimental design. First, using field-collected individuals hindered our ability to precisely control age or previous experience in the natural field site. We took this approach because two earlier pilot experiments using F1 and F2 generations reared from two different collection sites within the native range of this species resulted in such severe reduction in fecundity regardless of treatment that the experiment had to be aborted in each case. We thus cannot exclude the possibility that both traits respond plastically to density variation, but this response was masked by early adult life experiences uncontrolled for in our experiment. Second, our experiment only manipulated adult density and did so over a relatively short period during adult life prior to oviposition. In natural populations additional factors likely covary with adult density, such as adult nutritional status due to food availability. Therefore, we cannot exclude the possibility that interactions between population density and other environmental factors may be able to induce plastic responses in these two traits in natural populations, yet failed to do so in our experiment. Such interactions between population density and other environmental factors may also shape the nature of plastic responses observed in the remaining four traits; thus, the exact responses observed in the present study may vary depending on the precise environmental cue, or set of cues, experienced.

Despite these limitations, our density treatments elicited significant plasticity in the four remaining traits: number and weight of brood balls produced by mothers and the emergence success and body size of their offspring. Two of the traits examined in this study responded in a direction matching evolved divergences between natural populations. Mothers obtained from the ancestral Spanish range of the population produced more brood balls when exposed to high densities, similar to the higher number of brood balls produced by mothers obtained from the derived Western Australian population that typically experience high densities in nature. Offspring body size also decreased when mothers from Spain were exposed to high densities, again matching the consistently smaller body size observed among Western Australian beetles. Combined, these results support the hypothesis that for these two traits, evolution by genetic accommodation facilitated by pre-existing plasticity may have enabled subsequent canalized divergences among geographically isolated descendent populations (Pfennig et al., 2010). Interestingly, while we did not directly test whether derived Western Australian and eastern United States populations may have retained plasticity for either trait, previous studies have found that Western Australian beetles maintain relatively high brood ball numbers and low body sizes, even when reared under common garden, 'low-density' conditions (Beckers et al., 2015). This further supports a plasticity-first scenario, whereby ancestral plasticity present at the founding of at least one population (Western Australian) has since given way, via genetic accommodation, to the canalized differences observed today.

In contrast to body size and brood ball number, two of our traits exhibited plasticity in the opposite direction given what we had predicted based on ecological and phenotypic divergences among exotic populations. Both emergence success among offspring and average brood ball mass decreased on average when mothers were exposed to high adult densities, in contrast to the elevated emergence success and brood ball mass observed for Western Australian compared to eastern United States populations (Beckers et al., 2015). These results recall recent findings by Ghalambor et al. (2015) in Trinidadian guppies, *Poecilia reticulata*, whose plastic changes in brain gene expression in response to low predator conditions are most commonly in a nonadaptive direction, opposite to the observed evolved changes in natural populations. More generally, these results match theoretical predictions that because plasticity in response to a novel environment cannot anticipate adaptive variation, phenotypic responses may often be neutral or nonadaptive (Moczek, 2007). Note, however, that for ancestral plasticity to bias or facilitate adaptive evolution, it is not strictly necessary that average ancestral reaction norms generate phenotypic variation in a direction also favoured by selection, as long as variation among ancestral reaction norms encompasses at least some novel phenotypic variation that selection can then promote.

Behavioural plasticity has been hypothesized to play a critical guiding and enabling role in evolutionary diversification (Foster, Wund, & Baker, 2015; Foster, Wund, Graham et al., 2015), yet relatively few studies to date have been able to interrogate the potential interplay between behavioural plasticity and species or population divergence (Badyaev, 2009; reviewed in ; Foster, Wund, & Baker, 2015; Foster, Wund, Graham et al., 2015). Because many organisms are likely to confront environmental changes first and foremost through adjustments in their behaviour, addressing the role of behavioural plasticity in short-term as well as long-term evolution remains critical to better understand why and how population differentiation and species radiations unfold the way they do (Foster, Wund, & Baker, 2015; Foster, Wund, Graham et al., 2015), an objective made all the more urgent in a world marked by rapid anthropogenic changes (Campbell, Adams, Bean, & Parsons, 2017). In this study, we addressed three traits (brood ball weight, number, depth of burial) that reflect important aspects of maternal care and investment into offspring: each brood ball is a densely packed construct whose weight exceeds that of an average adult female by more than an order of magnitude. Brood balls are also constructed in tunnels approximately 8–12 cm in depth dug into compact soil by a beetle, which itself is merely about the size of a coffee bean. Larger brood balls provide more food for developing larvae (Moczek, 1998), while deeper burial ensures a more isothermic and less stressful developmental environment (Snell-Rood, Burger, Hutton, & Moczek, 2016), but the construction of both larger brood balls and deeper tunnels takes time (on the order of hours; Hunt & Simmons, 2002b), which trades off with the number of brood balls a given female may be able to produce. This trade-off is likely altered significantly under circumstances of extremely high resource competition as in Western Australian populations. In the present study, both brood ball weight and number responded plastically to our simulated high conspecific densities. We further broadened our focus to address plasticity in other types of traits, following the call of Foster, Wund, & Baker (2015), Foster, Wund, Graham et al. (2015) that studies must integrate multiple, diverse aspects of the phenotype to better understand how and why animals respond to environmental change the way they do, and showed that offspring body size (a morphological trait) and eclosion success (perhaps best understood as a developmental phenotype) also exhibit measurable plasticity in response to adult densities experienced by mothers. On one side, these results demonstrate that diverse trait classes possess the ability to respond

to environmental changes in ways that may bias subsequent population divergence. On the other, these results raise the possibility that disparate traits may interact in both their developmental and evolutionary responsiveness to environmental change. For example, *Anolis* lizards reared under narrow versus broad substrates differ in leg length, and this difference is mirrored by species that use broad surfaces compared to species that rely on narrow surfaces, consistent with a role of developmental plasticity in mediating the early stages of morphological diversification (Losos et al., 2000). At the same time, introduction of a terrestrial predator manifests in a behavioural response in some individuals to shift from a terrestrial habitat (broad substrate) in favour of an arboreal habitat (narrow substrate), suggesting that behavioural plasticity may constitute the initiating step in plasticity-mediated divergence, and that behavioural plasticity and developmental plasticity may build on each other in their contributions to subsequent morphological divergence (Losos et al., 2000, Losos, Schoener, Spiller, & 2004). Our results suggest a similar scenario where a maternal behavioural shift to produce lighter brood balls in the face of severe resource competition may initially yield, via larval developmental plasticity, reduced offspring body size, which may then become genetically stabilized in populations subject to consistently elevated levels of intra- and interspecific resource competition, resulting over time in significant divergences in mean adult body sizes between populations.

In summary, our study suggests that phenotypic plasticity may facilitate population-wide differentiation across diverse trait types. One of the reasons cited for the lack of consensus on the role of plasticity in evolutionary diversification is the wide diversity of responses that plasticity itself encompasses (Ghalambor et al., 2007). While plastic responses are clearly diverse (almost all traits are plastic in some shape or form on at least some levels of biological organization), this diversity may itself be evolutionarily significant: here we demonstrate that very different traits, despite their likely disparate genetic and developmental underpinnings, all share the ability to respond plastically to the same environmental factor, adult population density. This raises the possibility that plasticity may, at least in some contexts, mediate the early stages of population-wide differentiation of entire suites of traits regardless of their specific ontogenies, thereby fuelling rapid population-wide differentiation (Pfennig et al., 2010). Note that Western Australian and eastern United States populations have been in existence only for approximately 50 years or less (or approximately 100 generations), but have managed to diverge in the six focal traits studied here as well as several others (Beckers et al., 2015; Macagno et al., 2011; Macagno, Beckers, & Moczek, 2015, 2016; Moczek & Nijhout, 2002) to a degree normally only seen among closely related species. If the interpretations of our results are correct, this raises the possibility that at least in this species, plasticity-led evolution may have played a critical role in enabling extraordinarily rapid divergences in response to a single ecological parameter.

## CONFLICT OF INTEREST

We have no conflict of interest.

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## Appendix

**Table A1**

Chi-square values used to compare generalized linear mixed models for brood ball weight

Fixed effect	$\chi^2$	P
Density	4.023	0.045*
Maternal body size	10.919	0.001**
Sex	3.773	0.052
Maternal size $\times$ density	0.0264	0.871
Sex $\times$ density	0.1668	0.683
Maternal body size $\times$ sex	4.155	0.042*

Models include replicate container as a random factor and brood ball weight as the response variable, with population density treatment, maternal size, offspring sex and their interactions as fixed variables. \* $P < 0.05$ ; \*\* $P < 0.001$ .

**Table A2**

Chi-square values used to compare generalized linear mixed models for brood ball burial depth

Fixed effect	$\chi^2$	P
Density	0.09	0.765
Maternal body size	0	<0.001**
Sex	3.162	0.075
Maternal size $\times$ density	0	1
Sex $\times$ density	2.226	0.136
Maternal body size $\times$ sex	3.001	0.083

The models include replicate container as a random factor and brood ball burial depth as the response variable, with population density treatment, maternal body size, offspring sex and their interactions as fixed factors. \*\* $P < 0.001$ .

**Table A3**

Chi-square values used to compare linear mixed models for offspring body size

Fixed effect	$\chi^2$	P
Density	4.226	0.04*
Maternal body size	10.53	0.001**
Sex	0	1
Brood ball weight	5.845	0.016*
Maternal size $\times$ density	5.543	0.019*
Sex $\times$ density	4.688	0.096
Density $\times$ brood ball weight	8.419	0.004*
Maternal body size $\times$ sex	1.011	0.315
Maternal size $\times$ brood ball weight	5.2023	0.023*
Sex $\times$ brood ball weight	0.185	0.668

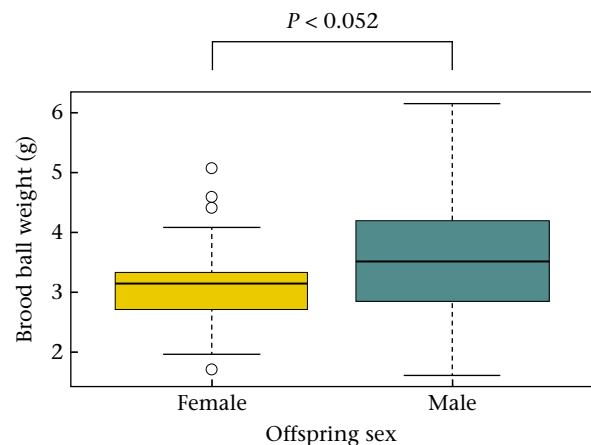
The models include replicate container as a random factor and offspring body size as the response variable, with population density treatment, maternal body size, offspring sex, brood ball weight and their interactions as fixed variables. \* $P < 0.05$ ; \*\* $P < 0.001$ .

**Table A4**

Parameter mean comparison of body size–horn length allometries across treatments

Parameter	P
Amplitude	1.0000
Slope	0.9096
Inflection point	0.8795
y intercept	0.9372

Welch's *t* tests were used to compare parameter means of a nonlinear regression fitted to each of the treatment groups.



**Figure A1.** Relationship between brood ball weight and offspring sex.