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A combination of developmental plasticity, parental effects, and genetic differentiation mediates divergences in life history traits between dung beetle populations

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SUMMARY The dung beetle, *Onthophagus taurus,* was introduced <50 years ago from its native Mediterranean range into Western Australia (WA) and the Eastern United States (EUS). The intensity of intra- and interspecific competition for dung as a breeding resource is substantially higher in WA. First, we tested whether differential resource competition in the two exotic ranges is associated with divergences in life history traits, which impact on resource use. We predicted that high levels of resource competition in WA should favor females that produce brood balls more efficiently and of altered size, and produce offspring more readily when a breeding opportunity arises. Furthermore, we predicted that larvae from WA populations may have evolved more efficient development and thus exhibit higher eclosion success, shorter development time, and altered body size under standardized conditions. Second, we examined the likely developmental mechanisms underlying these divergences, that is, genetic differentiation, developmental plasticity, or parental effects in a common garden experiment. Field-collected EUS and WA populations significantly differed, as predicted, in most of the traits examined. However, these differences are facilitated by a complex combination of proximate mechanisms. Developmental plasticity and (grand) parental effects mediated differences related to reproductive performance, whereas genetic differentiation mediated differences in the duration of larval development. Our study highlights that population divergences can be the product of a patchwork of proximate mechanisms, with each mechanism adjusting different traits in a way that the resulting composite phenotype may be better suited to its competitive environment.

INTRODUCTION

The causes, mechanisms, and consequences of population differentiation are of fundamental importance in evolutionary biology. Several basic, proximate mechanisms exist that enable populations to diverge in phenotype expression, each with unique implications for the evolutionary trajectory of a given population. On one extreme is allelic, or genetic, differentiation between populations, which arises via differential fixation of genetic variants driven by selection, mutation, or genetic drift. Population differentiation via genetic divergence is greatly influenced by the effective population size and gene flow, constrained by the amount of standing genetic variation in a population and mutation rates, and generates adaptive responses to novel environmental conditions at a comparatively slow pace (Pfennig et al. 2010).

On the other extreme is phenotypic, or developmental, plasticity, defined as the ability of individuals to express different phenotypes in response to changes in environmental conditions during their lifetime (West-Eberhard 2003). Plastic responses to environmental conditions may be simple or complex, continuous or discrete, reversible or irreversible, and adaptive or not (Schlichting and Pigliucci 1995). In contrast to genetic diversification, developmental plasticity enables individuals to respond to changes in the environment within their lifetime. Therefore, within a single generation, developmental plasticity can enable phenotypic divergence between populations without any underlying genetic differentiation. Furthermore, depending on the circumstances, this plasticity may either retard or accelerate genetic differentiation between populations (West-Eberhard 2003; Price et al., 2003; Crispo 2008; Pfennig et al. 2010).

Population differentiation may also be facilitated via parental effects. Parental effects enable trans-generational developmental plasticity, whereby the environment experienced by parents (most commonly mothers) influences offspring phenotypes, often in an adaptive manner (review in Mousseau and Fox 1998). Parental effects may exert their influence via parental behaviors (e.g., parental care, food provisions; Hunt and Simmons 2000), endosymbionts (e.g., gut microbiomes; Estes et al. 2013), or cytoplasmic factors (e.g., lipids, hormones, RNA transcripts; Mousseau and Fox 1998; Donohue and Schmitt

1998). In contrast to (within-generation) developmental plasticity, parental effects are the responses of offspring to environmental conditions experienced by their parents, that is, between-generation developmental plasticity (Mousseau and Dingle 1991). In some cases, the trans-generational nature of parental effects even extends beyond the immediate filial generation and can affect phenotype expression in subsequent generations, resulting in grand-parental effects (e.g., aphids: Blackman 1975). Parental effects and developmental plasticity have in common that both mechanisms enable rapid phenotypic responses to disparate environmental conditions, and that both can lead to the persistent expression of phenotypic differences across populations if the environmental conditions are recurrent. In this case the phenotype is said to be canalized by the environment (sensu Pfennig and Pfennig 2012) and is indistinguishable from genetic canalization without further investigation. Thus, consistent phenotypic differentiation among disparate populations may be explained by diverse mechanisms and distinguishing among these mechanisms is critical for evaluating their developmental and evolutionary origins (e.g., adaptive alleles or reaction norms) and evolutionary consequences (e.g., persistence or lability in the face of short-term environmental fluctuations).

Here, we investigate the proximate mechanisms of divergences in life history traits among exotic populations of the dung beetle *Onthophagus taurus*. This species reproduces by excavating tunnels underneath dung pads (mostly from cow, horse, sheep; Fincher and Woodruff 1975; Hanski and Cambefort 1991; Moczek 1996) and provisioning dung for offspring in the form of so-called 'brood balls' near the blind end of each tunnel (Halffter and Edmonds 1982). Each brood ball contains a single egg and represents the entire food available for a larva to complete all of larval growth and metamorphosis (Moczek and Emlen 1999). Thus, availability of fresh dung and tunneling space are critical for reproduction in *O. taurus*. *Onthophagus taurus* was introduced in the early 1970s from its native Mediterranean range into Western Australia (WA; Tyndale-Biscoe 1996) and the Eastern United States (EUS; Fincher and Woodruff 1975). Present-day EUS and WA populations differ substantially in the intensity of intra- and interspecific competition for breeding opportunities, that is, the availability of dung and tunneling space. Specifically, densities of *O. taurus* and other species that compete with *O. taurus* for dung and tunneling space are very low in the EUS, such that most naturally occurring dung pads contain none to only a few individuals and thus remain unutilized and decay above ground (Moczek 2003). In contrast, densities of *O. taurus* and competing species in WA populations are extremely high, such that most dung pads contain many hundreds to even thousands of beetles and are actively removed from pastures through the burial activity of *O. taurus* and its competitors, often over the course of hours (Moczek 2003). Male *O. taurus* are polyphenic and develop nutrition-dependent hornless (minor) and horned (major) morphs, separated by a sharp body size threshold (Moczek and Emlen 2000). Previous work suggested that the disparity in competition for breeding opportunities in EUS and WA populations has resulted in rapid, substantial, and heritable divergence in this male size threshold to a degree that parallels threshold divergences observed among *Onthophagus* species (Moczek 2003).

Here, we test whether differential resource competition between EUS and WA populations may have led to divergences in life history traits related to resource use, as well as the proximate developmental mechanisms underlying these divergences. Specifically, we predicted that high levels of resource competition in WA populations should favor females that (1) are more efficient in the production of brood balls (i.e., produce brood balls faster), allowing females to monopolize resources before they become depleted by competitors. Second, we predicted that limited breeding resources in WA may have selected for females that tradeoff brood ball number and size differently than EUS females (reviewed in Clutton-Brock 1991), that is, WA females may either (2a) produce larger, but fewer brood balls or, alternatively, (2b) produce more brood balls, but at a smaller size. Next, we predicted that heightened resource competition in WA may result (3) in a change in average adult body size in WA beetles because WA larvae may be able to complete development with fewer resources by eclosing into smaller adults (Shafiei et al., 2001) and/or WA mothers invest differently in offspring brood balls (see second prediction). More specifically, since brood ball size is directly and positively related to adult body size (Moczek and Emlen 1999), we predicted that offspring body size may track maternal investment into brood ball size, which could affect offspring size in either direction (see second prediction). Furthermore, we predicted that the very ephemeral nature of breeding resources in WA may favor (4) females that are physiologically ready to reproduce as soon as an opportunity to breed arises. Lastly, due to the elevated competition for dung prevalent in WA, we reasoned that WA larvae are likely to more frequently experience suboptimal feeding conditions, which may select for larval genotypes capable of completing development more efficiently (sensu MacArthur and Wilson 1967; Mueller 1988a; but Mueller 1990). We, therefore, predicted that (5) a larger percentage of WA larvae should eclose to adulthood requiring (6) less time to complete larval development compared to EUS larvae. Note that this last prediction also follows from hypothesis 2b, that is, reduced investment into brood balls should also result in a reduction in the time needed to complete larval development.

Next, we tested if any divergences in these life history traits may be the result of genetic differentiation, developmental plasticity, or parental effects as indicated by the persistence or loss of trait divergences between populations over four generations of rearing in a common garden environment (as discussed in Laugen et al. 2002). We predicted that any

divergences in life history traits observed between EUS and WA populations should persist under these rearing conditions if they were genetically canalized, disappear in the first lab generation if they were the result of developmental plasticity, or disappear in subsequent lab generations if they were the result of (grand) parental effects. We find that field-collected populations exhibit significant differences in nearly all life history traits examined, matching most of our initial predictions. At the same time, we find that these differences are underlain by a complex patchwork of genetic differentiation, developmental plasticity, and (grand) parental effects.

MATERIALS AND METHODS

Environmental conditions and natural history of *O. taurus* in exotic ranges

For our experiment, we collected beetles from both introduced ranges of *O. taurus*. Specifically, we collected approximately 400 adults each from pastures near Chapel Hill, NC in the eastern U.S. and Busselton in the south-western region of Australia (i.e., a total of 800 individuals). Both locations are situated in mild temperate climate zones with roughly similar environmental conditions (e.g., temperatures and humidity) on opposite sides of the Equator (sensu Köppen classification; Chen and Chen 2012). In both regions, *O. taurus* exhibits an obligate winter diapause, two reproductive generations per year, and is most commonly found on cow pastures where it feeds and reproduces primarily on cow dung. Similarly, in both regions *O. taurus* competes with dung breeding flies for access to dung, however, intraspecific resource competition, as well as competition imposed by other dung beetle species, is highly asymmetric: local densities of both *O. taurus* and competing dung beetle species are two to three orders of magnitude higher in WA compared to EUS populations (Moczek 2003). In summary, both WA and EUS populations of *O. taurus* inhabit roughly comparable ecological niches, but differ prominently in the intensity of intra- and interspecific resource competition, even though the existence of additional differences in other biotic and abiotic factors between the two populations cannot be fully excluded.

Animal husbandry—general

Animals were kept in an environmental chamber at 24°C constant ambient temperature, approximately 40% humidity, and a light:dark cycle of 16:8 h and were maintained following procedures previously described in Moczek and Nagy (2005). Prior to our breeding experiments (see below), females were kept with males at densities of approximately 60–150 individuals in colony containers for at least 2 weeks after adult eclosion to reach reproductive maturity and to provide opportunities for insemination. These values approximate intermediate densities that range between the very low natural densities observed in most locations in the EUS (where most dung pads contain none to few individuals; Moczek 2003) and the several orders of magnitude higher densities typical of WA populations (where dung pads commonly contain hundreds to thousands of individuals; Moczek 2003). We bred populations in a common garden environment for four consecutive generations that are referred to as F_0 to F_4 parental generations. Animal breeding took place in two contexts, (1) to maintain populations at comparable densities (non-experimental breeding) and (2) to assay life history traits (experimental breeding), both of which are described next.

Non-experimental breeding

To maintain populations for each generation at comparable densities, to minimize inbreeding, and to exclude the possibility of unintentional artificial selection imposed by some of our assays, we bred non-experimental beetles, drawn separately and at random from each colony, in parallel to our experimental breeding. Thus, each generation of laboratory colonies was composed of animals that were reared in the context of our experiment as well as additional rearing efforts to maintain colony size. Averaged across all generations, non-experimental breeding contributed about 650 brood balls to each generation in addition to the approximately 250–350 brood balls produced in each generation by our experiments. Emerged beetles of both breeding procedures were combined in multiple colony containers and experimental animals for each generation were drawn at random from these containers.

Experimental breeding

At the beginning of each round of breeding, we placed single females from each population/generation in cylindrical, light-impermeable breeding containers (pasta keeper 'HS-027', 1.5 L, 27 cm high, 7.2 cm diameter) filled to a height of approximately 20 cm with a moist soil/sand mixture (2:1 ratio). Each female was provided with approximately 200–250 g of defrosted, moist, organic cow manure. Containers were covered with window screen and perforated black plastic foil to enable ventilation and prevent escape. After 4 days, we collected each female and all brood balls that she had produced during this period. At this stage most of the initially provided dung remained unused, confirming that females were not resource limited during the preceding 4-day period. We excluded all data from females that died during the 4-day breeding period. All females were killed after experimental breeding and preserved in ethanol. We chose to quantify female breeding performance over 4 days because breeding opportunities (i.e., colonizable dung pads) in WA are very ephemeral, that is, they persist only for several hours to few days rather than weeks as found in the EUS (Moczek 2003; OMB and APM, personal observations). Thus, differences in breeding behavior as outlined above are

predicted to take place during the first few days when dung is available for both populations. Unfortunately, no data are available that would allow us to approximate female longevity in the field, or the possible existence of age-related changes in fecundity, egg size, or brood provisioning.

Adult body size

We measured adult thorax width as an estimate of body size (Emlen 1994; see Kijimoto et al., 2012 for details). To get a more robust population estimate of female body size and to include male sizes in the analyses for these generations, we measured additional males and females drawn at random from the colonies.

Reproductive performance

We compared reproductive performance of EUS and WA females using three approaches. First, we assessed the total number of brood balls that adult females were able to produce during a 4-day breeding period under *ad libitum* resource availability (see above). Second, we determined the percentage of females that produced at least one brood ball during each breeding bout, indicating female readiness to reproduce when the opportunity arises. Lastly, we determined the proportion of adult offspring emerging from brood balls during each breeding bout as a measure of larval eclosion success under laboratory conditions.

Maternal brood provisioning

After extracting brood balls from breeding containers (see above), we carefully removed soil from each brood ball using a paint brush (Yasumoto NB-28, size 6). After cleaning, we weighed brood balls to the closest 0.0001 g using a Mettler Toledo (AL 54) scale. After weighing, we placed each brood ball in a separate cylindrical plastic cup (Solo P101M, 30 ml, 4 cm tall, 1.5 cm diameter), covered it with a moist soil/sand mixture and sealed each cup with a plastic lid to reduce desiccation. We stored brood balls at 24°C constant ambient temperature and approximately 40% humidity in an environmental chamber.

Duration of larval development

We determined total developmental time (from egg to adult) by daily checking the plastic cups containing individual brood balls for adult emergence. Thus, total developmental time was defined operationally as the time period between the day at which brood balls were placed into individual plastic cups and until the day of adult emergence. Note that this measurement slightly underestimates the duration of development, yet does so equally for both populations, because oviposition may have occurred on any of the 4 days of the breeding experiment. Furthermore, the concordant difference in overall developmental time (this measurement) and the length of the third larval instar (see below) between populations strongly suggest that both populations do not differ in the onset of brood ball production during the 4-day experimental period. On the day of emergence, we determined sex, thorax width, and body mass of offspring as outlined above.

To obtain a separate and more specific estimate of the duration of larval development, we compared the duration of the third (=last) larval instar in a subset of animals. The third instar is the main feeding stage of onthophagine larvae (Moczek and Nijhout 2003) and its duration is scored as the number of days between the second-to-third instar molt and pupation. To measure the 3rd instar duration, we transferred second-instar F_1 larvae (i.e., offspring of the field-collected F_0 parents) from their brood balls into 12-well plates with dung as described in (Shafiei et al. 2001). We inspected larvae daily and recorded the date of molt to the 3rd instar as well as the date of pupation.

Statistical procedures

We used measurements from field collected (F_0) animals to test whether WA and EUS populations have diverged in life history traits. Data from subsequent laboratory generations were then used to test which proximate mechanism (i.e., developmental plasticity, parental effects, genetic canalization) may have resulted in the observed population differences. We used two-way ANOVAs and ANCOVAs and post-hoc Tukey tests to compare thorax width and brood ball mass between populations. We used Generalized Linear Models with Poisson distributions and post-hoc Tukey tests to compare the number of brood balls produced and larval developmental time between populations. The variance and distribution of all data sets justified the use of parametric testing procedures. All models had 'population' and 'generation' and the interaction between 'population and generation' included as fixed effects. For thorax width, we included 'sex' and all interactions between 'sex', 'population', and 'generation' in the model. We removed non-significant interactions from the model in a stepwise manner. The reduced model is presented in the results. Note that males and females within each generation and population did not differ significantly in thorax width. A previous study has shown (Hunt and Simmons 2000) and our initial analyses confirmed, that maternal size is correlated with brood ball number and size. We included therefore 'maternal thorax width' as a fixed effect for comparisons of brood ball numbers and brood ball mass. As females typically produced multiple brood balls, we included the identity of the mother producing each brood ball as a random factor in the model that compares the brood ball masses between populations.

Our analysis of larval development time was complicated by the fact that we lacked data on development duration for the F_3 generation and that we did not know which brood ball gave rise to which adult beetle of the F_4 generation. We were, therefore,

unable to correct for any potential effects of brood ball mass on the duration of larval development for this particular generation and therefore, analyzed larval developmental time in two ways. First, we used a Generalized Linear Model with Poisson distribution for the F_1 and F_2 generations with 'brood ball mass', 'generation', 'population', and 'generation x population' as fixed effects. Second, we repeated this analysis using a Generalized Linear Model with Poisson distribution for the F_1, F_2, and F_4 generations, but without 'brood ball mass' as fixed effect. Both analyses identified the same significant population differences and we, therefore, present the latter Generalized Linear Model in the results section.

We used Fisher's exact tests to compare the proportion of females that produced at least one brood ball as well as the proportion of successfully eclosing adults. We corrected for multiple comparisons using the Bonferroni–Holm correction (Holm 1979). Note that we lacked data for the proportion of eclosing adults for the F_3 generation of both populations. All statistical comparisons were performed using JMP (version 10.0, STATA Corp.).

RESULTS

Adult body size

We predicted that heightened resource competition may result in a change in average adult body size in WA beetles. We found that field-collected animals (F_0) from high-competition WA populations exhibited significantly smaller body sizes than individuals collected from low-competition EUS populations (Table 1 and Fig. 1; post-hoc Tukey HSD: $P < 0.001$). Body size differences between populations persisted in the F_2 and F_4 laboratory-reared generations (F_2 and F_4; both post-hoc Tukey HSDs: $P < 0.001$), but were marginally non-significant in the first ($P = 0.075$) and non-significant in the third lab generation (post-hoc Tukey HSD: $P = 0.926$), respectively. As detailed below, we found no support for the hypothesis that WA females may produce smaller brood balls resulting in smaller WA beetles. Taken together, these results suggest a heritable contribution to body size differences between populations that is independent of maternal provisioning behavior. However, the somewhat inconsistent pattern across generations suggests the existence of additional contributing factors.

Reproductive performance

Next, we compared reproductive performance between populations using three measurements, predicting that elevated resource competition may have selected for increased reproductive efficiency in WA females. First, we compared the number of brood balls produced by individually-reared females over four days under *ad libitum* resource availability. Consistent with our prediction, we found that field collected females from high-competition WA populations (F_0) produced nearly twice as many brood balls compared to females collected from low-competition EUS populations (Table 2 and Fig. 2, post-hoc Tukey HSD: $P < 0.001$). We detected the same substantial difference between common garden-reared F_1 females from the two populations (post-hoc Tukey HSD: $P = 0.010$), but failed to detect a corresponding difference in the F_2 and F_3 generations (Fig. 2; both post-hoc Tukey HSDs: $P = 1.0$). This lack of difference in the last two generations was due to a significant increase in brood ball production in F_2 and F_3 EUS females, whereas brood ball production among WA females remained constant across generations (EUS post-hoc Tukey HSD between F_1 and F_2: $P = 0.018$; EUS F_1 and F_3: $P = 0.004$; WA post-hoc Tukey HSD between F_1 and F_2: $P = 1.0$; F_1 and F_3: $P = 0.913$; Fig. 2).

Second, we compared the proportion of field-collected females (F_0) that produced at least one brood ball during the 4-day breeding period as a measure of readiness to reproduce when the opportunity arises. Consistent with our prediction, we found that 97.1% of WA females produced one or more brood balls compared to only 62.9% of EUS females (Fisher Exact test: $P < 0.001$, adjusted $\alpha = 0.0167$; Fig. 3). This difference persisted through the first two lab generations (both Fisher Exact tests: $P < 0.001$, adjusted $\alpha \geq 0.008$), but was no longer detectable in the F_3 generation (Fisher Exact test: $P = 1.0$). This lack of difference was due to a significant increase in the proportion of EUS females producing brood balls (Fisher Exact

Table 1. Two-way ANOVA comparing thorax width of adult beetles

Fixed effects	DF (factor)	DF (error)	F-ratio	*P*
Population	1	1212	194.08	<0.0001
Generation	4	1212	32.86	<0.0001
Sex	1	1212	6.85	0.009
Population x Generation	4	1212	55.01	<0.0001

Indicated are the degrees of freedom, the degrees of freedom of the error, the F-ratio of the test statistic, and the *P*-value for each factor and interaction of the model.

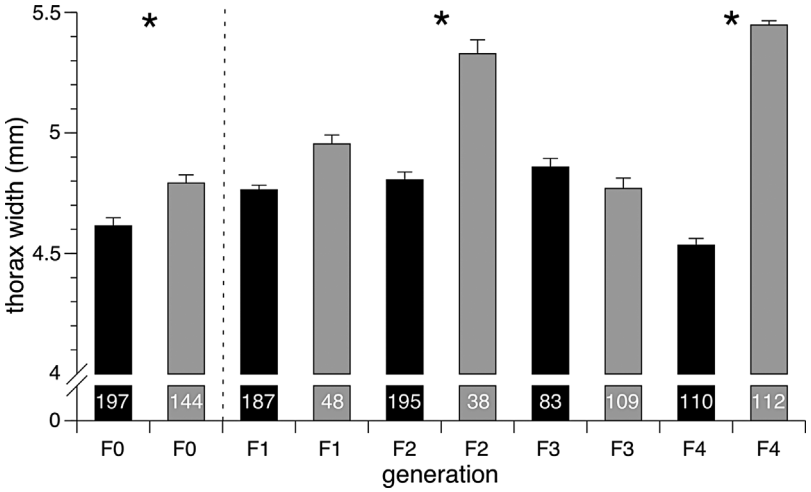

Fig. 1. Average thorax width (+ s.e.m.) of beetles from Western Australian and Eastern U.S. populations. Black bars indicate thorax widths of western Australian beetles and grey bars those of eastern U.S. beetles. Note that male and female thorax widths did not differ significantly within each generation/population combination and were pooled for graphical presentation. Numbers at bottom of each bar indicate sample sizes and asterisks indicate significant differences between populations within generation. Dashed line indicates transition from field-collected (F_0) to laboratory-reared generations (F_1–F_4).

test between F_1 and F_3: $P < 0.001$, adjusted $\alpha = 0.0125$) and a concomitant decrease in the proportion of WA females producing brood balls (Fisher Exact test between F_1 and F_3: $P = 0.009$, adjusted $\alpha = 0.025$) after the second lab generation. These results suggest that the striking reproductive differences observed between EUS and WA females collected in the field or reared under common garden conditions in the first one to two generations may be influenced by parental effects (number of brood balls) or grand parental effects (proportion of females producing brood balls) rather than genetic differentiation or within-generation developmental plasticity.

Lastly, we compared the proportion of WA and EUS brood balls from which adult offspring emerged as a measure of larval eclosion success (Fig. 4). We found that a significantly larger proportion of offspring emerged from brood balls produced by field-collected WA females (89.1%) compared to EUS females (64.4%; Fisher Exact test $P < 0.001$, adjusted $\alpha = 0.0125$), supporting the prediction that elevated resource competition in WA may favor elevated reproductive performance and/or efficiency of WA offspring. However, eclosion success of subsequent lab generations failed to show any differences between populations (Fisher Exact tests: $P \geq 0.384$) partially due to a significant decrease in eclosion success of WA offspring in the F_2 generation (Fisher Exact test between the F_1 and F_2 from WA: $P < 0.001$, adjusted $\alpha = 0.01$; F_1 and F_2 from EUS: $P = 0.71$). These results suggest that differences in eclosion success between WA and EUS populations are the product of developmental plasticity rather than parental effects or canalized genetic differences between populations.

Maternal brood provisioning

Next, we examined the average mass of brood balls produced by EUS and WA females. We predicted that high levels of resource competition in WA favor altered parental investment into individual brood balls, resulting in differential adjustment of the brood ball size and number tradeoff. As previous measurements indicated that WA females produce more brood balls than EUS females, we specifically predicted that WA brood balls should be smaller. Our results strongly reject this hypothesis. Instead, we observed that average brood ball mass did not differ between populations in the F_0 generation (Table 3 and Fig. 5; post-hoc Tukey HSD: $P = 0.998$), whereas in the subsequent F_1 and F_2 laboratory generations, brood balls produced by WA females were significantly heavier than those produced by the

Table 2. Generalized Linear Model comparing the number of brood balls produced by females

Fixed effects	DF (factor)	DF (Pearson)	χ^2	P
Population	1	188	25.96	<0.0001
Generation	3	188	33.42	<0.0001
Maternal size	1	188	13.98	0.0002
Population x Generation	3	188	25.91	<0.0001

Indicated are the degrees of freedom, Pearson's degrees of freedom, the χ^2 value of the test statistic, and the P-value for each factor and interaction of the model.

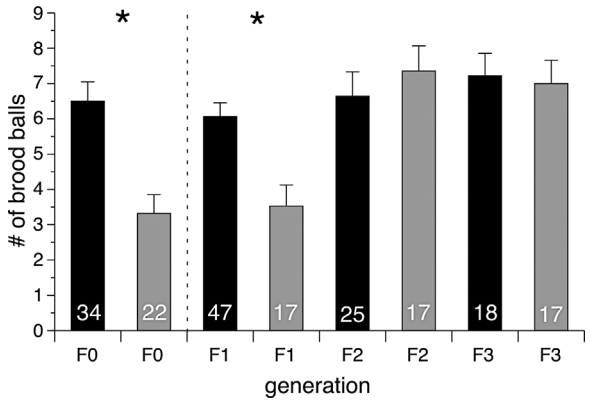

Fig. 2. Average number of brood balls produced per female (+ s.e. m.) over 4 days of breeding. Black bars indicate average brood ball numbers of Western Australian beetles and grey bars those of Eastern U.S. beetles. Numbers at bottom of each bar indicate sample size and asterisks indicate significant differences between populations within generation. Dashed line indicates transition from field-collected (F_0) to laboratory-reared generations (F_1–F_4).

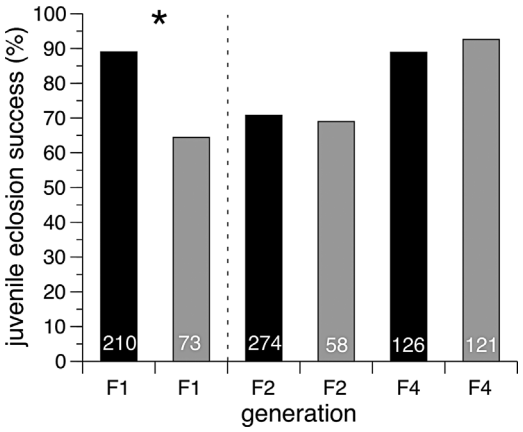

Fig. 4. Eclosion success of adult beetles measured as the percentage of brood balls producing adult offspring. Black bars indicate the eclosion successes of western Australian beetles and grey bars those of eastern U.S. beetles. Numbers at bottom of each bar indicate sample size and asterisks indicate significant differences between populations within generation. Dashed line indicates the transition from offspring of field-collected parents (= F_1 in graph) to generations of offspring of laboratory-reared parents ($F_2 + F_4$).

corresponding EUS females (both post-hoc Tukey HSDs: $P \leq 0.013$). Brood balls exhibited a similar, albeit statistically non-significant mass difference in the same direction in the F_3 generation (post-hoc Tukey HSD: $P = 0.260$). It is not clear whether the significant difference between brood balls produced by EUS F_1 and WA F_1 females was attributable to an increase in the mass of WA brood balls and/or a decrease of the mass of EUS brood balls from the F_0 to the F_1 generation (post-hoc Tukey tests between F_0 and F_1 for WA: $P = 0.092$; and EUS F_0 and F_1: $P = 0.998$). These data suggest that our common garden conditions may have revealed developmental plasticity in brood ball provisioning, which, however, may not be expressed in field-collected individuals.

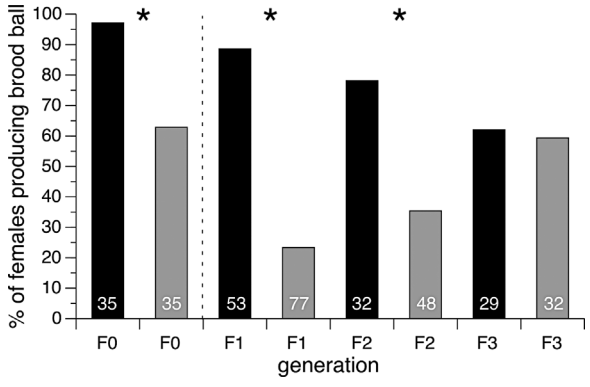

Fig. 3. Percentages of females that produced at least one brood ball during the experimental breeding period. Black bars indicate percentages of western Australian females and grey bars those of eastern U.S. females. Numbers at bottom of each bar indicate sample size and asterisks indicate significant differences between populations within generation. Dashed line indicates transition from field-collected (F_0) to laboratory-reared generations (F_1–F_4).

Duration of larval development

Lastly, we compared the duration of larval development, i.e., the duration between brood ball deposition and adult emergence, between EUS and WA populations. We predicted that the elevated level of resource competition in the WA population may have selected for larval phenotypes that are able to complete development faster and thus more efficiently. We first examined total development time (from egg to adult emergence). In contrast to our prediction, offspring of field-collected WA females (F_1 in Fig. 6a) took significantly more time to eclose than offspring of field-collected EUS females (Fig. 6a and Table 4; post-hoc Tukey HSD: $P < 0.001$). The difference in the duration of larval development was approximately 4 days for the F_1 generation and persisted throughout all subsequent laboratory generations (all post-hoc Tukey HSDs: $P < 0.001$). In a separate experiment, we focused specifically on the duration of the 3rd (=last) larval instar (Fig. 6b), which constitutes the main feeding stage of the larva (Moczek and Nijhout 2003). We found that WA larvae from field-collected mothers required approximately four more days (Fig. 6b) to complete the 3rd instar compared to EUS larvae (T-test: t-ratio= −5.497, $P < 0.001$). These results suggest that the difference in the 3rd instar duration fully explains population-specific differences in total development time, that EUS and WA populations have diverged genetically in 3rd instar duration, and that the direction of

Table 3. Two-way ANCOVA comparing the mass of brood balls produced by females

Fixed effects	DF (factor)	DF (Denominator)	F-ratio	*P*
Population	1	169	30.41	<0.0001
Generation	3	168.4	16.83	<0.0001
Maternal size	1	171	44.67	<0.0001
Population x Generation	3	169.8	3.04	0.0307

Indicated are the degrees of freedom, the degrees of freedom of the denominator, the F-ratio of the test statistic, and the *P*-value for each factor and interaction of the model.

divergence is opposite to what we predicted given the elevated levels of resource competition present in WA.

DISCUSSION

Several basic mechanisms allow populations to cope with disparate ecological conditions. Here, we test (a) whether exotic populations of the beetle *O. taurus* subject to highly disparate levels of resource competition may have diverged in important life history traits and (b) determine whether population divergences are mediated by parental effects, developmental plasticity, or genetically canalized differences. We find that field-collected EUS and WA populations have diverged phenotypically, and in part substantially, in a variety of life history traits and that the directions of most of these divergences match predictions based on the intensity of resource competition prevalent in each population. We also find, however, that these divergences are facilitated by a complex combination of developmental plasticity, parental effects, and genetic differentiation. Below, we discuss the most important implications, as well as limitations, of this study.

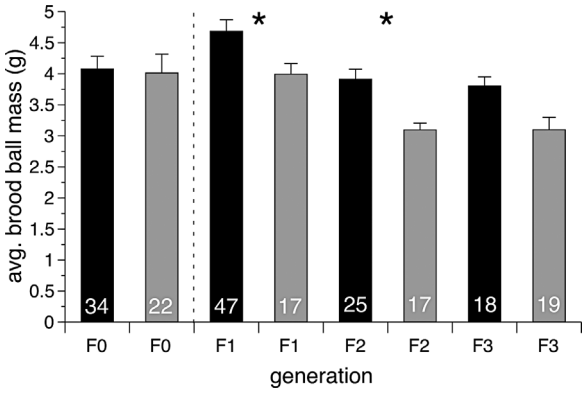

Fig. 5. Grand average mass of brood balls (+ s.e.m.) produced by females from the two exotic populations. For graphical presentation, brood ball masses were averaged for each female and then averaged for all females in each generation. Black bars indicate grand averages of brood ball masses of western Australian beetles and grey bars those of eastern U.S. beetles. Numbers at bottom of each bar indicate the number of females producing brood balls in each generation and asterisks indicate significant differences between populations within each generation. Dashed line indicates transition from field-collected (F_0) to laboratory-reared generations (F_1–F_4).

Phenotypic divergences between exotic *O. taurus* populations are mostly consistent with predictions based on differential resource competition

Populations of *O. taurus* from WA are subject to up to three orders of magnitude higher levels of resource competition in the field than their EUS counterparts (Moczek 2003). We predicted that the extremely high levels of resource competition prevalent in WA populations should favor females that (1) produce brood balls more efficiently, (2) invest differently into individual brood balls, (3) produce offspring more readily when a breeding opportunity arises and (4) with higher adult eclosion success, (5) eclose to adulthood at a different body size, and (6) require less time to complete larval development compared to their low-competition EUS counterparts. We find that field-collected WA and EUS populations have diverged phenotypically in all of these traits with the sole exception of brood ball mass. Furthermore, we find that the majority of these divergences are in the direction consistent with our initial predictions: high-competition WA produce more brood balls under *ad libitum* conditions, have a greater proportion of reproductively active females, and have a greater proportion of offspring emerging to adulthood, than low-competition EUS females. The only exception was the duration of larval development: rather than showing a reduction in the duration of development, WA individuals took significantly longer to complete larval development, even though larval feeding conditions (measured as brood ball mass) were indistinguishable between populations in the F_0 generation. Overall, these results suggest that field-collected WA and EUS populations exhibit remarkable differences in many important life history traits, with the direction of the majority of differences being consistent with what we would predict given the disparate levels of resource competition present in both populations.

However, two important limitations are inherent in our experimental design, which limit the strength of the conclusions stated above. First, even though the direction of trait divergence matched our initial predictions in most cases, we did not directly quantify the adaptive significance, if any, of these divergences, which, therefore remains to be examined experimentally. Secondly, our experiment only contrasted one field-collected population per exotic range. When designing the experiment, we had to choose between maintaining multiple populations per range for single generations or one population per range for

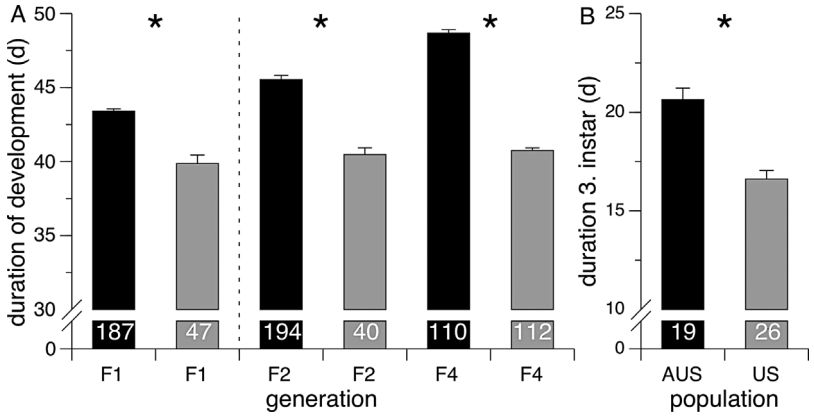

Fig. 6. Average durations or larval development (+ s.e.m.) of the two exotic beetle populations. (A) Duration of larval development was measured as the time period between brood ball production and adult emergence from the brood ball. Black bars indicate development times of western Australian beetles and grey bars those of eastern U.S. beetles. Dashed line indicates the transition from offspring of field-collected parents (= F_1 in graph) to generations of offspring of laboratory-reared parents ($F_2 + F_4$). (B) Average duration of third larval instar (+ s.e.m.) of offspring of field-collected parents from Western Australia (black bars) and Eastern US (grey bars). Numbers at bottom of each bar indicate sample size and asterisks indicate significant differences between populations within generation.

multiple generations. We choose the latter design because it enabled us to focus on the main objective of this study, namely to investigate the basic proximate mechanisms underlying trait divergences. However, by choosing this design we had to leave open the possibility that similar divergences in life history traits may exist within exotic ranges rather than reflect range-specific differentiation. It is worth noting, however, that earlier studies on EUS and WA populations, as well as populations within eastern Australia (a third, independent introduction) and studies on *O. taurus* within its native Mediterranean range failed to detect significant within-range geographic differentiation in mean body size, body size thresholds, local densities of intra-, and interspecific competitors, as well as sex ratios, suggesting that within-range differentiation may be rare, subtle, or absent (Moczek et al., 2002; Moczek 2003; Moczek and Nijhout 2003) .

A combination of developmental plasticity, parental effects, and genetic differentiation facilitates phenotypic divergences between EUS and WA populations

We used four generations of common garden rearing to distinguish the proximate mechanisms underlying the phenotypic divergences observed in wild-caught (F_0) animals from WA and EUS. We predicted that under common garden rearing conditions divergences based on genetic differentiation would persist across all laboratory generations, but disappear after the first laboratory generation or subsequent generations if the divergences were based on developmental plasticity, or (grand) parental effects, respectively. We found that divergences in the duration of larval development remained significant for all generations tested, and thus most likely reflect canalized, genetic differences between populations (Table 5). In contrast, population differences in larval eclosion success appeared to be mediated by within-generation developmental plasticity and were eliminated after a single generation of rearing under common garden conditions (Table 5). Lastly, population differences in brood ball number and the percentage of reproducing females persisted for a subset of common garden laboratory generations, indicative of parental and possibly grand-parental effects (Table 5). Importantly, changes in reproductive performance in laboratory conditions can also be brought about by inbreeding depression which, however, generally results in a decline in reproductive performance, in contrast to the (differential) increase in reproductive performance observed in our laboratory populations (see EUS in Figs. 2 and 3). These results are thus more in line with a recent study on maternal effects showing that *O. taurus* mothers produce sons with longer horns when they perceive higher population densities (Buzatto et al. 2012). Collectively, our results illustrate that population differentiation can be underlain by a surprisingly diverse patchwork of proximate mechanisms.

More specifically, our results suggest that within-generation developmental plasticity, trans-generation parental effects, and genetically canalized population differences may each mediate putatively adaptive adjustments in diverse, yet interrelated life history traits in response to changes in the competitive environment (see below for a possible adaptive role of the genetically canalized difference in development). If correct, our results have important implications for the persistence of population differences in the face of environmental changes. For example, changes in the competitive regimes in one or both of our focal populations would result in the immediate loss of

Table 4. Generalized Linear Model comparing juvenile developmental time

Fixed effects	DF (factor)	DF (Pearson)	χ^2	*P*
Population	1	684	82.81	<0.0001
Generation	2	684	18.14	0.0001
Population x Generation	2	684	8.73	0.0127

Indicated are the degrees of freedom, Pearson's degrees of freedom, the χ^2 value of the test statistic, and the *P*-value for each factor and interaction of the model.

Table 5. Differences in life history traits between EUS and WA *O. taurus* populations and their underlying mechanisms

Trait	F_0	F_1	F_2	F_3	F_4	Proximate mechanism
Body size (thorax width)	Yes	No	Yes	No	Yes	N/A
No. of brood balls	Yes	Yes	No	No	–	Parental effect
Brood ball mass	No	Yes	Yes	No	–	N/A
% females producing brood balls	Yes	Yes	Yes	No	–	Grand parental effect
% offspring eclosing	–	Yes	No	–	No	Developmental plasticity
Duration of larval development	–	Yes	Yes	–	Yes	Genetic differentiation

First column indicates each life history traits measured and the last column indicates the most likely proximate mechanism mediating a given difference between (F_0) field-collected beetles. 'Yes/No' indicates presence/absence of a significant trait difference between WA and EUS populations, '–' indicates that no data is available. Note that F_0 indicates field-collected beetles and F_1–F_4 indicate lab-reared generations.

phenotypic differentiation between these populations in some, but not all of the traits examined here, possibly resulting in the expression of maladaptive trait combinations and lower fitness under such circumstances (sensu Price et al. 2003). In contrast, if differences in the competitive environments of WA and EUS populations continue to persist, as they seem to have over the past more than 2 decades (Moczek 2003; Buzatto et al. 2012), this would result in the recurrent expression and environmental canalization (sensu Pfennig and Pfennig 2012) of the whole suite of differences in life history traits between WA and EUS populations identified in this study, no matter whether trait differences are currently underlain by developmental plasticity, parental effects, or genetic differentiation. This in turn would provide the opportunity for genetic accommodation to follow recurrent trait expression and to stabilize currently plasticity- or parental effects-based population differences via the selective fixation of genetic modifiers (West-Eberhard 2003; Pfennig et al. 2010). In fact, it is tempting to speculate that the present study may have caught WA and EUS populations mid-way through a process of divergence in a large suite of interrelated traits using whatever proximate means were initially available for a given trait.

Alternative explanations

We interpreted the changes in trait differences between populations across generations as indicative of developmental plasticity or parental effects. However, in principle, two other mechanisms could potentially account for such observations. For example, laboratory evolution due to inadvertent selection imposed by experimental procedures can bring about changes in mean trait values across laboratory-bred generations. However, the inclusion of large numbers of individuals in each generation that were not bred in the context of our experimental assays (though otherwise maintained identically; see Materials and Methods section) makes it unlikely that this could explain our results. Furthermore, several trait measures changed rather drastically across generations, which in order to be attributable to inadvertent laboratory based selection would require unrealistically high trait heritabilities. For example, the average number of brood balls produced (Fig. 2) by EUS females between the F_1 and the F_2 generation doubled from an average of about 3.5 (F_1; $n = 17$; except for three individuals, no individual produced more than four brood balls) to an average of more than seven (F_2; $n = 17$; except for two individuals, all individuals produced at least four and at most 11 brood balls) within one generation.

Another possible factor that could have influenced our results could be inbreeding depression of laboratory populations. If inbreeding depression had taken place in our experiment, we would generally expect fitness-relevant life history traits to deteriorate over time. However, many trait values improved over the course of our experiment for one or both lab populations. For example, both the number of brood balls produced by EUS females (Fig. 2) and the percentage of EUS females producing at least one brood ball (Fig. 3) significantly increased across lab generations, and eclosion success (Fig. 4) improved for both EUS and WA populations while maintained in the lab (Fisher exact test: F_2 vs. F_4 for both WA and EUS: $P < 0.0001$).

Canalized differences in development time and size may suggest physiological mechanisms underlying divergences in reproductive performance

The finding that under standardized conditions WA larvae grow longer yet eclose most of the time at a smaller adult body size than EUS larvae was unexpected and contradicts the common observation that development time and adult size are strongly positively correlated in invertebrates (e.g., Kingsolver et al. 2012). Importantly, WA females produce same-sized or even heavier brood balls than EUS females (Fig. 5), indicating that the observed smaller adult size of WA offspring in most generations (Fig. 1) cannot be explained by reduced larval nutrition. It is possible, however, that maternal contributions

mediated through the egg are of lower quality or quantity for WA mothers, thereby negatively affecting the developing embryo (review in Mousseau and Dingle 1991; Labeyrie 1967, 1988), or that WA larvae are less efficient in metabolizing and converting resources into body mass (Mueller 1990; Joshi and Mueller 1996). Such physiological or developmental differences between populations could reflect non-adaptive differences between populations, possibly due to founder effects dating back to the original introduction (Moczek et al., 2002). However, it is also conceivable that the prolonged development and mostly smaller body size of WA individuals may be adaptive in the context of the increased reproductive performance observed in WA females. Specifically, WA females invest far more resources into egg production (as indicated by the higher number of brood balls per female), and are capable of producing much larger numbers of eggs as young adults compared to EUS females (Fig. 2). This raises the possibility that WA females may invest more heavily into ovarian development as late-instar larvae, possibly at the expense of body size, and doing so by delaying eclosion to a developmental time point (Fig. 6b) able to accommodate the increased ovarian investment. This hypothesis is at least indirectly supported by the observation that recently emerged WA females weigh more over a broad range of body sizes (i.e., they are 'denser') than EUS females (OMB, unpublished data). Experiments are currently underway to investigate the timing and extent of ovarian maturation in both populations.

CONCLUSIONS

We demonstrated that two exotic dung beetle populations have diverged phenotypically in multiple life history traits as predicted by the substantial differences in competition for breeding resources prevalent in both populations. At the same time, we find that these putatively adaptive, phenotypic divergences across populations are facilitated by a surprisingly complex combination of developmental plasticity, (grand) parental effects, and genetic differentiation (Table 5). Our results illustrate that phenotypic differentiation in the field can be the result of a diversity of proximate mechanisms and that different traits may be able to diverge in concert via different developmental-genetic means. Lastly, our findings raise the possibility that plasticity and parental effects may constitute important short-term stepping-stones for the evolution of genetically canalized differentiation.

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