

Incipient hybrid inferiority between recently introduced, diverging dung beetle populations

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Understanding why and how certain clades emerge as speciation hotspots is a fundamental objective of evolutionary biology. Here we investigate divergences between exotic *Onthophagus taurus*, a dung beetle introduced into the USA and Australia in the 1970s, as a potential model for the widespread recent speciation events characterizing the genus *Onthophagus*. To do so, we hybridized *O. taurus* derived from Eastern US (EUS) and Western Australian (WA) populations, and assessed fitness-relevant trait expression in first- and second-generation hybrids. We found that dams invest more in offspring provisioning when paired with a sire from the same population, and that WA dams crossed with EUS sires produce smaller and lighter F1 hybrids, with an unexpectedly male-biased sex ratio. Furthermore, fewer F2 hybrids with vertically inherited WA cytoplasm and microbiome emerged compared with WA backcrosses with WA cytoplasm/microbiome, suggesting that combinations of nuclear genome, cytoplasm and/or microbiome may contribute to hybrid viability. Lastly, we document a dominance of WA genotypes over body size at the point of inflection between minor and major male morphs, a trait of significance in mate competition, which has diverged remarkably between these populations. We discuss our results in light of the evolutionary ecology of onthophagine beetles and the role of developmental evolution in clade diversification.

ADDITIONAL KEYWORDS: allopatry – crossbreeding – cytonuclear incompatibility – fitness – insect introduction – *Onthophagus taurus* – reproductive isolation – speciation.

INTRODUCTION

Understanding the ecological causes and developmental–genetic mechanisms underpinning the formation of new species are foundational objectives of evolutionary biology and allied fields. In particular, understanding why and how certain clades, but not others, emerge as speciation hotspots has been of major interest to evolutionary biologists and ecologists, and increasingly to the field of evolutionary developmental biology. With over 2300 species described so far, the subcosmopolitan dung beetle genus *Onthophagus* (Scarabaeidae: Onthophagini) is one of the most speciose genera in the animal kingdom (Tarasov & Solodovnikov, 2011). The clade containing this genus is estimated to have originated around 37 Mya and to have subsequently diversified greatly in the Oligocene and Miocene, paralleling the radiation of mammals and the expansion of grassland habitats (Emlen *et al.*, 2005; Breeschoten *et al.*, 2016). The genus

Onthophagus also comprises species that diverged in the Plio-Pleistocene (Breeschoten *et al.*, 2016), and many closely related sister species and species-complexes of difficult systematic attribution (e.g. Pizzo *et al.*, 2006; Macagno *et al.*, 2011b; Roy *et al.*, 2016; Joaqui *et al.*, 2019), broadly underlining the relatively recent diversification of these taxa, alongside speciation events that are putatively still ongoing (e.g. Pizzo *et al.*, 2011, 2013). Several processes have been proposed to account for the rapid rate of speciation within this genus, including, but not limited to, speciation following either dispersal or vicariance and the development of physical barriers due to changes in climate (Davis *et al.*, 2002), and sexual selection due to intense reproductive competition for ephemeral breeding resources (Emlen *et al.*, 2005; Simmons & Ridsdill-Smith, 2011).

In Afro-Eurasia, rapid Plio-Pleistocenic cooling and drying cycles are believed to have driven the recent diversification of several beetle taxa as a consequence of repeated range expansions and fragmentations following climate fluctuations (Davis *et al.*, 2002;

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Carisio *et al.*, 2004; Ribera & Vogler, 2004). Within the genus *Onthophagus*, the sister species *O. taurus* (Schreber, 1759) and *O. illyricus* (Scopoli, 1763) are a well-studied example of this diversification pattern, having last shared a common ancestor 3–4 Mya, and currently showing reproductive isolation even in their extensive area of syntopy (Pizzo *et al.*, 2006). However, while *O. illyricus* presently only occurs in its native Turanic–European range, *O. taurus* has been the subject of recent intercontinental introduction events beyond its original Turanic–European–Mediterranean distribution (reviewed by Silva *et al.*, 2016). Specifically, in the 1970s *O. taurus* was introduced from an unknown location into the eastern United States as part of an accidental release (Fincher & Woodruff, 1975), and from Spain, Italy, Greece and Turkey into Australia as part of a deliberate introduction programme aimed at limiting pasture degradation and controlling noxious flies (Tyndale-Biscoe, 1996; Edwards, 2007). These introductions have since resulted in the successful establishment of invasive, exotic *O. taurus* populations, thereby possibly recapitulating the very early stages of population differentiation assumed to have characterized the divergence of present-day onthophagine sister species such as *O. taurus* and *O. illyricus*. Importantly, Eastern US and Australian *O. taurus* populations have diverged in a variety of morphological, physiological, developmental, behavioural and life-history traits (reviewed by Hu *et al.*, 2020). Some of these traits mirror interspecific differentiation between *O. taurus* and *O. illyricus*, including: internal and external morphology (Pizzo *et al.*, 2008; Macagno *et al.*, 2011a, 2016); soil depth at which oviposition occurs (Macagno *et al.*, 2016); the body size at the switchpoint between minor and major male morphs (Moczek *et al.*, 2002; Moczek & Nijhout, 2003), a phenotypic outcome underlain by developmental changes in the degree and timing of sensitivity to the juvenile hormone (Moczek & Nijhout, 2002); and climatic conditions in their respective areas of occurrence. Specifically, in their native ranges *O. illyricus* reaches higher latitudes than *O. taurus* (Ljungberg, 2002), while in their respective exotic ranges Eastern US populations of *O. taurus* expanded their climatic range into substantially colder and more humid areas compared to both native and Australian populations (Silva *et al.*, 2016). While an expansion of climatic range may also stem from biotic filtering, collectively these observations suggest that evolutionary modifications similar to those characterizing differentiation between closely related species can occur within a relatively narrow time frame in isolated populations. Additionally, previous studies have hypothesized that ecological factors as simple as population densities, and the resulting diverging competitive environments, may

have been sufficient to bring about the phenotypic and developmental diversification across *O. taurus* populations we see today (Moczek, 2003; Moczek & Nijhout, 2003; Beckers *et al.*, 2015; Macagno *et al.*, 2015; Casasa & Moczek, 2018). However, it is entirely unclear whether this population-wide diversification in a large suite of diverse traits would be sufficient to also mediate a reduction in hybrid fitness were these populations to re-establish contact, a condition critical to relate the divergence events marking present-day *O. taurus* populations to the widespread speciation events that characterize the recent evolutionary history of the genus *Onthophagus*.

Hybrid inferiority may evolve between isolated populations due to dysfunctional interactions between loci that accumulate between differentiating lineages and act as an isolating barrier once these populations establish contact, thereby providing a fundamental contribution to the evolution of reproductive isolation and speciation (Dobzhansky, 1937; Müller, 1942; Coyne & Orr, 2004; Moyle & Nakazato, 2010). These interactions can arise either neutrally via drift or can be a side effect of adaptation to divergent ecological conditions. Additionally, if hybrids possess lower fitness than either parental form, natural selection against maladaptive hybridization following secondary contact may lead to the evolution of reinforcement, namely any mechanism that would allow parents to avoid, or lessen, the investment in gametes and potentially other resources into hybridization events yielding low-fitness offspring (Hoskin *et al.*, 2005). Here, we sought to investigate whether exotic *O. taurus* populations may already be showing signs of incipient reproductive isolation, by testing whether artificial hybridization in the laboratory results in potentially maladaptive hybrid phenotypes. In particular, we aimed to cross individuals belonging to a Western Australian (WA) and an Eastern US (EUS) population assumed to have resulted from introduction events of Mediterranean beetles around the same time in the early 1970s, to quantify phenotype expression and assess possible signatures of reduced hybrid fitness due to negative epistatic interactions within hybrid genomes. We hypothesized that mothers may invest differently in reproduction depending on the provenance of their mate. We additionally predicted that hybrids may express traits that are either closer to one parental population (in the event of major parental effects/dominance), intermediate (consistent with additive genetic effects) or outside both parental values (consistent with difficulties integrating ontogenetic mechanisms inherited from divergent parental strains in the case of transgressive segregation: Rieseberg *et al.*, 2003). In this framework, we assessed evidence of hybrid inferiority (or, conversely, hybrid vigour) by evaluating phenotypic traits linked to fitness, as detailed in Table 1 and the corresponding legend. Moreover, when incipient reproductive isolation

Table 1. Morphological and life-history traits considered for the study

Generation	Trait	Measured as:
P	Body size	Thoracic width of dam
F1	Brood ball mass	Weight of brood ball upon harvesting, after brushing off soil
	Brood ball depth	Distance (cm) from surface of ovipositing container to deeper end of the brood ball
	Brood ball count	Brood balls produced by each P dam, including those producing 0 brood balls
	Body size	Thoracic width of F1
	Developmental time	Time elapsed between setup of P dam in ovipositing container and F1 emergence
	Horn fluctuating asymmetry	Absolute value of the difference between left and right side of the horn in males
	Individual count	F1 produced by each P dam, including those producing 0 offspring
	Mass at emergence	Weight of F1 upon emergence from a brood ball
	Horn length	Length of right and left side of the horn of each F1 male, averaged
	Sex ratio	Number of female and male offspring emerged per dam
F2	Brood ball count	Brood balls produced by each F1 dam, including those producing 0 brood balls
	Body size	Thoracic width of F2
	Developmental time	Time elapsed between setup of F1 dam in ovipositing container and F2 emergence
	Female mass at emergence	Dry weight of female F2s fixed upon emergence
	Horn fluctuating asymmetry	Absolute value of the difference between left and right side of the horn in males
	Individual count	F2 produced by each F1 dam, including those producing 0 offspring
	Horn length	Length of right and left side of the horn of each F2 male, averaged
	Sex ratio	Number of female and male offspring emerged per dam

Brood ball mass, count and depth are measures of maternal investment (Macagno *et al.*, 2018). Offspring count is a direct measure of reproductive success, as fecundity is a fundamental determinant of fitness in natural populations (Kingsolver *et al.*, 2001). Smaller body size, which is tightly correlated with mass at emergence in *O. taurus* (Macagno *et al.*, 2016), is frequently associated with reduced fitness in insects (e.g. Allen & Hunt, 2001; Kolluru & Zuk, 2001; Kingsolver & Huey, 2008). The growth rate of a population decreases if developmental and therefore generation times are longer (Kingsolver & Huey, 2008). Horn fluctuating asymmetry was measured as a possible indicator of developmental instability (Dongen, 2006).

between populations is present, second- (or later) generation hybrids may show reduced fitness compared to the parental lineages even when the first-generation hybrids do not. This is widely recognized as an effect of cytonuclear incompatibility: co-adaptation within natural populations shapes optimal intergenomic interactions between organellar and nuclear genetic material, while recombination disrupts them (Burton *et al.*, 2003). However, maternal inheritance need not be limited to the cytoplasm, but rather encompasses a suite of vertically transmitted elements (Badyaev, 2013) which in the case of *O. taurus* include a functionally significant component of the microbiota that supports normal development of offspring (reviewed by Parker *et al.*, 2020). Co-adaptation between the genome of individuals and their microbiome, and its disruption following hybridization, may therefore bring about phenotypic consequences similar to those seen in the case of cytonuclear incompatibility. Based on these considerations we therefore crossed the EUS and WA populations of *O. taurus* for two generations,

predicting that with hybrid incompatibility the fitness of hybrid strains may be lower than that of purebred strains. Furthermore, in the second generation, such fitness deficits would be restored with *maternal* backcrosses introducing a full parental cytonuclear complement, but not with *paternal* backcrosses that would cause a mismatch between nuclear and cytoplasmic genomes (Burton *et al.*, 2003), between nuclear genome and the microbiome, or both. We discuss the implications of our results in light of the evolutionary ecology of onthophagine beetles and of the role of developmental evolution in clade diversification.

MATERIAL AND METHODS

EXPERIMENTAL DESIGN

Common garden rearing

The entire crossbreeding experiment was performed in a walk-in environmental chamber set at 24 °C, with

a 16:8-h light–dark cycle, using sterilized soil (i.e. a mixture of one part sifted topsoil and two parts sand) and thawed, drug-free dung from grass- and hay-fed cows. All dung used was collected year-round at Marble Hill Farm (Bloomington, IN, USA) and kept frozen at -20°C for at least 1 month. Dung was homogenized before each feeding and ovipositing round (see below) to avoid differences in offspring size and fitness that might be due to differences in dung quality (Hunt & Simmons, 2002).

Parental generation (P)

We generated virgin parental strains from lab-reared *O. taurus* collected from pastures in Western Australia (Busselton area: WA) and in the eastern US (Chapel Hill, NC: EUS), by either harvesting brood balls constructed within rearing containers or using the breeding protocol detailed by Macagno *et al.* (2016). We collected parental individuals (P) upon emergence from brood balls and separated them by sex and population of origin (55 EUS females, 75 EUS males, 71 WA females, 86 WA males) in 15-L plastic containers with shallow (10 cm in depth), loose soil and unlimited supply of dung. Beetles were fed once a week and kept separate by sex and population for 2 weeks after the addition of the last newly emerged individual, to ensure sexual maturity by the time the crosses were established.

First hybrid generation (F1)

We crossed virgin WA and EUS parental (P) beetles in order to generate two purebred F1 lines and two reciprocal F1 hybrid lines, specifically: (1) WA(WA) (individuals with WA nuclear genetic makeup and cytoplasm inherited from WA mothers); (2) EUS(EUS) (with EUS nuclear genetic makeup and cytoplasm inherited from EUS mothers); (3) hybr(WA) (with hybrid nuclear genome and cytoplasm inherited from WA mothers); and (4) hybr(EUS) (with hybrid nuclear genome and cytoplasm inherited from EUS mothers) (Fig. 1). While we refer to the nuclear/cytoplasmic makeup of the strains throughout the Material and Methods and Results sections for simplicity, maternal inheritance also includes a broad array of elements that are transmitted vertically from mother to offspring, including the symbionts that support normal development (Badyaev, 2013; Parker *et al.*, 2020).

We mated each of ten randomly selected P males to two randomly selected P females per line. For this, each individual sire and its dams were placed into a 1-L container, one-third filled with moist soil, and kept in it for at least 1 week. Mating containers were provided with 150 g of homogenized cow dung that

was replaced once a week. We measured thoracic width (a proxy for body size) of sires and dams and horn length of males using a digital camera (Scion, USA) connected to a stereoscope (Leica MZ-16, USA) and ImageJ (Schneider *et al.*, 2012). During four consecutive weeks (=ovipositing rounds), we transferred five mated females per line to individual ovipositing containers filled with sterilized soil to a height of 21 cm and provided with 200 g of homogenized cow dung (Macagno *et al.*, 2018). Dams were left in these ovipositing containers for 5 days, after which we sifted the soil for brood balls. We weighed all brood balls to the closest 0.001 g using a Mettler Toledo (AL 54) scale and incubated them individually in 30 mL SOLO cups loosely filled with soil. Upon emergence, F1 beetles were weighed to the closest 0.0001 g and their thoracic width and horn length were measured as detailed above. Individuals were then marked by family (i.e. with their P mother's unique code, PDamID), by puncturing both elytra (da Silva & Hernández, 2015; Supporting Information, Figure S1).

Second hybrid generation and backcrosses (F2)

Purebred and crossbred F1 beetles were kept separate by line, in single-sex maturation containers as described above. Once all individuals had reached sexual maturity, crosses were established by placing at least 12–15 females and 12–15 males in 8.5-L mating containers with shallow, loose soil and unlimited access to dung. Microclimatic conditions within each mating container were carefully standardized by regularly checking soil moisture, providing individual containers with the same amount of homogenized dung, and changing the placement of containers within the incubator weekly. Selected hybrid crosses and backcrosses were executed as reported in Figure 1. Because individuals were marked by family, we were able to avoid inbreeding in the F2 beetles by excluding any possibility of mating between siblings. Beetles were provided unlimited access to dung that was replaced once a week, and allowed to mate for at least 1 week, following which we started transferring dams to individual ovipositing containers for 5 days. Specifically, two or three females per cross were used at each ovipositing round, for five consecutive weekly rounds as described above. We collected all brood balls, incubated them individually, and fixed F2 adults in 70% EtOH upon emergence. We measured both maternal size and offspring size and horn length as above. Female offspring were subsequently air-dried for 10 days at 25°C and weighed to the nearest 0.0001 g.

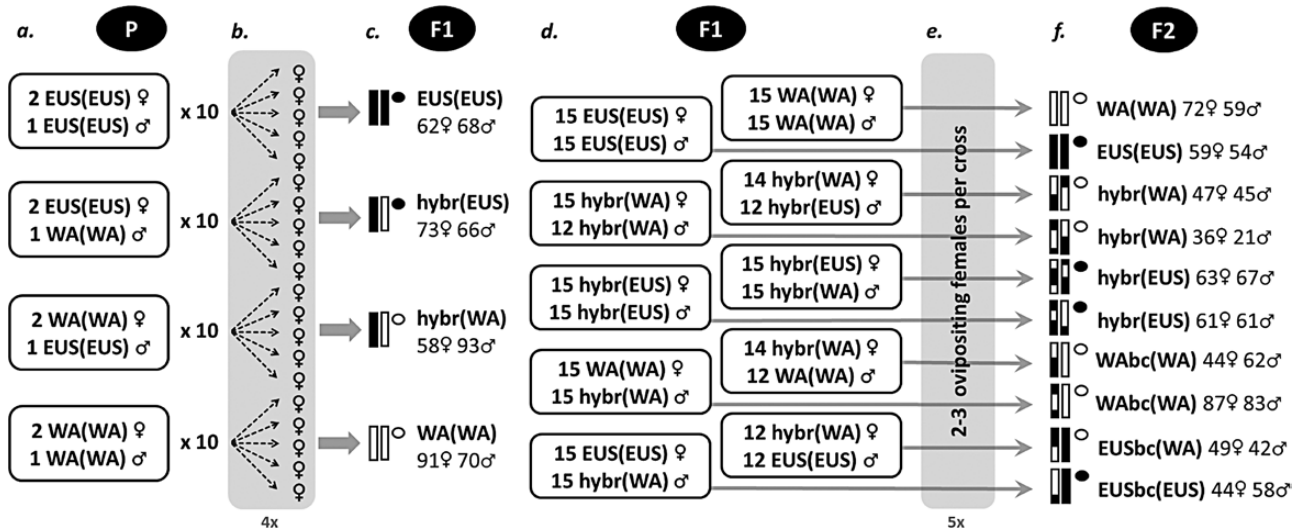


Figure 1. Experimental design. A, two virgin parental (P) females were paired with one P male in ten replicate mating containers per line for at least 1 week. B, over the course of four consecutive weekly rounds, five dams from different replicates were transferred into individual ovipositing containers, and kept in these for 5 days. C, their brood balls were harvested and F1 offspring reared to adulthood in individual containers. Once emerged, F1 beetles were marked by family as detailed in the Methods. D, after all F1 reached sexual maturity, crosses were established by placing 12–15 F1 females and 12–15 F1 males in mating containers, avoiding any possibility of sibling matings. Beetles were allowed to mate for at least one week. E, two or three dams per cross were then transferred to ovipositing containers for 5 days. This protocol was repeated with different females for five consecutive weekly ovipositing rounds. F, we collected all brood balls, incubated them individually, and fixed F2 beetles in 70% EtOH upon emergence. In the diagrams, nuclear and cytoplasmic inheritance is schematically represented by one pair of autosomes (vertical lines) and a circle, respectively, with black = Eastern US (EUS) and white = Western Australia (WA). The nuclear/cytoplasmic makeup of individuals is noted as nucleus(cytoplasm). EUSbc, Eastern US backcross; WAbc, Western Australia backcross; hybr, hybrid. Numbers of generated male and female offspring are reported for each line.

STATISTICAL ANALYSES

Morphology and life history of hybrid crosses

We measured a number of morphological and life-history traits linked to fitness in both parents and offspring, as listed in Table 1. We then used them in general (for normal, continuous variables) or generalized linear mixed models (for all other variables, specifying an appropriate distribution and link function: Crawley, 2007) as implemented in the lme4 package (Bates *et al.*, 2015) in R 3.5.2 (R Core Team, 2018). We obtained type II ANOVA tables with the Anova() function in the car package (Fox & Weisberg, 2019), and summary tables for model fits with the library lmerTest (Kuznetsova *et al.*, 2017).

In the F1 generation, we modelled each trait as a function of the dam's population (PDamPop), sire's population (PSirePop) and their interaction (PDamPop:PSirePop), as well as the dam's size (PDamSize). We included ovipositing round as a random factor [(1 | Round)]. In the analysis of traits where multiple measures from each dam were considered (e.g. offspring size), we grouped observations by dams nested within sires by entering these terms

in the models as nested random factors [(1 | PSireID/PDamID)]. For traits with only one measure per dam (e.g. offspring count) we instead entered PSireID as a random factor [(1 | PSireID)]. To simplify these models, we removed non-significant interactions as determined by χ^2 tests (Bolker *et al.*, 2009).

In the F2 generation, we modelled each response variable as a function of the nuclear/cytoplasmic makeup of the offspring [F2nucl(cytopl)] (Fig. 1) and the dam's size (F1DamSize). To account for grandparental effects, we grouped observations by dams nested within granddams [(1 | PDamID/F1DamID)] in the case of traits where multiple measures from each dam were considered (e.g. offspring size). For traits with only one measure per dam (e.g. offspring count), we instead only modelled PDamID as a random factor [(1 | PDamID)]. We again included ovipositing round as a random factor [(1 | Round)]. The significance of fixed effects was determined by χ^2 tests (Bolker *et al.*, 2009). Significant differences between specific contrasts were determined using post-hoc Tukey's HSD tests on estimated marginal means (library emmeans, Lenth, 2019), after setting the covariate (F1DamSize) to its mean value.

In the case of horn fluctuating asymmetry, the horn length of the offspring was also included as an explanatory variable in the models, with all other terms being equal, as it was expected that the difference in length between the left and right side of the horn would be positively affected by overall horn length.

In the event a model did not converge, we increased the number of iterations. When R returned a singular fit error, we typically removed the random effects explaining 0 variance, starting from the higher order (Barr *et al.*, 2013; Pasch *et al.*, 2013). We also fitted ovipositing round as a fixed effect instead of removing it, but the results did not change (data not shown). All models used in the analysis of each trait, before dropping non-significant interactions, are reported in the Supporting Information (Appendix S1).

Horn static allometries

Using the R package *drc* (Ritz *et al.*, 2015), we fitted the body size/horn length distribution a four-parameter log-logistic (Hill) function in the form $y = c + \frac{d - c}{1 + \exp(b(\log(x) - \log(a)))}$ where x is body size, y is horn length, a specifies the body size at the point of inflection of the sigmoid curve, b is its slope, and c and d are the minimum and maximum asymptotic horn lengths, respectively (Moczek, 2002). We then used the Akaike Information Criterion (AIC: Akaike, 1974) to infer whether four sigmoidal regressions (two for each hybrid strain and two for each purebred strain, i.e. a complex model) fitted our data better than one including the whole sample (i.e. a simpler model). The AIC is a measure of relative model fit, and the lower its value, the better the model fits to the experimental data (Burnham *et al.*, 2011). Upon finding the complex model more appropriate for our data, we used repeated Welch's T-tests and the Holm–Bonferroni sequential correction for multiple comparisons to examine the extent to which differences in each regression parameter (as returned by the *drc* fitting procedure) explained the allometric differences found (Moczek *et al.*, 2002). F1 hybrid strains were compared with F1 purebred strains. Due to the very small number of EUS F2 minor males (only three), the horn allometries of the F2 hybrid strains were compared with those of the first- and second-generation purebred individuals combined.

RESULTS

Past work has documented measurable, heritable divergences in morphological, physiological, developmental and life-history traits among EUS and WA populations of *O. taurus*, consistent with population differentiation following establishment in

novel geographical areas (Moczek & Nijhout, 2003; Rohner & Moczek, 2020). Here we sought to assess trait expression in first- and second-generation hybrids to determine possible evidence for incipient reproductive incompatibilities.

FIRST-GENERATION HYBRIDS

Size of the dam positively affected a number of traits in the subsequent generation (Table 2), specifically brood ball mass (estimate 1.662 ± 0.228 , $P < 0.0001$), brood ball count (estimate 0.466 ± 0.172 , $P < 0.01$), size of F1 offspring (estimate 0.279 ± 0.068 , $P < 0.001$), F1 count (estimate 0.365 ± 0.179 , $P < 0.042$), F1 mass (estimate 0.009 ± 0.002 , $P < 0.001$) and duration of development of the F1 (estimate 1.469 ± 0.586 , $P = 0.015$). These results match previous findings on the correlated effects of maternal size on brood ball mass and offspring size (cf. Hunt & Simmons, 2002; Beckers *et al.*, 2015).

Parental population also influenced several traits, but in a more complex manner. Both WA dams and WA sires lengthened the duration of development of the F1 (Table 2; Fig. 2D; estimates 1.479 ± 0.314 , $P < 0.0001$, and 2.051 ± 0.315 , $P < 0.0001$, respectively). Offspring of WA dams were smaller (Table 2; Fig. 2B; estimate -0.103 ± 0.038 , $P < 0.01$), while offspring of WA sires were heavier (Table 2; Fig. 2C; estimate 0.005 ± 0.001 , $P < 0.0001$). Furthermore, both brood ball mass and F1 sex ratios were influenced by an interaction between parent populations (Table 2). Specifically, dams made heavier brood balls when paired with a sire from the same population (Fig. 2A; estimate 0.590 ± 0.288 , $P < 0.05$), while F1 sex ratios were male-biased when WA dams mated with EUS sires, and were female-biased when WA dams mated with WA sires (Fig. 2E; estimate 0.708 ± 0.353 , $P < 0.05$). Lastly, we found no evidence of an effect of either parent's population on horn fluctuating asymmetry of the offspring (a potential indicator of developmental instability: Dongen, 2006), as this metric was influenced only by F1 horn length (Table 2).

SECOND-GENERATION HYBRIDS AND BACKCROSSES

Larger F1 dams had larger offspring with longer development times and heavier daughters (Table 3; estimates, respectively: 0.149 ± 0.052 , $P < 0.01$; 15.923 ± 5.906 , $P < 0.01$; 0.710 ± 0.345 , $P < 0.05$). The combination of nuclear and cytoplasmic makeup influenced body size and developmental time of the F2 significantly, and F2 count nearly significantly (Table 3). In particular, pair-wise comparisons on estimated marginal means showed that both the purebred EUS and the EUS backcross with EUS

Table 2. Effect of either parent's population (PDamPop, PSirePop) and their interaction on several morphological and life-history traits of the F1 strains (cf. Table 1); simplification of the models was carried out by removing non-significant interactions (-)

Dependent variable	Explanatory variable – test statistics					F1 HL*
		PDamPop	PSirePop	PDamSize	PDamPop × PSirePop	
BB mass	χ^2_1	2.919	0.770	53.092	4.206	NA
	<i>P</i>	0.087	0.380	<0.0001	0.040	NA
BB count	χ^2_1	0.793	0.312	7.340	–	NA
	<i>P</i>	0.373	0.576	<0.01	–	NA
BB depth	χ^2_1	0.090	0.109	0.121	–	NA
	<i>P</i>	0.764	0.741	0.728	–	NA
F1 body size	χ^2_1	7.536	1.257	16.554	–	NA
	<i>P</i>	<0.01	0.262	<0.0001	–	NA
F1 mass at emergence	χ^2_1	1.585	18.268	16.769	–	NA
	<i>P</i>	0.208	<0.0001	<0.0001	–	NA
F1 count	χ^2_1	0.650	0.264	4.143	–	NA
	<i>P</i>	0.420	0.607	0.042	–	NA
F1 development time	χ^2_1	22.236	42.439	6.281	–	NA
	<i>P</i>	<0.0001	<0.0001	0.012	–	NA
F1 sex ratio	χ^2_1	0.324	7.589	2.206	4.052	NA
	<i>P</i>	0.569	<0.01	0.137	0.044	NA
sqrt(F1 horn FA)	χ^2_1	1.536	2.787	1.022	–	5.204
	<i>P</i>	0.215	0.095	0.312	–	0.022

*Horn length (HL) of the offspring was only included in the analysis of horn fluctuating asymmetry.

PDamSize (maternal thoracic size) was included as a covariate in all models.

BB = brood ball; FA = fluctuating asymmetry; NA = not available; sqrt = square root. Significant results are shown in bold type.

cytoplasm were larger than the purebred WA ($P = 0.015$ and $P = 0.025$ respectively, Fig. 3A). Purebred EUS individuals developed faster than purebred WA individuals, consistent with previous studies (Fig. 3C; Moczek & Nijhout, 2002; Beckers *et al.*, 2015). Developmental durations of hybrid strains were intermediate between purebred strains, while the backcrosses were closer to the purebred strains than to the hybrids, and confidence intervals were consistently narrow, in line with additive genetic effects (Fig. 3C). Regarding offspring count, fewer F2 hybrids with WA cytoplasm emerged compared with WA backcrosses with WA cytoplasm (Fig. 3B). However, no other pairwise comparison was significant. Importantly, we found no evidence of an effect of nuclear/cytoplasmic makeup on brood ball count, mass of females at emergence, sex ratio and horn fluctuating asymmetry of F2 individuals (Table 3).

HORN STATIC ALLOMETRIES

Four sigmoidal curves fit the horn length–body size data better than one both when comparing first-generation hybrids with purebred F1 individuals (Fig. 4A: $AIC_{4\text{sigmoid}} = 469.77$, $AIC_{1\text{sigmoid}} = 553.52$),

and when contrasting second-generation hybrids to purebred F1 + F2 individuals (Fig. 4B: $AIC_{4\text{sigmoid}} = 850.70$, $AIC_{1\text{sigmoid}} = 1013.89$). In particular, body size at the point of inflection of the sigmoid curve was consistently smaller in the purebred EUS strains (F1: 5.09 ± 0.03 ; F1 + F2: 5.08 ± 0.02) compared to both purebred WA (F1: 5.26 ± 0.03 ; F1 + F2: 5.26 ± 0.01) and hybrid strains [F1 hybr(EUS): 5.22 ± 0.03 ; F1 hybr(WA): 5.20 ± 0.03 ; F2 hybr(EUS): 5.27 ± 0.02 ; F2 hybr(WA): 5.25 ± 0.02] (Welch's T tests with sequential Bonferroni correction, $P < 0.05$ in all instances) (Fig. 4). The slope of the WA population (-74.19 ± 14.45) was also significantly steeper than that of the EUS population (-38.59 ± 6.66) (F1 + F2 combined, $P < 0.05$; Fig. 4B). This phenotypic pattern confirms previously highlighted differentiation in horn allometry between these populations, and suggests a dominance of the WA population with respect to the body size at the switch point between minor and major morphs.

DISCUSSION

In this study we sought to investigate possible parallels between the divergences marking present-day

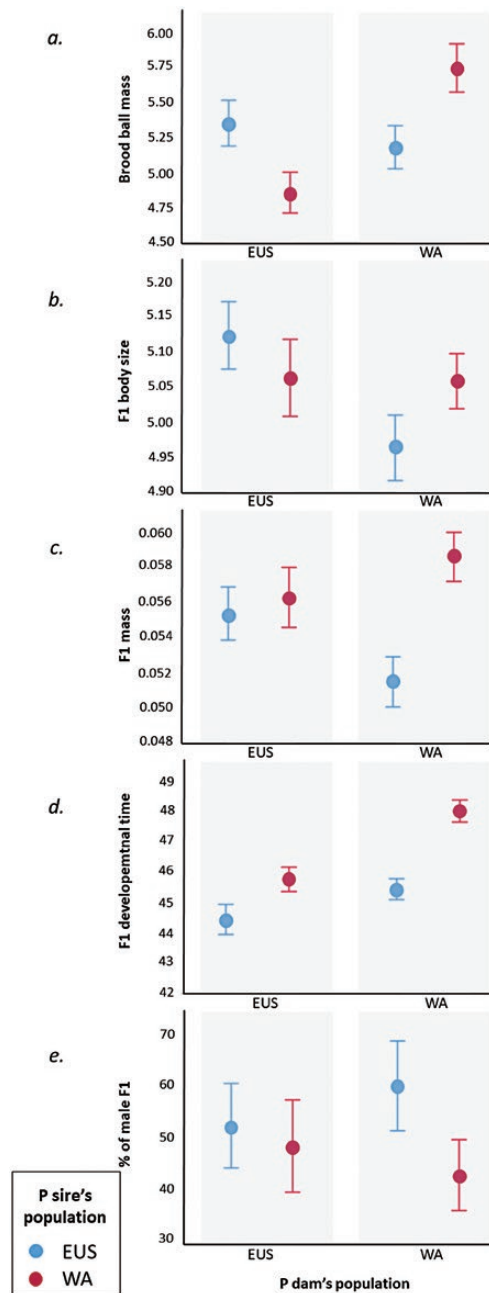


Figure 2. Mean values and 95% confidence intervals of (A) brood ball mass, (B) body size, (C) mass at emergence, (D) developmental time, and (E) sex ratio in the first-generation strains (F1), as a function of parental (P) population of dam (x-axis) and sire (colour coding: blue, EUS; red, WA). Significance is reported in Table 2.

O. taurus populations and the widespread speciation events that characterize the recent evolutionary history of the genus *Onthophagus*. Specifically, we evaluated the extent to which population-wide diversification in a large suite of diverse traits is sufficient to also mediate a reduction in hybrid fitness

were these populations to re-establish contact. To this aim, we assessed trait expression in first- and second-generation hybrids between diverging EUS and WA populations of *O. taurus*, to determine possible evidence for incipient hybrid inferiority. We found that dams invest more in offspring provisioning by constructing heavier brood balls when paired with a male from the same population, and that population origin has a significant influence on population mean values of several traits. In particular, we observed an overall negative effect of WA dams on F1 size, and a positive effect of WA sires on F1 mass, which resulted in relatively smaller and lighter first-generation hybrids deriving from a cross between a WA dam and an EUS sire. Because important fitness components correlate positively with adult body size in male (fertilization success: Hunt & Simmons, 2001) and female *O. taurus* (brood provisioning: Hunt & Simmons, 2007; survival: Hunt *et al.*, 2002; number of offspring produced: Hunt & Simmons, 2002), such a reduction in size and mass is therefore predicted to reduce fitness in hybrids. We further observed that crossing a WA dam with an EUS sire produces an unexpected male-biased F1 sex ratio, and that the WA genotype appears dominant over the body size at the point of inflection between minor and major morph. Additionally, in the second generation, fewer hybrids with WA cytoplasm and microbiome emerged compared with WA backcrosses with WA cytoplasm and microbiome. Below we discuss the most important implications of these findings.

MODEST EVIDENCE OF HYBRID INFERIORITY IN THE FACE OF ENHANCED MATERNAL INVESTMENT IN OFFSPRING PROVISIONING IN PUREBRED CROSSES

EUS and WA *O. taurus* have diverged in a remarkable diversity of traits (reviewed by Hu *et al.*, 2020), often to a degree that matches divergences between extant, closely related yet reproductively isolated species. Population divergences include, among others, heritable differences in the degree and timing of sensitivity to juvenile hormone, and correlated propensities for the induction of the horned male morph and the length of larval development (Moczek & Nijhout, 2002). We hypothesized that divergences in traits such as these, driven by divergent natural and sexual selection (Moczek, 2003) and/or neutral processes, might be conducive to bring about sufficient correlated divergences to result in compromised ontogenies when instructed by hybrid genomes (e.g. via negative epistasis). If so, life-history theory predicts that mothers of one or both populations should adjust their reproductive investment in a current breeding attempt based on the relative fitness benefits provided by their mate (Pryke & Griffith, 2010). Consistent with this prediction, we found that *O. taurus* dams

Table 3. Effect of nuclear and cytoplasmic makeup of the F2 strains [F2nucl(cytopl)] on several morphological and life-history traits (cf. Table 1).

Dependent variable	Explanatory variable – test statistics			
		F2nucl(cytopl)	F1DamSize	F2 HL*
BB count	χ^2 (d.f.)	8.761 (6)	2.303 (1)	NA
	<i>P</i>	0.187	0.129	NA
F2 count	χ^2 (d.f.)	12.222 (6)	0.215 (1)	NA
	<i>P</i>	0.057	0.643	NA
F2 body size	χ^2 (d.f.)	17.591 (6)	8.236 (1)	NA
	<i>P</i>	<0.01	<0.01	NA
ffF2 dry mass	χ^2 (d.f.)	7.144 (6)	7.269 (1)	NA
	<i>P</i>	0.308	<0.01	NA
F2 development time	χ^2 (d.f.)	207.519 (6)	4.224 (1)	NA
	<i>P</i>	<0.0001	0.040	NA
F2 sex ratio	χ^2 (d.f.)	4.499 (6)	0.631 (1)	NA
	<i>P</i>	0.609	0.427	NA
sqrt(F2 horn FA)	χ^2 (d.f.)	4.482 (6)	0.0370 (1)	35.796 (1)
	<i>P</i>	0.612	0.848	<0.0001

*Horn length (HL) of the offspring was only included in the analysis of horn fluctuating asymmetry.

F1DamSize (maternal thoracic size) was included in all models as a covariate. BB = brood ball; d.f. = degrees of freedom; FA = fluctuating asymmetry; ffF2 = female F2; NA = not available; sqrt = square root. Significant results are shown in bold type.

invest more in offspring provisioning by constructing heavier brood balls after mating with a male from the same population, even though sires were not present during brood ball construction. On the one hand, this observation suggests either a preference of females for males of the same population, or male manipulation resulting from sexual conflict (e.g. Hollis *et al.*, 2019), which warrants further investigations into the potential for pre- and post-copulatory female choice in mixed-population settings and the mechanisms therein. On the other, differential provisioning may exacerbate any detrimental fitness effect in hybrids, thereby setting the stage for potentially rapid reinforcement should divergent populations such as these re-establish contact. Here, it is worth noting that we did detect potentially suboptimal phenotypic outcomes in first-generation offspring deriving from a cross between WA dams and EUS sires, but not in the reciprocal F1 hybrid strain. Previous studies have shown that Western Australian individuals take longer to complete larval development and emerge into smaller, yet heavier, adults than size-matched Eastern US individuals. This larger mass-by-volume ratio is attained alongside enhanced ovarian maturation and fecundity, whereby females are able to produce more eggs, produce larger eggs, and start ovipositing earlier compared to their Eastern US counterparts (Beckers *et al.*, 2015; Macagno *et al.*, 2015). However, in this study we show that a cross between a WA dam and an EUS sire instead produces offspring that are relatively smaller *and* lighter compared those of

other first-generation crosses, thereby disrupting a successful combination of resource allocation to diverse biological functions (growth, somatic maintenance, reproduction) in a potentially deleterious way. Additionally, this same cross produced offspring with a male-biased sex ratio, while sex ratio was female-biased when WA females mated with males from their same population. This result was not explainable on the basis of differential larval mortality, because emergence rates were comparably high in all crosses. House *et al.* (2010) reported that WA females adjust the sex of their offspring based on the sire's attractiveness: females produce an excess of daughters when mating with smaller, less attractive males. Because EUS *O. taurus* develop horns at smaller body sizes (Moczek, 2003; Fig. 4), it is possible that the male-biased sex ratio detected in our study may have resulted from a perceived higher attractiveness of EUS sires to WA dams due to the expression of relatively larger horns (Moczek & Emlen, 2000). Nevertheless, the effect of sex ratio on offspring fitness would be context-dependent. In high-density conditions, it may be adaptive for parents to produce a male-biased sex ratio, as intense competition for dung may limit female breeding success more than reduce male reproductive success via increased male–male competition (Moczek, 2003). A male-biased sex ratio would in turn be disadvantageous in low-density conditions, where males might encounter difficulties finding mates.

Crossbreeding WA and EUS *O. taurus* populations for two generations further allowed us to detect

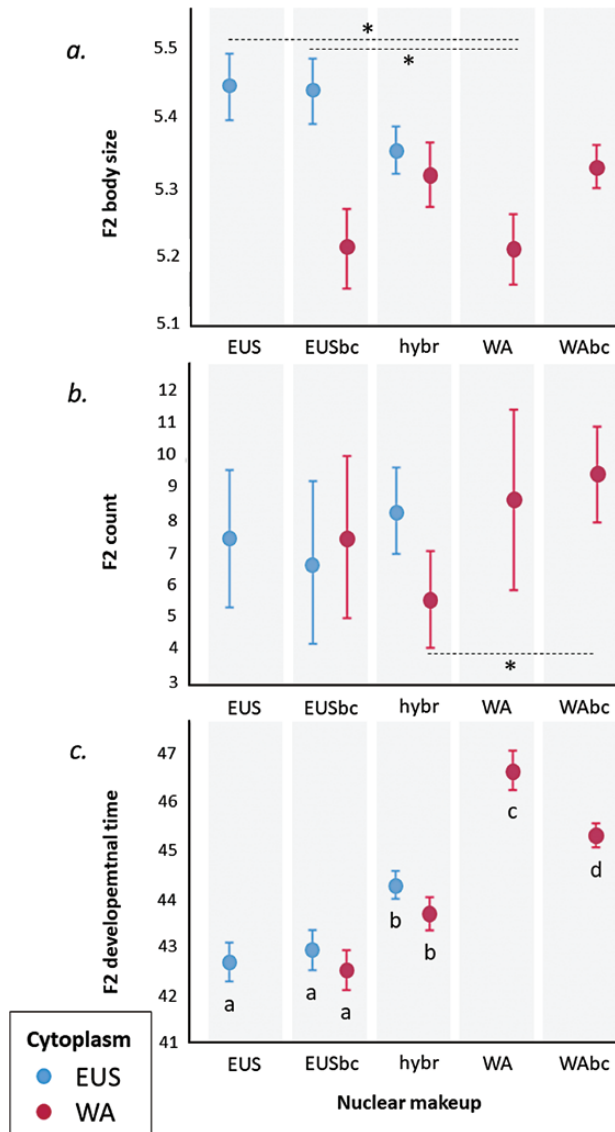


Figure 3. Mean values and 95% confidence intervals of (A) body size, (B) count and (C) developmental time of the second-generation strains (F2), ordered by nuclear (x-axis) and cytoplasmic makeup (also specifying provenance of vertically inherited microbiome; colour coding: blue, EUS; red, WA). Significance of pair-wise comparisons is set at $P < 0.05$, and reported with asterisks in A and B. In C, values flagged with the same letter do not differ significantly.

an apparent dominance of the WA population with respect to horn allometry, resulting in a larger-than-intermediate body size at the switch point between minor and major morphs in all hybrid strains. While horns aid in fights (Moczek & Emlen, 2000), whether horn possession indeed improves fitness of a given male is a function of his body size *relative* to that of most competitors, as well as of population densities, which determine the average number of competitors a

horned male guarding a female will have to contend with simultaneously (status-dependent selection model *sensu* Hunt & Simmons, 2001; Moczek, 2003). It is therefore conceivable that if EUS and WA populations were to re-establish contact, hybrid male larvae may undergo developmental trajectories based on their nutritional environment that do not yield optimal fitness returns given their future adult competitive status. Additionally, we found that fewer F2 hybrids possessing both WA cytoplasm and microbiome emerged compared with WA backcrosses with WA cytoplasm and microbiome. Whether this may be due to nuclear/cytoplasmic or nuclear/microbiome incompatibilities clearly warrants further investigation. Overall, however, we found no obvious, major effects of the nuclear/cytoplasmic makeup on nearly all fitness measures examined by our study in the second generation. While we cannot exclude the possibility that additional small fitness effects may not have been detectable given the experimental design and sample size, or may have been detectable under different environmental/rearing conditions, our results nevertheless reject the hypothesis that population differentiation among exotic *O. taurus* populations has been able to accrue obvious and immediate barriers to hybridization, at least when reared under standardized and benign conditions in the laboratory.

ONTOGENETIC INTEGRATION AND DEVELOPMENTAL EVOLUTION IN A CHANGING ENVIRONMENT: FUTURE DIRECTIONS

The overall maintenance, or only modest impairment, of hybrid fitness, symmetry and other measures of developmental performance documented here suggest the existence of mechanisms able to integrate divergent parental genomes or parental genome/cytoplasm/microbiome combinations during ontogeny. By extension, this implies that hybrid populations can simultaneously exhibit robust development *and* harbour significant genetic variability among their members. In natural zones of secondary contact, such a sudden increase in genetic variability may be able to fuel especially rapid responses to selection, in particular if the timing of secondary contact coincides with significant environmental changes, as would probably have been the case during repeated Plio-Pleistocene glaciation cycles. If such events occur with regularity, it is tempting to speculate that species formation in *Onthophagus*, rather than be driven solely by reduced hybrid fitness and subsequent evolution of reinforcement measures, could also be facilitated by localized population hybridization in secondary contact zones, followed by rapid responses to selection in these zones but not the remainder of the original population distribution. Further investigations into hybrid performance should therefore include the evaluation

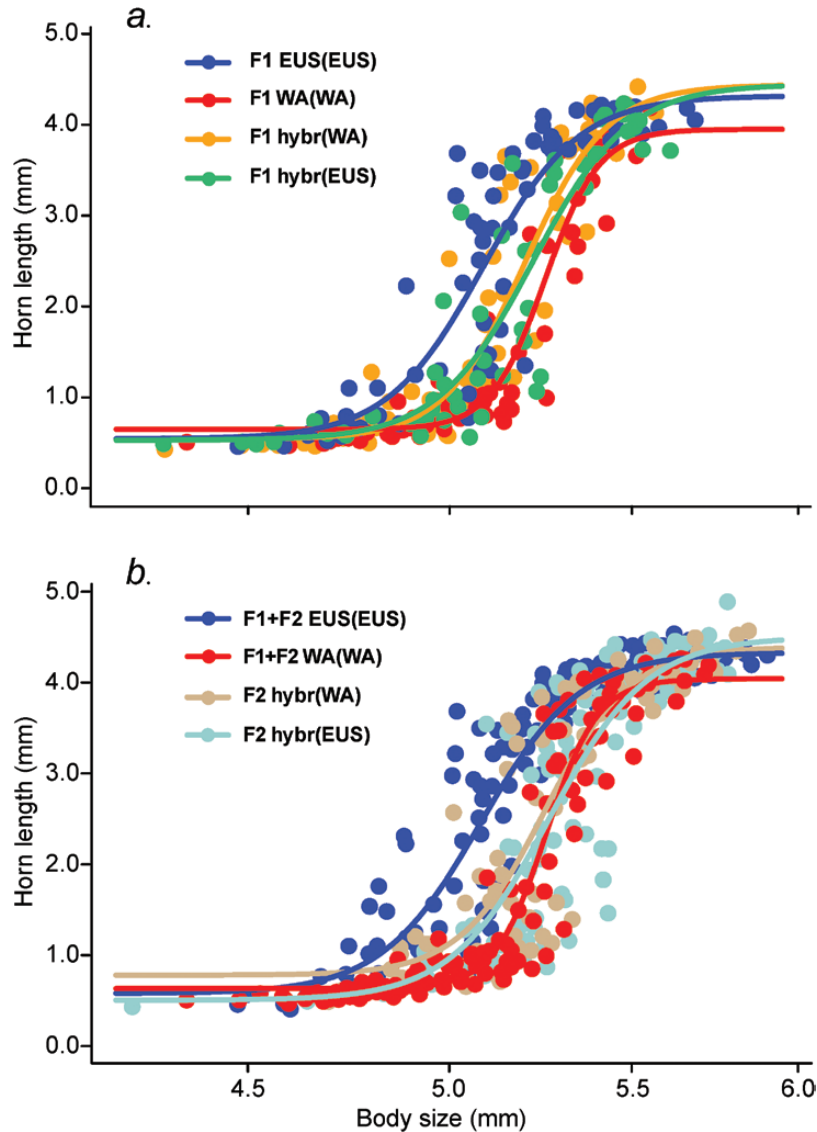


Figure 4. Horn length–body size scaling relationships in the first- (F1) and second-generation (F2) purebred [EUS(EUS), WA(WA)] and hybrid strains [hybr(EUS), hybr(WA)]. The nuclear/cytoplasmic makeup of individuals is noted as nucleus(cytoplasm). In both A and B, body size at the inflection point of the curve of the EUS(EUS) strain is significantly lower than those of both the WA(WA) and the hybrid strains (see Results). In both graphs, a few individuals with body size smaller than 4.2 cm are not shown.

of developmental outcomes under stressful conditions, incorporate environmental variability encountered in invasive areas (cf. Rohner & Moczek, 2020), and assess responsiveness to artificial selection. Additionally, molecular-based insights into the exact introduction dynamics of this species would help greatly to better assess the true speed with which population divergences manifest in nature, and the degree to which they may precede, parallel or follow speciation events. Lastly, given our growing understanding of the importance of maternally transmitted microbiota for normative development (reviewed by Parker *et al.*,

2020), crossing diverging populations of *O. taurus* affords a promising opportunity to experimentally disrupt successful co-adaptations of hosts and their microbiomes and to study their contributions to the evolution of reproductive barriers.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. How individual beetles were marked.

Appendix S1. Models used in data analyses.