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Author for correspondence:
David M. Linz
e-mail: linzd@iu.edu

†Both authors contributed equally to this study.

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The origins of novelty from within the confines of homology: the developmental evolution of the digging tibia of dung beetles

David M. Linz†, Yonggang Hu† and Armin P. Moczek

Department of Biology, Indiana University, Bloomington, IN 47405, USA

DML, 0000-0003-2096-3958; YH, 0000-0002-3438-7296

Understanding the origin of novel complex traits is among the most fundamental goals in evolutionary biology. The most widely used definition of novelty in evolution assumes the absence of homology, yet where homology ends and novelty begins is increasingly difficult to parse as *evo devo* continuously revises our understanding of what constitutes homology. Here, we executed a case study to explore the earliest stages of innovation by examining the tibial teeth of tunnelling dung beetles. Tibial teeth are a morphologically modest innovation, composed of relatively simple body wall projections and contained fully within the fore tibia, a leg segment whose own homology status is unambiguous. We first demonstrate that tibial teeth aid in multiple digging behaviours. We then show that the developmental evolution of tibial teeth was dominated by the redeployment of locally pre-existing gene networks. At the same time, we find that even at this very early stage of innovation, at least two genes that ancestrally function in embryonic patterning and thus entirely outside the spatial and temporal context of leg formation, have already become recruited to help shape the formation of tibial teeth. Our results suggest a testable model for how developmental evolution scaffolds innovation.

1. Introduction

How novel complex traits originate is among the most fundamental questions in evolutionary biology [1]. The most widely used definition of novelty in evolution assumes the absence of homology or homonomy (serial homology), i.e. a trait is considered an evolutionary novelty when it is neither homologous to any structure in the ancestral species nor homonomous to any other structure in the same organism [2]. This definition establishes a strict boundary condition, yet has also invited significant criticism. First, studies accumulating over the past two decades have forced a revision of the homology concept, away from dichotomous and towards a layered understanding of homology (reviewed in [3–5]). Accordingly, homology may now exist on the level of genes, gene networks or cell types, but not on the level of strict morphology [6–8]. The inverse also emerged as common: clearly homologous traits may be underlain by clearly non-homologous developmental processes, a phenomenon now recognized as developmental systems drift [9,10]. Second, by defining novelty as the absence of homology, evolutionary biologists are provided no conceptual framework with which to investigate the initiation of novelty. Yet our conception of the evolutionary process is fundamentally grounded in descent with modification where everything new must, ultimately, come from the old. As a consequence, how novel traits and functions emerge from within the confines of homology remains poorly understood. Here we explore the initial stages of morphological and functional innovation focusing on the front tibia of dung beetles, a trait homologous to the tibiae found in other insects and homonomous to the tibia of other leg-bearing body regions of the same organism. Specifically, we investigate how the fore tibia has become remodelled into a powerful digging apparatus enabling its bearers to use an ecological niche otherwise inaccessible to insects—compacted soil.

Insects possess three pairs of serially homologous legs, and strict homology extends further to the level of individual leg segments, such as the femur, tibia and tarsal segments [11]. This strict homology notwithstanding, different legs or leg segments have diversified in different lineages in significant ways, thereby opening up new ecological space within which insects were able to radiate, from the raptorial grasping appendages of mantids or the oar-like leg elongation of many aquatic insects to the pollen basket on the hind tibia of bees [12]. In comparison to classic cases of innovation such as the evolution of the vertebrate eye or the insect wing, innovations within and along the insect leg are modest. Yet, just like the evolution of the eye or wing, each leg innovation sparked subsequent radiations, allowing their bearers to conquer previously inaccessible habitats, or develop novel ways of resource acquisition [13,14]. Moreover, because many leg modifications occur within well-established modules whose own homology status is without doubt, they facilitate unambiguous comparisons across homonomous traits in the same organism or homologous traits in other taxa. Such modest innovations may thus provide experimentally tractable and conceptually interpretable means to investigate the earliest phases of morphological and functional innovation.

Here we use a combination of behavioural and developmental genetic approaches to assess the function and formation of the front tibia of dung beetles, a shovel-like enlarged digging tool that is presumed to have allowed dung beetles to access soil as a habitat, and to evolve tunnelling and subterranean reproduction as novel life-history strategies [15] (electronic supplementary material, figure S1). The front tibia of dung beetles is an enlarged, flattened, concave segment whose outer margin is typically characterized by four to five prominent tibial teeth (figure 1*a–c* and electronic supplementary material, figure S1). Mid and hind tibiae in contrast possess a much more conventional, tubular shape, lack elaborate teeth, but do possess minor, pointy projections. Furthermore, all tibiae regardless of segmental origin also possess a so-called tibial spur, a singular or sometimes two-pronged projection found on the distal end of the tibia of a wide range of insects (figure 1*b* and red arrows in figure 1*c–e*) [12]. Tibial teeth show significant wear as dung beetles age [16] and are assumed to play a critical role in digging, yet to the best of our knowledge this potentially adaptive significance has never been assessed experimentally. Here we show that tibial teeth indeed facilitate more efficient and deeper digging in two behavioural contexts—escape from threats and subterranean reproduction. We then turn to the developmental genetic mechanisms that enable the formation of the characteristic size and shape of the front tibia in general and the formation of tibial teeth in particular. We do so by contrasting two hypotheses. First, we hypothesized that the evolution of the digging tibia was made possible through the specific redeployment and modification of genes and pathways that were already involved in components of leg formation prior to the origin of the digging tibia. Such a result would be expected if locally available developmental and genetic mechanisms constitute the primary substrate for moderate morphological innovations. To test this hypothesis, we determined the functional significance of 16 candidate genes previously implicated in insect leg formation in other taxa. Second, we hypothesized that the evolution of the digging tibia was made possible through the differential recruitment of genes and pathways outside of a leg or even general appendage formation context. Such an outcome would support the hypothesis that innovation even well within existing morphological modules may draw upon developmental and genetic mechanisms far outside module boundaries and that locally available developmental machinery need not be a constraint on the initial stages of innovation. To test this hypothesis, we explored the functional significance of 13 genes involved in patterning the insect embryo. We chose this context for two reasons. First, embryonic patterning genes operate by definition at a developmental stage completely decoupled from late post-embryonic, metamorphic development and recent work suggests that embryonic patterning genes may thus be especially deconstrained to evolve novel functions at later stages (e.g. [17]). Our second motivation is methodological: embryonic patterning is one of the best-studied developmental processes in insects and accordingly detailed information on the underlying genes is available from a variety of insect taxa. Here we show that repurposing of diverse genes and pathways ancestrally already involved in leg formation has indeed enabled the evolution of the digging tibia, but that at least two embryonic patterning genes have also acquired novel and critical functions in the formation of tibial teeth.

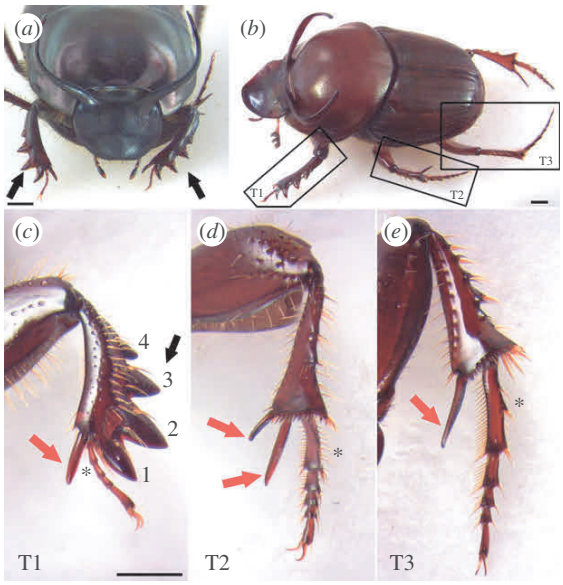


Figure 1. The legs of *Onthophagus taurus* dung beetles. (*a*) Anterior view. (*b*) Lateral view. (*c–e*) First (*c*), second (*d*) and third (*e*) thoracic segment (T1–3) distal legs. Numbers and black arrow in (*c*) indicate each prominent tibial tooth. Red arrows indicate tibial spurs on each leg. Asterisks indicate proximal tarsi on each leg. All scale bars are 0.5 mm. Scale in (*c*) applies to (*d*,*e*).

2. Results

(a) Functional significance of tibial teeth

We first sought to assess the functional significance of tibial teeth in the context of digging. To do so, we generated adult females of the same age and size (electronic supplementary material, figure S2A) whose tibial teeth had either been removed bilaterally through ablation or alternatively whose fore tibiae received comparable damage to the anterior surface (electronic supplementary material, figure S3A–C). We then experimentally replicated two specific behavioural contexts in which digging performance is probably fitness relevant in nature. First, we assayed the natural escape response of

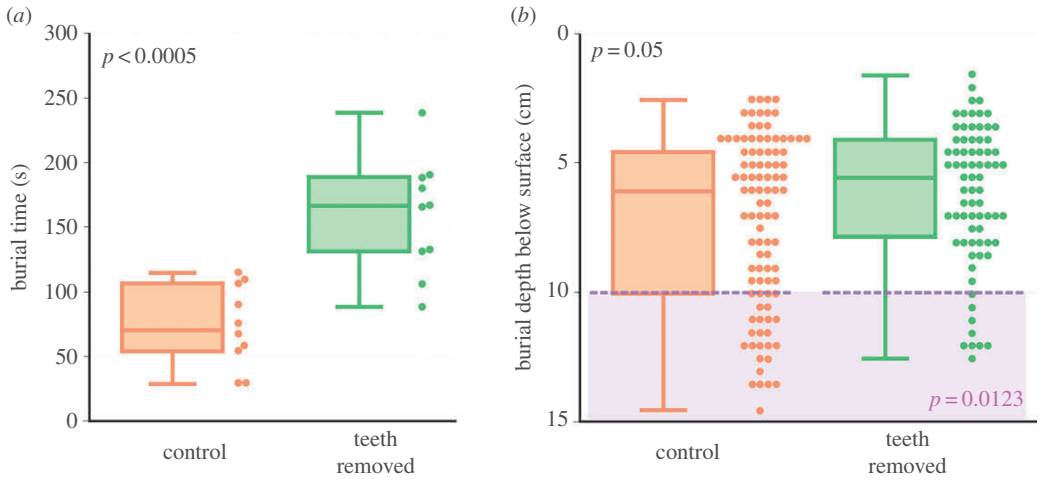


Figure 2. Ablation of tibial teeth affects two distinct digging behaviours. (*a*) Boxplot showing the effects of teeth removal on escape digging ($n = 10$ each). Tibial teeth removal increases the time required for complete burial ($t_{18} = 2.1$; $p < 0.0005$). (*b*) Boxplot showing the effects of teeth removal on brood ball burial depth ($n = 15$ control-ablated; $n = 12$ teeth removed). Tibial teeth ablation resulted in a marginally significant decrease in average depth of brood ball burial when all brood balls were included in the analysis (Mann–Whitney $U = 2875.5$, $p = 0.05$) and a significant reduction of the fraction of brood balls buried in the deepest of three 5 cm layers (Fisher's exact test, $p = 0.0123$; brood balls below purple dash and within the purple boxed region). Dots in (*a*) represent each individual. Dots in (*b*) represent each brood ball depth measured.

Onthophagus beetles: following disturbance, individual beetles will rapidly bury themselves until they are completely covered, then remain motionless. Beetles lacking tibial teeth took nearly three times as long to bury themselves completely compared to control beetles ($t_{18} = 2.1$; $p < 0.0005$; figure 2*a*), consistent with a role of tibial teeth in facilitating effective escape. Second, we assessed the potential functional significance of tibial teeth in the context of subterranean reproduction. *Onthophagus* reproduce by digging tunnels underneath dung pads. Once a certain depth is reached mothers move dung into these tunnels and use it to construct discrete brood balls. Females oviposit a single egg into each brood ball, which serves as the sole food source for the developing larva. Number and depth of brood balls that adults are able to construct contribute significantly to beetle fitness [18], and here we tested whether the presence or absence of tibial teeth influences these measures during a 5 day breeding period. Tibial teeth ablation did not affect the number of brood balls produced (electronic supplementary material, figure S2B), but resulted in a marginally significant decrease in average depth of brood ball burial when all brood balls were included in the analysis (Mann–Whitney $U = 2875.5$, $p = 0.05$; figure 2*b*). We then categorized burial depth into three 5 cm layers (shallow, intermediate and deep; after [18]) to contrast the proportion of brood balls buried at the deepest layer to those produced in shallower layers. We found that females whose tibial teeth had been experimentally removed buried a significantly smaller number of brood balls in the deepest layer (Fisher's exact test, $p = 0.0123$; purple box, figure 2*b*). Combined, these data support the hypothesis that tibial teeth enhance digging performance across behavioural contexts.

(b) Thirteen of 16 appendage patterning genes are functionally required for the correct formation of tibial teeth

We next investigated the regulation of tibial teeth formation during development. Specifically, we first sought to test the hypothesis that tibial teeth formation was facilitated by the specific redeployment and modification of genes already involved in medio-distal leg formation prior to the evolution of tibial teeth (table 1) (for review of general leg patterning, see [19]). Of the 16 genes we examined, three produced defects during leg formation *without* affecting the formation of tibial teeth, seven affected tibial teeth formation most likely as a secondary by-product of their larger regulatory role in tibial or distal leg specification, while six genes appeared to have acquired specific functions in facilitating the formation of tibial teeth, alongside their traditional roles in leg patterning.

Specifically, downregulation of the functionally redundant paralogues *bric-a-brac1* and *bric-a-brac2* (collectively *bab*) as well as *spineless* (*ss*) caused fusions of the tarsal segments as previously described in other Coleoptera [20], but as far as we were able to discern did not disrupt tibial teeth formation (electronic supplementary material, figures S4A–R, S5). By contrast, experimental downregulation of *dachshund* (*dac*), *lim1*, *Serrate* (*Ser*) and four members of the *odd-skipped* gene family—*odd-skipped* (*odd*), *brother of odd with entrails limited* (*bowl*), *sister of odd and bowl* (*sob*) and *drumstick* (*drm*)—disrupted tibial teeth formation, but most likely did so as part of their larger role in patterning the medio-distal leg (table 1). In particular, downregulation of *dac*, a well-studied leg gap gene critical for patterning the medial leg components [20–23], heavily truncated the *Onthophagus* tibia on all thoracic segments and caused tibial teeth on the first thoracic segment to be shortened and reduced in number (arrow, figure 3*a–d*; and electronic supplementary material, figure S6A–E). Downregulation of *lim1*, a lim-homeodomain transcription factor activated by EGFR signalling and critical in the patterning of the tibia in *Tribolium* [20], resulted in a reduction and fusion of the femur and tibia (figure 3*e,f* and electronic supplementary material, figure S6F,G). This defect was paralleled by tibial teeth becoming irregularly spaced and distributed (arrow, figure 3*e,f*). Similarly, when we used Ser^{RNAi} to modulate Notch signalling, which is known to be critical for the formation of leg joints [20,24–27], we observed a

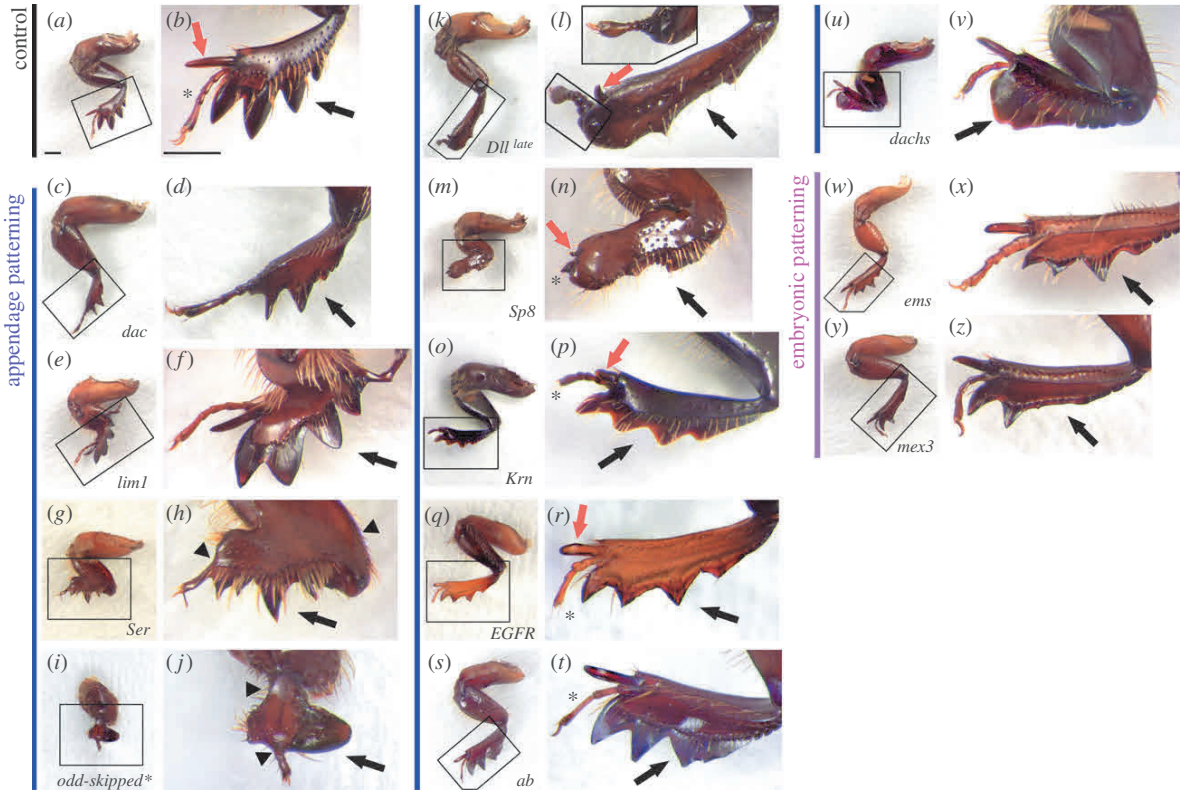
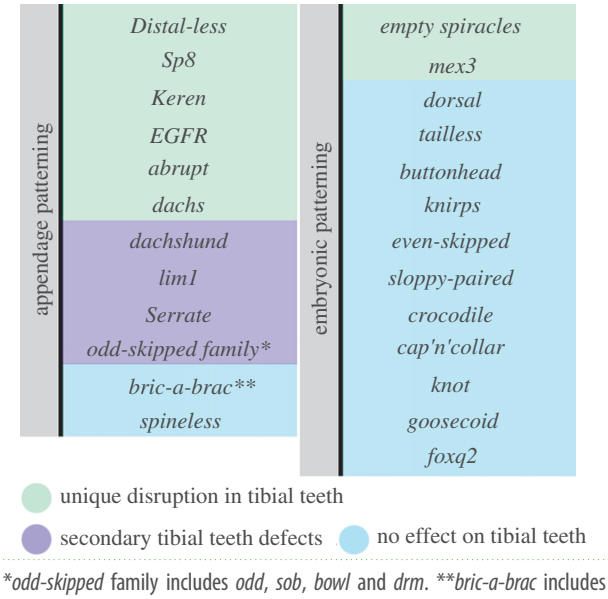


Figure 3. Defects in the tibial teeth and first thoracic leg formation induced by appendage and embryonic patterning genes. (*a*,*b*) Buffer injected control. The distal region of the leg (box in (*a*)), the tibial teeth (black arrow in (*b*)), the tibial spur (red arrow in (*b*)), and tarsi and tarsal claw (asterisk in (*b*)) are indicated. (*c*–*v*) Appendage patterning gene RNAi. (*c*–*j*) Downregulation of several appendage patterning genes caused truncation and fusion of leg segments. RNAi for *dac* (*c*,*d*), *lim1* (*e*,*f*), *Ser* (*g*,*h*) and *odd-skipped* family genes* (*i*,*j*) caused tibial teeth defects (black arrows in *d*,*f*,*h*, and *j*) as a by-product of truncation or fusion (arrowheads in *h* and *j*). (*k*–*v*) Additional appendage patterning gene RNAi caused unique defects in the tibial teeth. RNAi for *Dll*^{late} (*k*,*l*), *Sp8* (*m*,*n*), *Krn* (*o*,*p*), *EGFR* (*q*,*r*), *ab* (*s*,*t*), and *dachs* (*u*,*v*) disrupted tibial teeth formation (black arrows in *l*,*n*,*p*,*r*,*t*, and *v*) as well as irregularities in the tibial spur (red arrows in *l*, *n*, *p* and *r*) and tarsi (asterisks in *n*,*p*,*r* and *t*). (*w*–*z*) Embryonic patterning gene RNAi. RNAi for *ems* (*w*,*x*) and *mex3* (*y*,*z*) disrupted tibial teeth formation (black arrows in *x* and *z*). Scale in (*a*,*b*) is 0.5 mm and applies to left and right panes for all genes, respectively. **odd-skipped* family genes includes *odd*, *sob*, *bowl* and *drm* simultaneous knockdown.

Table 1. Genes examined in this study and their respective roles in tibial teeth formation.



**odd-skipped* family includes *odd*, *sob*, *bowl* and *drm*. ***bric-a-brac* includes both *bric-a-brac1* and *bric-a-brac2*.

corresponding fusion of all leg segments (arrowheads, figure 3*g*,*h*; and electronic supplementary material, figure S6H,I). This fusion of leg segments was accompanied by a reduction of tibial teeth at the most proximal and distal regions of the T1 tibia where they are closest to the nearest joint, while those in the medial tibia were less affected (arrow, figure 3*g*,*h*). Lastly, we investigated *odd*, *bowl*, *sob* and *drm*, four members of the *odd-skipped* gene family which act downstream of the Notch signal to further refine leg joint formation [28,29]. Owing to the functional redundancy and sequence similarity between *odd-skipped* family genes [20,30] we generated a 600 bp chimeric nucleotide sequence containing partial fragments of all gene family members to target each paralogue simultaneously; with the caveat that simultaneous knockdown prevents us from determining the functions of individual family members as well as individual knockdown levels owing to intracellular competition for the RNAi machinery. Nevertheless, *odd-skipped* family knockdown produced RNAi phenotypes that largely recapitulated *Ser*^{RNAi}, i.e. loss of joints via a fusion and reduction in length of leg segments (arrowheads, figure 3*i*,*j*; and electronic supplementary material, figure S6 J,K) and loss of most tibial teeth as a by-product of these effects (arrow, figure 3*i*,*j*).

Intriguingly, we also identified a group of six genes, including *Distal-less* (*Dll*), *Sp8* (called *Sp1* in *Drosophila*), *abrupt* (*ab*), as

well as members of the EGFR and Hippo signalling pathways, whose downregulation revealed a conserved role in leg patterning alongside novel, specific roles in the patterning of tibial teeth. Specifically, *Dll* is a key transcription factor regulating distal aspects of leg formation, and removing *Dll* function is known to eliminate distal leg segments, a role that has also been previously reported in *Onthophagus taurus* [21]. We first confirmed the role of *Dll* in patterning these leg regions by performing RNAi early in the last larval stage and confirmed that $Dll^{RNAi,\ early}$ truncates legs by removing distal leg segments (electronic supplementary material, figure S7A–L). Next, we adjusted the timing of our knockdown to the mid last larval stages to target possible additional roles of *Dll* in patterning leg structures. We observed that $Dll^{RNAi,\ late}$ leaves distal leg segments intact (figure 3*k*,*l* and electronic supplementary material, figure S6 L,M), yet completely eliminates tibial teeth on T1 legs (black arrow, figure 3*k*,*l*) as well as reduces the prominent tibial spurs present on T1, T2 and T3 legs (red arrow, figure 3*k*,*l*; and electronic supplementary material, figure S6 L,M).

Sp8 is a similarly conserved transcription factor critical for leg formation and elongation across bilaterians [31–35]. We found that $Sp8^{RNAi}$ truncates legs by eliminating tarsal segments while leaving remnants of the tarsal claw attached to the distal tibia (asterisks, figure 3*m*,*n*). This phenotype was observed in all three pairs of legs and parallels *Sp8* knockdown defects previously reported in other insect taxa (electronic supplementary material, figure S6N,O) [31,32]). At the same time, and again across all three pairs of legs, we observed the deletion of projections occurring on the tibia, including tibial teeth (black arrow, figure 3*m*,*n*) and tibial spurs (red arrow, figure 3*m*,*n*; and electronic supplementary material, figure S6N,O).

Prior work has also established the role of EGFR signalling in distal leg patterning [20,36,37]. To investigate if this pathway patterns tibial teeth formation, we knocked down the EGFR ligand *Keren* (*Krn*) as well as the receptor itself (*EGFR*). RNAi for both *Krn* and *EGFR* eliminated the most distal leg structure, the tarsal claw, from all legs (asterisks, figure 3*o*–*r*; and electronic supplementary material, figure S6P–S). In addition, both *Krn* and *EGFR* RNAi shortened and rounded all tibial teeth (black arrows, figure 3*o*–*r*), reduced the length of tibial spurs in all legs (red arrows, figure 3*o*–*r*; and electronic supplementary material, figure S6P–S), yet left the remainder of tibial morphology unaffected (figure 3*o*–*r* and electronic supplementary material, figure S6P–S).

Next, we assessed the function of the gene *ab*. In *Tribolium* beetles, the most closely related species in which *ab* function has been studied in detail, ab^{RNAi} fuses and reduces tarsi while leaving other aspects of leg patterning unaffected [20,38]. We observed similar fusions among *O. taurus* tarsi following ab^{RNAi} (asterisks, figure 3*s*,*t*; and electronic supplementary material, figure S6T,U), yet at the same time, observed fusion events during tibial teeth formation: specifically, the two most proximal teeth fused, resulting in a single tooth with a wider size and irregular shape, while more distal teeth were unaffected (arrow, figure 3*s*,*t*).

Lastly, we made similar observations for Hippo signalling. While not a standard component of insect leg axis specification, Hippo signalling nevertheless plays an integral role in shaping the growth and size relationships among a variety of appendages (for review, see [39]). We performed RNAi for *dachs*, a component of Hippo signalling known to affect tissue growth in multiple insect species [38,40]. $dachs^{RNAi}$ did not disrupt the general proximo-distal (P-D) patterning of leg segments; however, it did subtly shorten each individual segment causing the leg to become more compacted (figure 3*u*,*v* and electronic supplementary material, figure S6 V,W). Additionally, in T1 legs *dachs* RNAi also strongly and uniquely altered tibial teeth formation, fusing the individual teeth into a singular blade-like projection (arrow, figure 3*u*,*v*). Interestingly, *dachs* RNAi did not disrupt tibial spurs on the tibia of T1, T2 or T3 legs (figure 3*u*,*v* and electronic supplementary material, figure S6 V,W). Taken together, these data suggest that *Dll*, *sp8*, ab, alongside EGFR and Hippo signalling have acquired novel functions in the context of tibial teeth formation while maintaining their conserved roles in patterning diverse aspects of medio-distal leg formation.

(c) Two of 13 embryonic patterning genes have acquired functions in the formation of adult tibial teeth

We then sought to assess the role of genes and pathways outside of a leg or general appendage formation context in the origin of tibial teeth. To test this hypothesis, we explored the functional significance of 13 genes involved in patterning the insect embryo, a group of genes believed to be especially deconstrained to evolve novel functions at later developmental stages [41]. We chose 13 *O. taurus* genes orthologous to various members of the embryonic patterning network and then assessed their functions in *O. taurus* (table 1). Of these, eight exhibited no discernible morphological RNAi phenotypes anywhere, while three exhibited RNAi phenotypes entirely outside leg formation (electronic supplementary material, table S1). However, two genes—*empty spiracles* (*ems*) and *mex3*—revealed a patterning function during tibial teeth formation.

Specifically, when examining the post-embryonic function of the head gap-like genes *buttonhead* (*btd*), *sloppy-paired* (*slp*) and *ems*, we observed that btd^{RNAi} and slp^{RNAi} failed to reveal abnormalities in adult development, while ems^{RNAi} resulted in highly reproducible defects in the growth of tibial teeth, yielding teeth that were substantially smaller and more irregularly sized along the tibial P–D axis (arrow, figure 3*w*,*x*). Outside of the tibial teeth, ems^{RNAi} did not cause irregularities in the patterning of remaining leg segments nor in any other leg projections including the tibial spurs of T1–3 (figure 3*w*,*x* and electronic supplementary material, figure S6X,Y).

Similarly, we examined the post-embryonic function of *mex3*, which in *Tribolium* beetles functions akin to *bicoid* in *Drosophila* in the establishment of the early *caudal* posterior gradient critical for early embryogenesis across diverse bilateria [42,43]. $mex3^{RNAi}$ resulted in adult animals with a unique disruption of T1 tibial teeth: $mex3^{RNAi}$ eliminated proximal teeth while reducing the length of more distal teeth (arrow, figure 3*y*,*z*), yet without obvious alterations to length and width of the tibia (compare e.g. to dac^{RNAi} (figure 3*c*,*d*) to $mex3^{RNAi}$ (figure 3*y*,*z*)). Further, tibial spurs of all thoracic legs were similarly unaffected by $mex3^{RNAi}$, as was the patterning of the remaining leg segments except possibly for subtle irregularities in the length of the tarsi (figure 3*y*,*z* and electronic supplementary material, figure S6Z–AA). Combined, these data suggest that both *ems* and especially *mex3* have acquired novel functions outside their respective embryonic patterning domains in the shaping and patterning of tibial teeth.

3. Discussion

To meet the most widely used definition of evolutionary novelty, traits have to be neither homologous to other traits in ancestral taxa nor homonomous to traits in the other parts of the organism. Yet defining novelty through the absence of homology has become more complicated ever since evolutionary developmental biologists have uncovered the layered nature of homology, with homologous genes and pathways instructing the formation of non-homologous morphologies, and non-homologous developmental mechanisms enabling the formation of clearly homologous traits [3]. Determining where homology ends and novelty begins is thus more unclear than ever, causing some to question whether either concept remains useful to help guide future work and to conceptualize developmental evolution more generally [4,44]. Here we sought to move beyond definitional limitations by executing a case study that explores the origins of the tibial teeth of scarab beetles. Tibial teeth may be viewed as an example of a first phase of innovation, modest in morphological scope and nested well within a pre-existing homologous and homonomous morphological module, yet already endowed with significant novel, adaptive potential. In this study, we first aimed to confirm the long-standing expectation that the four prominent tibial teeth that characterize the outer margin of the *Onthophagus* fore tibia aid in digging and then sought to investigate the developmental genetic mechanisms that underlie their formation. Here, we specifically sought to contrast the roles of developmental mechanisms already tasked with instructing other aspects of leg development to those potentially recruited from outside developmental contexts to probe the developmental genetic origins of the earliest stages of morphological innovations as exemplified by tibial teeth. Our results show that tibial teeth functionally enhance digging performance and that their developmental evolution was facilitated through significant repurposing of diverse genes and pathways ancestrally already involved in leg formation, as well as the recruitment of at least two genes ancestrally tasked with instructing embryonic development. Below, we discuss the most important implications of our results in light of the genetic and developmental sources of biases in innovation during developmental evolution.

(a) The behavioural and ecological significance of tibial teeth

Tibial teeth enhance the shovel-like appearance of the *Onthophagus* fore tibia and are well known to undergo significant wear during adult life, so much so that they can be used to assess the adult age of burying scarabs [16]. These and other observations have fuelled a long-standing, but never directly tested, assumption that tibial teeth facilitate effective digging. Our results provide experimental support for this assumption by showing that tibial teeth function in digging in the context of at least two fitness-relevant behaviours. First, we found that the absence of tibial teeth greatly hindered the execution of a common escape response by reducing individuals' ability to bury themselves after release by an experimenter, compared to control-ablated individuals with intact tibial teeth. Second, the absence of tibial teeth caused adult females to bury relatively fewer brood balls in the deepest of three layers. This latter effect was more modest, yet over an adult lifetime may contribute significantly to fitness given the role of burial depth in enhancing offspring development by ensuring a more isothermic developmental environment [18]. Additionally, despite our best efforts, the compacted soil we generated for our experiments remained considerably less dense and compact than at least some of the soil types naturally colonized by *O. taurus*, suggesting that the importance of tibial teeth for deep burial may be more severe in natural populations than our experiment was able to detect.

(b) Tibial teeth formation relies substantially on the repurposing of conserved appendage patterning genes

We then sought to assess whether the evolution of tibial teeth was made possible through the redeployment of genes already involved in components of leg formation prior to the origin of the digging tibia. Such a result would support the hypothesis that initial and modest morphological innovation is facilitated primarily by locally available developmental and genetic mechanisms. Our findings support this hypothesis by showing that of the 16 leg genes examined, 13 are indeed required for the correct formation of tibial teeth. These genes can be broadly grouped into two categories: seven genes (*dac*, *lim1*, *Ser*, *odd*, *bowl*, *sob*, *drm*) exhibited RNAi phenotypes consistent with a maintenance of their ancestral role in patterning the leg including the tibia, and whose disruption of overall leg formation appears to secondarily affect tibial teeth formation. While these genes are in some sense functionally required for the formation of tibial teeth, this requirement is unlikely to reflect cooption events that specifically enabled the evolution of tibial teeth; rather, tibial teeth evolved within the larger, pre-existing functional domains of these genes.

However, we also identified a second group of six genes (*Dll*, *Sp8*, *ab*, *dachs*, *Krn*, *EGFR*), which in addition to maintaining their ancestral functions during leg formation, also appear to have acquired distinct additional roles in the context of tibial teeth formation. Intriguingly, of these, four (*Dll*, *Sp8*, *EGFR*, *Krn*) also disrupt the formation of tibial spurs—a separate projection on the tibia which, unlike tibial teeth, is taxonomically extremely widespread among extant insect orders including hemi- and holometabolous insects, and whose presence is probably reflective of a deeply ancestral character state. Similarly, the formation of more minute tibial projections present on mid and hind tibiae also appear affected by the same gene knockdowns; however, the small size of these structures precludes a more definitive analysis. If correct, our data raise the possibility that parts of the leg patterning gene network may have been recruited towards the formation of tibial spurs alongside minor projections, and then secondarily recruited again for forming tibial teeth. Interestingly, the precise developmental functions executed by these genes, in particular, *Dll* and *Sp8*, appear to recapitulate their broader function during leg formation: both genes play a major role in specifying the P-D axis during leg formation, and their experimental downregulation yields heavily truncated legs lacking distal elements. Similarly, hypomorphic knockdown of either gene in *Onthophagus* leaves overall P-D axis formation of the leg intact but results in the truncation of tibial teeth and spurs.

By contrast, *ab* and the Hippo signalling member *dachs* affected tibial teeth without affecting tibial spurs, suggesting that their recruitment may have occurred specifically to refine

tibial teeth formation, such as the precise spacing of teeth and the depth of the valleys between them, morphological aspects that do not pertain to tibial spurs. More generally, our results support the hypothesis that the repurposing of locally available genes and pathways has played a critical role in the developmental evolution of tibial teeth.

(c) Two embryonic patterning genes have evolved novel functions in the context of tibial teeth formation

We alternatively hypothesized that the evolution of tibial teeth was facilitated through the differential recruitment of genes and pathways outside an appendage formation context. If correct, such an outcome would support the hypothesis that even modest innovation occurring well within existing morphological modules may draw upon developmental and genetic mechanisms outside module boundaries and that locally available developmental machinery need not be a constraint on such innovation. To test this hypothesis, we explored the functional significance of 13 genes involved in patterning the insect embryo. Of those, two—*ems* and *mex3*—both uniquely affected size, shape and spacing of tibial teeth, notably without disrupting tibial spurs or the remainder of the leg's morphology. These findings support our hypothesis that the evolution of tibial teeth relied, on at least two occasions, on differential recruitment of genes well outside the context of appendage formation. By extension, these results show that the evolution of even modest forms of novelty, while clearly shaped by developmental mechanisms already in place, may readily co-opt genes from very different spatial and temporal contexts.

(d) Re-evaluating the origins of novelty

Defining evolutionary novelty through the absence of homology to pre-existing traits has been increasingly difficult to reconcile with empirical findings on innovation in evolution, which are dominated by the differential redeployment of conserved developmental building blocks outside their traditional developmental context. Striking examples include the repurposing of genes normally involved in outgrowth formation in the specification of butterfly wing spots [45], the reuse of pigmentation genes in firefly lantern development [46], or the cooption of hedgehog signalling genes normally involved in establishing anteroposterior polarity in the nutrition-dependent growth of beetle horns [47]. All of these traits are considered true novelties, except for the developmental mechanisms that produce them. Most importantly, however, defining novelty simply as the absence of homology provides no conceptual framework with which to guide an investigation into the nature of repurposing and the emergence of novelty from within the diversity of ancestral developmental processes. In this study, we find that the developmental evolution of tibial teeth was dominated by the redeployment of locally pre-existing gene networks, including genes whose precise developmental functions within the context of tibial teeth formation mirrors their broader function during leg formation. At the same time, we found that even at this very modest stage of innovation, genes that ancestrally function well outside the spatial and temporal context of leg formation—such as embryonic patterning—may already become recruited to help shape the formation of novel structures. Our results may thus suggest a possible model for how developmental evolution scaffolds innovation: first through the reuse of genes whose products are locally already available and whose ancestral functions are preadapted to support key aspects of the development of a given novel trait, followed by genes whose products are locally available yet which acquire additional functions alongside their traditional roles, followed lastly by genes whose products ancestrally function completely outside the context of a given novel trait, and thus have to evolve both novel domains of expression, and new functions therein. Whether this model indeed captures a dominant theme would be testable through the careful comparative analyses of independently evolved adaptations toward digging, as e.g. in mole crickets and nymphal cicadas, as well as other leg innovations of comparable morphological magnitude, such as the raptorial front legs of the praying mantis, or the hind-tibial elaborations of leaf-footed bugs. However, these findings also beg the larger question: what else may we be missing? How many genes from *other* contexts such as e.g. eye development or wing formation may also be repurposed in the early genesis of novelty, and even more importantly, what are the features of a gene or developmental context that influence the probability of cooption events? Comparative investigations into the nature of cooption in innovation will be key to address these and related questions in the future and may ultimately help us understand how novelty, rather than somehow emerge in the absence of homology, may instead be initiated through it.

4. Material and methods

(a) Beetle care

Adult *Onthophagus taurus* were collected around Durham, North Carolina and Busselton, Western Australia to establish laboratory colonies. Beetles were reared as described previously [48,49].

(b) Cloning, sequencing, dsRNA synthesis and injection of *Onthophagus taurus* candidate genes

Onthophagus orthologues of candidate genes were identified by reciprocal BLAST to *Tribolium* and *Drosophila* databases. dsRNA was synthesized as previously described [50]. Off-target checks with two non-overlapping fragments targeting the same gene were performed for embryonic patterning genes with previously undescribed defects in tibial teeth formation. See the electronic supplementary material, methods for details.

(c) Digging behaviour assay

Mated females of similar age and size were generated. Size was determined using thoracic width of adult females as a proxy of body size (see [51] for justification). Females in the experimental group had all four tibial teeth carefully ablated by micro-dissecting scissors, while control females received four point ablations using a fine needle (electronic supplementary material, figure S3A–C). See the electronic supplementary material, methods for additional details.

The first of two behavioural assays assessed the natural escape response of *Onthophagus* beetles, which entails that following disturbance beetles will rapidly bury themselves until they are completely covered and then remain motionless. To standardize soil packing, we used a homogenized sand/soil/water mixture, a standard pounding weight (5 lb) and number of tamps (5) to compact sand/soil mixture into a shallow, circular container (9 cm diameter, 28 cm depth). We observed in pilot runs that beetles placed into our experimental set-up will use a subtle indentation as a starting point to initiate digging. Thus, for our

experimental replicates, we added four standardized, shallow indentations (depth less than 1 mm) to the soil surface. At the start of the assay, an individual adult female was released into the arena, and in most cases, walked quickly away from the experimenter until encountering a depression, then initiated digging. The time from the start of digging to the beetle's complete disappearance in the soil was recorded. Experimental and control animals were alternated as we executed this assay.

The second behavioural assay then tested the same females already used in the first assay with respect to their tunnelling and brood provisioning ability. Using established protocols [18], each female was placed individually in cylindrical pasta containers (9 cm diameter, 28 cm depth) densely packed with sand/soil mixture and provisioned with a standard amount of dung. Females were given five days to tunnel and produce brood balls. After five days, pasta containers were inverted. The number of brood balls produced by each female as well as the burial depth of each brood ball was recorded to the nearest 0.5 cm.

(d) Statistical analysis

(i) Escape response behaviour

The duration from the start of digging to a beetle's complete disappearance in the soil was compared across treatments using a *t*-test.

(ii) Brood ball depth

Previous work [18] has shown that adult female body size can influence number and depth of brood balls. We therefore first determined that adult females did not differ in body size across our treatments (Welch *T*-test, $t_{129.47} = 0.25$, $p = 0.80$), nor in the number of brood balls they produced (Wald $\chi^2_1 = 0.232$, $p = 0.63$). With both conditions satisfied, we first used a non-parametric Mann–Whitney *U*-test to compare the entire distribution of brood ball depths across the two treatments. Second, we reanalysed burial depth data by counting the number of brood balls buried in the lowest of three 5 cm layers (0–5 cm, 5–10 cm, 10–15 cm) to test whether intact tibial teeth may enable females to bury a greater fraction of their brood balls at deeper layers. A two-tailed Fisher's exact test was then used to compare the number of brood balls buried in the lowest layer relative to those buried in shallower layers across both treatment groups.

Data accessibility. The datasets supporting the conclusions of this article are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.460hn37 [52].

Authors' contributions. D.M.L., Y.H. and A.P.M. designed the experiments; D.M.L. and Y.H. performed the experiments; D.M.L., Y.H. and A.P.M. analysed the data and wrote the manuscript. All authors read and approved the manuscript for publication.

Competing interests. All authors declare that they have no competing interests.

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