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Recent advances in understanding horn formation in the Japanese rhinoceros beetle *Trypoxylus dichotomus* using next-generation sequencing technology

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The exaggerated horns of beetles are attractive models for studying the origin of novel traits and morphological evolution. Closely related species often differ profoundly in the size, number, and shape of their horns, and in the body region from which they extend. In addition, beetle horns exhibit exquisite nutrition-dependent phenotypic plasticity, leading to disproportionate growth of the horns in the largest, best-condition individuals and much smaller — even stunted — horn sizes in poor-condition individuals. These exciting phenomena in beetle horns have recently been revealed at the molecular level with the advent of next-generation sequencing. This section reviews the latest research on a horned beetle, the Japanese rhinoceros beetle *Trypoxylus dichotomus,* whose genome was recently sequenced.

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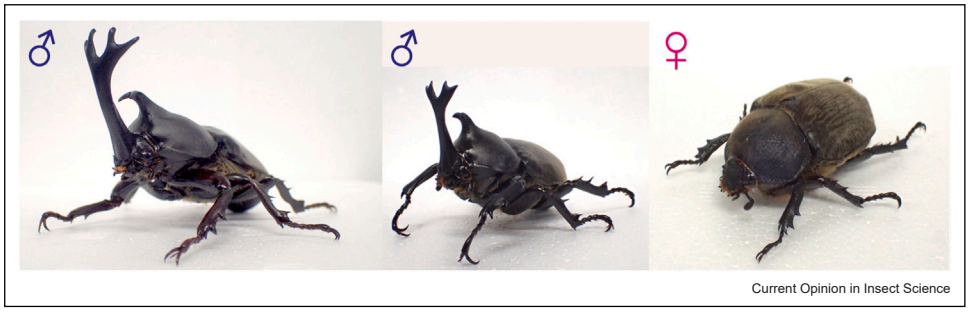
Introduction

Beetles (Insecta: Coleoptera) are the largest and most successful order, not only among insects but also among all animal species [1]. Beetles exhibit extraordinary morphological, ecological, and behavioral diversity [2], and many of their most charismatic character radiations involve the weapon trait of horns. Beetle horns have arisen multiple times independently within the Scarabs (e.g. dung beetles, Scarabaeinae; Dor beetles, Geotrupinae; rhinoceros beetles, and Dynastinae), where they typically project from the head or prothorax of males [3]. Relative to body size, beetle horns surpass even the largest weapons of ungulates [4]. Darwin (1871) [5] proposed that the exaggerated sizes of beetle horns were the result of sexual selection, and beetle horns are now known to be used for intraspecific combat between males as they battle to defend feeding territories attractive to females [3,6,7]. Beetle horns are also considered to be evolutionarily novel traits [8], making them exciting structures to study from the evo-devo perspective.

The Japanese rhinoceros beetle *Trypoxylus dichotomus* (Figure 1), one of the largest beetles in Japan, is popular as a pet with both children and adults [9] in part because of the large horn extending from the heads of males. This head horn can be up to 2/3 of the length of the body, and is bifurcated twice at the distal tip resulting in a distinct 'pitchfork' shaped morphology that is unique to this species. A smaller horn extending from the prothoracic region (thoracic horn) is bifurcated once at the distal tip. *T. dichotomus* horns are well adapted to the fighting style of these beetles, in which males attempt to place their head horn under the prothorax of an opponent, scooping up the rival and flipping him off of a tree. Biomechanical simulations demonstrate that the triangular cross-sectional shape of this horn is unusually resistant to breakage under vertical bending and twisting loads [10].

T. dichotomus has many advantages as a research model. (1) *T. dichotomus* has been established as a pet. As such, thousands of larvae can be purchased for around $ 1/animal. (2) Items necessary for breeding larvae to adults (breeding cages, food for larvae and adults, and artificial pupal chambers, etc.) can be purchased readily at a low cost. (3). Final instar larvae can be stored at low temperatures to delay development, permitting these animals to be used for research throughout the year [11]. (4) Precise staging of development during the period when

Figure 1



Sexual dimorphism and phenotypic plasticity in *T. dichotomus*. Male and female adults of *T. dichotomus*. *T. dichotomus* horns are a sexually dimorphic trait: horns are present only in males. *T. dichotomus* horns exhibit nutrition-dependent phenotypic plasticity and are considered to be dimorphic (major and minor) [21,41]. Scale bars are 10 mm.

horn formation occurs (prepupa to adult emergence) is possible because a system for breeding outside the soil has been established [12]. (5) Gene function analysis using larval RNAi is extremely efficient in *T. dichotomus* [11–16]. (6) Recently, an annotated genome has been published [17,18]. Due to these advantages, research using *T. dichotomus* has now been reported in fields as diverse as developmental biology, population genetics, genomics, animal behavior, evolution, biomechanics, and biochemistry [2–4,10–12,15,17–25]. In this review, we describe the developmental mechanism of horn formation, as recent genomics technologies have enabled genome-wide gene expression analysis in these exaggerated sexually dimorphic weapons.

T. dichotomus genome information

The *T. dichotomus* genome size is estimated to be 773.1 ± 24.6 Mb by flow cytometry [17]. Recently, detailed *T. dichotomus* genomic information using the 10× Chromium platform has been reported [17]. This haploid genome assembly was named the TdicSN1.0 and yielded a *de novo* assembly of 615 Mb, representing 80% of the estimated genome size. The TdicSN1.0 consists of 15 609 scaffolds with an N50 of 8.02 Mb. Twenty-three thousand nine hundred eighty-seven protein-coding genes are predicted in the *T. dichotomus* genome. Assembly completeness measured using benchmarking universal single-copy orthologs (BUSCO) is 99.4 % (98.9% complete, 0.2% fragmented) of the Insecta data set (version 4.0.6, n = 1367). Repetitive sequences accounted for 49.54% of the TdicSN1.0 (305 Mb). In addition, a different group recently reported the genome assembly of *T. dichotomus* generated from long-read data [18]. This genome assembly yielded a *de novo* assembly of 739 Mb, consisted of 2347 contigs and the BUSCO completeness was 99.6%. The difference in genome size between the two assemblies is probably due to repetitive sequences, because long-read technologies can capture more repetitive sequences than short-read-based platforms.

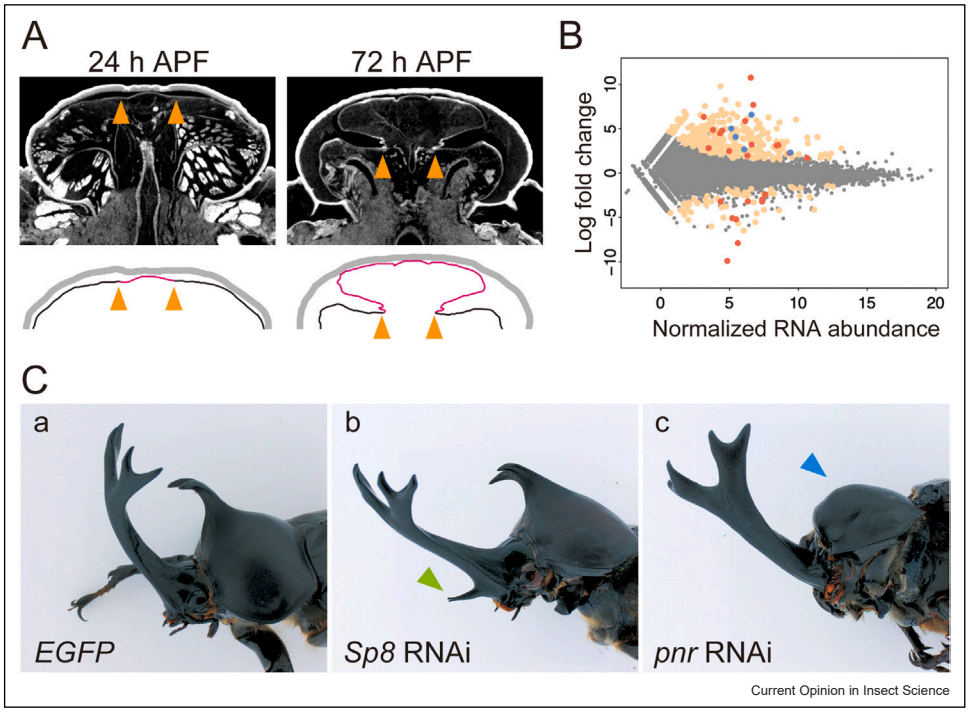
Thus, genomic information for *T. dichotomus* is currently well established and the high completeness and extensive annotations of this genome mean that it will be an excellent resource benefitting a variety of research areas.

T. dichotomus horn formation

T. dichotomus horns are sexually dimorphic (females do not develop horns). Sex differentiation in holometabolous insects is generally regulated by *transformer* (*tra*) — *doublesex* (*dsx*) in sex determination cascades. *tra* regulates sex-specific splicing of *dsx* pre-mRNA and induces sexual differentiation. These cascades drive sex determination in *T. dichotomus* as well [11,12]. In *T. dichotomus*, *tra* RNAi causes females to produce male-specific splice forms of *dsx*. This results in morphological sex transformation into males, including ectopic horn formation on both the head and prothorax [12], and implicates this pathway in the molecular mechanism of sexually dimorphic horn growth.

Because beetle horns are considered to be 'novel' traits — arising *de novo* rather than through modification of an existing structure — they are especially exciting to study mechanistically. Often novel traits evolve through the co-option of existing developmental pathways in new regions of the epidermis, but if true in this case, which genes are responsible? We focused on the sexual differences that first appear in horn primordia during the prepupal period (Figure 2A) [12,15] by assembling four cDNA libraries comprising male head horn primordia (HH), male thoracic horn primordia (TH), and corresponding HH and TH epidermal regions from females, and then used RNA-seq analyses to look at the onset of sex differentiation (Figure 2B). We also performed larval RNAi experiments on 49 candidate horn formation genes identified from these analyses, focusing on genes encoding for transcription factors (TFs) or signaling molecules. These studies identified 11 TFs with clear functional roles in horn development. These 11 genes

Figure 2



Morphological change of horn primordia and RNAi-mediated loss-of-function phenotypes.**(A)** Micro-CT images of the male head horn primordium during development. *T. dichotomus* forms large horn primordia in only 5 days. The left panel shows 24 hours APF, the right panel shows 72 hours APF for the head horn primordium. The orange arrows and magenta lines indicate the horn primordium regions. The gray line indicates the head capsule. APF, after pupal-chamber formation. **(B)** MA-plots comparing RNA-seq datasets of male head horn versus male thoracic horn. Gray dots indicate transcripts. Tan dots show genes differentially expressed at an FDR of < 0.05. Orange dots show transcription factors. Blue dots show signaling molecules. FDR, false discovery rate. **(C)** Extreme horn phenotypes by RNAi. Lateral view of the adult from *EGFP* (a), *Sp8* (b), and *pnr* (c) RNAi beetles. (b) The green arrow indicates an ectopic horn. (c) The blue arrow indicates a complete loss-of-horn phenotype.
Adapted from Ohde et al., *PLOS Genet*., 14: e1007651, 2018 [15••].

are mostly categorized as embryonic head patterning genes and appendage patterning genes (Figure 2C) [15].

The mechanism of horn development has also been analyzed in the beetle *Onthophagus* [26–28], a dung beetle likely to represent an independent evolutionary origin of horns from *T. dichotomus*. In both lineages, parts of the head-patterning and appendage-patterning gene networks are deployed in developing horns. This deep mechanistic parallel could mean that horns arose in the common ancestor of all scarabs, predating the divergence of these beetle families. Indeed, several clues suggest that the ancestral scarab beetles may have been horned [4]. However, a more likely possibility is that parallel origins of these extravagant structures each involved co-option of the same underlying developmental processes. Elucidating the molecular details of parallel evolution is an exciting theme in evo-devo.

Horn development as folded primordia

Beetle horns develop during the prepupal period as folded primordia under the larval head capsule (Figure 2A). Epidermal folding is not unique to the horns of beetles; it also occurs generally in insects as the body grows through molting [29–32]. But several features of beetle horns — their large size, rapid growth, sex specificity, and complex branched structure — all make these traits ideal for elucidating the developmental mechanisms of folding. In particular, structures that undergo significant changes in morphology via molting show dense folding and characteristic folding patterns [4,33–36]. The local density of folds and the orientation of the folded furrows combine to determine the size and shape of the structure after the molt. However, the developmental mechanisms determining the density and direction of furrows are not well understood.

T. dichotomus horns have now been used as a model to study how the 3D folding of epidermal primordia results in the morphogenesis of a large 3D structure [37]. These studies showed that extending, or unfurling the folded primordium is a surprisingly simple physical process, with little contribution of cytological factors such as cell proliferation or migration [23]. Taking advantage of the large size of the horn primordium, researchers were able to create virtual horn primordia from serial sections of

actual primordia. These virtual primordia made it possible to approach problems that are difficult to examine with actual organisms, such as understanding the positional relationship between the primordium before and after its extension, and how the final form changes when a particular folding structure is removed [23,38].

T. dichotomus horns have also proven useful for studying the molecular mechanisms regulating epidermal folding, using results based on RNA-seq screening. In the case of the horn primordium, there are two types of folding structures: those that define final size of the entire primordium, and those that are found on the surface of the primordium. Both types of folding structures likely contribute to the size and shape of the horn [16]. It is thought that each of these two is at least partially controlled by a different mechanism. For example, folds of the first type differ markedly from male to male, and are strongly correlated with the overall body size of the animal. These folds likely determine the overall size of the horn. In contrast, the depth and interval of the second type of folding, the surface furrows, are largely invariant across male body sizes; these folds likely determine the 'pitchfork' shape of the final horn [16].

Consistent with this, knockdown of *dachsous* (*ds*) by RNAi, a factor in the Fat-Hippo pathway that regulates cell polarity and proliferation, alters the first type of folding and changes the size of the resulting horn, but it does not affect the pattern of surface furrows [39]. Conversely, knockdown of *Notch*, which is involved in intercellular signaling, and *CyclinE*, which is involved in the cell cycle, did not significantly change the folds contributing to horn size, but did affect the surface furrows and, consequently, the resulting horn shape. Interestingly, the surface furrows were similarly affected in both *Notch* and *CyclinE* knockdown individuals, but the specific parameters of the furrows that were changed varied between the knockdown genes. In the case of *Notch* knockdown, the furrow direction and pattern were not affected, but the depth of the furrows became shallower [16]. On the other hand, in *CyclinE* knockdown, the furrow depth was not changed, but the furrow direction and pattern changed significantly [16]. Consequently, knockdown of *ds*, *Notch*, and *CyclinE*, all changed the final morphology of the horn, but the precise aspect of folding affected by each gene was different. This suggests that there are various 'developmental routes' for changing horn shape and size.

Differences among *T. dichotomus* males: extreme variation in horn length considered from the perspectives of morphology, ecology, and genomics

The horns of *T. dichotomus* are extremely sensitive to the nutrient environment experienced by larvae (Figure 1). In fact, the horns are significantly *more sensitive* to larval nutrition than are other morphological body structures. For example, Johns et al. [40] manipulated the amount of food available to male larvae and then quantified the effect of this diet perturbation on the growth of various adult structures. The length of the head and thoracic horns responded more dramatically to altered larval diet than did the elytra, femur, eyes, and genitalia [40]. This 'heightened' condition-sensitive expression in *Trypoxylus* horns has been studied from the aspect of molecular genetics. RNAi knockdown of the insulin receptor impacted the growth of the horns more than it did wings or genitalia, suggesting that tissue-specific increases in sensitivity to insulin or insulin-like growth factors might underlie the evolution of heightened condition sensitivity in weapons like beetle horns [13].

Zinna et al. [24] then investigated global changes in gene expression in developing horns, wings, and genitalia using RNA-seq. Specifically, they manipulated larval nutrition and compared expression levels in animals fed high-nutrition and low-nutrition food amounts. Traits extra sensitive to nutrition (i.e. head and thoracic horns) displayed greater numbers of differentially expressed genes, and more kinds of differentially expressed genes than did the less plastic traits (i.e. wings and genitalia) [24]. Although the overall number of differentially expressed genes responding to nutrition was small (fewer e.g. than differed between males and females), the number of differentially expressed genes varied according to the degree of nutritional dependence of the traits (head horn > thoracic horn > wings > genitalia). Furthermore, the authors identified 13 genes whose nutrition-sensitive response was sex specific. Interestingly, some of these genes have not been reported as genes that function in weapon growth or to have nutritional sensitivity. This result shows a potentially novel function for these genes.

The heightened condition sensitivity of *T. dichotomus* horns suggests that these structures may function as honest signals, either to choosy females or rival males, of the body size and/or physiological condition of a male. There is no evidence that females use the horn as a basis for mate choice. However, whether males use horns to size up an opponent has yet to be investigated. Field studies by Hongo [21] and del Sol et al. [25] clearly show that horn length and body size contribute to fighting and mating success, although the strength of selection acting on horns differed among populations. Thus, long-horned and large-bodied individuals do tend to win fights and these males are often likely to succeed at mating with females, but additional studies will be needed to explore whether the exaggerated horns are used as signals at any stage in this process.

Conclusions

In this review, we described the developmental mechanisms of horn formation based on genome-wide gene

expression analysis using next-generation sequencing technology. Recently reported high-quality *T. dichotomus* genomic resources will aid substantially in further clarification of the genetic mechanisms of horn formation. For example, it will be possible to analyze enhancer and promoter sequences on the horn-formation genes. In addition to the field of developmental biology, genomic resources for this species should enable rapid progress in diverse fields such as population genetics, genomics, ethology, evolution, biomechanics, and biochemistry.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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