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The Fat-Dachsous signaling pathway regulates growth of horns in *Trypoxylus dichotomus*, but does not affect horn allometry

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ABSTRACT

Males of the Asian rhinoceros beetle, *Trypoxylus dichotomus*, possess exaggerated head and thoracic horns that scale dramatically out of proportion to body size. While studies of insulin signaling suggest that this pathway regulates nutrition-dependent growth including exaggerated horns, what regulates disproportionate growth has yet to be identified. The Fat signaling pathway is a potential candidate for regulating disproportionate growth of sexually-selected traits, a hypothesis we advanced in a previous paper (Gotoh et al., 2015). To investigate the role of Fat signaling in the growth and scaling of the sexually dimorphic, condition-dependent traits of the in the Asian rhinoceros beetle *T. dichotomus*, we used RNA interference to knock down expression of *fat* and its co-receptor *dachsous*. Knockdown of *fat*, and to a lesser degree *dachsous*, caused shortening and widening of appendages, including the head and thoracic horns. However, scaling of horns to body size was not affected. Our results show that Fat signaling regulates horn growth in *T. dichotomus* as it does in appendage growth in other insects. However, we provide evidence that Fat signaling does not mediate the disproportionate, positive allometric growth of horns in *T. dichotomus*.

1. Introduction

Despite the ability of an organism to adaptively vary its body size depending on condition, its overall shape is maintained because the size of individual traits typically co-varies strongly with the size of surrounding traits, generally referred to as allometric growth (Shingleton and Frankino 2013). However, there are traits that do not grow in proportion to body size in a predictable and allometric fashion but instead exhibit exaggerated growth, growing much larger than expected and scaling radically out of proportion with body size; this phenomenon is known as hyper-allometric growth or positive allometry. In insects, condition-dependent, exaggerated traits can be found in a number of diverse species, ranging from the elongated eye stalks of stalk-eyed flies (Burkhardt and de la Motte 1985; Burkhardt and de la Motte 1988) to the enlarged head and mandibles of termite soldiers (Koshikawa et al., 2002). A recent review (Lavine et al., 2015) identified three unique ecological pressures that favor the evolution of exaggerated traits in insects: 1) reproductive competition, 2) social insect caste differentiation, and 3) natural selection favoring locomotor or prey-capture capabilities. However, while the evolution and consequences of exaggerated traits have been the focus of much research, the proximate mechanisms that modulate condition-dependent, exaggerated trait growth are not well understood.

The Japanese rhinoceros beetle (*Trypoxylus dichotomus*) is a particularly tractable model insect for investigating the molecular mechanisms mediating exaggerated trait growth (Emlen et al., 2007; Emlen et al., 2012; Ito et al., 2013; Johns et al., 2014; Zinna et al., 2016). These beetles produce some of the most dramatic and visually impressive exaggerated traits within the animal kingdom. Specifically, adult male beetles possess a long forked horn that extends outward from the top of their head, and a shorter forked horn that extends outward from the middle of the prothoracic tergum. Males use these horns as weapons in fights against other males for reproductive access to females (Siva-Jothy 1987; Hongo 2003), and males possessing the largest horns experience the greatest fighting and mating success (Hongo 2007; Karino et al., 2005; Siva-Jothy 1987; Hongo 2003). Horn size scales dramatically out of proportion with body size, such that

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large males may have horns that reach up to 2/3 of their total body length (Iguchi 1998; Karino et al., 2004; Emlen et al., 2012; Johns et al., 2014). Further, horn growth in *T. dichotomus* is largely condition-dependent – well-fed males express horns that grow to full size and reach extreme proportions, while poorly-fed males develop much smaller, less functional weaponry (Iguchi 1998; Karino et al., 2004; Emlen et al., 2012).

Exaggerated horn growth is at least partly achieved through tissue-specific differences in response to common whole-body circulating growth signals from the insulin signaling pathway: RNAi-mediated knockdowns of the insulin receptor gene (*InR*) show that head horns are approximately eight times more sensitive to insulin signaling than are traits that scale isometrically with body size, such as wings (Emlen et al., 2007). Although the insulin signaling pathway has been shown to mediate the condition-dependent growth of the exaggerated horns in rhinoceros beetles, it alone does not mediate the positive allometry of head horns, as evidenced by the fact that knockdown of *InR* affects the size of horns much more than of other appendages, yet larger individuals still have disproportionately larger horns (Emlen et al., 2012). Whole-animal signals such as the insulin pathway must therefore interact with local developmental genetic pathways to yield the tissue-specific characteristics of appendages, such as the positive allometric scaling of horns.

We have proposed (Gotoh et al., 2015) that the Fat-Dachsous signaling pathway is a potential mechanistic link between whole-body signals such as the insulin signaling pathway and tissue-specific morphogens that determine the final size of phenotypically plastic traits in insects. The Fat pathway is part of an evolutionarily conserved signaling network that is a major determinant of cell growth and polarity (Mahoney et al., 1991; Irvine 2012; Matis and Axelrod 2013). It has been shown to regulate the size and shape of legs in both *Drosophila* (Mao et al., 2006) and field crickets (Bando et al., 2011). In *Drosophila*, Fat signaling appears to be initiated through the interaction between the atypical transmembrane cadherins Fat (Ft) and Dachsous (Ds), whose extracellular regions can bind to each other across adjacent cells (Clark et al., 1995; Yang et al., 2002; Thomas and Strutt 2012; Mahoney et al., 1991). In *Drosophila*, reduced expression of *ft* and *ds* results in a number of phenotypic abnormalities in adults, including shortening and widening of structures such as the wings and legs among other cell polarity phenotypes (Mao et al., 2006). Gotoh et al. (2015) found that RNAi-mediated knockdown of *ft* reduced the size of the exaggerated horns of rhinoceros beetles (*T. dichotomus*) and knockdown of *ds* reduced the size of the exaggerated mandibles of stag beetles (*Cyclomamatus metallifer*). This led Gotoh et al. (2015) to the proposal that the Fat pathway is a link between the insulin signaling pathway, which mediates condition-sensitive growth of exaggerated traits and the final form and size of those traits.

In this study, we sought to explicitly test the hypothesis that Fat signaling is the missing mechanistic link coordinating environmentally sensitive insulin signaling with the hyper-allometric growth of head horns in the Japanese rhinoceros beetle, *T. dichotomus*. We knocked down *ft* and *ds* expression during exaggerated head horn growth in larval beetles using RNAi. Based on the predictions of Gotoh et al. (2015), we predicted that knockdown of *ft* and *ds* would result in a decrease in the hyper-allometric scaling of the head and thoracic horns. Our results show that *ft* (and to a lesser degree *ds*) regulate growth to full size of the *T. dichotomus* horns, and all other measured appendages, but do not regulate hyper-allometric growth of the horns. That is, RNAi against *ft* shortens and widens the head horn, prothoracic horn, and all appendages, but does not change the rate at which they scale to body size. Fat and Dachsous are thus shown to be important regulators of horn growth, as part of their general regulation of cell polarity during appendage development. However, Fat and Dachsous do not mediate the allometric scaling of the horns, at least not autonomously.

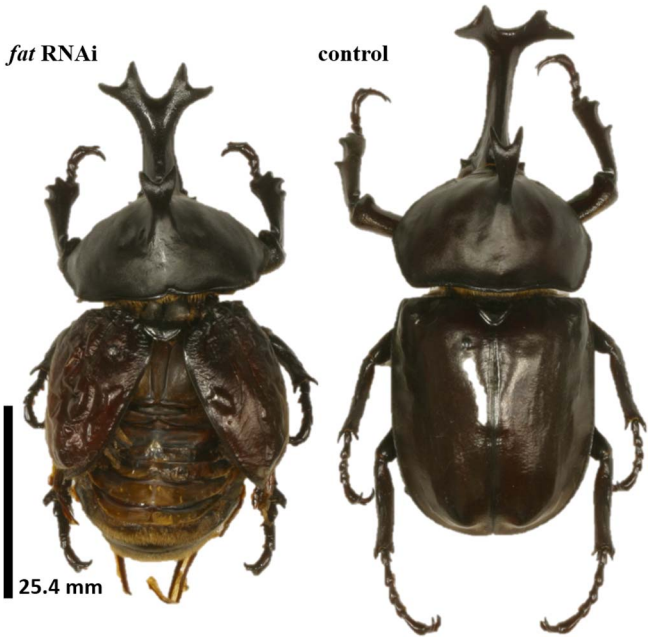

Fig. 1. *Trypoxylus dichotomus ft* knockdown phenotype compared to control. RNAi against *ft* resulted in short and fat head horn, prothoracic horn and appendages.

2. Results

2.1. Morphological effects of RNAi knockdown of *ft*

T. dichotomus larvae injected with *ft* dsRNA did not show differences in prothoracic width when compared with control larvae (One-way ANOVA, $F = 0.012$, $P = 0.913$). Prothoracic width was thus used as a measure of total body size. However, *T. dichotomus* larvae injected with *ft* dsRNA did show other morphological abnormalities as adults (Fig. 1). Head horn length (*ANCOVA:* $F_{1,19} = 48.336$, $P < 0.05$) and base width (*ANCOVA:* $F_{1,19} = 44.395$, $P < 0.05$) were significantly different between groups (Fig. 2A, B). There was a 36.0% reduction of head horn length and a 19.6% increase in head horn base width. RNAi against *ft* did not affect the scaling of the head horn to body size, as the slopes for head horn length (*ANCOVA:* $F_{1,19} = 0.067$, $P = 0.79$) and base width (*ANCOVA:* $F_{1,19} = 0.004$, $P = 0.951$) were not significantly different between groups. Based on these results, RNAi against *ft* perturbs overall growth of the head horn in *T. dichotomus*, but does not affect scaling (i.e. allometric slope).

In response to RNAi against *ft*, prothoracic horn length was significantly different between groups (*ANCOVA:* $F_{1,19} = 33.48$, $P < 0.05$) (Fig. 3), resulting in a 28.3% reduction of prothoracic horn length. RNAi against *ft* did not affect scaling of prothoracic horn length to body size (*ANCOVA:* $F_{1,19} = 1.767$, $P = 0.199$).

Tibia length (*ANCOVA:* $F_{1,19} = 126.63$, $P < 0.05$) was significantly different between groups (Fig. 4 A), resulting in a 33.0% reduction of tibia length and a 25.5% increase in tibia width. The allometric slope for tibia length (*ANCOVA:* $F_{1,19} = 0.32$, $P = 0.577$) was not significantly different between groups. Interestingly, *ft* RNAi removed the scaling relationship between tibia width and body size ($r^2 = 0.001$, $p = 0.897$) when compared to controls ($r^2 = 0.66$, $p < 0.05$) (Fig. 4 B).

Male genital, known as the aedeagus, length (*ANCOVA:* $F_{1,19} = 37.665$, $P < 0.05$) and width (*ANCOVA:* $F_{1,19} = 5.362$, $P < 0.05$) were significantly different between groups (Fig. 5 A, B), resulting in a 7.5% reduction in aedeagus length and a 6.5% increase in aedeagus width. The allometric slopes for aedeagus length (*ANCOVA:* $F_{1,19} = 0.152$, $P = 0.701$) or width (*ANCOVA:* $F_{1,19} = 0.14$, $P = 0.71$) were not significantly different between groups.

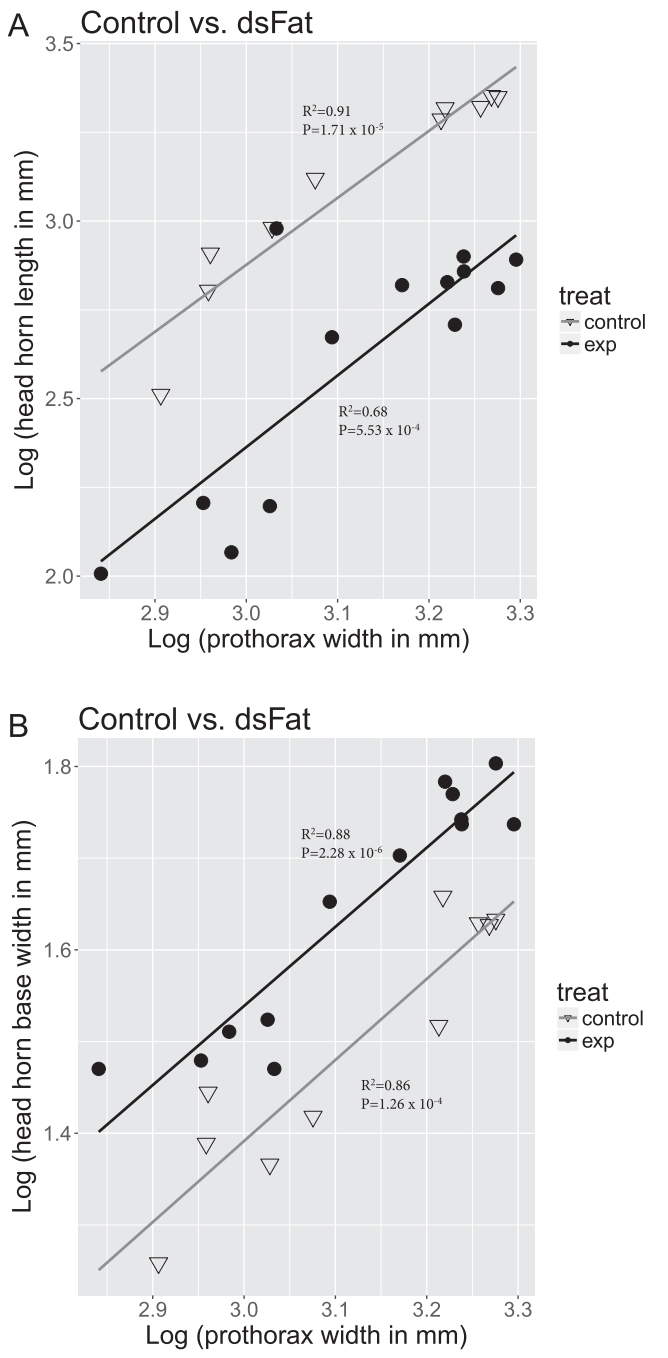

Fig. 2. RNAi against *T. dichotomus ft* resulted in a 36.0% reduction of head horn length (A) and 19.6% increase in head horn width (B). Allometric slopes for head horn length and width were not significantly different between control and *ft* treatment groups; dsRNAi against *ft* does not affect scaling of head horn to body size.

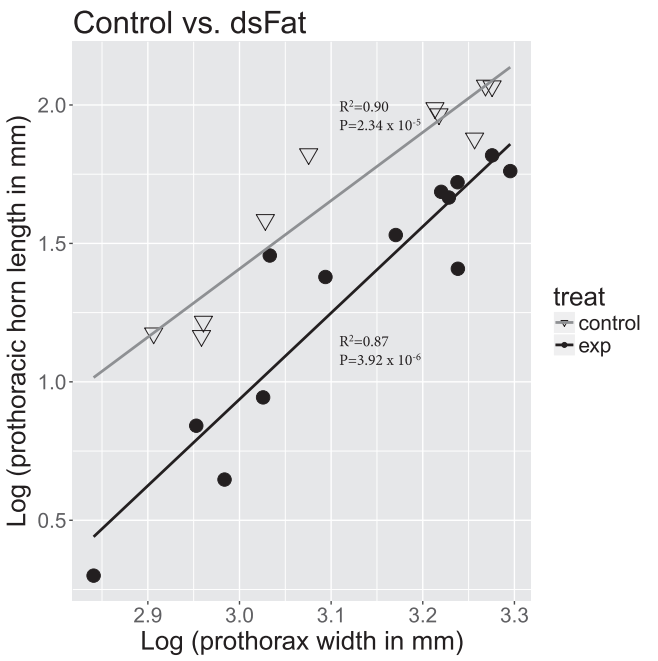

Fig. 3. RNAi against *T. dichotomus ft* resulted in a 28.3% reduction of prothoracic horn length. Allometric slope for prothoracic horn length was not significantly different between control and *ft* treatment groups; dsRNAi against *ft* does not affect scaling of prothoracic horn length to body size.

2.2. Morphological effects of RNAi knockdown of *ds*

T. dichotomus larvae injected with *ds* dsRNA did not show differences in prothoracic width from control larvae (One-way ANOVA, F = 0.472, $P = 0.5$). Prothoracic width was thus used as a measure of total body size. Head horn base width was significantly different between groups (*ANCOVA:* $F_{1,19} = 16.65$, $P < 0.05$), but head horn length did not differ significantly between groups (*ANCOVA:* $F_{1,19} = 3.21$, $P = 0.08$) (Fig. 6A, B). In response to RNAi against *ds*, there was a 21.3% reduction of head horn base width. RNAi against *ds* did not affect scaling of head horn length (*ANCOVA:* $F_{1,19} = 1.115$, $P = 0.3$) or base width (*ANCOVA:* $F_{1,19} = 0.37$, $P = 0.55$).

Prothoracic horn length was not significantly different between groups (*ANCOVA:* $F_{1,19} = 0.002$, $P = 0.996$) (Fig. 7), and the allometric slope for prothoracic horn length (*ANCOVA:* $F_{1,19} = 2.573$, $P = 0.125$) did not differ from that of controls.

Tibia length (*ANCOVA:* $F_{1,19} = 15.282$, $P < 0.05$) was significantly different between groups (Fig. 8A) demonstrated by a 6.3% reduction of tibia length. Additionally, the allometric slopes for tibia length (*ANCOVA:* $F_{1,19} = 4.21$, $P = 0.05$) was significantly different between groups. The allometric slope of *ds* treated animals increased, that is, the effect of *ds* knockdown on decreasing tibia length was proportionally greater in smaller individuals than in larger ones. Interestingly, as seen in the *ft* knockdowns, RNAi treatment of *ds* caused a loss of the scaling relationship between body size and tibia width (*ds* RNAi correlation between prothorax width and tibia width: $r^2 = 0.21$, $p = 0.11$) compared to controls (control correlation between prothorax width and tibia width: $r^2 = 0.66$, $p < 0.05$) (Fig. 8B).

Aedeagus length (*ANCOVA:* $F_{1,19} = 1.66$, $P = 0.21$) and width (*ANCOVA:* $F_{1,19} = 1.67$, $P = 0.211$) were not significantly different between groups (Fig. 9A, B), neither were the allometric slopes for aedeagus length (*ANCOVA:* $F_{1,19} = 0.553$, $P = 0.466$) or width (*ANCOVA:* $F_{1,19} = 0.085$, $P = 0.77$).

2.3. Electron microscopy analysis

To determine if cell polarity differences were responsible for the increased width of the effected traits in the knockdown individuals, we observed the knockdown phenotypes microscopically. Horns are produced from a thin, hollow layer of epidermis directly underneath the cuticle. Adult head horn gross morphology and epicuticular microstructure was different at all levels of magnification between individuals injected with *ft* dsRNA versus controls. The lateral aspect of the head horn from the *ft* knockdown treatment was substantially expanded along the lateral and antero-posterior axis compared to the control (Fig. 10). Also, there was a difference in the appearance of sensillae between groups, which appear obscured or absent in *ft* knockdown individuals (Fig. 10). Further, observations of the anterior head horn indicate differences in clypeal rise and reveal substantial lateral overgrowth at the head horn base (Fig. 10).

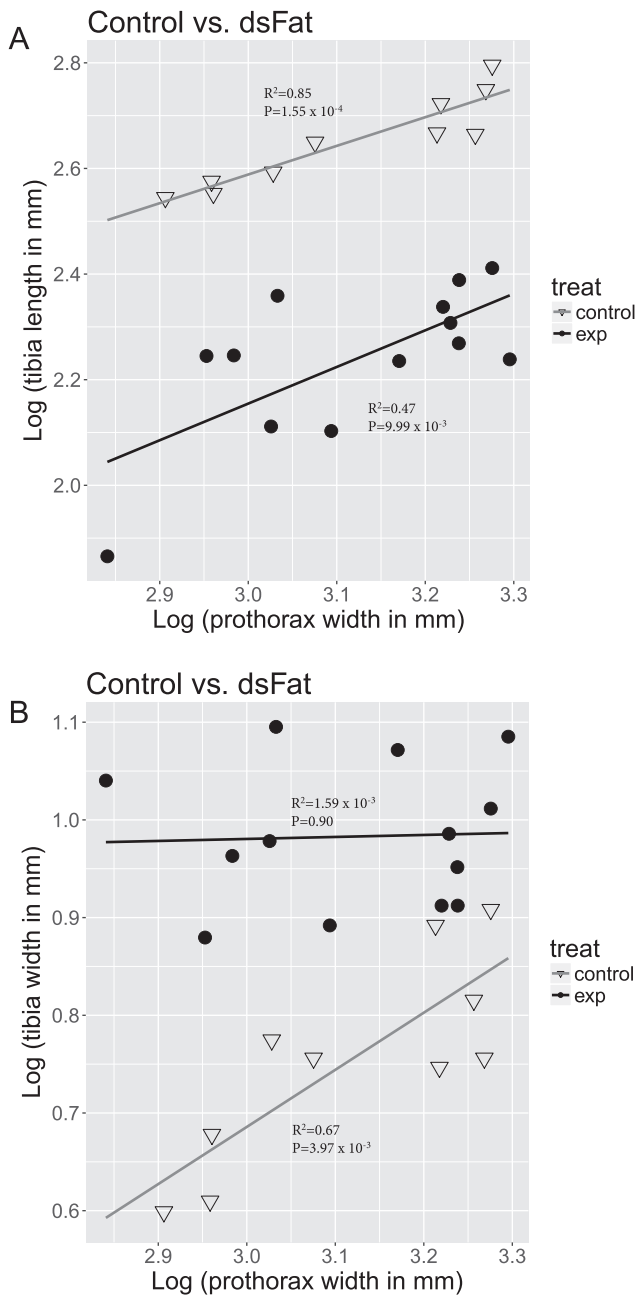

Fig. 4. RNAi against *T. dichotomus ft* resulted in a 33.0% reduction of tibia length (A) and loss of scaling to body size in tibia width (B). Allometric slope for tibia was significantly different between control and *ft* treatment groups; dsRNAi against *ft* does not affect scaling of tibia length to body size, but does affect scaling of tibia width.

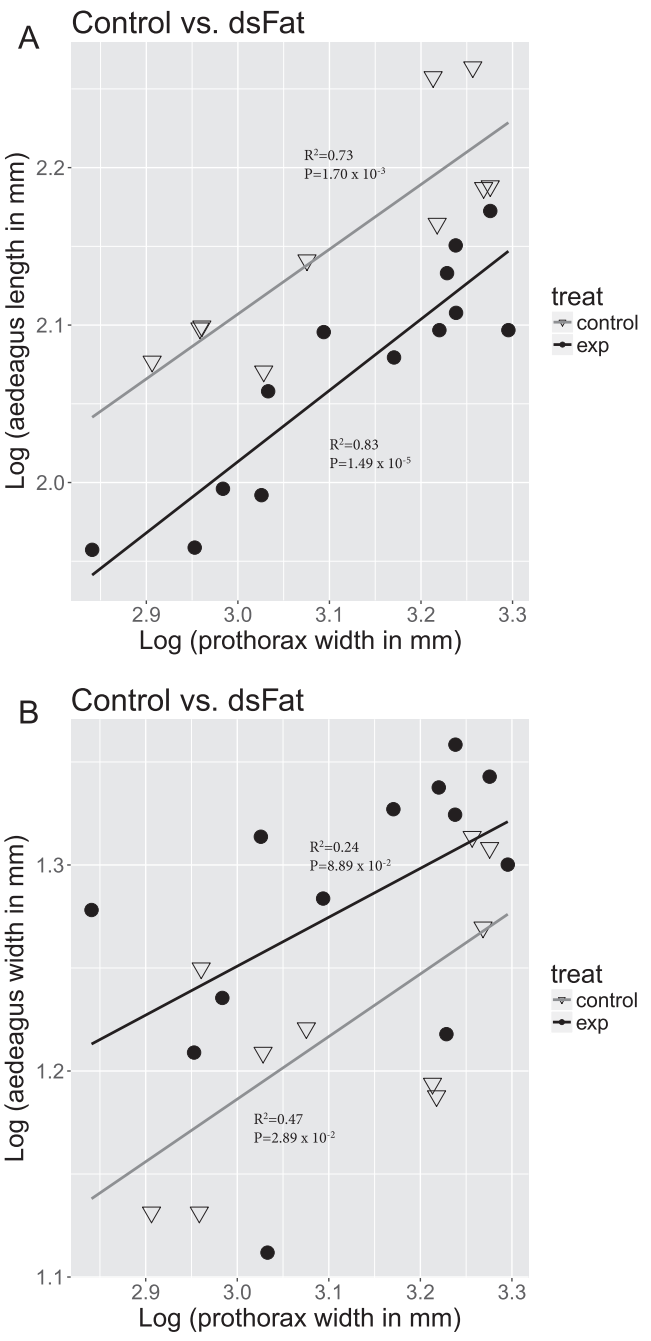

Fig. 5. RNAi against *T. dichotomus ft* resulted in a 7.5% reduction of aedeagus length (A) and 6.5% increase in aedeagus width (B). Allometric slopes for aedeagus length and width were not significantly different between control and *ft* treatment groups; dsRNAi against *ft* does not affect scaling of aedeagus to body size.

Epicuticular microstructure indicating cell shape was visually distinct between groups. In response to RNAi against *ft*, an epicuticular spike originates from the apical boundary of the regularly patterned, roughly hexagonal scales observed on the insect integument (Fig. 11). Also, scales from the *ft* knockdown beetle appear shorter and wider, and have less clearly defined ridges demarking their boundaries. Based on these results, RNAi against *ft* changes the formation of the epicuticular microstructure in the head horn. Further, these results indicate that *ft* regulates the shape of underlying epithelial cells during growth and development of the *T. dichotomus* head horn.

Tarsomere shape and segmentation was different between groups. Tarsomeres were shorter and fatter in response to *ft* knockdown (Fig. 12). RNAi against *ft* resulted in near complete fusion of the t2/t3 tarsomeres, and the partial fusion of the t3/t5 tarsomeres. Additionally, the hairs normally located on the distal tip of each tarsomere are absent from the shortened t2 segment, and are apparently located near the middle of t3. Based on these results, *ft* is critical for normal appendage growth and regulates normal segmentation in the metathoracic tarsus (Fig. 12).

3. Discussion

Our results demonstrate that *ft* is involved in modulating the growth of the *T. dichotomus* head horns and prothoracic horns, as well as appendages such as legs and male external genitalia. *ds* also modulates growth of the horns and legs, but does not affect growth of the aedeagus. These phenotypes seem largely consistent with the two other

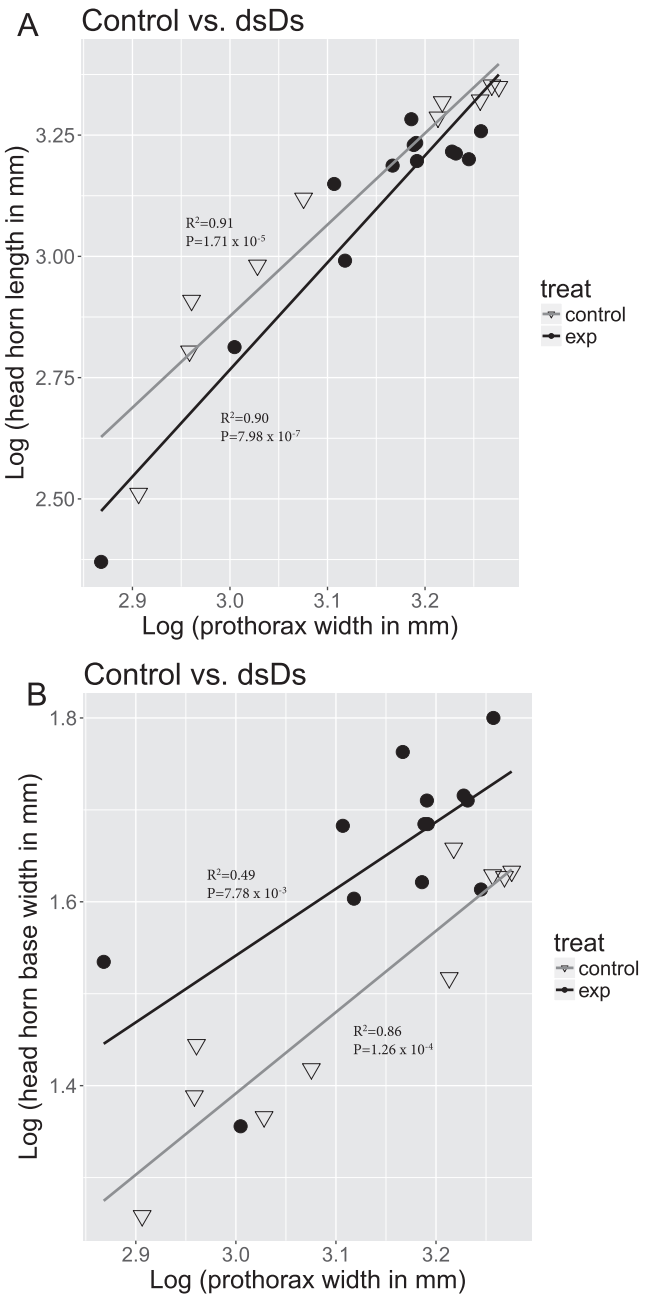

Fig. 6. RNAi against *T. dichotomus ds* resulted in a no significant change to head horn length (A) but caused a 21.27% increase in head horn width (B). Allometric slopes for head horn length and width were not significantly different between control and *ds* treatment groups; dsRNAi against *ds* does not affect scaling of head horn to body size.

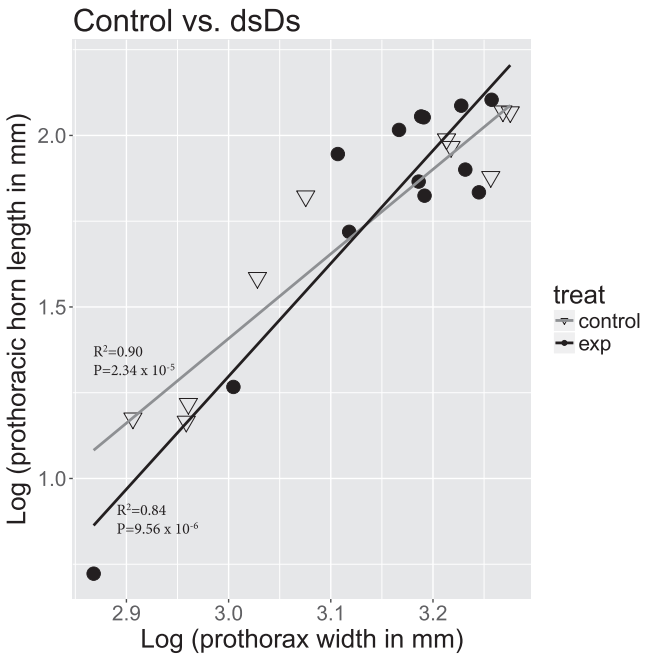

Fig. 7. RNAi against *T. dichotomus ds* resulted in no significant change to prothoracic horn length. Allometric slope for prothoracic horn length was not significantly different between control and *ds* treatment groups; dsRNAi against *ds* does not affect scaling of prothoracic horn length to body size.

insect species where the effects of *ft* and *ds* knockdown have been investigated, *Drosophila melanogaster* and the field cricket *Gryllus bimaculatus*. *Drosophila* mutants with reduced *ft* expression (true knockouts are lethal) show widened and shortened appendages such as wings and legs (Mao et al., 2006). Mutants with reduced *ds* expression show a similar, though less severe, phenotype (Mao et al., 2006). In limb regeneration in *G. bimaculatus*, *ft* or *ds* knockdown by RNAi causes similar phenotypes in the newly regenerated legs (Bando et al., 2009).

Beetle horns lack segmentation or joints, and as such are different from other segmented insect appendages such as legs and mouthparts. However, previous research indicates that beetle horn growth is regulated in part by the proximo-distal outgrowth module of the appendage-patterning pathway (Moczek and Nagy 2005; Wasik and Moczek 2011; Moczek et al., 2006). RNAi knockdown of *distal-less*, a major player in the proximo-distal outgrowth module, resulted in a reduction in horn length in the dung beetle *Onthophagus taurus*, but only in high-condition males (Moczek and Nagy 2005). More importantly, *decapentaplegic* (*dpp*) RNAi knockdown resulted in shorter horns in a second dung beetle species, *Onthophagus binodis* (Wasik and Moczek 2011). This is critical, as *dpp* signaling through its receptor Thickveins phosphorylates the transcription factor MAD, ultimately affecting localization of Dachsous in fruit fly wing imaginal discs in a Fat-dependent manner (Wartlick et al., 2011; Rogulja et al., 2008). It seems that the Fat pathway also functions similarly in the development of rhinoceros beetle horns as it does in the development of true insect appendages. *ft* knockdown caused similar percentage decreases in length and increases in width of horns and legs (Figs. 2–4). The aedeagi also showed this phenotypic effect due to *ft* knockdown. However, the effects were proportionally much smaller for the aedeagi. It should be noted that the aedeagus sizes are relatively invariant across rhinoceros beetle body sizes, as opposed to the isometric scaling of legs and the positive allometric scaling of horns (Emlen et al., 2012). Thus the effects of *ft* knockdown on aedeagus length and width may be influenced by the condition-independence of this trait.

As previously mentioned, the *ft* knockdown phenotype was much stronger than that for its ligand *ds*, in which only the width of the head horns and length of the tibia were affected (and to a lesser degree than in *ft* knockdowns). This is consistent with the phenotypes of *Drosophila ft* and *ds* mutants (Mao et al., 2006), and indicates that either some degree of Fat activity is ligand independent, or that there are other (as yet unidentified) ligands for Fat (Reddy and Irvine 2008).

Fat is known to function in the establishment of planar cell polarity (PCP) (Matis and Axelrod 2013). This response has been hypothesized to be responsible for the widening of appendages seen in *ft* mutants (Mao et al., 2006). Although Bando et al. (2009) observed widening of regenerated limbs in *ft* knockdown crickets, they did not see alterations in cuticular bristles and processes, leading them to hypothesize that any PCP defects did not extend to the development of surface structures. However, electron micrographs of horns from *ft* knockdown rhinoceros beetles show obvious changes in the position of sensillae and shape of epicuticular scales, indicating that unlike with cricket limbs Fat signaling affects surface structures in the developing horn.

Despite the pronounced effects of *ft* knockdown on horn and

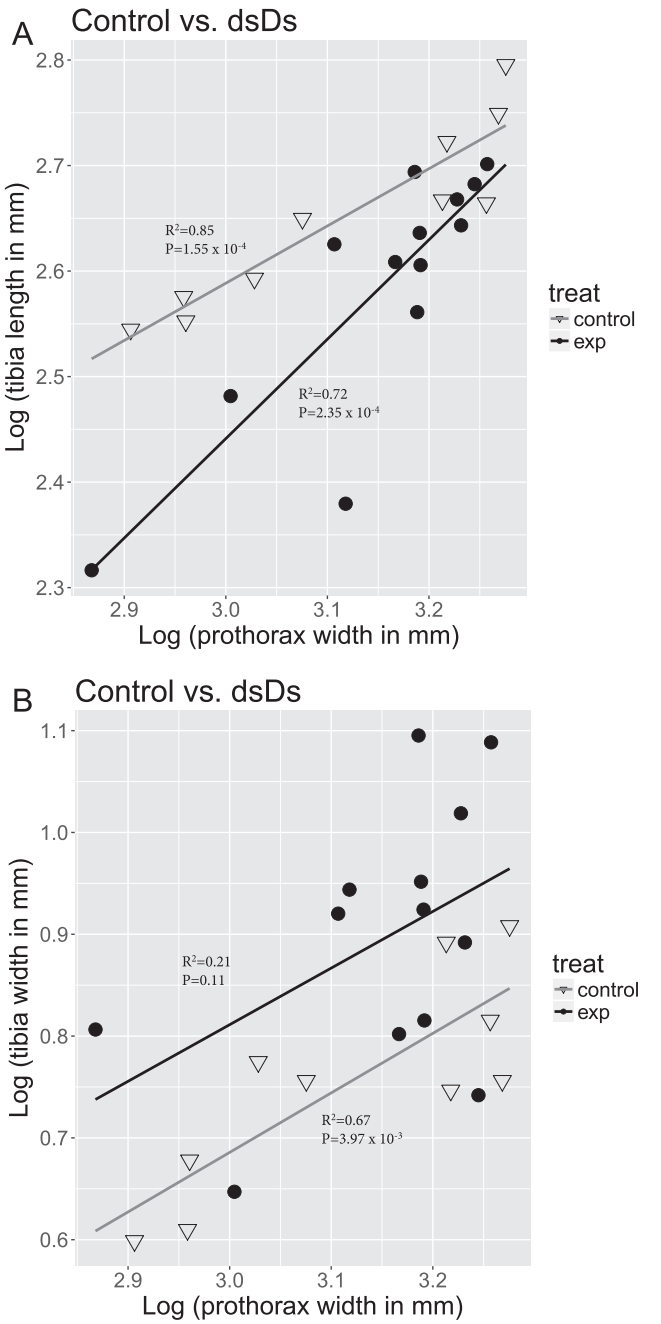

Fig. 8. RNAi against *T. dichotomus ds* resulted in a 6.3% reduction of tibia length (A) and loss of scaling of tibia width to body size (B). In addition, the allometric slope for tibia length was significantly different between control (slope = 0.54) and *ds* (slope = 0.94) treatment groups; dsRNAi against *ds* affects scaling of tibia length to body size, and disrupts the relationship between tibia width and body size.

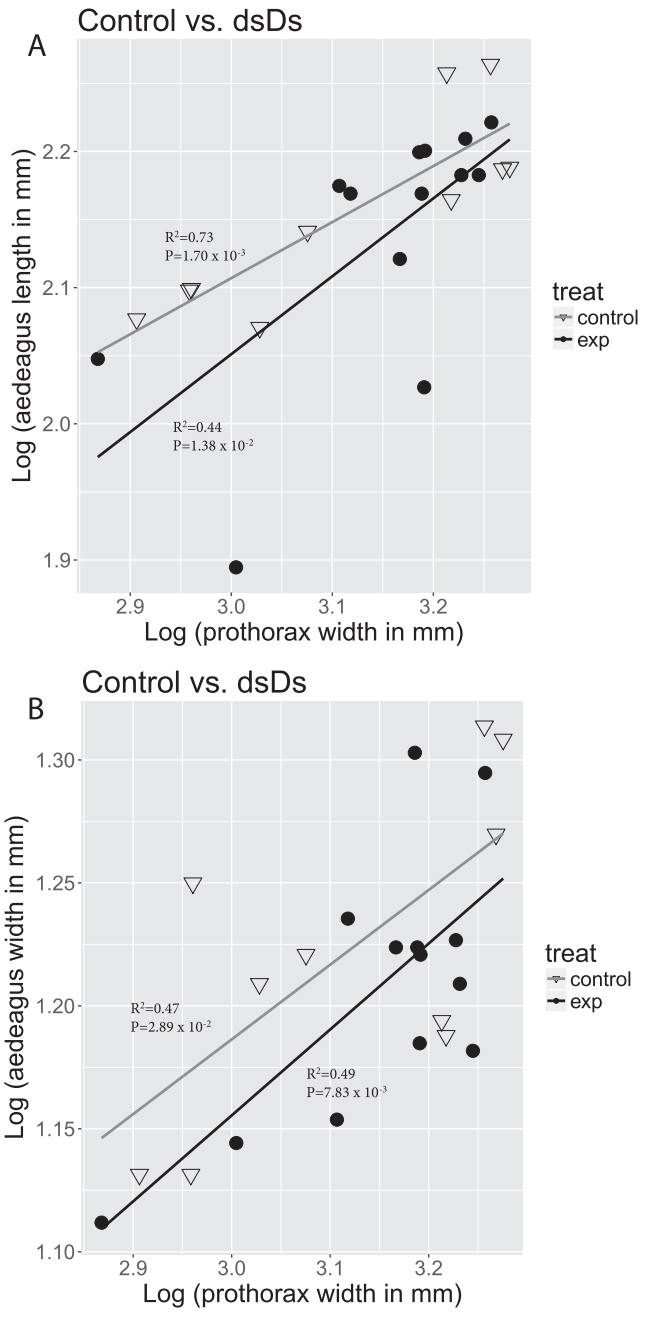

Fig. 9. RNAi against *T. dichotomus ds* did not have a significant effect on aedeagus length (A) or width (B). Allometric slopes for aedeagus length and width were not significantly different between control and *ds* treatment groups; dsRNAi against *ds* does not affect scaling of aedeagus to body size.

appendage growth in the beetles, *ft* does not appear to influence exaggerated growth of the horn. There was no effect on the allometric slopes (body size vs trait size) for head or prothoracic horns in either *ft* or *ds* knockdown. Horns were smaller but the allometry remained the same as in control individuals (i.e. more exaggerated horns in larger individuals). In fact, the allometric slopes were not affected for any trait except for one surprising exception: tibia length in *ds* knockdowns. In this case a trait that was normally isometric developed a positive allometry in *ds* knockdown individuals, i.e. the effect of knockdown was proportionately greater in smaller than in larger individuals. Why allometry of only this single trait was affected, and why only in *ds* knockdowns, is unclear, but it points to the complexity of the Fat-Dachsous interaction, and the evidence that some functions of Fat appear to be independent of Dachsous. Another important note is that RNAi knockdown of both *ft* and *ds* disrupted the typical isometric scaling of tibial width (Fig. 4B and Fig. 8B, respectively). While both *ft* and *ds*-treated tibia are wider than controls, in both treatments tibia width does not correlate with body size, unlike in control animals. It is possible that the disruption of PCP caused by RNAi knockdown in this trait affected limb growth in a non-linear fashion, thus leading to poor correlation of tibial width to body size.

Our results show the critical role of Fat signaling during larval/pupal development in the rhinoceros beetle, both in appendages such as legs and in the sexually dimorphic horns. At least part of the effect of *ft* knockdown is due to changes in planar cell polarity leading to wider, shorter appendages; the separate effect of Fat on cell proliferation may

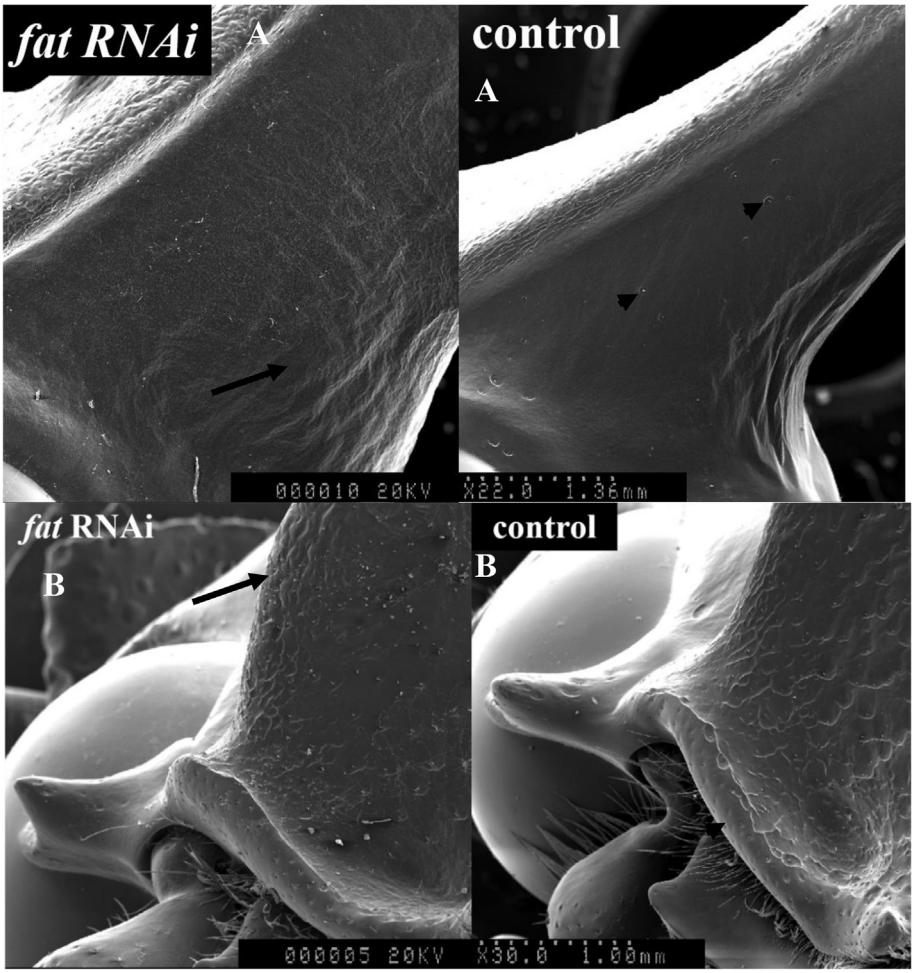

Fig. 10. SEM of *T. dichotomus ft* knockdown head horn (A) Lateral and (B) anterior aspect of horn appears 'bulged-out' on the *ft* knockdown. Arrows indicate irregular lateral outgrowth of underlying tissues. Images at same magnification from size matched individuals. Also, sensillae (arrowheads in control) appear to be obscured or absent in *ft* knockdown.

also contribute to this phenotype. However, we found no evidence that the Fat pathway specifically affects the exaggerated growth of the horns, at least by itself. Future studies will more specifically examine interactions between the Fat pathway and other signaling pathways, especially the insulin signaling pathway, which are known to mediate horn growth. It may be that effects on horn hyper-allometry will only be manifest when multiple interacting developmental pathways are manipulated at once.

4. Materials & methods

4.1. Larval rearing

T. dichotomus larvae were purchased as third-instar larvae from a Japanese company that raises beetles for commercial sale (Tamura-city Tokiwa Promotion Agency, Fukushima, Japan). Individual larvae were maintained in clear plastic cups filled with 25% compost material and 75% fermented hardwood chips. This substrate was prepared by soaking Pres-to-Logs® hardwood chips in water for 30 days, then mixing them with NuLife organic planting compost and allowing the mixture to compost for two weeks. Larvae were reared on a 16:8h light/dark cycle at 25 °C and monitored daily until they developed into adults.

4.2. Cloning and dsRNA synthesis of ft and ds

Transcripts of *ft* and *ds* were amplified out of head horn imaginal disc tissue from late third instar male *T. dichotomus* larvae. Larvae were anesthetized by being placed in a CO_2 chamber for 2 min. Larval head horn tissue was then dissected and stored in 3 ml of RNAlater® (Applied Biosystems) at −80 °C until RNA extraction was performed. Tissues were thawed and homogenized in TRiZOL® (Invitrogen) prior to RNA extraction following the manufacturer's protocol. RNA from larval tissues was converted into cDNA using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, USA). The cDNA transcript was then used as a template to amplify a 413 bp partial segment of the *ft* gene and a 404 bp partial segment of the *ds* gene for dsRNA synthesis. Using the partial *ft* and *ds* sequences identified by RNA-seq analyses in *T. dichotomus* (Niimi et al., in prep), 100% match primers were designed for *ft* and *ds* amplification from larval tissue. Fragments of *ft* were amplified by PCR with the following primers (containing a T7 polymerase promoter sequence added to the 5′ end): forward, 5′-(TAATACGACTCACTATAGGG) CGTCGCGATGTTGTTGGTGGTCGG-3′; reverse, 5′-(TAATACGACTCACT ATAGGG)CGGAGAAAGACACGGCTATCAC CGC-3′. Fragments of *ds* were amplified by PCR with the following primers (containing a T7 polymerase promoter sequence added to the 5′ end): forward, 5′-(TA ATACGACTCACTATAGGG)CGTCAGGGAACTCGAGGTATCCGAAGG-3′; reverse, 5′-(TAATACGACTCACTATAGGG)CCAGATCTAAATCCTTAGCC GGATGC-3′). PCR parameters for transcript amplification were as follows: 95 °C for 2 min; 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min; terminating with a final extension at 72 °C for 7 min. Reactions were conducted in a total volume of 20 µL, consisting of a final concentration of 0.2 mM each dNTP, 1.5 mM $MgCl_2$, 1.25 µL GoTaq® DNA Polymerase, and 0.5 µL 10 µM primer stock. The exact concentration of the cDNA template was unknown; 0.5 µL of cDNA was added to each reaction as template. A 413 bp fragment of *ft* and 404 bp fragment of *ds* were isolated by agarose gel electrophoresis and purified from the gel using GeneJet Gel Extraction Kit (Thermo Scientific) according to the manufacturer's protocol. The PCR product was further

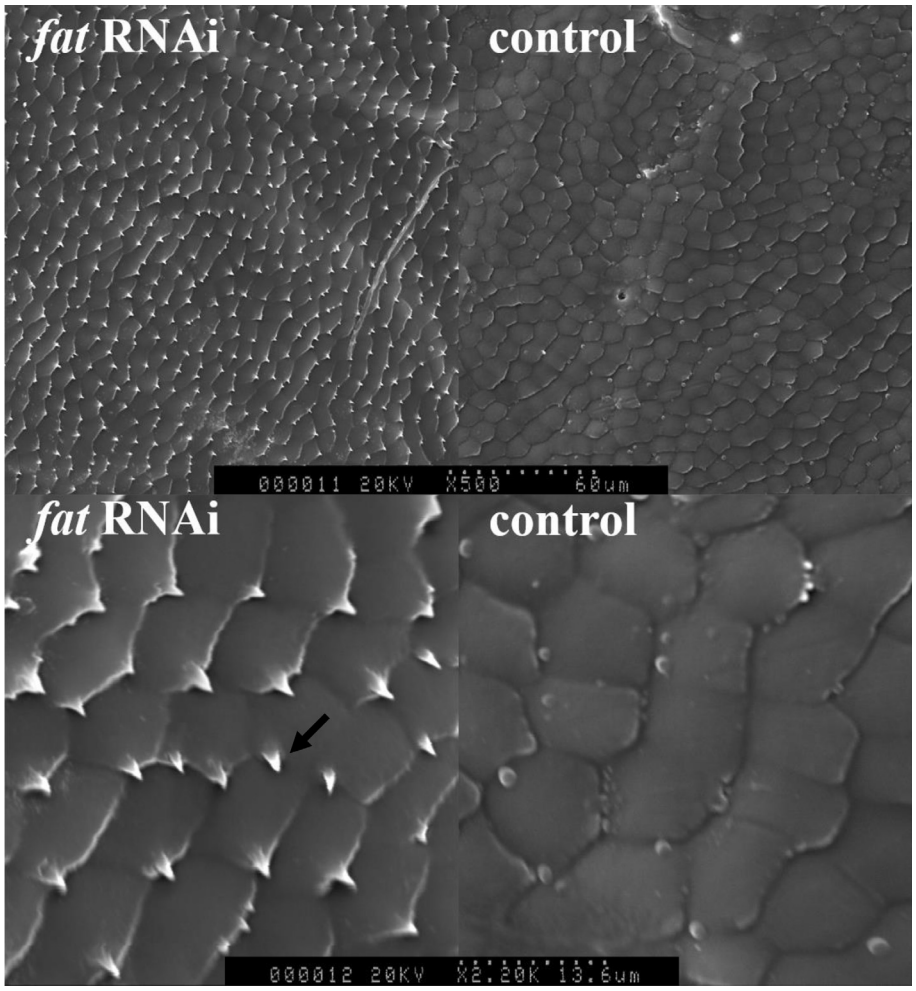

Fig. 11. SEM of *T. dichotomus ft* knockdown head horn cell shape phenotype. (A) 500x and (B) 2200× magnification of head horn lateral aspect. Pronounced differences in the epicuticular microstructure of the head horn are observed. The hexagonal delineations likely indicate underlying epithelial cell positions. Epicuticular spikes (arrow) are pronounced in *ft* knockdowns, and epicuticular plates are shorter and wider in *ft* knockdowns. Changes in epicuticular microstructure likely result from abnormal regulation of underlying epithelial cell shape, and/or cellular division processes.

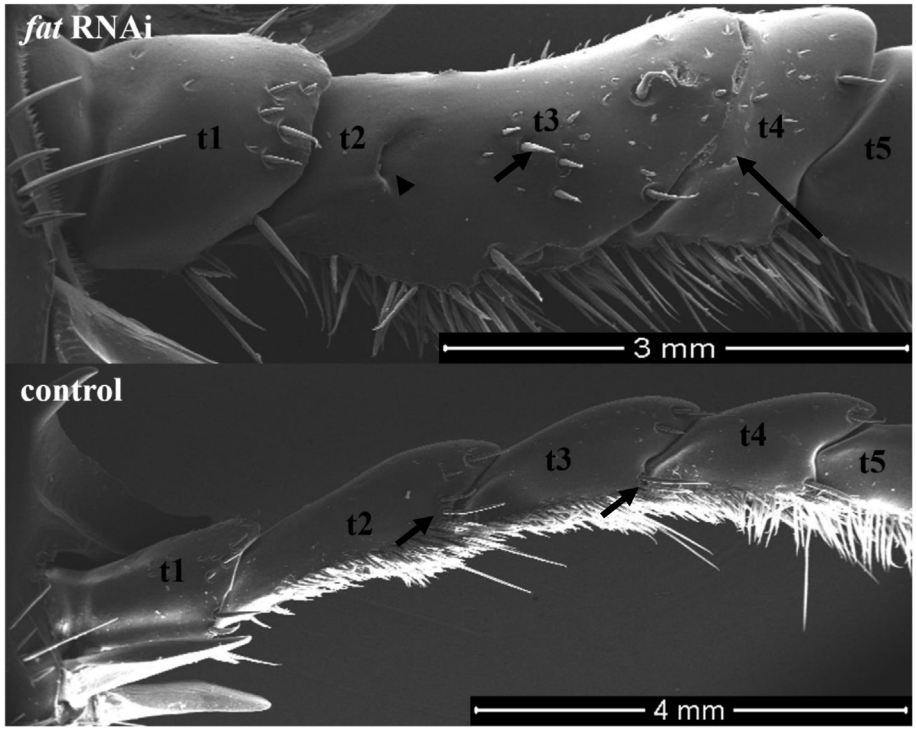

Fig. 12. SEM of *T. dichotomus ft* knockdown tarsormere fusion phenotype. Tarsomere t2 exhibited near complete fusion with t3; a small cleft may indicate the location of normal segmental separation (arrowhead). Hairs that typically locate on the apical end of each tarsomere of the control organism were not observed near the fusion site, but were instead found near the middle of t3 (short arrow). Partial tarsal fusion was observed at the t3/t4 boundary (long arrow). All tarsomeres of the *ft* knockdown organism were irregularly shaped, appearing shorter and fatter than the control tarsomeres. (t-number indicates tarsomere identity. There are 5 tarsomeres in the normally developed tarsus of *T. dichotomus*).

purified using a standard sodium acetate/ethanol precipitation and 70% ethanol wash and quantified using a NanoDrop 2000 (Thermo Scientific) spectrophotometer. The PCR product was then used as a template in the synthesis of double stranded RNA using the Ambion MEGAscript® T7 RNAi Kit, and purified with TURBO™ DNase, followed by a wash with binding buffer and ethanol according to the manufacturer's protocol. Final dsRNA concentrations were quantified using the NanoDrop 2000 (Thermo Scientific) spectrophotometer.

4.3. Injection of ft and ds dsRNA

RNAi was performed during the late larval period at the end of gut purge to functionally characterize the role of *ft* and *ds* during exaggerated trait growth. A 10 μL solution containing 1 μg of dsRNA against either *ft* (n = 15) or *ds* (n = 15) in nuclease free water was injected into the T2 segment of larvae at the end of gut purge stage. GFP dsRNA controls were also injected, but data from GFP controls are not included in the study due to high mortality of the beetle cohort used for of the GFP group. It is unlikely that the increased mortality was related to GFP injection per se, as un-injected larvae from this cohort experienced similarly high mortality. However, previous studies conducted by this laboratory have conclusively shown that GFP dsRNA does not affect tissue growth or mortality in *T. dichotomus* (Emlen et al., 2012). Thus, the control group used for this study is 11 male larvae injected with 10 μL nuclease free water. Quantitative PCR was used to verify RNAi-mediated transcript knockdown (Fig. S1). After injection, beetles were placed in their individual containers and allowed to develop into adults.

4.4. Morphometric analysis

After eclosion, morphological measurements were conducted on adult traits to examine the effect of *ft* or *ds* knockdown. Morphometric analyses were performed on the length and width of head horns, prothoracic horns, metathoracic tibiae, and aedeagi of male adult beetles (*ft: n = 15; ds:* n = 15; control: n = 11). Head horn length was measured as the distance between lateral margin of the clypeus and the distal tip of the head horn. Head horn width was measured as the widest portion of the base of the head horn immediately above the clypeus. Prothoracic horn length was measured as the distance between the horn's insertions into the prothoracic tergum and the distal end of the horn. Prothoracic horn width was not measured due to the lack of a reliable anatomical landmark at the base of the horn, where it attached to the prothorax in a graded manner. Metathoracic tibia length was measured as the distance between the joint connecting the femur and the tip of the most distal tibial spine. Tibial width was measured as the widest portion of the tibia midway between the femur joint and distal tibial spine. Aedeagus length was measured as the greatest proximo-distal distance from the base of the shaft and the distal tip. Aedeagus width was measured as the widest distance approximately midway up the shaft. All measurements were performed with UltraTech digital calipers (General®).

We assessed the magnitude of RNAi effect by comparing the mean trait size of individuals from the control group to those from the RNAi treatment group using percent change in trait size. Percent change in response to RNAi was calculated as: [(mean trait size of treatment group – mean trait size of control group)/mean trait size of control group]. Allometric relationships between trait size and body size were determined using the power equation $Y = \alpha X^{\gamma}$, where Y is the natural log of trait size, X is the natural log of prothorax width (an index for body size), α is the allometric coefficient, and γ is the slope. Allometric coefficients were estimated using an analysis of covariance (ANCOVA) in R, testing the effects of the categorical factor of RNAi treatment on our focal trait of interest using body size as a continuous co-variable. Separate regression lines for each factor were fit and tested for differences in intercept between the lines (representing a change in overall trait size between treatments), or the presence of a significant interaction between the regression lines, indicating a change in slope due to our treatments. One-way ANOVA was performed to test for differences in body size (i.e. prothorax width) between treatment groups and the control group. All statistical analyses were performed using the statistical software program R 3.3.1 (2012).

4.5. Electron microscopy

Scanning electron microscopy was used to help identify potential perturbations of regular growth and development in head horns and other appendages. Male adult beetles (control or injected with *ft* dsRNA) had their heads, prothoraxes, elytra, metathoracic legs, and genitalia excised and dried in a desiccator for 7 days. Excised body parts were mounted on aluminum pegs using double-sided carbon tape, and approximately 1.5 nm of argon-gold coating was applied with a Technics Hummer V Sputter Coater (Technics, Arlington, Va). Argon-gold plated body parts were imaged with a Hitachi S-570 Scanning Electron Microscope (Hitachi, Tokyo, Japan) at 20 kV. Metathoracic tarsi were also imaged with an FEI Quanta 200F Scanning Electron Microscope.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2018.01.006.

References

Bando, T., et al., 2009. Regulation of leg size and shape by the Dachsous/Fat signalling pathway during regeneration. Development (Cambridge, England) 136 (13), 2235–2245 Available at: http://www.ncbi.nlm.nih.gov/pubmed/19474149.
Bando, T., et al., 2011. Regulation of leg size and shape: involvement of the Dachsous-fat signaling pathway. Dev. Dyn. 240 (5), 1028–1041.
Burkhardt, D., de la Motte, I., 1985. Selective pressures, variability, and sexual dimorphism in stalk-eyed flies (diopsidae). Naturwissenschaften 72 (4), 204–206.
Burkhardt, D., de la Motte, I., 1988. Big "antlers" are favoured: female choice in stalk-eyed flies (Diptera, Insecta), field collected harems and laboratory experiments. J. Comp. Physiol. A 162 (5), 649–652.
Clark, H.F., et al., 1995. Dachsous encodes a member of the cadherin superfamily that controls imaginal disc morphogenesis in Drosophila. Genes Dev. 9 (12), 1530–1542.
Emlen, D.J., et al., 2012. A mechanism of extreme growth and reliable signaling in sexually selected ornaments and weapons. Science 337 (6096), 860–864.
Emlen, D.J., Lavine, L.C., Ewen-Campen, B., 2007. On the origin and evolutionary diversification of beetle horns. Proc. Natl. Acad. Sci. 104 (suppl 1), 8661–8668.
Gotoh, H., et al., 2015. The Fat/Hippo signaling pathway links within-disc morphogen patterning to whole-animal signals during phenotypically plastic growth in insects. Dev. Dyn. 1039–1045.
Hongo, Y., 2003. Appraising behaviour during male-male interaction in the japanese horned beetle Trypoxylus dichotomus septentrionalis (Kono). Behaviour 140 (4), 501–517.
Hongo, Y., 2007. Evolution of male dimorphic allometry in a population of the Japanese horned beetle Trypoxylus dichotomus septentrionalis. Behav. Ecol. Sociobiol. 62 (2), 245–253.
Iguchi, Y., 1998. Horn dimorphism of allomyrina dichotoma septentrionalis (coleoptera: scarabaeidae) affected by larval nutrition. Ann. Entomol. Soc. Am. 91 (6), 845–847.
Irvine, K.D., 2012. Integration of intercellular signaling through the Hippo pathway. Semin. Cell Dev. Biol. 23 (7), 812–817.
Ito, Y., et al., 2013. The role of doublesex in the evolution of exaggerated horns in the Japanese rhinoceros beetle. EMBO Rep. 14 (6), 561–567 Available at: http://embor.embopress.org/cgi/doi/10.1038/embor.2013.50.
Johns, A., et al., 2014. Heightened condition-dependent growth of sexually selected weapons in the rhinoceros beetle, Trypoxylus dichotomus (Coleoptera: Scarabaeidae). Integr. Comp. Biol. 54 (4), 614–621.

Karino, K., Seki, N., Chiba, M., 2004. Larval nutritional environment determines adult size in Japanese horned beetles Allomyrina dichotoma. Ecol. Res. 19 (6), 663–668.
Karino, K., Niiyama, H., Chiba, M., 2005. Horn length is the determining factor in the outcomes of escalated fights among male japanese horned beetles, Allomyrina dichotoma L. (Coleoptera: Scarabaeidae). J. Insect Behav. 18 (6), 805–815.
Koshikawa, S., Matsumoto, T., Miura, T., 2002. Morphometric changes during soldier differentiation of the damp-wood termite Hodotermopsis japonica (Isoptera, Termopsidae). Insectes Sociaux 49 (3), 245–250.
Lavine, L., et al., 2015. Exaggerated trait growth in insects. Ann. Rev. Entomol. 60 (1), 453–472.
Mahoney, P.A., et al., 1991. The fat tumor suppressor gene in Drosophila encodes a novel member of the cadherin gene superfamily. Cell 67 (5), 853–868 Available at: http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WSN-4C6BN7H-3R&_user=10&_rdoc=1&_fmt=&_orig=search&_sort=d&_docanchor=&view=c&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=5cb5683c0a7bc039234faf7ddcd02031%5Cnpapers2://publication/uuid/.
Mao, Y., et al., 2006. Dachs: an unconventional myosin that functions downstream of Fat to regulate growth, affinity and gene expression in Drosophila. Development (Cambridge, England) 133 (13), 2539–2551 Available at: http://www.ncbi.nlm.nih.gov/pubmed/16735478.
Matis, M., Axelrod, J.D., 2013. Regulation of PCP by the fat signaling pathway. Genes Dev. 27 (20), 2207–2220.
Moczek, A.P., et al., 2006. Conservation, innovation, and the evolution of horned beetle diversity. Dev. Genes Evol. 216 (11), 655–665.
Moczek, A.P., Nagy, L.M., 2005. Diverse developmental mechanisms contribute to different levels of diversity in horned beetles. Evol. Dev. 7 (3), 175–185.
Reddy, B.V., Irvine, K.D., 2008. The Fat and Warts signaling pathways: new insights into their regulation, mechanism and conservation. Development 135 (17), 2827–2838 Available at: http://www.ncbi.nlm.nih.gov/pubmed/18697904.
Rogulja, D., Rauskolb, C., Irvine, K.D., 2008. Morphogen control of wing growth through the fat signaling pathway. Developmental Cell 15 (2), 309–321.
Shingleton, A.W., Frankino, W.A., 2013. New perspectives on the evolution of exaggerated traits. BioEssays 35 (2), 100–107.
Siva-Jothy, M.T., 1987. Mate securing tactics and the cost of fighting in the Japanese horned beetle, Allomyrina dichotoma L. (Scarabaeidae). J. Ethol. 5 (2), 165–172.
Thomas, C., Strutt, D., 2012. The roles of the cadherins Fat and Dachsous in planar polarity specification in Drosophila. Dev. Dyn. 241 (1), 27–39.
Wartlick, O., et al., 2011. Dynamics of Dpp signaling and proliferation control. Science (New York, N.Y.) 331 (6021), 1154–1159 Available at: http://www.ncbi.nlm.nih.gov/pubmed/21385708.
Wasik, B.R., Moczek, A.P., 2011. Decapentaplegic (dpp) regulates the growth of a morphological novelty, beetle horns. Dev. Genes Evol. 221 (1), 17–27.
Yang, C.H., Axelrod, J.D., Simon, M.A., 2002. Regulation of Frizzled by Fat-like cadherins during planar polarity signaling in the Drosophila compound eye. Cell 108 (5), 675–688.
Zinna, R., et al., 2016. Endocrine control of exaggerated trait growth in rhinoceros beetles. Integr. Comp. Biol. 247–259.