The Influence of *bantam* MicroRNA on the Evolution of Size

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Abstract

How do changes in developmental programs cause evolution of organismal size? Beetles of the genus *Tribolium* provide a useful tool for elucidating this question. The goal of this study was to measure the expression of an effector of the Hippo pathway, which regulates growth. Expression of the microRNA *bantam* was assayed by realtime PCR in several species of closely related beetles that vary in average total body size. By measuring the activity of this pathway in closely related organisms, the results should reflect changes directly related to size evolution. The results show that the Hippo pathway has differential activity between several of the species testesd; however, in some species higher expression was associated with larger body size, while in others higher expression led to smaller body size. This project has shown that the Hippo pathway is involved in the regulation of size, but its exact mechanism remains unknown.

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How do changes in developmental programs cause evolution of organismal size? Growth of tissues and organs during development involves careful coordination of the rates of cell proliferation and cell death over space and time. How these cellular processes are coordinated with pattern formation during development and how this developmental program can evolve between species is a challenging question in evolutionary developmental biology. Beetles of the genus *Tribolium* (Coleoptera: Tenebrionidae) provide a useful tool for elucidating these questions. This study assayed the expression of an effector, bantam microRNA, of a growth control pathway, the Hippo pathway. By measuring the activity of the pathway in closely related organisms, the results should reflect changes directly related to size evolution. The results show that the Hippo pathway is differentially regulated between several of the organisms studied and there is a possible correlation between pathway expression and total body size. This project provides information regarding the control of size during development and how changes in one developmental pathway can contribute to size differences between species.

Introduction

The coordination of cell and tissue growth with pattern formation is complicated, requiring integration of cell proliferation and apoptosis to create a developmental program, which can change over time. Changes to the developmental program may reflect divergent selection and are observed in phenotypic changes, such as the variation in total body size of beetles closely related to *Tribolium castaneum*. Recently characterized in *Drosophila*, the Hippo pathway is a growth regulatory cascade that could bear on the evolution of varying body sizes. One effector of this pathway is the microRNA *bantam*, which has been shown to simultaneously promote cell proliferation and suppress apoptosis, thus promoting tissue growth through RNA interference (RNAi). As *bantam* has not yet been characterized in *Tribolium*, this study will outline its expression in several species to assess the possibility that it contributes to the evolution of size in these species.

The Hippo Pathway

The Hippo tumor suppressor pathway has emerged as a key signaling pathway in *Drosophila* tissue size control and has been extensively studied in relation to eye patterning. Hippo signaling restricts tissue size by promoting apoptosis and limiting cell proliferation. The action of the Hippo pathway is signaled through the Fat cadherin (Bennett and Harvey 2006).

Though the exact nature of Fat's interactions are as yet unknown, it has been shown to be activated by high cell density in culture, and it is thought to interact heterotypically with Dachsous, another cadherin (Harvey and Tapon 2007; Zhao, Li et al. 2010). Fat signals Merlin (Mer), Expanded (Ex), and Kibra, which form a complex that interacts with downstream pathway components (Genevet, Wehr et al. 2010). The exact nature of the Mer/Ex/Kibra interaction is unknown, and it has been proposed that Mer and Ex act as separate branches of Hippo signaling, with Fat acting through Ex, while Mer is controlled separately (Zhao, Li et al. 2010).

No matter the exact mechanism, Kibra, Ex, and Mer control the phosphorylation status of the core complex of the Hippo pathway, which includes the Ser/Thr kinases Hippo (Hpo) and Warts (Wts), the kinase cofactor Mob as tumor suppressor (Mats), and the scaffolding protein Salvador (Sav) (Grusche, Richardson et al. 2010). The role of the Hpo/Wts/Mats/Sav core complex is to control the phosphorylation status of transcriptional coactivator Yorkie (Yki, also known as YAP) (Oh and Irvine 2010). When phosphorylated, Yki is inhibited, making it unable to enter the nucleus to promote transcription of its target genes (Grusche, Richardson et al. 2010).

bantam MicroRNA

One of the genes for which Yki is a coactivator is *bantam*, a 21 nucleotide microRNA that promotes tissue growth through pro-cell proliferation and anti-apoptosis targets (Brennecke, Hipfner et al. 2003). *bantam*-induced tissue growth results from and increased number of cells, not from an increased cell size (Hipfner, Weigmann et al. 2002). During normal development, *bantam* is expressed in proliferating cells, but not in those that are quiescent, indicating that *bantam* is necessary for normal growth (Thompson and Cohen 2006). *bantam* is a powerful effector of the Hippo pathway because of its ability to coordinate stimulation of cell proliferation with suppression of apoptosis, making it a balanced growth regulator (Nolo, Morrison et al. 2006).

While the Hippo pathway is a key regulator of *bantam* expression, *bantam* is also induced by other pathways and may act as a regulatory integration point for several growth and patterning pathways (Nolo, Morrison et al. 2006; Thompson and Cohen 2006). Importantly, *bantam* and the Hippo pathway are also not the only mechanisms by which growth can be signaled, and cells mutant for *hippo* with decreased *bantam* levels can still overgrow (Nolo, Morrison et al. 2006). Based on the knowledge of *bantam* and its roles in *Drosophila*, the hypothesis for this study is

that species with a larger total body size will have higher expression of *bantam* that smaller species.

MicroRNA Mechanisms

MicroRNAs, such as *bantam*, work through RNAi to post-transcriptionally modify target mRNA levels in the cell. When a miRNA is transcribed as part of the normal transcription process, its characteristic self-complementary sequence causes it to fold into a hairpin shape. This small portion of double-stranded RNA is recognized by the protein Drosha and is cut from the mRNA. This pre-miRNA is exported from the nucleus, where it is further processed by Dicer and incorporated into an RNA-induced silencing complex (RISC). This RISC uses the miRNA sequence to find complementary sequences in target mRNAs and represses the expression of these genes (Zamore, Tuschl et al. 2000).

There are currently two known mechanisms by which miRNAs regulate gene expression: if the complementarity between the miRNA and the target mRNA is extensive, the mRNA is degraded, whereas if the complementarity is weak, the mRNA is not degraded, but translation is blocked (Zamore, Tuschl et al. 2000). microRNAs do not function in an "all or nothing" mechanism, but rather act as modulators of gene expression to ensure proper mRNA levels during developmental transitions or to shape spatial expression of a gene (Takacs and Giraldez 2010).

Tenebrionidae as Model Organisms

Beetles of the family Tenebrionidae are particularly useful in analysis of the effects of a miRNA because they exhibit a robust systemic RNAi system, as opposed to *Drosophila* (Roth and Hartenstein 2008; Tomoyasu, Miller et al. 2008). They are also apt for this project because they exhibit wide size variation (Figure 1), ideal for comparative assessment of the activity of a growth

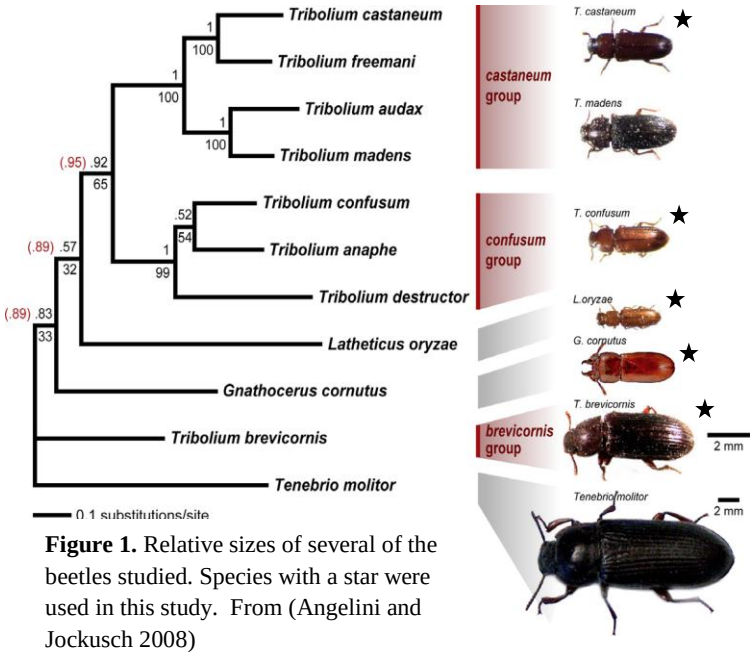

Figure 1. Relative sizes of several of the beetles studied. Species with a star were used in this study. From (Angelini and Jockusch 2008)

pathway. Finally, the genome of *Tribolium castaneum* is sequenced, which simplifies primer design and comparison to *Drosophila* sequences (Richards, Gibbs et al. 2008).

The Hippo pathway has not yet been characterized in Tenebrionidae, making *Tribolium castaneum*'s sequenced genome especially important. The sequence of *bantam* is not known for all of the species being studied, but the sequence is highly conserved between *Drosophila* and *Tribolium castaneum*, which are much more divergent than any of the other species in this study (Figure 2). As such, the assumption that *bantam* is conserved among Tenebrionidae has a logical base and seems to be true based on the results of this study.

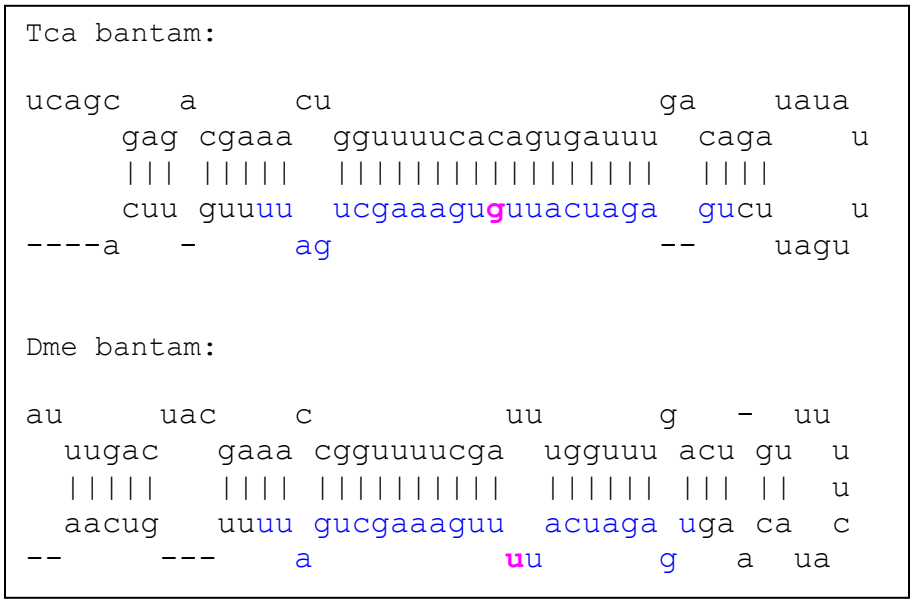

Figure 2. *Tribolium castaneum* and *Drosophila melanogaster bantam* sequences. The hairpin folding patterns are shown, with the active microRNA sequences highlighted in blue. The single base difference between the sequences is shown in pink.

Material and Methods

Beetle Colonies

Tribolium castaneum, *T. castaneum Goliath* and, *T. castaneum pygmy* were maintained on Carolina medium and incubated at 30°C. *T. confusum*, *T. freemani*, *T. brevicornis*, and *Gnathocerus cornutus* were maintained on Carolina medium at room temperature. *Latheticus oryzae* were maintained on a mixture of Carolina medium and rice flour at room temperature.

Prepupae were identified by characteristic apolytic inactivity. For *T. castaneum* and *T. confusum*, day 3 pupae were identified based on the number of pigmented ommatidia (Sokoloff 1972). For all other species, prepupae and early pupae were isolated from the colony and allowed to grow for approximately 3 days into day 3 pupae.

Total RNA extraction

For total RNA extraction, individuals of each species and life stage were pooled to a total mass of ~30mg. The PureLink RNA Mini Kit (Invitrogen) protocol was followed, including the optional DNase purification. All total RNA was stored at -80°C.

RNA standards

A *bantam* standard was synthesized using 50nt overlapping primers as templates for the pre-miRNA. PCR was performed with these primers at Tm=40°C for 10 cycles and Tm=68˚C for 10 cycles.

The *bantam* encoding DNA was cloned using the TOPO TA Cloning Kit for Sequencing (Invitrogen), isolated by PureLink Quick Plasmid Miniprep Kit (Invitrogen), and sequenced. *bantam* RNA was then transcribed using the Ambion T7 MegaScript Transcription System. The RNA was purified with an ethanol/ammonium acetate precipitation and stored at -80°C.

Real Time qPCR

Real time qPCR was performed using the qScript One-Step SYBR Green qRT-PCR Kit (Quanta BioSciences). All unknown RNA was diluted to 100ng/μL, and standards were diluted to 10^7, 10^6, 10^5, and 10^4 transcripts. Primer pairs were as follows: forward 5'-CGAGACGAAACTGGTTTTCACAG-3' and reverse 5'-TGAACAAAATCAGCTTTCACAATG-3'. The conditions used were Tm=66°C. Each sample was tested in triplicate, with biological replicates when available.

Results and Discussion

Real time qPCR results determined the initial copy number of *bantam* in each unknown RNA sample (see Table 1). These results were analyzed in R to determine significant differences between samples. Results are unavailable for *T. confusum* prepupae because the total RNA was poor quality and for *G. cornutus* day 3 pupae because no Ct value was determined for that sample.

The statistical analysis program R from C-RAN was used for analysis of all results. All results were log transformed, as this is how the standard curve is constructed during real time qPCR. ANOVA tests and Tukey's Honestly Significant Difference tests were employed to determine differences between the data and to ensure their significance. All differences are significant at the $p<5\%$ level.

Table 1. Initial transcript quantity for standards and unknowns as determined from the standard curve and Ct values.

Well Name	Quantity (copies)
Standard (E4)	1.00E+04
Standard (E5)	1.00E+05
Standard (E6)	1.00E+06
Standard (E7)	1.00E+07
T. castaneum Prepupae	3.05E-03
T. castaneum Day 3 Pupae	1.80E-02
pygmy Prepupae	9.42E-02
pygmy Day 3 Pupae	4.93E+03
Goliath Prepupae	1.48E-01

Well Name	Quantity (copies)
Goliath Day 3 Pupae	4.27E-02
T. freemani Prepupae	6.83E+00
T. freemani Day 3 Pupae	6.47E+00
T. confusum Day 3 Pupae	1.03E+03
L. oryzae Prepupae	1.74E-01
L. oryzae Day 3 Pupae	8.34E-01
G. cornutus Prepupae	8.18E+01
T. brevicornis Prepupae	4.25E-03
T. brevicornis Day 3 Pupae	2.34E+00

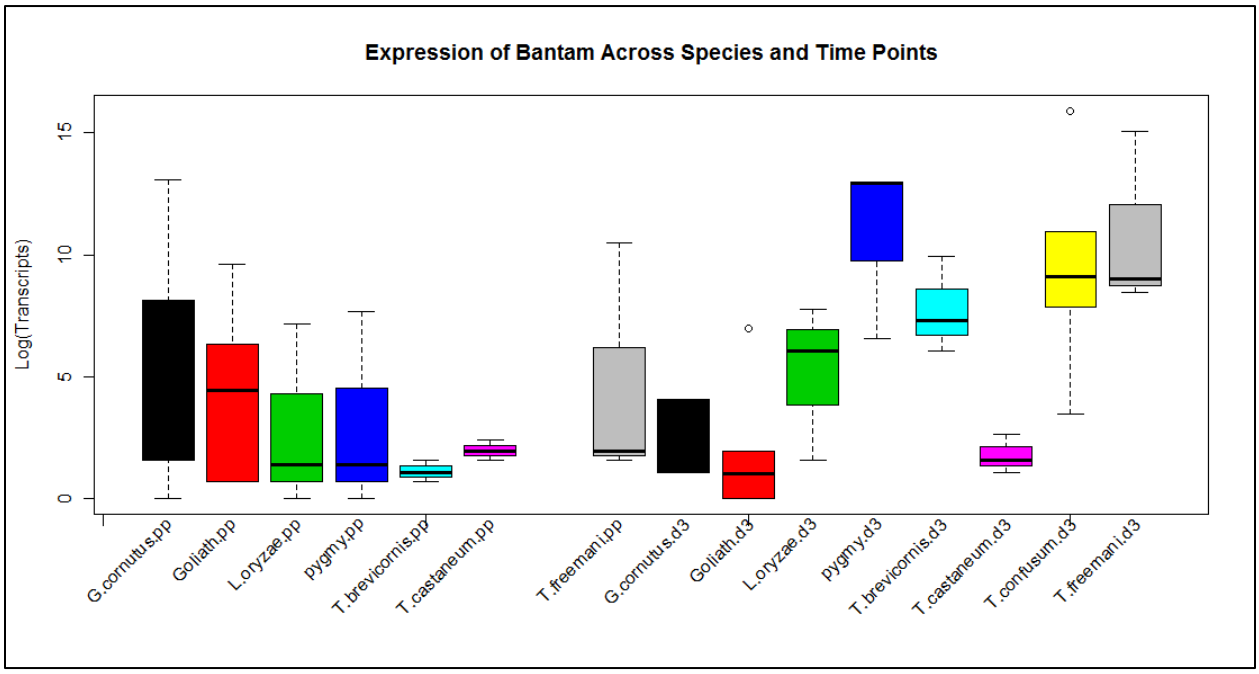

Figure 3. *bantam* expression across all species and time points tested. There is very little variation in expression at the prepupal stage and much greater variation at the day 3 pupal stage.

Tenebrionid Beetles have Differential bantam *Expression*

The real time qPCR results for expression of *bantam* show that there is variation in expression between species and across time points (see Figure 3). There are several general trends that can be observed. First, there is relatively little variation in *bantam* expression at the prepupal stage and increased variation in expression in the day 3 pupae (see Figure 4). In some species the expression is up-regulated, while in others it is down-regulated (Figure 4), indicating that *bantam* has greater effects at the day 3 pupal stage.

T. castaneum and Size Mutants Vary in bantam *Expression*

Tribolium castaneum and its two size mutants, *pygmy* and *Goliath*, delivered interesting results. These organisms are all the same species, but *pygmy* and *Goliath* contain mutations somewhere in the genome that result in proportional total body sizes that are much smaller or much larger,

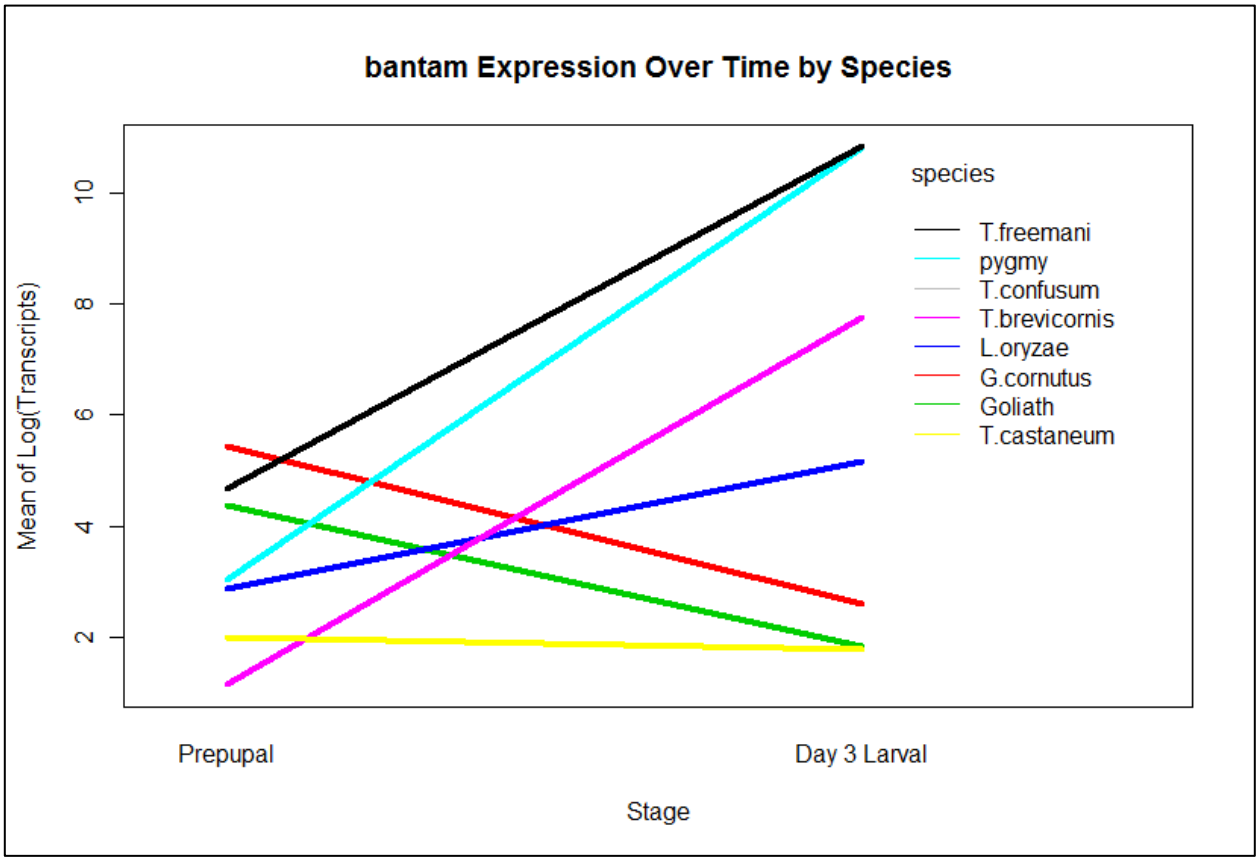

Figure 4. The interaction plot for all of the species and time points tested. *T. castaneum* maintains constant *bantam* expression over time while *T. freemani*, *T. castaneum pygmy*, *T. brevicornis*, and *L. oryzae* had increased *bantam* expression over time and *G. cornutus* and *T. castaneum Goliath* had decreased *bantam* expression over time. Data for *T. confusum* is unavailable for the prepupal stage, but the day 3 pupal expression is comparable to *T. castaneum pygmy* day 3 pupal expression.

respectively, than the wild type *T. castaneum*. Since the locations of these mutations are unknown, it is possible that they involve the Hippo pathway or its interactions. The results show that *T. castaneum* wild type maintains steady, low *bantam* expression across the stages, while *pygmy bantam* expression is significantly up-regulated between the prepupal and day 3 pupal stages (Figure 5). Conversely, *bantam* expression appears to decrease in *Goliath* over time,

though this change was not significant. Expression of *bantam* is also significantly higher in *pygmy* than in either wild type *T. castaneum* or *Goliath* at the day 3 pupal stage, indicating that there is some regulatory or signaling change for *bantam* between these size mutants. These results are at odds with the known growth promoting roles of *bantam* (Brennecke, Hipfner et al. 2003). A possible explanation for the up-regulation of *bantam* in *pygmy* is a feedback loop from a downstream target of *bantam* that causes further up-regulation of *bantam* to compensate for the apparent lack of growth. No matter the exact cause of *bantam* up-regulation in *pygmy*, it appears that this mutation does affect the Hippo pathway and determining the exact interactions would be a useful follow up study.

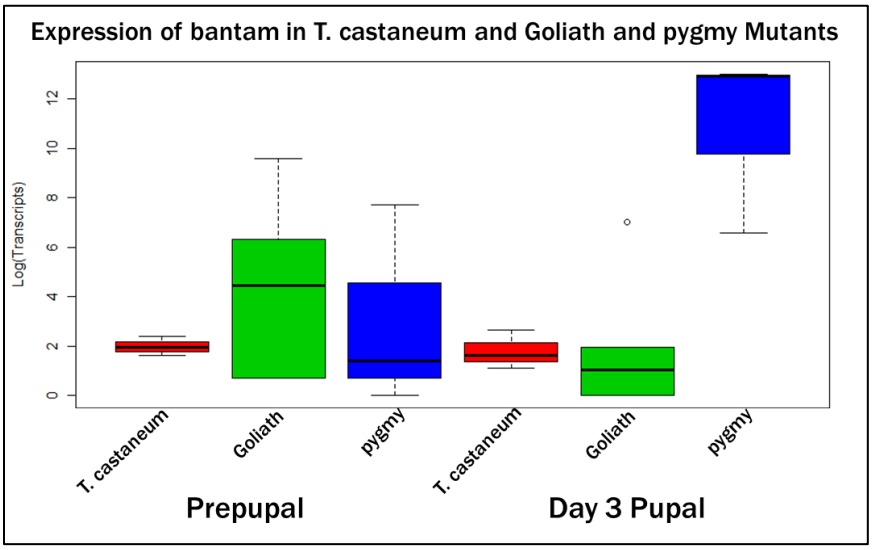

Figure 5. A comparison of *bantam* expression in *T. castaneum* and size mutants. Expression in *pygmy* mutants is significantly upregulated at the day 3 pupal stage when compared to *pygmy* prepupal, *T. castaneum* day 3 pupal, and *Goliath* day 3 pupal RNA.

Higher Expression Trends in Larger Beetles

The second trend observable from the data involves *T. castaneum*, *T. freemani*, and *T. brevicornis*. *T. castaneum* is smaller than both *T. freemani* and *T. brevicornis* and has low, constant *bantam* expression between time points (Figure 4), while *T. freemani* and *T. brevicornis* exhibit *bantam* up-regulation at the day 3 pupal stage (Figure 6). There is a significant expression difference between *T. freemani* and *T. castaneum* at the day 3 pupal stage, which is particularly remarkable because these are the most closely related species of the beetles studied (Angelini and Jockusch 2008). This significant difference in Hippo pathway expression indicates that it may contribute to the size differences between these beetles. *T. brevicornis* is the furthest species from *T. castaneum* and *T. freemani* phylogenetically, and it is as yet unclear if the similarities in *bantam* expression between *T. freemani* and *T. brevicornis* are due to convergent or homologous evolution or some other source (Angelini and Jockusch 2008).

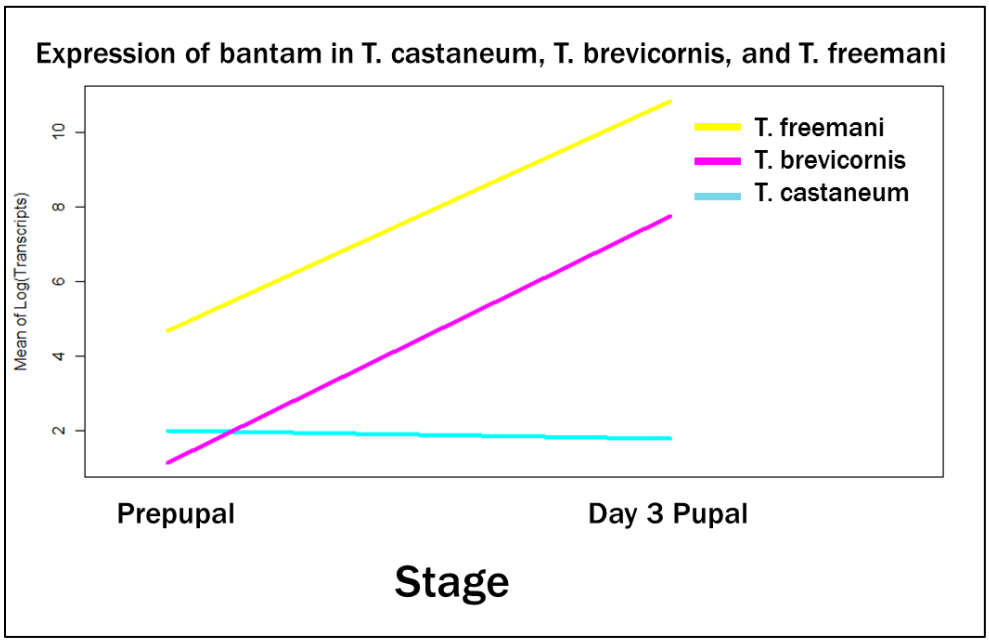

Figure 6. *bantam* expression in T. *castaneum*, *T. freemani*, and *T. brevicornis*. A trend of increasing *bantam* expression between time points is observable for *T. freemani* and *T. brevicornis*, though only the change in *T. freemani* is significant compared to T. *castaneum* expression.

Conclusions and Future Directions

The goal of this study was to assess whether differential activity of the Hippo pathway, as assayed through expression of the miRNA *bantam*, could explain the variation in total body size among *Tribolium* beetles. The results show that the Hippo pathway may contribute to size differences between some species, most notably *T. freemani* and *T. brevicornis*. There was also an interesting up-regulation of *bantam* in the *T. castaneum* size mutant *pygmy* that may be explained by feedback from inoperative downstream targets.

This project has confirmed that the Hippo pathway is active in *Tribolium* beetles and has shown that further exploration is merited, but the exact targets of *bantam* are still unknown, which is a major block to characterizing the pathway. Other useful information could be gained from testing more time points and different tissue to determine exactly when and where the Hippo pathway is active. Finally, injection of a *bantam* mimic could provide insights into how body size may have evolved through changes in the developmental program. Explaining the evolution and development of size over space and time in individuals and among populations will continue to challenge to evolutionary developmental biologists for many years to come.

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