American Journal of Developmental Biology 1(1), 1-6 (2010)

Allometry and Insulin: The Insulin Receptor Pathway and it's Affect on the Scaling Growth of *Tribolium castaneum*

Abigail LaBella

Abstract The insect insulin receptor (InR) is a likely candidate for the control of allometry. Allometry is the growth of an appendage or organ in relation to the whole body size. Regulation of allometry ensures that all parts of the body reach the proper size. By knocking down *InR* using RNAi in the red flour beetle *Tribolium castaneum*, we were able to illustrate that this significantly decreases the absolute appendage length in the beetles. In all the groups including the control there was no correlation between appendage length and body volume revealing that appendage length is consistent regardless of body volume. It is clear from this data that InR does play a major role in determining appendage growth in *T. castaneum*.

KEY WORDS: Insulin, *T. castaneum*, Allometry, InR,

INTRODUCTION

The study of insect development has yielded the discovery of many "patterning" pathways. These include evolutionarily conserved pathways such as Wingless/Wnt which was originally identified as a recessive mutation affecting wing development in *Drosophila melanogaster* (Sharma and Chopra, 1976). Another evolutionarily conserved patterning pathway is the Hedgehog signaling pathway responsible for the development of body segments, which was discovered in *Drosophila* embryos (Nusslein-Volhard and Wieschaus, 1980). The majority of known pathways are responsible for inducing the development of organs, segments and appendages and placing them in their proper position. Little attention, however, has been paid to how traits grow to the proper size (Johnston and Gallant, 2002). The study of the relative size of an appendage or organ in relationship to the entire body size is known as allometry. While little has been revealed about the molecular mechanisms behind this property, it has long been recognized as an important feature of growth.

In 1932 Julian Huxley published *Problems of Relative Growth* in which he queried as to how body parts scale with the total body size (Huxley, 1932). Since 1932 relatively less progress has been made in the field of allometry than in other developmental fields such as limb patterning or segmentation (Emlen et al., 2006). What has been accomplished is the separation of allometry into different categories based on species and pattern of development. Measurements made within one species during one developmental time point are static allometric relationships. Allometry studied during the growth of a single individual is ontogenetic allometry. Finally, evolutionary allometry is the change in allometry in one species over time or between two diverging populations of one species (Stern and Emlen, 1999).

Analysis of allometry is typically preformed by plotting the log of the whole body size against the log of the appendage

AMERICAN JOURNAL OF DEVELOPMENTAL BIOLOGY

size. This comparison often reveals linear relationships with different slopes (Shingleton et al., 2007). If the slope of the line is close to 1 the relationship is isometric and is the result of a trait that is typically independent of body size. Traits that exhibit a steep slope are traits that become relatively larger with increasing body size. Shallow slopes or flat slopes indicate that a trait becomes relatively smaller with increasing body size. A flat slope would also indicate that the absolute trait size was consistent across all body sizes. This is not to be confused with a negative slope where the absolute size of the trait decreases with body size (Shingleton et al., 2007). The goal of the molecular study of allometry is to determine what controls the growth of traits to the appropriate size.

The end result of growth is often easy to measure, however, it is the mechanism by which trait size is coordinated to the whole body size is still not well understood. The first step of this understanding is to determine what controls trait and body size. Two factors determine the size of any part of an organism: cell number and cell size. Experiments by Alpatov in 1930 and Robertson in 1955 and 1959 verified that both cell size and cell number contribute to trait size. Robertson showed that wing size to body size differences in *D. melanogaster* populations were the function of genetic differences in cell number while cell size remained similar between individuals (Robertson, 1959). When flies, however, were exposed to increased temperatures, size difference in the wings was due solely to changes in the cell size. This result indicates that regulations of cell size and cell number are independent (Robertson, 1959). Typically, however, appendage growth is determined by the rate of cell division because cell size remains relatively the same in one organ or appendage (Johnston and Gallant, 2002).

The regulation of cell division, therefore, determines the size of individual traits, indicating that the regulation of cell division is not constant throughout the body.

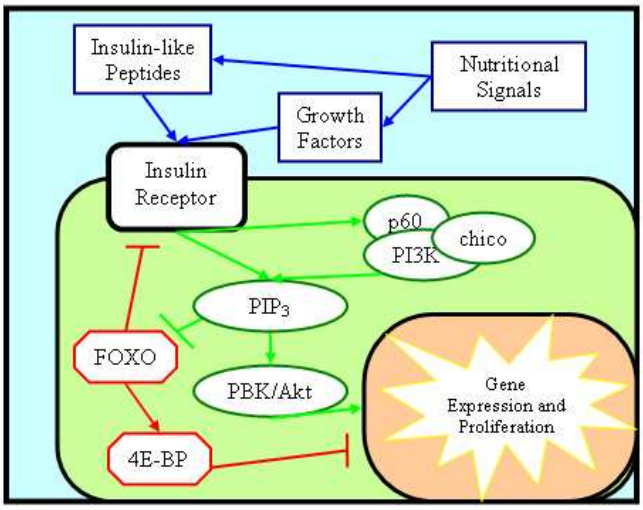

Figure 1: Insulin Receptor Pathway in Insects. The extracellular space is shown in blue, the intracellular space in green and the nucleus in red. The pathway activated by insulin receptor binding is shown in green. The repressive pathway regulated by FOXO is shown in red.

One pathway that has been identified as a regulator of cell division, and therefore allometry, is the insulin pathway. It is known to regulate protein synthesis in both vertebrates and insects (Kozma and Thomas, 2002). Because cell proliferation requires high levels of protein synthesis, the insulin pathway is a good candidate for allometric control (Emlen et al., 2006). Experiments in *Drosophila*, which have only one insulin-receptor (dInR,) have shown that binding of insulin-like peptides (ILPs) to dInR activates a conserved signal transduction cascade that controls the activity of protein translation machinery. Mutations in any step of the insulin pathway have resulted in a reduction in cellular and overall organismal growth. Additionally, it has been observed that the over-expression of components that inhibit the dInR pathway slow the rate of growth (Johnston and Gallant, 2002).

In all insects, insulin-like peptides are secreted by insulin-producing cells in the brain. These insulin-like peptides circulate the entire insect, but are targeted to specific tissues by the presence of the Insulin Receptor on the surface of cells. The amount of insulin-

like peptides circulating in the body is nutritionally dependent. Therefore, sensitive tissues will exhibit nutrition-dependent allometry whereas tissues that are less sensitive will exhibit nutrition-independent allometry (Emlen et al., 2006) Figure 1 illustrates the steps of the insulin pathway that will be discussed in detail.

The insulin pathway in insects is fairly well understood and begins with the binding of insulin-like peptides to the insulin receptor with the possible aid of growth factors. The Insulin Receptor is a receptor tyrosine kinase (RTK) that is composed of two heterodimers that each consist of an α and a β subunit (Claeys et al., 2002). The binding to the InR occurs on the extracellular α subunit at the cysteine-rich region. This results in the phosphorylation of the kinase regions in the membrane-spanning β-subunits. The InR can then phosphorylate down-stream intracellular proteins (Wu and Brown, 2006).

In insects the main protein activated by the InR is a phosphatidylinositol-3-Kinase (PI3K.) The p110 catalytic subunit of PI3K generates the membrane lipid messenger phosphatidylinositol-3,4,5-triphosphate (PIP3.) PIP3 in turn activates kinases such as phosphoinositide-dependent protein kinase 1 (PDK1) and Akt/protein kinase B. It is via these kinases that other proteins associated with glucose uptake, lipid synthesis and gene expression (all required for cell proliferation) are activated (Wu and Brown, 2006).

The Forkhead Transcription Factor (FOXO) is also involved in the InR pathway. In *Drosphila* dFOXO is phosphorylated by dAkt which is activated by the InR pathway. This phosphorylation results in retention in the cytoplasm and an inhibition of transcriptional activity. Un-phosphorylated dFOXO remains in the nucleus and regulates the transcription of dInR and d4EBp, a transcription regulator. *Drosophila* mutations of dFOXO that have no phosphorylation site, and therefore cannot be inactivated, result in growth arrest. FOXO therefore is one of the mechanism by which the insulin pathway has a transcriptionally induced feedback control that results in high levels of InR on cells that have not bound insulin-like peptides (Puig et al., 2006).

By looking at the effect of knocking down the insulin receptor in the red flour beetle *Tribolium castaneum* we hoped to further support the insulin receptor's role in the control of allometry. *Tribolium castaneum* is known as a stored product pest as it is found in stored grain products. Additionally, insulin-like peptides had been previously sequenced from *T. castaneum* (Li et al., 2007). By first isolating the InR sequence from *T. castaneum* we were able to create dsRNA to perform RNA interference (RNAi) to induce a knockdown of InR expression. The measurement of limb size in comparison to body size was studied to probe for effects of *T. castaneum* InR on allometry.

MATERIALS AND METHODS

Insect husbandry and care

Wild type *Tribolium castaneum* were obtained from Carolina Biological Supply Co. Strains termed CB2, CB3 and CB5 were used in the injections. Cultures were grown in a 30ºC incubator in media consisting of 45% sifted whole wheat pastry flour, 45% sifted white pastry flour, 10% sifted nutritional yeast, and 1:40,000 (w/w) of fumagilin, an anti-fungal compound (Carolina medium). Healthy cultures were sub-culturued about every month. Larvae were isolated for injection using a sieve and young larva were isolated by transferring the beetles to a Petri dish for sorting. Larvae were kept in Carolina medium post injection. After the injected larvae transitioned to pupae, each was placed in an Eppendorf tube closed with cotton until it reached the adult stage.

Table 1: Primer sequence and data.

Primer name	DNA sequence	size(bp)	position*	T_m	%GC	Product (bp)
cst'InR-f1	CGGCAGTTGGTTTTTGGACATC	22	67	58.0	50%	905
cst'InR-r1	TTGGCGTTACAGGAGTGTTTGC	22	971	56.6	50%	
cst'InR-f2	GTTCCTCCAGTTACTTCAACGACAAC	26	2323	56.4	46%	1051
cst'InR-r2	CCAAACGCTCCCTATTAGTGGC	22	3373	55.8	55%	

* Position is given relative to the start of translation for *T. castaneum InR* (GenBank Accession number XM_967677).

Table 2: T7 Primer Pairs. Primer pairs used for dsRNA creation

primer	DNA sequence	size (bp)	position*	Product (bp)
T7-cst'InR-f1	taatacgactcactataggg ACTTGCCAGTGTTTAGAAAC	40	22	218
T7-cst'InR-r1	taatacgactcactataggg AACACCCGGTTCGCATTTAG	40	199	
T7-cst'InR-f2	taatacgactcactataggg CCTTGAAAGCGTTATCGCCT	40	601	
T7-cst'InR-r2	taatacgactcactataggg TTTCTTCTTCTTGTAAAAAC	40		

* Position is given relative to the first nucleotide of the isolated *T. castaneum InR* fragment.

Isolation and cloning of orthologous genes

RNA extraction and cDNA synthesis were conducted to prepare *Tribolium castaneum* transcripts for PCR amplification. RNA was previously extracted by Dr. David Angelini as previously described (Angelini and Kaufman, 2004). Primers for the *Tribolium castaneum* InR were designed based on the alignments of published orthologue sequences, obtained using BLAST. Two exact primer pairs were created one positioned at the beginning of the mRNA transcript (Primer pair 1) and the other towards the middle of the mRNA transcript (Primer pair 2). Primer sequences are shown in Table 1.

Both primer pairs successfully amplified a single band of appropriate length. The PCR product was then cloned into the TOPO4 vector (Invitrogen) and transformed into One Shot® Top10 Competent cells (Invigtrogen) Cloning success was verified using PCR. Purified clone plasmids were sequenced by Beckman Coulter Genomics (Danvers, MA.) The exact sequences are shown in appendix 1.

RNA interference

interference (RNAi) was induced by injecting double-stranded RNA to reduce the gene activity of the *InR* gene. Double-stranded RNA was created by adding promoter sites for the RNA polymerase of the T7 virus to the 5' end of new InR primers (Table 2.) Double-stranded RNA was created through incubation with the T7 polymerase (Ambion Applied Biosystems). The double stranded RNA was then diluted to 2ug/ul in injection buffer with 0.05% green food coloring dye. Larval *Tribolium castaneum* were injected with a glass needle using a Märshäuser Wetzlar Gmblt & Co. KG micro-manipulator. The injection site was on the left side of the larva in between the thoracic segments. Larvae were injected until there was a persistent green coloring throughout the length of the body.

RNAi is induced when double stranded RNAs are taken in by the cells and digested into smaller fragments by a protein called Dicer. These smaller portions are then taken up by the RISC complex where one strand of the dsRNA is digested. The single stranded RNA in the complex then base pairs with the corresponding mRNA. This triggers degradation of the transcript or transcriptional inhibition. Control RNAi was preformed with GFP T7 primers which should not inhibit any protein synthesis as *T. castaneum* do not contain the GFP protein. This is a control for any effects of activation of the RNAi pathway. Another set of control beetles were injected with just control buffer. This is a control for injury during injection and any side effects of the injection buffer.

Fixation of Beetles

Adult beetles underwent a cuticle preparation to maintain body and appendage size. The beetles were heated overnight at 50ºC to 60ºC in a solution of 20% glycerol in aceic acid. After this incubation the beetles were washed with 20% glycerol in water for 5-10 minutes on a rocker. The prepared beetles were then stored in 70% glycerol in water at -10 ºC. Beetle appendages were then removed with forceps under a Zeiss dissecting microscope and placed into Aqua Poly/Mount (Polysciences, Inc.) on glass slides. The entire appendage including the coxa, the most proximal leg segment, was obtained.

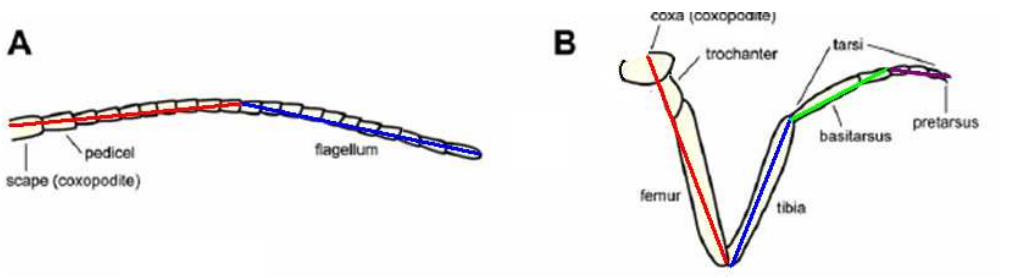

Figure 2. Measurements of the Appendages. (A) Antennae were measured in two sections. Red was the first and Blue was the second. (B) Leg Appendages were typically measured in 4 measurements. The first in Red from the coxa attachment to the end of the femur. The second in blue was the tibia length. The third in green was the all of the basitarsus consisting of most of the tarsi. The fourth in purple was the remaining tarsi and the pretarsus

Microscopy and Measurment

Whole beetle length and width were measured before dissection using a stage micrometer, to a precision of about 1mm. During this process the beetles were sexed using the identification of a patch of short bristles called sex patches on the ventral surface of the prothoracic legs. Appendages on the slide were measured using an ocular micrometer to a precision of about 10 µm. This difference in precision was due to a difference in possible magnification and focal differences between the Zeiss dissecting microscope and Opelco compound microscope.

Measurements were made as illustrated in Figure 2, which was adapted with permission of the authors from Angelini and Kaufman, 2004. Antennae were measured typically in two measurements to account for the curve of the antennae. Only antennae that could be measured from the scape to the end of the flagellum were considered for analysis. Legs were typically measured in 4 measurements. The first measurement was from the coxa to the end of the femur. The coxa was included from the point at which the coxa attached to the body wall in a direct line to the end of the femur. The second from the beginning of the tibia to the end of the tibia. The third was the proximal tarsi, including all the basitarsus. And the final was the remaining distal tarsi, if applicable, and the pretarsus. Appendages were only included if they could be measured all the way from the coxa to the end of the pretarsus.

Statistical Analysis

Statistical analysis was carried out with software package R prepared by the R-project of CRAN. The control data were fitted to the full model, the equal-slope/unequal-intercept model and the one-slope model. ANOVA analysis was run on the full model and equal slopes model to verify their fit. These models were also used to fit the data of the left prothoracic leg (L1). Microsoft Excel was used to plot these values on a graph and create a best fit line. Paired T-tests were performed to analyze the difference between the leg sets (L1 vs L2) within the experimental groups and between the left and right appendages (L1 vs R1) within the experimental groups. Left appendages and right appendages were not both included in this analysis because they would be a misrepresentation of the data as the left and right are taken from the same individual and we have determined that they

are synonymous i.e. they were not different. If we were to use both legs in the analysis it would be as though the data was duplicated which would be like counting the data point twice. Absolute appendage length was plotted in a box plot using R. A two-way ANOVA was used to determine the interaction terms of the different groups. The TukeyHSD was preformed to eliminate the possibility of overestimating difference between the groups because of the high amount of data used in the ANOVA analysis.

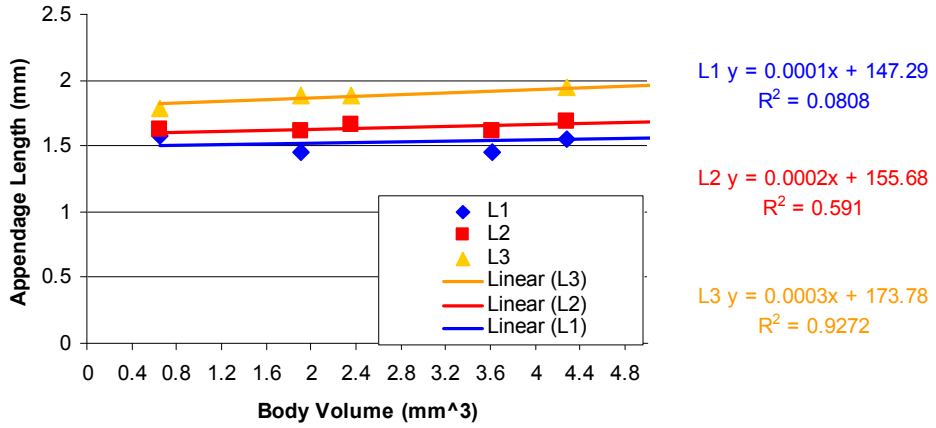

Figure 3. Body volume to Appendage Length Comparison in Control Injected beetles. Best-fit lines had slopes that varied between 0.0001 and 0.0003. The R-square values revealed a fit that accounted for 8% of the correlation in the L1 to 93% of the correlation in L3.

RESULTS

Comparison of Left and Right appendages within individuals and comparison of different appendages within individuals

A paired t-test was used to test if there was significant difference between the left and right appendage in the control injections. The paired T-test of the prothoracic legs on the left side (L1) to those on the right side (R1) resulted in a p-value of 0.39. Using a 95% confidence value of p=0.05 for rejection of the null hypothesis, we can say that the left and right first appendage are not significantly different from each other. Comparison of the left vs. right for meso- and metathoracic legs had p-values of 0.66 and 0.28 respectively. Therefore, for all appendages in the control there was no significant difference between the left and right appendage.

A paired T-test was also carried out on the left and right appendages of the dsRNA from the InR T7 primer pair1 (InR primer 1) and dsRNA from the InR T7 primer pair 2 (InR primer 2) injections. All p-values comparing the left and right appendages in both the InR1 and InR2 RNAi treatments were above 0.05. The lowest p-value was 0.091 which compared the left and right mesothoracic appendages in the InR1 primer.

In order to determine if all the appendages are the same size we used a paired t-test to compare each set of appendages on the left side to the other appendages in all experimental groups: control, InR primer pair 1 and InR primer pair 2. The highest p-value observed was 0.012 indicating that with >95% confidence that the appendages vary in length depending on their position on the body. This revealed that all future analyses would likely need to consider each appendage independently.

Lack of positive allometric relationship between traits and body size in the control

Appendage length was compared to body volume and then to body length. Body volume was calculated using the equation for a cylinder (half of the body width times the body length times π.) Both comparisons were highly similar (data not shown) and body volume was chosen for further analysis due to slightly higher correlation values in linear regression. The comparison of body volume to appendage size on the left side in beetles injected with control buffer or dsRNA encoding the exogenous gene *GFP* resulted in a nearly flat slope (close to 0) (Figure 3) in all of the

appendages studied. The antenna length was left out of this analysis due to a low percentage of complete antennae obtained. This was due mostly to breakage that occurred while obtaining the antennae.

Figure 3 plots appendage length against body volume. The correlation coefficient (R^2) revealed that the best fit line accounted for only 8% of the variation in the L1 but 93% of the variation in the L3 appendage. This variation is most likely due to a small sample size. Even though the correlation varied widely, the best fit lines revealed a slope that was nearly 0. A logarithmic transformation of the data did not result in a better linear fit (data not shown.) The statistical analysis conducted in the next section verifies that the slope of the control is virtually flat.

Comparison of the first left appendage between treatment groups revealed no allometric relationship

The data collected on the left prothoracic leg was analyzed using two different models: the full model (unequal slopes and unequal intercepts) and the equal-slopes/unequal intercept model. Using a confidence cut-off of 0.05, both models were able to account for the variance in the samples with a p-value of 0.03 for the full model and a p-value of 0.0068 for the unequal slopes model. The p-values and ANOVA analysis revealed that the equal slopes model better explains the variation in the data. This implies that there are three distinct experimental groups and that the equal slopes/unequal intercept model explains the variation significantly. The alternative model, which does not fit this data, is the model that shows all the data sharing one intercept and one slope. Even though this result was significant, the data had a low association with the fit with an adjusted R^2 value of only 0.246. Additionally the correlation of appendage length with body volume revealed a nearly flat slope of 0.0204.

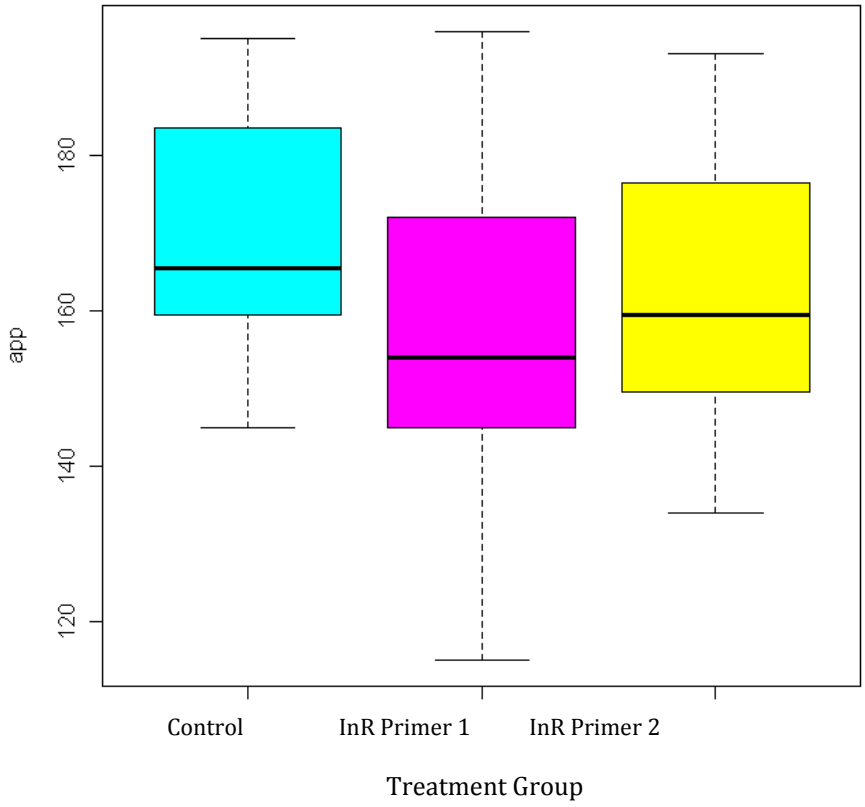

Figure 3. Box Plot of Absolute Appendage Length of the 1st through 3rd Appendage Versus the Treatment Group. Because the TukeyHSD revealed that we could make blanket comparisons all the appendages were grouped together to compare versus treatment. The median of the InR primer 1 is about 7% less than that of the control. The median of the InR primer 2 is about 4% less than that of the control.

Comparison of absolute appendage length between treatment groups

The lack of correlation between appendage length and body volume, as indicated by a nearly flat slope, prompted us to analyze the absolute appendage length between the treatment groups in the left appendages. We first used a TukeyHSD to determine if there were individual differences between treatments and limbs. A 95% group-wise

confidence level revealed that the groups of treatments were significantly different and we did not need to look at individual comparisons such as L1 control versus L2 InR primer pair 2, L1 control versus L3 InR primer pair 1. Instead we were able to group all of the limbs and treatments together to make blanket comparisons of absolute appendage length. This comparison is made in Figure 3.

The median leg length for the control was 1.65mm, it was 1.54mm in the InR1 and 1.59 in InR2 RNAi treatments. This represents almost a 7% decrease in the size of the appendages in InR1 RNAi and almost a 4% decrease in InR2 RNAi. The largest appendages in the experimental groups also do not significantly exceed the control group: the largest appendage in the InR primer 1 group was only larger by 0.02mm. The lower bounds however 18% lower in the InR primer 1 group and 5% lower in the InR primer 2 group.

Body Size analysis

Simple analysis showed that median body volume in the treatments were as follows: 3.0 mm^3 in the control, 2.6 mm^3 in InR primer pair 1 RNAi and 3.6 mm^3 in InR primer pair 2 RNAi. The lower bound of the InR primer pair 2 RNAi group was smaller than the control lower bound by about 0.5 mm^3 but InR primer pair 2 RNAi's median was higher. Additionally the median of InR primer pair 1 RNAi was lower than the control but its highest body volume was twice the maximum of the control body volume. This indicates that body volume was not significantly affected by the treatment because while the median may have changed, the average body volume only ranged from 3.0 mm^3 in the control to 3.5 mm^3 in primer pair 1 RNAi. This could easily be accounted for by the range of ages that were sampled from.

DISCUSSION

Lack of positive allometric relationship between appendages and body volume in *Tribolium castaneum*

Figure 3 and the subsequent statistical analysis revealed that while the variation of the prothoracic leg length versus body volume could be accounted for by the equal-slopes/ unequal-intercept model, the slope of all of the data's variation was very close to flat. Additionally the low adjusted R^2 values indicated that the regression is not explaining a high level of the variation seen. The flat slope observed in all three data sets indicated that the absolute appendage length did not increase with an increase in body length. Instead the flat slope indicated that the relative size of the appendage actually decreased with an increasing body size in the control and experimental groups. Because the equal-slopes/unequal-intercept model significantly explained the variation in all three experimental groups, and not the equal-slopes/equal intercept model, it appeared as though the absolute appendage length decreased in the treatment groups versus the control.

RNAi induced reduction in Insulin Receptor resulted in decreased absolute appendage length

A two way ANOVA revealed that treatments could be analyzed without taking into consideration the specific limb because there was no interaction between any one limb type and treatment. The box plot revealed that the absolute leg length decreased by about 7% in the Primer pair 1 RNAi and 4% in the primer pair 2 RNAi. This could have resulted from a difference in efficiency of the sequences isolated by the primers to induce RNAi. This could be analyzed using quantitative PCR to determine the level of *InR* transcripts present. Additionally it is likely that RNAi was not induced to the same level in every larvae injected. Larvae that did not receive enough dsRNA to induce RNAi would have appendage lengths that resembled the control group. This indicates that our data may represent an underestimation of the decrease in appendage length. In the future, quantitative PCR could also allow investigators to exclude samples in

which RNAi was not induced and would likely result in elimination of the uppermost appendage measurements. Because two-way ANOVA revealed that there was no correlation between the position of the appendage and the treatment it can be assumed that reducing the amount of InR present on cells affects all of the appendages in the same way.

Possible mechanism by which InR RNAi decreased appendage length

Our research so far has located homologs of the insulin pathway common to most insects in both the *Insulin Receptor* and *FOXO.* It is possible that FOXO is the mechanism by which RNAi of the insulin receptor results in decreased appendage length. Decreased activation of the insulin receptor could result in lower levels of PIP3 activation and hence lower activation of PBK/Akt that would allow FOXO to remain active and un-phosphorylated. Un-phosphorylated FOXO remains in the nucleus and regulates the transcription of *InR* and a master transcription regulator. In *Drosophila* mutations of DFOXO with no phosphorylation site, and the protein cannot be inactivated, resulting in growth arrest (Puig et al., 2006). Therefore low levels of InR activation would result in increased FOXO activity.

Additionally it appears that RNAi of the insulin receptor in *Tribolium castaneum* does not significantly affect the body size of the individuals. If the RNAi decreased both the appendage size and the body size it would not be revealed in the models we used. The average body volume was actually slightly higher by about 0.5 mm^3 in the experimental groups than in the control. This is even though the lowest value in the InR primer pair 2 RNAi treatment was 1 mm^3 lower than the lowest value in the control. There is little evidence to support any biologically significant change in body volume in the treatment groups as there was no consistent body volume range. It appears as though only the absolute appendage length is consistently decreased in RNAi of the *InR.*

CONCLUSION

By reducing the amount of *InR* transcript produced in *Tribolium castaneum* we were able to significantly decrease the absolute appendage length. This is likely the direct effect of reduced amounts of InR present on the surface of cells. Because the food available to the beetles was unlimited, this variation is not likely to be nutritionally dependent. Furthermore body size was not observed to consistently decrease or increase with injection. This indicates that body size is not likely under the control of InR.

In the future, these results could be verified using quantitative PCR to reveal the level of RNAi knockdown induced in the experimental groups. This would likely yield an even greater disparity between the appendage sizes in the experimental groups as it would eliminate those that had larger limbs due to lack of effective RNAi. Furthermore, more individuals in all groups need to be analyzed to create more robust statistical analyses. A parallel experiment which was attempted but was delayed due to lack of time would be to study the effects of *FOXO* RNAi and *InR, FOXO* simultaneous RNAi. It is possible a rescued phenotype could be observed by knocking down the expression of both *InR* and *FOXO.*

REFERENCES

Angelini D., Kaufman T.C. (2004) Functional analysis in the himipteran *Oncopeltus fasciatus* revealed conserved and derived aspects of appendage patterning in insects. Developmental Biology 271:306-321.

Claeys I., Simonet G., Poels J., Van Loy T., Vercammen L., De Loof A., Vanden Broeck J. (2002) Insulin-related peptides and their conserved signal transduction pathway. Peptides 23:807-16. DOI: S0196978101006660 [pii].

Emlen D.J., Szafran Q., Corley L.S., Dworkin I. (2006) Insulin signaling and limb-patterning: candidate pathways for the

origin and evolutionary diversification of beetle 'horns'. Heredity 97:179-91. DOI: 6800868 [pii]
10.1038/sj.hdy.6800868.

Huxley J.S. (1932) Problems of Relative Growth. Journal of Anatomy 67:484-485.

Johnston L.A., Gallant P. (2002) Control of growth and organ size in Drosophila. Bioessays 24:54-64. DOI: 10.1002/bies.10021 [pii]
10.1002/bies.10021.

Kozma S., Thomas G. (2002) Regulation of cell size in growth, development and human disease: PI3K, PKB and BioAssay 24.

Li B., Predel R., Neupert S., Hauser F., Tanaka Y., Cazzamali G., Williamson M., Arakane Y., Verleyen P., Schoofs L., Schachtner J., Grimmelikhuijen C.J.P., Park Y. (2007) Genomics, transcriptomics, and peptidomics of neuropeptides and protein hormones in the red four beetle *Tribolium castaneum*. Genome Res 18:113-122.

Nusslein-Volhard C., Wieschaus E. (1980) Mutations affecting segment number and polarity in Drosophila. Nature 287:795-801.

Puig O., Marr M.T., Ruhf M.L., Tjian R. (2006) Control of cell number by *Drosophila* FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes & Development 17:2006-2020.

Robertson F.W. (1959) Studies in Quantitative Inheritance. XII. Cell Size and Number in Relation to Genetic and Environmental Variation of Body Size in *Drosophila*. Genetics 44.

Sharma R.P., Chopra V.L. (1976) Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. Dev Biol 48:461-5. DOI: 0012-1606(76)90108-1 [pii].

Shingleton A.W., Frankino W.A., Flatt T., Nijhout H.F., Emlen D.J. (2007) Size and shape: the developmental regulation of static allometry in insects. Bioessays 29:536-48. DOI: 10.1002/bies.20584.

Stern D.L., Emlen D.J. (1999) The developmental basis for allometry in insects. Development 126:1091-101.

Wu Q., Brown M.R. (2006) Signaling and function of insulin-like peptides in insects. Annu Rev Entomol 51:1-24. DOI: 10.1146/annurev.ento.51.110104.151011.

APPENDIX 1

Insulin Receptor Cloned Sequences from

```
LOCUS       cst'InR                 1034 bp ss-DNA     linear   SYN 09-Dec-2009
DEFINITION  Tribolium castaneum insulin receptor, cloned cDNA fragment
ACCESSION   -
KEYWORDS    -
SOURCE      Tribolium castaneum (red flour beetle)
  ORGANISM  Tribolium castaneum
            Eukaryota; Metazoa; Arthropoda; Hexapoda; Insecta; Pterygota;
            Neoptera; Endopterygota; Coleoptera; Polyphaga; Cucujiformia;
            Tenebrionidae; Tribolium.
COMMENT     Isolated by exact PCR by Abigail Labella, 2009.
FEATURES             Location/Qualifiers
     source          <1..>1034
                     /db_xref=taxon:7070
                     /mol_type=mRNA
                     /organism=Tribolium castaneum
                     /strain=Carolina CB2
     gene            <1..>1034
                     /db_xref=GeneID:661524
                     /gene=LOC661524
     CDS             <1..>1034
                     /codon_start=2
                     /db_xref=GeneID:661524
                     /gene=LOC661524
                     /product=similar to insulin receptor
                     /protein_id=XP_972770.2
                     /translation=STTTSNDTCQCLETKPSTSSINEDVEKSRIDFEDELHNAVYVRKPN
                     FSESRKRRDVDSEQLNANRVFNETDEAGAYISFSTVVTGTEFYMPNLRHYTVYEINVQA
                     CREKTNDKLDTENPCSSKNMKTHRTLKKKGADDIKQIEVKNQSLGMVSLTWKEPDAPNG
                     LIYCYTIEYKKLEIENSKANEEYITHARFINQSRIYTLKALSPGNYSVRVSATTSGDYA
                     NFSPYAYFYIEERPSNTYVTLIVCMLILVIILALCAFCFYKKKKADKESMRLIPSVNPE
                     YVPSVYVPDEWEVPRKKIELIRELGQGSFGMVYEGIAQDVRGKAQIKCAVKTVNEHATN
                     RER
BASE COUNT       332 A       196 C       247 G       257 T         1 OTHER
ORIGIN      ?
        1 TTCAACGACA ACAAGTAACG ACACTTGCCA GTGTTTAGAA ACTAAACCGT CAACATCATC
       61 AATTAATGAA GATGTCGAAA AATCCCGAAT TGATTTTGAA GATGAATTAC ATAACGCGGT
      121 TTATGTGAGA AAGCCGAATT TTTCAGAATC TCGCAAAAGA CGTGACGTGG ACAGTGAGCA
      181 ACTAAATGCG AACCGGGTGT TTAATGAAAC CGATGAAGCC GGTGCTTATA TTTCGTTCAG
      241 TACTGTAGTC ACGGGGACAG AATTTTACAT GCCGAATTTG CGGCACTATA CGGTTTATGA
      301 AATAAACGTC CAAGCCTGTA GAGAAAAAAC CAACGACAAG CTTGATACCG AAAATCCCTG
      361 CAGTAGCAAA AATATGAAAA CGCATCGGAC TCTGAAGAAG AAAGGAGCCG ATGATATAAA
      421 ACAAATTGAA GTCAAAAACC AAAGTTTGGG AATGGTGTCA CTTACGTGGA AGGAACCGGA
      481 CGCTCCGAAC GGATTGATCT ATTGTTACAC GATAGAATAT AAAAAGTTGG AAATTGAAAA
      541 TTCCAAGGCT AATGAAGAGT ACATTACACA CGCGCGTTTT ATCAACCAGA GTAGGATTTA
      601 TACCTTGAAA GCGTTATCGC CTGGAAATTA CAGTGTTCGA GTTTCTGCAA CGACGTCAGG
      661 TGATTACGCC AACTTTTCTC CTTACGCGTA CTTCTATATC GAGGAGCGGC CGTCTAACAC
      721 TTACGTAACC TTGATTGTTT GTATGCTCAT TCTGGTGATA ATTTTGGCAC TCTGTGCGTT
      781 TTGTTTTTAC AAGAAGAAGA AAGCCGACAA GGAGAGTATG CGGCTGATTC CGTCGGTCAA
      841 TCCCGAGTAT GTTCCAAGTG TGTATGTTCC GGACGAATGG GAGGTGCCTC GCAAAAAAAT
      901 AGAACTGATT AGGGAGTTAG GACAGGGGAG TTTTGGCATG GTATACGAGG GCATCGCCCA
      961 AGACGTCCGC GGAAAGGCGC AAATCAARTG CGCTGTGAAG ACTGTTAATG AGCACGCCAC
     1021 TAATAGGGAG CGTT
//
```