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A Mechanism of Extreme Growth and Reliable Signaling in Sexually Selected Ornaments and Weapons

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Many male animals wield ornaments or weapons of exaggerated proportions. We propose that increased cellular sensitivity to signaling through the insulin/insulin-like growth factor (IGF) pathway may be responsible for extreme growth of these structures. We document how rhinoceros beetle horns, a sexually selected weapon, are more sensitive to nutrition and more responsive to perturbation of the insulin/IGF pathway than other body structures. We then illustrate how enhanced sensitivity to insulin/IGF signaling in a growing ornament or weapon would cause heightened condition-sensitivity and increased variability in expression among individuals—critical properties of reliable signals of male quality. The possibility that reliable signaling arises as a byproduct of the growth mechanism may explain why trait exaggeration has evolved so many different times in the context of sexual selection.

The most elaborate male ornaments and weapons of sexual selection grow to exaggerated proportions (Fig. 1), especially in the largest and best-conditioned individuals. The size and conspicuousness of these traits make them likely candidates for intraspecific signals, used either by males to assess the size, condition, or status of rival males, or by females to assess the relative genetic quality of potential mates (*1*, *2*). Not only are exaggerated traits easy to observe, they are unusually reliable signals of individual male quality (*2–4*) as their growth tends to be more sensitive to the nutritional histories and physiological conditions of individuals than is the growth of other traits (*5–7*). Exaggerated structures also tend to be more variable in their expression than other morphological structures (*8–10*). Hyper-variability in trait size can amplify otherwise subtle differences in the body size or condition of males, further enhancing the utility of these traits as signals. Combined, these structural characteristics – extreme size, heightened condition-sensitivity, and hyper-variability among individuals – are the foundation for 'handicap' and 'good genes' models of sexual selection and a central tenet of modern theories of sexual selection and animal communication (*2–4*, *11–15*). We offer a developmental explanation for this phenomenon. We suggest the evolution of trait exaggeration involves increased sensitivity to insulin/IGF signaling within a growing structure, and we show why such a change in mechanism should also confer both heightened condition sensitivity and hyper-variablity to expression of the trait (Figure 1B).

Insulin and IGFs are essential regulators of tissue growth and body size (*16*). Circulating levels of insulin and IGFs are sensitive to nutrition, as well as stress and infection, and the insulin/IGF pathway has emerged as the central mechanism integrating physiological condition with growth in multicellular animal taxa. Insulin and IGF levels within a growing animal reflect the nutritional state and physiological condition of that individual, and circulating levels of these signals modulate tissue growth via the insulin receptor pathway in a graded, or dose-dependent manner. Within an individual, growth will speed up or slow down in response to changes in nutritional or physiological state because of the action of this pathway. Across individuals, growth will differ between high-condition and low-condition individuals, resulting in population-level variation in body and trait sizes. Low-condition individuals have lower levels of these signals than higher condition individuals, and, as a result, they experience slower rates and lower overall amounts of tissue growth.

As long as the various organs and body parts (e.g., legs, eyes, wings) exhibit similar sensitivities to insulin/IGF signaling (*17*), their sizes will scale proportionally from individual to individual (*18–21*). But some traits deviate in their responsiveness to these signals, profoundly affecting the amount and nature of their growth. Genitalia are insensitive to circulating insulin/IGF signals in *Drosophila* (*20*, *21*). As a result, their growth is unresponsive to environmental conditions, such as nutrition, and genitalia size is largely invariant among individuals. In contrast, wings exhibit sensitivity to insulin/IGF signaling typical of the rest of the body; wing growth is sensitive to larval nutrition, and wing sizes scale isometrically with among-individual variation in body size (*21*).

We predicted that increased sensitivity to the insulin/IGF pathway might be a mechanism leading to the evolution of extreme growth in showy ornaments and weapons of sexual selection. In our model, individual males differ in their physiological state as a result of differences in their status, nutritional state, competitive ability, and/or health (parasite or pathogen loads), translating into among-individual variation in circulating levels of insulin/IGF signals (Fig. 1B). During their respective periods of growth, the adult structures in these animals would be exposed to insulin/IGF signals, and the sensitivity of cells within each growing structure to these signals would determine both how and by how much each trait grew. Just as wings are more sensitive to insulin/IGF signaling than genitalia in *Drosophila* (*20*, *21*), so we predicted that exaggerated ornaments or weapons of sexual selection would be even more sensitive to insulin/IGF signaling than wings or other non-sexually-selected body parts (Fig. 1B).

Male rhinoceros beetles (*Trypoxylus dichotomus*) wield a forked horn on their heads. During growth, horns in this species are more sensitive to larval nutrition than other body parts (wings, genitalia), and, among adult males, horn size is hyper-variable, ranging from tiny bumps to exaggerated structures two thirds the length of a male's body (*22*). We tested whether growing rhinoceros beetle horns were more sensitive to insulin/IGF signaling than wings or genitalia using RNA interference to perturb transcription of the insulin receptor (*InR*). Developing larvae were injected with a 398bp fragment of dsRNA of *T. dichotomus InR* as they commenced their transition from larval feeding to gut purge (the onset of the prepupal period and the beginning of metamorphosis). At this time all growth in overall body size had ceased, but adult structures (including genitalia, wings, and horns) were still growing. Thus, any effects of manipulation of insulin/IGF signaling would be visible as

reductions to genitalia, wing, or horn size relative to overall body size. If the evolution of exaggerated horn size resulted in part from an increase in cellular sensitivity to insulin/IGF signaling, then horns should be more sensitive than wings to perturbation of the activity of this pathway. We also predicted that genitalia would be relatively insensitive to pathway perturbation (sensu *20, 21*).

Injections significantly reduced *InR* transcript abundances for 48 hours near the end of the period of trait growth (i.e., before *InR* transcript abundance normally drops in these tissues; Fig. 2A-C). After metamorphosis was completed, we compared morphologies of treated and control animals. Genitalia did not respond to experimental perturbation of *InR* pathway activity (Wald statistic = 0.1245, 1 degree of freedom, p = 0.724; Fig. 2D). Wings, which exhibit nutrition-sensitive growth patterns typical of the majority of metric traits (e.g., eyes, legs, elytra, etc.), showed a significant reduction in size of ~ 2% (Wald statistic = 8.976, 1 df, p = 0.003; Fig. 2E). In contrast, male horns, the structures most sensitive to nutrition, were reduced by ~ 16% relative to controls (Wald statistic = 68.37, 1 df, p < 0.0001; Fig. 2F, G). Using response to *InR* knockdown as a metric, male horns were eight times more sensitive to insulin/IGF signaling than wings, consistent with our model for the evolution of disproportionate or exaggerated weapon size from enhanced tissue-specific sensitivity to the insulin/IGF pathway.

A growing body of research now implicates insulin/IGF signaling in the development of extreme animal structures (*23*). Insulin/IGF signaling is an ancient and conserved physiological pathway that has coupled rates of cell proliferation with available nutrients for at least 500 million years, and we suggest that this pathway has been co-opted repeatedly in lineages experiencing strong sexual selection to yield disproportionate growth in signaling structures. The insulin/IGF pathway would likely have controlled the rate of growth of these structures already; increased cellular sensitivity to these signals would therefore be an easy route to the evolution of accelerated growth if the structure came under directional sexual selection for increased size.

But such a route to exaggeration would only generate exaggerated trait sizes in high-condition individuals because low-condition individuals would have low circulating levels of insulin/IGF signals and attenuated rates of tissue and body growth. The same mechanism stimulating increased trait growth in high quality individuals would also repress trait growth in low quality individuals (Fig. 1B). This means that whenever exaggerated ornament or weapon size arises due to an increase in trait-specific sensitivity to insulin/IGF signaling, then the exaggerated trait should also show enhanced (or 'heightened') condition-sensitive expression and higher relative variability in trait size between low- and high-condition individuals (as compared to other, non-exaggerated, traits). Signal reliability would be an intrinsic property of these structures because of the developmental mechanism regulating their growth.

Theoretical considerations of sexual selection and animal signaling argue that escalated evolution of signals is most likely when signals are reliable, and it is difficult or impossible for low quality males to "cheat" by producing full-sized structures (Fig. 3). Signal reliability can be evolutionarily stable under two sets of conditions: either the signal is sufficiently costly to produce or wield that it is not cost-effective for low quality individuals to cheat ('handicap' signals), or the signal is intrinsically unfakable ('index' signals, 'good genes' signals) (*2–4*, *11–13*, *24–33*). The largest ornaments and weapons are generally assumed to be handicap signals of male quality, where the cost of these structures enforces signal reliability (*2–4*, *24–33*). However, for even the largest of structures, the process of escalation must have started when these structures were small, and at that early stage, these costs would likely have been minimal. Moreover, several recent studies of exaggerated male ornaments and weapons have failed to find significant costs (*34*, *35*), forcing a reconsideration of the question: why don't low quality males cheat?

We suggest that exaggerated animal structures may be unfakable signals of quality because of the developmental mechanism responsible for their accelerated growth. If true, then our hypothesis of 'intrinsic reliability' could help explain why so many different signal traits embark on an evolutionary trajectory of bigger and bigger size. We suggest that whenever receivers responded to variation in insulin/IGF-sensitive structures, they fared relatively well due to the intrinsic reliability of these traits as signals of underlying male quality. As these traits became larger under selection, their utility as signals would have increased, enhancing the benefits to receivers and accelerating the rate of signal evolution still further. Once these structures become large enough to be costly, they may also act as handicap signals and costs could contribute to signal reliability (Fig. 3). However, as long as the traits exhibit heightened sensitivity to insulin/IGF signals, costs may not be necessary for signal reliability (*36*). This means that subsequent evolution of compensatory structures alleviating costs to the signaling males (*37*) need not undermine the reliability of these traits as signals and could explain why some exaggerated sexually selected structures function as reliable signals even when no discernable costs are apparent (*34*, *35*).

References and Notes

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17. For this paper we define tissue sensitivity as the extent to which variations in the level of hormone signal influence the rate of cell proliferation via activity of the insulin/IGF pathway. Insensitive tissues grow to roughly the same final

size regardless of circulating insulin/IGF levels, whereas the amounts of growth of sensitive tissues are strongly regulated by signal levels. Tissue sensitivity is often equated with receptor density. However, in this case, altered expression of any number of downstream genes in the pathway could change the responsiveness of a tissue to insulin/IGF signals. Indeed, in the best-studied example to date, reduced insulin-sensitivity in a specific tissue (genitalia) in *Drosophila* resulted from lowered levels of expression of a "downstream" element of the insulin-signaling pathway, *FOXO*, and not from tissue-differences in expression of the insulin receptor (*21*).

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Supplementary Materials
www.sciencemag.org/cgi/content/full/science.1224286/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S3
Tables S1 and S2
References (*38–90*)

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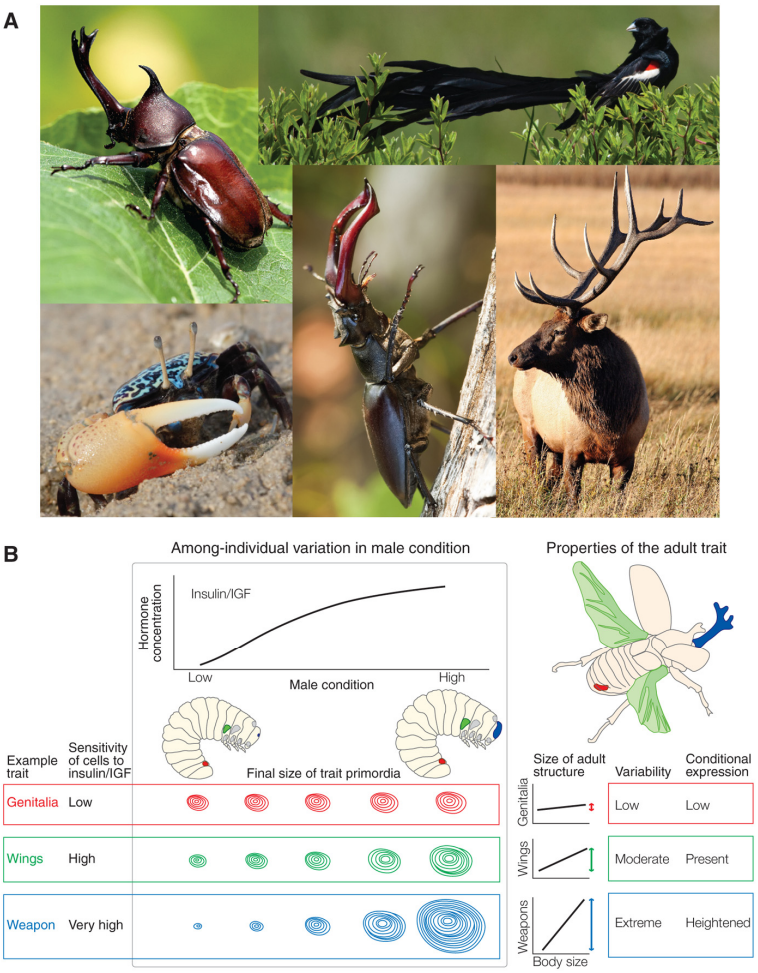

Fig. 1. A). Exaggerated growth of weapons and ornaments of sexual selection as exemplified by rhinoceros beetle horns (*Trypoxylus dichotomus*). B) Proposed mechanism for the evolution of trait exaggeration through increased cellular sensitivity to insulin/IGF signaling (shown for the disc-like appendage primordia of insects). Individual nutritional state and physiological condition are reflected in circulating levels of insulin-like peptides and IGFs, which modulate the rate of growth of each of the trait primordia. Traits whose cells are sensitive (*17*) to these signals (e.g., wings [green]) exhibit greater nutrition-dependent phenotypic plasticity and among-individual variability than other traits whose cells are less sensitive to these signals (e.g., genitalia [red]). An increase in the sensitivity of cells within a particular trait (e.g., horns [blue], see text) would lead to disproportionately rapid growth of that trait in the largest, best-condition individuals (i.e., exaggerated trait size) and smaller trait sizes in low-condition individuals.

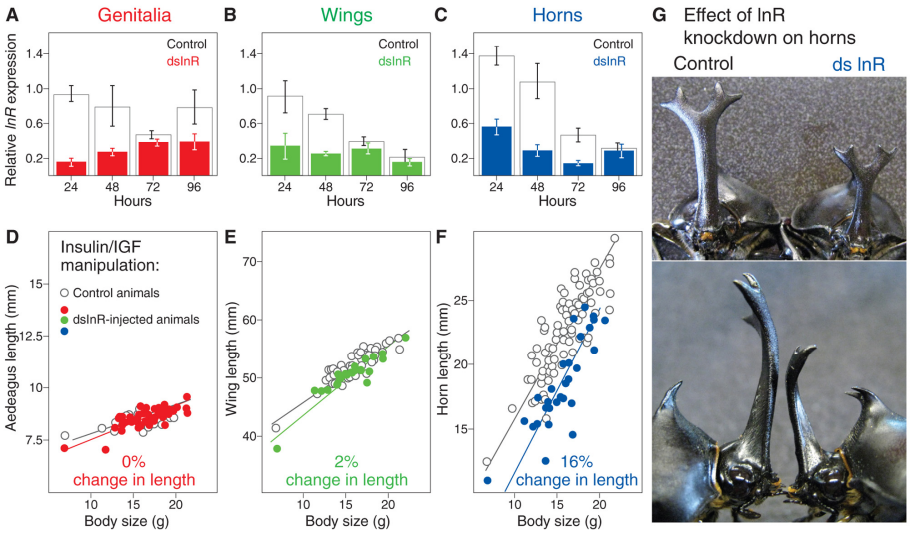

Fig. 2. Effect of *insulin receptor* (*InR*) knockdown on growth of adult structures in rhinoceros beetles. A-C) Relative transcript abundances for the *insulin receptor* (*InR*) gene in genitalia (A), wings (B), and horns (C), measured 24, 48, 72, and 96 hours after the onset of the prepupal period in control (open bars) and ds*InR*-injected (solid bars) animals. Injection with dsRNA significantly reduced transcript abundances for 48 hours following injection in all three tissues. D-F) Effects of ds*InR* knockdown on trait growth. Genitalia were insensitive (D); wings responded significantly but moderately to interrupted insulin/IGF signaling (E) (average reduction in wing length = 2%); and horns responded dramatically (F), with an average reduction in horn length of 16%. G. Head and thorax shown in two orientations (top and bottom) for same-sized control (left) and ds*InR*-injected (right) males.

Category of models:	Model conclusions:	Model predictions:	Contribution of the insulin/IGF mechanism:
Corroborated by an insulin/IGF mechanism for trait exaggeration:			
Index signal models Maynard Smith & Harper 2003 (*3*)	Sexually selected traits can evolve to exaggerated sizes if bigger versions of the trait are more effective signals than smaller versions, and if trait size is a physiologically or physically unfakable 'index' of the quality of the signaler. Trait size is a reliable signal because it is mechanistically impossible for low quality signalers to cheat.	Should be mechanistically impossible for low quality males to produce large signal traits. Why the size of a signal trait is unfakable is often unclear/unspecified.	Provides an explicit mechanism for unfakable signal expression in exaggerated morphological structures.
Good genes/indicator models Andersson 1986 (*26*) Iwasa & Pomiankowski 1999 (*13*) Lorch et al. 2003 (*27*)	Females benefit if they choose mates based on conditionally expressed ornaments because variation in the expression of these traits reliably indicates the overall quality of a male. Low quality males produce smaller signal traits because they are in poor condition.	Exaggerated traits should exhibit 'heightened' conditional expression.	Provides an explicit mechanism for heightened conditional expression of exaggerated morphological structures.
Genic capture models Rowe & Houle 1996 (*12*) Lorch et al. 2003 (*27*) Tomkins et al. 2004 (*28*)	Evolution of ornaments persists (genetic variation is not depleted) because in their expression these traits "capture" genetic variation for overall body condition, including health, resistance to parasites, competitive ability, nutrition, etc.	Genetic variation among males affecting their body condition, resistance to parasites, competitive ability, etc., should translate into differences in ornament size.	Provides an explicit mechanism for genic capture, since all of these aspects of body condition are channeled into a common endocrine signal regulating trait growth.
Assessment/arms race models Parker 1974 (*29*) Enquist & Leimar 1983 (*30*)	Male weapons can evolve to exaggerated sizes if weapon size reliably signals the fighting ability of a male.	Males should use relative weapon size as a basis for assessment; fights should be most likely to escalate if rival males are similarly armed. Not clear from the models why weapon size should remain reliable.	Suggests that weapons will become increasingly reliable signals of fighting ability as they increase in size, facilitating arms races.
Modified by an insulin/IGF mechanism for trait exaggeration:			
Handicap models Zahavi 1975 (*24*) Pomiankowski 1987 (*31*) Grafen 1990 (*25*) Johnstone 1995 (*11*) Iwasa & Pomiankowski 1999 (*13*)	Females benefit if they choose mates based on costly ornaments. A given increase in trait size costs low quality males more (or benefits them less) than it does high quality males, resulting in ornament sizes that reliably signal male quality.	Costs should be present, and they should be relatively highest for low quality males.	Suggests that costs may not be necessary for maintaining signal reliability*, and the handicap principle may only be relevant when exaggeration is extreme.
Allometry evolution models Bonduriansky & Day 2003 (*32*) Kodric-Brown et al. 2006 (*33*)	Exaggerated ornaments/ weapons will have steep allometry slopes when small males pay higher costs (or derive fewer benefits) than large males for the same increase in trait size.	Costs of ornaments/weapons should trade-off with allocation to overall growth or body maintenance, and these costs should be relatively highest for small males.	Suggests that costs may not be necessary for steep allometry slopes (they should arise as a byproduct of the mechanism of exaggeration)*. This should expand the conditions for which steep allometry slopes are expected.

*Costs will still work to ensure signal reliability in both cases (i.e., the models are not wrong); however, for insulin-sensitive exaggerated structures, costs may not be necessary. These structures should be intrinsically reliable due to the proximate mechanism regulating their growth.

Fig. 3. Sexual selection models whose relevance is affected by the proximate mechanism responsible for trait exaggeration.

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Supplementary Materials for

A Mechanism of Extreme Growth and Reliable Signaling in Sexually Selected Ornaments and Weapons

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Supplementary Text

Background on the insulin/IGF pathway:

In addition to their well-known roles regulating metabolism (*38*) and aging (*39*, *40*), insulin and insulin-like growth factors are essential for regulating tissue growth and body size (*41–43*). In mammals, both the insulin receptor (InR) and the insulin-like growth factor 1 receptor (IGFR) are receptor tyrosine kinases. Binding of either insulin or IGF to the receptor leads to de-repression of the kinase activity, activating signaling through a pathway that ultimately stimulates cell proliferation and tissue growth in a dose-dependent fashion (Figure S1). In insects, both insulin and IGF functions are mediated through the insulin receptor (*44*, *45*).

Although numerous insulin-like and IGF-like ligands have now been identified, the receptors and basic functions of this signaling pathway appear conserved across metazoans (*43*, *46*, *47*). This pathway is now recognized as the primary regulator of growth in mammals (*48*), birds (*49*), insects (*44*, *47*), and crustaceans (*50*). Since circulating levels of both insulin and IGFs are sensitive to nutrition, the insulin/IGF pathway is an important mechanism coupling tissue growth with the nutritional history of an individual (*18*, *19*, *51*). This pathway is responsible for blocking proliferation under starvation conditions (*52*), as well as modulating the rate of proliferation during normal periods of tissue growth (*53–56*). In addition, both stress (*57*) and infection by pathogens or parasites (*58*, *59*) depress insulin/IGF signaling, leading to reduced amounts of tissue and body growth.

For these reasons, the insulin/IGF pathway has emerged as the central mechanism integrating physiological condition with growth in multicellular animal taxa (*43*, *45*, *56*). Specifically, insulin and IGF levels within a growing animal reflect the nutritional state and physiological condition of that individual, and circulating levels of these signals modulate tissue growth via the insulin receptor pathway in a graded, or dose-dependent manner. Within an individual, growth will speed up or slow down in response to changes in nutritional or physiological state because of the action of this pathway. Across individuals, growth will differ between high condition and poor condition individuals, resulting in population-level variation in body and trait sizes. Poor condition individuals have lower levels of these signals than higher condition individuals, and as a result, they experience slower rates and lower overall amounts of tissue growth (*18*, *19, 41*).

Signaling through the insulin/IGF pathway is activated within cells of proliferating target tissues as circulating ligands bind to the membrane-bound receptors (*41–47*, *53*, *56*). How much each tissue responds (e.g. adjusts its rate of proliferation) depends on levels of expression of the receptor and downstream pathway genes in cells of that particular tissue. Trait-differences in expression of pathway genes can underlie their differential sensitivities to insulin/IGF signaling (*20*, *21*, *60*, *61*). For example, when individual structures are targeted with overexpression of pathway genes, the result is overgrowth of the affected structures (*62–64*).

As long as the various organs and body parts (e.g., legs, eyes, wings) exhibit similar sensitivities to insulin/IGF signaling, their sizes will scale proportionally from individual to individual (*17–19*, *60*). However, morphological traits need not respond

similarly to insulin/IGF signals (*20*, *21*, *60*, *61*). For example, in many insects genitalic structures are insensitive to environmental conditions such as nutrition, resulting in very low levels of among-individual variation and characteristically "flat" scaling relationships between genitalia size and body size in natural populations (Figure S2). In *Drosophila,* nutrition-insensitivity of genitalic growth results from a tissue-specific unresponsiveness to circulating insulin/IGF signals (*20*, *21*). In contrast, wings in *Drosophila* are sensitive to larval nutrition; wing sizes scale isometrically with among-individual variation in body size, and cell proliferation in growing wings exhibits sensitivity to insulin/IGF signaling typical of the rest of the body (*20*). Consequently, traits differ in their cellular responsiveness to insulin/IGF, and these differences affect the nutrition-sensitivity of their growth and the extent of among-individual variability in their final dimensions.

We predicted that increased sensitivity to the insulin/IGF pathway might be a mechanism leading to the evolution of disproportionate, or exaggerated, growth in the showy ornaments and weapons of sexual selection. In our model, individual males differ in their physiological state as a result of differences in their status, nutritional state, competitive ability, and/or health (parasite or pathogen loads), all of which translate into among-individual differences in circulating levels of insulin/IGF signals (Figure 1B). During their respective periods of growth, the adult structures in these animals would be exposed to insulin/IGF signals, and the sensitivity of cells within each growing structure to these signals would determine both how and by how much each trait grew. Just as wings are more sensitive to insulin/IGF signaling than genitalia in *Drosophila* (*20*, *21*), so we predicted that exaggerated ornaments or weapons of sexual selection would be even more sensitive to insulin/IGF signaling than wings or other non-sexually-selected body parts (Figure 1B). Specifically, we predicted that disproportionate growth of specific structures would be associated with enhanced sensitivity of these cells to insulin/IGF signals.

<u>Why we suspect increased sensitivity to insulin/IGF may be a general mechanism for the evolution of extreme animal structures:</u>

Although no studies have yet considered this pathway in the context of exaggerated growth *per se*, or considered its implications for animal signaling and sexual selection, a growing body of research now implicates critical roles for insulin/IGF signaling in the development of extreme animal structures. For example, altered signaling through the insulin/IGF pathway contributes to male-specific patterns of growth in sexually dimorphic salmon (*65*). The crustacean androgenic hormone, long known to stimulate growth of enlarged male chelae in crustaceans (*66*, *67*), was recently identified as a ligand of the insulin/IGF pathway (*50*). Both the *insulin receptor* (*68*) and *FOXO* (E. Snell-Rood personal communication) are differentially expressed in horn primordia of horned and hornless *Onthophagus nigriventris* dung beetles, consistent with involvement of the insulin/IGF pathway in regulation of weapon dimorphism in this species. Finally, insulin/IGF signaling appears critical for stimulating both season- and nutrition-dependent patterns of antler growth in red deer and fallow deer stags (*69–71*). Male stags vary in their nutritional history and body composition; this translates into among-individual variation in circulating concentrations of IGF, and IGF levels accurately

predict relative amounts of antler growth (*70*). Growing antler tips express receptors for IGF (*71*, *72*), and IGF stimulates proliferation of antler cells *in vitro* in a dose-dependent fashion (*73*, *74*). Consequently, this pathway appears critical for the development of a range of exaggerated animal structures. However, it is not yet clear whether these traits are more sensitive to signaling through this pathway than are other body structures.

Materials and Methods

Choice of Species:

Trypoxylus (formerly *Allomyrina*) *dichotomus septentrionalis* (Coleoptera: Dynastinae) is a large (~10g) nocturnal beetle native to East Asia. Males produce a long, forked horn that extends anteriorly from the dorsal surface of their heads, and a smaller curved horn that extends dorsally from their pronotum (see Fig. 1A in main text). In the largest male individuals the length of the head horn can be two-thirds the length of the rest of his body. In this species, and in beetles generally, horn growth is sensitive to the nutritional conditions experienced by larvae (*75–78*), and horn length scales positively and steeply with among-individual variation in body size. Long horns aid males in battles over reproductive access to females (*79*, *80*). Thus, male horns in this species are an exaggerated weapon that likely evolved in response to sexual selection.

Beetles undergo holometabolous development (complete metamorphosis). Scarab species undergo three larval instars before molting into a pupa, and then subsequently eclosing as an adult. In our laboratory, *T. dichotomus* require 211 ± 7 days to complete these larval stages. Structures characteristic to the adults and which are not present in larvae (e.g., genitalia, wings, compound eyes, and horns) develop late in the larval period, after the majority of overall body growth has been completed and coincident with the physiological transition from feeding to metamorphosis, when animals purge their guts and enter a 9-day 'prepupal' period.

In *T. dichotomus*, the adult structures first become visible as imaginal disc-like evaginations of epidermis (Fig. 1B) that have detached locally from the outer larval cuticle. The cells in these discs proliferate rapidly during the early stages of the prepupal period, though at this stage the structures they produce are not yet visible on the outside of the animal. They remain folded and compacted beneath the larval cuticle until the animal molts, at which point the discs unfurl into visible structures of the pupa.

We focused on three adult structures (genitalia, wings, and horns) and attempted to perturb their rates of growth in male larvae. We selected these traits because they are known to respond differently to heterogeneity in larval nutrition (Figure S2), because they undergo their proliferative growth simultaneously at the beginning of the prepupal period (necessary because we intend to compare the growth responses of the traits to each other), and because their physical location within larvae rendered them amenable to rapid surgical removal for measures of mRNA transcript abundance (necessary for validating the effects of dsRNA injection).

Nutrition manipulation experiment:

All experiments were conducted in the lab at the Fort Missoula Research Station of the University of Montana. Beetles were paired and mated using the methods described in Lai (*81*). Briefly, adult pairs were placed in gallon jars with substrate and sliced apples for food. After 7 days females were separated from the males and placed individually in substrate-filled jars for egg laying. Eggs were collected 7 days later, weighed, and placed

into separate plastic cups with 100% mulched maple leaves. Eggs were checked daily and hatching dates were noted.

Three days after molting into their 2nd instar, larvae were randomly assigned to either a standard or a low nutrition treatment and placed individually into substrate-filled jars kept in incubators set at 25 °C on a 16 hour light/ 8 hour dark cycle. In our laboratory we also rear larvae inside an indoor insectarium with deep mulch and abundant plants. Larvae move freely within this enclosure and presumably feed on plant roots in addition to the fermented sawdust. In this environment they often attain very large sizes (e.g., see data for 'background' animals in Figure S2). However, diet in this chamber is not controlled precisely, so for our experimental manipulation of diet we reared animals in jars filled with substrate. Larvae fed 'standard nutrition' were each provided with a mix of 25% leaf mulch and 75% fermented hardwood sawdust filling a 9 oz glass jar. Larvae fed 'low nutrition' were each given 100% fermented sawdust half filling a 9 oz glass jar. Substrate was kept moist and changed according to diet treatment. Low nutrition animals received new substrate when their frass filled 90% of the jar. Standard nutrition animals received new substrate as soon as frass content reached 40%. After molting into their third instar, standard nutrition animals were transferred into 1-gallon glass jars filled with 25% leaf mulch and 75% fermented hardwood sawdust substrate. Low nutrition animals remained in 9 oz jars and continued to be fed a diet of 100% fermented sawdust.

When larvae showed signs of approaching gut purge they were monitored daily for the presence of a pupal cell (a hard shell of cemented substrate that animals form around themselves prior to pupation). Pupal cells are constructed as animals purge their guts and enter the non-feeding prepupal stage, and they serve as a behavioral marker indicating that animals have begun the endocrine process of metamorphosis. At this developmental stage adult traits, such as genitalia, wings, and horns, have commenced their burst of growth. As soon as animals had begun to form a pupal cell, they were removed from their substrate-filled jars, weighed, and placed into artificial chambers for monitoring. Artificial pupal chambers were fashioned by pressing thumb-sized indentations into floral foam blocks, and kept moist for the duration of the pupal period. Once the pupae eclosed and the adults surfaced they were euthanized by freezing for later measurement.

Photographs of adult traits were taken after the beetles had been killed and disarticulated into body parts using a SPOT Insight Color camera mounted onto a Leica M26 stereomicroscope. All photos were taken with SPOT Advanced software and uploaded into ImageJ 64 (*82*) for measurement. Measurements were taken of the width of the thorax (a proxy for body size), and lengths of the head horn, wings, and genitalia (aedeagus). Measurements were compared for standard- and low-nutrition animals using unpaired t-tests.

Manipulation of larval diet amount significantly affected the final body sizes of adult beetles (Table S1), with larvae from the low nutrition treatment eclosing into adults approximately 24 % smaller than animals fed the standard nutrition diet. None of the animals in this experiment attained the largest sizes possible for these populations (Figure S2). Instead, our 'standard nutrition' animals eclosed as intermediate-sized adults. As predicted, genitalia were almost entirely insensitive to variation in larval nutrition, and

genitalia size was relatively invariant among males (coefficient of variation for aedeagus length = 0.09; Figure S2A, Table S1). Wings exhibited intermediate levels of nutrition-dependent phenotypic plasticity comparable to the effects on overall body size, and wing length varied among males similarly to variation in overall body size (C.V. for wing length = 0.13; C.V. for body width = 0.16; Figure S2B, Table S1). Male horns displayed the greatest sensitivity to larval nutrition and were the most variable among male individuals (C.V. for horn length = 0.43; Figure S2C, Table S1). Comparison of the effect sizes of diet manipulation on these traits illustrates their differential sensitivities to larval nutrition (percent change in aedeagus length = 7; percent change in wing length = 19; percent change in horn length = 58). As with previously published findings from *Drosophila* (*20*, *21*), genitalia are less sensitive to nutrition than wings, and here we show that the sexually selected weapon is substantially more sensitive to nutrition than wings. Compared with other traits, the sexually selected weapon (horns) exhibited both heightened condition-sensitive expression and hyper-variability in this species.

Insulin/IGF manipulation experiment:

Initially, two putative fragments of *insulin receptor* (*InR*) were cloned from *T. dichotomus*, a 438bp fragment using degenerate primers from (*83*), and a 444bp fragment from a 454 EST dataset for *Trypoxylus dichotomus*. A further 208bp connecting the two fragments and 298bp connecting the fragments to the 3' end of the sequence were cloned using the Clontech SMARTer™ RACE cDNA amplification kit (Clontech, USA). NCBI's BLASTx (http://blast.ncbi.nlm.nih.gov) was used to confirm identity and the sequence was deposited in Genbank (Accession number JX141307).

Third-instar larvae were kept in 1-gallon glass jars and given a diet of 25% leaf mulch and 75% fermented hardwood sawdust, and monitored daily for the presence of a pupal cell. At this point the larvae were removed, weighed, and randomly assigned to one of four treatment categories. 'Uninjected' larvae were placed back into their original pupal cells and monitored for the duration of their development. 'ds*GFP*-injected' larvae were injected with a control treatment containing 520bp of double stranded RNA (dsRNA) for *enhanced green fluorescent protein*, a gene that is not present in these animals. 'Sham-injected' animals were injected with the buffer solution used for the knockdown injections, absent of any dsRNA, and 'ds*InR*-injected' animals were injected with dsRNA for 398bp of *insulin receptor*. After injection, larvae were placed back into their pupal cells and monitored for the duration of their development.

dsRNA was synthesized using the Ambion MEGAscript® RNAi kit. DNA template for dsRNA-*eGFP* synthesis was made using primers GFPiF2 and GFPiR5 (*84*). DNA template for dsRNA-*InR* synthesis was produced with PCR using primers Td_InR_F1: AACAGTTAAATCCTCTGATACC and Td_InR_R1: TAATGAGCACGCAACTAAC with T7 binding sites attached to the 5' ends. The PCR product was purified and concentrated using a standard sodium acetate/ethanol precipitation, followed by 70% ethanol wash (*85*). Double-stranded RNA was synthesized using a T7-promoter following the manufacturer's instructions. dsRNA was quantified and diluted to 2µg/µl, aliquoted and stored at -80°C.

Injection treatments were performed on the first day that animals showed signs of making their pupal cells (this was the earliest consistent indicator that we had for the onset of metamorphosis). For all injection treatments, animals were given 15µl of fluid (buffer alone, or buffer plus dsRNA) between the head capsule and the 1st thoracic segment. Animals were then left undisturbed until they molted into pupae, at which point they were weighed and both thorax width and horn length measurements were collected.

After eclosion to adults, animals were euthanized and measured as described above. Some of the injected animals failed to fully eclose and died during the pupal-adult molt. Because they emerge from their pupal cuticle head-first, these animals often had fully formed heads and pronota, and with dissection it was sometimes possible to remove and measure the genitalia, but the wings in these individuals were wrinkled and unmeasurable. Body parts that did not molt properly were excluded from our analyses. Samples sizes of surviving animals with normal/measurable traits were: genitalia, 15 un-injected, 21 sham-injected, 16 ds*GFP*-injected, 28-ds*InR* injected; wings, 15 un-injected, 19 sham-injected, 13 ds*GFP*-injected, 17-ds*InR* injected; horns, 22 un-injected, 28 sham-injected, 27 ds*GFP*-injected, 29-ds*InR* injected.

To test for effective knockdown of *InR* expression, RNA was extracted from a subset of individuals and expression levels for the *InR* gene assessed using quantitative real-time PCR. Subsets of individuals from all four treatments were sacrificed at 24 hours, 48 hours, 72 hours, or 96 hours after they began making their pupal cell. Larvae were weighed and placed into a CO_2 chamber for 2 minutes for anesthetization. Larvae were then placed into a sterilized glass petri dish in sterile PBS. The primordia of the genitalia, wings, and horns were dissected and transferred into RNAlater® (Applied Biosystems) in 3ml vials within five minutes of the initial cut (to minimize RNA degradation within tissues) and stored at -80°C until analysis.

Tissues were thawed and homogenized in TRiZOL® (Invitrogen), stored overnight at -80°C before RNA extraction following manufacturer's instructions. Purified RNA was dissolved in 20µl nuclease-free water. After DNAse treatment with Ambion® DNA-*free* ™, RNA was quantified and qualified using a NanoDrop 2000 (Thermo Scientific). A subset of samples were run through an Agilent Bioanalyzer 2100 (Agilenet Technologies) to test for signs of RNAse activity. cDNA was synthesized from 1 µg of RNA using the Bio-Rad iScript cDNA synthesis kit (Bio-Rad, USA). Where possible, all extractions and cDNA synthesis were carried out simultaneously to reduce potential variation.

Primers were designed for *InR* and for three reference genes: *syntaxin* (syn; Genbank Accession JX141311), *ribosomal protein 27* (RPS27; Genbank Accession JX141309), and *ubiquitin* (ubi; Genbank Accession JX141310), and sequences confirmed using NCBI's BLASTx. Primer sequences for qPCR analyses were:

td_inr_qpcr_r3, CGAAAGGCCGACATTCTTAG

td_inr_qpcr_l3, AGCGGCACTATGGTAAAACG

td_syn_l1 ACTAAAGCGCCGAATTCTTT

td_syn_r1 TGGATCTGAGGGCTTTACAA

td_RPS27_l1 GAAAAACAGCGACGTTCTCA

td_RPS27_r1 ACTGCAGACTGTGCATGTGA

td_ubi_l1 GTTTGCCAGCGAAAATTAAC

td_ubi_r1 GAGGTCGAGCCCTCAGATAC

qPCRs were run on a BIO-RAD CFX96™ Real-Time PCR Detection System using SYBR-green. Standard curves were calculated to test efficiency and suitability of each primer pair. Normalized gene expression values ($\Delta\Delta C_q$) were calculated using the BIO-RAD CFX Manager™ Software. The most stable reference gene combinations were estimated for each comparison using the inbuilt qBase function (*86*). Statistical analyses were carried out in JMP®7.0.2 (SAS Institute Inc., USA).

qPCR of tissues confirmed that *InR* is naturally expressed within each tissue during the treatment period. All three traits showed high levels of *InR* expression during the first 48 hours of the prepupal period. After this point levels declined steadily in the horns and wings, and declined and then increased in genitalia (Figure 2A-C). qPCR also confirmed that ds*InR* injection successfully 'knocked down' transcript abundances for this gene in this experiment. *InR* transcript abundances for dsInR-injected animals were low for the entire period measured (i.e. up to 96 hours post injection) and this resulted in significant reductions below normal levels for the first 48 hours post injection (Figure 2). Thus, injection of dsRNA for *InR* effectively reduced expression levels of *insulin receptor* during the critical period of trait growth, potentially interfering with insulin/IGF signaling in the developing adult tissues.

We also measured transcript abundances for the *T. dichotomus* canonical histone gene *H2A* (Genbank Accession JX141308), in prepupal genitalia, wings, and horns. Canonical histones accumulate in cells before progression into and through the S-phase of the cell cycle, and can therefore be used as an indicator of active cell proliferation (*87*). Sequences were confirmed using NCBI's BLASTx. Primer sequences for qPCR analyses were: td_h2a_l1, ATTAGAATTGGCGGGAAATG and td_h2a_r1, CGTCGTTCCTGATGGCTAAT. qPCR measures of *H2A* transcript abundance confirmed that all three traits were growing at the time of our ds*InR* treatment (Figure S3).

<u>Effects of ds*InR* injection on trait growth:</u>

Because insulin/IGF signaling was perturbed after the onset of metamorphosis, and therefore after growth in overall body size had ceased, we expected any effects on trait growth to be manifest as differences in the sizes of structures *relative to overall body size*. That is, we predicted that treatment effects would be visible as departures from the baseline scaling relationship between trait size and body size. Measures of trait size (aedeagus length, wing length, or horn length) were regressed against a measure of body size (pupal weight) using standardized major axis (Type-II) regression, and treatments

compared using likelihood ratio tests (for differences in regression slope) and Wald tests (for differences in intercept) using the SMATR package in R (*88*). Separate analyses were conducted for each morphological trait. The three control treatments (uninjected, sham-injected, and ds*GFP*-injected) were not different from each other for any trait (genitalia: likelihood ratio test = 1.58, 2 degrees of freedom, p = 0.45; wings: LRT = 1.1, 2df, p = 0.58; horns: LRT = 0.13, 2df, p = 0.94), and were combined for subsequent analyses and figures.

dsRNA knockdown of the *T. dichotomu*s *InR* gene during the first 48 hours of the prepupal period did not affect final genitalia size (Figure 2D; Table S2) despite the fact that *InR* transcript abundances were significantly lower in treated than in control animals for this period (Figure 2A), and expression of the canonical histone gene *H2A* confirmed that genitalia were actively proliferating at this time (Figure S3). dsRNA knockdown of *InR* resulted in adult beetles with significantly shorter wings than control animals (Figure 2E; Table S2), although the magnitude of this effect was minimal. On average, wing sizes were reduced as a result of ds*InR* knockdown by 2% relative to control animals. Horn lengths of males were dramatically reduced by ds*InR* knockdown, with horn lengths of treated males an average of 16% shorter than the horns of control males (Figure 2F,G; Table S2). As with the nutrition-manipulation experiment, the effect sizes of ds*InR* knockdown differed between the three adult structures, with horns exhibiting by far the greatest response to this treatment. Because knockdown of the *InR* interrupts signaling though the insulin/IGF pathway, our results suggest that cells in developing horns are more sensitive to insulin/IGF signals than are cells in the other traits.

It is important to note that we are not implicating the *InR* gene *per se* in the evolution of trait exaggeration. Rather, we use perturbations of this gene to test for a functional role for the insulin/IGF *pathway*. In fact, we suggest that differential sensitivity of horns relative to other traits likely arises downstream of the insulin receptor, since overall levels of *InR* expression were similar for the three traits during the period of trait growth (i.e. during the first half of the prepupal period). This would be consistent with recent findings of Emlen et al. (*68*) and Snell-Rood (personal communication), which implicate insulin/IGF signaling in the mechanism of male horn dimorphism in *Onthophagus* dung beetles. These studies suggest that interruption of insulin/IGF signaling downstream from the insulin receptor blocks growth of the horn primordia for small males and females, which do not grow horns (e.g., horn tissues from horned and hornless males differ primarily in levels of expression of *FOXO*, rather than *InR*; E. Snell-Rood, personal communication). Similarly, the nutrition-insensitivity of genital imaginal discs in *Drosophila* also resulted from trait-specific differences in expression of *FOXO*, and targeted perturbations to *FOXO* expression in wing imaginal discs both enhanced and repressed nutrition sensitivity of this tissue, depending on the level of expression (*21*). Further studies will be needed to better characterize which elements in this pathway contribute to the enhanced sensitivity of horn cells to insulin/IGF signaling in these beetles.

It is also noteworthy that our treatment appears to have affected tissues at the very end of their period of nutrition-sensitive growth. Background transcript abundances for the *InR* gene dropped by 72 hours after the initiation of the pupal cells in all tissues (Figure 2A-C), meaning that dsRNA knockdown of the *InR* gene only reduced transcript

levels below background for 48 hours post injection. Furthermore, expression levels of the canonical histone gene *H2A* dropped by 72 hours after the onset of the prepupal period, indicating that cell proliferation had largely ceased by that stage (Figure S3). Finally, injection of animals after this stage (e.g. 48 hours after the onset of pupal cell construction) did not alter horn (or any other tissue) growth. Consequently, we conclude that dsRNA injection interrupted insulin/IGF signaling for the very end of the period of trait growth, rather than for the duration of trait growth.

We predict that had we been able to knock down transcription for the entire period of trait growth (e.g. by injecting animals 3 days earlier than we did), we would have truncated horn growth completely, dropping the slope of the horn length / body size scaling relationship for ds*InR* injected animals. However, we are unable at this point to reliably stage animals at this earlier time, as no behavioral or physical markers are apparent prior to the commencement of formation of the pupal cell (the preceding two months of the 3rd instar larval feeding period are characterized by relative stasis, often without appreciable increases or decreases in weight). Consequently, we were able to terminate the period of growth early (this experiment), but we were not able to prevent it altogether.

Nevertheless, the fact that traits differed predictably in how much they responded to this treatment is evidence for underlying differences in their relative sensitivity to this physiological pathway. What is less clear is why our treatment affected males of all body sizes similarly (i.e. why it decreased the intercept, rather than the slope, of the trait-size body size scaling relationships). Even considering the fact that we were truncating the period of trait growth early, we still predicted interrupted insulin/IGF signaling would decrease the slope of this relationship.

It is worth noting that our rationale for this prediction was based on the traditional view of cellular sensitivity to a hormone as mediated by levels of expression of the hormone receptor (*89*). In this situation, horn cells would be more sensitive to insulin/IGF signals than wings or genitalia *because they expressed more receptors*. If this were the case, then reducing transcription of *InR* should have caused the horn cells to behave more like the cells of wings or genitalia – it would have made them less sensitive to insulin/IGF signals, and, as a result, it would have decreased the slope of the horn size/body size scaling relationship. However, there is no reason to believe that trait differences in sensitivity result from differential expression of the insulin receptor. Indeed, there is compelling evidence that this is *not* what is happening, given our observation that *InR* transcription is not very different between traits (Fig. S3A), as well as the findings of Tang et al. (*21*) for *Drosophila* imaginal discs, and the preliminary results of E. Snell-Rood (personal communication) for the dung beetle *Ontophagus nigriventris*, all of which implicate the downstream gene *FOXO* in mediating trait-differences in sensitivity to insulin/IGF.

In fact, altered expression of any number of genes in the *InR* pathway can modify the extent and nature of cellular responses to a hormone. If differential trait sensitivity in these beetles is caused by downstream genes in the pathway, then our knock-down treatment could simply have restricted signaling through the pathway much as a reduction in hormone levels would have (e.g., by attenuating the number of hormone-receptor complexes). Our upstream perturbations to signaling (i.e., our knock-down of *InR*) could

have restricted overall levels of pathway activity without dramatically altering the already-present cellular differences in sensitivity to signaling. In this case, horn cells of all males would have been affected similarly, shifting the intercept, rather than the slope, of the horn length/body size scaling relationship. We are currently exploring relative transcription of numerous pathway genes in a large sample of beetles (50 males, 30 females) spanning the full range of body sizes, to better determine which genes might be responsible for the enhanced sensitivity of horn cells.

Although we do not yet understand why the slope of the scaling relationship between horn length and body size remained unchanged between control and ds*InR* animals, such a pattern is by no means unprecedented. For example, when Tang et al. (*21*) perturbed *FOXO* expression in imaginal discs they changed the slope of the wing size/body size scaling relationship at one temperature, but shifted intercept (and not the slope) at another. Perturbations to another gene in this pathway, *Dp110*, by Mirth et al. (*90*) also shifted the intercept without affecting the slope. Our study and these highlight the many ways that cellular sensitivity to a signaling pathway may be modified, and illuminate the need for a broader view of this phenomenon.

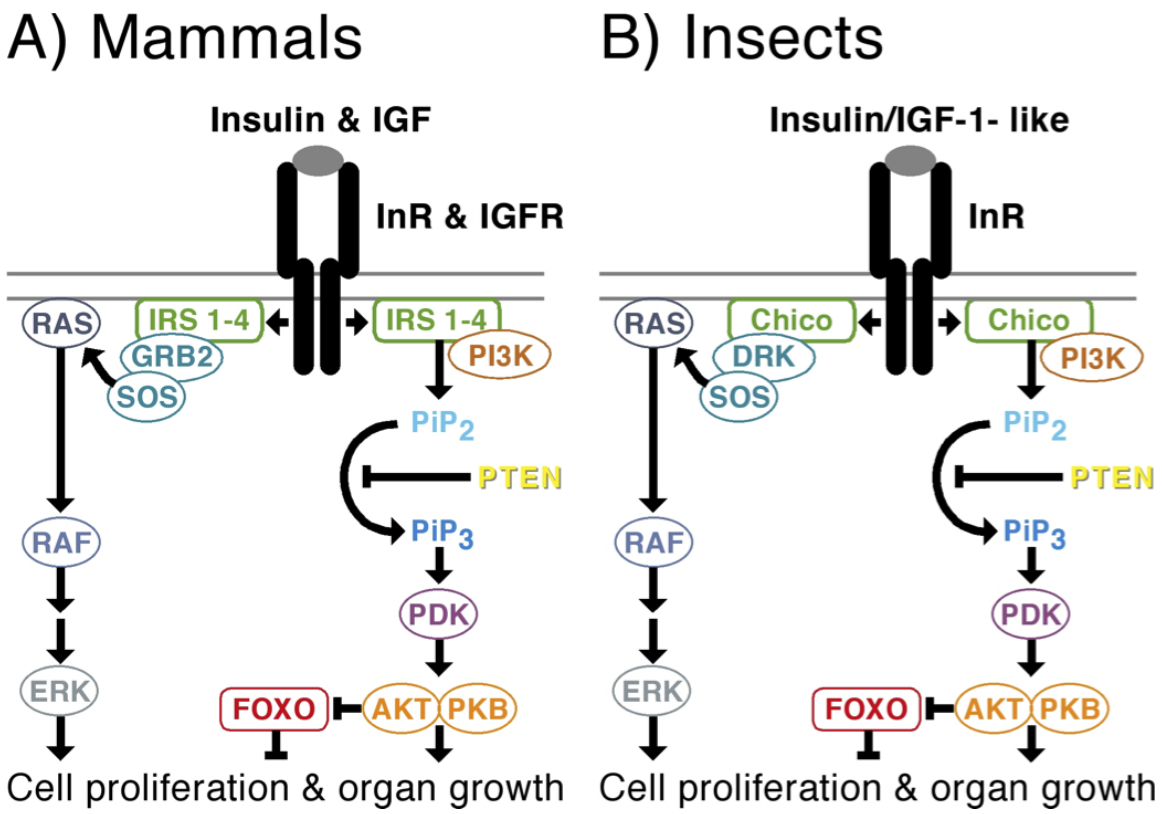

Fig. S1: Insulin/IGF signaling pathway in mammals (A) and insects (B). In all studied metazoans, this pathway incorporates long-range (whole-animal) circulating signals (insulin-like peptides, insulin-like growth factors) which, when bound to receptors within the different proliferating tissues, control the rate of proliferation that occurs in that tissue. Abbreviations: *InR*, insulin receptor; *IGFR*, insulin-like growth factor receptor; *IRS*, insulin receptor substrate, *PTEN*, protein tyrosine phosphatase; *PI3K*, phosphatidylinositol-3-OH-kinase; PIP_3, phosphatidylinositol (3,4,5) triphosphate; *PKB* (=*Akt*), protein kinase B; *FOXO*, forkhead-related transcription factor; *4E-BP*, 4E-binding protein; *S6*, S6 kinase; *GRB2*, growth factor receptor-bound protein 2; *RAS*, rat sarcoma protein; *RAF*, proto-oncogene serine/threonine-protein kinase; *ERK*, extracellular signal regulated kinase, *SOS*, son of sevenless;. Redrawn from (*40*, *43*, *46*, *53*).

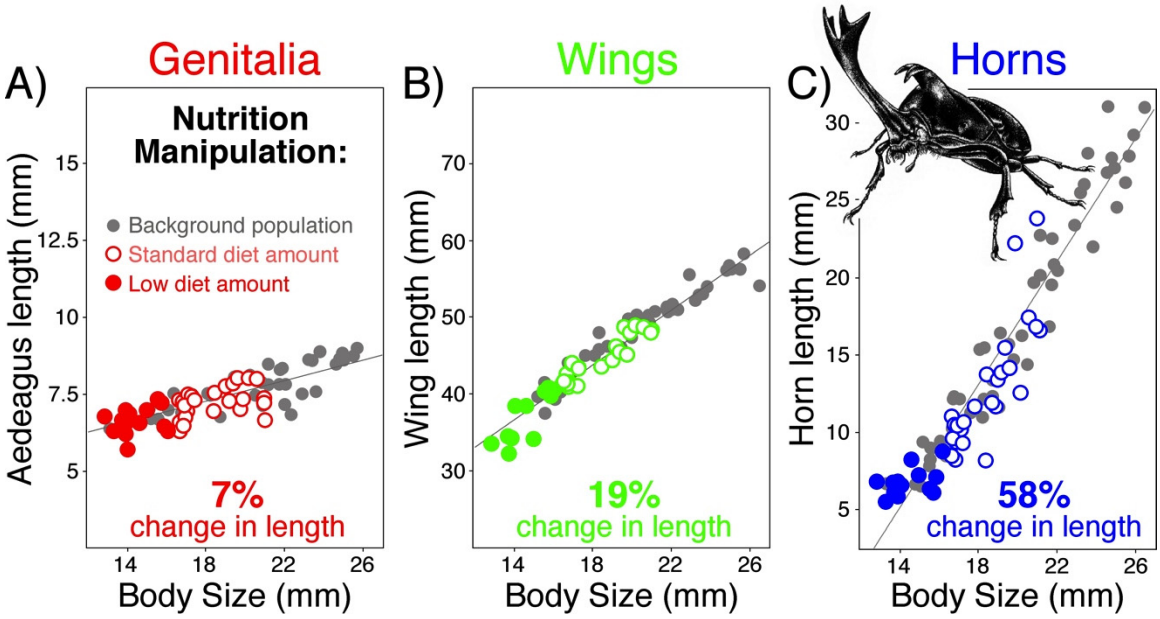

Fig. S2: Differential sensitivities of adult body parts to larval nutrition in the Japanese rhinoceros beetle *Trypoxylus dichotomous*. A) Genitalia (aedeagus) length is relatively invariant in males of this species (gray circles; coefficient of variation = 0.09). Larvae reared on standard (open circles) versus low (closed circles) nutrition diets differed in adult aedeagus length by an average of 7% ($t_{1,33} = 3.026$, $p = 0.0048$). B) Wing length is more variable than aedeagus length (C.V. = 0.13), and animals reared on standard versus low nutrition diets differed in wing length by 19% ($t_{1,32} = 7.263$, $p < 0.0001$), comparable to the nutrition-sensitivity of overall body size (mean difference in body size = 24%; $t_{1,40} = 9.251$, $p < 0.0001$). C) As predicted by sexual selection theory, the enlarged male weapon (cephalic horn) is more variable than wings or genitalia ('hyper-variability'; C.V. = 0.43), and horn length is more sensitive to variation in larval nutrition than the other traits (mean difference in body size between standard- and low-nutrition animals = 58%; $t_{1,38} = 5.736$, $p < 0.0001$). Beetle illustration: David Tuss.

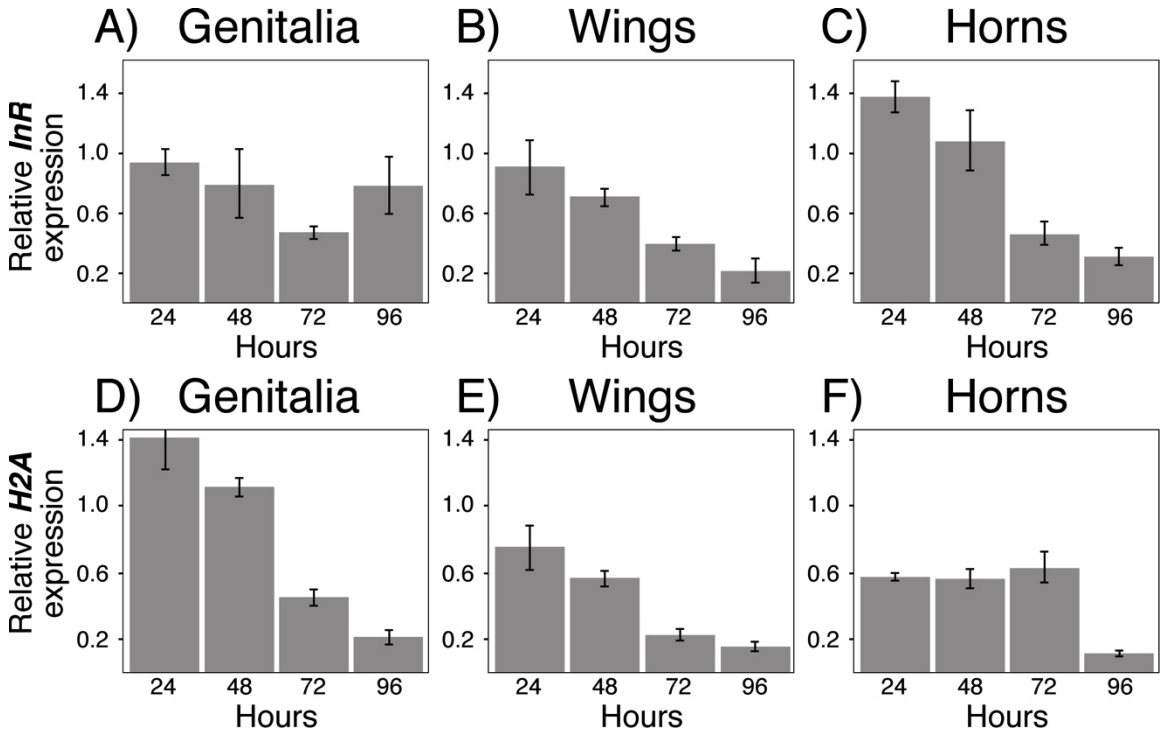

Fig. S3: A-C) Relative transcript abundances for the *T. dichotomus insulin receptor* (*InR*) gene in genitalia (A), wings (B), and horns (C), measured 24, 48, 72, and 96 hours after the onset of the prepupal period (the first stages of pupal cell construction) in uninjected control animals. D-F) Relative transcript abundances for the *T. dichotomus* canonical histone gene *H2A* in the same tissues, as an indicator of cell proliferation. Bars indicate standard errors. See SOM for methods of tissue extraction and qPCR.

Table S1: Results of nutrition manipulation experiment on overall body growth, and growth of genitalia, wings, and horns of *Trypoxylus dichotomus* (all traits measured in mm).

Trait	**Nutrition**	**Mean**	***Standard Deviation***	t_{df}	p	**Effect Size (% change in size)**
Genitalia (aedeagus length)	High	7.277	0.472	$t_{33} = 3.026$	0.0048	7%
	Low	6.753	0.483			
Wings (wing length)	High	44.779	2.811	$t_{32} = 7.263$	< 0.0001	19%
	Low	36.973	3.181			
Horns (horn length)	High	12.911	*4.053*	$t_{38} = 5.736$	< 0.0001	58%
	Low	6.805	*0.820*			
Body size (prothorax width)	High	18.515	0.303	$t_{40} = 9.251$	< 0.0001	24%
	Low	14.459	0.264			

Table S2: Results of ds*InR* injection on growth of genitalia, wings, and horns of *T. dichotomus*. For each trait, treatment and control populations were fitted with SMA regressions and compared for slope (A) and intercept (B) using log likelihood ratio and Wald tests, respectively (see text for methods; n.s. = not significant).

A – Effect on slope

Trait	**Treatment**	**Slope**	**95% Confidence intervals**	**Likelihood ratio**	***p***	**Effect size (% change in slope)**
Genitalia	Control	0.167	0.138 - 0.203	2.253	0.112	28% n.s.
	dsInR	0.121	0.085 - 0.172			
Wings	Control	0.999	0.873 – 1.143	2.866	0.090	20% n.s.
	dsInR	1.199	1.011 – 1.422			
Horns	Control	1.175	1.026 – 1.345	0.025	0.875	2% n.s.
	dsInR	1.198	0.971 – 1.479			

B – Effect on intercept

Trait	**Treatment**	**Intercept**	**95% Confidence intervals**	**Wald statistic**	***p***	**Effect size (% change in size)**
Genitalia	Control	8.493	8.398 – 8.587	0.13	0.724	0.02% n.s.
	dsInR	8.509	8.373 – 8.646			
Wings	Control	51.952	51.571 – 52.333	**8.98**	**0.003**	**2%**
	dsInR	50.949	50.314 – 51.584			
Horns	Control	22.401	21.952 – 22.851	**68.37**	**< 0.0001**	**16%**
	dsInR	18.815	18.048 – 19.582			

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