Article

CLONING AND CHARACTERIZATION OF AN mRNA ENCODING AN INSULIN RECEPTOR FROM THE HORNED SCARAB BEETLE *Onthophagus nigriventris* (COLEOPTERA: SCARABAEIDAE)

Laura Corley Lavine, Laura L. Hahn, and Ian A. Warren
Department of Entomology, Washington State University, Pullman, Washington

Stephen F. Garczynski
Yakima Agricultural Research Laboratory, USDA-ARS, Wapato, Washington

Ian Dworkin
Department of Zoology, Michigan State University, East Lansing, Michigan

Douglas J. Emlen
Division of Biological Sciences, University of Montana, Missoula, Montana

The insulin signaling pathway is the primary signaling pathway coupling growth with nutritional condition in all animals. Sensitivity to circulating levels of insulin has been shown to regulate the growth of specific traits in a dose-dependent manner in response to environmental conditions in a diversity of insect species. Alternative phenotypes in insects manifest in a

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Correspondence to: Laura Corley Lavine, Department of Entomology, Washington State University, Pullman, WA 99164–6382. E-mail: lavine@wsu.edu

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variety of morphologies such as the sexually dimorphic and male dimorphic horned beetles. Large males of the sexually dimorphic dung beetle Onthophagus nigriventris develop a thoracic horn up to twice the length of the body whereas small males and females never develop this horn. The regulation of this dimorphism is known to be nutrition dependent for males. We focused on the insulin signaling pathway as a potential regulator of this dimorphism. We sequenced a full-length gene transcript encoding the O. nigriventris insulin receptor (OnInR), which is the receptor for circulating insulin and insulin-like peptides in animals. We show that the predicted OnInR protein is similar in overall amino acid identity to other insulin receptors (InRs) and is most closely related phylogenetically to insect InRs. Expression of the OnInR transcript was found during development of imaginal tissues in both males and females. However, expression of OnInR in the region where a horn would grow of small males and female was significantly higher than in the horn tissues of large males at the end of growth. This variation in OnInR expression between sexes and morphs indicates a role for the InR in polymorphic horn development. © *2012 Wiley Periodicals, Inc.*

Keywords: *Onthophagus nigriventris*; insulin receptor; insulin signaling pathway; polyphenism

INTRODUCTION

Insect polyphenisms, defined as irreversible environment-specific alternative phenotypes (sensu West-Eberhard, 2003), are regulated by a complex interplay of environmental, nutritional, and hormonal factors. Variation in nutrition ultimately results in differences as extreme as the behavioral and morphological caste polymorphisms in social insects, and the remarkable sexual dimorphisms of horned beetles (Emlen, 1994; Iguchi, 1998; Moczek and Emlen, 1999; Karino et al., 2004; Wheeler et al., 2006; Emlen et al., 2007; de Azevedo and Hartfelder, 2008; Gotoh et al., 2011). The underlying genetic mechanisms responsible for these polyphenisms are still largely unknown, although studies of nutrition-dependent insect polyphenisms strongly support a key role of the insulin signaling pathway (Emlen and Allen, 2004; Emlen et al., 2006; Wheeler et al., 2006; de Azevedo and Hartfelder, 2008). The precise coordination of cell growth and proliferation in animals is regulated by the evolutionarily conserved insulin signal transduction pathway (Chan and Steiner, 2000; Claeys et al., 2002; De Meyts, 2004; Edgar, 2006; Wu and Brown, 2006; Rendell, 2008). The insulin signaling cascade in insects is initiated by the binding of insulin and insulin-like peptides to the insulin receptor (InR). The InR is a member of a large class of receptors known as receptor tyrosine kinases that have ligand-activated tyrosine kinases within their cytoplasmic domains (Hanks et al., 1988; Brogiolo et al., 2001; De Meyts, 2004). Insulin binding to the InR initiates a cascade of phosphorylation events resulting in the activation of glucose metabolism and protein synthesis and, importantly, cellular growth and proliferation (De Meyts, 2004; Edgar, 2006). Genetic manipulation of insulin signaling pathway components within specific imaginal discs in *Drosophila* have been shown to affect the final sizes of those traits by affecting the rate of cell proliferation (Chou et al., 1987; Potter et al., 2001; Goberdhan and Wilson, 2002, 2003; Oldham et al.,

2002; Colombani et al., 2003; Kramer et al., 2003; Puig et al., 2003; Puig and Tjian, 2005; Shingleton et al., 2005, 2008; Tang et al., 2011).

The large sword-like thoracic horn of the male dung beetle *Onthophagus nigriventris* is a striking example of an insect polyphenism. Male beetles of this species exhibit nutrition-dependent dimorphism for the thoracic horn, the length of which can extend up to twice the length of the body (Emlen et al., 2005). Males that reach a threshold body size during larval development produce a horn whereas males below the threshold body size never develop the thoracic horn. This trait is also sexually dimorphic via a nutrition-independent mechanism, as female *O. nigriventris* do not make the thoracic horn regardless of their nutritional history or body size. Physiological evidence from *Onthophagus acuminatus* and *O. taurus* (Emlen, 1997; Emlen and Nijhout, 1999, 2001) suggests that the insulin signaling pathway is either downstream or acting in concert with ecdysone and juvenile hormone to facilitate this variation in horn development and proliferation. The insulin signaling pathway has been correlated with nutrition-dependent insect polyphenisms in other species, especially in social insects (Wheeler et al., 2006; Ament et al., 2008; Azevedo and Hartfelder, 2008; Hunt et al., 2010; Wang et al., 2010; Mutti et al., 2011; Wolschin et al., 2011), has been implicated in the development of horn polymorphisms in dung beetles (Kijimoto et al., 2009; Snell-Rood et al., 2011; Snell-Rood and Moczek, 2012), and has been shown to regulate horn growth in the Asian rhinoceros beetle (Emlen et al., 2012).

The insulin signaling pathway has been suggested as a mechanism for modulating the amount of horn growth in scarab beetles in response to nutrition (Emlen et al., 2006, 2007). Emlen et al. (2006) predicted that if the insulin pathway was involved in differential horn growth, then horn discs in large males would be sensitive to circulating insulin signals while horn discs in small males and females would not. Indeed, recent studies of growth and allometry in the fruit fly *Drosophila melanogaster,* work by Tang et al. (2011), show organs differ in their size response to developmental nutrition because of differences in organ-specific specificity to circulating insulin. When faced with limited nutrition during development, male fruit fly genitalia do not respond to insulin signaling that direct the rest of the body to grow slowly (Tang et al., 2011). Based on the differential responsiveness of organs to insulin signaling, we predicted that cell proliferation in the horn discs of small males and females would be uncoupled from circulating insulin signals (Fig. 1). Indeed, we have shown differential responsiveness to insulin signaling between imaginal discs in the Asian rhinoceros beetle, *Trypoxylus dichotomus* (Emlen et al., 2012). Growing rhinoceros beetle horns are eight times more sensitive to insulin/IGF signaling than wings using RNA interference to perturb transcription of the InR (Emlen et al., 2012). Sensitivity to circulating insulin or insulin-like peptides is likely to be regulated first through the InR and then downstream by other members of the pathway (Tang et al., 2011; Emlen et al., 2012). Evidence from the development of other polymorphic animal structures such as the male-specific patterns of growth of the enlarged chelae of crustaceans and the growth of antlers in red deer (Charniaux-Cotton et al., 1966; Nagamine et al., 1980; Suttie et al., 1985, 1991; Elliot et al., 1996; Gu et al., 2007) show a critical role for insulin signaling in the development of these traits. As a first step in investigating the role of the InR in the regulation of nutrition-dependent horn polyphenisms in dung beetles, we cloned a full-length gene transcript encoding the *O. nigriventris* InR (OnInR) and examined its expression during the critical proliferative phase of horn development in males and females.

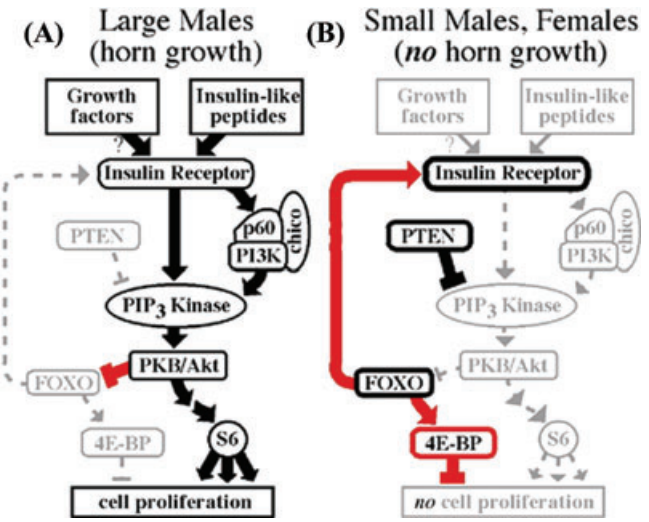

Figure 1. Diagram of the insulin signaling pathway and one model for its role in horn polyphenisms in the dung beetle *O. nigriventris*. (A) As signaling through the insulin pathway is increased overall in large males, the expression of InR decreases due to kinase-dependent inactivation of its transcriptional activator FOXO by PKB/Akt (red inhibitory bar). (B) In small males and females, insulin pathway activity is predicted to be truncated at some point downstream from the insulin receptor that would result in tissue "reprogramming" and no horn growth.

MATERIALS AND METHODS

Insects

Laboratory beetle colonies were established with *O. nigriventris* adults collected from cow and horse manure at approximately 1,000 m elevation at the Kahua Ranch, Kamuela, Hawaii 96743 in late May 2007, 2008, and 2009. Adults were kept and bred in cylindrical plastic containers 21-cm tall with a diameter of 7.5 cm containing approximately 600 ml of a sterilized soil/sand mixture and 200 ml of cow dung in an environmental chamber at 26°C with 80% humidity under a 16:8 light:dark cycle. One to two females were housed in each rearing cylinder with one male for optimal brood ball production. Brood balls were collected every 14 days from mating pairs. Brood balls were kept in 475 ml ventilated plastic deli containers on 3 cm of vermiculite using the environmental conditions described above. Beetle larvae were sexed during the third instar according to the method of Moczek and Nijhout (2002) and weights were recorded during the third instar and prepupal periods.

The transition from larva to pupa occurs in three distinct stages over a period of approximately 3 days in *O. nigriventris*. The third instar larva is the stage in which the transition to the pupal form occurs. In preparation for pupation, the third-instar larva stops feeding and initiates a purge of its gut contents. At the onset of this gut purge, the larva has entered the early prepupal period. The complete purging of the gut takes about 24 h. Other changes in the prepupa include the growth and proliferation of imaginal tissues for compound eyes, antenna, mouthparts, legs, wings, and genitalia. When the gut has completely purged, the larva has undergone many changes and has entered the late prepupal period that lasts from 24 to 48 h. Imaginal tissues thus initiate proliferation

during the early prepupal period and have completed growth and proliferation by pupation. Thus, proliferation at the early and late prepupal periods mark these as critical developmental time points when growth is regulated in these beetles and we targeted these in our experiments.

Larvae from the early and late prepupa periods were weighed and anaesthetized on ice. Under these conditions, day 2 late prepupa weight is an indicator of small (hornless) and large (horned) males and females (hornless). Larvae that will give rise to a large adult (horned male or hornless female) were at least 0.25 g on day 2 of the late prepupal period. Any individual below 0.24 g was considered to be below the threshold for a large individual and was categorized as small (hornless males and hornless females). Imaginal tissues were dissected from both large and small males and females of all sizes for RNA isolation. Imaginal tissues are easy to recognize at the early prepupal and late prepupal periods as obvious outgrowths of the larval epidermis—the horn imaginal tissues are under the thoracic dorsal region of the larval exoskeleton, wing imaginal tissues are located dorsally and laterally on the second and third thoracic segments, and the genital imaginal discs of males are found ventrally in the posterior abdominal segments.

RNA Extraction and cDNA Synthesis for Cloning

Total RNA was extracted from whole tissues dissected from late prepupal stage *O. nigriventris* stored in RNAlater® (Ambion, Austin, TX) using the RNeasy® Plus Mini kit (Qiagen, Valencia, CA) according to manufacturer's protocol for purification of total RNA from animal tissues. In order to convert RNA to cDNA, the SMART™ cDNA amplification kit (Clontech, Mountain View, CA) and the SuperScript III First Strand Synthesis SuperMix kit and protocol (Invitrogen, Carlsbad, CA) were used. Briefly, first-strand cDNA was generated from 4 μl total RNA primed with the CDSIII/3′PCR primer and SMART IV oligonucleotide (which is incorporated onto the 5′ end of the cDNA) in a 20 μl reaction with components supplied with the SuperScript® III First-Strand Synthesis SuperMix for 50 min at 50°C. Double-stranded (ds) cDNA was prepared by long distance (LD) PCR using the Advantage™ 2 Taq Polymerase (Clontech) and protocol supplied with the SMART™ PCR cDNA Synthesis Kit. Amplification of 2 μl of first-strand cDNA was done by LD PCR in a 100 μl reaction under the following conditions; initial denaturation for 3 min at 94°C, amplification for 20 sec at 94°C, 6 min at 68°C for 26 cycles followed by a 5 min 72°C incubation. The resulting ds cDNA was then purified using the High Pure PCR Product Purification Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's protocol and eluted with 100 μl of kit elution buffer.

The ds cDNA was normalized using duplex-specific nuclease (DSN; Axxora, LLC, San Diego, CA) according to the method of Zhulidov et al. (2004) with some modification. Denaturation and annealing was done in a total volume of 20 μl containing 500 ng of ds cDNA in hybridization buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.2 mM EDTA). This mixture was heated for 5 min at 98°C to denature the cDNA and annealing was done for 4 h at 70°C. After completion of the annealing step, a 10 μl mixture containing 3 μl 10× DSN buffer (yielding a final concentration of 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 1 mM dithiothreitol), 5.5 μl nanopure H_2O, and 1.5 μl DSN (0.5 Kunitz/μl) preheated to 70°C was added to the annealed cDNA and further incubated at 70°C for 20 min. To stop the reaction, 15 μl of 5 mM EDTA was added. Amplification of the normalized cDNA was done by (LD) PCR and purified as described above.

Cloning and Sequencing

In order to obtain the initial fragment of the OnInR, a PCR reaction was performed in a total volume of 50 μl using 2.0 μl of template cDNA from *RNA Extraction and cDNA synthesis Cloning*, 31.5 μl sterile water, 5 μl 10× PCR buffer, 5 μl 25 mM $MgCl_2$, 1 μl 10 mM dNTPs, 0.25 μl DMSO, 0.25 μl *Taq* DNA Polymerase (Invitrogen), and 2.5 μl of 10 μM the following degenerate primers: InR forward 5′-CTTYGGNATGGTNTAYGARGG-3′ and InR reverse 5′-CGTCATNCCRAARTCNCCRATYTT-3′ (Roovers et al., 1995). The cycling conditions were as follows: a single denaturation cycle of 95°C for 10 min, followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, with a final extension step of 72°C for 10 min. An amplicon of 450 bp was detected on agarose gels and this PCR product was excised and cloned using the TOPO TA Cloning® kit (Invitrogen) according to manufacturer's protocol. Plasmid DNA was extracted from individual colonies using the QIAprep mini prep kit (Qiagen, Valencia, CA) and the resultant cDNA clones were sequenced at MC Laboratories (MCLab, San Francisco, CA). The cDNA sequence data were used to design sequence-specific primers for subsequent RACE reactions using Primer 3 (http://primer3.sourceforge.net/; Rozen and Skaletsky, 2000). For 5′ and 3′ RACE, the 3′-RACE CDS Primer A and the Universal Primer Mix (UPM) from the SMART™ RACE cDNA Amplification kit were used along with the following sequence-specific primers in separate RACE reactions: OnInR 5′UTR2F 5′-GGAAGCTAGATCAACCAGAAACAATC-3′, OnInR 5'UTR2R 5′-GTTTCTACAGACGGAGCATTGTTTG-3′, OnInR F18F 5′-ATTTAT CGGCGAAGAAGTTTGTCCATCG-3′, and OnInR R22B 5′-AAGGAGGGCGGTTGCCC AATAATACCAT-3′ according to the manufacturer's instructions (Clontech). All PCR reactions were carried out in a Mastercycler gradient thermocycler (Eppendorf AG, Hamburg, Germany). Amplification products of the appropriate sizes were excised from agarose gels and the DNA was extracted in a GenElute™ Minus EtBr Spin column using the manufacturer's protocol (Sigma, St. Louis, MO). Cloning and DNA sequence analysis were performed as above.

Sequence Analysis and Phylogenetic Tree Construction

Putative OnInR cDNA sequences generated above were blast searched using tBlastx (NCBI) for comparison to known InR homologues. Seqman (Lasergene software, DNASTAR®, Inc.) was used to generate the full length OnInR gene transcript and produce a contiguous sequence from all sequencing projects. Each nucleotide was sequenced at least three times and up to 15 times. The complete open-reading frame (ORF) and translational start site of the OnInR was predicted using the ORF Finder on the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.htm). The signal peptide was predicted using Signal 3.0 (http://www.cbs.dtu.dk/services/SignalP/; Nielsen et al., 1997; Bendtsen et al., 2004). Other conserved domains were predicted using the Conserved Domain Database (CDD) on the NCBI website and comparison with *D. melanogaster* InR (DInR; ACL83551) (Fernandez et al., 1995; Ruan et al., 1995), molluscan insulin-related peptide receptor (MIPR; CAA59353) from *Lymnaea stagnalis* (Roovers et al., 1995) and mosquito InR from *Aedes aegypti* (AaeIR; AAB17094; Graf et al., 1997). A protein alignment was created by the Clustal W method using MegAlign (Lasergene software, DNASTAR®, Inc.) in order to determine similarity between the OnInR and other InRs including the human IR *Homo sapiens* (AAA59452), the mosquito *A. aegypti*, and the silkmoth *Bombyx mori* (NP_001037011).

A neighbor joining analysis of OnInR with InRs from different arthropods and mammals was performed using ClustalX 2.0.12 (Larkin et al., 2007). The entire prepropeptide of each InR was entered. A diversity of animal species were chosen for the analysis including: pacific oyster (*Crassostrea gigas* CAD59674), great pond snail (*L. stagnalis* CAA59353), freshwater snail (*Biomphalaria glabrata* AAF31166), human (*H. sapiens* AAA59452), mouse (*Mus musculus* AAA39318), fruitfly (*D. melanogaster* ACL83551), mosquitoes (*A. aegypti*, AAB17094 and *Anopheles gambiae,* XP_320130), hornworm (*Manduca sexta* ACI02334), silkmoth (*B. mori* NP_001037011), flour beetle (*Tribolium castaneum* EFA11583), nematode (*Caenorhabditis elegans* ACC47715), schistosome (*Schistosoma mansoni* AAV65745), and tapeworm (*Echinococcus multilocularis* CAD30260). A bootstrap analysis with 1,000 replicates was performed using MEGA 4 (Tamura et al., 2007).

Qualitative Reverse Transcription PCR and Quantitative Real-Time PCR

Qualitative reverse transcription PCR (RT-PCR) and quantitative real-time PCR (qPCR) were conducted to assess expression patterns of the OnInR transcript during the proliferative phase of prepupal development. Larvae were staged, sexed, and weighed to determine body size categories as described above. Imaginal tissues of interest included the horn area of large males, the anterior thoracic head epidermis in the region where a horn would be in small males and females, wings, legs, and male genital imaginal tissues.

Total RNA was extracted from imaginal tissues using the TRIzol® reagent and protocol (Invitrogen). In order to ensure no contamination from genomic DNA, the RNA was treated with DNase1 according to the manufacturer's protocol (Ambion). First-strand cDNA was made using 1 μg of total RNA and the SMART™ cDNA amplification kit and MMLV reverse transcriptase (Clontech). For all RT-PCR and qPCR experiments, single primer and no template controls were included to check for contamination. The cycling conditions for RT-PCR were as follows: 95°C for 1 min and 25 cycles of 95°C for 15 sec, 65°C for 30 sec, and 68°C for 6 min, hold at 4°C. SMART cDNAs from imaginal tissues were investigated for the presence of the OnInR transcript using sequence-specific forward and reverse primers: forward 5′-CCGAAAAGTCCAAATGGAAA-3′ and reverse 5′-TCGCCGTTAGCCATTAATTC-3′ and forward 5′-AACACGCAACAAACGCAGAAA-3′ and reverse 5′-TAATGTCGGTTGTCCTTGAGATAC-3′. Ribosomal 18S was used as a reference gene and primers used for *O. nigriventris* were: forward 5′-AGGGAAGACACG CTGATTCCTTCA-3′ and reverse 5′-ATTCTTGGATCGTCGCAAGACGGA-3′.

For qPCR experiments, OnInR expression was assessed using primers OnInR_for: 5′-ATGAACCATCCGATTCTTGG-3′ and OnInR_rev: 5′- GAGGACTTTCTTCTTGTTGACG-3′. The *O. nigriventris* ribosomal 28S gene was used as a reference gene (On28S_for: 5′-C GGATCCTCCCTAACACCACATTT-3′; On28S_rev: 5′-AACAAGGATTCCCTTAGTAGC GGC-3′). All qPCR experiments were conducted using the BioRad iCycler and BioRad Sybr-Green system (Bio-Rad). Standard curves for all reference genes and for the OnInR were generated. Due to constraints on tissue sampling and tissue availability, two separate qPCR experiments were conducted. First, an experiment was conducted in large males ($n = 3$) to compare OnInR expression in horns and wings and in early prepupa and late prepupal stages. Three technical replicates and three biological replicates were performed.

The second qPCR experiment conducted was to compare small male, large male, and female horn imaginal tissues from early and late prepupa using pooled samples. In the case of large males, horn imaginal tissues from four large males at the early prepupa stage were dissected, RNA was extracted and cDNA was synthesized as described, and then the

cDNA was pooled into one sample for qPCR. This was also done for horn imaginal tissues from four large males at the late prepupal stage, thoracic epidermis in the horn region for small males, small females, and large females at the early prepupal and late prepupal stages. Four technical replicates were performed with the 28S reference gene, at the time of these experiments, no other reference genes were available. The delta Ct method, (Livak and Schmitgen, 2001) was then used to calculate the relative expression in each tissue sampled and compared using two-sample *t*-tests assuming equal variance where appropriate or else the nonparametric Wilcoxon/Mann–Whitney U test was used. This rank-based test is a nonparametric replacement test for the *t*-test and is highly conservative in comparison to equivalent rank-based tests.

RESULTS

Cloning OnInR Gene Transcript and Sequence Analysis

Degenerate primer PCR was used to amplify a 450 bp fragment from *O. nigriventris* cDNA encoding a conserved region of the InR tyrosine kinase domain (data not shown). From this fragment, sequence-specific primers were designed and used in 5′ and 3′RACE reactions. Overlapping fragments generated by RACE were identified using SeqMan (Lasergene) yielding a gene transcript of 4,757 nt. The putative OnIR transcript has a 55 nt 5′UTR followed by an open-reading frame (ORF) of 4,162 nt encoding for 1,369 amino acids and a 3′UTR of 568 nt to the polyA tail (Figs. S1–S3 and S6–S8; Genbank accession JN711464). A phylogenetic analysis of known arthropod and mammalian InRs showed that the OnInR was in fact related to other animal InRs (Fig. S4). The unrooted phylogenetic tree also fully supported the NJ tree (Figs. S4 and S5).

OnInR mRNA Expression

The OnInR transcript was first assayed for its presence in developing horn, wing, and leg tissues from large males, large females, small males, and small females using qualitative reverse-transcription PCR (RT-PCR). Male genital discs were also tested. All tissues showed the presence of the OnInR transcript (Fig. S9). Weaker bands were observed in large male horn tissue and small female wing tissue; whether this reflects an actual change would require quantitative measurements.

Quantitative real-time PCR (qPCR) was used to assess relative expression of the OnInR transcript in large males (Fig. 2). Developing horn imaginal tissues and wing imaginal tissues from the early prepupa and late prepupa stages were assayed. OnInR transcript was expressed in relatively similar levels in early prepupal and late prepupal wing imaginal tissues with no significant difference observed (Fig. 2, Wilcoxon/Mann–Whitney $\chi^2 = 2.4$, $P = 0.1213$). OnInR transcript was 1.87 times higher in late prepupa individuals in horns compared to early prepupa individuals (Fig. 2, Wilcoxon/Mann–Whitney $\chi^2 = 5.4$, $P = 0.0201$). Comparison of horns versus wings was not significantly different for early prepupa tissues (*t*-test, $t_4 = 0.205$, $P = 0.8476$) or late prepupa tissues (*t*-test, $t_{10} = 0.431$, $P = 0.6753$).

We investigated OnInR expression more closely in horn imaginal tissues in large males compared to the thoracic epidermis (corresponding region of the horn of large males) of small males and females with qPCR (Fig. 3). Comparison of OnInR transcript relative expression in small male and female anterior thoracic epidermal imaginal tissues,

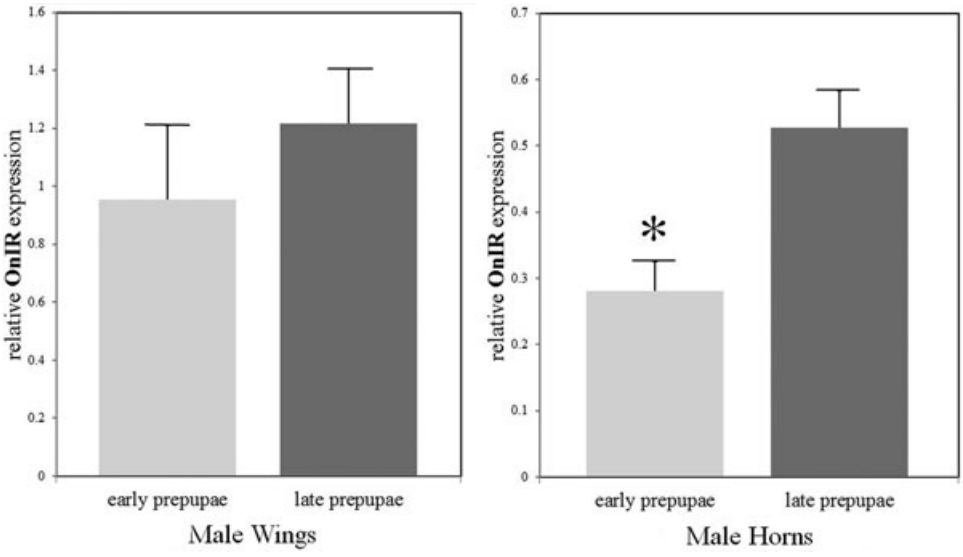

Figure 2. Relative expression of OnInR transcripts in large male prepupal wing and horn imaginal tissues from two developmental stages, the early and late prepupa periods.

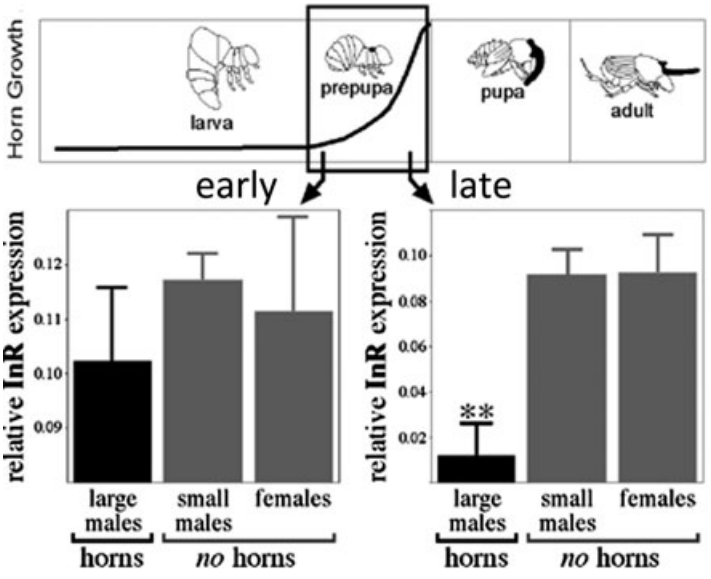

Figure 3. Differential expression of OnInR in horn imaginal tissues from large males, small males, and females during early and late prepupal development. Diagram of larval to adult development and graphs of the relative expression levels of OnInR during early prepupal and late prepupal development. Asterisks indicate significance of $P < 0.0001$.

to large male horn imaginal tissues during the early prepupal period was not significantly different (Fig. 3; large males to small males $t_8 = 2.315$, $P = 0.05$ and large males to females, $t_8 = 0.9425$, $P = 0.3735$). However, in the late prepupal period, the relative expression of OnInR was significantly different between small males and large males (Fig. S9; $t_8 = 9.74$,

$P < 0.0001$) and large males and females (Fig. 3; $t_8 = 8.19$, $P = 0.00011$). There were no significant differences in OnInR expression between small males and females (Fig. 3; $t_8 = 0.1474$, $P = 0.8864$). These results support what was observed in the qualitative RT-PCR assay (Fig. S9).

DISCUSSION

OnInR Protein Structure

The predicted OnInR protein is structurally similar to other known animal and insect InRs (Figs. S1–S8). Like InRs from other species, it appears that the mature OnInR protein is a tetramer composed of two α and two β subunits (Hanks et al., 1988). There are several important conserved features in the OnInR transcript that include potential insulin-binding domains, a cysteine-rich region, a tyrosine kinase domain, a furin-like site for proteolytic processing that potentially specifies the cleavage of the α from the β subunit, juxtamembrane and transmembrane domains (Figs. S6–S8). The potential α subunit contains the leucine-rich repeat domains (L1 and L2) and cysteine-rich domains (Figs. S1 and S6). Conservation of cysteine residues in the cysteine-rich domain may point to similar structural organization for the OnInR compared to other IRs (Figs. S1 and S6). Intramolecular di-sulfide bonds between these cysteines link the α subunits together and the α subunits to the β and therefore are implicated in the InR tertiary structure. The overall structure of the putative OnInR is homologous to the mammalian InR and InRs of other invertebrates (Figs. S4 and S5).

Localization of Expression

In order to determine the likelihood of the insulin signaling pathway's involvement in horn and trait growth, we investigated the distribution of OnInR mRNA in *O. nigriventris* imaginal discs during the early and late prepupal period, which encompasses the entire period of trait growth. OnInR mRNA was detected in all imaginal discs from both large and small males and females (Fig. S9). This result was expected because during the larval stages of *Drosophila*, prominent expression of InR mRNA is observed in the imaginal discs (Garofalo and Rosen, 1988). In this study, relative quantification of OnInR mRNA levels in large male horn and wing tissues during and at the end of imaginal disc growth revealed that there was essentially no difference in expression between the two stages (Fig. 2). However, when horned and hornless males were compared with each other and with females, OnInR expression differed between large males and both small males and females (Fig. 3). Specifically, by the end of the prepupal period that also marks the end of horn growth, OnInR levels in the horn tissues of large males (which had just finished growing a long horn) were significantly lower than in comparable tissues of small males and females (which did not grow horns; Fig. 3). Small males and females are distinct from large males in that they do not possess the sword-like thoracic horn.

Implications for the Mechanism of Horn Dimorphism

The insulin signaling pathway likely contributes to the nutrition-dependent modulation of growth of the exaggerated ornaments and weapons of sexual selection in animals generally (Emlen et al., 2012), and in the horns of scarab beetles specifically (Emlen et al.,

2005, 2006, 2007). This same pathway may also be part of the polyphenic mechanism shutting off horn growth in small males and females of dimorphic beetle species such as *O. nigriventris*. If the insulin signaling pathway is involved in horn dimorphism, then we predict that horn discs in large males will be sensitive to circulating insulin signals, while horn discs in small males and females should not be. Although the responsiveness of specific tissues to circulating insulin levels typically reflects the nutritional state of the individual in a dose-dependent manner, some traits are known to vary in their responsiveness to nutritional signals—through the insulin signaling pathway—affecting growth (Tang et al., 2011; Emlen et al., 2012).

As a first step toward testing the role of the insulin signaling pathway in the development of the sexually dimorphic dung beetle horn, we provide evidence that the *O. nigriventris* InR transcript is differentially expressed during horn growth and development between large horned males and hornless small males and females. Our data indicate that OnInR transcript is expressed in horn tissues during the period of horn growth, and that the levels of expression differ between individuals that have, and have not, experienced a rapid burst of proliferation in the horn imaginal tissues. Our results are therefore consistent with a role for this pathway in the polyphenic regulation of horn expression. What remains unknown is why OnInR transcript abundances were higher in the smallest males and females, rather than in the large males who were undergoing proliferative growth (Fig. 3). We suggest that this reflects an intrinsic and ancient feedback mechanism that acts to prime tissues for resuming growth when they have been exposed to starvation conditions. FOXO is a transcriptional activator for the InR (Fig. 1; Kramer et al., 2003; Puig and Tjian, 2005; Tang et al., 2011). Under starvation conditions, reduced signaling through the InR pathway can arrest cell proliferation throughout the body, but because of the feedback between FOXO and InR, starved cells begin to upregulate transcription of InR (Fig. 1). This is thought to "prime" the cells for a rapid return to full growth as soon as nutrient conditions improve and insulin-like peptides begin to circulate again (Puig and Tjian, 2005). In principle, if the OnInR pathway were interrupted at some point downstream of the OnInR in the horn tissues of small males and females, then the polyphenic mechanism of horn dimorphism would effectively mimic starvation within this tissue, activating FOXO and blocking proliferation in the horns (Fig. 1). However, activated FOXO would also signal an increase in OnInR transcription, and could generate the patterns of differential OnInR expression that we observe. While there is some evidence that the *O. nigriventris FOXO* transcript is differentially expressed in the pupal stages of male genitalia, no differences in *FOXO* expression was found in pupal horns between males and females (Snell-Rood and Moczek, 2012). However, because the pupal period marks the end of growth of the horn imaginal tissues in *O. nigriventris,* the role of FOXO during imaginal tissue growth and proliferation, when FOXO is predicted to have its primary effects, remains to be described in dung beetle horn polyphenisms.

Additional studies correlating receptor protein expression with transcript expression, as well as functional tests including insulin injections or receptor knockdown studies, will be needed in order to fully test this idea in the dung beetle. Comparative work in the Asian rhinoceros beetle, *T. dichotomus* (Emlen et al., 2012), suggests that the insulin signaling pathway has a major role in regulating polymorphic trait expression in dimorphic beetles. How widespread this signaling is in the development and evolution remains to be tested in scarab beetles. The results of this study of OnInR from the dung beetle are critical in elucidating the function of the insulin signaling pathway in the diversity of horned scarab beetle species specifically and its role in the control of horn growth and insect polyphenisms in general.

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