2020

Developmental Mechanisms for the Diversification of Polyphenic Morphs in the Head Horn of Onthophagine Beetles (Coleoptera: Scarabaeidae *Onthophagus taurus*): Plasticity through Nutrition

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**Developmental Mechanisms for the Diversification of Polyphenic Morphs in the
Head Horn of Onthophagine Beetles (Coleoptera: Scarabaeidae *Onthophagus*
taurus): Plasticity through Nutrition**

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Thesis submitted to the Davis College of Agriculture, Natural Resources and Design at West Virginia
University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Genetics and Developmental Biology

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2020

Keywords: (*Onthophagus taurus*, Gene Regulatory Networks, Nutrition, Polyphenism, Resource
Allocation, Development, Developmental Plasticity, Life-History, Evolutionary Novelties)

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Abstract

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Onthophagine Beetles (Coleoptera: Scarabaeidae *Onthophagus taurus*):

Plasticity through Nutrition

Logan Paul Zeigler

Developmental plasticity is the phenotypic variation between organisms that is caused by environmental interactions affecting the developmental systems of organisms. The research focused primarily on nutrition-responsive developmental plasticity. In this research we used the nutritionally determined head horn development of *Onthophagus taurus* to better understand the developmental mechanisms and genetic underpinnings of nutrition-responsive trait development. We focused specifically on altering the availability of specific nutrition-related primary metabolites, cholesterol and palmitic acid, identified in the activity of The Hedgehog pathway, a critical pathway in head horn development. By altering diet composition using cholesterol, reducing transcript expression of an acyltransferase gene, *rasp*, which is involved in Hedgehog pathway activity, and by reducing transcript expression of lipophorin receptors responsible in part for lipid and cholesterol resource allocation, this study used diverse approaches to determine the developmental significance of specific nutrients. As well, a pharmaceutical drug, atorvastatin, was used as an isoprenoid biosynthesis inhibitor, a signaling pathway which was identified to have possible impacts on known effectors of horn size. The results of this study indicated that nutrient modification and resource allocation play a role in regulating *O. taurus* body and horn development to maintain the distribution pattern of discrete morphs. Further, the results showed that statin supplementation may cause a shift in the evolved body/horn size relationship in an *O. taurus* population. Overall, we saw that resource mobilization and environmental changes impacted the developmental mechanisms regulating horn growth, which indicated that individual nutrients are involved in the developmental plasticity of specific traits.

Acknowledgements

Bennet Yunker (Undergraduate Student Researcher, Biochemistry Degree Candidate)

Callie Trainor (Undergraduate Student Researcher, Pre-Veterinary Degree Candidate)

Catherine Raines (Undergraduate Student Researcher, Pre-Veterinary Degree Candidate)

Victoria Bovard (Undergraduate Student Researcher)

Jeffrey Petty (Undergraduate Student Researcher)

Dr. Ida Holásková (Office of Statistics, Davis College of Agriculture, Natural Resources & Design)

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Chapter 1: The Evolutionary Significance of Onthophagine Horn Development

Abstract

Evolutionary developmental biology tries to connect evolution and development to more accurately and ubiquitously describe the fundamental change in organisms over time. A major goal for the progression of the field is to also account for the ecological and environmental impacts in the life-history of organisms which contributes to a large degree of phenotypic diversity within species. One of the significant contributions of evolutionary and developmental biology to the broader field of biology was the proposal of the concept that organismal development is controlled by Gene Regulatory Networks (GRNs). These networks are composed of a hierarchical array of modules. These modules are groupings of relatively autonomous interacting genes that perform a developmental function. Although GRNs control development, environmental influences can cause differences in expression of GRNs resulting in visible trait-specific phenotypic diversity between mature organisms of the same species. Nutrition is an environmental factor known to affect development and is fundamentally variable between developing organisms. However, current studies do not focus on the interaction of dietary components of nutrition with GRN modules involved in developmental plasticity. Environmental factors, specifically nutrition, determines the expressed phenotype of the head horn of *Onthophagus taurus* making it an ideal model for the study of how nutrition impacts the developmental plasticity of traits. Here, I have reviewed concepts of evolution and development, the ecology of *Onthophagus taurus*, and the known role of the environment and GRN modules in the development of the *O. taurus* head horn.

Introduction

As the role of the genome in development has become better understood, insight into how macroevolutionary changes occur has emerged (i.e. changes in morphology, speciation) (reviewed in Gilbert et al. 1996, Carroll 2008). Developmental pathways are highly conserved between distantly related organisms (McGinnis et al. 1984, Graham et al. 1989, Kusserow et al. 2005, Nichols et al. 2006, reviewed in Davidson 2006). As well, most proteins involved in the regulation of development are often involved multiple independent developmental pathways shaping the distinct morphology of different traits (e.g. the Sonic hedgehog protein plays a role in the development of limbs, eyes, brain as well as other body parts, reviewed in McMahon et al 2003). These proteins, which are mostly signaling proteins and transcription factors, are often functionally equivalent among diverged organisms. For example, Pax-6, the mouse homolog of *Drosophila* Eyeless has been used to induce ommatidium formation in *Drosophila* (Halder et al. 1995). Just as significant, the developmental pathways influenced by these proteins can be found in distantly related organisms performing similar developmental functions (reviewed in Davidson 2006). In both cases, the developmental patterning determined by the shared developmental pathways between organisms can result in radically diverse structures between organisms (i.e. as above, a mammalian eye versus ommatidium of the insect's compound eye). The integral role these signaling proteins and transcription factors seem to play in development has shown that they seem to make up a "genetic toolkit" of development.

The origin of novel traits often occurs as a result of changes in gene expression (Davidson 2006). *Cis*-regulatory regions (containing *cis*-regulatory elements such as promoters and enhancers) and the transcription factors that bind to them are responsible for the expression of genes (reviewed in Wittkopp and Kalay 2012). A single *cis*-regulatory element can be associated with tissue-specific gene expression therefore mutation or alteration of a *cis*-regulatory element can affect toolkit gene expression in a spatial/temporal manner without affecting protein function (reviewed in Wittkopp and

Kalay 2012). On a much larger scale Gene Regulatory Networks (GRNs) are logic maps that connect the regulation of the expression of genes and the functional interactions of genes to control development (reviewed in Levine and Davidson 2005). The comprehensive combination of regulators of toolkit genes, the target repertoire of toolkit proteins and how toolkit proteins affect downstream gene expression would create a GRN encompassing the overall development of an organism. A module of a GRN would therefore be the toolkit genes, their downstream targets and their expression that together regulate a developmental process. Evolutionary innovations can often be explained by changes in *cis*-regulatory regions causing modules to be recruited to function in a different body region/developmental context (i.e. co-option) (as described in Guinard 2014). How modules are co-opted, regulated and employed to alter morphology and subsequently diversify to develop a unique identity as an evolutionary novelty is a defining question of Evolutionary Developmental Biology (Wagner and Lynch 2010, Brakefield et al. 1996).

Environment impacts development. All organisms encounter variation in their environment, which influences the expression of regulatory network modules to produce a range of phenotypes for a single trait and this plasticity is now considered to be common in development (Gilbert and Epel 2009). Plasticity allows organismal development to adapt to environmental input. Consequently, under certain conditions, this can lead to evolutionary change (reviewed in Moczek 2007, Gilbert et al. 2015, Ehrenreich and Pfennig 2015). Currently, how developmental plasticity leads to the evolution of novel traits needs to be further investigated at a mechanistic level in order to understand exactly how the genetic underpinnings of developmental plasticity affects evolutionary outcomes.

Of all animals, insects are one of the most species-rich, and exhibit significant inter- and intra-specific diversity. Among this group, coleopterans (beetles) account for an estimable 40% of all insect species (Grimaldi and Engel 2005). Coleopterans are most prominently distinguished from other insect groups by the modified hardened forewing or elytra. Multiple beetle species have evolved head horns

independently within their phylogeny (reviewed in Emlen et al. 2007). These horns are major structures that are incredibly diverse in shape, number, and position on the head (Moczek 2010). Beetle horns can not only be used to morphologically distinguish horned beetles from other organisms but also can be used to distinguish different species of the same genus, different sexes of the same species, and be used to predict different behaviors within the same sex (Emlen 2001, Emlen and Simmons 2005, Moczek and Emlen 2000, Beckers et al. 2017). Other insect groups do not appear to have any obvious homologous structures to beetle horns (Moczek 2005). As well, beetle horns lack obvious homology to any other body part within the individual beetle (reviewed in Moczek and Rose 2009 and Kijimoto et al. 2013). Therefore, it is considered an evolutionary novelty. Thus, studying the genetic underpinnings of morphological variation of beetle horns would provide an optimal model to gain an insight into how morphologically novel traits develop and are evolutionarily diversified through changes in the expression of co-opted genes.

A model that provides a comprehensive perspective of the origin and diversification of a morphological novelty (horns) is the dung beetle genus, *Onthophagus*. Species of Onthophagine beetle generally have sexually dimorphic horn structures, where one sex (most often males) grows horn and the other sex does not or has different horn positioning or structure (Emlen et al. 2005a). As well, within a sex that develops horns there can be variability in horn size. Extreme examples of this occur in Onthophagine species that exhibit polyphenism, where the same genotype among the

same sex of the beetle species presents itself as multiple distinct phenotypes often in response to an

Figure 1: The sexes and sizes of *O. taurus*. Females do not develop head horns in this species. The top left image shows a small female. The top right image shows a large female. The bottom left image shows a small male with small, rudimentary horns. The bottom right image shows a large male with large, curved horns. Adapted from Kijimoto et al. 2014.

environmental cue (Nijhout 1999, Gilbert 2014). Among those species that exhibit this polyphenic relationship of the head horn is *Onthophagus taurus* (Coleoptera: Scarabaeidae). The male individuals have the ability to express one of two nearly discrete phenotypes as seen in Figure 1, and grow either small, rudimentary horns or large, curved horns in a bimodal fashion relative to their body size; if a male's body size grows past a threshold body size it will have large horns, described in further detail below (Moczek and Emlen 1999). In this research we have examined how alternative morphs are governed and constrained through the interaction of genetic mechanisms with developmental and ecological mechanisms and documented the effects of some environmental variables through nutrition that can be considered relevant to phenotypic expression.

Literature Review

The Modular Nature of Development and Evolution

Adult or matured morphology is determined during the different developmental stages of an organism. This development is governed by a body plan, which, as the name suggests, promotes and restricts organismal development ensuring that the organism develops within the confines of the taxonomic classifications it belongs to (Figure 2). Developmental processes or comparable characteristics shared phylogenetically among a group of related organisms are the components of homologous modules of development, groupings of which make up an organism's body plan (reviewed in Kuratani 2009 and Willmore 2012). Body plans are representative of shared patterns of development (modules) and can be viewed hierarchically, ranging from developmental traits conserved across broad taxonomic groups or traits specific to individual species (Figure 2). The grouping of traits unique to these classifications is controlled by the functional organization of GRNs (reviewed in Davidson and Erwin 2006, Peter and Davidson 2011, Smith et al. 2018). As mentioned above, a GRN is simply a functional grouping of transcription factors and *cis*-regulatory elements which regulate gene expression. Focusing

Figure 2: Conserved hierarchies of body plans. The phylogenetic relatedness of a clade of organisms can determine the shared body plan restrictions and characteristics of that clade. Broader spectrum patterning or general characteristics shared by a large group of organisms, therefore would be determined further down the ancestral lineages, before branching and divergence, and have more developmental similarities determined by shared modules of GRNs. More modern acquired developmental traits are shared by fewer organisms. Individual species have unique developmental traits, that have evolved more recently. The body plan at different levels is what determines the developmental restrictions of these categories of organisms.

on the groupings of functional interactions within GRNs, developmental modules higher level regulation is determined by morphogens. Morphogens are secreted signaling proteins that which cause the activation of downstream transcription factors. These transcription factors have a unique target repertoire of binding sites and genetic activation through which developmental fate is determined.

Figure 3: A simple GRN module regulating morphogenesis. GRNs control the development of organisms. Upstream in modules of GRNs, morphogens are secreted and bind to receptors on target cells. These receptors then activate a signal transduction pathway which causes large scale changes in the cell, determining a cell's developmental fate.

Developmental modules control morphogenesis terminally through regulation of cell migration, cell shape, and cell rearrangements (reviewed in Gilmour et al. 2017, Figure 3). To achieve this cellular level influence and coordination of development, GRNs are hierarchical in nature, correlating with the sequential nature of development. To put another way, developmental modules regulate development both spatially and temporally and earlier developmental events help determine those that follow (Peter and Davidson 2017). The complexity of GRNs is a focus of study in the field of developmental biology. The regulation of morphogens, differential spatial and temporal development, the target repertoires of transcription factors and the interactions between the different target repertoires in order to regulate development are some of the main focuses in progressing the field (Briscoe and Small 2015, Smith et al. 2018, Gilmour et al. 2017).

From Development to Evolution

The hierarchical and modular pattern of organismal development is widely conserved even among distantly related organisms (reviewed in the introduction), indicating that morphological evolution involves changes in developmental processes causing expression of heritable phenotypic change. Genetic accommodation is a theory that aims to explain the process through which this change occurs (reviewed in Moczek 2007). To better understand the process through which morphological evolution can occur through development, it is necessary to understand how phenotypic expression is influenced by genetic and environmental interactions.

It is common for developmental processes to produce a range of phenotypes for a single trait (reviewed in Gilbert and Epel 2009). Developmental processes have evolved to react reliably in response to environmental conditions and exhibit varying degrees of environmental sensitivity (adaptive phenotypic plasticity, Schlichting and Pigliucci 1998, von Dassow et al. 2000). The amount of environmental sensitivity of a trait is variable (Figure 4). A trait having reduced environmental sensitivity

is indicated when a developmental process expresses one phenotype when exposed to a range of environmental or genetic variation (canalization, Scharloo 1991). A canalized phenotype will develop as long as the environmental or genetic variation is not outside the range of the buffering effect of the canalization (reviewed in Moczek 2007). If canalization favors the development of a specific phenotype, selection does not act on the processes involved in the development of the

phenotypes that appear outside the range of the canalization and thus genetic variation can accumulate in the GRN module (cryptic genetic variation, West-Eberhard 2003). The expression of these traits following environmental or genetic perturbations beyond the buffering effect of canalization would then expose this variation phenotypically and put selective pressure on the trait. Developmental capacitance is the ability of each GRN to accumulate and express this cryptic genetic variation. Development mediates the phenotypic changes that occur due to environmental conditions, which can then be exposed to selection and stabilized genetically through selection (West-Eberhard 2005a).

Genetic changes appear to most often occur in the *cis*-regulatory regions of developmental genes in GRNs (Carroll 2008). *Cis*-regulatory elements in *cis*-regulatory regions contain binding sites for regulatory molecules and transcription factors to regulate transcription (Ong and Corces 2011). Changes more often occur in these sequences because *cis*-regulatory elements are often tissue/module specific and changes in these regions would affect the expression of genes in specific tissues/modules (reviewed

in Wittkopp and Kalay 2012, Carroll 2008). This is unlike mutations in protein coding regions which affects protein function which would affect all tissues/modules in which the protein is active. Divergence of these *cis*-regulatory regions is often seen as the result of simple nucleotide insertions, deletions, or substitutions that interfere with transcription factor binding. As well, the evolution of novel enhancer activity seems to most often occur with the co-option of ancestral transcription factor binding sites (reviewed in Wittkopp and Kalay 2012). The resulting phenotypic changes occur from GRNs activating at different spatial and temporal points (Kittelmann et al. 2018) otherwise referred to as the co-option of a GRN module. Depending on the component of the GRN in which changes occur, the resulting co-option can result in a spectrum of changes. GRNs reflect the hierarchical nature of development, and changes that occur in GRNs regulating more fundamental aspects of an organism's body plan can result in a more radical change as well as the reverse being true, which results in changes in GRNs that can have an impact that can range from sub-species level to phyla level (Davidson and Erwin 2006). As well because the evolutionary process takes advantage of pre-existing developmental machinery, these changes can occur rapidly under the proper environmental conditions. Further research into genetic accommodation could better integrate it with our understanding of existing evolutionary concepts.

Novel Traits

An antiquated concept of novelty is that a trait is considered novel only when it does not share homology with any structure in the ancestral species and it does not share homology with any other structure within the same organism (Müller and Wagner 1991). This concept has changed over time to leave behind the strict morphological restriction of evolutionary novelties, instead focusing on homology between genetic and developmental processes (Abouheif 1997, Shubin et al. 2009, Monteiro and Podlaha 2009, Mozcek and Rose 2009). Expanding the concept of novelty, some homologous structures have been found to originate from dramatically different developmental systems, a concept known as developmental systems drift (i.e. sex-determination pathways even among closely related species, True

and Haag 2001). As well, many well-known homologous developmental networks have been shown to be involved in the origination of novel trait through processes such as co-option (Linz et al. 2019). This study and others related to Onthophagine beetle horn development have shown that novelty can originate within developmental networks governing the development of traits that are already considered novel. For the purposes of this study, we will consider, broadly, the traits resulting from evolutionary changes that have no known homology to other traits as novel. The study of the characteristic modular and hierarchical nature of both development and evolution can utilize known novel traits in model organisms to better explain the developmental process and evolutionary patterns. Novelities are the product of the evolutionary co-option of genes and regulatory networks, the study of which can provide insights into Evolutionary and Developmental Biology. One such area of study into further understanding novelty is in how novelties themselves can be altered through the evolution of developmental processes.

The Scarab Beetles

The evolutionary novelty of interest for this research are the horns of the scarab beetle (Scarabaeidae). The vast majority of extant species of horned beetles lies within the Scarabaeoidea superfamily, with some of the most exaggerated structures appearing in the Lucanidae (stag beetles) family, the Dynastinae (rhinoceros beetles) subfamily, and the Scarabaeinae (dung beetles) subfamily (reviewed in Emlen et al. 2006). Beetle horns are exoskeletal projections existing mainly on the anterior pronotal thoracic segment and/or on the dorsal head, both body regions that typically do not develop structures in other insects (Emlen et al. 2006, Kijimoto et al. 2013). Horns are used in the competition of male beetles over access to reproductive females and thus horn size can be determinate of reproductive success based on mating behavior (Eberhard 1979, Eberhard 1987, Siva-Jothy 1987, Emlen 1997). There is no doubt that even by the strictest antiquated definition of novelty, beetle horns are a novel trait as they are functionally significant structures lacking visible homology to existing structures. While the

origin of head horns remains largely unknown, they appear to share developmental properties with that of traditional insect appendages (Moczek and Nagy 2005, Moczek et al. 2006b). The thoracic horn however may have developed as a failure to be resorbed after the pupal stage and then evolved to serve its current function (a process known as exaptation) (Kijimoto et al. 2010). There are many instances in which it has been found that the thoracic horn is extant in the pupal stage regardless of sex and in species that do not possess an adult morph with a thoracic horn. This structure is thought to aid in breaking through the tissue of the head capsule between the larval-to-pupal molt and after is resorbed in some species before the pupal-to-adult molt (Moczek et al. 2006a). Failure to resorb this pupal thoracic horn could potentially result in the development of an adult thoracic horn.

Phylogenetic inferences when examining head and thoracic horn development in the different subfamilies of Scarabaeidae indicate that the developmental modules that influence horn development originated ancestrally, not individually within each subfamily or species as there is widespread development of horns in each of the major clades (Emlen et al. 2006). This suggests that the so-called “hornless” species of scarab beetle (those that are uniquely hornless or, as in the case of many species, those that only develop rudimentary horns) are secondarily hornless having evolved the loss of horn development. This is further evidenced by some mutant individuals in completely hornless species having a horned phenotype (i.e. *Pterorthochaetes armatus* from the completely hornless subfamily Ceratocanthinae, Emlen et al. 2006). This capability to “lose” horns in species may explain another unique characteristic of beetle horns, dimorphism. Beetle horns are often sexually dimorphic, in many cases with females not having the ability to grow horns. As well, within male scarabs, beetle horns can be dimorphic (polyphenic, see below for polyphenism), with the potential to develop as exaggerated or rudimentary structures. Further between and within different species of beetle horns can vary in size, shape, number and location (Emlen et al. 2005b, reviewed in Emlen et al. 2007, Moczek 2010). Looking at one genus of horned beetle (*Onthophagus*) illustrates the diversity and dimorphism of beetle horns

(Emlen et al. 2005a, Emlen et al. 2005b).

Polyphenism

Within the genus *Onthophagus*, the developmental and phenotypic plasticity between and within beetle species is pronounced. Phenotypic plasticity is commonly seen as developmental processes affected by varying environmental conditions causing a range of phenotypes associated with a particular trait. Developmental plasticity is further of interest because it is thought to play a role in the origination and evolution of novel traits (Mozcek et al. 2011). As explained above (see the section From Development to Evolution), interaction with the environment directs development and exposure to environmental perturbations can reveal novel phenotypic variation. This outcome can be a plastic response. Subsequent genetic assimilation could then make the revealed variation a heritable trait.

An extreme case of phenotypic plasticity occurs when organisms with the same genotypes can develop discrete alternative phenotypes in response to environmental factors. This phenomenon is known as polyphenism. Some of the most pronounced and well known polyphenisms are that of insects with castes systems which can contain soldier, worker, and reproductive castes that can be phenotypically radically different, yet are genetically identical (Nijhout and Wheeler 1982, Luscher 1960). Polyphenism, through the development of discrete morphs, is thought to be an influencing factor in the evolution of plasticity and novel traits as well as in speciation (Pfennig et al. 2007, reviewed in Nijhout 2003, West-Eberhard 2005b). Polyphenism, as a requirement, needs to form distinct phenotypes meaning that canalization of the developmental networks responsible for polyphenic expression is needed to buffer environmental perturbations and form these distinct outputs (reviewed in Projecto-Garcia et al. 2017). This means that not only is polyphenism an end product of evolutionary mechanisms, it is also a vehicle for them, as canalized traits experience reduced selective pressure which could potentially become even more relaxed in the event certain morphs are developmentally biased

(Van Dyken and Wade 2010, Hu et al. 2019).

A linear relationship between beetle horn size and body size would be an example of traditional phenotypic plasticity (the reaction norm). Many of the species of Onthophagine beetles, as well as a majority of beetles that grow horns, exhibit this type of plasticity. Some species of Onthophagine beetle exhibit a different relationship of horn growth however, indicative of polyphenism, where horns will either be discretely rudimentary or discretely developed structures (Emlen et al. 2005a). Thus, demonstrating the developmental capacitance of Onthophagine horns to evolve as a polyphenic trait.

Model Species: *Onthophagus taurus*

Natively found in the Mediterranean region, *Onthophagus taurus* was purposefully introduced into Western and Eastern Australia between 1969 and 1983 to reduce and recycle cow dung and control the dung-breeding fly population (Tyndale-Biscoe 1990, Tyndale-Biscoe 1996). *O. taurus* were also introduced for this purpose in the Western United States between 1973 and 1977 (Anderson and Loomis 1998). *O. taurus* were introduced accidentally into the Eastern United States in the early 1970's (Fincher and Woodruff 1975), where they have since thrived and spread. The fecal matter of other organisms plays a critical role in *O. taurus* ecology and is used to feed on and complete the *O. taurus* life cycle. As larvae, these beetles develop in underground tunnels, enclosed inside a dung ball. This dung ball (otherwise known as a brood ball) is provisioned by the egg-laying female adult *O. taurus* beetle (Hunt and Simmons 1998) in a tunnel dug for this purpose (a breeding tunnel). Typically, these breeding tunnels are claimed by a single male who has won the tunnel through competition against other males. The female is assisted in brood ball provisioning by the male *O. taurus* beetle that guards the breeding tunnel from competing males (Hunt and Simmons 1998). The brood ball is the only resource available to a larva for food during this period and where the developing *O. taurus* will stay from egg to until it emerges as an adult (Halffter and Edmonds 1982). The larva undergoes three instars before transitioning

through a prepupal stage to become a pupa (Crowson 1981,). *O. taurus* spend their life cycle after emergence as adults mostly subterranean, emerging from dung piles or underground to travel to the next dung pile for food, digging tunnels underneath them, and mating (Figure 5).

As with other horned beetles, the head horn of *O. taurus* is a secondary sexual trait directly related to success during male-male competition over breeding tunnels and females. These horns are used as weapons in contest over females and male behavior will ecologically differ depending on competitive viability due to horn size. *O. taurus* males exhibit discrete horns sizes indicative of polyphenism and will utilize different tactics in order to mate depending on their horn size. Large horned males, having a competitive advantage over small horned males, will favor direct confrontation in which the larger horned male will fight off

Figure 5: Diagram of the larval stages and life cycle of *O. taurus*. The larval stages shown in A-E occur within a broodball. (A) shows an *O. taurus*: egg. (B) shows a first instar *O. taurus*. (C) shows a second instar *O. taurus*. (D) Shows a third instar *O. taurus*. (E) shows an *O. taurus* pupa. (F) shows an *O. taurus* emerging from a broodball as an adult. (G) shows the egg placement in broodball. (H) shows a larva feeding on a broodball. The chronological development of an *O. taurus* occurs from A to F with a prepupal stage occurring between D and E. Diagram produced by Estes et. al. 2013

smaller males, gaining or maintaining control over a breeding tunnel and access to a female beetle (Eberhard 1979, Moczek and Emlen 1999, Moczek and Emlen 2000). Although less competitive in direct confrontation, once expelled from the breeding tunnel by a competitor, small horned males will adopt another strategy. Using their increased underground mobility as compared to the large horned males, these “sneaking” males will dig access tunnels to a female and mate while the defending male is occupied (Moczek and Emlen 2000). These alternative morphs favor their respective tactics and both are

equally successful in producing progeny (Moczek and Emlen 2000).

Horn Polyphenism in *O. taurus*: A Threshold Body Size

The head horn is a developmentally plastic trait. In response to environmental factors the male *O. taurus* larvae encounter, the GRNs involved in horn development can either promote rudimentary or exaggerated horn development. Environmental factors can therefore alternatively regulate the developmental GRNs involved in horn development. As stated above, *O. taurus* spend their developmental period from egg until adulthood within a brood ball, keeping the environmental conditions that all *O. taurus* are exposed to relatively constant if undisturbed. The ecological condition that naturally varies between

Figure 6: The Development of beetle horns from larvae to adults. The grey highlighted region between the larval and prepupal stages indicates a 48-hour nutrition sensitive period. Depending on developmental signals received during this period, males with either grow exaggerated or rudimentary horns. These developmental signals stimulate cell proliferation in beetle horn primordia. In large males this stimulation causes rapid proliferation resulting in exaggerated horns present in the pupal and adult life stages. The blue line indicates the progression of the developing horn primordia from the larval to the pupal stage. Adapted from Emlen et al. 2005a.

brood balls is the nutrient quantity and quality, as brood ball size and composition vary between dung pile and due to parental allocation. This variation in larval diet has been found to be the determining factor for the growth of alternative head horn morphs of *O. taurus* (Moczek 1998). Just before the pupal stage *O. taurus* transition through a 48-hour prepupal stage. As well, horn tissue primordia develop just before the pupal stage (Figure 6). This period of stimulated cell growth determines whether a male will develop small horns, which indicates sub-optimal nutrition, or, if under conditions of optimal nutrition, large horns. The relationship of horn size compared to body size in the male shows a bimodal distribution where the majority of individuals have either rudimentary horns or elongated, curved horns, separated by a threshold body size (Figure 7). An organism's developmental response to varied

environmental conditions is coordinated by resource allocation to the many competing developmental structures and functions that are critical to the fitness of an organism (reviewed in Ng'oma et al. 2017). Resource allocation therefore influences context specific development. Differential gene expression is the central mechanism in which resource allocation is thought to alter context specific development. As a resource dependent trait, beetle horn polyphenism is regulated by differential gene expression to produce context specific development.

Figure 7: Measurements of a male population of West Virginia *O. taurus*. Each data point reflects the body width and average horn size of one adult male *O. taurus*. The shape of this population curve demonstrates a sigmoidal curve with a threshold body size typical of wild type *O. taurus* populations.

Genetically Determining the Threshold Body Size

Final body size and horn size is a direct response to larval food quality and quantity. As depicted in Figure 3, this type of environmental input has downstream effects on developmental regulators, particularly with the expression of the GRN modules involved in horn development. GRNs are responsible for trait development and, as such, the development of a novel morphological structure and its potential subsequent diversification (i.e. the innovation of the head horn and then the subsequent generation of a size threshold) should have a genetic basis. In other words, co-opted GRNs can result in the development of novel traits. Subsequent diversification of the relationship between co-opted GRNs may further contribute to the evolution of the novel trait, such as a novel trait evolving to express polyphenism. Differential gene expression is a central factor known to affect development and plasticity of structures (i.e. the gene expression profiles of different polyphenic variants will be different). Alternative nutritional input can cause fluctuations in the expression of genes that are responsible for the determination of the developmental fate and outcomes of traits (reviewed in Beldade et al. 2011).

Although adult horn size is a product of the nutritional state of a larva, the genome is the same between large and small *O. taurus* males, with final trait size being developmentally determined. Therefore, in order to understand the polyphenic development of horns, the underpinnings of the alternative morphs, nutritionally dependent gene expression differences, should be analyzed. *O. taurus*, as a prominent model for the origin of novel traits and developmental plasticity, has undergone a transcriptomic analysis in order to build a large-scale comprehensive database using expressed sequence tags (Choi et al. 2010). These large-scale efforts have also been conducted to determine transcriptomic differences between the head horn and other body tissues (Kijimoto et al. 2009, Ledon-Rettig et al. 2017), the modularity in developmental genetic networks in polyphenic development of horns in beetles (Snell-Rood et al. 2010), and the nutritionally responsive transcriptome of *O. taurus* (Kijimoto et al. 2014). Data from these analyses identified several candidate genes that indicated a potential impact on the development of the *O. taurus* head horn and regulation of the polyphenic expression of the horn.

Doublesex and The Sex-Determination Pathway

One of the candidate genes identified independently, but that repeatedly appeared in those transcriptomic analyses was *doublesex (dsx)*, an integral somatic sex-determination gene, that when translated into protein has the conserved function of being a transcription factor at the terminal end in the pathway that regulates the differential expression of downstream genes between males and females (reviewed in Baker 1989, Williams and Carroll 2009). The sex-determination pathway in general is responsible for the development of morphological differences between sexes and is active early in embryonic development (Bull 1983, Zarkower 2001). The sex-determination pathway has roughly the same basic structure in all insects studied thus far. Each sex is determined by upstream regulation of the sex-determination signal cascade. Although they have a similar function, genes involved in the initial signaling of the pathway and in the autoregulation of the pathway can vary between insects, however *dsx* is highly conserved and is the terminal factor of the sex-determination pathway in all cases of

studied insects with a similarly conserved function in orthologs of other organisms (for example *mab-3* in *Caenorhabditis elegans* and *Dmrt-1* in mammals, Raymond et al. 2000) (reviewed in Shukla and Nagaraju 2010, Ledon-Rettig et al. 2017).

One of the key characteristics of *dsx* is alternative splicing through the sex-determination pathway that generates sex-specific transcription factors which regulate the development of sexually dimorphic traits (Burtis and Baker 1989, reviewed in Shukla and Nagaraju 2010). The gene structure of *O. taurus dsx* is similar to other insects in this regard, as it has male and female specific mRNA isoforms (one identified male specific isoform and at least five female-specific isoforms) (Kijimoto et al. 2012). The regulation of sexually dimorphic traits by *dsx* is consistent with the sexual dimorphism seen in head horn development. Through transcriptomic analysis, it was found the *dsx* is enriched in the head horn of male *O. taurus* as compared to abdominal tissue, however it is not enriched in legs, an appendage which is not a secondary sexual trait (Kijimoto et al. 2009, Snell-Rood et al. 2010). It was also found that between the different morphs of *O. taurus* males there was differential expression of *dsx* in the head

horn. The large males had an increased expression of *dsx* in horn primordia as compared to the small males, suggesting that in large males, *dsx* was upregulated, potentially contributing to the development of increased horn size (Kijimoto et al. 2012). As seen in Figure 8, knockdown of this gene by RNA interference (RNAi) in male beetles

Figure 8: A comparison between *O. taurus* body and horn size in *dsx* knockdown and empty vector injected (control) individuals. In control males a normal bimodal distribution is seen between body size and horn size. However, when injected with *dsx* double-stranded RNA, horn development was dramatically reduced in large males whereas no change was detected in small male horn size. Figure adapted from Kijimoto et al. 2012.

reduced head horn development in large males while the small male horns remained largely unaffected.

As well, the horn reduction in large males was shown to have nutritional dependence (Kijimoto et al.

2012). Taken together, this indicates that *dsx* is responsible for influencing and promoting growth of horns to be larger in males that pass the body size threshold.

Hedgehog and Axis Patterning

Another identified candidate was a series of genes known to be involved in the Hedgehog pathway. This pathway is highly conserved within bilaterians (orthologs in mammals are *Sonic hedgehog*, *Indian hedgehog*, and *Desert hedgehog*, reviewed in Ingham and Placzek 2006). This pathway is best known for its role in anterior-posterior axis formation in various insect appendages (Nüsslein-Volhard and Wieschaus 1980, Mohler 1988, reviewed in Benazet and Zeller 2009). The Hedgehog (Hh) protein is a morphogen, which is a protein that is secreted and forms a gradient on the cell surface to direct cellular fate and tissue patterning (Briscoe and Therond 2013). The activity of Hh is largely

Figure 9: Diagram of the Hedgehog pathway. While the mechanism by which Ptc regulates Smo is not well understood, it is thought that without Hh binding, Ptc sequesters lipoproteins in endosomes and regulates them through its Sterol Sensing Domain. Endosomal interaction with Smo destabilizes the protein and interaction can either recycle the Smo protein back to the membrane or signal for Smo degradation. Secreted Hh binds to Ptc via the N-terminal palmitoyl modification. This modification signals for coreceptors of the Hh-Ptc complex to additionally bind (usually a second Ptc protein). The complex is then signaled for degradation, which derepresses Smo. Smo then activates downstream factors once activated by cholesterol binding. Figure designed using information from Khaliullina et al. 2009, Briscoe and Therond 2013, and Qi et al. 2018.

dependent on the concentration gradient, which decreases as distance increases from the secretory cell (Li et al. 2018). As seen in Figure 9, the Hh protein once secreted into the intercellular space will bind and inhibit its membrane bound receptor, Patched (Ptc). Ptc constitutively represses the G-Protein Coupled receptor, Smoothed (Smo). Hh binding to Ptc causes Smo to be derepressed, activating downstream factors (Briscoe and Thérond 2013).

Developmental regulatory networks that regulate the formation of limbs and appendages have been implicated in the control of horn development (Moczek and Rose 2009). The Hedgehog pathway, as one of these networks, has been shown to suppress horn development in sub-optimal nutrition males (Kijimoto and Moczek 2016). In males whose body size was below the threshold (indicating suboptimal nutrition as a larva), *hh* and *smo* RNAi resulted in an overall increase in horn size, while the maximum horn size did not change (Kijimoto and Moczek

Figure 10: Representation of body size and horn size measurements in *smo* and *hh* dsRNA injected beetles as compared to a control. The *hh* knockdown phenotype was only mildly affected. Conversely, the *ptc* knockdown phenotype was too severe to measure. Knockdown of *smo* resulted in small males developing large horn, while large male horn size remained unchanged. Figure adapted from Kijimoto and Moczek 2016.

2016, Figure 10). This result indicates that the growth of the head horn is reduced due to the activity of the Hedgehog pathway in individuals that are smaller than the body size threshold. Interestingly, the *smo* knockdown had a more dramatic effect on the development of the head horns, possibly because of the method of activation of the pathway as the Smo protein is the key signal transducer and terminal activator of the downstream effects of the pathway.

An Interactive Developmental Perspective

Both of these pathways perform their main function during embryogenesis and are profoundly developmentally important during this life stage. The redeployment of these pathways in a different

developmental context would not, therefore, be unusual because selective pressure should be reduced after embryogenesis and these pathways would then be developmentally available. In fact, redeployment of development pathways that are involved with novel trait development has been seen to occur in this way in several other studied models (Monteiro et al. 2013, Stansbury and Moczek 2014, Linz et al. 2019).

Taken together, *dsx* influences the development of large horns in large males but may not affect the horn growth of small males whereas the Hedgehog pathway influences the development of small horns in small males (Figure 11) but does not affect the horn growth of large males. Horn size is a direct consequence of the nutrition available to male larvae. Both the Doublesex (sex-determination) pathway and Hedgehog pathway affect horn size, but each only does so on one side of a threshold body size. Taken together, this indicates that both pathways could be nutritionally responsive. These two pathways have opposing developmental functions in the horn,

therefore in order for one pathway to be developmentally favored when determining horn size from a nutritional stimulus there should be some interaction (either direct or indirect) between the pathways that affects the expression of the pathways in the alternative variants. This means that upon co-option of the networks to horn development, these networks may have begun to regulate each other so that in response to nutrition the contextually favored variant can develop by way of the alternative pathways. Recent work has begun to show how the genetic networks of *hh* and *dsx* interact with each other to affect horn development and create a threshold (for a theoretical model see Figure 12). A transcriptomic analysis, comparing animals with the *dsx* transcript knocked down by RNA interference against control

Modularity in Populations

Figure 11: A representation of the role of the Hedgehog pathway and *dsx* in horn development in populations. The Hedgehog pathway is responsible for reducing horn growth in small males and *dsx* is responsible for enhancing horn growth in large males.

individuals, revealed that *dsx* expression downregulates *smo* expression in the head horn (Ledon-Rettig et al. 2017). This result suggests that the function of the Hedgehog pathway, activity of which is responsible for moderating growth to produce rudimentary horns, is repressed by *dsx*. In large animals with large horns and higher expression of *dsx* (Kijimoto et al. 2012), this may be a mechanism through which the threshold is generated and maintained. However, the effect the Hedgehog pathway has on the regulation of the Doublesex pathway to maintain this threshold body size has not yet been explored.

Interacting Pathways: Determining Head Horn Development Through Resource Allocation

Nutrition determines horn size. Nutrition also plays developmentally vital roles. The Hedgehog pathway is known to be nutritionally regulated and the Doublesex pathway is thought to be influenced by nutrition as well. Developmental changes could therefore be dependent on the nutritional allocation of resources in male larvae. During the development of males which are exposed to sub-optimal nutritional conditions, the Hedgehog pathway would then be favored for nutritional allocation.

Figure 12: A representation of the possible role of the Hedgehog pathway and the Doublesex pathway in horn development in individuals. Nutritional resource allocation may play a role in horn development. In individuals exposed to sub-optimal nutrition, the Hedgehog pathway could be preferentially upregulated by nutrition due to resource allocation, allowing the Hedgehog pathway to reduce horn growth. In individuals exposed to optimal nutrition, the Doublesex pathway could become nutrition sensitive causing resources to be allocated to the upregulation of Doublesex pathway and would cause the downregulation of the Hedgehog pathway. In this way, the Doublesex pathway could induce horn growth.

This would keep the pathway functional and provide a small level of upregulation to the Doublesex pathway, but not enough to promote the growth of large horns. In the occurrence of larvae exposed to abundant nutrition, the Hedgehog pathway could get saturated, or the Doublesex pathway could have more nutritional resources allocated to it. The resultant increase in *dsx* expression, could cause the inhibitory effects of *dsx* on the Hedgehog pathway to become much more pronounced, while also

becoming the prominent pathway for Horn Development.

The Nutritionally Responsive Head Horn

As stated above, the developmental pathways influencing the development of alternative morphs in the male *O. taurus* beetle are nutritionally responsive. All organisms are affected by the environmental resources available during their developmental periods and nutrition is one of the resources available to a developing organism. The concept of developmental pathways being influenced by nutrition is common among developing organisms (reviewed in Simpson and Raubenheimer 2012). The results of nutritional variance are often seen as developmental plasticity. Coordination of resource allocation therefore affects the developmental response in organisms (reviewed in Ng'oma et al. 2017). The developmental response to resource variance for different traits tends to affect morphology and allometry in one of three different ways. First, traits in which the functional effectiveness is dependent on the ratio to body size (such as legs and wings) can exhibit moderate sensitivity to nutrition and size tends to scale proportionally with the overall size of the organism. Second, traits that require more absolute sizes in order to remain functional (such as genitalia or the central nervous system) are not developmentally plastic and the size will remain consistent regardless of nutritional variances. Finally, traits with high nutrition sensitivity are developmentally plastic and have labile development corresponding to the relative amounts of resources available to the development of those traits versus other traits (i.e. some sexually selected traits usually weapons or ornaments such as deer antlers, avian color patterning, or beetle horns). These traits are affected by different patterns of resource allocation, in which the formation of traits has some energetic cost and the biological response determines the developmental pathway that individuals respond to in differential resource environments (Andersson 1986, Warren et al. 2013) In context this means that all male *O. taurus* are capable of developing horns, however only those in optimal nutrition environments will. No matter what nutritional environment however, genitalia size will be similar among all male *O. taurus* and elytra will scale with body size. The

capability of individual dietary components to affect different developmental processes is, as of yet, largely unexplored and has become a focus of research as well as identifying the key nutrition sensitive periods that regulate plastic development. Among dietary factors being explored in the regulation of the development shift between polyphenic beetle horn morphs, research has been done on the insulin signaling pathway (Snell-Rood and Moczek 2012, Casasa and Moczek 2018) which has a conserved function of regulating nutrition dependent growth. It has been found that the Insulin signaling plays a key role in regulating the development of male *O. taurus* horns in both polyphenic morphs. In this research we have examined other identified candidate dietary factors that are thought to play a role in beetle horn development, cholesterol and palmitic acid.

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Chapter 2: Characterization of Components of Nutrition that Influence Polyphenic Horn Development in *Onthophagus taurus*

Abstract

Nutrition is directly introduced routinely and because of the need for energy is arguably a subset of environmental factors with the greatest influence on development. How nutrition is processed by the body is governed by metabolic processes and resource allocation. As of yet, how specific factors of nutrition impact development are relatively unstudied. Here we examined the effects of cholesterol and palmitic acid on the development of an evolutionary novelty, not only for the purpose of examining how nutrition impacts development but also how it affects evolutionary outcomes. We did this by supplementing cholesterol to the diets of the beetle larva, reducing the transcript activity of the acyltransferase gene, *rasp*, that is involved in modification of the protein Hedgehog by palmitic acid, and by reducing the transcript activity of lipophorin receptors that are involved in the shuttling of lipids for use in energetic resource allocation. In parallel, we supplied a pharmaceutical drug, atorvastatin, to larval diets as an isoprenoid biosynthesis inhibitor which was identified to have possible impacts on known effectors of horn size. It was found that a slight increase in total cholesterol through cholesterol supplementation did not affect the body/horn size distribution of *O. taurus* males. Knockdown of *rasp* causes a significant increase in the relative percentage of beetles in the population with an intermediate horn size not typically seen in *O. taurus* populations. Knockdown of *lipophorin receptor* caused an overall increase in the percentage of large body size, large horned males in the population. Supplementation of the larval diet with atorvastatin resulted in a possible linearization of the population curve. These findings suggest that resource availability of individual nutrients maintains the typical bimodal distribution of horn size in *O. taurus* populations and that affecting resource availability can affect the proportion of expressed phenotypes in development plasticity.

Introduction

Polyphenic lability of the pathways controlling *Onthophagus taurus* horn size is regulated entirely by nutrition (Moczek 1998, Moczek and Emlen 2000, Kijimoto et al. 2014, Ledon-Rettig and Moczek 2016). One factor, insulin signaling, has already been implicated in the control of horn development (Emlen et al. 2006). Insulin signaling is a well-known mediator of physiological plasticity and is also known to be sensitive to changes in nutrition (glucose in particular) (reviewed in Nijhout 2003a, Mirth and Riddiford 2007). *Forkhead box, subgroup O (Foxo)*, as part of the insulin signaling pathway, is activated and arrests growth under nutrient stress and has been implicated as a regulator of beetle horn polyphenisms (Snell-Rood et al. 2011). When this theory was tested, knockdown of *Foxo* in *O. taurus* showed a moderate reduction of horn growth in large males and a moderate enlargement of horns in small males, taken together reducing the overall horn size distribution, while still maintaining the threshold body size (Casasa and Moczek 2018). This result indicates that *Foxo* plays a central role in nutritional resource allocation for determination of the head horn growth and possibly of other secondary sexual structures. Corroborating this possibility of *Foxo* regulating the growth of different tissues in response to nutritional variation in Onthophagine beetles, *Foxo* knockdown in *Onthophagus nigriventris* indicated that *Foxo* limits genitalia growth, another secondary sexual trait, and could play a more general role in regulating nutritionally linked scaling relationships (Snell-Rood and Moczek 2012).

Hormones, as signaling molecules that mediate responses to internal and external environmental signals, are involved in many cases of developmental plasticity (reviewed in Nijhout 2003b, Moczek et al. 2011, Ehrenreich and Pfennig 2015). Juvenile hormone has been implicated in having a role in *O. taurus* horn development. In insects, juvenile hormone in conjunction with the hormone ecdysone regulates molting and pupation during the lifecycle of insect larvae (reviewed in Jindra et al. 2013). The nutritional conditions which *O. taurus* larvae are exposed to potentially corresponds with juvenile hormone sensitivity in the horn primordia to determine horn size in mature

males (Emlen and Nijhout 1999). Interestingly, it has been found that juvenile hormone, an effector of horn size in *O. taurus*, interacts with the insulin signaling pathway in both *Drosophila* (Mirth et al. 2014) and *Manduca sexta* (Hatem et. al. 2015) to regulate nutrition-based growth. Further, when looking at a nutrition-dependent sexual dimorphism mediated by juvenile hormone in the stag beetle *Cyclommatus matallifer*, it was found that *dsx* regulates sensitivity of juvenile hormone in the dimorphic mandible in a sex-specific manner (Gotoh et al. 2014). However, although insulin signaling is a link between nutrition and developmental growth (Okamoto and Yamanaka 2015), it is unclear how this pathway regulates horn development alongside the known pathways that affect horn development, the Hedgehog pathway and The Doublesex pathway.

The protein Hedgehog (Hh) is a secreted morphogen and forms a dispersion gradient that directs tissue patterning. The range of Hh dispersion between its point of origin and the endpoint body distance is regulated by the post-translational modification of the protein that occurs within Hh secreting cells. The C-terminus is modified by cholesterol and the N-terminus is modified by palmitic acid (Briscoe and Therond 2013) for Hh to be in a fully active conformation. The sterol-recognition region of the Hh protein recruits cholesterol, and palmitic acid is added by the acyltransferase protein, Rasp (Briscoe and Therond 2013). Both modifications are required to produce a fully active Hh protein (reviewed in Eaton 2008). Recent research has shown cholesterol can directly activate Smoothed (Smo) (Ledon-Rettig et al. 2017). An extracellular cysteine-rich domain of Smo mediates cholesterol binding which dictates the activation of the protein in the presence of cholesterol or Hh ligands (Luchetti et al. 2016). As well, intracellular cholesterol modification occurs on the cytosolic C-terminal tail by covalent binding of the aspartic acid residue at the 95th position of the Smo protein (Figure 9) and is inhibited by Ptc and enhanced by Hh (Xiao et al. 2017). The modification at this residue has been shown to activate the downstream signaling in the pathway (Xiao et al. 2017). These findings may be linked, and it has been proposed that cholesterol binding at the cysteine-rich domain may occur before the

esterification reaction. Thus, the Hedgehog pathway, or more specifically how the Hh protein is modified to its fully active state and Smo activation indicates two components of nutrition (cholesterol and palmitic acid) that may impact horn growth. Based on the known functional interaction of the Hedgehog pathway, it was predicted that *rasp* knockdown would present a phenotype and measurement pattern similar to or more severe than that of the *hh* knockdown from the study done by Kijimoto and Mozcek (2016) as the protein Rasp is responsible for generating a fully functional Hh protein through palmitic acid modification. Moreover, secretion of the Hh protein may be impacted because of the impaired ability of the protein to be modified. As well, one of the ways the protein Hh can bind to its receptor, Patched (Ptc), is through a binding interface facilitated by the palmitoyl modification which derepresses Smo (as shown in Figure 9). Interestingly, by removing the palmitoyl moiety from Hh, it has been shown that formation of the Hh-Ptc complex is significantly impaired, however, some binding still occurs indicating another alternative mechanism of binding in the absence of a palmitoyl modification (Qi et al. 2018). Sequestering Hh and impaired binding to Ptc by knockdown of *rasp* would result in a decreased activity of Smo, similar to that of a *hh* knockdown, and so downstream factors influencing horn development cannot be activated.

Insects generally do not synthesize cholesterol *de novo* as they lack several enzymes downstream of mevalonate in the isoprenoid biosynthesis pathway required to synthesize sterols (Santos and

Figure 13: The isoprenoid/cholesterol biosynthesis pathway starting at acetyl-CoA. Farnesyl-PP marks a branch point, where resources are either converted to isoprenoids or sterols. The mammalian sterol branch is boxed. Insects lack homology to seven key enzymes in the sterol branch, which are marked by red arrows. Instead, insects must find an external sterol source to obtain functional amounts of cholesterol. One such known way insects do this is by converting plant sterols or phytosterols to cholesterol, as seen in the plant sterol conversion pathway. Notable phytosterols known to be converted into cholesterol are sitosterol and

Lehmann 2004, Figure 13), therefore they require an external sterol source in order to obtain cholesterol or similar molecular compound. The abundance of cholesterol in herbivore dung can be extremely low, and may be too low to fully supplement an *O. taurus* diet (Frank et al. 2017), thus it seems likely that they may rely on consumption of plant material and conversion of major plant sterols to cholesterol (Clayton 1964, reviewed in Svoboda et al. 1975, Ikekawa 1992, Frank et al. 2017) or bacterial symbiosis to fulfill dietary requirements of cholesterol (reviewed in Svoboda et al. 1975, Frank et al. 2017). Modification by cholesterol activates both Hh and Smo. This would indicate that the pathway should be sensitive to changes in the amount of available cholesterol. Therefore, addition of cholesterol would result in the loss of the small horn phenotype or an overall reduction in the number of small horned beetles, while keeping the same distribution of body size. Resource allocation, in response to an abundance of cholesterol (provided that it is a determining factor in horn size), would signal for the induction of beetle horns as it would biochemically appear that the beetles have enough free resources to dedicate to the development of nonessential factors like secondary sexual traits.

Upon ingestion, cholesterol is absorbed via the midgut and transported via High-Density Lipoprotein (HDLp) which is the sole carrier of free cholesterol in the hemolymph (Jouni et al. 2002b). Lipoproteins are insect lipoproteins. Several protein components of HDLp are homologous to that of mammalian Low-Density Lipoprotein (Babin et al. 1999). In insects, fat bodies are utilized in the storage of excess nutrients including cholesterol, lipids, and glycogen, they control the synthesis and utilization of energy reserves, and synthesize most of the circulating metabolites and hemolymph proteins and lipids (reviewed in Arrese and Soulages 2010). Carbohydrate metabolism also occurs in this organ (reviewed in Arrese and Soulages 2010). Cholesterol transfer from the midgut to HDLp, as well as to and from fat bodies by HDLp occurs through a mechanism of aqueous diffusion (Jouni et al. 2002a, Yun et al. 2002). Extrapolating from this information, introduction of additional cholesterol into larval diet should increase the overall amount of cholesterol available over the whole of the larval duration and elicit an

uptake response from HDLp lipoproteins resulting in an increased availability of hypothetically significant nutrient resources for beetle horn development (see materials and methods for more details). While the mechanisms through which circulating lipophorins provide lipids to cells are largely unknown, it is known that specific receptors mediate this exchange (Dantuma et al. 1996, Gondim and Wells 2000). The best characterized of these are the Low-Density Lipoprotein Receptor Family homologs denoted as lipophorin receptors (Dantuma et al. 1999, Rodriguez-Vazquez 2015, Matsuo et al. 2019). Through knockdown of an identified lipophorin receptor in *O. taurus*, *lipophorin receptor*, the mobilization of lipids and cholesterol in the insect body were expected to be affected, reducing the available resources for trait development. It was also predicted that this would cause an increased proportion of male *O. taurus* with a rudimentary horn size in the population of beetles because development of the large horned phenotype occurs in optimal nutrition environments.

Previous studies indicate that the isoprenoid biosynthesis pathway (Figure 13), which expresses a diverse and large group of cell-signaling and metabolic molecules (Gershenzon and Dudareva 2007), may have an effect on beetle horn development in much the same way as nutrition modification of Hh. Statin exposure or *3-Hydroxy-3-Methylglutaryl Coenzyme A* (hmg-CoA) *synthase* (*hmgcs*) knockdown (responsible for enzymatic synthesis of Hmg-CoA in Figure 13) would have a similar expected effect on the biological dispersion of the protein Hh as it too has been shown to result in the reduced dispersal and sequestering of Hh. Hmgcs has been implicated in having differential expression in *O. taurus* horn tissue (Kijimoto et al. 2009). Downstream of Hmgcs, in *Drosophila* HMG-CoA reductase (*hmgcr*, see Figure 13 for the position of this enzyme in the isoprenoid biosynthesis pathway) mutation causes abnormally high levels of Hh protein to accumulate within the cell and membranes of *hh* expressing cells, while causing a reduction in the amount of Hh protein that is transmitted to the receiving cells (Deshpande and Schedl 2005). As well, although the isoprenoid biosynthesis pathway does not contribute to the production of cholesterol in insects, the cholesterol modification of Hh appears to be

the determining factor of potentiation by *hmgcr* (Deshpande et al. 2013). In *Drosophila*, the ability of statins to inhibit hmg-CoA reductase (*hmgcr*) is conserved (Yi et al. 2006). Thus, by analogy, the efficacy of statins to inhibit *hmgcr* would be conserved in *O. taurus*. We would expect to see an effect on horn growth similar or possibly even more severe to that of the RNAi of the *rasp* transcript. Of note, however, is that *hmgcr* is also a key enzyme in the isoprenoid biosynthesis pathway of juvenile hormone (Cusson et al. 2013) and that statins have been shown to suppress juvenile hormone biosynthesis however this suppression does not appear to affect development (Debernard et al. 1994). It may affect development in *O. taurus* as the head horn is responsive to juvenile hormone.

In this research we have examined some of the known nutritionally influenced components of beetle horn development and attempted to alter nutritional availability in pathways know to affect horn size. More specifically, we have examined the effects of a reduction in the ability of Hh to be modified by palmitic acid through *rasp* knockdown and have increased the total cholesterol available during the larval stage of development. As well, we have examined the effects of lipid transport on *O. taurus* head horn development through knockdown of a lipophorin receptor (*lpr*). Finally, inhibition of the isoprenoid biosynthesis pathway was done through the inhibition of a key enzyme of the pathway using statins.

Results and Discussion

Knockdown of *rasp*

The partial sequence of the *O. taurus rasp* coding region is shown in Figure 14. This sequence data was compared and validated against a reference in the NCBI database

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CCACTATTATTCTTGGGCCCTTCATTCAATATAAAGATTTTAAAAAGTGTGCTGAGTTTAATA
ACGATCCTTTAAAATTACGTTTATTATTATTATGAAAAATTTAATTCGATTATTATTTTGGTG
GATTTTACTGATATTCTTTACATTATATTATGTAATTTTTTCCATTCTTCCCGAGTTA
GTAAAAACGTTTGATTCTGTTACTTTATATGGTTTGGGATATAGTATGGGTCATTTTTCATT
TAAATACGTTTATATATGGTTTGAGTACTACTTTTGCTCAATTTGATGGTATACCAACACC
AAATATTCAAAATGTATTGGAAGAATACATTTGTATTCAGATATGTGGAATATTTTGATAAT
GGATTGTATCAATTCTTATTAAAGTATATTATATTCCTGTAAGAACTTCACATCATCACCAT
ATGTTTCAAATATTATCTGTTTCTTATTTATTTTGTATGGCATGGTTTATGAGGATGGATAT
TTTGTGTGGGTACCTTAATTG
```

Figure 14: The *rasp* sequence assembly. Represented here is a 536 base-pair sequence derived from cDNA library of pupal RNA extraction. The region that is highlighted in green is the 195 base gene specific sequence used to create dsRNA for *rasp* the RNAi injection treatment. This sequence includes a potential variant region (highlighted in grey) that may indicate a second isoform of *rasp* so as to create a more comprehensive knockdown. The sequence highlighted in purple denotes a potential MBOAT superfamily domain.

(XP_022913834.1) and was then used to design primers to isolate a gene specific region for knockdown of *rasp*. This construct was injected into 168 *O. taurus* (both male and female) and we obtained 24 successful male knockdowns of *rasp*.

Figure 15: A comparison between *O. taurus* body and horn size in *rasp* knockdown and empty vector injected (control) individuals. (A) shows the comparison between *rasp* dsRNA knockdown males and empty vector control males. (B) shows the extrapolated sigmoidal curves of both the treatment and control groups based on a 4-parameter logistic regression analysis.

In both *rasp* knockdown and control treatments, the body/horn size relationship statistically can fit a sigmoidal curve based on a 4-parameter logistic regression analysis (Figure 15). There is no significant difference between the parameters defining the 4-parameter logistic curve between the two treatments within a 95% confidence interval (based on the difference between a single parameter compared between treatment groups, therefore, if 0 is within the range of the confidence interval there is no detectable significant difference between the treatments for that parameter. Difference in slope 95% confidence interval = [-5.1192, 11.3090], difference in inflection point 95% confidence interval = [-0.2815, 0.2811], difference in lower asymptote 95% confidence interval = [-3.2370, 4.9164], difference in upper asymptote 95% confidence interval = [-0.9025, 0.4800]). Although *rasp* knockdown did not result in any noticeable shift in absolute horn size of small or large males, there appears to be a change in the proportion of male body size in the population of treated animals as there are no beetles with a small body size in the *rasp* knockdown treatment group (Figure 15, Figure 16). As well there is a higher proportion of animals growing medium sized horns (Figure 15, Figure 16). By looking at Figure 16, it becomes more evident that we see the anticipated bimodal distribution of horn size in the control

Figure 16: Parallel plots of the relationship between body size and horn size in *rasp* knockdown and empty vector injected individuals. The plot on the left shows the control treatment and the plot on the right shows the *rasp* knockdown treatment. Each line indicates one male beetle and connects that beetle's body size to its horn size. On either side, the top indicates the largest size and the bottom indicates the smallest.

Figure 17: The cumulative distribution functions of control and *rasp* knockdown treatments. (A) shows the cumulative distribution function of horn size and (B) shows the cumulative distribution function of body size. The cumulative percentage of the population is shown on the y-axis. The line increases on the y-axis as the percentage of beetles at a particular horn size is added to the cumulative percentage of the data up to that horn size.

animals, however in the *rasp* knockdown treatment group there are no males with a small body size and there does not appear to be a gap in horn size as would normally be seen in a bimodal distribution. When looking at the empirical cumulative distribution functions (Figure

17) of the *rasp* knockdown and control injected *O. taurus*, the horn size distributions are trending towards a difference between the control and *rasp* knockdown treatments ($p > D^- = 0.0618$, Figure 17A) and there is a difference between the distributions of body size ($p > D^+ = 0.253$). Additionally, the data for horn size can be viewed as three distinct categories indicative of the bimodal distribution typically seen in male *O. taurus* beetles and the middle horn size. To further simplify the data, large and small horns were grouped together and considered the typical phenotype, where the middle phenotype would be considered the atypical phenotype and the relative percentage of beetles that fell into each of these clusters were analyzed (Figure 18). It was found that, in the control treatment, the typical trend

Figure 18: A mosaic plot of the different horn sizes between treatment groups. The typical phenotypes group is composed of all typical small and large horned beetles from the treatment specified. The atypical phenotypes group is composed of all beetles that fell into the intermediate horn size category.

was seen, where 90% of beetles fell into the typical phenotype category. In the *rasp* knockdown treatment, the phenotypes were much more evenly split. The typical phenotypes still had the majority of individuals (58.33%), however the atypical phenotype was significantly higher with 41.67% of individuals ($p = 0.0030$). This indicates that the palmitoyl modification of Hh by Rasp plays a role in the

regulation of the bimodal distribution of horn size. We see that the function of *rasp* appears necessary for the development of beetles with a small body size, as well as reducing/increasing horn size in beetles that developmentally might have produced medium sized horns otherwise. While not a completely cryptic variant, in a wild-type population the development of a medium horned phenotype is greatly reduced when compared to proportion of the large and small horned phenotypes. A medium horned phenotype would typically not be suited for the reproductively successful tactics that are seen in *O. taurus* colonies (Moczek and Emlen 2000). In this way, natural selection would favor the phenotypes that are best suited to carry out those tactics. The only known function of Rasp is as an acyltransferase (Micchelli et al. 2002, Shilo 2003). This suggests that the acyltransferase activity of Rasp is necessary to develop the reproductively favored phenotypes. In order to further examine what influence Rasp has on the horn and body size of male *O. taurus* beetles, *rasp* activity can be measured in small, medium and large horned animals using quantitative PCR. Also, to confirm the effect *rasp* has on body size, *rasp* knockdown animals can be exposed to caloric restriction to determine whether the small body size animals will develop or whether *rasp* knockdown causes a change in the lower limit of the absolute body size.

Figure 19: A comparison between *O. taurus* body and horn size in *rasp* knockdown individuals and the *O. sharpi* body and horn size relationship. (A) shows the horn and body size scaling relationship for the *rasp* knockdown treatment population. (B) Shows the relationship in another species of Onthophagine beetle, *O. sharpi*, that has a curved/bent dimorphic scaling relationship between the body and horn size. Visually the *rasp* dsRNAi relationship appears to be a bent relationship, though inverse that of *O. sharpi*. (B) is adapted from Emlen et al. 2005.

In other species of Onthophagine beetles, dimorphic relationships can appear as curved/bent (appears similarly to the isolated population curve in Figure 19, as in the case of *O. sharpi*) if not sigmoidal and there are species that lack dimorphism

and have a linear scaling relationship (as in the case of *O. pentacanthus*) (Emlen et al. 2005). Both sister tribes and many species of *Onthophagus* do not exhibit bimodal horn size relationships (Emlen et al. 2005) indicating that the linear relationship is the ancestral relationship, with species having evolved other relationships. The results from this *rasp* knockdown treatment would indicate that in *O. taurus*, *rasp* co-option into head horn development could have played a role in the evolutionary change from a linear relationship to a sigmoidal relationship. It may be worth exploring *rasp* protein interactions to further elucidate how this relationship change occurred evolutionarily.

It is important to note that *Rasp* acts as a palmitoyl acyltransferase for another protein as well, Spitz, which is required for normal embryonic development and is involved in the development of eyes, wings and legs of *Drosophila* (Shilo 2003). In response to palmitoylation, the local concentration of Spitz increases which is necessary for the normal function of the Epidermal Growth Factor Receptor (Miura et al. 2006). In many *O. taurus*, there were abnormalities seen in these body structures, indicating that knockdown of *rasp* may have caused several off-target effects. It is also important to that the horn size categories in Figure 18 and that were used in statistical analyses were determined roughly by approximating the linear portion of the sigmoidal curve in control animals. This group was considered medium horned beetles. More accurate categorical separation is needed.

Knockdown of *Lipophorin Receptor*

In *Drosophila*, there are two partially redundant *lpr* genes called *lipophorin receptor 1* and *lipophorin receptor 2* (*lpr1* and *lpr2*) and each of these genes has multiple isoforms when translated (Parra-Peralbo and Culi 2011). Sections of genome were identified in the present study that were thought to be homologous to those *lpr* genes. A putative conserved region between both genes and all of the isoforms was identified and used to perform a general *lpr* knockdown. Knockdown of *lpr* resulted

Figure 20: A comparison between *O. taurus* body and horn size in *lpr* knockdown individuals. (A) shows the comparison between *rasp* dsRNA knockdown males and empty vector control males. (B) shows the extrapolated sigmoidal curves of both the treatment and control groups based on a 4-parameter logistic regression analysis.

in the same logistic 4-parameter sigmoidal curve fitting the data as the control (Figure 20B). There is no significant difference between the parameters defining the 4-parameter logistic curve between the two treatments within a 95% confidence interval (based on the difference between a single parameter compared between treatment groups, therefore, if 0 is within the range of the confidence interval there is no detectable significant difference between the treatments for that parameter. Difference in slope 95% confidence interval = [-5.2274, 7.1043], difference in inflection point 95% confidence interval = [-0.1350, 0.0438], difference in lower asymptote 95% confidence interval = [-0.7394, 1.0646], difference in upper asymptote 95% confidence interval = [-0.4267, 0.2675]). However, when looking at Figure 20A we can see that although there is an overlap between the two treatment datasets, there are many fewer small horned males and even fewer males with small bodies. When looking at the empirical cumulative distribution functions for horn size between the two treatment groups (Figure 21A) we see

Figure 21: The cumulative distribution functions of control and *lpr* knockdown treatments. (A) shows the cumulative distribution function of horn size and (B) shows the cumulative distribution function of body size. The cumulative percentage of the population is shown on the y-axis. The line increases on the y-axis as the percentage of beetles at a particular horn size is added to the cumulative percentage of the data up to that horn size.

more clearly that there is a higher percentage of large horned males in the *lpr* knockdown. This treatment has been shown to cause a change in

the distribution of beetles between the two treatments ($p > D = 0.0327$). Interestingly, there is also a change in the distribution of body size (Figure 21B, $p > D = 0.0214$) where, in the *lpr* knockdown treatment group, we see that there are no beetles at the smallest body sizes of the control and a higher percentage of beetles have a larger body size. The categorical separation of beetles (Figure 22) partially reflects the changes we are seeing in the horn size distribution. We see represented in the mosaic plot that the large horned category is trending to have a higher percentage of beetles present in the *lpr* knockdown group than in the control group ($p = .0640$).

This data would seem to indicate that knockdown of *lpr* causes the overall body and horn size to increase so that a higher number of larger and fewer smaller beetles are seen along the same typical sigmoidal curve shown in a wild type population. As stated above, the roles of LpR in lipid transport are not fully understood, however LpR has been shown

Figure 22: A mosaic plot of the different horn sizes between the control and *lpr* knockdown treatment groups. The categorical separation is based on horn size. Beetles were grouped into small, medium, and large horned categories and each block represents the relative percentage of beetles within that category within a treatment group.

to have effects in the lipid transport system in insects. It would therefore be expected that knockdown would interfere with lipid mobilization in the insect body resulting in more lipids sequestered (in fat bodies or lipophorins) and unable to be utilized in the insect bodies. While this could possibly explain the increase in body size, it would not account for the increase in horn size because this would, in theory, be taking away a resource to be utilized in the development of nutritionally responsive traits. Mass spectrometry could be used to quantify lipids in the hemolymph between control and *lpr* injected pupa to determine if *lpr* knock down is causing a reduction in the utilization of lipids. One explanation for why we are seeing this change in horn size in conjunction with body size comes from the Hedgehog pathway as it is both nutrition responsive and affects the development of rudimentary horns in small males. It is also of interest to note that the protein, Ptc, has been shown to function as a lipophorin receptor and lipid internalization caused by Ptc has been shown to affect Hh gradient formation (Callejo et al. 2008). To determine if any changes are occurring in the Hedgehog pathway due to this knockdown quantitative PCR could be done measuring *ptc* expression in both the control and *lpr* knockdown animals. Another possibility to see the effect of lipid transport in horn development of *O. taurus* would be to examine the effects of Adipokinetic Hormones (AKH), a family of neuropeptides responsible for the mobilization of lipids and other molecules from fat bodies (reviewed in Gade and Auerswald 2003). Instead of targeting the shuttling of lipids, interference of AKH could sequester resources inside of fat bodies preventing utilization of lipids by lack of mobilization.

Cholesterol

The first method of cholesterol administration to larvae was injection. This method was chosen over dietary addition as the amount given to each larva could be standardized and controlled much more easily to produce consistent findings. However, cholesterol is not commonly soluble in many compounds, and many cholesterol solvents are harmful to organisms. It appears that the combination of solvents, larvae being developmentally sensitive organisms and the stress of injection, in most cases,

Figure 23: The results of the cholesterol injection trials. The trials were done with known cholesterol solvents. In all trials involving ethanol, 32.5 g/mL of cholesterol was dissolved in 100% ethanol, and then diluted. Cyclodextrin mixed with cholesterol had a stock value of 10 mg/mL and then was diluted as well. The total number of beetles that died was generated by subtracting the number of beetles injected that made it to adulthood from the total number of beetles injected.

Treatment	Cholesterol Concentration (mg/mL)	# injected	# alive at prepupa	# alive at pupa	# alive at adult
3 ul 0.01% Triton	0	9 (6 injected twice)	7	4	1
3 ul 0.1% Triton, 5% EtOH control	0	12 (8 injected twice)	3	3	3
3 ul 0.01% Triton, 5% EtOH	0	12 (4 injected twice)	2	2	1
3 ul PBS, 0.01% Triton	0	22 (10 injected twice)	12	9	2
3 ul 100% DMSO	0	30	5	5	0
3 ul 25% DMSO	0	25	7	5	2
3 ul 5% DMSO	0	21	4	2	1
3 ul 100% EtOH	0	6	0	0	0
3 ul 5% EtOH	0	97 (53 injected twice)	47	23	8
3 ul PBS, 0.01% Triton, 0.5% EtOH	0	6	0	0	0
3 ul 5% EtOH + Cholesterol	1.6	29 (17 injected twice)	15	7	3
3 ul PBS, 0.01% Triton, 0.5% EtOH + Chol	1.6	112 (42 injected twice)	3	2	0
3 ul Cyclodextrin	0	2	0	0	0
3 ul Cyclodextrin + Chol	12	24	1	1	1
3 ul Cyclodextrin + Chol, 20 x dil	0.2	35	18 (Later L3 injection)	0	0
3 ul Cyclodextrin + Chol, 50 x dil	0.1	24	0	0	0
3 ul Cyclodextrin + Chol, 100 x dil	0.5	8	0	0	0
3 ul Cyclodextrin + Chol, 500 x dil	0.02	3	0	0	0
Totals: 455 total dead/477 total injected <i>O. taurus</i> from cholesterol injection trials					

resulted in death as seen in Figure 23. While DMSO and ethanol are known to have harmful effects on organisms, cyclodextrin is a ring structure of glucose subunits, and death occurred even at incredibly low concentrations of solvent. The results and the lack of samples to measure of the injection study led to dietary addition of cholesterol. While this method resulted in reduced control over the amount of known cholesterol introduced into the system and is impacted by inconsistent homogeneity of the mixed dung with solid cholesterol crystals, the advantage is that the larvae are provided with supplemental cholesterol over the whole of the larval period, instead of introducing a sharp rise in available cholesterol around the critical period.

As seen in Figure 24, the introduction of additional cholesterol into the diet of the larvae from a period just after hatching from an egg resulted in very similar overlap in the body/horn size relationship between the treatment groups and the control groups. This relationship overlap is the most pronounced in Figure 24D, which indicates that there is no threshold shift or overall differences in maximum or minimum horn size on either side of the threshold caused by increased dietary cholesterol. Interestingly, the compilation of datasets so that all data points are on one graph forms a complete

Figure 24: A comparison between *O. taurus* body and horn size in individuals with dietary cholesterol added during the larval period and without cholesterol added (control). Each data point reflects the body width and average horn size of one adult male *O. taurus*. All experimental groups had the same concentration of cholesterol added. (A) (B) and (C) had different overall quantities of dung divided approximately equally among 12 wells. (A) 50 g total dung, (B) 20 g dung, (C) 12.5 g dung. (D) represents all data points combined into one graph.

Figure 25: The combined data sets body/horn size relationship fit to a Logistic 4-parameter (sigmoidal) curve. The triangles pointing up are data points from the 50 g treatment group, the circles are data points from the 50 g treatment group, and the triangles pointing down are data points from the 50 g treatment

sigmoidal curve (fit by a logistic 4-parameter model) (Figure 24D, Figure 25). As the quantity of dung only matters for growth in terms of nutritional content, this is unsurprising, and the combination of these treatments can be considered as an overall treatment of adjusted nutritional content. It has been shown starvation influences the growth of the beetle (Shafiei et al. 2001) and as such the reduction in size is to be expected when reducing the quantity of dung. Treatments can then show any altered expression

of phenotype, especially when compared to controls at the same amounts of dung across the whole sigmoidal curve. The parameters defining the logistic 4-parameter curve of the combined data set are the same between the combined cholesterol and control datasets within a 95% confidence interval (based on the difference between a single parameter compared between treatment groups, therefore, if 0 is within the range of the confidence interval there is no detectable significant difference between the treatments for that parameter. Difference in slope 95% confidence interval = [-4.4745, 5.7880], difference in inflection point 95% confidence interval = [-0.0810, 0.0417], difference in lower asymptote 95% confidence interval = [-0.2723, 0.3127], difference in upper asymptote 95% confidence interval = [-0.3986, 0.3658]). The fundamental differences between the different treatments of this combined dataset are such that the combined dataset is not suitable for further statistical analysis.

When looking at the categorical horn sizes of the treatment groups allocated 12.5 g of dung as larvae and the treatment groups allocated 20 g of dung, no statistical difference is found between the

Figure 26: Mosaic Plots of the different horn sizes between non-cholesterol and cholesterol supplemented treatment groups allocated varying amounts of dung as larvae. The categorical separation is based on horn size. Beetles were grouped into small, medium, and large horned categories and each block represents the relative percentage of beetles within that category within a treatment group. (A) Represents treatments allocated 12.5 g of dung as larvae. (B) Represents treatments allocated 20 g of dung as larvae.

treatments within these different groups (Figures 26A and 26B, $p = 0.5091$ and $p = 0.2174$ respectively). This could in part be due to the low sample size and categorical separation within

the treatment groups (12.5 g dung + cholesterol added: $n = 12$, 12.5 g dung control: $n = 19$, 20 g dung + cholesterol added: $n = 12$, 20 g dung control: $n = 12$) so we don't have an accurate view of the

distribution in each treatment. The number of non-cholesterol supplemented beetles in the 50 g dung treatment group was too low ($n = 5$) to do statistical analysis against the cholesterol supplemented treatment group. *O. taurus* should be sensitive to changes in nutrition for horn growth but it is possible that we didn't supplement enough cholesterol to have a noticeable effect. Pursuing this research further, using similar nutrition restriction conditions as a control, we could use the introduction of different concentrations of cholesterol to see if the large horned, large male phenotype is rescued with beetles allocated quantities of dung that mostly results in the development of small horned, small males (e.g. 12.5 g dung), which would indicate how key a component cholesterol is to the development of large horned males and could mean that the increase in cholesterol is influencing development to produce large horns when all other nutritional factors are kept constant.

Atorvastatin Supplementation

Figure 27A shows the body/horn size relationship data for a small population of statin treated beetles. When compared to the control population, there is no statistical difference between the values of the parameters of the relationship of the body and horn size of the statin supplemented beetle population and a population fed with non-supplemented dung (Figure 27B). The confidence interval for

Figure 27: (A) A comparison between *O. taurus* body and horn size in individuals with statins added to dung during the larval period and without statins added (control). Each data point reflects the body width and average horn size of one adult male *O. taurus*. **(B)** An equivalence test for the populations fit to the same Logistic 4-parameter curve. The non-supplemented treatment group is used as the reference. For each parameter, the line represents the confidence interval of the ratio between the treatment groups. UDL and LDL are the upper and lower decision limits of the ratio between the statin and non-statin supplemented groups. All confidence intervals cross the decision limits indicating no difference between the parameters.

Figure 28: A linear regression model of the statin supplemented male *O. taurus*. Each data point reflects the body width and average horn size of one adult male *O. taurus*.

the slope may be large because the slope of the non-supplemented control group was driven by the presence of only three rudimentary horned beetles (Figure 27A). More replication could reduce the confidence interval. Interestingly, in the statin treated group the regression curve that best fits is linear (Figure 28, based on AICc's: Linear – 18.99, Logistic 3-Parameter – 20.72,

Quadratic – 22.09, Logistic 4-Parameter – 27.46). This contrasts with the non-supplemented group, which maintains the sigmoidal curve typically seen in a population of *O. taurus* (AICc's: Logistic 4-Parameter – 26.45, Linear – 33.54, Logistic 3-Parameter – 36.00, Quadratic – 37.40). When looking at the cumulative distribution of horn size between the treatments (Figure 29), we see that the distribution is trending toward being different as well ($p > D = 0.0618$), indicating that there may be a difference between the proportional distributions of the different treatments, supporting a shift in prevalence of

Figure 29: The cumulative distribution function of control and statin treatments. The cumulative percentage of the population is shown on the y-axis. The line increases on the y-axis as the percentage of beetles at a particular horn size is added to the cumulative percentage of the data up to that horn size.

different body/horn size relationships. This data is from a small sample size and therefore needs more replication to verify the validity of the shown relationship. The isoprenoid biosynthesis pathway synthesizes a vast array of signaling molecules. As such, although the proximate cause of the any changes in the bimodal distribution of horn size would be part of this pathway. Should further replication confirm a change in the population body/horn size relationship, the known interactions of the pathway with effectors of horn size

could be examined in this pathway (Figure 30). Any change in the population body/horn size relationship could be seen to occur possibly due to the downstream changes in Hh Signaling, juvenile hormone titers or both and targets could be identified beyond the branch point of the isoprenoid biosynthesis pathway and knocked down so that each of these implicated factors are affected individually.

Figure 30: The isoprenoid/cholesterol biosynthesis pathway starting at acetyl-CoA. Farnesyl-PP marks a branch point, where resources are either converted to isoprenoids or sterols. Insects lack a cholesterol biosynthesis pathway. Juvenile hormone is a product of isoprenoid biosynthesis, as well as modification of the protein Gy1 which is known to affect Hh dispersal.

There is some significance to a linear model fitting the statin supplemented population body/horn size relationship better. Kijimoto et al. (2012) and Kijimoto and Moczek (2016) found that

co-option of two different pathways affected the development of individuals morphs of the *O. taurus* head horn. This co-option, in part, is involved in the evolution of the head horn from linear, continuous developmental plasticity, to a special case of plasticity, polyphenism. The linear body/horn size relationship is considered the ancestral condition with several Onthophagine beetle species having evolved the polyphenic relationship. The amount of development plasticity a trait exhibits is highly variable between traits. Here, the evolution of polyphenism in horn development is indicative of evolution of increased canalization (evolution of decreased plasticity). The linear curve being the model that best fits the statin supplemented treatment group indicates that more replication is needed to investigate the relationship between the isoprenoid biosynthesis pathway and *O. taurus* horn development.

The nature of the interaction in genetic regulatory systems is responsive. Specific signaling

molecules can have widespread regulatory impacts on genetic expression and the development of traits. Metabolites not only maintain homeostatic conditions, but signal for responses based on environmental input. Here we demonstrate and give evidence for specific metabolites playing a critical role in development, a field which is mostly unexplored and becoming a topic of interest in several fields of developmental biology.

Materials and Methods

O. taurus were collected at the Animal Sciences Farm in Morgantown, West Virginia. After collection, all *O. taurus* were kept in colonies of ~350 beetles per colony in a sand/soil mixture. Colonies were kept in an incubator at 26°C. Cow manure was used to feed twice a week. To obtain larvae, colonies were sifted using 1 cm² wire mesh and breeding containers containing packed, moist sand/soil mixture and cow manure were set up once a week. Breeding containers contained four males and six females. Brood balls were collected after seven-eight days. Larvae were collected after 10 days and transferred to 12-well plates. Dung was squeezed using cheese cloth to get rid of extra moisture to provide an optimal environment for larvae to grow in the 12-well plates. At ~45 days after the egg was laid, if alive, the beetles reached adulthood. Larvae were identified as male or female by examining genitalia primordia. This identification was confirmed by the development of horns as pupae and again as adults.

Isolating and sequencing *O. taurus* gene *rasp*

Sequence data was found from the genomic sequence of *O. taurus* from the i5k database (<https://i5k.nal.usda.gov/webapp/blast/>) which was compiled from transcriptomic data from previous studies and used to make primer sequences (Protein sequence corresponds with NCBI accession number: XP_022913834.1) RNA was extracted from pupal samples and reverse transcription was done using the QuantiTect Reverse Transcription Kit. RT-PCR was used to amplify cDNA fragments of interest which

were cloned into pSC-A vector from the Agilent StrataClone PCR Cloning Kit. Fragments were sent to Operon for sequencing. Sequence was verified against known *rasp* sequences (sequence length amplified - 538 bases). The full-length *rasp* sequence was analyzed and a gene specific region of *rasp* was isolated following the same protocol to amplify cDNA fragment of interest. Total sequence length of fragment amplified: 168-bp. dsRNA was synthesized from 168 bp fragment.

***rasp* Knockdown**

To generate dsRNA, the region of interest was determined using sequence data obtained from the protocol above. The region

was amplified using PCR and

cloned into pSC-A vector using the Agilent StrataClone PCR Cloning Kit. The vector was

purified using a miniprep kit. The product from the Miniprep

purification was then used as a

template for PCR using M13

primers. This PCR product was

used for in vitro transcription.

The MEGascript T7 and T3 Kit

were used to produce forward

and reverse RNA strands. Equal

amounts by weight of sample

from the T7 and T3 in vitro

transcription were mixed and

Map for the StrataClone PCR Cloning Vector pSC-A-amp/kan

Figure 31: Generated dsRNA products used for knockdown are derived from a non-conserved 150 – 300 bp fragment of the gene of interest that will be recognized as foreign short dsRNA when injected, reduced to shorter fragments by Dicer and used by the RISC complex to silence gene transcripts. Isolating the gene from cDNA and forming the dsRNA involves cloning of the gene into a vector. The dsRNA product will include additional bases from the T3 primer binding site to the T7 primer binding site, as seen above labeled in the figure, as well as the PCR Product because of this. To account for any possible effects caused by the additional bases, an empty vector control injection is used, which is dsRNA generated from the bases from the T3 primer binding site to the T7 primer binding site without the addition of the PCR product. Results are compared to the control. From StrataClone PCR Cloning kit manual.

then heated at 70°C for 10 minutes then put on ice. Samples were then incubated at in hot water at 80°C, allowing the water temperature to cool. Concentration was measured and a gel was run to confirm the result. A specialized syringe and needle were used for microinjection. Most of the larvae injected with 3 ul containing 1 ug of *rasp* dsRNA were injected once during the first five days of the third instar. This was done so that the RNAi would be effective during the critical stages that determine the outcome of horn development.

Empty vector cells containing an empty pSC-A vector were harvested from a glycerol stock stored at -80°C. These empty vector cells were prepared from cells that were not transformed during the above process using the StrataClone PCR Cloning Kit. Miniprep and M13 primers were used to create a sample ready for dsRNA synthesis. The above dsRNA synthesis process was repeated using the purified and M13 prepped pSC-A vector sample to synthesize empty vector dsRNA that would be used as a control injection to compare against the *O. taurus* injected with *rasp* dsRNA. Injections were done following the protocol above.

A total of 168 *O. taurus* were injected with *rasp* dsRNA and 108 *O. taurus* were injected with empty vector. Once injected, beetles were kept in a 12-well plate until adulthood with dung allocated per well by hand. The body and horn size of 24 *rasp* dsRNA injected males and 40 empty vector injected males were measured as described below.

***lpr* Knockdown**

dsRNA of *lpr* was synthesized and *O. taurus* larvae were injected using the same protocol described in the above *rasp* knockdown section.

A total of 168 *O. taurus* were injected with *rasp* dsRNA. Once injected, beetles were kept in a 12-well plate until adulthood with dung allocated per well by hand. The body and horn size of 31 *lpr* dsRNA injected males and 40 empty vector injected (the same animals as the *rasp* study) males were

measured as described below.

Cholesterol Injection

Various solvents were used to solubilize cholesterol. 1 mL of 100% Ethanol was used to dissolve 32.5 mg of cholesterol. attempts were made to dissolve cholesterol in 100% DMSO and 100% triton. A 10 mg/mL cholesterol in 181 mg/mL cyclodextrin solution was obtained from the company Aquaplex and compared against a 181 mg/mL control cyclodextrin stock. See Figure 23 above for all solvents used. 3 uL of solvent (control animals) or solvent + cholesterol (treatment animals) were injected into 477 day six larvae. The first 140 that lived two-three days after injection were injected a second time, two-three days after the first injection.

Cholesterol Feeding Study

A total of 187 day one-two *O. taurus* larvae were transferred into cholesterol supplemented dung. A total of 91 day one-two *O. taurus* larvae were transferred into dung without cholesterol supplementation. Male *O. taurus* that survived until adulthood had body and horn size measured as described below. A consistent concentration of 1.4 mg of cholesterol per g of dung was added for standard supplementation of the dung. Each 12-well plate (one larva per well) had a total of 50 g of dung (70 mg of cholesterol) allocated and split evenly into separate wells by eye (~4 g per well). The first 51 larvae with cholesterol supplementation died including 24 with reduced cholesterol supplementation (a total of 12 beetles supplemented with 0.28 mg cholesterol per g dung and a total of 12 beetles supplemented with 0.14 mg cholesterol per g dung). Dung was then squeezed on the day of transfer and mortality rates drastically decreased (only 32 of 136 larvae in cholesterol supplemented dung died after this point). A total of 28 male larvae were measured when they reached adulthood. A total of 55 larvae were transferred to another set of 12-well plates were made by dividing 50 g of transfer dung with no cholesterol added into a 12-well plate evenly by eye. A total of five males with no cholesterol

supplementation were measured and acted as a control group.

A second treatment of 36 cholesterol supplemented and 36 non-supplemented beetles were transferred using the above protocol, but with 12.5 g of transfer dung per plate (a quarter of the original amount). Dung used for transfer had 18 mg cholesterol added for a final concentration of 1.4 mg of cholesterol per g of dung. A total of 12 cholesterol supplemented males were measured. A total of 19 males with no cholesterol supplementation were measured and acted as a control group.

A third treatment of 24 cholesterol supplemented beetles were transferred using the above protocol, but with 20 g of transfer dung per plate. Dung used for transfer had 28 mg cholesterol added for a final concentration of 1.4 mg of cholesterol per g of dung. A total of 12 cholesterol supplemented males were measured. The same control males as the statin experiment were used for this treatment.

Statin Study

Dung was allocated into 12-well plates using the same methods as the Cholesterol study. The dung was supplemented with 0.2 mg of atorvastatin/g of dung. Each plate had a total of 20 g of dung and 24 *O. taurus* larvae were transferred into these statin supplemented plates. Dung was replaced using the same methodology every week, exactly seven days, after the initial plates were made until all larvae pupated. Dung without statins added was allocated into 12-well plates that were made using the same protocol and 24 *O. taurus* larvae were transferred into these plates. A total of 11 statin supplemented males were measured. A total of 12 males with no statin supplementation were measured and acted as a control group.

Measurement of Body and Horn Size

Adult horn and body size were measured using a Leica microscope at 1.6x magnification with a camera attachment to obtain an image. ImageJ software (<https://imagej.nih.gov/ij/>) was used to

measure. Measurements used a 5 mm calibration target to calibrate scale. Thorax width (at the largest point) was used to measure body size and horn size was measured from eye to the tip of the horn following the outer curvature of the horn. Data recorded and graphs made in excel.

Statistics

In all statistical analyses, significance criterion alpha for all tests was 0.05 and a statistical trend was declared when $p < 0.1$.

Data were analyzed using JMP and SAS software (JMP®, Version Pro 14.0, SAS Institute Inc., Cary, NC, Copyright ©2015; SAS®, Version 9.4, SAS Institute Inc., Cary, NC, Copyright ©2002-2012). Significance criterion alpha for all tests was 0.05.

In all regression analyses, the model that was chosen to best fit the data was determined by Akaike Information Criterion, corrected (AICc). The model with the lowest AICc was determined to best fit the data.

All cumulative distribution functions were compared using Kolmogorov Smirnov Asymptotic Test. Values less than 0.05 were considered to show a difference in distributions.

All Mosaic Plots were evaluated by creating a Contingency Table and evaluating the values using a Pearson Test. In the case of LPR this was evaluated using a Fisher's Exact Test to account for low cell counts (three small horned and three medium horned beetles total) in the LPR RNAi treatment. Mosaic plots were grouped by small, medium, and large horned beetles, not taking into account body size. Horn size categories were determined roughly by approximating the linear portion of the sigmoidal curve in control animals. This group was considered medium horned beetles. More accurate categorical separation is needed however in this case the linear portion was approximated to be the inflection point ± 1 mm. This resulted in a medium horn size category between 1.18 mm and 3.18 mm. All beetles with

a horn size smaller than this were considered to be in the small horned category and all beetles with a horn size larger than this were considered to be in the large horned category.

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Chapter 3: Conclusions and Perspectives

This research demonstrates that the Gene Regulatory Networks (GRNs) governing the developmental systems that influence polyphenic horn development are complex and involve numerous components. This research focuses on the regulatory control of only part of a vast schema of interacting components of the networks.

The understanding of co-option, deployment, and regulation of GRNs in novel evolutionary traits helps to solidify our perspective on evolution and development as well as how life-history can impact both systems. It is known that beetle horns are evolutionarily novel and in the case of Onthophagine beetles some have evolved even further from a traditional linear plasticity, to a nutrient responsive polyphenic plasticity. This shift in developmental ability occurs through the interaction of GRNs. The purpose of this research was to elucidate regulatory mechanisms of these networks through identified effectors of resource allocation, Rasp and lipophorin receptors, and specific nutritional factors, cholesterol and palmitic acid. It was found that the regulation of the allocation of specific nutrients, not only overall nutritional input can influence the development of this polyphenic trait.

How is resource allocation mediated in horn development? Casasa and Moczek (2018) gave us our first insights that insulin signaling, a known mediator of nutrition responsive development, may be partially responsible. We still must investigate if there is a link between insulin signaling and the regulation of cholesterol and palmitic acid allocation for horn development. As well if there are any other key metabolites influencing horn development. While palmitic acid and cholesterol were previously known to impact horn development through the Hedgehog pathway and there is a loose link between *doublesex* and the insulin signaling pathway, any other potential nutritional factors that influence horn development are unknown. A metabolomics study could clarify this on a broad scale now that known effectors have been exhausted.

Insights into how nutritional resource availability impacts developmental systems have only begun to show the impact of specific metabolites. As well, models of systems governing development of evolutionary novelties are finally gathering enough information to further support the theory of evolution by genetic accommodation by revealing the mechanisms of GRNs involved in novel trait development. The potential for co-option of GRNs under conditions of canalization necessary for evolutionary changes to occur are being further understood. The research in this paper reflects these modern perspectives of development and evolution in the pursuit of furthering them.

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