SEX, WAR AND DISEASE: THE EFFECTS OF INFECTION ON HORN SIZE AND INTRA-SEXUAL COMPETITION IN THE BROAD-HORNED FLOUR BEETLE, *GNATHOCERUS CORNUTUS*

by

AMRITA NAIDU

Presented to the Faculty of the Graduate School of
The University of Texas at Arlington in Partial Fulfillment
of the Requirements
for the Degree of

MASTER OF SCIENCE IN BIOLOGY

THE UNIVERSITY OF TEXAS AT ARLINGTON
December 2009

ACKNOWLEDGEMENTS

I am extremely grateful to Dr. Jeff Demuth for his advice and exceptional guidance throughout my project. I am thankful that I got an opportunity to learn under the supervision of such an awesome research scientist. I would like to convey my sincerest gratitude to Dr. Laura Mydlarz for her continued support and for allowing me to use her lab for my research work. I would like to thank Dr. Daniel Formanowicz for kindly serving on my committee.

I am especially thankful to Donna Kirkland, Eldon Prince, Brian Fontenot, Eric Watson and other fellow graduate students and friends for their invaluable assistance. Thank you to Dr. Sherman Hendrix at Gettysburg College who provided the parasite eggs to infect my beetles with.

I dedicate this work to my father, Raju Naidu, who wanted to see me enjoy a life of sensitivity, accomplishment, goodness and love but did not live to realize it. Had it not been for the continuous encouragement and unfailing support from my mother, Bela Naidu, I would never have made it to the US. Thank you mom for believing in my abilities and thank you to my sisters Archana Basnyat and Deepika Naidu for standing by me always.

Words are not enough for me to thank my husband, Shankar Bhandari, for going above and beyond anyone's expectations to see to it that I completed my degree. I am very proud of you and I love you.

November 20, 2009

ABSTRACT

SEX, WAR AND DISEASE: THE EFFECTS OF INFECTION
ON HORN SIZE AND INTRA-SEXUAL COMPETITION
IN THE BROAD-HORNED FLOUR BEETLE,
GNATHOCERUS CORNUTUS

Amrita Naidu, M.S.

The University of Texas at Arlington, 2009

Supervising Professor: Jeffrey P. Demuth

Sexual selection is widely used to explain the evolution of mating systems where most often it is manifest as the competition among males for access to females. This competition takes the form of direct male-male interactions in broad-horned flour beetle *Gnathocerus cornutus* and often results in exaggerated male phenotypes.

Sexually selected traits are interesting because in many cases their existence appears to contradict natural selection. For example, the "handicap hypothesis" suggests that there may be a trade-off between immune response and horn size because mounting an immune response necessary for survival (natural selection) may divert resources away from growing longer horns that are required to garner mating opportunities (sexual selection). In contrast, the "good genes hypothesis" suggests that the degree of expression in secondary sexual traits is indicative of males' overall fitness, and therefore should be positively correlated with other fitness related traits such as immunity.

I tested these opposing hypotheses about sexual selection using the eggs of *Hymenolepis diminuta* (the rat tapeworm) to infect *G. cornutus* larvae and then measuring the correlation between adult horn size and immune protein level. As a result of infection with *H. diminuta*, the beetles developed shorter horns that imposed a direct disadvantage to them in male-male competition. Growth and maintenance of secondary sexual trait in the form of beetle horns in males did not impose a trade-off in the constitutive levels of immune protein in their bodies but rather advertised their increased ability to resist the detrimental effects of parasitism.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Sexual Selection

Exaggerated, sex-specific traits are interesting because in many cases their existence appears to contradict natural selection. A difficulty for Darwin's theory of evolution by natural selection was trying to understand why males of many animals evolved conspicuous traits that probably reduced survival. Darwin developed the idea of sexual selection, wherein individuals of one sex, typically males, struggle amongst themselves to "possess" members of the opposite sex rather than the more general struggle of all individuals for limited resources and survival (Darwin, 1859, 1871). Since exaggerated sexual traits are typically lacking in one sex, it suggests that these traits do not enhance overall survival. If such traits did improve survivorship, we would expect them to be expressed in both sexes (Wallace, 1868). Wallace (1868) also observed that many male-limited phenotypes, such as the bright plumage of birds, the antlers of some deer and the calls of male frogs, are displayed only during the breeding season. Considering the cost of the growth and development of some exaggerated male structures, he reasoned that these traits would be displayed all year round if they were advantageous in ways other than mate acquisition.

Male reproductive success varies more than female reproductive success in polygamous breeding system where females invest many more resources in gametes and often also in parental care, and males compete to mate with these females; as from the viewpoint of males, females are a scarce resource. (Kodric-Brown and Brown, 1984; Bateman, 1948; Trivers, 1972; Shuster and Wade, 2003). Bateman (1948) showed why sexual selection in *Drosophila melanogaster* is usually strongest in males. There was a strong correlation in males

between the number of mates and number of progeny. Female fecundity, however, did not increase if she mated with more than one male.

Sexual selection can be manifest as either direct or indirect competition among males for access to females, and those who win are able to mate with more females than losing males and as a result sire more offspring (Darwin, 1859; Shuster and Wade, 2003; Andersson, 1994). Direct competition takes place when males combat each other for access to females. Antlers of some deer and other ungulates are used in head-to-head combat during the mating season where the winning male gets the opportunity of mating with does (Clutton-Brock, 1982). In the male fungus beetle, *Bolitotherus cornutus*, the pronotal horns are used in aggressive encounters where the males often aggressively chase each other away from fungi containing females (Conner, 1988). The winner of the encounter then courts the female and attempts to mate. Hence, antlers in deer (and other ungulates) and horns in beetles are used in male-male combat for mates and are pivotal component of male reproductive fitness (Shuster and Wade, 2003).

Indirect competition is involved when the exaggerated traits of some males do not have a direct and plausible connection to male-male combat. Petrie and Williams (1993) reported that in peafowl (*Pavo cristatus*), peahens preferred to mate with and produced more eggs for those peacocks with more elaborate trains. Red jungle fowl roosters (*Gallus gallus*) that have large combs do not behave differently from the ones that have small combs but females mate quickly and show a preference for large-combed males (Zuk et al., 1990). Besides the male courtship displays of traits that influence female choice, there are other mechanisms to increase male reproductive success. The decorative bowers built by male satin bowerbirds (*Ptilonorhynchus violaceus*) are used as sites for courting females and mating takes place in them. A significant relationship between bower quality and male mating success suggests that these bowers provide females with information regarding the relative quality of the males (Borgia, 1985). Both

direct and indirect competition result in exaggerated male phenotypes, which, Darwin postulated, were beneficial to male reproductive fitness but not survival.

There are two general hypotheses for the evolution of exaggerated male (or female) traits that arise as a consequence of sexual selection. First, the handicap hypothesis posits a conflict between sexual selection and natural selection where sexually selected traits benefit an individual's probability of successful mating at the expense of other fitness aspects (e.g. survival). The alternative hypothesis is that exaggerated traits serve as an indicator of genetic superiority. This so called good-genes hypothesis suggests, for example, that the offspring of a female who mates with a showy male will accrue an indirect benefit by inheriting his superior genes.

1.1.1 The Handicap Hypothesis

Several types of natural selection may limit secondary sex traits. For example, secondary sex traits are expressed at a cost in males where signaling or searching for mates can lead to higher predation (Andersson and Iwasa, 1996). These traits can also impose energetic and foraging costs. For example, fights over territories or females may cause injury or death and males that are sexually selected for larger body size in some birds and mammals are more prone to starve than females during the growth period (Andersson and Iwasa, 1996). Gustafsson et al. (1995) demonstrated that life-history traits and secondary sexual characters trade off against each other in male collared flycatchers (*Ficedula albicollis*). Males with large forehead patches mate with more females and have higher lifetime reproductive success. An intra-generational trade-off between parental effort and the size of the male's forehead patch (a secondary sexual character) in the following year was reported. The size of the forehead patch in first-year males was negatively related to the change in brood size of the nest in which they were raised, suggesting an inter-generational trade-off (Gustafsson et al., 1995).

Zahavi (1975) suggested that the development and maintenance of secondary sexual characters confers a handicap on survival, where the larger the effect of mate preference, the

more developed the character and the larger the handicap imposed. His “handicap hypothesis” proposes that the handicap be considered as a kind of a test imposed on the individual and hence, an individual with a well-developed sexually selected character has survived a test. The females that choose these males can be sure that they have selected from among the best genotypes of the male population.

1.1.2 The Good-genes Hypothesis

The good-genes hypothesis suggests that the degree of expression in secondary sexual traits is indicative of males’ ability to resist parasites or pathogens and therefore have important effects on fitness (Hamilton and Zuk, 1982). According to this hypothesis, females choose mates using male traits that are genetically correlated with total fitness and, as a result, greater elaboration of the preference and male trait will occur. Then a male who is unmistakably outstanding in health and vigor offers females that mate with him an inherited advantage in their offspring (Hamilton and Zuk, 1982). For example, female North American house finches prefer to mate with bright, colorful males. High parasite load in these finches is correlated with reduced development of bright male plumage. Hence, the preference for males with bright plumage suggests that mating with such males will increase offspring survival (Thompson et al, 1997). Moller (1994) showed that offspring viability was correlated to the degree of male ornamentation in barn swallows. Antler development and body mass may be associated with pathogen resistance in white-tailed deer (Ditchkoff et al, 2001). In addition, a negative relationship was observed between the degree of antler development and overall abundance of abomasal helminths (Ditchkoff et al, 2001). Hence, the development of secondary sexual characters can be an honest advertisement of male quality. Sexual selection and the potential role of parasites is an interesting research topic as the interaction between host and parasite can produce cycles of coadaptation, which ensure a continual source of fitness variation among genotypes.

1.1.3 Beetle Horns – An Example of a Sexually Selected Trait

Beetle horns are fascinating structures because of their evolutionary novelty and extraordinary diversification (Moczek et al., 2007). They are rigid outgrowths of the exoskeleton that may be located on the head or thorax either in the centre or on the sides (Figure 1.1). They may be straight, curved or branched and may also vary in allometry. Species may also differ in presence/absence and dimorphism (male dimorphism or sexual dimorphism) (Emlen et al., 2006). Out of the over 120 different families of beetle, the Scarabaeidae represents the vast majority of horned species (Emlen et al., 2006). A phylogenetic analysis of 48 species from the genus *Onthophagus* revealed 25 changes in the physical location of horns and extensive variation in horn size and shape (Emlen et al., 2005b).

Figure 1.1: Scarab beetles with horns. Sample taxa illustrating the extreme size, and some of the shapes, of beetle horns: top: *Dynastes hercules* (Dynastinae); middle, left to right: *Golofa porteri* (Dynastinae); *Onthophagus rangifer* (Scarabaeinae); *Enema pan* (Dynastinae); bottom: *Proagoderus tersidorsis* (Scarabaeinae); *Proagoderus lanista* (Scarabaeinae). Photos: O Helmy, D Emlen. (Emlen et al., 2006)

Strong sexual selection to increase horn size, fighting ability, and presumably access to mates, is typically inferred because horns represent a substantial proportion of beetles' body weights (Eberhard, 1982). Some dung beetle horns are so massive that they constitute more than 10 percent of total body mass in some scarab beetles, for example *Onthophagus nigriventris* (Emlen, 2000). Furthermore, use of the horns as weapons in intra-sexual combat between rival males over access to females has been demonstrated in several instances. In the milkweed longhorn beetle *Tetraopes tetraophthalmus*, males interlocked their mandibles and pushed until one male either retreated or was thrown from the host plant (McLain and Boromisa, 1987). The average size of the winner was significantly larger than the average size of the loser. Males of the fungus beetle *Bolitotherus cornutus* chased other males off fungi containing females and also attacked courting couples to disrupt courtship and prevent copulation (Conner, 1989). The longer-horned larger males significantly won in fights over females regardless of population density. The longer-horned males also had significantly greater access to females, resulting in greater insemination success, in low-density populations (Conner, 1989). In their experiment with the dung beetle *Euoniticellus intermedius*, Pomfret and Knell (2006) showed that males fought aggressively using their horns to push the rival out of the tunnel that contained the female. Both body size and horn size were important in determining the outcome when smaller males fought but in combat between larger males, horn size was the most important predictor of victory. Moczek and Emlen (2000) showed that male dung beetles (*Onthophagus taurus*) with longer horns were better able to aggressively defend tunnel entrances containing breeding females. Males of the sap beetle *Librodor japonicus* have sexually dimorphic enlarged mandibles that they use in fights for mates on a sap site (Okada et al, 2007). Larger males fought well and stayed in the sap site while the smaller ones were displaced. Enlarged mandibles of male stag beetles in the genus *Lucanus* are used to chase away rivals in intrasexual contests, during which the males fight with their opponents on the logs and tree trunks where females come to oviposit (Knell et al, 2004).

In *O. taurus,* the only species where horn development has been studied molecularly, horns originate in the prepupal stage of larval development during a period of rapid growth (Moczek, 2006). Most of the growth in the imaginal discs or undifferentiated larval epidermis that eventually forms horns, occurs during a period of resource limitation after larvae stop feeding. Finite resources accumulated to that point in development must be distributed among growing adult traits. Growth of horn precursors in this closed system must occur at the expense of reserves that would otherwise go to other aspects of the adult phenotype (Nijhout and Emlen, 1998; Tomkins et al, 2005). Exaggerated sexually selected ornaments or weapons may thus result in impaired immune response, impaired locomotion, increased risk of predation, reduced viability or survival and stunted growth of nearby organs (Emlen, 2001). For instance, in the genus *Onthophagus,* production of horns occurs at a cost to neighboring morphological structures such as antennae, eyes or wings depending on species-specific horn locations (Emlen, 2001).

1.2 Study System

1.2.1 Gnathocerus cornutus

The broad-horned flour beetle, *Gnathocerus cornutus* (Coleoptera: Tenebrionidae), is a minor pest of stored products found predominantly in mill machinery where it feeds on milled grains (Savvidou and Bell, 1994). It is of tropical origin and cosmopolitan in distribution (Savvidou and Bell, 1994). It is susceptible to cold and is found in warmer parts of the world or in temperate regions where it is protected from the cold. It is secondary pest of grain and causes fouling with its excreta and quinones. Adult beetles measure 3.5 – 4.5 mm in length and are reddish-brown in color. Development occurs between 15° and 32°C. Total development period (egg to adult) of *G. cornutus* is 57 days at temperatures between 24°C and 30°C (Pimental, 1949) and 47 days at 30°C (Tsuda and Yoshida, 1984). Larval and pupal stages last for 35 and 6 days respectively at 30°C and 70 – 75% relative humidity (Tsuda and Yoshida, 1984). Pupation in *G. cornutus* is inhibited under high larval density (Okada et al, 2006).

A marked sexual dimorphism is found in this beetle where males have enlarged mandibles, widened gena and a pair of small horns on the vertex, but females lack these traits completely (Figure 1.2).

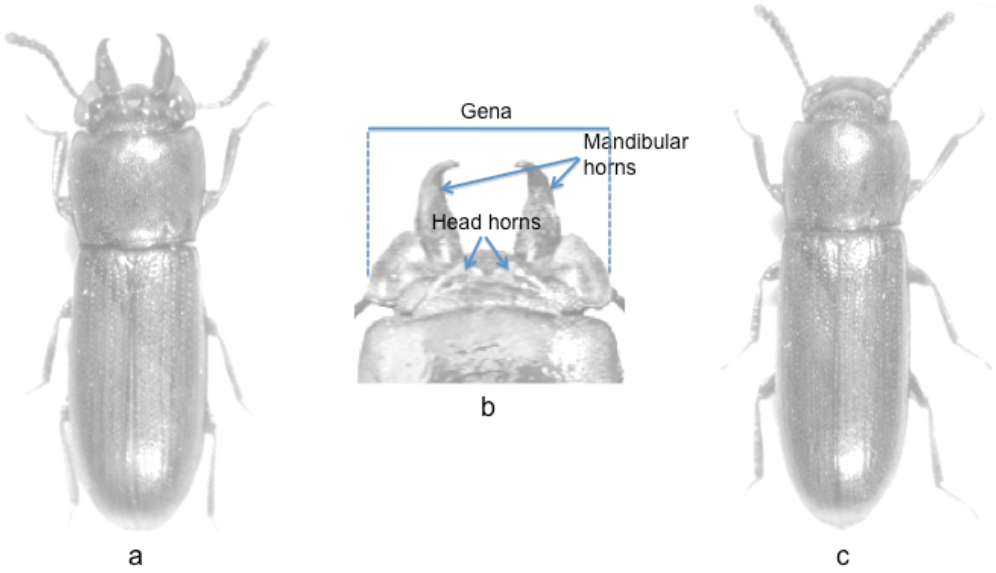

Figure 1.2: *Gnathocerus cornutus:* (a) Morphology of male, (b) Male secondary sexual structures, and (c) Morphology of female.

The enlarged mandibles of the males are used as weapons in male-male interactions where larger males with larger mandibles win in combat; the wide gena and the head horns are not used (Okada et al, 2006). Okada et al (2006) conducted male combat experiments using *G. cornutus* where the males fought each other in the absence of females in laboratory condition. The males interlocked their mandibular horns and shoved each other, lifted the opponent off the substrate with the mandibles and/or bit each other with their mandibles. Artificial selection on mandible size of *G. cornutus* conferred increased fighting endurance where lines selected for larger mandibles were able to fight for longer (Okada and Miyatake, 2009). Their study showed that the length of the male weapon has a heritable basis and can evolve in response to selection. Selection on mandible size for 10 generations also affected male morphology and behavior. The dimensions of compensatory or supportive trait for the weapon like genae width,

length and width of the head, length and width of the prothorax were positively genetically correlated with the size of the mandible. The exaggeration of the weapon results in an overall body shape that is more suited for fighting. There was a positive genetic relationship between how long a male fights and fighting body shape.

G. cornutus is easily reared in the laboratory. They grow well on a 95:5 (by weight) mixture of whole-wheat organic flour and brewer's yeast. Since rearing successive generations and observing male combat is easy, this beetle fits as a perfect candidate in our evolutionary studies of exaggerated male traits.

1.2.2 Hymenolepis diminuta

The rat tapeworm *Hymenolepis diminuta* is a cestode parasite of rodents and occurs in temperate zones worldwide. The adults average about 20 to 25 cm and occur in the small intestine of the host. Flour beetles are among the intermediate hosts for this parasite. The tapeworm eggs, which are excreted in the feces, need to be ingested by the beetle for onset of infection. These parasites are not horizontally or vertically transmissible from beetle to beetle (Zhong et al, 2005). Once ingested by the insect host, the oncospheres hatch and penetrate the intestinal wall. Cysticercoid larvae develop in the hemal cavity where they live until a new primary host eats the infected intermediate host.

1.2.3 Innate Immune Response

Invertebrates rely on the evolutionarily ancient innate immune system for resistance against pathogens. Insects lack true antibodies and rely on processes such as phagocytosis, nodule formation, encapsulation and production of extracellular molecules such as cytotoxic quinones for defense (Ratcliffe et al., 1985; Mullen and Goldsworthy, 2006). Blood cell counts, antibacterial activity in the haemolymph and the activity of phenoloxidase (PO) are the most important and frequently used assays that provide a general index of innate immunity (Armitage and Siva-Jothy, 2005). The activation of PO via proteolysis of its zymogen, pro-phenoloxidase (PPO), plays a crucial role during encapsulation and the subsequent melanization reaction

(Schwarzenbach and Ward, 2007; Newton et al, 2004) (Figure 1.3). After the cuticle, the PPO pathway represents a first generalized response against various immune challenges. Surface molecules of the invading parasite activates the conversion of PPO to active PO and insects with higher PO activity levels are thought to have increased resistance to immune insult (Schwarzenbach and Ward, 2007).

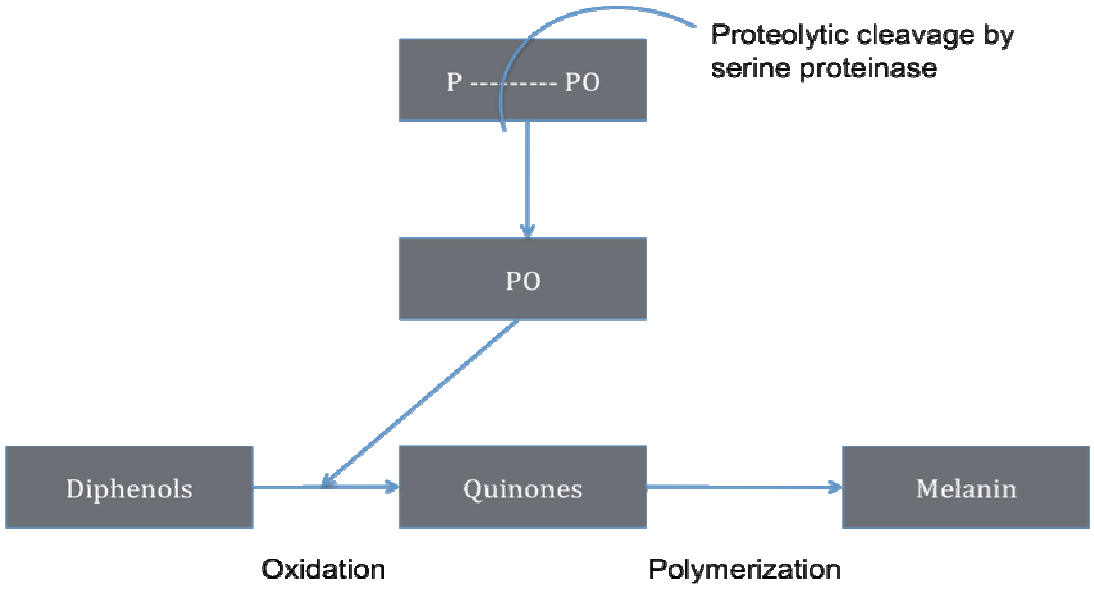

Figure 1.3: Schematic representation of innate immune response

The melanization reaction, which is a common response to parasite entry in invertebrates, is due to the activity of phenoloxidase. Phenoloxidase (PO) is toxic in its active state, so it is found in the form of prophenoloxidase (PPO) constitutively in the beetles. Serine proteinase is responsible for the proteolytic liberation of active PO from PPO. The activated PO in turn catalyses the oxidation of diphenols to cytotoxic quinones. Quinones are believed to be directly toxic to invading parasites and also to polymerize non-enzymatically to form insoluble melanin (Mullen and Goldsworthy, 2006). Localized activation of PPO resulting in the deposition

of melanin entraps the invading parasite and kills it after isolating it within the hemocoel (Schwarzenbach and Ward, 2007).

Resistance against pathogens is a critical determinant of fitness. It has been shown that host resistance to parasite infection is associated with fitness costs in the red flour beetle *Tribolium castaneum* where genome regions conferring resistance to tapeworm infection are partially responsible for fitness costs in the resistant beetle populations (Zhong et al., 2005). Mealworm beetle *Tenebrio molitor* selected for black cuticles had significantly higher pre-immune challenge activity of PO and higher haemocyte density post-immune challenge than beetles selected for tan cuticles (Armitage and Siva-Jothy, 2005). The basis of the correlation may be pleiotropic since cuticular color and the correlated immune traits are both dependent on PO activity, which ultimately resides in the haemocytes (Hoffman, 1995). Also, the degree of cuticular melanization was correlated with resistance to fungal infection in these beetles where the black beetles were more resistant than tan beetles (Barnes and Siva-Jothy, 2000). Higher degree of cuticular melanization was reported for beetles reared at higher larval densities than those reared solitarily but no significant difference in the haemolymph PO activity was found between the two rearing densities (Barnes and Siva-Jothy, 2000).

1.2.4 Cost of Mounting Immune Response

Host-parasite interactions are an increasingly interesting topic to address in ecology and evolutionary biology where researchers study the impact that parasites may have on life-history evolution, sexual selection and population dynamics of the host (Sheldon and Verhulst, 1996). In order to reduce the fitness costs associated with parasitism, hosts have evolved preventative measures, like maintaining a competent immune system, that come at a cost to growth, reproduction, temperature, work etc (Sheldon and Verhulst, 1996).

Several studies on different species have shown differing correlations between immune function and investment in sexually selected traits. In the dung beetle *Onthophagus taurus*, only the males produce horns and there is further dimorphism within the males where large males

produce large horns (major males) and small males produce rudimentary horns or are hornless (minor males) (Emlen and Nijhout, 1999). Cotter et al. (2008) found the investment in larval immune function, as measured by PO activity, to be highest in major males than females and minor males. Immune insulted males of the mealworm beetle *Tenebrio molitor* produced more attractive odors and showed significantly higher PO activity than controls (Sadd et al., 2006). Female *T. molitor* is attracted to male sex-pheromones and the modulation of sexual signaling by immune-challenged males is an example where honesty in advertisement is compromised as males increase terminal reproductive effort (investment in attractiveness) in response to a survival threat (immune insult).

A trade-off between sexual advertisement and immune activation in male blackbirds *Turdus merula* was demonstrated by Faivre et al. (2003), where birds immunized with a suspension of sheep red blood cells displayed a significant decrease in bill color, a sexually selected trait. Male blackbirds with orange bills have been shown to sire more offspring than males with yellow bills. In the drumming wolf spider *Hygrolycosa rubrofasciata*, drumming plays a crucial role in mate choice where no matings occur without male drumming. *H. rubrofasciata* males with increased investment in drumming rate had considerably lower lytic activities and encapsulation rates compared to control males (Ahtiainen et al, 2005). In another experiment, the courtship songs preferred by females of the Mediterranean field cricket *Gryllus bimaculatus* were positively correlated with encapsulation rate of nylon implants but negatively correlated with lytic activity of haemolymph of the male (Rantala and Kortet, 2003). Jacot et al (2004) showed that an immune insult using bacterial lipopolysaccharides causes a lasting reduction in sexual display as well as longevity of male field cricket *Gryllus campestris*. The LPS-induced reduction in daily calling rate of males, which affects female choice of mates, can reduce male conspicuousness to females and mating success. These studies suggest that there are direct immunological costs of sexual signaling in natural populations.

1.2.5 Are Gnathocerus cornutus Horns a Handicap or an Indicator of Good Genes?

In the present study I used the rat tapeworm (*Hymenolepis diminuta*) to infect the broad-horned flour beetle (*Gnathocerus cornutus*) to study the correlation between mandibular horn size and immune protein level in the haemolymph of *G. cornutus*. I hypothesize that infected males with elevated immune responses will consequently have smaller horns due to the diversion of resources from building horns to fighting infection. Alternatively, large horns may be indicative of "good genes", and larger horned males may also have higher immune protein levels; thus producing an extravagant sexual trait while maintaining their immune response.

Beetles were infected with the tapeworm eggs as larvae and allowed to mature into adults. The morphological measurements performed on the experimental and control groups were analyzed to report differences between the infected and uninfected populations. PPO assay was performed to quantify the immune proteins PPO and PO in all the beetles and those of infected versus uninfected populations were compared. Correlation between the immune protein levels and the horn size corrected for body size was then derived where a positive correlation would provide support for the good-genes hypothesis and a negative correlation for the handicap hypothesis. Male competition experiments were conducted to study the roles played by horn size and state of infection. It is interesting to address the cost-benefit relation of the immune proteins to the length of mandibular horns for understanding the evolution of beetle morphology. The cost of producing the trait rather than the reproductive benefit of the trait itself would be beneficial to the study of the diversity of sexually selected traits.

CHAPTER 2

METHODS

2.1 Parental Generation

An original stock of *G. cornutus* used for this study was provided by Dr. Michael Wade of Indiana University, Bloomington, IN. *G. cornutus* was easily reared in the laboratory on a 95:5 by weight mixture of whole-wheat organic flour (Arrowhead Mills, TX) and brewer's yeast (Frontier Natural Products Co-op, Norway, IA). Adults were allowed to lay eggs in plastic trays of dimensions 45L X 30W X 8D cm filled with standard media 3 centimeters deep. After 2 weeks the adults were transferred to fresh media. Trays containing eggs were left in a dark incubator at 30°C and 70% relative humidity until abundant larvae could be seen. Under crowded conditions, pupation of mature larvae is inhibited and the larval period is prolonged (Tsuda and Yoshida, 1985). *G. cornutus* larvae are cannibalistic on other larvae and pupae (Savvidou and Bell, 1994). To prevent the inhibition of pupation and cannibalism, the larval density was limited to 200-300 per tray by sifting out extra larvae using 850 um sieve, and transferring them to a new tray containing fresh media.

To ensure that beetles remained virgins for the present experiments, pupae were sifted out using a 850 um sieve and transferred to individual wells (3.5 cm diameter) of a 24-well tissue culture plate (Cellstar; Greiner Bio-One, Germany). Individuals were sexed upon eclosion into adults based on the conspicuous mandibular horns of males that are absent in females.

2.2 Controlled Crosses

I set up 124 mating pairs between virgin males and females that were between 15-30 days old. Individual mate pairs were transferred to glass vials 25Diam X 95H mm (Kimble Glass Inc.) with only enough media to cover the bottom of the vial (~5 mm deep). Mate pairs were

allowed to mate for 1 week, then transferred to new media for an additional week. This process was repeated so that each mate pair produced a total of two sets of progeny.

Larvae from the first and second weeks of mating were transferred to 6-well tissue culture plates (Cellstar; Greiner Bio-One, Germany). No more than 4 larvae were put in each well to avoid density effects on pupation time.

2.3 Morphological Measurements

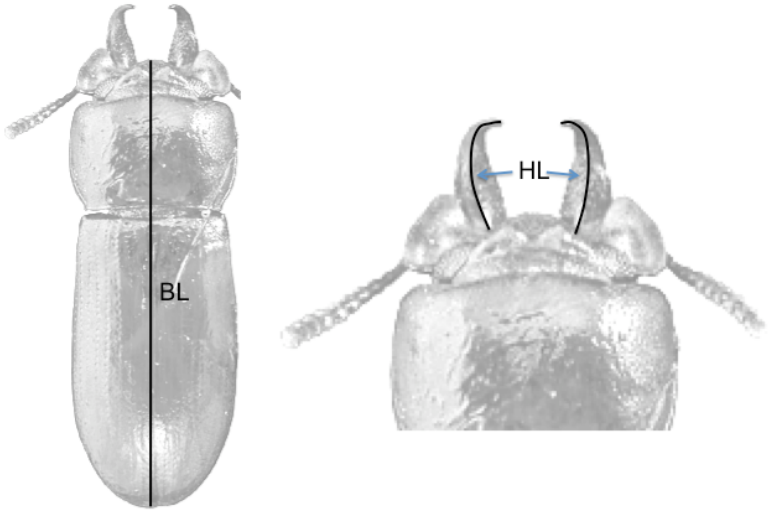

Figure 2.1: Morphological measurements of male *G. cornutus*. BL: body length; HL: horn length. (Figure not drawn to scale).

Morphological measurements for the males' and females' total body length, mandibular horn length (of males), and body mass were obtained. All length measurements were performed under dissecting microscope Nikon SMZ 1500 fitted with Nikon Digital Sight DS-Fi1 camera. Body length was measured as straight-line distances between the tip of the head and the tip of the elytra. Horn length was measured as a curved line along its median axis (Figure 2.1). Body mass was obtained using Mettler (AT261 DeltaRange) precision balance. Adult beetles were CO_2 anaesthetized at 10 psi for 2 mins to prevent them from moving while taking pictures under the microscope.

2.4 Larval Exposure to Parasites

Larvae from each mate pair that were collected in tissue culture plates were starved for 24 hours and then divided into 2 populations: Experimental group and Control group. The experimental group was exposed to rat feces containing *Hymenolepis diminuta* eggs while the control group was not exposed. *H. diminuta* eggs were acquired from Dr. Sherman Hendrix (Gettysburg College). The infected rat feces were collected and shipped overnight to ensure maximum survival of the oncospheres and so that beetles were infected within 36 hours of deposition of the parasite eggs in rat feces. Presence of *H. diminuta* eggs (Figure 2.2) in rat feces was verified by examination under a dissecting microscope (10X) prior to infection treatments. The same batch of rat feces containing *H. diminuta* was used for all infection treatments.

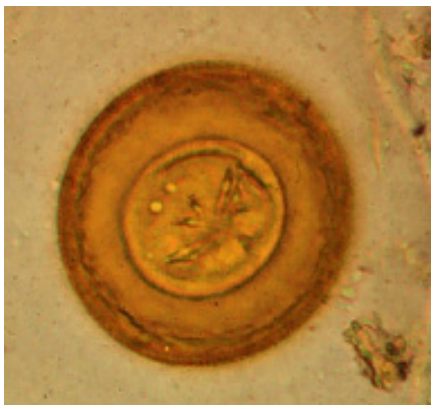

Figure 2.2: Egg of *Hymenolepis diminuta*. Diameter = 70-86 µm X 60-80 µm (pathmicro.med.sc.edu/ parasitology/hym2.jpg)

For infection treatments, 0.3 g of rat feces was weighed on Whatman filter paper (#3) 3.5 cm in diameter on Scout Pro SP601 scale (Ohaus, USA). 0.2 g distilled water was added to the feces (on the filter paper on the balance), mixed gently and worked into a thin layer that covered the surface of the filter paper. The infected filter paper was then placed in a well of a 6-well tissue culture plate (Cellstar; Greiner Bio-One, Germany). The experimental larvae were

then placed on the filter paper containing feces in each well. The number of larvae in each well was limited to six. The control group was provided 0.5 g by weight of distilled water on the filter paper and maintained in 6-well tissue culture plates. Both the experimental and control group larvae were allowed to feed for 48 hours. The larvae were then transferred to new 6-well plates containing standard flour and reared at 30°C and 70% relative humidity until they matured. Additional larvae from six mating pairs were selected at random from the population of offspring and exposed to the infected rat feces following the above protocol. Upon maturation, these test beetles were sacrificed to verify that beetles were infected and to quantify the numbers of *H. diminuta* cysticercoids that had developed from the ova consumed by the larvae during their exposure to rat feces. Each ovum develops into a cysticerciod larva that remains in the beetle body until a new primary host eats the infected beetle.

2.5 Male-male Competition

2.5.1 Effect of Horn Size

22 competition experiments were carried out where the beetles were selected randomly, using random number generator (http://www.randomizer.org/), from the population of uninfected males and females.

All male-male competition experiments were set up on Whatman filter paper discs 3.5 cm in diameter placed in a well (3.5 cm diamater) of a 6-well tissue culture plate (Cellstar; Greiner Bio-One, Germany). The two males for each type of competition were paired with a female and their behavior were video recorded with Sony handycam HDR-SR5 video recorder inside an incubator lit with red light and maintained at 30°C and 70% relative humidity. White out (Sanford, Bellwood, IL) was used on the elytra of one of the males to tell it apart from the other. The males were timed for being in contact with the female either in the mating position (male mounting the female) or any other form of contact (including head to head or side-by-side) that lasted more than 5 seconds. Males with the largest accumulated time were deemed the winner. Behavior was observed for 30 minutes for each competition experiment.

2.5.2 Effect of Parasite Infection

Siblings were used for this competition experiment to avoid variation introduced by any other factor besides the state of infection. There were 7 sibling pairs for this experiment. Competition experiments were set up as described in Section 2.5.1.

2.6 Immune Protein Measurement

Table 2.1: Recipe for beetle lysis/homogenizing buffer

Chemical	**Amount**
50 mM Tris-HCl pH 8.0	25 ml
Cocktail Protease Inhibitors	250 ul
1 mM DTT	25 ul
4 mM EDTA	1ml

{ pH again to 8.0 by adding NaOH }

Each individual offspring that was alive was placed in a 1.5 ml Eppendorf tube and the tube was dipped in liquid nitrogen for 5 seconds to freeze-kill the beetle. Body protein was extracted by grinding the frozen beetle in the tube with a pestle. 100 ul of lysis buffer (Table 2.1) was the added to it and proteins extracted in buffer for 60 mins. The protein extract was centrifuged at 10 rpm for 10 minutes, after which the supernatant was pipetted out into smaller, labeled sample tubes and kept on ice.

For PPO assay, 10 ul beetle protein along with L-DOPA (stock concentration 10 mM, final concentration 2.5 mM) and Trypsin (stock concentration 0.05 mg/ml, final concentration 0.0125 mg/ml) was loaded on a 96-well assay plate (Cellstar; Greiner Bio-One, Germany), whereas for the estimation of PO, only L-DOPA (stock concentration 10 mM, final concentration 2.5 mM) was added to 10 ul beetle protein. The volumes of both types of reactions were standardized with DI water (Sigma). The plate was immediately read on the Bio-Tek Synergy 2 plate reader to get the absorbance values every 2.5 minutes starting at time 0 through 30 minutes.

Quick Start Bradford Protein Assay protocol was used for the estimation of total protein concentration (Bradford, 1976). Quick Start Bradford dye (1X concentration) was added to the wells containing 5 ul of the standard protein concentrations as well as the ones containing 5 ul of beetle protein. The absorbance was read on Bio-Tek Synergy 2 plate reader and compared with the standard curve to estimate total beetle protein.

Average PPO and PO were calculated by dividing the difference between t=0 and t=10 by protein amount.

2.7 Statistical Analyses

To account for the potential confounding effect of the correlation between body size and horn size I used the residual of horn size regressed on body weight (corrected, or relative horn length). Independent Samples t-tests were used to test for differences among (i) means of corrected horn lengths, and (ii) means of the immune proteins PPO, PO and their difference (PPO-PO) with infection status as the grouping variable. Levene's test for equality of variances was not significant at $\alpha = 0.05$ unless otherwise stated. Means are reported ± 1 s.e. Correlation graphs for PPO, PO and PPO-PO versus horn length were generated and Pearson's r was calculated for each comparison. One-sample Kolmogorov-Smirnov test was performed to check the distribution of morphological measurements of the infected and uninfected offspring. Normality and variance assumptions were tested. Violations and alternative tests are reported in the results.

In order to get rid of the extreme differences between the times spent by winners in one trial versus the next trial, the cumulative time that each male spent with the female was ranked to consider all the competition experiments as a single sample. Mann-Whitney U test statistic was computed to check the distribution of the rank of time for the infected and the uninfected rivals in competition. Ranked time was also used to test whether there was any effect of white out on the outcome of male-male competition. Horn length was ranked and Wilcoxon's signed rank test was used to test the difference in mandible lengths between winners and losers.

Differences were considered significant at $\alpha = 0.05$. Data were analyzed using the statistical package SPSS version 17.0 for Windows and Microsoft Excel.

CHAPTER 3

RESULTS

3.1 Infection with *Hymenolepis diminuta*

Infection was present in 85.2% (23/27) of the test beetles as evidenced by the presence of cysticercoids in the haemolymph. Based on this I assume that a large majority of the experimental beetles that were exposed to the parasite became infected. The data that were averaged out among the experimental infected population were representative of infection. The ova had developed into cysticercoids in the haemolymph and each infected individual had 5 cysticercoids on average (mean ± s.e. = 4.91 ± 0.72, range = 1-13). Each cysticercoid was a clear or pale white oval structure with elongated ends and a darkish centre that represents the invaginated scolex. There was no evidence of the parasite being encapsulated with melanin by the host immune system (Figure 3.1). This observation is consistent with my additional finding that parasite infection did not affect levels of circulation immune proteins. Specifically, the amounts of PPO ($t = -0.266$, $p = 0.791$), PO ($t = 0.096$, $p = 0.923$) and PPO-PO ($t = -0.280$, $p = 0.780$) per mg of total body protein were not different between infected and uninfected beetles (Table 3.1).

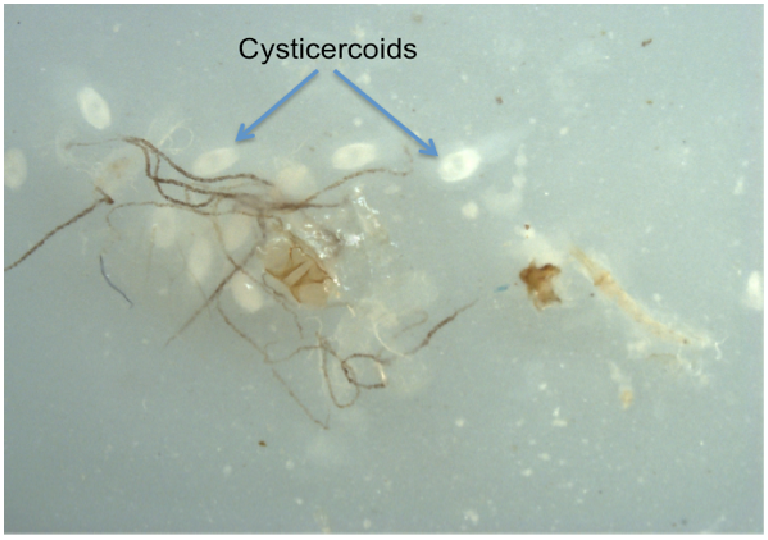

Figure 3.1: Cysticercoids of *Hymenolepis diminuta* in the beetle haemolymph exposed after dissection under microscope. They are seen as clear structures with no evidence of melanin encapsulation.

Table 3.1: Measurements of the immune protein levels in the infected and uninfected offspring

	PPO/mg total protein (Mean ± s.e.)	PO/mg total protein (Mean ± s.e.)	PPO – PO/mg total protein (Mean ± s.e.)
Infected	1.1832 ± 0.0800	0.0763 ± 0.0077	1.1069 ± 0.0787
Uninfected	1.2041 ± 0.0263	0.0754 ± 0.0034	1.1287 ± 0.0261

3.2 Morphological Measurements

The morphological measurements of mandibular horn length, body weights and body lengths were normally distributed for both the infected and the uninfected offspring (Figures 3.2 and 3.3). The sample sizes were 28 and 100 for the infected and uninfected offspring respectively.

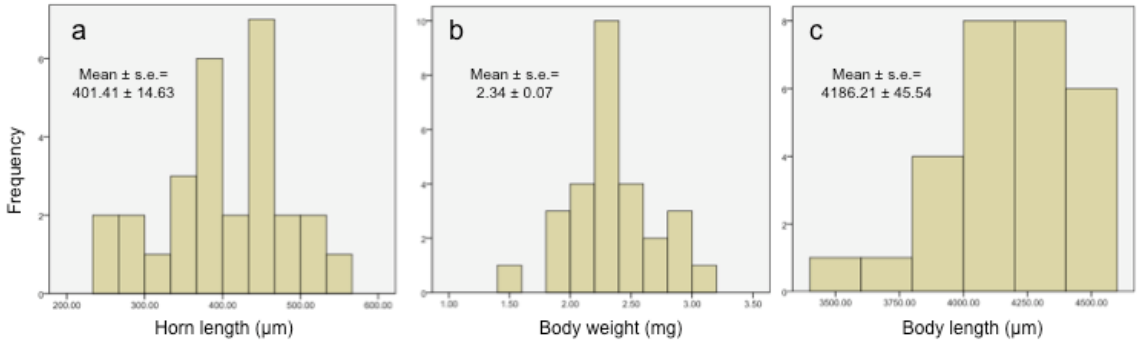

Figure 3.2: Frequency distribution of the morphological measurements of (a) mandibular horn length (mean ± s.e.= 401.41 ± 14.63), (b) body weight (mean ± s.e.= 2.34 ± 0.07)and (c) body length (mean ± s.e.= 4,186.21 ± 45.54) were normal for the infected offspring. N = 28.

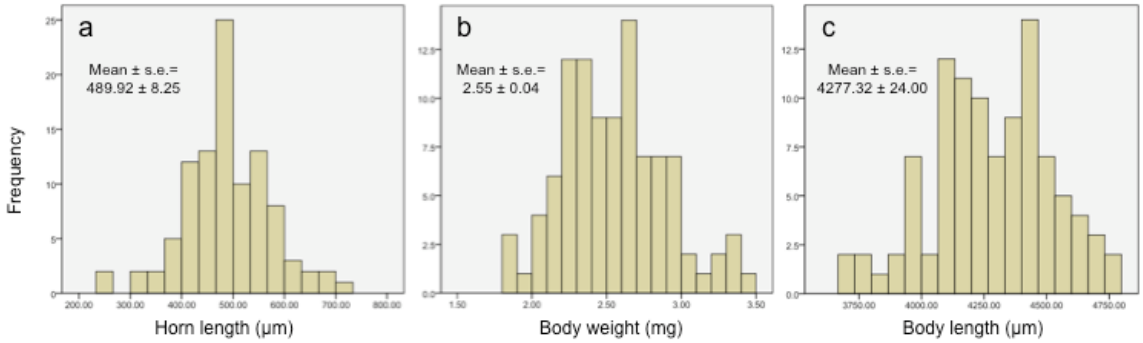

Figure 3.3: Frequency distribution of the morphological measurements of (a) mandibular horn length (mean ± s.e.= 489.92 ± 8.25), (b) body weight (mean ± s.e.= 2.55 ± 0.04)and (c) body length (mean ± s.e.= 4,277.32 ± 24.00) were normal for the uninfected offspring. N = 100.

Horn length is highly correlated with body length and body weight. The relationship holds for both infected ($r = 0.796$, $p = 0.000$ and $r = 0.661$, $p = 0.000$ respectively; Figure 3.4) and uninfected ($r = 0.761$, $p = 0.000$ and $r = 0.858$, $p = 0.000$ respectively; Figure 3.5) beetles.

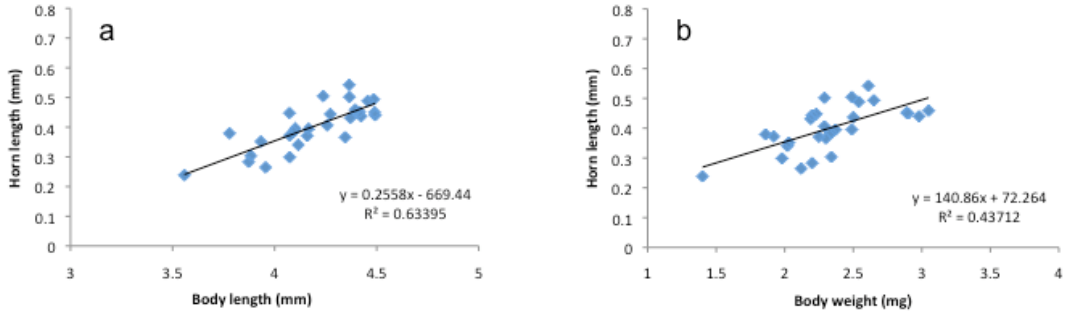

Figure 3.4: For the infected offspring (a) 63% of the variation and (b) 44% of the variation in horn length can be explained by (a) body length and (b) body weight respectively.

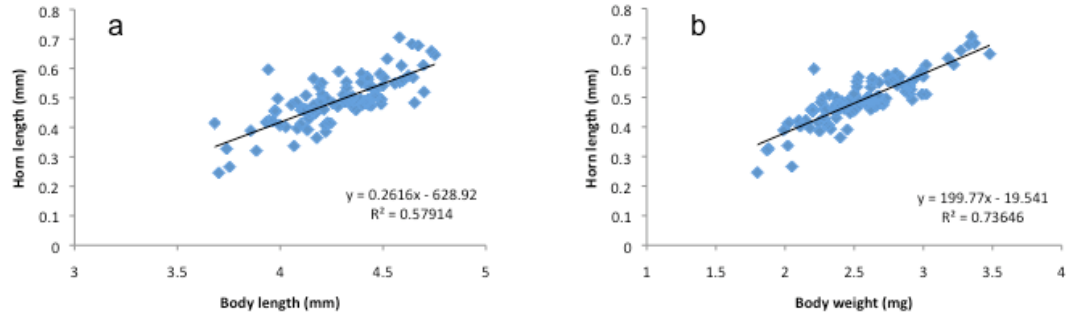

Figure 3.5: For the uninfected offspring (a) 58% of the variation and (b) 74% of the variation in horn length can be explained by (a) body length and (b) body weight respectively.

To account for the potential confounding effect of the correlation between body size and horn size I used the residual of horn size regressed on body weight (relative horn length) to assess the impact of infection on horn size and the effect of horn size on competitive ability.

Infection with *H. diminuta* affects body size and horn size in *G. cornutus*. Infected individuals weighed 8% lower and had 18% smaller horn sizes than their uninfected siblings on average (Table 3.2 and Figure 3.6). Not assuming equal variances, the horn lengths (corrected for body weights) were significantly different between the infected and the uninfected offspring ($t = -3.681$, $p = 0.001$) (Figure 3.6). Body weights varied significantly between the states of infection ($t = -2.802$, $p = 0.006$).

Table 3.2: Morphological measurements of the infected and uninfected offspring

	Body length (µm) (Mean ± s.e.)	Mandibular horn length (µm) (Mean ± s.e.)	Body weight (mg) (Mean ± s.e.)
Infected sons	4,186.21 ± 45.54	401.41 ± 14.63	2.34 ± 0.07
Uninfected sons	4,277.32 ± 24.00	489.92 ± 8.25	2.55 ± 0.04

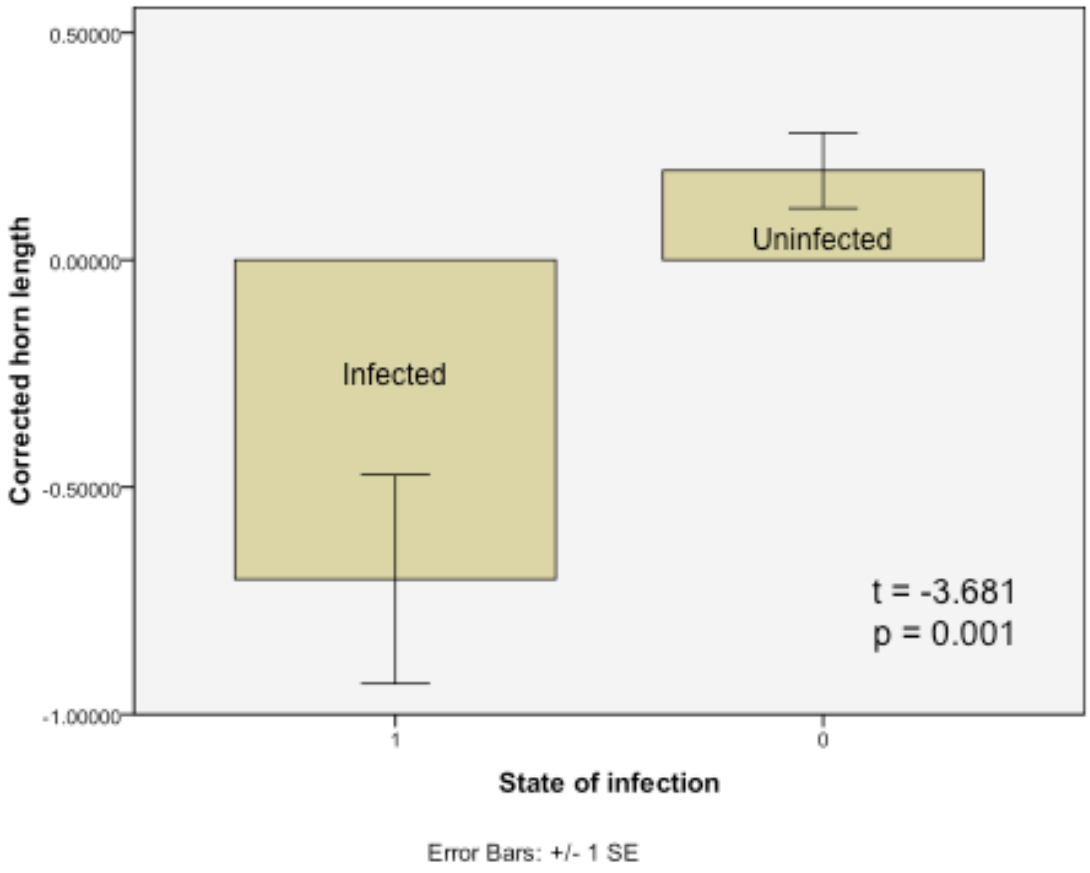

Figure 3.6: Comparison of the means of mandibular horn lengths corrected for body weights of infected (state of infection = 1) and uninfected (state of infection = 0) offspring assuming unequal variance. Levene's test for equality of variance was significant (p = 0.002). Error bars represent one standard error of the means.

3.3 Correlation of the Immune Proteins With Mandibular Horn Lengths

Males with larger relative horn sizes have significantly more PPO-PO ($r = 0.226$, $p = 0.035$). While not statistically significant, there is also a trend for increasing PPO with increasing relative horn size ($r = 0.208$, $p = 0.053$). However, the relationship does not hold for PO($r = -0.140$, $p = 0.197$) (Figure 3.7). The immune proteins PPO, PO and their difference PPO-PO are expressed per mg of total body protein.

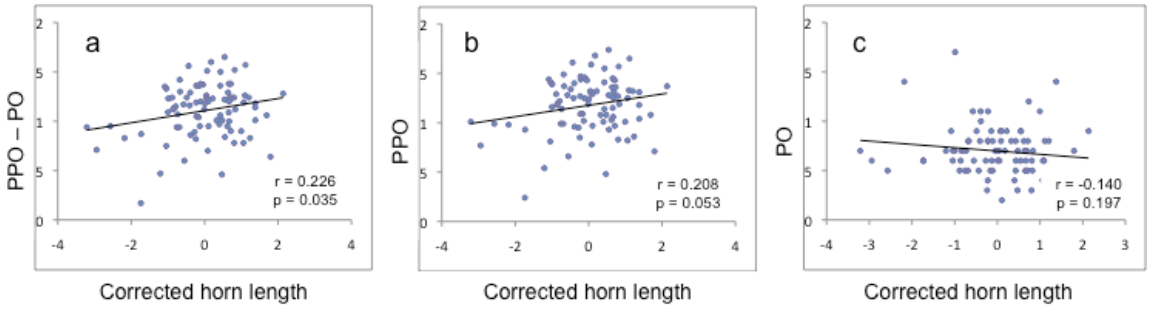

Figure 3.7: Correlation between (a) PPO-PO, (b) PPO, and (c) PO per mg total body protein and relative horn length. Horn lengths were corrected for body weights.

3.4 Male-male Competition

To correct for different variance across trials in mate competition experiments I used a non-parametric Wilcoxon's signed ranks test. Rank of horn lengths was significantly different between the winners and losers of competition ($Z = -2.303$, $p = 0.021$) (Figure 3.8). Similarly there was no direct effect of infection status (Mann-Whitney $U = 27.000$, $p = 0.749$), or the marking technique used to distinguish between competing males ($Z = -0.786$, $p = 0.432$).

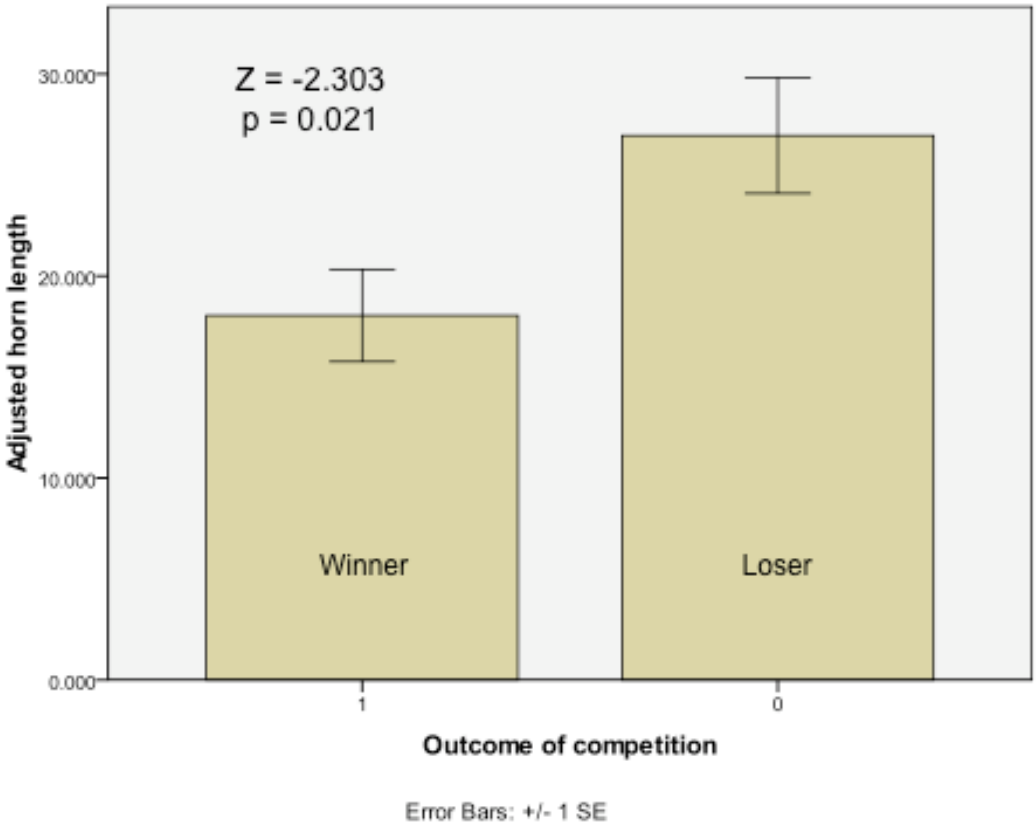

Figure 3.8: Adjusted horn length is significantly different between the winners and losers of competition. Horn lengths were ranked where rank 1 was assigned to the largest horn. Outcome of competition was 1 (winner) or 0 (loser). Error bars represent one standard error of the means.

CHAPTER 4

DISCUSSION

4.1 Immune Protein Measurements

My study showed no significant difference in the levels of PPO and PO between the infected and the uninfected beetles. The measure of PPO gives an estimate of the inactive reserve that is present in the beetles and the measure of PO shows how much enzyme is activated due to the infection. The difference between PPO and PO (true measure of PPO corrected for experimental activation) was also not significant between the states of infection. When the test beetles that were infected were dissected and observed under the microscope, the cysticercoid larvae of the parasite did not appear to be melanized (Figure 3.1). This suggests that PPO cascade might not have been initiated upon infection of these beetles with tapeworm eggs and that there may be another mechanism of immune response to this parasite. Other possible mechanisms of immune response in insects include opsonization, phagocytosis, coagulation etc in addition to the production of antimicrobial peptides and a range of other defense molecules (lysozyme, and proteolytic and hydrolytic enzymes) (Figure 4.1, Schmid-Hempel, 2005). Different pathways regulate the insect immune defense. Another mechanism of immune defense is the synthesis of proteins that neutralize toxins produced by a parasite (Schmid-Hempel, 2005). It remains to be investigated which of these different mechanisms might have played a role in my study system.

A potential explanation for similar immune protein levels between the infected and uninfected beetles could be that the immune protein measurements were not taken immediately following the infection, while the beetles were still larvae and trying to fight it off. This warrants further study infecting the beetles and quantifying immune proteins at specific stages in the life

cycle to understand if there are any temporal variations in the degree of expression of heightened immune response as a result of infection.

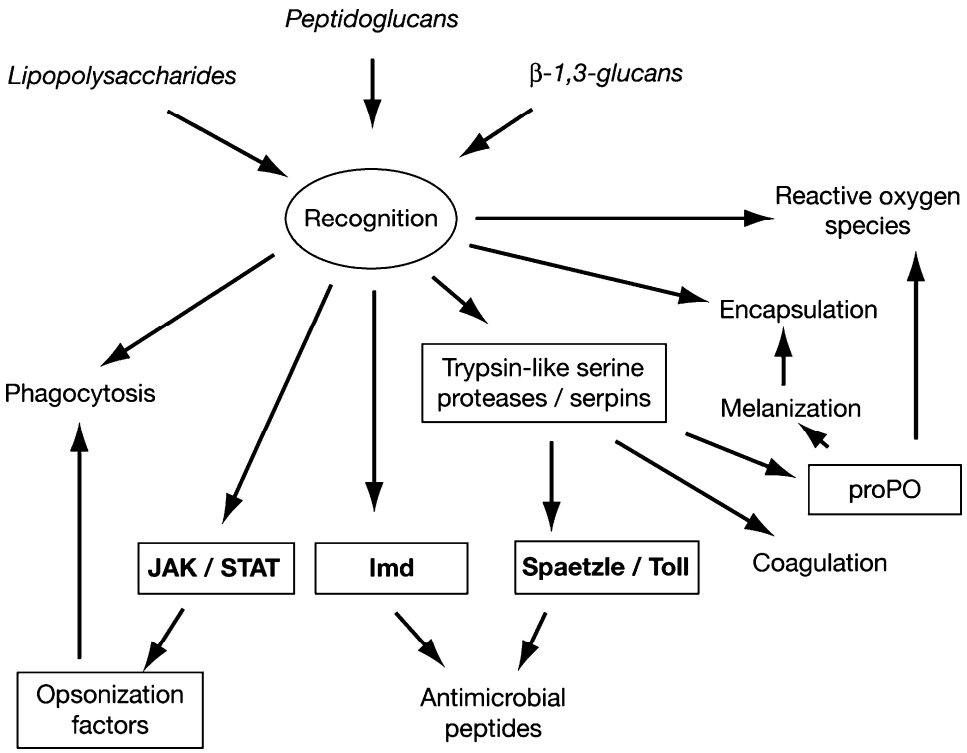

Figure 4.1: Typical responses of the insect immune defense after different antigens (lipopolysaccharides, peptidoglucans, and β-1,3-glucans) have been recognized (oval). Rectangles denote major receptors and signaling pathways (Schmid-Hempel, 2005).

There is relatively scarce and contradictory evidence showing positive correlations between the immune assay and resistance against pathogens (Schwarzenbach and Ward, 2007). In yellow dung flies *Scathophaga stercoraria*, lines were selected to have different mean PO levels. Exposure of these flies to mites and spores of *Metharizium anisopliae* produced infection that negatively affected fitness. However, the significant differences in PO levels between the selection regimes did not affect prevalence of the two pathogens significantly (Schwarzenbach and Ward, 2007). In their experiment with *Daphnia magna*, Mucklow et al. (2004) showed that the success rate of the parasite *Pasteuria ramosa* in infection were

negatively correlated with innate PO activity but these correlations disappeared when the data were corrected for the host population effect. In the cricket *Gryllus texensis*, there was no evidence that organisms with lower PO activity are more susceptible to bacteria than those with higher PO activity (Adamo, 2004a). The immune protein measures would give a false ranking of the males, with regard to their disease resistance, if they were used to rank a male's resistance to bacteria. Hence, inferences made about trade-offs between immunity and other physiological systems based on these measures of PO and lysozyme-like activity for this species would be incorrect. Leclerc et al. (2006) showed that, in *Drosophila*, PO activity in the haemolymph of adult flies is not required for survival to microbial infections and the role of PO in immune defense in *Drosophila* is vague. However, these examples in no way negate the important and well-documented role PO plays in invertebrate immunity but suggest that differences in PO activity levels may not always adequately reflect overall resistance (Schwarzenbach and Ward, 2007).

Marteilia sydneyi is a protistan parasite that causes the fatal disease QX in Sydney rock oysters *Saccostrea glomerata*. Newton et al. (2004) showed in their study that oysters selected for resistance to QX disease have significantly higher PO activities than wild type oysters. This increased activity is associated with the expression of a novel form of PO protein that enhances their PO enzymatic activities. In tsetse flies, PO activity was significantly higher for the refractory fly (*Glossina palpalis palpalis*) than it was for the susceptible fly (*Glossina palpalis morsitans*) when live procyclic parasites (*Trypanosoma brucei rhodesiense*) were used to activate the hemolymph PPO (Nigam et al., 1997). Pathogen resistance was found to be correlated with cuticular melanization in the noctuid *Spodoptera exempta* where melanized *S. exempta* larvae were more resistant than non-melanized forms, independent of rearing density (Reeson et al., 1998). Cuticular melanization was also correlated with PO levels providing evidence for a link between melanization and immunity. The black-bodied mutant of *Tribolium castaneum* has

significantly higher levels of PPO-PO than wild type suggesting that the inactive PPO is constitutively higher in black-bodied mutants (Unpublished data).

4.2 Beetle Morphology

Secondary sexual traits of *G. cornutus* namely the mandibles, the gena and the horns on the vertex are completely absent in females. In adult *G. cornutus* males, body length and body weight explained 58% and 74% of the variance in the mandibular horn length respectively (Figure 3.5). My study found the mandibular horn length to be a continuous variable with a normal distribution. Previous studies have shown that the mandibles of *G. cornutus* are intra-sexually dimorphic and the mandibles of larger males are relatively larger than that of smaller males (Okada et al, 2006). In their experiment involving artificial selection on mandible size, Okada and Miyatake (2009) have shown that the length of the mandible has a heritable basis and can evolve in response to selection. Selection on the weapon can also generate evolutionary changes in these beetles where selection on mandible size for 10 generations affected male morphology and behavior. The dimensions of compensatory or supportive trait for the weapon like genae width, length and width of the head, length and width of the prothorax were positively genetically correlated with the size of the mandible. The exaggeration of the weapon results in an overall body shape that is more suited for fighting (Okada and Miyatake, 2009).

The sample sizes for the morphological measurements were 28 and 100 for the infected and uninfected offspring respectively. This suggests that on average 70% of the larvae died due to infection with parasite since there were equal numbers of larvae in both the experimental and control groups. Infection with *H. diminuta* also caused the beetles to develop significantly shorter horns and smaller bodies than the uninfected, reducing their overall fitness.

4.3 Parasite Infection's Effect on Mandibular Horn Length

The length of the mandibular horns, corrected for body weight, of the infected beetles were found to be significantly shorter than those of the uninfected beetles. The infected beetles

were also significantly smaller than the uninfected beetles. This observation suggests that there is a cost that the beetles had to bear as a result of infection with parasite. Since there is no difference in the PPO or PO levels between the infected and uninfected beetles, the handicap hypothesis of a trade-off between mandibular horn length (a secondary sexual trait) and immune proteins (indicator of resistance to disease, a naturally selected trait) does not hold. The decrease in horn and body size appears to be a direct effect of infection rather than one that occurred as a result of a trade-off with elevated immune response.

There was a significant positive correlation when the level of PPO-PO (true measure of reserve PPO) per mg body protein was compared with the mandibular horn length (corrected for body weight), suggesting that males with larger horns also have constitutively higher levels of the zymogen PPO in their haemolymph. This result provides evidence for Hamilton and Zuk's (1982) good-genes hypothesis where the degree of expression in secondary sexual traits is indicative of the male's ability to resist parasites. Male *G. cornutus* with larger horns also had higher PPO levels so they were able to produce an extravagant sexual trait without compromising their immune response. This parasite-sexual selection hypothesis can also be considered as a modification of Zahavi's (1975) handicap hypothesis where even though the expression of secondary sexual traits may reduce survival, they also act as signal of quality by advertising parasite resistance under sexual selection (Hamilton and Zuk, 1982; Maynard Smith, 1985).

My results that showed increased PPO for larger horned males are also consistent with another model proposed by Kodric-Brown and Brown (1984) who developed the truth in advertising model by suggesting that sexual selection favors the evolution of costly, phenotypically variable traits in such a way that their expression is highly correlated with overall genetic fitness. This concept is based on Zahavi's (1975) handicap hypothesis but the cost of honest advertisements need not always be expressed as a handicap to survival. Sexual traits that honestly advertise male genetic quality need not reflect a trade-off between enhanced

reproductive success and reduced survival, and consequently need not be handicaps in the sense of Zahavi. The expression of sexual traits requires expenditure of limited energy and other resources that could otherwise be allocated to other structures and/or functions and hence, are costly to produce.

4.4 Male-male Competition

My study showed that there is a significant difference in the mandibular horn lengths between the winners and the losers of male-male competition. The length of the horns affected the outcome of fights but the state of infection did not significantly predict the winner of such intraspecific combat. This result suggests that being infected with parasite does not constitute a direct disadvantage in combat. Males were frequently seen interlocking their horns and pushing each other around in the arena. Some males also were seen lifting their rivals off the substrate with their mandibles. In their experiment with *G. cornutus*, Okada et al (2006) also found a significant tendency for males with larger mandibles to win in male combat. The male that pushed his opponent out of the fighting site and chased him was considered the winner, and the male that retreated from the fight site was considered the loser. The result Okada et al (2006) reported was that in male G. cornutus, the length of the mandible affected the outcome of male fights and this observation supports the results of my current experiment.

There have been numerous studies that suggest the horns of horned beetles are used as weapons in intermale contests. Palmer (1978) showed that in the burrowing beetle *Typhoeus typhoeus* the horns are specifically designed for fighting between males. The beetle *Doryphora* sp. uses its sternal horn as a weapon in intraspecific aggressive interactions (Eberhard, 1981) and *Ageopis nigicollis* uses its cephalic and thoracic horns as weapons in intraspecific battles (Eberhard, 1987). More recently, in *Onthophagus acuminatus*, males with relatively longer horns (controlled for body size) won significantly more fights over tunnel ownership than males with relatively shorter horns. So the males that were large enough to guard tunnels had longer horns than smaller males to ward off the smaller males that tried to sneak past them to get

access to the female in the tunnel (Emlen, 1997). Moczek and Emlen (2000) show that horns in male *Onthophagus taurus* are bimodal in distribution and are used in male-male fights over possession of tunnels containing breeding females, where alternative reproductive tactics of fighting or sneaking favored alternative horn phenotypes of being horned or hornless. I did not observe such alternative male phenotypes in *G. cornutus* in my study. The horns of many species of beetles represent a substantial proportion of the beetles' body weights and this supports the notion that natural selection in these species has strongly favored increased fighting ability (Eberhard, 1982).

In summary, my results show that in the broad-horned flour beetles *G. cornutus*, growth and maintenance of secondary sexual trait in the form of beetle horns do not impose a trade-off in the constitutive levels of immune protein in their bodies but rather advertise their increased ability to resist the detrimental effects of parasitism suggesting honesty of sexual ornaments. Such kind of truth in advertising implies that female choice may be present in these beetles. Several studies have shown cryptic female choice in the beetle order Coleoptera. Cryptic female choice was shown to be present in the red flour beetle *Tribolium castaneum* during sequential stages of sperm transfer and storage. In this hornless ancestor of *Gnathocerus,* fed males transferred significantly more sperm than starved males when mating with live females but not when mating with dead females (Fedina, 2007). The male phenotypic quality was manipulated by starvation and live females actively differentiated against starved males. Females of the spotted cucumber beetle (*Diabrotica undecimpunctata howardi*) discriminated among males after copulation had begun on the basis of antennal stroking displays that males performed where males that stroke quickly had a higher probability of being accepted as a mate (Tallamy et al., 2002). As a result of infection with *H. diminuta* in my study system, the beetles developed shorter horns that imposed a direct disadvantage to them in male-male competition. Okada and Miyatake (2009) showed that exaggeration of the *G. cornutus* mandibular horn leads to heritable changes in body shape resulting in one that is more suited for fighting. Although

there is evidence that sexual selection on this trait favors larger horns and increases success in direct competition with other males for mates, evidence for female choice for larger horned *G. cornutus* males is lacking. My study did not look into any aspect of female choice but future studies on this beetle will elucidate if the females of this species use a cryptic mechanism of female choice based on male phenotypic quality (length of mandibular horns).

REFERENCES

Adamo, S.A. (2004a) Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*, *Journal of Insect Physiology* 50 (2004), pp. 209–216.

Ahtiainen, J.J., Alatalo, R.V., Kortet, R., and Rantala, M.J. (2005). A trade-off between sexual signaling and immune function in a natural population of the drumming wolf spider *Hygrolycosa rubrofasciata*, J. Evol. Biol. 18: 985-991.

Akesson, M., Bensch, S., Hasselquist, D., Tarka, M., and Hansson, B. (2008). Estimating Heritabilities and Genetic Correlations: Comparing the 'Animal Model' with Parent-Offspring Regression Using Data from a Natural Population. PLoS ONE, v.3(3).

Andersson, M.B. (1994). Sexual Selection. Princeton University Press.

Andersson, M., and Iwasa, Y. (1996). Sexual selection. *Trends Ecol. Evol.* 11:53-58.

Armitage, S.A.O., and Siva-Jothy, M.T. (2005). Immune function responds to selection for cuticular colour in Tenebrio molitor, Heredity 94:650–656.

Barnes, A.I., and Siva-Jothy, M.T. (2000). Density-dependent prophylaxis in the mealworm beetle *Tenebrio molitor* L. (Coleoptera: Tenebrionidae): cuticular melanization is an indicator of investment in immunity. Proc R Soc Lond B Biol Sci 267: 1–6.

Bateman, A. J. (1948). Intrasexual selection in *Drosophila*. Heredity 2:349-69.

Borgia, G. (1985). Bower quality, number of decorations, and mating success of male satin bowerbirds (*Ptilonorhynehus violaceus*): an experimental analysis. Anim Behav 33 : 266-271.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72: 248-254.

Clutton-Brock, T.H. (1982). The functions of antlers. Behaviour, 70, 108–125.

Conner, J. (1988). Field measurements of natural and sexual selection in the fungus beetle, *Bolitotherus cornutus*. Evolution 42:736–749.

Conner, J. (1989). Density-dependent sexual selection in the fungus beetle, *Bolitotherus cornutus.* Evolution 43:1378–1386.

Cotter, S.C., Beveridge, M. and Simmons, L.W. (2008). Male morph predicts investment in larval immune function in the dung beetle, *Onthophagus taurus*. Behav. Ecol. 19: 331–337.

Darwin, C. (1859). On the Origin of Species by Means of Natural Selection. Murray; London, UK

Darwin, C. (1871). The Descent of Man, and Selection in Relation to Sex. Murray; London, UK.

Ditchkoff, S.S., Lochmiller, R.L., Masters, R.E., Hoofer, S.R., and Van Den Bussche, R.A. (2001). Major-histocompatibility-complex-associated variation in secondary sexual traits of white-tailed deer (*Odocoileus virginianus*): evidence for good-genes advertisement. *Evolution* **55 ,** 616-625.

Eberhard, W.G. (1979). The functions of horns in *Podischnus agenor* (Dynastinae) and other beetles. In: Sexual Selection and Reproductive Competition in Insects (Ed. by M. S. Blum & N. A. Blum), pp. 231–258. New York: Academic Press.

Eberhard, W.G. (1981). The natural history of *Doryphora* sp. (Coleoptera: Chrysomelidae) and the function of its sternal horn. Annals of the Entomological Society of America, 74, 445–448.

Eberhard, W.G. (1982). Beetle horn dimorphism: Making the best of a bad lot. The American Naturalist 119:420-426.

Eberhard, W.G. (1987). Use of horns in fights by the dimorphic males of *Ageopsis nigicollis* (Coleoptera, Scarabaeidae, Dynastinae). Journal of the Kansas Entomological Society, 60: 504–509.

Emlen, D.J. (1997). Alternative reproductive tactics and male-dimorphism in the horned beetle Onthophagus acuminatus (Coleoptera: Scarabaeidae). Behavioral Ecology and

Sociobiology 41:335–341.

Emlen, D.J. (2000). Integrating development with evolution: a case study with beetle horns. BioScience 50, 403-418.

Emlen, D.J. (2001). Costs and the diversification of animal structures. Science 291: 1534–1536.

Emlen, D.J., Marangelo, J., Ball, B. and Cunningham, C.W. (2005b). Diversity in the weapons of sexual selection: horn evolution in the beetle genus *Onthophagus* (Coleoptera: Scarabaeidae). Evolution, 59, 1060-1084.

Emlen, D.J., and Nijhout, H.F. (1999). Hormonal control of male horn length dimorphism in the dung beetle *Onthophagus taurus* (Coleoptera: Scarabaeidae). Journal of Insect Physiology 45:45–53.

Emlen, D.J., Szafran, Q., Corley, L.S. and Dworkin, I. (2006). Insulin signaling and limb-patterning: candidate pathways for the origin and evolutionary diversification of beetle 'horns'. Heredity, 2006, 1–13.

Faivre, B., Gregoire, A., Preault, M., Cezilly, F., and Sorci, G. (2003). Immune activation mirrored in a sexual trait. Science 300:103.

Faivre, B., Preault, M., Thery, M., Secondi, J., Patris, B. and Cezilly, F. (2001). Breeding strategies and morphological characters in an urban population of blackbirds, *Turdus merula*. Animal Behaviour 61:969–974.

Farrell, B.D. (1998). "Inordinate fondness" explained: Why are there so many beetles? Science 281:555–559.

Fedina, T.Y. (2007). Cryptic female choice during spermatophore transfer in *Tribolium castaneum* (Coleoptera: Tenebrionidae). J. Insect Physiol. 53: 93–98.

Gustafsson, L., Qvarnström, A., and Sheldon B.C. (1995). Trade-offs between life-history traits and a secondary sexual character in male collared flycatchers. Nature 375 (1995), pp. 311–313.

Hamilton, W.D., and Zuk, M. (1982). Heritable true fitness and bright birds: a role for parasites? Science 218: 384-387.

Hoffmann, J. (1995). Innate immunity of insects. Curr Opin Immunol 7: 4–10.

Jacot, A., Scheuber, H., and Brinkhof, M.W.G. (2004). Costs of an induced immune response on sexual display and longevity in field crickets. Evolution 58:2280–2286.

Knell, R.K., Pomfret, J.C., and Tomkins, J.L. (2004). The limits of elaboration: curved allometries reveal the constraints on mandible size in stag beetles. Proceedings of the Royal Society of London B 271:523–528.

Kodric-Brown, A. and Brown, J. H. (1984). Truth in advertising: the kinds of traits favored by sexual selection. Am. Nat. 124, 303−323.

Leclerc, V., Pelte, N., Chamy, L.E., Martinelli, C., Ligoxygakis, P., Hoffmann, J.A. and Reichhart, J.M. (2006). Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*. EMBO Rep. 7, 231-235.

Maynard Smith, J. (1985). Sexual selection, handicaps and true fitness. J Theor Biol 111 : 1-18.

McLain, D.K. and Boromisa, R.D. (1987). Male choice, fighting ability, assortative mating and the intensity of sexual selection in the milkweed longhorn beetle, *Tetropes tertraphtalmus* (Coleoptera, Cerambycidae). Behav Ecol Sociobiol 20:239–246.

Moczek, A.P. (2006). Pupal remodeling and the development and evolution of sexual dimorphism in horned beetles. American Naturalist 168:711-729.

Moczek, A.P., Andrews, J., Kijimoto, T., Yerushalmi, Y., and Rose, D.J. (2007). Emerging model systems in evo-devo: horned beetles and the origins of diversity. Evolution and Development, 9:323-328.

Moczek, A.P., and Emlen, D.J. (2000). Male horn dimorphism in the scarab beetle, *Onthophagus taurus*: do alternative reproductive tactics favour alternative phenotypes? *Anim Behav.* 59(2):459–466.

Moller, A.P. (1994). Sexual Selection and the Barn Swallow, Oxford University Press.

Mucklow, P.T., Vizoso, D.B., Jensen, K.H., Refardt, D. and Ebert, D. (2004). Variation in phenoloxidase activity and its relation to parasite resistance within and between populations of *Daphnia magna*. Proceedings of the Royal Society of London Series B – Biological Sciences, 271, 1175–1183.

Mullen, L.M., and Goldsworthy, G.J., (2006). Immune responses of locusts to challenge with the pathogenic fungus *Metarhizium* or high doses of laminarin. Journal of Insect Physiology, 52:389-398.

Newton, K., Peters, R., and Raftos, D. (2004). Phenyloxidase and QX disease resistance in Sydney rock oysters (*Saccostrea glomerata*). Developmental and Comparative Immunology, 28, 565–569.

Nigam, Y., Maudlin, L., Welburn, S. and Ratcliffe, N. (1997). Detection of phenoloxidase activity in the hemolymph of tsetse flies, refractory and susceptible to infection with Trypanosoma brucei rhodensie. J. Invertebr. Pathol. 69, 279–281.

Nijhout, H.F., and Emlen, D.J. (1998). Competition among body parts in the development and evolution of insect morphology. Proc Nat Acad Sci. 1998;95:3685–3689.

Okada, K., Miyanoshita, A., and Miyatake, T. (2006). Intra-sexual dimorphism in male mandibles and male aggressive behavior in the broad-horned flour beetle Gnatocerus cornutus (Coleoptera: Tenebrionidae). Journal of Insect Behavior, 19, 457–467.

Okada, K., and Miyatake, T. (2009). Genetic correlations between weapons, body shape and fighting behavior in the horned beetle Gnatocerus cornutus. Anim Behav 77:1057-1065.

Okada, K., Nomura, Y., and Miyatake, T. (2007). Relations between allometry, male-male interactions and dispersal in a sap beetle, *Librodor japonicus*. Anim Behav 74:749–755.

Palmer, T.J. (1978). A horned beetle which fights. Nature 274:583-584.

Petrie, M. and Williams, A. (1993). Peahens lay more eggs for peacocks with larger trains. Proc. R. Soc. London Ser. B251, 127–131.

Pimental, D. (1949). Biology of *Gnathocerus cornutus*. J. econ. Ent. 42, 229-231.

Pomfret, J. and Knell, R. (2006). Sexual selection and horn allometry in the dung beetle *Euoniticellus intermedius*. Animal Behaviour, 71, 567-576.

Rantala, M.J. and Kortet, R. (2003). Courtship song and immune function in the field cricket *Gryllus bimaculatus*. Biol. J. Linn. Soc. 79: 503–510.

Ratcliffe, N.A., Rowley, A.F., Fitzgerald, S.W., and Rhodes, C.P. (1985). Invertebrate immunity: basic concepts and recent advances. *Int. Rev. Cytol.* **97**, pp. 183–350.

Reeson, A.F., Wilson, K., Gunn, A., Hails, R.S. and Goulson, D. (1998). Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density. Proceedings of the Royal Society of London B, Biological Sciences 265:1787–1791.

Sadd, B., Holman, L., Armitage, H., Lock, F., Marland, R. and Siva-Jothy, M.T. (2006). Modulation of sexual signaling by immune challenged male mealworm beetles (*Tenebrio molitor*, L.): Evidence for terminal investment and dishonesty. *Journal Compilation* 19:321–325.

Saino, N., Ambrosini, R., Martinelli, R., Ninni, P., and Moller, A.P. (2003). Gape coloration reliably reflects immunocompetence of barn swallow (Hirundo rustica) nestlings. Behav. Ecol. 14: 16–22.

Savvidou, N., and Bell, C.H. (1994). The effect of larval density photoperiod and food change on the development of Gnatocerus cornutus (F.) (Coleoptera: Tenebrionidae). J. stored Prod. Res. 30: 17–21.

Schmid-Hempel, P. (2005) Evolutionary ecology of insect immune defenses. Annu Rev Entomol 50: 529–551.

Schwarzenbach, G.A., and Ward, P.I. (2007). Phenoloxidase activity and pathogen resistance in yellow dung flies *Scathophaga stercoraria*. J Evol Biol 20:2192–2199.

Sheldon, B.C. and Verhulst, S. (1996). Ecological immunology: costly parasite defenses and trade-offs in evolutionary ecology. - Trends Ecol. Evol. 11: 317-321.

Shuster, S.M., and Wade, M.J. (2003). Mating Systems and Strategies. pp:12-18. Princeton University Press; Princeton, NJ.

Söderhäll, K., and Cerenius, L. (1998). Role of the prophenoloxidase activating system in invertebrate immunity. *Curr. Opin. Immunol.* 10, pp. 23–28.

Tallamy, D.W., Powell, B.E. and McClafferty, J.A. (2002). Male traits under cryptic female choice in the spotted cucumber beetle (Coleoptera: Chrysomelidae). Behav. Ecol. 13, 511– 518.

Thompson, C.W., Hillgarth, N., Leu, M., and McClure, H.E. (1997). High parasite load in house finches (*Carpodacus mexicanus*) is correlated with reduced expression of a sexually selected trait. Am Nat 149:270–294.

Tomkins, J.L., Kotiaho, J.S., and LeBas, N.R. (2005). Phenotypic plasticity in the developmental integration of morphological trade-offs and secondary sexual trait compensation. Proceedings of the Royal Society of London. Series B. Biological Sciences, 272, 543–551.

Trivers, R.L. (1972). Parental investment and sexual selection. Pages 136-179 in B. Campbell, ed. Sexual selection and the descent of man. Aldine, Chicago.

Tsuda, Y. and Yoshida, T. (1984). Population biology of the broad-horned flour beetle *Gnathocerus cornutus* (F) (Coleoptera Tenebrionidae) I. Life table and population parameters. Appl Entomol Zool 19:129–131.

Tsuda, Y., and Yoshida, T. (1985). Population biology of the broad-horned flour beetles, *Gnathocerus cornutus* (F.) II. Crowding effects of larvae on their survival and development. Res. Popul. Ecol. 27: 77–85.

Wallace, A.R. 1868. *Journal of Travel*, Vol. 1, A. Murray (ed.), n.p.

Yan G., Stevens, L., and Schall, J. (1994). Behavioral changes in Tribolium beetles infected with a tapeworm: Variation in effects between beetle species and among genetic strains. American Naturalist. 143:830–847.

Zahavi, A. (1975). Mate selection – a selection for a handicap. Journal of Theoretical Biology 53:205-214.

Zhong, D., Pai, A., and Yan, G. (2005). Costly resistance to parasitism: evidence from simultaneous quantitative trait loci mapping for resistance and fitness in *Tribolium castaneum*. Genetics. 169:2127–2135.

Zuk, M., Johnson, K., Thornhill, R. and Ligon, J.D. (1990). Mechanisms of female choice in red jungle fowl. Evolution 44, 477-485.

BIOGRAPHICAL INFORMATION

Amrita Naidu is a Thomas F. Pyburn fellow studying animal behavior and ecology. She was born and brought up in Kathmandu, Nepal and came to the United States in 2003 in pursuit of higher education. After getting her Bachelor of Science degree in Microbiology from the University of Texas at Arlington in 2006, she worked as a laboratory coordinator for a food processing plant for several months before deciding to return to school to work on her Master's degree. She wants to work as a researcher in the field of health, food or environment after returning to her native Nepal upon completion of her Master's degree at the University of Texas at Arlington in December 2009.