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Declaration

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All other research described in this report is my own original work.

Shanta C. Nair

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Epigenetic regulation of immunity: Methylation profiling in the mealworm beetle, *Tenebrio molitor*

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Abstract

Immune responses incur a large energy cost for organisms. Hence, environmental conditions such as nutritional status may change how an organism's immune response genes are expressed, and these changes can be inherited by offspring. At the centre of this is the concept of epigenetics: the process by which environmental factors alter an organism's DNA expression. These changes can be heritable, and so it is widely hypothesized that epigenetics is the key mechanism driving the co-evolution of hosts and parasites. This study used *T. molitor* to investigate the regulatory role of a particular type of environmentally induced epigenetic change, methylation, on immune response levels. Beetles were subjected to different starvation treatments before being presented with a bacterial immune challenge. Immunoassays and methylation sensitive AFLP demonstrated that methylation patterns in this species are subject to change following a period of starvation, and that starvation downregulates phenoloxidase activity levels.

KEYWORDS: Epigenetics, methylation, phenoloxidase, *Tenebrio molitor*

Notes on Thesis Preparation

This thesis has been formatted according to the guidelines for the journal Developmental and Comparative Immunology, as a manuscript for publication. Line numbers have been included for ease of examination, and the Introduction section is slightly extended relative to a standard journal article.

1. Introduction

Preface

The ability of an organism to distinguish “self” and “not-self” at the cellular and molecular levels forms the heart of immune theory, and has directed immunological study throughout its history (Burnet, 1969). The majority of immunology centres on vertebrates, where the immune response takes the form of either non-specific (innate) or highly specific (adaptive) sets of defense mechanisms (Burnet, 1969). The non-specific response is primarily instigated by pattern recognition receptors (PRRs) recognizing pathogen-associated molecular patterns (PAMPs) on not-self cells (Shrestha and Kim, 2010). In contrast, the specific (adaptive) response relies on the accumulation of memory T-cells and B-cells to prime against future pathogenic exposure. Both systems rely heavily on the expression and recombination of host immune response genes (Shrestha and Kim, 2010). While vertebrate immunity has been studied in depth, analyses of invertebrates have thus far served primarily as model organisms to infer the evolution of vertebrate immunity. Despite the biodiversity and evolutionary success of insects (Vilcinskas, 2013), studies involving insects have traditionally asserted that this group possesses only non-specific immune systems, lacking an adaptive component (Dheilly et al., 2014; Vilmos and Kurucz, 1998). This reflects a paucity of information. The traditional anthropocentric approach ignores the majority of Earth’s multicellular biodiversity. It also ignores the true variety of highly specialized and effective invertebrate immune responses (Haine et al., 2008 B) and their taxon-specific ecological adaptations (Dheilly et al., 2014). In this context, the insect world must form the foundations for our concept of modern immune theory alongside our understanding of the vertebrate (primarily mammalian) immune system. Remedying this gap in knowledge will rely heavily on studying the genetic mechanisms underlying immunological adaptation in insects, with particular emphasis on environmental factors that alter gene expression. Among the many available options for the regulation of immunity, such studies may be able to characterize the potential for epigenetic changes as a functional mechanism for host-parasite co-evolution and/or evolutionary ecological immunity (Vilmos and Kurucz, 1998). This Introduction will focus on insect immune responses as a way to inform the next-generation of molecular studies that may transform our broad understanding of immunology. The Introduction focuses on studies of the mealworm beetle, *Tenebrio molitor*, as a model organism. It investigates the molecular and biochemical responses associated with environmental change and its putative effects on the beetle immune system.

1.1 Host-Parasite Coevolution

Implications for Ecology

Host-parasite coevolution theory, or the "Red Queen" hypothesis, is central to evolutionary immunology (Vilcinskas, 2013). The theory suggests that pathogens and their hosts are at odds in an ongoing evolutionary arms race, each seeking strategies to outcompete the other. If we consider the impressive biodiversity of insects as indicative of their evolutionary success in this context, the mechanisms that drive insect immunity appear to be highly efficient (Vilmos and Kurucz, 1998). From this, Vilmos (1998) proposed that the insect immune system is a homologue of the mammalian innate immune response. As insect immune systems had not previously been confirmed to have an adaptive component involving immunoglobulin antibodies, studies have since concentrated on documenting alternative systems utilized by insects (Dheilly et al., 2014).

If we accept that innate immunity must form the basis for the insect immune response, then it follows that maintaining an innate immune response against a pathogen would incur considerable fitness costs to the individual (Armitage, S.et al., 2003). Armitage and colleagues (2003) examined the costs of maintaining an investment in innate immunity in the mealworm beetle *T. molitor*, and found a longevity cost associated with producing an encapsulation response (part of the insect immune arsenal). Interestingly, there was no similar effect observed in association with a constitutive (or prophylactic) response against infection. This indicates that the ability to protect against infection is an efficient choice in terms of fitness costs, and suggests that there must be a mechanism for insects to adapt and respond to an infection that staves off re-infection (Armitage, S., et al., 2003).

Antimicrobial peptides (AMPs) have been put forward as a putative mechanism for such rapid adaptation (Vilcinskas, 2013). Vilcinskas (2013) suggests that insect AMPs may have a role beyond immune defense, functioning to regulate beneficial endosymbiotic species for the host. This argument stems from the author's assertion that, as insects are the most biodiverse group of organisms on earth, they show an unprecedented ability to adapt rapidly to pathogenic environments, which in turn are rapidly evolving (Vilcinskas, 2013). This extension of the host-pathogen "Red Queen" hypothesis suggests that insect AMP repertoires expand and diversify to reflect these host-parasite relationships. This concept weighs against arguments that predict the loss of immune response genes in favour of less costly adaptations, such as social immunity. Hence, the need for examination of insect AMPs and their roles, along with a broader re-examination of host-parasite coevolution, becomes apparent.

Insect Studies: Immunology and Ecology

In 2003, a new investigative field ("ecological immunology") began to emerge that sought to address the role of insect immunity within an ecological context (Rolff and Siva-Jothy, 2003). Ecological immunology represents a growing field in biology, in which ecology and immunology are synthesized to characterize variation in immune responses. By examining the causes and effects of immune system variability within ecosystems, immune responses themselves may be better characterized and their role in ecological processes better understood (Rolff and Siva-Jothy, 2003). The amount of interest generated by ecological immunology in the scientific community over the past decade is testament to its importance—and also highlights the need for molecular insights to reconcile immunological observation with evolutionary theory (Martin et al., 2011).

The search for genetic mechanisms to characterize immune variation at the molecular level has a long history. Early work speculated that immune responses in insects must be under frequency-dependent selection. This mechanism would account for and maintain high levels of variation in immune response genes even as natural selection seeks to minimize variation in other traits involved in fitness (Cotter et al., 2003). However, Cotter et al. (2003) cautioned against using broad heritability studies alone to characterize immune responses, citing it as being too indirect a method to accurately examine pathogen-induced responses (Cotter et al., 2003).

Studies since appear to have focused on just one component of immune responsiveness at a time, for example phenoloxidase activity. Phenoloxidase activity in particular has the ability to serve as a proxy for overall immune responsiveness (González-Santoyo and Córdoba-Aguilar, 2012). The consensus of the recent work is that the phenoloxicase cascade incurs considerable costs for the individual, with repercussions for host fitness. González-Santoyo and Córdoba-Aguilar (2012) suggest that future work needs to be focused on the complex chemical processes and interactions underlying phenoloxidase-based immune responses (González-Santoyo and Córdoba-Aguilar, 2012). Once again, it appears that more quantitative studies are required to reconcile the complex interactions of genotype, environment, and immune response.

An early quantitative study of insect immune responses proposed that females gain a genetic benefit from selecting males that are more resistant to parasites as mates (Hamilton and Zuk, 1982). From this premise, Siva-Jothy and Thompson set out in 2002 to assay immune effector activity in an insect species to identify the costs involved in producing an immune response. They investigated phenoloxidase immune responses in *T. molitor*, comparing the responses of starved beetles against

those raised under optimal conditions. It was found that immune responses were downregulated during short-term starvation, and that this effect was reversed after normal feeding was restored. This suggested a link between an environmental stress (starvation) and immune system regulation (Siva-Jothy and Thompson, 2002).

Subsequently, the effects of immune effector activity over time have been investigated by quantitatively measuring immune responses in the form of antimicrobial peptides and the phenoloxidase cascade (Haine et al., 2008 A). Over a period of time, *T. molitor* exposed to different live and dead pathogens were found to increase the production of haemolymph AMPs immediately following a bacterial challenge, and that this response was maintained for approximately fourteen days. No concurrent response in phenoloxidase activity was noted, suggesting that phenoloxidase is not solely responsible for maintaining a long-lasting immune response (Haine et al., 2008 B). This temporal regulation of an immune response again suggests a trade-off in terms of costs and benefits for maintaining a specific immune response. It also suggests that there is complex set of interactions between the environment and immunity at the molecular level, as a long-term immune response indicates both the presence of an environmental stimulus to elicit it and an underlying mechanism to respond to stimuli.

Similarly, Lee et al. (2008) examined the effect of differences in dietary protein on phenoloxidase activity and melanization in the caterpillar *Spodoptera littoralis*. Comparisons between treatment groups fed high-protein quality and low-protein quality diets showed higher antibacterial activity with a high-protein diet. Interestingly, the study found that phenoloxidase activity was not affected by dietary protein levels. These results do not concur with previous studies, such as those of Siva-Jothy and Thompson (2002). Such differences may be due simply to differences between taxa in metabolic processes and feeding habits. However, they highlight the need for further study into inducible immune responses in invertebrates, and raise the question of to what extent the environment dictates immune response activity.

1.2 Strain-Specific Immunity and Transgenerational Effects

Constitutive vs. Strain-Specific Defenses

The question of nonspecific responses vs. strain-specific immunity in insects has presented opportunities to expand our knowledge of immunology as a whole, as well as the opportunity to inform ecological studies in a broader context. For example, persistent antimicrobial activity vs. constitutive defense in *T. molitor* was evaluated by monitoring the success of bacterial clearance (Haine et al., 2008 A). The premise of this study was that the pathogens of insects rarely exhibit microbial resistance against insect immune responses. This implies that the induced immune responses of insects may work to suppress persistent bacterial infections within the host, rather than clearing the infection outright. By maintaining a suppressed population of bacteria, insects could employ a strategy that is entirely different to the approach taken by modern antibiotic medicine (Haine et al., 2008 A).

This emphasizes the importance of further insect studies. It also raises the question of whether forms of adaptive immunity exist in invertebrates. Clearly, insect species show the ability to respond to environmental conditions in terms of their immune responses (Haine et al., 2008 B). More specifically, the survival benefit of previous pathogenic infection through induced prophylactic immune response in *T. molitor* has been studied to test the principle that previous infection indicates a higher risk of reinfection in the near future. While acquired immunity involving immunoglobulin antibodies is not present in invertebrates, the presence of reinfection risk following initial exposure indicates the need for a prophylactic response. In this context, pre-exposed *T. molitor* show a survival benefit to subsequent fungal infections (Moret and Siva-Jothy, 2003).This led to the concept of immune priming as an analog of the specific immune memory found in vertebrate species (Schmid-Hempel, 2005). The need for further study into invertebrate host-parasite interactions is emphasized. This would allow us to isolate ecologically or evolutionarily significant relationships that may indicate such a response. Figure 1 (below) shows the author's representation of the insect immune system, reflecting a network of complex interactions that reflect a long evolutionary history (Schmid-Hempel, 2005).

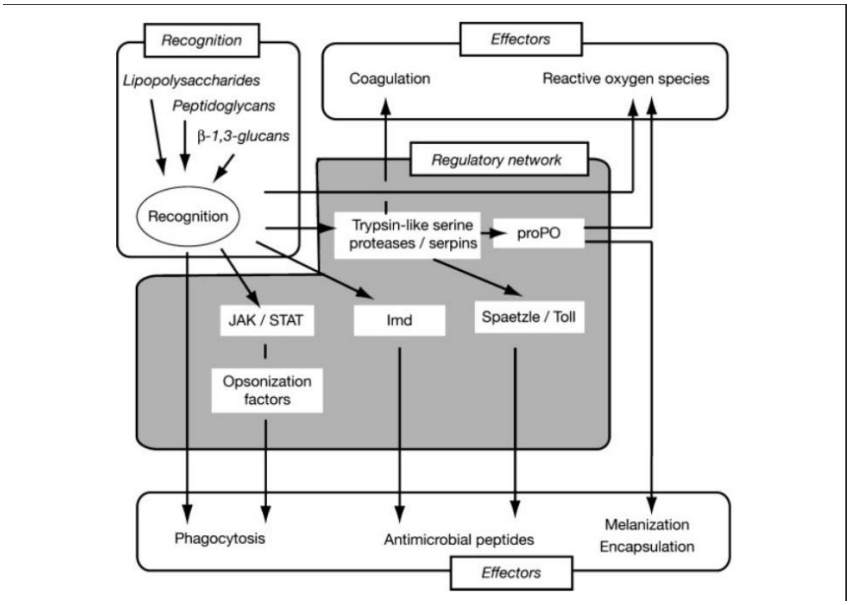


Figure 1. Summary of recognition, regulation, and effector mechanisms in the insect immune system, illustrating the complexity of the insect immune response as the result of a long evolutionary history. (from Schmid-Hempel, 2005).

This representation includes the new concept of immune priming and the function of specific immunity, laying the groundwork for future molecular studies to elucidate the mechanistic basis of this phenomenon (Schmid-Hempel, 2005).

Transgenerational Immune Priming (TGIP)

If we accept adaptive immunity as a newly discovered facet of the insect immune response, it follows that such adaptive changes may result in heritable trait changes as part of host-parasite coevolution. It had been assumed that invertebrates possess only innate immunity, as their lack of immunoglobulin antibodies, T-cell receptors and a major histocompatibility complex prevents adaptive immunity of the type seen in mammals (Little et al., 2003). This idea has since been challenged, with the argument that some invertebrates show forms of acquired strain-specific immunity as a result of sociality or population density. Maternally conferred strain-specific bacterial immunity in an invertebrate model was investigated by Little et al. (2003). They found increased survival of *Daphnia magna* infected with a bacterial strain when subjects' mothers had also been infected with the same strain prior to breeding (Figure 2; Little et al., 2003).

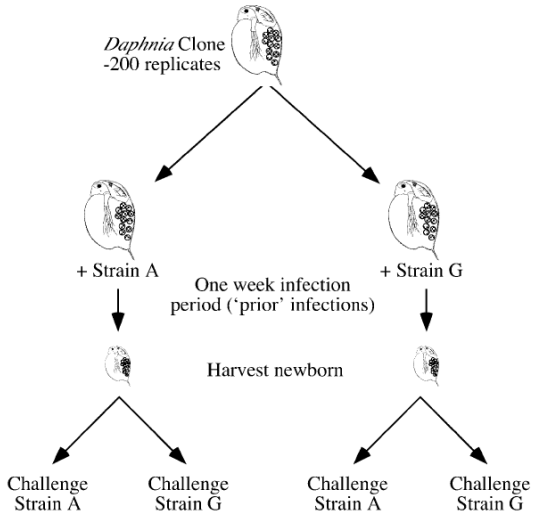


Figure 2. Experimental design to observe transgenerational immune priming via maternal transfer of resistance to infection. (from Little et al., 2003).

This represents one of the first studies investigating the transfer of immune response capabilities between generations, and gives an early example of transgenerational immune priming (TGIP). As such, it represents a synthesis of adaptive innate immunity and host-parasite coevolution (Little et al., 2003).

Building on the work of Moret and Schmid-Hempel, functional relationships concerning invertebrate immunity have been investigated in an evolutionary context (Sadd et al., 2005). Immunoassays on *Bombus terrestris* revealed higher levels of induced antibacterial activity in the offspring of queen bees that had been given an immune challenge (Sadd et al., 2005). Similarly, bacterial lipopolysaccharides were used to induce immune reactions in *Tenebrio* beetles over the course of two generations (Moret, 2006). Parental exposure to this pathogen-associated molecular pattern improved offspring immunity to the pathogen. Further examination of *T. molitor* TGIP in females found that smaller females invested more in their own immune response, at the expense of their egg production (Moreau et al., 2012). This indicates a trade-off between immunity and reproduction. Natural selection would suggest reproduction as the biological imperative, meaning that reproduction should receive the bulk of an individual's resource investment. However, in the case of *T. molitor*, short generation times plus the species' low dispersal rates favour transgenerational infection, and hence TGIP as a more efficient use of resources to ensure survival.

Following this, a study on the effects of TGIP on developmental stages of the tobacco hornworm, *Manduca sexta*, found that phenoloxidase activity of unchallenged larvae, pupae, and adult offspring differed, whereas no variation occurred in immunochallenged parents. This suggests that while transgenerational immune priming has significant, observable effects on insect immunity, these effects may not be obvious from current studies, and may form more complex interactions at the molecular level (Trauer and Hilker, 2013).

1.3 Epigenetics: A Mechanism for Immune Priming

Overview

Epigenetic change refers to modifications to the DNA or to histones that alter gene expression without changing the underlying genetic sequence. For example, the addition of methyl groups to cytosine bases (methylation) changes the shape of the DNA, and hence its interaction with transcription factors (Romanoski et al., 2015). Methylation alters the way a particular gene is expressed, without changing the gene itself, and so is reversible (Jaenisch and Bird, 2003). This makes methylation a key mechanism for modification of gene expression by environmental conditions. As early as 2003, it was widely accepted that epigenetic changes could dictate physiological responses to the environment (Jaenisch and Bird, 2003). More recently, methylation has been investigated in more detail, and has been established as a "silencing" mechanism (Jones, 2012). With improved genome-wide mapping techniques, it is now possible to examine methylation in different situations and determine its different effects. Particular emphasis has been placed on the role of methylation in regulatory elements, such as transcriptional start sites and repeat sequences (Jones, 2012).

Molecular Techniques and Insect Studies

In recent years, next-generation sequencing techniques have allowed more fine scale comparison between insect species and taxa at the molecular level (Dheilly et al., 2014). It also has the potential to revolutionize the study of epigenetic changes in invertebrate species. Recently, a whole-genome profile of methylation in the jewel wasp, *Nasonia vitripennis,* was published (Beeler et al., 2014). Similar analyses can be used to investigate the mechanism behind transgenerational immune priming and the environmental factors that influence this process.

1.4 Nutrition and Immunology in *Tenebrio molitor*

Previous studies involving nutrient deprivation and immune challenge showed not only that immune response in *T. molitor* change following an environmental stressor, but also that these changes are transferred to offspring (Moret, 2006; Siva-Jothy and Thompson, 2002). Further, *Tenebrio* beetles have a relatively short generation time and are easy to maintain in captivity, making them an ideal model organism for such studies (Siva-Jothy and Thompson, 2002). More detailed investigations of the effects of nutrient deprivation on immune responses would be able to make use of modern molecular techniques to assess methylation signatures at immunoregulatory regions of the genome as a way of resolving the molecular mechanism responsible for TGIP.

However, using *Tenebrio* as a model organism presents significant challenges, as DNA extraction from beetles is problematic. Different methods of DNA extraction in insects have been published in recent years, resulting in successful protocols for extracting PCR-competent DNA from several invertebrate taxa (Gillings, 2014; Chen et al., 2010). This has resulted in the genome sequencing of many insect species and has facilitated gene function and expression studies. *T. molitor* does not have a fully sequenced genome to date, even though a complete genome sequence exists for a closely related species (*Tribolium castaneum*), (Richards et al., 2008). Despite this, there has been considerable progress made in identifying and annotating *T. molitor* genes related to immune function, including the annotation of expressed sequence tags and cDNA libraries (Dobson et al., 2012) (Jeong et al., 2013). Studies such as these provide a basis for epigenetic analyses as the next phase in comparative immunology, and its potential to work alongside next-generation sequencing studies to link gene regulation and expression with environmental conditions (Zhu et al., 2013).

Parallel to studies of immune genes, work on the detection of methylation signatures has been carried out in recent years, with different methods of methylation analysis being developed. (Patterson et al., 2011). Methylation profiling has also been applied to recent insect studies. For instance, methylation has been shown to regulate the ability of the bee *Bombus terrestris* to alter its reproduction. In this case, methylation-sensitive amplified fragment length polymorphism (AFLP) was used to identify methylation sites across the genome and investigate the role of methylation in altering reproductive ability (Amarasinghe et al., 2014). However, the question remains as to whether methylation is present in species outside the social hymenoptera, such as *Tenebrio molitor.* The presence of environmentally triggered immune response changes in this species, and their ability to be transferred to subsequent generations, would suggest that this is the case. Furthermore, preliminary work conducted in 2015 has

indicated the presence of methylation signatures within the *Tenebrio* genome, confirming it as a candidate mechanism for TGIP (May, 2015).

1.5 Research Aims and Hypotheses

Previous research (described above) suggests that starvation leads to the downregulations of insect immune responses (Siva-Jothy and Thompson, 2002). However, the mechanistic basis of this downregulation has not been thoroughly investigated. This study addresses that deficit in our knowledge by testing whether changes in genomic DNA methylation signatures are associated with downregulation of the phenoloxidase response in *T. molitor* following a period of starvation. The study tests the hypothesis that *T. molitor* will show changes in DNA methylation after starvation. It also tests whether there is a concurrent downregulation of phenoloxidase enzyme activity in starved beetles.

2. Materials and Methods

2.1 Summary of experimental design

To investigate the effects of starvation on methylation patterns of immunoregulatory genes and phenoloxidase activity in the beetle, *T. molitor*, populations were subjected to a period of starvation and subsequent immune challenge. This was followed by immunoassays of phenoloxidase activity and molecular analysis to determine the methylation states of beetle DNA. A complete schematic of methods is provided in figure 3 below.

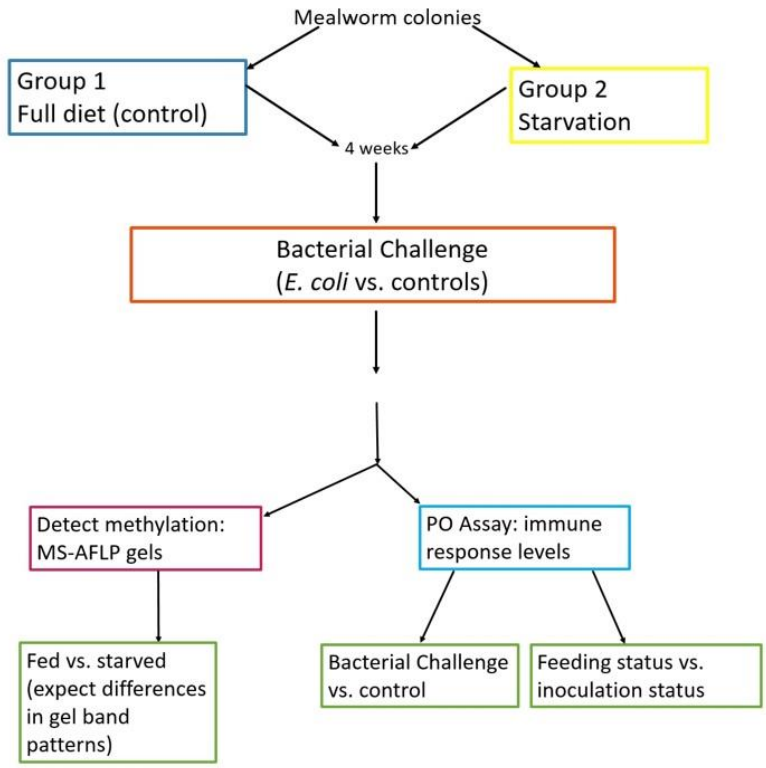


Figure 3. Flow chart of experimental methodology.

2.2 *T. molitor* culture and treatments

Starvation Protocols

To assay changes in methylation and phenoloxidase activity due to environmental stress, 4^{th}-5^{th} instar *T. molitor* larvae (obtained from Pisces Enterprises) were divided into two treatment groups subjected to different feeding conditions for four weeks:

1. Group One (control, fully fed group): *T. molitor* were given a substrate of approximately three cups of edible rolled oats per 40 larvae and supplied with 1 fresh leaf of Chinese cabbage per 40

larvae as a water and nutrient supplement three times a week. Additionally, populations received a protein and sugar supplement of one tablespoon of skim milk powder once a week.

2. Group Two (experimental, starved group): *T. molitor* were cultured on a substrate of approximately three cups of inedible pine shavings, with no ready source of protein or carbohydrate. They received a water and nutrient supplement of one Chinese cabbage leaf only once per week.

Each treatment group comprised five replicate populations in 220 × 140mm plastic containers with forty larvae in each replicate. Additionally, two stock colonies (comprising forty individuals each) were held under conditions mirroring each treatment group. That is, stock colony boxes were held under conditions identical to those of each treatment group. Treatment populations were closely monitored during progression to adulthood, and population density in each replicate was maintained by replacing any dead individuals with those from the stock colonies. Population density was maintained within each replicate to maintain adequate numbers for later division of replicate groups into inoculation treatments and to maintain the amount of food available per individual (see below).

All boxes were kept in a light- and temperature-controlled room, with a light/dark cycle of approximately 12 hours and a constant temperature of approximately 25°C. Arrangements of boxes on shelves were shuffled at random after each observation.

2.2 Bacterial Challenges and Phenoloxidase assays

Bacterial Challenges

At the end of the four-week starvation period, adult *T. molitor* from each treatment group (starved vs fully fed) were subjected to bacterial challenges before they were assessed for phenoloxidase activity and DNA methylation. To administer bacterial challenges, cultures of *Escherichia coli* (strain BMH 71-18 *mutS*) from Macquarie University's Department of Chemistry and Biomolecular Science stocks were grown from frozen competent cells. Bacteria were cultured by inoculating 50mL Falcon tubes containing prepared LB broth with 25µl of defrosted cell stock. Pipette tips used for inoculation were left inside the tubes, and cultures were left overnight to incubate with shaking at 37°C. After

incubation, 12mL aliquots were taken and transferred to 15mL falcon tubes. Cultures were then heat-killed by immersion in a water bath at 90°C for 30 minutes.

Nine adults taken from each replicate (5 x 9 fully fed and 5 x 9 starved) were inoculated with heat-killed *E. coli* using a pin dipped into the culture solution and inserted into the abdomen. A further nine individuals from each replicate were inoculated in the same manner with phosphate buffered saline (PBS) to act as a sham control, while a further nine individuals per replicate did not undergo any inoculation.

Haemolymph was extracted from three bacteria-inoculated, three PBS-inoculated, and three non-inoculated individuals from each replicate at five hours, 24 hours, and 48 hours following inoculation. Haemolymph was extracted by piercing each individual with a sterile pin inserted into the pleura between the head and thorax, with exuding fluid pipetted into PCR tubes and frozen immediately. Following haemolymph extraction, sampled individuals were frozen at -30°C.

Extracted haemolymph samples were then diluted 1:20 with a 0.5% Triton X-100 lysing agent in sodium dodecyl sulfate (SDS, 10% in water) prior to phenoloxidase and methylation assays.

Phenoloxidase assays

The phenoloxidase enzyme activity assay reported by Haine et al. (2008, A) was optimized as follows. Initially, PBS was used as a physiological buffer and for dilution of haemolymph samples, both as a 1:20 dilution and in serial dilutions across a microtitre plate. Both of these approaches resulted in inconsistencies in the lysis of haemocytes and in the conversion of L-DOPA to dopachrome, as reflected by inconsistent immunoassay results. Following this, SDS (10% in water) was trialed as a substitute for PBS. However, SDS resulted in bubbling within the microtitre plate wells, rendering samples unreadable by the plate reader. Finally, lysing agent Triton X-100 was used as a 0.5% solution in 10% SDS. This solution lysed haemolymph cells adequately without bubbling, as reflected in consistent assay results. Siva-Jothy and Thompson (2002) report using a freeze-thaw method at -90°C to disrupt haemocyte cells for phenoloxidase assays; however due to the need to also extract DNA from sampled individuals freezing at this temperature would have risked denaturing the DNA. Therefore SDS was used as a detergent to lyse cells as a modification to their protocol (Siva-Jothy and Thompson, 2002).

Subsequently, phenoloxidase activity in haemolymph samples was measured using a Multiskan plate reader (Thermo-Fisher Scientific), using a protocol adapted from Siva-Jothy and Thompson (2002). Eight µl aliquots of diluted (1:20) haemolymph in 0.5% Triton-X-100 were mixed in the wells of 96-well microtitre plates with 56µL distilled water, 8µl L-DOPA substrate (3mg/mL in distilled water) and 64µL 0.5% Triton X-100 in distilled water. Phenoloxidase activity was then measured continuously for 50 minutes, using a kinetic stepping measure with shaking at 490nm (Wilson et al., 2011). Enzymatic activity was represented as a maximum reaction rate for each sample, calculated as the mean slope in the first five minutes of each reaction. The maximum reaction rates for different treatments were compared using two-factor ANOVAs where the two factors were feeding treatment and inoculation treatment. Post-hoc analyses were conducted using paired t-tests. Differences between treatments were considered to be significant if $p < 0.05$.

2.3 DNA Extraction and Methylation Profiling

DNA Extraction

DNA extraction from adult *T. molitor* was optimized from the protocol of Chen et al. (2010) as follows. The initial DNA extraction protocol based on Chen et al. (2010) used TriZol (Phenol 30-60%, guanidine thiocyanate 15-40% ammonium thiocyanate 7-13%, Life Technologies) and a modified protocol using SDS yielded too little high quality DNA for subsequent analysis. Hence, a phenol:chloroform:isoamyl alcohol (25:24:1) extraction including a proteinase-K digestion step and a cleanup step with 1,3 bromo-chloro-propane (BCP) to remove excess phenol contamination was adopted. This resulted in consistently adequate DNA concentrations and minimal contamination from other reagents. The finalized DNA extraction protocol was as follows:

Adult *T. molitor* specimens that had been frozen following inoculation treatments and haemolymph sampling were dissected, with the head, legs, and elytra removed. Single beetles were then homogenized in a glass homogenizer with 1 mL of lysis buffer. The resulting liquid homogenate was removed to an Eppendorf tube using a glass pipette to minimize accidental transfer of pieces of whole tissue. 10µL of Proteinase-K (Sigma-Aldrich) was added to each tube, and then sample tubes were incubated overnight at 55°C.

Following homogenization and incubation, sample volumes were made up to 500µL with lysis buffer (10mM NaCl and 10mM tris-HCL in 0.5% SDS) and an equal volume of phenol:chloroform:isoamyl alcohol was added. Tubes were emulsified and centrifuged at 16,000 × g for 5 minutes, resulting in a

phase separation with DNA contained within the uppermost phase. The top aqueous phase was removed to a new tube, while the rest was discarded. Sample volumes were then made up to 1mL with 10mM tris-hydrochloride, and an equal volume of 1, 3 bromo-chloro-propane was added. Again, samples were emulsified and centrifuged at 16,000 × g for 5 minutes. This resulted in another phase separation, and once again the aqueous phase was removed to a new tube.

To the recovered aqueous phase, 10% of the total sample volume of 3M sodium acetate was added, along with 60% of the total sample volume of isopropanol. Samples were mixed via pipetting, before being centrifuged at 16,000 × g for 15 minutes. This resulted in the formation of a DNA pellet at the bottom of each tube. The aqueous material was discarded, and the remaining pellet was washed with 50μL of 70% ethanol. A short centrifugation recovered the pellet once more, and the ethanol wash step was repeated. Finally, any remaining ethanol was pipetted off and discarded, and sample tubes were left open for approximately 5 minutes to evaporate any remaining ethanol. The pelleted samples were then resuspended in 50μL of a pre-made resuspension buffer (10mM tris-HCL with EDTA) and stored at -30°C.

2.4 Methylation Profiling: MS-AFLP

To analyze changes in methylation states between fed and starved specimens, methylation-sensitive amplified fragment length polymorphism (MS-AFLP) was carried out on genomic DNA samples according to an established protocol (Amarasinghe et al., 2014). Two restriction enzymes were used, each with a different sensitivity to methylation states: MspI, which binds restriction sites regardless of methylation state, and HpaII, which does not cut at methylated cytosines. Together, the activity of the two enzymes can be compared to detect methylation patterns within a certain recognition site across a genome: 5’ CC/GG. As methylated cytosines tend to form C/G rich islands, this recognition site was used along with the two methylation-sensitive restriction enzymes to search for a genome-wide pattern of methylation without the need for a fully sequenced and annotated genome (Amarasinghe et al., 2014). Genome wide restriction fragments were then amplified with 12 sets of sequence specific primers to identify differences in methylation patters. Enzymes and primers for fragment amplification were selected based on Amarasinghe et al. (2014), where methylation was detected in the genomes of the bumblebee *Bombus terrestris.* Previous testing of this protocol in our laboratory has shown the same protocol amplifies as yet unsequenced regions of the *Tenebrio* genome, as confirmed by gel electrophoresis (see supplementary figures).

First, genomic DNA samples were digested with the two restriction enzymes, as two separate reactions each paired with EcoRI. The digest products were then ligated separately with HpaII-MspI adapters, and with EcoRI adapters. Two rounds of PCR followed, generating overhangs with sticky ends. The EcoRI adapter and HpaII-MspI adapters then bound to the sticky ends, facilitating PCR on an otherwise unknown DNA sequence, as the adapters function as universal primer sites. This process is summarized in figure 4 below.

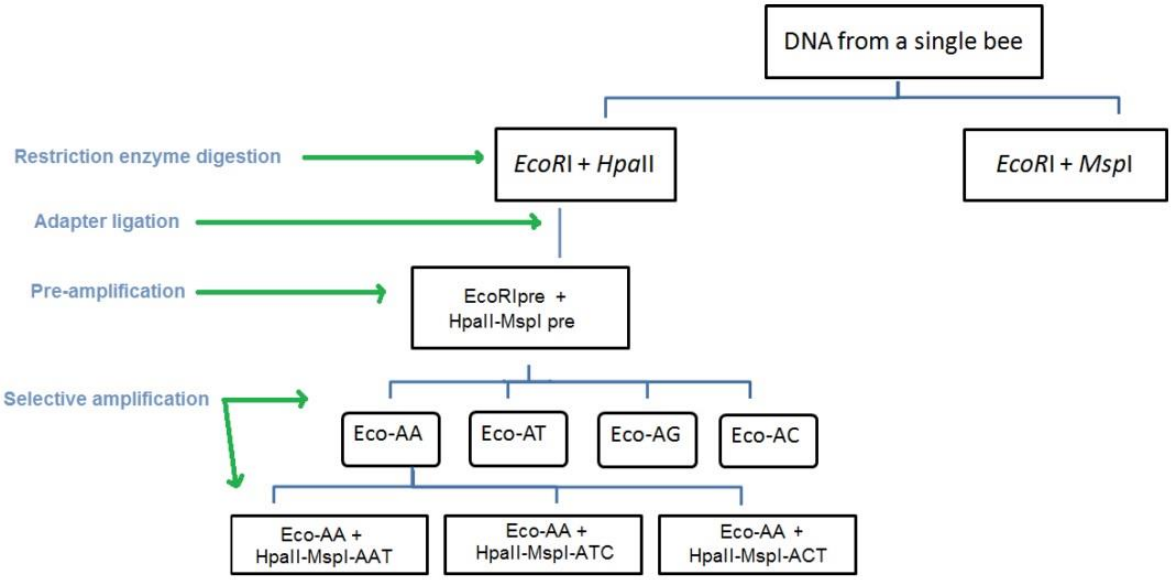


Figure 4. Flow chart of MS-AFLP process, including restriction digestion, ligation, and both rounds of PCR amplification (from Amarasinghe et al., 2014)

Adapters and primers used throughout the MS-AFLP process were as reported in Amarasinghe et al. (2014), and are summarized in Table 1 below.

Table 1. Summary of primers used throughout MS-AFLP (from Amarasinghe et al., 2014).

Adapter / Primer	Sequence (5' -3')
Ligation	
*EcoR*I-F	CTCGTAGACTGCGTACC

*EcoR*I-R	AATTGGTACGCAGTCTAC
HpaII-MspI–F	GACGATGAGTCTAGAA
HpaII-MspI –R	CGTTCTAGACTCATC
Preamplification	
EcoRIpre (EcoRI + 0)	GACTGCGTACCAATTC
HpaII-MspI pre (HpaII-MspI + A)	GATGAGTCTAGAACGGA
Selective amplification (sequence specific primers)	
Eco-AA	**GACTGCGTACCAATTCAA**
Eco-AT	**GACTGCGTACCAATTCAT**
Eco-AG	**GACTGCGTACCAATTCAG**
Eco-AC	**GACTGCGTACCAATTCAC**
HpaII-MspI-AAT	**GATGAGTCTAGAACGGAAT**
HpaII-MspI-ACT	**GATGAGTCTAGAACGGACT**
HpaII-MspI-ATC	**GATGAGTCTAGAACGGATT**

Restriction Digest

The protocol for MS-AFLP and subsequent PCR was modified from Amarasinghe et al. (2014), as follows. EcoRI and MSpI were used to digest the genomic DNA samples in two separate reactions: an MspI digest and an HpaII digest. MspI digest reactions consisted of at least 3µl target gDNA (at 500ng/µL) with 0.2µL EcoRI (200 units/µL), 0.25µL MspI (20,000 units/mL), 1µl CutSmart NEB buffer (New England Biolabs). HpaII digest reactions also used at least 3µL of 500ng/µL target gDNA and 0.02µL EcoRI (200units/µL), with 0.5µl HpaII and 1µL CutSmart NEB buffer. Reactions were made up to a total volume of 10µL with distilled water. Digests were then incubated at 37°C for one hour. Ligation

Following this, the digested products were ligated as individual reactions with the EcoR1 adapters. 3µl of digested product were ligated with 7µL of a ligation reaction mix containing:

1µL EcoRI adapter

0.25µL T4 DNA ligase

1µL 10X T4 ligase buffer

3.75µL distilled water

The ligation reaction was then incubated at 37°C for 3 hours and then overnight at room temperature. Following ligation, products were diluted with 100µl of distilled water, to use as a template for PCR.

PCR: Pre-Selective Amplification

PCR was carried out in two rounds. The first round, serving as a pre-amplification step, is as follows. 1µL ligation product was added to 24µL of a mastermix containing:

7.5µL dNTPs (10mM)

75µL Flexi reaction buffer (New England Biolabs)

37.5µL MgCl2 (25mM)

15µL EcoRI pre-primer

15µL HpaII-MspI pre-primer

1.5µLGoTaq polymerase (5units/µl) (New England Biolabs)

4.1µL distilled water

PCR was carried out under the following conditions:

95°C	3 minutes	
95°C	30 seconds	X29 cycles
55°C	30 seconds	
72°C	45 seconds	
72°C	3 minutes	

With a final holding step at 8°C.

PCR: Selective Amplification

Following the pre-amplification round, 7µL of the PCR product was diluted with 93µL of distilled water ready for selective amplification in a second round of PCR. This PCR round used a set of 12 sequence specific primer combinations targeting minor sequence differences. The products from the pre-amplification step were split into 12 separate reactions, with each reaction given a different primer combination. Four different kinds of EcoRI primer were used (each terminating in a specific binucleotide sequence, AA, AT, AG or AC), along with three different MspI/HpaII primers (each terminating with a specific trinucleotide, AAT, ACT or ATC). Primer combinations are summarized in table 2 below.

Table 2. Primer combinations used in MS-AFLP selective PCR. From Amarasinghe et al., 2014.

EcoRI primers	Eco-AA	Eco-AT	Eco-AG	Eco-AC
MspI-HpaII primers	MspI-HpaII-AAT	MspI-HpaII-AAT	MspI-HpaII-AAT	MspI-HpaII-AAT
	MspI-HpaII-ACT	MspI-HpaII-ACT	MspI-HpaII-ACT	MspI-HpaII-ACT
	MspI-HpaII-ATC	MspI-HpaII-ATC	MspI-HpaII-ATC	MspI-HpaII-ATC

This separated out different fragments, making it easier to isolate particular fragments and examine the fragments generated by MspI vs. those from HpaII. 1µL diluted PCR product was added to 1µL HpaII-MspI primer, 1µL EcoRI primer, and 7µL reaction mix (as above). PCR conditions were carried out as above.

The final PCR products were diluted with 100µL of distilled water, before gel electrophoresis.

2.5 Gel Visualisation and Image Analysis

PCR products were electrophoresed on 4-10% polyacrylamide TBE gels, using a mini-PROTEAN Tetra gel tank (BioRad). Approximately 15µl of selective PCR product was loaded into each well, with 2µL of 1kb ladder. Electrophoresis was performed at 90 volts for 40-50 minutes in a 1X TAE buffer solution (242g Tris-HCL, 57.1mL acetic acid, 0.5M EDTA at pH 8.0). Following electrophoresis, gels were post-stained in a solution of 3% GelRed (Biotium) in water for 30 minutes. Gels were then imaged under ultraviolet transillumination using a BioRad Gel Doc XR+ system, and analyzed using Image Lab 5.1 software (BioRad). The number of bands present in each well was recorded.

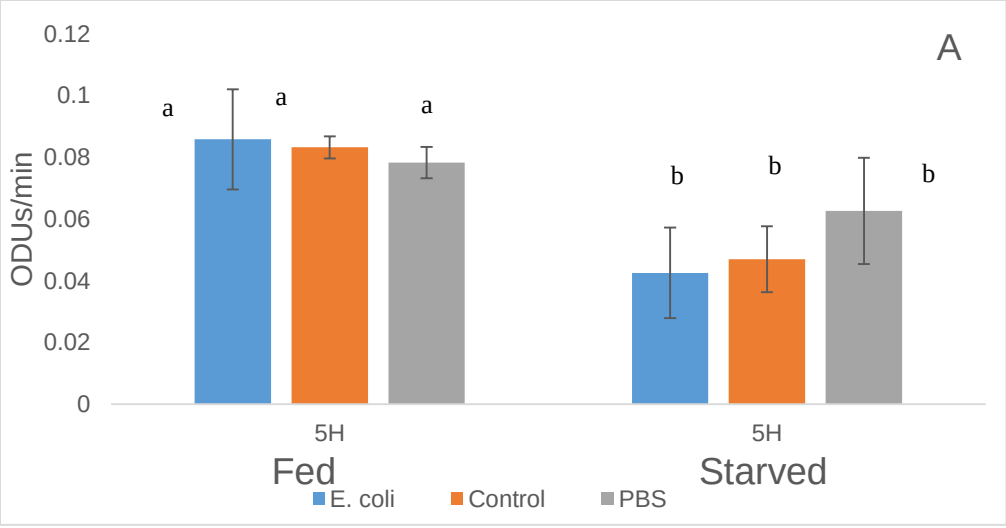
The number of bands present after gel electrophoresis were compared between group 1 (fully fed) samples and group 2 (starved) samples by one-way ANOVA ($p<0.05$). The mean number of bands for

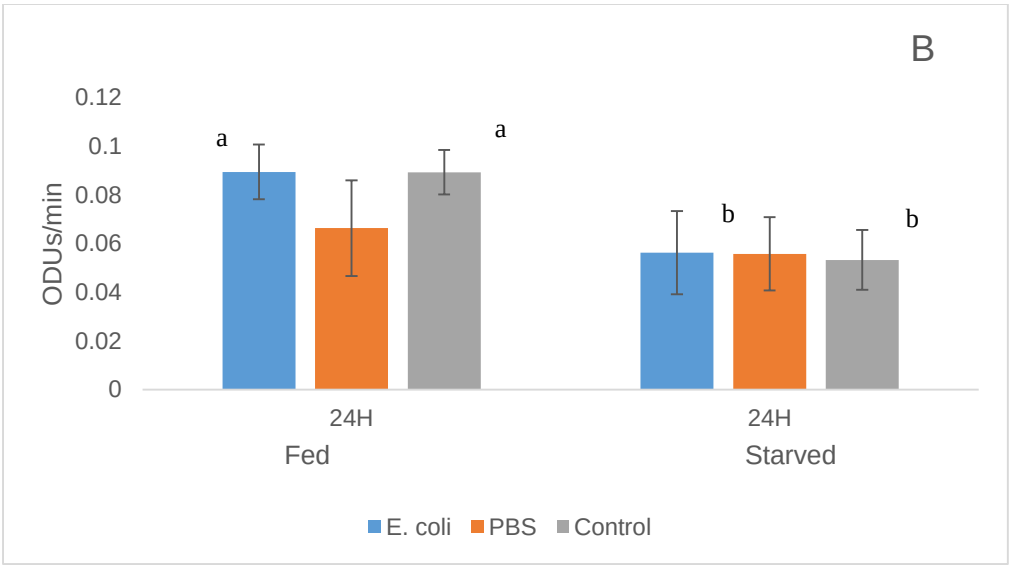
each primer combination was calculated as a ratio of HpaII bands to MspI bands and compared between group 1 and group 2 samples using a chi-squared test ($p<0.05$).

3. Results

3.1 Phenoloxidase Activity

Phenoloxidase assays were conducted on haemolymph samples collected at 5 hours, 24 hours, and 48 hours following inoculation (Figure 5, below). Here, phenoloxidase activity is shown as the maximum rate of L-DOPA conversion for each inoculation treatment. Within feeding regimes (fully fed or starved), no significant differences ($p > 0.05$) were evident between the *E. coli* inoculated treatments, PBS inoculated treatments and non-inoculated controls at any of the post-inoculation time points tested. However, phenoloxidase activity did differ significantly between feeding regimes. Five hours after inoculation, phenoloxidase activity among starved *T. molitor* for all three inoculation treatments (*E. coli* inoculated, PBS inoculated and non-inoculated controls) were approximately half that of fully fed beetles (two-factor ANOVA, $p < 0.05$). Full ANOVA tables for these analyses have been appended as supplementary data. Similar levels of inhibition were evident in starved treatments 24 and 48 hours after inoculation, although the L-DOPA reaction plateau meant that differences at these time points were not significant (two-factor ANOVA, $p > 0.05$). Overall, the plateau effect observed in the L-DOPA reaction after five hours meant that any differences observed in the 24 and 48 hour samples were not significant.





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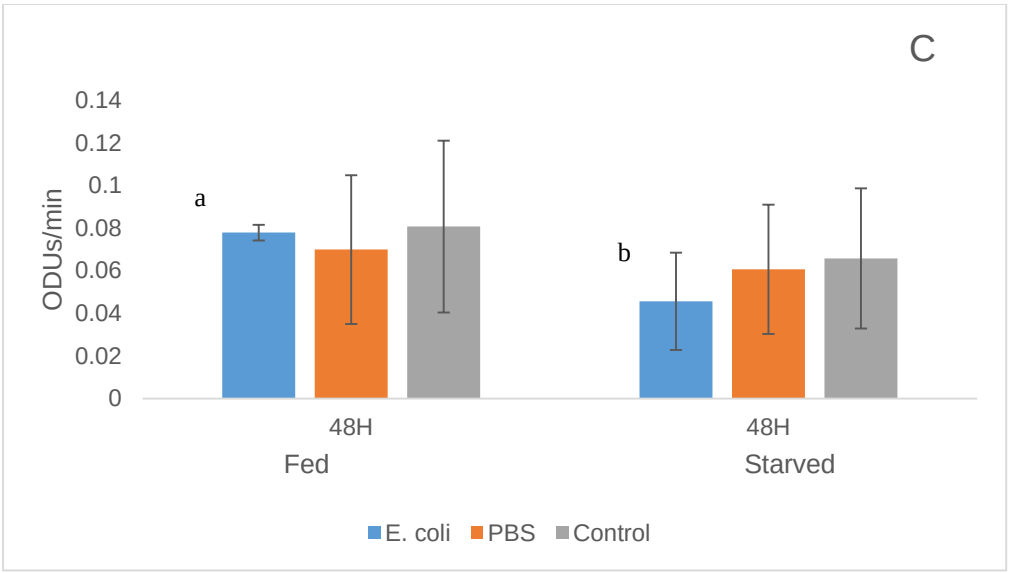


491

Figure 5. Phenoloxidase activities (ODU_{490}/min) of fully fed and starved *T. molitor* five hours (a), 24 hours (b), and 48 hours (c) after the three inoculation treatments (E.coli inoculated, PBS inoculated and non-inoculated (n = 45 per treatment, bars = SEM). Like letters show comparisons that did not differ significantly ($p > 0.05$).

492 3.3 Methylation Profiling

493 Gel electrophoresis resolved numerous discrete amplicons of different sizes in group 1 (fed) and group
494 2 (starved) treatments using different sequence specific primer combinations (Figure 6).

495 Primer pairs EAA-HAAT and EAA-HACT did not yield any detectable amplicons in samples from
496 starved beetles (group 2) digested with MspI, and so were removed from the analysis. Of the remaining
497 primer pairs, the highest number of bands was detected in samples from fully fed beetles (group 1)
498 digested with HpaII, whilst the fewest bands were evident in group 2 samples digested with MspI and

amplified with EAC-HATC. Figure 6 shows that fewer bands were detected among samples from starved individuals than those from fully fed beetles regardless of the restriction enzyme or primer pair used (one way ANOVA, $p<0.05$). Both HpaII and MspI digests yielded a significant difference in the number of bands present in fed beetles compared to starved beetles, shown in figure 7 (chi-squared test, $p<0.05$). A significant difference in amplification was also found in the HpaII and MspI enzymes between group 1 and group 2 beetles (chi-squared test, $p<0.05$), indicating a difference in methylation patterns between the two groups. In group 1 samples, primer combinations EAA-HACT, EAA-HATC, EAT-HATC, EAC-HAAT and EAC-HACT showed higher numbers of MspI bands than HpaII bands, indicating the presence of methylation at these sites. Similarly, in group 2 primer combinations EAT-HAAT, EAT-HACT, EAT-HATC, EAG-HACT, EAG-HATC, and EAC-HACT showed the same effect. In both groups, enzyme activity at the remaining primer combinations indicated higher numbers of HpaII bands than MspI bands, indicating no statistically significant methylation at these sites (figure 8; chi-squared test, $p<0.05$).

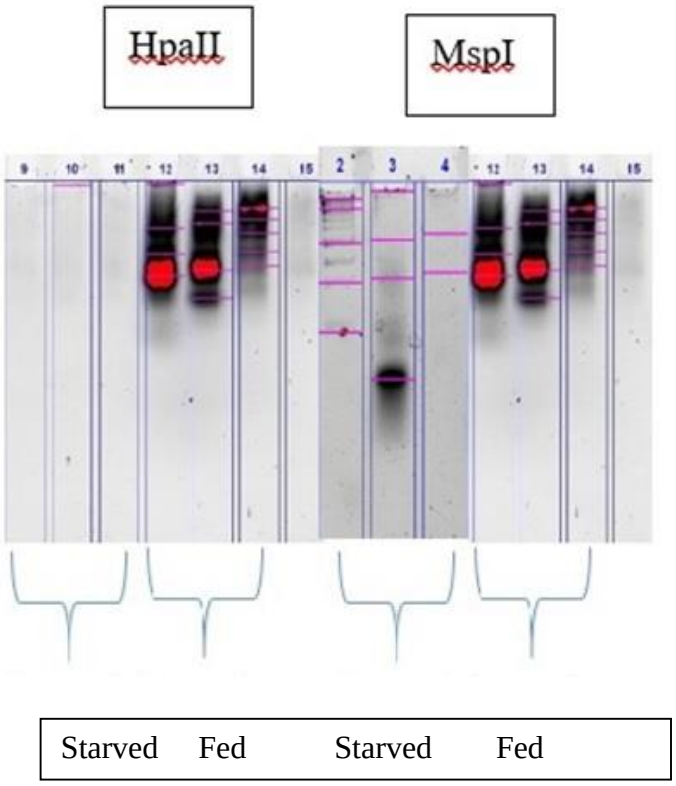
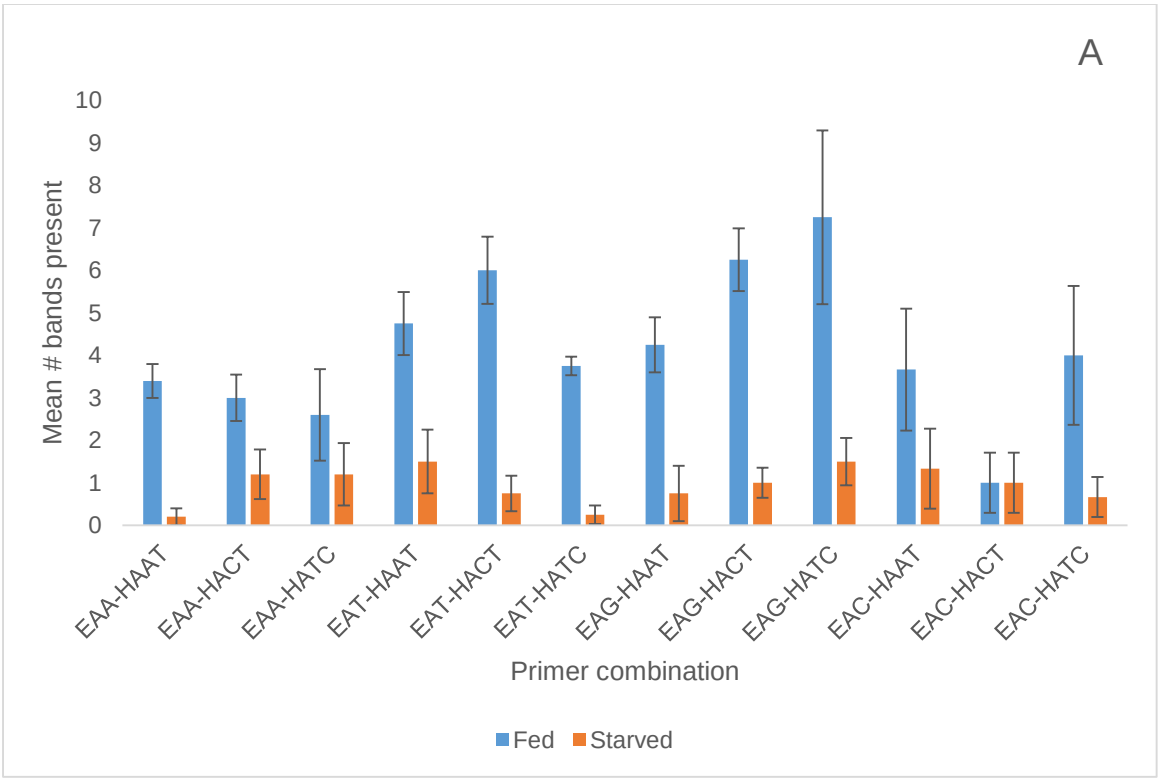


Figure 6. Composite images of gel results for MspI (A) and HpaII (B) for group 1 samples vs. group 2 samples.



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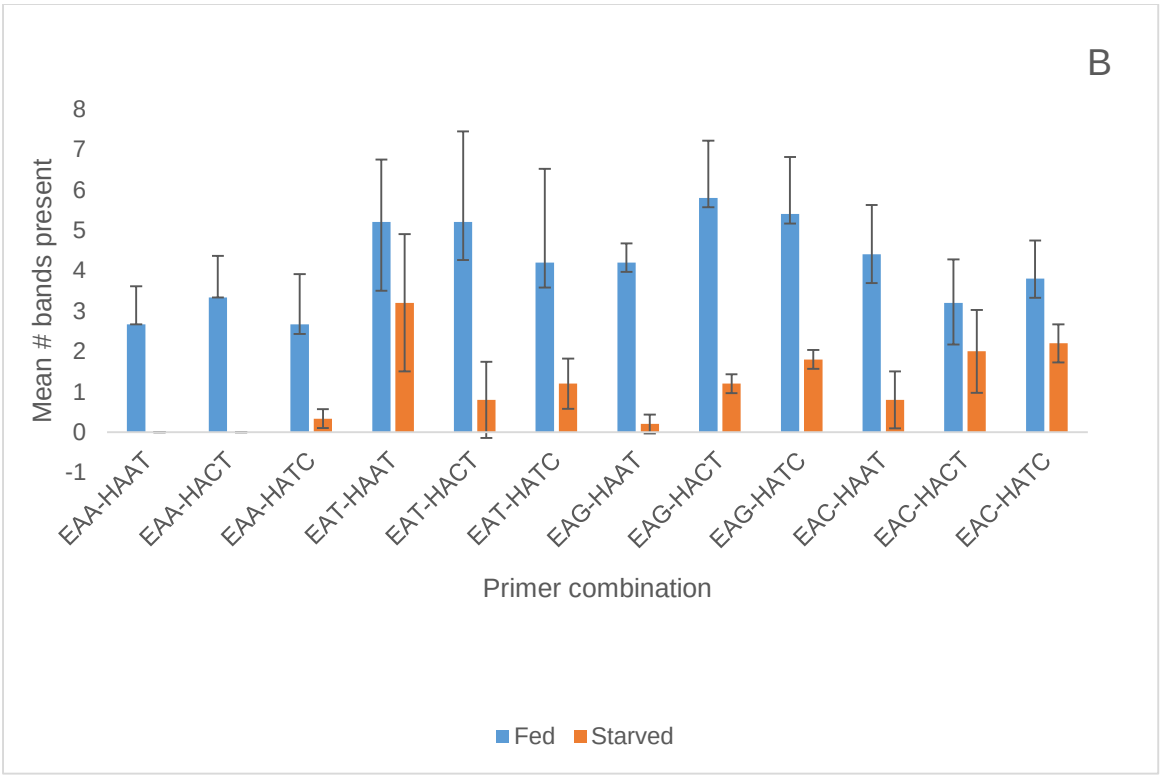

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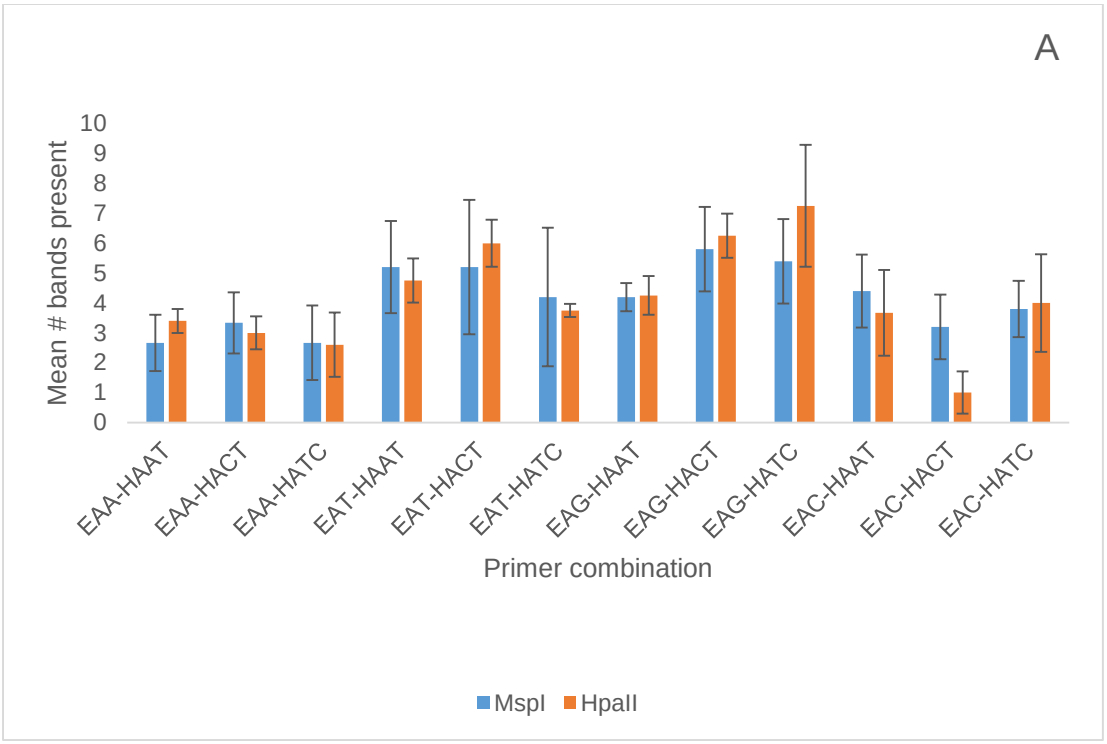
Figure 7. Comparison of HpaII (A) and MspI (B) restriction enzyme amplification in mean number of bands present, showing MS-AFLP results between fed and starved groups.



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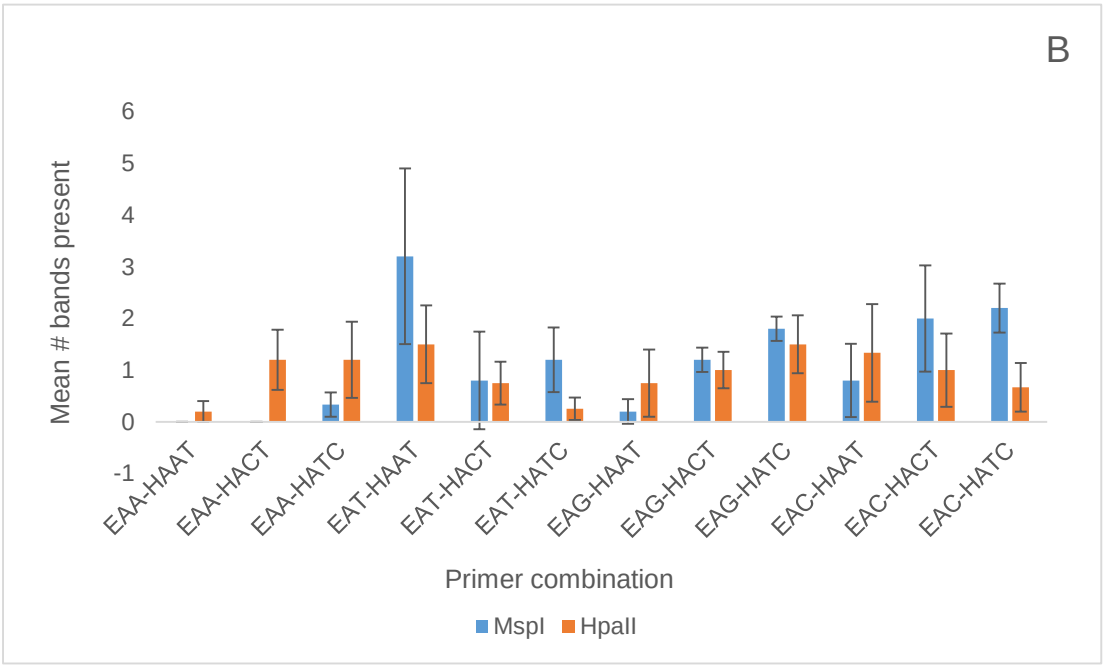

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Figure 8. Comparison of primer combination results in group 1(A) and group 2 (B) MS-AFLP results. Significant differences in the number of amplicons present were detected between HpaII and MspI digests (chi-squared test, $p < 0.05$, bars=SEM).

521

522

523

524 The mean number of bands present for each primer combination and restriction enzyme are shown in
525 Table 3 below, as a summary of findings.

Table 3. Mean number of bands present for primer combinations and restriction enzymes in each treatment group.

	Group 1		Group 2	
	MspI	HpaII	MspI	HpaII
EAA-HAAT	2.7	3.4	0	0.2
EAA-HACT	3.3	3	0	1.2
EAA-HATC	2.7	2.6	0.3	1.2
EAT-HAAT	5.2	4.75	3.2	1.5
EAT-HACT	5.2	6	0.8	0.75
EAT-HATC	4.2	3.75	1.2	0.25
EAG-HAAT	4.2	4.25	0.2	0.75
EAG-HACT	5.8	6.25	1.2	1
EAG-HATC	5.4	7.25	1.8	1.5
EAC-HAAT	4.4	3.7	0.8	1.3
EAC-HACT	3.2	1	2	1
EAC-HATC	3.8	4	2.2	0.7

526

4. Discussion

This study explored the association between immune system downregulation following a period of starvation and changes in genome-wide methylation patterns in the mealworm beetle *Tenebrio molitor*, and has shown that starvation changes the DNA methylation patterns and phenoloxidase activities of *T. molitor*. However, the lack of responsiveness of beetles to immunological challenge meant that the changes detected in DNA methylation patterns could not be related to altered immunocompetence. As no genome sequence is yet available for this species, this study represents a preliminary study into methylation as a molecular mechanism for downregulation in the insect immune system.

Starvation Treatments

Treatment groups for starvation periods and general beetle maintenance were designed to minimize confounding factors such as tank and handling effects. However, it was impossible to rule out the potential for cannibalism confounding starvation conditions, a documented behaviour in this species (Siva-Jothy and Thompson, 2002). To further reduce or eliminate these issues, a larger study must be conducted, involving a much larger sample size and a series of nesting replicates within treatment groups. Furthermore, it must be noted that starvation may trigger a myriad of stress responses unrelated to immunity, with resulting methylation changes. This may be an avenue for future study, benefiting from a fully sequenced genome and transcriptome to cross-reference methylation loci with gene functions.

Immune Challenges and Immunoassays

Immunoassay results showed a significant difference in phenoloxidase levels between the starved and fed treatment groups, while there was no significant difference between inoculation treatments. This concurs with the background literature for the design of this study (Siva-Jothy and Thompson, 2002; Schmid-Hempel, 2005), and further supports the hypothesis that a period of starvation results in a downregulated immune response compared to that of a fully fed control group. There were also no significant results seen in the 24 and 48 hour inoculation groups, due to an observed plateau in the L-DOPA reaction. However, it must be noted that interactions at different time points within the reaction

have the potential to override single factor effects such as feeding treatment or inoculation treatment. The study needs to be repeated, with a much larger sample size, to further investigate these effects.

While there was a significant difference between the fed and starved groups, there was no significant difference between inoculation treatments in the assay results. It must be conceded that methylation cannot be directly associated with immune response. The lack of a significant phenoloxidase activity increase in *E. coli*-treated samples may also be due to procedural error in experimental methods, which still require optimization in future work: due to the culture methods used in maintaining beetle stocks, an undetected baseline immune reaction may have been present at the time of sampling. Additionally, the immunoassay used in this study may not be an optimal method of measuring phenoloxidase activity in this species, particularly if the phenoloxidase cascade relies on exocytosis and subsequent activation rather than simply genetic upregulation (Raftos, 2015). It may also be the case that an alternative cell lysis buffer needs to be found, as SDS may have introduced an element of error into the assays in itself through its role as a detergent.

Lastly, phenoloxidase responses were used in this experiment to efficiently and reliably assay immune response levels, given the time restraints and equipment available during the course of this study. Future work would benefit from examining antibacterial activity responses in this species in conjunction with methylation changes, given the bacterial challenge described here, as this may give a more accurate picture of the relationship between immune response and methylation changes.

Methylation-Sensitive AFLP

To compare patterns of methylation across the genome of starved and fully-fed individuals, methylation-sensitive AFLP was conducted according to an established protocol (Amarasinghe et al., 2014). Imaging of selective PCR products via polyacrylamide gel electrophoresis found significant differences in the number of bands present between group 1 and group 2 samples. This indicates that the amplification of methylation patterns by the selected primer pairs is changed as a result of starvation. These observations are supported by previous studies showing environmentally-driven changes in methylation patterns in other species(Amarasinghe et al., 2014). Future studies must be undertaken to investigate the specific interactions of immune responses with methylation states.

Both HpaII and MspI amplification levels were significantly higher in fed samples than in starved samples. These significant differences between the two restriction enzymes in each group at particular

primer locations indicate differences in methylation patterns at these loci between fed and starved samples. However, the differences in banding patterns seen between fed and starved samples in MspI digests may be due to errors in the imaging software and band resolution methods used, which will require a process of optimization.

Again, while these results cannot be directly associated with immune function, there is further support that environmentally-induced methylation states are present in *T. molitor*. Additionally, given that the MS-AFLP protocol used ensures an equal concentration of DNA per microlitre of sample before proceeding, this study's design ensured that any differences in bands resolved from gel electrophoresis are due to amplification differences.

Given the lack of a fully sequenced genome, it must be noted that all pertinent information about gene expression patterns may not able to be resolved by MS-AFLP. It has been noted that *Tenebrio molitor* gene expression patterns differ under certain stress conditions (Pedersen et al., 2006), and in particular transcribe differently following an immune challenge (Dobson et al., 2012). Although this study did not examine gene expression, the mechanisms responsible for these effects may also have an observable effect on amplification at the genomic level. While supporting this study's hypothesis, previous research indicates that methylation may be only one of many factors responsible for the observed changes in immune response levels and AFLP amplification results in this study. A transcriptomic analysis paired with a starvation treatment and subsequent immune challenge could yield new perspectives not only on mediators of gene expression within *T. molitor* (Wang et al., 2009), but also on genomic immunoregulatory regions that may be conserved between taxa (Chambers and Schneider, 2012; Hirahara et al., 2011).

This study has demonstrated methylation signatures across particular amplified regions within a whole genome. However, as a rule MS-AFLP is only able to illustrate a correlation between the presence of methylation and starvation treatments, and cannot show enough detail to give insight into the silencing of phenoloxidase genes. Again, we must note that this is impossible given the lack of a sequenced genome. This study represents a preliminary investigation into the presence or absence of methylation and its response to immune system downregulation in this species, and much more work needs to be done here to accurately describe these findings. A q-PCR study will enable more direct examination of any correlation between methylation states and immune gene expression, while amplicon sequencing of isolated DNA fragments would yield a more finely tuned understanding of the genetic expression

patterns underlying the observed effects on methylation patterns and amplification. (Grolleau-Julius et al., 2010; Pennisi, 2005).

Further Applications

Similar studies across a variety of insect species would allow future studies to search for the conservation of particular immune gene regions that dictate effector mechanisms (Little et al., 2005; Schaefer and Lyko, 2007), and hence for the conservation of associated methylation patterns. This methylome analysis in turn would give ecological immunologists a greater understanding of the nature of epigenetic change and its effects on gene expression in invertebrate species —a field that has until now been largely overlooked in favour of vertebrate studies (Schaefer and Lyko, 2007). In particular, the extended relevance of insect studies as a proof-of-concept method for exploring epigenetic mechanisms gives a new perspective on host-parasite coevolution (Robertson, 2005).

It is clear that invertebrates exhibit an adaptive immune system that is capable of producing adaptive specificity (Kurtz, 2004), as is suggested by both evolutionary theory and previous studies in ecological immunology (Kurtz, 2005). This study represents the first step towards using epigenetic analyses and next generation genomic techniques to generate a bigger, clearer picture of the interaction of the environment with molecular mechanisms that drive the immune system effectors of both vertebrates and invertebrates (Lister et al., 2009). While there is at this point a need for more work to be done in this area, this and the studies discussed above have provided the first steps towards the synthesis of immunology and molecular biology as they relate to insect studies.

5. Conclusion

The concept of innate vs. adaptive immunity in invertebrates has long been the subject of discussion and research, with the consensus gradually shifting to one of acceptance regarding the presence of adaptive immune change in invertebrates (Ziauddin and Schneider, 2012). Modern molecular

techniques have provided support for this, showing remarkable plasticity in insect immune effectors that in turn creates considerable variation within species (Ziauddin and Schneider, 2012). Having accepted adaptive immunity in invertebrate species, research has also focused extensively on the relationship between environmental factors and immune responses, with special emphasis placed on the role of nutrition in dictating how much of an individual's resources are invested in the immune response (Ponton et al., 2013). This has highlighted the role of environmental stressors in catalyzing adaptive changes in immune response, and raised the question of epigenetic change as the mechanism driving such changes. This study has addressed these questions by examining the effects of a period of starvation on phenoloxidase effector responses in *T. molitor* haemolymph in the face of a bacterial infection. This study then analyzed methylation patterns of starved and fully-fed *T. molitor* specimens at the genomic level. It was found that starvation significantly downregulates the levels of phenoloxidase present in haemolymph samples five hours after initial immune challenging, and results in changes in the amplification of methylation patterns by the selected primer pairs at certain loci. While these findings do not directly relate methylation to immune response function, this study confirms the presence of methylation in the *T. molitor g*enome and establishes methylation as a possible mechanism for adaptive specificity in insect immune responses. Future research will be able to examine other types of epigenetic modification as mechanisms for immune system adaptations as a direct result of host-parasite coevolution.

These findings therefore have the potential to inform future invertebrate studies as well as immunological studies involving epigenetics (Moret, 2006; Siva-Jothy and Thompson, 2002). Transgenerational immune priming as a result of nutrient deprivation in *T. molitor* presents a crucial first step into this new view of immunology and into the emergent field of epigenetics: not as separate fields of inquiry, but as an integrative approach comprising a synthesis of evolution, immunology, ecology, and epigenetics.

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Supplementary Figures

E. coli vs. control:

Anova: Two-Factor With Replication

SUMMARY	e coli	control	Total
group 1			
Count	5	5	10
Sum	0.430834	0.417895	0.84873
Average	0.086167	0.083579	0.084873
Variance	0.001318	6.51E-05	0.000616
group 2			
Count	5	5	10
Sum	0.214714	0.236923	0.451638
Average	0.042943	0.047385	0.045164
Variance	0.001074	0.000569	0.000736
Total			
Count	10	10	
Sum	0.645549	0.654819	
Average	0.064555	0.065482	
Variance	0.001582	0.000646	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0.007884	1	0.007884	10.42338	0.005253	4.493998
Columns	4.3E-06	1	4.3E-06	0.005681	0.940855	4.493998
Interaction	6.18E-05	1	6.18E-05	0.081666	0.778719	4.493998
Within	0.012102	16	0.000756			
Total	0.020052	19				

PBS vs. control:

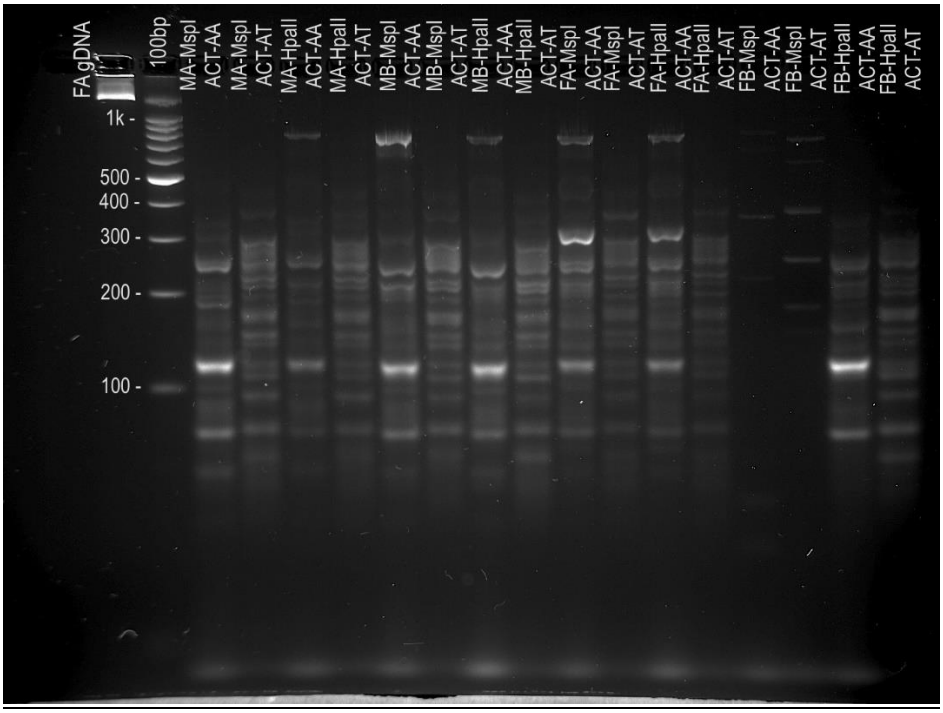
Anova: Two-Factor With Replication

SUMMARY	pbs	control	Total
fed			
Count	5	5	10
Sum	0.393108	0.417895	0.811003
Average	0.078622	0.083579	0.0811
Variance	0.000128	6.51E-05	9.26E-05
starved			
Count	5	5	10
Sum	0.315002	0.236923	0.551926
Average	0.063	0.047385	0.055193
Variance	0.001475	0.000569	0.000976
Total			
Count	10	10	
Sum	0.70811	0.654819	
Average	0.070811	0.065482	
Variance	0.00078	0.000646	

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Sample	0.003356	1	0.003356	6.002562	0.026172	4.493998
Columns	0.000142	1	0.000142	0.253977	0.621157	4.493998
Interaction	0.000529	1	0.000529	0.946285	0.345145	4.493998
Within	0.008946	16	0.000559			
Total	0.012973	19				

Supplementary figure 1. ANOVA tables for 5 hour phenoloxidase assays: two-factor ANOVAs with replication.



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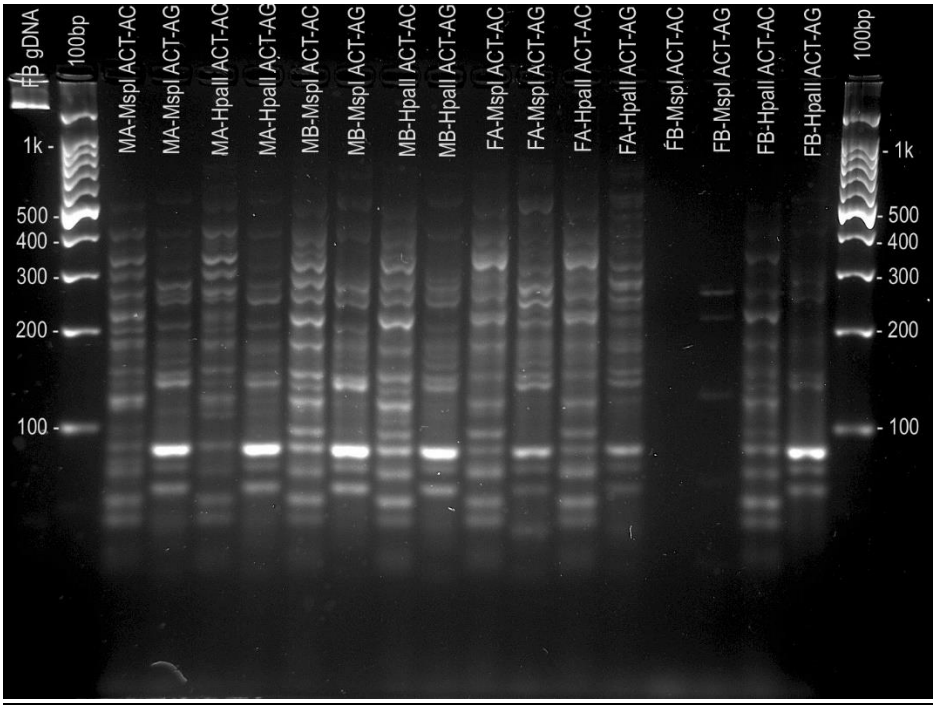

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Supplementary figure 2. Amplification testing of primer combinations and restriction enzymes used in this study. Tested by J. May, 2014.

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