UNIVERSITY OF CALIFORNIA, SAN DIEGO

# Feeding larval honey bees (*Apis mellifera*) dead *Nosema* spores improves their ability to resist *Nosema* infection as adults

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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2014

The Thesis of Matthew B. Endler is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2014

## EPIGRAPH

"The bee's life is like a magic well: the more you draw from it, the more it fills with water." *Karl Von Frisch*

TABLE OF CONTENTS

Signature Page……...iii

Epigraph……iv

Table of Contents……...vi

List of Tables and Figures…….……...vii

Acknowledgements……..viii

Abstract of the Thesis……..…….....ix

Introduction…….1

Material and Methods…….5

Results……...10

Discussion……14

Tables and Figures…….……...18

References…….23

LIST OF TABLES AND FIGURES

Table 1: Treatment Groups……...18

Figure 1: Spore Counts of Pre-pupae and Adults infected as Larvae…….……..19

Figure 2: Larval Survival to Adulthood……20

Figure 3: Adult Weight at Emergence……..21

Figure 4: Spore Counts of Bees Infected as Adults…….....……..22

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to James Nieh for giving me the opportunity to work in lab, for his guidance through my project, and for his patience as I learned and developed my lab techniques. I am incredibly grateful for his time, advice, and teachings he has provided me throughout my undergraduate and graduate work.

In addition to my advisor, I would like to thank my other thesis committee members, Joshua Kohn and David Holway, for their availability and assistance throughout my research.

I would like to individually thank Daren Eiri for his time mentoring me during my introduction into the lab, and for him teaching me the proper protocol for *in vitro* honey bee rearing.

I would also like to thank my fellow lab members: Jacalyn Ho, Adam Yen, Joanna Lee, Kristi Choi, Alena Nabidrek, Sabhyata Sharma, Alexander Neskovic, and Danielle Nghiem for their assistance of *in vitro* rearing of honey bee larvae, adult care, dissections, and morphology measurements.

I would like to thank the North American Pollination Protection Campaign (NAPPC), for the grant it provided to fund my research.

Finally, I would like to thank my friends and family for supporting me, for all their teasing and interest, and for their unconditional love and encouragement.

The introduction, in part, is currently being prepared for submission for publication of the material. Eiri, Daren M.; Suwannapong, Guntima; Endler, Matthew; Nieh, James C. Daren M. Eiri was the primary investigator and author of the material.

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ABSTRACT OF THE THESIS

Feeding larval honey bees (*Apis mellifera*) dead *Nosema* spores improves their ability to resist *Nosema* infection as adults

by

Matthew B. Endler

Masters of Science in Biology

University of California, San Diego, 2014

Professor James Nieh, Chair

*Nosema ceranae* (Microsporidia) is an important honey bee pathogen that is thought to only affect adult bees and which currently has a single effective treatment, the antibiotic fumagillin. However, this treatment may be losing its efficacy.

Previously, we showed that *N. ceranae* can infect *Apis mellifera* larvae. These data suggested that a sufficiently high dose triggers a larval immune response that conveys some protection. We therefore tested if feeding larvae dead *N. ceranae* spores can protect bees subsequently exposed to *Nosema* as adults. In addition, probiotics fed to larvae can activate larval immune genes. However, it was not known if probiotics could actually help bees resist infection. We therefore tested the prophylactic effect of two larval feeding treatments: autoclaved *Nosema* spores and probiotics. Probiotics were not beneficial: they significantly reduced larval and adult longevity and did not help bees subsequently resist adult infection (measured as the number of midgut *Nosema* spores). However, *Nosema* treatment reduced adult infection levels by 57% without significantly altering larval or adult longevity as compared to controls. *Nosema* treatment also had no significant effect on adult morphology, though it slightly increased bee mass at adult emergence. These data provide the first evidence that honey bee disease resistance can be boosted by larval exposure to a dead pathogen, a promising approach that deserves further study.

## Introduction

Honey bees are a valuable pollinator for many commercially important crops. (Morse & Calderone 2000; Winfree et al. 2011). As a result, there is global demand for their services (Aizen & Harder 2009). However, problems with honey bee heath have contributed to reductions in the number of managed colonies (Potts et al. 2010). The United States has experienced annual overwintering colony losses of approximately 30% of managed colonies since 2006 (Spleen et al. 2013). Some European countries have reported similar losses (Higes et al. 2010; Genersch et al. 2010). Researchers have identified multiple factors contributing to the decline in honey bee health: pesticides, *Varroa* mites, pathogens, and interactions between these factors (Pettis et al. 2012; Rennich et al. 2012; Pettis et al. 2013).

The pathogen, *Nosema ceranae,* is a microsporidian that reduces honey bee health and is associated with global declines in managed honey bee colonies (Higes et al. 2008; Dainat et al. 2011). *Nosema ceranae* originally infected the Asian honey bee species, *Apis cerana* (Huang et al. 2007), but switched hosts to the European honey bee *Apis mellifera* as early as 1996 (Fries et al. 1996; Huang et al. 2007; Paxton et al. 2007).

*Nosema* infects via spores that inject sporoplasm into the epithelial cells of the host midgut, where they replicate (Chen et al. 2008; Fries 2010). *Nosema* only infects the bee midgut (Huang & Solter 2013). After approximately seven days, these gut cells become full of spores and burst, releasing spores that are then excreted. Transmission is assumed to be mainly fecal-oral but may also be oral-oral (Smith 2012). *Nosema* infection decreases host immune function (Antúnez et al. 2009;

Bromenshenk et al. 2010; Chaimanee et al. 2012). Specifically, *Nosema* inhibits genes involved in homeostasis and renewal of intestinal tissues at the histological level and triggers host immunosuppression, resulting in malnutrition and eventually death (Malone et al. 1995; Dussaubat et al. 2012; Chaimanee et al. 2012).

*Nosema ceranae* is associated with Colony Collapse Disorder (Higes et al. 2008). In the United States, 57% to 85% of honey bees sampled from 2009 to 2011 showed *N. ceranae* infection, including colonies previously treated for *Nosema* infection with fumagillin (Rennich et al. 2013). Recently, researchers demonstrated that *N. ceranae* can resist the most common and effective treatment, fumagillin (Huang et al. 2013). Thus, it is increasingly important to find alternative *Nosema* treatments, particularly ones that will be resistant to pathogen evolution.

Activating the honey bee immune system against *Nosema* would provide one such solution. Although insect immune systems does not employ antibodies to fight infection like the vertebrate immune system, the two systems share some broad similarities (Evans et al. 2006). The honey bee immune system exhibits innate responses that are non-specific and always present and induced responses that are more specific and take time to develop (Laughton et al. 2011). The induced responses are triggered via four interconnected routes that are activated by parasite exposure or infection: Toll, Imd, Jak/STAT, and Jnk pathways (Evans & Spivak 2010).

Bee immunity against *Nosema* infection can be enhanced. Danish populations selectively bred for *Nosema* resistance showed lower mortality and an up-regulated immune response associated with the Toll pathway (Huang et al. 2012). Bees can also be treated to activate these induced responses. For example, a probiotic mixture of

bacteria fed to larvae can activate larval immune genes (Evans & Lopez 2004). However, no studies have shown that activating larval immunity will increase resistance to *Nosema* infection.

Recently, we demonstrated that honey bee larvae can be infected with *N. ceranae* (Eiri et al. 2014). We fed live spores to larvae (0, $10^4$, or $4x10^4$ spores) and then reared them to adulthood. Adults fed $10^4$ or $4x10^4$ spores had significantly elevated midgut spore counts as compared to those of controls (which had few spores). This study yielded an intriguing result. A larger spore dose ($4x10^4$) fed to larvae resulted in significantly lower adult infection levels than did a lower spore dose ($10^4$) fed to larvae. We suspected that this result was due to the larger dose activated larval immunity. However, this larger dose also significantly decreased adult survival (Eiri et al. 2014). Is it possible to activate larval immunity without infecting larvae? We decided to try using dead (autoclaved) spores.

In addition, our original experiment raised questions about whether the spores counted in adults were simply residual spores from the single dose fed to larvae or spores that had truly infected and replicated in larval tissue. To address this question, we decided to feed autoclaved spores to larvae. If we were not counting residual spores, then the autoclaved spore treatment should yield adults with no midgut spores.

Our research therefore had three goals. First, we sought to provide a control experiment for the previous results that suggested live *Nosema* spores can reproduce in larvae (Eiri et al. 2014). Second, we wished to test the efficacy of using autoclaved spores fed to larvae as a way of increasing adult resistance to *Nosema* infection. Third, we wished to test if a mixture of several bacteria—probiotics—fed to larvae can

actually confer protection against *Nosema* infection in adult bees.

This introduction contains, in part, material that is currently being prepared for submission for publication. Specifically, Eiri *et al*. (2014) is cited for the original experiment testing if honey bee larvae can be infected by *N. ceranae*. Eiri, Daren M.; Suwannapong, Guntima; Endler, Matthew; and Nieh, James. Daren M. Eiri is the primary investigator and author of the submission. Matt Endler contributed significantly to this publication by generating some of the data on control, 10K, and 40K treatment data and by generating all of the data on the autoclaved spores. The material that will be published in Eiri *et al*. (2014) is contained in this thesis because Matt Endler contributed to significantly to this paper and is therefore a co-author on this paper. This material also provides the rationale for the main part of Matt Endler's thesis.

## Materials and Methods

### Spore Preparation

*Nosema ceranae* spores were originally obtained from infected *A. florea* and *A. ceranae* workers in Chon Buri, Thailand and fed to *A. mellifera* workers in La Jolla, California to ensure a fresh stock, which was constantly renewed, for our experiments. Spore-producing bees were only fed 2.0M sucrose solution (no pollen), ensuring gut contents consisted mainly of spores. To obtain spores, we dissected out adult honey bee midguts, homogenized them in sterile double distilled water ($ddH_20$), and vacuum-filtered them through Fisherbrand P5 filter paper with 20-25 μm pores (modified from Webster et al. 2004). We measured spore concentrations using a hemocytometer in a compound microscope (Zeiss Axioskop), making two independent measures of each sample and recording the average spore count (Cantwell 1970).

### Infecting honey bee larvae

*In vitro* rearing

The standard developmental timeline for honey bees is as follows: a bee becomes a larvae, prepupae, and adult 1, 17, and 19 days after egg hatching (Winston 1987). We grafted (transferring of larvae from comb to 24-well cell cultured plate) first instar larvae (1 day post egg hatching) and treated them with either inactivated *Nosema* or probiotics 3 days of age post egg hatching. We followed standard methods for *in vitro* rearing of larvae (Huang 2009; Eiri et al. 2014). Larvae were collected from combs obtained from colonies either at the University of California, San Diego Biological Field Station or at the Elliot Chaparral Reserve between March 2013 and

March 2014. We used seven total colonies.

We maintained sterile conditions, carried out all grafting, feeding, and transferring in a sterile laminar flow hood (AirClean 600). All equipment, including cages for holding adults, cell culture plates, glassware, and pipettes were regularly sterilized with 10% bleach solution (soaked for 30 minutes followed by repeated rinses with deionized water), then 70% ethanol, followed by one hour UV treatment, and concluding with drying in the hood. All pipette tips and tissues were autoclaved prior to use, and all researchers wore gloves. Additionally, we collected and autoclaved all waste to eliminate potential *Nosema* contamination.

Upon adult emergence, we weighed each bee (Mettler AE 200) and painted it with bee-safe, water-based paint according to the day of emergence. Bees were placed in sterile plastic cages (12cm x 8cm x 12cm), one cage per treatment group (Table 1). All adults, regardless of treatment, were given dd$H_20$, 2.0M sucrose, and bee bread (30% 2.0M sucrose and 70% pollen w/w) *ad libitum* (Williams et al. 2013). Live *Nosema* was suspended in 2.0M sucrose at a concentration of 250 spores/ $\mu$ L (Rortais et al. 2005). Cages were maintained in a dark environment at 34°C and 70% humidity. Adults were allowed to live as long as possible. Each day, we removed dead bees, recorded mortality, and changed the bee bread. Dead bees were kept at -18°C until dissections.

Treatments

We used three larval treatments: (1) control, (2) probiotics, and (3) autoclaved *Nosema*. Because we also applied treatments to adult bees, we have a total of six

treatment groups (Table 1).

All larvae were randomly assorted into one of the treatment groups on day 3 post egg hatching. *Control* larvae were given 10 $\mu$ L double-distilled water ($ddH_20$). The probiotic treatment was based upon the experiment of Evans & Lopez (2004). We used the same manufacturer (Jarro Formulas, Institut Rosell-Lallemand, Inc, Canada) and a similar formulation, EPS probiotic supplement, which contained the same species as Evans & Lopez (2004), *Lactobacillus rhamnosus* R0011, *L. acidophilus* R0052, *L. casei* R0215, *L. plantarum* R1012, *Bifidobacterium longum* BB536, and *B. breve* R0070. Our mixture contained two additional non-pathogenic species: *Pediococcus acidilactici* R1001, which occurs in bee pollen (Belhadj et al. 2014), and *Lactococcus lactis* ssp. *lactis* R1058, which is found naturally in the bee gut (Ahn et al. 2012). We used two doses: $5x10^7$ and $5x10^6$ bacteria per larva (given on day 3 post egg hatching). Evans & Lopez (2004) used a dose of $5x10^7$ bacteria per larva, but we noticed very strong detrimental effects of this dose on larval survival in our preliminary trials (<7% of larvae survived to adulthood). We therefore used the reduced dose of $5x10^6$ bacteria per larva in all subsequent trials and focus on this dose in our analyses.

The autoclaved *Nosema* treatment consisted of each larvae receiving a dose of $4x10^4$ inactivated *Nosema* spores. This dose is the same one that showed a potentially immune activating effect in our prior study (Eiri et al. 2014). To obtain dead *Nosema* spores, we ran the samples through an autoclave heat cycle (30 min liquid cycle, 121°C). Each sample was subsequently recounted and its volume adjusted because autoclaving slightly reduced the solution volume.

**Morphology measurements and adult infection levels**

We tested the effect of larval treatment on bee morphology. We measured adult morphology upon death so that we could separately measure the mass of different body parts and make size measurements without causing stress to living bees. Using a scalpel, we removed and separately weighed the head and thorax (Mettler AE 200). We also measured the intertegular distance (IT) and abdominal length, measures of bee size, with a digital micrometer. To measure abdomen length, we carefully thawed out the bee, allowing it assume a relaxed shape, without stretching or compressing the abdomen. To determine adult infection levels, we dissected out the midgut and counted spores (method of Cantwell 1970). We made two spore counts per bee and averaged these counts to determine the total spore count per individual.

**Effect of probiotics fed to adult bees**

Even at the reduced dose, probiotics severely decreased larval survival (see below). We therefore decided to test the effect of probiotics on adult bees. To ensure that all adults were the same age, we collected newly emerged bees from a comb placed in an incubator and divided these bees into two groups that received either a probiotics treatment ($5x10^6$ bacteria per bee fed in sucrose solution in a cage) or a control treatment (pure sucrose solution).

**Statistical analyses**

We used non-parametric tests, Wilcoxon-Signed Ranks and Steel-Dwass multiple comparison tests (which control Type I error), to determine the effect of

treatment on spore counts because spore counts are not parametrically distributed, with many bees showing no infection (zero inflated data). In this non-parametric model, we are not able to include colony as a model effect. To test the effect of the potential prophylactic treatments on adult spore counts, we therefore ran separate tests for each of the seven colonies used.

The effect of treatment on the number of larvae surviving to adulthood is tested with a Chi-square goodness of fit test, with our expected number of surviving bees calculated based upon an equal proportion of bees surviving in all treatments. For adult survival data, we used a Cox Proportional Hazards model because we knew the day that each bee died. We report the Log-Rank (L-R) chi-square value for the effects of treatment, log spore counts, and colony (Cox 1972).

For the experiment testing how probiotics fed to adults affect adult longevity, we only examined the effects of treatment and colony because these bees were not exposed to spores. We used Analysis of Variance (ANOVA) REML algorithm to test the effect bee age on spore count, with colony as a random effect.

We used ANOVA with Tukey Honestly Significant Difference (HSD) tests (which control Type I error) to conduct multiple pairwise comparisons. In our multifactor models, we begin with a parameter-rich model that includes all factors and interactions. We then used stepwise elimination and report the minimal adequate model, one that includes only significant factors and interactions.

## Results

### *Nosema* can infect honey bee larvae

Treatment significantly affected spore counts in prepupae ($\chi^2_3$ =72.29, $P$<0.0001, $n$=220 bees from 5 colonies) and adults ($\chi^2_3$ =137.01, $P$<0.0001, $n$=386 bees from 8 colonies). In prepupae the control and autoclaved spore group had essentially no spores and did not significantly differ ($Z$=-0.51, $P$=0.96), but larvae fed 10K or 40K spores had significantly elevated spore counts in comparison to these controls ($Z$≥4.15, $P$≤0.0002). There is no significant difference between spore counts in the 10K and 40K treatment groups ($Z$=0.40, $P$=0.98).

In adults, the control and autoclaved spore groups also had essentially no spores and did not significantly differ ($Z$=-1.31, $P$=0.56), but larvae fed 10K or 40K spores had significantly elevated spore counts in comparison to these controls ($Z$≥5.72, $P$<0.0001). Furthermore, 10K treated bees had significantly more spores compared to 40K treated bees ($Z$=7.84, $P$=0.013, see Fig. 1).

### Effect of treatments on larval survival to adulthood

There is a strong effect of treatment on larval survival to adulthood ($\chi^2_2$ =13.94, $P$=0.0009, $n$=593 bees from seven colonies, see Fig. 2). However, this finding was driven by the lower survival of the probiotics treatment group. Compared to controls, the probiotics treatments reduced the number of larvae surviving to adulthood by 61%. There is no significant difference between the control and autoclaved spore treatments ($\chi^2_1$ =0.68, $P$=0.40).

**Effect of larval treatments on adult longevity**

Adult longevity was strongly influenced by colony identity. In the control group (0K/0K vs. 0K/10K), there is a significant effect of colony (L-R $\chi^2_6$ =46.79, $P$<0.0001) and spore count (L-R $\chi^2_1$ =13.90, $P$=0.0002), but no effect of treatment (L-R $\chi^2_1$ =3.62, $P$=0.06). There is no significant effect of treatment*spore count (L-R $\chi^2_3$ =3.20, $P$=0.36). In the 0K/10K treatment, the longer a bee survived, the more likely it was to be heavily infected ($F_{1,147}$=28.3, $P$<0.0001, colony accounts for 46% of model variance).

In the autoclaved spore (AC/0K vs. AC/10K) and probiotic (PB/0K vs. PB/10K) groups, there is a strong effect of colony (L-R $\chi^2_6 \geq$48.65, $P$<0.0001), no effect of treatment (L-R $\chi^2_1 \leq$1.39, $P\geq$0.24), and no effect of spore count (L-R $\chi^2_1 \leq$2.06, $P\geq$0.15).

Because there is no effect of adult *Nosema* treatment within each treatment group, we calculated median survival for each treatment groups. The autoclaved spore and control groups both had a median survival of 7 days. The probiotics group had a median survival of 2 days, a 71% decrease compared to the control and autoclaved spore groups.

**Effect of probiotics fed to adult bees upon adult survival and longevity**

As with larvae, probiotics strongly reduced adult survival. There is a significant treatment effect (L-R $\chi^2_1$ =24.17, $P$<0.0001, $n$=184 bees from 3 colonies) but no significant colony effect (L-R $\chi^2_2$ =2.86, $P$=0.24). Probiotics reduced the

median age of death by 27% (from 15 to 11 days).

**Effect of larval treatments on adult mass and morphology**

There is a significant effect of the three treatments (control, autoclaved spores, and probiotics) upon adult mass at emergence ($F_{2\ 546}$=6.28, $P$=0.002, colony accounts for 7.1% of model variance). Bees fed autoclaved spores are significantly heavier than bees in the other two treatment groups, which did not significantly differ in mass from each other (Tukey HSD test, $P$<0.05, Fig. 3). Bees fed autoclaved spores had 16% and 3% higher masses compared to that of probiotic bees and control bees, respectively.

However, there is no significant effect of treatment upon head mass, thorax mass, IT distance, or abdominal length ($F_{5,\ 418}$≤1.84, $P$≥0.10, colony effects account for ≤6.1% of model variances).

**Effect of treatments on adult infection levels**

The larval treatments significantly affected spore counts in bees exposed to *Nosema* as adults ($\chi^2_5$ =336.86, $P$<0.0001, $n$=550 bees from 7 colonies, Fig. 4). The effect of treatment is also significant when each colony is separately analyzed ($\chi^2_5$ ≥65.74, $P$<0.0001).

As expected, the 0K/10K treatment resulted in significantly higher spore counts than in any of the controls ($Z$≥4.94, $P$<0.0001) and than in the AC/10K treatment ($Z$≥9.12, $P$<0.0001).

Probiotics were detrimental. The PB/10K treatment resulted in significantly higher spore counts than any of the controls ($Z$≥4.92, $P$≤0.0001) and than the AC/10K

treatment ($Z$=4.93, $P$<0.0001). The PB/10K and 0K/10K treatments are not significantly different ($Z$=2.39, $P$=0.16).

However, the autoclaved spore treatment was beneficial. On average these bees had significantly lower spore counts than the 0K/10K and PB/10K treatments ($Z$≥5.46, $P$<0.0001, Fig. 4). No bees in the control groups (0K/0K, AC/0K, and PB/0K) had any spores.

The Results section entitled "*Nosema* can infect honey bee larvae" contains the combined results of Matt Endler and his co-authors. Eiri, Daren M.; Suwannapong, Guntima; Endler, Matthew; and Nieh, James. Daren M. Eiri is the primary investigator and author of the submission. Matt Endler contributed significantly to this publication by generating some of the data on control, 10K, and 40K treatment data and by generating all of the data on the autoclaved spores. All other Results sections are exclusively the product of Matt Endler's thesis research. Specifically, only Matt Endler and James Nieh contributed intellectually to the idea of developing and testing the efficacy of a *Nosema ceranae* vaccine in *Apis mellifera*.

## Discussion

These results demonstrate that (1) honey bee larvae can be infected with live *Nosema* spores (Fig. 1), (2) probiotics can be harmful (Fig. 2), and (3) larvae fed dead *Nosema* spores have decreased infection levels per bee after they are exposed to *Nosema* as adults (Fig. 4). The dead spore treatment reduced the average level of adult infection by 57% as compared to the control. We thus provide the first demonstration that honey bee exposure to a dead pathogen is an effective prophylaxis, raising the interesting possibility that this treatment may be effective for reducing *N. ceranae* infections in full colonies. Current treatment largely uses a single antibiotic, fumagillin, which has limited efficacy against *N. ceranae* and whose utility may decrease as this pathogen evolves resistance (Huang et al. 2013). A "vaccine-like" treatment is therefore a useful development.

## Bee mass and morphology

Feeding larvae dead *Nosema* spores resulted in slightly (3%) heavier adult bees at emergence than controls. This dead *Nosema* treatment may have increased the level of larval food consumption. Naug & Gibbs (2009) showed that adults infected with *Nosema* displayed higher hunger levels than adults in a control group. However, we were not able to find any significant effects of our dead spore treatment upon adult morphology at death. There are two potential explanations. First, the weight effect is small (3%) and weight differences upon emergence may stem not from increased size but from increased food or fat stores. Second, we measured adult morphology upon death to avoid stressing living bees, but weighed bees upon emergence (a more

reliable measure than weighing bees after death when their mass could be influenced by dehydration). We found no effect of probiotics on bee weight and adult morphology as compared to the control treatment. Probiotics strongly decreased larval survival to adulthood, but this limited the sample size, and survivors may have been bees that were better able to resist the effects of probiotics.

**Infection levels**

The levels of infection found in pre-pupae and adults infected as larvae are relatively low. However, we only found spores when larvae were fed live, not dead, *Nosema* spores (Fig. 1). Thus, these pre-pupal and adult spore counts reflect spore propagation, not the presence of residual spores from the larval treatment. In general, the level of *Nosema* infection increases as bees age, as we (see above) and other authors have shown (Meana et al. 2010; Smart & Sheppard 2011). Even a low level of infection in young bees may therefore have strong consequences in later life.

**Survival**

In total, 81% of controls survived to adulthood. These results are similar to natural mortality, in which 85% of larvae natural tended by nurse bees survive to adulthood (Fukuda & Sakagami 1968; Crailsheim et al. 2013). Similarly, 75% of larvae fed autoclaved spores survived to adulthood; this level of survival was not significantly different from that in the control treatment. In the larval treatment experiment, the lack of a treatment effect on subsequent adult longevity is puzzling, but may be due to the strong colony effects, which has also reported in another

*Nosema* study (Huang et al. 2012).

**Probiotics**

Probiotics, even at a dose 10 times lower than previously published (Evans & Lopez 2004), significantly reduced larval survival to adulthood to 31%. It is possible that our results differ from Evans and Lopez (2004) because we used a slightly different mixture of probiotic bacteria (see Methods). Two of our species, *P. acidilactici* R1001 and *Lactococcus lactis* ssp. *lactis* R1058, were not used by Evans and Lopez (2004). However, *Pediococcus* occurs in the pollen that honey bees collect (Belhadj et al. 2014), and *Lactococcus* is naturally found in the guts of *A. mellifera* (Ahn et al. 2012). Moreover, *Lactobacillus* and *Bifidobacterium* fed to larvae reduced the number of larvae infected by American Foul Brood, a common honey bee bacterial disease (Forsgren et al. 2009). Our probiotic data suggest that the use of probiotics in food given to bees should be closely scrutinized. In our study, even adult bees exhibited decreased longevity when fed probiotics.

**Treatment with inactivated *Nosema* spores**

Our "vaccine-like" treatment of feeding larvae autoclaved *N. ceranae* spores reduced the severity of subsequent adult infection by 57%, a strong effect. Future studies should focus on testing this treatment in the field with full colonies and refining the method to increase its preventative efficacy. In addition, it would be useful to determine if feeding adult bees inactivated *Nosema* spores also confers protection. Of potentially greater interest, however, is the basic biology underlying

this effect. Does exposure to autoclaved spores activate honey bee immunity? If so, how does it do so? A better understanding of the mechanisms involved is important for enhancing our knowledge of honey bee immunity and for implementing this method with other important pathogens such as the multiple bacteria known to infect honey bees and reduce honey bee health (Evans & Schwarz 2011).

**Tables and Figures**

**Table 1**. The six treatment groups used in these experiments.

| **Treatment** | **3-Day Old Larvae** | **1-Day Old Adult** |
|---|---|---|
| **0K/0K** (Complete Control) | 90 µL BLD, 10 µL water | 2.0M sucrose |
| **0K/10K** (*Nosema* Control) | 90 µL BLD, 10 µL water | $1x10^4$ live *Nosema* spores in 2.0M sucrose |
| **AC/0K** (Autoclaved *Nosema* Control) | 90 µL BLD, 10 µL $4x10^4$ inactivated *Nosema* solution | 2.0M sucrose |
| **AC/10K** (Autoclaved *Nosema* Treatment) | 90 µL BLD, 10 µL $4x10^4$ inactivated *Nosema* solution | $1x10^4$ live *Nosema* spores in 2.0M sucrose |
| **PB/0K** (Probiotics Control) | 100 µL BLD mixed with probiotics ($5x10^7$ bacteria) | 2.0M sucrose |
| **PB/10K** (Probiotics Treatment) | 100 µL BLD mixed with probiotics ($5x10^7$ bacteria) | $1x10^4$ live *Nosema* spores in 2.0M sucrose |

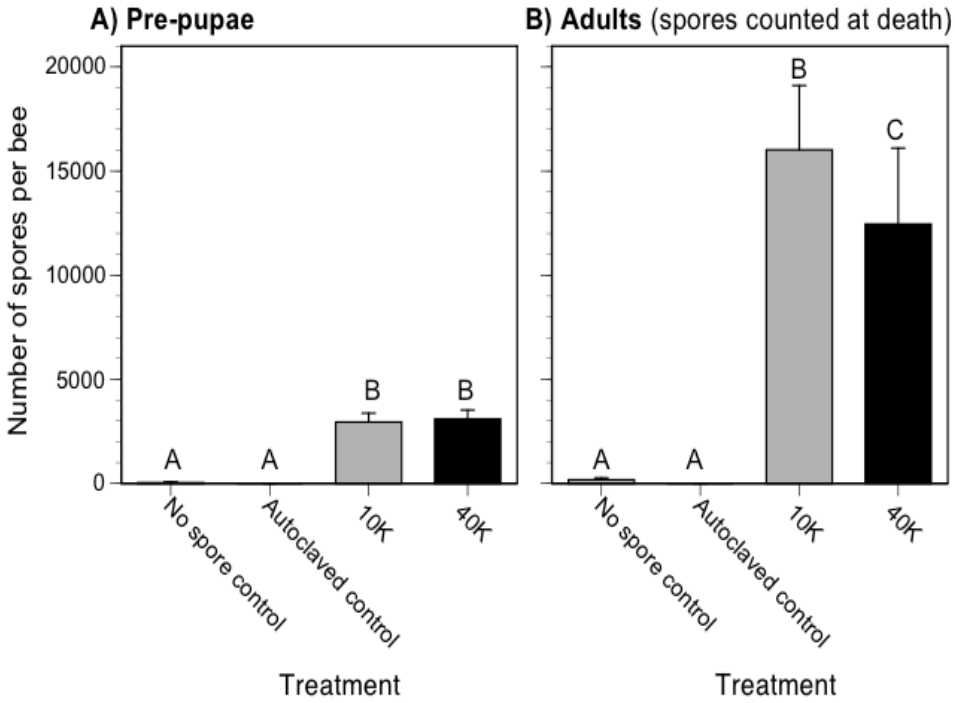


**Figure 1**: Effect of treating larvae with live (10K and 40K) *Nosema* spores vs. controls (no spores and autoclaved spores). Results are shown for (a) pre-pupae and (b) adults. Mean spore counts with standard error bars are shown. Different letters indicate significant differences.

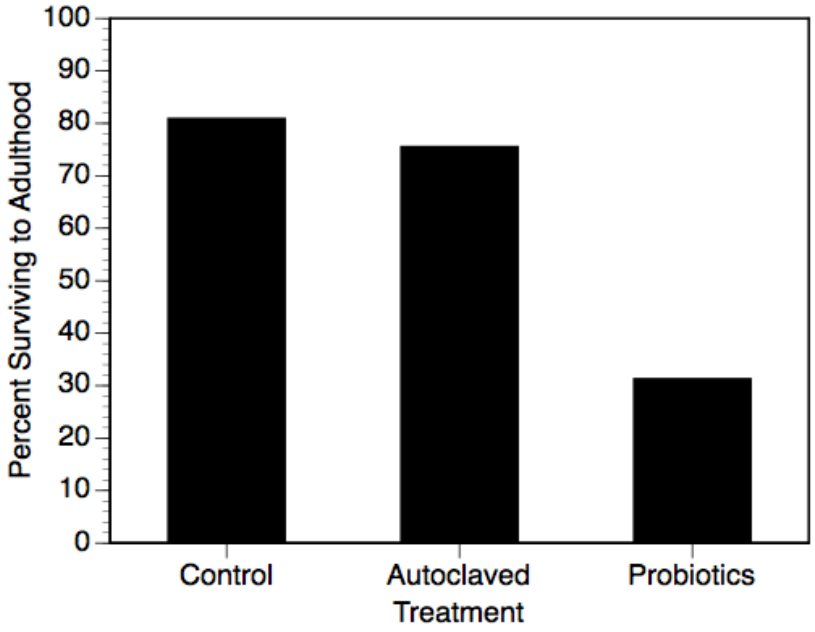


**Figure 2**: Effect of treatments on the percentage of bees surviving to adulthood.

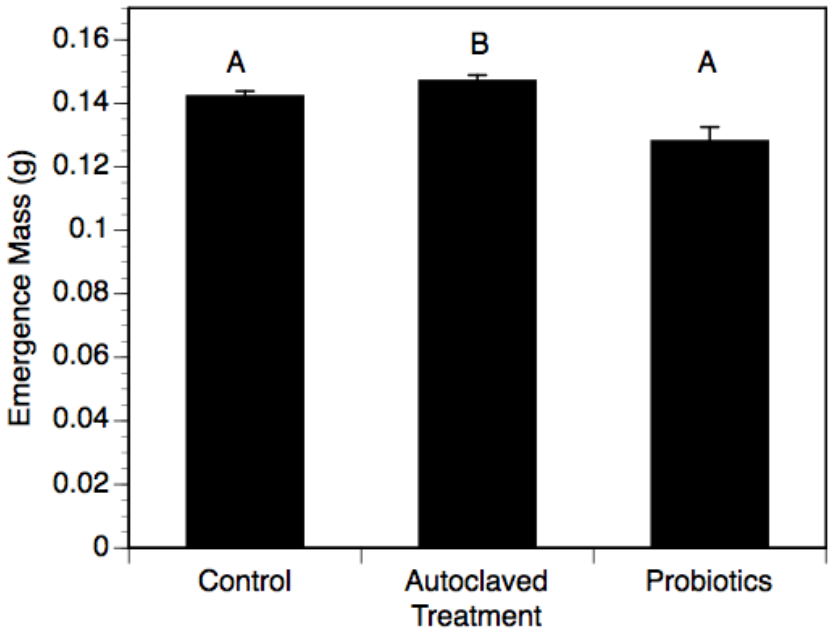


**Figure 3**: Effect of larval treatment on bee mass upon adult emergence. Standard error bars shown. Different letters indicate significant differences.

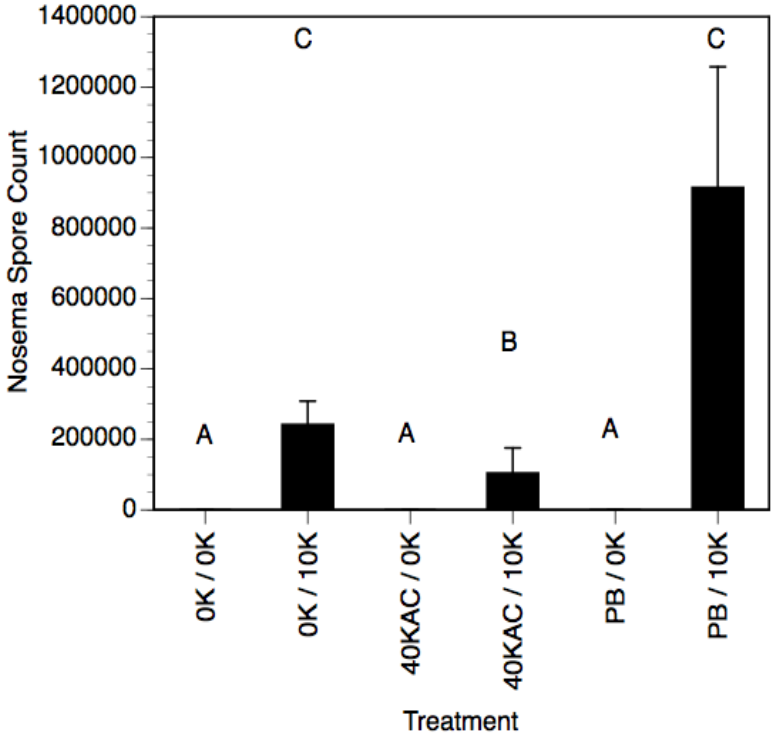


**Figure 4**: The effect of the different treatments (Table 1) on the average level of infection in adult bees. Different letters indicate significant differences. Standard error bars are shown.

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