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Survival and health improvement of *Nosema* infected *Apis florea* (Hymenoptera: Apidae) bees after treatment with propolis extract

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ABSTRACT

Nosema ceranae is now considered to be an emerging infectious disease of the European honey bee *Apis mellifera*. Only one antibiotic, Fumagillin, is commercially available to combat *Nosema* infections. This antibiotic treatment is banned from use in Europe and elsewhere there is a high probability for antibiotic resistance to develop. We are therefore interested in investigating the effects of a natural propolis extract on its ability to reduce *N. ceranae* infection loads in the dwarf honey bee, *Apis florea*, a native honey bee with a range that overlaps with *Apis cerana* and *Apis mellifera* that is at risk of infection. Experimentally infected caged bees were fed a treatment consisting of 0%, 50%, or 70% propolis extract. All 50% and 70% propolis treated bees had significantly lower infection loads, and the 50% treated bees had higher survival in comparison to untreated bees. In addition, propolis treated bees had significantly higher haemolymph trehalose levels and hypopharyngeal gland protein content similar to levels of uninfected bees. Propolis ethanolic extract treatment could therefore be considered as a possible viable alternative to Fumagillin to improve bee health. This natural treatment deserves further exploration to develop it as a possible alternative to combat *N. ceranae* infections distributed around the world.

Introduction

Nosemosis, is a disease caused when honey bees are infected with *Nosema ceranae* or *Nosema apis*, and it is now currently distributed around the world (Higes et al., 2013; Paxton et al., 2007; Williams et al., 2008a; Suwannapong et al., 2011a; Fries, 2010). Nosemosis is also implicated as one of the possible factors responsible for the recent decline in honey bee health (Higes et al., 2013; Higes et al., 2010a; Higes et al., 2008). *N. ceranae* is much more prevlanant and is suspected to be replacing N. apis throughout the world. In the European honey bee *Apis mellifera*, N. apis appears to have a competitive disadvantage when co-infected with the relatively new N. ceranae (Natsopoulou et al., 2015; Williams et al., 2014). The widespread invasive nature of N. ceranae is concerning because it is suspected to be a larger threat to sustaining honey bee health than previously thought. *Apis florea* can potentially get a *N. ceranae* infection from shared flower use of contaminated flowers or other food sources because it has foraging areas that overlap with *Apis cerana* and *Apis mellifera*. For this reason, there is potential for *N. ceranae* to jump from its original host, *A. cerana*, to other bee species like it has done with *A. mellifera*. If this is the case there is potential for the lowering of bee health of *A. florea* due to increased virulence in this new host like what has been found with *A. mellifera* (Higes et al., 2013; Suwannapong et al., 2011a; Higes et al., 2010b; Botías et al., 2013).

Maintaining honey bee health is critical to sustaining current food production practices. Honey bees provide important ecosystem and agricultural services as pollinators, and thus maintaining honey bee health is paramount to aid high agricultural output in order to meet the growing demand of food consumption (Breeze et al., 2014; Brittain et al., 2013; Breeze et al., 2011; Potts et al., 2010; Klein et al., 2007). *A. florea* in particular is valuable for local economic development in Thailand because this species of honey bee is the primary pollinator of many crops and wild plants (Suwannapong et al., 2011b). Although *Nosema* infected bees do not exhibit obvious external disease symptoms, they can have digestive disorders resulting in malnutrition, reduced hypopharyngeal glands, and shortened life spans (Goblirsch et al., 2013; Woyciechowski and Lomnicki, 1995; Woyciechowski and Kozlowski, 1998; Wang and Moeller, 1971; Wang and Moeller, 1969). In general, malnutrition and energetic stress have emerged to be one of the main pathological effects from a *N. ceranae* infection in *Apis*

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mellifera (Dussaubat et al., 2012; Vidau et al., 2014; Mayack and Naug, 2009; Naug and Gibbs, 2009; Alaux et al., 2010). Furthermore, hives with Nosemosis demonstrate lower honey yields and depopulation of worker bees (Fries et al., 1984; White, 1919). One of the possible mechanisms suggested for the depopulation of hives is due to forager energetic stress (Mayack and Naug, 2010; Mayack and Naug, 2013; Wolf et al., 2014), as infected bees have lower haemolymph trehalose, which is the sugar used to power flight when foraging out away from the hive (Thompson, 2003; Blatt and Roces, 2001). Accompanying this reduction in trehalose is an increase in bee mortality (Mayack and Naug, 2009; Mayack and Naug, 2013; Martín-Hernández et al., 2011). The pathological effects of *Nosema* do not cause immediate death, but still can reduce pollination effectiveness and increase the likelihood of a colony collapsing (Higes et al., 2008; Wolf et al., 2014; Naug, 2014). Thus, there is a need for effective treatment control measures in order to combat *Nosema* infections on a regular basis.

The antibiotic Fumagillin was the first cost effective treatment identified for combating *N. apis* infections (Goodman et al., 1990; Moffett et al., 1969). However, Fumagillin is only effective at killing the vegetative stage of the *N. apis* life cycle, and mature spores are resistant to Fumagillin treatment (Katznelson and Jamieson, 1952; Liu, 1973). Furthermore, Fumagillin has been shown to be only temporarily effective at reducing *N. ceranae* parasite burdens in honey bee colonies (Williams et al., 2008b) and is banned from use in Europe (Higes et al., 2014). This is a pressing concern because the spread of *N. ceranae* is on the rise and this pathogen is also potentially more virulent than *N. apis* (Paxton et al., 2007; Williams et al., 2014; Martín-Hernández et al., 2011). Therefore, the use of Fumagillin, despite its limited effectiveness against *N. ceranae*, will continue to increase and consequently there is a high probability for Fumagillin resistance to develop rapidly in *N. ceranae*.

In response to this, other antibiotics, including sulpha drugs have been tested for the control of *N. ceranae* with limited success (Roussel et al., 2015). There are additional drawbacks to the use of antibiotics, including sulpha drugs as well, as they pose potential health risks for humans when consuming contaminated honey. Increased exposure to these antibiotics and their residues are likely to confer increased bacterial antibiotic resistance to human diseases such as tuberculosis (Kochansky et al., 2001). Indeed, recently it has been shown that Fumagillin and its counterpart dicyclohexylamine, which are both highly toxic to mammals, does not completely degrade in contaminated honey held under typical hive conditions, even after one year (van den Heever et al., 2015). Therefore, it has been realized that a natural product and perhaps a less toxic one to humans that kills *Nosema,* is desirable (Maistrello et al., 2008).

Given that Nosema lives primarily in the gut, a number of natural treatments have been developed and tested that are administered orally by mixing the substance in sugar syrup that is bulk fed to bees. One treatment, Nozevit works by keeping the midgut pH low and thereby prevents the midgut from becoming rigid that is detrimental for absorption of nutrients (Higes et al., 2014). In the same vein, prebiotics and probiotics have been investigated to maintain this low pH required to determine if it reduces the parasite burden in the midgut, but actually an increase in *Nosema* loads have been observed (Ptaszynska et al., 2016), these increases however can be negated with Fumagillin treatment (Maggi et al., 2013). On the other hand, natural products such as Zeolite and BeeCleanse significantly reduce *Nosema* loads, but the magnitude of the effect was marginal, with millions of spores remaining in bees after many days of treatment (Gajger et al., 2013; Gajger et al., 2015). Essential oils and other plant extracts have been found to have a more dramatic and targeted effect at reducing *Nosema* loads and extending the life-span of infected bees, providing evidence for exploring treatments along these lines to be more promising for developing alternative treatment methods to combat *N. ceranae* (Damiani et al., 2014; Costa et al., 2010; Strachecka et al., 2015; Porrini et al., 2011).

Previous work supports the notion that propolis, as a natural product obtained from plant resins by bees, can be effective at inhibiting microsporidian development and improve infected honey bee survival (Suwannapong et al., 2011b; Krol et al., 1993). Therefore, in this study we not only evaluate the potential of propolis to control *Nosema* development in *Apis florea*, but we also measure the extent in which the treatment can ameliorate its associated pathological effects by measuring trehalose levels in the haemolymph and the protein content of the hypopharyngeal gland that are known to decrease in infected bees (Wang and Moeller, 1969; Mayack and Naug, 2010; Suwannapong et al., 2010). The aim of this study was to investigate the effect of propolis on experimentally infected *A. florea* workers inoculated with *Nosema* spores due to its ability to spread to other native bee species in the local area and cause increased virulence in a new host.

Materials and methods

Preparing propolis extractions

Propolis was obtained from three colonies of the stingless bee *Trigona apicalis* in an apiary located in Chanthaburi Province, Thailand. The propolis obtained was collected from plants growing in this local area collected by managed stingless bees from a central research station. Propolis was first dried in a hot air oven at 80 °C for 72 h, and then 60 g of it was shaken with a 100 ml of 70% ethanol, followed by gravity filtration using a Whatman No. 4 filter. This crude extract was stored in a dark bottle and was considered as a stock solution of 100% propolis extract. The stock propolis extract was then diluted with distilled water to make 50% and 70% concentrations (v/v) that were used in the following experiment as propolis ethanoic extraction treatments.

Nosema spore preparation

Nosema spores were isolated from three heavily infected colonies of *Apis cerana* located in the Samut Songkhram Province, in southern Thailand. Honey bee midguts were each placed in a microcentrifuge tube containing 200 µl distilled water and homogenized using a sterile pestle. These tubes were then spun at 6000*g* for 10 min three times or until pollen grains could be separated. Spores were counted using a hemocytometer. Spores were then re-suspended in 50% (w/v) sucrose solution at a concentration required to feed 8×10^4 spores per bee. The sucrose solution containing spores was kept at 4 °C until it was needed for inoculation.

Nosema inoculation and propolis extract treatment

Bee brood comb from tree branches were obtained from three colonies of *A. florea* free of *Nosema*. To provide newly emerged worker bees for caged experiments, this comb was incubated at 34 ± 2 °C with relative humidity maintained between 50 and 55%. The newly emerged bees were carefully removed and placed in a cage (50 bees per cage). Two days after eclosion they were divided into six treatment groups (50 bees per group), each treatment group was placed in one bee cage. The first three treatment groups were randomly selected to be inoculated with *Nosema* and this was accomplished by individually force-feeding 2 µl of the 50% sucrose solution (w/v) containing 8×10^4 *Nosema* spores. These treatment groups were then provided with 0%, 50% and 70% propolis extract mixed with 20 ml 50% sucrose solution (v/v), defined as 0P, 50P and 70P, respectively. The last three treatment groups were deemed as controls. The negative control (CO) was not infected with *Nosema*, was not treated with propolis, and did not receive any ethanol. The propolis control bees (CP), were not infected with *N. ceranae*, but instead were treated with 70% propolis, without ethanol. The last control group was infected with *N. ceranae*, but treated with 49% ethanol (CE), which was based on the amount used during the extraction of the 70% propolis extraction process. All treatment groups