Prolyl-tRNA synthetase inhibition reduces microsporidia infection intensity in honey bees

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Abstract – *Nosema ceranae* is a microsporidian parasite that is pathogenic to honey bees, causing disease at the individual and colony level. *N. ceranae* infection can be controlled by treatment with Fumagillin, but as this the future of this drug is uncertain, alternative treatment strategies are critical. Genomic examination of proteostasis pathways in *N. ceranae* and its host, *Apis mellifera*, has revealed key differences in component conservation. One example is the amino acid response (AAR), which responds to amino acid limitation through the sensing of uncharged tRNA molecules. The AAR is conserved in the honey bee, but not in *N. ceranae*. Pharmacological inhibition of prolyl-tRNA synthetase activity resulted in a substantial reduction in *N. ceranae* infection intensity. Evident toxicity to bees suggests further work is required to make this approach safe and feasible. However, these results provide proof of principle that tRNA synthetase inhibition could be an effective future treatment strategy.

Nosema ceranae / Microsporidia / Honey bee / Infection / tRNA synthetase

1. INTRODUCTION

Colonies of the western honey bee, *Apis mellifera*, have suffered from increased mortality in recent years that is likely caused by a complex set of interacting stresses (Goulson et al. 2015). There has been intensifying focus on the role of microbial attack on honey bee health (Evans and Schwarz 2011). The microsporidian species *Nosema ceranae* and *Nosema apis* can cause individual mortality in honey bees and have been implicated in colony disease (Weiss and Becnel 2014; Goblirsch 2017; Martín-Hernández et al. 2018). *N. ceranae* is now one of the most common pathogens of the honey bee which infects the

midgut and causes energetic stress, epithelial damage, and, when untreated, death.

N. ceranae infection has traditionally been controlled by treatment with the drug Fumagillin, a methionine aminopeptidase 2 inhibitor (reviewed in (van den Heever et al. 2014)). Yet, the effectiveness of Fumagillin treatment in controlling *N. ceranae* at the colony level appears limited in scope and duration (Mendoza et al. 2017). High doses of this drug impact host cell function, and evidence suggests that *N. ceranae* can evade suppression in some circumstances (Huang et al. 2013). Most critically, this drug was temporarily unavailable due to production problems, leading to doubts about its future. Thus, efforts to find alternative treatment strategies are critical to protect honey bees from this parasite (van den Heever et al. 2014; Holt and Grozinger 2016).

As comparative genomics indicates that microsporidia have lost many of the cellular processes and pathways found in free-living

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eukaryotes (Nakjang et al. 2013), it appears likely that these organisms could possess fewer genes encoding proteins involved in protecting cells from stress. Examination of proteostasis pathways in *Nosema* species and its host revealed key differences in component conservation and function. One such proteostasis pathway is the amino acid response (AAR) activated by amino acid deprivation. Genome analysis revealed that the components of the AAR are conserved in the honey bee, but not in *Nosema* species, suggesting that stresses that trigger this pathway might provide an attractive therapeutic strategy. Pharmacologic inhibition of aminoacyl-tRNA synthetases (AARSs), which catalyze the attachment of the appropriate amino acid to its cognate tRNA to form an aminoacyl-tRNA, cause proteotoxic stress, and activate the AAR. Recent discoveries have prompted a significant interest in targeting tRNA synthetases in a wide variety of eukaryotic infectious organisms that cause disease in humans and livestock (Pham et al. 2014). Most notably, Halofuginone inhibits prolyl-tRNA synthetase activity in malaria-causing parasites (Khan 2016). Comparison of the honey bee bifunctional glutamyl/prolyl-tRNA synthetase (EPRS) and the *Nosema* spp. prolyl-tRNA synthetase (PRS) with homologs known to be inhibited by Halofuginone suggested that they would be inhibited by this drug as well. The impacts of prolyl-tRNA synthetase inhibition on *N. ceranae* infection and host function and survival were evaluated.

2. MATERIALS AND METHODS

2.1. Honey bee tissue collection

Honey bees were collected from outbred colonies consisting of a typical mix of *Apis mellifera* subspecies found in North America at different times during the months of April–October in New York, New York. Only visibly healthy bees were collected, and all source colonies were visually inspected for symptoms of common bacterial, fungal, and viral diseases of honey bees. For most experiments, bees were collected from the landing board of the colony with the entrance blocked and likely represent primarily foragers. Gut tissue was

removed from abdomens, and midguts were dissected.

2.2. Ortholog screening

AAR pathway and aminoacyl-tRNA-synthetase gene candidates from *Drosophila melanogaster* and *Caenorhabditis elegans* were used to find orthologs in the honey bee genome using the BLAST family of search functions (www.ncbi.nlm.nih.gov). AAR pathway and aminoacyl-tRNA-synthetase gene candidates from *Saccharomyces cerevisiae* were used to find orthologs in the *N. ceranae* as well as other available microsporidian genomes using the same methods. The KEGG (Kyoto Encyclopedia of Genes and Genomes) database was also used as a guide for comparing pathways between species (Kanehisa and Goto 2000).

2.3. Caging and chemical treatments

For caging experiments, honey bees were selected as above and kept in 177.4-mL (6 oz.) square-bottomed *Drosophila* stock bottles (VWR) with modified foam tube plugs (Jaece Industries). Approximately 15 bees were added to each cage, and cages were maintained in incubators at 35 °C in the presence of PseudoQueen (Contech, Victoria, British Columbia, Canada) as a source of queen mandibular pheromone (QMP). Bees were fed 33% sucrose via a modified 1.5-ml screw-cap tube and allowed to feed ad libitum with or without Halofuginone (Sigma), Febrifugine (Sigma), or Fumagillin-B (Medivet) as noted in the text. When DMSO was used as the chemical solute, equivalent amounts of DMSO were added to the food of the control group. No differences in the amount of food consumed per bee were observed in any treatment group.

2.3.1. *N. ceranae* infection

Infection intensity was quantified by counting spore number per bee using light microscopy (Cantwell 1970). *N. ceranae* spores for infecting bees were obtained from infected individuals. In this case, midguts from infected or uninfected bees were individually crushed in 0.5-ml H₂O

and washed 3 times with water and resuspended in 33% sucrose syrup at a concentration of 1×10^6 spores per ml. Bees were allowed to consume food ad libitum for 24 h before food was replaced with 33% sucrose syrup alone. Bees in the uninfected group always received sucrose syrup containing a midgut from an uninfected bee processed in the same way as the midgut containing spores.

2.3.2. Newborn caging, infecting, and chemical treatments

When newly emerged bees were used, they were collected after hatching from a capped brood frame overnight in an incubator at 35 °C in the presence of PseudoQueen (Contech, Victoria, British Columbia, Canada) as a source of queen mandibular pheromone (QMP). Approximately 30 newly eclosed bees were placed in each 4.8 × 3.4 × 8.4-in. acrylic cage with sliding door machined at Carleton Labs, Columbia University, New York, NY, USA. Approximately 4 foragers from the same source colony (marked with a spot of paint (Testors, Vernon Hills, IL, USA)) were added to each cage. Bees were fed sucrose solution and supplied with a ~5-g pollen substitute patty (1:1 mix of BeePro and sucrose solution). On day 2 post-eclosion, *N. ceranae* spores (5×10^6 /ml) were fed to bees in sucrose solution ad libitum (Fries et al. 2013) for 48 h. At 3-day postinfection, honey bees were fed sucrose solution containing one of the pharmacologic agents or vehicle control alone at the indicated doses. After 4 days of drug feeding, honey bee midguts were dissected, pooled, and crushed in 0.5-ml water/bee, and the number of mature spores was counted by light microscopy as previously described (Fries et al. 2013). Survival during drug treatment was monitored in parallel.

2.4. RNA isolation, reverse transcription, and quantitative PCR gene expression

RNA was prepared from bees from the described populations as before (Johnston et al. 2016). Briefly, the midgut was manually crushed with a disposable pestle in TRIzol Reagent

(Invitrogen, San Diego, CA). The RNA was extracted as per the manufacturer's instructions, and contaminating DNA was removed through treatment with RQ1 RNase-Free DNase (Promega, Madison, WI). RNA was then quantified, and cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Subsequently, 1 µl of cDNA was then used as a template for quantitative PCR to determine the transcript levels for genes of interest using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Primer sequences for transcripts of honey bee genes of interest developed for this study are *lactate dehydrogenase* (F: 5'-AAACAGAAGGCTGG GAGCAG-3', R: 5'-ACGGTGACTTTGTG CGTACT-3') and *asparaginyl-tRNA synthetase* (F: 5'-CCTGGTGGTGCAGATTCAT-3', R: 5'-ATACATCGCCCATTGCTGGA-3'). For honey bee genes, the difference between the threshold cycle (Ct) number for honey bee β -actin (primer sequence from (Johnston et al. 2016) and that of the gene of interest was used to calculate expression levels relative to β -actin using the $\Delta\Delta C_T$ method.

2.5. DNA extraction and quantitative PCR for infection quantitation

DNA extraction was performed using a modified Smash & Grab DNA Miniprep protocol (Rose et al. 1990). Subsequently, 1 µl of DNA was used as a template for quantitative PCR to determine the levels of infection for *Nosema sp.* using the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) in a LightCycler 480 thermal cycler (Roche, Branchburg, NJ). Primer sequences for *N. ceranae* β -actin and honey bee ATP synthase F1 subunit alpha (*ATP5a*) gene were from Snow et al. (2019). The *ATP5a* gene measures honey bee genome equivalents by amplifying only genomic DNA. The difference between the threshold cycle (Ct) number for honey bee *ATP5a* and *N. ceranae* β -actin was used to calculate the level infection relative to *ATP5a* using the $\Delta\Delta C_T$ method. A sample was considered negative for a specific *Nosema* species if it did not amplify any product by 35 cycles, and zero was entered as the value in these cases.

2.6. Statistical analysis

Data is presented as means $\pm$ SEM shown. For gene expression analysis, data was log10 transformed. For two groups, data was compared using unpaired *t* tests with Welch's correction when values fit normal distributions or Mann-Whitney U nonparametric tests when they did not fit normal distributions. Normality was assessed using Shapiro-Wilk tests. When more than two groups were being compared, data was compared using one-way ANOVA with Tukey's multiple comparison test when values fit normal distributions or a Kruskal-Wallis test when they did not. For survival analysis, treated versus untreated groups were compared using the Gehan-Breslow-Wilcoxon test.

3. RESULTS

AAR pathway components are conserved in the honey bee, but not Nosema ceranae or other microsporidia We previously showed that the

AAR pathway (Figure 1 and Suppl Figure 1) is conserved in honey bees (Johnston et al. 2016) and Suppl Table 1. However, similar analysis revealed that *N. ceranae* does not appear to possess genes encoding general control nonderepressible 2 (GCN2) or activating transcription factor 4 (ATF4) (general control nonderepressible 4 (GCN4) in yeast) homologs. In addition, no other microsporidia have either of these AAR components. All three subunits of eIF2 are found in *N. ceranae* (Suppl Table 2), and alignment of the alpha subunit from *N. ceranae* with that of *S. cerevisiae* shows that Ser51 is conserved, suggesting that eIF2 regulation by phosphorylation is possible (Suppl Figure 2).

Apis mellifera bifunctional glutamyl/prolyl-tRNA synthetase and *N. ceranae* prolyl-tRNA synthetase predicted to bind HalofuginoneHalofuginone, a well-characterized inhibitor of prolyl-tRNA synthetase activity, was used to trigger the AAR in both species. In unicellular eukaryotes such as microsporidia (Lee et al. 2010), the ERS and

Figure 1. Honey bee and *N. ceranae* amino acid response (AAR) pathway. Diagram of components of the AAR pathway conserved in honey bees (blue) and both honey bees and *Nosema* spp. (green).

PRS activities exist in two proteins, while in higher eukaryotes, such as the honey bee, these functions have been combined in a single fusion protein (Guo et al. 2010). Two recent studies showed the key amino acids involved in binding of Halofuginone to *Plasmodium* prolyl-tRNA synthetase (PRS) and its inhibition (Jain et al. 2015; Herman et al. 2015). The bifunctional glutamyl/prolyl-tRNA synthetase (EPRS) protein sequence for the honey bee (XP_026301491.1) and the prolyl-tRNA synthetase (PRS) sequence for *N. ceranae* (XP_024331292.1) were identified (Figure 2a). Regions containing critical Halofuginone binding site for the honey bee EPRS and the *N. ceranae* PRS proteins were aligned with the corresponding regions from the PRS proteins of *Plasmodium falciparum* and

S. cerevisiae (Figure 2b). Based on this comparison, both honey bee and *N. ceranae* proteins are predicted to bind Halofuginone (critical amino acid residues highlighted in red). In addition, both have a threonine in position 512 (numbering based on *Pfc*PRS) instead of the mutation to serine that is thought to make *S. cerevisiae* unaffected by Halofuginone (residue highlighted in orange). Examination of the PRS sequences for other microsporidia with available genomes (Suppl Table 3) through sequence alignment suggests that most species are sensitive to Halofuginone inhibition (data not shown).

Halofuginone controls existing infections by N. ceranae in experimentally and naturally infected bees To determine how PRS inhibition would

Figure 2. *Apis mellifera* bifunctional glutamyl/prolyl-tRNA synthetase and *N. ceranae* prolyl-tRNA synthetase predicted to bind Halofuginone. A schematic representation of the domain structures of the bifunctional glutamyl/prolyl-tRNA synthetase (EPRS) protein of the honey bee (XP_016772424.1) and the prolyl-tRNA synthetase (PRS) protein for *N. ceranae* (XP_002996195.1) (a). Sequence alignment of the region containing critical Halofuginone binding site for the honey bee EPRS and the *N. ceranae* PRS protein with the corresponding regions from the PRS proteins of *Plasmodium falciparum* and *Saccharomyces cerevisiae* (b). Critical amino acids are shaded in red while the resistance mutation found in *S. cerevisiae* is shaded in yellow.

impact *N. ceranae* infection in honey bees, experimentally infected bees were fed either sucrose syrup or sucrose syrup containing 200- μ M Halofuginone for 2 days starting on 6-day postinfection. On day 8 postinfection, spore levels were measured using light microscopy to determine the effects of PRS inhibition on *N. ceranae* infection intensity. Feeding infected bees Halofuginone or the canonical anti-*Nosema* agent Fumagillin for 48 h both resulted in a reduction in infection intensity in infected bees (Figure 3a). Similar results were observed for bees captured on the landing board of a heavily infected colony (80% prevalence) (Figure 3b). As spore counts do not quantify all life stages of microsporidia, the amounts of

N. ceranae β -actin gene relative to honey bee *ATP5a* gene by qPCR were also used to assess relative infection intensity. Similar reductions in infection intensity were found through measuring spore counts or genome equivalents (Suppl Figure 3). There were no decreases in survival to bees over this 2-day treatment. To assess the impacts of Halofuginone treatment on honey bees, uninfected bees from the landing board were fed various concentrations of Halofuginone (40 or 200 μ M), vehicle control, or Fumagillin. Honey bees had reduced survival by 5 days after starting treatment with 200- μ M Halofuginone ($p < 0.01$) (the dose required to reduce *N. ceranae* infection) (Figure 3c).

Figure 3. Prolyl-tRNA synthetase inhibition by Halofuginone reduces *N. ceranae* infection intensity in experimentally or naturally infected honey bees. *N. ceranae* levels in midguts as determined by spore count using light microscopy 8 days postinfection in uninfected or infected bees fed sucrose syrup containing DMSO or Halofuginone for the final 2 days $a \neq b \neq c \neq d$, $p < 0.05$ (**a**). *N. ceranae* levels as determined by spore count using light microscopy in individual bees from an infected colony fed sucrose syrup containing DMSO, Fumagillin, or Halofuginone for 2 days $**p < 0.01$ (**b**). Survival of individual uninfected honey bees fed sugar solution containing no drug ($n = 58$), 1.25-mg/ml Fumagillin ($n = 39$), or Halofuginone (40 μ M ($n = 55$) or 200 μ M ($n = 35$)) $**p < 0.01$ (**c**).

Febrifugine controls existing infections by N. ceranae in experimentally and naturally infected bees Halofuginone is the halogenated derivative of

Febrifugine (Figure 4a), the biologically active antimalarial found in the *Dichroa febrifuga* herb (Jiang et al. 2005). To determine if PRS inhibition

Figure 4. Prolyl-tRNA synthetase inhibition by Febrifugine reduces *N. ceranae* infection intensity in experimentally or naturally infected honey bees. Chemical structures of Halofuginone and Febrifugine (**a**). *N. ceranae* levels in midguts of newly eclosed bees as determined by spore count using light microscopy 8-day postinfection in uninfected or infected bees fed sucrose syrup containing DMSO, Fumagillin, Halofuginone, or Febrifugine for the final 2 days $a \neq b$, $p < 0.05$ (**b**). *N. ceranae* levels in midguts as determined by spore count using light microscopy (**c**) or by qPCR (**d**) 8-day postinfection in uninfected or infected bees fed sucrose syrup containing DMSO, Fumagillin, or Febrifugine for 5 days $a \neq b$, $p < 0.05$. Survival of individual honey bees fed sugar solution containing no drug ($n = 42$), 1.25-mg/ml Fumagillin ($n = 38$), or Febrifugine (40 μ M ($n = 25$) or 200 μ M ($n = 42$)) $**p < 0.01$ (**e**). *N. ceranae* levels in midguts as determined by spore count using light microscopy 11-day postinfection in uninfected or infected bees fed sucrose syrup containing DMSO, Fumagillin, or Febrifugine for 4 days $a \neq b$, $p < 0.05$ (**f**).

by this naturally occurring compound would impact *N. ceranae* infection in honey bees, experimentally infected bees were fed either sucrose syrup or sucrose syrup containing 200- μ M Febrifugine for 2 days starting on 6-day postinfection. On day 8 postinfection, spore levels were measured using light microscopy to determine the effects of PRS inhibition on *N. ceranae* infection intensity. Feeding infected bees Febrifugine for 48 h resulted in reduction in infection intensity in infected bees by spore count (Figure 4b). Experimentally infected bees were fed either sucrose syrup or sucrose syrup containing 200- μ M or 40- μ M Febrifugine for 5 days starting on 3-day postinfection. Longer treatment regimens resulted in almost complete ablation of *N. ceranae* in those bees fed either Febrifugine (40 or 200 μ M) or Fumagillin (1.25 mg per ml) relative to bees fed vehicle control (DMSO) by spore count (Figure 4c) or genome equivalents (Figure 4d). To assess the impacts of Febrifugine treatment on honey bees, uninfected bees were gathered from the landing board and fed them Febrifugine (40 or 200 μ M), vehicle control, or Fumagillin. Here, it was found that honey bees had reduced survival by 5 days after starting treatment with 200- μ M Febrifugine ($p < 0.01$), but no change in survival over the 8-day time course using treatment with 40- μ M Febrifugine (Figure 4e).

The impact of Febrifugine treatment on infected of age-matched, newly eclosed bees was also assessed. Newly emerged bees were infected on day 3 post-eclosion and then fed Febrifugine (40 or 200 μ M) or Fumagillin (40 μ M) or vehicle control for 4 days starting on day 7 post-eclosion. In these newly eclosed bees, honey bees only had reduced infection intensity as assessed by spore count when treated with the higher dose of Febrifugine (Figure 4f). There were no differences in survival between groups over the 4 days of treatment (Suppl Figure 3c).

Robust AAR induction after Halofuginone and Febrifugine treatment in the honey bee To determine whether inhibition of prolyl-tRNA synthetase activity would result in induction of the AAR in the honey bee, the expression of putative target genes was examined. As transcriptional activation by the transcription factor ATF4 is the primary

mechanism regulating AAR-induced gene expression (Figure 2 and Suppl Figure 1), genes that have been shown to be dependent on ATF4 after its activation by the related unfolded protein response (UPR) pathway in the fruit fly (Lee et al. 2015) were tested. The glucose metabolism enzyme *lactate dehydrogenase* (*Ldh*) (Figure 5a) and the *asparaginyl-tRNA synthetase gene* (*NARS*) (Figure 5b) were upregulated significantly in the honey bee digestive tract after 48 h of Halofuginone treatment relative to bees consuming sucrose syrup alone, while the level of the reference gene (*β -actin*) was not altered by treatment (Figure 5c). After Febrifugine treatment, *Ldh* was upregulated significantly in the honey bee digestive tract after 48 h of Febrifugine treatment relative to bees consuming sucrose syrup alone (Figure 5d), while the level of the reference gene (*β -actin*) was not altered by treatment (Figure 5e).

4. DISCUSSION

Components of the AAR are conserved in the honey bee, but not in *N. ceranae*. The absence of a recognizable AAR in *N. ceranae* and other microsporidia species might be expected, as microsporidia do not possess the biosynthetic pathways required to synthesize most amino acids and must import amino acids from the host cell through the use of transporters (Nakjang et al. 2013). However, the apparent conservation of Ser51, which is phosphorylated in yeast to regulate translation (Dever et al. 2016), suggests that novel mechanisms may be involved in regulating translation in this species despite the absence of homologs of kinases predicted to phosphorylate eIF2. In the absence of a canonical AAR pathway, it is interesting to speculate about whether microsporidia respond to amino acid limitation in host cells during infection. The impact of *N. ceranae* infection on honey bee health and productivity is still debated, and regional and strain-specific differences may complicate these issues (Goblirsch 2017; Martín-Hernández et al. 2018). When treated, *N. ceranae* infection of honey bee colonies in the USA has traditionally been controlled with Fumagillin. The future of

Figure 5. Honey bee AAR target genes are induced by Halofuginone and Febrifugine. Transcript levels of AAR target genes lactate dehydrogenase (*Ldh*) (a) and asparaginyl-tRNA synthetase gene (*NARS*) (b) relative to β -actin in midgut tissue from individual bees with (H, $n = 4$) or without (U, $n = 4$) 200- μ M Halofuginone treatment after 48 h. Threshold cycle number for β -actin from these bees (c). Transcript levels of AAR target gene *Ldh* (d) relative to β -actin in midgut tissue from individual bees with (F, $n = 8$) or without (U, $n = 8$) 200- μ M Febrifugine treatment after 48 h. Threshold cycle number for β -actin from these bees (e). Data is represented as Mean $\pm$ SEM. Statistical significance is noted as * $p < 0.05$ and ** $p < 0.01$.

this drug is uncertain, making the discovery of novel therapeutic agents of significant importance. Eukaryotic pathogens can be challenging to combat using chemical antibiotics due to the phylogenetic closeness with their hosts, and attempts to find other treatments have not yet resulted in effective alternatives (reviewed in (Holt and Grozinger 2016)). However, a number of recent studies have found that compounds targeting members of the AARS family within eukaryotic pathogens can be highly efficacious treatment options for eukaryotic infectious organisms that cause disease in humans and livestock (reviewed in (Pham et al. 2014; Khan 2016)). For example, Halofuginone acts by inhibiting the prolyl-tRNA synthetase protein, which attaches proline amino acids to their cognate tRNA molecules (Keller et al. 2012; Zhou et al. 2014).

Halofuginone is a promising antimalarial drug (Herman et al. 2015) and has been used in veterinary medicine as a coccidiostat (Zhang et al. 2012) and appears to have activity against *Cryptosporidium parvum* (Trotz-Williams et al. 2011) and *Toxoplasma gondii* (Jain et al. 2015).

These studies showed that *N. ceranae* intensity was effectively reduced by treatment with Halofuginone. However, longer survival assays with bees revealed that the drug is toxic to bees at the concentrations most effective at killing *N. ceranae*. While Halofuginone has been found to be less toxic than Febrifugine in mammals, Febrifugine is equally potent in killing *N. ceranae* with less apparent toxicity to bees than Halofuginone. However, these results also show that at higher doses, toxicity of Febrifugine to bees is dramatically increased. Interestingly, the

dose required for reducing infection intensity was higher in newly eclosed bees than in the landing board bees, perhaps suggesting age-related factors that impact efficacy of this drug against *N. ceranae* infection. Taken together, the results described here likely represent promising but early steps in using prolyl-tRNA synthetase inhibition as an anti-*Nosema* strategy. For example, novel Febrifugine derivatives have been developed (Kikuchi et al. 2006) with increased efficacy in killing multiple eukaryotic pathogens (Jain et al. 2017). Further, multiple groups have explored the key amino acids involved in binding of Halofuginone to *Plasmodium* prolyl-tRNA synthetase (PRS) and its inhibition (Jain et al. 2015; Herman et al. 2015). Further work could be done with the relevant prolyl-tRNA synthetases to pursue rational drug design to modify the base molecule Febrifugine to develop derivatives with a higher relative affinity to *N. ceranae* prolyl-tRNA synthetase than to the honey bee EPRS, leading to a better efficacy to toxicity ratio. However, the impacts of Febrifugine (or other derivatives) on different life stages and on the colony as a whole will need to be rigorously field tested before they can be used in the management of *Nosema* infection (Holt and Grozinger 2016). Thus, a more comprehensive analysis of the doses and long-term effects of such a treatment strategy on honey bee health, garnered through rigorous field trials, is imperative.

A number of additional points need to be considered in evaluating tRNA-synthetase inhibitors as drugs for use to control *N. ceranae* infection in honey bees. Resistance to Halofuginone does occur in malaria parasites (Herman et al. 2014; Herman et al. 2015) through homeostatic increase in proline synthesis machinery and mutation or amplification of the *PRS* gene. Changes in the *PRS* are the most likely pathway to potential resistance in *N. ceranae*. As no AAR machinery or proline metabolic enzymes are evident in *N. ceranae*, alterations in proline synthesis are unlikely. However, microsporidia do possess a significant number of transporters for amino acids (Nakjang et al. 2013), and this is likely how proline is acquired by the parasitic cell. Therefore, mechanisms to increase host proline synthesis or microsporidia proline transport could change

sensitivity to these compounds. In a related point, a recent study showed that *N. ceranae* loads eclipsed pre-treatment levels after cessation of Fumagillin treatment through an unknown mechanism (Huang et al. 2013). Thus, future studies to examine the stability of suppression should also be undertaken.

Microsporidia infect diverse invertebrate species playing important roles throughout our food production system, including many types of beneficial invertebrates such as pollinators (Weiss and Becnel 2014). In addition to insects, microsporidian infections are also problematic in other agriculturally important species. In the aquaculture industry, microsporidia are a significant source of pathology in multiple fish species, leading to loss of productivity (Weiss and Becnel 2014). Microsporidia also cause significant losses among aquatic invertebrates, such as crustaceans, that represent an important food and income source for humans (Weiss and Becnel 2014). Microsporidian infection is also a health issue for humans. While not a major problem in healthy individuals, immunocompromised individuals are highly susceptible to infection by a variety of microsporidian parasites, such as *Encephalitozoon* species (Weiss and Becnel 2014). Most microsporidia species that cause disease in agriculturally important animals and humans are predicted to possess PRS proteins that bind Halofuginone (Suppl Table 3). Thus, the results from these studies might be applicable in agriculture and human health.

Amino acid deprivation or AARS inhibition can be considered to negatively affect proteostasis, the homeostasis of protein synthesis, folding, function, and degradation both within a cell and in an organism as a whole (Klaips et al. 2017). A number of normal and pathologic conditions can lead to disruption of proteostasis, which is in turn sensed by a number of signaling systems in the cell. Comparison of the AAR of honey bees of those in other invertebrates, such as the fly (Santoyo et al. 1997; Olsen et al. 1998) and nematode (Nukazuka et al. 2008), suggests that it is quite similar in form and function. Interestingly, GCN2 and the AAR pathway have been linked to metabolism, development, and aging in these organisms (for example, see (Kang et al. 2015;

Kang et al. 2017)), suggesting that these data will help add to our understanding of honey bee cellular stress responses and honey bee health.

Febrifugine is also found in the common garden plants of the genus *Hydrangea* (Ishih et al. 2006), which do serve as a nectar source for honey bees (Suppl Figure 4). Animals are known to employ self-medication or zoopharmacognosy (de Roode et al. 2013) and many invertebrates (Schmid-Hempel 2017), including honey bees (Erler and Moritz 2016), and participate in this behavior. Honey bees infected with *N. ceranae* prefer honey that possessed higher antibiotic activity (Gherman et al. 2014). Bumble bees ingest more nectar containing the anti-parasitic compound anabasine when parasitized by a specific trypanosome (Anthony et al. 2015). While Febrifugine levels in nectar are unknown (Ishih et al. 2006), it is tempting to speculate that honey bees may preferentially consume *Hydrangea* nectar when parasitized by microsporidia.

In summary, perturbing the cellular process linked to the AAR through pharmacological inhibition of prolyl-tRNA synthetase activity results in reduction in *N. ceranae* infection intensity. Evident toxicity to bees suggests further work is required to make this approach safe and feasible. However, these results may provide proof of principle that tRNA synthetase inhibition could eventually be an effective treatment and suggest that targeting cellular stress pathways is a promising strategy for identification of potential novel therapeutics.

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L'inhibition de la prolyl-tRNA synthétase réduit l'intensité de l'infection par les microsporidies chez les abeilles.

Nosema ceranae / microsporidia / abeille domestique / infection / ARNt synthétase.

Die Hemmung der Prolyl-tRNA-Synthetase reduziert den Infektionsgrad mit Mikrosporidien bei Honigbienen.

Nosema ceranae / Mikrosporidien / Honigbienen / Infektion / tRNA-Synthetase.

REFERENCES

- Anthony W.E., Palmer-Young E.C., Leonard A.S. et al (2015) Testing Dose-Dependent Effects of the Nectar Alkaloid Anabasine on Trypanosome Parasite Loads in Adult Bumble Bees. *PLoS ONE* **10**, e0142496. <https://doi.org/10.1371/journal.pone.0142496>
- Cantwell G.E. (1970) Standard methods for counting nosema spores. *Am Bee J* **110**, 222–223
- de Roode J.C., Lefèvre T., Hunter M.D. (2013) Self-Medication in Animals. *Science* **340**, 150–151. <https://doi.org/10.1126/science.1235824>
- Dever T.E., Kinzy T.G., Pavitt G.D. (2016) Mechanism and Regulation of Protein Synthesis in *Saccharomyces cerevisiae*. *Genetics* **203**, 65–107. <https://doi.org/10.1534/genetics.115.186221>
- Erler S., Moritz R.F.A. (2016) Pharmacophagy and pharmacophory: mechanisms of self-medication and disease prevention in the honeybee colony (*Apis mellifera*). *Apidologie* **47**, 389–411. <https://doi.org/10.1007/s13592-015-0400-z>
- Evans J.D., Schwarz R.S. (2011) Bees brought to their knees: microbes affecting honey bee health. *Trends Microbiol* **19**, 614–620. <https://doi.org/10.1016/j.tim.2011.09.003>
- Fries I., Chauzat M.-P., Chen Y.P. et al (2013) Standard methods for Nosema research. *Journal of Apicultural Research* **52**, 1–28. <https://doi.org/10.3896/IBRA.1.52.1.14>
- Gherman B.I., Denner A., Bobiş O. et al (2014) Pathogen-associated self-medication behavior in the honeybee *Apis mellifera*. *Behav Ecol Sociobiol* **68**, 1777–1784. <https://doi.org/10.1007/s00265-014-1786-8>
- Goblirsch M. (2017) *Nosema ceranae* disease of the honey bee (*Apis mellifera*). *Apidologie* **49**, 131–150. <https://doi.org/10.1007/s13592-017-0535-1>
- Goulson D., Nicholls E., Botías C., Rotheray E.L. (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science* **347**, 1255957. <https://doi.org/10.1126/science.1255957>
- Guo M., Yang X.-L., Schimmel P. (2010) New functions of aminoacyl-tRNA synthetases beyond translation. *Nat Rev Mol Cell Biol* **11**, 668–674. <https://doi.org/10.1038/nrm2956>
- Herman J.D., Rice D.P., Ribacke U. et al (2014) A genomic and evolutionary approach reveals non-genetic drug

- resistance in malaria. *Genome Biol* **15**, 413. <https://doi.org/10.1186/s13059-014-0511-2>
- Herman J.D., Pepper L.R., Cortese J.F. et al (2015) The cytoplasmic prolyl-tRNA synthetase of the malaria parasite is a dual-stage target of febrifugine and its analogs. *Science Translational Medicine* **7**, 288ra77. <https://doi.org/10.1126/scitranslmed.aaa3575>
- Holt H.L., Grozinger C.M. (2016) Approaches and Challenges to Managing Nosema (Microspora: Nosematidae) Parasites in Honey Bee (Hymenoptera: Apidae) Colonies. *Journal of Economic Entomology* **109**, 1487–1503. <https://doi.org/10.1093/jee/tow103>
- Huang W.-F., Solter L.F., Yau P.M., Imai B.S. (2013) Nosema ceranae Escapes Fumagillin Control in Honey Bees. *PLoS Pathog* **9**, e1003185. <https://doi.org/10.1371/journal.ppat.1003185>
- Ishih A., Miyase T., Suzuki T. et al (2006) Seasonal variation in the content of a febrifugine and isofebrifugine alkaloid mixture in aerial parts of Hydrangea macrophylla var. Otaksa, with special reference to its antimalarial activity. *J Nat Med* **61**, 213–216. <https://doi.org/10.1007/s11418-006-0124-5>
- Jain V., Yogavel M., Oshima Y. et al (2015) Structure of Prolyl-tRNA Synthetase-Halofuginone Complex Provides Basis for Development of Drugs against Malaria and Toxoplasmosis. *Structure* **23**, 819–829. <https://doi.org/10.1016/j.str.2015.02.011>
- Jain V., Yogavel M., Kikuchi H. et al (2017) Targeting Prolyl-tRNA Synthetase to Accelerate Drug Discovery against Malaria, Leishmaniasis, Toxoplasmosis, Cryptosporidiosis, and Coccidiosis. *Structure* **25**:1495–1505.e6. <https://doi.org/10.1016/j.str.2017.07.015>
- Jiang S., Zeng Q., Gettayacamin M. et al (2005) Antimalarial activities and therapeutic properties of febrifugine analogs. *Antimicrob Agents Chemother* **49**, 1169–1176. <https://doi.org/10.1128/AAC.49.3.1169-1176.2005>
- Johnston B.A., Hooks K.B., McKinstry M., Snow J.W. (2016) Divergent forms of endoplasmic reticulum stress trigger a robust unfolded protein response in honey bees. *J Insect Physiol* **86**, 1–10. <https://doi.org/10.1016/j.jinsphys.2015.12.004>
- Kanehisa M., Goto S. (2000) KEGG: kyoto encyclopedia of genes and genomes. *Nucleic Acids Research* **28**, 27–30. <https://doi.org/10.1093/nar/28.1.27>
- Kang K., Ryoo H.D., Park J.-E. et al (2015) A Drosophila Reporter for the Translational Activation of ATF4 Marks Stressed Cells during Development. *PLoS ONE* **10**, e0126795. <https://doi.org/10.1371/journal.pone.0126795.s005>
- Kang M.-J., Vasudevan D., Kang K. et al (2017) 4E-BP is a target of the GCN2–ATF4 pathway during Drosophila development and aging. *The Journal of Cell Biology* **216**, 115–129. <https://doi.org/10.1083/jcb.201511073.dv>
- Keller T.L., Zocco D., Sundrud M.S. et al (2012) Halofuginone and other febrifugine derivatives inhibit prolyl-tRNA synthetase. *Nature Chemical Biology* **8**, 311–317. <https://doi.org/10.1038/NCHEMBIO.790>
- Khan S. (2016) Recent advances in the biology and drug targeting of malaria parasite aminoacyl-tRNA synthetases. *Malar J* **15**, 1–10. <https://doi.org/10.1186/s12936-016-1247-0>
- Kikuchi H., Yamamoto K., Horoiwa S. et al (2006) Exploration of a New Type of Antimalarial Compounds Based on Febrifugine. *J Med Chem* **49**, 4698–4706. <https://doi.org/10.1021/jm0601809>
- Klaips C.L., Jayaraj G.G., Hartl F.U. (2017) Pathways of cellular proteostasis in aging and disease. *The Journal of Cell Biology* **217**, 51–63. <https://doi.org/10.1083/jcb.201709072>
- Lee S.C., Corradi N., Doan S. et al (2010) Evolution of the sex-Related Locus and Genomic Features Shared in Microsporidia and Fungi. *PLoS ONE* **5**, e10539. <https://doi.org/10.1371/journal.pone.0010539.s005>
- Lee J.E., Oney M., Frizzell K. et al (2015) Drosophila melanogaster activating transcription factor 4 regulates glycolysis during endoplasmic reticulum stress. *G3 (Bethesda)* **5**, 667–675. <https://doi.org/10.1534/g3.115.017269>
- Martín-Hernández R., Bartolomé C., Chejanovsky N. et al (2018) Nosema ceranae in Apis mellifera: a 12 years postdetection perspective. *Environmental Microbiology* **20**, 1302–1329. <https://doi.org/10.1111/1462-2920.14103>
- Mendoza Y., Diaz-Cetti S., Ramallo G. et al (2017) Nosema ceranae Winter Control: Study of the Effectiveness of Different Fumagillin Treatments and Consequences on the Strength of Honey Bee (Hymenoptera: Apidae) Colonies. *Journal of Economic Entomology* **110**, 1–5. <https://doi.org/10.1093/jee/tow228>
- Nakjang S., Williams T.A., Heinz E. et al (2013) Reduction and Expansion in Microsporidian Genome Evolution: New Insights from Comparative Genomics. *Genome Biology and Evolution* **5**, 2285–2303. <https://doi.org/10.1093/gbe/evt184>
- Nukazuka A., Fujisawa H., Inada T. et al (2008) Semaphorin controls epidermal morphogenesis by stimulating mRNA translation via eIF2 in Caenorhabditis elegans. *Genes Dev* **22**, 1025–1036. <https://doi.org/10.1101/gad.1644008>
- Olsen D.S., Jordan B., Chen D. et al (1998) Isolation of the gene encoding the Drosophila melanogaster homolog of the Saccharomyces cerevisiae GCN2 eIF-2 α kinase. *Genetics* **149**, 1495–1509
- Pham J.S., Dawson K.L., Jackson K.E. et al (2014) Aminoacyl-tRNA synthetases as drug targets in eukaryotic parasites. *Int J Parasitol Drugs Drug Resist* **4**, 1–13. <https://doi.org/10.1016/j.ijpddr.2013.10.001>
- Rose M.D., Winston F., Hieter P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual* (Plainview, New York). American Psychological Association, Washington
- Santoyo J., Alcalde J., Méndez R. et al (1997) Cloning and characterization of a cDNA encoding a protein synthesis initiation factor-2 α (eIF-2 α) kinase from Drosophila melanogaster - Homology to yeast GCN2 protein kinase. *J Biol Chem* **272**, 12544–12550. <https://doi.org/10.1074/jbc.272.19.12544>

- Schmid-Hempel P. (2017) Parasites and Their Social Hosts. *Trends Parasitol* **33**, 453–462. <https://doi.org/10.1016/j.pt.2017.01.003>
- Snow J.W., Ceylan Koydemir H., Karınca D.K. et al (2019) Rapid imaging, detection, and quantification of *Nosema ceranae* spores in honey bees using mobile phone-based fluorescence microscopy. *Lab on a Chip* **19**, 789–797. <https://doi.org/10.1039/C8LC01342J>
- Trotz-Williams L.A., Jarvie B.D., Peregrine A.S. et al (2011) Efficacy of halofuginone lactate in the prevention of cryptosporidiosis in dairy calves. *Veterinary Record* **168**, 509–509. <https://doi.org/10.1136/vr.d1492>
- van den Heever J.P., Thompson T.S., Curtis J.M. et al (2014) Fumagillin: An Overview of Recent Scientific Advances and Their Significance for Apiculture. *J Agric Food Chem* **62**, 2728–2737. <https://doi.org/10.1021/jf4055374>
- Weiss L.M., Becnel J.J. (2014) *Microsporidia*. John Wiley & Sons, Chichester
- Zhang D.-F., Sun B.-B., Yue Y.-Y. et al (2012) Anticoccidial effect of halofuginone hydrobromide against *Eimeria tenella* with associated histology. *Parasitol Res* **111**, 695–701. <https://doi.org/10.1007/s00436-012-2889-7>
- Zhou H., Sun L., Yang X.-L., Schimmel P. (2014) ATP-directed capture of bioactive herbal-based medicine on human tRNA synthetase. *Nature* **494**, 121–124. <https://doi.org/10.1038/nature11774>

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