EDGE FGT: Bee Functional Genomics Using Engineered Symbionts

Overview and Objectives

Insects are the most widespread and diverse animals on our planet. While genetic techniques are well established for studying certain insects (e.g., *Drosophila*), such tools do not exist for millions of other species. Honey bees (*Apis mellifera*), bumble bees (*Bombus* spp.), and other bee species are model organisms in a variety of research fields and also economically important pollinators, yet we lack effective functional genomics tools for bees. This project will develop and disseminate a toolkit that fills this gap. Our approach is based on engineering bacteria that naturally reside in the co-evolved gut communities of bees to induce an RNA interference (RNAi) response in their insect hosts. Colonizing bees with these symbionts knocks down expression of target genes throughout the bee body so that the roles of these genes in a biological process of interest can be tested (**Fig. 1**).

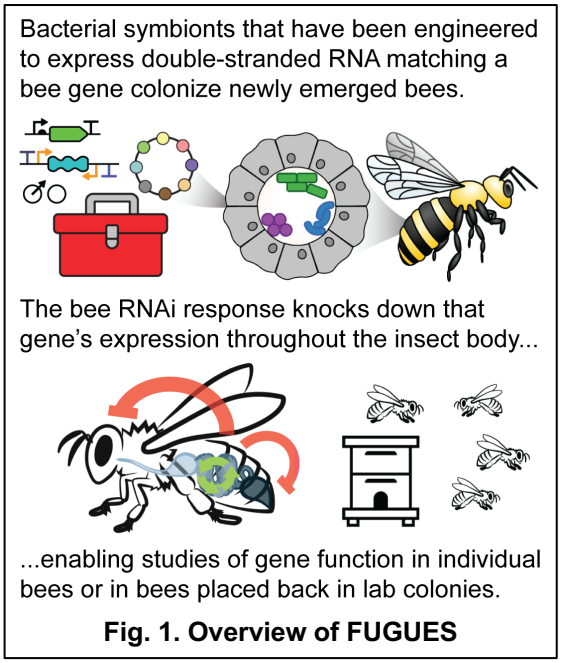

Fig. 1. Overview of FUGUES

We have achieved proof of principle of this **FUnctional Genomics Using Engineered Symbionts (FUGUES)** method and have the experience and networks needed to optimize it further and broadly disseminate it to other researchers with this EDGE project. The Barrick lab has expertise in bacterial synthetic biology and experimental evolution. The Moran lab has studied the molecular mechanisms of host-microbe associations between insects and their symbionts, including in the bee gut microbiota. Our labs have collaborated on bee, aphid, and leafhopper symbiont engineering projects.

The current proposal has the following objectives:

1. **Improve core tools for applying FUGUES to any organism**
   A. Simplify cloning and eliminate reliance on antibiotics
   B. Engineer systems for increasing and controlling ds RNA production
   C. Improve the genetic stability of FUGUES constructs
2. **Optimize FUGUES for studying bee gene function**
   A. Establish colorimetric and behavioral assays for assessing gene silencing in bees
   B. Enhance knockdown efficiency by co-targeting bee nucleases and immunity genes
   C. Characterize and control transmission of engineered bee symbionts
3. **Disseminate FUGUES resources for bee functional genomics**
   A. Simplify engineering *S. alvi* to knock down expression of a target bee gene
   B. Achieve symbiont colonization and gene knockdown in additional bee species

Expected Significance

Bees are longstanding models for researchers worldwide, who have characterized many aspects of their behavior and physiology related to sociality, learning and cognition, communication, navigation, and development, and who are investigating causes of ongoing population declines. The eusocial life cycles of bees, in which most individuals are nonreproductive, make traditional genetic approaches difficult or impossible. *Our EDGE project will provide diverse communities of bee researchers, ranging from ecologists to neurobiologists, with a simple and effective tool for testing hypotheses about the functions of bee genes.* We expect these tools will have positive impacts by making new types of studies possible in these fascinating insects that are ecologically and economically important. **Furthermore, we expect the FUGUES protocols and resources we create will also be of use for functional genomics studies of many of the other insect and arthropod species that harbor co-evolved microbiomes or intracellular symbionts.**

Research Community Impact

Justification for the Selection of Bees as the Focal Organisms

Honey bees (*Apis mellifera*) and bumble bees (*Bombus sp.*) are important pollinators in agriculture and in natural ecosystems that are facing threats to their health [1]. They are beset by environmental stressors (e.g., pesticides) and pathogens (e.g., *Varroa* mites) which, in honey bees, contribute to sudden colony collapse and high mortality in overwintering hives [2,3]. Bumble bees are wild species that are also important pollinators and undergoing population declines, with some approaching extinction [4]. In addition to their important ecological roles, bees have been a key insect system for studying the evolution of sociality [5,6], life-history transitions [7,8], animal communication [9], and memory and learning [10] for decades. The bee genome was completed in 2006 [11] and was recently upgraded to a chromosome level assembly [12]. *Yet, this resource has not enabled direct tests of gene function, for reasons detailed below.*

Bottlenecks to Answering Functional Genomics Questions in Bees

Honey bees, and to a lesser extent bumble bees, pose difficulties for standard genetics approaches because the reproductive unit is a queen supported by an entire hive. Thus, controlled genetic crosses are difficult. Though it is possible to create mini-nucleus honey bee colonies with "only" a few hundred workers, these are cumbersome to maintain and do not fully replicate aspects of bee biology (e.g., behavior) found in full hives. Thus, bee research has generally relied on natural polymorphisms that have developed from bee breeding or analogies to other insect species to understand gene function.

No causality from gene expression and genetic association studies. Transcriptomics has been used to discover differential gene expression (DGE) patterns in the brains of bees related to social behavior [13], to investigate developmental responses (e.g., changes in insulin signaling in response to diet and age) [14], and to study immune responses to pathogens (e.g., chalkbrood) [15]. *However, DGE studies are limited to discovering correlations and not causality.* For example, an age-related transition in honey bee foraging behavior is associated with increased expression of the *foraging* gene, and *Drosophila* mutants show a related phenotype, but this function has never been directly tested in *A. mellifera* [8]. In another case, DGE associated with aggression, resilience against pesticides, and immune responses was found by comparing bees reared in different hives [16], but the contributions of different genes to these responses is unknown. Recently, low sequencing costs have also enabled genome-wide association (GWA) studies aimed at linking particular genomic loci with traits, such as so-called hygienic behavior, in which worker bees clean hives of dead larvae and debris, reducing parasite loads [17]. *On their own, DGE and GWA studies only generate candidate loci and cannot verify causality; new approaches would empower these research efforts.*

***Genome editing of bees faces unique challenges*.** RNAi-mediated gene knockdown and genome editing via engineered transposons or CRISPR/Cas9 are common approaches for functional genomics studies in insects [18]. These technologies have only seen sporadic success in bees. A major obstacle to using genome editing (transgenesis) for studies of bee gene function is the need to rear queens and then give them colonies (hives) for proper development and genetic isolation of the next generation. Gene knock-in has been reported with 20% efficiency of editing *A. mellifera* males using *piggyBac* transposons [19]. Targeted gene knockout using CRISPR-Cas9 cleavage and error-prone repair by non-homologous end joining has also been reported [20]. However, both procedures are resource intensive and have not been used by other labs to our knowledge. They require tracking multiple mosaic queens placed in their own hives in flight rooms and then mating queens twice over two generations with mutant haploid males to achieve a mixture of heterozygous and homozygous workers. *Tools that could assess gene function directly in worker bees would allow much more rapid, flexible, and scalable studies of bee function than genome editing methods, as tens of thousands of these genetically uniform workers are present per honey bee hive.*

Injecting or feeding dsRNA can silence bee genes but has major limitations. Bees have an RNAi response as part of their innate immune system [21,22]. RNAi in honey bees can be induced by feeding or injecting purified dsRNA [23,24]. Feeding of dsRNA has also been shown to protect honey bees against arthropod pathogens (*Varroa* mites) and viruses [25–27], including in field trials against Israeli acute paralysis virus [28]. RNAi induction is systemic in honey bees [29], meaning that gene knockdown occurs throughout the insect body, and not just around the gut, at an injection site, or in a subset of tissues, as is the case in some insects [30–32]. As with other insects [30,33], the efficacy of RNAi varies widely between

different methods of delivery. Injection is more effective overall, but it is tedious and causes a traumatic wound that complicates interpreting the results of an experiment. Feeding is generally less effective because dsRNA must survive nucleases present in the insect gut [34]. Both types of treatments rely on expensive and finicky production of dsRNAs by *in vitro* transcription, must contend with the chemical instability of RNA before it is administered, and only induce a transient response after treatment. *These limitations have prevented the widespread use of RNAi for functional genomics studies of bees.*

How the Bottlenecks Will Be Addressed

We will engineer microbes that colonize insects to continuously produce and deliver dsRNAs to alter host gene function by inducing a robust RNAi response. We will apply our platform technology for **FUnctional Genomics Using Engineered Symbionts (FUGUES)** to bees in the current proposal. We recently showed that engineering the highly abundant, co-evolved bee gut symbiont *Snodgrasella alvi* to express dsRNA induces robust and persistent silencing of bee genes and can also be used to protect bees against viruses and *Varroa* mites [35] (see **Preliminary Data**). We expect that the FUGUES approach will be generalizable to other microbes associated with other arthropod hosts and the production of chemicals and biomolecules other than dsRNAs that can impact host gene function and phenotypes. The genetic tools and experimental protocols that we develop in this project will inform employing the FUGUES approach to other host species.

How Scientific Progress Will be Accelerated

Due to the ease of engineering bacterial plasmids and genomes compared to the insect germline, FUGUES enables more rapid and higher-throughput tests of gene function than insect genome editing. Our approach significantly reduces the overhead and infrastructure needed for these experiments in bees, as one does not need to create and then maintain transgenic hives/lines to employ the technique. In fact, the genetic relatedness of worker bees in a hive and their large number actually become a boon for genetic studies. For example, one could realistically construct libraries of bacterial strains designed to knock down hundreds to thousands of different bee genes and then screen the effects of each one on a phenotype or biological process of interest by feeding each strain to a set of multiple co-housed bees. One can even imagine new and powerful experimental designs, such as testing knockdown of different genes in different individuals co-housed in one colony to disentangle the complex environmental factors in a hive from its genetics.

Also, since our FUGUES approach can be applied to many other arthropod and insect species, it will complement existing EDGE awards focused on germline editing of arthropods. J. Rasgon and collaborators (NSF EDGE 1645331) are developing approaches for gene editing of arthropod eggs or embryos, using small peptide ligands that target germline cells to deliver CRISPR/Cas9. Some success has been reported for mosquito, parasitoid wasp, and whitefly species [36–39]. Benefits of their technology include the stability of germline-edited individuals and flexibility to create mutation types other than loss-of-function. However, our FUGUES approach is more useful for rapidly altering many insects for large-scale functional genomics screens. Also, by colonizing with the engineered symbionts later in life, FUGUES enables functional studies of genes whose knockout would be lethal or have pleiotropic effects in embryos or juveniles. Finally, its implementation requires less equipment (e.g., no microinjection) and expertise. Thus, *FUGUES offers an alternative means of interrogating arthropod gene function and complements other funded EDGE projects.*

Research Communities That Will Benefit

Bees are widely studied. They are a central model for behavioral scientists, who study learning [40], developmental shifts associated with roles in the hive, communication [41,42], behaviors with parallels to human behavioral disorders (e.g., autism [43]), and the interplay of genes and environment underlying behavioral variation [44]. Honey bees are also intensively investigated by chemical ecologists, insect physiologists, and developmental biologists; and by agricultural researchers working to improve pollinator efficacy. Many such researchers have identified candidate genes for particular processes. To give just a few examples: the SNARE protein gene *syntaxin* has been hypothesized to be key for sociality in bees [45]; a slate of genes have been proposed as candidates for detoxification of insecticides and other toxins in the bee gut [46–48]; AmOAR (an octopamine receptor) is hypothesized to be required for learning and remembering olfactory stimuli associated with sugar rewards [49]; and several candidate genes were recently proposed to underlie shifts in behavioral tasks performed by bees within a hive [50]. Research on honey bees is increasing even more recently, in response to colony declines that have been severe during the last decade.

Scientific Gaps and Scientific Questions That Can Be Addressed with FUGUES

Rather than editing the DNA of unborn progeny, FUGUES delivers persistent knockdown of genes in adult insects, following normal (wild-type) development. For social bees specifically, this has the advantage of eliminating the cumbersome step of injecting queens in order to obtain edited worker progeny, an approach that is very challenging given the eusocial bee reproductive system. FUGUES can be employed to alter gene expression in a cohort of individuals—without entirely eliminating function—to study genes that are essential, such as those in innate immune pathways or underlying neurotransmitter delivery. FUGUES also allows gene function to be studied at specific life cycle phases (adult stages). Most candidate gene targets are of interest specifically in adult workers, as these are the focus of most honey bee and bumble bee research, including behavioral, developmental, ecological, and toxicology investigations.

Social behavior and communication. Honey bees are among the most social of animal species, with sophisticated mechanisms for communication, including the waggle dance for communicating the locations of resources; alarm pheromone for signaling and responding to danger; and queen pheromone and brood pheromone for indicating reproductive status of the hive and nutritional needs of larvae, nestmate recognition for maintaining colony identity, and other adaptations. This inter-individual communication is interwoven with reproductive and developmental controls mediated by hormones. Researchers have been intensely interested in identifying the functions of genes underlying these processes. However, despite long-standing and extensive research programs using bees as a model, gene functional assignments remain minimal, due to the difficulties presented by the honey bee life cycle for performing standard genetics.

Learning and cognition. Bees are a model for learning, as they can retain spatial and olfactory memory for long periods [51], and brain regions underlying memory have been experimentally identified [52,53]. Despite the history of outstanding neurobiology and behavioral research on honey bee cognition, the genetic underpinnings are largely unknown. For example, proteomic work has identified hundreds of brain neuropeptides predicted from the genome sequence, but their functions remain largely undetermined [54].

Development and metabolism. Adult worker bees go through a succession of developmental stages characterized by different behaviors within the hive. These behaviors are modulated by hormones and are accompanied by shifts in expression of a large number of genes [55]. These interactions are mediated by social interactions enabling coordination between the social environment and worker tasks in the hive [56]. There is some genetic variation in the tendency to perform different behavioral tasks [57]. Researchers would also like to understand the foraging behavior of bees, but no efficient tools are available for testing the functions of the 170 odorant receptor genes in the honey bee genome [58]. Related to foraging is the developmental, reproductive, and aging program of bees that can be manipulated via hormones [59,60]. A study on reproductive parasitism, in which normally sterile worker females asexually produce viable eggs, used molecular evolution analyses to identify a set of candidate genes, including genes underlying the production of juvenile hormone and dopamine and genes encoding proteins that localize to centromeres and likely regulate chromosome segregation during meiosis [61], but their functions need to be tested.

Bee health and toxicology. Bees encounter diverse challenges in their environments. Understanding how they succeed or fail to meet these challenges is of intense interest in efforts to improve bee health. Among these environmental stressors are agrochemicals, such as pesticides [62]. Numerous bee genes are activated in response to pesticides (e.g., imidacloprid) [47], but the functions of these genes are unknown. Potentially, they provide protection to bees and might be induced before exposure or to higher levels to better protect them. Further, there is an interaction between toxins and bee cognition, as studies show that a major basis for harmful effects of insecticides is an impact on learning and memory, causing bees to lose their way back to hives and thus to die [63–65]. Immune system function in response to bee pathogens, such as Israeli acute paralysis virus and deformed wing virus, is implicated in colony collapse disorder.

Innate immunity. Innate immune pathways were originally genetically characterized in *Drosophila melanogaster*, and gene functional roles are best established for that species. Honey bees and bumble bees harbor orthologs of most genes in these pathways, but some differences are apparent, including, in honey bees, an activation of the Toll-pathway in response to Gram-negative bacteria and some differences in signaling genes [66]. Functionally characterizing bee innate immune genes would give insight into how immune pathways vary during evolution and could be key to developing strategies to improve bee health.

Impediments the Communities May Face in Employing FUGUES

Most bee researchers involved in behavioral, nutritional, toxicology, or ecological research are not also microbiologists or necessarily even molecular biologists. We will disseminate protocols and materials that we have tested in an undergraduate experiential learning environment, so that we can be sure that researchers with diverse backgrounds can readily use FUGUES to study their genes of interest. Some impediments can be overcome by simplifying the equipment needs for growing these bacteria and creating strains with known mechanisms and rates of bee-to-bee transmission. Other barriers to entry can be made less intimidating by creating FUGUES control strains that induce visual or behavioral bee phenotypes that are easy to assay as positive and internal controls for gene knockdown. Finally, we will also create and disseminate a library of FUGUES strains with pre-made and tested genetic constructs targeting widely studied bee genes to relieve many researchers of the burden of needing to construct plasmids.

Experimental Approach

Our overall goal is to create a straightforward and effective procedure for employing symbionts in the service of functional genomics. We will optimize and improve the usability of this FUGUES approach in honey bees and bumble bees in this proposal, but the methodology will be applicable to many other insects and arthropods that have gut-associated and/or vertically inherited bacterial symbionts, especially those that can be cultured, engineered, and reintroduced into animals.

Review of Relevant Literature

Bee gut microbiome. Honey bees and bumble bees have a characteristic community of gut symbionts that are specialized to their diets of pollen and nectar [67,68]. Bacterial strains within each of these core species have co-evolved with and are largely restricted to their host species (e.g., *S. alvi* from honey bees generally cannot colonize bumble bees and vice versa) [69,70]. Representatives of all of the major species have been cultured and their localization within the gut is known [71]. These bacterial symbionts are important for bee health. Bees without a microbiota are developmentally abnormal, lack appetite, and fail to gain weight normally [72]. Bees with a gut microbiota depleted due to antibiotic exposure experience higher mortality in hives [73], and the microbiota also protects bees from intestinal pathogens [74] and from parasites [75].

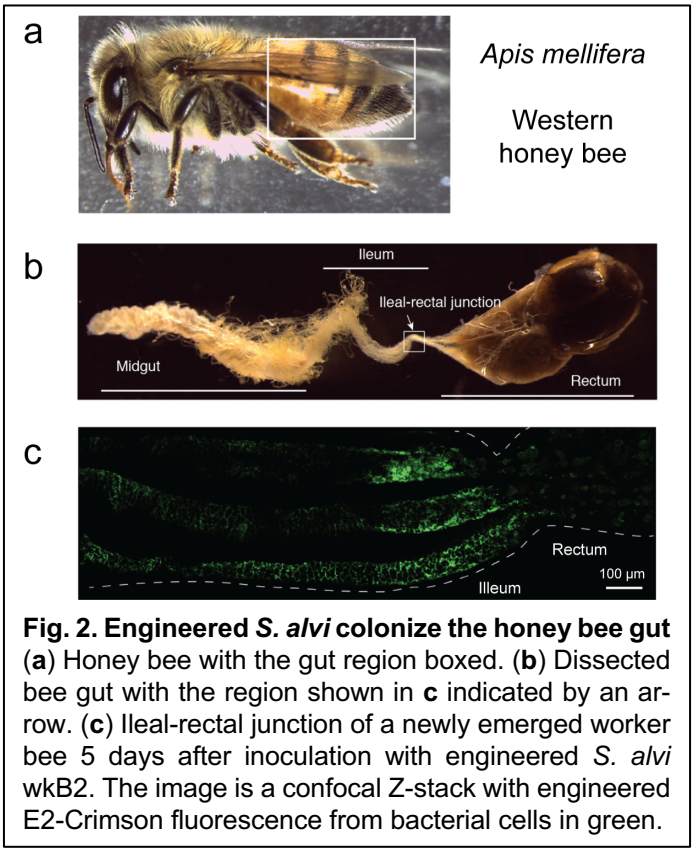

Fig. 2. Engineered *S. alvi* colonize the honey bee gut (**a**) Honey bee with the gut region boxed. (**b**) Dissected bee gut with the region shown in **c** indicated by an arrow. (**c**) Ileal-rectal junction of a newly emerged worker bee 5 days after inoculation with engineered *S. alvi* wkB2. The image is a confocal Z-stack with engineered E2-Crimson fluorescence from bacterial cells in green.

***Genetic engineering of insect symbionts*.** Synthetic biology tools are improving to engineer diverse bacterial species that can be cultured or to engineer them *in situ* when they cannot be cultured [76]. *Sodalis* is a group of insect symbionts that has been engineered in several ways, including addition of plasmids expressing markers, and knock out and complementation of genes affecting establishment of symbiosis [77]. Engineered *Sodalis* strains can be reestablished in hosts for biological experiments [78]. Other insect symbionts in which plasmids bearing fluorescent markers have been introduced include *Arsenophonus* [79] and *Asaia* [80]. *Serratia marcescens* AS1 can be fed to mosquitos and is vertically inherited. It has been engineered to impact the malarial parasite [81]. However, much of this work is in its infancy compared to toolkits developed for engineering common human gut bacterial taxa such as the *Bacteroidetes* [82,83]. In bees, genetic engineering of *Lactobacillus kunkeei* has been proposed for improving bee health and studying gene functions, but the efficacy of this approach has never been shown [84,85]. *L. kunkeei* does not typically

colonize the bee gut and is mostly an environmental species present in the hive and nectar [67], so this organism seems unlikely to be a promising platform for altering bee biology.

Symbiont-mediated RNAi. Microbial genetic engineering has recently been combined with RNAi to impact insect host gene expression. One way of implementing this is to engineer a microbe (e.g., *E. coli*) to produce dsRNA and then feed it to a host in lieu of directly administering dsRNA [86]. Symbiont-mediated RNAi in which one engineers a microbe that colonizes the body of the host insect has the potential to continuously deliver RNAi for stronger and more persistent knockdown [87]. These approaches have primarily been developed for pest control, but they are attractive for functional genomics because one can tailor the dsRNA sequence to silence a specific insect gene of interest to study its function. Symbiont-mediated RNAi has been used in *Rhodnius prolixus* (kissing bug) and *Frankliniella occidentalis* (western flower thrips) [88]. We have established that engineering native coevolved gut symbionts to produce dsRNA is extremely effective in *A. mellifera*, as described below.

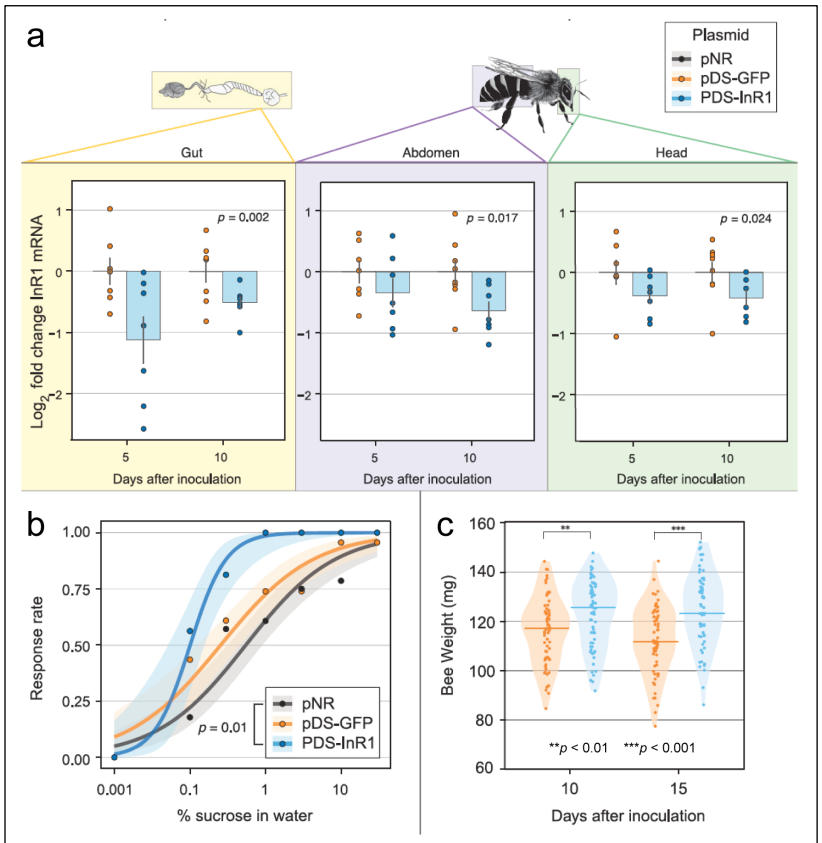

Fig. 3. Symbiont-mediated RNAi affects bee gene expression and behavior. (**a**) Bees colonized with engineered *S. alvi* wkB2 expressing *InR1*-targeting dsRNA show reduced expression of *InR1* compared to the off-target control (pDS-GFP). Knockdown occurs in other regions of the bee body, not just in the gut (*p*-values for dsRNA plasmid effect in a two-way ANOVA). (**b**) These bees are more responsive to sugar solutions at lower concentrations in the proboscis extension response assay (*p*-value for dsRNA plasmid effect in a binomial mixed-effects generalized linear model). (**c**) They also show increased weight gain (*p*-values for Mann-Whitney U-tests).

Preliminary Studies

Study 1. Stable emplacement of engineered *Snodgrassella alvi* in the bee gut. We developed a broad-host-range bee microbiome toolkit (BTK) of genetic constructs and used it to engineer several bacterial species native to the honey bee gut [89]. *Snodgrassella alvi* wkB2 is found in high abundance in the ileum along the epithelial wall. By expressing a fluorescent protein from a plasmid we can visualize *S. alvi* colonization (**Fig. 2**). When we inoculate newly emerged worker bees that are naturally microbiota-free, engineered *S. alvi* reliably colonizes to a titer of ~10^7 CFU per bee after 5 days. When bees are reared on a sucrose solution containing antibiotic, expression of the fluorescent protein is stable in the gut for at least 15 days.

Study 2. Knockdown of bee genes using symbionts that express dsRNA. We have shown that honey bees colonized with *S. alvi* wkB2 expressing dsRNA targeting an insulin receptor (*InR1*) exhibit systemic knockdown of this gene, affecting at least the gut, abdomen, and head (**Fig. 3a**) [35]. Bees treated with these bacteria also exhibited expected phenotypes: greater weight gain (**Fig. 3b**) and increased sensitivity to sucrose (**Fig. 3c**) [35]. In unpublished experiments, we have used this same symbiont-mediated RNAi approach to knock down DOPA metabolism enzymes (tyrosine hydroxylase, DOPA decarboxylase). We observed reduced expression of these genes in the head and the expected impairment of aversive learning.

Study 3. Colonization of bumble bees with co-evolved *S. alvi* strain wkB12. The wkB2 strain of *S. alvi* used for symbiont-mediated RNAi of honey bees does not effectively colonize bumble bees (*Bombus impatiens*) [70]. However, other *S. alvi* strains are consistently found in the guts of bumble bees and in most species of stingless bees (tribe Meliponini). As expected, we find that *S. alvi* isolates from *Bombus* spp., such as wkB12, are capable of colonizing their cognate bee hosts. Furthermore, *S. alvi* wkB12 can be engineered using the same toolkit we used for wkB2 [89]. Therefore, we expect that other *S. alvi* can be engineered and used to implement symbiont-mediated RNAi in a wide variety of bee species.

Proposed Studies

Overview. Objective 1 addresses the core genetic tools used to implement FUGUES. These tools will also be useful for engineering symbionts and microbiomes of other organisms, especially for producing dsRNA to induce gene silencing in insect hosts. **Objective 2** comprises studies that create controls and optimize methods for using FUGUES to silence bee genes. **Objective 3** addresses disseminating this approach to bee research communities. It integrates the development and testing of FUGUES with undergraduate experiential learning streams that create DNA constructs and tutorials to share with researchers.

Objective 1: Improve core tools for applying FUGUES to any organism

We first propose improvements to FUGUES aimed at reducing its reliance on costly and cumbersome methods and at optimizing its power and stability within a host organism (**Fig. 4**). While we focus on implementing symbiont-mediated RNAi, these genetic platforms could also be used to engineer symbionts to affect their hosts in other ways (e.g., producing enzymes, peptides, or small molecules).

A. Simplify cloning and eliminate reliance on antibiotics

Rationale. The current broad-host range symbiont-mediated dsRNA expression system based on the BTK requires complex DNA assembly steps and feeding bees antibiotics to select for plasmid maintenance. We need to simplify cloning of dsRNA sequences and develop alternative genetic platforms to obviate the need for administering antibiotics. These changes will ensure FUGUES is effective for longer-term experiments.

Experimental design. First, we will expand and simplify our BTK system of plasmids compatible with Golden Gate assembly (GGA) [89]. GGA avoids PCR steps that have trouble with the inverted promoters used to express dsRNA. We will construct new GGA dsRNA expression 'dropout vectors' with the RSF1010 origin to make the successful cloning of a targeted bee gene sequence directly visible because it leads to loss of a fluorescent protein stuffer sequence [90]. We will also construct GGA part plasmids that make it possible to create and test multi-dsRNA expression cassettes with all dsRNA sequences between one promoter pair or such that each one has its own promoter pair. Second, we will develop vectors for integrating DNA constructs into the chromosome via conjugation and either site-specific Tn*7* transposition or non-specific Tn*5* transposition [91,92]. Generally, integration is most useful for fluorescent protein (FP) genes that can be used to monitor successful colonization of the insect host but need only be expressed at a low level to be visible. Genomic integration also obviates the need for administering antibiotics to select for plasmid maintenance. We will also test whether integrating dsRNA expression cassettes in the chromosome is sufficient for effective bee gene knockdown (via qPCR or bee phenotypes such as those shown in the **Preliminary Data** or developed in **Objective 2A**). Third, we will test adding the auxotrophic and counterselectable gene *pyrF* [93] to the broad-host-range RSF1010 plasmid as an alternative to using antibiotic selection for stabilizing this high-level dsRNA expression configuration that we already know is effective.

Expected results. We expect to develop a coordinated set of broad-host-range vectors and genetic parts for GGA that enables researchers to add combinations of FPs and multiple dsRNA expression cassettes to *S. alvi* or other bacteria, either integrated into their chromosomes or maintained on plasmids. We will eliminate the need for adding antibiotics to select for retention of dsRNA expression.

Potential pitfalls and alternative approaches. New methods for site-specific integration, using CRISPR-targeted Tn*7* transposons for example [94,95], could also be adopted as these technologies mature. Complementation of amino acid (AA) auxotrophy is a backup approach to using *pyrF* for antibiotic-free plasmid maintenance. Fourteen genes can be knocked out to give *E. coli* auxotrophies with robust growth restored when a single AA is added to media [96], and many of these genes are important for fitness of *S. alvi* in the bee gut based on a Tn-Seq study we conducted [97]. Therefore, it is likely that we could use AA

antimetabolites to select for loss-of-function mutations in specific genes in AA biosynthesis pathways (e.g. aminoethylcysteine for lysine), which is important because the bee gut bacteria can only be grown in rich nutrient broth in which there may not be a strong fitness cost for loss of AA synthesis. Then, we can complement the auxotrophy by adding an active copy of the mutated gene to the RSF1010 plasmid.

B. Engineer systems for increasing and controlling dsRNA production

Rationale. We can improve FUGUES symbionts to make them more likely to induce a strong RNAi gene silencing response and also engineer them to first colonize the bee and then induce dsRNA expression only at a specific time during an experiment. The *E. coli* lab strain HT115 provides a partial roadmap [98]. It contains an inducible T7 RNA polymerase in the genome and has RNAse III inactivated to prevent degradation of dsRNA within the bacterial cell. Knocking out RNase III has also proven effective for improving symbiont-mediated RNAi in other insect symbionts [88].

Experimental design. To stabilize RNAs that induce RNAi, we will (1) attempt to disrupt RNase III (the *rnc* gene) in *S. alvi* wkB2 by using Cas9-assisted allelic exchange to integrate an antibiotic resistance gene in its place [89]; and (2) test co-expressing a plant tombusvirus p19 protein, which binds to and stabilizes accumulation of ~21-bp siRNA-like products of RNase III degradation that induce RNAi [99,100]. We will monitor improvements by separating total RNA from cultured *S. alvi*. By separating and staining on gels, we can visualize both the originally transcribed dsRNAs that are hundreds of bp in size and the ~21-bp cleavage products [99]. To validate that improvements in RNase III mutants persist in bacteria colonizing bee guts, we will use RT-qPCR (normalized to *S. alvi* 16S RNA copy number). In both cases, we will examine whether the new hosts/vectors improve gene knockdown as described in **Objective 2A**.

To improve and control dsRNA production, we will test: (1) screening native *S. alvi* promoters from >20 operons that are highly expressed in an existing RNAseq dataset [97]; (2) integrating T7 RNA polymerase into the *S. alvi* genome via methods described in **Objective 1A**; and (3) optimizing IPTG-inducible (LacI) systems for regulation that showed reasonable induction in our prior work [87] and testing arabinose-inducible (AraC) systems [101]. We will initially test the relevant promoters and regulators cloned into our RSF1010-based vector to drive FP expression, using flow cytometry to measure their relative expression per cell. Then, we will test using the promoters to drive dsRNA production as described above. We will also determine whether potential inducer compounds are toxic to bees, by measuring survival curves on cohorts of exposed bees, and whether feeding can activate these promoters in the bee gut by monitoring induction of the FP on a plasmid in *S. alvi* that constitutively express a different FP from the chromosome.

Expected results. We expect to increase dsRNA/siRNA by a factor of ≥10× through a combination of one or more of these approaches. We also expect to implement temporal control, such that silencing of a target gene is triggered and sustained by feeding bees a chemical inducer.

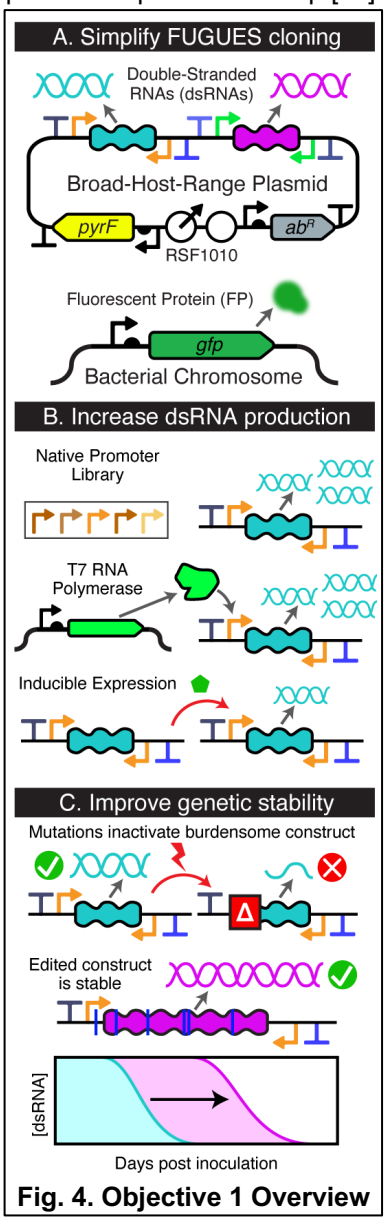

Fig. 4. Objective 1 Overview

Potential pitfalls and alternative approaches. RNase III function is essential to some bacteria; knockouts can be inviable or have compromised fitness [102–104]. If we cannot create an *S. alvi* Δ*rnc* mutant or find that it is defective for FUGUES (e.g., cannot robustly colonize bees), we will test whether we can permanently or transiently reduce RNase III expression to increase dsRNA accumulation. We can pilot this alternative approach using a dCas9-based CRISPR interference (CRISPRi) plasmid we developed that uses the same RSF1010 backbone as our other FUGUES constructs [105], optionally integrated with chemical induction of dCas9 expression. If these results are promising, we will follow up by constructing and testing hypomorphic *rnc* alleles (e.g., with promoter or catalytic mutations).

We note that *S. alvi* with wild-type *rnc* already delivers sufficient dsRNA to knock down bee genes, so these efforts to improve efficacy are not essential for our general approach.

C. Improve the genetic stability of FUGUES constructs

Rationale. Genetically engineering bacteria often reduces their fitness. It may compromise their ability to robustly colonize a host organism and lead to strong selection for mutations that "break" engineered functions such as dsRNA production. Stochastic failure of FUGUES constructs due to evolution would hamper the adoption and the reproducibility of gene function studies. Therefore, we need to characterize the burden of our plasmids and FP/dsRNA expression constructs and then implement methods to reduce the chances of evolutionary failure if this is a significant problem.

Experimental design. We will measure the burden of FP/dsRNA expression using an *S. alvi* strain with a GFP gene integrated into its chromosome as a 'burden monitor' [35]. Using a microplate reader, we will determine if these constructs reduce maximum exponential growth rates *in vitro* (one measure of fitness). We can ascertain if burden is due to a construct sequestering gene expression capacity (e.g., ribosomes) away from cellular replication versus using other limiting factors (e.g., metabolites) or producing a toxin by monitoring the correlation between GFP production (a proxy for native gene expression) and growth rate [106]. RNAi gene silencing studies in insects often use dsRNAs that are a few hundred base pairs in length, but longer constructs may be more powerful [30]. One barrier to producing longer dsRNAs in bacteria may be a cost from "cryptic" translation. The Barrick lab has shown in other systems that spurious transcription/translation of eukaryotic sequences can greatly burden bacteria. Using software (CryptKeeper) developed in these projects, we will computationally screen targeted bee gene exon sequences to choose those with low potential for cryptic translation. Our aim is to increase the success rates of dsRNA cloning and the sizes of constructs that can be stably maintained in *S. alvi* by avoiding burden. We will test, in turn, whether longer (and now cloneable) dsRNA constructs improve RNAi silencing (see **Objective 2A** for methods).

The use of inverted promoters for dsRNA expression is common in bacterial plasmids, and it is what we propose in **Objective 1A**, but these types of repeats may lead to mutational hotspots in DNA [107]. We have also previously seen the evolution of 'satellite plasmids' that act as molecular parasites from the main RSF1010 vectors used in the BTK under some conditions [108]. To more generally understand whether these and other failure modes limit plasmid stability, we will propagate FP/dsRNA expression plasmids in *S. alvi* cultured *in vitro* and *in vivo* and deep sequence plasmids isolated from these cell populations using unique molecular identifier error-correction methods that can detect mutations at frequencies < 0.01% from Illumina data [109]. We will determine what types of mutations and what parts of the plasmid are responsible for failure most often (e.g., as we have in [110]). This information will be used to redesign part sequences and the overall layout of these plasmids, for example creating promoters that have balanced expression—but distinct sequences that cannot recombine—for expressing dsRNA, to achieve the necessary stability.

Expected results. As the main outcome, we expect to be able to reliably clone dsRNA expression plasmids that target any bee gene and ensure that they are stable for ≥30 days in *S. alvi* in the bee gut, long enough to conduct most studies of bee gene function, as this is roughly the maximum lifespan of adult bees in the lab. If burden from cryptic translation limited what dsRNA expression plasmids were clonable in the past, we expect to also be able to more highly express longer dsRNAs to achieve stronger gene knockdown.

Potential pitfalls and alternative approaches. *S. alvi* genomes encode many insertion sequences (e.g., 23 in strain wkB2 [70]) that can be a major source of failure mutations in bacteria [111,112]. If these transposable elements are responsible for instability, we will test employing our CRISPRi system (see **Objective 1B**) to silence their activity, as we have used successfully for increasing the stability of constructs in *E. coli* and *Acinetobacter baylyi* [105]. Another option for managing burden we can implement, if necessary, is a feedback-based genetic circuit in which dsRNA is produced by a native or synthetic promoter that is down-regulated when cell growth decreases, so that dsRNA is produced up to the highest stable level [113].

Objective 2: Optimize FUGUES for studying bee gene function

Introduction. While we have shown that a first-generation FUGUES design functions in honey bees, achieving widespread adoption of this new technology requires more robust workflows. For example, we need to supply reagents for simple positive controls and understand how FUGUES operates in different experimental setups. In this objective we will optimize gene silencing efficiency specifically in honey bees

(**Fig. 5**). We will also characterize and, if necessary, develop ways of controlling how engineered *S. alvi* are transmitted between co-housed bees.

A. Establish colorimetric and behavioral assays for assessing gene silencing in bees

Rationale. Developing straightforward ways to read out the effectiveness of FUGUES will accelerate our ability to further improve this technology. Targeting bee phenotypes that are easily assayed will also let us create reliable positive/internal controls for when we disseminate our kit to the research community. We will target conserved insect pathways so that the same gene knockdowns and phenotypic assays can be readily used to test FUGUES in other insect species in the future.

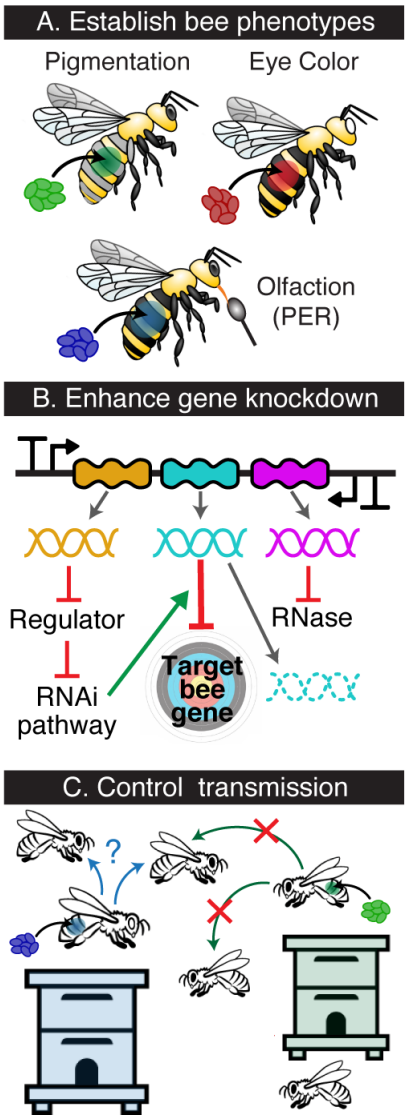

Fig. 5. Objective 2 Overview

Experimental design. We will target three phenotypes:

• ***Body pigmentation.*** Melanin is the primary black pigment of insects, and forms the warning coloration on the bee abdomen [114]. By silencing orthologs of conserved insect genes that synthesize melanin through oxidizing and polymerizing tyrosine or by redirecting melanin precursors toward alternative sclerotization pathways that result in lighter coloration, we expect to change the appearance of adult bees, as has been achieved via dsRNA injection in other insects [115,116]. We will target single-copy genes early in these pathways, including phenoloxidase (*PPO*) [117], tyrosine hydroxylase (*TyHyd*), DOPA decarboxylase (*LOC410638*), dopamine *N*-acetyltransferase (*LOC409361*), *N*-β-alanyldopamine synthase (*LOC724495*), and β-alanyl-dopamine hydrolase (*LOC408592*). We targeted TyHyd with FUGUES in a preliminary experiment aimed at altering bee learning and, anecdotally, observed the expected darker pigmentation. To judge success, we will use RT-qPCR to validate that there is effective gene knockdown with the dsRNA constructs we design, visually score changes in bee coloration, and finally measure levels of eumelanin and pheomelanin in specific bee body parts by HPLC with UV detection [118]. We can also measure PPO enzyme activity in hemolymph with a simple colorimetric assay [119].

• ***Eye color.*** Ommochromes are eye pigments that are synthesized from tryptophan [120]. Mutations that alter bee eye color are known to disrupt steps in ommochrome synthesis [121,122], and knockout of orthologous genes in other insects changes eye color [120,123]. We will use FUGUES to target bee orthologs of the *Drosophila* genes *vermilion* (tryptophan 2,3-dioxygenase, *LOC410828*), *cinnabar* (kynurenine 3-monooxygenase, *LOC551858*), and *white* (ABC-transporter subunit for ommochrome and pteridine precursors, *LOC726508*). After scoring eye color next to controls under a microscope, we will again use RT-qPCR for validation. We can quantify ommochrome modification directly by dissecting eyes and using HPLC with UV detection [124], if necessary.

• ***Chemosensing.*** ORCO is a highly conserved protein coreceptor of odorant receptor (OR) function that is commonly targeted for gene silencing in insects [125]. Reducing or knocking out ORCO expression, including by ingestion/injection of dsRNA, changes feeding and other behaviors in diverse species [126–131], many of which can be relatively easily assayed. FUGUES targeted to the bee insulin receptor (*InR1*) resulted in altered feeding behavior, as measured by the proboscis extension assay (PER) (see **Preliminary Data**). We will knock down ORCO orthologs in *A. mellifera* (AmelR2) [132]. and monitor knockdown using the PER. While behavioral assays are not as simple as visual inspection, performing the PER on bees is straightforward, and does not require expensive equipment [133]. We will also test using a newer variant of the PER (the Free-Moving Proboscis Extension Response) that does not require harnessing bees and can be used to test for changes in feeding preference [134].

Expected results. These experiments will provide us with (i) an improved platform for rigorously testing improvements in induction of bee RNAi by symbionts (for **Objectives 1** and **2**) and (ii) one or more non-lethal phenotypes that can be scored by non-experts who are testing the system for the first time as a positive control before using it on their genes of interest (**Objective 3**). Ideally, they will also enable (iii) a researcher to co-express dsRNAs targeting this phenotype and their gene of interest in tandem in the same symbiont as an internal control showing that the FUGUES system is operational in each inoculated bee.

Potential pitfalls and alternative approaches. Silencing of some of these genes may have pleiotropic effects that will complicate using them as benchmarks and controls. For example, melanin synthesis by hemocytes has roles in innate immunity and wound healing [114,135–137], and bee pigmentation can be affected by non-genetic factors, including whether a bee is colonized by the gut bacterium *Frischella perrara* [138]. Silencing ORCO is expected to impair chemical communication and foraging, as it does in other insects [126]. While these strong phenotypes are appropriate for optimizing knockdown efficiencies, we will also want subtler phenotypic changes for internal controls that researchers can co-target with their gene of interest. Therefore, we expect to refine our search to look at subfunctionalized genes that act downstream within these processes in the later stages of the project. For example, we can test pigmentation genes that are likely to only be expressed in some tissues (e.g., among the eight copies of *yellow* in bees) [139,140] or specific chemoreceptors (e.g., the ten gustatory receptors of bees) [58].

B. Enhance knockdown efficiency by co-targeting bee nucleases and immunity genes

Rationale. Using FUGUES to co-target bee genes that affect dsRNA stability or the RNAi response may result in more potent suppression of a gene of interest. This approach is similar to treating insects with multiple dsRNAs to improve RNAi responses [141–143] and mimics the use of RNAi-sensitive *Caenorhabditis elegans* mutants [144–146]. In particular, most applications of FUGUES will rely on the systemic RNAi response, but the genes that mediate this response in bees are not well-characterized.

Experimental design. We will use the approaches of **Objective 1A** to engineer *S. alvi* to express multiple dsRNAs, co-targeting candidate enhancement genes and a gene with an easily assayed phenotype as described in **Objective 2A**. We will compare these constructs to similar ones containing off-target sequences in place of the candidate enhancement genes (e.g., *gfp*). We will score any relevant phenotype and quantify differences in gene knockdown as described in earlier sections, with the addition of testing the tissue specificity of changes by dissecting bees [35].

• ***Nucleases.*** Many insects express nucleases in their guts or other tissues that degrade dsRNA and thereby dampen the RNAi response [147]. A systematic search for similar nucleases has not been carried out in honey bees, but they do possess orthologs to ribonucleases targeted in studies that have improved RNAi responses in other insects [141–143]. We will target these nucleases (*LOC100577681*, *LOC551715*, *LOC408336*) one at a time and in combination with FUGUES.

• ***RNAi response.*** We may also be able to enhance the activity of bee RNAi by silencing negative regulators of this pathway. For example, in *Drosophila*, *dFOXO* positively regulates RNAi expression of RNAi pathway genes [148]. *dFOXO* is suppressed by *Akt* in *Drosophila*, and orthologs of Akt in honey bees include *LOC413430* and *LOC724607*. Therefore, suppressing these genes may upregulate FOXO and enhance the RNAi response in honey bees. ERI-1 is an exoribonuclease that negatively regulates RNAi in *C. elegans* [100], and there is a honey bee ortholog (*LOC100576286*). We will test co-targeting this gene and also genes mutated in other RNAi-sensitive *C. elegans* strains [146,149] that have orthologs in honey bees, including *eri-3* (*LOC102655994*), *eri-5* (*LOC727113*), *eri-9* (*LOC413825*), and *eri-12* (*LOC725024*).

Expected results. These experiments will reveal whether any bee genes can be suppressed to enhance the RNAi response to FUGUES. We expect to be able to incorporate co-expression of dsRNAs targeting one or more of the best targets into new versions of the default FUGUES genetic constructs to further improve the reliability and robustness of gene knockdown in bees.

Potential pitfalls and alternative approaches. The RNAi pathway is poorly understood in bees, compared to *Drosophila* and other model organisms, and it is unclear how dsRNA produced in the symbiont is transferred into bee cells. Despite the high conservation of RNAi pathway components across organisms, the immune pathways characterized in other organisms may differ in bees. Because FUGUES is a facile platform for screening gene function, especially when coupled to the visual phenotypes in **Objective 2A**, we

can relax the homology requirements to cast a wider net to test other immunity pathways and paralogs of the genes listed here if we see no appreciable improvements from co-targeting these top candidates.

C. Characterize and control transmission of engineered bee symbionts

Rationale. Much progress in understanding honey bee gene function can be made using our typical approach of colonizing newly emerged worker bees (NEWs) with engineered *S. alvi*. Our previous results show that engineered *S. alvi* can spread between co-housed bees in the laboratory [35], but we do not know if there are limitations that will prevent expanding the scope of bee FUGUES to social and hive-level phenotypes. Will engineered *S. alvi* remain stable in a bee's gut once it is exposed to bees with normal microbiomes? Is engineered *S. alvi* capable of colonizing those other bees? Can we engineer it to limit its spread? Do different *S. alvi* strains differ in these characteristics?

Experimental design. We will test the dissemination and persistence of *S. alvi* strains, with FPs and antibiotic resistance genes (e.g., $Spec^R$, Kan^R) integrated into their genomes, from colonized NEWs to other workers. We will co-house these bees with cohorts of bees that have a normal microbiota (emerged in the hive) or a conventionalized microbiota (emerged in the lab and exposed to gut homogenate from a normal bee). We will monitor engineered *S. alvi* transmission to other bees and persistence in inoculated bees by quantifying antibiotic resistant and fluorescent CFUs in guts of sacrificed bees of both types over time.

Next, we will use transposon insertion sequencing (Tn-Seq) to identify genes that are required for viability in quiescent *S. alvi* cells (by examining survival during stationary phase in the lab) and for transmission (by examining what mutants are passed to co-housed NEWs and which are not). We already have a Tn-Seq library for *S. alvi* and have used it to identify genes needed to colonize inoculated bees [97]. We will validate that a Tn-Seq depleted gene is involved in these processes and create less-transmissible *S. alvi* strains to use as chassis by knocking genes out via allelic exchange or repressing them via our existing broad-host-range CRISPRi system (see **Objective 1B**) [105].

We will further test transmission and newly created non-transmissible *S. alvi* mutants using experiments involving multiple mini-nucleus hives maintained in the lab. This experiment will be conducted using multiple *S. alvi* strains that have different fluorescent and antibiotic markers and will compare our restricted mutants to normal *S. alvi* head-to-head to collect as much information as possible. We will sample bees over time to determine whether these *S. alvi* strains are transmitted within one bee colony with a differentiated worker population and a queen, and also whether they are transmitted to other colonies.

Expected results. We will characterize the chances that engineered *S. alvi* will be transmitted to other bees, a critical piece of knowledge for planning experiments that will employ this technology to study bee function. We will also engineer alternative *S. alvi* strains that have a reduced chance of spreading to other co-housed bees. These studies are a step toward an eventual long-term goal (outside of the scope of this proposal) of understanding whether FUGUES can be safely used in bees living in hives outside of the lab.

Potential pitfalls and alternative approaches. The Moran lab has conducted many studies of bee microbiome resilience and transmission, so we do not anticipate problems with characterizing these processes. The Tn-Seq experiments testing transmission between bees may lose power due to random loss of mutants depending on the size of this bottleneck. If they cannot identify candidate genes to target, we will fall back to knocking out genes encoding Type IV secretion system effectors (*rhs* toxins) that appear to mediate competition between *S. alvi* strains [150] and conserved genes needed by many bacteria for survival of long-term quiescence such as σ^S, 6S RNA, and ribosome hibernation factors [151,152], to attempt to rationally create S. *alvi* strains that exhibit improved biocontainment properties.

Objective 3. Disseminate FUGUES resources for bee functional genomics

Introduction. A primary objective of our project is to share FUGUES tools for studying bee genes with the research community. We will integrate this goal with broader impacts and research training by leveraging Freshman Research Initiative (FRI) streams associated with the Barrick and Moran labs. FRI is a multi-semester experiential learning research course/lab for undergraduates, with a high rate of enrollment of underrepresented groups and a high rate of success in retaining students in STEM fields [153,154]. These students will develop and debug our protocols and initiate outreach to bee researchers.

A. Simplify engineering *S. alvi* to knock down expression of a target bee gene

Rationale. Culturing *S. alvi* requires a low-oxygen environment, and CO_2 incubators to maintain this atmosphere are not commonly available to bee researchers. Applying FUGUES for gene knockdown via RNAi also requires molecular biology and microbiology procedures that may be unfamiliar and expensive for some laboratories. We can improve dissemination of FUGUES for studying gene function by providing researchers with premade dsRNA-expressing *S. alvi* strains targeting their genes of interest.

Experimental design. We will use the **Microbe Hackers** FRI Stream (Mishler-Barrick) to simplify and debug these steps. The undergraduates will first characterize growth of *S. alvi* in different types of disposable CO_2 generation bags, unshaken flasks, and other environments that can reduce O_2 levels. Next, they will test and document the steps needed for cloning a fragment of a bee gene being targeted into a plasmid in *E. coli* and then conjugating it into *S. alvi* to transform it with a new construct (as described in **Objective 1A**). Critically, they will also describe how one chooses dsRNA sequences derived from a bee gene, such that they do not have off-target effects on expression of other bee genes, by using software such as E-RNAi [155], and such that they do not burden *S. alvi*, by using CryptKeeper (see **Objective 1C**).

Expected results. These FRI students will build a library of tens to hundreds of "ready-made" dsRNA-expressing *S. alvi* strains targeting important bee genes. They will select these based on reading the literature as part of their research experiences. The students will be required to contact the bee researchers who have written these papers, to improve their understanding of the research questions and as a way of letting the community know about FUGUES resources (see **Dissemination Plan**). The students will create video protocols covering dsRNA construct design, plasmid cloning, and transformation of *S. alvi*. We will integrate a Responsible Conduct of Research component into their training materials, since bee researchers need to be aware of proper guidelines for working with recombinant DNA and engineered microbes. Their protocols will be developed in a versioned GitHub repository and permanently archived.

Possible pitfalls and alternative approaches. The Moran lab will manage distributing FUGUES genetic resources for the duration of this project (see **Dissemination Plan**). Since we cannot maintain the resources indefinitely, we will also submit the plasmids/strains needed in these protocols and for specific sets bee genes to a non-profit plasmid/strain repository such as Addgene or ATCC. If we find that existing dsRNA design tools are not effective for bees, the Barrick lab has experience implementing and sharing computational tools [107,156] and will develop custom web-based software to remedy these deficiencies.

B. Achieve symbiont colonization and gene knockdown in additional bee species

Rationale. *S. alvi* is a core bee gut symbiont of honey bees, bumble bees, and some corbiculate bees. The proposed work described to this point has focused on *S. alvi* strain wkB2, which is specific to honey bees [70,157], but we can colonize bumble bees with *S. alvi* strain wkB12 that has co-evolved with this host insect species (see **Preliminary Data**). Extending FUGUES to bumble bee species is important for reaching additional research communities that study their unique adaptations and behaviors.

Experimental design. We will use the **Bugs in Bugs** FRI stream (Moran-Holley) to test gene silencing by FUGUES in *Bombus impatiens,* a native North American bumble bee species that is commercially available (due to its use in agricultural pollination). *B. impatiens* is commonly used to study learning, foraging, physiology, and social behavior. We have used established methods [158] for inoculating microbiota-free *B. impatiens* with *S. alvi* wkB12. These FRI students will apply them to *S. alvi* wkB12 that have been engineered by the Microbe Hackers FRI stream or other researchers on our team to express dsRNAs targeting bumble bee genes. They will test colonization dynamics (CFUs at different times) and measure gene silencing (using assays from **Objective 2A**). They will also test to what extent the timing of when microbiota-free bees are inoculated affects the efficacy of gene silencing, both in bumble bees and in honey bees, to flesh out our understanding of what parameters are crucial for effective studies of bee gene function.

Expected results. We expect to establish an alternative *S. alvi* strain that can be used as a platform for robust studies of bumble bee gene function using FUGUES. As in **Objective 3A**, the students in this FRI stream will create video protocols—for both honey bees and bumble bees—describing how to perform bee inoculations, monitor colonization, measure relevant phenotypes, and conduct molecular assays that confirm gene knockdown. We will also employ these students as ambassadors, having them contact bee labs

to understand their research questions and bridge the gap between development of our tools and their use in projects elsewhere (see **Dissemination Plan**).

Possible pitfalls and alternative approaches. If *S. alvi* wkB12 does not give robust bumble bee gene silencing, we will test other strains from the Moran lab's collection of *S. alvi* isolates (e.g., Pens 2-2-5, Snod 2-1-5) [70]. Like wkB12, we have shown that several of these strains can be engineered with the BTK [89]. The Bugs in Bugs stream also isolates bacterial symbionts from other insect species (e.g., they have characterized *Bifidobacterium* and *Orbaceae* strains from honey wasps and *Drosophila* populations near Austin). Depending on student interests, we will also guide them in experiments applying the FUGUES toolbox to symbionts that they culture from these insects, in conjunction with Microbe Hackers students.

Timeline

FUGUES Generation 1 Generation 2	Year 1				Year 2				Year 3				Year 4			
1A Simplify cloning and eliminate reliance on antibiotics																
1B Engineer systems for increasing and controlling dsRNA production																
1C Improve the genetic stability of FUGUES constructs																
2A Establish colorimetric and behavioral assays for assessing gene silencing in bees																
2B Enhance knockdown efficiency by co-targeting bee nucleases and immunity genes																
2C Characterize and control transmission of engineered bee symbionts																
3A Simplify engineering *S. alvi* to knock down expression of a target bee gene																
3B Achieve symbiont colonization and gene knockdown in additional bee species																

Our goal is to iterate through two main generations of FUGUES tools (blue and purple shading). We will immediately begin disseminating the current version of these tools with new documentation. Then over Years 2-3 we will test a majority of the proposed improvements. In Year 4, we will finalize and archive the updated tools and concentrate on testing and controlling transmission of the engineered symbionts.

Intellectual Merit

Genetic engineering of a microbe associated with a plant or animal to affect its phenotype is a promising approach due to the ease of manipulating bacterial genomes and creating large libraries of strains for perturbing different genes in a host organism. For superorganisms, such as bees, that may have thousands of individuals in a colony, our **FUnctional Genomics Using Engineered Symbionts (FUGUES)** method will fill a gap in functional genomics studies of these fascinating and important insects. Even in other arthropod species in which genome editing is possible, the simplicity and speed of FUGUES has advantages for large-scale studies of gene function. Thus, we expect the tools that we create and share in this project will be broadly enabling for the bee research community and studies of other organisms that lack genetic tools.

Broader Impacts

Honey bees, bumble bees, and other bee species are critical pollinators in natural ecosystems and agriculture worldwide. They currently face population declines and, in some cases, are at risk of extinction. Our project will develop tools that can be used to identify threats to bees and possibly improve bee resiliency. Bees are also model organisms for studies of social behaviors, learning, and communication, and have been proposed as models of human diseases such as autism. This project will develop tools that will empower studies of these complex behaviors. Understanding bee biology can help to continue and improve the long-standing beneficial association of bees with humans in agriculture, apiculture, and nature.

An integrated component of this proposal involves professional research educators and undergraduates in testing and disseminating the new FUGUES tools for bees using experiential learning courses. Specifically, this project will positively impact education at UT Austin through partially supporting two Freshman Research Initiative (FRI) streams. Collaboration between these streams is a way to massively replicate testing

of the FUGUES toolkit, use students as ambassadors to interface with the bee research community, and also potentially to try FUGUES in new bacterial-insect species combinations in student-initiated research projects. Furthermore, by leveraging the existing FRI program infrastructure (there are 29 FRI streams at UT Austin), we have a proven pathway for involving underrepresented and first-generation college students in this impactful experience that will prepare them for careers in STEM research [154]. The FRI actively recruits for diversity. For example, Hispanic and Black students make up 36.3% of entering FRI students in 2020. Studies of student success metrics show the benefits of scientific inquiry experiences [159] and specifically FRI [153], such as a higher 4 year graduation rate and improved retention in STEM majors.

Results of Prior NSF Support

Jeffrey Barrick (PI)

CAREER: Preventing Evolutionary Failure in Synthetic Biology

CBET-1554179, 05/16/2016 – 05/15/2021, $500,000 total costs

Intellectual Merit Summary. This project tests new approaches for stabilizing genetically engineered microbes and plasmids against unwanted evolution. Results to date include: demonstrating a Periodic Reselection for Evolutionarily Reliable Variants (PResERV) directed evolution scheme by isolating *E. coli* strains with lower-than-natural plasmid mutation rates; engineering a CRISPR interference against mobile elements (CRISPRi-ME) system that can be used to silence multicopy selfish DNA elements that disrupt engineered functions in diverse bacterial species; and the Evolutionary Failure Mode (EFM) calculator software that predicts mutational hotspots so that they can be avoided when designing DNA constructs.

Broader Impacts Summary. Barrick has mentored 5 iGEM synthetic teams with ~12 undergraduate participants each year. iGEM students have produced blog posts and web pages explaining how evolution impacts the reliability of synthetic biology. Eight undergrads from underrepresented groups have participated in iGEM, and this award has supported training three Hispanic graduate students in the Barrick lab.

Research products. Seven publications to date are indicated (+) in the **References Cited**. The EFM Calculator is freely available online as source code and a web tool. Plasmids and strains for CRISPRi-ME and improved tools for editing the genome of *A. bayli* ADP1 have been deposited in Addgene.

Nancy Moran (co-PI)

Dimensions: Genomics, functional roles, and diversity of the symbiotic gut microbiota of honey bees and bumble bees (PI: Moran; co-PI: Evans)

DEB-1046153 (Yale), DEB-1415604 (transfer to UT), 01/01/2011-09/30/2016, $2,006,000 total costs

Intellectual merit summary. This research showed that social bees have a highly specific core microbiota that is socially transmitted within colonies and present in honey bees, bumble bees, and stingless bees, following acquisition from a common ancestor >80 Mya. The project enabled formal description and genome sequencing of core gut species, establishing culture methods for all gut species, understanding their spatial and temporal distribution within bee gut, and the first results on beneficial roles of these bacteria in hosts. This provided a foundation for later work on bee microbiota, including work in the Barrick/Moran labs on developing genetic tools, and thus a foundation for the current proposal. For *S. alvi* (the focus of this proposal), culture methods were established, many isolates were obtained from *Apis* and *Bombus* hosts, and broad surveys revealed that every honey bee worker contains *S. alvi* in the ileum region of the hindgut.

Broader impacts summary. The results provide basic knowledge of factors affecting bee health, and this may be useful in developing new approaches for preventing colony declines. This award was the basis for developing the Bugs in Bugs FRI stream, which has trained ~120 UT undergrads, with >50% from underrepresented groups, largely Hispanic, first-generation college students.

Research products. In all, 39 publications were supported by this award, including 31 with Moran as an author, marked (++) in the **References Cited**. The award generated genome sequences for members of the bee gut microbiota, and metagenomic profiles for gut communities from *Apis* and *Bombus* populations (data all in NCBI, cited in publications). Type strains for newly described bee gut bacteria were deposited in culture collections. Supported trainees (one graduate student and two postdoctoral researchers) have acquired permanent independent positions pursuing work on bee-associated bacterial communities.

References

1. Pollinator Health Task Force. (2015) National strategy to promote the health of honey bees and other pollinators. *White House* 1–54.

2. Cox-Foster, D. L., Conlan, S., Holmes, E. C., Palacios, G., Evans, J. D., Moran, N. A., Quan, P., Briese, T., Hornig, M., Geiser, D. M., Martinson, V., VanEngelsdorp, D., Kalkstein, A. L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S. K., Simons, J. F., Egholm, M., Pettis, J. S., Lipkin, W. I. (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* **318**: 283–287. doi:10.1126/science.1146498

3. Evans, J. D., Schwarz, R. S. (2011) Bees brought to their knees: microbes affecting honey bee health. *Trends Microbiol.* **19**: 614–620. doi:10.1016/j.tim.2011.09.003

4. Cameron, S. A., Sadd, B. M. (2020) Global trends in bumble bee health. *Annu. Rev. Entomol.* **65**: 209–232. doi:10.1146/annurev-ento-011118-111847

5. Michener, C. D. (Belknap Press, 1974). *The social behavior of the bees: A comparative study*.

6. Kocher, S. D., Paxton, R. J. (2014) Comparative methods offer powerful insights into social evolution in bees. *Apidologie* **45**: 289–305. doi:10.1007/s13592-014-0268-3

7. Elekonich, M. M., Roberts, S. P. (2005) Honey bees as a model for understanding mechanisms of life history transitions. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **141**: 362–371. doi:10.1016/j.cbpb.2005.04.014

8. Ben-Shahar, Y., Robichon, A., Sokolowski, M. B., Robinson, G. E. (2002) Influence of gene action across different time scales on behavior. *Science* **296**: 741–744. doi:10.1126/science.1069911

9. Barron, A. B., Plath, J. A. (2017) The evolution of honey bee dance communication: A mechanistic perspective. *J. Exp. Biol.* **220**: 4339–4346. doi:10.1242/jeb.142778

10. Hammer, M., Menzel, R. (1995) Learning and memory in the honeybee. *J. Neurosci.* **15**: 1617–1630. doi:10.1523/jneurosci.15-03-01617.1995

11. Weinstock, G. M., Robinson, G. E., Gibbs, R. A., Worley, K. C., Evans, J. D., Maleszka, R., Robertson, H. M., Weaver, D. B., Beye, M., Bork, P., Elsik, C. G., Hartfelder, K., Hunt, G. J., Zdobnov, E. M., Amdam, G. V., Bitondi, M. M. G., Collins, A. M., Cristino, A. S., Lattorff, H. M. G., Lobo, C. H., Moritz, R. F. A., Nunes, F. M. F., Page, R. E., Simões, Z. L. P., Wheeler, D., Carninci, P., Fukuda, S., Hayashizaki, Y., Kai, C., Kawai, J., Sakazume, N., Sasaki, D., Tagami, M., Albert, S., Baggerman, G., Beggs, K. T., Bloch, G., Cazzamali, G., Cohen, M., Drapeau, M. D., Eisenhardt, D., Emore, C., Ewing, M. A., Fahrbach, S. E., Forêt, S., Grimmelikhuijzen, C. J. P., Hauser, F., Hummon, A. B., Huybrechts, J., Jones, A. K., Kadowaki, T., Kaplan, N., Kucharski, R., Leboulle, G., Linial, M., Littleton, J. T., Mercer, A. R., Richmond, T. A., Rodriguez-Zas, S. L., Rubin, E. B., Sattelle, D. B., Schlipalius, D., Schoofs, L., Shemesh, Y., Sweedler, J. V., Velarde, R., Verleyen, P., Vierstraete, E., Williamson, M. R., Ament, S. A., Brown, S. J., Corona, M., Dearden, P. K., Dunn, W. A., Elekonich, M. M., Fujiyuki, T., Gattermeier, I., Gempe, T., Hasselmann, M., Kadowaki, T., Kage, E., Kamikouchi, A., Kubo, T., Kucharski, R., Kunieda, T., Lorenzen, M., Milshina, N. V., Morioka, M., Ohashi, K., Overbeek, R., Ross, C. A., Schioett, M., Shippy, T., Takeuchi, H., Toth, A. L., Willis, J. H., Wilson, M. J., Gordon, K. H. J., Letunic, I., Hackett, K., Peterson, J., Felsenfeld, A., Guyer, M., Solignac, M., Agarwala, R., Cornuet, J. M., Monnerot, M., Mougel, F., Reese, J. T., Schlipalius, D., Vautrin, D., Gillespie, J. J., Cannone, J. J., Gutell, R. R., Johnston, J. S., Eisen, M. B., Iyer, V. N., Iyer, V., Kosarev, P., Mackey, A. J., Solovyev, V., Souvorov, A., Aronstein, K. A., Bilikova, K., Chen, Y. P., Clark, A. G., Decanini, L. I., Gelbart, W. M., Hetru, C., Hultmark, D., Imler, J. L., Jiang, H., Kanost, M., Kimura, K., Lazzaro, B. P., Lopez, D. L., Simuth, J., Thompson, G. J., Zou, Z., De Jong, P., Sodergren, E., Csurös, M., Milosavljevic, A., Osoegawa, K., Richards, S., Shu, C. L., Duret, L., Elhaik, E., Graur, D., Anzola, J. M., Campbell, K. S., Childs, K. L., Collinge, D., Crosby, M. A., Dickens, C. M., Grametes, L. S., Grozinger, C. M., Jones, P. L., Jorda, M., Ling, X., Matthews, B. B., Miller, J., Mizzen, C., Peinado,

M. A., Reid, J. G., Russo, S. M., Schroeder, A. J., St. Pierre, S. E., Wang, Y., Zhou, P., Jiang, H., Kitts, P., Ruef, B., Venkatraman, A., Zhang, L., Aquino-Perez, G., Whitfield, C. W., Behura, S. K., Berlocher, S. H., Sheppard, W. S., Smith, D. R., Suarez, A. V., Tsutsui, N. D., Wei, X., Wheeler, D., Havlak, P., Li, B., Liu, Y., Jovilet, A., Lee, S., Nazareth, L. V., Pu, L. L., Thorn, R., Stolc, V., Newman, T., Samanta, M., Tongprasit, W. A., Claudianos, C., Berenbaum, M. R., Biswas, S., De Graaf, D. C., Feyereisen, R., Johnson, R. M., Oakeshott, J. G., Ranson, H., Schuler, M. A., Muzny, D., Chacko, J., Davis, C., Dinh, H., Gill, R., Hernandez, J., Hines, S., Hume, J., Jackson, L. R., Kovar, C., Lewis, L., Miner, G., Morgan, M., Nguyen, N., Okwuonu, G., Paul, H., Santibanez, J., Savery, G., Svatek, A., Villasana, D., Wright, R. (2006) Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* **443**: 931–949. doi:10.1038/nature05260

12. Wallberg, A., Bunikis, I., Pettersson, O. V., Mosbech, M. B., Childers, A. K., Evans, J. D., Mikheyev, A. S., Robertson, H. M., Robinson, G. E., Webster, M. T. (2019) A hybrid de novo genome assembly of the honeybee, *Apis mellifera*, with chromosome-length scaffolds. *BMC Genomics* **20**: 275. doi:10.1186/s12864-019-5642-0

13. Zayed, A., Robinson, G. E. (2012) Understanding the relationship between brain gene expression and social behavior: lessons from the honey bee. *Annu. Rev. Genet.* **46**: 591–615. doi:10.1146/annurev-genet-110711-155517

14. Ament, S. a, Corona, M., Pollock, H. S., Robinson, G. E. (2008) Insulin signaling is involved in the regulation of worker division of labor in honey bee colonies. *Proc. Natl. Acad. Sci. U.S.A.* **105**: 4226–4231. doi:10.1073/pnas.0800630105

15. Chen, D., Guo, R., Xu, X., Xiong, C., Liang, Q., Zheng, Y., Luo, Q., Zhang, Z., Huang, Z., Kumar, D., Xi, W., Zou, X., Liu, M. (2017) Uncovering the immune responses of *Apis mellifera ligustica* larval gut to *Ascosphaera apis* infection utilizing transcriptome sequencing. *Gene* **621**: 40–50. doi:10.1016/j.gene.2017.04.022

16. Rittschof, C. C., Rubin, B. E. R., Palmer, J. H. (2019) The transcriptomic signature of low aggression in honey bees resembles a response to infection. *BMC Genomics* **20**: 1029. doi:10.1186/s12864-019-6417-3

17. Harpur, B. A., Guarna, M. M., Huxter, E., Higo, H., Moon, K. M., Hoover, S. E., Ibrahim, A., Melathopoulos, A. P., Desai, S., Currie, R. W., Pernal, S. F., Foster, L. J., Zayed, A., Eyre-Walker, A. (2019) Integrative genomics reveals the genetics and evolution of the honey bee's social immune system. *Genome Biol. Evol.* **11**: 937–948. doi:10.1093/gbe/evz018

18. Huang, Y., Liu, Z., Rong, Y. S. (2016) Genome editing: from *Drosophila* to non-model insects and beyond. *J. Genet. Genomics* **43**: 263–272. doi:10.1016/j.jgg.2016.04.007

19. Schulte, C., Theilenberg, E., Muller-Borg, M., Gempe, T., Beye, M. (2014) Highly efficient integration and expression of piggyBac-derived cassettes in the honeybee (Apis mellifera). *Proc. Natl. Acad. Sci. U.S.A.* **111**: 9003–9008. doi:10.1073/pnas.1402341111

20. Kohno, H., Suenami, S., Takeuchi, H., Sasaki, T., Kubo, T. (2016) Production of knockout mutants by CRISPR/Cas9 in the European honeybee, *Apis mellifera* L. *Zoolog. Sci.* **33**: 505–512. doi:10.2108/zs160043

21. Brutscher, L. M., Flenniken, M. L. (2015) RNAi and antiviral defense in the honey bee. *J. Immunol. Res.* **2015**: 941897. doi:10.1155/2015/941897

22. Dowling, D., Pauli, T., Donath, A., Meusemann, K., Podsiadlowski, L., Petersen, M., Peters, R. S., Mayer, C., Liu, S., Zhou, X., Misof, B., Niehuis, O. (2016) Phylogenetic origin and diversification of RNAi pathway genes in insects. *Genome Biol. Evol.* **8**: 3784–3793. doi:10.1093/gbe/evw281

23. Marco Antonio, D. S., Guidugli-Lazzarini, K. R., Do Nascimento, A. M., Simões, Z. L. P., Hartfelder, K. (2008) RNAi-mediated silencing of vitellogenin gene function turns honeybee (*Apis mellifera*) workers into extremely precocious foragers. *Naturwissenschaften* **95**: 953–961. doi:10.1007/s00114-008-0413-9

24. Amdam, G. V., Simões, Z. L. P., Guidugli, K. R., Norberg, K., Omholt, S. W. (2003) Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. *BMC Biotechnol.* **3**: 1. doi:10.1186/1472-6750-3-1

25. Maori, E., Paldi, N., Shafir, S., Kalev, H., Tsur, E., Glick, E., Sela, I. (2009) IAPV, a bee-affecting virus associated with colony collapse disorder can be silenced by dsRNA ingestion. *Insect Mol. Biol.* **18**: 55–60. doi:10.1111/j.1365-2583.2009.00847.x

26. Desai, S. D., Eu, Y. J., Whyard, S., Currie, R. W. (2012) Reduction in deformed wing virus infection in larval and adult honey bees (*Apis mellifera* L.) by double-stranded RNA ingestion. *Insect Mol. Biol.* **21**: 446–455. doi:10.1111/j.1365-2583.2012.01150.x

27. Garbian, Y., Maori, E., Kalev, H., Shafir, S., Sela, I. (2012) Bidirectional transfer of RNAi between honey bee and *Varroa destructor*: *Varroa* gene silencing reduces *Varroa* population. *PLoS Pathog.* **8**: e1003035. doi:10.1371/journal.ppat.1003035

28. Hunter, W., Ellis, J., Vanengelsdorp, D., Hayes, J., Westervelt, D., Glick, E., Williams, M., Sela, I., Maori, E., Pettis, J., Cox-Foster, D., Paldi, N. (2010) Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, hymenoptera: Apidae). *PLoS Pathog.* **6**: e1001160. doi:10.1371/journal.ppat.1001160

29. Aronstein, K., Pankiw, T., Saldivar, E. (2006) SID-1 is implicated in systemic gene silencing in the honey bee. *J. Apic. Res. Bee World* **45**: 20–24.

30. Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., Zhu, K. Y., Douglas, A. E. (2013) Towards the elements of successful insect RNAi. *J. Insect Physiol.* **59**: 1212–1221. doi:10.1016/j.jinsphys.2013.08.014

31. Miller, S. C., Brown, S. J., Tomoyasu, Y. (2008) Larval RNAi in Drosophila? *Dev. Genes Evol.* **218**: 505–510. doi:10.1007/s00427-008-0238-8

32. Tomoyasu, Y., Miller, S. C., Tomita, S., Schoppmeier, M., Grossmann, D., Bucher, G. (2008) Exploring systemic RNA interference in insects: A genome-wide survey for RNAi genes in *Tribolium*. *Genome Biol.* **9**: 1–22. doi:10.1186/gb-2008-9-1-r10

33. Yu, N., Christiaens, O., Liu, J., Niu, J., Cappelle, K., Caccia, S., Huvenne, H., Smagghe, G. (2013) Delivery of dsRNA for RNAi in insects: an overview and future directions. *Insect Sci.* **20**: 4–14. doi:10.1111/j.1744-7917.2012.01534.x

34. Liu, J., Swevers, L., Iatrou, K., Huvenne, H., Smagghe, G. (2012) *Bombyx mori* DNA/RNA non-specific nuclease: expression of isoforms in insect culture cells, subcellular localization and functional assays. *J. Insect Physiol.* **58**: 1166–1176. doi:10.1016/j.jinsphys.2012.05.016

35. Leonard, S. P., Powell, J. E., Perutka, J., Geng, P., Heckmann, L. C., Horak, R. D., Davies, B. W., Ellington, A. D., Barrick, J. E., Moran, N. A. (2020) Engineered symbionts activate honey bee immunity and limit pathogens. *Science* **367**: 573–576. doi:10.1126/science.aax9039

36. Chaverra-Rodriguez, D., Dalla Benetta, E., Heu, C. C., Rasgon, J. L., Ferree, P. M., Akbari, O. S. (2020) Germline mutagenesis of *Nasonia vitripennis* through ovarian delivery of CRISPR-Cas9 ribonucleoprotein. *Insect Mol. Biol.* imb.12663. doi:10.1111/imb.12663

37. Chaverra-Rodriguez, D., Macias, V. M., Hughes, G. L., Pujhari, S., Suzuki, Y., Peterson, D. R., Kim, D., McKeand, S., Rasgon, J. L. (2018) Targeted delivery of CRISPR-Cas9 ribonucleoprotein into arthropod ovaries for heritable germline gene editing. *Nat. Commun.* **9**: 1–11. doi:10.1038/s41467-018-05425-9

38. Macias, V. M., McKeand, S., Chaverra-Rodriguez, D., Hughes, G. L., Fazekas, A., Pujhari, S., Jasinskiene, N., James, A. A., Rasgon, J. L. (2020) Cas9-mediated gene-editing in the malaria mosquito *Anopheles stephensi* by ReMOT Control. *G3: Genes, Genomes, Genet.* **10**: 1353–1360. doi:10.1534/g3.120.401133

39. Heu, C. C., McCullough, F. M., Luan, J., Rasgon, J. L. (2020) CRISPR-Cas9-based genome editing in the silverleaf whitefly (*Bemisia tabaci*). *Cris. J.* **3**: 89–96. doi:10.1089/crispr.2019.0067

40. Farooqui, T., Robinson, K., Vaessin, H., Smith, B. H. (2003) Modulation of early olfactory processing by an octopaminergic reinforcement pathway in the honeybee. *J. Neurosci.* **23**: 5370–5380. doi:10.1523/jneurosci.23-12-05370.2003

41. Mattila, H. R., Burke, K. M., Seeley, T. D. (2008) Genetic diversity within honeybee colonies increases signal production by waggle-dancing foragers. *Proc. R. Soc. B Biol. Sci.* **275**: 809–816. doi:10.1098/rspb.2007.1620

42. Guzman-Novoa, E., Hunt, G. J., Page, R. E., Uribe-Rubio, J. L., Prieto-Merlos, D., Becerra-Guzman, F. (2005) Paternal effects on the defensive behavior of honeybees. *J. Hered.* **96**: 376–380. doi:10.1093/jhered/esi038

43. Shpigler, H. Y., Saul, M. C., Corona, F., Block, L., Cash Ahmed, A., Zhao, S. D., Robinson, G. E. (2017) Deep evolutionary conservation of autism-related genes. *Proc. Natl. Acad. Sci. U.S.A.* **114**: 9653–9658. doi:10.1073/pnas.1708127114

44. Alaux, C., Sinha, S., Hasadsri, L., Hunt, G. J., Guzmán-Novoa, E., DeGrandi-Hoffman, G., Uribe-Rubio, J. L., Southey, B. R., Rodriguez-Zas, S., Robinson, G. E. (2009) Honey bee aggression supports a link between gene regulation and behavioral evolution. *Proc. Natl. Acad. Sci. U.S.A.* **106**: 15400–15405. doi:10.1073/pnas.0907043106

45. Kocher, S. D., Mallarino, R., Rubin, B. E. R., Yu, D. W., Hoekstra, H. E., Pierce, N. E. (2018) The genetic basis of a social polymorphism in halictid bees. *Nat. Commun.* **9**: 4338. doi:10.1038/s41467-018-06824-8

46. Berenbaum, M. R., Johnson, R. M. (2015) Xenobiotic detoxification pathways in honey bees. *Curr. Opin. Insect Sci.* **10**: 51–58. doi:10.1016/j.cois.2015.03.005

47. Alptekin, S., Bass, C., Nicholls, C., Paine, M. J. I., Clark, S. J., Field, L., Moores, G. D. (2016) Induced thiacloprid insensitivity in honeybees (*Apis mellifera* L.) is associated with up-regulation of detoxification genes. *Insect Mol. Biol.* **25**: 171–180. doi:10.1111/imb.12211

48. Mao, W., Schuler, M. A., Berenbaum, M. R. (2011) CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). *Proc. Natl. Acad. Sci. U.S.A.* **108**: 12657–12662. doi:10.1073/pnas.1109535108

49. Farooqui, T. (2007) Octopamine-mediated neuromodulation of insect senses. *Neurochem. Res.* **32**: 1511–1529. doi:10.1007/s11064-007-9344-7

50. Hamilton, A. R., Traniello, I. M., Ray, A. M., Caldwell, A. S., Wickline, S. A., Robinson, G. E. (2019) Division of labor in honey bees is associated with transcriptional regulatory plasticity in the brain. *J. Exp. Biol.* **222**: jeb200196. doi:10.1242/jeb.200196

51. Maleszka, R., Helliwell, P. (2001) Effect of juvenile hormone on short-term olfactory memory in young honeybees (*Apis mellifera*). *Horm. Behav.* **40**: 403–408. doi:10.1006/hbeh.2001.1705

52. Menzel, R. (2001) Searching for the memory trace in a mini-brain, the honeybee. *Learn. Mem.* **8**: 53–62. doi:10.1101/lm.38801

53. Menzel, R. (1996) Learning and memory in honeybees: From behavior to neural substrates. *Annu. Rev. Neurosci.* **19**: 379–404. doi:10.1146/annurev.neuro.19.1.379

54. Hummon, A. B., Richmond, T. A., Verleyen, P., Baggerman, G., Huybrechts, J., Ewing, M. A., Vierstraete, E., Rodriguez-Zas, S. L., Schoofs, L., Robinson, G. E., Sweedler, J. V. (2006) From the genome to the proteome: uncovering peptides in the *Apis* brain. *Science* **314**: 647–649. doi:10.1126/science.1124128

55. Whitfield, C. W., Cziko, A. M., Robinson, G. E. (2003) Gene expression profiles in the brain predict behavior in individual honey bees. *Science* **302**: 296–299. doi:10.1126/science.1086807

56. Huang, Z. Y., Robinson, G. E. (1992) Honeybee colony integration: Worker-worker interactions mediate hormonally regulated plasticity in division of labor. *Proc. Natl. Acad. Sci. U.S.A.* **89**: 11726–11729. doi:10.1073/pnas.89.24.11726

57. Robinson, G. E., Page, R. E. (1988) Genetic determination of guarding and undertaking in honey-bee colonies. *Nature* **333**: 356–358. doi:10.1038/333356a0

58. Robertson, H. M., Wanner, K. W. (2006) The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res.* **16**: 1395–1403. doi:10.1101/gr.5057506

59. Slessor, K. N., Winston, M. L., Le Conte, Y. (2005) Pheromone communication in the honeybee (*Apis mellifera* L.). *J. Chem. Ecol.* **31**: 2731–2745. doi:10.1007/s10886-005-7623-9

60. Page, R. E., Peng, C. Y.-S. (2001) Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. *Exp. Gerontol.* **36**: 695–711. doi:10.1016/S0531-5565(00)00236-9

61. Wallberg, A., Pirk, C. W., Allsopp, M. H., Webster, M. T. (2016) Identification of multiple loci associated with social parasitism in honeybees. *PLOS Genet.* **12**: e1006097. doi:10.1371/journal.pgen.1006097

62. Blacquière, T., Smagghe, G., Van Gestel, C. A. M., Mommaerts, V. (2012) Neonicotinoids in bees: A review on concentrations, side-effects and risk assessment. *Ecotoxicology* **21**: 973–992. doi:10.1007/s10646-012-0863-x

63. Muth, F., Francis, J. S., Leonard, A. S. (2019) Modality-specific impairment of learning by a neonicotinoid pesticide. *Biol. Lett.* **15**: 20190359. doi:10.1098/rsbl.2019.0359

64. Henry, M., Beguin, M., Requier, F., Rollin, O., Odoux, J.-F., Aupinel, P., Aptel, J., Tchamitchian, S., Decourtye, A. (2012) A common pesticide decreases foraging success and survival in honey bees. *Science* **336**: 348–350. doi:10.1126/science.1215039

65. Schneider, C. W., Tautz, J., Grünewald, B., Fuchs, S. (2012) RFID Tracking of Sublethal Effects of Two Neonicotinoid Insecticides on the Foraging Behavior of Apis mellifera. *PLoS ONE* **7**: e30023. doi:10.1371/journal.pone.0030023

66. Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J. L., Jiang, H., Kanost, M., Thompson, G. J., Zou, Z., Hultmark, D. (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol. Biol.* **15**: 645–656. doi:10.1111/j.1365-2583.2006.00682.x

++67. Kwong, W. K., Moran, N. A. (2016) Gut microbial communities of social bees. *Nat. Rev. Microbiol.* **14**: 374–384. doi:10.1038/nrmicro.2016.43

68. Zheng, H., Steele, M. I., Leonard, S. P., Motta, E. V. S., Moran, N. A. (2018) Honey bees as models for gut microbiota research. *Lab Anim. (NY).* **47**: 317–325. doi:10.1038/s41684-018-0173-x

++69. Martinson, V. G., Moy, J., Moran, N. A. (2012) Establishment of characteristic gut bacteria during development of the honeybee worker. *Appl. Environ. Microbiol.* **78**: 2830–2840. doi:10.1128/AEM.07810-11

++70. Kwong, W. K., Engel, P., Koch, H., Moran, N. A. (2014) Genomics and host specialization of honey bee and bumble bee gut symbionts. *Proc. Natl. Acad. Sci. U.S.A.* **111**: 11509–11514. doi:10.1073/pnas.1405838111

++71. Kwong, W. K., Moran, N. A. (2013) Cultivation and characterization of the gut symbionts of honey bees and bumble bees: description of *Snodgrassella alvi* gen. nov., sp. nov., a member of the family *Neisseriaceae* of the *Betaproteobacteria*, and *Gilliamella apicola*. *Int. J. Syst. Evol. Microbiol.* **63**: 2008–2018. doi:10.1099/ijs.0.044875-0

72. Zheng, H., Powell, J. E., Steele, M. I., Dietrich, C., Moran, N. A. (2017) Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. *Proc. Natl. Acad. Sci.*

114: 4775–4780. doi:10.1073/pnas.1701819114

73. Raymann, K., Shaffer, Z., Moran, N. A. (2017) Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. *PLoS Biol.* **15**: e2001861. doi:10.1371/journal.pbio.2001861

74. Raymann, K., Coon, K. L., Shaffer, Z., Salisbury, S., Moran, N. A. (2018) Pathogenicity of *Serratia marcescens* strains in honey bees. *mBio* **9**: e01649-18. doi:10.1128/mBio.01649-18

75. Koch, H., Schmid-Hempel, P. (2011) Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proc. Natl. Acad. Sci. U.S.A.* **108**: 19288–19292. doi:10.1073/pnas.1110474108

76. Sheth, R. U., Cabral, V., Chen, S. P., Wang, H. H. (2016) Manipulating bacterial communities by *in situ* microbiome engineering. *Trends Genet.* **32**: 189–200. doi:10.1016/j.tig.2016.01.005

77. Dale, C., Young, S. A., Haydon, D. T., Welburn, S. C. (2001) The insect endosymbiont *Sodalis glossinidius* utilizes a type III secretion system for cell invasion. *Proc. Natl. Acad. Sci. U.S.A.* **98**: 1883–1888. doi:10.1073/pnas.98.4.1883

78. De Vooght, L., Van Keer, S., Van Den Abbeele, J. (2018) Towards improving tsetse fly paratransgenesis: stable colonization of *Glossina morsitans morsitans* with genetically modified *Sodalis*. *BMC Microbiol.* **18**: 165. doi:10.1186/s12866-018-1282-9

79. Nadal-Jimenez, P., Griffin, J. S., Davies, L., Frost, C. L., Marcello, M., Hurst, G. D. D. (2019) Genetic manipulation allows in vivo tracking of the life cycle of the son-killer symbiont, *Arsenophonus nasoniae*, and reveals patterns of host invasion, tropism and pathology. *Environ. Microbiol.* **21**: 3172–3182. doi:10.1111/1462-2920.14724

80. Damiani, C., Ricci, I., Crotti, E., Rossi, P., Rizzi, A., Scuppa, P., Esposito, F., Bandi, C., Daffonchio, D., Favia, G. (2008) Paternal transmission of symbiotic bacteria in malaria vectors. *Curr. Biol.* **18**: 1087–1088. doi:10.1016/j.cub.2008.10.040

81. Wang, S., Dos-Santos, A. L. A., Huang, W., Liu, K. C., Oshaghi, M. A., Wei, G., Agre, P., Jacobs-Lorena, M. (2017) Driving mosquito refractoriness to *Plasmodium falciparum* with engineered symbiotic bacteria. *Science* **357**: 1399–1402. doi:10.1126/science.aan5478

82. Mimee, M., Tucker, A. C., Voigt, C. A., Lu, T. K. (2015) Programming a human commensal bacterium, *Bacteroides thetaiotaomicron*, to sense and respond to stimuli in the murine gut microbiota. *Cell Syst.* **1**: 62–71. doi:10.1016/j.cels.2015.06.001

83. Wu, M., McNulty, N. P., Rodionov, D. A., Khoroshkin, M. S., Griffin, N. W., Cheng, J., Latreille, P., Kerstetter, R. A., Terrapon, N., Henrissat, B., Osterman, A. L., Gordon, J. I. (2015) Genetic determinants of in vivo fitness and diet responsiveness in multiple human gut *Bacteroides*. *Science* **350**: aac5992. doi:10.1126/science.aac5992

84. Rangberg, A., Diep, D. B., Rudi, K., Amdam, G. V. (2012) Paratransgenesis: An approach to improve colony health and molecular insight in honey bees (*Apis mellifera*)? *Integr. Comp. Biol.* **52**: 89–99. doi:10.1093/icb/ics089

85. Rangberg, A., Mathiesen, G., Amdam, G. V., Diep, D. B. (2015) The paratransgenic potential of *Lactobacillus kunkeei* in the honey bee *Apis mellifera*. *Benef. Microbes* **6**: 513–523. doi:10.3920/BM2014.0115

86. Abrieux, A., Chiu, J. C. (2016) Oral delivery of dsRNA by microbes: beyond pest control. *Commun. Integr. Biol.* **9**: e1236163. doi:10.1080/19420889.2016.1236163

87. Whitten, M., Dyson, P. (2017) Gene silencing in non-model insects: overcoming hurdles using symbiotic bacteria for trauma-free sustainable delivery of RNA interference. *BioEssays* **39**: 1600247. doi:10.1002/bies.201600247

88. Whitten, M. M. A., Facey, P. D., Del Sol, R., Fernández-Martínez, L. T., Evans, M. C., Mitchell, J. J., Bodger, O. G., Dyson, P. J. (2016) Symbiont-mediated RNA interference in insects. *Proc. R.*

Soc. B **283**: 20160042. doi:10.1098/rspb.2016.0042

89. Leonard, S. P., Perutka, J., Powell, J. E., Geng, P., Richhart, D. D., Byrom, M., Kar, S., Davies, B. W., Ellington, A. D., Moran, N. A., Barrick, J. E. (2018) Genetic engineering of bee gut microbiome bacteria with a toolkit for modular assembly of broad-host-range plasmids. *ACS Synth. Biol.* **7**: 1279–1290. doi:10.1021/acssynbio.7b00399

90. Lee, M. E., DeLoache, W. C., Cervantes, B., Dueber, J. E. (2015) A highly characterized yeast toolkit for modular, multipart assembly. *ACS Synth. Biol.* **4**: 975–986. doi:10.1021/sb500366v

91. Wiles, T. J., Wall, E. S., Schlomann, B. H., Hay, E. A., Parthasarathy, R., Guillemin, K. (2018) Modernized tools for streamlined genetic manipulation and comparative study of wild and diverse proteobacterial lineages. *mBio* **9**: e01877-18. doi:10.1128/mBio.01877-18

92. Schlechter, R. O., Jun, H., Bernach, M., Oso, S., Boyd, E., Muñoz-Lintz, D. A., Dobson, R. C. J., Remus, D. M., Remus-Emsermann, M. N. P. (2018) Chromatic bacteria – a broad host-range plasmid and chromosomal insertion toolbox for fluorescent protein expression in bacteria. *Front. Microbiol.* **9**: 3052. doi:10.3389/fmicb.2018.03052

93. Freed, E., Fenster, J., Smolinski, S. L., Walker, J., Henard, C. A., Gill, R., Eckert, C. A. (2018) Building a genome engineering toolbox in nonmodel prokaryotic microbes. *Biotechnol. Bioeng.* **115**: 2120–2138. doi:10.1002/bit.26727

94. Klompe, S. E., Vo, P. L. H., Halpin-Healy, T. S., Sternberg, S. H. (2019) Transposon-encoded CRISPR–Cas systems direct RNA-guided DNA integration. *Nature* **571**: 219–225. doi:10.1038/s41586-019-1323-z

95. Rubin, B. E., Diamond, S., Cress, B. F., Crits-christoph, A. (2020) Targeted Genome Editing of Bacteria Within Microbial Communities. *bioRxiv* doi:10.1101/2020.07.17.209189

96. Mee, M. T., Collins, J. J., Church, G. M., Wang, H. H. (2014) Syntrophic exchange in synthetic microbial communities. *Proc. Natl. Acad. Sci. U.S.A.* **111**: E2149–E2156. doi:10.1073/pnas.1405641111

97. Powell, J. E., Leonard, S. P., Kwong, W. K., Engel, P., Moran, N. A. (2016) Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. *Proc. Natl. Acad. Sci. U.S.A.* **113**: 13887–13892. doi:10.1073/pnas.1610856113

98. Timmons, L., Court, D. L., Fire, A. (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* **263**: 103–112. doi:10.1016/S0378-1119(00)00579-5

99. Huang, L., Jin, J., Deighan, P., Kiner, E., McReynolds, L., Lieberman, J. (2013) Efficient and specific gene knockdown by small interfering RNAs produced in bacteria. *Nat. Biotechnol.* **31**: 350–356. doi:10.1038/nbt.2537

100. Kaur, G., Cheung, H. C., Xu, W., Wong, J. V., Chan, F. F., Li, Y., McReynolds, L., Huang, L. (2018) Milligram scale production of potent recombinant small interfering RNAs in *Escherichia coli*. *Biotechnol. Bioeng.* **115**: 2280–2291. doi:10.1002/bit.26740

101. Guzman, L. M., Belin, D., Carson, M. J., Beckwith, J. (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol.* **177**: 4121–4130.

102. Takiff, H. E., Chen, S. M., Court, D. L. (1989) Genetic analysis of the *rnc* operon of *Escherichia coli*. *J. Bacteriol.* **171**: 2581–2590. doi:10.1128/jb.171.5.2581-2590.1989

103. Durand, S., Gilet, L., Condon, C. (2012) The essential function of *B. subtilis* RNase III Is to silence foreign toxin genes. *PLoS Genet.* **8**: e1003181. doi:10.1371/journal.pgen.1003181

104. Saramago, M., Robledo, M., Matos, R. G., Jiménez-Zurdo, J. I., Arraiano, C. M. (2018) Sinorhizobium meliloti RNase III: catalytic features and impact on symbiosis. *Front. Genet.* **9**: 350.

doi:10.3389/fgene.2018.00350

[+]105. Geng, P., Leonard, S. P., Mishler, D. M., Barrick, J. E. (2019) Synthetic genome defenses against selfish DNA elements stabilize engineered bacteria against evolutionary failure. *ACS Synth. Biol.* **8**: 521–531. doi:10.1021/acssynbio.8b00426

106. Ceroni, F., Algar, R., Stan, G.-B., Ellis, T. (2015) Quantifying cellular capacity identifies gene expression designs with reduced burden. *Nat. Methods* **12**: 415–418. doi:10.1038/nmeth.3339

107. Jack, B. R., Leonard, S. P., Mishler, D. M., Renda, B. A., Leon, D., Suárez, G. A., Barrick, J. E. (2014) Predicting the genetic stability of engineered DNA sequences with the EFM calculator. *ACS Synth. Biol.* **4**: 939–943. doi:10.1021/acssynbio.5b00068

[+]108. Zhang, X., Deatherage, D. E., Zheng, H., Georgoulis, S. J., Barrick, J. E. (2019) Evolution of satellite plasmids can prolong the maintenance of newly acquired accessory genes in bacteria. *Nat. Commun.* **10**: 5809. doi:10.1038/s41467-019-13709-x

109. Schmitt, M. W., Kennedy, S. R., Salk, J. J., Fox, E. J., Hiatt, J. B., Loeb, L. A. (2012) Detection of ultra-rare mutations by next-generation sequencing. *Proc. Natl. Acad. Sci. U.S.A.* **109**: 14508–14513. doi:10.1073/pnas.1208715109

[+]110. Deatherage, D., Barrick, J. (2020) Profiling the initial burst of beneficial genetic diversity to anticipate evolution of a cell population. *bioRxiv* doi:10.1101/2020.07.10.196832

111. Sleight, S. C., Bartley, B. A., Lieviant, J. A., Sauro, H. M. (2010) Designing and engineering evolutionary robust genetic circuits. *J. Biol. Eng.* **4**: 12.

112. Rugbjerg, P., Myling-Petersen, N., Porse, A., Sarup-Lytzen, K., Sommer, M. O. A. (2018) Diverse genetic error modes constrain large-scale bio-based production. *Nat. Commun.* **9**: 787. doi:10.1038/s41467-018-03232-w

113. Ceroni, F., Boo, A., Furini, S., Gorochowski, T. E., Borkowski, O., Ladak, Y. N., Awan, A. R., Gilbert, C., Stan, G.-B., Ellis, T. (2018) Burden-driven feedback control of gene expression. *Nat. Methods* **15**: 387–393. doi:10.1038/nmeth.4635

114. Whitten, M. M. A., Coates, C. J. (2017) Re-evaluation of insect melanogenesis research: views from the dark side. *Pigment Cell Melanoma Res.* **30**: 386–401. doi:10.1111/pcmr.12590

115. Liu, J., Lemonds, T. R., Marden, J. H., Popadić, A. (2016) A pathway analysis of melanin patterning in a hemimetabolous insect. *Genetics* **203**: 403–413. doi:10.1534/genetics.115.186684

116. Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R., Kramer, K. J. (2005) Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning. *Proc. Natl. Acad. Sci. U.S.A.* **102**: 11337–11342. doi:10.1073/pnas.0504982102

117. Lourenço, A. P., Zufelato, M. S., Gentile Bitondi, M. M., Paulino Simões, Z. L. (2005) Molecular characterization of a cDNA encoding prophenoloxidase and its expression in *Apis mellifera*. *Insect Biochem. Mol. Biol.* **35**: 541–552. doi:10.1016/j.ibmb.2005.01.013

118. Ito, S., Nakanishi, Y., Valenzuela, R. K., Brilliant, M. H., Kolbe, L., Wakamatsu, K. (2011) Usefulness of alkaline hydrogen peroxide oxidation to analyze eumelanin and pheomelanin in various tissue samples: application to chemical analysis of human hair melanins. *Pigment Cell Melanoma Res.* **24**: 605–613. doi:10.1111/j.1755-148X.2011.00864.x

119. Wilson-Rich, N., Dres, S. T., Starks, P. T. (2008) The ontogeny of immunity: development of innate immune strength in the honey bee (*Apis mellifera*). *J. Insect Physiol.* **54**: 1392–1399. doi:10.1016/j.jinsphys.2008.07.016

120. Figon, F., Casas, J. (2019) Ommochromes in invertebrates: biochemistry and cell biology. *Biol. Rev.* **94**: 156–183. doi:10.1111/brv.12441

121. Dustmann, J. H. (1968) Pigment studies on several eye-colour mutants of the honey bee, *Apis*

mellifera. *Nature* **219**: 950–952. doi:10.1038/219950b0

122. Dustmann, J. H. (1987) Eye-colour mutants of the honeybee. *Bee World* **68**: 124–128.

123. Xue, W. H., Xu, N., Yuan, X. B., Chen, H. H., Zhang, J. L., Fu, S. J., Zhang, C. X., Xu, H. J. (2018) CRISPR/Cas9-mediated knockout of two eye pigmentation genes in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). *Insect Biochem. Mol. Biol.* **93**: 19–26. doi:10.1016/j.ibmb.2017.12.003

124. Riou, M., Christidès, J. P. (2010) Cryptic color change in a crab spider (*Misumena vatia*): identification and quantification of precursors and ommochrome pigments by HPLC. *J. Chem. Ecol.* **36**: 412–423. doi:10.1007/s10886-010-9765-7

125. Mansourian, S., Fandino, R. A., Riabinina, O. (2019) Progress in the use of genetic methods to study insect behavior outside Drosophila. *Curr. Opin. Insect Sci.* **36**: 45–56. doi:10.1016/j.cois.2019.08.001

126. Fandino, R. A., Alexander Haverkamp, Bisch-Knaden, S., Zhang, J., Bucks, S., Tu Anh Thi Nguyen, Schröder, K., Achim Werckenthin, Rybak, J., Monika Stengl, Knaden, M., Hansson, B. S., Groβe-Wilde, E. (2019) Mutagenesis of odorant coreceptor Orco fully disrupts foraging but not oviposition behaviors in the hawkmoth *Manduca sexta*. *Proc. Natl. Acad. Sci. U.S.A.* **116**: 15677–15685. doi:10.1073/pnas.1902089116

127. Liu, Q., Liu, W., Zeng, B., Wang, G., Hao, D., Huang, Y. (2017) Deletion of the *Bombyx mori* odorant receptor co-receptor (*BmOrco*) impairs olfactory sensitivity in silkworms. *Insect Biochem. Mol. Biol.* **86**: 58–67. doi:10.1016/j.ibmb.2017.05.007

128. Trible, W., Olivos-Cisneros, L., McKenzie, S. K., Saragosti, J., Chang, N.-C., Matthews, B. J., Oxley, P. R., Kronauer, D. J. C. (2017) orco mutagenesis causes loss of antennal lobe glomeruli and impaired social behavior in ants. *Cell* **170**: 727-735.e10. doi:10.1016/j.cell.2017.07.001

129. Franco, T. A., Oliveira, D. S., Moreira, M. F., Leal, W. S., Melo, A. C. A. (2016) Silencing the odorant receptor co-receptor RproOrco affects the physiology and behavior of the Chagas disease vector *Rhodnius prolixus*. *Insect Biochem. Mol. Biol.* **69**: 82–90. doi:10.1016/j.ibmb.2015.02.012

130. Zhang, R., Gao, G., Chen, H. (2016) Silencing of the olfactory co-receptor gene in *Dendroctonus armandi* leads to EAG response declining to major host volatiles. *Sci. Rep.* **6**: 23136. doi:10.1038/srep23136

131. Fan, J., Zhang, Y., Francis, F., Cheng, D., Sun, J., Chen, J. (2015) Orco mediates olfactory behaviors and winged morph differentiation induced by alarm pheromone in the grain aphid, *Sitobion avenae*. *Insect Biochem. Mol. Biol.* **64**: 16–24. doi:10.1016/j.ibmb.2015.07.006

132. Krieger, J., Klink, O., Mohl, C., Raming, K., Breer, H. (2003) A candidate olfactory receptor subtype highly conserved across different insect orders. *J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol.* **189**: 519–526. doi:10.1007/s00359-003-0427-x

133. Van Nest, B. N. (2018) The olfactory proboscis extension response in the honey bee: a laboratory exercise in classical conditioning. *J. Undergrad. Neurosci. Educ.* **16**: A168–A176.

134. Muth, F., Cooper, T. R., Bonilla, R. F., Leonard, A. S. (2018) A novel protocol for studying bee cognition in the wild. *Methods Ecol. Evol.* **9**: 78–87. doi:10.1111/2041-210X.12852

135. Dubovskiy, I. M., Whitten, M. M. A., Kryukov, V. Y., Yaroslavtseva, O. N., Grizanova, E. V, Greig, C., Mukherjee, K., Vilcinskas, A., Mitkovets, P. V, Glupov, V. V, Butt, T. M. (2013) More than a colour change: insect melanism, disease resistance and fecundity. *Proc. R. Soc. B* **280**: 20130584. doi:10.1098/rspb.2013.0584

136. González-Santoyo, I., Córdoba-Aguilar, A. (2012) Phenoloxidase: a key component of the insect immune system. *Entomol. Exp. Appl.* **142**: 1–16. doi:10.1111/j.1570-7458.2011.01187.x

137. Schmid, M. R., Brockmann, A., Pirk, C. W. W., Stanley, D. W., Tautz, J. (2008) Adult honeybees

(*Apis mellifera* L.) abandon hemocytic, but not phenoloxidase-based immunity. *J. Insect Physiol.* **54**: 439–444. doi:10.1016/j.jinsphys.2007.11.002

++138. Engel, P., Bartlett, K. D., Moran, N. A. (2015) The bacterium *Frischella perrara* causes scab formation in the gut of its honeybee host. *mBio* **6**: e00193-15. doi:10.1128/mBio.00193-15

139. Drapeau, M. D., Albert, S., Kucharski, R., Prusko, C., Maleszka, R. (2006) Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees. *Genome Res.* **16**: 1385–1394. doi:10.1101/gr.5012006

140. Ferguson, L. C., Green, J., Surridge, A., Jiggins, C. D. (2011) Evolution of the insect *yellow* gene family. *Mol. Biol. Evol.* **28**: 257–272. doi:10.1093/molbev/msq192

141. Tayler, A., Heschuk, D., Giesbrecht, D., Park, J. Y., Whyard, S. (2019) Efficiency of RNA interference is improved by knockdown of dsRNA nucleases in tephritid fruit flies. *Open Biol.* **9**: 190198. doi:10.1098/rsob.190198

142. Spit, J., Philips, A., Wynant, N., Santos, D., Plaetinck, G., Vanden Broeck, J. (2017) Knockdown of nuclease activity in the gut enhances RNAi efficiency in the Colorado potato beetle, *Leptinotarsa decemlineata*, but not in the desert locust, *Schistocerca gregaria*. *Insect Biochem. Mol. Biol.* **81**: 103–116. doi:10.1016/j.ibmb.2017.01.004

143. Guan, R. B., Li, H. C., Fan, Y. J., Hu, S. R., Christiaens, O., Smagghe, G., Miao, X. X. (2018) A nuclease specific to lepidopteran insects suppresses RNAi. *J. Biol. Chem.* **293**: 6011–6021. doi:10.1074/jbc.RA117.001553

144. Kennedy, S., Wang, D., Ruvkun, G. (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* **427**: 645–649. doi:10.1038/nature02302

145. Simmer, F., Tijsterman, M., Parrish, S., Koushika, S. P., Nonet, M. L., Fire, A., Ahringer, J., Plasterk, R. H. A. (2002) Loss of the putative RNA-directed RNA polymerase RRF-3 makes *C. Elegans* hypersensitive to RNAi. *Curr. Biol.* **12**: 1317–1319. doi:10.1016/S0960-9822(02)01041-2

146. Zhuang, J. J., Hunter, C. P. (2011) Tissue specificity of *Caenorhabditis elegans* enhanced RNA interference mutants. *Genetics* **188**: 235–237. doi:10.1534/genetics.111.127209

147. Katoch, R., Thakur, N. (2012) Insect gut nucleases: a challenge for RNA interference mediated insect control strategies. *Int. J. Biochem. Biotechnol.* **1**: 198–203.

148. Spellberg, M. J., Ii, M. T. M. (2015) FOXO regulates RNA interference in *Drosophila* and protects from RNA virus infection. *Proc. Natl. Acad. Sci. U.S.A.* **112**: 14587–14592. doi:10.1073/pnas.1517124112

149. Pavelec, D. M., Lachowiec, J., Duchaine, T. F., Smith, H. E., Kennedy, S. (2009) Requirement for the ERI/DICER complex in endogenous RNA interference and sperm development in *Caenorhabditis elegans*. *Genetics* **183**: 1283–1295. doi:10.1534/genetics.109.108134

150. Steele, M. I., Kwong, W. K., Whiteley, M., Moran, N. A. (2017) Diversification of Type VI Secretion System Toxins Reveals Ancient Antagonism among Bee Gut Microbes. *mBio* **8**: e01630-17. doi:10.1128/mBio.01630-17

151. Wassarman, K. M., Storz, G. (2000) 6S RNA Regulates E. coli RNA Polymerase Activity. *Cell* **101**: 613–623. doi:10.1016/S0092-8674(00)80873-9

152. Pletnev, P., Osterman, I., Sergiev, P., Bogdanov, A., Dontsova, O. (2015) Survival guide: *Escherichia coli* in the stationary phase. *Acta Naturae* **7**: 22–33. doi:10.32607/20758251-2015-7-4-22-33

153. Rodenbusch, S. E., Hernandez, P. R., Simmons, S. L., Dolan, E. L. (2016) Early engagement in course-based research Increases graduation rates and completion of science, engineering, and mathematics degrees. *CBE—Life Sci. Educ.* **15**: ar20. doi:10.1187/cbe.16-03-0117

154. Beckham, J. T., Simmons, S. L., Stovall, G. M., Farre, J. (World Scientific, 2015). The Freshman Research Initiative as a model for addressing shortages and disparities in STEM engagement. in *Dir. Math. Res. Exp. Undergraduates* **40**: 181–212. doi:10.1142/9789814630320_0010

155. Horn, T., Boutros, M. (2010) E-RNAi: A web application for the multi-species design of RNAi reagents-2010 update. *Nucleic Acids Res.* **38**: 332–339. doi:10.1093/nar/gkq317

156. Deatherage, D. E., Barrick, J. E. (2014) Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using *breseq*. *Methods Mol. Biol.* **1151**: 165–188. doi:10.1007/978-1-4939-0554-6_12

⁺⁺157. Kwong, W. K., Medina, L. A., Koch, H., Sing, K. W., Soh, E. J. Y., Ascher, J. S., Jaffé, R., Moran, N. A. (2017). Dynamic microbiome evolution in social bees. *Sci. Adv.* **3**: e1600513. doi:10.1126/sciadv.1600513

158. Gill, R. J., Ramos-Rodriguez, O., Raine, N. E. (2012) Combined pesticide exposure severely affects individual-and colony-level traits in bees. *Nature* **491**: 105–108. doi:10.1038/nature11585

159. Hanauer, D. I., Dolan, E. L. (2014) The project ownership survey: measuring differences in scientific inquiry experiences. *CBE—Life Sci. Educ.* **13**: 149–158. doi:10.1187/cbe.13-06-0123

⁺160. Suárez, G. A., Renda, B. A., Dasgupta, A., Barrick, J. E. (2017) Reduced mutation rate and increased transformability of transposon-free *Acinetobacter baylyi* ADP1-ISx. *Appl. Environ. Micriobiol.* **83**: e01025-17. doi: 10.1128/AEM.01025-17.

⁺161. Deatherage, D. E., Leon, D., Radriguez, Á. E., Omar, S. K., Barrick, J. E. (2018) Directed evolution of *Escherichia coli* with lower-than-natural plasmid mutation rates. *Nucleic Acids Res.* **46**: 9236-9250. doi: 10.1093/nar/gky751.

⁺162. Gutierrez, A. E., Shah, P., Rex, A. E., Nguyen, T. C., Kenkare, S. P., Barrick, J. E., Mishler, D. M. (2019) Bioassay for determining the concentrations of caffeine and individual methylxanthines in complex samples. *Appl. Environ. Microbiol.* **85**: e01965-19. doi: 10.1128/AEM.01965-19.

⁺163. Suárez, G. A., Dugan, K. R., Renda, B. A., Leonard, S. P., Gangavarapu, L. S., Barrick, J. E. (2020) Rapid and assured genetic engineering methods applied to *Acinetobacter baylyi* ADP1 genome streamlining. *Nucleic Acids Res.* **48**: 4585-4600. doi: 10.1093/nar/gkaa204.

⁺⁺164. Powell, J. E., Eiri, D., Moran N. A., Rangel J. (2018) Modulation of the honey bee queen microbiota: Effects of early social contact. *PLoS One* **13**: e0200527. doi: 10.1371/journal.pone.0200527.

⁺⁺165. Kwong, W. K., Zheng, H., Moran, N. A. (2017) Convergent evolution of a modified acetate-driven TCA cycle in bacteria. *Nat. Microbiol.* **2**: 17067.

⁺⁺166. Zheng, H. A., Nishida, W. K., Kwong, H., Koch. P., Engel, M. I., Steele, M. I., Moran, N. A. (2016) Metabolism of toxic sugars by the bee gut symbiont, *Gilliamella apicola*. *mBio* **7**: e01326-16.

⁺⁺167. Powell, J. E., Ratnayeke, N., Moran, N. A. (2016) Strain diversity and host specificity in a specialized gut symbiont of honey bees and bumble bees. *Mol. Ecol.* **25**: 4461-4471.

⁺⁺168. Schwarz, R. S., Moran, N. A., Evans, J. D. (2016) Early gut colonizers shape parasite susceptibility and microbiota composition in honey bee workers. *Proc. Natl. Acad. Sci. U.S.A.* **113**: 9345-9350.

⁺⁺169. Tian, B., Moran, N. A. (2016) Genome sequence of *Hafnia alvei* bta3_1, a bacterium with antimicrobial properties isolated from honey bee gut. *Genome Announc.* **4**: e004399-16.

⁺⁺170. Dainat, B. et al. 2016 (33 authors total). The bee microbiome: Impact on bee health and model for evolution and ecology of host-microbe interactions. *mBio* **7**: 02184-15.

⁺⁺171. Kwong, W. K., Moran, N. A. (2016) *Apibacter adventoris* gen. nov., sp. Nov., a member of the phylum Bacteroidetes isolated from honey bees. *Int. J. Syst. Evol. Microbiol.* **66**: 1323-1329.

++172. Moran, N. A., Sloan, D. B. (2015) The hologenome concept—helpful or hollow? *PLoS Biol.* **13**: e1002311. doi: 10.1371/journal.pbio.1002311.

++173. Li, J., Powell, J. E., Guo, J., Evans, J. D., Wu, J., Williams, P., Lin, Q., Moran, N. A., Zhang, Z. (2015) Two gut community enterotypes recur in diverse bumblebee species. *Curr. Biol.* **25**: R652-R653.

++174. Moran, N. A. (2015) Genomics of the honey bee microbiome. *Curr. Opin. Insect Sci.* **10**: 22-28.

++175. Kwong, W., Moran, N. A. (2015) Evolution of host specialization in gut microbes: The bee gut as a model. *Gut Microbes* **6**: 214-220.

++176. Kwong, W., Manenido, A., Moran, N. A. (2014) Genome sequences of *Lactobacillus* spp. Strains wkB8 and wkB10, members of the 'Firm-5' clade, from honey bee guts. *Genome Announc. **2***: e01176-14. doi: 10.1128/genomeA.01176-14.

++177. Powell, J. E., Martinson, V. G., Urban-Mead, K., Moran, N. A. (2014) Routes of acquisition of the gut microbiota of *Apis mellifera*. *Appl. Environ. Microbiol.* **80**: 7378-7387. doi: 10/1128/AEM.01861-14.

++178. Engel, P., Stepanauskas, R., Moran, N. A. (2014) Hidden diversity in honey bee gut symbionts revealed by single-cell genomics. *PLoS Genet.* **10**: e1004596. doi: 10.1371/journal.pgen.1004596.

++179. Martinson, V., Majoc, T., Koch, H., Salzberg, G. L., Moran, N. A. (2014) Genomic features of a bumble bee symbiont reflect its host environment. *Appl. Environ. Microbiol.* **80**: 3793-3780. doi: 10.1128/AEM.00322-14.

++180. Moran, N. A. (2015) Genomics of gut microbial communities in honey bees and bumble bees. *Antenna: Bulletin of the Royal Entomological Society of London*.

++181. Cariveau, D., Powell, J. E., Koch, H., Winfree, R., Moran, N. A. (2014) Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*). *ISME J.* **8**: 2369-2379. doi: 10.1038/ismej.2014.68.

++182. Engel, P. E., James, R., Koga, R., Kwong, K., McFrederick, Q., Moran, N. A. (2013) Standard methods for research on *Apis mellifera* gut symbionts. *J. Apicultural Research*. **37**: 699-735.

++183. Engel, P. E., Kwong, W., Moran, N. A. (2013) *Frischella perrara*, gen. nov., sp. Nov. a gammaproteobacterium isolated from the gut of *Apis mellifera*. *Int. J. Syst. and Evol. Microbiol.* **63**: 3646-3651.

++184. Engel, P., Moran, N. A. (2012) Functional and evolutionary insights into the simple yet specific guy microbiota of the honey bee from metagenomic analysis. *Gut Microbes* **4**: 60-65.

++185. Tian, B., Fadhil, N. H., Powell, J. E., Kwong, W. K., Moran, N. A. (2012) Long-term exposure to antibiotics has caused accumulation of resistance determinants in the gut microbiota of honey bees. *mBio* **3**: e00377-12.

++186. Sabree, Z. L., Hansen, A. K., Moran, N. A. (2012) Independent studies using deep sequencing resolve the same set of core bacterial species dominating gut communities of honey bees. *PLoS ONE* **7**: e41250.

++187. Moran, N. A., Hansen, A. K., Powell, J. E., Sabree, Z. (2012) Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLoS ONE*. **7**: e36393.

++188. Engel, P., Martinson, V. G., Moran, N. A. (2012) Functional diversity within the simple gut microbiota of the honey bee. *Proc. Natl. Acad. Sci. U.S.A.* **109**: 11002-11007.