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Standardized protocol for collecting bee samples for internal parasite and pathogen data

James P. Strange[1*], Margarita M. López-Uribe[2], Liam Whiteman[3], Bryan N. Danforth[4], Shalene Jha[5], Hannah K. Levenson[6], Brianne Du Clos[7], Jonathan Berenguer Uhuad Koch[8], & S. Hollis Woodard[9]

Abstract. Internal parasites and pathogens have not been a focus of wild bee systematic data collection efforts to-date but are important to document because they have been strongly linked to bee declines. Here, we provide a standardized protocol for collecting fresh bee tissue samples for generating parasite and pathogen data. The protocol emphasizes appropriate handling and storage conditions and data standards. It can be embedded within bee health monitoring projects or used by individual data collection efforts that aim to generate parasite and pathogen data now and in the future. This protocol is part of a series developed in association with the U.S. National Native Bee Monitoring Network to standardize bee monitoring practices.

INTRODUCTION

A wide diversity of parasites and pathogens infect bees (Fünfhaus *et al.*, 2018; Hristov *et al.*, 2020; Evans *et al.*, 2023; Figueroa *et al.*, 2023;) and there is increasing interest in the roles they play in influencing wild bee population dynamics, including decline (López-Uribe *et al.*, 2020). Parasite and pathogen population dynamics are also closely aligned with host population dynamics; thus, co-monitoring of bee hosts and

[1] Department of Entomology, The Ohio State University, Columbus, OH, 43210, USA (strange.54@osu.edu)
[2] Department of Entomology, Pennsylvania State University, University Park, PA, 16802, USA (mml64@psu.edu)
[3] Department of Entomology, The Ohio State University, Columbus, OH, 43210, USA (whiteman.70@osu.edu)
[4] Department of Entomology, Cornell University, Ithaca, NY, 14850, USA (bnd1@cornell.edu)
[5] Integrative Biology Department, University of Texas at Austin, Austin, TX, 78712, USA (sjha@austin.utexas.edu)
[6] Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC, 27695, USA (hklevens@ncsu.edu)
[7] Louisiana Universities Marine Consortium, Chauvin, LA, 70344, USA (bduclos@lumcon.edu)
[8] U.S. Department of Agriculture, Agricultural Research Service, Pollinating Insects Research Unit, Logan, UT, 84322, USA (jonathan.koch@usda.gov)
[9] Department of Entomology, University of California, Riverside, CA, 92521, USA (hollis.woodard@ucr.edu)
*corresponding Author
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their parasites and pathogens can bring about a fuller understanding of changes in populations. In addition to wild bee species-specific parasites and pathogens, disease outbreaks in commercially managed bees in North America (including the non-native honey bee, *Apis mellifera* L., and native bee species such as *Bombus occidentalis* Greene) have raised concerns regarding pathogen spillover into wild bee populations (Colla *et al.*, 2006; Arbetman *et al.*, 2013: Fürst *et al.*, 2014; Deutsch *et al.*, 2023; Strange *et al.*, 2023). Although the connection is not fully understood, higher prevalence of parasites and pathogens have been detected in declining bee species (Cameron *et al.*, 2011; Cordes *et al.*, 2012; Hristov *et al.*, 2020; Figueroa *et al.*, 2023).

Here, we first briefly outline the diversity and natural history of common internal macro- and micro- bee parasites and pathogens that occur across a broad diversity of bee taxa. We emphasize that future work is warranted on this topic, specifically on how particular parasites and pathogens impact wild species, for which there is a paucity of data. We provide a protocol and associated data standards to aid with collecting individual bee tissue for parasite and pathogen analysis. The protocol specifically addresses sample collection and storage methods, rather than sampling design or downstream handling or analyses, as these details can differ greatly depending on the aims of a specific project or study. Although it can be used as part of any wild bee data collection effort, the protocol is specifically provided with an eye towards wild native bee monitoring and not for sampling managed bees where disease monitoring protocols already exist (*e.g.*, Dietemann *et al.*, 2013). Similarly, we focus on primarily internal parasites and not kleptoparasites or social parasites in detail as these are often handled as individual specimens.

OVERVIEW OF PATHOGENS AND PARASITES AFFLICTING WILD BEES

VIRUSES: To date, nearly all bee-associated viruses were first described in honey bees (Grozinger & Flenniken, 2019; Figueroa *et al.*, 2023). Although detections of these viruses frequently occur in other bee species, there is little information regarding the route of transmission, symptoms, or consequences of infection for most non-*Apis* species (McArt *et al.*, 2014; Figueroa *et al.*, 2019). Indeed, non-*Apis* bees generally show significantly lower prevalence and titers of viruses than honey bees (Evison *et al.*, 2012; Jones *et al.*, 2021; Levenson & Tarpy, 2022). Cross-transmission is likely, given that co-occurring bee species share similar viral profiles (McMahon *et al.*, 2015; Tehel *et al.*, 2020), and transmission dynamics within and among species are still being discovered (Alger *et al.*, 2019a,b; Burnham *et al.*, 2021). Common bee viruses found in both honey bees and wild bees include deformed wing virus, sacbrood virus, and black queen cell virus. Because many of the viruses are single-stranded RNA viruses that break down quickly and can become undetectable in a deceased host (Grozinger & Flenniken, 2019), particular care must be taken when sampling bees for viral presence (see Sample Collection and Storage below).

FUNGAL PARASITES: Several groups of fungal symbionts can form pathogenic relationships with bees (Evison *et al.*, 2012; Evison & Jensen, 2018). Two primary groups have received the most attention in bees. The Microsporidian genus *Nosema* (Microsporidia: Nosematidae) is an important group of pathogens found in both commercial (*Apis* Linneaus, *Bombus* Latreille, etc.) and wild bees. A recent generic name change to *Vairimorpha* was proposed; however, we refer to this group as *Nosema* in this document following the suggestion of the Society for Invertebrate Pathology

(Bartolomé *et al.,* 2024). Several *Nosema* species are known to infect bees, chiefly *N. bombi* Fantham & Porter, *N. apis* Zander, and *N. ceranae* Fries (Grupe & Quandt, 2020; Deutsch *et al.,* 2023; Figueroa *et al.,* 2023). Another microsporidian, *Antonospora scoticae* Fries *et al.,* has been isolated from *Andrena scotica* Perkins, underscoring the potential for continued discovery of novel parasites and limited knowledge of parasite diversity across bee taxa (Fries *et al.,* 1999). The genus *Ascosphaera* (Ascomycota: Eurotiomycetes: Ascosphaerales) has several members with associations with numerous bee species (Klinger *et al.,* 2013; Evison & Jensen, 2018). Both *Nosema* and *Ascosphaera* are primarily known from studies of managed bees, but research into associations with and impacts on wild bees is growing (Deutsch *et al.,* 2023; Figueroa *et al.,* 2023; LeCroy *et al.,* 2023). *Nosema* spp. are primarily detected through dissection of gut tissues followed by microscopic observation, PCR based screening, or qPCR quantification. *Ascosphaera* spp. infections largely impact developing brood (Klinger *et al.,* 2013), thus detections are generally made via inspection of the nest; however, PCR detection has been implemented with adult bees (Evison *et al.,* 2012).

Bacterial parasites: Bee-associated bacteria appear to be largely commensal or beneficial; however, some important diseases of honey bees are bacterial and thus it is reasonable to expect that other bee species encounter pathogenic bacteria. American and European Foulbrood (*Paenibacillus larvae* White and *Melissococcus plutonius* White, respectively) are devastating bacterial infections of honey bees (Fünfhaus *et al.,* 2018) and the causative agents have recently been reported in other bees, although disease has not (Deutsch *et al.,* 2023). *Spiroplasma* spp. have been described in honey bees, bumble bees, mason bees, and squash bees (Schwartz *et al.,* 2014; Fünfhaus *et al.,* 2018; Jones *et al.,* 2022). These bacteria may occur intra- or extracellularly with corresponding differences in pathology. Detection is generally done with a combination of microscopy, PCR-based detection, or qPCR quantification (Schwartz *et al.,* 2014).

Protozoan parasites: Several groups of Protozoa impact bee populations. *Apicystis bombi* (Liu, Macfarlane & Pengelly) (Apicomplexa: Neogregarinorda: Lipotrophidae) is highly virulent and occurs in all life stages of bumble bees (Lipa & Triggiani, 1996). Introduced in South America by commercial *Bombus terrestris* L. colonies, it spilled over to wild *B. dahlbomii* Guérin-Méneville populations, contributing to their rapid decline (Rutrecht & Brown, 2008; Arbetman *et al*., 2013). Furthermore, undetermined Neogregarines have been described in a diversity of wild bees in North America (Figueroa *et al.,* 2021), emphasizing the need for new species discovery in a diversity of host taxa. Three genera of trypanosomes, *Crithidia* Léger, *Leptomonas* W. S. Kent, and *Lotmaria* spp. (Euglenozoa: Kinetoplastea: Trypanosomatidae), are highly prevalent in bees viscera (Jones *et al.,* 2022; Figueroa *et al*., 2023). Detection of protozoan parasites is typically done with microscopy, PCR-based detection, or qPCR quantification.

Other parasites: A variety of multicellular organisms are known to cause pathology in bees (Sammataro *et al.,* 2000; Evans *et al.,* 2023). A rich fauna of mites, both internal and external, is known to inhabit bees and their nests. Several parasitic wasps, nematodes, and flies have been recorded from across bee taxa (Evans *et al.,* 2023). Detection varies, but is generally through microscopic examination, either of the external cuticle of the bee or the bee nest, or through dissection and microscopic examination. PCR is frequently used to verify species identity of immature parasitoids.

SAMPLING DESIGN

The sampling design employed for generating parasite and pathogen data is dependent on the goals of a study or project. Consultation with both an insect pathologist and the laboratory performing subsequent analyses is highly recommended prior to carrying out any sampling. For example, sample sizes are dependent on the prevalence of a pathogen within the study population, which should be estimated prior to collecting because this will influence the depth of recommended sampling. Low prevalence pathogens may require large sample sizes for detection; for example, if prevalence is low (*e.g.*, < 5%), numbers of individuals in the hundreds would be required to quantify parasite or pathogen prevalence accurately. Pathogens and parasites can also be life stage- (Klinger *et al.*, 2013), caste- (Poinar & Van der Laan, 1972; Kapheim *et al.*, 2015), and tissue-specific (Larsson, 2007; Otti & Schmid-Hempel, 2007), making it important to collect the appropriate samples for the target pathogen. The time of year in which samples are collected is also important when designing studies to include pathogen monitoring. This is especially true for social or semi-social species with discrete brood cycles (Graystock *et al.*, 2020). Due to the dynamic nature of host-parasite interactions, pathogen and parasite monitoring, paired with host-population monitoring, using repeated longitudinal sampling (*e.g.*, sampling of a population at regular temporal intervals across a season) is necessary to reveal true patterns of prevalence and host choice over time (Moussy *et al.*, 2022; Cardoso *et al.*, 2022). A single sampling event is a snapshot that offers little context; regular sampling events provide finer resolution to the host-parasite dynamic. Moreover, individual laboratory protocols for pathogen or parasite analysis can vary (Levenson & Tarpy, 2022) and may require special handling considerations in the field to ensure data quality. Note that there are also important considerations about the functional significance of pathogens that require guidance from insect pathologists. For example, detection of a parasite does not necessarily equate to a fitness effect on the host (Tehel *et al.*, 2020), and this needs to be considered when interpreting the findings of pathogen quantification studies based on prevalence data.

SAMPLE COLLECTION AND STORAGE

LETHAL SAMPLING: Proper handling and storage of specimens is critical to achieve accurate detection and quantification of pathogens and parasites. Passive traps, such as bowl traps, which often collect multiple individual bees, should not be used for studies focused on pathology. This is because these traps create conditions that do not adhere to the standards outlined in this protocol, which are the minimum standards for treating tissue intended for generating high-quality parasite and pathogen data. Moreover, traps that capture multiple individuals of a species or multiple different species create a risk of contamination for pathology work, if the goal is to quantify pathogens per individual. A core component of this protocol is that individual bees are collected with tubes, vials, or forceps that are sterilized using 10% bleach solution, > 70% ethanol, flame, or another appropriate method of sterilization. Freshly sterilized tools need to be used for each bee specimen to avoid cross-contamination, and all samples (whole bees or tissue) collected into plastic bags or sterile tubes or vials (*e.g.*, glass scintillation vials for ease of observation and storage). Repeated use of uncleaned nets is a potential source of contamination of specimens, although no specific evidence of this exists. We recommend disinfecting nets (*e.g.*, bleach, hot water, detergent)

regularly to avoid pathogen spread between sites and to minimize potential specimen contamination; a simple method for this is spraying ethanol on nets and laundering nets between site visits. This is also a best practice for minimizing the spread of invasive weeds among sites.

Most tissue collection methods for parasite and pathogen analysis involve collecting live tissue, maintaining it under some temporary storage conditions, then processing it immediately with microscopy, nucleic acid (DNA/RNA) extraction, or placing it at ultra-cold (*e.g.*, frozen at -80°C or below) temperatures for long-term storage. Diseased tissue may be collected from nests and care should be taken to limit transfer of disease to healthy brood in the nest site. Ultra-cold temperatures can be achieved by placing tissue on dry ice (~-78°C), directly into liquid nitrogen (~-196°C), into a liquid nitrogen-charged dry shipper, or placed directly into an ultra-cold freezer (~-80°C). The time between when samples are first collected and subsequently transferred to their long-term storage conditions, and the temperature at which they are kept during this intermediate time, are especially important for parasite and pathogen studies. A core component of this protocol is to record and report this information. As a rule of thumb, the more one reduces the time to freezing and the temperature of storage, the more accurate detection and quantification are both with microscopy and PCR-based techniques. The optimal method, when using a lethal sampling approach, is to flash-freeze the entire bee as early as possible so that there is no time between field collection and long-term storage. This minimizes the suffering of the bees (Gibbons *et al.*, 2022) and results in tissue that can be used for the maximum number of purposes (*e.g.*, isolation of high-quality DNA, RNA, and RNA viruses). A notable exception is the detection and study of trypanosomes using microscopy, which is best done with fresh, unfrozen tissue because the movement of live trypanosomes greatly aids in detection and quantification. If immediate freezing is not possible, bees can be kept alive between 0–10°C for up to 24 hours before long-term storage. For bees that have been collected and are temporarily stored at room temperature the maximum is 12 hours until they enter long-term storage; note that in warmer temperature conditions, or in direct sunlight, bees can perish quickly (< 1 hour) in collection vials if they are not kept on ice.

As an alternative to handling and storing tissue at cold temperatures, tissue can also be collected and stored in fixing substances that preserve them for parasite and pathogen analyses. These other methods can be more practical in some situations, such as remote fieldwork, where ultra-cold temperatures are not accessible. Ethanol (> 95%) can be used for long-term storage, but samples stored in ethanol cannot be reliably used for RNA extraction, visualization of some, or culturing of most pathogens in the future, whereas flash-freezing followed by maintenance at or below -80°C would allow for the future use of these methods. Additionally, specimens stored in ethanol for microscopy benefit from cold storage at -20°C or below to ensure high quality tissue for dissections. As an alternative to ethanol, tissues can be collected into and stored in quaternary ammonium salt solutions such as RNA*later*™ (Ambion, Applied Biosystems), which preserves RNA for one day at 37°C, one week at 25°C, one month at 4°C, or indefinitely at -20°C. RNA*later* also preserves DNA for a longer duration than RNA and is compatible with microscopy and potentially cell culture (Van Eijsden *et al.*, 2013). Note that when entire bees are collected into or stored in these fixing substances (ethanol, RNA*later*), specimen curation and species identification can be difficult because these substances can alter tissue integrity over time. Ethanol storage can make tissues brittle and indistinguishable, whereas RNA*later* will precipitate salt

crystals onto the bee cuticle in storage and may make subsequent identification difficult (Strange *pers. obvs.*). Crystals can be dissolved again by heating to 37°C, but this may impact downstream molecular analyses of pathogens.

NON-LETHAL SAMPLING: Effective, high-throughput methods are being developed for collecting tissue non-lethally for parasite and pathogen analyses (Tissier *et al.*, 2024). At this time, however, these non-lethal methods are still in development and are not field-ready for most projects, and there are some costs that must be considered. First, sampling time will be much greater than if collecting whole bee specimens and may be prohibitive based on sampling design and objectives. Second, the methodology for non-lethal pathogen sampling is not fully developed, although active research is being conducted (Tissier *et al.*, 2024). Equipment for sampling non-lethally may also vary from studies collecting lethal samples. The utility of fecal samples to detect parasitism by nematodes, parasitic wasps, and parasitoid flies is unknown; thus non-lethal samples may provide an incomplete picture of parasitism. Finally, while pathogens have been detected in non-lethal samples, correlation of pathogen loads in fecal samples to pathogen loads in the gut tissue is not known. Given the overwhelming evidence that parasites and pathogens can be key regulators of bee population dynamics, including those in decline (Cameron *et al.*, 2011; Cordes *et al.*, 2012; Hristov *et al.*, 2020; Figueroa *et al.*, 2023), we advise that protocol users consider balancing the considerable value of collecting samples lethally for parasite and pathogen analysis with any downsides of lethal collection, such as unintentionally over-collecting and causing harm to wild populations.

If used, non-lethal tissue sampling should follow the sample collection and storage requirements outlined above, with the exception that the live bee being sampled is released once the non-lethal sample (*e.g.*, tissue, fecal matter) is collected. Upon collection, non-lethal tissue samples should be processed for microscopy or DNA/RNA extraction or placed in storage (frozen, ethanol, RNA*later*) as directed for lethal sampling.

VOUCHERING: Vouchering is especially important for studies where bees are not maintained as specimens in their entirety both in lethal and non-lethal sampling. If specimens are sampled "non-destructively" (*i.e.*, if a single body part or tissue type is removed and destroyed, but the remainder of the sampled specimen has been maintained), and can still be used to confirm species identification, then the remaining specimen can become the voucher. In this case, vouchers should be pinned and labeled to be clearly associated with the tissue sample used in the analysis and their deposition information reported in publications (Montero-Castaño *et al.*, 2022). Alternatively, extracted DNA can be stored as a voucher that can be used to determine species identity. If samples are used destructively or tissue samples are collected non-lethally (see above), photographic vouchers can be used instead. In this case, collecting photographs of the entire individual and parts of the body that will confirm identification is critical (see Cariveau *et al.*, 2024). We also recommend collecting a set of individuals of the same species to be maintained as pinned voucher specimens that are representatives of the species sampled during the collection event.

ADDITIONAL BEST PRACTICES

Where possible, we recommend 'banking' a subset of samples in ultra-cold storage (at or below -80°C) for potential future use, rather than using all samples destructively.

This is because novel pathogens can be difficult to detect in historical samples that are not stored appropriately.

We also recommend collecting and reporting information about honey bees or other managed bees from any sites included in wild bee pathogen studies. This may include the presence or density of managed bees, or the quantification of shared resource use (as per Page & Williams, 2023). Additionally, we recommend collecting a sample of managed bees present at a site, as these can be used for pathogen or parasite detection.

METADATA SPECIFIC TO THIS PROTOCOL

Metadata to record for pathogen samples include information about the sterilization methods of collecting equipment, type of tissue(s) sampled, sample handling conditions prior to final storage, time until final storage, final storage conditions, and final storage location (Table 1). The final storage conditions, particularly the temperature at which the sample is stored, should be provided in **dwc:preparations**. All other information can be provided together in the term **dwc:materialEntityRemarks**. When providing multiple pieces of information for one Darwin Core term, separate them with a vertical bar; for example: "collecting equipment sterilized with 10% bleach | sample stored on ice between collection and final storage | 0.8 hour between collection and final storage | stored in Sample Lab" is an appropriate entry for **dwc:materialEntityRemarks**. Although it may seem counterintuitive to provide this much text in one spreadsheet cell, adhering to these practices aligns with the Darwin Core standard (Wieczorek *et al.*, 2012) and promotes reproducibility and utility of the data. Lastly, please be sure to cite this protocol in **dwc:samplingProtocol**. Full details on using these Darwin Core terms are provided in The Wild Bee Data Standard (Du Clos *et al.*, 2024a); examples of proper data entry in spreadsheet templates can be found on Zenodo (Du Clos *et al.*, 2024b).

Table 1. Required data to be recorded when implementing the protocol to adhere to the Wild Bee Data Standard (Du Clos *et al.*, 2024a).

Required Data	Description	Darwin Core Term
Sterilization method	Method used to sterilize equipment between samples. Methods can include bleach (10% is recommended), ethanol (>70% recommended), or another appropriate method of sterilization	dwc:materialEntityRemarks
Sample conditions prior to final storage	Report how the specimen was handled between collection and final storage	dwc:materialEntityRemarks
Time until final storage	Report how long between specimen collection and final storage	dwc:materialEntityRemarks
Final storage conditions	Report how the specimen is stored, particularly the temperature	dwc:preparations
Final storage location	Report where the specimen is stored	dwc:materialEntityRemarks
Type of tissue stored	Report the type of stored tissue (whole body, abdomen, gut, brain, etc.)	dwc:preparations

DISCUSSION

Disease monitoring is a major focus of wildlife monitoring efforts (Morner *et al.*, 2002), and has been performed in managed honey bees (Lee *et al.*, 2015), but has not yet been a focus in wild bee monitoring. This is despite concerns of pathogen spillover (Colla *et al.*, 2006; Strange *et al.*, 2023) and implications of disease in species declines (Cameron *et al.*, 2011; Cordes *et al.*, 2012; Cameron & Sadd, 2020). As interactions between managed bees and wild bees continue to increase, and documented declines of wild bees continue, the urgency for understanding the underlying mechanisms of pathogen and parasite dynamics in bee communities grows. To facilitate this understanding, disease monitoring becomes most powerful when it is paired with population monitoring (Cardoso *et al.*, 2022) and fully integrated into comprehensive bee monitoring programs. Effective monitoring should consider a more targeted approach that prioritizes monitoring wild bee parasites and pathogens based on their ease of transmission and virulence for a given focal bee species, if this is known. Note, however, that surveillance monitoring, which can detect emerging and lesser-understood parasites and pathogens, is also an important component of comprehensive disease monitoring programs.

The urgency of expanding our knowledge and increasing baseline data on pathogens and parasites in bee communities is underscored by past declines in some wild bee species (Cameron *et al.*, 2011). Specifically, declines in several North American bumble bee species in the late 1990s and early 2000s are associated with the pathogen outbreaks in commercial bumble bees during that time (Flanders *et al.*, 2003; Cameron *et al.*, 2011). Despite intensive efforts to link directly the declines to these pathogens (Cordes *et al.*, 2012; Cameron *et al.*, 2016), the lack of baseline data prior to the population declines has prevented definitive answers to the cause of decline. Given that many additional bee species are threatened, or potentially declining, there is an urgent need to collect tissue samples from other bee groups to obtain the information needed to link parasites and pathogens better to declines in these species, if these relationships exist.

There are also foundational knowledge gaps in our understanding of bee symbionts, their functions and/or pathologies, and their dynamics in wild bee populations (Figueroa *et al.*, 2023). For example, recent studies have shown that many parasites and pathogens move among species in bee communities. Yet, little is known about these types of transfers outside of a few case studies (Evison *et al.*, 2012; McArt *et al.*, 2014; McMahon *et al.*, 2015; Evison & Jensen, 2018; Figueroa *et al.*, 2019; Deutsch *et al.*, 2023), and a few well-studied, managed bee species (*A. mellifera*, *B. terrestris*, *B. impatiens* Cresson, *Megachile rotundata* (Fabricius), and *Osmia* spp.) (Graystock *et al.*, 2013; Grupe & Quandt, 2020). Other valuable pieces of information, such as the geographic distribution, favorable environmental conditions, preferred suite of hosts, and impacts of infection, are also unknown for most pathogens found in wild bees. This information is essential to understand population dynamics properly, manage recovery of endangered species, or implement habitat restoration for most bee species. This further underscores the need for more research on parasites and pathogens in wild bees, and greater integration of the sampling approaches we outline into wild bee monitoring projects.

In the future, we anticipate that wild bee monitoring programs will increasingly incorporate pathogen and parasite monitoring. We provide this protocol to support

those efforts by giving researchers information about the best ways to sample tissue to maximize its use for parasite and pathogen analyses. We note also that although our protocol was developed with the goal of advancing wild bee monitoring, it holds equal value for any data collection effort aimed at generating data on wild bee parasites and pathogens. We also foresee an increasing emphasis on non-lethal sampling approaches for the study of wild bee parasites and pathogens, although at present we urge caution using these methods because of their current efficacy and practicality. Ultimately, the goal is to predict and prepare for potential disease outbreaks in at-risk populations and to reduce the role pathogens play in wild bee population declines.

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