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## FECAL SAMPLING PROTOCOL TO ASSESS BUMBLE BEE HEALTH IN CONSERVATION RESEARCH

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**Abstract**—An increasing number of wild bee species are declining or threatened with extinction worldwide. Decline has been proposed to be caused by a combination of threats, including increasing wild bee disease prevalence and pathogen spillover from managed bees that can reduce health of wild bees. Most approaches aiming at characterizing bee health, however, require sacrificing tens to hundreds of individual bees per site or species, with reports of several thousand individuals collected per study. Considering the widespread need to assess bee health, this sampling approach is not sustainable, especially for endangered populations or species. Here, we present a non-destructive protocol to collect bumble bee faeces and assess parasite loads of wild-caught individuals. The standard protocol consists of net-capturing individual bumble bees and placing them in a 10 cm (diameter) petri dish to collect faeces. This fecal screening approach is frequently used in laboratory settings, but much less in the field, which can impair conservation research. When placing bumble bees in a previously refrigerated cooler, we successfully collected faeces for 86% individuals, while the standard protocol, as used in laboratory settings, yielded 76% success in collecting faeces. We also identified cells and spores of two common gut parasites *Crithidia* spp. and *Vairimorpha* spp. in faecal samples. The faecal sampling presented here opens future avenues to assess bee pathogen loads using molecular techniques, while collected faeces could also be used to assess bee health more broadly, including bee microbiota and bee diet.

**Keywords**—wild bees, parasites, feces, non-lethal sampling, *Trypanosomatidae*, *Nosematidae*

### INTRODUCTION

An increasing number of wild bee species are declining worldwide, likely from a combination of stressors, including habitat loss, pesticides, malnutrition, climate change, invasive species, an increasing prevalence of wild bee diseases and

pathogen spillover from managed bees (Cameron et al. 2011; Colla et al. 2012; Graystock et al. 2013; Goulson et al. 2015; Baron et al. 2017; Cameron & Sadd 2020; Botías et al. 2021; Burnham et al. 2021; Aldercotte et al. 2022; Jackson et al. 2022). The impacts of environmental changes on bumble bees (*Apidae: Bombus*), an important group of wild bee

pollinators, are increasingly documented (Cameron et al. 2011; Botías et al. 2021; Guzman et al. 2021; Siviter et al. 2021; Jackson et al. 2022; Su et al. 2022). A growing number of investigations are assessing health of wild bumble bee individuals, colonies, and populations (Giacomini et al. 2018; Cameron & Sadd 2020; McNeil et al. 2020; Pislak Ocepek et al. 2021; Trillo et al. 2021; Tsvetkov et al. 2021; Garlin et al. 2022) and more than 50% of studies examining causes of bumble bee decline considered parasitic infections (Cameron & Sadd 2020).

Conventional approaches to assess the health and pathological state of wild bees consists of assessing disease prevalence and intensity. These approaches increasingly consider other physiological markers of health, using either macroscopic and morphological (e.g., wing morphology, ectoparasites, body mass or size) or physiological and molecular approaches (e.g., assessment of body fat, prevalence and loads of intestinal parasites or presence of viruses in body tissues) (Giacomini et al. 2018; Lopez-Uribe et al. 2020; McNeil et al. 2020; Garlin et al. 2022; Parreno et al. 2022). Whereas morphological approaches provide information about general body condition and overall physical health status of individuals, physiological approaches can assess the identity, prevalence, and abundance of pathogens (pathological status) as well as nutritional, immune, or metabolic markers of health (Lopez-Uribe et al. 2020; Parreno et al. 2022). These approaches, however, require killing a significant number of individuals ( $N = 20$  to 300/site or species) to accurately measure wing or body size/mass, to extract the gut or to grind bodies, before conducting nutrient, microbial or pathogen assessments using microscopic, molecular or biochemical analyses (Blaker et al. 2014; Giacomini et al. 2018; Graystock et al. 2020; Lopez-Uribe et al. 2020; McNeil et al. 2020; Tsvetkov et al. 2021; Babin et al. 2022; Garlin et al. 2022). Considering the increasing number of studies assessing bee health, with around 400 conservation-based studies per year on bumble bees alone (Cameron & Sadd 2020), collecting as many as several thousand individuals per study, this approach is not sustainable and raises conservation concerns (Miller et al. 2022; Montero-Castaño et al. 2022). The conservation impact of repeated and widespread lethal sampling is rarely studied and

merits further investigation (Montero-Castaño et al. 2022). The few studies on the topic report contrasting effects of repeated lethal sampling (Gezon et al. 2015; Gibbs et al. 2017). Although the impact of scientific research may be small compared to human-mediated impacts such as habitat loss, pesticides, pathogen spillover or climate change (Sánchez-Bayo & Wyckhuys 2019; Wagner 2020; Miller et al. 2022; Montero-Castaño et al. 2022), the effects of such repeated destructive samplings are mostly unknown and likely to vary between populations, species or even castes of bees (Montero-Castaño et al. 2022). Furthermore, sampling still remains an additional pressure on pollinators, which could be especially damaging to rare or endangered wild bee species and communities (Miller et al. 2022).

It is thus paramount to develop and implement non-destructive, widely accessible, and reproducible practices that work across species and castes, to monitor bee health in ecology, conservation and agriculture research. A common non-destructive approach to monitor individual health in ecology and conservation consists of focusing on their pathological status, by collecting and screening their faeces to assess the presence of parasites or other pathogens (Darimont et al. 2008; Biswas et al. 2019). Fecal samples could not only be used to assess diseases, but also microbial communities or nutritional state (Koch & Schmid-Hempel 2011; Parreno et al. 2022). In wild bees, two pathogens that are commonly monitored to assess health through pathological status are *Crithidia spp.* (Trypanosomatidae) and *Nosema/Vairimorpha spp.* (Nosematidae) (Cameron & Sadd 2020; Graystock et al. 2020; Grupe & Quandt 2020). Cells or spores of these parasites are horizontally transmitted (i.e., via an oral-faecal route), either through contamination of flowers during foraging or through contamination of the nest (Graystock et al. 2015; Graystock et al. 2020; Grupe & Quandt 2020). They have been identified in a large variety of bee species and genera (Gillespie 2010; Cameron & Sadd 2020; Grupe & Quandt 2020; Figueroa et al. 2021). In bumble bees, infection with parasites have been linked to a reduction in gyne production, queen fitness and colony growth (Brown et al. 2000; Goulson et al. 2018), impair learning and foraging ability of workers (Gegear et al. 2005; Goulson et al. 2018), increase mortality of both males and workers (Otti

& Schmid-Hempel 2007; Grupe & Quandt 2020) and ultimately lead to colony decline under field-realistic stressful conditions (Brown et al. 2000). In addition, *Crithidia* and *Vairimorpha* are common parasites found in managed bees (i.e., commercial bumble bees and domestic honey bees) that raise important concerns regarding pathogen spillover to native and/or wild bees (Grupe & Quandt 2020; Strange et al. 2023), in which they are able to replicate (Ngor et al. 2020), though their pathogenicity remain to be determined for several bee species. Finally, rare and endangered bumble bees are at higher risk of pathogen spillover and show greater prevalence (i.e., rate of infection) of *Vairimorpha* than common and non-threatened species (Gillespie 2010; Cameron et al. 2011; Averill et al. 2021), increasing the need to monitor parasites without causing harm to threatened populations or endangered bumble bee species, such as *B. affinis* or *B. terricola* (Colla & Packer 2008; Jacobson et al. 2018). Here we present the step-by-step development of a non-destructive fecal screening approach to assess the presence of the gut parasites *Crithidia spp.* and *Vairimorpha spp.*, both transmitted through faeces (Cameron & Sadd 2020; Graystock et al. 2020; Grupe & Quandt 2020). The prevalence and loads of these parasites are commonly assessed either on macerated gut extract or on full grinded bodies of worker bees, which requires killing the bees (Blaker et al. 2014; Giacomini et al. 2018; McNeil et al. 2020). We thus adapted a laboratory-validated faecal screening protocol, which consists of placing individual bees inside petri dishes until they defecate (Chen et al. 2006; Gomez-Moracho et al. 2021), to field conditions. Although commonly used under laboratory conditions (Chen et al. 2006; Bailes et al. 2020; Gomez-Moracho et al. 2021) and occasionally under field or semi-natural conditions (Jones & Brown 2014), this method is not commonly deployed in ecological and conservation studies. One reason is that there is no detailed step-by-step protocol of the fecal screening in the field available to allow its use by stakeholders in conservation or agriculture research, and to ensure reproducibility. We present two alternative approaches in this paper: (i) a standard protocol (as used in laboratory settings) where bees are placed in a petri-dish until faeces collection and (ii) an “updated cooler protocol” in which individuals are placed in a dark and cool low-sensory

environment. We measured defecation probability and confirmed the presence of *Crithidia spp.* and *Vairimorpha spp.* in each sample and for each protocol. This methodological paper is a step in the development of a unified non-destructive approach to assess bumble bee health in the field through faeces collection.

## MATERIALS AND METHODS

### EXPERIMENTAL DESIGN: BEE CAPTURES AND FAECES COLLECTION

We gathered data on faecal collection success using two protocols (*standard protocol* or *updated cooler protocol*, two samplings per protocol) implemented during four independent studies conducted on bumble bee health. Although it would have been ideal to compare the success of both methods within the same study, each of these studies was conducted for a separate additional purpose, constraining us to use a single protocol per study for consistency. Nonetheless, all four studies were conducted in summer and under similar meteorological conditions (non-rainy days, similar range of ambient temperatures as detailed below). In addition, we corrected for between-studies variation by including several control variables in our statistical models (see below).

The *standard protocol* consisted of placing bees in a shady environment during faeces collection to prevent thermic stress and limit risk of death (Fig. 1; studies 1 and 2). The *updated cooler protocol* consisted of placing bees in a dark and cool low-sensory environment (studies 3 and 4; see Fig. 2). In all four studies, adult bumble bees were net-captured by hand in the field (see details below). Bees were placed individually in transparent, 10 cm diameter, petri dishes for faeces collection and species identification. Net capturing is an efficient and minimally invasive approach to catch bumble bees (Montero-Castaño et al. 2022; Tronstad et al. 2022). We decided to use petri-dish to minimize the risks of pollen (which can host spores of the investigated parasites) being mixed with fecal samples, which is likely to occur in smaller containers such as vials.

For each bumble bee and in each study, we recorded the site and date of captures (Fig. 1). Each bumble bee was released from the petri dish after all faecal samples were collected. Faecal samples were immediately placed in a standard, low-cost



**Figure 1:** A gyne (*B. impatiens*) placed in a petri dish to obtain a faecal sample (standard protocol).

and widely available portable hard case cooler (12-17 liters, Coleman, USA) with ice packs or on ice (either works for short-term storage of samples) until screening under the microscope (see details for each study). In all four studies described below, if bees did not defecate after a maximum of one hour, they were considered as not having defecated. These bees were released or transferred into a vial and placed on ice until completion of the faecal sampling session at each site, after which all captured bees were released. Most bees defecated within 20min after being placed inside the petri dish, the threshold of one hour was fixed as a common limit to each study after which we could exclude the bee from the study and release it.

#### STUDIES AND SITES

##### a) STANDARD PROTOCOL

###### Study 1

Captures were conducted once a week at five agricultural sites in the vicinity of Lac Brome (Southern Québec, Canada; 45° 12' 18.4752" N and 72° 33' 32.8320" W) from August 6-17, 2020, between 10:00 and 17:00. We conducted three capture events in total: one capture per site per week, and the order of the sites was randomized each week. Ambient temperatures ranged from 17-34°C during sampling sessions. A total of 48 bumble bees were captured during this period. Upon capturing, each bee was immediately placed in an individual petri dish, identified to species, and placed in a shady environment, avoiding

direct exposure to the sun to prevent thermic stress and risk of death. At an ambient temperature of 30°C, bumble bees already have difficulty maintaining their body temperature below the lethal threshold of 45°C (Heinrich 1977). Placing a bee inside a petri dish in the sun when ambient temperatures are above 30°C, without ventilation, exposes the individual to extreme temperatures, likely to be lethal. Bees were maintained in the petri dish in this shady environment until they defecated or for up to 1 hour. None of the bees placed in the shade died during the study, but one bee that had been forgotten in the sun for several minutes by the experimenter died, likely because of overheating. We inspected petri dishes for faeces every 10 min. When faeces were confirmed, we circled the area where faeces were observed on the outside of the petri dish using a marker and then we placed the side with faeces on top by gently turning the petri dish over, to limit destroying the sample due to the bumble bee walking inside. Of the 48 bees captured, we successfully collected faecal samples from 30 bees (62.5% success). Samples were screened for parasites under the microscope within 24 hours after collection as detailed in section “*Identification of Crithidia and Vairimorpha parasites*” below.

###### Study 2

Captures were conducted every other week from 08:00 to 16:00 inside and surrounding commercial greenhouses around Compton

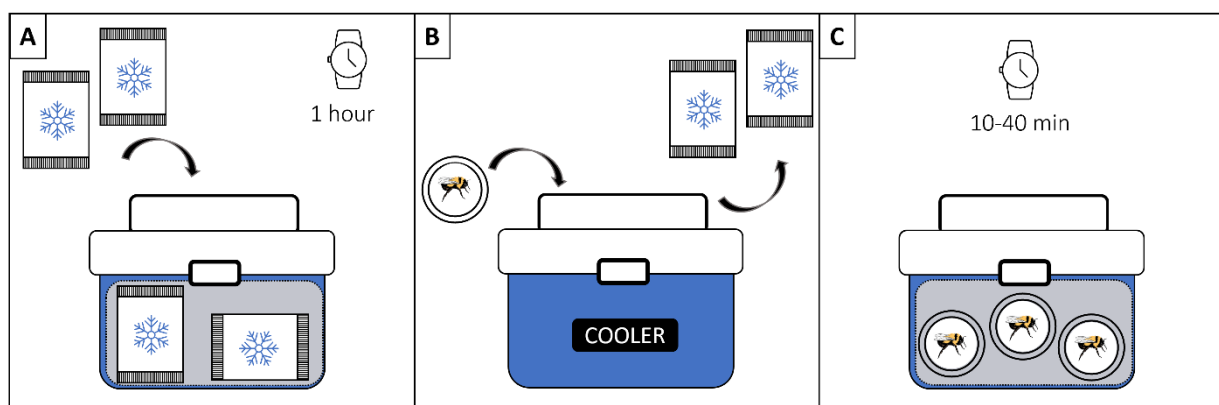
(Southern Québec, Canada, 33° 53' 45.0492" N and 118° 13' 12.2520" W) from April 22<sup>nd</sup> to August 13<sup>th</sup>, 2021. Upon being placed in a petri dish, bumble bees were placed in a shaded environment outside of the greenhouses. Bees were handled as in the standard protocol described in study 1. Two bees died in the petri-dishes during the experiment, likely because of over-heating (these two bees were not in full shade and sunlight reached the petri-dish during faeces collection). Ambient temperatures ranged from 15 to 32°C during sampling sessions. A total of 398 bumble bees were captured, from which we collected 288 faecal samples (72.4% success). Faecal samples were screened for parasites under the microscope within 24 hours after collection using a Neubauer hemocytometer.

b) UPDATED COOLER PROTOCOL: OPTIMIZING FAECAL COLLECTION SUCCESS

Study 3

Captures were conducted once every other week at four agricultural sites in the vicinity of Alma (Québec, Canada; 45° 12' 18.4752" N and 72° 33' 32.8320" W) from June 1<sup>st</sup> to September 9<sup>th</sup>, 2021, between 10:00 and 17:00. Ambient temperatures ranged from 13 to 32°C during sampling sessions. In study 1, faeces collection seemed especially limited in the afternoon, when ambient temperatures could exceed 30°C and some bees displayed signs of thermic stress. We thus developed an updated protocol to optimize faecal screening success (i.e., the probability that a bee

will defecate), using two coolers (Fig. 2) to provide the bumble bees with a cool and dark environment. We first placed two icepacks in one of the coolers for at least 1 hour (Fig. 2A, B). We then started net-capturing bumble bees in the field. As soon as the first bumble bee was captured, we removed the two icepacks from the cooler and placed the petri dish with the bumble bee inside (Fig. 2C). Removing the icepacks is especially important; if bumble bees were placed on the icepacks, no faeces would be obtained due to a major decrease in metabolism. At that step, the icepacks were placed in the second cooler, subsequently used to store faecal samples upon collection. As for the standard protocol described in study 1, we checked the cooler for faeces every 10 min, or every time we placed a new petri dish in the cooler (Fig. 2C). As soon as we observed faeces in a petri dish, we placed the section of the petri dish with the faeces on top by gently turning the petri dish, since we could not release bees immediately to avoid re-capturing them. We marked the area where faeces were observed using a marker. Once faecal collection was completed, all bees were released. A total of 298 bumble bees were captured in this study, on which we collected 256 faecal samples (85.9% success; 6 samples with no information on success collection – marked as NA). No bees died during this experiment. Faecal samples were frozen at -80°C within 24 hours after collection and subsequently analyzed within 1 hour after being defrosted using a Neubauer hemocytometer.



**Figure 2.** Experimental design to collect faeces in the field using coolers. A) Cover the bottom of the cooler with icepacks before initiating bee captures and wait one hour to ensure that they effectively cool the compartment. B) Remove the icepacks as soon as the first bee is captured and placed in a petri dish, and then place the petri dish in the cooler. Store the cooler away from direct sunlight. Removed icepacks can then be placed in a second cooler, which will be used to store petri dishes with faeces upon collection. C) Inspect petri dishes for faeces every 10 min maximum. When faeces are confirmed, place the side with faeces on top by turning the petri dish over, to protect it from trampling by the bee still inside the petri dish.

#### Study 4

Captures were conducted following the updated cooler protocol (Fig. 2) at 13 sites (one survey per site) around Toronto (Southern Ontario, Canada; 43° 39' 3.8520" N and 79° 20' 49.2540" W) from May 1<sup>st</sup> to June 27<sup>th</sup>, 2022. Ambient temperatures ranged from 10 to 26°C during sampling sessions, which lasted up to three hours. No bees died during this experiment. A total of 194 bumble bees were net captured, from which we collected 163 individual faecal samples (84.0% success). Samples were analyzed under the microscope on the day of collection using a Neubauer hemacytometer.

#### IDENTIFICATION OF CRITHIDIA AND VAIRIMORPHA PARASITES

Faecal samples were either frozen at -80°C (study 3) or preserved on icepacks or in the fridge at +4°C until being processed (see details for each study above). While snap-freezing did not seem to influence cell/spore integrity (they were intact under the microscope), it could influence the experimenter's ability to detect *Crithidia* cells, as they are no longer mobile. We thus recommend using the same conservation method (freezing or at +4°C) within the same study, and to adapt it to the experimenter's experience in recognizing *Crithidia* cells. When processing a sample, we added 100 µL of distilled water into the petri dish with a micropipette and homogenized (stirring with the pipette). We then collected the faecal solution, placed it in a 0.5 ml Eppendorf tube on ice, and then shook this sample with a Vortex mixer for 15 seconds. We immediately conducted faecal screening by taking 4 µL of the faecal solution and mounting it on a slide with a coverslip. We analyzed the sample under a compound microscope (phase-contrast, x40 magnification). We recorded the presence/absence of cells and spores of *Crithidia* and *Vairimorpha*, respectively, to assess parasite presence by looking at the entire sample mounted (0 spores = absence and >1 spore = presence). Methods for measuring number of spores/cells varied between studies so it is not possible to compare intensity, whereas presence/absence was analyzed similarly between studies and can be used to validate that cells and spores of these parasites can be detected with this protocol.

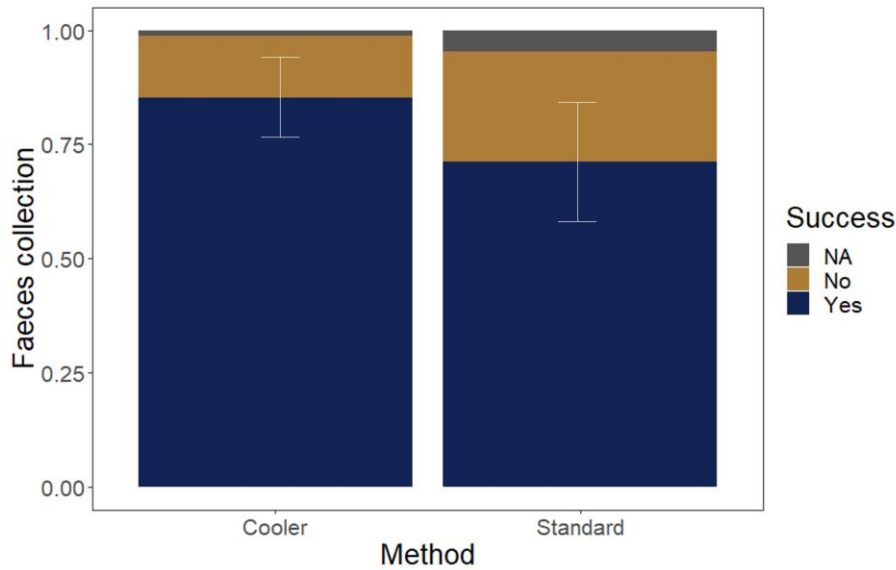
#### STATISTICAL ANALYSES

Using a Generalized Linear Mixed Model (GLMM) with a binomial distribution, we investigated the effect of the protocol (standard *versus* cooler) on the faeces collection success (binary response). Sampling protocol and Julian date (accounting for ambient temperature) were included as fixed effects, while the site and the species were included as random effects. As data presented here were collected as part of four independent studies, compositions of species and castes vary between each study (Table S1). This paper intended to validate a non-destructive protocol and assess the efficacy of the updated cooler protocol to collect adult bumble bee faeces in the field, increase the rate of faeces collection, and validate the presence of *Crithidia* and *Vairimorpha* in faecal samples. For those reasons, we did not perform analyses of protocol vs species or caste, or compare parasite loads or prevalence. However, including the species and sites as a random effect in our model allowed us to control for potential species or landscape effects on faecal collection success. In addition, we ran an alternative model looking at caste effect on faeces collection success, to ensure that results of the model testing for the method were not driven by a difference of defecation rate between queens and workers, considering the different proportion of queens and workers in each study (Table S1). Caste could not be added in the same model because of autocorrelation issues. This alternative model showed no effect of caste on faeces collection success (Table S2) and had a greater AIC than the model we present for testing the protocol ( $\Delta$ AIC = 1.66). Analyses were conducted using SPSS software (IBM SPSS Statistics for WINDOWS v. 24.0. IBM Corp.). Fig. 3 was prepared using the package ggplot2 (function ggplot; R; v4.3.2). The significance threshold was set at  $\alpha < 0.05$ . Data presented are means  $\pm$  SE.

#### RESULTS

##### SUCCESS IN FAECES COLLECTION

We captured and released a total of 938 bumble bees, belonging to 14 species plus cuckoo bumble bees (subgenus *Psithyrus*) that were not identified to species (Table S1). Overall, faeces were collected for 737 of 932 bumble bees (79.1% success rate; information was missing for 6 bees).



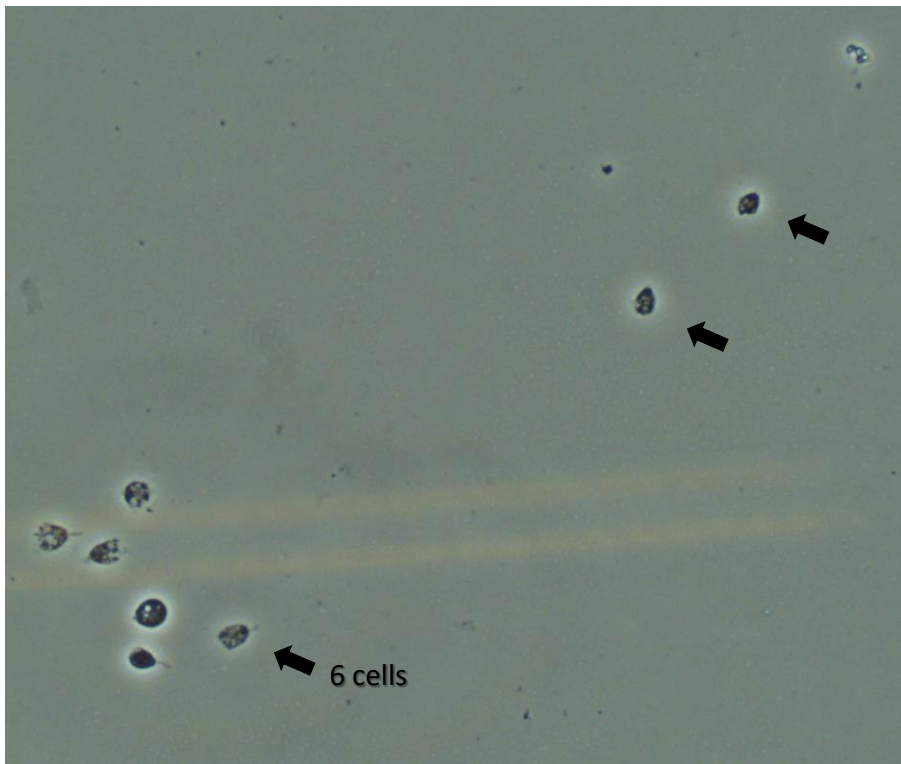
**Figure 3: Probability to collect faeces depending on the protocol.** In the standard protocol, individuals were placed into a petri-dish and set out in a shaded environment in ambient conditions, while in the updated cooler protocol, they were placed in a dark, cool, and low-sensory environment as described in Fig. 2. Raw data are presented as means  $\pm$  SE. The probability to collect faeces was of  $0.76 \pm 0.11$  and  $0.86 \pm 0.07$  in the Standard and Cooler protocols, respectively ( $P = 0.036$ ).

We found no effect of the Julian date on the probability of collecting faeces ( $F_{1,938} = 0.04$ ,  $t = 0.19$ ,  $P = 0.85$ ). However, results of the GLMM revealed a significant effect of the protocol: the cooler protocol significantly increased the probability of collecting faeces compared to the standard protocol ( $F_{1,938} = 4.41$ ,  $t = 2.10$ ,  $P = 0.036$ ; Fig. 3). After accounting for potential species, site and daily variations between each study, faeces collection success probability was 76% ( $0.76 \pm 0.11$ ,

$N = 496$ ) with the standard protocol, and 86% ( $0.86 \pm 0.07$ ,  $N = 436$ ) with the updated cooler protocol.

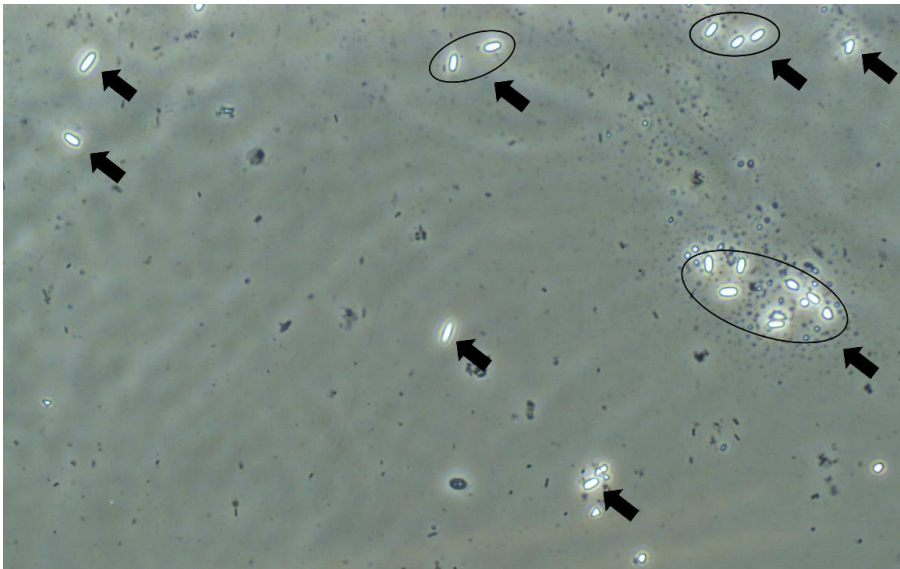
#### IDENTIFICATION OF GUT PARASITES

Cells of *Crithidia* (Fig. 4) and spores of *Vairimorpha* (Fig. 5) were detected in bumble bee faecal samples. The use of the phase contrast was especially important in observing *Vairimorpha* spores. The number of *Crithidia* cells ranged from 0 to 159 per sample, while the number of *Vairimorpha* spores ranged from 0 to 45 per sample.



**Figure 4: *Crithidia* cells observed under a compound microscope.** In this example image, eight cells are observed (Phase contrast,  $\times 40$  magnification).





**Figure 5:** *Vairimorpha* spores observed under a compound microscope. In this example image, 17 spores are observed (Phase contrast, x40 magnification).

## DISCUSSION

We validated a non-destructive approach commonly used in laboratory conditions to collect bumble bee faeces in the field, and proposed a detailed “cooler protocol” to increase faecal screening success in conservation research. We also confirmed the presence of cells of *Crithidia* and spores of *Vairimorpha* in faeces by microscopy. The detection of these pathogens is often used as a marker of bee disease status. However, since we did conduct a direct fecal/gut comparison for infection intensity, caution when using this method to assess pathogen load is warranted, as discussed below.

### OPTIMAL PROTOCOL AND COMPARISON WITH OTHER STUDIES

Using petri dishes to collect bee faeces is a commonly used approach in laboratory settings (Chen et al. 2006; Gomez-Moracho et al. 2021), and more sporadically on wild bees (Jones and Brown, 2014). We adapted this approach to field conditions, and provide a step-by-step protocol in which bumble bees are placed in a dark, cool, and low-sensory environment using a previously refrigerated cooler with no ice in it (Fig. 2). Faeces collection success was estimated at  $76\pm 11\%$  with the standard protocol by our model, and at  $86\pm 7\%$  with the cooler protocol. The updated cooler protocol thus increased our collection success by 10% point and reduced variation compared to the standard protocol. Additionally, the low-cost and widespread availability of the equipment should promote its use in field studies and improve

willingness to sample bees using non-destructive methods. As there are calls to reduce destructive sampling methods in bee research where possible (Miller et al. 2022; Montero-Castaño et al. 2022; Tronstad et al. 2022), especially when studying wild and/or threatened species of bees, using the updated cooler protocol will contribute to achieving this goal.

The number of *Crithidia* cells was highly variable and ranged from 0 to 159 in 0.02  $\mu\text{L}$  of faeces extract diluted by 100  $\mu\text{L}$  of water, which is of the same order of magnitude than what has been reported in several studies on bumble bees using macerated gut extract (Giacomini et al. 2018; LoCascio et al. 2019; Malfi et al. 2023). *Vairimorpha* spore counts ranged between 0 and 45 spores per 0.02  $\mu\text{L}$  diluted faecal sample, thus around 225,000 spores/ $\mu\text{L}$  of undiluted faecal sample, thus within the range of what is usually recorded (Blaker et al. 2014). Nonetheless, using faeces to assess prevalence of *Vairimorpha* spores (i.e., the number of bee individuals infected) has been shown to be reliable in laboratory conditions, although faecal counts yielded more variation than PCR in infection intensity, i.e. number of spores recorded (Gomez-Moracho et al. 2021). In addition, the risk to have false negative cannot be excluded. This non-destructive approach could thus be used when studying wild bees and especially declining species, for which we want to avoid over-collecting specimens, as soon as it is standardized within each study. For instance, if infection intensity is assessed by counting the cells and spores under the microscope, this must be performed using a

Neubauer hemocytometer (Giacomini et al. 2018; Gomez-Moracho et al. 2021), to standardize data and allow between-studies comparisons. However, considering that faecal screening may underestimate infection intensity (Wolmuth-Gordon et al. 2023) caution should be applied regarding the assessment of pathogen load with this technique, which could be complemented by gut sampling when no conservation concerns apply.

#### LIMITATIONS AND PERSPECTIVES

Our studies did not test for species differences in defecation probability or parasite loads, which could be the focus of future work. However, we accounted for species variability in our models, and even though our samples were dominated by common bumble bee species (e.g., *B. impatiens* and *B. ternarius*), we also tested the updated cooler protocol on few specimens of less common or even endangered species (e.g., *B. fervidus* and *B. terricola*, with success rates of 67% and 83.3%, respectively).

Furthermore, this non-destructive method screens for two gut parasites commonly found in bumble bees, *Crithidia* and *Vairimorpha* (Graystock et al. 2020; Grupe & Quandt 2020), but it cannot be used to look at other pathogens that infect different parts of the body (e.g. body fat, brain or the reproductive system) (Figueroa et al. 2023). However, faecal sampling can not only be used for visual screening of pathogens as done here, but also for molecular detection, which allows great sensitivity and detection of viruses (Babin et al. 2022), also transmitted through an oral-faecal route in Hymenoptera (Yañez et al. 2020). Conducting molecular analyses on faecal samples has been done in laboratory settings to assess the prevalence of important viruses found in bees (Chen et al. 2006). In their study, Chen et al. (2006) highlighted that faecal samples collected using petri dishes were amongst the best tissues to assess the prevalence of Black Queen Cell Virus (BQCV) and Deformed Wing Virus (DWV) by RT-PCR in the honey bee, but were less effective for Chronic Bee Paralysis Virus (CBPV) and Sacbrood Bee Virus (SBV) (Chen et al. 2006). Both BQCV and DWV are common and important threats to bumble bees (McNeil et al. 2020; Burnham et al. 2021; Tsvetkov et al. 2021); thus, assessing their prevalence using a non-destructive faecal screening approach could be of particular

importance, especially for endangered species such as *B. terricola* or *B. affinis*. In addition, faeces could be used to study bee microbiota and bee diet or nutritional status (through pollen metabarcoding or morphological analysis), thus expanding our ability to assess bee health through a non-destructive approach (Koch & Schmid-Hempel, 2011; Parreño et al. 2022).

The choice of the non-destructive approach described here will however depend on the research questions and the risks/benefits trade-offs. Some questions will require to continue using gut screening instead of fecal screening. If the non-destructive faecal sampling approach was used in molecular analyses, the protocol would have to be adjusted to ensure the proper sample collection and storage for such analyses. This includes using double-distilled nuclease free water, keeping samples on ice and freezing them (at  $-20^{\circ}\text{C}$  for analyses on DNA and at  $-80^{\circ}\text{C}$  for analyses on RNA) within hours of collection and until being processed (McNeil et al. 2020; Gomez-Moracho et al. 2021; Tsvetkov et al. 2021).

The protocol described in this study was developed focusing mostly on workers, queens and gynes, as the main casts of focus in studies related to bee health (Brown et al. 2000; Fowler et al. 2020; Malfi et al. 2023). Male sample size was thus very low in our study, and the efficiency of the method remains to be confirmed for this cast. In addition, since the protocol presented here was developed for bumble bees, it remains to be confirmed whether it can be adapted to other species of wild bees, especially smaller solitary bees. Nonetheless, a recent study on *Osmia bicornis* used a similar faecal screening approach in laboratory conditions (Tian et al. 2018), suggesting that the non-destructive approach presented here could be applied to some smaller bee species, which have been identified as hosts of *Crithidia* (Figueroa et al. 2021). Considering the growing interest and need to assess the health of social and solitary wild bees, especially endangered or declining species that are more exposed and impacted by diseases than common and non-endangered species (Gillespie 2010; Cameron et al. 2011; Averill et al. 2021), the development of non-destructive sampling methods is crucial. The protocol presented here is a step that should help achieving sustainable sampling in bee research, by

reducing potential damages of repeated sampling on wild bees when assessing their health.

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#### AUTHOR CONTRIBUTION

M.L.T and V.F. obtained funding, with contribution from L.A. and G.L. for study 1, from S.M., L.A., P.B., C.C. and S.C. for study 3 and from JS.M. and S.M. for study 4. M.L.T. conceived the theoretical framework and design of these studies, with feedback from other authors. Data collection and curation was conducted by M.L.T. and C.B., with help from S.M. Finally, M.L.T. conducted statistical analyses and wrote the first draft of the manuscript with edits from all authors.

#### DISCLOSURE STATEMENT

Authors declare no competing interests.

#### DATA AVAILABILITY STATEMENT

All data will be made available on OSF.

#### APPENDICES

Additional supporting information may be found in the online version of this article:

Appendix I. Detailed sample sizes per species, cast and study (Table S1).

Appendix II. Output of the alternative GLMMs (Table S2).

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