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ARTICLE

Soda maker for field anesthesia as a step towards a non-lethal identification of wild bees and other flower visitors

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Abstract
Species identification is currently a strong limitation to wild pollinator studies. It requires killing specimens for laboratory analyses, which can pose ethical issues in some programs of conservation biology and citizen science. The recent development of image-based identification tools using machine learning could challenge the current paradigm of required specimen euthanasia for species identification. However, to be accurate, these new methods call for standardized images or images of precise characters that are difficult or even impossible to obtain on live specimens. To facilitate the acquisition of these pictures, we tested two *in-situ* CO_2 anesthesia protocols using material easily available, even in citizen science programs. We measured the time of anesthesia of 196 flower visitors belonging to the Hymenoptera and Diptera orders. The most efficient protocol enabled us to anesthetize 90 % of the specimens for more than a minute with a marginal mortality (1.5 %). Anesthesia time increased with specimen size in Hymenoptera and decreased with air temperature. Diptera were less sensitive to anesthesia. Further analyses would be required to investigate the potential sublethal effects of these anesthesia. These preliminary results suggest nonetheless that CO_2-based anesthesia could help the development of non-lethal methods of wild pollinator identifications.

Keywords | anesthesia duration • pollinators • Hymenoptera • Diptera • CO_2 • citizen science

L'anesthésie sur le terrain par machine à soda, un moyen de faciliter le développement de méthodes non létales d'identification des abeilles sauvages et autres visiteurs floraux

Résumé
L'une des limites actuelles à l'étude des pollinisateurs sauvages est la difficulté d'identifier ces insectes au niveau de l'espèce. Le développement d'outils d'identification sur images par intelligence artificielle ouvre de nouvelles perspectives par rapport au paradigme actuel d'euthanasie des spécimens pour les identifier en laboratoire. Cependant, l'obtention d'images de référence standardisées ou de caractères morpho-anatomiques précis nécessaires à ces outils est difficile, voire impossible sur un spécimen actif. Pour faciliter l'obtention de ces photos, nous avons testé deux protocoles d'anesthésie au CO_2 de spécimens sur le terrain avec un matériel accessible au grand public. Nous avons mesuré le temps d'anesthésie sur 196 visiteurs de fleurs, hyménoptères et diptères. Avec le protocole le plus performant, 90 % des insectes étaient anesthésiés pendant plus d'une minute. La mortalité due au traitement était marginale (1,5 %). La durée de l'anesthésie augmentait avec la température de l'air, ainsi qu'avec la taille des spécimens chez les hyménoptères. Les diptères étaient moins sensibles à l'anesthésie que les hyménoptères. Des études complémentaires seraient nécessaires pour appréhender les effets sublétaux potentiels de ces anesthésies. Néanmoins, l'anesthésie au CO_2 sur le terrain pourrait faciliter le développement de méthodes non-létales d'identification des pollinisateurs.

Mots-clefs | durée d'anesthésie • pollinisateurs • Hymenoptera • Diptera • CO_2 • science participative

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Table S1. Data of specimens studied [saved at https://doi.org/10.5281/zenodo.6581063]
Table S2. Anesthesia duration for specimens exposed more than 60 seconds to the CO_2 [saved at https://doi.org/10.5281/zenodo.6581110]

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INTRODUCTION

Insect identification is of paramount importance in many fields of study and applications such as the monitoring of ecosystem status. The identification of flower visitors in particular is key to understand the population trends of these ecosystem service providers in a context of global decline (OLLERTON *et al.*, 2011; POTTS *et al.*, 2016). Such data are required to develop the legislative tools in reaction to these declines and to apply them and build efficient conservation actions. Species identification is also crucial to build accurate plant-pollinator networks and to identify the levels of specialization and maintenance of these networks (SOARES *et al.*, 2017). Currently, species identification of flower visitors often requires the sacrifice of the specimens. The minute diagnostic criteria to distinguish the species force people to collect the specimens, to kill them and to mount them on a pin so that they can be studied under a microscope (GILL *et al.*, 2016). The current necessity to kill specimens for identification through microscopic observations is not trivial. While there is currently no known effect of lethal sampling on pollinator communities (GEZON *et al.*, 2015), it raises ethical issues when developing citizen science programs aimed at environmental awareness and conservation at large scales (*e.g. Spipoll*; see DEGUINES *et al.*, 2012). The advent of DNA barcoding and environmental DNA could provide a non-lethal option (SCHMIDT *et al.*, 2015; THOMSEN & SIGSGAARD, 2019). However, while the technology is improving at a high pace, the method is still too costly to be applied outside of dedicated programs of restricted sizes (*e.g.* VILLALTA *et al.*, 2021), and it still requires the development of complete reference databases for all pollinator species. Image analysis provides a more accessible solution for the public.

Image-based automated identification of live insects can be developed using deep learning through convolutional neural networks (WÄLDCHEN & MÄDER, 2018; HØYE *et al.*, 2021). Such methods have been boosted by citizen science programs providing hundreds of thousands of *in situ* habitus images or annotations to train such algorithms (HORN *et al.*, 2018). For wild bees, these methods are still limited. They require a lot of images, and their efficiency can be impaired by the morphological similarity of many species. This limit can be circumvented by using more standardized pictures of diagnostic characters, such as that of the wing venation (BUSCHBACHER *et al.*, 2020). However, it is almost impossible to take standardized pictures of minute structures such as wings on live insects in the field. A potential solution could be to anesthetize the specimens for the time of the pictures, directly in the field. Insect anesthesia through high carbon dioxide (CO_2) exposure is a common practice for many laboratories manipulating insects for surgery, injections, sexing and specimen identification of flying insects, ranging from cockroaches to flies, including honey bees (*e.g.* NICOLAS & SILLANS, 1989, KOYWIWATTRAKUL *et al.*, 2005). However, it is still not widely used by field ecologists and citizen scientists. One possible explanation could be the lack of access to the necessary material for anesthesia. In this study, we test the use of a cheap and widely available source of CO_2 for anesthesia of flower visitors in field conditions.

Insect anesthesia using carbon dioxide has a long history in entomology. A strong increase in ambient CO_2 from a gas source or using dry ice, puts many insects to a state of immobility (TUTUN *et al.*, 2020; EBADI *et al.*, 1980; COOPER, 2011; BAHNEY, 1996). Such methods have been used both for precise imaging using CT-scan (POINAPEN *et al.*, 2017) and on flower visitors such as honeybees (KOYWIWATTRAKUL *et al.*, 2005). Unlike freezing, another insect anesthesia method which can be difficult to control in field conditions, CO_2 can easily be transported and applied directly in the field. A low-cost and widely available source of CO_2 is soda makers such as *Sodastream* (KILLICK-KENDRICK, 1993). While originally used to kill the specimens and considered cumbersome for transport in the field (KILLICK-KENDRICK, 1993), its format can be transported and it contains enough CO_2 for repeated anesthesia of dozens of bumblebees (MARTIN *et al.*, 2006).

However, it has not been tested yet in field conditions, nor on a wide diversity of flower visitors (figure 1). The objective of this study is to evaluate whether a soda-maker could be used for a non-lethal identification system of flower visitors based on pictures.

Our first question is whether CO_2 anesthesia can be performed in a standardized way in the field without killing specimens. Fieldwork induces a variation of many factors likely to affect the anesthesia duration and effects. Our hypothesis is that CO_2 specimen anesthesia can be safely performed in the field for long enough to take pictures of flower visitors with a standardized CO_2 injection protocol. In particular, temperature is known to affect insect mobility and could interact with CO_2 induced anesthesia in field conditions (ALINIAZEE, 1971; NICOLAS & SILLANS, 1989). Is the CO_2 anesthesia impacted by the air temperature? We suggest that anesthesia is more efficient at lower temperature, where insects are already chilled out.

Finally, the system has been used on bumblebees, but flower visitor species vary drastically in terms of size and physiology. Do size and taxonomic differences affect the anesthesia duration? We think that not every species will react the same to the anesthesia, with smaller species more sensitive to the CO_2 saturation.

To test these hypotheses, we anesthetized flower-visitors using a soda-maker under field conditions following two ways of injecting the CO_2 in the jar. We then measured the duration and lethality of these anesthesia, and we tested the influences of the air temperature, the specimen size and the taxonomic order on these measurements.

MATERIAL AND METHOD

Data acquisition

Insect specimens were collected in three localities between March 1st and April 16th 2021: the Faculté de Pharmacie (48.8439 °N, 2.3366 °E), the Jardin des Plantes in Paris (48.8442 °N, 2.3619 °E), and the Station d'Écologie forestière

de Fontainebleau Avon (48.4213 °N, 2.7287 °E). They were captured while visiting flowers with an insect sweep net and locked into jars with a screened opening on the lid. In order to expose them to carbon dioxide, we used a soda maker (*Spirit* model of the *Sodastream* brand – Israel), a common, affordable and transportable CO_2 source. The CO_2 dispenser of the soda maker was connected to the screen opening of the jars using a plastic pipe to facilitate handling (figure 1).

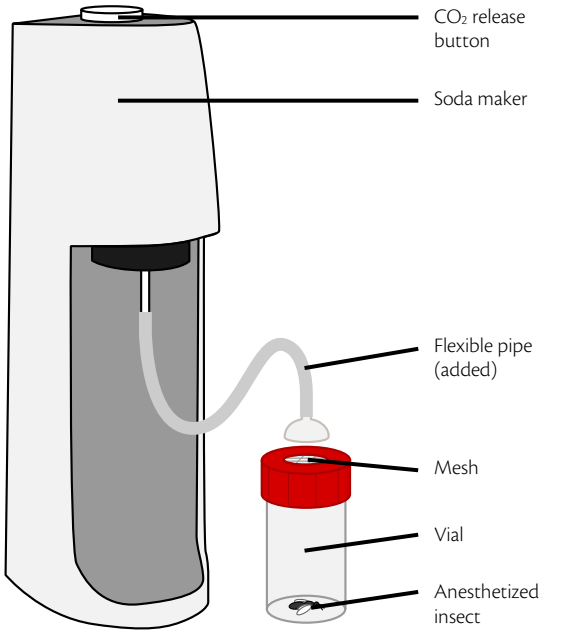

Figure 1. Device used for anesthesia. The soda maker was expanded with a pipe to facilitate the manipulation. The cap of the vial was pierced with a glued mesh to let the gas in with no risk of insect escaping.

The CO_2 was injected using two different protocols. In "protocol 1", we applied a single full pressure on the *Sodastream* machine's button. As this pressure created an abrupt release of gas which projected some of the specimens on the walls of the jars, we tested a second protocol using a seven-seconds one softer pressure, pressing the button only up to the point that we could hear the sound of the gas, hereafter "protocol 2".

In order to increase the volume of CO_2 breathed by the insect, and thereby, its anesthesia duration, we let it rest one minute in the jar with the screened opening clogged after CO_2 injection to avoid leaks. After one minute, we could remove the insect from the jar to stop the CO_2 exposure and to measure its anesthesia duration. To simulate a stimulation of the specimen as it would happen during the process of taking a standardized wing picture, we grabbed one of its wings with a tweezer (figure 2).

The anesthesia duration of every individual was measured with a stopwatch and was bounded between 0 and 301 seconds. The 300-seconds limit was selected to optimize the number of specimens that can be processed in one fieldwork session, considering that 300 seconds is more than enough to take standardized pictures. Any immobilization longer than 300 seconds was therefore recorded as "301 seconds". Specimens still immobilized after this time were put back in ventilated jars.

Specimen awakening was estimated using either one of the two following criteria for the specimens who slept less than 300 seconds: wing flapping or leg movements trying to get rid of the tweezer. If one of this behavior occurred, the stopwatch was stopped and the insect was put back in a ventilated jar. To estimate the lethality of the anesthesia, we verified that specimens were able to fly in their jar. If specimens were only able to stand or walk, they were considered dead as they would not have been able to survive long if released.

Other parameters were measured in order to test their influence on anesthesia duration. We measured the air temperature during every anesthesia with a liquid thermometer.

Specimens were subsequently killed using ethyl acetate, then mounted for identification. Hymenoptera identification to the morphospecies was performed using a stereomicroscope and identification guides (FALK, 2019; MICHEZ *et al.*, 2019; PAULY, 2019). The identification of Diptera to the genus level was made with the help of an online community of naturalists (*iNaturalist*). Specimen size was estimated on pinned specimens by the ITD (inter-tegular distance, an estimator of dry weight and classically used to estimate size of bees). We measured the ITD of every specimen in the lab using an optical microscope associated with a digital imagery system and *ZEN* software (Oberkochen, Germany).

Analyses

Flower visitor anesthesia in field conditions

To determine whether the tested protocols could be used to anesthetize specimens long enough to take standardized pictures, we looked at the proportion of specimens which slept at least 60 seconds. This threshold was considered a sufficient time, given some practice, to take minute or

Figure 2. Some field images during the study (with M. TOULZAC). **a.** General view. **b.** Anesthetized *Osmia*. **c.** Anesthetized *Bombus*. Photos M. BRAULT [a, c] & M. TOULZAC [b].

standardized picture of insects, such as standardized pictures of the wing venation used in previous accurate image-based species identification studies. Since the Diptera sample size was low and its anesthesia duration did not follow a normal distribution, we tested the anesthesia duration difference between Diptera and Hymenoptera with a WILCOXON test. We also compared the anesthesia duration obtained with protocols 1 and 2 for Diptera and Hymenoptera specimens separately using a STUDENT's *t*-test for Hymenoptera (n = 179) and a WILCOXON test for Diptera (n = 17).

Effects of specimen size, taxonomy and air temperature on anesthesia duration

In order to understand the origin of variation in anesthesia duration observed, we tested the influence of parameters that could affect the anesthesia: specimen taxonomic group, specimen size (estimated by the ITD) and air temperature at the time of the anesthesia. Since our data were double-bounded temporal data, we used COX regressions from survival analyses in order to test for the influence of ITD and temperature over anesthesia duration (ANDERSEN & GILL, 1982) using the *coxph* function of the *Survminer R package* (KASSAMBARA *et. al*, 2021). We then checked the three main assumptions for using this test. The proportional hazard assumption was tested using a chi-square test on scaled SCHOENFELD residuals. The linearity of the covariates was visually assessed using plots of the martingale residuals against the null cox proportional hazards model. The absence of influential data was also visually assessed using the *dfbeta* values which quantifies the amount of regression coefficient changes when the datapoint is removed from the model.

The regression was applied on the entire dataset obtained using protocol 2 to understand the influence of these factors on the most efficient protocol. Temperature data were log-transformed to improve their linearity for the test. Since the proportional hazard assumption was not met for this regression, statistical results could not be interpreted. We therefore performed another regression on Hymenoptera data only to test the effects of temperature and specimen size with a simpler model that would meet the regression assumptions. Four data points appeared very influential in this second regression. Since it could have biased the statistical results, we verified that the results of the second regression were still significant when performing the regression without these four points.

Analyses and plot were made using the R software (R CORE TEAM, 2021) and *R package* "ggplot2" (WICKHAM, 2016). Statistical significance was assessed using a risk α = 5%.

RESULTS

Flower visitor anesthesia in field conditions for standardized pictures

We realized a total of 196 anesthesias in field conditions using a soda maker (115 using protocol 1 and 81 using protocol 2) on 196 different specimens (179 Hymenoptera and 17 Diptera) belonging to 23 genera. Overall, 86.2 % of the specimens slept at least 60 seconds (figure 3 + supplementary material, table S1). Diptera slept significantly less than Hymenoptera (mean anesthesia duration: Diptera = 90 ± 88 s; Hymenoptera = 175 ± 90s; W = 722.5, N = 196, p-value = $3.409 \cdot 10^{-4}$). Lethality amounted to three deaths over the 196 individuals anesthetized, for a death rate of 1.5 %.

Protocol 2 was slightly more efficient to immobilize flower visitors for at least 60 seconds (83.48 % for protocol 1 and 90.12 % for protocol 2). This difference is particularly obvious with the Diptera order (figure 4) with 20 % of anesthesia duration below 60 seconds for protocol 2 against 60 % for protocol 1. The WILCOXON test to compare the anesthesia duration between protocol in Diptera is not significant (W = 20.5, N = 17, p-value = 0.204), but it may be related to the extremely low sample size ($N_{protocole1}$ = 7 and $N_{protocole2}$ = 10). The difference of efficiency between protocols is less visible in the Hymenoptera order, and the STUDENT test shows that the difference is not significant (t = 0.245, N = 179, p-value = 0.807). However, a higher proportion of anesthesia lasted 60 seconds or more in Hymenoptera with protocol 2 (91.55 %) than with protocol 1 (87.04 %). In addition, the results of protocol 1 appeared influenced by the level of remaining CO_2 in the bottle.

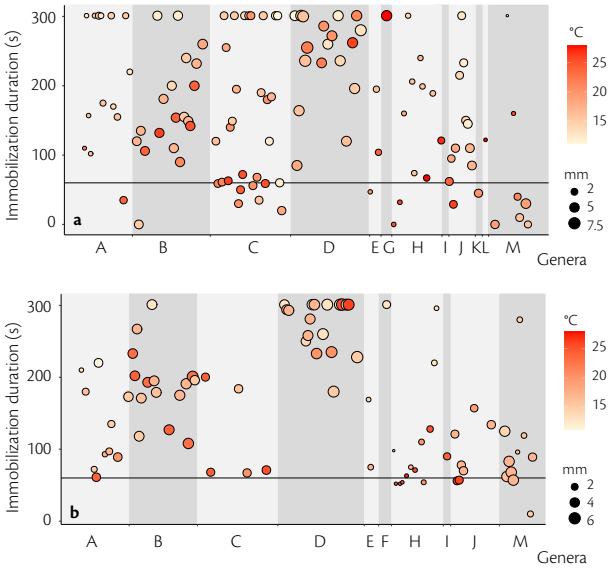

Figure 3. Immobilization duration (seconds) of anesthesias performed with protocol 1 (**a**) or with protocol 2 (**b**). Datapoints are sorted by taxon on the *X*-axis. A = *Andrena*. B = *Anthophora*. C = *Apis*. D = *Bombus*. E = *Nomada*. F = *Halictus*. G = *Xylocopa*. H = *Lasioglossum*. I = *Sphecodes*. J = *Osmia*. K = *Cynipoidea*. L = *Abia*. M = Diptera. Point color indicates air temperature (°C) at the moment of the anesthesia and point size indicates the size of the specimen (estimated by the intertegular distance, in mm).

Effects of specimen size, taxonomy and air temperature on anesthesia duration

Temperature had a significant negative effect and size a significant positive effect on the immobilization duration in the first model using protocol 2 data for Hymenoptera and

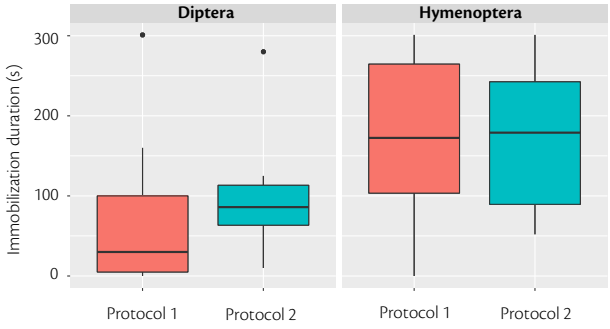

Figure 4. Immobilization duration (s) in Diptera and Hymenoptera orders, depending on protocol.

Diptera specimens combined (table I). The taxonomic order covariable did not have a significant *p*-value in the model, a surprising result considering the results of the STUDENT'S *t*-test (figure 4). However, this first model violated the proportional hazard assumption and three Diptera specimens had high *dfbeta* values.

The second COX regression model focusing on Hymenoptera data anesthetized with protocol 2 supported these results with ITD and temperature having significant effects, respectively positive and negative (table II). This model was in accordance with the proportional hazard assumptions for all factors, but four data points appeared to have high dfbeta values, indicating a strong influence of these points on the regression results. Another analysis without these specimens returned similar effects, confirming that temperature and individual size have a significant influence the immobilization time in Hymenoptera (figure 5).

Table I. Results of the COX Regression and the associated proportional hazards test on influences of taxonomic order, air temperature and insect size on immobilization duration. The data are from the dataset of Hymenoptera and Diptera immobilized using protocol 2. Temperature was estimated as the log-transformed air temperature (°C) and size was estimated by the ITD (mm). Sample size: $N = 81$. Likelihood ratio test: 47.52, $df = 3$, $p = 2.695 \cdot 10^{-10}$.

Variable	Cox regression				Proportional hazards assumption	
	Parameter estimate	Standard	*Z*-value	*p*-value	χ^2	*p*-value
Taxonomic order	– 0.610	0.377	– 1.619	0.106	4.224	0.040
Temperature	2.685	0.544	4.933	$8.09 \cdot 10^{-7}$	1.555	0.212
Size	– 0.390	0.076	– 5.140	$2.75 \cdot 10^{-7}$	0.506	0.477
Global	–	–	–	–	7.474	0.058

Table II. Results of the COX regression and the associated proportional hazards test on influences of taxonomic order, air temperature and insect size on immobilization duration. The data are from the dataster of Hymenoptera immobilized using protocol 2. Temperature was estimated as the log-transformed air temperature (°C) and size was estimated by the ITD (mm). Sample size: $N = 71$. Likelihood ratio test: 57.8, $df = 2$, $p = 2.815 \cdot 10^{-13}$.

Variable	Cox regression				Proportional hazards assumption	
	Parameter estimate	Standard error	*Z*-value	*p*-value	χ^2	*p*-value
Temperature	3.129	0.569	5.494	$3.93 \cdot 10^{-8}$	1.520	0.217
Size	– 0.550	0.084	– 6.584	$4.57 \cdot 10^{-11}$	1.980	0.159
Global	–	–	–	–	5.870	0.053

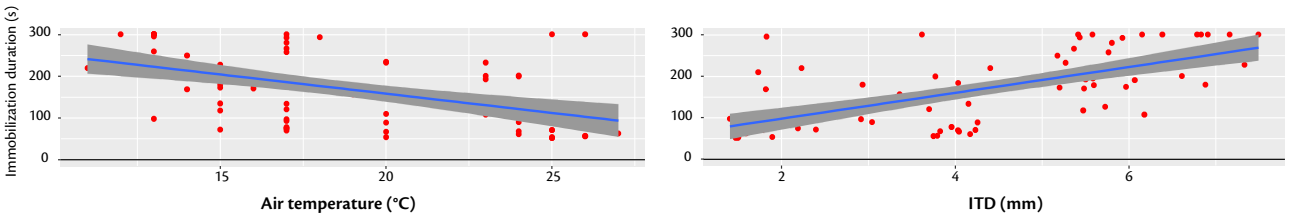

Figure 5. Immobilization duration of Hymenoptera relative to air temperature (°C) during the anesthesia, and to size estimated by the intertegular distance (ITD, in mm). Trends are represented by linear regressions in blue, with their 95 % confidence intervals in grey.

DISCUSSION

Flower visitor anesthesia in field conditions

We chose to use a soda maker as our CO_2 source because of its affordability, transportability (relatively light weight and not fragile) and simplicity of use in a standardized protocol. Previous studies already used soda makers on insects with success for global insect euthanasia or as a bumblebee anesthetic in laboratory conditions (KILLICK-KENDRICK, 1993; MARTIN *et al.*, 2006). Here, we tested its use as an anesthetic in field conditions on a more representative community of flower visitors including bumblebees, but also other wild bees and flies.

Our results show that most flower visitors can be immobilized for 60 seconds or more, in field conditions, using a largely available CO_2 source. The system appeared to be sufficient to anesthetize a wide array of flower visitors, our samples including 10 bee genera, two other Hymenoptera (one parasitoid wasp and one sawfly) and a few brachyceran Diptera, including Syrphid flies.

A progressive introduction of the gas into the jar enabled a more stable and reproducible anesthesia, especially for Diptera (figure 3b). We assume that the insects were exposed to less stress and that more carbon dioxide stayed into the jar with protocol 2. Another asset of protocol 2 over protocol 1 is that the gas pressure entering the jar did not depend on the level of gas remaining in the cylinder. Hence, protocol 2 seems a better option to anesthetize flower visitors for at least 60 seconds.

With only three deaths over 196 specimens tested, the protocols resulted in a very low direct lethality. In addition, these deaths may have resulted from mishandling rather than a direct effect of CO_2: the dead specimens were among the few specimens left in jars exposed to the sun when recovering from the anesthesia, which could have dehydrated them. Preliminary tests of the experiment (longer CO_2 exposure, see supplementary material, table S2) suggested that bees could also die following anesthesia if the temperature of recovery was below 12 °C. In case of a cold environment, warming the specimen or the jar once the manipulation is over could help minimize such fatalities.

Here, we made sure that the insects could recover and fly, but additional tests would be required to assess the safety of our protocol in the long term. Indirect lethality and sublethal effects are described in the literature (see NICOLAS & SILLANS, 1989) and include negative effects such as oviposition behavioral changes in Apidae, with the inhibition of ovary activation in honey bee workers (KOYWIWATTRAKUL *et al.*, 2005). Negative effects on long and short-term memory were noticed in honey bees (STEC & KUSZEWSKA, 2020), along with their ability at gathering pollen and their longevity (RIBBANDS, 1950; EBADI *et al.*, 1980; OLSZEWSKI *et al.*, 2012). Studies on *Drosophila melanogaster* also describe detrimental effects over insect mating behavior due to rough handling and CO_2 anesthesia (BARRON, 1999), along with impaired motor functions (COLINET & RENAULT, 2012; BARTHOLOMEW *et al.*, 2015). Positive effects of CO_2 treatments were also detected, such as the oviposition activation in virgin honeybee queens (MACKENSEN, 1947) or post-diapausing *Bombus terrestris* queens with higher and faster colony founding success and higher percentage of queens laying eggs (GUREL & KARSLI, 2013).

Our experimental design did not enable us to test for these additional effects of CO_2 anesthesia. Further observations would be required, but two points suggest that sublethal effects should be minimal in our case compared to the literature cited above. First, these studies showed an effect of exposure time (*e.g.* BARTHOLOMEW *et al.*, 2015) and were based on much higher exposures to CO_2 than our protocol (exposure to CO_2 saturated air from two to 30 minutes of exposure, against 60 seconds in our protocol). There was only one study using 60 seconds or less of CO_2 exposure (EBADI *et al.*, 1980). According to their experiments on honeybee workers, this level of CO_2 exposure induced no effect for orientation and mortality but an effect on the pollen collection frequency. Second, a mixture of air and CO_2 was found to diminish sub-lethal effects of the anesthesia (CZEKOŃSKA, 2009). Our system may benefit from a similar mixture, since the vial was not airtight. These sublethal effects could therefore be reduced in our protocol in comparison with these other studies.

Other anesthetics could be explored too as a replacement for CO_2. For example, nitrous oxide is also cheap and easy to find, and widely used in vertebrate anesthesia (BECKER & ROSENBERG, 2008). However, it has been much less studied in insects, and the rare studies demonstrate other sublethal effects than CO_2 such as a drastic increase of aborted egg production in cockroaches (*e.g.* BROOKS, 1965).

These potential sublethal effects also have to be contrasted with the current context, where the only alternative for accurate specimen identification is to kill the specimens.

Factors influencing the anesthesia duration

Our results show a clear influence of taxonomy, temperature and size over anesthesia duration of pollinators. Anesthesia lasted longer at colder temperatures and in larger insects. While some model assumptions were not validated when using the entire dataset, we could apply the model on Hymenoptera data and it confirmed the effects suggested by the first analysis. Flower visitors were immobilized for a shorter time at higher temperatures, a trend highly visible when illustrating the data with linear models (figure 5). This result is coherent with previous studies on Drosophila melanogaster (NICOLAS & SILLANS, 1989). Larger individuals were also immobilized for a longer time than smaller ones.

Our data also show that the two insect orders reacted differently to CO_2 and to its interaction with the other parameters. While we did not have enough data to test this among genera or species, our data suggested that the sensitivity to CO_2 varied between these genera. For example, *Lasioglossum* and *Osmia* specimens were immobilized for a shorter time than specimens of *Anthophora* and *Bombus* (figure 3). Unfortunately, our data were insufficient to disentangle these effects from other effects such as size or sociality, *Anthophora* and *Bombus* being larger than *Osmia* and *Lasioglossum*, and *Bombus* and some of the *Lasioglossum* living in underground colonies with different CO_2 conditions. In addition, the nature of the data, double-bounded at 0 s and at 301 s, makes it unsuitable to classical linear analyses that would enable a statistical test of these interactions. While the survival analysis enabled us to detect effects of some of the parameters on anesthesia time, other data, for example not bounded at 300 seconds, would be required to better assess the multifactorial nature of this anesthesia duration.

Other parameters such as whether the individual had already drunk nectar or its level of excitation before its anesthesia could affect these results. Flower nectar can contain pollinator stimulating molecules such as caffeine (COUVILLON *et al.*, 2015), or can have the opposite effect (STEVENSON *et al.*, 2017). The excitation level of the pollinator could also have a major effect on anesthesia duration, for example by influencing its ventilation and how much CO_2 enters its organism (EVEN *et al.*, 2012). Preliminary data on these factors, using a scale of specimen excitability and record of visited flowers, suggest non-significant tendencies, but our protocol was not designed to test these effects and these preliminary results would need further exploration. Stress may also be an important component in the differences in anesthesia times observed between the two protocols tested here. The first protocol strongly agitated

the specimens in the vials, certainly creating a highly stressful situation. This may have induced stress responses such as a closing of the stigma in some specimens, especially in Diptera. Such a response would explain why these insects were much less sensitive to the CO_2 with this protocol (figure 4).

Towards a non-lethal solution to the flower visitor identification problem

In a context of pollinator decline, we need long term data over large areas to monitor pollinator populations. Such programs require the support of the public and operators that can be reluctant to kill specimens, especially insects considered as beneficial (DRINKWATER *et al.*, 2019). Even though repeated massive collections appear to have minimal impacts on wild bee populations (GEZON *et al.*, 2015), euthanasia seems not sustainable for large-scale pollinator monitoring programs. The development of non-lethal species identification methods is therefore critical.

Being able to immobilize flower visitors for one minute tackles two challenges faced by picture-based solutions for non-lethal insect identifications: 1) the ability to take standardized pictures of specific criteria and 2) the ability to photograph live specimens in their environment then to capture them afterward to confirm their species identification.

Standardized pictures are necessary for methods such as species identification based on image analysis of wing venations. This criterion is known to be efficient to distinguish species and even populations (PERRARD *et al.*, 2014; BUSCHBACHER *et al.*, 2020). However, end-users need to be able to take the required pictures on live specimens if this is to become a non-lethal method of species identification. The affordability and the wide availability of the CO_2 source suggested here opens this solution to most potential end-users. Other sources of widely available compressed CO_2 could be explored, such as the smaller cartridges of CO_2 used for tire inflators. We chose soda makers because of the larger amount of CO_2 available in one bottle and of the ability to reuse the bottles, since smaller cartridges are designed for single use. Although the protocol of wing-picture has yet to be tested on anesthetized specimens in the field, the 60-seconds anesthesia is a promising advance. Standardized wing pictures are usually taken on cut wings (BUSCHBACHER *et al.*, 2020), but it can be obtained without removing the wings of specimens (HOULE *et al.*, 2003; PERRARD *et al.*, 2012). These wing preparations and pictures on entire specimens lasted less than a minute. So far, this process has been applied on live insects anesthetized with CO_2 only on *Drosophila* in laboratory conditions (HOULE *et al.*, 2003). Now that we know we can anesthetize wild pollinators in the field, the challenge is to translate the picture system into an easy-to-use, portable format that could be applied in the field by anyone.

The uses of our field anesthesia are going further than just non-invasive population monitoring by professionals. One of the main barriers in developing efficient automated species identification systems from regular flower visitor pictures is the training dataset deep learning requires. This reference database consists of pictures of identified specimens in situ. These pictures have to be taken preferably in situ, since collection specimens have a pin and a background that would be different from the pictures taken on live specimens. In addition, specimen appearance can be different between live and dead individuals due to post-mortem variations in eye colors, hairs and body position, further weakening the ability of the system to recognize live specimens based on collection ones.

Hundreds of *in situ* pictures can be required for each species to obtain an efficient system (SHAHINFAR *et al.*, 2020). It is already not easy to take a good photograph of a flower-visitor, but it is even rarer to be able to capture the specimen afterwards. By knocking out the specimens, anesthesia makes it much easier to photograph the specimens, then to capture them for reliable species identification.

It is nonetheless important to acknowledge that non-lethal sampling methods have limits and are not meant to replace specimen-based studies and collections. Non-lethal sampling can provide a testimony of the presence of a species at a time and date, and can dramatically increase the pace of collection of occurrence data by demanding less material and less time investment than specimen pinning. However, it provides only a partial testimony of the specimen (TROUDET *et al.*, 2018). Unlike specimens, pictures do not enable posterior measurements of molecular or morphological traits. Picture-based records are therefore much more limited for taxonomy, evolutionary or functional ecology studies than specimens (ROCHA *et al.*, 2014). It is therefore essential to keep collecting specimen-based occurrence data.

CONCLUSION

The non-lethal identification of flower visitors is an emerging concern for which picture-based solutions are promising but limited by the difficulty to obtain good pictures from live insects. Our results on 196 flower visitors show that soda makers can provide a cheap, accessible anesthesia device that can be applied in field conditions to a wide variety of flower visitors with low mortality. Our result show that this system performs best when the gas is injected slowly, without disturbing the captured insect, and that for a same dose, bees appear to stay anesthetized longer than flies, that larger bees are more sensitive than smaller bees, and that warmer conditions decrease the duration of the anesthesia.

Further studies are still needed to explore the potential sublethal effects that such an anesthesia can induce on flower visitor fitness, but the device could help to develop picture-based recognition systems. It could enable end-users to picture directly in the field minute details necessary for identification. It could also help entomologists to develop training datasets to develop deep-learning systems for insect identification that cannot be identified from pictures by a human eye. The development of such non-lethal options to study the diversity of pollinators could speed up our data acquisition by harnessing the full potential of citizen science programs.

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