Effects of chronic exposure to the new insecticide sulfoxaflor in combination with a SDHI fungicide in a solitary bee

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HIGHLIGHTS
- Chronic oral exposure to 100 ppb of sulfoxaflor reduces the survival of Osmia bicornis.
- The chronic exposure up to 60,000 ppb of fluxapyroxad does not reduce bee survival.
- Syrup consumption increases at 20 ppb of sulfoxaflor and decreases at 100 ppb.
- We found no synergistic interaction between sulfoxaflor and fluxapyroxad.

GRAPHICAL ABSTRACT

ABSTRACT

The recent EU ban of the three most widely used neonicotinoids (imidacloprid, thiamethoxam and clothianidin) to all outdoors applications has stimulated the introduction of new insecticides into the market. Sulfoxaflor is a new systemic insecticide that, like neonicotinoids, acts as a modulator of nicotinic acetylcholine receptors. In agro-environments, bees can be exposed to this compound via contaminated pollen and nectar for long periods of time. Therefore, it is important to assess the potential effects of chronic exposure to sulfoxaflor, alone and in combination with fungicides, on pollinators. In this study, we tested the effects of chronic exposure to two field concentrations of sulfoxaflor (20 and 100 ppb) alone and in combination with four concentrations of the fungicide fluxapyroxad (7500, 15,000, 30,000 and 60,000 ppb) on syrup consumption and longevity in females of the solitary bee Osmia bicornis L. Exposure to 20 ppb of sulfoxaflor, alone and in combination with the fungicide, stimulated syrup consumption, but did not affect longevity. In contrast, syrup consumption decreased in bees exposed to 100 ppb, all of which died after 2–6 days of exposure. We found no evidence of synergism between the two compounds at any of the two sulfoxaflor concentrations tested. Comparison of our findings with the literature, confirms that O. bicornis is more sensitive to sulfoxaflor than honey bees. Our results highlight the need to include different bee species in risk assessment schemes.

1. Introduction

The last decades have seen dramatic declines in wild bee diversity at local and regional scales (Bartomeus et al., 2013; Biesmeijer et al., 2006; Ollerton et al., 2014) together with unusual honeybee colony losses in various parts of the world (Chauzat et al., 2013; Lee et al., 2015). Although
these declines are caused by a combination of factors, evidence suggests that the use of pesticides is one of the main drivers (Goulson et al., 2015). Among the various pesticide groups, neonicotinoid insecticides, which have been widely used to control insect pests since the 1990s, have raised particular concern (Godfray et al., 2015; Goulson, 2013; Maini et al., 2010; Sgolastra et al., 2020; Woodcock et al., 2017). As a result, the outdoors use of three neonicotinoids -clothianidin, imidacloprid, and thiamethoxam- has been banned in the EU and restricted in some areas of the US. Along with the appearance of pest resistance to neonicotinoids (Bass et al., 2015), these restrictions have prompted the search for new insecticides. One of the substances that is being considered as a potential alternative to neonicotinoids is sulfoxaflor, a newly registered sulfoximine insecticide. Like neonicotinoids, sulfoxaflor targets the insect nervous system, acting as a selective agonist of Nicotinic Acetyl Choline Receptors (NACHRs) (Sparks et al., 2013; Zhu et al., 2011). However, based on different NACHR binding sites and the absence of an sp² nitrogen (Brown et al., 2016; Casida, 2018; Matsuda et al., 2020), sulfoxaflor and neonicotinoids belong to different IRAC (Insecticide Resistance Action Committee) categories (4C and 4A, respectively).

At the time this study was conducted (2019), regulation on the use of sulfoxaflor varied widely across European countries. The product was banned in France (ANSES, 2019) and could not be used outdoors in the UK (Corteva UK, 2021). However, in other countries, such as Ireland, Italy and Spain, its outdoors use was allowed up to 5–6 days before bloom (Corteva Ireland, 2021; Corteva Italy, 2021; Corteva Spain, 2021). Later, a report from the European Food Safety Authority report on sulfoxaflor risk assessment (EFSA, 2020a) identified this compound as highly hazardous to two social bee species, the western honey bee (*Apis mellifera* L) and the buff-tailed bumblebee (*Bombus terrestris* L.). Then, in April 2022, the European Commission imposed further restrictions on the use of sulfoxaflor which is now only allowed in permanent greenhouses (OJEU, 2022). In the USA, sulfoxaflor applications during flowering are forbidden in some crops, such as pome fruits, citrus and berries, but allowed in others, such as cucurbits, tomato and strawberries (Corteva US, 2022; US EPA, 2019). In Australia and South Africa applications are also allowed during bloom (Corteva Australia, 2022; Corteva South Africa, 2022).

Some studies have shown that long term exposure to field-realistic doses of sulfoxaflor, can affect egg-laying rates, colony growth, foraging performance, and reproductive success in bumblebees (Siviter et al., 2019, 2018; Tamburini et al., 2021a). However, the impact of sulfoxaflor on solitary bees has received less attention. A recent study has shown that the solitary bee *Osmia bicolor* L. is much more sensitive to acute sulfoxaflor exposure than both honey bees and bumblebees (Azpiazu et al., 2021). Another study, using the same bee species in the laboratory, shows that long-term exposure to sulfoxaflor at field-realistic doses impairs survival, foraging performance and flower visitation rate (Boff et al., 2021).

In agricultural environments, bees are often exposed to pesticide combinations, rather than single compounds. Pollen and nectar from insect-pollinated crops (Heller et al., 2020; Kubik et al., 2000; Skerl et al., 2010), and adjacent wildflowers (Botías et al., 2015; Krupke et al., 2012) often contain residues of multiple pesticides. Yet, risk assessment protocols target single compounds, thus potentially underestimating the risks posed by pesticide mixtures, which may result in synergistic toxic effects (Gill et al., 2012; Sgolastra et al., 2017, 2018; Siviter et al., 2021). Bee exposure to pesticide mixtures is particularly likely to occur when insect-pollinated crops whose pollen and nectar contain residues of pre-bloom systemic insecticides (such as sulfoxaflor) are sprayed during bloom with fungicides. Fluxapyroxad is a succinate dehydrogenase inhibitor fungicide (SDHI) that is considered non-harmful to bees. For this reason, it is routinely sprayed during bloom on many insect-pollinated crops that are also treated with sulfoxaflor, including apple, pear, peach, melon, tomato or strawberry (EFSA, 2020b; US EPA, 2019). Therefore, co-exposure with sulfoxaflor is likely in those countries in which outdoor application is still allowed. Fluxapyroxad has experienced a rapid growth in the agricultural pesticide market (Chaulagain et al., 2019; Fernández-Ortuño et al., 2017; Sierotzki and Scalliet, 2013) and has been found in several bee matrices (Simon-Delso et al., 2017).

In a previous study we showed that oral exposure of sulfoxaflor in combination with fluxapyroxad resulted in synergistic effects in *O. bicolor* and *A. mellifera*, but not in *B. terrestris* (Azpiazu et al., 2021). Bees in that study were acutely exposed. However, under field conditions bees may be exposed to pesticides for long periods of time, either due to persistence of the compounds in the environment or to multiple applications in the same or in different fields within the bee foraging range. In the current study, we test the combined toxicity effects of sulfoxaflor and fluxapyroxad through chronic oral exposure, by means of evaluation of two endpoints: longevity and syrup consumption. Longevity is ecologically relevant because it is strongly related to fecundity in *Osmia* spp. (Bosch and Vicens, 2006; Sgolastra et al., 2016). Syrup consumption, on the other hand, is a necessary measure to calculate the amounts of active ingredient ingested. In addition, pesticides may have a repellent effect (Barascou et al., 2021; Siviter et al., 2019; Thompson and Wilkins, 2003), but also an attractant effect (Baron et al., 2017; Kessler et al., 2015; Sgolastra et al., 2018), thus affecting syrup consumption. We have three objectives: 1) To measure the effects of chronic exposure to sulfoxaflor on syrup consumption and longevity in a solitary bee; 2) To determine whether the synergistic effects between sulfoxaflor and fluxapyroxad observed in previous studies are maintained when the two compounds are administered chronically *ad libitum*; 3) If so, to establish whether synergism is dose-dependent.

2. Materials and methods

2.1. Bee population and test conditions

We worked with a local *O. bicolor* population reared in a semi-natural area of La Garrotxa (Girona, NE Spain). Palynological analysis of the provisions showed that females collected mostly oak pollen. Adult-containing cocoons were wintered at 3–4 °C until May when large cocoons (presumed to contain females) were incubated in the laboratory at 22–23 °C until emergence. Because emergence time influences sensitivity to pesticides (females taking longer to emerge are more sensitive; Sgolastra et al., 2018), we only used bees that emerged over three consecutive days during the peak of the emergence period (days 5–7 of incubation). Upon emergence (<24 h), bees were transferred to a Plexiglas holding cage (50 × 50 × 50 cm) for ca. 4 h so they could deposit their meconium. Then, bees were individually placed in plastic ice cream cups (Maximum diameter: 8 cm; height: 7 cm) capped with transparent pin-perforated lids. Each cup contained a 1-ml calibrated syringe (Tuberculin Beroject® III, Beromed; accuracy: 0.01 ml) filled with syrup and inserted through the lid. The syrup was prepared by diluting sucrose in distilled water (33 % w/w). A pellet of *Euryaps* (Asteraceae) was attached to the tip of the syringe to enhance the prompt location of the feeder by the bee. Cups were maintained at 22 ± 2 °C and 50–70 % relative humidity and received indirect natural light throughout the experiment.

 Females emerging on any given day were evenly distributed among 16 treatments (compounds added to the syrup): water control (water), solvent control (acetone 1.3 %), two sulfoxaflor concentrations (20 and 100 ppb), four fluxapyroxad concentrations (7500, 15,000, 30,000 and 60,000 ppb; geometric series factor of 2), and the combinations of the two sulfoxaflor and the four fluxapyroxad concentrations (eight combinations). The test concentrations of sulfoxaflor correspond to residue levels found in honey-bee collected nectar 1–5 days after spray application in cotton (20 ppb) (US EPA, 2016), and to residue levels found in cucumber flowers 6 days after application (100 ppb) (Cheng et al., 2018). The two concentrations are also within the range of concentrations found in nectar in other field studies (US EPA, 2010, 2019). The fluxapyroxad concentrations tested range from mean levels of SDHI fungicides found in different bee matrices (7500 ppb; Stejskalová et al., 2018; Wallner, 2010) to the application rate of fluxapyroxad commercial formulative Sercadis® (60,000 ppb). Bees were exposed *ad libitum* to their respective treatments until they died.
The fluxapyroxad stock solution was prepared by diluting 100 μL of Sercadis® (300 g of a.i./L, BASF S.A.) in 50 ml of purified distilled water. For sulfoxaflor (purity 98 %; A2S Analytical Standard Solution) a stock solution with a concentration of 0.88 mg sulfoxaflor/mL acetone was used. These solutions were diluted in the syrup to reach the desired concentrations. The final concentration of acetone in the feeding solution was adjusted to 1.3 % (v/v) in all treatments (except for the water control) by adding pure acetone.

Cups were inspected daily to monitor syrup consumption (assessed by checking the level of syrup in the calibrated syringes) and bee mortality. Three additional containers without bees were used as controls to measure account for changes in syrup levels due to evaporation. Bees that consumed <10 μL during the first 2 days of the exposure were considered non-feeders and were excluded from the analyses. The initial sample size was 15 females per treatment. However, as many as 26.6 % of the bees exposed did not reach the established feeding threshold, which reduced sample sizes to an average of 11 bees per treatment (range: 7–15; Table 1). Future studies should increase initial sample sizes to account for non-feeding individuals. The syrup was renewed every 3 days. Once a bee died, it was frozen (−20 °C) for later measurement of the head width under a stereomicroscope at 240 ×. Head width is a good proxy of body size in Osmia (Bosch and Vicens, 2002).

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>SUL ingested (Total mean ng·bee−1)</th>
<th>Daily (mean ng·bee−1·day−1)</th>
<th>FLU ingested (Total mean μg·bee−1)</th>
<th>Daily (mean μg·bee−1·day−1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUL 20</td>
<td>12</td>
<td>16.52 ± 6.24 a</td>
<td>0.91 ± 0.16 ab</td>
<td>6.62 ± 2.31</td>
<td>0.28 ± 0.05</td>
</tr>
<tr>
<td>SUL 20 + FLU 7500</td>
<td>13</td>
<td>17.73 ± 6.19 a</td>
<td>0.74 ± 0.13 a</td>
<td>13.26 ± 2.09</td>
<td>0.66 ± 0.10</td>
</tr>
<tr>
<td>SUL 20 + FLU 15000</td>
<td>14</td>
<td>17.68 ± 3.99 a</td>
<td>0.88 ± 0.13 a</td>
<td>23.15 ± 5.97</td>
<td>1.42 ± 0.18</td>
</tr>
<tr>
<td>SUL 20 + FLU 30000</td>
<td>13</td>
<td>15.46 ± 3.99 a</td>
<td>0.95 ± 0.12 ab</td>
<td>62.64 ± 10.32</td>
<td>2.48 ± 0.27</td>
</tr>
<tr>
<td>SUL 20 + FLU 60000</td>
<td>15</td>
<td>20.87 ± 3.44 a</td>
<td>0.83 ± 0.09 a</td>
<td>50.28 ± 9.70</td>
<td>2.50 ± 0.32</td>
</tr>
<tr>
<td>SUL 100</td>
<td>10</td>
<td>20.25 ± 5.98 a</td>
<td>1.59 ± 0.19 c</td>
<td>13.75 ± 3.38</td>
<td>5.05 ± 0.72 c</td>
</tr>
<tr>
<td>SUL 100 + FLU 7500</td>
<td>12</td>
<td>11.78 ± 2.97 a</td>
<td>3.09 ± 0.64 bc</td>
<td>1.01 ± 0.25</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>SUL 100 + FLU 15000</td>
<td>9</td>
<td>11.60 ± 2.89 a</td>
<td>2.71 ± 0.44 bc</td>
<td>3.49 ± 0.87</td>
<td>0.93 ± 0.19</td>
</tr>
<tr>
<td>SUL 100 + FLU 60000</td>
<td>12</td>
<td>16.08 ± 2.95 a</td>
<td>3.43 ± 0.72 c</td>
<td>9.67 ± 1.77</td>
<td>2.75 ± 0.43</td>
</tr>
<tr>
<td>FLU 7500</td>
<td>10</td>
<td></td>
<td></td>
<td>8.84 ± 2.18</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>FLU 15000</td>
<td>9</td>
<td></td>
<td></td>
<td>11.27 ± 4.23</td>
<td>0.45 ± 0.09</td>
</tr>
<tr>
<td>FLU 30000</td>
<td>7</td>
<td></td>
<td></td>
<td>31.20 ± 11.90</td>
<td>1.03 ± 0.26</td>
</tr>
<tr>
<td>FLU 60000</td>
<td>10</td>
<td></td>
<td></td>
<td>38.18 ± 13.98</td>
<td>1.87 ± 0.27</td>
</tr>
</tbody>
</table>

Different letters within a column indicate significant differences (p < 0.05) according to Dunn's test with Bonferroni correction.
ppb), but no interaction with fluxapyroxad at any tested concentration. Mean longevity of bees chronically exposed to sulfoxaflor at 20 ppb (13–23 days) was not significantly different from that of the controls (20–26 days), and similar to mean longevity of Osmia females nesting in greenhouses (16.2 days; Sgolastra et al., 2016) and in the field (17.3–30.5 days; Bosch and Vicens, 2006). By contrast, the longevity of Osmia females exposed to sulfoxaflor at 100 ppb was drastically reduced (100 % mortality after 2–6 days). Bees exposed to the 100 ppb treatments ingested total amounts of sulfoxaflor (13.31–23.22 ng) that were similar to those ingested by bees exposed to the 20 ppb treatments (17.67–23.91 ng), but over a
shorter period of time. Mean daily dose of sulfoxaflor ingested by bees of the 100 ppb treatment (3.11–5.25 ng; Table 1) did not exceed acute LD50 values found in O. bicornis for sulfoxaflor (5.9 ng/bee at 96 h; Azpiazu et al., 2021). However, daily syrup consumption fluctuated widely (see also Azpiazu et al., 2019), and as a result most bees of the 100 ppb treatment ingested levels of sulfoxaflor well above this dose (Fig. 3). On the other hand, bees of the 20 ppb treatment never reached ingestion levels over the LD50 value (Fig. 3).

We detected no effects on longevity at any of the five flupyradroxad tested concentrations (7500, 15,000, 30,000 and 60,000 ppb), alone or in combination with sulfoxaflor at 20 ppb. Depending on the treatment, the total amount of flupyradroxad ingested was 6.62–62.64 μg/bee and the mean daily ingested dose was 0.28–2.48 μg/bee (Table 1). These doses are much lower than the acute oral LD50 of flupyradroxad reported for honey bees (110.9 μg/bee; EFSA, 2012).

Longevity of bees exposed to sulfoxaflor, either at 20 or 100 ppb, did not decrease with the addition of flupyradroxad at any of the concentrations tested. Therefore, our results provide no evidence of interaction between these two compounds. This is in contrast to a previous study in which we found synergism between these two pesticides in O. bicornis after acute oral exposure (Azpiazu et al., 2021). In that study, synergism was tested with sulfoxaflor doses ranging from 2.75 to 88 ng/bee combined with a single flupyradroxad dose (1200 ng/bee) and synergism was observed only at intermediate insecticide doses (11 ng/bee). Our ability to detect potential synergism under chronic exposure in the current study may have been hindered by the choice of test concentrations, with 20 ppb being too low to cause mortality, and 100 ppb quickly yielding high levels of mortality irrespective of the addition of flupyradroxad. The various flupyradroxad concentrations tested, on the other hand, did not influence mortality at any of the two sulfoxaflor concentrations. Therefore, future studies should address intermediate insecticide concentrations at the expense of fungicide concentrations.

Our study shows that sulfoxaflor affects feeding behaviour in O. bicornis by increasing (at 20 ppb) and decreasing (at 100 ppb) syrup consumption compared to control bees. This result is in partial agreement with previous studies which found inhibitory effects of sulfoxaflor in bumblebees (Sliviter et al., 2019) and honey bees (Barascou et al., 2021; Li et al., 2021). Similar results have been found in A. mellifera (Zhu et al., 2017), B. terrestris (Laycock et al., 2012; Thompson et al., 2015) and O. bicornis (Azpiazu et al., 2019) exposed to neonicotinoids. For this group of insecticides, some studies have demonstrated that bees prefer solutions containing low concentrations of neonicotinoids indicating that feeding behaviour is dose-dependent (Kessler et al., 2015; Sgolastra et al., 2018).

Bees in our study were chronically exposed to pre-determined concentrations of sulfoxaflor and flupyradroxad, an approach that does not account for pesticide degradation, which effectively decreases pesticide concentration over time (Cheng et al., 2018; Kyriakopoulou et al., 2017; US EPA, 2019). However, levels of sulfoxaflor higher than 20 ppb (the lowest concentration we tested) have been found in the nectar of several crop flowers up to 14 days after bloom application (US EPA, 2019). Bees of the 100 ppb treatment in our study lived for 2–6 days and, again, levels of sulfoxaflor above this concentration have been found in the nectar of some crop flowers up to one week after application (US EPA, 2019). A recent study (Schwarz et al., 2022) in which Phacelia plants were treated with sulfoxaflor before bloom showed levels of 74–129 ppb in pollen 5–8 days after the application. Sulfoxaflor levels in nectar were not measured in that study, but they are usually lower than levels found in pollen (US EPA, 2019). The use of sulfoxaflor during bloom is still allowed in many countries (Corteva Australia, 2022; Corteva South Africa, 2022; Corteva US, 2022) and wild flowers growing near field crops may also become contaminated from sprays conducted outside of the blooming period of the target crop (Botias et al., 2015).

Comparisons of pesticide sensitivity across species need to be interpreted with caution because, due to differences in biological traits, experimental laboratory conditions cannot be exactly the same for different species (Sgolastra et al., 2019). For example, honey bee toxicity tests are usually conducted under group feeding conditions at 25 ± 2 °C in darkness (OECD, 2017). In contrast, A. mellifera spp. need to be individually fed and tests are usually conducted at lower temperatures (22 ± 2 °C) under light (Ladurner et al., 2003; Sgolastra et al., 2018; this study). Accepting this caveat, and assuming that experimental conditions have been tailored to mimic appropriate environmental conditions for each species, Osmia spp. appear to be more sensitive to sulfoxaflor than honey bees and bumblebees. Chronic exposure to 100 ppb strongly reduced O. bicornis survival in our study. By contrast, no increased mortality was observed in A. mellifera workers chronically exposed to 500 ppb for 10 days (EFSA, 2020a). This conclusion holds when the daily doses of sulfoxaflor ingested by the two species are compared (O. bicornis: 2.71–5.35 ng·bee−1·day−1; A. mellifera: 11.5 ng·bee−1·day−1, EFSA, 2020a), and after correcting for body weight (O. bicornis: 29.46–58.15 ng·g−1·day−1; A. mellifera: 125 ng·g−1·day−1). Other studies on honey bees also found no effects on survival after 6 days of exposure to either 20 ppb or 2000 ppb (Barascou et al., 2021), and
to 47 ppb for 30 days (Al Naggar and Paxton, 2021). A previous study calculated the LD₅₀ of *O. bicincta* acutely exposed to sulfoxaflor and also concluded that this species was more sensitive than *A. mellifera* and *B. terrestris* (Azpiazu et al., 2021). Similarly, sulfoxaflor applications 6 days before bloom reduced colony growth, colony size and foraging performance in bumblebees (Tamburini et al., 2021a), but not in honey bees (Tamburini et al., 2021b). Studies on neonicotinoid insecticides have also found sensitivity differences between bee species, indicating that *Osmia* are more sensitive than honey bees to compounds that target NACHr receptors (Arenà and Sgolastra, 2014; Biddinger et al., 2013; Sgolastra et al., 2017; Uhli et al., 2019).

Sulfoxaflor has been proposed as an alternative to the use of neonicotinoids and its use has been increasing worldwide since its approval in 2013 and 2015 in US and EU, respectively. A previous study showed that, although less toxic to bees than nitro-substituted neonicotinoids, sulfoxaflor was much more toxic than cyano-substituted neonicotinoids (Azpiazu et al., 2021). Neonicotinoid toxicity is greatly enhanced by the presence of EBI fungicides (Biddinger et al., 2013; Gill et al., 2012; Sgolastra et al., 2018, 2017; but see Haas and Nauen, 2021). By contrast, our results and those of previous studies (Azpiazu et al., 2021; Tamburini et al., 2021a) indicate that synergism between sulfoxaflor and SDHI is weak and highly dose-dependent (although this could not be confirmed in the current study). Because both neonicotinoids and sulfoxaflor act on Nicotinic Acetyl Choline Receptors (NACHr) (Sparks et al., 2013; Zhu et al., 2011), future studies should address potential synergism between sulfoxaflor and EBI fungicides. Ideally, these studies should be conducted on *Osmia* spp. given that these species are more sensitive to sulfoxaflor than honey bee and bumblebees. Notwithstanding the recent restrictions on outdoors use of sulfoxaflor in the EU, this active ingredient is still allowed in many other countries, even in bloom applications of entomophagous crops (Corteva Australia, 2022; Corteva South Africa, 2022; Corteva US, 2022).

In conclusion, our data showed that the highest tested concentration of sulfoxaflor causes a decline in syrup consumption and a marked decrease in longevity. By contrast, the low concentration stimulated syrup consumption, but did not affect longevity. In addition, we found no interaction between sulfoxaflor and fluxapyroxad administered chronically *ad libitum* at any of the concentrations tested and, therefore, no evidence of dose-dependent synergism.

**CRediT authorship contribution statement**

Celeste Azpiazu: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft; Jordi Bosch: Conceptualization, Funding acquisition, Writing - review & editing; Catia Martins: Methodology, Data Curation, Writing - review & editing; Fabio Sgolastra: Conceptualization, Methodology, Investigation, Data Curation, Funding acquisition, Supervision, Writing - review & editing.

**Data availability**

Data will be made available on request.

**Declaration of competing interest**

The authors declare they have no conflict of interest.

**Acknowledgements**

This study was supported by a grant from the University of Bologna to FS (Grant RFO2019.SGOLAstra), the Spanish Ministry of Science, Innovation and Universities (Grant R + D RTI2018-098399-R-B00) and the postdoctoral fellowship from the French Association POLLINIS (www. pollinis.org) to CA. We appreciate the technical assistance of G. Zanetti, T. Brandolini and E. Cacciatori. We also thank Dr. Molowny-Horas for his statistical advice.

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